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BIOLOGY OF THE FIRE BLIGHT PATHOGEN *Erwinia amylovora* IN OLIGOTROPHIC ENVIRONMENTS: SURVIVAL RESPONSES AND VIRULENCE

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La Dra. Elena González Biosca, Profesora Titular (Acreditada a Catedrática de Universidad) del Departamento de Microbiología y Ecología de la Facultad de Ciencias Biológicas de la Universitat de València,

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Ricardo Delgado Santander, licenciado en Biología por la Universitat de València, ha realizado bajo su dirección el presente trabajo, titulado: "BIOLOGY OF THE FIRE BLIGHT PATHOGEN *Erwinia amylovora* IN OLIGOTROPHIC ENVIRONMENTS: SURVIVAL RESPONSES AND VIRULENCE", y que hallándose concluido, autoriza su presentación a fin de que pueda ser juzgado por el tribunal correspondiente y optar así a la obtención del grado de Doctor por la Universitat de València, con la Mención de "Doctor Internacional", dentro del Programa de Doctorado en Biomedicina y Biotecnología.

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A mis padres,
a mi hermana,
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“Hunger, love, pain, and fear are some of those inner forces which rule the individual's instinct for self preservation.”

Albert Einstein

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Acrónimos y abreviaturas / Acronyms and abbreviations

σ	Sigma
Ác. Glc	Ácido glucurónico
ADN / DNA	Ácido desoxirribonucleico / Deoxyribonucleic acid
AFLP	Amplified Fragment Length Polymorphism
ARN / RNA	Ácido ribonucleico / Ribonucleic acid
ARNm	ARN mensajero
ARN Pol.	ARN polimerasa
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
ºC	Grados Celsius / Degrees Celsius
CABI	Centre for Agriculture and Bioscience International
CCT	Cycloheximide Crystal violet Thallium nitrate
CDS	Coding DNA Sequence
CBGP	Centro de Biotecnología y Genómica de Plantas
CFBP	Collection Française de Bactéries Phyto-pathogènes
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
cv.	Cultivar
Da	Daltons
DASI	Double-Antibody Sandwich Indirect
dpi	Days post-inoculation
DsRed	Discosoma red fluorescent protein
ECF	Extracytoplasmic factors
EEUU	Estados Unidos
EFM	Epi-Fluorescence Microscopy
ELISA	Enzyme-Linked Immunosorbent Assay

EPPO	European and Mediterranean Plant Protection Organization
EPS	Exopolisacáridos / Exopolysaccharides
Fe^{2+}	Hierro (II) / Iron (II)
Fe^{3+}	Hierro (III) / Iron (III)
FeOOH	Óxido-hidróxido de Hierro (III)
FPM	Fuerza protomotriz
Gal	Galactosa
GDP	Guanosin Disfosfato / Guanosine diphosphate
GFP	Proteína verde fluorescente /Green fluorescent protein
GTP	Guanosin T rifosfato / Guanosine triphosphate
h	Hours
H^+	Protón
H_2O_2	Peróxido de hidrógeno / Hydrogen peroxide
HAE	Hrp-Associated Enzymes
HEE	Hrp Effectors and Elicitors
HR	Hypersensitive Response
IG	Islas Genómicas
IT	Island Transfer
IVIA	Instituto Valenciano de Investigaciones Agrarias
Kpb	Kilopares de bases
LC-MS/MS	Liquid Chromatography-Mass Spectrometry
LMG	Laboratory of Microbiology Gent Bacteria Collection
LPS	Lipopolisacárido / Lipopolysaccharyde
Lsc	Levanosacarasa
LSCM	Microscopía láser confocal / Laser Scanning Confocal Microscopy
MAGRAMA	Ministerio de Agricultura, Alimentación y Medio Ambiente
Mb	Megabases
MIC	Minimal Inhibitory Concentration
min	Minutes

mL	Milliliter
MLSA	Multi-Locus Sequence Analysis
MLVA	Multi-locus variable number of tandem repeats analysis
mM	Millimolar
NH_3	Amoniaco
O-Ac	*O*-acetilo
OH^-	Ión hidróxido
·OH	Radical hidroxilo
Oxid.	Oxidative
OMV	Outer Membrane Vesicle
PAGE	PolyAcrylamide Gel Electrophoresis
Pathog.	Pathogenicity
PCR	Polymerase Chain Reaction
PGA	Poli-β-1,6-acetil-D-glucosamina
PM	Peso Molecular
Pol	Polimerasa
ppGpp	Guanosina 3',5'-bispirofosfato / guanosine 3',5'-bispyrophosphate
ppm	Parts per million
Pyr	Piruvato
RAPD	Random amplification of polymorphic DNA
Rep-PCR	Repetitive element palindromic PCR
ROS	Especies Reactivas del Oxígeno / Reactive Oxygen Species
RT-PCR	Reverse Transcriptase PCR
SNA	Sucrose Nutrient Agar
SST6	Sistema de Secreción de Tipo VI
SST3 / T3SS	Sistema de Secreción de Tipo III / Type III Secretion System
TEM	Transmission Electron Microscopy
UFC / CFU	Unidades Formadoras de Colonias / Colony Forming Units
var.	Variedad

VBNC	Viable no cultivable / Viable but nonculturable
Vir.	Virulence
WT	Wild Type
ZP	Zona Protegida

Resumen

Erwinia amylovora es una bacteria fitopatógena de la familia *Enterobacteriaceae*, responsable del fuego bacteriano de las rosáceas. Los efectos destructivos de este patógeno sobre frutos, flores y prácticamente todos los órganos de las plantas hospedadoras afectadas constituyen una amenaza importante para la producción de pera y manzana, y suponen graves pérdidas económicas anuales en todo el mundo. *E. amylovora* está clasificada como un organismo de cuarentena en la Unión Europea y en otros países pertenecientes a la EPPO (del inglés, *European and Mediterranean Plant Protection Organization*). España, ha sido considerada durante muchos años como un área protegida de fuego bacteriano por la Unión Europea. No obstante, dicha enfermedad ha acabado estableciéndose en muchas provincias, mientras que otras mantienen, total o parcialmente, el estatus de Zona Protegida.

El nombre fuego bacteriano se refiere al aspecto ennegrecido, como quemado por el fuego, característico de las plantas u órganos afectados por *E. amylovora*. A diferencia de otras bacterias fitopatógenas, esta sintomatología no es dependiente de la liberación de exoenzimas. *E. amylovora* utiliza el sistema de secreción de tipo tres (SST3) para inducir el estallido oxidativo y la muerte celular programada de las células vegetales asociada la respuesta hipersensible (HR, del inglés, *Hypersensitive Response*). Esta estrategia es inusual entre las bacterias patógenas de plantas, que precisamente utilizan el SST3 para evitar el estallido oxidativo y la HR, que son sistemas de defensa del hospedador para bloquear la progresión de los patógenos dentro de los tejidos vegetales. *E. amylovora* debe poseer, por lo tanto, mecanismos antioxidantes para lidiar con el

estrés oxidativo provocado durante su avance por los tejidos. Algunos elementos que se han relacionado con la protección de este patógeno frente al estrés oxidativo son los sideróforos, el lipopolisacárido (LPS) y los exopolisacáridos (EPSs), aunque el papel de éstos últimos ha sido discutido debido a resultados dispares en distintos trabajos.

Los brotes de fuego bacteriano son irregulares y difíciles de controlar y, una vez el patógeno se ha establecido en una zona, la erradicación de la enfermedad resulta compleja. El ciclo del fuego bacteriano ocurre en paralelo al desarrollo estacional de la planta hospedadora. Durante la primavera *E. amylovora* entra en el hospedador a través de los nectarios en las flores, así como otras aberturas naturales y/o heridas en brotes y otros órganos susceptibles. El movimiento de *E. amylovora* dentro de la planta se produce a través de los espacios intercelulares del parénquima cortical y los haces del xilema. A día de hoy, aún no existe consenso entre los investigadores sobre cuáles son los sitios iniciales de multiplicación del patógeno dentro del hospedador, o las rutas óptimas para la infección sistémica del mismo.

Los síntomas más característicos del fuego bacteriano son la necrosis, los exudados (compuestos por bacterias inmersas en una matriz de EPSs) y el marchitamiento. Dichos síntomas pueden desarrollarse en flores, frutos, brotes, ramas, el tronco y/o el portainjerto, por lo que el fuego bacteriano se considera principalmente una enfermedad de la parte aérea de las plantas. En algunas ocasiones se han detectado signos de fuego bacteriano en raíces, en condiciones de campo. No obstante, la infección primaria de las raíces no se considera una vía importante para la infección del hospedador.

E. amylovora puede detectarse también como organismo endófito en plantas asintomáticas, aunque el papel de las

poblaciones endófitas en las epidemias de fuego bacteriano se desconoce. En plantas enfermas el avance de los síntomas puede acabar matando la planta entera. En general, las infecciones más graves se producen en los periodos en los que el hospedador presenta un crecimiento activo. Las altas y bajas temperaturas durante el verano y el invierno, respectivamente, conducen a la aminoración del crecimiento de las plantas, lo que suele provocar una ralentización del desarrollo de los síntomas de fuego bacteriano. Sin embargo, en países con condiciones climáticas especiales, la progresión de los síntomas durante el invierno puede ser también importante. En relación a esto, la virulencia de muchas bacterias fitopatógenas en campo está determinada por las temperaturas ambientales. En el caso de *E. amylovora*, son necesarias temperaturas ambientales de 18-29ºC para el desarrollo de brotes de fuego bacteriano en flores. No obstante, el efecto de la temperatura sobre la capacidad del patógeno para causar síntomas en tejidos sensibles, distintos a las flores, no ha sido evaluada.

La infección de tejidos lignificados suele conducir a la formación de chancros (zonas hundidas y usualmente agrietadas que aparecen, generalmente, en el tronco y/o las ramas), en los que el patógeno sobrevive durante el invierno. Estas estructuras se consideran una de las principales fuentes de inóculo primario para el desarrollo de brotes de fuego bacteriano. Durante la primavera, las condiciones de temperatura y humedad favorables permiten la multiplicación de *E. amylovora* en los chancros, lo que favorece la producción de exudados. Las moscas y las hormigas son probablemente los vectores más importantes para la propagación del patógeno desde chancros con exudados a otras plantas, órganos o tejidos susceptibles. Los insectos polinizadores y perforadores parecen jugar un papel más importante en la diseminación secundaria de *E. amylovora* (de flor a flor, de brote a brote, etc). El viento, la lluvia y las aves

también contribuyen de forma importante a la diseminación de las células del patógeno.

En plantas enfermas la presencia de poblaciones epífitas de *E. amylovora* en hojas o la superficie de otros órganos se ve favorecida por ciertas condiciones ambientales. *E. amylovora* también es capaz de sobrevivir en suelo, insectos, o herramientas de poda. La persistencia de este y otros patógenos no obligados en ambientes fuera del hospedador depende de su tolerancia y/o adaptación a las condiciones de inanición que prevalecen en la naturaleza. La temperatura es otro factor ambiental que afecta directamente a la supervivencia de los microorganismos en el medio ambiente. Sin embargo, sus efectos varían en función del estado nutricional de las células, y las temperaturas que promueven la multiplicación celular en condiciones nutricionales óptimas pueden ser perjudiciales para células en estado de inanición. Las estrategias de *E. amylovora* para sobrevivir en condiciones naturales de limitación nutricional, así como los efectos de la temperatura sobre dichos mecanismos aún no han sido caracterizados.

Dos respuestas características de las bacterias no esporuladas a la oligotrofía ambiental son la supervivencia en estado de inanición y el estado viable no cultivable (VBNC, del inglés *viable but nonculturable*). El desarrollo de ambas estrategias está fuertemente influido por la temperatura, y en muchos casos, determina el ciclo de vida, así como el de la enfermedad de muchos patógenos de animales y de plantas. El estado fisiológico de las células en estado de supervivencia en inanición tiene muchas similitudes con el de las células en fase estacionaria. En ambos casos, las bacterias experimentan cambios en la morfología y tamaño celular, la movilidad, la composición bioquímica, la expresión génica, desarrollan resistencia cruzada a múltiples factores de estrés, etc., lo que les

permite adaptarse a las nuevas condiciones nutricionales.

A diferencia de las células en estado de supervivencia en inanición, las bacterias en estado VBNC pierden la capacidad para formar colonias en medio de cultivo sólido y, por lo general, también la patogenicidad. No obstante, las células en estado VBNC siguen siendo metabólicamente activas. Además de la inanición, otros tipos de estrés pueden inducir la respuesta VBNC. Tanto la cultivabilidad como la patogenicidad en células VBNC pueden recuperarse mediante la eliminación del agente estresante, la incubación en medio rico líquido, la exposición a ciertos factores de resucitación secretados por algunas especies de bacterias, y/o mediante el pase a través de un hospedador susceptible. *E. amylovora*, por ejemplo, entra en el estado VBNC y pierde la patogenicidad en frutos inmaduros tras la exposición a cobre. La recuperación de la cultivabilidad y la patogenicidad puede lograrse mediante diversas estrategias, aunque la intensidad de los tratamientos y/o el período transcurrido desde el inicio de la respuesta VBNC determinan el éxito de dicha recuperación.

En muchas especies bacterianas los mecanismos moleculares que controlan la adaptación a la limitación nutricional y/o la entrada en la fase estacionaria implican la participación del factor sigma alternativo RpoS. Tras la exposición de las células a condiciones de inanición u otros estreses, esta proteína se une a la ARN polimerasa y modifica los patrones de transcripción, favoreciendo la expresión de genes relacionados con el metabolismo, la resistencia al estrés, etc., que posibilitan la supervivencia de las células bacterianas en estas nuevas condiciones. En muchas especies bacterianas, RpoS también regula genes de virulencia. Dicha regulación puede ser directa, a través del control de genes de virulencia, o también indirecta, mediante la modulación de genes implicados en la supervivencia

del patógeno dentro del hospedador.

Dado que el estrés por inanición es uno de los principales factores de inducción del estado VBNC, el factor sigma RpoS también podría estar relacionado con la regulación de esta respuesta de supervivencia, aunque todavía existe poca información al respecto. Curiosamente, RpoS controla genes relacionados con la resistencia al estrés oxidativo, y la disminución progresiva de actividad catalasa se ha relacionado con la pérdida de cultivabilidad en medios sólidos durante la entrada en el estado VBNC.

La posible contribución de RpoS en la regulación de los estados de supervivencia en inanición y VBNC en *E. amylovora* aún no ha sido investigada. Por otra parte, también se desconoce el papel de RpoS en la regulación de importantes factores de virulencia/patogenicidad como la movilidad o la síntesis de EPS. Finalmente, aunque distintos estudios sugieren la relación entre el estrés oxidativo, la actividad catalasa y el desarrollo de la respuesta VBNC, la conexión entre dichos factores se ha establecido, en la mayoría de los casos, de forma indirecta y se desconoce la contribución exacta de las catalasas al fenotipo VBNC. La contribución de las catalasas a la virulencia de *E. amylovora* tampoco se ha estudiado todavía.

De acuerdo con lo anteriormente mencionado, muchos aspectos de la biología de *E. amylovora* aún siguen siendo poco conocidos, aunque de su estudio podrían derivarse mejoras en las estrategias de prevención y control del fuego bacteriano. En base a esto, el objetivo principal de esta Tesis Doctoral ha sido caracterizar los mecanismos de adaptación de *E. amylovora* a condiciones naturales de inanición, los cuales serían responsables de la capacidad del patógeno para persistir en el ambiente y diseminarse a través del agua, vectores, etc. Este objetivo global, puede subdividirse en los siguientes objetivos

particulares:

1. Caracterizar las respuestas de *E. amylovora* a la limitación nutricional, así como el efecto de la temperatura en dichas respuestas y la virulencia del patógeno.

2. Investigar la supervivencia y la transmisión de *E. amylovora* a través de la mosca mediterránea de la fruta *Ceratitis capitata*.

3. Determinar la posible infección de plantas de *Pyrus communis* por *E. amylovora* a través de las raíces mediante riego y caracterizar las rutas de colonización e invasión de la raíz.

4. Estudiar el papel de RpoS en el desarrollo de las respuestas a condiciones de inanición en *E. amylovora*, y caracterizar la participación de este regulador en la protección cruzada, el control de factores de virulencia, o la virulencia en diferentes tipos de material vegetal.

5. Explorar el papel funcional de las catalasas KatA y KatG de *E. amylovora* durante la exposición a condiciones limitantes en nutrientes y las interacciones planta-patógeno.

Para alcanzar estos objetivos se llevaron a cabo diferentes estudios, lo que ha llevado a la publicación de cuatro artículos científicos (anexo I, III, IV, VI) y la preparación de tres manuscritos adicionales que han sido o serán enviados a publicar en breve (anexo II, V, VII). Los resultados de estos estudios se recopilan en los cinco capítulos que forman parte de esta Tesis Doctoral.

El **Capítulo 1** resume los resultados de tres publicaciones (Anexo I, II y III). En todas ellas se prepararon microcosmos de agua oligotrófica de distintos tipos, que se inocularon con *E. amylovora* e incubaron a una o más temperaturas ambientales,

entre 4ºC y 28ºC. En el artículo 1 (Anexo I; Santander *et al*., 2014b) se seleccionaron condiciones de temperatura que favorecieran el mantenimiento de la cultivabilidad en *E. amylovora,* con el fin de analizar los efectos de la inanición sobre la movilidad, la expresión de genes de virulencia, la morfología o la virulencia. Parte de este estudio se llevó a cabo en la Facultad de Biología de la Universidad de Carolina del Norte en Charlotte (EEUU). En el artículo 2 (Anexo II; Santander & Biosca, 2016, no publicado) también se investigó el efecto de distintas temperaturas (4ºC, 14ºC y 28ºC) sobre las respuestas de *E. amylovora* a inanición, así como sobre la morfología, el tamaño celular, la virulencia y algunos rasgos fenotípicos relacionados con la supervivencia y/o virulencia. Por último, el artículo 3 (Anexo III; Santander *et al*., 2012a) se centró en el estudio de la respuesta VBNC inducida por condiciones de inanición y por exposición a cloro, así como de las condiciones que favorecen la recuperación de dichas células.

Los principales resultados de este capítulo son:

i) Las temperaturas entre 14ºC y 20ºC favorecen el mantenimiento de la cultivabilidad en células de *E. amylovora* sometidas a condiciones de inanición, mientras que temperaturas por encima o por debajo de este rango inducen la respuesta VBNC. Esta respuesta implica cierta pérdida de viabilidad a temperaturas por encima de 20ºC. Dicha reducción de viabilidad no se observa durante la entrada en el estado VBNC a temperaturas por debajo de 14ºC.

ii) La exposición de *E. amylovora* a condiciones de inanición por periodos prolongados induce la adquisición de forma cocoide, una reducción de tamaño (*dwarfing*), el desarrollo de vesículas de superficie y la pérdida de movilidad, aunque algunos flagelos permanecen unidos a la

superficie celular. Los cambios de tamaño, pero no los de morfología, también son dependientes de la temperatura, viéndose favorecidos a 14ºC con respecto a 4ºC o 28ºC.

iii) La exposición de *E. amylovora* a condiciones de inanición induce cambios en la expresión de genes relacionados con las respuestas a inanición (*cstA*, *dps*, *relA*, *rpoS*, *spoT*), la virulencia o patogenicidad (*flgN*, *hrpL*, *rcsB*, *rlsA*) y la resistencia al estrés oxidativo (*katA, katG, oxyR*). Los niveles de ARNm de estos genes son detectables a lo largo de un período de inanición de 40 días.

iv) Las células de *E. amylovora* en estado de supervivencia en inanición son patógenas en frutos inmaduros (peras y nísperos) y plántulas de peral. Sin embargo, tras la inducción del estado VBNC mediante inanición o exposición a cloro se observa la pérdida tanto de cultivabilidad como de patogenicidad en frutos inmaduros. No obstante, estas células VBNC pueden volver a ser cultivables y patógenas en frutos inmaduros tras su pase a través de plántulas de peral.

v) *E. amylovora* es patógena a 4ºC, 14ºC y 28ºC. La virulencia disminuye en paralelo a la temperatura de incubación. De forma similar, las tasas de crecimiento en medio rico, la movilidad y la secreción de sideróforos también disminuyen junto con las temperaturas de incubación. Sin embargo, otros factores de virulencia/patogenicidad se inducen a bajas temperaturas. Por ejemplo, la producción de amilovorano y levano se incrementa a 4ºC y 14ºC, respectivamente. La incubación a 14ºC también favorece la formación de biopelículas y la resistencia a peróxido de hidrógeno (H_2O_2), con respecto a 4ºC o 28ºC.

El **Capítulo 2** contiene los resultados de una publicación sobre la supervivencia y transmisión de *E. amylovora* por la mosca mediterránea de la fruta *C. capitata* (Anexo IV; Ordax *et al.,* 2015), que se realizó en colaboración con el Instituto Valenciano de Investigaciones Agrarias (IVIA, España). *C. capitata* es una plaga común en los huertos de manzanos y perales, y ha sido descrita como un vector potencial de patógenos humanos, por lo que también podría desarrollar un papel importante en la diseminación y transmisión de *E. amylovora* a nuevos hospedadores. Una condición imprescindible para que un insecto pueda transmitir un patógeno es que dicho patógeno sea capaz de sobrevivir en el insecto y que dicho insecto sea capaz de transmitir el patógeno a un hospedador susceptible. Con el fin de determinar la supervivencia de *E. amylovora* en/sobre la mosca de la fruta, grupos de insectos se expusieron durante 48 h a manzanas contaminadas con *E. amylovora* en el interior de jaulas. Posteriormente, dichos insectos fueron trasladados a nuevas jaulas con comida artificial (para determinar la supervivencia del patógeno a lo largo del tiempo) o con material vegetal susceptible como frutos, brotes o plántulas (para estimar la capacidad de transmisión de *E. amylovora* por *C. capitata*). La supervivencia de *E. amylovora* se evaluó mediante recuentos en placa. Cuando dichos recuentos disminuyeron por debajo de los niveles de detección, la posible existencia de células VBNC se evaluó mediante la inoculación de extractos de mosca en material vegetal susceptible. Durante los ensayos de transmisión, tanto el desarrollo de síntomas de fuego bacteriano en el material vegetal expuesto a las moscas como el aislamiento de *E. amylovora* de la superficie de dicho material en medios de cultivo fueron considerados como eventos de transmisión positivos. La supervivencia y localización de *E. amylovora* en/sobre los insectos también se analizó mediante el uso de cepas transformantes de *E. amylovora* marcadas con la proteína verde

fluorescente GFP, o la proteína roja fluorescente DsRed, y su posterior análisis por microscopía de epifluorescencia (EFM). Los principales resultados de este capítulo son:

i) *E. amylovora* puede ser adquirida por *C. capitata* y sobrevivir en el insecto durante, al menos, 28 días. La detección del patógeno en medio sólido sólo fue posible durante los primeros 14 días. Las dos semanas siguientes *E. amylovora* dejó de detectarse en medio de cultivo, aunque la inoculación de extractos de *C. capitata* en material vegetal susceptible indujo el desarrollo de síntomas de fuego bacteriano, indicando la presencia de células de *E. amylovora* en el estado VBNC.

ii) *C. capitata* es capaz de transmitir *E. amylovora* a diferentes tipos de material vegetal. La contaminación de la superficie de las plantas expuestas a las moscas ocurre por el contacto de las mismas con células de *E. amylovora,* que forman agregados y biopelículas sobre las moscas (principalmente sobre la parte distal de las alas, el abdomen y, en el caso de las hembras, también sobre el ovopositor). La transmisión de *E. amylovora* a través de heces con células del patógeno previamente ingeridas por la mosca no se descarta. Las hembras de *C. capitata*, además, son capaces de inocular células de *E. amylovora* en el interior de tejidos vegetales susceptibles mediante el ovopositor.

iii) Tras la adquisición del patógeno, *E. amylovora* persiste 8 días dentro de la mosca de la fruta. El análisis por EFM de secciones hechas con vibratomo de moscas expuestas a cepas de *E. amylovora* transformantes de color rojo fluorescente reveló la internalización del patógeno mediante la alimentación. Se detectaron células de *E. amylovora* en las partes internas de los órganos bucales,

el buche y el tracto digestivo.

En el **Capítulo 3** se detallan los resultados de un trabajo no publicado sobre la viabilidad de la transmisión por el agua e infección de *P. communis* por *E. amylovora* a través de las raíces (anexo V; Santander *et al.,* 2016, no publicado). Para llevar a cabo este estudio, se adaptó un protocolo para la obtención de plántulas de peral por cultivo *in vitro* de embriones. Para determinar la capacidad de *E. amylovora* para migrar desde las raíces hasta las partes superiores de la planta, se realizó primero un ensayo de inoculación directa en raíz. Posteriormente se ensayó la inoculación por riego de plantas intactas y plantas con las raíces dañadas por trasplante, utilizando agua contaminada con *E. amylovora*. Las etapas de la infección de las raíces y las rutas de migración del patógeno dentro de los tejidos del hospedador se caracterizaron utilizando una cepa de *E. amylovora* marcada con GFP y análisis por EFM y microscopía laser confocal (LSCM, del inglés *Laser Scanning Confocal Microscopy*). Los principales resultados de este trabajo son:

i) El cultivo *in vitro* de embriones de peral permite la germinación de semillas almacenadas a largo plazo, que son difíciles de germinar mediante procedimientos estándar.

ii) La inoculación directa de *E. amylovora* en las raíces de plántulas de peral permite la invasión sistémica de la plántula por el patógeno, que es capaz de generar síntomas de fuego bacteriano en la parte aérea de la planta, lejos del sitio de inoculación.

iii) La infección de raíces de *P. communis* por *E. amylovora* a través del agua de riego es posible en condiciones

controladas. Los porcentajes de plantas con síntomas fueron del 52.5% en el caso de plantas con heridas en las raíces (por trasplante), y del 20% en el caso de plantas intactas. Las plantas desarrollaron síntomas de fuego bacteriano en el tallo, las hojas y los pecíolos.

iv) Las fases de la colonización de las raíces por *E. amylovora* son muy similares a las descritas en bacterias fitopatógenas del suelo. La entrada del patógeno en el sistema radicular se produce a través de heridas, y/o de grietas en el lugar de emergencia de las raíces secundarias. Las células de *E. amylovora* se multiplican en el apoplasto del córtex de la raíz, alcanzan el sistema vascular y se desplazan a través del xilema a las partes aéreas de la planta, donde son capaces de generar síntomas típicos de fuego bacteriano.

En el **Capítulo 4** se describen los resultados de un estudio sobre el papel de RpoS en las respuestas de *E. amylovora* a condiciones de inanición, la protección cruzada frente a distintos tipos de estrés, la movilidad, la producción de EPSs, y la virulencia (Anexo VI; Santander *et al.,* 2014a), que se realizó, en parte, en el Centro de Biotecnología y Genómica de Plantas (CBGP, Madrid). Para ello se obtuvo un mutante de *E. amylovora* en el gen *rpoS* junto con la correspondiente cepa complementada, que se caracterizaron mediante distintos ensayos junto con la cepa silvestre. Los resultados más importantes de este estudio son:

i) El mutante de *E. amylovora* deficiente en RpoS es más sensible a condiciones de inanición: muestra una inducción más rápida de la respuesta VBNC acompañada de una pérdida de viabilidad y lisis celular no observadas

en la cepa silvestre.

ii) En *E. amylovora* el factor sigma RpoS participa en la protección cruzada de células en fase estacionaria frente a estrés osmótico, ácido y oxidativo, y juega un papel esencial en la respuesta a choque térmico. La protección frente a choque ácido, sin embargo, es independiente de RpoS.

iii) RpoS aumenta la supervivencia de *E. amylovora* en tejidos de tabaco (planta no susceptible). La mutación en el gen *rpoS* genera un aumento de la virulencia de *E. amylovora* en frutos inmaduros, un aumento significativo de la producción de levano, una ligera disminución en el caso de amilovorano y una reducción de la movilidad por *swimming*.

Por último, en el **Capítulo 5** se resumen los resultados correspondientes a un estudio no publicado sobre el papel de las catalasas de *E. amylovora* KatA y KatG durante las respuestas a condiciones de inanición y las interacciones planta-patógeno (Anexo VII; Santander *et al.,* 2016, no publicado). Para ello, se construyeron mutantes de *E. amylovora* (dos simples y uno doble) en los genes que codifican las catalasas, y también las correspondientes cepas complementadas. La caracterización del papel funcional de las catalasas se llevó a cabo mediante: estudios de expresión, mediciones *in vivo* e *in vitro* de actividad catalasa, la detección de isoenzimas por zimografía después de electroforesis en gel de poliacrilamida (PAGE, del inglés *PolyAcrylamide Gel Electrophoresis*) en condiciones nativas, identificación de proteínas mediante cromatografía líquida-espectroscopía de masas (LC-MS/MS, del inglés *Liquid Chromatography – Mass Spectometry*), monitorización de las

dinámicas poblacionales de células cultivables, viables y totales durante la exposición a condiciones de inanición, ensayos de inhibición de crecimiento, estudios de supervivencia en tejidos de plantas no hospedadoras y ensayos de virulencia. Los resultados más relevantes de este trabajo son:

i) Los genes *katA* y *katG* de *E. amylovora* se inducen en las fases de crecimiento estacionario y exponencial, respectivamente. Ambos genes se inducen también en presencia de H_2O_2 y en los tejidos del hospedador. El análisis de la actividad catalasa reveló que la mayor parte de dicha actividad en *E. amylovora* corresponde a KatA. KatA y KatG son las únicas fuentes de actividad catalasa en *E. amylovora*.

ii) Es posible detectar KatA, pero no KatG mediante zimografía estándar y LC-MS/MS.

iii) KatA, y en segundo lugar KatG, son necesarias para la protección de *E. amylovora* frente a H_2O_2 y menadiona.

iv) La ausencia de actividad catalasa en el doble mutante (sin KatA ni KatG) de *E. amylovora* induce un desarrollo más rápido de la respuesta VBNC que en el resto de cepas en condiciones de inanición. Este fenómeno no se observa ni en la cepa silvestre ni en los mutantes simples (sin KatA o KatG), que se comportan de manera equivalente a la cepa silvestre. La adición de catalasa a las placas de medio de cultivo o la complementación de las cepas mutantes con una copia funcional de los genes que codifican las catalasas, mejora la cultivabilidad, pero no evita la entrada en el estado VBNC.

v) Las catalasas de *E. amylovora* juegan un papel importante en la supervivencia de la bacteria en los tejidos de una

planta no susceptible. En plantas sensibles al patógeno, KatA y KatG desempeñan diferentes funciones durante el proceso infeccioso. KatG parece más importante en los estadios iniciales de la infección, mientras que KatA parece contribuir a la progresión de *E. amylovora* en los tejidos del hospedador.

Las principales conclusiones de esta Tesis Doctoral se pueden resumir en:

1) Las temperaturas ambientales entre 14ºC y 20ºC favorecen la persistencia en estado cultivable de células de *E. amylovora* expuestas a condiciones de inanición. Las temperaturas por encima y por debajo de este rango inducen una entrada progresiva en el estado VBNC. Las temperaturas más bajas, además, mejoran el mantenimiento de la viabilidad celular durante la entrada en el estado VBNC. Las adaptaciones de *E. amylovora* a condiciones de escasez nutricional incluyen cambios morfológicos, reducción de tamaño (*dwarfing*), modulación de la movilidad y también de la expresión génica. La reducción del tamaño de las células es dependiente de la temperatura de incubación.

2) Las células de *E. amylovora* en estado de supervivencia en inanición son patógenas. La inducción del estado VBNC por exposición a inanición o cloro conduce a una pérdida de patogenicidad en frutos inmaduros, que puede ser revertida mediante pase a través de plántulas de peral susceptibles.

3) En condiciones controladas de laboratorio, *E. amylovora* es patógena incluso a 4ºC. Además, muchos factores de virulencia son inducidos a temperaturas inferiores a la óptima de crecimiento.

4) *E. amylovora* es capaz de sobrevivir en *C. capitata* durante, al menos, 28 días, y las condiciones en el insecto inducen la entrada del patógeno en el estado VBNC. *E. amylovora* forma agregados celulares y/o estructuras similares a las biopelículas en la superficie de *C. capitata* (en la parte distal de las alas, el abdomen y el ovopositor), aunque también puede ser internalizada por el insecto y persistir hasta 8 días en su interior (en el aparato bucal, el buche y el tracto digestivo).

5) La transmisión de *E. amylovora* por *C. capitata* ocurre, principalmente, por contacto de la mosca con la superficie de las plantas. Las hembras, además, pueden inocular *E. amylovora* en el interior de tejidos susceptibles mediante el ovopositor. Por tanto, *C. capitata* podría ser un vector potencial de *E. amylovora.*

6) *E. amylovora* es capaz de invadir plántulas de peral a través de las raíces, que desarrollan síntomas característicos de fuego bacteriano en la parte aérea. El patógeno, además, en condiciones controladas es capaz de infectar plántulas de peral a través de las raíces mediante inoculación por riego, y el porcentaje de infección aumenta en plantas con raíces dañadas por trasplante. Las etapas de infección de la raíz de peral por *E. amylovora* son muy similares a las de otras bacterias fitopatógenas del suelo.

7) El factor sigma RpoS contribuye al mantenimiento de la cultivabilidad y la viabilidad en células de *E. amylovora* en condiciones de inanición. También participa en la protección cruzada de células de *E. amylovora* en fase estacionaria frente a diversos tipos de estrés que el patógeno puede encontrar en la naturaleza, contribuye a la supervivencia de *E. amylovora* en los tejidos de plantas no hospedadoras, y modula la producción de EPSs, la movilidad y también la virulencia en frutos inmaduros.

8) La expresión de los genes que codifican las catalasas

KatA y KatG de *E. amylovora* es dependiente de la fase de crecimiento, y se induce en presencia de H_2O_2 y tejidos del hospedador. KatA y KatG también poseen diferente actividad catalasa, lo que indica papeles funcionales diferenciados para cada una de estas catalasas. Ambas contribuyen al mantenimiento de la cultivabilidad en células de *E. amylovora* expuestas a condiciones de inanición, retrasando, por lo tanto, la entrada en el estado VBNC, además, contribuyen también a la supervivencia del patógeno en tejidos de hospedadores no susceptibles, y son necesarias para una virulencia completa en frutos inmaduros.

En resumen, la investigación realizada en esta Tesis Doctoral proporciona nueva información acerca de los mecanismos que permiten a *E. amylovora* sobrevivir fuera del hospedador. De acuerdo con los resultados aquí expuestos, tanto los estados de supervivencia en inanición como VBNC podrían formar parte del ciclo de vida de *E. amylovora,* contribuyendo a su persistencia en el ambiente, diseminación y probablemente también al desarrollo de la enfermedad en condiciones de campo. Estos resultados podrían explicar, en parte, la distribución global de *E. amylovora* por distintos países en diferentes zonas climáticas. Así mismo, dado que el conocimiento del ciclo de vida de los patógenos es imprescindible para establecer medidas de prevención y control de las enfermedades, los datos aportados en esta Tesis podrían contribuir a la mejora de las existentes.

Summary

Erwinia amylovora is a plant pathogenic bacterium of the family *Enterobacteriaceae*, responsible for fire blight of rosaceous plants. The destructive activity of this pathogen on fruits, flowers and almost all the host plant organs is a major threat for pear and apple production, and causes serious economic losses worldwide. *E. amylovora* is of quarantine concern in the European Union and other countries pertaining to the European and Mediterranean Plant Protection Organization (EPPO). Spain was considered a protected area with respect to fire blight for many years. However, the disease has finally been established itself in several provinces, while others still maintain a total or partial Protected Zone status.

The name "fire blight" refers to the characteristic appearance of affected plants or plant organs, which is black and shriveled as if burnt by fire. The development of these characteristic fire blight symptoms is not dependent on the release of exoenzymes, as occurs in other plant pathogens. In this case, *E. amylovora* uses the type three secretion system (T3SS) to provoke an oxidative burst and trigger the programmed cell death linked to the hypersensitive response (HR). This strategy is uncommon among plant pathogens, which use the T3SS to avoid this plant response which, in most cases, blocks the progression of pathogens within plant tissues. As a result of *E. amylovora* infections, however, the HR progresses continuously, and is used by the pathogen to invade neighboring tissues. Therefore, based on this information, antioxidant mechanisms to deal with oxidative stress are necessary for a successful development of symptoms. Siderophores, the lipopolysaccharide (LPS) and the exopolysaccharides (EPSs) amylovoran and levan (important virulence and pathogenicity factors in *E. amylovora,* respectively) have been linked to the

protection of the pathogen against this stress, although the role of EPSs remains unclear due to inconsistent outcomes in different studies.

Fire blight outbreaks are irregular and difficult to control, and once *E. amylovora* is established in an area, the eradication of the disease becomes an arduous task. The disease cycle occurs in parallel with the seasonal development of the host. During spring the pathogen enters the host plant through the nectarthodes in flowers, and other natural openings, and/or wounds in shoots and other susceptible young organs. The movement of *E. amylovora* within susceptible plants occurs through the intercellular spaces of the cortical parenchyma and xylem vessels. However, the initial multiplication sites within host tissues or the optimal routes allowing the systemic spread of the pathogen are still a subject of discussion.

Necrosis, ooze (composed of bacteria in a hygroscopic EPS matrix) and/or wilting are characteristic fire blight symptoms, and can develop in flowers, fruits, shoots, twigs, branches, the trunk and/or the rootstock, hence fire blight is considered a disease of the aerial parts of the plant. Root blight has also occasionally been reported under field conditions, although the primary infection of these organs is not considered an important route for host invasion.

Endophytic populations of the pathogen in asymptomatic hosts are usually reported, and their role in fire blight epiphytotics is also still debated. When symptoms are developed, the systemic spread of the pathogen can sometimes lead to the death of the entire host. In general, the most severe infections occur in actively growing host plants, along with the high and low temperatures during summer and winter, leading to a decay in plant growth, usually provoking a slow-down of symptom development. However, in countries with special climatic conditions, the progression of symptoms during winter can also be important. Related to this, the virulence of many plant pathogens is determined by environmental

temperatures. In the case of *E. amylovora*, temperatures of 18-29ºC seem to be necessary for the occurrence of blossom blight outbreaks under field conditions; however, the effect of temperature on the pathogen's ability to cause disease in susceptible tissues other than flowers has not been assessed.

Infections of lignified tissues often lead to the formation of cankers (sunken and usually cracked areas, mainly on the trunk and/or shoots), where the pathogen overwinters. These structures are considered one of the main sources of primary inoculum for the development of fire blight outbreaks. During spring, favorable temperature and humidity conditions allow the multiplication of *E. amylovora* in cankers, which leads to ooze production. Flies and ants are probably the most important vectors spreading the fire blight pathogen from oozing cankers to other susceptible plants, organs or tissues, while pollinating and piercing-sucking insects seem to play a more relevant role in the secondary dissemination of the pathogen (flower to flower, shoot to shoot, etc). Wind, wind-driven rain and/or birds also contribute greatly to the dissemination of bacterial cells from oozing cankers to other susceptible tissues, organs and hosts.

In diseased plants the presence of epiphytic populations of the pathogen on leaves or other plant surfaces is favored by certain environmental conditions. Moreover, *E. amylovora* is able to survive in soil, insects, or on pruning tool surfaces. The persistence of this and other non-obligated pathogens outside the host is compromised by their tolerance and/or adaptation to the starvation conditions prevailing in nature. Temperature is another key factor directly affecting the survival of microorganisms in natural environments. However, its effects vary depending on the nutritional state of cells, and temperatures promoting cell multiplication under optimal nutrient conditions might be deleterious for starved cells. How *E. amylovora* deals with starvation or the effects of temperature on the

pathogen survival under natural nutrient limiting conditions is still a scarcely explored subject.

Two common responses of nonsporulating bacteria to environmental oligotrophy, strongly influenced by temperature, are the starvation-survival and the viable but nonculturable (VBNC) states. These two survival strategies determine the life cycle and disease development in many plant and animal bacterial pathogens. The physiology of bacterial cells developing the starvation-survival response has many similarities to that of cells in stationary-phase. Bacteria in this state experience changes in morphology, cell size, motility, biochemical composition, gene expression, develop cross-resistance to multiple stresses, etc., in order to adapt to this new nutrient limiting condition.

As opposed to bacteria in the starvation-survival state, bacterial cells adopting the VBNC state lose their ability to form colonies on solid conventional media, and they also usually lose their pathogenicity, but remain metabolically active. Apart from environmental nutrient scarcity, other stresses can induce the VBNC response. The recovery of both culturability and pathogenicity in VBNC cells can occur after removing the stressing agent, by incubation in rich medium, by exposure to certain resuscitation factors secreted by some bacterial species, and/or by passage through a susceptible host. In *E. amylovora,* for example, cells become VBNC after exposure to copper, and this response is accompanied by a loss of pathogenicity in immature fruits. The recovery of culturability and pathogenicity is possible throug a variety of strategies, but the intensity of treatments and/or the elapsed period since the onset of the VBNC response may determine the success of the recovery.

In many bacterial species the molecular mechanisms controlling adaptation to nutrient limiting environments and/or the entry into the stationary-phase involve the participation of the

alternative sigma factor RpoS. This protein changes the transcription patterns of the RNA polymerase, favoring the expression of genes related to metabolism, stress resistance, etc., which enhance the survival of the challenged bacterial cells in these new conditions. In some bacterial species, RpoS has also been described as being able to control virulence. The regulation of virulence can occur directly, via the control of virulence genes, or also indirectly, by modulating the genes involved in the survival of the pathogen under host conditions.

Given that starvation is one of the main VBNC-inducing stresses, RpoS might also be linked to the regulation of this survival response, although there is still little information in this regard. Interestingly, RpoS controls genes related to oxidative stress resistance, and the loss of catalase activity has been reported as an important factor determining culturability on solid media in starved cells.

In the case of *E. amylovora,* the potential roles of RpoS in the regulation of the starvation-survival and the VBNC responses have not yet been investigated. Moreover, the control exerted by this master regulator on virulence/pathogenicity factors such as motility, or EPS synthesis in the fire blight pathogen is still unknown. Furthermore, despite the fact that a link response has been established between oxidative stress, catalase activity and the development of the VBNC, mainly by indirect observations, the exact contribution of catalases to the VBNC phenotype has never been explored in *E. amylovora,* nor in any other bacterial pathogens. In addition, the contribution of catalases to *E. amylovora* virulence has not yet been assessed.

There are still many aspects of the *E. amylovora* life cycle that remain poorly understood. The main objective of this Doctoral Thesis was focused on the characterization of the mechanisms allowing *E. amylovora* to adapt to natural nutrient limitation

conditions, which are responsible for the pathogen's persistence in the environment, and its spread by water, vector insects, etc. The main objective of this Doctoral Thesis can be specified in the following partial objectives:

1. Characterize *E. amylovora*'s responses to starvation, as well as the effect of environmental temperatures on these responses and virulence.

2. Investigate the survival and transmission of *E. amylovora* by the medfly *Ceratitis capitata.*

3. Determine the potential water-borne infection of *Pyrus communis* plants by *E. amylovora* through the roots by soil-irrigation, and characterize the root colonization, invasion and migration routes of the pathogen.

4. Study the role of the alternative sigma factor RpoS in the development of starvation-survival responses by *E. amylovora,* as well as characterize its functions as a regulator of cross-protection, virulence factors and virulence in different types of plant material.

5. Explore the functional roles of the *E. amylovora* catalases KatA and KatG during exposure to nutrient limiting conditions and also during plant-pathogen interactions.

To reach these objectives different studies were performed leading to the publication of four scientific articles (Annex I, III, IV, VI) and the preparation of three additional manuscripts that will be submitted shortly (Annex II, V, VII). The results from all these studies are collected and summarized in five chapters in this Doctoral Thesis.

Chapter 1 summarizes the results of three publications (Annex I, II and III). In all of them oligotrophic water microcosms were prepared, inoculated with *E. amylovora* and incubated at one or more environmental temperatures, ranging from 4ºC to 28ºC. In Article 1 (Annex I; Santander *et al.,* 2014b) temperature conditions favoring the maintenance of culturability over time were selected to further analyze possible morphological, motility, gene expression and virulence changes occurring during the exposure of *E. amylovora* to starvation. Part of this study was performed in the Faculty of Biology, in the University of North Carolina at Charlotte (USA). In Article 2 (Annex II; Santander & Biosca, 2016, unpublished), the effect of incubation at low, temperate and warm temperatures on morphology, cell size, virulence and some phenotypic traits related to the survival and/or virulence of *E. amylovora* was also investigated. Finally, Article 3 (Annex III; Santander *et al.,* 2012a) was focused on the induction of the VBNC response in *E. amylovora* by exposure to starvation or chlorine, in order to test several methodologies to recover VBNC cells.

The main results in this chapter are:

i) Temperatures between 14ºC and 20ºC favor the maintenance of *E. amylovora* starved cells in a culturable state, while temperatures above or below this range induce the VBNC response. Higher temperatures (>20ºC) induce a loss of viability not observed at lower temperatures (<14ºC).

ii) Long-term starved *E. amylovora* cells acquire coccoid shapes, reduce their size (dwarfing), develop outer membrane vesicles and lose motility, although some flagella remain attached to the cell surface. Changes in cell size but not in morphology are also dependent on temperature, with dwarfing being favored at 14ºC with respect to 4ºC or 28ºC.

iii) *E. amylovora* cells in the starvation-survival state modulate the expression of genes related to starvation (*cstA, dps, relA, rpoS, spoT*), virulence or pathogenicity (*flgN, hrpL, rcsB, rlsA*) and oxidative stress resistance (*katA, katG, oxyR*), to reach stable levels in short periods after the exposure to starvation conditions. The levels of mRNA of these genes were detectable throughout a starvation period of 40 days.

iv) *E. amylovora* cells in the starvation-survival state are pathogenic in immature fruits (loquats and pears) and in pear plantlets. On the contrary, starvation- and chlorine-induced VBNC cells lose pathogenicity together with pathogenicity on immature fruits over time. However, these VBNC cells can recover their culturability and pathogenicity on immature fruits by passage through pear plantlets.

v) *E. amylovora* is pathogenic at 4ºC, 14ºC and 28ºC, and virulence decreases in parallel with incubation temperature. Growth rates in rich medium, motility and siderophore secretion also decreased along with incubation temperatures. By contrast, other virulence/pathogenicity factors were induced at temperatures below 28ºC. The EPSs amylovoran and levan, for example, were overproduced at 4ºC and 14ºC, respectively. Incubation of cells at 14ºC also enhanced biofilm formation and hydrogen peroxide (H_2O_2) resistance, with respect to 4ºC or 28ºC.

Chapter 2 contains results from a publication on *E. amylovora* survival in the Mediterranean fruit fly, or medfly, *C. capitata*, and on its potential transmission by this insect (Annex IV; Ordax *et al.,* 2015). This work was a collaboration with *Instituto Valenciano de Investigaciones Agrarias* (IVIA, Spain). *C. capitata*, is a common pest in pear and apple orchards, which has been

described as a potential vector for human pathogens. Hence, it might also develop an important role in host-to-host transmission and the long distance dispersal of *E. amylovora.* For an insect to act as a vector of a plant pathogen, the latter has to be able to survive in/on the insect, and the insect has to be able to transmit the pathogen to susceptible plant material. In order to monitor the survival of *E. amylovora* in/on medflies and the potential of these insects to transmit the pathogen, groups of medflies were exposed to apples contaminated with the pathogen over 48 h in cages. Afterwards the insects were transferred to new cages with artificial food (to monitor *E. amylovora* survival over time) or containing susceptible plant material such as fruits, shoots or potted plants (to monitor *E. amylovora* transmission by the medflies). Survivability was monitored on solid media. When plate counts decreased below the detection limit, the possible survival of *E. amylovora* in the VBNC state was tested by inoculating medfly extracts in susceptible plant material. In transmission experiments, transmission events were considered positive when fire blight symptoms were observed in the plant material exposed to the flies and/or by the isolation of *E. amylovora* cells from such material on culture media. Survival and location of *E. amylovora* in/on insects was also analyzed by using *E. amylovora* strains labeled with the green fluorescent protein GFP, or the red fluorescent protein DsRed followed by epifluorescence microscopy (EFM) analysis. The main results in this chapter are:

i) *E. amylovora* can be acquired by, and survive on/in medflies, for at least, 28 days with the detection of the pathogen on solid media being possible during the first 14 days. In the following two weeks, the pathogen was not isolated by culture-dependent procedures, but the inoculation of medfly extracts into susceptible plant material led to the development of fire blight symptoms, indicating the presence of *E. amylovora* cells in the VBNC state.

ii) The medfly *C. capitata* is able to transmit *E. amylovora* to different types of plant material. EFM analysis of medflies exposed to GFP-tagged bacteria led to the conclusion that plant surfaces are contaminated by contact with *E. amylovora* cells forming aggregates and biofilm-like structures on insect surfaces (mainly on the distal section of wings and the abdomen, as well as on the ovipositor surface of female medflies). The transmission via faeces containing *E. amylovora* cells previously ingested cannot be discarded. Female medflies, additionally, are able to inoculate *E. amylovora* cells inside susceptible plant tissues while using the ovipositor.

iii) *E. amylovora* persists 8 days within medflies. The EFM analysis of vibratom sections of medflies exposed to DsRed-tagged *E. amylovora* cells revealed that the pathogen can be internalized by feeding, with bacterial cells being located in internal parts of the mouth and the crop, but mainly in the digestive tube.

In **Chapter 3** the results of an unpublished work on the feasibility of the water-borne root infection of *P. communis* by *E. amylovora* are summarized (Annex V; Santander *et al.,* 2016, unpublished). To conduct this study, we first adapted a protocol to obtain pear plantlets by *in vitro* culture of embryos. To determine *E. amylovora*'s ability to migrate from roots to upper parts of the plant, we first carried out direct root inoculation assays. Afterwards, we performed inoculations of plants with intact *versus* wounded roots (by transplanting) by means of soil irrigation with *E. amylovora* contaminated water. The stages of root infection, and the migration pathways of the pathogen within host tissues, were characterized using a GFP-tagged strain together with EFM and LSCM analysis. The main results of this work are:

i) The *in vitro* culture of pear embryos allows the germination of long-term stored pear seeds, which are difficult to germinate by standard procedures.

ii) The inoculation of *E. amylovora* into the roots of pear plantlets leads to the development of fire blight symptoms in the aerial part of the plant, far from the inoculation site.

iii) Water-borne infection of *P. communis* roots by *E. amylovora* is possible under laboratory conditions, leading to the development of characteristic fire blight symptoms (in stem, leaves and petioles) in 20 % and 52.5 % of the inoculated intact and transplanted plants, respectively.

iv) The stages of root colonization followed by *E. amylovora* are very similar to those described in well-known soil-borne plant pathogens. The entry of the pathogen into the radicular system occurs via wounds and/or cracks at the site of emergence of secondary roots. Bacterial cells multiply within the intercellular spaces of the cortex, reach the vascular system and migrates through xylem vessels to the aerial parts of the plant, where they are able to generate typical fire blight symptoms.

In **Chapter 4,** results from a study on the role of the sigma factor RpoS in *E. amylovora* responses to starvation, cross-protection, motility and the production of EPS, and virulence are summarized (Annex VI; Santander *et al.,* 2014a). Part of this work was performed at *Centro de Biotecnología y Genómica de Plantas* (CBGP, Spain). To this aim, an *E. amylovora* mutant in the *rpoS* gene together with the corresponding complemented strain were obtained and characterized along with the wild type strain. The results of this study are:

i) The *E. amylovora* mutant in the *rpoS* gene showed defective development of starvation responses, with a faster induction of the VBNC response, accompanied by a loss of viability and cell lysis not observed in the wild type strain.

ii) In *E. amylovora,* the alternative sigma factor RpoS is required for full stationary-phase cross protection against osmotic, acid and oxidative stresses, and it is essential for a heat-shock response; however, apparently is not required for acid shock resistance.

iii) RpoS enhances *E. amylovora* survival in non-host tissues, and a mutation in the *rpoS* gene leads to an increase in virulence in immature fruits, a significant increase in levan production, a slight decrease in amylovoran production, and a reduction of swimming motility.

Finally, in **Chapter 5** results of an unpublished study on the role of the *E. amylovora* catalases KatA and KatG during responses to starvation and plant-pathogen interactions are summarized (Annex VII; Santander *et al.,* 2016, unpublished). To this purpose, *E. amylovora* single and double catalase mutants were constructed, together with the corresponding complemented strains. The different assays carried out to characterize the functional roles of these catalases included: expression studies, *in vivo* and *in vitro* measurements of catalase activity, detection of catalase isozymes by zymography after native polyacrylamide gel electrophoresis (PAGE), identification of catalases by liquid chromatography – mass spectrometry (LC-MS/MS), determination of culturable, viable and total cell population kinetics, growth inhibition assays, survival experiments in non-host tissues and virulence assays. The results obtained in this work are:

i) *E. amylovora* catalase genes *katA* and *katG* are induced in the stationary and exponential growth phase, respectively. Both genes are also induced in the presence of H_2O_2 and in host tissues. The analysis of catalase activity revealed that KatA accounts for most of the catalase activity in *E. amylovora* cells. KatA and KatG were the only sources of catalase activity in *E. amylovora*.

ii) The detection of KatA, but not KatG can be achieved by standard zymography and LC_MS/MS after native PAGE.

iii) KatA, followed by KatG are required for full protection against H_2O_2 and menadione.

iv) The absence of catalase activity in the *E. amylovora* double catalase mutant induces a faster development of the starvation-induced VBNC response. This phenomenon is not observed in the single catalase mutants or the wild type strain, which behave similarly. The addition to catalase to solid media, or the complementation of mutants with a functional copy of catalase genes improves culturability of starved cells.

v) The *E. amylovora* catalases are required for full survival in non-host tissues. In susceptible plants, KatA and KatG play different roles during infections, with KatG probably being involved in the onset of symptoms and KatA in the progression of bacterial cells throughout host tissues.

The main conclusions of this Doctoral Thesis can be summarized as:

1) Temperate environmental temperatures (14ºC – 20ºC) favor the persistence of *E. amylovora* starved cells in a culturable state, with warmer or lower temperatures inducing a progressive entry into the VBNC state, and low temperatures, moreover, enhancing a greater maintenance of viability. Adaptations to starvation include morphological changes, dwarfing, modulation of motility and gene expression, with temperature further modulating changes in cell size.

2) *E. amylovora* starved cells are pathogenic. By contrast, the starvation- or chlorine-induction of the VBNC response leads to a loss of pathogenicity on immature fruits which can be reverted by inoculation into susceptible pear plantlets.

3) Under controlled conditions, *E. amylovora* remains pathogenic even at low incubation temperatures, with many virulence factors also being induced at temperatures below the optimal for growth.

4) *E. amylovora* survives in *C. capitata* for, at least, 28 days and insect conditions induce the entry of the pathogen into the VBNC state. Bacterial cells form aggregates and/or biofilm-like structures on the insect's surface (on the distal part of wings, the abdomen and the ovipositor), but can also persist within the insect for 8 days (internal parts of the mouth, the crop and the digestive gut).

5) The *E. amylovora* transmission by *C. capitata* occurs mainly through the contact of the medfly with plant surfaces, although female medflies can directly inoculate *E. amylovora* into plant material with the ovipositor. These results suggest that *C. capitata* can act as a potential vector for *E. amylovora.*

6) *E. amylovora* is able to invade pear plantlets through the roots, with normal fire blight symptoms being developed in the aerial part of the plant. Under controlled conditions, root infection of pear plantlets by soil-irrigation with *E. amylovora* contaminated water is feasible, with infection percentages increasing in plants with damaged roots. The *E. amylovora* root infection stages are very similar to those of well-known soil-borne plant pathogens.

7) The sigma factor RpoS contributes to the maintenance of culturability and viability in *E. amylovora* starved cells. It is also required for full stationary-phase cross protection against a variety of stresses which the pathogen can encounter in nature, and for proper survival of *E. amylovora* in non-host tissues. RpoS also modulates virulence and pathogenicity factors and virulence in immature fruits

8) The *E. amylovora* catalases KatA and KatG are differently regulated by growth phase, induced by exposure to H_2O_2 and host tissues and possess different specific catalase activities, indicating differentiated functional roles for each catalase. Both of them contribute to the maintenance of culturability in *E. amylovora* starved cells, delaying the entry into the VBNC state. Furthermore, they contribute to the pathogen's survival in non-host tissues, and are required for full virulence in immature fruits.

Overall, the research carried out in this Doctoral Thesis provides new knowledge of the mechanisms allowing the *E. amylovora* survival in environments outside the host. Results in this Thesis suggest that the starvation-survival and the VBNC responses are part of the *E. amylovora*'s life cycle, contributing to its persistence in the environment and probably determining the development of fire blight in field conditions. Furthermore, *E. amylovora*'s adaptations to survive outside the host, be transmitted

by different means, and remain pathogenic at a wide range of temperatures, something which has probably contributed to its spread to a variety of countries worldwide, in different climatic areas. Finally, the results in this Doctoral Thesis may provide clues to help improve the control of fire blight and/or preventive measures against it.

I. INTRODUCCIÓN

I. Introducción

1. El fuego bacteriano, la enfermedad

El fuego bacteriano de las rosáceas es una enfermedad devastadora que afecta a plantas frutales y ornamentales de gran importancia económica. El agente causal es la bacteria *Erwinia amylovora* (Burrill 1882) Winslow *et al*. (1920). Las hojas y brotes de las plantas afectadas por este microorganismo adquieren un aspecto ennegrecido, como quemadas por el fuego, característica a la que alude el nombre de la enfermedad. Desde el primer brote en Estados Unidos, descrito en el siglo XIX, el fuego bacteriano se ha extendido prácticamente por los cinco continentes, estableciéndose en muchos países con cultivos de frutales susceptibles, y originando cada año graves pérdidas económicas. La rápida diseminación del fuego bacteriano por todo el mundo, así como la dificultad para su control son un reflejo de la complejidad de la enfermedad y de los aspectos que aún se desconocen de la misma.

1.1. Rango de hospedadores

Se han identificado alrededor de 180 especies de 39 géneros de plantas susceptibles al fuego bacteriano, todas ellas pertenecientes a la familia *Rosaceae*. La mayoría de cepas de *E. amylovora* infectan plantas de la subfamilia *Spiraeoideae,* que engloba las antiguas subfamilias *Maloideae* y *Amigdaloideae.* En dicha subfamilia encontramos árboles frutales de gran importancia económica, como el peral (*Pyrus* communis) y el manzano (*Malus domestica*), con variedades muy sensibles al fuego bacteriano. Otros frutales afectados por esta enfermedad

son el membrillero (*Cydonia oblonga*), el níspero común o europeo (*Mespilus germanica*), el níspero japonés (*Eriobotrya japonica*) y el ciruelo japonés (*Prunus salicina*). La subfamilia *Spiraeoideae* también engloba un extenso número de especies de plantas ornamentales y silvestres, entre las que destacan por su susceptibilidad a *E. amylovora* las de los géneros *Cotoneaster, Crataegus, Pyracantha* y *Sorbus*. El fuego bacteriano también afecta a rosáceas de la subfamilia *Rosoideae*, que incluye otros frutales y/o plantas ornamentales de los géneros *Fragaria, Potentilla, Rosa* y *Rubus* (van der Zwet *et al.,* 2012). Curiosamente las cepas aisladas de *Rubus* (zarzamora, frambueso) son incapaces de producir síntomas en manzano (Ries & Otterbacher, 1977; Braun & Hildebrand, 2005), y tan solo algunas de ellas producen síntomas leves en pera inmadura (Powney *et al.,* 2011). Del mismo modo, las cepas de *E. amylovora* aisladas de rosáceas de la subfamilia *Spiraeoideae* raramente infectan plantas del género *Rubus* (Braun & Hildebrand, 2005; Rezzonico *et al.,* 2012). Aunque se han detectado diferencias entre estos dos grupos de cepas en la composición del exopolisacárido (EPS) amilovorano, las fuentes de carbono utilizadas y los perfiles de proteínas secretadas, ninguna de estas parece determinar la especificidad por el hospedador. Dicha especificidad parece estar determinada por una (Asselin *et al.,* 2013) o probablemente varias (Mann *et al.,* 2013) proteínas secretadas durante la interacción con el hospedador.

1.2. Sintomatología

Los síntomas de fuego bacteriano son generalmente fáciles de reconocer y, salvo excepciones, distinguibles de los causados por otras enfermedades de las rosáceas. No obstante, aunque constituyen un elemento importante para la realización

de prospecciones a gran escala, la presencia de síntomas, por sí solos, no es suficiente para el diagnóstico del fuego bacteriano en campo. Las infecciones causadas por *E. amylovora* pueden darse en plantas de cualquier edad, y los primeros síntomas suelen observarse en primavera, durante la floración y/o brotación (Palacio-Bielsa & Cambra, 2009; van der Zwet *et al.,* 2012). El síntoma más característico de la enfermedad y al que hace referencia su nombre consiste en una necrosis progresiva que confiere a los órganos afectados un aspecto similar al que observaríamos si hubiesen sido quemados por el fuego. Esta necrosis es muy llamativa en peral, donde adquiere una coloración muy oscura o negra intensa. Puede estar presente en flores, brotes, ramas, frutos, tronco, cuello de la raíz e incluso raíces (Palacio-Bielsa & Cambra, 2009; van der Zwet *et al.,* 2012) (Fig. 1a, b). En muchas ocasiones la necrosis de los brotes va acompañada de marchitez, que otorga a los brotes infectados la forma característica de cayado de pastor (Fig. 1c). Otro síntoma típico del fuego bacteriano es la presencia de exudados (Fig. 1d). Estos se componen de savia del hospedador, bacterias y sus EPS (Geider, 2000), y desempeñan un papel muy importante como fuente de inóculo para el desarrollo de infecciones secundarias durante el ciclo del fuego bacteriano.

Las plantas afectadas por *E. amylovora* también pueden presentar chancros, que son lesiones longitudinales en tejido lignificado, usualmente necróticas y que tienden a extenderse (Fig. 1e). Estos suelen originarse al finalizar el ciclo infeccioso y constituyen un reservorio en el cual las células de *E. amylovora* persisten durante el invierno, hasta que finaliza la parada vegetativa de las plantas hospedadoras y las condiciones se tornan favorables para la multiplicación del patógeno (van det Zwet *et al.,* 2012).

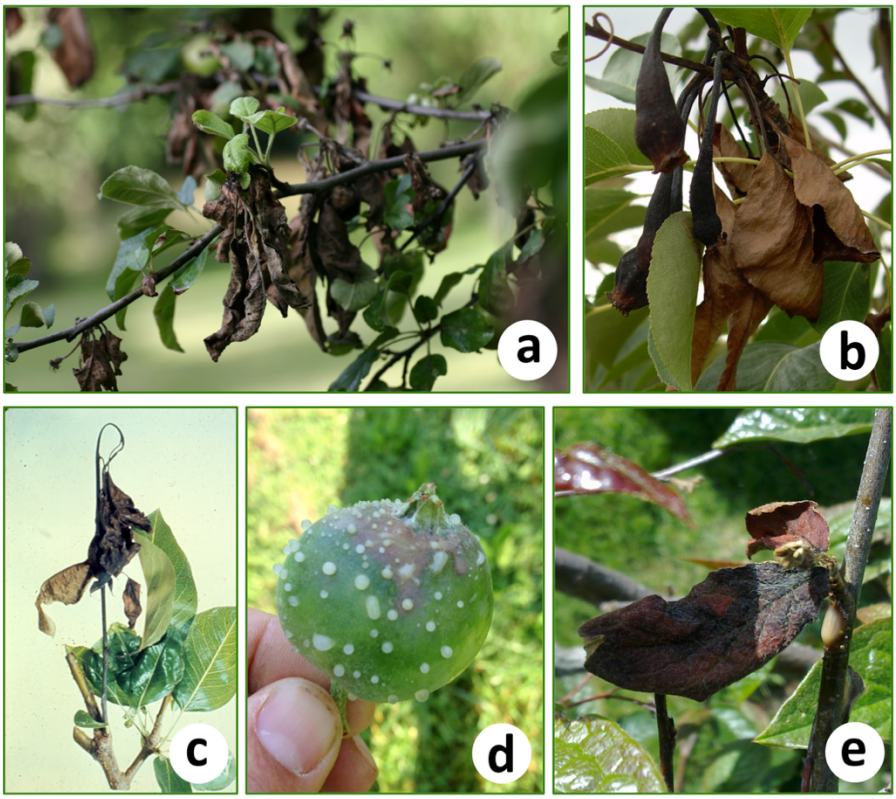


Fig. 1. Síntomas característicos de fuego bacteriano en distintos hospedadores. (a) rama de manzano con necrosis extendida a hojas y flores (autor: Sebastian Stabinger); **(b)** detalle de una inflorescencia de peral con necrosis (autor: Ninjatacoshell); **(c)** rama de peral con el aspecto característico de cayado de pastor causado por *E. amylovora* (autor: Scot Nelsen); **(d)** manzana inmadura con exudados (autor: Scot Nelsen); **(e)** detalle de rama de *Cotoneaster bullatus* con un chancro de fuego bacteriano activo y exudados (autor: Elintarviketurvallisuusvirasto Evira). Fotografías libres de derechos de autor de Wikimedia Commons, Flickr y Flickriver.

1.3. El ciclo de la enfermedad

El ciclo del fuego bacteriano está estrictamente ligado al desarrollo estacional de la planta hospedadora, y se resume en la Fig. 2.

Los hospedadores de *E. amylovora* sufren una parada vegetativa en invierno. Durante este periodo el patógeno sobrevive en el tejido del córtex de alrededor de los chancros formados en ramas y tronco durante la temporada anterior. En general, tan solo algunos de los chancros permiten la supervivencia de la bacteria durante el invierno, principalmente aquellos formados en los tejidos lignificados más viejos, y aquellos con límites entre tejido sano y enfermo poco definidos. Con la llegada de la primavera y la subida de la temperatura, las plantas reinician su actividad y se reactivan algunos chancros. Las bacterias comienzan a multiplicarse y afloran a la superficie en forma de exudados. Dichos exudados, compuestos de bacterias, EPS y savia, permiten a *E. amylovora* sobrevivir en condiciones de baja humedad durante periodos prolongados, y constituyen la principal fuente de inóculo primario para la diseminación de la bacteria a nuevos hospedadores o a partes sanas de la misma planta (van der Zwet *et al*., 2012).

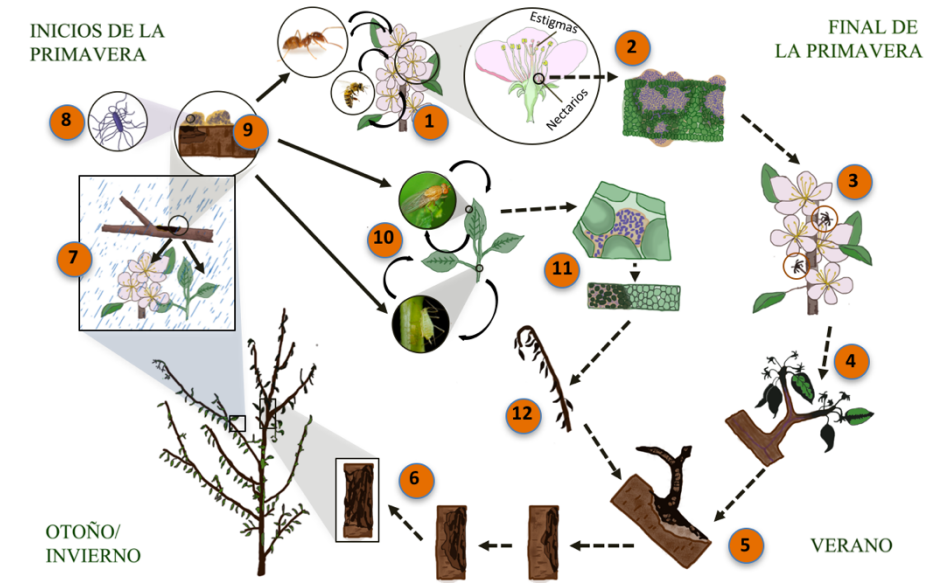
Una vez aparecen los exudados, estos son transportados por la lluvia o el viento a órganos susceptibles como las flores o los brotes. Otros vectores de la bacteria son moscas, hormigas e insectos perforadores, que son atraídos por los exudados y participan activamente en la distribución del patógeno por la planta (Thomson, 2000). Una vez en las flores la bacteria es capaz de crecer en los estigmas, los estambres y los nectarios, alcanzando niveles poblacionales elevados y posibilitando una muy efectiva dispersión del patógeno a nuevas flores mediada por insectos polinizadores como las abejas (Thomson, 2000;

Alexandrova *et al.,* 2002a). En estos órganos la entrada de *E. amylovora* al interior del hospedador ocurre de forma natural a través de los nectarios, que se encuentran situados en la base de la flor (Bubán *et al.,* 2003). La lluvia también juega aquí un papel importante, arrastrando las células epífitas de *E. amylovora* de estambres y estigmas a los nectarios (Pusey, 2000). En el caso de los brotes y las hojas el patógeno penetra en la planta principalmente a través de heridas, por lo que los insectos perforadores y las granizadas en primavera y verano favorecen este tipo de infección (Thomson, 2000). También se ha descrito la entrada a través de hidátodos y estomas. Tanto las flores como los brotes, una vez infectados, producen más exudados, que sirven de inóculo secundario para órganos susceptibles aún no infectados (flores, brotes, frutos inmaduros) (Thomson, 2000).

Una vez la bacteria penetra en los tejidos de la planta, esta es capaz de avanzar a través del apoplasto del parénquima cortical sin emplear enzimas degradadoras de la pared vegetal o los tejidos (Zamski *et al.,* 2007; Billing, 2011). Pese al número de estudios al respecto, actualmente aún no existe consenso entre los investigadores para explicar los mecanismos que posibilitan este proceso (Vanneste & Eden-Green, 2000; Billing, 2011; van der Zwet *et al.,* 2012). Una vez en el apoplasto, las células de *E. amylovora* promueven a su paso la muerte de las células de la planta hospedadora, originando cavidades en los tejidos. Este patógeno también puede migrar a otros órganos de la planta a través de los haces vasculares del xilema (Billing, 2011). La capacidad para diseminarse sistémicamente permite a *E. amylovora* alcanzar nuevos tejidos dentro del hospedador y producir síntomas de fuego bacteriano en brotes, frutos y flores sanos, más o menos alejados de órganos enfermos.

Con el descenso de las temperaturas, los brotes del hospedador dejan de crecer y se vuelven cada vez menos

susceptibles al fuego bacteriano. Al mismo tiempo, la progresión de la infección causada por *E. amylovora* se ralentiza y, en tejidos lignificados, produce chancros, donde la bacteria pasa el invierno hasta la temporada siguiente.



1. Insectos polinizadores transportan *E. amylovora* de flores infectadas a flores sanas.
2. Las bacterias penetran en los tejidos del hospedador a través de los nectarios, se multiplican y avanzan a través de los espacios intercelulares.
3. Las flores infectadas se necrosan y mueren.
4. La infección avanza a través de los tejidos internos del hospedador, incluyendo los haces vasculares del xilema, y afecta a otras flores, hojas, brotes y frutos inmaduros.
5. Se forman chancros en tallos, ramas y la base del tronco.
6. En algunos casos, los chancros pueden crecer hasta estrangular el órgano al que afectan, contribuyendo a la muerte de tejidos por encima del chancro.
7. Al llegar la primavera, las bacterias que han sobrevivido al invierno en los chancros producen exudados. Las lluvias y el viento favorecen la dispersión de estos exudados a tejidos sanos del mismo u otro hospedador.
8. Estos exudados contienen bacterias, EPS y savia.
9. Hormigas, moscas, e insectos succionadores y/o perforadores sienten atracción por los exudados, y participan en la dispersión de *E. amylovora* a tejidos sanos.
10. Los mismos insectos contribuyen, como las abejas, a las infecciones secundarias de hojas, brotes, tallos, frutos y flores.
11. La bacteria se multiplica en el interior de dichos tejidos, avanza por los espacios intercelulares.
12. La infección de brotes puede generar los "callados de pastor", un signo característico de las infecciones causadas por *E. amylovora.*

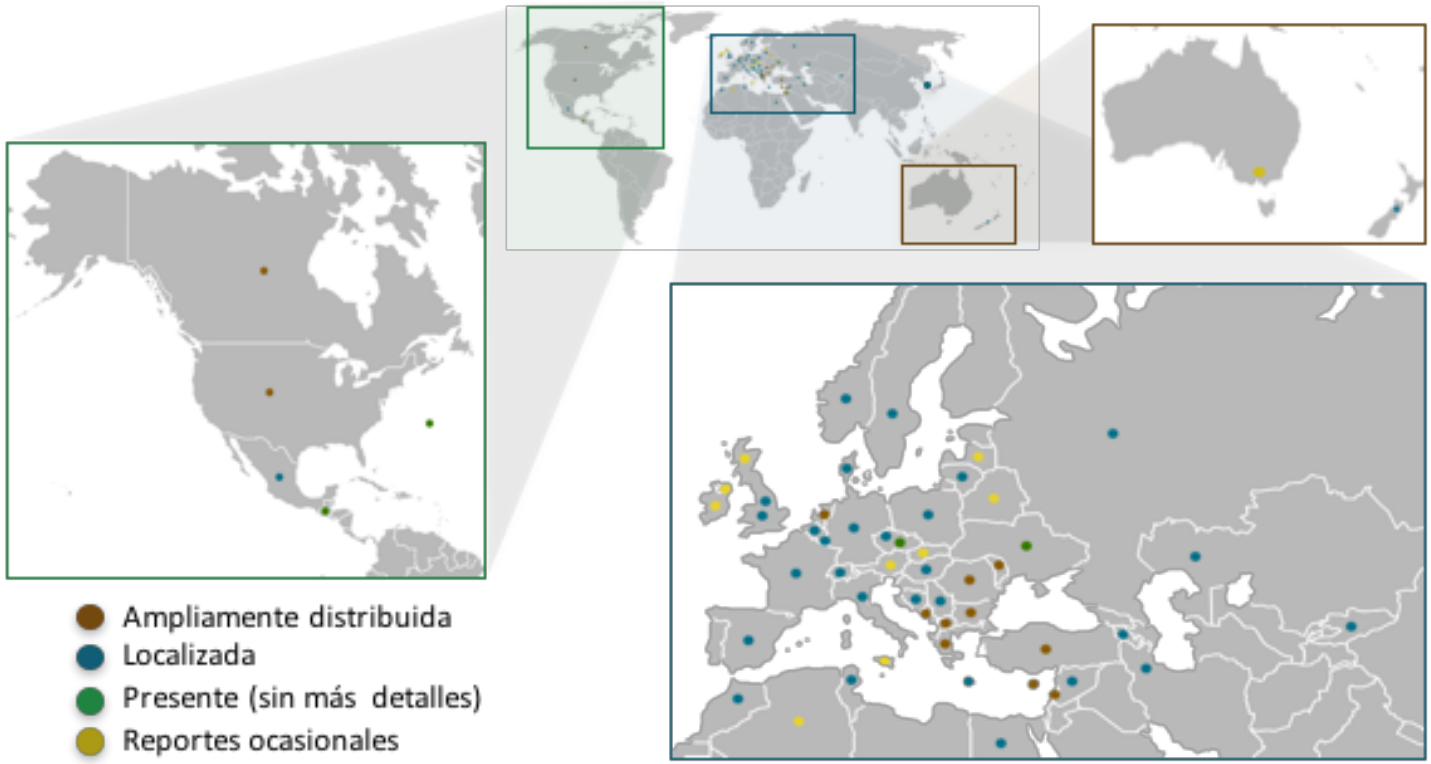
Fig. 2. **Ciclo del fuego bacteriano causado por *E. amylovora*.** Las flechas sólidas y discontinuas representan el movimiento de la bacteria y la dispersión de la enfermedad fuera y dentro del hospedador, respectivamente. Modificado de van der Zwet *et al* (2012).

1.4. Distribución geográfica

El origen del fuego bacteriano se sitúa en EEUU. Se cree que su agente causal, *E. amylovora,* se encontraba ya presente en hospedadores silvestres norteamericanos, como el espino albar o el serbal, y pasó a variedades sensibles de peral, manzano y membrillo cuando estas fueron introducidos por los colonos en Nueva Inglaterra a finales del siglo XVIII (Griffith *et al.,* 2003; van der Zwet *et al.,* 2012). Tras el primer caso de fuego bacteriano descrito en Nueva York (Burrill, 1883; Winslow *et al.,* 1920), esta enfermedad se diseminó por los alrededores, llegando a Canadá, otros estados de la zona atlántica y también a la costa del Pacífico. En 1919 se identificaron casos de fuego bacteriano en Nueva Zelanda, y pocos años después en el Reino Unido (1957), Egipto (1964) o los Países Bajos (1966). Desde entonces se ha diseminado por más de 40 países (EPPO, 2013) (Fig. 3), incluyendo la mayoría de países del centro y norte de Europa, el área del Mediterráneo y Oriente Medio (EPPO, 2016), y recientemente, Korea (Park *et al.,* 2016).

Tanto en España como en el resto de países europeos, *E. amylovora* recibe el trato de organismo nocivo de cuarentena, y existe una legislación específica sobre las medidas para la prevención de la introducción y/o diseminación del patógeno en países o zonas, dentro de los mismos, donde no está presente (RD 58/2005). En España, además, existe un programa específico para la erradicación y control del fuego bacteriano de las rosáceas (RD 1201/1999).

Fig. 3. Distribución mundial de *E. amylovora* (basado en Taylor & Hale (1998) y las webs del CABI (del inglés, *Centre for Centre for Agriculture and Bioscience International,* 2016) y EPPO (2016).



1.4.1. Estado actual en España

A pesar de la detección de focos de fuego bacteriano desde 1995, España ha sido considerada durante mucho tiempo Zona Protegida (ZP) de la enfermedad por la Unión Europea (Real Decreto 2071/1993), debido a la adopción de medidas de erradicación y de reconversión varietal de las zonas afectadas, que han evitado numerosas pérdidas tanto en la producción como económicas (Palacio-Bielsa & Cambra, 2009).

También se han establecido medidas para evitar la introducción y propagación de este patógeno de cuarentena (Directiva Europea 2000/29 y su modificación 2003/116). No obstante, pese a los esfuerzos realizados, el fuego bacteriano ha acabado llegando a la mayoría de Comunidades Autónomas del estado español, estableciéndose en muchas de ellas, como es el caso de Castilla y León, Extremadura, Castilla la Mancha, la Rioja, Navarra, País Vasco (Guipúzcoa), Aragón, Murcia, Comunidad Valenciana (comarcas de L'alt Vinalopó y el Vinalopó Mitjà en la provincia de Alicante y los municipios de Turís y Alborache en Valencia) y, más recientemente, Andalucía y Cataluña (comarcas de Garrigues, Noguera, Segrià, Pla d'Urgell i Urgell en la provincia de Lleida). Por este motivo, muchas de estas zonas han solicitado su salida de la Zona Protegida y, como consecuencia, sus acciones han cambiado, dirigiéndose ahora a la convivencia con la enfermedad. El resto de Comunidades Autónomas, parcial o totalmente, continúan manteniendo el estatus de Zona Protegida ante el fuego bacteriano (Fig. 4).

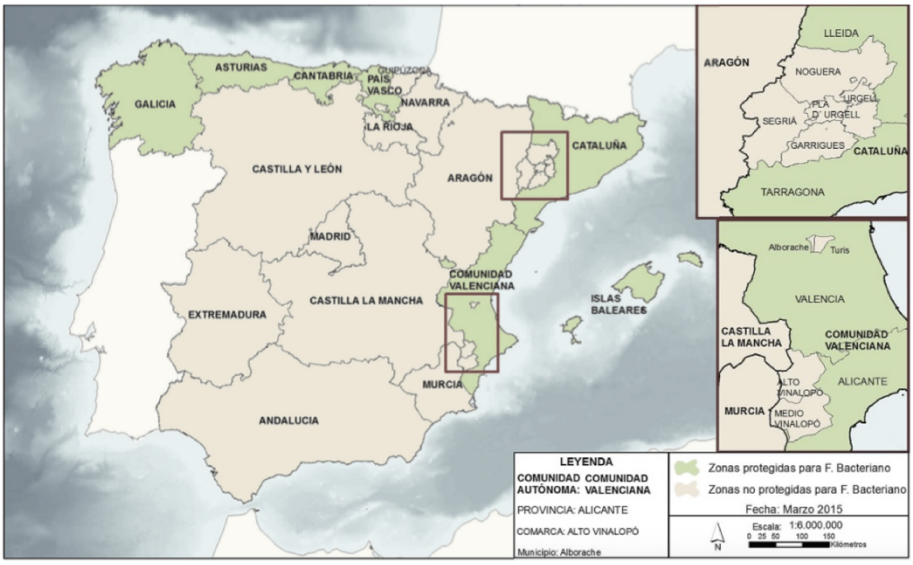


Fig. 4. Distribución de *E. amylovora* en España. En verde y marrón se indican las zonas protegidas y no protegidas de fuego bacteriano, respectivamente. Información actualizada según el Reglamento (CE) nº690/2008 y Disposición adicional segunda del RD 1201/1999 (web del Ministerio de Agricultura, Alimentación y Medio Ambiente - MAGRAMA, 2016).

1.5. Importancia económica e impacto sociocultural

Los efectos del fuego bacteriano sobre peral, manzano, níspero y algunas ornamentales son devastadores, y representan cuantiosas pérdidas económicas cada año en numerosos países. Tan solo en EEUU, las pérdidas anuales debidas a esta enfermedad rondan los 100 millones de dólares (Norelli *et al.,* 2003). En el caso de los frutales, cuando la enfermedad se manifiesta no sólo se pierde la producción de ese año, si no que ramas e incluso árboles enteros quedan afectados por completo, lo que conlleva pérdidas más graves para las plantaciones y los viveros. Además, los países en los cuales la enfermedad está

presente no solo ven reducidos el número de árboles y/o la producción anual, sino que, una vez sustituidos los ejemplares enfermos, también sufren pérdidas económicas durante los años posteriores, mientras los nuevos árboles alcanzan los niveles máximos de producción (web del CABI, 2016).

Dado que en la Unión Europea *E. amylovora* está considerada un organismo de cuarentena, existe una legislación específica para evitar su introducción y diseminación, lo que supone pérdidas económicas adicionales para los países en los que el patógeno está presente, relacionadas con las limitaciones en el comercio internacional de plantas susceptibles (Palacio-Bielsa & Cambra, 2009; van der Zwet *et al.,* 2012).

Además de las consecuencias económicas, el fuego bacteriano de las rosáceas también puede causar un importante impacto sociocultural, haciendo del cultivo tradicional de frutales, seña de la identidad de determinadas regiones, algo tremendamente difícil y/o inviable económicamente. Este ha sido el caso de la región de Emilia-Romagna (Italia), con cientos de años de tradición en el cultivo del peral y seriamente afectada por el fuego bacteriano; o de los emblemáticos perales y manzanos integrados en los prados y que forman parte de los paisajes característicos del sur de Alemania, difíciles de proteger de la enfermedad y cuya desaparición alteraría la identidad de la región, lo que repercutiría también sobre el turismo (CABI, 2016).

1.6. Epidemiología

La incidencia y la severidad de los síntomas de fuego bacteriano en una región pueden ser desde fulminantes y con consecuencias nefastas para la producción y para las plantas afectadas, a pasar prácticamente inadvertidas. Tanto el número

de plantas afectadas, como la magnitud con la que se manifiestan los síntomas dependen, en gran medida, de la interacción entre tres variables: la sensibilidad y receptibilidad de la planta, la abundancia de inóculo u otros factores asociados al patógeno como la virulencia de la cepa, y parámetros ambientales como el clima o la presencia de plagas y/o vectores de *E. amylovora* (Thomson, 2000; Palacio-Bielsa & Cambra, 2009; van der Zwet *et al.,* 2012) (Fig. 5). Por este motivo, la gravedad de la enfermedad varía mucho de unos lugares a otros y según los años.

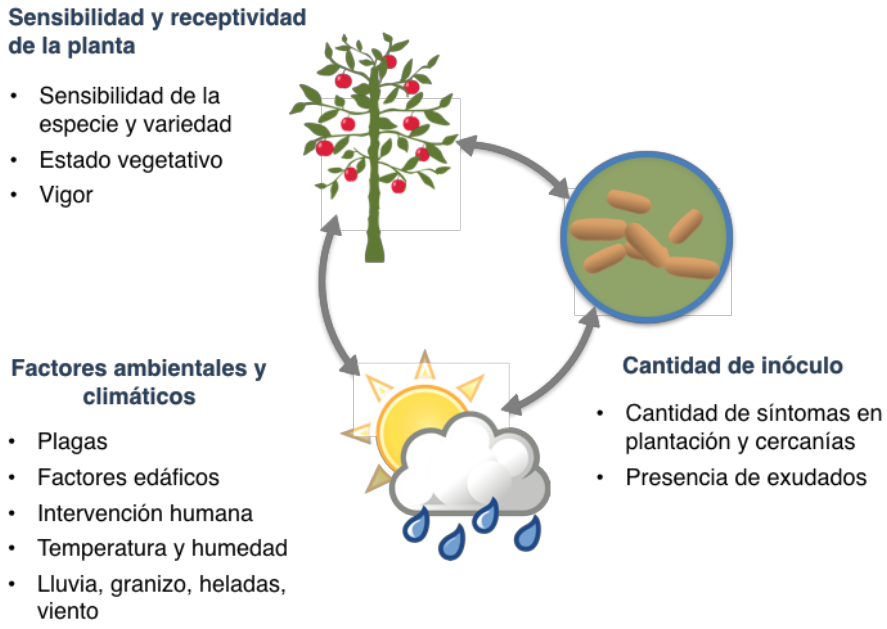


Fig. 5. Factores condicionantes del fuego bacteriano.

1.6.1. Factores condicionantes asociados al hospedador

No todas las especies de rosáceas son sensibles al fuego bacteriano, y entre las que lo son, existen también diferencias de sensibilidad varietal. Por ejemplo, entre los frutales de pepita, el peral es más susceptible a la enfermedad que el manzano (Maroofi & Mostafavi, 1996), pero entre ambas especies de rosáceas existen variedades poco sensibles (*P. communis* var Ercolini o *M. x domestica* var Golden Delicious), muy sensibles (*P. communis* var Passe Crassane o *M. x domestica* var Reina de Reinetas) y otras de sensibilidad intermedia (*P. communis* var Limonera o *M. x domestica* var Fuji) (Palacio-Bielsa & Cambra, 2009). Lo mismo ocurre en el caso de plantas ornamentales y silvestres. La sensibilidad varietal viene determinada por el genotipo de la planta, aunque también está influenciada por factores ambientales y de cultivo. Por otro lado, independientemente de la sensibilidad de la especie y/o variedad, la receptividad de la planta hospedadora a *E. amylovora* también varía en función de su estado nutricional y fenológico. Así pues, la susceptibilidad al patógeno aumenta durante los periodos de floración y/o de crecimiento vegetativo intenso, y es reducida o nula durante la parada vegetativa en invierno (Thomson, 2000; van der Zwet *et al.,* 2012).

1.6.2. Factores condicionantes asociados al patógeno

No todas las cepas de *E. amylovora* capaces de multiplicarse en una especie o variedad vegetal son igual de virulentas o agresivas, es decir, no todas requieren de la misma dosis de inóculo para causar infección, o causan síntomas con la misma intensidad (Momol & Aldwinckle, 2000; Cabrefiga & Montesinos, 2005). Las cepas de *E. amylovora* que requieran

dosis infectivas elevadas tendrán menores probabilidades de infectar con éxito nuevos hospedadores, o lo harán más lentamente que las cepas más virulentas. Del mismo modo, las cepas más agresivas producirán síntomas más severos y de forma más rápida, lo que facilitará la propagación de la enfermedad en campos o viveros. En relación a esto, la cantidad de inóculo presente en una plantación o en áreas circundantes también influye en la severidad de las epidemias de fuego bacteriano. La enfermedad se manifestará de forma más violenta si en los alrededores existe un número elevado de plantas con síntomas, especialmente exudados. Por lo tanto, todas las causas que favorezcan el aumento de las fuentes de inóculo bacteriano incrementarán también la intensidad de las infecciones causadas por *E. amylovora*.

1.6.3. Factores condicionantes asociados al ambiente

Existen factores ambientales que influyen sobre el patógeno y la planta hospedadora, lo que repercute sobre la epidemiología del fuego bacteriano (Palacio-Bielsa & Cambra, 2009; van der Zwet *et al.,* 2012):

Factores edáficos. El tipo de suelo, así como su contenido en agua y nutrientes puede influir tanto en la receptividad del hospedador a *E. amylovora* como en la severidad de los síntomas del fuego bacteriano. Por ejemplo, los suelos de tipo arcilloso, con mal drenaje y fertilizantes en exceso, al igual que los desequilibrios nutricionales o los abonados nitrogenados, facilitan la predisposición al desarrollo del fuego bacteriano.

Factores climáticos. Los factores ambientales más importantes para el desarrollo del fuego bacteriano son aquellos que favorecen el crecimiento del patógeno. Temperaturas entre

22ºC y 25ºC y condiciones de humedad relativa entre el 90% y el 95% son óptimas para el desarrollo de *E. amylovora* sobre el hospedador. En general, los límites de temperatura para el desarrollo de brotes fuego bacteriano en campo en periodos de floración son 18ºC como mínimo y 30ºC como máximo, y la humedad relativa debe ser como mínimo del 70%. El rocío o la presencia de lluvias también facilitan las infecciones causadas por el patógeno. Condiciones climáticas como granizo o viento, que dañan hojas, frutos, brotes y ramas en las plantaciones, pueden incrementar la virulencia de los ataques por *E. amylovora*, que puede utilizar las heridas frescas como vía de entrada a la planta hospedadora. Del mismo modo, la lluvia y el viento pueden favorecer la dispersión de exudados bacterianos, desde tejidos infectados a tejidos sanos en la misma planta, o a tejidos sanos en nuevas plantas. Las heladas, sin embargo, pueden reducir el inóculo primario en tal proporción que no se produzcan infecciones.

En relación a esto, existen modelos matemáticos de predicción de riesgo principalmente basados en datos climáticos y fenológicos (Billing, 2000; van der Zwet *et al.,* 2012). Algunos de los modelos de predicción más conocidos son el Maryblyt (Steiner, 1990) y el Cougarblight (Smith, 1999). Dichos modelos se emplean para estimar el riesgo de infección en regiones adyacentes a zonas afectadas, o los periodos más favorables para la infección dentro de una región donde *E. amylovora* ya se ha establecido (van der Zwet et al., 2012). También se utilizan para optimizar la aplicación de tratamientos en el tiempo, reduciendo costes e incrementando la eficacia de los mismos (van der Zwet et al., 2012). En general, los valores de los parámetros en los que se sustentan dichos modelos pueden variar en función de factores ambientales, prácticas de los agricultores, etc., lo que muchas veces da lugar a fallos en las predicciones de fuego bacteriano, o invalida el uso de dichos

modelos en zonas con condiciones climáticas particulares (van der Zwet *et al.,* 2012; Shtienberg *et al.,* 2015).

Otros factores ambientales. Hay otros factores que pueden modular la incidencia y la virulencia de las epidemias de fuego bacteriano, siendo de especial importancia aquellos que favorecen la diseminación de *E. amylovora* a cortas, medias y largas distancias. El ser humano puede transportar y diseminar el patógeno de plantas infectadas a plantas sanas, a través de herramientas de poda, útiles de cultivo, manos, ropa, etc. De hecho, es frecuente encontrar fuego bacteriano en las distintas parcelas de un mismo agricultor. Los vectores más importantes para el transporte de *E. amylovora* a corta y media distancia son los insectos. Se han asociado hasta 77 géneros distintos con la diseminación del patógeno, siendo de especial relevancia las abejas (Alexandrova *et al.,* 2002a; Sabatini *et al.,* 2006). *E. amylovora* puede sobrevivir en abejas contaminadas durante 48 h, y durante 72 h en polen, lo que convierte a estos insectos en eficientes vectores capaces de dispersar el patógeno en áreas de cultivo de gran tamaño (hasta 7 Km^2) en breves periodos de tiempo (Sabatini *et al.,* 2006). Otros insectos como moscas u hormigas, fuertemente atraídos por los exudados procedentes de chancros u órganos afectados (Hildebrand *et al.,* 2000), también se han relacionado con el transporte de *E. amylovora* a flores sanas (van der Zwet *et al.,* 2012). Finalmente, insectos como los áfidos y otros perforadores/succionadores, tienen un papel muy importante en la inoculación de células de *E. amylovora* en plantas sanas a través del aparato bucal (Hildebrand *et al.,* 2000).

1.7. Control

En la Unión Europea *E. amylovora* es un organismo para el cual existe legislación fitosanitaria específica, cuyo fin es evitar

su introducción y propagación. Dicha legislación incluye el establecimiento de cuarentenas, inspecciones, muestreos, y/o erradicación de ciertas plantas susceptibles a la enfermedad. En el caso de España, las medidas de control frente a *E. amylovora* adquirieron carácter obligatorio tras la introducción del patógeno en el país, y se recogen en la normativa específica de los Reales Decretos RD 58/2005, RD1201/1999 y RD 1512/2005, complementados por la Ley de Sanidad Vegetal (Montesinos *et al.,* 2009).

El control oficial se realiza en los viveros productores de plantas hospedadoras, y se hace una distinción entre Zonas Protegidas (países o regiones libres de fuego bacteriano) y el resto del territorio. La circulación de material vegetal por países miembros de la Unión Europea requiere de un Pasaporte Fitosanitario, que asegura que el material vegetal ha sido producido por entidades inscritas en el Registro Oficial y sometidas a medidas de control adecuadas. La importación de material vegetal a una Zona Protegida solo puede realizarse si dicho material posee un pasaporte tipo ZP, que certifica la producción en otra Zona Protegida, o en su defecto, en una Zona Tampón oficial (una zona libre de fuego bacteriano dentro de un área que no lo está) (Montesinos *et al.,* 2009; MAGRAMA, 2016).

Una vez establecido en una región, el control del fuego bacteriano se realiza mediante aproximaciones integradas que incluyen la reducción de la cantidad de inóculo, el uso de barreras para evitar el establecimiento de *E. amylovora* sobre las plantas y, finalmente, la sustitución de las plantas hospedadoras por otras de variedades menos susceptibles a la infección por este patógeno (Norelli *et al.,* 2003; van der Zwet *et al.,* 2012). No obstante, es importante señalar que no existen métodos de control realmente eficaces, por lo que las estrategias empleadas actualmente para combatir la enfermedad son, más bien,

medidas de convivencia para evitar o reducir sus efectos (Montesinos *et al.,* 2009). Los métodos preventivos y/o de control más empleados son:

Control mecánico. Consiste en la reducción de las fuentes de inóculo primarias o secundarias mediante la eliminación de los chancros durante la poda de invierno, de ramas y/o brotes con síntomas, o incluso de árboles enteros si se encuentran seriamente afectados por la enfermedad (Norelli *et al.,* 2009; van der Zwet *et al.,* 2012).

Control químico. Se aplica para evitar la colonización de la planta hospedadora por el patógeno (no existen tratamientos curativos de la enfermedad). Los productos más utilizados tienen efecto microbicida (compuestos cúpricos, antibióticos) o ayudan a combatir el fuego bacteriano de forma indirecta (fosetil-Al, prohexadiona de calcio, benzotiadiazol y harpinas) estimulando los sistemas de defensa de la planta hospedadora. Suelen emplearse combinados. Cabe destacar que el uso de antibióticos en la Unión Europea está prohibido (Montesinos *et al.,* 2009; van der Zwet*et al.,* 2012).

Control biológico. La posibilidad de controlar el fuego bacteriano mediante la acción de microorganismos se ha estado investigando desde hace más de 40 años. No obstante, la aparición de cepas de *E. amylovora* resistentes a antibióticos, así como la prohibición de los mismos en la mayoría de países de la Unión Europea ha impulsado en las últimas décadas la búsqueda de estrategias de control alternativas a los antibióticos (van der Zwet *et al.,* 2012). En la actualidad existen diversas marcas de biopesticidas basadas en agentes de biocontrol, como BloomTime Biological, cuyo componente activo es la cepa de *Pantoea agglomerans* E325; BlightBan, cuyo componente activo es la cepa de *Pseudomonas fluorescens* A506; y Blossom-

Protect, cuyo componente activo es la levadura *Aureobasidium pullulans*. En todos los casos, su aplicación es más efectiva si se combinan con otras estrategias de control de fuego bacteriano.

Uso de portainjertos resistentes al fuego bacteriano. No previenen la infección de las variedades injertadas, pero sí evitan o reducen las pérdidas asociadas a la infección de portainjerto y raíces, que suelen conducir a la pérdida del árbol entero (Norelli *et al.,* 2003; van der Zwet *et al.,* 2009).

Otras medidas preventivas y de control. Para evitar las contaminaciones de origen antrópico con *E. amylovora* se recomienda a los agricultores la desinfección de las herramientas de poda con lejía o etanol. Otras prácticas habituales para combatir el fuego bacteriano son la limitación del abonado nitrogenado para evitar el vigor excesivo en los árboles, controlar las refloraciones de otoño o evitar la poda en verde (Montesinos *et al.,* 2009; van der Zwet *et al.,* 2012). Por otra parte, dada la capacidad de supervivencia de *E. amylovora* en abejas y polen (Alexandrova, 2002b; Sabatini *et al.,* 2006), el transporte de colmenas en la Unión Europea está restringido mediante directivas fitosantitarias como la 2000/29/EC, para limitar diseminación a larga distancia de la enfermedad.

2. *Erwinia amylovora*, el patógeno

E. amylovora fue el primer patógeno bacteriano asociado a una enfermedad de plantas (Burrill, 1883) y también la primera especie bacteriana cuya diseminación se asoció a insectos (Baker, 1971). Durante décadas se ha hecho un gran esfuerzo por dilucidar los mecanismos de patogénesis en *E. amylovora,* así como las estrategias de defensa de las plantas frente a este microorganismo. No obstante, pese a la gran cantidad de trabajos realizados al respecto, muchos de los aspectos relacionados con la interacción de *E. amylovora* con sus hospedadores siguen siendo poco conocidos.

2.1. Taxonomía

El género *Erwinia* (clase *Gammaproteobacteria*, orden *Enterobacteriales,* familia *Enterobacteriaceae*), cuyo nombre hace honor al fitopatólogo Erwin F. Smith, se empleó inicialmente para agrupar a las enterobacterias patógenas de plantas y/o asociadas a estas. Dicha clasificación inicial, basada principalmente en el tipo de síntomas causados en sus hospedadores y un pequeño número de rasgos fenotípicos (Dye, 1968, 1969a, b, c; Komagata *et al*., 1968), subdividía a las erwinias en tres grupos principales: amylovora (agrupaba bacterias causantes de necrosis y marchitez), carotovora (patógenos responsables de podredumbres blandas) y herbícola (bacterias saprófitas, usualmente con pigmentación amarilla), así como un cuarto grupo adicional para clasificar algunas erwinias con perfiles bioquímicos atípicos. No obstante, reclasificaciones filogenéticas posteriores, basadas en aproximaciones polifásicas (análisis microbiológicos, perfiles bioquímicos, y de ácidos grasos, hibridación ADN-ADN y secuenciación de ácidos

nucleicos) condujeron a la reasignación de distintas especies y la creación de los géneros *Brenneria, Pantoea, Pectobacterium, Dickeya* y *Lonsdalea* (Gavini *et al.,* 1989; Hauben *et al*., 1998; Samson *et al*., 2005; Brady *et al*., 2010, 2012).

Según indican algunos estudios (Yarza *et al.,* 2010; Smits *et al.,* 2011), los géneros *Erwinia* y *Pantoea* tendrían un ancestro común, mientras que los géneros *Brenneria, Lonsdalea, Pectobacterium* y *Dickeya,* junto con otros géneros de enterobacterias, provendrían de linajes diferentes al de *Pantoea/Erwinia.*

Actualmente, estudios de MLSA (del inglés *multi-locus sequence analysis*) indican el origen monofilético del género *Erwinia,* que se compone de 18 especies (Rezzonico *et al*., 2016). La mayoría de estas son fitopatógenas y/o epífitas (*E. amylovora E. billingiae, E. gerundensis, E. mallotivora, E. oleae, E. papayae, E. persicina, E. piriflorinigrans, E. psidii, E. pyrifoliae, E. rhapontici, E. tasmaniensis, E. toletana, E. tracheiphila, E. uzenensis*) (Octavia & Lan, 2014; Rezzonico *et al*., 2016). De las tres especies restantes, *E. aphidicola* se detectó inicialmente en el tracto digestivo del áfido del guisante *Acyrthosiphon pisum* (Harada *et al.,* 1997), aunque más tarde se identificó como el agente causal de enfermedades con diferente sintomatología en *Pisum sativum* y en *Phaseolus vulgaris* (Santos *et al.,* 2009). Las especies *E. iniecta* y *E. typography,* por el momento, únicamente han sido aisladas del áfido ruso del trigo *Diuraphis noxia* (Campillo *et al*., 2015) y del escarabajo *Ips typographus* (Skrodenytee-Arbaciauskiene *et al*., 2012), respectivamente.

Tabla 1. Características fenotípicas que permiten diferenciar las distintas especies dentro del género *Erwinia*

	E. amylovora	*E. aphidicola*	*E. billingiae*	*E. gerundiensis*	*E. iniecta*	*E. mallotivora*	*E. oleae*	*E. papayae*	*E. persicina*	*E. piriflorinigrans*	*E. psidii*	*E. pyrifoliae*	*E. rhapontici*	*E. tasmaniensis*	*E. toletana*	*E. trachelphila*	*E. typhographi*	*E. uzenensis*
Glicerol	-	+	+	+	+	-	-	-	+	-	+	+	+	+	+	+	-	-
L-Arabinosa	+	+	+	+	ND	-	+	+	+	+	+	+	+	+	+	+	+	+
D-Manosa	-	+	+	+	+	+	+	+	+	-	+	-	+	-	+	+	+	-
L-Ramnosa	-	+	+	+	ND	-	+	-	+	-	+	-	+	-	-	-	-	-
Inositol	+	+	+	+	ND	-	-	-	+	-	-	+	+	+	+	-	-	+
D-Manitol	+	+	+	+	ND	+	+	+	+	+	+	+	+	+	+	-	+	+
Maltosa	-	+	+	+	+	-	-	-	+	-	-	-	+	-	+	-	-	-
Melibiosa	-	+	+	+	+	-	-	-	+	-	-	-	+	-	+	+	-	-
Sacarosa	+	+	-	+	+	+	-	+	+	+	+	+	+	+	-	+	-	+
Rafinosa	-	+	-	+	+	-	-	-	+	-	-	-	+	-	-	-	-	-
Xilitol	-	+	-	-	+	-	-	-	-	-	-	-	+	+	-	-	-	-
D-Arabinol	-	-	+	-	+	-	+	-	-	-	-	-	-	-	+	+	-	-
Gluconato potásico	-	+	-	+	ND	-	+	+	-	-	-	-	-	-	-	-	-	-
2-Cetogluconato potásico	-	+	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-

Adaptada de Rezzonico *et al* (2016)
+, positivo; -, negativo; ND, no determinado.

2.2. Características fenotípicas

Las células de *E. amylovora* son bacilares, con un tamaño entre 0,3 y 1-3 μm (Fig. 6) y móviles, con flagelación peritrica (Fig. 6). Presentan una cápsula de exopolisacáridos que cumple con importantes funciones tanto en el proceso de patogénesis (Geider, 2000; van der Zwet *et al*., 2012) como en la formación de biopelículas (Koczan *et al.,* 2009; 2011) y/o la supervivencia dentro y/o fuera del hospedador (Geider, 2000; Venisse *et al.,* 2003; Ordax *et al.,* 2010; Roach *et al.,* 2014). Tanto el tamaño, como la movilidad y la presencia de una cápsula más o menos abundante depende en gran medida de las condiciones de incubación y/o componentes del medio de cultivo (Raymundo, 1980; 1981; Eastgate, 2000; Geider, 2000; van der Zwet *et al.,* 2012).

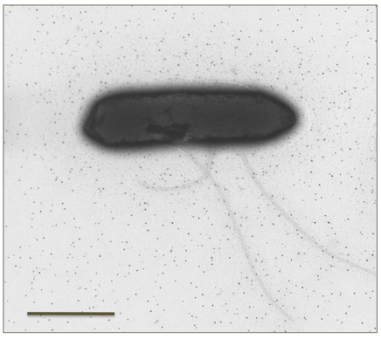


Fig. 6. Imagen de microscopía electrónica de transmisión de una célula flagelada de la cepa de *E. amylovora* ATCC 49946 teñida con ácido fosfotúngstico. La barra horizontal representa la escala, y equivale a 1 μm.

E. amylovora es una bacteria considerada mesófila, con una temperatura óptima de crecimiento de alrededor de 28ºC, aunque puede crecer a 35ºC - 37ºC. No obstante, dicho patógeno es capaz de multiplicarse a temperaturas alrededor de 3ºC - 4ºC (van der Zwet *et al*., 2012). Por lo tanto, según la clasificación de los microorganismos en base a sus rangos de crecimiento, *E. amylovora* entraría dentro de la definición de bacteria

psicrotolerante (crece a bajas temperaturas y las temperaturas óptima y máxima se encuentran por encima de los 15ºC y 20ºC, respectivamente) (Moyer & Morita, 2007). En cuanto al pH, el óptimo de crecimiento está en torno a 6 – 7,5, el mínimo entre 4,0 – 5,9 y el máximo entre 7,6 y 8,8 (van der Zwet *et al*., 2012).

Como otras enterobacterias, *E. amylovora* es un microorganismo anaerobio facultativo, aunque su crecimiento en condiciones de anaerobiosis es débil. La utilización de glucosa, ya sea en presencia o en ausencia de oxígeno produce ácido, pero no gas (Holt *et al.,* 1994). La mayoría de cepas de la especie también producen ácido a partir de ribosa y trehalosa y, en muchos casos, también a partir de sorbitol y arabinosa (Paulin, 2000; van der Zwet *et al.,* 2012).

Las cepas de *E. amylovora* presentan una elevada homogeneidad bioquímica (Paulin, 2000; van der Zwet *et al.,* 2012). Como fuente de carbono son capaces de utilizar los compuestos orgánicos citrato, lactato y formato, pero no el tartrato, el galacturonato o el malonato. Como fuente de nitrógeno *E. amylovora* puede utilizar distintos aminoácidos, siendo de especial relevancia el aspartato, que es uno de los aminoácidos más abundantes en brotes de manzano (uno de sus principales hospedadores) (Lewis & Tolbert, 1964). Al contrario que otras bacterias de la familia *Enterobacteriaceae*, *E. amylovora* es incapaz de reducir nitratos a nitritos (Paulin, 2000), y posee una auxotrofía para el ácido nicotínico, por lo que resulta imprescindible suplementar los medios de cultivo mínimos con esta vitamina para posibilitar su crecimiento (Starr & Mandel, 1950).

El agente causal del fuego bacteriano posee diferentes determinantes antigénicos, entre los que destacan: el lipopolisacárido (LPS) liso o rugoso (con o sin cadena lateral); el

antígeno termoestable GAI, de probable naturaleza polisacarídica, aunque distinta del LPS; el antígeno TV, que constituye una parte de los EPS capsulares y está presente, tan solo, en cepas virulentas de *E. amylovora*; y, finalmente, el antígeno GAJ, detectado en el material mucoso extracelular procedente de cultivos puros (van der Zwet *et al.,* 2012). Cabe destacar la elevada homogeneidad serológica entre cepas de *E. amylovoroa*, lo cual ha permitido la obtención de anticuerpos monoclonales específicos para su detección (Lin *et al.,* 1987; Gorris *et al.,* 1996a, b).

2.3. Características genómicas y genotípicas

2.3.1. Variabilidad intraespecífica

Aunque la especie *E. amylovora* es bastante homogénea a nivel bioquímico y fisiológico, encontramos diferencias importantes a nivel de cepa en cuanto a virulencia, movilidad, secreción de EPS, tipo de hospedador susceptible (*Rubus* o subfamilia *Spiraeoideae*), e incluso resistencia a antibióticos (principalmente estreptomicina), por lo que cabría esperar cierta variabilidad genética intraespecífica (Puławska *et al.,* 2006; Lee *et al.,* 2010; Puławska & Sobiczewski, 2012). No obstante, el nivel de heterogeneidad genética entre las cepas de *E. amylovora* es paradójicamente bajo (Mann *et al.,* 2013), motivo por el cual muchas de las técnicas habitualmente empleadas para la caracterización genética de distintas cepas son poco útiles en el caso de este patógeno.

Estrategias *a priori* interesantes como el análisis de genes de patogenicidad y/o de mantenimiento celular, el análisis por PCR de secuencias repetitivas palindrómicas (Rep-PCR), el

ribotipado, o los RAPDs (del inglés *Random Amplified Polymorphic DNA*), han permitido únicamente agrupar las cepas en función del tipo de planta hospedadora a la que afectan (*Rubus* versus plantas de la subfamilia *Spiraeoideae*) (Kim *et al*., 1996; Waleron *et al*., 2002; Giorgi & Scortichini, 2005; Puławska *et al*., 2006), o identificar pequeñas diferencias entre cepas originarias de Norte América (lugar donde se describió por primera vez el fuego bacteriano). No obstante, estas técnicas se han mostrado poco (McManus & Jones, 1995; Gürtler & Stanisich, 1996; Momol *et al*., 1997; Jeng *et al*., 1999) o nada resolutivas (Jock & Geider 2004; Giorgi & Scortichini, 2005) en la discriminación entre cepas de origen europeo.

Las técnicas independientes de secuenciación más efectivas para la caracterización intraespecífica de *E. amylovora* son el análisis por AFLP (del inglés *Amplified Fragment Lenght Polymorphism*), y la electroforesis por campo pulsante tras una macrorestricción del ADN genómico con *Xba*I. Ambas técnicas permitieron establecer una correlación entre el perfil genético de distintas cepas españolas y su origen geográfico (Rico *et al*., 2004; Donat *et al.,* 2007). No obstante, mientras que el análisis por AFLP no ha dado siempre buenos resultados en la diferenciación entre cepas europeas (Keck *et al*., 2002), la electroforesis de campo pulsante ha permitido caracterizar con éxito la variabilidad intraespecífica de aislados tanto americanos como europeos, permitiendo establecer rutas hipotéticas de dispersión del patógeno por la Unión Europea (Zhang & Geider, 1997; Zhang *et al*., 1998; Jock *et al*., 2002; 2013).

En los últimos años, los avances en las técnicas de secuenciación han permitido la comparación de genomas enteros de varias cepas de *E. amylovora* (Powney *et al.,* 2011; Zhao & Qi, 2011; Mann *et al.,* 2013), y la aplicación de nuevas estrategias para la caracterización de la variabilidad

intraespecífica mucho más resolutivas, como el análisis multilocus MLVA (del inglés *Multi-Locus Variable number of tandem repeats Analysis*) (Bühlmann *et al.,* 2014), o el estudio de las secuencias CRISPR (del inglés *Clustered Regularly Interspaced Short Palindromic Repeats*) (Rezzonico *et al.,* 2011; McGhee & Sundin, 2012), lo que ha permitido avanzar tanto en la caracterización genética intraespecífica como en el estudio de la epidemiología molecular de *E. amylovora* (Rezzonico *et al.,* 2011; McGhee & Sundin, 2012; Förster *et al.,* 2015; Tancos & Cox, 2016).

2.3.2. Características del pangenoma

En la actualidad hay disponibles en las bases de datos secuencias completas y borradores de secuencias de los genomas de 15 cepas de *E. amylovora,* incluyendo patógenas de *Rubus* y de *Spiraeoideae,* de distintos orígenes geográficos y aisladas en diferentes periodos (Sebaihia *et al.,* 2010; Smits *et al.,* 2010; Powney *et al.,* 2011; Mann *et al.,* 2013). El análisis de los genomas de dichas cepas ha permitido identificar algunas de las características del pangenoma (porción del genoma común en todas las cepas) de este patógeno (Mann *et al.,* 2013). *E. amylovora* posee un solo cromosoma, con un tamaño medio de aproximadamente 3,8 Mb y un contenido en G+C de entre el 53,3% y el 53,6%. Aproximadamente un 86% de las secuencias que conforman el genoma de *E. amylovora* corresponden a regiones codificantes (CDS), con una densidad de alrededor de 1 CDS por Kb. Un porcentaje muy elevado de secuencias de ADN codificante, alrededor de un 89%, es común en todas las cepas, lo que resulta sorprendente si lo comparamos con el de otras bacterias fitopatógenas (un 48% en *Ralstonia solanacearum,* un 64% en *Pseudomonas syringae* o un 75% en *Xanthomonas campestris*) (Mann *et al.,* 2013). El análisis de la identidad de

aminoácidos en la región codificante del pangenoma revela también un elevado nivel de conservación, con más de un 99% de identidad de aminoácidos entre todas las cepas analizadas (Mann *et al.,* 2013).

2.3.3. El genoma accesorio

El genoma accesorio de una especie bacteriana es la secuencia o conjunto de secuencias de ADN presente/s en una o algunas de las cepas que conforman dicha especie, pero no en todas. Entre los elementos más importantes del genoma accesorio de *E. amylovora* encontramos plásmidos, islas genómicas, determinantes de patogenicidad y de especificidad por el hospedador, sistemas de secreción de tipo VI y genes relacionados con el metabolismo y/o transporte de carbohidratos (Mann *et al.,* 2013).

Plásmidos. En la actualidad se han identificado hasta 12 plásmidos distintos en *E. amylovora* (Tabla 2), que contribuyen de forma importante a la diversidad intraespecífica en este patógeno, sobretodo en el caso de las cepas patógenas de *Spiraeoideae* (Smits *et al.,* 2010; Mann *et al.,* 2013; Ismail *et al.,* 2014). De entre todos estos plásmidos, cabe destacar el pEA29, que durante mucho tiempo se consideró ubicuo en *E. amylovora,* hasta que se aislaron cepas que carecían del mismo (Llop *et al.,* 2006). En general, los plásmidos en patógenos animales y vegetales suelen contener genes relacionados con resistencia a antibióticos, producción de toxinas, hormonas, genes de virulencia, etc (Llop *et al.,* 2012). Dada la elevada homogeneidad genética, bioquímica y serológica que encontramos en *E. amylovora*, cabría esperar que, al menos parte de las diferencias en virulencia, secreción de EPS o incluso en la especificidad de hospedadores observada entre distintas cepas de este patógeno

(Cabrefiga & Montesinos, 2005; Wang *et al.,* 2009; Powney *et al.,* 2011) se debiese al contenido plasmídico. No obstante, la realidad es que, pese que en algunos casos parece existir una relación entre plásmidos y virulencia (Llop *et al.,* 2011; Llop *et al.,* 2012), no se han encontrado genes asociados directamente con patogénesis. Puntualmente también se han identificado genes de resistencia a antibióticos (Chiou & Jones, 1991; Palmer *et al.,* 1997; McGhee *et al.,* 2002), pero se desconoce la función de otros muchos genes codificados en estos plásmidos, y su eliminación no produce cambios metabólicos o de virulencia apreciables (Llop *et al.,* 2012; Ismail *et al.,* 2014).

Islas genómicas. Las islas genómicas (IG) son grupos de genes, probablemente adquiridos por transmisión horizontal, que incluyen profagos, plásmidos integrados, elementos integrativos conjugativos, integrones y transposones conjugativos. En *E. amylovora* se han identificado un total de 12 regiones del genoma cuyo contenido en IGs varía en función de la cepa analizada. La mayoría de CDS identificadas en dichas IGs codifican proteínas hipotéticas o relacionadas con la replicación, transferencia e integración de elementos móviles. En otros casos se trata de sistemas de protección frente al ADN extraño, que codifican tanto proteínas que protegen el ADN bacteriano como enzimas que degradan el ADN exógeno. Salvo alguna excepción, no se ha establecido relación alguna entre el contenido en IGs y la planta hospedadora (Mann *et al.,* 2013).

Determinantes de patogenicidad y especificidad por el hospedador. Una de las regiones del genoma de *E. amylovora* que mayor variación presenta entre cepas patógenas de *Rubus* y plantas de la subfamilia *Spiraeoideae* es la isla de patogenicidad Hrp (del inglés, *Hypersensitive response and pathogenicity*), que codifica un sistema de secreción de tipo III (SST3) así como proteínas efectoras secretadas a través de dicho sistema. Otras

secuencias que presentan variación son la región IT (del inglés, *Island Transfer*), anexa a la isla de patogenicidad Hrp, y proteínas efectoras secretadas a través del SST3, pero situadas fuera de la isla Hrp (Mann *et al.,* 2013).

Sistemas de secreción de tipo VI (SST6). La comparación de varios genomas de *E. amylovora* ha permitido identificar tres grupos de genes que codifican un SST6 (Smits *et al.,* 2010), aunque se desconoce su función en esta especie bacteriana. Tal y como ocurría en el caso anterior, la variabilidad observada en las secuencias de dichos SST6 se correlaciona con diferencias en el hospedador de las cepas analizadas (*Rubus* versus *Spiraeoideae*), pero no permite discriminar entre las cepas patógenas de *Spiraeoideae* (Mann *et al.,* 2013).

Metabolismo y/o transporte de carbohidratos. En base a los genomas anotados de *E. amylovora* se han determinado diferencias entre cepas en genes que codifican proteínas necesarias para metabolizar y transportar activamente L-arabinosa y otras fuentes de carbono, aunque no hay correlación entre la variabilidad entre cepas y su hospedador (Mann *et al.,* 2013).

Tabla 2. Plásmidos detectados en *E. amylovora,* cepas representativas de las cuales se han aislado y características más relevantes asociadas a dichos plásmidos

Plásmido	Tamaño (Kpb)	Cepa representativa	Planta hospedadora	Origen de las cepas que contienen el plásmido	Función más relevante
pEA72	71,5	ATCC 49946	Manzano	EEUU	Desconocida
pEA68*	68,8	LMG 28361	*Sorbus* sp.	Polonia, Bélgica	Desconocida
pEI70	65,9	IVIA 1614-2a	*Crataegus* sp.	Europa (11 países distintos)	Su inserción en cepas poco virulentas incrementa su virulencia, probablemente debido a un incremento del éxito biológico de la cepa que lo contiene
pEL60	60,1	LebB66	Manzano, peral membrillo	Líbano	Alberga genes que codifican sistemas de protección del ADN, que confieren tolerancia a la radiación UV, lo que podría incrementar el éxito biológico de la cepa que lo contiene
pEA34	~34	CA11	Manzano, peral membrillo	EEUU (Michigan)	Confiere resistencia a estreptomicina
pEU30	30,3	UTRJ2	Manzano, peral membrillo	EEUU (Washington, Utah, Oregon)	Desconocida
pEA29	28,3	CFBP 1430	*Crataegus sp.*	Europa, Norte América, Oceanía, Norte de África, Asia	Casi ubicuo; confiere prototrofia para la tiamina; cepas curadas del plásmido muestran producción alterada de EPSs y una reducción de virulencia y agresividad
pEA8.7	~9	CAR3	Manzano	EEUU (California)	Confiere resistencia a estreptomicina
pEAR5.2	5,3	ATCC BAA-2158	*Rubus* sp.	EEUU (Illinois)	Desconocida
pEAR4.3	4,4	ATCC BAA-2158	*Rubus sp.*	EEUU (Illinois)	Desconocida
pEA2.8	2,8	IL-5	*Rubus sp.*	EEUU (Illinois)	Confiere resistencia a ampicilina
pEA1.7	1,7	IH3-1	*Crataegus sp.*	EEUU (Louisiana)	Desconocida

Tabla modificada de Llop *et al* (2012).
* Plásmido identificado por Ismail *et al* (2014).

2.4. Aislamiento, detección e identificación

El diagnóstico del fuego bacteriano (Fig. 7) comienza con la identificación de síntomas característicos en plantas, aunque puede requerirse el análisis de muestras asintomáticas. Existen varios medios de cultivo para el aislamiento de *E. amylovora* (Paulin, 2000), pero los más empleados son los medios generales B de King (King *et al.,* 1954), y SNA (del inglés *Sucrose Nutrient Agar*) (Lelliot *et al.,* 1966) y el medio semiselectivo CCT (del ínglés *Cycloheximide, Crystal violet and Thallium nitrate*) (Ishimaru y Klos, 1984). Para la detección de *E. amylovora* también suelen emplearse técnicas serológicas y moleculares, con o sin enriquecimiento previo en medio de cultivo líquido (López *et al.,* 2005; 2009; Kałużna *et al.,* 2013). Dicho enriquecimiento suele ser necesario para el análisis de muestras asintomáticas, dado que en este tipo de muestras *E. amylovora* suele estar en números por debajo del nivel de detección de la mayoría de técnicas.

Las pruebas serológicas y moleculares permiten una detección mucho más rápida del patógeno, ya sean células vivas, muertas o en estado viable no cultivable (VBNC, del inglés, *Viable But NonCulturable*). Entre las técnicas serológicas destacan el análisis DASI-ELISA (del inglés *Double-Antibody Sandwich Indirect ELISA*) (Gorris *et al.,* 1996b) o las tiras reactivas *Ea* AgriStrip (Duffy *et al.,* 2007; Braun-Kiewnick *et al.,* 2011). En cuanto a las técnicas moleculares de detección, podemos destacar diversas PCR convencionales y a tiempo real (RT-PCR) específicas frente a regiones del plásmido casi ubicuo pEA29 (Bereswill *et al.,* 1992; McManus & Jones, 1995; Llop *et al.,* 2000; Salm & Geider, 2004; De Bellis *et al.,* 2007), así como PCR y RT-PCR cromosómicas (Bereswill *et al.,*1995; Guilford *et al.,* 1996; Maes *et al.,* 1996; Taylor *et al.,* 2001; Geider *et al.,* 2007; Pirc *et al.,* 2009; Gottsberger, 2010) que permiten la

detección de regiones específicas del cromosoma de *E. amylovora,* evitando los falsos negativos por ausencia de plásmido.

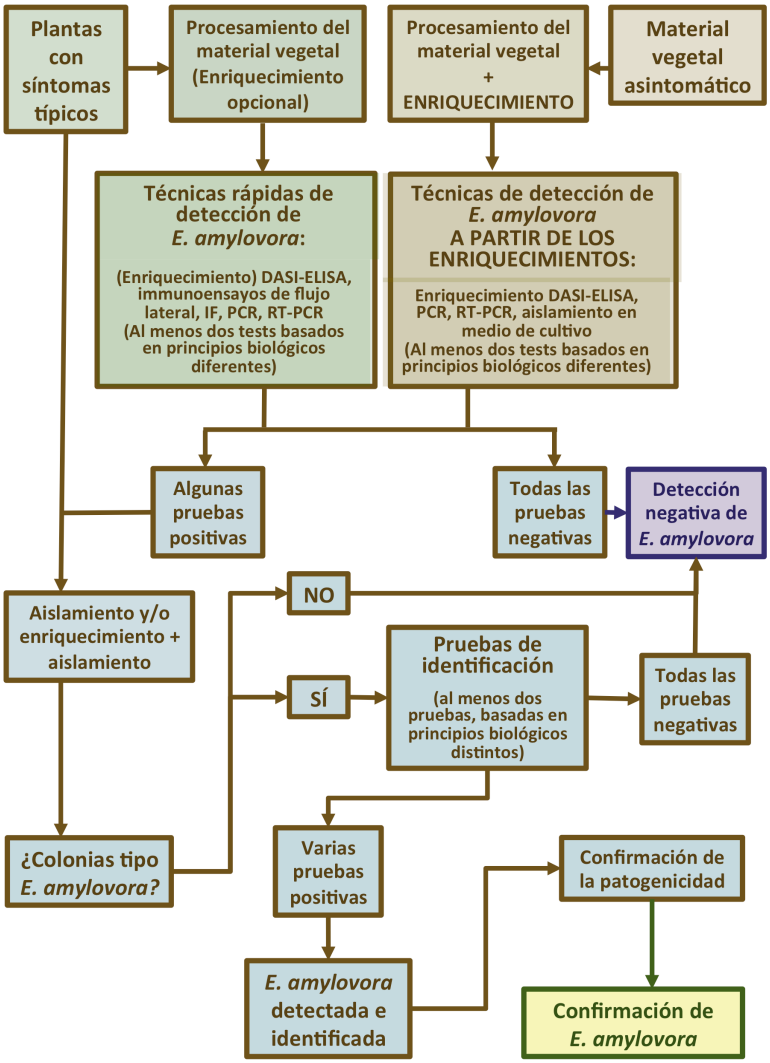


Fig. 7. Pasos a seguir para el diagnóstico de *E. amylovora* a partir de muestras sintomáticas y asintomáticas. Adaptado de EPPO (2013).

Finalmente, la identificación de los microorganismos aislados en medio de cultivo con morfología colonial tipo *E. amylovora* se realiza mediante pruebas bioquímicas, fisiológicas, serológicas, moleculares y de patogenicidad. En la Figura 7 se muestra un diagrama de flujo con la metodología a seguir durante el diagnóstico de fuego bacteriano a partir de muestras sintomáticas y asintomáticas, siguiendo la norma PM 7/20 de la EPPO (EPPO, 2013).

2.5. Factores de patogenicidad y virulencia

El desarrollo de sistemas de mutagénesis ha permitido identificar tres clases de genes implicados en la patogenicidad de *E. amylovora*, es decir, genes cuya mutación impide el desarrollo de síntomas de fuego bacteriano en plantas susceptibles. Dichos genes se relacionan con: *i)* la biosíntesis de amilovorano, *ii)* la formación de un SST3 y, finalmente, *iii)* las proteínas secretadas a través de dicho sistema de secreción (Bugert & Geider, 1995; Bereswill & Geider, 1997; Oh & Beer, 2005; Zhao *et al.,* 2009). También se han identificado genes que codifican factores de virulencia, de naturaleza mucho más diversa y cuya mutación disminuye la intensidad de los síntomas de fuego bacteriano o retrasa su aparición (van der Zwet *et al.,* 2012; Vrancken *et al.,* 2013; Piqué *et al.,* 2015). Finalmente, para que se pueda dar con éxito el proceso infeccioso, son necesarias complejas redes de regulación que coordinan la expresión de los genes anteriormente mencionados en respuesta a señales ambientales relevantes. Dichos mecanismos de regulación involucran sistemas de transducción de dos componentes y señalización mediada por ARN regulador pequeño y la chaperona Hfq, la alarmona ppGpp, GMP cíclico y *quorum sensing,* entre otros (Gao *et al.,* 2009; Zhao *et al.,* 2009; Edmunds *et al.,* 2013; Zeng *et al.,* 2013; Ancona *et al.,* 2015; Piqué *et al.,* 2015). La mutación

de cualquiera de estos elementos de regulación conlleva también la pérdida de patogenicidad o virulencia (Piqué *et al,.* 2015).

2.5.1. Factores de patogenicidad

Amilovorano. Uno de los principales signos de fuego bacteriano es la aparición de exudados en las plantas infectadas, formados mayoritariamente por savia, bacterias y sus EPS. Estos exudados dificultan el reconocimiento del patógeno por la planta hospedadora, obstruyen el sistema vascular (Vrancken *et al.,* 2013) y facilitan el movimiento de la bacteria por los espacios intercelulares del parénquima cortical (Zamski *et al.,* 2007). Además, se ha sugerido su papel protector frente a las especies reactivas del oxígeno (ROS, del inglés *Reactive Oxigen Species*) durante la invasión de los tejidos (Venisse *et al.,* 2001), y también frente al cobre (Ordax *et al.,* 2010) y la desecación (Denny, 1995), así como su papel como fuente exógena de alimento en condiciones de escasez de nutrientes (Ordax *et al.,* 2010), en la formación de biopelículas (Koczan *et al.,* 2009; 2011), o en la protección frente a algunos tipos de bacteriófagos (Roach *et al.,* 2014).

El componente mayoritario de los exudados es el amilovorano. Se trata de un heteropolisacárido cuyas unidades repetitivas constan de tres residuos de galactosa a los que se une un residuo de ácido glucurónico y, a este, una galactosa terminal con grupos piruvato y, en ocasiones, uno o dos grupos acetilo (Nimtz *et al.,* 1996; Langoltz *et al.,* 2011). En las cepas de *E. amylovora* que infectan plantas de la subfamilia *Spiraeoideae* una fracción de los monómeros (10-50%) contiene una cadena lateral constituida por un residuo de glucosa (Fig. 8) (Nimtz *et al.,* 1996; Langoltz *et al.,* 2011). Esta cadena no se encuentra presente en las cepas de *E. amylovora* aisladas de *Rubus*

(Busson *et al.,* 2001). Cada molécula de amilovorano, de aproximadamente 10^6 Da, contiene unos 1000 monómeros, aunque este número puede variar en función de las condiciones de cultivo y de otros factores (Geider, 2000; Vrancken *et al.,* 2013).

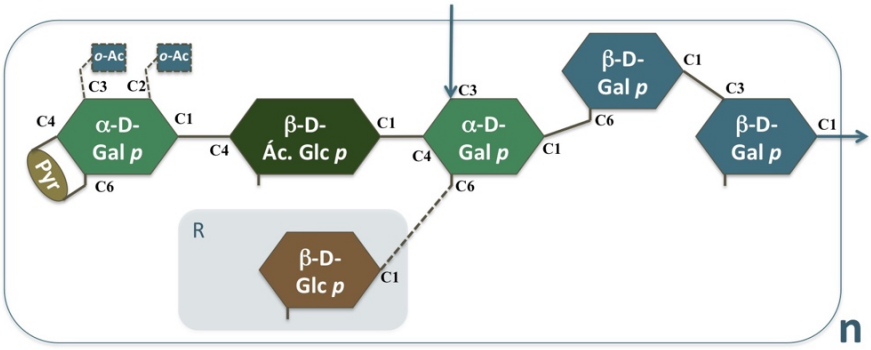


Fig. 8. Representación esquemática de la unidad estructural del amilovorano (Basado en Nimtz *et al.,* (1996). Se trata de un pentasacárido compuesto de galactosa (Gal) y ácido glucurónico (Ác. Glc). La galactosa terminal se encuentra unida a un residuo de piruvato (Pyr) y en ocasiones, presenta sustituciones con uno o dos grupos *O*-acetilo (*O*-Ac). Una fracción de los monómeros del amilovorano pueden presentar una cadena lateral (R) constituida por un residuo de glucosa. Las líneas discontinuas señalan enlaces o sustituciones cuya presencia es variable. Las flechas azules indican los átomos de carbono que sirven de unión entre los distintos monómeros que conforman el amilovorano, así como la dirección de los enlaces.

La biosíntesis del amilovorano depende de las condiciones ambientales, como la temperatura, el pH, la salinidad, la presencia de cobre o la fuente de carbono (Geider, 2000). En la regulación de este proceso intervienen sistemas de transducción de señales de dos componentes, como el sistema de transferencia de fosfatos Rcs (del inglés *Regulatory of capsule synthesis*), o los sistemas GrrS/GrrA (controla la producción de metabolitos secundarios y enzimas implicadas en patogenicidad) y EnvZ/OmpR (participa en la adaptación a cambios osmóticos)

(Zhao *et al.,* 2009). La síntesis de amilovorano también está regulada por la proteína tipo histona H-NS, que es un regulador transcripcional global que modula la expresión de múltiples genes en respuesta a estímulos ambientales (Hildebrand *et al.,* 2006), y por otros reguladores (Ma *et al.,* 2001; Wang *et al.,* 2012).

La mutación de genes implicados en la síntesis de amilovorano o en su regulación afecta drásticamente la capacidad de *E. amylovora* para generar síntomas de fuego bacteriano en plantas susceptibles (Belleman & Geider, 1992; Bernhard *et al*, 1993; Bereswill & Geider, 1997; Hildebrand *et al.,* 2006; Zhao *et al.,* 2009; van der Zwet *et al.,* 2012), así como de formar biopelículas (Koczan *et al.,* 2009).

Sistemas de secreción de tipo III (SST3) y proteínas asociadas. Se trata de una estructura proteica similar a una jeringa, cuya función principal es la inyección de proteínas en el citosol de las células hospedadoras. Algunas de las consecuencias de las interacciones entre las bacterias fitopatógenas y las plantas mediadas por el SST3 son la supresión de las defensas basales de la planta, el desarrollo de síntomas en plantas susceptibles, y el inicio de la respuesta hipersensible (HR, del inglés *Hypersensitive Response*) durante interacciones incompatibles con plantas no susceptibles (Alfano & Collmer, 2004; Fones & Preston, 2012; Khan *et al.,* 2012). Los principales componentes de los SST3 son las proteínas Hrc (relacionadas con la HR, conservadas) y Hrp (relacionadas con la HR y patogenicidad) (Alfano & Collmer, 2004; Vrancken *et al.,* 2013). En *E. amylovora* los genes *hrp* y *hrc* se encuentran agrupados, junto con otros genes que codifican proteínas efectoras e inductoras de la HR, en una sección del genoma denominada complejo génico *hrp/dsp*. La mutación de los genes *hrp* en *E. amylovora* tiene como consecuencia la pérdida de patogenicidad en plantas

susceptibles y de la capacidad de inducir la HR en hospedadores no susceptibles como el tabaco. La mutación de los genes *dsp*, sin embargo, afecta solo a la patogenicidad en hospedadores susceptibles, pero no a la HR (Oh & Beer, 2005). El complejo génico *hrp/dsp* forma parte de la isla de patogenicidad Hrp, de unas 62 Kb, constituida por unos 60 genes y organizada en cuatro regiones bien definidas, denominadas *hrp/hrc*, HEE (del inglés *Hrp Effectors and Elicitors*), HAE (del inglés *Hrp-Associated Enzymes*) e IT (del inglés *Island Transfer*) (Fig. 9).

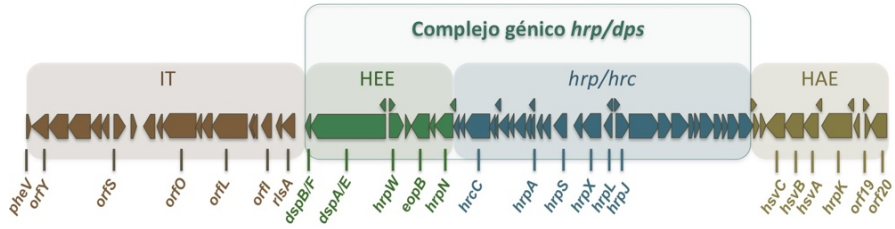


Fig. 9. Esquema representativo de la isla de patogenicidad Hrp de *E. amylovora.* Modificado de Oh & Beer (2005).

La expresión del SST3 en *E. amylovora* se activa *in planta,* aunque las condiciones que inducen dicha activación (ciertas fuentes de carbono o nitrógeno, ácido nicotínico, amonio o condiciones de temperatura, pH y nutrientes similares a las del apoplasto) no son específicas de las plantas, por lo que puede inducirse artificialmente (Wei *et al.,* 1992). En la actualidad se conocen 12 proteínas secretadas a través del SST3 (Nissinen *et al.,* 2007), entre las cuales encontramos proteínas efectoras, proteínas ayudadoras, harpinas, chaperonas, proteínas estructurales, entre otras funciones (Tabla 3).

Tabla 3. Principales proteínas secretadas por el SST3 de *E. amylovora* (descritas en la cepa Ea 273) (modificada de Nissinen *et al.,* 2007).

Proteína	PM aparente	Función, propiedades, homólogos
DspA/E	200	Proteína efectora requerida para patogénesis; homólogos ampliamente distribuidos entre otras especies de *Erwinia* y *Pseudomonas*
DspA/E'	100	Fragmento de DspA/E
HrpK	80	Función desconocida; similar a HrpK de *P. syringae*
Eop2	68	El extremo C-terminal posee un dominio pectato liasa; similar a la proteína ayudadora de tipo III HopAK1 de *P. syringae* pv. tomato y otros miembros de la familia HopPmaH
HrpW	60	Harpina con un dominio harpina N-terminal y un dominio similar a una pectato liasa en el extremo C-terminal; no se requiere para patogenicidad o HR
Eop1	44	Miembro de la familia de proteínas YopJ/AvrTxv/HopZ (cisteínproteasas efectoras), con homólogos conservados en patógenos animales y vegetales
HrpN	40	Harpina, factor de virulencia
HrpJ	40	Factor de patogenicidad; similar a YopN de *Yersinia* spp. Donde se requiere para una translocación eficiente de proteínas efectoras de tipo III
TraF(Ea)	40	Similar a las proteínas tipo TraF, implicadas en la transferencia de plásmidos y formación de pilus
Eop3	33	Homólogo de proteínas efectoras de tipo III de tipo AvrPphE (HopX)
FlgL	33	Similar a una proteína flagelar de unión al filamento
HrpA	7,5	Pilina de tipo III, esencial para la formación del pilus *hrp* y la secreción a través de él.

El principal mecanismo de regulación de la transcripción de los genes *hrp* que codifican el SST3 es a través del factor sigma alternativo HrpL (Wei & Beer, 1995; McNally *et al.,* 2012), también codificado por un gen del complejo *hrp/dsp.* Adicionalmente, la regulación de la expresión del SST3 y/o de *hrpL* depende también de varios sistemas de transducción de señales de dos componentes (Wei *et al.,* 2000; Zhao *et al.,* 2009) y proteínas reguladoras adicionales (Wei *et al.,* 2000; Ancona *et al.,* 2014; Piqué *et al.,* 2015).

2.5.2. Factores de virulencia

Levano. Se trata de uno de los principales EPS producidos por *E. amylovora.* Es un homopolímero de residuos de fructosa (Fig. 10), cuya síntesis es llevada a cabo por la levanosacarasa. Esta enzima es una exoproteína que escinde moléculas de sacarosa (el principal azúcar de transporte en la mayoría de plantas, y presente también en las rosáceas) en fructosa, que sirve como sustrato para la formación de levano, y glucosa, que puede ser aprovechada como fuente de carbono. Las deficiencias en la síntesis de levano afectan a la formación de biopelículas *in vitro* e *in vivo* (Koczan *et al.,* 2009), y ralentizan la aparición de síntomas de fuego bacteriano en la planta hospedadora (Geider, 2000), aunque su papel concreto durante el proceso infeccioso se desconoce (Piqué *et al.,* 2015).

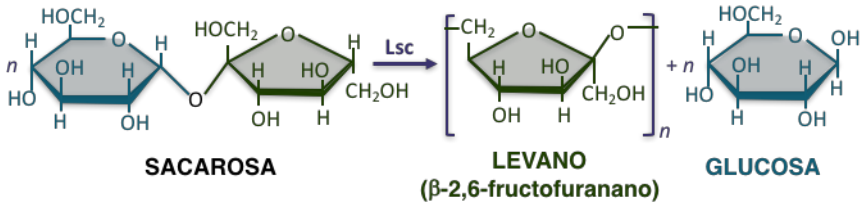


Fig. 10. **Esquema de la síntesis de levano a partir de sacarosa en el medio extracelular por la enzima levanosacarasa (Lsc)** (Modificado de Geider, 2000).

Metabolismo del sorbitol y la sacarosa. El sorbitol es un azúcar de transporte complementario a la sacarosa, cuyo uso es característico de plantas de la familia de las rosáceas y que, por dicho motivo, se ha propuesto como un factor determinante de los hospedadores de *E. amylovora* (Geider, 2000). Este azúcar alcohol es un buen inductor de la producción de amilovorano y la suplementación de los medios de cultivo con esta fuente de carbono incrementa su síntesis (Geider, 2000). La capacidad

para metabolizar el sorbitol en este patógeno está codificada en distintos genes dentro del operón *srl.* Mutantes deficientes en dicho operón presentan alterada su capacidad para colonizar tejidos de manzano, lo que conlleva una disminución de los síntomas de fuego bacteriano (Aldridge *et al.,* 1997).

En cuanto a la sacarosa, se trata de otro azúcar de transporte, presente en la mayoría de plantas, incluidas las rosáceas. Mutantes de *E. amylovora* en el regulón *src,* que posibilita el metabolismo de este disacárido, también poseen una virulencia disminuida y una menor capacidad de colonización que la cepa parental (Bogs & Geider, 2000).

Sideróforos. *E. amylovora* produce sideróforos de tipo trihidroxamato, de la familia de la desferrioxamina, así como receptores para los mismos. Estos sistemas de unión de hierro de alta afinidad participan en la captación de hierro de los tejidos del hospedador y contribuyen a la protección frente a los efectos tóxicos de dicho metal, relacionados con la generación de ROS. Deficiencias tanto en la producción de sideróforos como de sus receptores repercuten negativamente en la capacidad de *E. amylovora* para iniciar síntomas de fuego bacteriano en plantas susceptibles. Además, la alteración de la síntesis de desferrioxamina afecta también la tolerancia del patógeno al peróxido de hidrógeno (H_2O_2) (Dellagi *et al*., 1998; Venisse *et al.,* 2003; van der Zwet *et al.,* 2012).

Plásmidos pEA29 y pEI70. La presencia de los plásmidos pEA29 y pEI70 en *E. amylovora* se ha relacionado con un incremento de la virulencia, aunque aparentemente no contienen genes específicamente relacionados con patogenicidad o virulencia (Llop *et al.,* 2011; 2012). No obstante, cabe destacar que el plásmido pEA29 contiene en su secuencia el gen *hns,* que codifica la proteína tipo histona H-NS (McGhee & Jones, 2000).

Este tipo de proteínas, junto con factores sigma alternativos y otros reguladores transcripcionales globales, se encargan de la regulación de la transcripción de numerosos genes en respuesta a cambios ambientales. En *E. amylovora,* entre todos los genes regulados por H-NS se incluyen los de la síntesis de amilovorano. Curiosamente este patógeno contiene otra copia del gen *hns* en el cromosoma, y la mutación de la copia plasmídica y/o la cromosómica tiene efectos negativos sobre la virulencia (Hildebrand *et al.,* 2006).

Formación de biopelículas. La formación de biopelículas parece ser necesaria para la infección sistémica de plantas susceptibles por *E. amylovora*. Su formación depende tanto de los EPS amilovorano y levano (Koczan *et al.,* 2009), como de estructuras de adhesión (flagelos, fimbrias tipo I, *pili* tipo IV y *curli*) (Koczan *et al.,* 2011).

El amilovorano es necesario para la adhesión a superficies y entre células. Este EPS, además, es de vital importancia para la supervivencia en los tejidos vegetales. El levano, sin embargo, aunque también contribuye a los fenómenos de adhesión y agregación, parece tener un papel más relevante en la orientación de las células en los tejidos vegetales, y mutantes deficientes en el enzima levanosacarasa muestran deficiencias en la colonización del xilema (Koczan *et al.,* 2009). Mutaciones en genes relacionados con la formación de estructuras de adhesión tipo flagelos, fimbrias, etc., también repercuten en la formación de biopelículas y, por lo tanto, en la virulencia de *E. amylovora* (Koczan *et al.,* 2011).

El lipopolisacárido (LPS). La composición del LPS de *E. amylovora* se ha relacionado con la especificidad por el hospedador, y permite diferenciar a las cepas patógenas de *Rubus* de las que infectan plantas de la familia *Spiraeoideae*

(Rezzonico *et al.,* 2012; Mann *et al.,* 2013). La mutación del gen *waaL,* relacionado con la síntesis de LPS, induce en *E. amylovora* un aumento de la sensibilidad a H_2O_2 y a polimixina B, defectos en movilidad por *twitching* y también una disminución de la virulencia (Berry *et al.,* 2009).

Otros factores de virulencia. Aparte de los anteriormente mencionados, se han descrito factores de virulencia adicionales, como la metaloproteasa PrtA (Zhang *et al.,* 2009) y las bombas de expulsión multidroga AcrAB, MdtABC y MdtUVW (Burse *et al.,* 2004; Pletzer & Weingart, 2014).

La metaloproteasa PrtA es una de las pocas enzimas secretadas por *E. amylovora*. Aunque su actividad se ha caracterizado y su implicación en la capacidad del patógeno para colonizar los tejidos del hospedador ha sido demostrada, se desconoce su contribución exacta en este proceso (Zhang *et al*., 1999).

En cuanto a las bombas de expulsión multidroga AcrAB, MdtABC y MdtUVW, estas participan en la protección de *E. amylovora* frente a fitoalexinas y probablemente otros compuestos antimicrobianos sintetizados por las plantas en respuesta a los patógenos. Mutantes en cualquiera de estos sistemas de transporte tienen menor capacidad para colonizar los tejidos de la planta hospedadora (Burse *et al.,* 2004; Pletzer & Weingart, 2014).

3. Interacciones con el hospedador

3.1. Mecanismos de patogénesis y respuestas de la planta hospedadora

Uno de los aspectos más intrigantes de la relación de *E. amylovora* con sus hospedadores es el mecanismo empleado por dicho patógeno para invadir los tejidos infectados. En la mayoría de fitopatógenos estudiados, las interacciones incompatibles con plantas no susceptibles inducen: *i)* la liberación de ROS (estallido oxidativo), que actúan como moléculas señal y poseen acción bactericida/bacteriostática (Camejo *et al.,* 2016); *ii)* la inducción de la HR. Esta última consiste en la muerte celular programada de las células en contacto con el patógeno, lo que contribuye a su aislamiento y evita su propagación por el resto de tejidos vegetales (Senthil-Kumar & Mysore, 2013). Por el contrario, durante las interacciones compatibles, los patógenos bacterianos suelen desactivar las defensas del hospedador mediante proteínas efectoras secretadas a través del SST3, lo que evita o reduce el estallido oxidativo y la HR, y les deja vía libre para la invasión de los tejidos (Fones & Preston, 2012). La estrategia empleada por *E. amylovora* para invadir los tejidos de plantas sensibles implica también la secreción de moléculas efectoras a través del SST3 (Venisse *et al.,* 2003), pero en este caso, la consecuencia inmediata es precisamente la contraria a la esperada en interacciones compatibles. *E. amylovora* induce la liberación de ROS y una HR indistinguible bioquímicamente de la que se produce en interacciones incompatibles, por ejemplo, en tabaco. No obstante, lejos de frenar el avance de la infección, tanto el estallido oxidativo como la HR parecen ser necesarios para el éxito de *E. amylovora* en la invasión de los tejidos de la planta hospedadora (Venisse *et al.,* 2001; 2003) (Fig. 11).

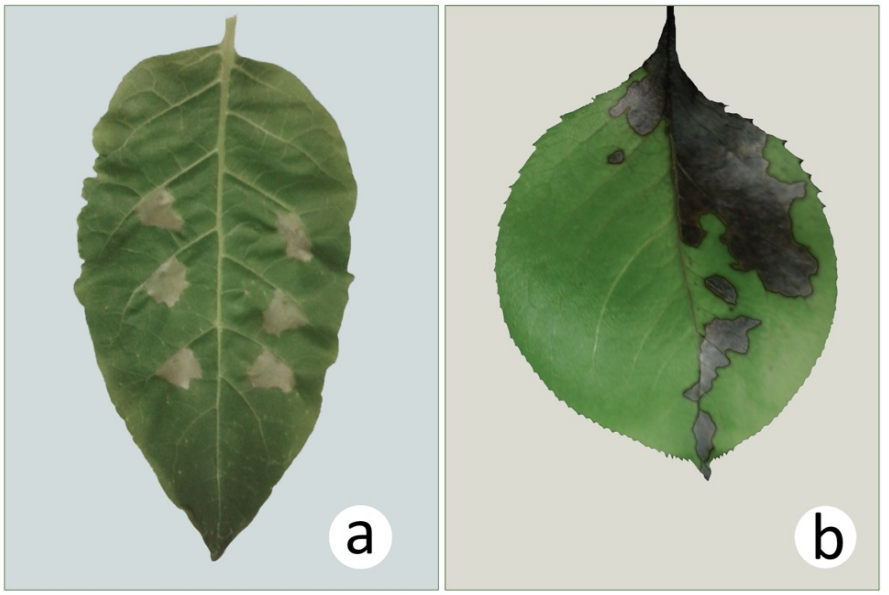


Fig. 11. Interacción de *E. amylovora* con hojas de un hospedador incompatible (*Nicotiana tabacum* var. Xanthi) (a) y otro compatible (*P. communis* var. Passe Crassane) (b). En el primer caso, la bacteria se inoculó en seis puntos distintos mediante infiltración con jeringa. Como resultado se observan necrosis localizadas correspondientes a una HR típica. En el segundo caso, la inoculación se realizó en peciolo. Se observan necrosis que progresan del nervio central a los tejidos circundantes.

La manera en que este patógeno se protege del estallido oxidativo no ha sido prácticamente caracterizada. En muchas bacterias fitopatógenas (Fones & Preston, 2012), incluida *E. amylovora* (Király *et al.,* 1997; Dellagi *et al.,* 1998; Venisse *et al.,* 2001), se ha sugerido el papel los sideróforos y los EPSs como elementos importantes en la lucha contra el estrés oxidativo. Por otra parte, *E. amylovora* posee distintas actividades enzimáticas antioxidantes (catalasa, peroxidasa, superóxido dismutasa) (Keck *et al.,* 1997), aunque su papel durante la patogénesis y otros aspectos de la biología de la bacteria todavía no ha sido investigado. Sin embargo, algunos estudios parecen indicar que, durante las interacciones con el hospedador, *E. amylovora*

expresa una cantidad importante de proteínas relacionadas con, la protección frente al estrés oxidativo y la reparación de elementos dañados por las ROS (Thoelen *et al.,* 2008).

Debido a la complejidad de las interacciones entre *E. amylovora* y sus hospedadores, los mecanismos moleculares que condicionan la resistencia o susceptibilidad de los mismos frente a *E. amylovora* son en su mayoría desconocidos. La reciente secuenciación del genoma del manzano (Velasco *et al.,* 2010) ha permitido identificar el perfil transcripcional de dicho hospedador durante la interacción con *E. amylovora* (Kamber *et al.,* 2016). Este estudio ha confirmado datos previos (Vrancken *et al.,* 2013; Warabieda *et al.,* 2015) sobre la relevancia del ácido jasmónico, el etileno, varios compuestos del metabolismo secundario, proteínas de defensa, etc., frente a este patógeno. Adicionalmente, ha permitido la identificación de nuevos genes, marcos abiertos de lectura y secuencias conservadas no codificantes, que se expresan diferencialmente en respuesta a *E. amylovora.* Entre estos destaca un gen que codifica una proteína de la superfamilia de las peroxidasas, aunque se desconoce si el producto de dicho gen desempeñaría funciones similares a otras peroxidasas durante las infecciones (Kamber *et al.,* 2016).

Las infecciones causadas por *E. amylovora* promueven en la planta hospedadora cambios de expresión de un gran número de genes involucrados en metabolismo, transducción de señales y respuesta al estrés. La infección de flores de manzano por *E. amylovora,* por ejemplo, afecta a la expresión de genes que codifican quitinasas, fitohormonas y algunas proteínas de resistencia, lo que afecta negativamente a la defensa de la planta frente hongos y bacterias (Kamber *et al.,* 2016). Así mismo, tras la liberación de ROS y la HR, la interacción con el patógeno produce en el hospedador un incremento de actividades antioxidantes ascorbato peroxidasa, glutatión reductasa,

glutatión-S-transferasa y catalasa (Venisse *et al.,* 2003); cambios en los niveles de metabolitos secundarios como los flavonoides, fenolaminas y lignina; se altera la expresión de genes relacionados con la fotosíntesis; se estimula la acumulación de ácido salicílico y etileno, y la reducción de los niveles de ácido jasmónico y se induce la producción sistémica de diferentes proteínas con acción antimicrobiana (Vrancken *et al.,* 2013; Kamber *et al.,* 2016) (Fig. 12).

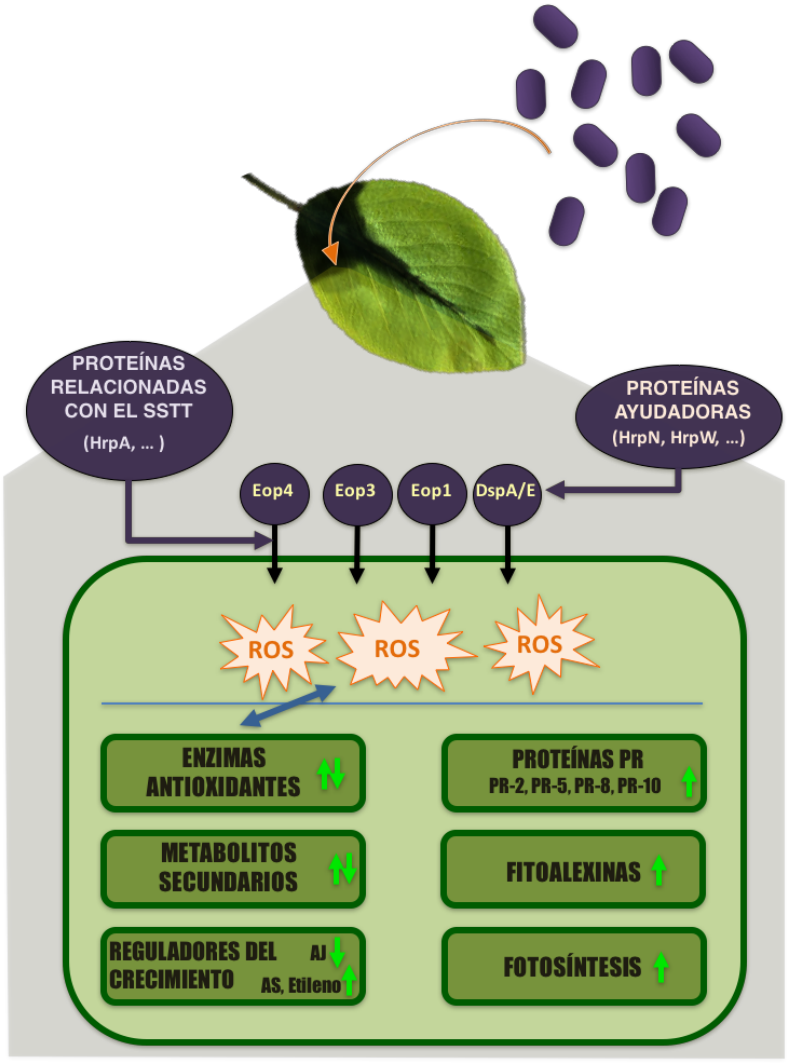


Fig. 12. Mecanismos moleculares de patogenicidad de *E. amylovora*. Durante las infecciones *E. amylovora* secreta a los tejidos de la planta las proteínas relacionadas con efectores DspA/E, Eop1, Eop3 y Eop4, junto con proteínas ayudadoras y otras proteínas relacionadas con el SST3. Como resultado se incrementan los niveles de ROS junto con otras respuestas de las células de la planta. Adaptado de Vrancken *et al* (2013).

3.2. Colonización del hospedador por *E. amylovora*

Las infecciones naturales causadas por *E. amylovora* suelen comenzar a través de las flores. Los puertos de entrada para la bacteria son los nectarios, y también los estomas y otras aberturas naturales de la superficie de la planta. No obstante, la entrada de la bacteria en el hospedador a través de heridas generadas de forma artificial o por causas naturales también ha sido ampliamente documentada (Billing, 2011; van der Zwet *et al.,* 2012).

Una vez dentro de la planta, el patógeno puede infectar los tejidos circundantes y/o moverse dentro de la misma, pudiendo llegar a producir una infección sistémica. No obstante, tanto la identidad de los tejidos en los que el patógeno comienza a multiplicarse como las rutas empleadas por el mismo para la invasión sistémica del hospedador han sido fruto de controversia desde los inicios del estudio del fuego bacteriano. Ello se debe a la disparidad de resultados obtenidos según el tipo de material vegetal analizado, la edad de los tejidos inoculados, el sistema de inoculación o los métodos empleados para la detección de la bacteria en los tejidos infectados (Billing, 2011; van der Zwet *et al.,* 2012).

En general se admite que, tanto el punto de entrada como el método de inoculación condicionan el tejido donde *E. amylovora* comienza a multiplicarse. Una de las principales vías de este patógeno para la infección sistémica de plantas sensibles son los tejidos de la corteza, donde se multiplica ocasionando los signos clásicos de fuego bacteriano. La infección sistémica a través del floema parece carecer de evidencias experimentales sólidas (Billing, 2011; van der Zwet *et al.,* 2012).

El avance de *E. amylovora* a través de tejido parenquimático puede proporcionarle acceso a los conductos xilemáticos. En estos últimos puede multiplicarse también y desplazarse de forma veloz a otras localizaciones dentro de la planta. No obstante, la salida de los conductos xilemáticos puede plantear problemas. *E. amylovora* puede persistir viable en los conductos del xilema durante varios periodos vegetativos, hasta que, de forma natural (tormentas, viento, etc.) o artificial (poda, rotura manual, etc.), se producen roturas (Billing, 2011), y es entonces cuando tiene acceso a los tejidos de la corteza, donde puede producir signos característicos de fuego bacteriano.

Según un estudio realizado por Bogs *et al* (1998), el desplazamiento más favorable de *E. amylovora* en el tallo sería en sentido descendente, pudiendo llegar a invadir las raíces primaria y secundarias. En relación a esto, *E. amylovora* se considera un patógeno de la parte aérea de la planta, por lo que, aunque se han detectado síntomas de fuego bacteriano en raíz en campo, la invasión a través de las raíces, no se considera una vía principal de entrada para el patógeno (Bogs *et al.,* 1998; van der Zwet *et al.,* 2012).

4. Supervivencia de *E. amylovora* dentro y fuera de la planta hospedadora

Además del carácter sistémico de las infecciones y el rápido desarrollo del fuego bacteriano, otro de los factores que contribuyen al difícil control de esta enfermedad es la capacidad de su agente causal para sobrevivir dentro y fuera del hospedador en condiciones subóptimas para el crecimiento, lo que favorece su persistencia en el ambiente, y facilita su diseminación a través de múltiples vías (Thomson, 2000; van der Zwet *et al.,* 2012). En muestras de campo *E. amylovora* puede aislarse tanto de chancros, como de hojas y otros órganos vegetales (en fase epífita), así como de vectores y otros elementos de origen biótico y abiótico (herramientas de poda, insectos, etc.,) (Miller & Sroth, 1972; Hildebrand *et al.,* 2000; van der Zwet *et al.,* 2012). A continuación se describen los aspectos más relevantes de la supervivencia del patógeno en algunos de estos elementos.

4.1. Supervivencia en fase epífita

Además de en los chancros, *E. amylovora* puede estar presente y ser detectada en la superficie de hojas, flores y frutos sin síntomas aparentes de fuego bacteriano (Thomson, 2000; van der Zwet *et al.,* 2012).

Supervivencia en hojas. En general las poblaciones del patógeno en hojas suelen ser muy bajas, excepto justo antes, durante y poco después de periodos de lluvia (Ockey & Thomson, 2006). *E. amylovora* coloniza con mayor facilidad las hojas más jóvenes, posicionándose cerca de hidátodos y tricomas glandulares (Norelli & Brandl, 2006). Las condiciones de elevada

temperatura y baja humedad disminuyen rápidamente sus poblaciones epífitas. Por otra parte, los cambios bruscos de temperatura durante las tormentas de verano favorecen el establecimiento del patógeno en la superficie de las plantas. Esto coincide con el aumento del riesgo de epidemias de fuego bacteriano asociado a las tormentas estivales. El incremento del número de células de *E. amylovora* en la superficie de la planta junto con la rotura de ramas y hojas ocasionada por la lluvia, el viento y/o el granizo garantizan el acceso del patógeno a un mayor número de sitios de entrada, lo que incrementa el poder destructivo de la enfermedad (Norelli & Brandl, 2006; Ockey & Thomson, 2006; van der Zwet *et al.,* 2012). Este fenómeno es extensible a las poblaciones epífitas del patógeno en otros órganos de la planta.

Supervivencia en flores. En las flores la supervivencia y proliferación de *E. amylovora* dependen de factores relacionados con la propia flor, como el órgano floral infectado, la edad de la flor, o la posición de esta con respecto al resto, y también de factores ambientales, siendo la humedad relativa y la temperatura los más importantes (Hasler & Mamming, 2002; Bubán *et al.,* 2003; Farkas *et al.,* 2012; van der Zwet *et al.,* 2012). A diferencia de las hojas o los chancros, *E. amylovora* puede multiplicarse en varios órganos florales sin que ello implique la aparición de síntomas, siendo los estigmas los órganos que permiten una mayor proliferación de las poblaciones del patógeno. Las anteras, aunque menos propicias para el crecimiento microbiano, ofrecen un ambiente ideal para la transmisión del patógeno, a través de la contaminación del polen y la dispersión mediada por insectos polinizadores (Bubán *et al.,* 2003; Azegami *et al.,* 2008). La lluvia, en este caso, favorece el lavado y/o dilución del néctar, lo que facilita la multiplicación e invasión de los nectarios por *E. amylovora,* constituyendo la

principal vía de entrada del patógeno en la planta (Pusey, 2000; Bubán *et al.,* 2003).

Supervivencia en frutos. En campos con un elevado índice de colonización de flores por *E. amylovora* es posible también detectar la bacteria en frutos (en los restos de los estigmas, en el cáliz, y rara vez en superficie) (Thomson, 2000). La capacidad de *E. amylovora* para generar síntomas de fuego bacteriano en frutos maduros es reducida (Dueck, 1974; Hale & Taylor, 1998; Roberts *et al.,* 1998; Taylor *et al.,* 2003; Tsukamoto *et al.,* 2005; Roberts & Sawyer, 2008; Ordax *et al.,* 2009). La detección de *E. amylovora* en la superficie de frutos maduros ocurre principalmente en parcelas severamente afectadas por fuego bacteriano, siempre en las proximidades de plantas con síntomas (Thomson, 2000). El estudio de la supervivencia de *E. amylovora* en frutos maduros se ha llevado a cabo en numerosas ocasiones, aunque el papel de estos órganos como vía de transmisión de fuego bacteriano sigue generando debate entre los expertos (Dueck, 1974; Hale & Taylor, 1998; Roberts *et al.,* 1998; Taylor *et al.,* 2003; Tsukamoto *et al.,* 2005; Roberts & Sawyer, 2008; Ordax *et al.,* 2009). Se ha demostrado la supervivencia de *E. amylovora* en el cáliz de manzanas maduras durante tiempos variables, dependiendo de las condiciones climáticas (en estudios de campo) y/o de temperatura (en ensayos *in vitro*). En general, la supervivencia se prolonga a bajas temperaturas (4ºC) y condiciones de elevada humedad (Ordax *et al.,* 2009). No obstante, e independientemente de lo factible de la dispersión de *E. amylovora* a través de estos órganos, la coexistencia de frutos maduros contaminados y árboles con síntomas de fuego bacteriano en campo ocurre (Thomson, 2000), por lo que las poblaciones epífitas del patógeno también en este caso podrían contribuir a la dispersión de la bacteria a plantas sanas, mediada por insectos atraídos por estos frutos, lluvia, etc.

4.2. Supervivencia en fase endófita

Supervivencia en chancros. Los chancros se consideran una de las principales fuentes de inóculo de *E. amylovora* (Thomson, 2000; Kielak *et al.,* 2002; van der Zwet *et al.,* 2012; Aćimović *et al.,* 2014). Por lo tanto, la capacidad de *E. amylovora* para sobrevivir en estas estructuras durante el invierno es una característica del patógeno, esencial para el desarrollo de brotes de fuego bacteriano en los años siguientes a la formación de los chancros (Thomson, 2000; Kielak *et al.,* 2002).

Se han descrito dos tipos de chancros en base al aspecto de los bordes que los limitan: definidos o indefinidos. Los primeros se suelen originar como fruto de las infecciones tempranas, a inicios de la primavera. La multiplicación de *E. amylovora* en flores, frutos inmaduros y/o brotes tiernos, origina los primeros signos de fuego bacteriano en brotes. El patógeno avanza a través de los tejidos suculentos de la planta y/o del xilema, y llega hasta tejidos lignificados de brotes y ramas. Una vez allí, y como consecuencia de la respuesta del hospedador a la infección, se produce una callosificación de los tejidos y la zona afectada por el patógeno queda rodeada por una barrera lignificada y suberizada de peridermo. Estos chancros suelen caracterizarse por poseer los bordes bien definidos y frenar eficientemente el avance de *E. amylovora*. La detección del patógeno en estos chancros en momentos posteriores a su formación suele ser negativa (van der Zwet *et al.,* 2012; Aćimović *et al.,* 2014).

Las infecciones tardías, al final de la primavera y durante el verano y/u otoño, originan chancros con características diferentes a los mencionados anteriormente. Tanto las condiciones climáticas, como el estado fenológico de la planta hospedadora en esta época favorecen la aparición de chancros

de bordes indefinidos, sin formación de callos, más pequeños (pueden llegar a tener 2-5 mm de diámetro) y más difíciles de detectar que los chancros con bordes definidos (van der Zwet *et al.,* 2012; Aćimović *et al.,* 2014). La localización de las bacterias en este tipo de chancros suele quedar restringida a los bordes, y las condiciones en los mismos permiten, generalmente, la supervivencia de *E. amylovora* durante meses, hasta la llegada de condiciones favorables para su crecimiento (Thomson, 2000; van der Zwet *et al.,* 2012; Aćimović *et al.,* 2014).

Supervivencia en brotes y yemas. La presencia de *E. amylovora* como organismo endófito en brotes y yemas asintomáticos ha sido ampliamente documentada (Thomson, 2000; van der Zwet *et al.,* 2012; Tancos *et al.,* 2016). Este modo de vida se considera uno de los mecanismos que permiten la supervivencia del patógeno durante la parada vegetativa de la planta hospedadora en invierno. No obstante, se desconoce si la bacteria en estas condiciones es capaz de multiplicarse, o simplemente sobrevive, y también si estas células endófitas serían capaces de generar síntomas de fuego bacteriano en respuesta a algún estímulo (Crepel *et al.,* 1996; Thomson, 2000; Billing, 2011; van der Zwet *et al.,* 2012). La información con respecto a esto último es contradictoria. Existen datos de campo que sugieren que las infecciones latentes en brotes y/o yemas asintomáticas podrían ser la única fuente de inóculo primario en algunas epidemias de fuego bacteriano. Sin embargo, los datos experimentales parecen indicar la aparente incapacidad de las células endófitas de *E. amylovora* para producir síntomas en la planta hospedadora (Thomson, 2000). En cuanto al origen de estas infecciones latentes, mediante estudios de inoculación artificial se ha determinado que las poblaciones endófitas de *E. amylovora* en brotes ocurre durante o tras la formación de chancros. La primavera siguiente, el patógeno se puede detectar

en brotes originados a partir de yemas situadas debajo de dichos chancros (Ge & van der Zwet., 1996).

Además del modo de vida endófito en material vegetal asintomático, *E. amylovora* es capaz de sobrevivir en frutos momificados (Thomson, 2000). Recientemente también se ha demostrado la capacidad del patógeno para sobrevivir durante periodos prolongados en hojas del hospedador ya necrosadas, principalmente en el interior del raquis o nervio central y, en un porcentaje más reducido, en el interior de nerviaciones laterales (Sobiczewski *et al.,* 2014). Aunque en este último caso, los autores del trabajo sugieren la clasificación de *E. amylovora* como organismo necrotrofo, tal afirmación solo sería posible si, aparte de sobrevivir, se confirmase también su capacidad para obtener nutrientes y multiplicarse a partir del tejido necrosado.

4.3. Supervivencia en insectos

Los insectos son, probablemente, los vectores más importantes de *E. amylovora,* habiéndose relacionado con la transmisión del patógeno alrededor de 90 especies distintas (van der Zwet *et al.,* 2012). Las abejas, junto con insectos perforadores y/o succionadores juegan un papel importante en la transmisión secundaria del patógeno, de flor a flor, o de órganos sintomáticos a órganos sanos (Thomson, 2000; Sabatini *et al.,* 2006; van der Zwet *et al.,* 2012; Peusens *et al.,* 2013). Las moscas y las hormigas, que se sienten fuertemente atraídas por los exudados, tendrían un papel más importante en la transmisión primaria de *E. amylovora,* desde los chancros a tejidos sanos de la misma u otra planta (Thomson, 2000; van der Zwet *et al.,* 2012).

Dada la importancia de las flores en el desarrollo de brotes de fuego bacteriano, desde los primeros estudios de la enfermedad las abejas y otros insectos polinizadores se consideraron potenciales reservorios del patógeno (van der Zwet *et al.,* 2012). No obstante, la supervivencia de *E. amylovora* en estos insectos (un máximo de 48 h) y distintas partes de las colmenas (30 h, 36 h y 60 h en miel, cera de abejas y polen, respectivamente) es reducida, por lo que su posible papel como reservorios del patógeno durante el invierno se ha descartado (Thomson, 2000; Alexandrova *et al.,* 2002a, b).

La temperatura ambiental parece influir significantemente en la supervivencia de *E. amylovora* en abejas, que se ve favorecida a temperaturas bajas y/o templadas, con respecto a temperaturas iguales o superiores a 28ºC (Alexandrova *et al.,* 2002b). Estos resultados probablemente sean extrapolables a otros insectos vectores.

La supervivencia de *E. amylovora* también se ha determinado en otros insectos de distintos géneros. Por ejemplo, el patógeno sobrevivió durante 3 días en un colémbolo del suelo (*Folsomia candida*), 5 días en la crisopa verde (*Chrysoperla carnea*) y hasta 12 días en el áfido del manzano (*Aphis pomi*) (Hildebrand *et al.,* 2000; 2001).

4.4. Supervivencia en suelo

E. amylovora se considera un patógeno de la parte aérea de la planta, aunque la lluvia, viento, etc., podrían conducirlo al suelo. La presencia de *E. amylovora* en este medio se ha sugerido en diversas ocasiones, tras el aislamiento de bacteriófagos activos frente al patógeno del suelo, debajo de árboles afectados por fuego bacteriano (Thomson, 2000; van der

Zwet *et al.,* 2012; Born *et al.,* 2014). La supervivencia de *E. amylovora* en suelo depende de diversos factores, y se reduce considerablemente en presencia de la microbiota autóctona de este ambiente (Hildebrand *et al.,* 2001). La opinión más extendida entre los investigadores es que la probabilidad de transmisión del patógeno desde el suelo a la planta hospedadora es reducida, aunque podría incrementarse en viveros y otro tipo de instalaciones donde las hojas de las plantas se encuentran cerca del suelo (Bogs *et al.,* 1998; Thomson, 2000; Hildebrand *et al.,* 2001; van der Zwet *et al.,* 2012).

5. Mecanismos de supervivencia bacteriana en ambientes oligotróficos

La distribución de las especies vegetales en la naturaleza hace del entorno un ambiente nutritivo discontinuo para las bacterias fitopatógenas. Sin embargo, los campos de cultivo y viveros, aunque también ofrecen separación entre los distintos cultivos, proporcionan a los patógenos una mayor densidad de plantas susceptibles, lo que facilita su persistencia y también su transmisión. Los cambios de estación y/o climáticos, que afectan tanto a la planta hospedadora como al patógeno, así como el estado fenológico de la planta, repercutirán también sobre el crecimiento del patógeno y su persistencia en el ambiente. En relación a esto, determinadas prácticas agronómicas como el cultivo en invernadero ofrecerán a los patógenos condiciones más estables y favorables para su multiplicación. Otro factor igualmente importante que determinará el éxito de un patógeno será su capacidad para sobrevivir fuera del hospedador (que también dependerá de sus propias características fisiológicas) y/o la presencia de vectores. Finalmente, y teniendo en cuenta todas las condiciones mencionadas anteriormente, el éxito de las bacterias fitopatógenas en la naturaleza dependerá también de la cantidad de inóculo que son capaces de producir cuando las condiciones lo permiten. En general, dados los breves tiempos de generación en bacterias, la supervivencia de una pequeña población bacteriana puede producir en poco tiempo una epidemia, si las condiciones son propicias (Schuster & Coyne, 1974).

5.1. Características de los ambientes naturales

Los patógenos no obligados, como *E. amylovora,* pasan por periodos en los que encuentran condiciones ideales para su multiplicación, y otros en los que tienen que enfrentarse a condiciones ambientales adversas para sobrevivir. Uno de los factores ambientales que influyen de manera más determinante en la supervivencia de las bacterias es la escasez de nutrientes. De hecho, dadas las características oligotróficas de la mayoría de ambientes naturales, los patógenos, y los microorganismos en general, difícilmente encuentran condiciones óptimas para su crecimiento, siendo la inanición la condición fisiológica predominante (Roszak & Colwell, 1987; Morita, 1997; Edwards, 2000; Navarro-Llorens *et al.,* 2010).

La presión selectiva ejercida por la escasez nutricional, junto con la competencia por los recursos entre los microorganismos heterótrofos que cohabitan en un mismo nicho ecológico han favorecido, a lo largo de la evolución, la aparición de nuevas especies con distintos requerimientos nutricionales, así como interacciones sintróficas entre las mismas que disminuyen el solapamiento en el uso de los escasos recursos. Adicionalmente, la oligotrofia ambiental ha sido también uno de los factores clave para la aparición de estrategias alternativas para la obtención de nutrientes, como la depredación o las simbiosis mutualistas, comensalistas o parasíticas (Morita, 1997). En el caso de los patógenos no obligados, además, la escasez nutricional ambiental juega un papel importante en la diversificación de la estructura de las poblaciones, así como en la evolución de las estrategias de virulencia (Walther & Ewald, 2004; Sundberg *et al.,* 2014; Cressler *et al.,* 2016).

En función de los requerimientos nutricionales podemos clasificar las especies bacterianas en *oligotrofas*, si son capaces de proliferar utilizando los nutrientes presentes en bajas concentraciones; *copiotrofas*, si necesitan concentraciones elevadas de nutrientes para multiplicarse; u *oligotrofas facultativas*, si son capaces de crecer en ambas condiciones (Schut *et al.,* 1997). De acuerdo con esta clasificación, parece lógico pensar que las concentraciones de nutrientes presentes en el ambiente supondrán un problema, principalmente, para las bacterias copiotrofas. No obstante, la presencia de este tipo de bacterias en la naturaleza no es nada despreciable (Morita, 1997; Schut, 1997), y son las mismas condiciones ambientales las que han determinado la aparición de mecanismos de adaptación que posibilitan su persistencia en el entorno durante periodos más o menos prolongados, hasta que encuentran condiciones adecuadas para su crecimiento. Dichas estrategias, también presentes en arqueas, abarcan desde cambios en la composición química, adaptaciones morfológicas, de tamaño y moleculares, que permiten la subsistencia en el ambiente mediante la adopción de un estado de supervivencia en inanición, o por la producción de esporas o células en estado de dormición (células VBNC, persistentes, etc) (Morita, 1997; Byrd, 2000; Gauthier, 2000; Chen *et al.,* 2009; Fida *et al.,* 2013; Winters *et al.,* 2015), que funcionan a modo de banco de semillas y permiten a las bacterias sobrevivir durante periodos prolongados, resistiendo la inanición y otros tipos de estrés.

5.2. Supervivencia en estado de inanición

Los cambios metabólicos, celulares y genéticos asociados a las respuestas bacterianas a la oligotrofia natural son muy variados, y equiparables en muchos aspectos a los observados durante la entrada en la fase estacionaria de crecimiento

(Navarro-Llorens *et al.,* 2010). En este apartado se describen algunas de las respuestas bacterianas más importantes a la escasez nutricional característica de la mayoría de ecosistemas y que constituye una de las principales limitaciones para el crecimiento y la supervivencia de los patógenos en el medio ambiente (Morita, 1997; Edwards, 2000; Hobbie & Hobbie, 2013).

5.2.1. Patrones de respuesta a condiciones de inanición

Basándose en datos de cultivabilidad, Morita (1985) describió cuatro patrones bacterianos de respuesta a condiciones de inanición (Fig. 13):

i) El primer patrón (A) consiste en un incremento progresivo del número de células cultivables (por un proceso de división reductiva) durante las siguientes horas a la exposición a escasez de nutrientes, hasta alcanzar un número de células estable, superior al inicial, y que se mantiene a lo largo del tiempo.

ii) El segundo patrón (B) es el menos frecuente, y consiste en el mantenimiento inalterado de la cultivabilidad y el tamaño celular, a lo largo del tiempo.

iii) El tercer patrón (C) consiste en un incremento inicial del número de células por un proceso de división reductiva, seguido de una progresiva pérdida de cultivabilidad, hasta alcanzar niveles estables por debajo de los iniciales.

iv) El cuarto patrón (D) consiste en una pérdida de cultivabilidad inicial y una estabilización del número de células cultivables que se mantiene a lo largo del tiempo (o que decrece muy lentamente).

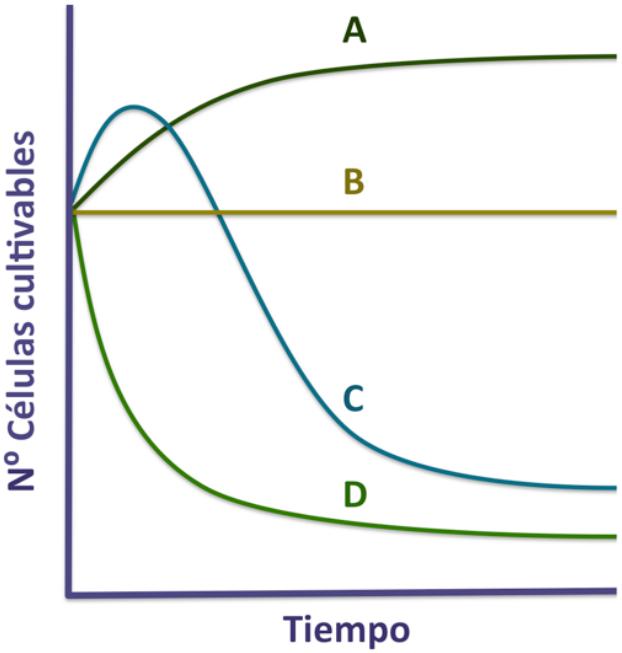


Fig. 13. Patrones de supervivencia en condiciones de inanición. Modificado de Morita (1997).

5.2.2. Cambios morfológicos y de tamaño

El acceso a los nutrientes es uno de los principales factores que han influido evolutivamente sobre el tamaño y la forma de las bacterias (Morita, 1997; Young, 2006; 2007). Muchas especies bacterianas modifican su tamaño y/o morfología en función de la fase de crecimiento y/o de las condiciones nutricionales del medio extracelular (Young, 2006; Yang *et al.,* 2016). Además de las ventajas nutricionales, los cambios morfológicos pueden ofrecer también ventajas de otro tipo, como una mayor protección frente a la depredación o, en el caso de los patógenos, frente a las defensas del hospedador (Byrd, 2000; Nyström, 2004; Yang *et al.,* 2016). Algunas de las respuestas celulares más importantes a la limitación nutricional

son la reducción de tamaño, la adquisición de forma cocoide, la formación de vesículas (McBroom & Kuehn, 2006; Kulp & Kuehn, 2010) y/o la formación de filamentos (Young, 2006; 2007).

Reducción de tamaño o "dwarfing" y adopción de forma cocoide. Muchas especies bacterianas sufren división reductiva al ser expuestas a condiciones oligotróficas (Morita, 1997). La reducción del tamaño tiene ventajas con respecto a la captación de nutrientes, ya que permite reducir el volumen de la célula sin una disminución considerable de la superficie. Esta relación entre superficie y volumen se maximiza cuando las células adoptan forma cocoide, un fenómeno que acompaña frecuentemente la reducción de tamaño (Young, 2006; 2007). Algunas especies bacterianas incapaces de multiplicarse en ambientes limitantes en nutrientes reducen su tamaño mediante la digestión de la pared celular y la membrana interna, lo que genera pliegues en la membrana externa (Nyström, 2004). Esta estrategia tiene la ventaja añadida del aprovechamiento de los recursos de la propia célula como fuente de energía. Además, los pliegues o vesículas originados en la superficie permiten multiplicar el área de absorción de nutrientes sin incrementar el volumen de la célula, lo que es especialmente práctico en ambientes pobres en nutrientes.

Formación de vesículas. La liberación de vesículas de membrana externa, OMVs (del inglés *Outer Membrane Vesicles*) en bacterias Gram negativas se considera un tipo de respuesta al estrés (McBroom & Kuehn, 2006, Kulp & Kuehn, 2010). Estas OMVs tienen un tamaño que oscila entre 20 y 300 nm, están compuestas de membrana externa y material periplásmico y se liberan de la superficie celular sin afectar a la integridad de la membrana. El estrés nutricional puede inducir la formación de OMVs, a las que se atribuye un papel en el transporte de nutrientes esenciales o la producción de enzimas generadoras

de nutrientes (Kulp & Kuehn, 2010). Una vez liberadas, las OMVs también pueden actuar como vehículos de transporte intercelular. Los componentes asociados a ellas, como proteínas activas, pueden intervenir en distintos procesos biológicos (Kulp & Kuehn, 2010; Tashiro *et al.,* 2012).

Filamentación. Otra respuesta característica de muchas especies bacterianas a las concentraciones limitantes en uno o varios nutrientes es la filamentación o elongación de las células sin incrementar el diámetro. Para ello las células crecen como si fueran a dividirse, pero no se produce el proceso de septación, con la consecuente formación de células elongadas con varias copias de su material genético (Young, 2006; Justice *et al.,* 2008). Como resultado de la filamentación se incrementa la superficie de la célula destinada a la captación de nutrientes sin que se modifique notablemente la relación superficie/volumen (Young, 2006).

5.2.3. Uso de las reservas metabólicas y cambios en la composición de lípidos de membrana

El metabolismo endógeno (sin incorporación de elementos del exterior) permite a las células reordenar su estructura interna e iniciar procesos fisiológicos que favorecen la supervivencia en el ambiente durante periodos prolongados, en ausencia de nutrientes (Kieft, 2000).

Tanto los azúcares simples como los polímeros de reserva son consumidos rápidamente en entornos pobres en carbono (Hood *et al.,* 1986; Kadouri *et al.,* 2005). Tras el consumo de azúcares y polímeros de reserva tiene lugar la degradación de parte del contenido en lípidos, ácidos nucleicos y proteínas (Hood *et al.,* 1986; Kieft, 2000; Zundel *et al.,* 2009). El grado en que el

ADN y ARN endógenos son degradados varía mucho en función de las especies analizadas (Kieft, 2000), no obstante, en la mayoría de los casos ambos tipos de ácidos nucleicos son consumidos hasta llegar a unos niveles basales estables trascurridos días o semanas de exposición a condiciones de limitación nutricional (Kieft, 2000). Finalmente, el estrés nutricional induce también cambios en la composición de fosfolípidos de membrana: se incrementa el porcentaje de ácidos grasos de tipo ciclopropilo, y también de ácidos grasos saturados con respecto a los insaturados (Guckert *et al.,* 1986; Fida *et al.,* 2013). Además, dentro de los ácidos grasos monoinsaturados, aumenta la proporción de ácidos trans- con respecto a los cis- (Guckert *et al.,* 1986; Rice & Oliver, 1992). La función de estos cambios es, probablemente, la estabilización de la estructura de las membranas en condiciones de estrés, aunque se relacionan también con los cambios morfológicos y/o de tamaño de las células que ocurren en respuesta a estrés nutricional (Kieft, 2000).

5.2.4. Optimización de la captación de nutrientes y los recursos metabólicos

Las bacterias en ambientes oligotróficos ralentizan su metabolismo y destinan prácticamente toda la maquinaria celular a la optimización de la captación de nutrientes. En estas condiciones las células reducen el nivel de represión catabólica, lo que permite la utilización de múltiples rutas metabólicas para el aprovechamiento simultáneo de distintos tipos de sustratos. Como consecuencia las células sometidas a inanición poseen la habilidad de utilizar una mayor variedad de sustratos que las células no estresadas (Morita, 1997).

Adsorción de nutrientes a la superficie celular. Para incrementar la captación de nutrientes las bacterias emplean

proteínas de membrana, algunas de las cuales son capaces de funcionar con concentraciones muy bajas de sustrato. Aunque entre estas encontramos receptores de quimiotaxis o transportadores transmembrana, la mayoría de proteínas implicadas en la captación de nutrientes carecen de actividad enzimática, y su función principal es la unión específica a determinados sustratos, con la consecuente formación de reservas de los mismos en la superficie o el periplasma de las células (Morita, 1997).

Reducción de la difusión pasiva de nutrientes al medio extracelular. Para evitar la pérdida de nutrientes como el NH_3 (capaz de atravesar membranas) por difusión pasiva, las bacterias sintetizan sistemas de transporte específicos que permiten la reincorporación de estos compuestos al interior de la célula (Shimizu, 2014).

Incorporación de moléculas complejas. Cuando las concentraciones de azúcares y/o aminoácidos son limitantes, las células intensifican el transporte de sustratos al interior celular e incrementan la variedad de sustratos que son capaces de utilizar (Morita, 1997), mostrando predilección por aminoácidos, que pueden utilizar como fuente de carbono y nitrógeno y cuya incorporación directa para la síntesis de proteínas supone un ahorro energético importante para la célula (Ayo *et al.,* 2001). Para ello, las bacterias sometidas a oligotrofia sobreexpresan sistemas de transporte de los que ya poseen normalmente (Morita, 1997; Schut *et al.,* 1997), y también otros diferentes, con especificidad por nuevos sustratos, muchos de los cuales no son asimilables en condiciones nutricionales óptimas (Morita, 1997).

5.2.5. Fenómenos de movilidad y quimiotaxis

Las bacterias móviles están presentes en prácticamente todos los ecosistemas. Generalmente poseen múltiples sistemas quimiotácticos, que se componen de uno o varios flagelos, motores, bombas de iones y receptores. Dichos sistemas consumen gran cantidad de energía, y su expresión depende de señales externas como la luz o la presencia de nutrientes (Mitchell, 2002). Precisamente por su gran demanda energética, aunque pueden permitir la rápida localización de fuentes de nutrientes, los costes de la movilidad en ambientes oligotróficos pueden ser demasiado elevados. Se han descrito varias estrategias para optimizar el uso de la movilidad en ambientes pobres en nutrientes:

Sistemas alternativos de energía flagelar. Los sistemas flagelares de la mayoría de bacterias funcionan con energía proveniente de la fuerza protomotriz (FPM), o Na^+-motriz. No obstante, se ha descrito un sistema alternativo que dota de movilidad al motor flagelar en situaciones en las que la FPM es cero. Dicho sistema emplea energía procedente de la cadena respiratoria y/o la ATPasa, y podría servir para huir de zonas pobres en nutrientes, hasta encontrar condiciones más favorables para el crecimiento (Eisenbach, 1990).

Búsqueda activa de nutrientes. En muchas bacterias el estrés nutricional estimula tanto la síntesis de flagelos como el quimiotactismo positivo hacia zonas con una mayor concentración de nutrientes (Terracciano & Canale-Parola, 1984; Wei & Bauer, 1998; Zhao *et al.,* 2007; Stocker *et al.,* 2008). Este comportamiento es especialmente interesante en entornos donde los nutrientes se distribuyen de forma heterogénea o en parches y posibilita el crecimiento y la multiplicación de las

bacterias, aun cuando la cantidad media de nutrientes en el entorno es baja (Blackburn *et al.,* 1998; Stocker *et al.,* 2008).

Activación momentánea de la movilidad. En otros casos la oligotrofia activa la síntesis de flagelos y las respuestas quimiotácticas, pero solo durante un periodo más o menos prolongado. Conforme avanza el tiempo el número de células móviles disminuye, y aparecen subpoblaciones de células con flagelos inmóviles y/o con un número menor de flagelos, hasta la pérdida total de movilidad y/o flagelos (Wei & Bauer, 1998).

Inhibición de la movilidad. Existen especies bacterianas en las que la escasez nutricional inhibe directamente la movilidad flagelar, y las células estresadas aguardan inmóviles hasta detectar nutrientes en el entorno, momento en el cual recuperan la movilidad (Morita, 1997).

5.2.6. Adhesión a superficies y formación de biopelículas

La oligotrofia ambiental induce en muchas bacterias marinas, del suelo y epífitas la modificación de sus características celulares para incrementar la adsorción a superficies (Morita, 1997; Kieft, 2000; Monier & Lindow, 2004). La ventaja de vivir en superficies o en agregados bacterianos con respecto al modo de vida libre radica en la tendencia de los nutrientes a acumularse sobre las superficies o en torno a material particulado en suspensión (Schut *et al.,* 1997; Kieft, 2000; Azúa *et al.,* 2007).

La unión de las bacterias al sustrato o a otras bacterias comienza con el incremento de la hidrofobicidad celular, y puede volverse irreversible con la producción extracelular de

polisacáridos (Morita, 1997; Kieft, 2000). En muchas bacterias del suelo que pasan parte de su vida ancladas al sustrato, no obstante, el agotamiento de los recursos estimula la eliminación de las matrices exopolisacarídicas, que se emplean como fuente de alimento. Como consecuencia, las células pueden dispersarse en busca de nutrientes (Kieft, 2000).

La adhesión de las bacterias a las superficies es el paso previo a la formación de biopelículas. Dichas estructuras suelen estar compuestas por varias especies bacterianas englobadas por una matriz polisacarídica y tanto flagelos como fimbrias y curli son necesarios para su formación (Pratt & Colter, 1998; Koczac *et al.,* 2011; Castiblanco & Sundin, 2016). Durante la formación de las biopelículas también se dan uniones entre células, mediadas por adhesinas y EPS como el PGA (poli-β-1,6-N-acetil-D-glucosamina) (Shimizu, 2014). Las biopelículas proporcionan protección frente a la predación, condiciones ambientales adversas y/o compuestos tóxicos. Los integrantes de las biopelículas también pueden beneficiarse de capacidades nutricionales cooperativas o sintróficas, lo que favorece un mayor aprovechamiento de los recursos disponibles, aunque sean escasos (Morita, 1997; Young, 2006). En el caso de bacterias simbiontes (mutualistas, comensales o parasíticas) de animales y de plantas, además, la adhesión a superficies y la formación de biopelículas constituyen importantes elementos para la supervivencia en el ambiente y/o durante el proceso infeccioso, incluyendo la protección frente a los sistemas de defensa del hospedador (Parsek & Singh, 2003; Danhorn & Fuqua, 2007; Castiblanco & Sundin, 2016).

5.3. Control genético de la respuesta a la inanición

El éxito de las bacterias en la naturaleza reside en su capacidad para responder rápidamente ante fluctuaciones ambientales. Esto es posible gracias a sistemas de transducción de señales y reguladores intracelulares que permiten la adaptación a las condiciones del entorno mediante cambios en los patrones de expresión génica.

Las adaptaciones de las bacterias a la oligotrofia natural tienen como consecuencia una resistencia aumentada a múltiples tipos de estrés, lo que puede tener importantes implicaciones en estudios con patógenos y en la correcta evaluación de métodos de erradicación, compuestos antimicrobianos, etc.

Gran parte de los cambios o adaptaciones que ocurren en las células ante determinados estímulos se deben a la acción de factores sigma alternativos. Estas proteínas, presentes tanto en bacterias Gram positivas como en Gram negativas, se expresan en función de condiciones ambientales o intracelulares concretas, se unen a la ARN polimerasa y modifican su afinidad por los promotores génicos, redirigiendo la expresión en función de las necesidades de la célula. Algunos ejemplos de factores sigma alternativos en *Escherichia coli* se recogen en la Tabla 4. La Fig. 14. muestra un esquema del funcionamiento de los factores sigma alternativos.

Tabla 4. Factores sigma (σ) en *E. coli* y sus funciones (basada en Navarro-Llorens *et al.,* 2010, y Cook & Ussery, 2013).

Gen	Producto	Función
rpoD	σ^{70} (σ^{D})	Mantenimiento celular
rpoH	σ^{H} (σ^{32})	Choque térmico
rpoE	σ^{E} (σ^{24})	Choque térmico; estrés periplásmico (ECF)
fliA	σ^{F} (σ^{28})	Movilidad mediada por flagelos
rpoS	σ^{S} (σ^{38})	Adaptación a condiciones de inanición
rpoN, glnF	σ^{N} (σ^{54})	Genes regulados por nitrógeno
fecI	σ^{fecI}	Captación de citrato férrico (ECF)

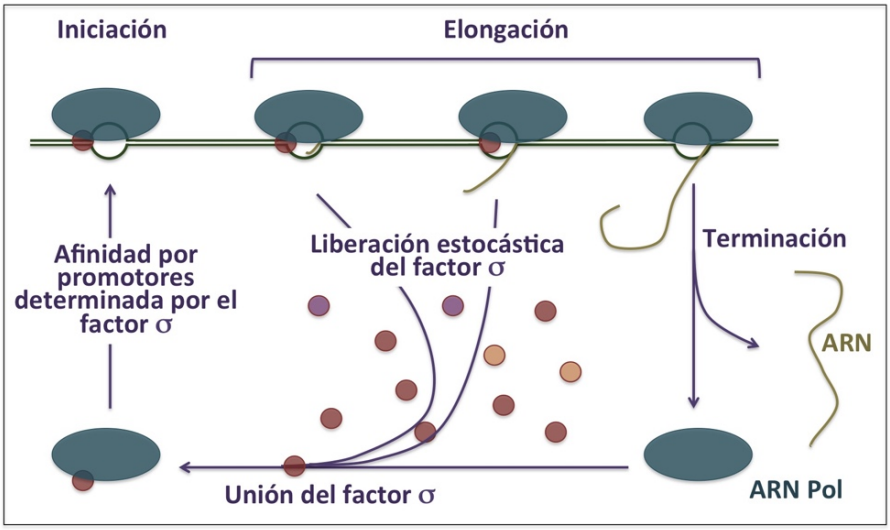


Fig. 14. Reprogramación de la ARN polimerasa (ARN Pol) mediada por factores sigma (σ). Distintos factores σ (círculos de colores) compiten por la unión a la ARN Pol. Las condiciones ambientales y/o fisiológicas determinarán la abundancia de uno u otro factor σ. La unión del factor sigma modula la afinidad de la ARN Pol por promotores de genes determinados, favoreciendo el inicio de su expresión. Durante la fase de elongación de la síntesis de ARN mensajero, el factor σ se libera y se incorpora al pull de factores σ en la célula, mientras la ARN Pol finaliza la transcripción del gen y se libera del ADN para dar comienzo a un nuevo ciclo. Modificado de Österberg *et al* (2011).

5.3.1. El factor sigma alternativo RpoS

Muchos de los cambios celulares y fisiológicos observados en las células bacterianas sometidas a inanición o durante la transición a la fase estacionaria son promovidos por el factor sigma alternativo RpoS, también denominado sigma S o sigma 38 (Hengge-Aronis, 2000; 2002; Navarro-Llorens *et al.,* 2010; Battesti *et al.,* 2011). En *E. coli,* este factor controla, entre otros, genes implicados en las respuestas celulares a diversos tipos de estrés, incluyendo el estrés osmótico, el choque ácido o térmico (por altas y bajas temperaturas) o el estrés oxidativo (Loewen *et al.,* 1998). Además, RpoS modula la expresión de genes relacionados con la morfología y la división celular, proteínas de unión y transporte de sustratos, el metabolismo, la regulación génica, la replicación, recombinación, modificación y reparación del ADN, la producción de polímeros de reserva, la virulencia, etc (Ishihama, 1997; 2000; Hengge-Aronis, 2002; Vijayakumar *et al.,* 2004; Kazmierczak *et al.,* 2005). Como consecuencia, debido a los cambios de expresión mediados por RpoS, las células bacterianas expuestas a oligotrofia o en fase estacionaria no sólo son capaces de adaptarse a condiciones ambientales desfavorables si no que, además, incrementan su resistencia a otros tipos de estrés a los cuales todavía no han sido expuestas. Este rasgo fenotípico, denominado respuesta general al estrés, ha sido descrito en un gran número de especies bacterianas, aunque principalmente en patógenos humanos (Morita, 1997; Hengge-Aronis, 2002; Weber *et al.,* 2005; Battesti *et al.,* 2011).

5.3.2. Señalización intracelular mediada por ppGpp

Otra molécula importante en las respuestas celulares a inanición es el nucleótido modificado ppGpp (guanosina-3,5-bispirofosfato). Esta molécula, considerada una hormona de alarma celular o alarmona, se acumula en el citoplasma celular ante una multitud de señales ambientales, incluyendo la limitación en aminoácidos, carbono, fósforo, etc., y promueve el cese del crecimiento, reajustes metabólicos y la puesta en marcha de mecanismos de defensa frente al estrés y de adaptación a las condiciones ambientales (Fig. 15).

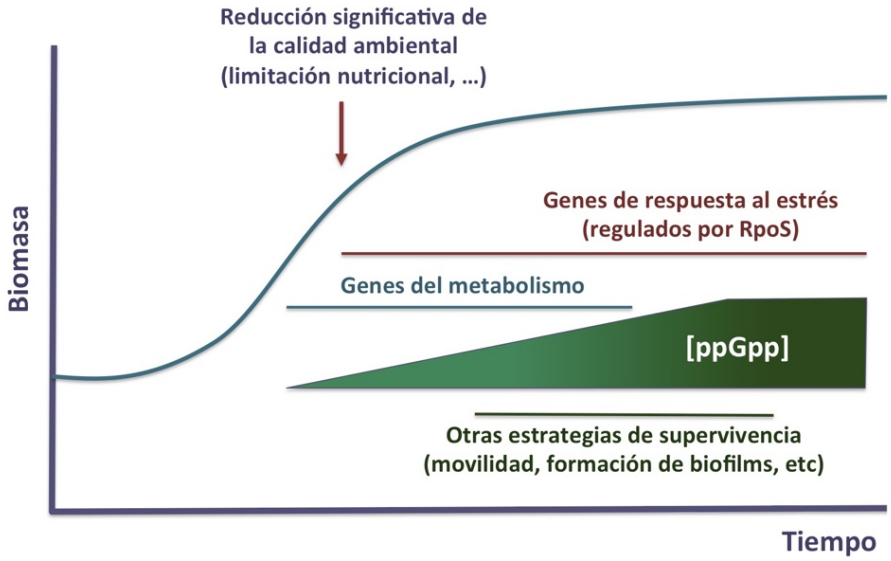


Fig. 15 Modelo de la inducción secuencial de distintas funciones celulares mediada por ppGpp, tras una reducción significativa de la calidad ambiental. La expresión de genes del metabolismo y de respuesta al estrés se activa de forma secuencial con el incremento en la concentración de la alarmona ppGpp en el citoplasma. Otras funciones relacionadas con la adaptación al entorno son reguladas por niveles intermedios de la alarmona. Modificado de Balsalobre (2011).

En *E. coli,* las proteínas encargadas de ajustar las concentraciones de la alarmona ppGpp son RelA y SpoT. Ante una limitación nutricional en aminoácidos RelA sintetiza ppGpp a partir de ATP y GDP o GTP. SpoT, sin embargo, tiene una actividad basal como ppGpp hidrolasa, lo cual favorece el mantenimiento de unos niveles mínimos de la alarmona en condiciones óptimas de crecimiento. Ante una limitación en carbono, fósforo, hierro o ácidos grasos se inhibe la actividad ppGpp hidrolasa de SpoT y la misma proteína adquiere actividad ppGpp sintetasa, lo cual incrementa los niveles citoplasmáticos de la alarmona. El nucleótido modificado ppGpp participa en la regulación de infinidad de procesos en múltiples especies bacterianas, como la esporulación, la movilidad, la formación de biopelículas, etc (Navarro-Llorens *et al.,* 2010; Balsalobre, 2011).

5.3.3. Defensa frente al estrés oxidativo: OxyR y Dps

Una de las consecuencias más importantes de la inanición sobre la fisiología de las bacterias es la acumulación de ROS a nivel intracelular (McDougald *et al*., 2002; Boaretti *et al*., 2003; Nyström, 2004). Las células bacterianas, por tanto, deben poseer mecanismos para proteger las biomoléculas diana (ADN, ARN, fosfolípidos, proteínas) de la oxidación. Un elemento clave en la protección frente a las ROS intracelulares son los enzimas antioxidantes como las catalasas, peroxidasas, etc. En *E. coli* y otras enterobacterias, muchos de estos enzimas son regulados por RpoS y, de forma independiente, por el factor transcripcional OxyR (Ivanova *et al.,* 1994). Esta proteína, controlada también por RpoS durante la entrada a la fase estacionaria, se induce junto a RpoS en condiciones de escasez nutricional. OxyR controla diversos genes relacionados con la defensa frente al estrés oxidativo y se sobreexpresa tras la exposición de las células a agentes oxidantes como el H_2O_2 (Storz & Tartaglia,

1992; Schellhorn, 1995; Nyström, 2004). Adicionalmente, OxyR activa la síntesis del ARN no codificante *oxyS,* lo que posibilita la regulación indirecta de alrededor de 40 productos génicos (Altuvia *et al.,* 1997).

Otro elemento importante para la protección de las bacterias de los efectos negativos de la inanición es la proteína Dps (del inglés *DNA-binding Protein during Starvation*). Dicha proteína se sobreexpresa en condiciones de limitación nutricional y también durante la exposición a estrés oxidativo, y su función es la protección del ADN de las ROS producidas durante el estrés. El control de la expresión de Dps es llevado a cabo por varias proteínas, incluyendo el factor sigma alternativo RpoS y el factor transcripcional OxyR (a través del factor sigma 70) (Fig. 16).

La acción protectora de Dps se efectúa por tres vías: *i)* afecta la regulación de la expresión de diversos genes; *ii)* una vez sintetizada forma dodecámeros con otros monómeros de Dps y promueve la condensación del ADN mediante la unión inespecífica con esta molécula, originando un complejo nucleoproteico altamente estable y denominado biocristal; *iii)* Dps posee homología estructural con las ferritinas, las cuales, por un lado, secuestran átomos de ion ferroso (Fe^{2+}) con la consecuente reducción de la formación de ROS vía la reacción de Fenton ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \cdot OH$) y, por otro lado, neutralizan peróxidos peligrosos gracias a su actividad ferroxidasa ($2\ Fe^{2+} + H_2O_2 + H_2O_2 \rightarrow 2FeOOH + 4H^+$) (Navarro-Llorens *et al.,* 2010).

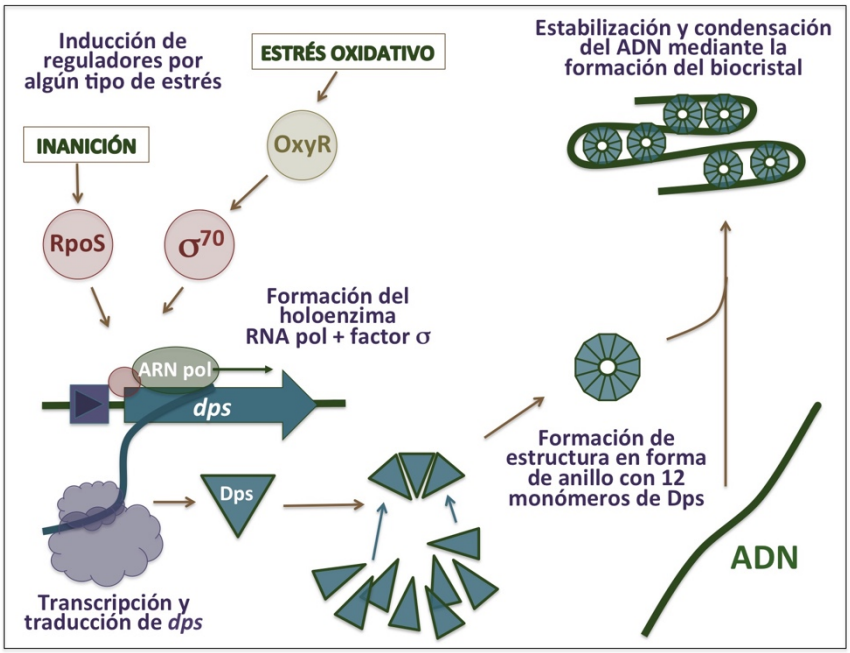


Fig. 16. Modelo simplificado de la regulación de la expresión del gen *dps* y la formación del biocristal. Tanto la inanición como la exposición a estrés oxidativo estimulan la expresión del gen *dps.* En el primer caso, el responsable de la regulación es el factor sigma RpoS. En el segundo, el control de la expresión de *dps* lo lleva a cabo OxyR a través del factor sigma 70. Tras la formación de Dps, tiene lugar la asociación de 12 monómeros y la unión al DNA, formando el biocristal, una nucleoproteína muy estable que permite la condensación del material genético y evita daños en el material genético. Basado en Navarro-Llorens *et al* (2010).

5.4. Dormición bacteriana

Aunque las bacterias responden de múltiples formas a la escasez nutricional, la duración de los periodos de inanición en muchas ocasiones es impredecible. La consecuencia evolutiva ha sido la aparición de formas bacterianas de resistencia que actúan de forma similar a los bancos de semillas en las plantas, es decir, como reservorios ambientales de individuos resistentes

al estrés, capaces de recuperar su estado fisiológico normal tras ser expuestas a determinados estímulos ambientales (Lennon & Jones, 2011).

A parte de la formación de esporas, característica de importantes géneros bacterianos como *Bacillus* o *Clostridium,* los mecanismos bacterianos de resistencia o estados de dormición más conocidos entre las bacterias no esporuladas son la persistencia y el estado VBNC. Aunque ambos estados son diferenciables experimentalmente (Orman & Brynildsen, 2013b), son más sus similitudes que sus diferencias, y actualmente se ha propuesto la hipótesis de que, tanto la persistencia bacteriana como el estado VBNC constituyen varios grados de un mismo proceso de dormición bacteriana (Ayrapetyan *et al.,* 2015).

5.4.1. Persistencia bacteriana

La existencia de células persistentes se describió por primera vez en un estudio con bacterias del género *Staphylococcus* (Bigger, 1944). Se identificó una subpoblación de células en cultivos en crecimiento activo, que toleraban tratamientos letales con penicilina. Estas células no crecían en medio con antibiótico, pero sí cuando este se eliminaba de la composición del medio. Además, al multiplicarse en ausencia del antibiótico, la composición de las poblaciones resultantes era idéntica a las iniciales, es decir, una gran mayoría de células sensibles al antibiótico, y una pequeña fracción de células persistentes.

Las células persistentes se detectan rápidamente mediante la elaboración de curvas de supervivencia durante el tratamiento con antibióticos (Fig. 17). Si dichos tratamientos tuvieran el mismo efecto bactericida sobre todas las células, las

gráficas resultantes mostrarían un descenso lineal del número de células a lo largo del tiempo, más pronunciado cuanto mayor fuera el tratamiento. La presencia de células persistentes, no obstante, provoca la aparición de curvas bifásicas. La primera parte de dichas curvas posee una pendiente decreciente muy pronunciada, y corresponde al patrón de muerte de células "normales", en crecimiento activo. La segunda parte de la curva posee una pendiente mucho menor, lo que indica la tolerancia de una fracción de las células (persistentes) de la población al tratamiento con el antibiótico. (Fig. 17) (Lewis, 2010; Maisonneuve & Gerdes, 2014).

El estudio de las células persistentes es complejo debido al bajo número de células en este estado fisiológico y a su similitud en multitud de aspectos a las células VBNC, que son mucho más abundantes. La mayoría de trabajos sugieren el origen estocástico de las células persistentes, como resultado de fluctuaciones aleatorias en la expresión génica en poblaciones de células en crecimiento activo (Ayrapetyan *et al.,* 2015). Su formación también es favorecida en condiciones de inanición, o por exposición a estrés oxidativo, antibióticos, etc (Ayrapetyan *et al.,* 2015). Recientemente, además, se ha propuesto un modelo matemático que explicaría la formación de células persistentes como un proceso evolutivo en el que interviene la herencia epigenética (Day, 2016).

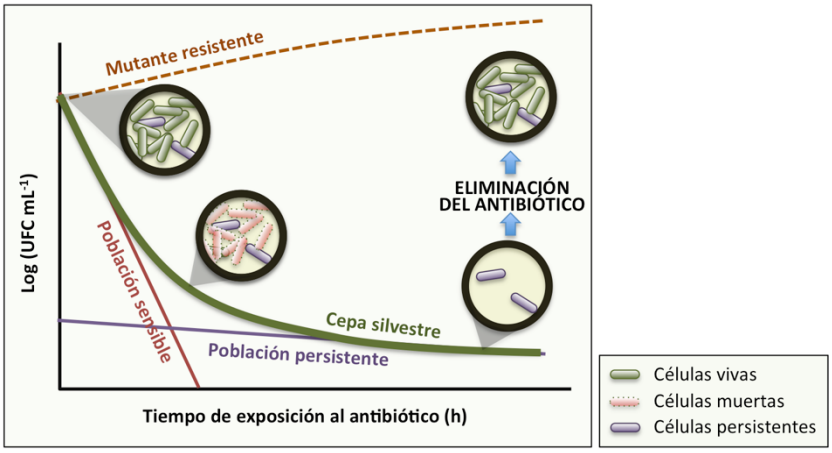


Fig. 17. Ejemplo de curva de supervivencia durante un tratamiento con un antibiótico bactericida. A tiempo 0 se añade una dosis letal del antibiótico a un cultivo en crecimiento de una población genéticamente homogénea de bacterias sensibles al mismo. El experimento revela una curva bifásica característica. La pendiente inicial indica una cinética exponencial de muerte celular (línea roja). La segunda parte de la curva, con una pendiente mucho más reducida indica la presencia de una subpoblación de células persistentes, cuya cinética de muerte es mucho más lenta (línea morada). Si transferimos células persistentes a medio sin antibiótico, estas originan una nueva población compuesta mayoritariamente por células sensibles, y una pequeña fracción de persistentes. Como ejemplo comparativo, en línea discontinua se muestra la curva de supervivencia de una cepa resistente al antibiótico bajo las mismas condiciones. En este caso, todas las células sobreviven al tratamiento, se dividen y generan de nuevo una población de células resistentes. Modificado de Maisonneuve & Gerdes (2014).

La tolerancia a los antibióticos en estas células se debe a la inactividad de funciones celulares esenciales (Lewis, 2010). No obstante, parece haber excepciones, pues un estudio reciente ha demostrado la capacidad de crecimiento de micobacterias persistentes en el interior de macrófagos (Wakamoto *et al.*, 2013). Además, aunque los ensayos iniciales parecían indicar que para que se diera la persistencia bacteriana era necesaria la presencia de células en estado de dormición previa al tratamiento con el antibiótico, en un trabajo reciente se ha demostrado que

la dormición previa no es esencial ni necesaria (Orman & Brynildsen, 2013a).

Las células persistentes forman parte, junto a las células activas y las células VBNC, de biopelículas bacterianas, y se consideran una de las principales razones de las infecciones recalcitrantes causadas por algunos microorganismos (Ayrapetyan *et al.,* 2015). La importancia de la persistencia bacteriana en cuestiones de la salud pública se refleja en el gran número de estudios y revisiones alrededor de este tema que existen en la actualidad.

5.4.2. El estado VBNC

Las bacterias en el estado VBNC se caracterizan por su incapacidad para crecer en medios de cultivo convencionales, pese a ser viables y mantener activo su metabolismo (Roszak & Colwell, 1987; Morita, 1997; Oliver, 2010; Ramamurthy *et al.,* 2014; Pinto *et al.,* 2015) (Fig. 18). Este estado fisiológico ha sido descrito en aproximadamente 40 especies bacterianas de 17 géneros distintos (Pinto *et al.,* 2015). Del mismo modo que las células persistentes recuperan la cultivabilidad al retirar el antibiótico del medio, las células VBNC son capaces de resucitar de dicho estado tras ser expuestas a condiciones favorables, aunque, generalmente, las células en este estado fisiológico suelen precisar de condiciones más específicas (Caruso *et al.,* 2005; Oliver, 2010; Golmohammadi *et al.,* 2013; Li *et al.,* 2014) y tratamientos promotores de la resucitación más prolongados que en el caso de las células persistentes (Ayrapetyan *et al.,* 2015).

Dado que muchos protocolos para la detección de patógenos utilizan técnicas dependientes de cultivo, el estado VBNC, aparte de una estrategia de supervivencia bacteriana ante

condiciones adversas, constituye un importante fenómeno a tener en cuenta a la hora de estudiar los reservorios de los patógenos en el medio ambiente. En relación a esto, el descubrimiento de estrategias de dormición bacteriana como el estado VBNC han abierto una nueva vía para los microbiólogos en la investigación de las dinámicas poblacionales de los patógenos en ambientes complejos como los ecosistemas asociados al hospedador.

Los ciclos de vida de muchos patógenos en estos ambientes se caracterizan por periodos de crecimiento asociados a los procesos infecciosos, con un metabolismo muy activo y un consumo rápido de los recursos, seguidos de periodos de inanición en los que los estados de dormición como el VBNC ofrecen múltiples ventajas. Muchos patógenos, por ejemplo, causan infecciones latentes con números muy bajos de células VBNC que evitan la detección por el sistema inmunitario del hospedador. Cuando se dan las condiciones apropiadas, las células VBNC resucitan, se multiplican y reinician de nuevo el ciclo infeccioso (Lennon & Jones, 2011).

Aunque entre las células en crecimiento activo podemos encontrar una pequeña subpoblación de células VBNC (Orman & Brynildsen, 2013b), este estado fisiológico se induce considerablemente en condiciones de limitación nutricional (Morita, 1997; Oliver, 2010). Otros factores químicos y medioambientales que favorecen la entrada en el estado VBNC incluyen: la exposición a estrés térmico, osmótico, oxidativo, lumínico, sustancias empleadas en la conservación de alimentos, metales pesados, etc (Oliver, 2010; Pinto *et al.,* 2015).

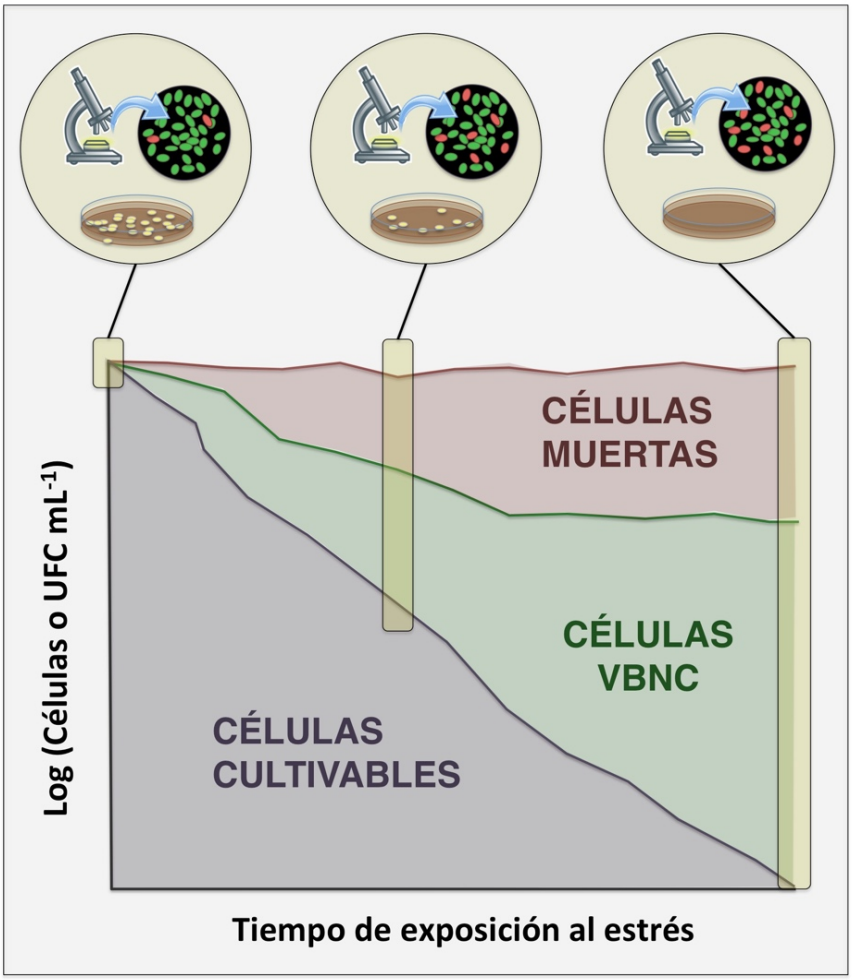


Fig. 18. Inducción del estado VBNC por exposición a estrés. Los recuentos de células cultivables se realizan mediante siembra en placa. La estimación de células viables, muertas y totales se realiza mediante tinción de viabilidad (por ejemplo, con el kit *Baclight* Live/Dead, de Life Technologies) y recuento vía microscopía de epifluorescencia o citometría de flujo. A tiempo 0 prácticamente todas las células están vivas y son cultivables. Conforme incrementa el tiempo de exposición al estrés se reduce la cultivabilidad celular, y también la viabilidad celular, pero esta última en menor grado, lo que indica que una subpoblación de células es VBNC. Finalmente, toda la población de células viables es VBNC.

Fenotípicamente, con la salvedad de su incapacidad para crecer en medios de cultivo, las células VBNC poseen características similares a las descritas para el estado de supervivencia en inanición: reducción de las tasas de respiración y de síntesis de macromoléculas, mantenimiento del potencial de membrana, cambios en la composición de las membranas celulares, resistencia incrementada a distintos tipos de estrés, capacidad para incorporar aminoácidos, división reductiva, producción de EPSs, adhesión a partículas o superficies y/o formar parte de biopelículas bacterianas (Morita, 1997; Byrd, 2000; Kieft; 2000; Navarro-Llorens *et al.,* 2010; Oliver, 2010; Trevors, 2011; Nowakowska & Oliver, 2013). En otros estudios se han identificado reajustes en los patrones de expresión (Meng *et al.,* 2015), modificaciones de la pared celular (Costa *et al.,* 1999; Signoretto *et al.,* 2002) y perfiles proteicos (Heim *et al.,* 2002; Lai *et al.,* 2009) característicos del estado VBNC. No obstante, la mayoría de estudios de caracterización fisiológica y/o molecular de células VBNC emplean un solo estrés para producir células en ese estado. Como consecuencia es difícil identificar si las características anteriormente mencionadas son producto de las respuestas celulares al estrés empleado para generar células VBNC, o se trata de un fenotipo asociado a este estado (Pinto *et al.,* 2015).

En los últimos años se han publicado varios trabajos cuyo objetivo era la caracterización molecular del estado VBNC. Según indican algunos de ellos el crecimiento bacteriano en medios de cultivo depende de la capacidad de las células para detoxificar ROS producidas en dichos medios de cultivo, y la entrada en el estado VBNC se ha correlacionado con una pérdida de actividad catalasa (Kong *et al.,* 2004; Tanaka *et al.,* 2014). Estos resultados permiten explicar que, mutantes de varias especies bacterianas en OxyR (un regulador de genes relacionados con la respuesta a estrés oxidativo), sean

incapaces de crecer en medios de cultivo sólido, a no ser que estos sean suplementados con catalasa o algún agente reductor (Miguel *et al.,* 2000; Kong *et al.,* 2004; Flores-Cruz & Allen, 2011). Otros trabajos, además, han mostrado que las bacterias sometidas a estrés por inanición expresan diferentes genes relacionados con la respuesta a estrés oxidativo, lo que sugiere la necesidad de mecanismos antioxidantes para combatir las ROS producidas por el propio metabolismo celular (Hengge-Aronis, 2000; Nyström, 2001; McDougald *et al.,* 2002).

Aun así, a día de hoy los mecanismos moleculares que determinan la entrada en dicho estado siguen siendo un misterio (Pinto *et al.,* 2015).

II. OBJECTIVES

II. Objectives

Based on what has been discussed in the introduction, it is evident that there are still many aspects of *E. amylovora's* life cycle that remain poorly understood. The main objective of this Doctoral Thesis was focused on the characterization of the mechanisms that allow *E. amylovora* to adapt to natural nutrient limitation conditions, which are responsible for the pathogen's persistence in the environment, its spread by water, vector insects, etc. The main objective of this Doctoral Thesis can be specified in the following partial objectives:

1. Characterize *E. amylovora*'s responses to starvation, as well as the effect of environmental temperatures on these responses and virulence.

2. Investigate the survival and transmission of *E. amylovora* by the medfly *Ceratitis capitata.*

3. Determine the potential water-borne infection of *Pyrus communis* plants by *E. amylovora* through the roots by soil-irrigation, and characterize the root colonization, invasion and migration routes of the pathogen.

4. Study the role of the alternative sigma factor RpoS in the development of starvation-survival responses by *E. amylovora,* as well as characterize its functions as a regulator of cross-protection, virulence factors and virulence in different types of plant material.

5. Explore the functional roles of the *E. amylovora* catalases KatA and KatG during exposure to nutrient limiting conditions, and also during plant-pathogen interactions.

III. SUMMARY OF METHODS, RESULTS AND DISCUSSION

Chapter 1

Characterization of *Erwinia amylovora* responses to starvation and the effect of temperature on survival responses and virulence

(articles I, II and III of the Annex)

E. amylovora is a non-obligated pathogen, which means that it can survive in environments outside the host (plant surfaces, pruning tools, vector insects, soil, etc.) (Thomson, 2000; van der Zwet *et al.,* 2012), allowing its spread by a variety of means and contributing to the difficulty in the control of fire blight (Thomson, 2000).

Given the oligotrophic conditions prevailing in most natural environments (Morita, 1997), the survival of *E. amylovora* in nature will depend on its tolerance and adaptability to starvation. In fact, adaptation to non-host environments might have been an important factor contributing to the evolution of phytopathogenicity and affecting the epidemic potential of *E. amylovora,* as reported for other bacterial plant pathogen (Bartoli *et al*., 2015).

Another crucial factor influencing bacterial survival in oligotrophic environments is temperature. According to Moyer & Morita's (2007) classification of microorganisms by growth temperature ranges, *E. amylovora* is a psychrotrophic species,

able to grow at temperatures ranging between 4ºC and 37ºC (van der Zwet *et al.,* 2012). However, despite the wide *E. amylovora* temperature growth range, a minimum temperature of 18ºC is necessary for the development of blossom blight epidemics under field conditions. This is why most studies on the temperature regulation of *E. amylovora* virulence genes/factors have focused on this temperature, and not on lower ones, to determine the expression of virulence genes, or other aspects of the biology of this species (Raymundo & Ries, 1980; Wei *et al*., 1992; Bereswill *et al.,* 1997; Goyer & Ullrich, 2006).

Interestingly, given the variety of factors influencing the development of fire blight under field conditions, sometimes, when environmental variables other than temperature are optimal, fire blight outbreaks and/or the progression of symptoms can occur at suboptimal low temperatures (van der Zwet *et al.,* 2012; Shtienberg *et al.,* 2015).

In many plant pathogens the expression of virulence determinants is restricted to a certain range of temperatures (Smirnova *et al.,* 2001; du Raan *et al.,* 2016), but in the case of *E. amylovora* knowledge concerning how low temperatures affect its pathogenicity is still scarce.

Also noteworthy is that the effects of temperature on bacterial physiology also depend on nutrient availability, and such effects can vary depending on the analyzed microorganism (Morita, 1997; Gauthier, 2000; Arana *et al.,* 2010). During spring or summer, for example, environmental temperatures allow *E. amylovora* growth inside the host tissues and on flower surfaces. Nevertheless, these temperatures, could negatively affect bacterial survival under starvation conditions. In other pathogens the effect of environmental temperatures on their physiology during starvation determines their life cycle and the seasonal development of the disease they cause (Oliver *et al.,* 1995;

Armada *et al.,* 2003; Caruso *et al.*, 2005; Vattakaven *et al.,* 2006; Lutz *et al.,* 2013).

Two common responses to starvation in nonsporulating bacteria, which are strongly influenced by temperature, are the starvation-survival (Morita, 1997) and the VBNC states (Oliver, 2010; Pinto *et al.,* 2015). The former is characterized by the maintenance of culturability over time, among other phenotypic traits (Morita, 1997). Bacterial responses to starvation also include changes in morphology, cell size, flagellation, motility, etc. (Morita, 1997; Young, 2006; Chen *et al.,* 2009; Fida *et al.,* 2013), but none of these responses have been previously characterized in the fire blight pathogen. Furthermore, despite the fire blight pathogen's ability to grow at low temperatures, the effect of such temperatures on starvation survival has not been yet explored.

The VBNC state is considered a long-term-survival mechanism by which cells overcome environmental stresses that could otherwise be lethal (Gauthier, 2000). In this state, cells remain viable and metabolically active, but they are unable to grow on solid media, being thus undetectable by conventional culture-dependent methods (Gauthier, 2000; Oliver, 2010).

The description of the VBNC strategy in an increasing number of human and plant pathogenic species (Oliver, 2010; Pinto *et al.,* 2015) raises a concern about the retention of virulence by cells in this state, which every year is translated into a huge number of publications on this topic. Virulence can sometimes be lost in parallel to the entry of bacterial cells into the VBNC state, but the recovery of both culturability and virulence can be achieved in some cases by, among others, removing the stressing agent, by culture in a liquid medium, or by passage through a susceptible host (Oliver, 2010; Pinto *et al.,* 2015). The existence of resuscitation factors in some pathogens has also been described (Oliver, 2010; Ayrapetyan *et al.,* 2015; Pinto *et*

al., 2015). In the case of *E. amylovora,* the induction of the VBNC state in response to copper treatments, and the recovery of such VBNC cells by different procedures, has been demonstrated (Ordax *et al.,* 2006; 2009). However, our experience in the laboratory with the recovery of *E. amylovora* VBNC cells induced by other stresses, using the previous described strategies, has not always been successful.

This chapter summarizes and discuses results from three studies on different aspects of *E. amylovora* responses to starvation (Annex I, II and III; Santander *et al.,* 2014b; Santander & Biosca, 2016, unpublished; Santander *et al.,* 2012a, respectively).

1.1. Temperatures below the optimal for growth favor the maintenance of culturability in starved *E. amylovora* cells

The survival strategies adopted by *E. amylovora* during exposure to starvation conditions were assessed using oligotrophic water microcosms, by monitorization of culturable, viable and total cell population dynamics. Culturability was determined by plate counts on solid medium. Viable and total cells were counted by epifluorescence microscopy (EFM) or flow cytometry, after the staining of microcosm samples with the *Baclight* Live/Dead viability kit (Life Technologies) (Annex I and II).

Assays with oligotrophic water microcosms demonstrated that *E. amylovora* responses to starvation over time vary depending on the incubation temperature (Fig. 19). A general conclusion of articles 1 and 2 is that incubation temperatures near or equal to the optimal for growth (28ºC) enhance the entry of viable cells into the VBNC state. This response is reduced at lower temperatures, with a greater maintenance of culturability being observed at 20ºC and 14ºC. Interestingly at 4ºC, an induction of the VBNC response was also observed, but in a slower manner than at 28ºC. Moreover, starvation caused cell lysis and the loss of viability of a small fraction of cells, which was more evident at 28ºC, reduced at 14ºC and almost null at 4ºC. In addition, in a short study (Santander *et al.,* 2012b), incubation of *E. amylovora* in microcosms at 37ºC revealed the fastest entry into the VBNC state, with the greatest reduction of cell viability.

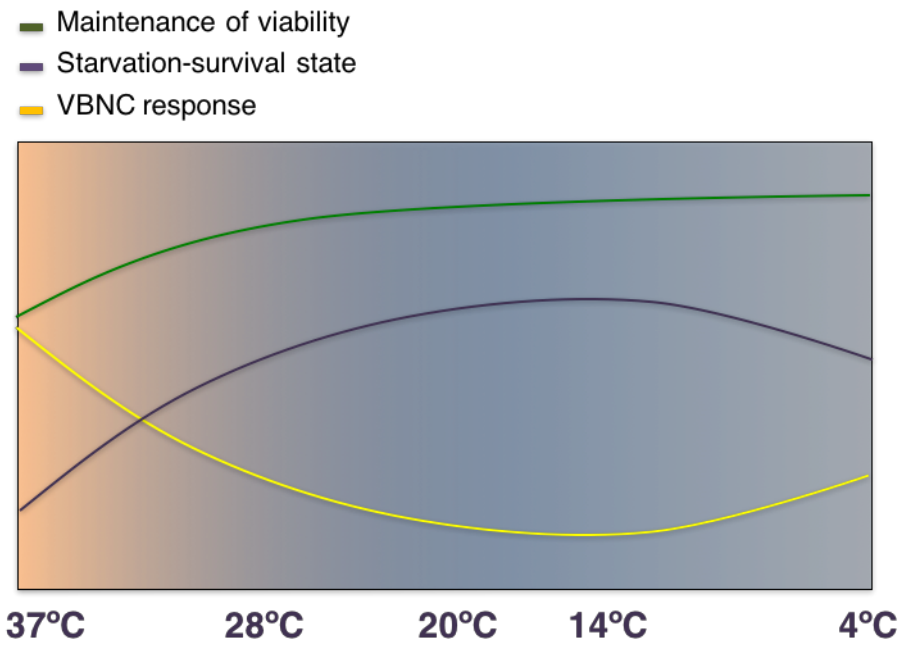


Fig. 19. Scheme of temperature-dependent *E. amylovora* responses to starvation. The maintenance of the culturability characteristic of the starvation-survival response is enhanced at 14ºC – 20ºC. The proportion of cells entering the VBNC state at these temperatures is low. At higher and lower temperatures, the VBNC response is induced. However, there is a reduction of viability at 28ºC – 37ºC that is not produced at 4ºC. Curves in the scheme are based on Santander *et al.,* 2012a, b; Santander *et al.,* 2014b and Santander & Biosca, 2016, unpublished.

In cells under starvation conditions, the increased maintenance of culturability at low temperatures has been related to the slow-down of metabolism (Arana *et al.,* 2010). However, exposure to low temperatures outside the growth range has also been reported to induce the VBNC response (Pinto *et al.,* 2015).

The induction of the VBNC response has been related to the activity of antioxidant enzymes (Kong *et al.,* 2004; Asakura *et al.,* 2007; Masmoudi *et al.,* 2010), and factors controlling oxidative stress resistance genes (Kong *et al.,* 2004; Kusumoto *et al.,* 2012). However, the regulation of this physiological response is

complex, and much investigation is required to understand the mechanisms involving this survival strategy (Pinto *et al.,* 2015).

In general, the modulation of bacterial responses to starvation by temperature can vary radically depending on the analyzed microorganism (Morita, 1997; Gauthier, 2000; Archuleta *et al.,* 2002; Besnard *et al.,* 2002; Caruso *et al.,* 2005; Vattakaven *et al.,* 2006; Arana *et al.,* 2010; Wu *et al.,* 2016), and in some cases this has been directly related to the pathogen's life cycle (Caruso *et al.,* 2005; Vattakaven *et al.,* 2006; Ayrapetyan *et al.,* 2015).

Our results might indicate that temperate environmental temperatures would facilitate *E. amylovora*'s long-term persistence in the environment in a culturable state. This would coincide with periods in which fire blight symptoms occur under field conditions, thus favoring environment enrichment with the bacterium. Warmer and colder temperatures, however, would induce a VBNC response in starved cells, allowing the survival of the pathogen until temperatures and/or conditions would allow the recovery of cell growth in parallel to the renewal of the vegetative growth of the hosts. In connection with this, the detection of *E. amylovora* in cankers and terminal shoots has usually been reported to be possible only by culture-independent methods (Kielak *et al.,* 2002; Sobiczewski *et al.,* 2006), these results suggesting the presence of the pathogen in the VBNC state.

1.2. Starvation causes morphological changes in *E. amylovora,* which also depend on temperature

Morphological changes occurring in starved *E. amylovora* cells were determined either by transmission electronic microscopy (TEM) (Annex I) or by EFM (Annex II), after staining them with phosphotungstic acid or the green fluorophore Syto9,

respectively.

In "Santander *et al* (2014a)" (Annex I), *E. amylovora* cells were exposed to starvation conditions for 40 days at a temperature favoring the maintenance of culturability over time (20ºC). Then, possible morphological, cellular and motility changes occurring during exposure to starvation were monitored by TEM over time. Cells at time 0 showed a typical rod shape, peritrichous flagella and, in some cases, a surrounding layer of EPS. During exposure to starvation both the EPS layer and the peritrichous flagella were maintained in many cells. In addition, an increasing fraction of cells over time experienced dwarfing (a reduction of cell size) and/or acquired rounded shapes (Fig. 20). A very small percentage of cells, however, developed the opposite response, showing elongated shapes about 2.5 times longer than cells at time 0. Finally, most of the analyzed cells showed small flame-shaped vesicles all over the outer membrane surface (OMVs).

Morphological changes of shape and size are common features observed in a variety of bacterial species (Morita, 1997; Álvarez *et al.,* 2008a; Chen *et al.,* 2009; Oliver, 2010; Yang *et al.,* 2016). The adoption of rounded shapes has been linked to the optimization of the cells' energetic resources through an increase in the surface/volume ratio (Roszak & Colwell, 1987; Morita, 1997; Byrd, 2000; Nyström, 2004). The production of OMVs has also been described as a mechanism to deal with starvation, involved in nutrient acquisition and other biological functions (McBroom & Kuehn, 2007; Kulp & Kuehn, 2010; Tashiro *et al.,* 2012). Filamentation is also a common phenomenon occurring in many bacterial species in response to starvation and other conditions (Wainwright *et al.,* 1999; Young, 2006; Álvarez *et al.,* 2008a; Justice *et al.,* 2008; Yang, 2016). In this case, bacteria increase their total surface area without a significant increase in

the surface-to-volume ratio, which has been related to an improvement for nutrient uptake (Young, 2006). Other phenomena related to morphological changes of cell shape are avoidance of predation, resistance to antibiotics, etc (Byrd, 2000; Young, 2006; Justice *et al.,* 2008).

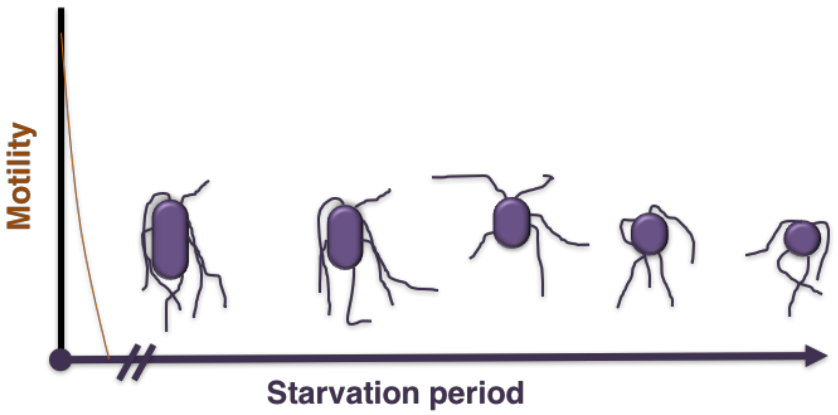


Fig. 20. *E. amylovora* morphological and motility responses during starvation. A few hours after exposure to nutritional stress *E. amylovora* cells lose motility, but maintain some flagella attached to the cell surface. With extended exposure to starvation *E. amylovora* cells turn from rods to cocci and experience a reduction in cell size (dwarfing).

Regarding the EPS layers observed in some of the *E. amylovora* cells exposed to starvation, these molecules have been described as carbon or energy sources in conditions of nutrient shortage in *E. amylovora* (Ordax *et al.,* 2010), and in other pathogens (Sheng *et al.,* 2010), and can also act as a barrier against desiccation, bacteriophages and toxic compounds such as copper (Jock *et al.*, 2005; Ordax *et al.,* 2010; Born *et al.,* 2014).

The starvation-induced reduction of cell size in some bacterial species is linked to reductive division (Morita, 1997; Byrd, 2000; Nyström, 2004; Álvarez *et al.,* 2008a), but this is not the case of *E. amylovora.* In some organisms, dwarfing occurs due to the degradation of the cell wall and the cytoplasmic membrane, leading to a contraction of the outer membrane (Nyström, 2004) or the formation and/or release of OMVs (Mårdén *et al*., 1985; Byrd, 2000; Nyström, 2004; Álvarez *et al*., 2008a). This might explain the mechanism by *E. amylovora* cells subjected to starvation reduce cell size. Other ecological roles attributed to these vesicles are the delivery of substances to favor interspecific communication, the attack of competitors, or biofilm formation (Kulp & Kuehn, 2010; Tashiro *et al*., 2012).

In nutrient limiting environments both the induction of motility and chemotactic responses to improve the search for nutrients, and the loss of motility to save energy are considered two common survival strategies in bacteria (Morita, 1997; Blackburn *et al.,* 1998; Wei & Bauer, 1998; Stocker *et al.,* 2008). *E. amylovora* cells exposed to oligotrophic conditions at 20ºC progressively lost motility during the first 72 h of exposure to the stress, although flagella remained attached to many cells throughout the 40-day experimental period (Fig. 20). This phenotypic trait in *E. amylovora* can be rapidly recovered under favorable conditions, e.g. when water films are formed under host surfaces (Raymundo & Ries, 1981).

In many bacterial species changes in cell size and morphology occur in a temperature-dependent manner (Chen *et al.,* 2009; Yang *et al.,* 2016). In Santander & Biosca (2016, unpublished) (Annex II), the effects of temperature on the morphology and size of *E. amylovora* cells starved for about 4 months were analyzed by EFM. This study revealed that in *E. amylovora* the intensity of starvation induced changes in cell size,

but not in cell shape, was dependent on temperature. Dwarfing was enhanced at the temperatures improving the maintenance of culturability under starvation conditions (14ºC > 4ºC > 28ºC). The acquisition of rounded shapes, however, occurred regardless of the incubation temperature.

1.3. Starvation modulates but does not inhibit the expression of housekeeping, starvation-related and virulence/ pathogenicity genes in *E. amylovora*

As part of the characterization of the starvation-survival state in *E. amylovora,* the expression of a variety of genes was analyzed by end-point RT-PCR throughout a 40-day starvation period at 20ºC. Additionally, the modulation of such genes during the first 24 h of exposure to the above mentioned conditions was assessed by semi-quantitative RT-PCR. The analyzed genes were related to starvation and oxidative stress responses (*cstA, dps, katA, katG, oxyR, relA, rpoS, spoT*), virulence/pathogenicity (*flgN, hrpL, rcsB, rlsA*) and housekeeping (*rrs*) (Annex I).

End-point RT-PCR allowed the detection of all the assayed genes throughout the entire experimental period, demonstrating the transcriptional activity of *E. amylovora* cells entering the starvation-survival state. The semi-quantitative RT-PCR analysis of gene expression, moreover, revealed three different regulation patterns occurring during the first 24 h of starvation (Fig. 21). The first pattern, observed in gene *dps,* consisted of the maintenance of expression levels overtime. The product of this gene is involved in protection of DNA during starvation and other stresses, and its continuous expression might be a reflection of its importance in stationary phase cells (the physiological state of the *E. amylovora* cells inoculated in water microcosms at time 0) and cells under starvation conditions.

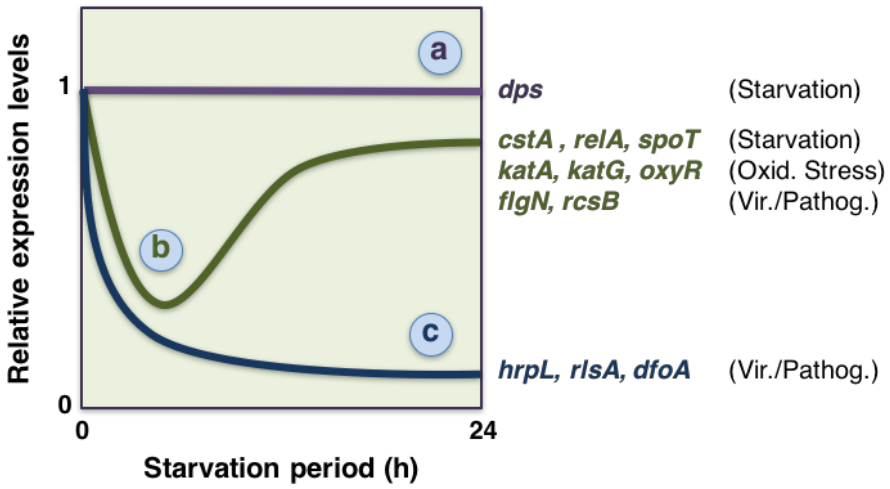


Fig. 21. Expression patterns of different *E. amylovora* genes during the first 24 h of exposure to starvation. Pattern "a" consists of the maintenance of expression levels over time, regardless of the exposure to the stress; pattern "b" consists of a shift of expression levels, which is followed by the partial or total recovery of initial values; pattern "c" was characterized by a sharp decrease of gene expression to reach a steady state which was maintained until the end of the experimental period.

The second pattern was the most common amongst the analyzed genes, which included the starvation-related genes *cstA* (encodes the carbon starvation protein A), *relA* and *spoT* (encode regulators of the stringent response alarmone (p)ppGpp), and *rpoS* (encodes the stationary phase alternative sigma factor rpoS); the oxidative stress related genes *katA, katG* and *oxyR* (encode the catalases KatA and KatG, and the regulator of H_2O_2-inducible genes OxyR); and the virulence/pathogenicity related genes *flgN* (encodes a protein involved in flagellar biosynthesis) and *rcsB* (encodes a positive regulator of amylovoran biosynthesis). The second expression pattern consisted of an initial shift of gene expression (usually a down-regulation) during the first hours of exposure to the stress, followed by the partial or total recovery of initial expression levels. This pattern, usually

observed in cells exposed to environmental changes (González-Escalona *et al*., 2006; Chechik & Koller, 2009), correlates with the activation of emergency responses after exposure to stress, allowing cells to reach a homeostatic adaptation to the new environment (Chechik & Koller, 2009).

Finally, the third pattern was observed in the virulence/pathogenicity genes *dfoA* (related to the synthesis of the siderophore desferrioxamine), *hrpL* (an alternative sigma factor that regulates the expression of pathogenicity genes) and *rlsA* (related to levan synthesis), and consisted of a decrease of gene expression during the first hours, reaching lower expression values than the initial ones that were maintained until the final sampling period. This response indicates the downregulation of these genes under the assayed conditions, which might be linked to an optimization of the energetic resources in starved cells.

1.4. Culturable starved *E. amylovora* cells are pathogenic on immature fruits, but VBNC cells can recover their culturability and pathogenicity in fruits after passage through susceptible host plantlets

In order to determine how starvation affects the virulence/pathogenicity of *E. amylovora,* bacterial cells of this pathogen subjected to starvation for 40 days at 20ºC, were inoculated into immature pears (*P. communis* cv. Williams) and loquats (*E. japonica* cv. Tanaka), and in one-month-old pear plantlets (*P. communis* cv. Passe Crassane). *E. amylovora* cells from overnight cultures were used as control. Results revealed the maintenance of pathogenicity under starvation conditions, and also a similar virulence of starved cells to that observed in control cells.

In order to investigate the pathogenicity of *E. amylovora* VBNC cells, and/or the recovery of culturability of such cells, the VBNC state was induced by exposure to starvation or chlorine (Annex III). These stresses have usually been reported as inducers of the VBNC state in different works (Morita, 1997; Oliver *et al.,* 2005; Moreno *et al.,* 2007; Oliver, 2010). Starvation conditions were recreated using oligotrophic water (rainwater or distilled water) microcosms, incubated at 26ºC. In the case of chlorine, the selected concentration (0.7 ppm) was chosen based on the ranges of this compound in regular tap water.

The entire population of *E. amylovora* cells exposed to nutrient limiting conditions in rainwater and distilled water entered the VBNC state after 60 and 30 days post-inoculation (dpi), respectively. Cells exposed to 0.07 ppm chlorine developed the VBNC response in less than 5 min.

Resuscitation of VBNC cells was tested by inoculation of *E. amylovora* VBNC cells into liquid medium, immature fruits (*P. communis* cv. Williams) or one-month-old pear plantlets (*P. communis* cv. Passe Crassane). The recovery of VBNC cells in liquid medium was positive only in chlorine-treated cells, 30 min after VBNC induction. The VBNC recovery of cells exposed to this compound for prolonged periods, or starvation-induced VBNC cells in liquid medium was negative. Similarly, the use of immature fruits to recover VBNC cells resulted unsuccessful, except in chlorine-treated cells 30 min after the entry into the VBNC state. However, the inoculation of VBNC cells into one-month-old pear plantlets allowed the recovery of both culturability and pathogenicity of *E. amylovora* VBNC cells, regardless of the time elapsed after the adoption of this response by the entire cell population (Fig. 22).

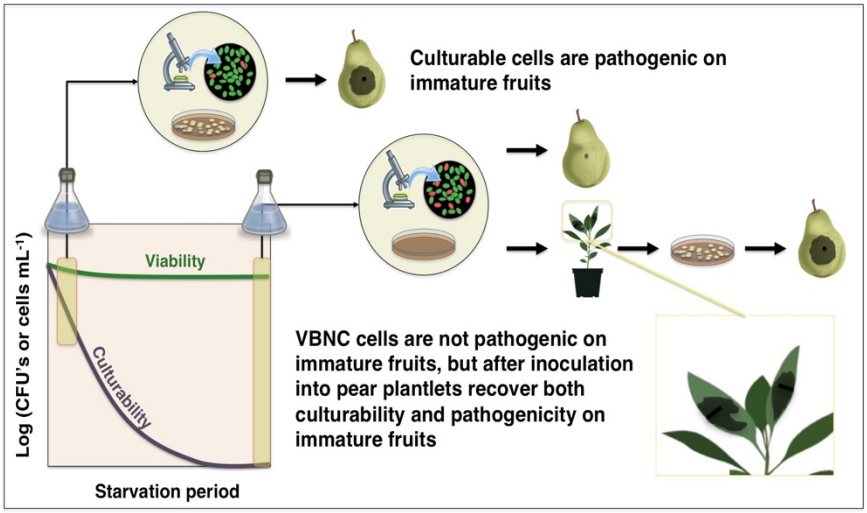


Fig. 22. Scheme summarizing the *E. amylovora* modulation of pathogenicity during starvation. *E. amylovora* cells starved for short periods remain culturable and able to cause fruit blight on immature pears. With extended exposure to the stress, the entire viable cell population becomes VBNC and loses pathogenicity on immature fruits. However, after passage through a one-month-old pear plantlet of a susceptible cultivar, stressed cells recover both culturability on solid media and pathogenicity on fruits.

Results in this work confirmed that chlorine concentrations in the range of those present in tap water have a bacteriostatic, but not a real bactericidal effect on *E. amylovora*, agreeing with that described for other pathogens (Dusserre *et al.,* 2008; Pawlowski *et al.,* 2011). Moreover, the survival of *E. amylovora* in multiple types of water (Annex I, II, III), together with the recovery of starvation- and chlorine-induced *E. amylovora* VBNC cells by passage through a susceptible host plant, might pose a risk in nurseries and orchards, where the pathogen could also be spread *via* irrigation water.

Furthermore, VBNC cells constitute a potential and hard-to-detect inoculum source of pathogens in the environment, which might have important implications in diagnostic procedures where culturability is considered as the only indicator of viability (López

et al. 2005; Oliver, 2005). Knowledge of the roles that the starvation-survival and the VBNC responses develop in the *E. amylovora* life cycle, hence, might allow the improvement of control and/or prevention measures against fire blight.

1.5. *E. amylovora* is pathogenic on immature fruits at temperatures below 18ºC, which correlates with the production of virulence/pathogenicity factors at those temperatures

To determine if low temperatures inhibit fire blight symptom development, the effects of low (4ºC), temperate (14ºC) and warm (28ºC) temperatures on *E. amylovora* virulence were assessed on immature fruits, as described in the previous section. Additionally, the effect of the same temperatures on some virulence/survival factors was also analyzed *in vitro*. The factors analyzed were growth in rich medium, motility, siderophore secretion, EPS production, biofilm formation, and oxidative stress resistance (Annex II).

E. amylovora was able to cause disease in detached immature fruits at the three temperatures assayed, with a slow-down of symptom development correlating with colder temperatures and slower growth rates. Moreover, the *in vitro* analysis of the effect of temperature on virulence/survival factors revealed the production of such factors at 4ºC, 14ºC and 28ºC. However, despite the fact that some of the phenotypes analyzed (siderophore secretion and motility) also decreased in parallel to incubation temperatures, others showed a clear enhancement at temperatures below 28ºC. Amylovoran and levan, for example, were overproduced at 4ºC and 14ºC, respectively, and biofilm formation and H_2O_2 resistance were also enhanced at 14ºC with respect to the other assayed temperatures.

The fact that *E. amylovora* was pathogenic even at 4ºC is not a common trait among animal and plant pathogens, in which pathogenicity is restricted to a certain range of temperatures (Serfontein *et al.*, 1991; Durand *et al.,* 2000; du Raan *et al.*, 2016). This is due to the repression of temperature-regulated virulence and/or pathogenicity genes (Konkel & Tilly, 2000; Smirnova *et al.,* 2001).

Our results indicate that *E. amylovora* has the potential to cause fire blight at a very wide range of temperatures as long as susceptible tissues are present, which agrees with the observed progression of fire blight symptoms in perennial pear branches during winter in Israel, following autumn infections (Shtienberg *et al.*, 2015). Also, these results might explain, at least in part, the effective spread and adaptation of the fire blight pathogen to different climate areas in multiple countries worldwide (EPPO, 2016).

Chapter 2

Living outside the host: Survival and transmission of *Erwinia amylovora* by the medfly *Ceratitis capitata,* a potential host vector

(article IV in the Annex)

As in many other non-obligated phytopathogenic bacteria (Purcell, 1982; Agrios, 2008), the presence of insects greatly contributes to the dispersal and epiphytotics of *E. amylovora* under field conditions (Miller & Schroth, 1972).

Since the first studies on fire blight, an enormous diversity of insect vectors have been involved in the spread of the disease (Miller & Schroth, 1972; van der Zwet & Keil, 1979; Johnson *et al.,* 1993; Hildebrand *et al.,* 2000; van der Zwet *et al.,* 2012; Peusens *et al.,* 2013). In fact, when fire blight is present in orchards *E. amylovora* can be isolated from a variety of insect species (Miller & Schroth, 1972; Hildebrand *et al.,* 2000; van der Zwet *et al.,* 2012), although the multiplication of the bacterium on/in insects has not been demonstrated (Purcell, 1982; van der Zwet *et al.*, 2012). In this regard, ants and flies, seem to be linked to the primary infection cycle, transferring cells of the pathogen from oozing activated cankers to healthy flowers (Thomson, 2000; van der Zwet *et al.,* 2012). Pollinating and piercing-sucking insects, however, have been related to the secondary spread of *E. amylovora*, from flower to flower (e.g. honeybees) or from

infected tissues to healthy ones (e.g. stink bugs), respectively (Johnson *et al.,* 1993; Thomson, 2000; Peusens *et al.,* 2013).

From all the insects present in pome fruit orchards, fruit flies (family *Tephritidae*), and in particular the Mediterranean fruit fly, or medfly, *Ceratitis capitata,* might play an important role in host-to-host transmission and long distance dispersal of *E. amylovora,* but its role in the transmission of the pathogen has not yet been investigated.

C. capitata is a cosmopolitan pest affecting more than 400 commercial and wild plant species. Because of its successful spread worldwide, and characteristics of its behavior, *C. capitata* has also been previously considered a potential vector for human bacterial pathogens (Sela *et al.,* 2005). Adult medflies have a polyphagous diet, feeding on a variety of protein sources (including animal faeces). Females inject clusters of eggs with the ovipositor into wounds in fruits, performed with the same organ (Sela *et al.,* 2005). The exportation of egg-contaminated fruits together with the flying abilities of this insect (it can travel up to 20 Km) enhance the local and long dispersal of *C. capitata*, which greatly complicates efforts to control this pest (Israeli *et al.,* 2005; Meats & Smallridge, 2007).

In this chapter results corresponding to a study on the survival and potential transmission of *E. amylovora* by the medfly *C. capitata* (Ordax *et al.,* 2015; see Annex IV) are summarized and discussed.

2.1. *E. amylovora* can be acquired by, and survive on/in medflies for, at least, 28 days

In order to analyze the survival of *E. amylovora* cells in/on medflies, caged insects were exposed for 48 h to *E. amylovora*

contaminated and artificially wounded ripe apples. This kind of plant material was selected because in nature medflies are odor attracted to wounded ripe fruits (Aluja & Norrbom, 2000). Furthermore, although *E. amylovora* does not develop well in ripe fruits, such fruits can become surface-contaminated with *E. amylovora* when located near of blighted trees (Thomson, 2000). Finally, the low level of multiplication of *E. amylovora* in apple ripe fruit tissues facilitates the standardization of initial inoculum doses, avoiding increasing overexposure of caged medflies to bacterial cells throughout time.

Once the 48-h acquisition period had finished, medflies were transferred to clean cages where the pathogen was not present, and analyzed to determine the presence and/or survival of *E. amylovora* cells in/on the insect over a 28-day period. Survivability was analyzed by culture of insect extracts on different media. When pathogen detection on solid media was negative, the possible presence of VBNC cells was assessed by specific PCR and recovery assays using liquid medium or susceptible plant material (similar to that described in Santander *et al.,* 2012a, Annex III). The identification of *E. amylovora*-like colonies after re-isolation from insects was carried out by specific PCR and pathogenicity tests.

Plate counts after the 48-h acquisition period (from now, this will be considered time 0 in survival and transmission assays), allowed the detection of *E. amylovora* in challenged insects, in concentrations ranging from 10^4 – 10^6 CFUs per medfly. Counts during the following days revealed the continuous decrease in *E. amylovora* culturable cell numbers over time, with positive isolations being possible up to 14 days after the start of the survival experiment but not later (< 1 CFU per medfly). Interestingly, the molecular detection of the pathogen by specific PCR at times 21 and 28 days was positive, indicating the potential

presence of VBNC *E. amylovora* cells in/on insects. Recovery assays using susceptible plant material evidenced the presence of *E. amylovora* cells in the analyzed samples, with medfly extract inoculation in fruits and shoots leading to fire blight symptom development. Presumptive *E. amylovora* colonies were isolated from diseased plant material and confirmed by PCR.

An important feature for pathogens to be disseminated by insects is their survivability in these environments. Our results indicate that *E. amylovora* survives for 28 days in/on medflies, with a progressive loss of culturability corresponding, at least in part, to the entry of a part of the pathogen population into the VBNC state. These values are surprisingly higher than those reported in other insects, with average survival values of about 6 days (Ark & Thomas, 1936; Hildebrand *et al.,* 2000; Hildebrand *et al.,* 2001; Alexandrova *et al.,* 2002b). Furthermore, it is remarkable that studies on bacterial survival are classically performed by culture-dependent procedures, indicating the probable underestimation of the number of viable cells, i.e. not including data on nonculturable but recoverable VBNC cells, when inoculated in susceptible plant material.

It is also noteworthy that *E. amylovora*-contaminated medflies showed similar behaviors and lifespans to the control medflies not exposed to the pathogen. This means that *E. amylovora* is not pathogenic on *C. capitata,* which is required for an effective spread of the pathogen to new hosts by the insect.

2.2. The medfly *C. capitata* transmits *E. amylovora*

In order to assess the potential host-to-host transmission of *E. amylovora* by *C. capitata*, batches of medflies (separated by sexes) exposed to *E. amylovora*-contaminated apples for 48 h were transferred to new cages containing either non-

contaminated wounded apples, detached shoots or potted plantlets of susceptible *E. amylovora* host plant species. In the case of shoots and plantlets, variations of the experiment with wounded plant material were also included to mimic natural wounds caused by wind, hail, etc. After the transmission period (from 5 to more than 14 days, depending on fire blight symptom development), the plant material was analyzed for the presence of *E. amylovora* by specific PCR and culture on different media. To characterize the insect organs potentially involved in *E. amylovora* transmission, some repeats of this assay were performed using GFP-marked strains.

Results revealed that the medfly *C. capitata* was able to transmit *E. amylovora* from contaminated fruits to other fruits, young shoots and potted plantlets. The times required for *C. capitata* to transmit *E. amylovora* varied depending on the plant material assayed (less than 5 days in the case of fruits, less than 5-7 days in the case of shoots and less than 10-14 days in the case of potted plants).

In the case of fruits, as expected, they did not develop fire blight symptoms (Dueck, 1974; Thomson, 2000; Ordax *et al.,* 2009), but epiphytic and endophytic *E. amylovora* cells were re-isolated from all the fruits exposed to *E. amylovora*-contaminated *C. capitata* for 5 days. Male and female medflies similarly transmitted the pathogen to the fruit's peel surface, except in the area surrounding the peduncle, where females transmitted greater bacterial cell numbers than males. Females, moreover, were more effective than males in contaminating the flesh of the challenged fruits. The presence of medfly eggs in tunnels within the apple flesh suggested a link between *E. amylovora* flesh contamination and the use of the ovopositor by females, which was confirmed by the observation of groups of fluorescent cells on the ovopositor surface of females exposed to GFP-marked *E.*

amylovora cells during the 48-h acquisition period (Fig. 23). Groups of green fluorescent cells were also observed on the distal part of wings and the abdomen of many of the analyzed flies (Fig. 23), suggesting that both the acquisition and the transmission of *E. amylovora* might occur due to the position of such organs during feeding and/or defecating on plant material surfaces.

C. capitata also transmitted *E. amylovora* cells from contaminated fruits to detached shoots and potted plantlets regardless the presence of wounds, with positive transmissions notified by the development of fire blight symptoms 5-14 days after the exposure of susceptible plant material to contaminated medflies. Also in these cases, *E. amylovora* was re-isolated from diseased plant material. Similar to that occurring in fruits, necrotic spots or greater necrotic lesions surrounding some egg clusters embedded in leaf tissues, corresponding to wounds performed by female medflies with the ovopositor were also observed. *E. amylovora* cells were re-isolated from such necrosed tissues, but not from eggs.

According to these results, the medfly *C. capitata* (especially females) satisfies the minimal conditions necessary to act as an *E. amylovora* vector: *i*) the pathogen can be acquired by medflies; *ii) E. amylovora* is not pathogenic on *C. capitata,* as demonstrated by the normal behavior and lifespan of both control and challenged medflies carrying the pathogen; *iii)* is able to survive in/on the insect and the medflies efficiently transmit the pathogen to susceptible hosts by the normal behaviors of feeding, defecating and ovipositing.

2.3. *E. amylovora* persists for 8 days within medflies

With the aim of determining the possible internalization and survival of the fire blight pathogen into *C. capitata*, batches of medflies were subjected to the 48-h acquisition protocol described above, in the presence of *E. amylovora* cells tagged with the red fluorescent protein DsRed. The use of red fluorescent labelling instead of green was necessary due to the green background of internal medfly tissues. *E. amylovora*-contaminated medflies were transferred to new cages where the pathogen was not present, and randomly selected individuals (males and females) were processed at different periods by being cut into semi-fine sections before EFM analysis.

The EFM analysis of medfly sections allowed the location of *E. amylovora* cells on the surface and inner parts of the mouth of medflies after the 48-h acquisition period. Red fluorescent *E. amylovora* cells were also observed in the crop, oesophagus and throughout the digestive tube (Fig. 23). In the latter, bacterial cells appeared as aggregates occupying the lumen and/or coating the internal wall of the tube. No *E. amylovora* cells were observed inside the female medflies ovopositor. EFM detection of *E. amylovora* cells within medfly tissues was positive up to 8 days after the acquisition, but not for extended periods. In addition, the enrichment in liquid medium of semi-fine sections where red fluorescent bacterial cells were absent, was negative. The detection of *E. amylovora* in faeces found all over the cages at the end of the experimental period was also negative.

The fluorescence of the proteins GFP and DsRed is linked to cell viability (Lehtinen *et al.,* 2004; Jhingran *et al.,* 2012). The absence of red cells in the gut of medflies for periods greater than 8 days, together with the null *E. amylovora* enrichment in liquid media in time periods at which *E. amylovora* was detectable by

culture dependent methods in survival assays (8 – 14 days), suggested the absence of *E. amylovora* within *C. capitata* tissues 8 days after the acquisition. Accordingly, results in this work suggest the potential role of *C. capitata* as an active vector of *E. amylovora,* with most of the transmission of the pathogen occurring by bacterial cells on the insect's surface. These can be easily spread during the contact of the carrier insect with *E. amylovora* hosts, during feeding, defecating and/or ovipositing. The spread of the pathogen via contaminated faeces cannot be discarded because, although the detection of *E. amylovora* from faeces was negative, this assay was only performed at the end of the experimental period of assays with DsRed-marked *E. amylovora* strains.

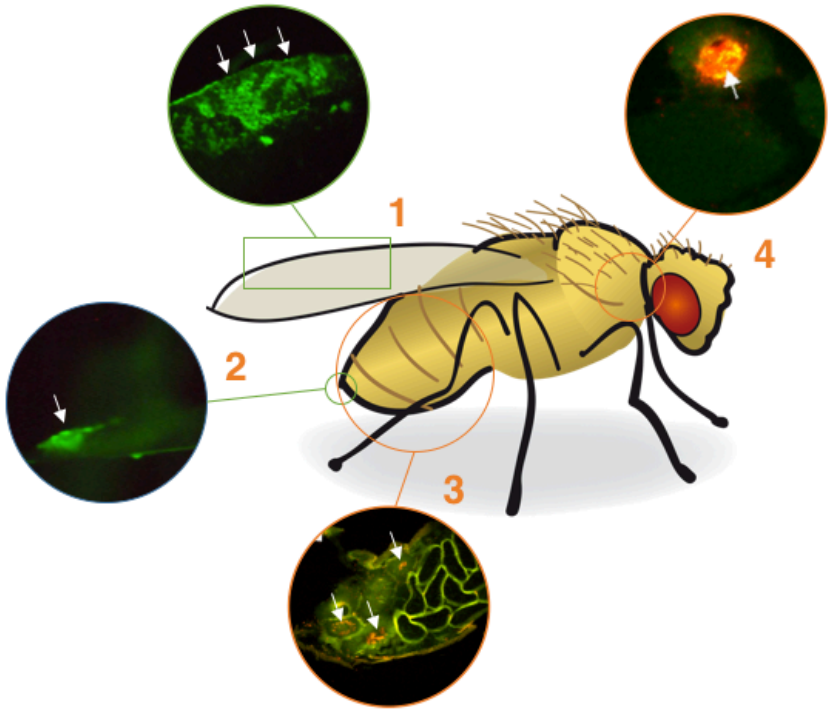


Fig. 23. Representation of the location of *E. amylovora* cells on/in the medfly *C. capitata* after the acquisition. *E. amylovora* GFP-tagged strains were used to monitor the presence of cells on medfly surfaces. Green fluorescent cells were located on the distal part of wings (1), the abdomen and, in the case of females, also on the ovipositor surface (2). To locate *E. amylovora* cells within the insect, DsRed-tagged strains were used. Red fluorescent cells were easily located in the digestive tube in the abdomen (3), but also in the thorax (4). EFM images from Ordax *et al.,* 2015; fly scheme from B. Nuhanen (http://commons.wikimedia.org).

Chapter 3

Studying the feasibility of the water-borne root infection of *Pyrus communis* by *Erwinia amylovora* and characterization of the root infection routes with a green fluorescent protein (GFP)-tagged strain

(Article V in the Annex)

E. amylovora has been traditionally classified as a pathogen of the aerial part of plants. It enters the host plant through natural openings such as nectarthodes in flowers, or stomata on leaves, or also via wounds performed by hail, wind, birds, insects, pruning tools, etc. In the latter case, young and vigorous tissues are usually more susceptible to infection than older or slow growing ones, although factors like the host species or cultivar, weather conditions, etc., are important in determining the success of infections (Thomson, 2000; van der Zwet *et al.,* 2012).

The invasion pathways followed by *E. amylovora* inside the host have been heatedly discussed (Billing, 2011) due to the variability of results depending on inoculation procedures, the type of plant material employed, etc. However, it is generally accepted that once inside the host, *E. amylovora* multiplies and moves throughout the intercellular spaces of the cortex and occasionally invades the vascular vessels, presumably the xylem, which are also used to reach other parts of the host plant. In most cases, infections by *E. amylovora* lead to the production of

characteristic fire blight symptoms (ooze, necrosis and wilting), which are called, depending on the affected organ: leaf blight, fruit blight, shoot blight, twig blight, trunk blight, collar blight or rootstock blight. During severe infections, moreover, the pathogen systemically invades the host, eventually leading to the death of the entire plant (Thomson, 2000; van der Zwet *et al.,* 2012).

Interestingly, *E. amylovora* cells inoculated in the shoot tip or in the middle stem of apple plants migrate to the roots (Gowda & Goodman, 1970; Bogs *et al.,* 1998). Furthermore, cases of root blight under field conditions have sometimes been reported (van der Zwet *et al.,* 2012) although roots are not considered primary infection sites of *E. amylovora* (Bogs *et al.,* 1998).

E. amylovora survives in a variety of types of water (Santander *et al.,* 2012a; Santander *et al.,* 2014b), some of which can be used for irrigation in nurseries and orchards, as well as in soil and in soil-inhabiting insects (Hildebrand *et al*., 2000; Hildebrand *et al*., 2001). According to this, and the above-mentioned information, the root-infection of susceptible plants via contaminated water and/or root-damaging insects, or nematodes, could be possible.

It is also remarkably that, despite pear cultivars being more sensitive to fire blight than apple cultivars (Maroofi & Mostafavi, 1996), most of the studies analyzing tissue sensitivity, or *E. amylovora* migration routes within the host have used the apple as the host model system (Billing, 2011).

In this chapter we summarize and discuss results from a study on the potential of *E. amylovora* to infect plants through the radicular system, which included the *in vitro* culture of pear embryos to obtain pear plantlets and assays of direct root inoculation, root inoculation by soil irrigation, as well as the use of

a green fluorescent protein (GFP)-tagged strain combined with EFM and laser scanning confocal microscopy (LSCM) analysis to characterize the colonization, invasion and *E. amylovora* migration routes throughout the roots (Santander *et al.,* 2016, unpublished; Annex V).

3.1. The *in vitro* culture of pear embryos improves the germination of long-term stored seeds

To analyze the ability of *E. amylovora* to infect roots whole plants were required. The easiest way to obtain this kind of plant material is from seeds. From our own experience, the percentage of germination of pear or apple seeds rapidly decreases during storage under classical conditions of low temperatures and low relative humidity (James, 1967). As a result, experiments involving pear plants are usually restricted to periods in which pears of the needed cultivars are available in the market. In fact, we attempted to germinate a surplus of pear seeds from a previous assay that had been stored for two years, by a standard protocol used in our laboratory (stratification at 4ºC in wet river sand), but none of these seeds germinated over a two month period.

To break seed dormancy, responsible for low germination percentages and elapsed germination periods, we adapted a protocol for *in vitro* culture of embryos, successfully employed with two plant species, one of them pertaining to the family *Rosaceae* (Arrillaga *et al.,* 1992). As a result, about an 85% of the two-year-period stored seeds germinated in only 2-7 days after the *in vitro* culture of excised embryos. Accordingly, the strategies for seed preservation and obtaining plants which were used in this study ensure the availability of whole plants at any time of the year, as long as a sufficient number of seeds are properly collected and stored.

3.2. *E. amylovora* migrates from the roots to the aerial parts of the plant

In order to standardize experimental procedures, most of the studies on *E. amylovora* tissue invasion/sensitivity have been carried out using the apple as the preferred plant material model (Billing, 2011). In an attempt to determine the *E. amylovora* migration pathways in a different host model (*P. communis* cv. Passe Crassane), one-month-old pear plantlets were root-inoculated with a hypodermic needle previously dipped into a colony of a wild type reference strain of the pathogen (CFBP 1430). About 2-3 days after the inoculation, 100 % of inoculated pear plantlets developed fire blight symptoms in aerial organs, in most cases, far from the inoculation site (sometimes 10-12 cm upwards). The tip of the stem and/or petioles of the youngest expanded leaves were usually the most sensitive organs where the first fire blight symptoms were observed (ooze droplets and darkening of tissues, corresponding to incipient necrosis). The progression of fire blight symptoms occurred downwards, with necrosis extending to the rest of the stem, petioles and leaves.

Interestingly, when pear plantlets were inoculated in the tip of the stem for comparison, the onset of symptoms was similar, but they started near to the inoculation site and progressed downwards. Once again, the organs where the first fire blight symptoms were observed were the tip of the stem and petioles.

E. amylovora was re-isolated from symptomatic plant material on semis-selective solid medium, and the identity of randomly selected colonies was confirmed by specific PCR.

Similar results were obtained after the inoculation of plantlets of a different cultivar (cv. Blanquilla), with other two *E. amylovora* strains from different geographical origins and isolated from different hosts (IVIA 1892.1, a Spanish strain isolated from

P. communis; and ATCC 49946 a North American strain isolated from *M. domestica*).

In previous studies performed on apple (Gowda & Goodman, 1970; Bogs *et al.,* 1998), after the direct inoculation of *E. amylovora* into the stem bacterial cells migrated to the radicular system. However, when the challenged organ was the root, the movement of bacterial cells upwards was slow and limited in one case (Gowda & Goodman, 1970), and non-existent in the other one (Bogs *et al.,* 1998). Differences between these results, and the ones in the present study, might be linked to the different fire blight sensitivities of apple and pear (Maroofi & Mostafavi, 1996), but also to the convenience of incubation conditions of inoculated plants. Given the requirement of high relative humidity for *E. amylovora* to cause fire blight symptoms (Thomson, 2000; van der Zwet *et al.,* 2012), the usual procedure to increase the relative humidity surrounding the plants is to put them inside closed plastic bags after inoculation (Wilson & Lindow, 1993; Bogs *et al.,* 1998; Cabrefiga *et al.,* 2007). In our experiments, however, to create high humidity conditions inoculated plants were placed into petri dishes inside trails containing water. In some pathogens and endophytic bacterial species, the translocation of bacteria from roots to aerial parts of the plants occurs via the xylem, moved by the transpiration stream (James *et al.,* 2002; Lelis *et al.,* 2014). Accordingly, enclosing plants inside plastic bags probably stops or highly reduces transpiration currents, preventing bacterial cells from moving from roots to the stem.

3.3. The water-borne infection of *P. communis* roots by *E. amylovora* is possible under laboratory conditions

To evaluate *E. amylovora*'s ability to colonize and invade the radicular system of intact plants, a set of potted one-month-old pear plantlets (*P. communis* cv. Passe Crassane) were soil irrigated with *E. amylovora*-contaminated water (about 10^8 CFUs per g of substrate). For comparative purposes another set of pear plantlets was subjected to a treatment mimicking root lesions after a transplant (unpotting, slightly shaking to remove part of the soil, and potting again) and inoculated by soil irrigation as mentioned above. Both sets of plants were incubated in a climatic chamber with a 12-h photoperiod, at 26ºC, and soil-irrigated periodically (each 3-5 days) with *E. amylovora* contaminated water. These assays were repeated in two independent experiments with the French wild type *E. amylovora* strain CFBP 1430, and the results confirmed in one off assays with the strains IVIA 1892.1 and ATCC 49946.

The average percentages of intact and transplanted plants developing fire blight symptoms after water-borne infections were of 20 % and 52.2 %, respectively. The onset of symptoms in the former case occurred about 25 days after the inoculation. In the case of transplanted plantlets, however, the first symptoms were observed from 5 to 14 days after the inoculation.

Similar to direct root-inoculation assays, the first fire blight symptoms occurred in the aerial parts of the challenged plants, mainly in the tip of the stem and petioles. Furthermore, a delay in the development of fire blight symptoms in roots was observed with respect to the green parts of the plant.

E. amylovora was successfully re-isolated on semi-selective medium from symptomatic plant material. Interestingly, only *E. amylovora*-like colonies were isolated from symptomatic

aerial organs of diseased plants, but a mixture of *E. amylovora*-like colonies and fluorescent pseudomonad-like colonies grew when root samples were analyzed. The presumptive isolation of *E. amylovora* from plants showing fire blight symptoms was further confirmed by specific PCR of randomly selected colonies.

Differences in the infection percentages between intact and transplanted plants were probably due to the presence of wounds in the radicular system in transplanted plants. These results, thus, suggested wounds as sites of entry of *E. amylovora* into roots, similar to that described in the aerial parts of the plant (Thomson, 2000; van der Zwet *et al.,* 2012). With respect to differences between plants directly inoculated in the roots (100 % of successful infections after inoculation, earlier onset of symptoms) and soil-irrigated plants with *E. amylovora*-contaminated water (20 - 52.5% of successful infections, delayed onset of symptoms), they might be due to multiple factors. Some of these factors are: *i)* the effective inoculum size, which was diluted in soil irrigated plants, and highly concentrated in plants directly inoculated into the roots; *ii)* directly reaching susceptible tissues and/or vascular vessels in *E. amylovora* cells inoculated with a needle, which does not occur during water-borne infections, which probably involve colonization of root surfaces, multiplication to reach infective doses and invasion of the host tissues; *iii)* antagonistic interactions of *E. amylovora* cells inoculated by soil-irrigation with microbiota present in the rhizosphere and/or the substrate. In this regard, strains of fluorescent pseudomonads, which were frequently co-isolated with *E. amylovora* from root samples, have been frequently reported as effective biocontrol agents against this pathogen (Wilson & Lindow, 1993; Cabrefiga *et al.,* 2007).

3.4. Characterization of the root-colonization, invasion and migration pathways using a GFP-tagged *E. amylovora* strain

Once the ability of *E. amylovora* to invade host plants through the roots had been demonstrated, we aimed to characterize the pear root colonization and invasion patterns by the pathogen, and also the migration of bacterial cells within host tissues. For this purpose, a GFP-tagged transformant (GFP1) of the wild type strain CFBP 1430 was used (Ordax *et al.,* 2009). Previous to inoculation assays, the stability of the plasmid in water, soil and in plants was confirmed, indicating the usefulness of strain GFP1 for microscopic studies.

To facilitate root infections, one-month-old pear plantlets (*P. communis* cv. Passe Crassane) were inoculated after removing the soil and rhizosphere, and cleaning the roots with tap water. The roots were then subjected to a brief surface disinfection with ethanol, rinsed and inoculated with *E. amylovora* GFP1 by immersion in a cell suspension in distilled water. Afterwards, inoculated plantlets were potted using sterile soil, incubated as described above and periodically processed for their observation by EFM or LSCM.

Despite the huge amount of studies on *E. amylovora,* topics such as the preferred initial multiplication sites or the optimal routes for a systemic spread within the host are subject to controversy (Billing, 2011).

Assays with the green fluorescent *E. amylovora* strain GFP1 allowed the identification of different stages during pear root colonization and invasion, and also the preferred routes to migrate upwards in the plant (Fig. 24):

i) Colonization of root surfaces by the formation of cell aggregates and biofilm-like structures. These structures were observed on the surface of both the main and lateral roots, as well as in areas surrounding the sites of emergence of lateral roots. Individual cells and small aggregates were also observed covering root hairs.

ii) Invasion of the radicular system via wounds performed by root manipulation, as well as through cracks at the points of emergence of lateral roots. An important accumulation of fluorescent cells was observed in such entry sites, probably indicating the multiplication of the pathogen in the intercellular spaces of the cortical parenchyma.

iii) Access of bacterial cells to the vascular cylinder. Green fluorescent bacteria were observed inside the vascular vessels in both wounded and unwounded roots. In the former, vessels were usually exposed in wounded areas, facilitating the bacteria's ability to reach vascular tissues. In the latter, however, despite a clear tendency for bacteria to grow on the endodermis surface, the entry of bacteria through the Casparyan strip was not observed in any of the preparations. This might occur *via* collapsed cells of the endodermis or by other unknown mechanisms.

iv) Migration from the root upwards to the stem and other aerial organs of the host plant. Fluorescent *E. amylovora* cells were observed in the middle stem of non-symptomatic root-inoculated pear plantlets, inside xylem tracheids, with the characteristic helicoidal cell wall thickenings. Furthermore, in none of the analyzed

samples were bacterial cells occupying the phloem observed, suggesting that the main *E. amylovora* migration route from the roots to the stem is the vascular system.

The observed stages of root colonization, invasion and migration in *E. amylovora* were similar to those of well-known plant soil-borne pathogens such as *R. solanacearum* (Vasse *et al.,* 1995; Álvarez *et al.,* 2008b), *Clavibacter michiganensis* subsp. *michiganensis* (Lelis *et al*., 2014) and *Dickeya* ssp (Czajkowski *et al.,* 2010). Furthermore, the visualization of *E. amylovora* cells inside the xylem vessels in asymptomatic plants shortly after their inoculation in the roots agrees with the results of direct-root inoculation assays, in which the first observed fire blight symptoms were developed in the upper parts of the plants. These results together indicate that *E. amylovora* uses the xylem for a rapid systemic spread in the same direction of the transpiration current, as described in other pathogens or endophytic bacterial species (Vasse *et al*., 1995; James *et al.,* 2002; Liu *et al*., 2006; Quecine *et al.,* 2016).

Once in the tip of the stem and/or petioles (the first organs that developed ooze or necrosis after root inoculation), *E. amylovora* is able to break out of the xylem and gain access to the cortex, and starts to multiply in the intercellular spaces. The movement of the bacterium in the cortex occurred in both directions, agreeing with the observations of Zamski *et al* (2007). These authors hypothesize that this movement is possible due to bacterial growth and ooze production in the cortical apoplast, which leads to an increase in pressure that favors the leakage of water, sugars and sorbitol to the intercellular spaces by degenerated plant cells. The later phenomenon contributes to the increase of pressure, which pushes bacterial cells in all directions.

Finally, according to our results, water-borne infection with *E. amylovora* might be possible in nurseries, containing high densities of young host plants, as well as in orchards where pear trees are cultivated. Also, given the ability of *E. amylovora* to colonize and invade the whole radicular system, roots could act as a reservoir of the pathogen under field conditions. However, the feasibility of *E. amylovora* root-infection in host plants other than pear and/or lignified trees, or the possible function of roots as reservoirs for the pathogen must be determined.

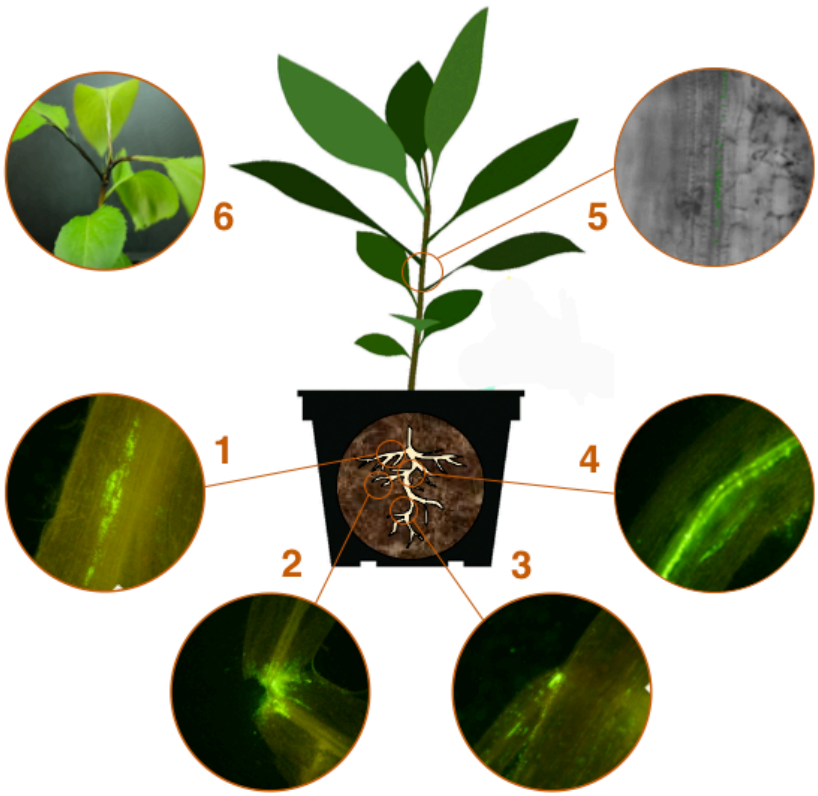


Fig. 24. Representation of the root infection stages by *E. amylovora* in *P. communis*. *E. amylovora* colonizes root surfaces forming cell aggregates and biofilm-like structures (1). The invasion of the radicular system occurs via wounds performed by root manipulation (2), as well as through cracks at the points of emergence of lateral roots (3). The pathogen multiplies at the site of entry and advances through the apoplast of the cortex. *E. amylovora* enters the vascular system through wounds or other unknown mechanisms (4). Bacterial cells migrate from the root upwards to the stem and other aerial organs of the host plant via the xylem (5). Once there, bacteria translocate from xylem vessels to the apoplast of the cortex, and fire blight symptoms develop (6). Pictures 1-4 correspond to EFM images of pear plantlets inoculated with the *E. amylovora* strain GFP1. Picture 5 is an overlay of GFP fluorescence and a transmitted light image of a section of stem taken by LSCM (*E. amylovora* cells can be seen inside a xylem vessel with the characteristic helicoidal cell wall thickening).

Chapter 4

Surviving starvation and other harsh conditions inside and outside host plants: Role of the alternative sigma factor RpoS

(Article VI in the Annex)

E. amylovora, like other non-obligated pathogens, experiences short periods of feasting (i.e. when growing on flower surfaces and within host tissues) interspersed by more or less prolonged periods of starvation (i.e. during epiphytic stages on leaves or other plant surfaces, not sustaining growth, when transported by rainwater or insect vectors, on the surface of contaminated pruning tools, etc.) during its life cycle.

The ability of pathogens to persist in the environment, hence, will depend on their strategies to face nutrient scarcity. Linked to this, the perception of starvation by actively growing bacteria is a signal of entry into the stationary phase (Lazazzera, 2000). In fact, starved and stationary-phase cells share a variety of physiological and genetic features (Lange & Hengge-Aronis, 1991; Kolter *et al.,* 1993; Navarro-Llorens *et al.,* 2010).

In *E. coli* and other bacterial species, a key regulator of starvation responses, and also entry into stationary phase, is the alternative sigma factor of the RNA polymerase RpoS, encoded by the gene *rpoS* (Ishihama, 1997; Hengge-Aronis, 2002; Navarro-Llorens *et al*., 2010; Battesti *et al.,* 2015). This sigma factor changes the transcription program of the RNA polymerase

from one allowing growth to one promoting survival (Battesti *et al.,* 2015). The RpoS-mediated reprogramming of cell functions during starvation occurs via the regulation of metabolic genes (Battesti *et al.,* 2015), genes related to stress resistance (Edwards, 2000; Hengge-Aronis, 2000), DNA protection and repair (Vijayakumar *et al*., 2004), nutrient recycling, correct protein folding (Dong & Schellhorn, 2009), etc.

A group of RpoS regulated genes of special interest are those encoding catalases and other antioxidant enzymes (Eisenstark *et al.,* 1996; Robbe-Saule *et al.,* 2001; Tarassova *et al.,* 2009). These enzymes neutralize the intracellular ROS produced by aerobic metabolism, whose levels increase during starvation (McDougald *et al*., 2002; Boaretti *et al*., 2003), or growth on solid media (Kong *et al.,* 2004; Tanaka *et al.,* 2014). On the other hand, the loss of culturability characteristic of the VBNC state has been connected to the decrease of catalase activity in stressed cells (Kong *et al.,* 2004). It can therefore be expected that bacterial strains impaired in RpoS will be defective in both the neutralization of ROS and/or the development of the VBNC response, but the role of this sigma factor in the starvation responses of *E. amylovora* has not yet been assessed.

As a result of the action of RpoS, starved and stationary-phase cells become protected against stresses to which they were not previously exposed, a phenomenon also known as cross-protection (Hengge-Aronis, 2002). In addition, RpoS can be induced by other stresses, including host defenses during host-pathogen interactions. In fact, RpoS participates in the regulation of pathogenicity and/or virulence in a variety of bacterial pathogens via the direct regulation of genes involved in disease development, or by controlling those enhancing the biological fitness of the bacterium within the host (Kazmierczak *et al.,* 2005; Dong & Schellhorn, 2010).

In this chapter, results from a study on the participation of RpoS in *i) E. amylovora* responses to starvation, including the development of the VBNC state; *ii)* stationary-phase cross-protection against multiple stresses; *iii)* synthesis of EPSs; *iv)* swimming motility; *v)* survival in non-host tissues; and *vi)* virulence in different types of plant material are summarized and discussed (Santander *et al.,* 2014a; article VI of the Annex).

4.1. The sigma factor RpoS plays an important role in *Erwinia amylovora*'s ability to adapt to starvation, enhancing the maintenance of both culturability and viability

In order to characterize novel functions of the sigma factor RpoS in *E. amylovora,* an insertional mutant of the reference strain CFBP 1430 (wild type, WT) defective in *rpoS* (*rpoS*$^-$) was constructed by marker ex-change mutagenesis*,* together with a complemented strain carrying a plasmid copy of the wild type gene (*rpoS*$^+$). To determine the possible contribution of RpoS to *E. amylovora* responses to starvation, natural water microcosms were prepared, inoculated separately with strains WT, *rpoS*$^-$ and *rpoS*$^+$, incubated at 28ºC, and the kinetics of entry into the VBNC state and the duration of viability were measured for 12 days.

The analysis of culturable data showed a typical starvation-survival response in strains with a functional copy of the *rpoS* gene (the WT and the strain *rpoS*$^+$*)* with a slight loss of culturability during the first week, and a sharpened decay of culturable cell populations during the following 5 days. Viable and total cell populations remained in similar numbers as at time 0, thus indicating that the observed loss of culturability corresponded to a progressively increasing number of viable cells over time entering the VBNC state. However, the *E. amylovora* mutant *rpoS*$^-$ developed an aberrant response to starvation, consisting of a faster entry into the VBNC state linked to the phenomena of cell death and cell lysis, not observed in the WT strain.

The defective adaptation to starvation in *rpoS* impaired strains has been attributed to an elevated mutation frequency due to deficient protection against ROS (Tarassova *et al.,* 2009), and also failing to shoot down metabolic programs by a misregulation of the stringent response (Trigui *et al.,* 2015).

The enhanced induction of the VBNC state observed in the *E. amylovora* mutant *rpoS*$^{-}$ has been reported in similar mutants of other gammaproteobacteria of different orders (Boaretti *et al.,* 2003; Kusumoto *et al.,* 2012; Trigui *et al.,* 2015). The loss of culturability occurring during the VBNC response seems to be correlated to a decrease in catalase activity, and hence to the defective ability of stressed cells to detoxify the H_2O_2 (McDougald *et al*., 2002; Boaretti *et al*., 2003; Kong *et al*., 2004). However, it is necessary to point out that entry into the VBNC state in *E. amylovora* WT and *rpoS*$^{+}$ strains occurred without an apparent loss of viability, which means that RpoS might be involved not only in bacterial cell adaptation to starvation, but also in molecular coordination for safe development of the VBNC response.

4.2. RpoS is necessary for full *E. amylovora* stationary phase cross-protection against diverse stresses

To characterize the participation of RpoS in *E. amylovora* stationary phase cross-protection against a variety of stresses (osmotic, oxidative and acid stress, heat shock and acid shock), the resistance to such stresses of stationary-phase and log-phase cells of *E. amylovora* WT, *rpoS* mutant and complemented strains was compared.

RpoS was required for complete stationary-phase cross protection against osmotic, acid and oxidative stresses. *E. amylovora* has to deal with these stresses during nectary surface colonization, while growing in the apoplast of host plants, and when exposed to plant defenses, respectively (Grignon & Sentenac, 1991; Pusey, 2000; Venisse *et al.,* 2001). Interestingly, although log-phase cells were less resistant to the above-mentioned stresses than stationary-phase cells, the sigma factor RpoS also conferred enhanced stress resistance to *E. amylovora*

log-phase cells, especially in the cases of osmotic and acid stresses. The RpoS regulated expression of a variety of genes in *E. coli* during the exponential growth phase has been described (Dong *et al.,* 2008), also suggesting a possible function of the sigma factor RpoS in log-phase cells. However, the exposure of *E. amylovora* to these stresses might have been delayed enough to increase RpoS levels in the challenged log-phase cells.

The sigma factor RpoS was essential for *E. amylovora* stationary-phase cells to develop proper resistance to heat-shock. Proteins related to resistance to this stress have been involved in the adaptation of pathogens to the environment, including the host (Mantis & Winans, 1991; Feder & Hofmann, 1999). In fact, heat-shock proteins are induced during *E. amylovora* infections of immature pears (Zhao *et al.,* 2005).

Finally, except for cross-protection against heat-shock, where only RpoS determined the increased resistance to this stress in stationary-phase cells, the cross resistance against the remaining stresses in stationary phase cells possessed an RpoS independent component. This phenomenon was particularly important in the case of stationary phase cross protection against acid shock, which occurred regardless of the presence or not of a functional copy of the *rpoS* gene. Acid shock (pH 3.5) was assayed because this was the pH inside the wounds performed on immature fruits prior to inoculating the pathogen. The stress protection of stationary phase cells occurring in an RpoS-independent manner has been reported in other pathogens (Jørgensen *et al*., 1999; Rosche *et al*., 2005) and it has been attributed to factors other than RpoS controlling some genes during the entry into the stationary phase.

4.3. RpoS enhances *E. amylovora* survival in non-host plant tissues, modulates virulence in immature fruits and participates in the regulation of EPS synthesis and swimming motility

To determine the possible role of RpoS in different aspects of *E. amylovora*'s interactions with plants, the effect of *rpoS* mutation on bacterial survival in a non-host (*N. tabacum*), swimming motility, EPS production and fire blight symptom development in immature loquats (*E. japonica*) and pear plantlets (*P. communis* cv. Passe Crassane) was evaluated.

During incompatible plant-pathogen interactions the host triggers a HR, in which pathogenesis-related (PR) genes and localized cell death are induced at the site of infection. This HR is orchestrated, in part, by ROS produced in the recognition site of the pathogen. These ROS act thus as signaling molecules, and also contribute to block pathogen progression by their toxicity (Zurbriggen *et al.,* 2010). The *E. amylovora* mutant *rpoS*$^{-}$ elicitated an apparently normal HR on *N. tabacum* leaves, but the survival of this strain in leaf tissues was lower than that observed in strains possessing a wild type copy of the gene (WT and *rpoS*$^{+}$). The high H_2O_2 sensitivity of the *E. amylovora* mutant *rpoS*$^{-}$, hence, might have contributed to the observed phenotype.

However, one of the characteristics distinguishing *E. amylovora* from other plant pathogenic bacteria is the use of the TTSS to induce plant cell death and/or modify host responses to infection, so that a typical HR, with the corresponding localized cell death and oxidative burst, is produced regardless of the compatibility or incompatibility of the host (Venisse *et al.,* 2001; Iakimova *et al.,* 2013; Vrancken *et al.,* 2013). Surprisingly, the reduced survival of the *E. amylovora rpoS* mutant in *N. tabacum* was not evidenced in assays with compatible plant material

(immature fruits and plantlets of *E. amylovora* susceptible host plants). In fact, the mutation of *rpoS* led to increased virulence in immature fruits, and symptom development/progression in plantlets which was indistinguishable from that observed in WT and *rpoS*$^{+}$ strains.

Differences between the survival of the *E. amylovora* mutant *rpoS*$^{-}$ in incompatible and compatible hosts might be explained either by *i)* the sensitivity of the *rpoS*$^{-}$ strain to toxic compounds other that ROS in non-host tissues, not present in compatible hosts, and/or *ii)* the induction of bacterial protective molecules against ROS and other toxic molecules (e.g. siderophores or EPSs) under compatible host conditions.

In this regard, despite amylovoran production in the *E. amylovora* mutant *rpoS*$^{-}$ being slightly lower than in the WT strain, the biosynthesis of levan was about two times higher. Levan is an important virulence factor in *E. amylovora* (Geider, 2000; van der Zwet *et al.,* 2012; Piqué *et al.,* 2015), and is also involved in biofilm formation inside host plant tissues (Koczan *et al.,* 2009). Levan production depends on levansucrase, an exoenzyme that synthesizes levan using sucrose as a substrate (Geider, 2000). Defects in levan production are linked to a slow development of fire blight symptoms in host plants (Geider, 2000). The overproduction of this virulence factor by the *E. amylovora* mutant *rpoS*$^{-}$ might explain the increased virulence of this strain in immature fruits. Furthermore, given the sucrose-dependence of levan production, virulence differences in plantlets and fruits might be linked to different sucrose concentrations in these two types of plant material. Host species/organ-related differences in plant defense elicitation might also explain the differences observed.

Finally, the *E. amylovora* mutation of the *rpoS* gene led to reduced swimming motility, indicating the involvement of RpoS in the control of this phenotypic trait, which is required for the pathogen to move to wounds or other sites of entry into the host plant (Raymundo & Ries, 1981).

Chapter 5

An insight into the link between survival and oxidative stress resistance: Role of the *Erwinia amylovora* catalases during responses to starvation and plant-pathogen interactions

(Article VII in the Annex)

E. amylovora, like other bacterial pathogen inhabiting oxic environments, has to deal with ROS throughout its life cycle. Some sources of ROS are aerobic metabolism as well as external biotic and abiotic sources, such as antagonistic microbiota or plant host defenses (Cabiscol *et al*., 2000; Kong *et al*., 2004; Trias *et al.,* 2008; Torres, 2010; Kashmiri & Mankar, 2014). Nowadays, it is known that *E. amylovora* possesses different antioxidant enzymes (Keck *et al.,* 1997), but they have only just been detected and their functions have not yet been analyzed. Other known elements related to *E. amylovora* protection against oxidative stress are siderophores (Dellagi *et al.,* 1998), LPS (Berry *et al.,* 2009) and EPSs (Király *et al.,* 1997; Venisse *et al.,* 2001). However, the participation of the latter in protection against oxidative stress is not yet clear (Venisse *et al.,* 2003).

An important ROS is H_2O_2, a compound able to penetrate cell walls and membranes which is highly reactive with all kinds of biomolecules (Linley *et al.,* 2012). To deal with H_2O_2, *E. amylovora* and other phytopathogenic bacteria possess

catalases and/or catalase-peroxidases, antioxidant enzymes which decompose H_2O_2 into innocuous compounds. These enzymes might be important mechanisms when facing the oxidative stress occurring during both compatible and incompatible plant-pathogen interactions (Venisse *et al.,* 2001; 2003; Iakimova *et al.,* 2013; Abdollahi *et al.,* 2015), and also in periods of starvation, when the neutralization of the ROS produced by metabolism is essential for survival (McDougald *et al*., 2002; Boaretti *et al*., 2003). However, despite the potential role that catalases might play during these important stages of the pathogen's life cycle, their functional roles have yet to be characterized.

According to annotated *E. amylovora* genomes, this pathogen possesses a catalase (KatA) and a catalase-peroxidase (KatG). In this chapter results from a study (Santander *et al.,* 2016, unpublished; Annex VII) on the characterization of the functional roles of the *E. amylovora* catalatic enzymes KatA and KatG during both plant-pathogen interactions and starvation survival responses are summarized and discussed. A discussion of results on the role of the *E. amylovora* EPSs against H_2O_2 is also provided.

5.1. *E. amylovora* catalases KatA and KatG possess different regulation patterns and specific activities *in vivo* and *in vitro*

To characterize the *E. amylovora* catalases KatA and KatG, catalase mutants (*katA*$^-$, *katG*$^-$, *katAG*$^-$) of the reference strain CFBP 1430 (WT) were obtained using the lambda Red Recombinase System. Complemented strains (*katA*$^+$, *katG*$^+$) were also obtained by introducing a functional plasmid copy of *katA* and *katG* genes into the corresponding single mutants. Catalases were characterized by:

i) Determination of the expression of the genes *katA* and *katG* in the WT strain, in log and stationary phase cells, in the presence of H_2O_2, and in host tissues, by semi-quantitative RT-PCR.

ii) Measurement of the catalase activity of the above-mentioned strains (WT, *katA*$^-$, *katA*$^+$, *katG*$^-$, *katG*$^+$, *katAG*$^-$).

iii) Detection of catalase isozymes by polyacrylamide gel electrophoresis under native conditions (native PAGE) followed by zymographic detection of catalase bands and identification of proteins by liquid chromatography - tandem mass spectrometry (LC-MS/MS).

iv) Growth inhibition halo assays on semi-solid agar plates using H_2O_2 and menadione as oxidizing agents, and minimal inhibitory concentration (MIC) assays in liquid medium, using H_2O_2 as oxidizing toxic compound.

The results of this part of the study revealed that catalase gene expression in *E. amylovora* is growth phase dependent, with *katA* and *katG* gene expression being induced in stationary and in log phase, respectively. Moreover, the expression of both genes, but especially *katG*, were induced by exposure to H_2O_2 and host tissues. These results may indicate the contribution of KatA and KatG to neutralizing ROS during growth within the host, with probable differentiated roles during growth and when bacterial cells reach the stationary growth phase, a physiological state also resembling that of microorganisms under starvation conditions (Navarro-Llorens *et al.,* 2010).

The *in vitro* analysis of the specific catalase activity in the different *E. amylovora* strains demonstrated that KatA accounts for most of the catalase activity*,* followed by KatG. The null catalase activity of the double mutant *katAG*$^-$ indicated that KatA and KatG are the only sources of catalase activity in *E. amylovora.* Surprisingly, it was only possible to detect KatA, and not KatG, by catalase zymography, regardless of the conditions assayed or the strain analyzed (e.g. total protein concentrations from 1 to 150 μg of log and stationary phase cultures, induction of catalases by exposure of cells to H_2O_2). Additionally, LC-MS/MS analysis of proteins of catalase activity bands in zymograms confirmed the presence of KatA, but not KatG, in samples corresponding to cultures of the strains containing a functional copy of the corresponding gene. Peroxidase activity detection by zymography was null in all the strains and conditions described above.

Our results agree with those of Keck *et al* (1997), who only detected one catalase isozyme in four *E. amylovora* strains in a study on the zymographic detection of antioxidant activities. Nevertheless, the same authors described a peroxidase band when protein extracts of 40-day-old cultures were analyzed. We

were able to reproduce this result with the *E. amylovora* WT strain. The peroxidase band was the same size as KatA, and the LC-MS/MS analysis revealed the presence of KatA and a peroxiredoxin in this band (amongst other proteins with no catalatic and/or peroxidatic activity described), but not KatG. The LC-MS/MS analysis of the entire gel portion above KatA confirmed the absence (or the presence in numbers below the detection limit of both peroxidase zymography and LC-MS/MS) of KatG within samples. The degradation of KatG during native PAGE and/or zymographic staining and/or the limited induction of this protein under the assayed conditions are other possible explanations for these results.

The growth inhibition halo assays revealed the functional participation of KatA and KatG in H_2O_2 and menadione detoxification. The lack of the two enzymes caused extreme sensitivity of the double mutant *KatAG*$^-$ to oxidative stress. However, the presence of one of the two enzymes, but mainly KatA, improved resistance to the above-mentioned compounds. Accordingly, these results confirm the need for catalases for the *in vivo* protection of *E. amylovora* against oxidative stress. The MIC of H_2O_2 determined for each mutant agreed with the above mentioned results: WT, 20 mM < MIC ≤ 40 mM; *katA*$^-$, MIC ≤ 0.6 mM; *katG*$^-$, MIC ≤ 20 mM; *katAG*$^-$, MIC ≤ 0.1 mM.

5.2. *E. amylovora* catalases play a role in the maintenance of culturability under starvation conditions, delaying entry into the VBNC state

The role of the *E. amylovora* catalases during starvation-survival was assessed by comparison of culturable, viable and total cell population dynamics of the *E. amylovora* WT, catalase mutants and complemented strains in oligotrophic water

microcosms incubated at 28ºC for 33 days. Culturability was periodically assessed in parallel on rich media supplemented, or not, with catalase. Viable and total cell numbers were counted by flow cytometry at initial and final times, after staining microcosm samples with the Live/Dead viability kit.

The results of this part of the study revealed an accelerated entry into the VBNC state (i.e. a marked loss of culturability not accompanied by a decrease of viable or total cell numbers) of the double catalase mutant *katAG*$^-$, in comparison with the WT strain and mutants lacking just one catalase (*katA*$^-$ and *katG*$^-$). Interestingly, single catalase mutants behaved statistically indistinguishably from the WT strain. In any case, a catalase-dependent decrease of viable or total cells was reported. The overexpression of catalase genes, mainly *katA*, in complemented strains induced an enhanced maintenance of culturability with respect to the WT and the corresponding mutants. The addition of catalase to culture media increased culturable cell numbers of all the strains, compared to plate counts on regular medium without catalase. However, progressive entry into the VBNC state occurred regardless of culture medium amendment with catalase.

These results, hence, indicate a link between catalase activity and the VBNC response. Related to this, Kong *et al* (2004) observed in *Vibrio vulnificus* a decrease in catalase activity occurring in parallel with the entry into the VBNC state. However, these authors used an *oxyR* mutant to conclude that catalases were key enzymes for the maintenance of culturability. This mutant completely lacked catalase activity, and was unable to grow on solid media unless it was supplemented with an external source of catalase, due to the production of H_2O_2 during media preparation. Nevertheless, *oxyR* controls a variety of antioxidant enzymes (Storz & Tartaglia, 1992; Pomposiello & Demple, 2001), so the recovery of culturability of *oxyR* mutants by the exogenous

addition of catalase is probably a more complex phenomenon, which is difficult to explain based only on catalase activity. In fact, the double *E. amylovora* catalase mutant, also lacking catalase activity, grew similar to the WT strain on solid media, but developed faster entry into the VBNC state.

In a previous work (Santander *et al.,* 2014a; Annex VI) we reported an enhanced starvation-induced VBNC response of an *E. amylovora* mutant impaired in the alternative sigma factor RpoS. Interestingly, the specific catalase activity in this mutant (analyzed in the present study) was comparable to that of the single catalase mutant *katA*$^{-}$*,* which developed a normal VBNC response, similar to the WT strain. The sigma factor RpoS also controls a variety of genes related to oxidative stress resistance, amongst others (Eisenstark *et al.,* 1996; Robbe-Saule *et al.,* 2001; Tarassova *et al.,* 2009), and the deficient responses of *rpoS* mutants to starvation are, in part, induced by cell damage caused by ROS (Tarassova *et al.,* 2009).

According to this, the *E. amylovora* catalases KatA and KatG contribute to the maintenance of culturability during starvation, with their expression delaying entry into the VBNC state. However, the "culturable" phenotype must be the result of a combined action of multiple antioxidant activities, which explains the unaltered ability of the double catalase mutant *katAG*$^{-}$ to form colonies on solid media.

5.3. Catalases contribute to *E. amylovora* survival in non-host tissues, and are required for full virulence in immature fruits

To determine the potential functional roles of *E. amylovora* catalases during plant-pathogen interactions, survival and virulence assays were performed on leaves of a non-host plant (*N. benthamiana*) and in immature fruits of two susceptible hosts

(*E. japonica* cv. Tanaka and *P. communis* cv. Devoe), respectively.

Although many of the assays performed with *E. amylovora* catalase mutants revealed KatA as the main catalase in this pathogen, the KatG deficient mutant was more affected than the one impaired in KatA during plant-pathogen interactions. This suggests a strong induction of KatG activity under plant conditions and/or other functions for this protein different and/or complementary to H_2O_2 neutralization.

The *E. amylovora* catalase mutants generated a HR in *N. benthamiana* apparently similar to that observed in the WT strain. However, the survival of these mutants within the leaf apoplast of this non-host plant was compromised, with strain *katAG*$^-$ being the most sensitive to non-host conditions, followed by *katG*$^-$ and *katA*$^-$.

Similarly, catalase mutants showed reduced virulence in immature fruits with respect to the WT and complemented strains. In this regard, a delay in the onset of symptoms was observed in the *E. amylovora* mutants *katAG*$^-$ and *katG*$^-$, but not in *katA*$^-$. Moreover, the necrosis extent in the three mutants was reduced compared with that of the WT or the complemented strains. In the case of mutants *katG*$^-$ and *katAG*$^-$, this was probably a consequence of the delayed onset of necrosis. Nevertheless, the mutant *katA-,* showed a similar onset of symptoms but reduced necrotic areas compared to the WT strain.

Based on these results, *E. amylovora* catalases contribute to the survival in non-host tissues, probably via detoxification of the H_2O_2 produced by plant cells during interactions with the pathogens. A similar contribution of catalases might be deduced from virulence assays. Interestingly, and in connection with expression studies, our results suggest that the *E. amylovora*

catalase KatG is probably required at the initiation of the infection, while KatA may contribute to a faster symptom development.

5.4. EPSs are induced by H_2O_2 but apparently do not enhance *E. amylovora* protection against this compound

The contribution of EPSs in *E. amylovora* protection against H_2O_2 has been discussed (Venisse *et al.,* 2003). In our study, we obtained catalase mutants of this pathogen which were highly sensitive to oxidative stress. If the protective role of EPSs is real, then it would be expected that the sensitivity of the *E. amylovora* catalase mutants to this compound under EPS inducing conditions would be reduced. In order to test this hypothesis, we repeated MIC and growth inhibition halo assays using H_2O_2 as oxidizing compound and culture media with carbon sources inducing either amylovoran or levan. Additionally, we measured EPS production in the wild type strain in the presence or absence of H_2O_2.

These results demonstrated that the H_2O_2 sensitivity of *E. amylovora* catalase mutants was not reduced under EPS inducing conditions. However, EPS biosynthesis was enhanced in cells grown in the presence of H_2O_2, a phenomenon hitherto not described.

The release of H_2O_2 by plant cells is one of the first host responses to bacterial infections. This compound has a toxic effect on bacteria, participates in the stiffening of the plant cell walls and contributes to the orchestration of plant defenses as an important signaling molecule (De Gara *et al.,* 2003; Zurbriggen *et al.,* 2010). Our results suggest that H_2O_2 might also act as a signaling molecule for *E. amylovora,* ensuring a rapid deployment of virulence/pathogenicity factors such as EPSs, which develop important roles during the invasion of host tissues (Geider, 2000;

van der Zwet *et al.,* 2012). The role of EPSs in protecting against ROS other than H_2O_2 also cannot be ruled out.

IV. FINAL CONCLUSIONS

IV. Final conclusions

To round off the presentation of the research carried out in this Doctoral Thesis many conclusions can be drawn from all the results obtained, some of which are novel with respect to the survival, the infection process and other aspects of the biology of the fire blight pathogen. Among these conclusions we highlight the following:

1. Temperate environmental temperatures (14ºC – 20ºC) favor the persistence of *E. amylovora* starved cells in a culturable state. This coincide with periods in which fire blight symptoms can occur under field conditions, thus favoring the environment's enrichment with the bacterium. Temperatures above and below this range induce a progressive entry of the pathogen into the VBNC state, with lower temperatures, moreover, improving the maintenance of viability in starved cells. Recovery from the VBNC state may occur in parallel with the renewal of the vegetative growth of the hosts.

2. *E. amylovora* responds to starvation through morphological changes (including the adoption of rounded shapes and the development of OMVs), dwarfing, and modulation of motility and gene expression. These are strategies which are also described in other bacterial pathogens, and demonstrate that *E. amylovora* is endowed with mechanisms to adapt to environmental nutrient limitation.

3. Temperature modulates both starvation responses and changes in cell size in *E. amylovora,* suggesting that responses to nutrient scarcity in nature may depend on environmental conditions, probably contributing to the

seasonal development of fire blight disease.

4. *E. amylovora* starved cells are pathogenic. The induction of the VBNC response by starvation or chlorine leads to a loss of pathogenicity in immature fruits. However, this can be reverted by inoculation of VBNC cells into susceptible pear plantlets. These results support the VBNC state as a survival strategy, and the possible contribution of VBNC cells to fire blight epiphytotics should be investigated. Furthermore, these data highlight the importance of complementing *E. amylovora* detection protocols with culture-independent techniques, to detect VBNC cells.

5. Under controlled conditions, *E. amylovora* remains pathogenic, even at very low temperatures. Furthermore, many virulence factors are induced at temperatures below the optimal for growth. These results reveal *E. amylovora* as a psychrotolerant bacterium. The former, together with starvation adaptations, might explain the successful spread of this pathogen to multiple countries in different climatic areas worldwide.

6. *E. amylovora* survives in *C. capitata* for at least, 28 days, and insect conditions induce the entry of the pathogen into the VBNC state. These results indicate that the VBNC state may also contribute to the dissemination of the fire blight pathogen, enhancing its tolerance to stress and, therefore, the probability of reaching a new host throughout vectors.

7. *E. amylovora* cells form aggregates and/or biofilm-like structures on the insect surface, mainly on the distal part of wings, the abdomen and the ovipositor. *E. amylovora* can also be internalized by *C. capitata* and persist within the insect for 8 days. The location of cells within the insect are the internal parts of the mouth, the crop and the digestive gut.

These results suggest bacterial adaptations to insect conditions, and reveal *E. amylovora* as a nonpathogenic bacterial species of *C. capitata.*

8. *E. amylovora* transmission by *C. capitata* occurs mainly through contact of the medfly with plant surfaces, during normal feeding or defecating behaviors, although female medflies can directly inoculate *E. amylovora* into plant material with the ovipositor. This supports the idea that the transmission of *E. amylovora* by *C. capitata* may also occur under field conditions.

9. *E. amylovora* is able to invade pear plantlets through the roots, with normal fire blight symptoms being developed in the aerial part of the plant. These results have never been described before, and might indicate that part of the fire blight symptoms observed under field conditions might be due to infections which started in the radicular system. Moreover, given the *E. amylovora*'s ability to colonize roots, these organs may act as a reservoir of the pathogen.

10. Under controlled conditions, the root infection of pear plantlets by soil-irrigation with *E. amylovora* contaminated water is feasible, and infection probabilities increase in plants with damaged roots, for example, after transplanting. This may raise a concern in nurseries and/or orchards using rainwater or other kinds of natural water for irrigation, about an unforeseen risk of water-borne transmission of *E. amylovora*. Although these results should be confirmed with lignified plants, or plants of other susceptible species, this information might be used to improve preventive and control measures against fire blight.

11. The *E. amylovora* root infection stages are very similar to those of well-known soil-borne plant pathogens, indicating

adaptations to root infection similar to those present in its pathogenic counterparts.

12. The sigma factor RpoS contributes to the maintenance of culturability and viability in *E. amylovora* starved cells. This indicates that RpoS may act by conditioning *E. amylovora* cells for starvation, allowing proper starvation responses including regular development of the VBNC state without a loss of viability or cell integrity.

13. RpoS is required for full stationary-phase cross protection of *E. amylovora* against a variety of stresses that the pathogen can encounter in nature, for example, osmotic stress during flower colonization or acidic pH of the apoplast during host tissue invasion.

14. The sigma factor RpoS is required for proper survival of *E. amylovora* in non-host tissues. It modulates virulence and pathogenicity factors such as motility and EPS production (mainly levan) as well as virulence in immature fruits, but not in pear plantlets. These results demonstrate a not yet described regulation of the *E. amylovora* EPSs by RpoS in *E. amylovora,* and suggest a key role for this sigma factor during plant-pathogen interactions, for example during flower colonization.

15. The *E. amylovora* catalases KatA and KatG are regulated differently by growth phase, induced by exposure to H_2O_2 and host tissues and possess different specific catalase activities, indicating differentiated functional roles for each catalase.

16. Both catalases contribute to the maintenance of culturability in *E. amylovora* starved cells, hence delaying the entry into the VBNC response. However, the absence of catalase

activity does not inhibit growth on solid media, indicating the contribution of these enzymes to H_2O_2 detoxification, but with the participation of other antioxidant enzymes in the development of the VBNC phenotype. These results provide new insights into the link between survival and oxidative stress resistance.

17. The *E. amylovora* catalases contribute to the pathogen's survival in non-host tissues, and are required for a full virulence in immature fruits. These results indicate catalases as not yet described virulence factors in *E. amylovora,* which probably contribute to the adaption of the pathogen to host conditions by H_2O_2 neutralization. However, the different expression patterns, activities and roles of catalases in different stages of the infection might suggest more complex roles for these enzymes during plant-pathogen interactions which require more investigation.

Finally, the results obtained through the research in this Doctoral Thesis expand our knowledge on the hidden life of *E. amylovora* involving survival, spread and virulence under different environmental conditions, and opening new research lines on this topic. Furthermore, it provides new and valuable information to improve control and/or preventive strategies against fire blight.

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V. Bibliography

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VI. ANNEX

Annex I

Santander *et al.,* 2014b

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RESEARCH ARTICLE

Cellular, physiological, and molecular adaptive responses of *Erwinia amylovora* to starvation

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Abstract

Erwinia amylovora causes fire blight, a destructive disease of rosaceous plants distributed worldwide. This bacterium is a nonobligate pathogen able to survive outside the host under starvation conditions, allowing its spread by various means such as rainwater. We studied *E. amylovora* responses to starvation using water microcosms to mimic natural oligotrophy. Initially, survivability under optimal (28 °C) and suboptimal (20 °C) growth temperatures was compared. Starvation induced a loss of culturability much more pronounced at 28 °C than at 20 °C. Natural water microcosms at 20 °C were then used to characterize cellular, physiological, and molecular starvation responses of *E. amylovora*. Challenged cells developed starvation-survival and viable but nonculturable responses, reduced their size, acquired rounded shapes and developed surface vesicles. Starved cells lost motility in a few days, but a fraction retained flagella. The expression of genes related to starvation, oxidative stress, motility, pathogenicity, and virulence was detected during the entire experimental period with different regulation patterns observed during the first 24 h. Further, starved cells remained as virulent as nonstressed cells. Overall, these results provide new knowledge on the biology of *E. amylovora* under conditions prevailing in nature, which could contribute to a better understanding of the life cycle of this pathogen.

Introduction

Erwinia amylovora is the etiological agent of fire blight, a necrotic destructive disease affecting rosaceous plants such as pear, apple, and many ornamental species. In the last two centuries, this pathogen has spread worldwide causing serious economic losses (Vanneste, 2000; van der Zwet *et al.*, 2012). For this reason, *E. amylovora* has been cataloged as a quarantine organism in the European Union, where it is subject to phytosanitary legislation (Anonymous, 2000, 2003).

Erwinia amylovora belongs to the *Enterobacteriaceae* family, which contains such well-known animal pathogens as *Salmonella enterica* and some strains of *Escherichia coli*. Similar to these species, *E. amylovora* is not an obligate pathogen and is able to survive in the environment and to be spread by a variety of means, including rainwater, wind, insects, birds, or contaminated pruning tools (Thomson, 2000; van der Zwet *et al.*, 2012). These features are related to the serious difficulties in controlling fire blight once established in an orchard (Thomson, 2000). They also reveal the pathogen's life cycle, alternating periods of active growth, mainly when causing disease, with periods when growth is hindered by nutrient limitation, either in host or nonhost environments.

Persistence of pathogens in nature is determined by their ability to withstand fluctuations in nutrient availability in that most natural environments, including soil, water, or the phyllosphere, are oligotrophic (Morita, 1997; Colwel & Grimes, 2000; Parangan-Smith & Lindow, 2013), and it is assumed that heterotrophic bacterial species inhabit natural ecosystems in a starvation state, showing biochemical and morphological features very different to those displayed under laboratory conditions (Edwards, 2000). In this sense, bacteria have evolved means to adapt to starvation, involving considerable changes in their physiology,

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which are precisely regulated at the genetic level (Matin *et al.*, 1989; Kjelleberg, 1993; Lauro *et al.*, 2009). Many nonsporulating bacteria trigger a starvation-stress response characterized by the maintenance of culturability over time (Roszak & Colwell, 1987; Morita, 1997). Nutrient scarcity can also induce entry into the viable but nonculturable (VBNC) state, in which viable, stressed cells lose their ability to grow on rich media (Colwel & Grimes, 2000; Oliver, 2010). These physiological states are influenced by incubation temperature, although the effects of this stressing factor vary depending on the species analyzed (Gauthier, 2000; Arana *et al.*, 2010). In some cases, reductive division is observed after exposure to oligotrophic conditions (Oliver *et al.*, 1991; Byrd, 2000; Álvarez *et al.*, 2008). Regulation of morphology and motility in nutrient-poor environments has also been described in a variety of bacterial species to optimize the sequestering of available nutrients or the preservation of energy (Mårdén *et al.*, 1985; Wei & Bauer, 1998; Álvarez *et al.*, 2008).

Previous studies have demonstrated the ability of *E. amylovora* to withstand nutrient scarcity in different environments, such as the calyx of mature apples (Ordax *et al.*, 2009), in mineral medium lacking carbon and phosphate sources (Ordax *et al.*, 2006), or in distilled water or rain water (Santander *et al.*, 2012). Nevertheless, little is known about the cellular and molecular mechanisms underlying *E. amylovora* adaptive responses to starvation. Knowledge of factors affecting the persistence of this pathogen in the nutrient-poor conditions characteristic of most natural environments would contribute to understanding its hidden life when it is not causing disease.

In this work, we showed the adaptive responses of *E. amylovora* to the low-nutrient conditions that the pathogen may encounter in most environments outside the host, such as soil or water, or even in the host, as an epiphyte in the phyllosphere or as an endophyte during host dormancy when bacterial cells do not find enough resources to multiply (Vanneste & Eden-Green, 2000). Given the importance of water for *E. amylovora* dissemination and epiphytic colonization or flower infection (Pusey, 2000; van der Zwet *et al.*, 2012), as well as its oligotrophic characteristics, natural water microcosms were used to mimic the multiple nutrient starvation regime prevailing in nature. We first studied the effect of incubation temperature on the survival responses developed by the fire blight pathogen when subjected to nutrient limitation in different waters. From these experiments, we selected one temperature to characterize *E. amylovora* responses to starvation by temporal monitoring of population dynamics, cell morphology, motility, pathogenicity/virulence, and gene expression. We included genes related to starvation (*cstA*, *dps*, *relA*, *rpoS*, and *spoT*) and oxidative stress detoxification (*katA*, *katG*, and *oxyR*). The expression of genes related to the ability of *E. amylovora* to cause disease (*dfoA*, *hrpL*, *rcsB*, and *rlsA*) or to move (*flgN*) was also analyzed.

Materials and methods

Bacterial strains and culture conditions

Two *E. amylovora* strains isolated from different geographical areas and hosts, whose genomes have been sequenced and annotated (Sebaihia *et al.*, 2010; Smits *et al.*, 2010), were used in all the experiments: CFBP 1430, isolated from *Crataegus* sp. in France and ATCC 49946, isolated from *Malus domestica* in North America. Strains were cryopreserved at −80 °C in 25% (v/v) glycerol and streaked on King's B (KB) agar plates (King *et al.*, 1954) to obtain starter inocula for the experiments. *Erwinia amylovora* strains employed in this study were grown at 28 °C. Overnight cultures were prepared by inoculating a single 48 h colony into liquid KB with shaking (200 r.p.m.) for 16–18 h. Culturable cell counts were carried out on KB agar plates.

Water microcosm preparation

For water microcosms, Turia's river water (Valencia, Spain) and mineral water from Segovia (Spain) were filtered through a pore size of 0.22 μm in a Stericup Vacuum System (Millipore, St Quentin en Yvelines, France) and autoclaved at 121 °C for 20 min. The pH of these water samples was around neutrality (7.3 in the case of river water and 6.6 in mineral water). Mineral nutrient composition of river water was (values per liter) as follows: carbonates, 11.6 mg; chlorides, 176 mg; nitrates, 30 mg; and sulfates, 330 mg. In the case of mineral water, nutrient contents were as follows: carbonates, 10.4 mg; chlorides, 0.7 mg; and nitrates, 2.8 mg. Organic matter contents in river and mineral waters estimated by biochemical oxygen demand were below 1.8 and 0.1 mg L^{-1}, respectively. All water microcosms were prepared with the same samples, although some samples from Toby creek (Charlotte, NC) and other mineral waters with similar characteristics to the above mentioned were used.

To inoculate microcosms, overnight cultures of each *E. amylovora* strain obtained as specified above were diluted 100-fold in 110 mL fresh liquid KB and incubated at 28 °C (200 r.p.m.) until an $A_{600\ nm}$ of 0.13 ± 0.03 (about 2×10^8 CFU mL^{-1}) was reached. A volume of 100 mL of these cultures was centrifuged at 4424 ***g*** for 7 min at room temperature in a JS-5.3 rotor (Beckman Coulter, Santa Clara, CA) with the pellet washed twice with the initial volume of the natural water being tested (100 mL). Once inoculated, mineral and river water microcosms were immediately incubated at the desired temperatures for the

required time in the dark, without shaking. Temperatures selected in this study were 20 and 28 °C, which are temperatures at which this bacterial species is able to grow and cause disease under laboratory and field conditions. Aliquots from these microcosms, prepared in duplicate in three independent experiments, were periodically taken to perform different analyses, as detailed below.

Monitoring of bacterial population dynamics

Culturable, viable, and total cell population dynamics were followed overtime after the inoculation of river and mineral water microcosms. Cell counts were determined in three independent experiments performed in duplicate, and average values ± standard deviations (SD) calculated.

Culturable cell counts

To study the effect of the incubation temperature on the culturability of starved *E. amylovora* cells, river and mineral water microcosms incubated at 20 and 28 °C were sampled for plate counts on KB agar at time 0 and then every 2 days for 10 days. To monitor culturability for an extended period, new microcosms incubated at 20 °C were prepared and sampled periodically by plate count for up to 40 days postinoculation (dpi). Culturable cell counts were assessed by the drop plate method, as previously described (Santander *et al.*, 2012).

Total and viable cell counts

The number of viable and total cells was determined overtime after staining of 1 mL aliquots of each microcosm with the Baclight Live/Dead (Invitrogen) viability kit as described by the manufacturer. Cell counts were assessed by epifluorescence microscopy (EFM) with a Nikon Eclipse E800 microscope (Nikon Instruments, Melville, NY) equipped with a fluorescence B-2A filter (EX450–490, BA520). Briefly, stained aliquots were diluted 10-fold in 10 mM phosphate-buffered saline pH 7.0 (PBS), and cells collected onto a 0.22-μm pore-size black polycarbonate filter (Millipore, Bedford, MA). A minimum of 300 cells were counted in at least 20 fields at a magnification of × 1250. In initial experiments at 20 and 28 °C, total and viable cell counts were assessed at times 0, 2, 6, and 10 dpi. In subsequent experiments at 20 °C, inoculated microcosms were sampled for viable and total cell counts at 0, 2, 5, 10, 20, and 40 dpi.

Cell morphology and motility analysis

Both the morphology and the motility of *E. amylovora* cells starved in river water microcosms for 40 days at 20 °C were analyzed overtime in three independent experiments performed in duplicate.

Morphology changes were monitored by transmission electron microscopy (TEM). For this purpose, 10 mL aliquots were collected at times 0, 2, 10, and 40 dpi. Cells were pelleted by centrifugation for 7 min at 4424 ***g*** at room temperature and fixed with 2.5% glutaraldehyde for 2 h in the dark. To remove the fixing agent, cells were centrifuged at 10 500 ***g*** for 2 min, washed twice with filtered-sterilized deionized water ($_{di}H_2O$), and finally resuspended in 0.1 mL $_{di}H_2O$. Samples were stored at 4 °C in the dark until examined. For TEM observations, 10 μL drops from fixed samples were deposited over a 500 mesh copper TEM grid for 1 min, dried with filter paper, and negatively stained for 5 s with 2% phosphotungstic acid. Samples taken at time 0 were used as controls. Images were acquired with a JEOL JEM-1010 transmission electron microscope (JEOL, Tokyo, Japan) with a CCD MegaViewIII digital camera (Soft Imaging System, Boulder, CO) and the capture program AnalySIS 3.2. A minimum of 200 cells per sample were photographed and analyzed with 'Particle Analysis' of ImageJ software (http://rsb.info.nih.gov/ij/).

To monitor motility, starved cells were observed at a magnification of × 1250 by phase-contrast microscopy with a Leica DM LB microscope (Leica GmbH, Heidelberg, Germany) at times 0, 1, 4, and 8 h postinoculation (hpi) and at 1, 2, 3, 4, 5, 6, 7, 10, 20, 30, and 40 dpi.

Temporal analysis of gene expression

The expression of genes related to starvation (*cstA*, *dps*, *relA*, *rpoS*, and *spoT*), oxidative stress (*katA*, *katG*, and *oxyR*), pathogenicity or virulence (*dfoA*, *hrpL*, *rcsB*, and *rlsA*), and motility (*flgN*) was monitored overtime in cells starved in Spanish river water microcosms at 20 °C for 40 days. Two genes were tested as controls for sample normalization in the RT-PCR: *rrs* (16S subunit of rRNA) and *gapDH* (glyceraldehyde 3-phosphate dehydrogenase), although only *rrs* showed constitutive expression under the conditions assayed (Fig. 1). Thus, this gene was

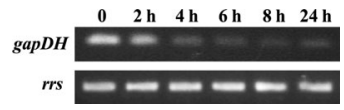

Fig. 1. Representative 1% agarose gel showing expression profiles of the two genes tested as controls for the semi-quantitative RT-PCR in *Erwinia amylovora* CFBP 1430 within the first 24 h of exposure to oligotrophic conditions in river water microcosms at 20 °C. Each lane contains 10 μL of the RT-PCR mastermix. Gels were stained for 10 min with 1 μg mL^{-1} ethidium bromide. Similar expression profiles were observed in the North American strain ATCC 49946.

chosen to normalize results in both *E. amylovora* strains. Expression analyses were repeated at least three times with each strain, in independent experiments. Some repeats were also performed with river water collected in the USA (Charlotte, NC) or mineral waters from different geographical areas. Mean values and SD were calculated after normalization to control RNA.

Primer design

Primers used for RT-PCR are shown in Table 1. Primer design was conducted with Primer3 software (Rozen & Skaletsky, 2000). Different annealing temperatures (T_a) were tested with each pair of primers, and finally, a single T_a, 54.5 °C, was used for the analysis of all genes.

RNA sampling and processing

To sample RNA, 0.5 mL aliquots were periodically taken from water microcosms. All aliquots were immediately mixed with 1 mL of RNA Protect Bacteria Reagent (Qiagen, Courtaboeuf, France), processed according to the supplier's instructions, and stored at −80 °C prior to the RNA extraction.

Total RNA was isolated from RNA-protected samples with the RNeasy mini kit (Qiagen) following the manufacturer's specifications. RNA extracts were treated with DNase I (DNA*free* kit; Ambion, Austin, TX) to remove residual genomic DNA (gDNA). Before reverse transcription PCR (RT-PCR), a conventional *E. amylovora*-specific PCR (Taylor *et al.*, 2001) was carried out to examine for the presence of contaminant gDNA, using 2 μL of each RNA sample as template.

RT-PCR

An endpoint RT-PCR analysis was carried out at times 0, 1, 3, 5, 7, 10, 20, and 40 dpi with the Access RT-PCR System (Promega, Madison, WI). Total RNA was quantified with a Nanodrop 2000 spectrophotometer (Thermo

Table 1. RT-PCR primers designed for this study

Genes related to	Gene	Oligonucleotide sequence (5′→3′)	Product size (bp)
Starvation	*cstA*	ACACCACGACCAGAAACAGAATAC	173
		GTTCAGCCAGAATCCACAGATG	
	dps	TCAGGTGGTGAGTGACAGAAC	145
		GTCTTCGTCTTCAACTTCGCTG	
	relA	CATCTGCGTGAAGTGAAGGAC	157
		ACTCATCCGGGTGCAGATAG	
	rpoS	CCTCAACGAACGCATTACCTCA	215
		CCGCTTCATAGCCTAACAGG	
	spoT	CTTATCTTGTCGCCCGTGATG	154
		TCTTCGATAACGTCGTGCAGCA	
Oxidative stress	*katA*	AGTGGGTAAACTGGTGCTGGA	152
		GGTGAGCATCCGCATAGGC	
	katG	CCTCAAGTGGGCTTTCAGTC	209
		ATCCGCTAACGACATTTTGCCG	
	oxyR	CTGGTGAAAGAGTCGGAAGC	154
		AGTGACCATCCTCCAGCATC	
Motility	*flgN*	GAAAAGAACGCCGGACTGTA	128
		TGATTGAGCAGCATCCCGTT	
Virulence/Pathogenicity	*dfoA*	AACTGACGCTGGAAATGACCTC	200
		CTCCGAATGGGTAAAGAGATTGAC	
	hrpL	GACGATCCCAGCCATATCAC	112
		CTTTCTCGACCAGCATGTTCAAC	
	rcsB	CCGACGACCATCCTATTGTTC	181
		GTGATGCCGTCACCGTATTTTTC	
	rlsA	ACCTCCCGTCTCACGAATGTT	193
		GGGAGAAGCAATCAAAAACTCTTGC	
Internal controls*	*gapDH*	CCTGACTGCACGTCTGGAAAA	187
		GTTATCGTTCAGGGCGATACC	
	rrs	CAGCCACACTGGAACTGAGA	196
		GTGCTTCTTCTGCGGGTAAC	

*Both the *gapDH* and *rrs* genes were initially used as internal controls for expression studies, but based on initial results, only *rrs* was ultimately employed for this purpose.

Scientific, Wilmington, DE), and 50 ng were used for the analysis of each gene. Concentrations of the reaction buffer, $MgCl_2$, dNTPs, primers, AMV reverse transcriptase, and *Tfl* DNA polymerase employed in each RT-PCR reaction were those recommended by the manufacturer's instructions, in a final volume of 25 μL. A negative control consisting of 2 μL nuclease-free water was used in every round of RT-PCR to check for possible nucleic acid contamination. For the RT, an initial incubation at 45 °C for 45 min was performed, followed by a 2 min step at 94 °C to inactivate the AMV reverse transcriptase and to denature cDNA for the subsequent PCR. Amplification of cDNA was carried out using 44 cycles of denaturation at 94 °C for 30 s, annealing at 54.5 °C for 30 s, and extension at 68 °C for 30 s, followed by a final extension step at 68 °C for 7 min.

Additionally, to study the regulation of gene expression during the first hours of exposure to nutrient-stressing conditions at 20 °C, a semi-quantitative RT-PCR was performed similar to that described by Rezzonico & Duffy (2007). For this purpose, new river water microcosms were prepared and RNA collected at times 0, 2, 4, 6, 8, and 24 hpi. The number of cycles employed to amplify target cDNA was optimized individually for each primer pair, to ensure they fell within the exponential phase of the amplification reaction (Rezzonico & Duffy, 2007). All primer pairs showed exponential amplification of the cDNA in the range of 24–36 cycles, using 25, 50, and 100 ng tRNA as template. Then, semi-quantitative RT-PCRs with all primer pairs were carried out with 33 amplification cycles at the temperature cycles and times specified above.

Aliquots of 10 μL from RT-PCR reactions were run on a 1% agarose gel and stained for 10 min with 1 μg mL^{-1} ethidium bromide. Band densities were analyzed with ImageJ software (http://rsb.info.nih.gov/ij/). The expression level of each target gene overtime was normalized to the expression of the constitutively expressed *rrs* gene. Expression levels at a given time were also represented as a percentage, relative to the expression at time 0.

Pathogenicity and virulence assays on immature fruits and pear plantlets

The pathogenicity of *E. amylovora* cells starved at 20 °C in river and mineral water microcosms was determined at 0, 20, and 40 dpi, inoculating direct aliquots of water microcosms into immature pears (*Pyrus communis* cv. Williams), loquats (*Eriobotrya japonica* cv. Tanaka), and 4-week-old pear plantlets (*P. communis* cv. Passe Crassane). Additionally, the virulence of *E. amylovora* cells starved at 20 °C in river water microcosms was studied at 0 and 40 dpi by inoculation of 10-fold serial dilutions of water microcosms in pear fruits and plantlets.

Prior to inoculation, fruits were washed with tap water, surface disinfected with 2% (w/v) sodium hypochlorite for 5 min, rinsed with sterile distilled water, and allowed to dry under aseptic conditions. To obtain pear plantlets, seeds were manually extracted from mature fruits, surface disinfected with 3% (w/v) sodium hypochlorite for 5 min and rinsed with sterile distilled water. Disinfected seeds were stratified in wet river sand at 4 °C until their germination (3–6 weeks). Seedlings were transferred to soil and grown in a greenhouse for 3 weeks with natural illumination, 65–70% relative humidity, and maximum day/night temperatures of 26 and 23 °C, respectively.

For pathogenicity assays, immature fruits were inoculated similar to that described by Cabrefiga & Montesinos (2005). Briefly, pears and loquats were wounded four times with a sterile 100 μL pipet tip. Then, 2 μL aliquots from water microcosms were inoculated into each wound (four wounds per fruit). Pear plantlets were inoculated according to Ruz *et al.* (2008), by cutting two young expanded leaves (two cuts per leaf and four cuts per plantlet) with scissors previously dipped into aliquots of water microcosms. In virulence assays, immature pear fruits were inoculated as described above, with either direct aliquots or 10-fold dilutions of *E. amylovora*-inoculated river water microcosms. To inoculate pear plantlets with known doses of *E. amylovora* starved cells, the upper part of the stem (about 2 cm in length) was cut off and, similar to fruit inoculation, 2 μL aliquots from microcosms (or their dilutions) were placed on the wound generated by the cut.

Positive and negative controls were inoculated as described above, with overnight cultures of each strain or sterile PBS, respectively. All assays were carried out with each *E. amylovora*-inoculated water microcosm, prepared in duplicate in three independent repeats (a total of six microcosms per type of water, strain, and starvation period assayed). One pear, one loquat, and two pear plantlets were inoculated per microcosm in pathogenicity tests. One pear and two pear plantlets were inoculated per microcosm in virulence assays.

Inoculated fruits were incubated in the dark at 28 °C in a moist chamber for 1–3 weeks. Challenged plantlets were incubated under controlled conditions (16 h of light at 28 °C, 8 h of dark at 24 °C, and high relative humidity) for 3 weeks. Fruits and plantlets were periodically monitored for development of fire blight symptoms. To confirm our results, symptomatic fruits and plantlets were processed to re-isolate *E. amylovora* on culture media and to confirm identification by specific PCR. Protocols for plant material processing, bacterial isolation, DNA extraction, and PCR conditions to detect *E. amylovora* from extracted DNA were performed according the diagnostic protocol PM7/20 of the European Plant Pathogen Organization (EPPO) standards (EPPO, 2013).

Statistical analysis

Culturable, viable, and total cell counts were log transformed prior to the statistical analysis. All quantitative data were represented as means ± SD. Statistical significance of differences between means was assessed by analysis of variance (ANOVA). Depending on the experiment, different factors were compared: strain, type of water, incubation temperature, time, and/or experiment. Comparison of cell-shape descriptors in starved cells and control fresh cultures at time 0 was determined using a Student's *t* test. A *P*-value ≤ 0.05 was considered significant.

Results

Temperature affects the survival strategies adopted by *E. amylovora* cells starved in natural waters

Results of *E. amylovora* (strains CFBP 1430 and ATCC 49946) responses to oligotrophy in two types of water incubated at two temperatures (20 and 28 °C) are shown in Fig. 2. Statistically significant differences between strains were detected in colony counts ($P < 0.05$) in both types of microcosms and incubation temperatures assayed (Fig. 2a and b vs. Fig. 2c and d). Nevertheless, trends in cell population dynamics of both the European and the North American strains were similar, likely describing the general behavior of *E. amylovora* in response to the conditions assayed. The analysis of incubation temperature effects on population dynamics of *E. amylovora* cells starved in natural waters revealed a greater decrease in colony counts ($P < 0.05$) in microcosms incubated at 28 °C compared with those at 20 °C (Fig. 2), regardless of the type of water employed in the microcosms. At 28 °C, a decline in culturability of about 2–3 log units was observed in both strains during the 10-day period, reaching values around 10^6–10^5 CFU mL^{-1}. Conversely, *E. amylovora* cells incubated at 20 °C maintained culturability at high numbers, with only slight decreases (< 0.5 log units) after 10 days of starvation. The European strain CFBP 1430 showed small but significantly ($P < 0.05$) higher culturable counts (Fig. 2a and b) than the North American strain ATCC 49946 (Fig. 2c and d) within the assayed period.

Total and viable cell counts of both *E. amylovora* strains remained nearly constant in river and mineral water microcosms at the two incubation temperatures assayed (Fig. 2). Thus, incubation temperature did not affect bacterial viability or cell integrity during the 10-day period. Considering that culturability gradually decreased below initial levels without a reduction in viability, it is concluded that a subpopulation of *E. amylovora* culturable cells adopted the VBNC state, while another fraction of cells persisted in a nongrowing but culturable state, developing a starvation-survival response. These two survival strategies were observed regardless of the assayed strain or the type of water. The maintenance of culturability was strongly temperature dependent, being favored at 20 °C and markedly reduced at 28 °C. Based on these results, we selected the temperature (20 °C) allowing an

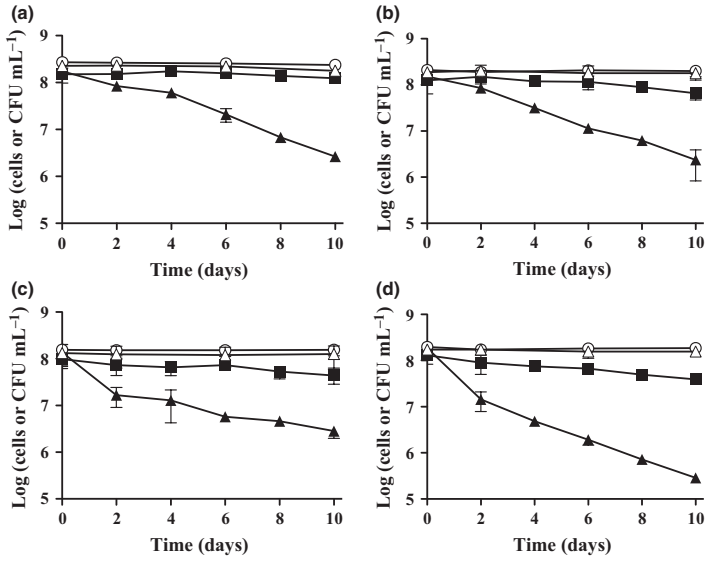

Fig. 2. Effect of incubation temperature on population dynamics of *Erwinia amylovora* strains CFBP 1430 (a, b) and ATCC 49946 (c, d) starved in river water (a, c) and mineral water microcosms (b, d) for 10 days. Closed squares and triangles represent culturable cell counts from microcosms incubated at 20 and 28 °C, respectively. Open circles and triangles represent total and viable cell counts, respectively. As no differences were observed between total and viable cell counts of cells starved at 20 °C and at 28 °C, only those corresponding to 28 °C are shown. Each data point shown corresponds to the average value of at least three independent experiments performed in duplicate. Bars represent the SD.

extended survival in culturable state to study *E. amylovora* responses to nutrient scarcity for a longer period of time.

Characterization of *E. amylovora* adaptive responses to starvation at 20 °C over a 40-day period

Population dynamics of starved cells

Figure 3 shows survivability of *E. amylovora* cells (strains CFBP 1430 and ATCC 49946) starved in river or mineral water microcosms incubated at 20 °C for 40 days. No significant differences in colony counts were observed between the two types of water in the two assayed strains, but the European strain CFBP 1430 again showed higher culturable cell numbers ($P < 0.05$; Fig. 3a and b) than the North American one ATCC 49946 (Fig. 3c and d) during the entire assay period. Plate counts slightly decreased during the first 10 dpi (Fig. 3), about 0.28 log units in the case of strain CFBP 1430 and 0.37 log units in the case of strain ATCC 49946, similar to the results observed in the culturability studies of Fig. 2. At the end of the experimental period (40 dpi), culturable cell counts were about 2×10^7 and 5×10^6 CFU mL^{-1} in the European and North American *E. amylovora* strains, respectively. Total and viable cells remained at initial levels (*c.* 10^8 cells mL^{-1}) over the entire 40-day experimental period in both strains and in the two types of water assayed. Hence, starvation conditions at 20 °C triggered a slow entry into the VBNC state, although high culturable cell numbers were still present at the end of the experiment.

Morphology and motility changes in starved cells

TEM photographs of *E. amylovora* strains CFBP 1430 (Fig. 4a–e) and ATCC 49946 (Fig. 4f–j) starved in river water microcosms at 20 °C over a 40-day period are shown in Fig. 4. Control cells at time 0 (Fig. 4a and f) showed the typical rod-shaped morphology (circularity values of 0.49 ± 0.05 SD in CFBP 1430 and 0.45 ± 0.17 SD in ATCC 49946) and exhibited long peritrichous flagella attached to the bacterial surface (Fig. 4a and f). Lengths of the European and the North American *E. amylovora* strains at time 0 were 1.84 ± 0.27 and 2.22 ± 0.48 μm SD long, respectively. Exposure to nutrient scarcity in river water at 20 °C induced the acquisition of a rounded shape in subpopulations (between 30% and 40%) of both strains (Fig. 4b and g), reaching the highest circularity values at 40 dpi (0.74 ± 0.10 SD in the strain CFBP 1430 and 0.60 ± 0.25 SD in the strain ATCC 49946). In addition, cells reduced their cell size (to 1.73 ± 0.23 μm SD in the case of strain CFBP 1430 and 1.82 ± 0.33 μm SD in that of strain ATCC 49946) at time 40 dpi with respect to time 0 ($P < 0.05$). These responses were observed in a small percentage of cells incubated even after 2 dpi. Elongated cells reaching lengths up to 5 μm were also observed at different times (Fig. 4c and h). Most cells of the two studied strains subjected to starvation conditions showed small flame-shaped vesicles of *c.* 42 nm in length at the surface of the outer

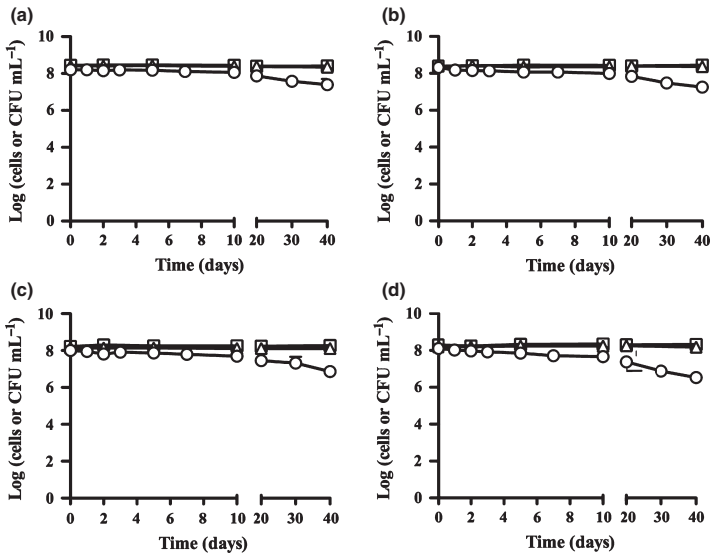

Fig. 3. Population dynamics of *Erwinia amylovora* strains CFBP 1430 (a, b) and ATCC 49946 (c, d) in river (a, c) and mineral (b, d) water microcosms incubated at 20 °C (starvation-survival inducing conditions) for 40 days. Total, viable, and culturable cell counts are represented with squares, triangles, and circles, respectively. Each data point corresponds to the average value of at least three independent experiments performed in duplicate. Bars represent the SD.

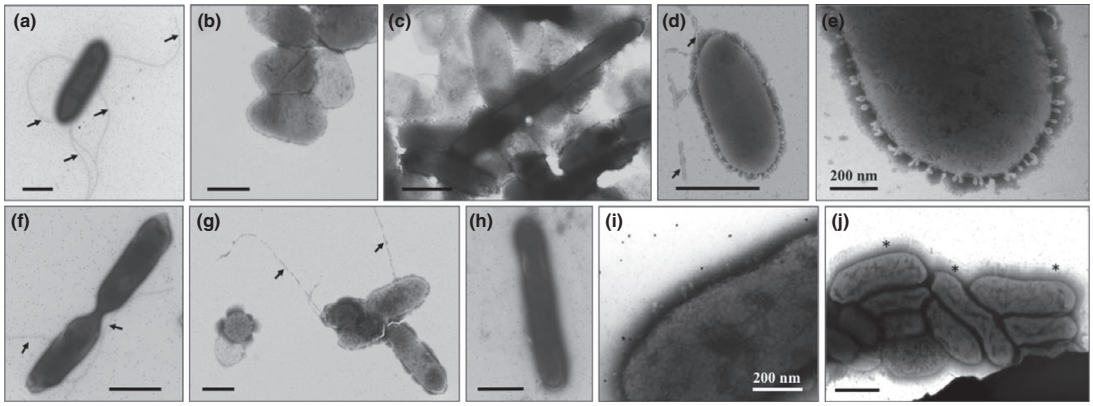

Fig. 4. TEM photographs of *Erwinia amylovora* strains CFBP 1430 (a–e) and ATCC 49946 (f–j) starved in river water microcosms at 20 °C. Cells at time 0 (a, f); rounded (b, g) or elongated (c, h) cells starved for 10 or 40 dpi; surface vesicles in challenged cells after a 10 day (d, at higher magnification in e) and a 40-day period (i); 10-day starved cells showing an external layer of EPS (j). Bars correspond to 1 μm, unless a different measure is specified. Flagella are indicated with black arrows.

membrane (Fig. 4d, e and i). These vesicles were not observed in cells at time 0 (Fig. 4a and f), but were visualized in starved cells between 2 and 40 days. An exopolysaccharide (EPS) layer was visualized surrounding *E. amylovora* cells in samples at time 0 and at different periods of starvation (Fig. 4j).

Regarding motility, direct observation of *E. amylovora* cells (strains CFBP 1430 and ATCC 49946) from microcosms inoculated in independent experiments revealed a high number of motile cells at time 0 that decreased progressively during the following 72 hpi. No motile cells were observed in samples analyzed from time 4 to 40 dpi. However, TEM observations revealed the presence of long peritrichous flagella attached to the surfaces of many bacterial cells over the entire experimental period (Fig. 4a, d, f and g). Unattached, broken flagella were also observed in many of the analyzed samples (Fig. 4g).

Gene expression changes in starved cells

The endpoint RT-PCR analysis of selected genes in *E. amylovora* cells starved in river water microcosms at 20 °C at different times revealed equivalent expression profiles in both the European and the North American strains, with transcripts of all analyzed genes detected at time 0 (data not shown). The endpoint RT-PCR expression analysis allowed the detection of defined bands corresponding to the expression of all genes studied in the two *E. amylovora* strains at all the assayed periods in all types of environmental water microcosms analyzed (data not shown).

Graphs corresponding to the semi-quantitative analysis of gene expression during the first 24 h of exposure of the two *E. amylovora* strains to starvation at 20 °C are shown in Fig. 5. Three different regulation patterns were observed, based on normalized band densities at each assayed time. Both strains developed equivalent genetic regulation patterns in the three microcosms (inoculated in independent experiments) analyzed.

Two of the three different expression profiles were observed in starvation-related genes (Fig. 5a). The first one consisted of the maintenance of expression levels overtime, as was detected in the *dps* gene in both the European and the North American *E. amylovora* strains. The second regulation pattern was the most common among all analyzed genes and consisted of a shift of expression levels in the first hours after the inoculation (usually a decrease, corresponding to a down-regulation), with a partial or total recovery of initial values in the following hours. This was seen in *cstA*, *relA*, *rpoS*, and *spoT* in the two strains.

With minor differences, expression analysis of oxidative stress-related genes (*katA*, *katG*, and *oxyR*) revealed a regulation pattern similar to that observed with *cstA*, *relA*, *rpoS*, or *spoT* genes in both the European and the North American strains (Fig. 5b).

Regarding to the regulation of motility and pathogenicity/virulence genes included in this work, *flgN* and *rcsB* showed the second regulation pattern described above (Fig. 5c). Genes *hrpL*, *rlsA*, and *dfoA* showed a third regulation pattern, different to all observed in the rest of the genes analyzed (Fig. 5c). Transcripts of these genes exhibited a drastic depression of their levels within the first 2–6 hpi, with their expression values then being maintained or slightly reduced throughout the following 18–22 hpi. This regulation pattern was very similar in the two

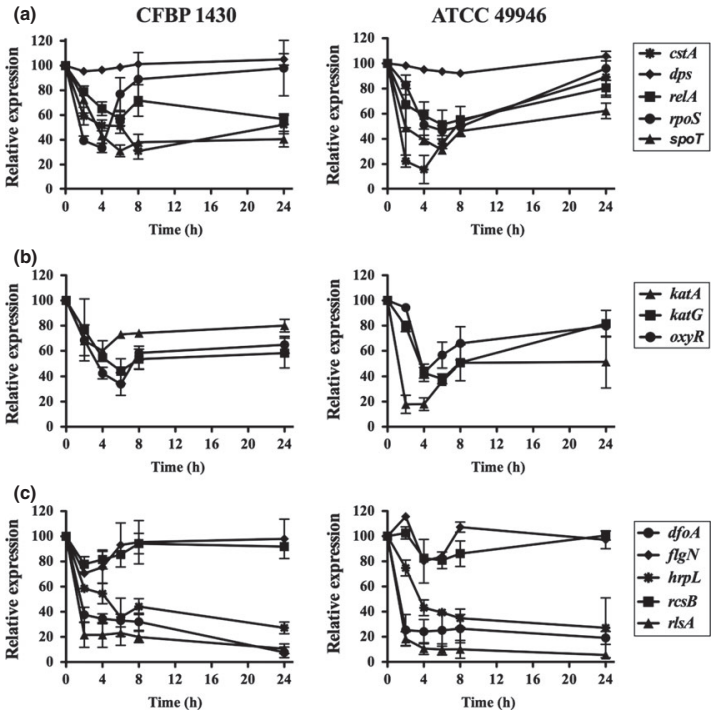

Fig. 5. Semi-quantitative expression analysis of starvation (a), oxidative stress (b), and motility and virulence/pathogenicity (c) related genes in *Erwinia amylovora* strains CFBP 1430 and ATCC 49946 over a 24 h-starvation period in river water microcosms incubated at 20 °C. Expression values at each time were calculated relative to the endogenous control (*rrs*). The densitometric value for the expression of each gene at time 0 was arbitrarily set at 100 and band densities corresponding to samples taken at different times after the inoculation calculated as a percentage with respect to their initial values. Data shown correspond to the mean of three independent determinations. Bars represent the SD.

E. amylovora strains. Finally, the control gene *rrs* (analyzed in parallel, together with the other genes) maintained its expression in the initial levels throughout the entire experimental period.

Erwinia amylovora 40-day starved cells remained pathogenic and as virulent as control cells

Representative pictures showing characteristic fire blight symptoms in all types of inoculated plant material in pathogenicity and virulence assays are shown in Fig. 6.

In pathogenicity assays, immature fruits (pears and loquats) and pear plantlets inoculated with direct aliquots of mineral and river water microcosms containing cells of both *E. amylovora* strains starved for 0, 20, and 40 dpi developed characteristic fire blight symptoms within 2–3 days of inoculation (Fig. 6a–d), regardless of the inoculated strain, the type of water microcosm, or the starvation period. Symptoms in immature pears consisted of progressive necrotic lesions and abundant exudates (Fig. 6a). Blighted loquats mainly showed necrosis surrounding the inoculated wound (Fig. 6b), although exudates were also observed in some cases. Symptoms in pear plantlets inoculated by cuts in leaves consisted of an initial necrosis surrounding the inoculation site that progressed to the rest of the leaf, petiole (Fig. 6c), and the stem, which finally wilted (Fig. 6d) and became completely necrotic. In a few cases, light brown exudates were observed in some necrosed petioles. Identical symptoms were also observed in positive controls, but not in negative controls inoculated with sterile PBS (Fig. 6f–i).

Results corresponding to virulence assays with 0- and 40-day starved *E. amylovora* cells in river water at 20 °C are shown in Table 2 and Fig. 6. All pear fruits and plantlets inoculated with decreasing 10-fold dilutions of 40-day starved cells (from about 2–4 $\times$ 10^4 to 2–4 CFU per wound) developed similar fire blight symptoms to those observed in plant material inoculated with equivalent numbers of cells at time 0, regardless of the inoculated strain. In some cases, 40-day starved cells of strain CFBP 1430 were able to cause fire blight in plantlets (but not in fruits) inoculated with < 1 CFU per wound (Table 2). Representative disease symptoms in pear plantlets inoculated after cutting off the upper part of the stem are shown in Fig. 6e. These started with an initial necrosis at the inoculation site, with abundant exudates in plantlets inoculated with high bacterial doses. Necrosis progressed to tissues below the inoculation site, reaching petioles and leaves (Fig. 6e). Negative controls did not show any of these symptoms (Fig. 6j).

Erwinia amylovora was re-isolated on culture media from diseased fruits and plantlets, either from exudates or

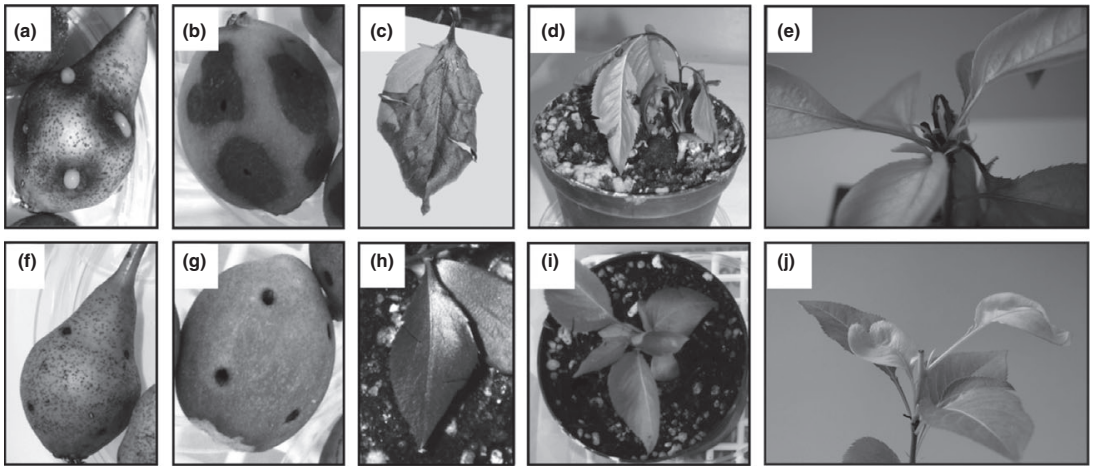

Fig. 6. Representative pictures showing immature pears and loquats, and pear plantlets inoculated with *Erwinia amylovora* cells (CFBP 1430) starved for 40 days in river water (a–e) or sterile PBS (f–j) in pathogenicity (a–d, f–i) and virulence (e, j) assays. Pears and loquats showing necrosis (a, b) and exudates (a) surrounding the inoculation sites; detail of a pear plantlet leaf with necrosis progressing from cuts in the margins to the rest of the lamina and the petiole (c); pear plantlet inoculated by cuts with scissors showing advanced necrosis and wilting in the stem and petioles (d); detail of a pear plantlet inoculated after cutting off the end of the stem (e, j), with leaves still healthy and necrosis progressing from the inoculation site to lower parts of the stem and petioles, which start to wilt (e).

Table 2. Virulence analysis of *Erwinia amylovora* cells starved in river water microcosms at 20 °C for 0 and 40 dpi

	E. amylovora strain					
	CFBP 1430			ATCC 49946		
		% Symptomatic wounds			% Symptomatic wounds	
Starvation period (days)	Inoculum (CFU's per wound)*	Pear fruits†	Pear plantlets‡	Inoculum (CFU's per wound)*	Pear fruits†	Pear plantlets‡
0	$4.07 \pm 0.26 \times 10^5$	100	100	$1.87 \pm 0.12 \times 10^5$	100	100
	$\approx 4.07 \times 10^4$	100	100	$\approx 1.87 \times 10^4$	100	100
	$\approx 4.07 \times 10^3$	100	100	$\approx 1.87 \times 10^3$	100	100
	$\approx 4.07 \times 10^2$	100	100	$\approx 1.87 \times 10^2$	100	100
	$\approx 4.07 \times 10$	100	100	$\approx 1.87 \times 10^1$	100	100
	≈ 4.07	100	100	≈ 1.87	100	100
	≈ 0.41 (4.94 ± 0.76)§	0	0	≈ 0.19 (2.86 ± 0.42)§	0	0
40	$3.32 \pm 0.32 \times 10^4$	100	100	$2.04 \pm 0.12 \times 10^4$	100	100
	$\approx 3.32 \times 10^3$	100	100	$\approx 2.04 \times 10^3$	100	100
	$\approx 3.32 \times 10^2$	100	100	$\approx 2.04 \times 10^2$	100	100
	$\approx 3.32 \times 10^1$	100	100	$\approx 2.04 \times 10^1$	100	100
	≈ 3.32	100	100	≈ 2.04	100	100
	≈ 0.33 (4.66 ± 0.70)§	0	8.3	≈ 0.20 (2.76 ± 0.35)§	0	0

*Calculated from average values of three independent experiments performed in duplicate ± SD.
†A percentage of 100% corresponds to fire blight symptoms in a total of 24 inoculated wounds (four wounds per fruit in six fruits).
‡A percentage of 100% corresponds to fire blight symptoms in a total of 12 inoculated wounds (one wound per plantlet in 12 plantlets).
§Average number of viable cells per wound ± SD inoculated, calculated from three independent experiments performed in duplicate.

necrotic lesions, and the identity of random selected colonies was confirmed by species-specific PCR. In challenged plants, the pathogen was also detected by PCR from tissues or organs different to the inoculation site.

Discussion

As is the case with other phytopathogenic bacteria, *E. amylovora* is a heterotrophic species adapted to grow

at favorable temperatures when nutritional resources are abundant. However, bacteria rarely find these idyllic conditions in nature, not even in the host, some of the main causes being the oligotrophic nature of most ecosystems (Morita, 1997) and/or fluctuations of temperature (Gauthier, 2000). In our study, *E. amylovora* cells subjected to starvation at two temperatures favorable for growth (20 and 28 °C) developed two survival strategies, the starvation-survival and the VBNC responses. However, the adoption of these survival strategies was dependent upon temperature, with the starvation-survival response being favored at 20 °C and the VBNC response at 28 °C. Our results are similar to those reported for *E. coli* cells starved in river water (Flint, 1987) or in sterile saline solution (Arana *et al.*, 2010). In both cases, incubation temperatures below the optimal for cell growth in rich media (< 28 °C in *E. amylovora* and < 37 °C in *E. coli*) favored maintenance of culturability overtime, probably via a reduction in cellular damage as a consequence of a slowing down of their metabolism at these temperatures (Arana *et al.*, 2010).

A subpopulation of *E. amylovora* starved cells experienced a reduction in cell size and/or the adoption of coccoid shapes. This phenomenon has been attributed to the optimization of the cells' energetic resources through the increase in the surface/volume ratio (Roszak & Colwell, 1987; Morita, 1997; Byrd, 2000; Nyström, 2004). The enhanced biological fitness of small bacteria has also been suggested by avoiding predation by protists in nonhost environments (Byrd, 2000). Reductive division is a common bacterial response to starvation that usually explains the decrease in cell size (Morita, 1997; Byrd, 2000; Nyström, 2004). However, *E. amylovora* did not increase cell numbers after its exposure to oligotrophy, so reductive division may not be an explanation for this phenomenon. The process of size reduction in nondividing cells, also called dwarfing, is a mechanism regulated by starvation that consists in the degradation of endogenous material, including cell envelopes (Nyström, 2004). In some bacterial species, a preferential degradation of the cell wall and the cytoplasmic membrane results either in a contraction of the outer membrane (Nyström, 2004) or in the release of outer membrane small vesicles (Mårdén *et al.*, 1985; Byrd, 2000; Nyström, 2004; Álvarez *et al.*, 2008). In our study, TEM analysis revealed the formation of small vesicles on the outer membrane of *E. amylovora* starved cells, which may explain the cell size reduction observed and suggesting a type of adaptation to starvation in this pathogen. Some ecological roles attributed to these vesicles include the delivery of substances to favor interspecific communication, attack of competitors, or biofilm formation (Álvarez *et al.*, 2008; Tashiro *et al.*, 2012).

In natural systems with a low-nutrient content bacterial motility may represent an advantage, allowing cells to search for additional nutrients. However, at the same time, this trait is energy expensive, so the loss of motility may be considered as another form of adaptation to enhance the survival of starved bacteria. The direct observation of *E. amylovora* cells by phase-contrast microscopy indicated the existence of motile cells no longer than 3 days after microcosm inoculation, although TEM images revealed the presence of peritrichous flagella in many cells during the 40-day starvation period. These results are similar to that observed in rhizobia or vibrios, although different behaviors have been described in other bacterial species exposed to similar conditions (Wei & Bauer, 1998).

Molecular and physiological responses to oligotrophy have been widely studied in different bacterial pathogens (Matin *et al.*, 1989; Kjelleberg, 1993; Lauro *et al.*, 2009). However, such studies in *E. amylovora* are very scarce. In this work, we analyzed the expression of a selection of genes related to starvation (*cstA*, *dsp*, *relA*, *rpoS*, and *spoT*), oxidative stress (*katA*, *katG*, and *oxyR*), motility (*flgN*), and pathogenicity and virulence (*dfoA*, *hrpL*, *rcsB*, and *rlsA*) in *E. amylovora* cells starved at 20 °C. Endpoint RT-PCR analyses revealed the continued expression of all analyzed genes, including the control gene *rrs*, throughout the entire experimental period, indicating an active metabolism of *E. amylovora* cells while starved. Some of the studied genes, for example *rpoS*, *katG*, and *oxyR*, have been directly related to the maintenance of culturability in different pathogens exposed to similar conditions (Kong *et al.*, 2004; Oliver, 2010; Flores-Cruz & Allen, 2011; Kusumoto *et al.*, 2012). The gene *rpoS* encodes an alternative sigma factor of the RNA polymerase that regulates the expression of multiple genes, including the starvation-related genes *cstA* and *dps* (Dubey *et al.*, 2003; Nair & Finkel, 2004), and has an important role during the general response to stress (Anderson *et al.*, 1998; McDougald *et al.*, 2002; Hengge, 2011). The functions of *oxyR* (encoding a regulator of genes in response to hydrogen peroxide) and *katA* and *katG* (encoding catalases) are linked to oxidative stress detoxification, which appears to have a key role in the ability of starved bacteria to form colonies on solid media (Kong *et al.*, 2004; Oliver, 2010; Flores-Cruz & Allen, 2011). Other starvation-related genes which expression was detected during the entire experiment were *dps*, which encodes an unspecific protein that binds to DNA during starvation and other stresses, *cstA*, encoding a peptide transporter induced by carbon starvation, and *relA* and *spoT*, which participate in the synthesis of the alarmone ppGpp, which accumulates in cells subjected to amino acid starvation and favors transcription of genes related to starvation and virulence.

The continued expression of *flgN* (a gene involved in flagellar biosynthesis) might be related to the presence of peritrichous flagella attached to *E. amylovora* cells' surface during the 40-day starvation period. Interestingly, these cells lost motility 3 days after their inoculation into oligotrophic microcosms. In other bacterial models, this phenomenon is a consequence of the reversible nature of motility inactivation during starvation. While the entire bacterial cell population loses motility, flagellar integrity is lost only in a fraction of cells, enabling a rapid recovery of motility in a subpopulation of cells when adequate stimuli are present (Wei & Bauer, 1998).

The detection of transcripts of virulence and pathogenicity genes during the entire study period was consistent with the *in vivo* virulence and pathogenicity results. *E. amylovora* cells starved for 40 days in oligotrophic water microcosms were able to infect and to cause disease similar to cells at time 0. The maintenance of pathogenicity and/or virulence in cells subjected to oligotrophy has also been reported in other plants and fish pathogenic bacteria (Larsen *et al.*, 2004; Álvarez *et al.*, 2008) and, together with the adoption of different survival strategies, changes in cell size and shape (including the formation of outer membrane vesicles), the regulation of motility, and the continued expression of all analyzed genes, reflects a variety of *E. amylovora* adaptations to starvation. Further, the continuous expression of the pathogenicity genes *rcsB* (involved in the biosynthesis of amylovoran, the main EPS of *E. amylovora*) and *rlsA* (encoding a regulator of the biosynthesis of levan, another major component of the EPS of *E. amylovora*) may also be linked to a strategy to enhance bacterial survival under nutrient-limited conditions. In fact, both EPSs have been shown to favor the survival of *E. amylovora* in carbon-free mineral medium (Ordax *et al.*, 2010) and to protect cells from drying out (Jock *et al.*, 2005) in nutrient-depleted conditions.

Semi-quantitative RT-PCR analyses allowed the monitoring of gene expression regulation throughout a 24-h period after the exposure of growing cells to an oligotrophic environment. Three different gene expression regulation patterns were observed during nutritional challenge, revealing rapid molecular responses to the new environmental conditions. The first pattern consisted of a minor variation in expression levels overtime and was only observed in the *dps* gene, which is involved in protection of DNA during starvation and other stresses. The second regulation pattern was observed in most of the analyzed genes, including starvation (*cstA*, *relA*, *rpoS*, *and spoT*) and oxidative stress (*katA*, *katG*, and *oxyR*) related genes, a gene linked to bacterial motility (*flgN*), and another one related to pathogenicity (*rcsB*). This pattern involved a two-step regulation process that consisted of an initial up- or down-regulation of expression levels and a second phase where transcripts reached a steady state. This type of pattern has been described in different organisms subjected to environmental changes, including bacteria exposed to starvation at environmental temperatures (González-Escalona *et al.*, 2006; Chechik & Koller, 2009) and would correspond to the activation of emergency responses after exposure to stress, allowing cells to reach a homeostatic adaptation to the new environment (Chechik & Koller, 2009). The third regulation pattern consisted of a sharp depression of gene expression followed by the maintenance of transcription at low levels. This pattern was observed in the rest of the virulence/pathogenicity genes analyzed: *dfoA* (participates in the synthesis of the siderophore desferrioxamine), *rlsA* (related to levan synthesis), and *hrpL* (an alternative sigma factor that regulates the expression of pathogenicity genes). In *E. amylovora,* the expression of *hrpL* is modulated by environmental conditions and enhanced during host infection (Gaudriault *et al.*, 1997; Pester *et al.*, 2012). Our results revealed a partial repression of the transcription of this gene and others related to virulence during the first 24 h of exposure to starvation, probably triggered by conditions unlikely to be encountered by this pathogen while causing disease.

Overall, results in this study have revealed different mechanisms exhibited by the fire blight pathogen to adapt to the nutrient-limiting conditions present in most natural environments, including soil, rain water, host surfaces, or overwintering cankers. The significance of bacterial responses to starvation in both the transmission and the persistence of the fire blight pathogen in natural ecosystems has long been underestimated. Our study provides new data concerning those stages of the *E. amylovora* life cycle in which it has to face nutrient scarcity, and contributes to a better understanding of the behavior of this bacterium under conditions more resembling those found in nature.

Acknowledgements

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Statement

We describe *Erwinia amylovora* responses to starvation, including changes in population dynamics, cell morphology, cell shape, motility, and gene expression. No change in virulence.

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Annex II

Santander & Biosca, 2016, unpublished

(it will be submitted to the journal *PeerJ*).

Title

Temperature-regulated virulence and starvation-survival responses in *Erwinia amylovora*: a cold-temperature adapted pathogenic species, with enhanced survival at low environmental temperatures.

Running title

Thermoregulation of virulence and starvation-survival responses in *Erwinia amylovora*

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Abstract

The fire blight pathogen *Erwinia amylovora* can grow at temperatures ranging from 4ºC to 37ºC, but blossom blight epidemics under field conditions mainly occur at temperatures above 18ºC. It is for this reason that many studies have focused on this temperature to determine the expression of virulence genes, or other aspects of the biology of this species. In many plant pathogens the expression of virulence determinants is restricted to a certain range of temperatures, but in the case of *E. amylovora* it is still unknown how low temperatures affect pathogenicity despite the fact that some fire blight outbreaks have been reported at lower temperatures. There is also scarce information on how such temperatures affect the starvation-survival responses of this microorganism, which might determine its persistence in the environment and probably contribute to the seasonal development of the disease, as occurs in other pathogens. To characterize the virulence and survival of *E. amylovora* at low temperatures we evaluated the effect of three temperatures (4ºC, 14ºC, 28ºC) on disease development, different parameters linked to starvation survival and a variety of virulence/survival determinants. *E. amylovora* was pathogenic at the three assayed temperatures, with a slow-down of symptom development correlating with colder temperatures and slower growth rates. Siderophore secretion and motility also decreased in parallel to incubation temperatures. However, production of the exopolysaccharides amylovoran and levan was enhanced at 4ºC and 14ºC, respectively. Similarly, biofilm formation, and oxidative stress resistance were improved at 14ºC, with this temperature also favoring the maintenance of culturability, together with a reduction in cell size and the acquisition of rounded shapes in *E. amylovora* cells subjected to long-term starvation. Starvation at 28ºC and 4ºC, however, induced a progressive loss of culturability over time (to a lesser extent at 4ºC), with a greater number of cells remaining viable, thus indicating the development of the viable but nonculturable (VBNC) strategy. This work reveals new data on fire blight pathogen biology, revealing an efficient adaptation to low temperatures including the ability to cause fire blight disease even at 4ºC, or improved exopolysaccharide synthesis, biofilm formation or oxidative stress resistance at 14ºC, with respect to the optimal growth temperature (28ºC). Finally, our results also demonstrate the thermoregulation of starvation responses in *E. amylovora,* suggesting that the starvation-survival and the VBNC states are part of its life cycle. These adaptations have probably contributed to the successful spread of the fire blight pathogen to countries with different climates

where susceptible hosts are cultivated, and knowledge of these might improve preventive and control measures against fire blight.

Key words

Fire blight; cold; EPS; biofilms; oxidative stress; siderophores; motility; VBNC

Introduction

Erwinia amylovora is a Gram-negative bacterium that causes fire blight, a destructive plant disease affecting economically important fruit trees such as pear and apple, as well as ornamental plants of the family *Rosaceae.* Among the environmental factors affecting the development of fire blight, temperature is critical, directly affecting bacterial growth, the phenologic development of the host (and interactions among these factors) and determining the presence of vectors responsible for the spread of the pathogen (Thomson, 2000; van der Zwet, Orolaza-Halbrendt & Zeller, 2012).

Compared to other phytopathogens of the family *Enterobacteriaceae* (predominantly mesophilic), the fire blight pathogen possesses an unusually wide range of growth temperatures (4ºC - 37ºC), the optimal temperature being 28ºC (van der Zwet, Orolaza-Halbrendt & Zeller, 2012). It is for this reason that *E. amylovora* can be considered a psychrotrophic microorganism, i.e., able to grow at low temperatures but with optimal and maximal growth temperatures above 15ºC and 20ºC, respectively (Moyer & Morita, 2007). Nevertheless, it is noteworthy that despite this phenotypic trait, temperatures equal or higher than 18ºC are usually needed for epidemic blossom blight under field conditions (van der Zwet, Orolaza-Halbrendt & Zeller, 2012). This temperature, hence, has been taken into account for the study of different aspects of the biology of *E. amylovora* (Raymundo & Ries, 1980; Wei, Sneath & Beer, 1992; Bereswill *et al.,* 1997; Goyer & Ullrich, 2006). However, little is known about the effects of low temperatures on the physiology and survival of the pathogen, or the link of such responses with the *E. amylovora* life cycle.

The effect of temperature on bacterial physiology depends on the nutritional state of cells. When inside the host, or as an epiphyte on floral organs, nutrient availability together with appropriate environmental temperatures favor *E. amylovora* multiplication. This is usually followed by the invasion of plant tissues and fire blight symptoms development (Thomson, 2000). Accordingly, the increase of bacterial cell numbers under these conditions is linked to the expression of pathogenicity and/or virulence factors allowing entry sites into the host to be reached, and/or plant nutrients to be acquired while avoiding host defenses, etc. Interestingly, particular temperature fluctuations between the environment and the host usually constitute a signal triggering the synthesis of virulence determinants mainly during host-pathogen interactions (Konkel & Tilly, 2000; Smirnova *et al.,* 2001). In fact, the pathogenicity of many bacterial species is restricted to a certain range of temperatures (Serfontein *et al.*, 1991; Durand *et al.,* 2000; du Raan, Coutinho & van der Waals, 2015).

In contrast to animal pathogens, temperatures favoring the expression of virulence genes (16ºC-24ºC) in phytopathogenic bacteria are usually lower than the optimal for growth and do not coincide with the host temperature (Konkel & Tilly, 2000; Smirnova *et al.,* 2001). These temperature conditions favor the formation of water aerosols or films near/on plant surfaces, which are usually required for efficient infection of plant hosts, or coincide with temperatures in water films (Smirnova *et al.,* 2001). In *E. amylovora,* for example, rain and/or heavy dew at the end of warm periods promote infection events under field conditions (Johnson & Stockwell, 1998). Related to this, the expression of many genes related to virulence, pathogenicity or other functions in *E. amylovora* is induced at 18ºC (Wei, Sneath & Beer, 1992; Goyer & Ullrich, 2006). However, there is scarce information on the minimal temperatures at which this pathogen is able to cause symptoms under appropriate conditions.

The effect of temperature on starvation responses is a less studied phenomenon in the fire blight pathogen. When *E. amylovora* experiences nutrient limitation (on plant surfaces, rainwater, soil, vector insects, etc.) this stress induces growth arrest, morphological changes and the development of a starvation-survival response with subpopulations of cells entering the viable but nonculturable (VBNC) state (Santander *et al.,* 2012; Santander, Oliver & Biosca, 2014; Ordax *et al.,* 2015). However, these VBNC cells are able to recover culturability under certain conditions (Ordax *et al.,* 2009; Santander *et al.,* 2012), thus constituting potential and hard-to-detect inoculum sources of the pathogen in the environment. In other bacterial a role for starvation-survival and VBNC responses in their life cycles has been described (Biosca *et al*., 1996; Armada *et al.,* 2003; Caruso *et al*., 2005; Vattakaven *et al.,* 2006; Lutz *et al.,* 2013; Ayrapetyan *et al*., 2015), but their role in the *E. amylovora* life cycle has not been well established. Similar to virulence factors, starvation responses are temperature-dependent (Santander, Oliver & Biosca, 2014), but their regulation at temperatures below 18ºC has not yet been assessed.

In this work, we investigated the effect of warm (28ºC), temperate (14ºC) and low (4ºC) temperatures on *E. amylovora* virulence and survival under natural starvation conditions, as well as on different factors related to virulence and/or environmental survival. Our results provide new knowledge on scarcely studied aspects of the *E. amylovora* life cycle, such as the temperature-regulated virulence and starvation responses which have probably contributed to the successful spread of this bacterial species to numerous countries worldwide with different climate areas (EPPO, 2016).

Material and methods

Bacterial strains and culture conditions

Five *E. amylovora* strains from different hosts and geographical origins frequently used in studies on fire blight were employed in this work: Ea 1/79 (Falkenstein *et al.,* 1988), Ea 1189 (Burse, Weingar & Ullrich, 2004), CFBP 1430 (Paulin & Samson, 1973), NCPPB 2080 (Giorgi & Scortichini, 2005) and ATTC 49946 (Sebaihia *et al.,* 2010). Unless otherwise specified, bacterial cultures were grown in liquid LB or on LB agar (LBA) plates at 28ºC with shaking (150 rpm). Strains were cryopreserved at -80ºC in 25 % glycerol.

Virulence

The virulence of the five *E. amylovora* strains included in this study was tested using green loquats (cv Tanaka). One-off assays were additionally performed on green pears (cv Devoe) with the reference strain CFBP 1430. To reduce fungal growth during prolonged incubation periods, particularly at low temperatures, fruits were first surface disinfected with 2 % sodium hypochlorite as previously described (Santander *et al.,* 2014; Santander, Oliver & Biosca, 2014) and then treated with 21.6 mg L^{-1} natamycin (Nataproq-G, Proquiga Biotech, Spain) for 5 min. The final natamycin concentration was selected based on Perdersen (1992). Fruits were let to dry in aseptic conditions under the hood, and then wound-inoculated with 10^3 CFU of *E. amylovora* per wound according to Santander *et al* (2014)*,* incubated in a wet chamber at 4ºC, 14ºC and 28ºC, and periodically monitored for fire blight symptoms development. *E. amylovora* was re-isolated from symptomatic fruits and identified by specific PCR as previously described (Santander *et al.,* 2012, 2014; Santander, Oliver & Biosca, 2014). Ten fruits were inoculated per strain and temperature, and the experiment was performed in two independent repeats.

Growth curves

Growth curves were performed at 4ºC, 14ºC and 28ºC in LB broth under static conditions. For this purpose, overnight cultures in LB were adjusted to an OD_{600} nm of 1.0 (ca. 2 10^9 CFU mL^{-1}), diluted 1:50 into fresh medium and incubated in parallel under static conditions at the above-mentioned temperatures. Aliquots of 0.5 mL were periodically taken from cultures and the OD_{600} nm measured. This experiment was performed in duplicate, per strain and temperature.

Siderophore detection

To induce the biosynthesis/secretion of siderophores, AB minimal medium (Chilton *et al.,* 1974) containing 0.05 g L^{-1} nicotinic acid (ABN) to allow *E. amylovora* growth (Starr & Mandel, 1950) and lacking $FeSO_4$ (ABN-Fe) was employed. *E. amylovora* overnight cultures in LB were pelleted, washed twice, resuspended in fresh ABN-Fe medium to an OD_{600} nm of 0.3, and incubated in parallel at 28ºC, 14ºC and 4ºC. Siderophore levels were quantified after 0, 1, 3 and 7 days post-inoculation (dpi) by mixing 0.4 mL supernatants with 0.3 mL of a fresh solution of 0.12 M $FeCl_3$ in 5 mM HCl (Holzberg & Artis, 1983). The abundance of siderophores in culture supernatants was spectrophotometrically determined at A_{425} nm, and normalized to the OD_{600} nm of the analyzed cultures. This experiment was performed in duplicate, in two independent experiments.

Motility assays in soft agar

Swimming motility was evaluated similar to Santander *et al* (2014), using TG (1 % tryptone, 0.5 % glucose, pH 7.0) soft agar plates (0.3 % agar). Briefly, a sterile toothpick previously dipped into an overnight culture of the *E. amylovora* strain to test was used to inoculate soft agar plates, which were sealed with parafilm and incubated at 4ºC, 14ºC and 28ºC. This assay was performed in two independent experiments, each with three technical repeats.

EPS relative quantification

To induce the production of the main *E. amylovora* EPSs amylovoran and levan overnight cultures were washed and diluted to an OD_{600} nm of 0.4 in LB plus 1 % sorbitol (LB Sor) or 5 % sucrose (LB Suc), respectively (Geider, 2000). Cultures were incubated at 4ºC, 14ºC and 28ºC and EPS contents in supernatants were measured at times 1, 3 and 7 dpi.

Amylovoran was measured (OD_{600} nm) after a 10 min reaction of 0.8 mL LB Sor supernatants with 40 μL of 50 mg mL^{-1} cetylpyridinium chloride (CPC) (Ordax *et al.,* 2010; Santander *et al.,* 2014). Levan production was measured as an increase in turbidity (OD_{580} nm) in LB Suc culture supernatants (Ordax *et al.,* 2010; Santander *et al.,* 2014).

Amylovoran and levan levels were normalized to the OD_{600} nm of the analyzed culture in each case (Santander *et al.,* 2014). This assay was performed in duplicate in two independent experiments.

202

203 **Biofilm quantification by a microtiter assay**

204 *E. amylovora* biofilms were quantitated using a microtiter assay based on
205 Chelvam, Chai & Thong (2014). To this aim, *E. amylovora* overnight cultures were
206 diluted to an OD_{600} nm of 0.5 into 0.5x LB broth, and transferred (160 µL per well) to
207 polystyrene (surface-treated, hydrophylic) Nunc™ MicroWell™ 96-well microplates
208 (Thermo Scientific). Sterility controls consisting of non-inoculated 0.5 x LB were
209 included in each assay. Inoculated plates were incubated in parallel at 4ºC, 14ºC and
210 28ºC for 48 h. After this period, the plates were left to reach room temperature for 10
211 min, and planktonic cells and medium were removed by inversion, gentle tapping and
212 incubation upside down for 5 min, inside a biosafety cabinet. Then, biofilms were heat-
213 fixed in a Pasteur oven at 80ºC for 30 min and let to cool down to room temperature.
214 Afterwards, 220 µL of 1 % crystal violet (CV) were added to each well and incubated
215 for 15 min at room temperature. The CV was removed by inversion, and the plates
216 were thoroughly rinsed with distilled water and let to dry upside down inside the hood.
217 Biofilm formation was determined spectrophotometrically (A_{600} nm) with a plate reader
218 (FLUOstar OPTIMA, BMG Labtech), after solubilizing the CV from biofilms with an 8:2
219 mixture of absolute ethanol and acetone (220 µL per well) for 20 min. This assay was
220 carried out with, at least, seven replicates per strain and temperature, in two
221 independent experiments.

222

223 **Oxidative stress resistance**

224 Oxidative stress sensitivity at 4ºC, 14ºC and 28ºC was assessed by an agar
225 dilution assay, based on Wiegand, Hilpert & Hancock (2008). Briefly, plates of LBA
226 (control) and LBA + 0.75 mM H_2O_2 were prepared and let to solidify under the hood for
227 30 min. The H_2O_2 concentration was selected based on preliminary assays. *E.*
228 *amylovora* overnight cultures in LB were adjusted to an OD_{600} nm of 1.0 (ca. 10^9 CFU
229 mL^{-1}), serially tenfold diluted in sterile saline, and 5 µL drops of each dilution (from 10^{-1}
230 to 10^{-6}) were plated on the above-mentioned media. Plates were then sealed and
231 incubated in parallel at 28ºC, 14ºC and 4ºC for 6, 18 and 41 days, respectively. The
232 minimal dilution at which each strain grew on each medium at each temperature was
233 recorded. This assay was performed in two independent repeats.

234

235 **Microcosms preparation, monitoring of population dynamics and**
236 **morphology analysis of starved cells**

Oligotrophic water microcosms were prepared as previously described (Santander *et al.,* 2012; Santander, Oliver & Biosca, 2014), inoculated with *E. amylovora* at a final density of ca. 2 10^7 CFU mL^{-1}, and incubated in parallel at 4ºC, 14ºC and 28ºC. Culturable cell populations at different periods were determined by drop-plate on LBA, as described elsewhere (Santander *et al.,* 2012; 2014; Santander, Oliver & Biosca, 2014). Viable and total cell population dynamics were monitored by flow cytometry, using a BD FACSVerse™ flow cytometer (BD Biosciences), with 488 nm excitation. To this aim, microcosm aliquots were taken at different intervals throughout a 122-day period, and stained with the components of the Baclight Live/Dead viability kit (Life Technologies), according to the manufacturer's instructions. Red and green fluorescence were measured with 700/54 and 527/32 filters, respectively, the trigger was set on FSC (forward scatter), and data were analyzed using the BD FACSuite™ software (BD Biosciences). Each strain was inoculated in three independent microcosms per temperature.

Morphological changes occurring in *E. amylovora* cells starved in water microcosms were monitored by epifluorescence microscopy similar to Álvarez, López & Biosca (2008). Briefly, 20 μL microcosm aliquots were taken at times 0 and 122 dpi, fixed on a glass slide and stained for 10 min with 12 mM SYTO9 (Life technologies). Afterwards, the stained cells were covered with a glass slide and photographed at a magnification of x 1000, using a digital camera adapted to a Nikon Eclipse E800 epifluorescence microscope, and the automatic camera tamer software ACT-1. Microscope images were thresholded to create binary images with IMAGEJ software (Schneider, Rasband & Eliceiri, 2012), and the parameters Feret diameter, area and circularity were measured to determine possible changes in cell size and shape due to starvation and/or temperature.

Statistical analysis

Prior to statistical analysis a log transformation of culturable, viable, and total cell counts was performed. In general, differences between means were determined by analysis of variance (ANOVA). The factors compared were temperature and strain, although the time factor was also included in certain analyses. In some experiments replicate means were compared using Bonferroni post-tests. P-values ≤ 0.05 were considered significant.

Results & Discussion

Natamycin allows the performance of long-term virulence assays by delaying fungal growth on inoculated fruits

To reduce the growth of filamentous fungi on *E. amylovora* inoculated loquats and pears, fruits disinfected by a standardized procedure (Santander *et al*., 2012; 2014; Santander, Oliver & Biosca, 2014) were additionally treated with natamycin. This compound is a broad-spectrum fungicide, with low toxicity for animals, plants and bacteria, and is successfully used in multiple applications (Pedersen, 1992; Pengfei, Juxin & Shaobo, 2013; Biosca *et al*., 2016). Applying natamycin to immature fruits prior to their inoculation efficiently delayed fungal growth, allowing the incubation of inoculated fruits for prolonged periods, particularly at the low assayed temperatures.

E. amylovora remains pathogenic even at 4ºC, with virulence, growth rates, siderophore secretion and motility decreasing in parallel to incubation temperature

Results corresponding to virulence assays at 4ºC, 14ºC and 28ºC in green loquats are shown in Fig. 1. All the *E. amylovora* assayed strains were able to cause typical fruit blight symptoms (necrosis and exudates) at the three tested temperatures. However, the onset of symptoms and the progression of necrosis throughout time were strongly dependent on temperature ($p < 0.001$). Fruit blight symptoms developed earlier at 28ºC (2 dpi) (Fig. 1a), followed by 14ºC (7-9 dpi) (Fig. 1b) and 4ºC (35-79 dpi) (Fig. 1c). In the same way, the fastest necrosis expansion over time was observed at 28ºC, followed by 14ºC and 4ºC (Fig. 1). These results were similarly reproduced in virulence assays with green pears (cv. Devoe) inoculated with the *E. amylovora* strain CFBP 1430 (Fig. S1).

It is noteworthy that *E. amylovora* retained its pathogenicity even at an extremely low temperature such as 4ºC. This is not common behavior amongst animal and plant pathogens. For example, in bacterial species of the genera *Shigella, Pectobacterium* and *Dickeya*, the ability to cause disease is restricted to a certain temperature range (Serfontein *et al*., 1991; Durand *et al.,* 2000; du Raan, Coutinho & van der Waals, 2015). This is due to the repression of virulence and/or pathogenicity genes by temperatures above or below this range (Konkel & Tilly, 2000; Smirnova *et al.,* 2001). Our results indicate that *E. amylovora* has the potential to cause fire blight at a very wide range of temperatures as long as susceptible tissues are present, which

agrees with the observed progression of fire blight symptoms in perennial pear branches during winter in Israel, following autumn infections (Shtienberg *et al.*, 2015).

The effect of incubation temperature on disease development was similar to that observed in growth rates (Fig 2), siderophores secretion (Fig. 3) and motility (Fig. 4). Coinciding with fire blight symptom development, these virulence determinants reached lower values the lower the assayed incubation temperature was.

Regarding growth rates, some virulence factors in *E. amylovora* are regulated in a cell population dependent manner via *quorum sensing* (Molina *et al.,* 2005). Therefore, it is reasonable that fruit blight symptoms appear and develop faster at temperatures allowing *E. amylovora* to reach appropriate cell densities in shorter periods, which correlates with the higher growth rates observed at 28ºC, followed by 14ºC and 4ºC (Fig.2). Interestingly, Pusey & Curry (2004) demonstrated that the *E. amylovora* proliferation on stigma surfaces of crab apple flowers only occurs at temperatures above 12ºC, with temperatures ranging from 20ºC to 32ºC allowing the pathogen to reach cell densities greater than those of antagonistic bacteria. This fact, among others, might explain why blossom blight in field is observed at temperatures above 18ºC (van der Zwet, Orolaza-Halbrendt & Zeller, 2012). However, growth curves and virulence assays in immature fruits indicate that *E. amylovora* not only grows at 4ºC, but is also able to cause fire blight symptoms once it reaches susceptible host tissues such as immature fruits.

With respect to siderophores, these iron chelating compounds have long been recognized as important virulence factors in *E. amylovora*, contributing to iron acquisition inside the host and survival under oxidative stress (Dellagi *et al.,* 1998; 1999; Venisse, Gullner & Brisset, 2001; Venisse *et al.,* 2003). In this study, we identified a decrease in siderophore production occurring in parallel with the diminution of incubation temperature, with the relative levels of these iron chelating compounds at 28ºC being 2.5 ($p < 0.001$) and 5.4 ($p < 0.001$) times greater than those determined at 14ºC and 4ºC, respectively (Fig. 3). The lower siderophore production together with the reduced virulence at low temperatures, might thus support the important role of these iron-uptake molecules as *E. amylovora* virulence factors.

Motility in *E. amylovora* is necessary for full virulence, aiding bacterial cells to reach natural openings in apple blossoms (Bayot & Ries, 1986) or other plant organs (Cesbron *et al.,* 2006). This phenotypic trait is modulated by temperature, pH and other environmental factors (Raymundo & Ries, 1980; Goyer & Ullrich, 2006). Once inside host tissues, *E. amylovora* cells lose flagella (Raymundo & Ries, 1981; Cesbron *et al.,*

2006), avoiding the elicitation of plant defense responses by flagellins (Cesbron *et al.,* 2006; Holtappels *et al.,* 2015). However, non-motile cells from infected tissues can recover their motility if water films on plant surfaces are formed (Raymundo & Ries, 1981). Furthermore, cells expressing flagella can remain motile even under starvation conditions (Santander, Oliver & Biosca, 2014) and for variable periods, also depending on incubation temperatures (Raymundo & Ries, 1981). This probably makes it easier to reach new host entry sites and/or other nutrient sources.

As shown in Fig. 4, all the assayed *E. amylovora* strains were able to move throughout soft agar regardless of the tested temperature, with the greatest motility observed at 28ºC, followed by 14ºC and 4ºC. These results suggest the expression of motility-related genes at low temperatures, and hence the potential ability of the fire blight pathogen to move towards new host entry sites or other nutrient sources even at suboptimal environmental temperatures such as 4ºC.

EPS production, biofilm formation and oxidative stress resistance are enhanced at temperatures below the optimal for growth

Two important virulence/pathogenicity factors in the fire blight pathogen are the production of EPSs and the formation of biofilms (van der Zwet, Orolaza-Halbrendt & Zeller, 2012; Piqué *et al.,* 2015). EPSs contribute to pathogen spread and host colonization, by obstruction of the plant vascular system and masking the bacterial cell surface elicitors of plant defenses (Geider, 2000; Maes *et al.,* 2001; Venisse, Gullner & Brisset, 2001; Ordax *et al.,* 2015). Moreover, they might favor pathogen survival in the environment, acting as alternative carbon sources during starvation periods, and as a barrier against desiccation, bacteriophages and toxic compounds such as copper (Geider, 2000; Jock, Langlotz & Geider, 2005; Ordax *et al.,* 2010; van der Zwet, Orolaza-Halbrendt & Zeller, 2012; Born *et al.,* 2014). With regard to biofilms, their role during the systemic invasion of plant hosts has been reported (Koczan *et al.,* 2009; 2011), as well as their relevance for the survival of the pathogen on plant surfaces and vectors (Ordax *et al.,* 2009; 2015).

In our work, we provide for the first time data on the ability of *E. amylovora* to produce EPSs (Fig. 5) and biofilms (Fig. 6) at temperatures ranging from 4ºC to 28ºC, with these virulence factors being more intensely induced at temperatures below the optimal for growth ($p < 0.0001$).

Our results also revealed an interesting differentiated regulation of the two main EPSs in *E. amylovora,* amylovoran and levan, by temperature (Fig. 5). Amylovoran

was more intensively produced at 4ºC, followed by 14ºC and 28ºC, reaching maximum levels at 1 dpi (Fig. 5a). Levan production, however, increased over time regardless of the assayed temperature, with levels of this EPS reaching maximum values at 3 and 7 dpi, and being greater at 14ºC, than at 28ºC or 4ºC (Fig. 5b). These results might suggest a not yet described role for EPSs in *E. amylovora* facilitating growth at low temperatures and/or enhancing cold tolerance, as described in bacteria from cold environments (Nichols *et al.,* 2005; Chrismas *et al.,* 2016).

In some cases, a reduction in EPS levels (mainly amylovoran) over time was observed (Fig. 5). This phenomenon has been linked to their use as alternative carbon sources under nutrient limiting conditions (Ordax *et al.,* 2010). In our case, transitory nutrient starvation of *E. amylovora* cells due to their incubation for a week under static conditions could explain the results obtained.

Analogous to levan production, biofilm formation in all the assayed *E. amylovora* strains was enhanced at 14ºC, followed by 28ºC and 4ºC (Fig. 6). This demonstrates the potential of *E. amylovora* to form biofilms in plant tissues at 14ºC and even at 4ºC, suggesting that the systemic invasion of host plants is possible even at very low temperatures. Furthermore, the need of both amylovoran and levan for proper biofilm development in *E. amylovora* has been described (Koczan *et al.,* 2009). Accordingly, the observed differences in the thermoregulation patterns of these two EPSs might indicate that their degree of contribution to biofilm formation might also vary depending on environmental temperatures.

Apart from producing EPSs and forming biofilms, the *E. amylovora* invasion of host tissues depends on other virulence/pathogenicity factors (Piqué *et al.,* 2015) such as a type three secretion system (TTSS) and TTSS-related effector proteins, which are used to kill host plant cells by means of a strategy that distinguishes *E. amylovora* from other phytopathogens (Venisse, Gullner & Brisset, 2001; Venisse *et al.,* 2003). While many plant pathogens avoid or reduce host responses via deployment of the TTSS (Grant *et al.,* 2006), *E. amylovora* uses the same mechanism to provoke an oxidative burst, which is used to kill plant cells (Venisse, Gullner & Brisset, 2009; Venisse *et al.,* 2003). Accordingly, antioxidant mechanisms are required to face the reactive oxygen species (ROS) released by host cells. Our results indicate that *E. amylovora* resistance to oxidative stress is also thermoregulated, with resistance to H_2O_2 being enhanced at 14ºC, with respect to 28ºC and 4ºC (Fig. 7). Interestingly, the production of EPSs was also more efficiently induced at 14ºC than at 28ºC (Fig. 5). Although, the role of these macromolecules as protecting agents against H_2O_2 has been discussed (Venisse *et al.,*

2003), they are induced by oxidative stress (R.D. Santander, À. Figàs-Segura & E.G. Biosca, unpublished data), suggesting their possible contribution to the protection against other ROS, or toxins secreted by plant cells, which indirectly might cause oxidative stress.

It is remarkable that, despite the clear enhancement of H_2O_2 sensitivity at 4ºC and 28ºC (Fig. 7), all the *E. amylovora* strains were able to cause regular fire blight symptoms in immature fruits (Fig. 1), with the strains which are more sensitive to oxidative stress (NCPPB 2080 and 49946) (Fig. 7) being similarly or even more virulent than some of the strains showing better resistance to H_2O_2. A strain-dependent production of factors reducing the effects of ROS on bacterial cells probably also contributes to the phenotypes observed.

Starvation at 14ºC favors the persistence in a culturable state, while the VBNC response is enhanced at 28ºC and, to a lesser extent, at 4ºC

As part of its life cycle, *E. amylovora* has to deal with periods of starvation, either within host cankers, as an epiphyte, inside vectors, or when spread by rainwater, etc. (Thomson, 2000; Santander, Oliver & Biosca, 2014). In a previous work we showed the temperature-dependent nature of *E. amylovora* responses to nutrient limitation (Santander, Oliver & Biosca, 2014). However, the effect of temperatures below 18ºC on starvation responses had not yet been assessed. Results in this work demonstrated the modulation of *E. amylovora* long-term starvation responses by temperature (Fig. 8). A progressive drop of culturability over time was observed at 28ºC and, to a lesser extent, at 4ºC. This phenotype, however, was not observed at 14ºC, a temperature at which *E. amylovora* cells developed a characteristic starvation-survival response, with culturability values being similar to those at initial time, with only a slight reduction of culturability at final time (Fig. 8).

Flow cytometry analyses showed a temperature-dependent loss of viability ($p < 0.05$) between initial and final (122 days) times, which was greater at 28ºC, followed by 14ºC, but nonsignificant at 4ºC (Fig. S2). Nevertheless, compared to culturable cell counts, this decrease of viability was very reduced, indicating that the drop in culturability observed at 28ºC and 4ºC actually corresponded to a progressive entry into the VBNC state, with the number of VBNC cells at the end of the experiment at 14ºC being reduced compared to that observed at 28ºC or 4ºC (Fig. 8d). The flow cytometric analysis of *E. amylovora* total cell populations also revealed a certain cell

lysis at final time, which was greater at 28ºC, followed by 14ºC and 4ºC ($p < 0.01$) (Fig. S2).

As demonstrated in previous works, *E. amylovora* cells starved for long periods remain fully virulent while culturable (Santander *et al.,* 2012; Santander, Oliver & Biosca, 2014). Starved cells entering the VBNC state, however, lose their ability to cause disease in immature fruits, although virulence can be recovered by passage through a susceptible host (Santander *et al.,* 2012; Ordax *et al.,* 2015). Interestingly, the loss of culturability characteristic of the VBNC state has been linked to a reduced resistance to oxidative stress (Oliver, 2010) probably due to a simultaneous drop in different antioxidant activities (R.D. Santander, À. Figàs-Segura & E.G. Biosca, unpublished data), which agrees with results obtained in this work.

Alternating periods of VBNC and starvation-survival responses are characteristic stages of the life cycle of many non-obligated pathogens, and determine the seasonal incidence of the disease they cause (Armada *et al.,* 2003; Caruso *et al.*, 2005; Smith & Oliver, 2006; Vattakaven *et al.,* 2006; Ayrapetyan *et al.*, 2015). The knowledge of the roles that these survival responses play in the *E. amylovora* life cycle, may, indeed, allow for an improvement in control and/or prevention measures against fire blight.

Taken together, the results in this work and a previous one (Santander, Oliver & Biosca, 2014) indicate that temperatures ranging from 14ºC to 20ºC would favor the persistence of *E. amylovora* in the environment in a culturable and pathogenic state, coinciding with those ensuring a greater abundance of pollinating insects (or other biotic vectors), and enhancing the epiphytic growth on blossoms or fire blight symptom development (van der Zwet, Orolaza-Halbrendt & Zeller, 2012). Lower and higher temperatures in other periods of the year, however, would induce the development of the VBNC response in starved cells, for example inside host cankers, until favorable conditions (e.g., resumption of the host's growth) occur.

Starvation induces morphological changes in a temperature-dependent manner

Similar to other bacteria that change their shape during their life cycle (Morita, 1997; Álvarez, López & Biosca, 2008; Chen *et al.,* 2009; Oliver, 2010; Yang, Blair & Salama, 2016), *E. amylovora* responds to starvation with a reduction in cell size (dwarfing) and the acquisition of rounded shapes, probably via the degradation of endogenous material, cell walls and envelopes through the formation of surface

vesicles (Santander, Oliver & Biosca, 2014). In many pathogens, morphological changes occurring under starvation conditions are dependent on temperature (Chen *et al.,* 2009; Yang, Blair & Salama, 2016), however, the influence of this environmental factor on *E. amylovora* starved cells had not yet been explored.

At time 0, *E. amylovora* cells showed a typical rod shape (Fig. 9). However, in general, prolonged exposure to starvation provoked the acquisition of rounded shapes and dwarfing (Fig. 9). Except for strain NCPPB 2080, the acquisition of rounded shapes in response to oligotrophy was observed at all the temperatures assayed ($p < 0.05$). However, a strong effect of incubation temperature on the cell size of long-term starved *E. amylovora* cells was observed ($p < 0.001$). Most strains experienced enhanced dwarfing at 14ºC, while the effect of the other temperatures on size reduction varied depending on the assayed strain (Fig. 9).

The reduction of bacterial cell size and the acquisition of coccoid shapes have been related to the optimization of energetic resources in starved cells, but also as a strategy to avoid predation by protists (Byrd, 2000; Nyström, 2004). Moreover, for some pathogens morphological transitions occur in parallel to the colonization of different host tissues or cell types, transmission between hosts and/or shifts to environmental reservoirs (Yang, Blair & Salama, 2016). *E. amylovora* cells during infections are rod-shaped, similar to what occurs during growth in liquid medium. Our results indicate that prolonged starvation conditions such as in reservoirs outside the host, or also within the host in cankers, would induce size reduction and the adoption of circular shapes, with this response being favored at temperatures enhancing the persistence of the pathogen in a culturable and pathogenic state, such as 14ºC and also 20ºC (Santander, Oliver & Biosca, 2014).

Overall, this study provides, for the first time data on the pathogenic potential, survival and expression of different virulence and/or survival factors of *E. amylovora* at a wide range of environmental temperatures (4ºC - 28ºC) shedding light on scarcely known aspects of the biology of this pathogen.

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Figures

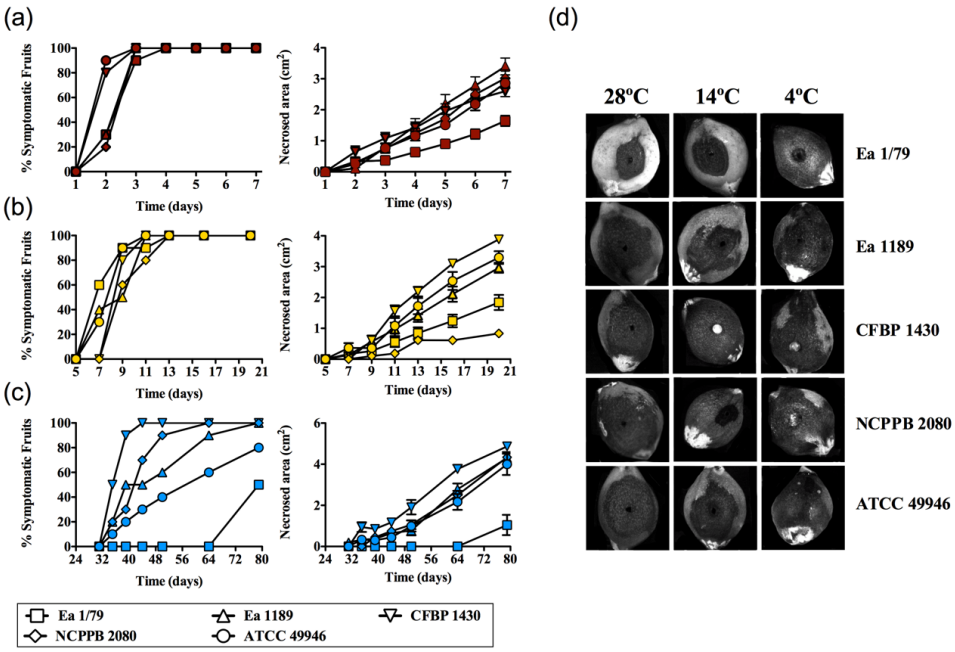
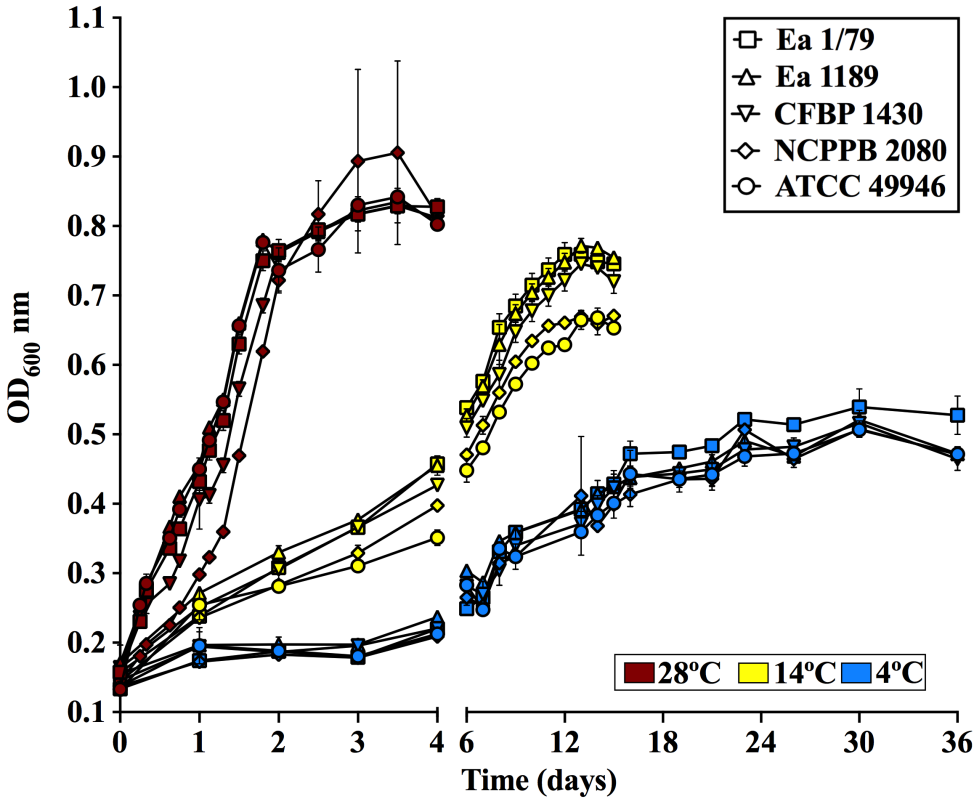


Fig. 1. *E. amylovora* virulence assays in immature loquats (cv. Tanaka) at 28ºC (a), 14ºC (b) and 4ºC (c). Graphs on the left and the right show the percentage of symptomatic fruits (total values) over time and the expansion of necrotic lesions (mean values of two experiments with 10 technical repeats) over time, respectively. Representative pictures of fruits showing fire blight symptoms at the end of the experimental period (28ºC, 7 dpi; 14ºC, 20 dpi; 4ºC, 79 dpi) (d).



767

768 **Fig. 2. *E. amylovora* growth curves in LB at 28ºC, 14ºC and 4ºC under**
769 **static conditions.** Data in this graph correspond to mean values of one experiment
770 with two replicates. Vertical lines indicate the SD.

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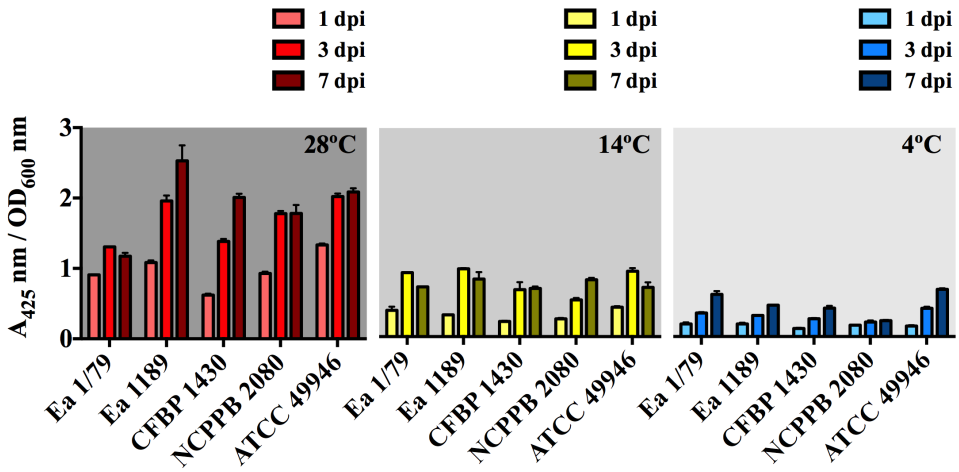

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776 **Fig. 3. *E. amylovora* siderophore production at 28ºC, 14ºC and 4ºC in**
777 **minimal medium ABN-Fe after 1, 3 and 7 dpi.** Data correspond to mean
778 measurements of two independent experiments performed in duplicate. Vertical lines
779 mark the SD. The two-way ANOVA analysis of these results showed that differences of
780 siderophore production among the three temperatures were very significant ($p < 0.0001$).
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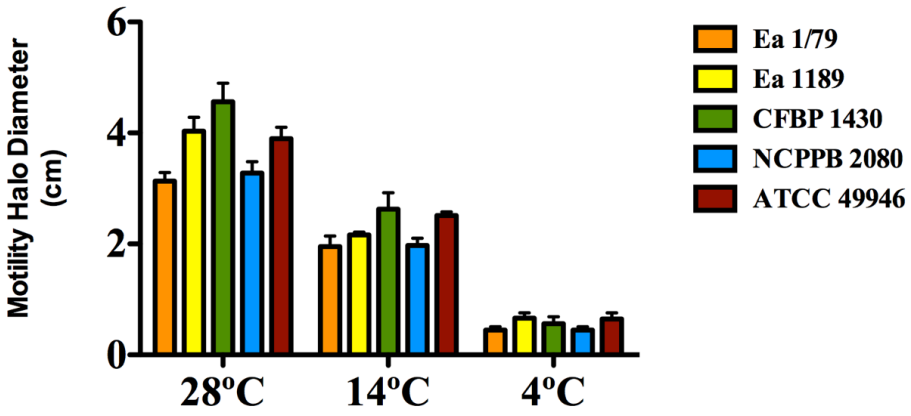


Fig. 4. Motility of *E. amylovora* in soft agar plates at 28ºC, 14ºC and 4ºC. Data correspond to mean measurements of two independent experiments performed in triplicate. Vertical lines mark the SD. Swimming motility differences at the three temperatures assayed were very significant ($p < 0.001$), regardless of the tested strain.

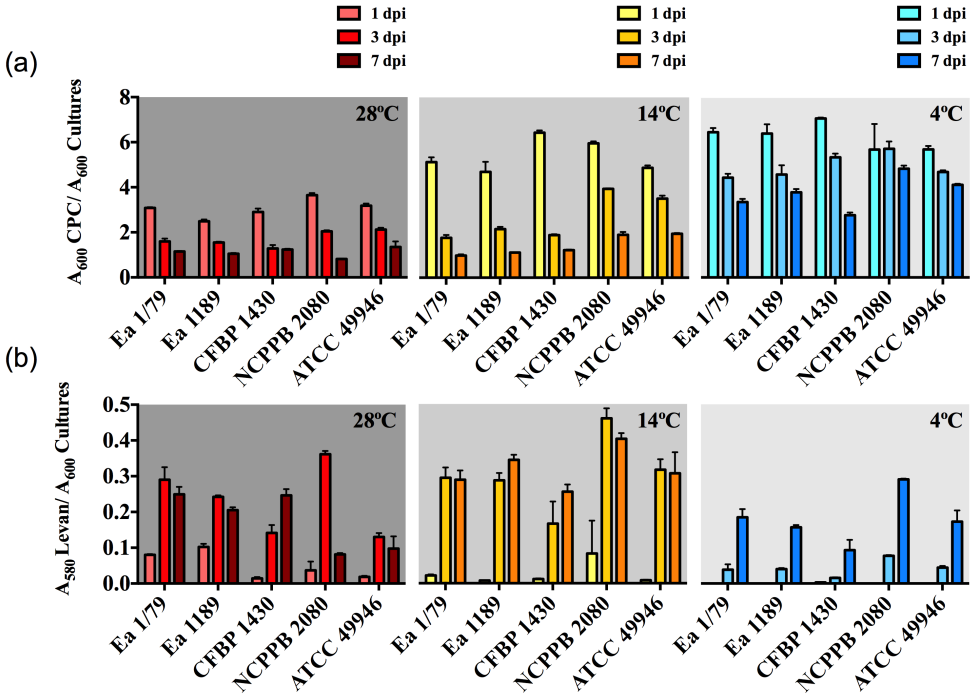


Fig. 5. Relative production of amylovoran (a) and levan (b) by *E. amylovora* at 28ºC, 14ºC and 4ºC. Data in graphs correspond to mean values of two experiments performed in duplicate. Vertical lines denote the SD. A two-way ANOVA analysis of data revealed that differences in EPS production at 28ºC, 14ºC and 4ºC were very significant ($p < 0.0001$), regardless of the assayed strain

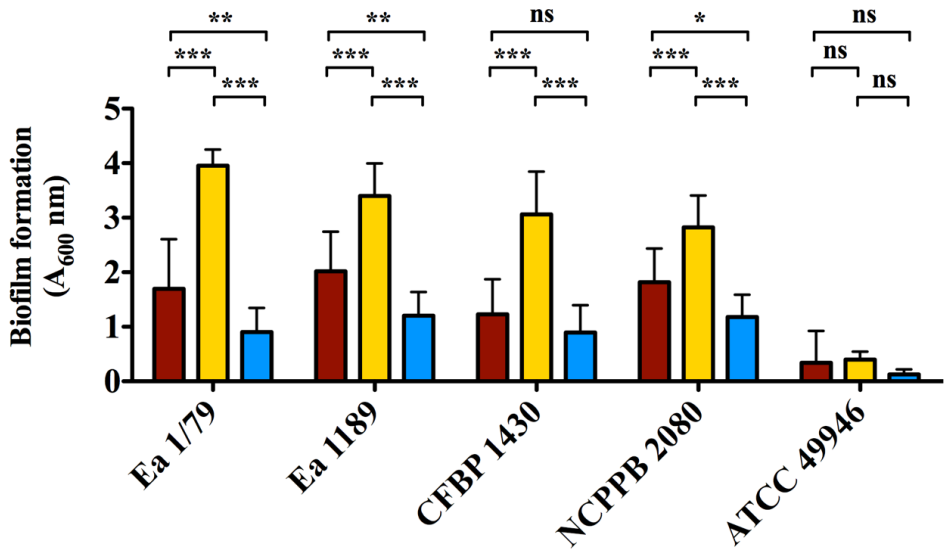
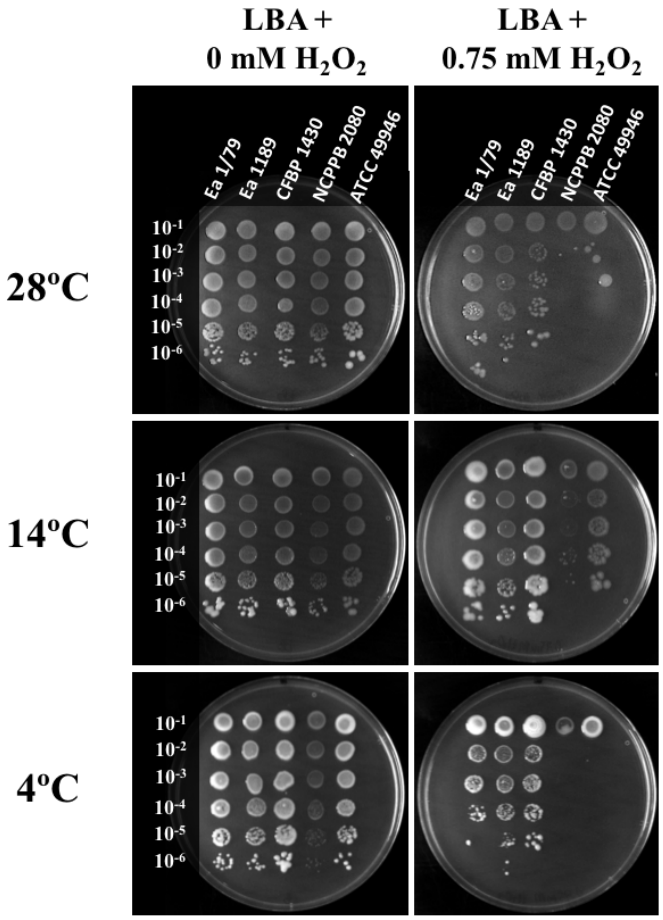
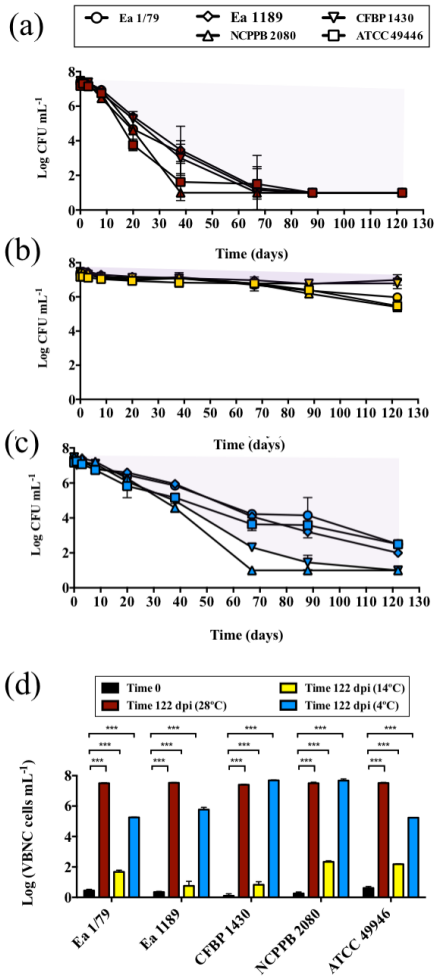


Fig. 6. Quantification of biofilm formation by *E. amylovora* after 48 h of incubation at 28ºC (red), 14ºC (yellow) and 4ºC (blue). Represented data correspond to average values of two independent experiments analyzed in septuplicate. Vertical lines indicate the SD. Asterisks above horizontal lines denote statistically significant differences between the two signaled columns. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns, non-significant differences.



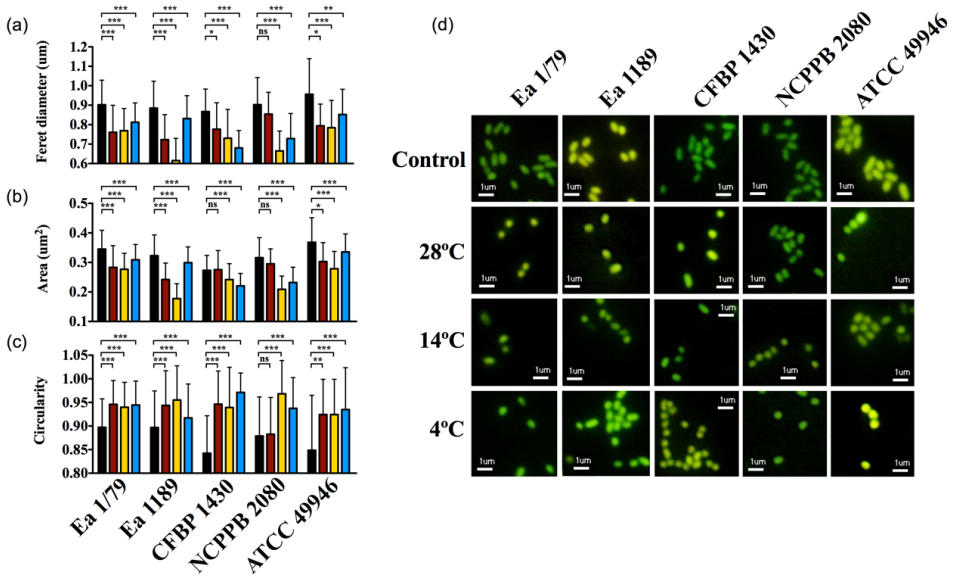
826

827 **Fig. 7. Quantification of the oxidative stress resistance of *E. amylovora* at 28ºC,**
828 **14ºC and 4ºC by an agar dilution assay using LBA plates containing 0.75 mM**
829 **H_2O_2**. Plates inoculated with serial tenfold dilutions of *E. amylovora* overnight cultures
830 were sealed, incubated at each temperature and photographed at 6, 18 and 41 dpi,
831 respectively. This assay was performed in two independent experiments with
832 equivalent results. Oxidative stress resistance was greater at 14ºC than at 28ºC or 4ºC.
833



834

835 **Fig. 8. Analysis of *E. amylovora* culturable and VBNC cell populations**
836 **during a long-term starvation period (122 days) at 28ºC (a), 14ºC (b) and 4ºC (c).**
837 Culturable cell counts are represented with symbols (a-c). Purple areas indicating
838 VBNC cell subpopulations are drawn (a-c). Given the similar trends in the VBNC
839 induction of all the assayed *E. amylovora* strains, only results corresponding to strain
840 CFBP 1430 are shown. VBNC cell populations at times 0 and 122 dpi are summarized
841 in (d). In all the graphs represented data correspond to average values of three
842 independent experiments. Vertical lines indicate the SD, and asterisks denote
843 statistically significant differences between temperatures (***, $p < 0.001$).



844 **Fig. 9. *E. amylovora* morphological changes induced by starvation at**
845 **different temperatures.** Data correspond to mean values of about 300 measures (per
846 strain and temperature), from three independent experiments. Variations in cell length
847 (Feret diameter) (a), area (b) and circularity (c) in cells at time 0 (black columns) with
848 respect to cells starved for 122 days at 28ºC (red columns), 14ºC (yellow columns) and
849 4ºC (blue columns). Representative pictures of the previous data (d). Asterisks above
850 horizontal lines denote statistically significant differences between two conditions (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns, non-significant differences).
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Supplementary Information

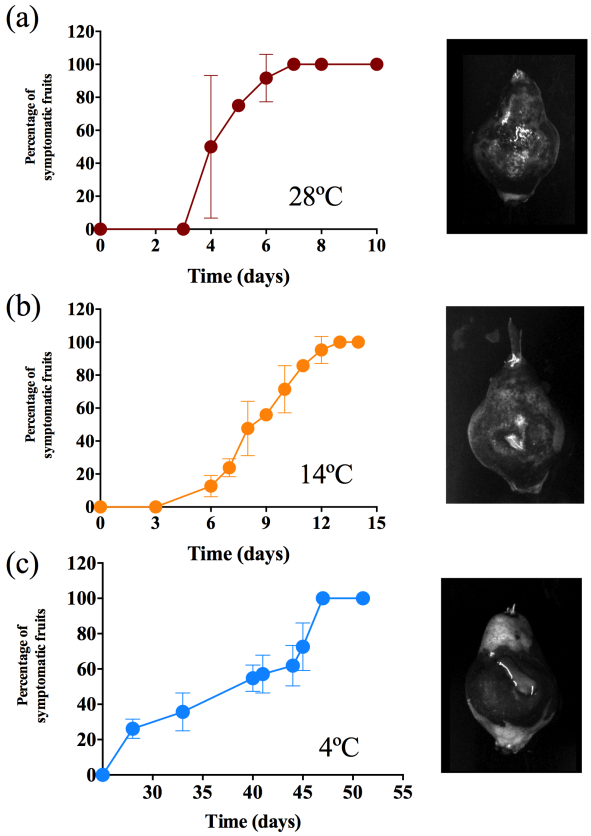

Fig. S1. Virulence assays in immature pears (cv. Devoe) with the *E. amylovora* strain CFBP 1430 at 28ºC (a), 14ºC (b) and 4ºC (c). Graphs show the percentage of symptomatic fruits over time (mean values of two experiments with 7-9 fruits per temperature). Representative pictures of fruits showing fire blight symptoms at the end of the experimental period (28ºC, 10 dpi; 14ºC, 15 dpi; 4ºC, 51 dpi) are represented besides the corresponding graph. Vertical lines indicate the SD.

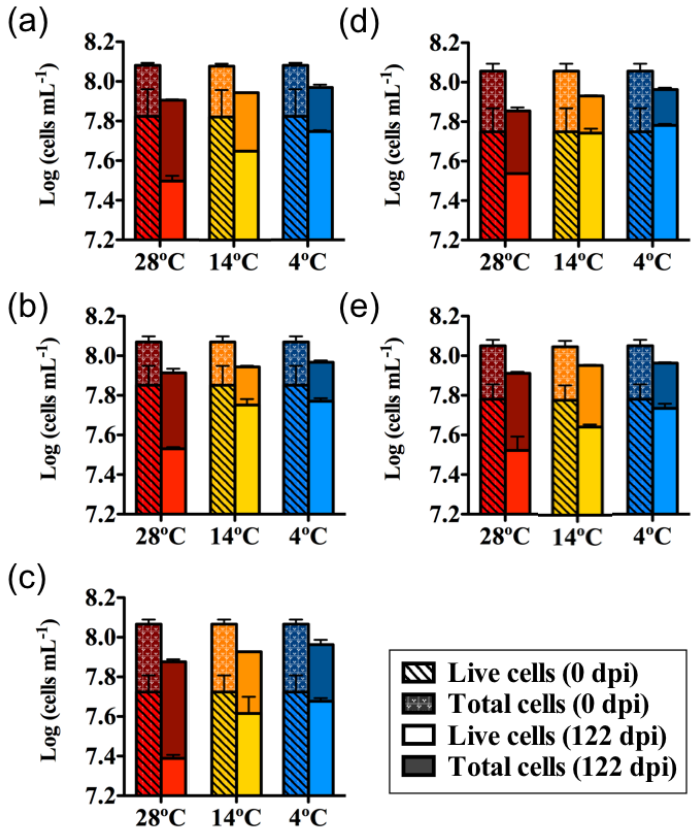

Fig. S2. Flow cytometry analysis of total and viable cell counts in *E. amylovora* starved cells at times 0 and 122 dpi. Graphs show mean values of three independent experiments with strains Ea 1/79 (a), Ea 1189 (b), CFBP 1430 (c), NCPPB 2080 (d) and ATCC 49946. Vertical lanes indicate the SD. An ANOVA analysis of data revealed an effect of temperature on viability ($p < 0.05$) and total cell integrity (total cell counts) ($p < 0.01$)

Annex III

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ORIGINAL PAPER

In planta recovery of *Erwinia amylovora* viable but nonculturable cells

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Abstract Little is known about the survival mechanisms of *Erwinia amylovora* outside its hosts. It has been demonstrated that it enters the viable but nonculturable state (VBNC) when exposed to different types of stress. In the VBNC state, bacterial cells remain viable but unable to grow on the solid general media where they usually do, and are thus undetectable by conventional culture-dependent methods. In this work, we have evaluated the recovery of *E. amylovora* VBNC cells by passage through pear plantlets, in comparison with other recovery methods commonly used for this pathogen: incubation in KB broth and inoculation of immature fruits. VBNC cells were obtained by exposure of bacterial cells to different types of stress (oligotrophy, nutrient deprivation and chlorine), and recovery assays were performed at 26°C. In all cases, the recovery of VBNC cells was more effective in plantlets than in liquid KB or immature fruits. In fact, when cells were exposed to chlorine for more than 30 min, only passage through host plant gave positive result, enabling recovery of *E. amylovora* cells few days after inoculation of plants. These results suggest a higher effectiveness of in planta recovery than those performed with liquid KB or detached fruits. Our results support the hypothesis of the VBNC state being part of the *E. amylovora* life cycle. The potential existence of this physiological state in nature should be taken in consideration in epidemiological studies of fire blight, with the aim to optimize the management and control of this disease.

Keywords Fire blight · Oligotrophy · Nutrient deprivation · Chlorine · VBNC · Recovery

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A contribution to the Special Issue: Pome Fruit Health.

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Introduction

Erwinia amylovora (Burrill) (Winslow et al. 1920) is a gram-negative plant pathogenic bacterium of the family *Enterobacteriaceae,* which causes fire blight in a wide range of rosaceous plants. Susceptible hosts include economically important pome fruit trees, like pear and apple, and many ornamental and wild rosaceous plants (van der Zwet and Beer 1995). The name of the disease is due to the distinctive appearance of twigs, branches, flowers and leaves of infected plants, as if burnt by fire. Since its first detection in Europe in southern England in 1957, fire blight has spread across Europe (van der Zwet 2000). Nowadays, *E. amylovora* is classified as a quarantine organism in the European Union (Anonymous 2000). It is present in more than 40 countries (Bonn and van der Zwet 2000), and causes important economic losses in pome fruit crops worldwide.

Fire blight is a highly contagious disease. When introduced into a crop area, it causes an extensive dieback of flowers, branches, fruits and even entire plants of susceptible cultivars, dramatically affecting crop production. Difficulties to control this disease have been related with

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the ability of the pathogen to survive in different conditions and to use multiple and diverse dissemination routes (Thomson 2000). Nevertheless, there is scarce information about the survival mechanisms of the bacterium outside susceptible hosts under unfavorable conditions.

One of the main characteristics of most natural environments is the limited availability of nutrients (Morita 1997). In recent years, it has been shown that *E. amylovora* is able to survive and maintain pathogenicity during exposure to both oligotrophic conditions and nutrient deprivation, in different types of environmental water and distilled water, respectively (Biosca et al. 2006). *E. amylovora* cells overcome these conditions by adopting two physiological states. The starvation–survival state (Morita 1997), which allows their persistence in a culturable state (Biosca et al. 2006), and the viable but non-culturable state (VBNC) (Biosca et al. 2006), in which bacterial cells remain viable but unable to grow on general solid culture media, being undetectable by conventional culture methods (Oliver 2005, 2010; Roszak and Colwell 1987). These two physiological states are different from the stage of growth, and both are considered survival strategies in many non-sporulating bacterial species in response to stressing conditions (Morita 1997; Oliver 2005, 2010; Roszak and Colwell 1987). Initial studies have demonstrated that *E. amylovora* can also survive treatments by disinfectants such as chlorine (Santander et al. 2011a). It can persist in the presence of metals such as copper (Ordax et al. 2006, 2009, 2010), which is widely used for the control of fire blight. In both cases, the bacterium survives by adopting the VBNC state. *E. amylovora* cells in this physiological state are undetectable by culture-based methods and, although only pathogenic for few days (Ordax et al. 2006, 2009), they could be involved in the transmission of this pathogen.

In some animal pathogenic bacteria, where the VBNC has been more extensively studied, recovery of the culturability and pathogenicity of VBNC cells was possible after exposure to favorable conditions, such as the passage through a susceptible host (Cappelier et al. 1999; Oliver 2005, 2010). In the case of *E. amylovora,* previous studies have demonstrated that copper induced VBNC cells can recover their culturability and pathogenicity by enrichment in a liquid medium (Ordax et al. 2006, 2009) or by inoculation in detached plant organs (Ordax et al. 2006, 2009). But these recovery methods are not always successful (Santander et al. 2009).

Given the potentially significant epidemiological implications of the VBNC state on the management and control of fire blight, the aim of this study was to evaluate the in planta recovery of VBNC *E. amylovora* cells by passage through susceptible host, using pear plantlets, and compare it with commonly used enrichment in liquid medium and inoculation in detached plant fruits.

Materials and methods

Bacterial strains, culture conditions and inocula preparation

Erwinia amylovora strain CFBP1430 (CFBP, Collection Française de Bacteries Phytopathogènes), isolated from *Crataegus* sp. in France, was used in all experiments. The Spanish *E. amylovora* strain IVIA1892-1 (IVIA, Instituto Valenciano de Investigaciones Agrarias, Spain), isolated from *Pyrus* sp., was included in some of the assays for comparative purposes. Inocula were prepared in sterile saline solution (0.9% NaCl, pH 7.0) from cultures incubated at 26°C for 48 h on King's B (KB) agar plates (King et al. 1954). Inocula concentrations were adjusted spectrophotometrically to an A_{600} of 1.0, an equivalent of 10^9 cfu/ml, and then diluted 1/100 in filter-sterilized rainwater or distilled water, depending on the experiment (see below), to obtain a final concentration of 10^7 cfu/ml.

Induction of the VBNC state in *E. amylovora* with different stressing factors

The VBNC state was induced by exposure of *E. amylovora* cells to different stresses: oligotrophy in rainwater, nutrient deprivation in distilled water and chlorine exposure. For this purpose, microcosms were prepared using filter-sterilized samples of rainwater, distilled water (pH adjusted to 7.0) and distilled water plus chlorine [0.7 ppm of free chlorine; value within the range of drinking water (Anonymous 1984)]. These microcosms were separately inoculated with strains CFBP1430 and IVIA1892-1 to a final concentration of 10^7 cfu/ml. Natural and distilled water microcosms were maintained at 26°C (Biosca et al. 2006), at least up to 75 and 45 days, respectively. For chlorine stress, the bacterium was exposed to chlorine at least up to 24 h, also at 26°C.

To examine the entry into the VBNC state, 1–2 ml aliquots from each microcosm were periodically sampled to carry out culturable, viable and total cell counts (Biosca et al. 2006). Sampling from natural and distilled water microcosms was performed at time zero, daily during the first 7 days, and then weekly until the complete loss of culturability. Distilled water microcosms treated with chlorine were sampled at 0, 5, 15, 30, 60 and 120 min, and thereafter, every 2 h during 8 h, with a final sampling time at 24 h. After each sampling time, chlorine was removed from sampled aliquots by neutralization with sodium thiosulfate (1% *w/v*) as described (Santander et al. 2011a). Bacterial counts for culturable cells were performed by the drop plate (Hoben and Somasegaran 1982) and spread plate methods. Briefly, at initial sampling points, each sample was serially tenfold diluted in

sterile saline solution and 10 µl drops of each dilution were plated on KB agar plates in triplicate. When culturable cell counts decreased below the drop-plate method detection limit (10^2 cfu/ml), culturable cell counts were carried out by spreading 0.1 and 1 ml of each sample (detection limit <1 cfu/ml) on KB agar plates (three plates per sample). Plates were incubated at 26°C for 48 h, and up to 7 days when culturability dropped below 1 cfu/ml. To perform viable and total cell counts, samples were first stained with the LIVE/DEAD Baclight bacterial viability kit (Molecular Probes Inc., Eugene, OR, USA) (Boulos et al. 1999) according to the manufacturer instructions, and then counted with a Nikon ECLIPSE E800 epifluorescence microscope (20 random fields per sample) at a magnification of ×1,250. For the purpose of this study, we considered the whole bacterial cell population to have entered into the VBNC state when plate counts dropped below the detection limit (<1 cfu/ml) on KB agar plates.

Each experiment of induction of the VBNC state by each stressing factor assayed was done in triplicate microcosms and repeated in an independent experiment, again in triplicate.

E. amylovora VBNC cells recovery assays

When culturable cell numbers dropped below the detection limit (<1 cfu/ml), aliquots of each type of VBNC-induced cells were collected at different times and subjected to different recovery methods as follows:

1. In vitro recovery assay in liquid KB: One milliliter of each sample was inoculated into sterile tubes with 9 ml of KB broth. Tubes were incubated with shaking (200 rpm), at 26°C for 1–7 days as previously described (Ordax et al. 2006, 2009; Santander et al. 2009). Turbidity was considered as indicative of positive recovery after PCR confirmation (see below).
2. Recovery assays in immature pear fruits: Immature pear fruits (2 × 4 cm approx) cv. Williams were collected from an orchard and stored at 4°C in the dark until use. Pear fruits were surface disinfected by immersion into twofold diluted bleach (17.5 g/L of active chlorine) for 5 min, rinsed three times with sterile distilled water and dried in laminar air flow. Inoculation was performed according to Cabrefiga and Montesinos (2005), with modifications. Briefly, pear fruits were wounded in four places with a sterile 1,000 µl pipette tip. Then, 50 µl aliquots of the different types of VBNC-induced cells were inoculated in each of the four wounds per fruit (four repeats of the same treatment by fruit). Inoculated fruits were incubated at 26°C in a moist chamber for 1–3 weeks and monitored periodically for fruit blight symptoms (drops of bacterial exudates and/or necrotic areas around or inside the inoculation point).
3. In planta recovery assays: Experiments were performed on 1-month-old pear plantlets cv. Passe Crassane. Pear plantlets were obtained from seeds manually extracted from commercial fruits, surface-disinfected, stratified, germinated and grown, according to Santander et al. (2011b). Inoculations were performed according to Ruz et al. (2008), by wounding two young expanded leaves with scissors, previously dipped into 2 ml aliquots of each type of sample. Plants were incubated with 16 h of light at 26°C, 8 h of darkness at 24°C and high relative humidity (≥85%) under quarantine conditions, and examined daily for 3 weeks to check for symptoms of fire blight.

Recovery assays were considered positive when *E. amylovora* was isolated from inoculated KB broth and plant material and PCR-identified. The isolation of this bacterium from KB broth grown tubes and blighted fruits was carried out by spread-plating diluted samples of turbid medium or bacterial exudates, respectively, on KB agar plates. Symptomatic leaves or necrotic pear tissue were processed according to the diagnostic protocol PM7/20 on the EPPO standards following symptomatic plant material procedure (Anonymous 2004). Bacteria were isolated by direct plating plant extracts on two general media, KB and sucrose nutrient agar (SNA, Lelliot 1967), and the semi-selective medium for *E. amylovora,* CCT (Ishimaru and Klos 1984). In all cases, randomly selected *E. amylovora*-like colonies were identified by *E. amylovora*-specific PCR (Taylor et al. 2001). Additionally, direct PCR detection of *E. amylovora* from plant material was performed following DNA extraction (Llop et al. 1999).

All recovery methods were assayed with the triplicate microcosms at least in duplicate for each independent experiment, sampling point and VBNC inducing stressing factor. *E. amylovora* overnight cultures in liquid KB, and sterile saline solution (0.9% NaCl pH 7.0) were used as positive and negative controls, respectively, in all the assays.

Statistical analysis

Data of total, viable and culturable *E. amylovora* cell counts were analyzed by using mean values of log-transformed data from independent experiments. Null data from culturable counts below the detection limit were not included in the analysis. Significancy of differences was assessed by variance analysis (ANOVA). Factors considered for the analysis of the induction of the VBNC state in *E. amylovora* were: stress factor, day, strain and

experiment. Differences were recorded as significant at p values below 0.05.

Results

Induction of VBNC state in *E. amylovora* cells by exposure to different stresses

The effect of oligotrophy, nutrient deprivation and chlorine on the induction of the VBNC state in *E. amylovora* is shown in Fig. 1. Since no significant differences on cell population trends were observed between the two tested strains, only survival curves of the reference strain CFBP1430 are represented in Fig. 1. Regardless of the assayed stress, total cell numbers remained constant (10^8 cells/ml) throughout the experiment (Fig. 1). Viable cell populations also remained at the same level, around 10^7 cells/ml, during incubation in oligotrophy (Fig. 1a) and under nutrient deprivation (Fig. 1b). However, *E. amylovora* viable cells decreased significantly from 10^7 to 10^6 cells/ml during the first 6 h of exposure to chlorine (Fig. 1c), at which level they remained until the end of the experiment.

In general, culturability declined more and faster than viability, falling below the detection limit (<1 cfu/ml) with all stressing factors assayed, but at different times (Fig. 1). Induction of the VBNC state seems to be significantly faster in distilled water than in rainwater. Hence, during incubation under these conditions culturable and VBNC cells coexisted, but the proportion of VBNC cells increased until the whole population became VBNC (after approximately 60 and 30 days in rainwater and distilled water, respectively) (Fig. 1a, b). *E. amylovora* cells treated with 0.7 ppm of free chlorine rapidly lost culturability, with less than 1 cfu/ml recovered by plating on media after only 5 min treatment (Fig. 1c). Therefore, all assayed stressing factors induced the entry of whole bacterial populations into the VBNC state. Exposure times inducing VBNC transition depended on the stress factor assayed, with no significant differences between the two assayed strains.

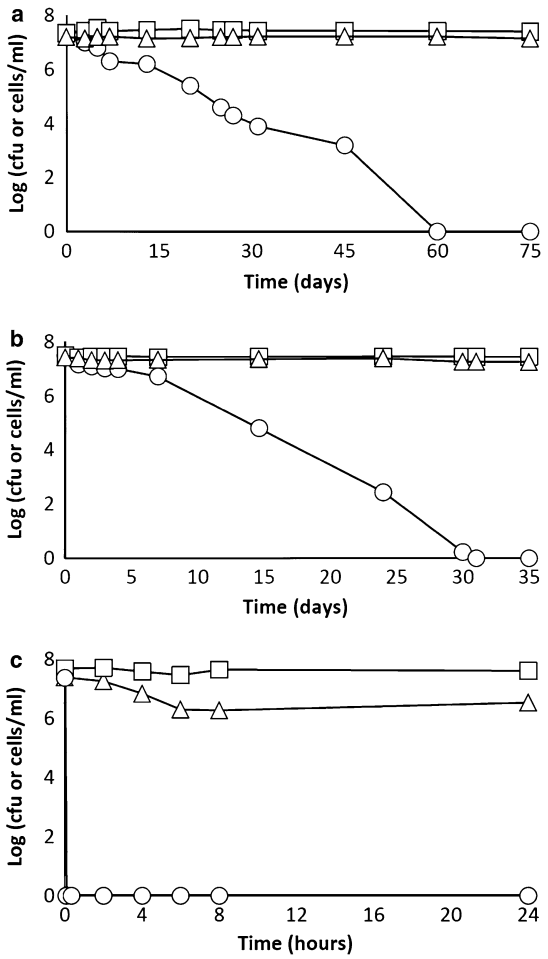

Fig. 1 Induction of the VBNC state in *E. amylovora* (strain CFBP1430) by different stressing conditions: **a** oligotrophy (rainwater); **b** nutrient deprivation (distilled water); **c** chlorine exposure (0.7 ppm). Total cells (*squares*), viable cells (*triangles*); and culturable cells (*circles*). Each point corresponds to the mean of two independent experiments in triplicate microcosms. The minimum standard deviation was 0.09 and the maximum 0.51

Recovery of *E. amylovora* VBNC cells with different methods

Results of the three assayed recovery methods with the different types of *E. amylovora* VBNC-induced cells (strain CFBP1430) are summarized in Table 1. Similar results were obtained with the Spanish strain that was included in some of the assays. Positive controls produced turbidity in liquid KB, exudates and/or necrosis in immature fruits and necrosis and wilting in pear seedlings. Negative controls did not show any of these features.

The in vitro method (recovery in KB broth) was unable to recover the culturability of *E. amylovora* VBNC cells after 65 and 75 days under oligothrophy and 35 and 45 days under nutrient deprivation (Table 1). Recovery of chlorine-induced VBNC cells was possible with this method after exposure times of 30 min, but not longer (Table 1). Turbidity appeared between 24 and 72 h after KB broth inoculation. Similar results were obtained after inoculation of VBNC cells into immature pear fruits, that when were positive (Table 1) showed typical exudates and necrotic lesions along the three weeks of incubation.

Table 1 Result of the different methods assayed for the recovery of *E. amylovora* VBNC cells

Stressing factor	Recovery sampling time	Recovery method		
		In vitro[a]	In vivo[a]	
		Incubation in KB broth	Inoculation of green pears	Inoculation of pear plantlets[c]
Oligotrophy	65 days	−(0/12)	−(0/12)	+(12/12)
	75 days	−(0/12)	−(0/12)	+(12/12)
Nutrient deprivation	35 days	−(0/12)	−(0/12)	+(12/12)
	45 days	−(0/12)	−(0/12)	+(12/12)
Chlorine (0.7 ppm)	30 min	+(6/12)	+(12/12)[b]	+(12/12)
	1 h	−(0/12)	−(0/12)	+(8/12)
	2 h	−(0/12)	−(0/12)	+(12/12)
	4 h	−(0/12)	−(0/12)	+(8/12)
	6 h	−(0/12)	−(0/12)	+(12/12)
	24 h	−(0/12)	−(0/12)	+(12/12)
Positive controls[d]		+(12/12)	+(12/12)	+(12/12)
Negative controls[d]		−(0/12)	−(0/12)	−(0/12)

[a] It was considered as a positive result when at least one of the replicates was positive. Number of positive tubes, pears or plantlets out of total number of tubes, pears or plantlets are shown in brackets

[b] In 100% of cases, the four inoculation points on each fruit showed blight symptoms

[c] In 93% of cases, the four inoculation points per seedling showed blight symptoms

[d] To simplify, all positive and negative controls for all treatments are shown as a single representative experiment

Unlike KB enrichment and inoculation of immature pear methods, inoculation in pear plantlets allowed the recovery of all types of induced VBNC cells in all tested times (Table 1). As shown in Fig. 2, *E. amylovora* VBNC cells inoculated in pear plantlets were able to multiply and produce an initial necrosis surrounding the inoculation site, which progressed to the central nerve of the leaf (Fig. 2a), and finally to the entire plant (Fig. 2b), within 3 weeks.

E. amylovora-like colonies were isolated from all positive recovery assays (turbid liquid KB medium, bacterial exudates from pears and plantlets and necrotic plant tissues) and subsequently identified by *E. amylovora* specific PCR. Semiselective and differential properties of CCT and SNA media, respectively, provided easier discrimination of *E. amylovora*-like colonies from epiphytic microbiota than KB. In most cases, it was also possible the PCR detection of the pathogen directly from symptomatic plant material.

In summary, the in vivo recovery of *E. amylovora* VBNC cells by passage through a susceptible whole plant host was possible regardless of the assayed VBNC inducing factor or the exposure time to the stress. Enrichments in liquid KB medium or inoculation in immature pear fruits allowed the recovery of chlorine-induced VBNC cells after exposure time of 30 min, but not after longer chlorine exposures and long-term oligotrophy and nutrient deprivation.

Discussion

The VBNC state has been described as a long-term-survival mechanism of non-sporulating bacteria by which cells overcome environmental stresses that could otherwise be lethal (Gauthier 2000). In most bacterial pathogens, the entry into the VBNC state is linked to the progressive incapacity to induce disease. Nevertheless, VBNC cells can recover their pathogenic potential, at the same time as culturability, when exposed to certain favorable conditions (Oliver 2005, 2010). As reported by Biosca et al. (2006), *E. amylovora* cells enter into the VBNC state when exposed to different levels of nutrient limitation. Moreover, treatments with chlorine or copper, assumed to be bactericidal, can also induce the VBNC state in this bacterium (Ordax et al. 2006, 2009, 2010; Santander et al. 2011a). However, attempts to recover *E. amylovora* VBNC cells induced by long-term incubation in oligotrophic or nutrient deprivation conditions or by exposure to chlorine or copper, are not always successful (Ordax et al. 2006, 2009; Santander et al. 2009).

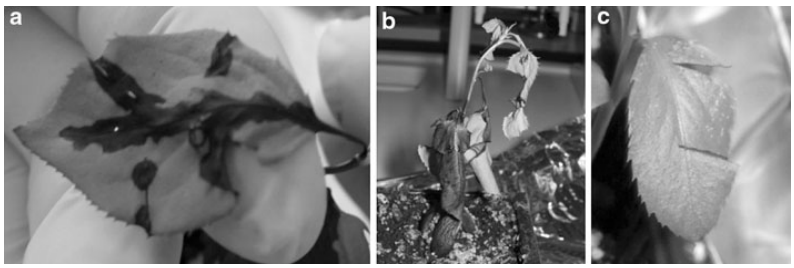

Fig. 2 Recovery of *E. amylovora* (strain CFBP1430) VBNC cells induced by a 60 min. chlorine treatment by inoculation in pear plantlets. **a** Necrotic lesions surrounding the inoculation sites and the central nerve in a leaf, **b** inoculated plant showing necrosis in the stem and leaves, **c** negative control inoculated with sterile saline solution

Some described strategies to revert VBNC bacteria to a culturable state, described for human pathogens include reversal of the stressing factor, enrichment in liquid media, or direct inoculation into susceptible hosts (Oliver 2005, 2010). Some of these methodologies have also been employed for the recovery of VBNC cells in plant pathogenic bacteria (Alexander et al. 1999; del Campo et al. 2009; Grey and Steck 2001; Ordax et al. 2006, 2009; Santander et al. 2009). For *E. amylovora* most common recovery strategies are the enrichment in liquid media or the inoculation in detached plant organs (Ordax et al. 2006, 2009; Santander et al. 2009). However, these strategies do not always allow the recovery of VBNC cells, which has been related to the type of VBNC-inducing factor, the exposure time and/or the time-period after the entry into the VBNC state.

The objective of this study was to investigate the effectiveness of the in planta recovery of *E. amylovora* VBNC cells. To this aim, we induced the entry of *E. amylovora* into the VBNC state by exposure to previously described VBNC-inducing factors such as oligotrophy, nutrient deprivation and chlorine (Biosca et al. 2006; Santander et al. 2011a). Then, we compared the recovery of *E. amylovora* VBNC cells by passage through a whole host plant (1-month-old pear plantlets) with the two routinely used recovery methods employed with this pathogen: enrichment in liquid KB and inoculation in immature pears.

All stressing conditions assayed caused a decrease in culturability that was not related with a loss of viability. The rate at which cells entered into the VBNC state varied depending on the exposure time or the type of stress employed, but in all cases increased throughout the experimental time. Chlorine exposure proved to be the strongest VBNC inducing factor, followed by nutrient deprivation, and then by oligotrophy. Total cell numbers remained constant throughout the experiment, regardless of the stress condition and/or the exposure time, indicating that the decrease in viable and culturable cells was not related to lytic effect of stressor. Viable cell numbers under natural oligothrophy in rainwater and nutrient deprivation in distilled water microcosms remained at the same level throughout the experimental time. In contrast, the concentration of viable cells in chlorine-treated samples decreased from 10^7 to 10^6 cells/ml during the first 6 h, and remained at that level until the end of the experiment. Chlorine is considered a powerful bactericidal substance due to its high oxidizing capacity (Virto et al. 2005). Then, as might be expected, chlorination had a lethal effect on part of the population of viable cells. However, according to our viable cell counts data under our experimental conditions, the chlorine effect was mostly bacteriostatic. These results are similar to those obtained by other authors in other pathogenic bacteria (Oliver et al. 2005; Wang et al. 2010).

Regarding culturable cell counts, in all cases, part of the bacterial cell populations entered into the VBNC state, and the rate of entry into this state was related with the type of VBNC-inducing stress assayed. In natural water, the loss of culturability below the detection limit was observed around 60 days post-inoculation, while in distilled water it was earlier, about 30 days post-inoculation. These significant differences in the point of entry into the VBNC state between cells exposed to natural oligotrophy in rainwater or nutrient deprivation in distilled water microcosms could be due, at least partially, to the difference in the nutrient content, since distilled water completely lacks any carbon source (Biosca et al. 2006). As previously pointed out by Morita (1997), bacterial cells are exposed to oligotrophic conditions most of the time in almost all types of environments. Then, bacterial adaptations to these conditions increase bacterial survival. According to our results, *E. amylovora* cells respond to long-term oligotrophy and nutrient deprivation by progressively adopting a VBNC physiological state as a survival strategy. These results are consistent with previous studies on *E. amylovora* (Biosca et al. 2006) and other plant pathogenic bacteria such as *Agrobacterium tumefaciens* (Byrd et al. 1991; Manahan and Steck 1997), *Xanthomonas campestris* (Ghezzi and Steck 1999) and *Ralstonia solanacearum* (Álvarez et al. 2008). *E. amylovora* cells also survived chlorine exposure by entering into the VBNC state few minutes after contacting with this compound, according to our previous work (Santander et al. 2011a) and other bacterial pathogens stressed by chlorine (Moreno et al. 2007; Oliver et al. 2005).

The adoption of the VBNC state allows bacterial cells to survive under unfavorable conditions, maintaining, in most cases, the potential to revert to an infectious and culturable state when conditions turn favorable (Oliver 2005, 2010; Weichart 1999). Ordax et al. (2006, 2009) were able to recover copper-induced VBNC *E. amylovora* cells by enrichment in KB broth and, in some specific cases, by inoculation into detached plant organs. In the case of chlorine-induced VBNC cells, recovery by KB enrichment was possible only after very short treatments, but not by inoculation of immature pears (Santander et al. 2009).

The VBNC state has important implications in diagnostic procedures where culturability is considered as the only indicator of viability (López et al. 2005; Oliver 2005), and on the epidemiology of bacterial diseases. It might contribute to the persistence of plant pathogens in nature (Grey and Steck 2001; Marco-Noales et al. 2008) and, in the case of human pathogens, to some recurrent infections (Oliver 2005; Sardessai 2005). Similarly, the VBNC state could contribute, in some manner, to the apparent disappearance of *E. amylovora* during some months in infected

orchards. Then, we decided to evaluate the effectiveness of in planta VBNC recovery, successfully applied in other important plant pathogenic bacteria (Grey and Steck 2001). We employed 1-month-old pear plantlets cv. Passe Crassane, described as a highly sensitive cultivar to the fire blight causal agent. Results in this study have shown that only the in planta inoculation method allowed the recovery of VBNC cells in all cases, regardless of the VBNC-inducing factor or the exposure time. Enrichment in KB broth or inoculation in immature pears only allowed the recovery of VBNC cells after 30 min chlorine treatments or shorter. Similarly, Ordax et al. (2009) inoculated immature pears and loquat fruits with copper-induced VBNC cells and fire blight symptoms only were observed after exposure times shorter than 6 days.

The basis for the recovery of VBNC cells in liquid medium or immature pears has not been well described yet. Ordax et al. (2006) were able to recover copper-induced VBNC cells by using copper chelating compounds, such as EDTA, citric acid and asparagine only after 18 or 75 days, while KB broth or pear juice recovered VBNC cells even after 9 months. These results suggest that the removal of the stressing factor could not be as important as certain compounds provided, in this case, by KB broth or pear juice. In our case, chlorine was neutralized with sodium thiosulfate. Then, it seems that some factors present in growing plantlets could be involved in the recovery of all types of VBNC-induced *E. amylovora* cells assayed in this work. In this respect, it is noteworthy that in a recent review by Oliver (2010), it has been reported that host environment could elicitate the recovery of VBNC cells in some human pathogenic bacteria.

In summary, the results of this study further support the hypothesis that *E. amylovora* can survive under different types of stress by adopting the VBNC state as a survival strategy. Further, we have shown the high efficacy of the in planta recovery method compared to KB broth and immature fruit strategies. Finally, our results reinforce the proposal of the VBNC state as a part of the *E. amylovora* life cycle. The potential existence of this state in nature should be taken into consideration in epidemiological studies of fire blight, which eventually may improve measures for management and control of this disease.

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Annex IV

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RESEARCH ARTICLE

Medfly *Ceratitis capitata* as Potential Vector for Fire Blight Pathogen *Erwinia amylovora*: Survival and Transmission

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Abstract

Monitoring the ability of bacterial plant pathogens to survive in insects is required for elucidating unknown aspects of their epidemiology and for designing appropriate control strategies. *Erwinia amylovora* is a plant pathogenic bacterium that causes fire blight, a devastating disease in apple and pear commercial orchards. Studies on fire blight spread by insects have mainly focused on pollinating agents, such as honeybees. However, the Mediterranean fruit fly (medfly) *Ceratitis capitata* (Diptera: Tephritidae), one of the most damaging fruit pests worldwide, is also common in pome fruit orchards. The main objective of the study was to investigate whether *E. amylovora* can survive and be transmitted by the medfly. Our experimental results show: i) *E. amylovora* can survive for at least 8 days inside the digestive tract of the medfly and until 28 days on its external surface, and ii) medflies are able to transmit the bacteria from inoculated apples to both detached shoots and pear plants, being the pathogen recovered from lesions in both cases. This is the first report on *E. amylovora* internalization and survival in/on *C. capitata*, as well as the experimental transmission of the fire blight pathogen by this insect. Our results suggest that medfly can act as a potential vector for *E. amylovora*, and expand our knowledge on the possible role of these and other insects in its life cycle.

Introduction

Phytopathogenic bacteria cause annually very important losses in major crops and fruit trees, producing serious economical damage. Epidemiological studies have been mostly focused on plant-pathogen interactions, excluding the role of other organisms in disease dissemination. Insects are often neglected as ecological players, but many plant diseases become more severe and detrimental in the presence of specific or nonspecific insect vectors that spread the pathogen to new hosts [1]. Nowadays, there is a trend to investigate plant diseases at a community

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level [2], looking for a better understanding of the interactions and associations of the bacteria with other organisms in the environment.

The bacterium *Erwinia amylovora* is the causal agent of fire blight, a destructive and highly infectious disease of apple, pear and other rosaceous plants. The name of the disease is derived from the characteristic dark discoloration of affected plant tissues, as if they were burnt. Fire blight causes dramatic losses worldwide, and remains as a disease difficult to control due to the lack of fully efficient chemical and biological treatments and the ability of *E. amylovora* to persist in nature and to spread in diverse ways [3]. *E. amylovora* cells are usually disseminated by insects, rain, wind or wind-driven rain (as aerosols) to open blossoms, and also to shoots, tender leaves and fruits [3, 4]. In a recent review on fire blight by Billing [5], the author concludes that some aspects of *E. amylovora* life cycle and fire blight epidemiology rest on uncertainty. Although it is considered that *E. amylovora* uses blossoms as the main route of infection, natural openings or wounds can also provide entryways into the plants [6]. Further, there are many insects presumably associated with the spread of fire blight [7, 8, 9, 10], although their exact role and the *E. amylovora* survival in/on these insects is poorly understood, specially in non-pollinating agents [4, 11].

There is a wide presence of non-pollinating insects in pome fruit orchards throughout summer, late spring and early autumn [12]. An important group of these insects are the fruit flies (family *Tephritidae*), which have a particular relevance in agriculture [13]. The Mediterranean fruit fly, or medfly, *Ceratitis capitata* (Wiedemann) is one of the most destructive fruit pests worldwide and is considered the most important invasive species throughout the world [14, 15, 16, 17]. The medfly has been extremely successful at invading and settling new areas, particularly due to its polyphagous diet, causing damage in nearly 400 plant species, and its liberal host acceptance behavior, rapid population growth, and high tolerance for a wide range of climates [15]. In addition, the medfly can be spread via the local sale or exportation of fruit, and it can fly at least 20 km, which greatly complicates the efforts to control this insect [18, 19]. Thus, the medfly is now spread throughout more than 70 countries, and it is considered a quarantine pest in many of them [16]. However, up to date scarce reports on fruit flies as vectors of plant and human diseases are available [20, 21, 22]. Since high densities of *C. capitata* are usually found in apple orchards [12], our main objective was to investigate whether *E. amylovora* can survive and be transmitted by *C. capitata*. Because the predilection of medfly for apple fruit as the most suitable host [23], we selected these fruits, which could act as a vehicle for *E. amylovora* dissemination [24, 25, 26]. In the light of results obtained, we consider that medfly can not be neglected as a potential vector for *E. amylovora*, since this pathogen can survive in/on *C. capitata* and be transmitted to plant material causing disease symptoms.

Materials and Methods

Bacterial strains and growth conditions

Two reference strains of *E. amylovora*, CFBP1430 (from *Crataegus oxyacantha*, France) and NCPPB2080 (from *Pyrus communis*, New Zealand), and their respective green fluorescent protein (GFP) and red fluorescent protein (DsRed)-labeled transformants were used. Green fluorescent transformants 1430-GFP1 and 2080-GFP3 were previously obtained [24]. Transformants 1430-DsRed2 and 2080-DsRed1 were obtained in this study by transformation of competent cells with plasmid pDs-Red, which confers ampicillin resistance and red fluorescence. The colony morphology of DsRed-tagged strains on growth media was the same of the parental strains but with a pink-red colour provided by the pDs-Red plasmid. There were not differences in pathogenicity and severity of symptoms when DsRed-transformants were inoculated in immature pears cv. Devoe. Green-fluorescent transformants were used to monitor the

bacteria on external surfaces of flies, while red-fluorescent ones were used to monitor them inside the insects, since in the preliminary experiments it was observed that fly tissues show a green fluorescence interfering with that of GFP-bacteria.

The semi-selective media CCT [27] and RESC (Recovery-*Erwinia amylovora*-Stressed-Cells) [28], and the general media King's B (KB) [29], Luria-Bertani (LB) and sucrose nutrient agar (SNA) [30], all of them in solid and liquid formulation, were used for growth of *E. amylovora*. In the case of GFP or DsRed-tagged strains, media were supplemented with tetracycline (12.5 μg/ml) [24] or ampicillin (100 μg/ml), respectively. Incubation conditions were 26°C for 48h, at 50 rpm of shaking in the case of liquid media. For the isolation and/or recovery of challenged *E. amylovora* cells, incubation period was extended up to 7 days.

C. capitata strain and rearing conditions

Medfly pupae were obtained from the Entomology Laboratory of IVIA, from colony IVIA2002 [31]. Adults were maintained in a 20x20x20 (cm) Perspex cage at 25 ± 40°C, 75 ± 5% RH and a 16:8 h (L:D) photoperiod in an environmental chamber (MLR-350, Sanyo). Standard food consisted of a mixture of sugar (from sugar beet, *Beta vulgaris* L.; Azucarera Ebro, SL, Madrid, Spain), hydrolyzed yeast (Biokar Diagnostics Co., Pantin, France) (4:1; wt:wt) and water. Sexually mature medflies (5–7 days old) were used for all assays.

Plant material

Royal Gala apple fruits from organic culture were used in acquisition and transmission experiments (see below). They were disinfected with a 60% sodium hypochlorite solution for 5 min, washed 3 times for 15 min each with sterile distilled water, and then dried [24]. Detached young pear shoots of cv. Conference were disinfected with 50% ethanol for a few seconds, followed by 3 washings of 10 min each with sterile distilled water, left to dry and their bases introduced into sterile 1.5% agar [24]. Whole pear seedlings used for transmission experiments were obtained from Conference pear seeds. Following the protocol of Santander et al. [32], the seeds were disinfected with sodium hypochlorite 3% (wt:vol) for 5 min, washed with sterile water, dried, and stratified in wet river sand at 4°C. After 1–3 months, the seeds were transferred to an autoclaved nutritive substrate (black and white peat, sand and perlite) and incubated in an environmental chamber (MLR-350, Sanyo) for two months (stem length 8–16 cm). Immature apple fruits of cv. Golden Delicious and loquats cv. Argelino (2–4 cm diameter) were disinfected with a 30% sodium hypochloride for 1 min, washed 3 times for 10 min each, and then dried [24, 33,] before inoculation.

Acquisition of *E. amylovora* by the medfly from inoculated mature apples

Two disinfected mature apples were challenged in cages (20x15x10 cm) in which they were placed in opposite positions (peduncle or calyx face up). Several wounds were made in each apple: six cuts of 1.5 cm in the central area of the fruit and five cuts in the area surrounding the peduncle or calyx. Each cut was inoculated with 20 μl of an *E. amylovora* suspension at 10^7–10^8 CFU/ml in PBS buffer. Afterwards, medflies were introduced into the cages: 25 males and 25 females, 5 males and 5 females, 2 males and 3 females, or only one male or one female, depending on the assay. For 48 h, all cages were maintained under conditions favorable for both *E. amylovora* and *C. capitata* (26 ± 2°C, 12-hour light/dark cycle, 75–85% RH) in an environmental growth chamber (MLR-351, Sanyo). Acquisition experiments were performed in duplicate and repeated independently for each *E. amylovora* strain and batch of medflies. After the period for acquisition of the bacterium by the medfly, a) the inoculated apples were removed from half of the cages containing 50 medflies for being used in survival studies (see the next section),

and b) in the remaining cages, the medflies were captured and transferred to other cages with healthy plant material for transmission assays (see further below for the two sections on transmission).

To verify acquisition of the pathogen, some insects were analyzed for the presence of *E. amylovora* culturable cells. Groups of 3–5 medfly individuals were crushed in 2.5 ml TNES buffer [31], and these extracts and their dilutions were plated onto solid CCT medium or enriched with 1 ml CCT broth. In addition, 300 μl aliquots of each medfly extract were subjected to an insect DNA extraction protocol [31] before performing a specific PCR analysis to detect *E. amylovora* [34].

Survival of *E. amylovora* on/in medflies and cages

Immediately after the acquisition stage and after removing the inoculated apples from 16 cages with 50 medflies per cage, we began to monitor the survival of the bacterium on the medfly over 28 days. The survival of *E. amylovora* populations was monitored at 7, 14, 21 and 28 days after contact with the inoculated *C. capitata*. Throughout the challenge period, the medflies were fed with 10% sterile sucrose, as lifespan of starved flies is of 1–2 days only [35]. Weekly, living medflies (approximately 10–15 insects) were analyzed for the presence of *E. amylovora* by the cultural and PCR methods described above, plating up to 1 ml of medfly extract to improve the detection limit (<1 CFU/fly). *E. amylovora*-like colonies were identified by PCR, and the pathogenicity of representative colonies was verified by inoculation into immature apples, loquats, and pear shoots [36]. If the culturability analysis was negative but PCR was positive, the corresponding medfly extracts were subjected to recovery assays (see further below). The survival experiments were performed in duplicate and repeated independently for each *E. amylovora* strain.

Food and drinking water from cages containing flies were analyzed 8 days after the acquisition period to discard their possible contamination with *E. amylovora* and, therefore, the continuos acquisition of the bacterium by the medflies over time. For this purpose, samples of food, drinking water, and also aborted eggs, regurgitated food, and from the walls of the cages, were taken with sterile swaps, which were immersed in liquid CCT medium (supplemented with antibiotics when required) and incubated at 26°C.

Integrated recovery protocol for non-culturable *E. amylovora* cells

When no *E. amylovora* colony was found on solid medium after plating 1 ml of medfly extract, recovery assays were carried out *in vitro* and *in vivo* in accordance with previous works [24, 33, 37]. Thus, an enrichment of the extracts was performed by adding KB and CCT broth (1:1), followed by plating onto solid CCT medium, for the *in vitro* assays. *In vivo* recovery was based on the inoculation of susceptible plant material, either immature apples or loquat fruits, or detached pear shoots. A volume of 15 μl of medfly extract was inoculated per cut (four cuts per immature fruit and one cut per young leaf) and the plant material was regularly examined throughout 15 days. A suspension of the strain CFBP1430 at 10^8 CFU/ml in PBS buffer was used as a positive control and PBS and TNES buffers as negative ones.

Transmission assays of *E. amylovora* through the medfly

(i) Transmission to mature apples. Medflies that had the opportunity to acquire the bacterium from inoculated apples were transferred to other cages with two healthy mature apples in opposite orientations. These apples had been injured by performing wounds (1–1.5 cm), distributed as described above, to mimic fruits in orchards where they are subjected to injuries from birds, insects, worms, hail, rain, wind, and other biotic or abiotic factors. Throughout the

five-day transmission period, the cages were maintained under the same conditions as those used for the acquisition period. Transmission assays were performed in duplicate and repeated independently for each *E. amylovora* strain. Once the transmission period had elapsed, live medflies, eggs, and apples were analyzed to detect *E. amylovora*. Insects were processed as bulk samples as described above, in groups of one to five. Eggs were frozen, crushed in 300 μl TNES buffer, and analyzed by PCR after DNA extraction as described for the medflies.

Apple fruits were analyzed individually. First, each fruit was washed in 10 ml PBS buffer to detect external *E. amylovora* cells. Second, flesh layers (approximately 0.5 cm beneath each wound) were removed, crushed in 2.5 ml AMB buffer [38] and processed for detection of internal *E. amylovora* cells. Washings and flesh extracts from each apple were analyzed separately by plating directly onto CCT medium or onto KB after enrichment in KB or CCT broth in proportion 1:1 and by specific PCR amplification [34] after DNA extraction [39]. If *E. amylovora*-like colonies were observed, they were PCR identified and their pathogenicity verified [36] as indicated above. The remaining washings and extracts were frozen at—20°C in 30% (vol: vol) glycerol.

(ii) Transmission to pear shoots and plants. Batches of approximately 50 medflies that had the opportunity to become contaminated with *E. amylovora* from inoculated apples were transferred to cages containing detached pear shoots or whole plants of cv. Conference. These assays were performed with either intact leaves or ones that had been cut to the main vein following an inoculation standard procedure [36]. Incubation conditions were as in the acquisition stage. The transmission period ranged between 5 and 14 days, depending upon the evolution of symptoms. After the incubation period, the live medflies were analyzed by cultural techniques and by PCR as previously described. Plant material (shoots or whole plants) was processed individually. The symptomatic parts of each leaf were processed by comminuting them in 1–1.5 ml AMB buffer [36], and after few minutes, the extracts were plated onto solid media, enriched with broth or analyzed by PCR as for mature apples. *E. amylovora*-like colonies were also confirmed by PCR.

Monitoring of *E. amylovora* on/in medflies by fluorescence microscopy

Over 20 live medflies from the survival and transmission assays that were challenged with the fluorescent transformants of *E. amylovora* strains were visualized using a Nikon ECLIPSE E800 epifluorescence microscope with filters B-2A (EX 465–495 nm, DM 505 nm, BA 515–555 nm) and/or G-2A (EX 450–490 nm, DM 505 nm, BA 520 nm). To detect *E. amylovora* cells on medfly surfaces, intact individuals were placed on hollow slides and examined under epifluorescence microscope. The same flies were also observed after being slightly crushed with cover slips. To evaluate the possible internal location of *E. amylovora* in medflies through the time, groups of three medfly individuals challenged with DsRed-tagged *E. amylovora* cells (since native fluorescence of fly tissues interferes with GFP fluorescence) were analyzed at 4, 8 and 24 h throughout the acquisition period, and also 1, 4, 8 and 15 days after that period. Medflies were processed and sectioned with a cryostat Leica CM1510 S (Leica Biosystems) as follows. Wings and legs were removed, heads detached from the body and both of them immersed by separate into a sterile 30% (wt/vol) sucrose solution in 10 mM PBS pH7.2, at 4°C for 48-72h. Previously to cut abdomen and thorax sections with the cryostat, medfly bodies were embedded in Tissue-Tek® O.C.T.™ Compound (Sakura Finetek) and frozen to -30°C. Tissue sections were observed with the epifluorescence microscope.

An enrichment in CCT broth supplemented with ampicillin was performed in those tissue samples that were negative for red-fluorescent bacterial cells by microscopic observation. Thus, slides with these samples were placed into sterile Petri dishes, covered with the semi-selective

medium and incubated under favorable conditions for 16 h before plating the enriched liquid on KB.

Statistical analysis

E. amylovora cell counts (after log-transformation) were analyzed as the means of two replicate samples from at least two independent experiments ($n \geq 4$). Significant differences were determined by analysis of variance (ANOVA). Fixed main factors considered in the survival studies were the experiment, strain and duration of insect contact. For the apple transmission assays, the factors were the experiment, strain, part of the apple fruit, medfly sex and location (inside/outside) in/on the fruit. For the shoot and plant transmission assays the factors were the strain, shoot or plant and leaf necrosis. Two assumptions to apply this test were checked. First, the sample should meet the assumption of normality of the quantitative response variable (CFU), so it was normalized transforming it logarithmically. Second, the assumption of homogeneity of variances was verified. Data below the detection limit of the plate counts were not included in the analysis. Differences were considered significant for p values <0.05.

Results

E. amylovora can be acquired by and survive on/in medflies

The medflies were observed walking near the wounds made on mature apples inoculated with *E. amylovora* and feeding on the inoculum drops, as expected by its feeding behavior and attraction by odor clues [14, 15, 35, 40]. These contaminated apples did not show any fire blight symptom throughout the challenge period, despite the fact that *E. amylovora* colonies were always re-isolated from the apples at levels similar to the inoculum doses. Analyses of the medflies after 48 h of contact with the apples showed positive results for *E. amylovora* detection by cultural (in all media assayed) and specific PCR methods, with pathogen concentrations ranging from 10^4 to 10^6 CFU/medfly.

After 7 and 14 days of pathogen being in contact with the medflies, *E. amylovora*-like colonies were recovered from medflies on CCT solid medium and then identified by PCR (Fig 1). There were no significant differences among the assayed strains ($p>0.05$) (S1 Fig). Colonies of *E. amylovora* were easily distinguishable on CCT and RESC media from native bacteria of the medflies, such as *Serratia marcescens* and *Providencia rettgeri*, identified by partial 16S ribosomal DNA sequencing. Inoculation of *E. amylovora* cells re-isolated from medflies into immature apples and loquats showed that the bacterium maintained its pathogenic potential after contact with the insect (data not shown).

However, no *E. amylovora*-like colonies were observed in medfly extracts after 21 and 28 days of contact between the bacteria and the medflies (detection limit <1 CFU/medfly) (Fig 1) despite PCR results were positive. To determine whether *E. amylovora* had died or lost the culturability, these medfly extracts were subjected to *in vitro* and *in vivo E. amylovora* recovery assays. *In vitro* assays based on initial enrichments in KB and CCT broth were unsuccessful in recovering culturability of nonculturable *E. amylovora* cells, even after incubation periods longer than one week. In contrast, recovery was achieved by extract passage through susceptible plant material such as immature apple and loquat fruits or detached young pear shoots. After inoculation with these medfly extracts, both shoots and fruits showed fire blight symptoms at 7–14 days post-inoculation (dpi) (Fig 1A and 1B). Approximately 10^3 *E. amylovora*-like CFU/lesion were recovered from small necrotic areas and 10^7 CFU/lesion from extensive necroses, regardless of the type of plant material or extract inoculated. Presumptive *E. amylovora* colonies were confirmed by PCR. In all cases, positive controls showed severe fire blight symptoms at 5–7 dpi, and negative controls showed no signs of disease.

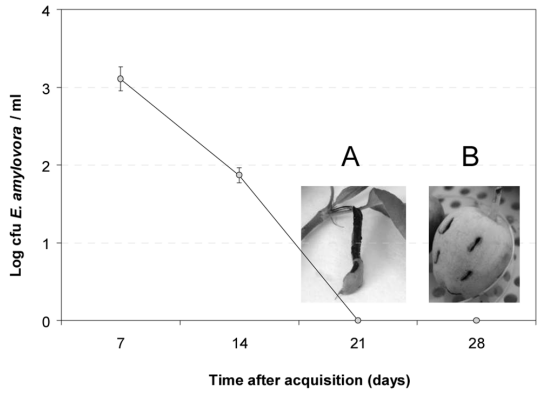

Fig 1. Survival of *E. amylovora* strain CFBP1430 on *C. capitata*. Culturable cells of *E. amylovora* were recovered up to 14 days after contact with medflies (detection limit < 1 CFU/medfly). However, *C. capitata* extracts containing non-culturable *E. amylovora* cells obtained after 21 or 28 days produced symptoms in detached pear shoot (A) (picture taken at 7 days post inoculation, dpi, showing necrosis) and immature apple (B) (picture taken at 14 dpi, showing necrosis and sinking of wound edges). Bars corresponding to SD are less than 0.19.

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Interestingly, *E. amylovora* was not detected, after direct isolation or after enrichment in CCT, neither in food, water for drinking, aborted eggs or regurgitated food, nor in the walls of the cages used for challenges.

The medfly transmits *E. amylovora*

(i) Transmission to mature apples. Medflies with *E. amylovora* cells acquired from inoculated apples were enclosed with healthy apples for 5 days. The numbers of *E. amylovora* cells carried per medfly after that period ranged from 10^3 to 10^5 CFU, and the plant pathogen was never detected in the medfly eggs. Challenged apples did not develop fire blight symptoms either on the peel or in the flesh during the assayed period; however, cultural and PCR analysis of these apples after 5 days revealed that *E. amylovora* had been transferred to the fruits. External and internal *E. amylovora* populations transmitted to the mature apples were quantified as approximately 10^5–10^4 and 10^4–10^3 CFU/fruit, respectively. Furthermore, *E. amylovora* cells recovered from the recipient apples were pathogenic when inoculated into susceptible plant material, which developed typical fire blight symptoms.

Pathogen transmission to the different fruit parts and the possible relationship between transmission and the sex of the medfly were further studied. Regardless of the medfly sex, the majority of the *E. amylovora* external population transmitted was found in the peel surrounding the calyx and in the area of the greatest diameter of the fruit (Fig 2A). In contrast, the internal population was mainly found in the peduncle area (Fig 2A). The males transmitted *E. amylovora* cells in significantly higher numbers ($p<0.05$) to the peel than to the flesh of the fruit, but no significant differences ($p>0.05$) between these two parts were found for the females (S2 Fig). Moreover, significant differences ($p<0.05$) between the sexes were also observed inside the distal parts of the fruit (Fig 3), where the transmission was mainly due to females, either in the peduncle area or in the calyx region (Fig 2A). Data on the incidence of detection are not provided in Fig 2 because *E. amylovora* was detected in all the samples with positive results and in none with negative ones.

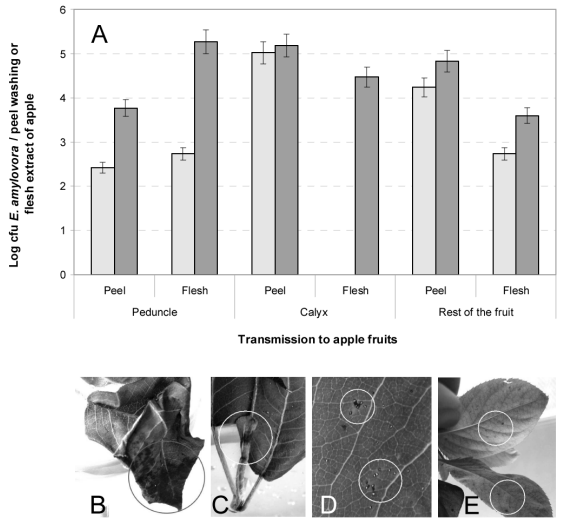

Fig 2. Transmission of *E. amylovora* to mature apples by *C. capitata*. Number of CFBP1430 strain CFUs counted after processing washings of fruits (one piece/10 ml PBS) or flesh extracts (obtained by crushing flesh layers in 2.5 ml AMB buffer) after transmission by both males (light grey bars) or females (dark grey bars) (SE is represented by vertical lines) (A). Transmission to detached young pear shoots, showing necrotic lesions in intact (not pre-injured) leaves (B, C). Medfly eggs embedded in the leaf tissues (D). Transmission to potted pear plants showing black spots on intact leaves (E).

doi:10.1371/journal.pone.0127560.g002

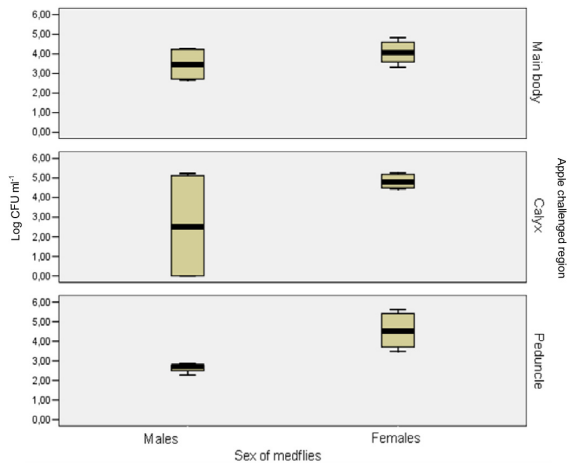

Fig 3. Boxplot of CFU mL^{-1} of *E. amylovora* from calyx, peduncle or the rest of the fruit of mature apples after transmission by *C. capitata*. Colonies of CFBP1430 strain were counted from mature apples after transmission experiments by males or females *C. capitata* flies from *E. amylovora* contaminated mature apples to healthy ones. Data are from two independent experiments with two replicates each. The females transmitted *E. amylovora* cells in significantly higher numbers ($p<0.05$) to the distal parts of the fruit, either the peduncle area or the calyx region.

doi:10.1371/journal.pone.0127560.g003

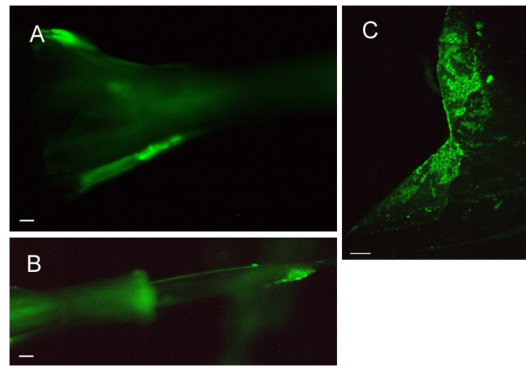

Fig 4. Location of *E. amylovora* cells on *C. capitata* female. CFBP1430 strain cells tagged with GFP protein monitored on a female of *C. capitata* after the transmission period (5 days) on the 7th abdominal segment (A), the 9th abdominal segment (the end of the ovipositor) (B), and the wings (C). White bar represents 10 μm in A-B and 100 μm in C.

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(ii) Transmission to pear shoots and plants. In transmission assays of *E. amylovora* from contaminated apple fruits to detached pear shoots, irregular necrotic lesions of various sizes (0.5–4 cm) were observed on several leaves of each shoot after 5–7 days of contact with the medflies (Fig 2B and 2C). The severity of the lesions was apparently independent of whether the leaves had been injured or not. Some egg clusters were occasionally noticed as being embedded in the leaf tissues and surrounded by a necrotic margin (Fig 2D). The presence of *E. amylovora* in the leaf necrotic areas and in the transmitting medflies was confirmed by cultural and PCR techniques, with higher populations of *E. amylovora* recovered from the pear leaf lesions (10^5–10^7 CFU/necrotic lesion) than from the transmitting medflies (less than 10 CFU/fly). The results were negative for egg samples.

In assays of transmission to potted pear plants, slight necrotic symptoms were observed on several leaves of each plant, showing between 1–4 dark necrotic spots (approximately 0.2 cm diameter) per leaf after 10–14 days in contact with contaminated medflies (Fig 2E). Some leaves were punctured by female medflies to lay their eggs. Spots showed a random distribution, being observed in either young or adult, injured or intact leaves, and on the upper or the underside of leaf surfaces, and *E. amylovora* was isolated from these spots. No *E. amylovora*-like colonies were recovered from the medflies after 14 days of contact with the bacterium, which is in accordance with the survival results. However, the PCR results for *E. amylovora* were positive for both pear leaves and transmitting medflies from all of the samples assayed, but not for the eggs deposited by the flies.

Green fluorescent *E. amylovora* cells, regardless of the assayed strain, were mostly found as cellular aggregates on the ovipositors of medfly females (Fig 4A and 4B) and on the distal parts of the wings of both sexes (Fig 4C) after transmission challenge.

Location of *E. amylovora* into medflies

Red fluorescent cells were observed in the mouth of medflies challenged with red-fluorescent tagged bacteria, not only on the surface but also in the inner parts; they were not found in medflies fed with wild type bacterial strains (data not shown). Fig 5A shows a diagram of the thorax and abdomen of medflies. In contrast to the observations in the negative control medflies (Fig 5B and 5C), red-fluorescent bacteria were observed in thorax and abdomen sections of

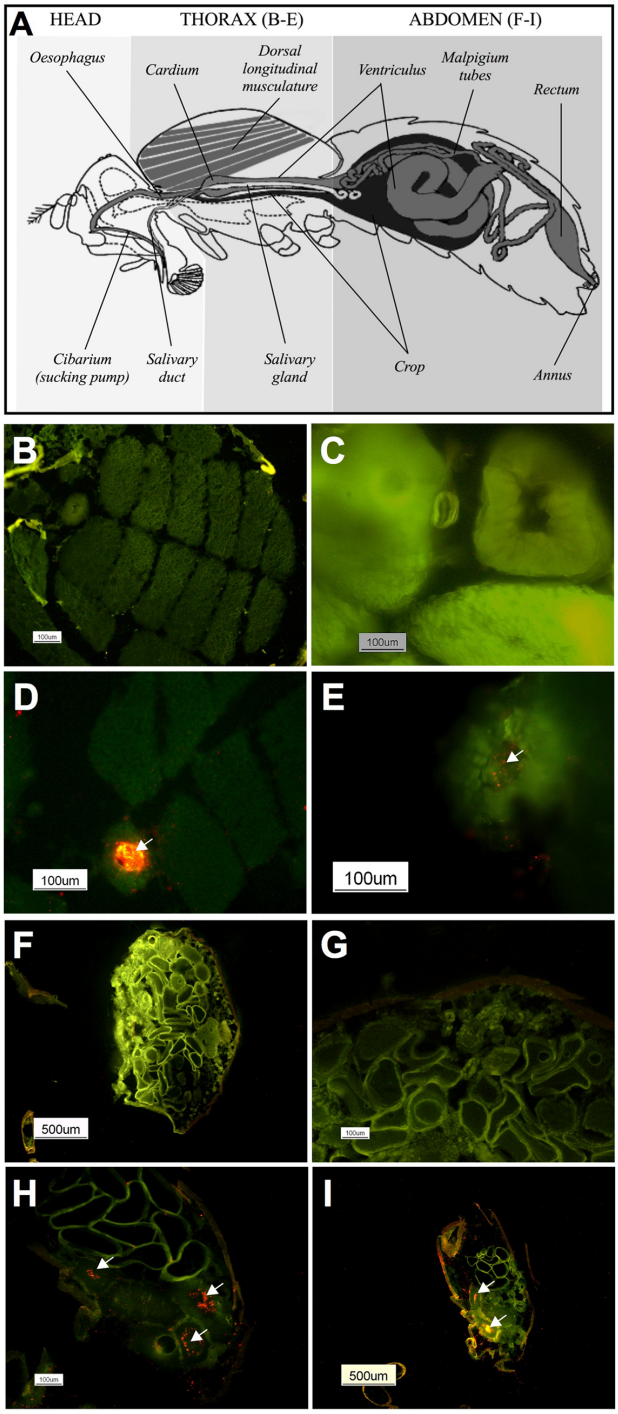
A
HEAD
THORAX (B-E)
ABDOMEN (F-I)
Oesophagus
Cardium
Dorsal longitudinal musculature
Ventriculus
Malpigium tubes
Rectum
Cibarium (sucking pump)
Salivary duct
Salivary gland
Crop
Annus
B
C
100um
D
100um
E
100um
F
500um
G
H
I
500um

Fig 5. Location of *E. amylovora* cells into the *C. capitata* body. The dorsal longitudinal thoracic flight muscles and the digestive system in an adult fly (A) and cross sections of thorax (B-E) and abdomen (F-I) of control (B, C, F, G) or challenged (D, E, H, I) female medflies fed with red fluorescent *E. amylovora* NCPPB 2080 cells for 24h (D, H) and 4 days (E, I) after the acquisition period.

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challenged insects, inside the digestive tract (Fig 5D and 5E), both in the lumen (Fig 5D) and/or coating the internal wall of the tube (Fig 5E). Moreover, red-fluorescent bacteria were also located in the crop of challenged medflies (data not shown). Fig 5F and 5G show control flies with no *E. amylovora* cells in the abdomen; in this structure, in general, the amount of red-fluorescent bacteria in challenged flies was higher than in thorax (Fig 5H and 5I), and mostly the bacterial cells appeared as aggregates and extended by wide areas along the abdomen. In all cases, red bacteria were observed in these structures, both in thorax and abdomen, until 8 days after the acquisition period. At 15 days, all analyzed samples were negative for the presence of red-fluorescent bacteria, and the enrichment did not allow the detection of the target pathogen. Interestingly, red-fluorescent cells were not observed inside ovipositor structure.

Discussion

The spread of plant pathogens to new hosts by insect vectors can cause increased damage and economic losses [1]. Although there are many insects associated with *E. amylovora* dissemination [10], their exact role and the bacterial location and survival in/on the insects has not been studied in depth and only by isolation techniques. More specifically, the role of non-pollinating insects is poorly understood [4]. Consequently, there is a lack of knowledge on the role of these insects as potential fire blight vectors, in spite they are recognized as a contributing factor in the epidemiology of this disease [3]. Past research on flies showed that the vinegar fly (*Drosophila melanogaster*) could be involved in the spread of *E. amylovora* [7]. Recently, the transport of *E. amylovora* cells on greenbottle flies to wounded young fruit and pear shoot was demonstrated under experimental conditions, but no information on the bacterial location in/on the flies was provided [3]. Despite *C. capitata*'s relevance as worldwide fruit pest of high economic importance [13, 14, 15], its potential role on *E. amylovora* transmission has not been studied before.

Pathogen transmission by contaminated insects depends not only on the pathogen's dose carried by them but also on the pathogen's survival period in/on the insect [8]. In this study, *E. amylovora* showed a notable ability to persist in a culturable state in/on *C. capitata* for up to 14 days. On honeybees [7, 8, 41, 42, 43] *E. amylovora* was reported to survive only up to 6 days [7]. Nevertheless, the persistence of *E. amylovora* in/on insects is likely to have been underestimated due to two reasons: survival has been assessed only by culturability on solid media and no studies have investigated its survival inside insects. In our experiments, after 14 days in contact with the fly, *E. amylovora*-like colonies were not isolated. This could indicate either bacterial cell death or loss of culturability. It is remarkable that the lack of *E. amylovora* colonies ruled out the possibility that repetitive contacts of the flies with the sucrose solution, used for feeding, led to its contamination with the bacterium. Consequently, this excludes the feed as a source of re-introduction or redistribution of the pathogen on flies over time. Moreover, the negative result of the enrichment in CCT from the challenged cages (food, water, walls) also indicated that insects could not acquire the target bacteria continuously, supporting the significance of the data on *E. amylovora* acquisition by *C. capitata*.

To elucidate whether non-culturable *E. amylovora* cells were dead or alive after 14 days of bacterium-medfly contact, a recovery method previously described [24, 33, 37] was challenged to demonstrate regained culturability. Although the KB liquid medium had provided excellent

results for recovering non-culturable *E. amylovora* cells induced by certain stress conditions, neither it nor CCT broth were successful here. The passage through susceptible host plant material [37], however, resulted an appropriate method. The inoculation of medfly extracts containing non-culturable *E. amylovora* cells into susceptible pear plants caused necrotic lesions from which the pathogen was subsequently re-isolated. These results, which confirm *E. amylovora*'s survival ability in non-host environments [32, 33, 37, 44, 45, 46, 47, 48, 49, 50], also demonstrate that stressed or injured bacterial cells [28, 37, 51] can remain viable on the medfly, for at least 28 days, and can be potentially pathogenic. This is the longest survival period reported for *E. amylovora* on any insect [7, 41]. Consequently, this integrated protocol, based on isolation, molecular and bioassay approaches, not applied before to insects, is highly useful to recover bacterial cells and should be further applied to honeybees and other insects.

Furthermore, transmission experiments revealed that *C. capitata* was able to efficiently acquire *E. amylovora* from contaminated mature apples (inoculated with approximately 10^5–10^6 CFU per fruit) and transmit it to healthy ones, consistently in all the assays performed. The assayed *E. amylovora* strains (CFBP1430 and NCPPB2080) had been described as highly and moderately aggressive, respectively [52], and one viable cell of *E. amylovora* is enough to develop an infection under highly favorable conditions [52]. The fact that the bacterium-recipient apples remained asymptomatic was most likely due to *E. amylovora*'s inability to multiply on ripe fruit [24, 36, 53]. Nevertheless, the pathogen was recovered from both the apple's peel and flesh, suggesting a pathogen-insect relationship probably involving insect reproductive and digestive processes [20, 54, 55]. This hypothesis was confirmed by microscopic examinations where *E. amylovora* red-fluorescent cells were observed in the digestive tract along the thorax and abdomen of the medflies 8 days after the acquisition period. This finding is unknown up to now and very relevant for fire blight epidemiology, as there was no previous evidence of *E. amylovora* internalization by insects [2].

Both medfly sexes contributed equally to pathogen transmission in the apple peel. The presence of fluorescent (GFP-marked) cells on the wings of flies of both sexes is most likely related to the fly's position during feeding, defecating, or laying eggs (females), enabling contact between the distal parts of the wings with the fruit or leaf. The transmission to the interior of the fruit was mainly due to female insects. The observation of GFP cells on the external surface of ovipositors of transmitting medflies coincided with the detection of *E. amylovora* cells inside the apple flesh. These were probably introduced by laying of eggs and/or fecal contamination [54, 56]. The female medfly may lay approximately 20 eggs per day, in egg-clutches of 1 to 10 eggs, by puncturing the fruit up to 0.5 cm deep with its ovipositor [57]. Indeed, some tunnels containing eggs were found in the flesh of several bacterium-recipient apples. However, *E. amylovora* was never detected in eggs. These results agree with the findings of other studies where vertical transmission of plant pathogens (mother to egg or sperm to egg) is unusual in insect vectors [58, 59]. The contamination was most likely external, on the surface of the ovipositor. In fact, red-fluorescent *E. amylovora* cells were not found inside the ovipositor structure in any sample. Moreover, it is known that *C. capitata* usually produces two ceratoxins to prevent the presence of bacteria in the ovaries [56].

Assays of transmission to susceptible plant materials other than fruit, including detached young shoots and whole pear plants, revealed that the transmitted pathogen could effectively reach host tissues, causing leave necrosis of variable extent. Although egg laying on leaves or penetration in leaf tissue is not the normal behavior of medflies, when fruit is not available flies can lay eggs on other plant material [14].

One of the goals of this work was to elucidate the ability of medflies to transmit fire blight pathogen to shoots or other organs, since it is less understood than the blossom blight transmission [4]. Under our experimental conditions, *E. amylovora* can survive in/on *C. capitata*

and be efficiently transmitted from mature apples to different healthy plant materials, without requiring artificial wounds in plant tissue. This transmission could go unnoticed because of the absence of fire blight symptoms [24] or the presence of atypical disease symptoms in leaves. Medfly should not be neglected as a key ecological player, as it is likely to have repeated encounters with *E. amylovora* in different parts of the host plants, so flies travelling from plant to plant could act as vectors, as shown for other insects [60]. There are many species of flies that may be present in apple or pear orchards, some considered non-pests and others causing damage, such as *Rhagoletis pomonella* (Walsh) [17] or *Drosophila suzukii* (Diptera: Drosophilidae) [61], but *C. capitata* remains the pest most widely distributed in these orchards throughout the year [15]. Although the role of other fly species in fire blight epidemiology is still unknown, our results with *C. capitata* suggest other fly species could also be efficient vehicles for *E. amylovora* transmission.

Overall, the presence of *C. capitata* in fruit-growing areas with fire blight should not be disregarded as a potential threat for dissemination of *E. amylovora*. The exceptional ability of *E. amylovora* to survive in *C. capitata*, both in the medfly surface and inside its digestive tract, the efficiency of this insect in acquiring and transmitting this bacterium, which remains pathogenic, and the relatively high cell numbers of the plant pathogen carried by each medfly in our experimental system demonstrate its potential as a fire blight vector. A clear understanding of medfly role in dissemination of *E. amylovora* and on the epidemiology of fire blight, with field studies in orchards with infected hosts, will contribute to design more optimized control strategies.

Supporting Information

S1 Fig. Boxplot of CFU mL^{-1} of *E. amylovora* from *Ceratitis capitata* flies after acquisition experiments. *E. amylovora*-like colonies, confirmed by PCR, were recovered after 7 and 14 days of contact of medflies with *E. amylovora* contaminated apples. Data are from two independent experiments with two replicates each. There were no significant differences among the four assayed strains ($p>0.05$).
(TIF)

S2 Fig. Boxplot of CFU mL^{-1} of *E. amylovora* from surface or flesh of mature apples after transmission by *C. capitata* flies. Colonies of CFBP1430 strain counted from apple surface or flesh after transmission by male or female flies from *E. amylovora* contaminated mature apples to healthy ones. Data are from two independent experiments with two replicates each. The males transmitted *E. amylovora* cells in significantly higher numbers ($p<0.05$) to the peel than to the flesh of the fruit.
(TIF)

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Author Contributions

Conceived and designed the experiments: MO JEPS RDS BSM EGB MML EMN. Performed the experiments: MO JEPS RDS. Analyzed the data: MO RDS BSM EGB MML EMN.

Contributed reagents/materials/analysis tools: BSM EGB MML EMN. Wrote the paper: MO RDS BSM EGB MML EMN.

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Annex V

Santander *et al.,* 2016, unpublished

(It will be submitted to the journal *PeerJ*).

Title

Monitoring the root colonization and invasion routes of *Erwinia amylovora* in *Pyrus communis* using a GFP-tagged strain: feasibility of water-borne infections

Running title

Water-borne root infection of *Pyrus communis* by *Erwinia amylovora*

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Abstract

Background. *Erwinia amylovora* is the bacterial pathogen responsible for fire blight of rosaceous plants, which causes devastating effects in pear and apple crops worldwide. The pathogen can survive in water, soil and other environments outside the host for variable periods, and it is able to invade host plants through natural openings such as the nectarthodes in flowers and stomata in leaves, as well as through wounds. The invasion of internal host tissues is often accompanied by the production of symptoms such as necrosis, exudates and/or wilting, which can occur in flowers, fruits, shoots, branches, the trunk and the rootstock; hence fire blight is often considered a disease of the aerial part of plants. Root blight symptoms under field conditions have also occasionally been reported, but root infection is considered a less important host invasion route. Consequently, the *E. amylovora* mechanisms for root colonization and invasion remain virtually unexplored.

Methods. Pear plantlets were obtained by *in vitro* culture of pear seed embryos. The potential invasion of pear plantlets by *Erwinia amylovora* through the roots was assessed by direct inoculation assays. Afterwards, we determined the feasibility of water-borne root infections by this pathogen in pear plantlets using a GFP tagged strain to characterize the colonization, invasion and migration routes followed by the pathogen within the hosts.

Results. The *in vitro* culture of embryos allowed the successful germination of long-term stored seeds that did not germinate by other procedures. Direct root inoculation assays demonstrated the rapid migration of *E. amylovora* from roots to the aerial parts of the inoculated plants, in the same direction as the water flux inside xylem vessels. Fire blight symptoms in root-inoculated plants usually first developed in the tip of the stem and in petioles. The inoculation of plants by soil irrigation with *E. amylovora*-contaminated water led to the development of fire blight symptoms in higher percentages in plants with roots damaged by transplanting compared to intact plants. The stages of host plant invasion through the roots consisted of *i)* colonization of root surfaces, *ii)* invasion of the root cortex through damaged roots and/or the sites of emergence of lateral roots, *iii)* penetration into the xylem (favored in damaged roots) and migration to aerial tissues.

Discussion. *E. amylovora* is able to grow in the pear root system, migrate and cause fire blight in aerial organs, which contrasts with similar studies using apple plants. This indicates that part of the fire blight symptoms observed in orchards or nurseries might be due to infections initiated in the roots. The stages of *E. amylovora* root-infection

were similar to those reported in well-known soil borne plant pathogens or endophytes. The results in this work suggest that the water-borne infection of *E. amylovora* susceptible plants through roots in nurseries or orchards might be possible, but more studies on the root-infection susceptibility of other hosts and/or lignified older plants are required to confirm this hypothesis.

Introduction

Erwinia amylovora is a non-obligated plant pathogen pertaining to the family *Enterobacteriaceae.* It is the etiological agent of fire blight, one of the most devastating diseases affecting rosaceous plants such as pears (*P. communis* L.) and other economically important fruit trees and ornamental species, mainly of the subfamily *Spiraeoideae* (van der Zwet, Orolaza-Halbrendt & Zeller, 2012).

The fire blight disease cycle depends on the seasonal development of the host. The epiphytic growth of *E. amylovora* on flowers is favored by warm temperatures during spring, and bacterial cells are easily spread by pollinating and other flower-visiting insects, birds, the wind and/or rainwater to non-infected flowers in the same or in other plants (Thomson, 2000). Warm temperatures and rain or high humidity favor *E. amylovora*'s ability to reach and/or grow in nectaries, where the invasion of internal host tissues occurs via the nectarthodes in the floral cup (hypanthium) (Bubán, Orosz-Kovács & Farkas, 2003). Wounds produced by wind, hail or insects, as well as natural openings such as stomata can also lead to infections through leaves or growing shoots. *E. amylovora* multiplies in the intercellular spaces of the cortical parenchyma, reaches the xylem vessels, and moves downwards to supporting branches, limbs and the main trunk. Infected tissues can develop fire blight symptoms such as wilting (due to parenchyma collapse), ooze droplets and/or necrosis (Thomson, 2000). The severity of infections depends on diverse factors (e.g. host species, cultivar, environmental conditions, the age of the infected tissues, etc.), with young and vigorous tissues usually being more affected than older or slow growing ones (Thomson, 2000; van der Zwet, Orolaza-Halbrendt & Zeller, 2012). The pathogen can cause symptoms in almost every plant organ, and its systemic spread during severe infections may eventually cause the death of the entire host (Thomson, 2000; van der Zwet, Orolaza-Halbrendt & Zeller, 2012). The progression of infections, especially those occurring during late summer or autumn, leads to the formation of cankers in lignified tissues of branches, the trunk or the rootstock. The bacterium overwinters in cankers until the host's growth is activated the next year (Thomson, 2000). Under warm favorable conditions at the end of winter and the beginning of spring, *E. amylovora* cells start to multiply in canker margins, and ooze droplets containing high bacterial numbers are released to the host surface. This ooze attracts insects such as flies or ants, which are responsible, together with other biotic and abiotic vectors, for the spread of the pathogen to flowers or natural openings and/or wounds in other susceptible host organs, starting a new cycle of the disease (Thomson, 2000).

How *E. amylovora* spreads systemically throughout host tissues is still a controversial topic, due to the variety of factors affecting the *in situ* study of the infection pathways followed by the pathogen (Billing, 2011). Fire blight affects blossoms, shoots, leaves, fruits, limbs, the trunk, the collar or the rootstock (van der Zwet, Orolaza-Halbrendt & Zeller, 2012), hence it is considered a disease of the aerial part of plants. Interestingly, *E. amylovora* cells inoculated in the stem of apple seedlings migrate to roots (Bogs *et al.,* 1998), and roots showing fire blight symptoms have been occasionally observed in infected trees under field conditions (van der Zwet, Orolaza-Halbrendt & Zeller, 2012). However, the colonization and invasion of the host radicular system is not considered a primary infection route of the pathogen (Bogs *et al.,* 1998).

E. amylovora is able to survive in different types of water, e.g. distilled water, rain water, mineral water and river water (Santander *et al.,* 2012; Santander, Oliver & Biosca, 2014), some of which are used for irrigation in orchards and/or nurseries. The pathogen's survival in soil and in soil-inhabiting insects for different periods has also been demonstrated (Hildebrand, Dickler & Geider, 2000; Hildebrand, Tebbe & Geider, 2001). Accordingly, and given the reported cases of root blight (Billing, 2011), the infection of host plants through the roots under certain circumstances (e.g. via contaminated water and/or root-damaging insect or nematodes) cannot be discarded.

In order to determine the potential ability of *E. amylovora* to colonize and invade host roots we used one-month-old pear (*P. communis* L.) plantlets and different strains of *E. amylovora*, including a green fluorescent protein (GFP)-labelled transformant, to monitor bacterial migration patterns within host tissues. Furthermore, we determined the feasibility of host infections through the radicular system by watering plants, either intact or with roots damaged by transplanting, with *E. amylovora* contaminated water. In this work, we describe for the first time the ability of *E. amylovora* to migrate from the root system to the aerial part of root-inoculated plants, where it causes typical fire blight symptoms. Furthermore, we also provide new knowledge concerning the routes followed by the pathogen to colonize and invade host root tissues. We also demonstrate the feasibility of the water-born infection of pear plantlets by the fire blight pathogen *via* the radicular system under controlled conditions. This might raise concern about the not yet described transmission pathways of *E. amylovora* under field conditions or in nurseries, which might be taken into consideration for preventive and control measures of the disease.

Material and methods

Bacterial strains and culture media

Assays in this study were performed with the *E. amylovora* reference strain CFBP 1430, isolated from *Crataegus* sp. in France, and/or a transformant of this strain, GFP1 (Ordax *et al.,* 2009), carrying the plasmid pHC60 (Cheng & Walker, 1998), which contains the gene encoding the jellyfish GFP. In addition, results were confirmed with other two *E. amylovora* strains isolated from different hosts and geographical origins: IVIA 1892.1, from *Pyrus communis* in Spain; and ATCC 49946, from *Malus domestica* in USA.

Unless otherwise specified, all the *E. amylovora* strains were routinely grown either in liquid LB or on LB agar (LBA) plates at 28ºC. For the growth of strain GFP1, media were amended with 12.5 μg mL^{-1} tetracycline ($Tc^{12.5}$).

E. amylovora isolation from inoculated plant material was performed on the semi-selective CCT medium (Ishimaru & Klos, 1984) in which the fungicide cycloheximide was substituted by natamycin (Proquiga, Spain) (NCT), which is less toxic and a possesses greater anti-fungal activity (Pedersen, 1992; Biosca *et al.,* 2016). The final natamycin concentration in the medium was 21.6 mg L^{-1}, according to Pedersen (1992). The null differences between CCT and NCT in the recovery, growth or colony morphology of the *E. amylovora* tested strains was confirmed prior to using NCT in subsequent experiments.

Seed preservation and obtaining pear plantlets by *in vitro* embryo culture

Plant material in this study consisted of one-month old pear plantlets of *P. communis* cv. Passe Crassane. Additionally, in some assays plantlets of *P. communis* cv. Blanquilla were also included.

Pear plantlets were obtained from pear seeds which were manually extracted from mature fruits, slightly cleaned with $_{di}H_2O$ and dried under the hood. For seed preservation, these were packaged inside filter paper envelopes in groups of 40 individuals, and stored at 4ºC until use inside 50 mL tubes with a volume of about 20 mL of silica gel grains.

Due to problems with the germination of long-term preserved seeds following a protocol based on seed stratification in wet river sand at 4 °C (Santander *et al.,* 2012; Santander, Oliver & Biosca, 2014), plantlets were obtained by *in vitro* embryo culture, adapting the protocol of Arrillaga, Marzo & Segura (1992), to pear seeds. Briefly, seeds stored for 2 years under the above-mentioned conditions were first surface disinfected (70% ethanol, 30 s; 1.2 % NaOCl plus 0.1% Tween-20, 30 min) and rinsed several

times with sterile distilled water ($_{di}H_2O$). Afterwards they were imbibed in sterile $_{di}H_2O$ for 48 h inside a glass beaker under stirring conditions. Seeds were then subjected to a slight surface disinfection treatment (0.4 % NaOCl with 0.1% Tween-20 for 15 min), rinsed several times with sterile $_{di}H_2O$, and embryos aseptically excised by removing the seed coats (testa) with sterile scalpels and forceps. Embryos were placed into 90-mm diameter Petri dishes (ca. 10 embryos per plate) containing 25 mL of sterile germination medium (3 % sucrose in $_{di}H_2O$, pH 5.7-5.8, 0.7 % agar), and cultured under dark conditions at room temperature (about 25ºC) for 3 – 6 days, until the first signs of radicle elongation were observed. Plates were then placed for 7 days into a plant growth chamber (model MLR-352-PE, Panasonic) at 26ºC with a 12-h photoperiod at 100 μmol m^{-2} s^{-1}. Afterwards, when radicles were about 1-2 cm long, the pear plantlets were individually transferred to pots containing a mixture 3:1 of substrate (Kekkilä 50/50, Kekkilä Ltd., Finland) and perlite, and grown under greenhouse conditions for one month under natural illumination.

Initial characterization of the GFP labeled strain

To determine the stability of the plasmid pHC60 carrying the GFP gene in the *E. amylovora* strain GFP1, overnight cultures were prepared in LB+$Tc^{12.5}$, washed twice with fresh LB, adjusted to an OD_{600} nm of 1.0 (ca. 2 10^9 CFU mL^{-1}), diluted 1/1000 in fresh medium (without any antibiotics) and let to grow for 18 h at 28ºC (150 rpm). The latter step was repeated multiple times to reach 100 generations of growth without the selective pressure of the antibiotic. Aliquots of 10 μL each 18-h culture were inoculated into LB+$Tc^{12.5}$ to check the loss/maintenance of the plasmid carrying both the GFP and the tetracycline resistance encoding genes. The stability of the plasmid in soil and water was also evaluated as follows. An overnight culture in LB+$Tc^{12.5}$ was washed twice with sterile saline, adjusted to an OD_{600} nm of 1.0, and inoculated into sterile soil or $_{di}H_2O$ microcosms to a final cell concentration of about 10^8 CFU g^{-1} or mL^{-1}, respectively. Bacterial counts were then performed throughout a 15-day period simultaneously on LB and LB + $Tc^{12.5}$. Plasmid stability, in this case, was calculated by dividing the plate counts on LB+$Tc^{12.5}$ by those on LB. Values below 1 thus indicated the loss of the plasmid in a number of cells, and/or a mutation inactivating the tetracycline gene in plasmid pHC60. These assays were performed in triplicate.

In order to compare the virulence of the *E. amylovora* strain GFP1 with that of the parental strain CFBP 1430, one-month-old pear plantlets (*P. communis* cv. Passe Crassane) were inoculated with each strain by cutting a leaf with scissors previously dipped into a cell suspension of 2 10^7 CFU mL^{-1} (in $_{di}H_2O$). Virulence was assessed

198 comparing the extent of the necrotic areas caused by each strain. Additionally, to
199 determine the stability of the GFP bacterial labeling in plant material, leaves inoculated
200 with the strain GFP1 were processed and observed by epifluorescence microscopy
201 (EFM) (see below) at different periods over 15 days. This assay was performed thrice
202 in three independent experiments.
203
204
205 **Plant inoculation and processing of plant material for *E. amylovora* detection**
206 To assess the *E. amylovora* infection pathways throughout the radicular system
207 one-month old pear plantlets were inoculated as follows. A group of 8 plants were
208 directly inoculated in the main root, between the two first lateral roots (about 2-3 cm
209 below the stem), using a sterile syringe needle previously dipped into an *E. amylovora*
210 colony on LBA. For comparison, a control group of 8 plants were inoculated by placing
211 a 10 µL drop containing 10^3 CFUs of *E. amylovora* on the surface of the wound
212 resulting from cutting the upper part of the stem as previously described (Santander,
213 Oliver & Biosca, 2014). These experiments were performed with the *E. amylovora*
214 strain CFBP 1430, and bacterial tracking was performed by monitoring fire blight
215 symptom development followed by isolation and PCR-identification of *E. amylovora*-like
216 colonies on NCT medium as previously described (EPPO, 2013; Santander *et al.,*
217 2012; Santander, Oliver & Biosca, 2014).
218 In additional experiments, to determine the ability of *E. amylovora* to colonize roots
219 and invade susceptible host plants throughout the radicular system, different
220 inoculation methods were tested. To favor root wounding, a group of 10 pear plantlets
221 was unpotted, shaken to detach part the soil surrounding the roots, and potted again in
222 non-sterile substrate. These transplanted plants were then inoculated by immersing ¾
223 of the pot for 20 min into a suspension of *E. amylovora* adjusted to an OD_{600} nm of
224 0.450 (about 4 10^7 CFU per g of soil). A second group of 10 plantlets with intact roots
225 were irrigated similar to the first group without any manipulation. In both cases, the
226 plantlets were periodically inoculated with *E. amylovora* as explained above every 3-5
227 days (depending on the substrate wetness). Finally, in order to characterize the
228 pathways employed by *E. amylovora* to invade host plants through the roots, the
229 infection of a third group of 10 pear plantlets with the *E. amylovora* strain GFP1 was
230 favored as follows. Plantlets were unpotted, and the soil and rhizosphere carefully
231 removed from the roots with tap water. Afterwards, to avoid possible antagonistic
232 effects of the root and rhizosphere native microbiota on *E. amylovora*, the radicular

system was slightly surface disinfected with 70 % ethanol for 30 seconds. Thereafter, roots were rinsed with sterile $_{di}H_2O$ and immersed for 20 min in a suspension of *E. amylovora* strain GFP1 (OD_{600} of 0.450) in sterile $_{di}H_2O$. Plants were then potted using wet sterile substrate.

Inoculated plants were transferred to a growth chamber (model MLR-352-PE, Panasonic) at 26ºC (12 h of light) at 150 µmol m^{-2} s^{-1}. To increase the relative humidity around the plants they were placed into individual Petri dishes inside a 5-cm-high tray containing a thin layer of tap water covering the base of the tray, but not touching the pots.

The tracking of *E. amylovora* cells within plant tissues was performed as described above and, in the case of plants inoculated with the GFP-labeled strain, also by EFM and/or laser scanning confocal microscopy (LSCM) (see below). These assays were repeated twice with strain CFBP 1430, and the results were confirmed with strains ATCC 49946 and IVIA 1892.1.

Processing of plant material for EFM and LSCM

In order to monitor the *E. amylovora* progression through pear plant tissues after root inoculation with the strain GFP1, challenged plants were processed at time 0 and at different periods over 15 days for EFM and CM as follows. Plants were unpotted, immersed into sterile $_{di}H_2O$ and slightly shaken to carefully disaggregate soil and detach the rhizosphere from the roots. Randomly selected secondary roots, and the thinnest section of the main root, were separated from the radicular system and mounted on a glass slide with $_{di}H_2O$ and a cover slip for their direct observation with a Nikon Eclipse E800 fluorescence microscope (Nikon Instruments, Melville, NY). Plant leaves were cut into ca. 4 x 4 mm square sections and directly mounted on a glass slide as mentioned above, prior to EFM.

To prepare the thicker sections of the stem of processed plants for microscopy, they were chopped using a razor-blade. In the case of the main root, 0.5-cm-long root pieces were imbibed in a sterile 30 % sucrose solution in 10 mM PBS pH 7.2 inside sterile 15 mL tubes, and stored at 4ºC for 24-48 h until the fragments sank to the bottom of the tubes. Afterwards, plant sections were embedded into melted 5 % agarose, which was then left to solidify and then cut with a vibrating blade microtome (Leica VT1000S, Nussloch, Germany) into 150-µm-thick sections. Samples were then mounted on glass slides with $_{di}H_2O$ and observed with an epifluorescence microscope (Nikon Eclipse E800, Melville, New York), using the B-2A medium band blue excitation long pass filter set (450-490 nm excitation filter, 500 nm dichromatic mirror cut-on, 515 nm cut-on barrier filter). Some samples were also observed with a laser scanning confocal microscope (Leica TCS-SP, Lasertechnik, Heidelberg, Germany). The GFP was excited with the 488-nm laser line from an argon laser, and the barrier filter had a 525-nm cutoff. Confocal

images and/or transmitted light images were reconstructed and combined with the LCS lite (Leica) software.

Results

In vitro culture of embryos of pear seeds improves the percentage of germination of long-term stored seeds

The pear seeds (*P. communis* cv. Passe Crassane and cv. Blaquilla) used in this study had been stored for 2 years at 4ºC under low humidity conditions inside closed tubes containing silica gel. After the *in vitro* culture of embryos, about a 85 % of the seeds successfully germinated in periods as short as 2-7 days after embryo excision, with the time required to obtain one-month-old pear plantlets being about 1 month and 1 week in the worst cases. Interestingly, none of the seeds from the same stock germinated using a routine protocol based on the stratification of seeds at 4ºC over a period of 2 months.

Migration of *E. amylovora* from the roots to the upper parts of the plant

To determine the ability of *E. amylovora* to migrate from roots to the aerial parts of plant hosts, the main root of one-month-old pear plantlets (cv. Passe Crassane) was inoculated with a hypodermic needle previously dipped into a colony of *E. amylovora* strain CFBP 1430. In Fig. 1 representative pictures of a negative control (Fig. 1a) and fire blight symptoms in plants challenged with *E. amylovora* in the roots (Fig. 1b-h) are shown. All plants inoculated with *E. amylovora* in the main root developed characteristic fire blight symptoms, in most cases only 2-3 days after the inoculation. It is worth mentioning that the first visible disease symptoms usually developed in plant organs far from the inoculation site (up to 10-12 cm) such as the tip of the stem (Fig. 1b, c), and/or petioles connecting the stem with the newest expanded leaves. In general, the first symptoms observed consisted of exudate droplets (Fig. 1b) and a progressive darkening of infected organs, indicative of the necrosis of plant tissues (Fig. 1c, d). The progression of necrosis usually advanced from the tip of the stem and/or petioles of the youngest leaves downwards (Fig. 1d). However, in some cases fire blight symptoms started at the base of the stem and/or cotyledons (Fig. 1e) and extended upwards. In several cases, a simultaneous development of symptoms at the base and in upper parts of the stem was also observed. Interestingly, one of the most sensitive plant organs to *E. amylovora* infections was the petioles. In fact, in most of the inoculated plants the first observed necrotic lesions occurred in these organs (Fig.

1f-h), with a subsequent expansion of necrosis to the stem and/or the limb of the leaf *via* parenchymatic tissues or the vascular vessels (Fig. 1f-h). By contrast, severe fire blight symptom development in roots tended to occur in the latest phase of the disease, when most of the aerial part of the plant was necrosed.

For comparative purposes, a set of pear plantlets was inoculated in wounds in the tip of the stem. The results corresponding to these assays are shown in Fig. 2. Control plants inoculated with sterile $_{di}H_2O$ after cutting the tip of the stem continued growing, and even sprouting during the incubation period (Fig. 2a, b). However, plants challenged with *E. amylovora* started to develop characteristic fire blight symptoms about 2-3 days after their inoculation (Fig. 2c-e). In contrast to plants inoculated in the main root (Fig. 1), the first ooze droplets and/or necrosis were observed in tissues close to the inoculation site, advancing downwards (Fig. 2c-e). Also in this case, petioles showed a particular sensitivity to *E. amylovora* infections (Fig. 2c-e), and necrosis easily extended from these organs towards the stem and/or the limb of the connected leaf (Fig. 2c-e).

The results obtained with pear plantlets of the cv. Passe Crassane, were reproducible in plants of the cv. Blanquilla. In the same way, one off assays repeated with the *E. amylovora* Spanish and North American strains IVIA 1892.1 and ATCC 49946, respectively, confirmed the results with the French reference strain CFBP 1430.

Plasmid conferring green fluorescence is stable under different conditions, and allows the progression of the *E. amylovora* infection in pear plant to be monitored

An initial approach to determine the stability of the plasmid carrying the *gfp* gene (pHC60) in the transformant *E. amylovora* strain GFP1 consisted of growing this strain in rich medium (LB) without selective pressure (i.e. non-amended with tetracycline) to check the maintenance of the plasmid. The strain GFP1 retained resistance to tetracycline after growing 100 generations without the antibiotic. Moreover, in order to characterize the stability of plasmid pHC60 under conditions encountered by bacterial cells during experiments in this study, the strain GFP1 was inoculated in water and soil microcosms, and the stability of the plasmid measured over time as the ratio of cells growing on LB+$Tc^{12.5}$ with respect to those on LB (Fig. 3a, b). As revealed by culturable cell counts, strain GFP1 was unable to grow under the conditions present in soil (Fig. 3a) or in $_{di}H_2O$ (Fig. 3b), developing typical starvation-survival responses, with a progressive loss of culturability over time more which was pronounced in soil (Fig. 3a) than in water microcosms (Fig. 3b). The analysis of the ratio of cells growing on

LB+Tc$^{12.5}$ *versus* LB in soil microcosms revealed plasmid stability values of about 1.0 over 8 days, with a progressive loss of the plasmid (and/or mutation of the plasmid tetracycline gene) during the following days, reaching values of ca. 0.6 at the end of the experiment (15 days) (Fig. 3a). In the case of water microcosms, however, data revealed that plasmid pHC60 was more stable than in soil, with average stability values never decreasing below 0.81 (Fig. 3b).

Finally, in order to compare the virulence of the transformant strain GFP1 with that of the parental strain CFBP 1430, as well as its usefulness in monitoring the progression of the infection in the *P. communis/E. amylovora* pathosystem, one-month-old pear plantlets were inoculated with both strains by cutting leaf limbs with *E. amylovora* contaminated scissors. Virulence assays in pear plantlet leaves demonstrated a similar extent of necrosis in strains GFP1 and CFBP 1430 (Fig. 3c). Furthermore, the EFM analysis of leaves inoculated with strain GFP1 confirmed the validity of this strain to monitor disease progression in pear plants, allowing the identification of *E. amylovora* cells in necrosed tissues surrounding the inoculation site (Fig. 3d), in tissues previous to necrosis development (Fig. 3d, e), as well as inside vascular vessels (Fig. 3d, f). Depending on the analyzed plant, it was possible to discriminate GFP-labeled cells from time 0, to up to 10 days after the inoculation.

***E. amylovora* is able to colonize root surfaces, enter the radicular system through artificial and/or natural wounds, and systemically spread within pear plant tissues**

To evaluate the ability of *E. amylovora* to colonize and invade host plants through the radicular system, one-month-old pear plantlets, either transplanted or with intact roots, were irrigated with $_{di}H_2O$ contaminated with *E. amylovora*. Moreover, to easily visualize the colonization of root surfaces and/or the invasion pathways employed by the pathogen in this plant organ, a group of plants transplanted into sterile soil after rinsing the roots with tap water was irrigated with the green fluorescent *E. amylovora* transformant strain GFP1. On average, 20 % of intact (i.e. not transplanted) plants irrigated with *E. amylovora* developed fire blight symptoms within an average incubation period of 25 days. In the case of transplanted plantlets, however, the percentage of positive infections increased to an average value of 52.5 %, with the first symptoms observed from 5 to 14 days after the inoculation.

Similar to direct root-inoculation assays, the first fire blight symptoms observed in most diseased intact and transplanted plants irrigated with *E. amylovora*-contaminated water, occurred in the upper parts of the plant, mainly in the tip of the stem and

374 petioles. In adddition, in a reduced number of cases, atypical fire blight symptoms
375 consisting of non-progressing localized necrotic spots on the leaf limbs, became visible
376 in apparently healthy plants.

377 *E. amylovora* pear plantlets inoculated by soil irrigation showed different degrees
378 of fire blight symptoms in the radicular system, depending on the stage of symptom
379 development in the aerial part of the plant. In general, the main root of healthy plants
380 was brown, and lateral roots were white (Fig. 4a). However, most plants showing
381 intermediate necrosis in stem, petioles and leaves, also showed a necrosis (slight or
382 significant, depending on the analyzed plant) in part or the entire main root, and
383 sometimes the color of lateral roots turned from white to red (Fig. 4b). In pear plants
384 showing severe symptoms in aerial organs, a reduction of the root diameter and an
385 intense blackening of the main and secondary roots were also observed (Fig. 4c)

386 *E. amylovora* was successfully re-isolated on semi-selective NCT medium from
387 symptomatic petioles, leaves, stems and roots of diseased plants, with no fungal
388 growth observed during the time required to identify *E. amylovora*-like colonies on this
389 medium (48-72h). In general, only *E. amylovora*-like colonies were isolated from
390 symptomatic aerial organs of host plants on NCT. During root isolations, however, a
391 mixture of *E. amylovora*-like colonies and fluorescent pseudomonad-like colonies grew
392 on NCT. The presumptive isolation of *E. amylovora* from plants showing fire blight
393 symptoms was further confirmed by specific PCR of colonies.

394 The EFM and LSCM analysis of plants inoculated with the *E. amylovora* strain
395 GFP1 by a procedure favoring root infections allowed the characterization of the *E.*
396 *amylovora* colonization and host invasion pathways throughout the roots, as well as its
397 movements throughout host plant tissues. 100 % of root-inoculated plants with the
398 GFP tagged strain developed fire blight symptoms in the stem, petioles and/or leaves.
399 The time required for these plants to develop fire blight symptoms fluctuated between 3
400 and 13 days after their inoculation.

401 Bacterial aggregates and/or biofilms were observed on the surface of the main
402 and/or lateral roots of inoculated plants after removing soil and the rhizosphere and
403 careful washing with $_{di}H_2O$ (Fig. 5a). Surface bacterial aggregates were also usually
404 observed surrounding the site of emergence of lateral secondary roots (Fig. 5b).
405 Furthermore, in few cases, it was possible to visualize individual or small groups of
406 cells on the protective mucilaginous layer covering root hairs (Fig. 5c, d), or also the
407 caliptra (the tip) of some lateral roots.

408 The direct observation of roots by EFM allowed the identification of two potential
409 sites of entry of *E. amylovora* into the radicular system, wounds occurring during
410 transplants (Fig. 6a) and also natural wounds originated by the emergence of lateral
411 roots (Fig. 6b). In both cases an intense fluorescence corresponding to high amounts
412 of bacterial cells inside and/or around wounds was observed (Fig. 6a, b), probably
413 indicating the multiplication of bacterial cells in such entry sites.
414 Regarding the movement of *E. amylovora* through root tissues, the EFM
415 observation of the roots of pear plantlets inoculated with the GFP labelled *E. amylovora*
416 strain allowed the identification of green fluorescent bacteria inside the vascular
417 vessels of the main and lateral roots (Fig. 7a), as well as in the intercellular spaces of
418 the cortical parenchyma (Fig. 7b, c), with a certain tendency of cells to grow, aggregate
419 and/or migrate around the root endodermis (Fig. 7d).
420 The LSCM analysis of GFP1-infected plants allowed the identification of *E.*
421 *amylovora* green fluorescent cells inside the xylem vessels, with the characteristic
422 helical cell wall thickening of xylem tracheids, even in plants without any visible disease
423 symptoms (Fig. 8a, b). Plants showing incipient necrosis in the base or the tip of the
424 stem several days after root-inoculation, also contained large amounts of bacterial cells
425 growing within the cortical parenchymatic tissues of the stem (Fig. 8c, d). In several
426 samples, it was possible to observe individual or groups of fluorescent cells within the
427 apoplast of mesophyll cells close to a necrosed area (data not shown).
428 Delayed onset of symptoms in inoculated pear plantlets (10-13 days) correlated
429 with the loss of the GFP-labeling, and with the location of GFP tagged cells by EFM
430 (but not their isolation on NCT) being difficult, or not possible.
431
432 **Discussion**
433 *E. amylovora* is able to spread systemically within host tissues causing
434 characteristic fire blight symptoms in almost all of the organs of infected plants,
435 including roots (Thomson, 2000; van der Zwet, Orolaza-Halbrendt & Zeller, 2012).
436 However, root blight under field conditions has received little attention and only a few
437 works have included root analysis in *E. amylovora* migration studies within hosts
438 (Gowda & Goodman, 1970; Bogs *et al.,* 1998), and only in apple seedlings. In fact,
439 much of the research on the progression of pathogen infection within the host and/or
440 on the susceptibility of plant tissues to *E. amylovora* has been carried out with young
441 apple shoots as models of plant material (Billing, 2011).

The use of whole plants is essential for a complete analysis of the progression of fire blight throughout plant tissues. The easiest way to obtain such plant material is from seeds. The genetic heterogeneity of plants obtained by this procedure may provide additional strength to certain results. However, based in our experience, one of the main problems linked to acquiring pear or apple plants from seeds is that they need to be used just after their extraction from fruits, due to the rapid inhibition of seed germination during storage by routine procedures. In our study, we successfully employed an embryo culture technique previously used to break seed dormancy in two different plant species, one of them pertaining to the family *Rosaceae* (Arrillaga, Marzo & Segura, 1992). This methodology allowed pear plantlets to be obtained in short periods of time, using long-term stored seeds that would not germinate by other methodologies. Hence, the strategies for seed preservation and acquisition of the plants used in this study ensure the availability of whole plants at any time of the year, as long as a sufficient number of seeds are properly collected and stored.

With regard to direct root inoculation assays, an interesting result from this work was the rapid development of the first fire blight symptoms in the stem, petioles and leaves of pear plantlets from different cultivars (*P.communis* cv. Passe Crassane and cv. Blanquilla). These data demonstrate that *E. amylovora* is able to grow in the pear root system and migrate to aerial organs, which contrasts with similar studies using apple plants, where a slow and limited (Gowda & Goodman, 1970) or a null (Bogs *et al.,* 1998) invasion of aerial tissues after root inoculation was observed. These results agree with the greater sensitivity of pear cultivars to fire blight compared to apple ones (Maroofi & Mostafavi, 1996). Furthermore, our results might suggest that part of the symptoms observed in the aerial part of pear trees under field conditions might be a consequence of infections initiated in the roots. It is remarkable that in our study we observed delayed damage of roots in *E. amylovora* root-inoculated plants compared to the plant organs located upwards. This result, together with the apparent ability of *E. amylovora* to invade the entire radicular system of pear plantlets and apple seedlings (Bogs *et al.,* 1998), might also suggest roots as potential reservoirs and/or inoculum sources of the pathogen within the host, although more research is required to demonstrate this hypothesis.

With regards to water-borne root infection assays, the infection percentage of pear plantlets after irrigation inoculation was lower than that of plants directly inoculated in the main root with *E. amylovora*. This could be explained, at least in part, by the abundance in soil and in pear rhizosphere of fluorescent pseudomonads, which have

been classically reported, together with other species of *Pseudomonas*, as important *E. amylovora* antagonists (Wilson & Lindow, 1993; Cabrefiga, Bonaterra & Montesinos, 2007; Stockwell & Stack, 2007; Mikiciński *et al.,* 2016).

In spite of the abundance of studies concerning the tracing of *E. amylovora* within the host, there is still no consensus, for example, on which sites are the preferred ones for initial multiplication, or to allow the optimal systemic spread of the pathogen (Billing, 2011). This is due to multiple factors, such as the inoculation method, tissue maturity, the degree of tissue (or cultivar, or species) susceptibility, etc (Billing, 2011). The labeling of bacteria with GFP has been successfully used to monitor cell populations during interactions with plants (including parasitic, commensal and mutualistic relationships) or vectors (Gage, Bobo & Long, 1996; Bogs *et al.,* 1998; Liu, Zhao & Chen, 2006; Ordax *et al.,* 2009, 2015; Czajkowski *et al.,* 2010; Lelis *et al.,* 2014; Quecine *et al.,* 2016).

In our study, we used a GFP-marked *E. amylovora* strain to characterize, for the first time, the *E. amylovora* root colonization patterns, using pear plantlets as the model plant material. Root colonization was accompanied by the formation of cell aggregates and/or biofilm-like structures on the surface of primary and secondary roots, and also around the sites of emergence of secondary roots. Interestingly, such colonization patterns were very similar to those described in *Ralstonia solanacearum* (Vasse, Frey & Trigalet, 1995), a well-known soil borne plant pathogen, and *Bacillus megaterium* C4 (Liu, Zhao & Chen, 2006), a bacterial diazothophic endophyte of maize. We further observed a noticeable accumulation of green fluorescent bacterial cells within lesions in roots, including the wounds caused by lateral root emergence. These observations suggest that artificial and natural wounds of the root cortex could be initial multiplication sites and points of entry of *E. amylovora* into the roots, as described for *R. solanacearum* (Vasse, Frey & Trigalet, 1995, Álvarez *et al.,* 2008), *Dickeya* spp. (Czajkowski *et al.,* 2010) and *Clavibacter michiganensis* subsp. *michiganensis* (Lelis *et al.,* 2014). We also identified large amounts of green fluorescent bacteria inside the xylem vessels of the main and lateral roots. This fact might indicate the potential ability of *E. amylovora* cells to multiply using the nutrients present in pear xylem, as previously discussed by Bogs *et al* (1998) in apple seedlings.

Interestingly, groups of GFP-tagged cells inside vascular vessels were observed in roots with clear breaks reaching the vascular cylinder, but also in apparently undamaged roots. Moreover, similar to that reported by Bogs *et* al (1998) in the apple model, a clear tendency for *E. amylovora* cells to grow in the intercellular spaces

adjacent to the endodermis was noticed. The entry of the pathogen through the Casparyan strip in intact roots might occur *via* collapsed cells of the endodermis, or by other unknown mechanisms. According to our observations, both the invasion of the root cortex and the translocation of *E. amylovora* cells to the vascular cylinder are probably facilitated by damage of the roots, which might explain the higher percentages of infection of transplanted *versus* intact plants.

Regarding the routes used by the fire blight pathogen for its transport from the root system to the stem, petioles and/or leaves, conclusions from direct inoculation assays and experiments with the *E. amylovora* GFP1 strain agree with the hypothesis that xylem vessels, but not phloem, are used by *E. amylovora* to migrate to different tissues (Billing, 2011). This is also the case of other bacterial pathogens and/or endophytes (Vasse, Frey & Trigalet, 1995; James *et al.,* 2002; Liu, Zhao &Chen, 2006; Quecine *et al.,* 2016). It is noteworthy that, in a previous work, Bogs *et al* (1998) described the movement of *E. amylovora* via the xylem against the water stream. According to our results, however, the transport of the pathogen in the same direction of the xylem water flow was demonstrated by the location in the stem of green fluorescent *E. amylovora* cells inside characteristic xylem tracheids, with helicoidal cell wall thickenings after their root inoculation with the GFP-marked strain. This migration pathway might be favored by root pressure and the transpiration of challenged plants (James *et al.,* 2002; Lelis *et al.,* 2014), which would explain the rapid movement of *E. amylovora* cells from roots to the upper parts of the stem (about 6 cm per day in the best cases in plants directly inoculated in the roots with the pathogen). Apart from the different host plant model employed, a possible explanation for differences between our results and those of Bogs *et al* (1998) is that they covered the apple plants with bags after inoculation to increase the relative humidity, which probably affects the transpiration stream as well as the movement of the pathogen throughout xylem vessels.

In our work, we also demonstrated the ability of *E. amylovora* cells to break out of the vascular cylinder and enter the parenchymatic tissues, mainly in the tip of the stem and in petioles, which matches observations in other works (Billing, 2011). It is worth mentioning that *E. amylovora's* initial multiplication in vascular vessels did not cause visible fire blight symptoms, while this did occur when the pathogen grew in the cortex of the stem. Also, the movement of the pathogen throughout the cortical intercellular spaces occurred both upwards and downwards, probably due to an increase in pressure in the apoplast of the cortex as a consequence of bacterial growth, ooze

546 production and leakage of water, sugars and sorbitol by degenerated plant cells
547 (Zamski *et al.,* 2007).

548 In summary, in this work we successfully applied and optimized an *in vitro* embryo
549 culture method to rapidly obtain pear plants. We demonstrated the rapid migration of
550 *E. amylovora* cells from the roots to the aerial part of the plant, mainly *via* the xylem,
551 something not yet reported in any *E. amylovora*-host plant pathosystem. We also
552 evidenced that the water-borne infection of pear plantlets *via* the roots is feasible, with
553 plant infection percentages being enhanced by transplant-related root damage. Water-
554 borne infection with the fire blight pathogen might be possible in nurseries, where great
555 densities of young *E. amylovora* host plants are present, and also in orchards where
556 pear trees are cultivated. However, the feasibility of *E. amylovora* root-infection in host
557 plants other than pear and/or lignified trees must be determined. Finally, the use of a
558 GFP-marked strain of *E. amylovora* allowed monitoring of the stages of pear root
559 colonization and invasion, which can be summarized as: *i)* colonization of root
560 surfaces and junctions of the main and lateral roots; *ii)* invasion of the cortex through
561 wounds and cracks at the points of lateral root emergence; and *iii)* penetration via
562 wounds (or by other unknown mechanisms) into the vascular cylinder, and *iv)*
563 migration through xylem vessels to aerial parts of the host plant.
564

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679 **Figures**

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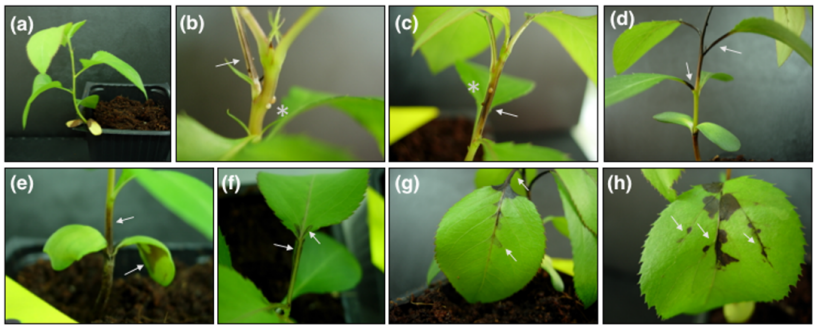

681

682 **Fig. 1. Representative images of one-month-old pear plantlets root-**
683 **inoculated with a hypodermic needle previously dipped into an *Erwinia***
684 ***amylovora* CFBP 1430 colony**. Control plant inoculated with a sterile needle (a);
685 plant showing initial fire blight symptoms in the stem and petioles 3 days after the
686 inoculation (b); progression of fire blight symptoms from the upper parts of the
687 stem downwards 4 and 5 days post-inoculation (dpi) (c, d); pear plantlet showing
688 initial necrosis in the base of the stem and petioles 4 dpi (e); detail of the onset of
689 necrosis in petioles (f) and the progression of this symptom to the limb of affected
690 leaves, corresponding with the advance of the pathogen throughout the vascular
691 system, breaking out of vessels and invading surrounding parenchymatic tissues
692 (g, h). Asterisks indicate the presence of exudates, and arrows mark necrosed
693 areas or incipient necrosis.

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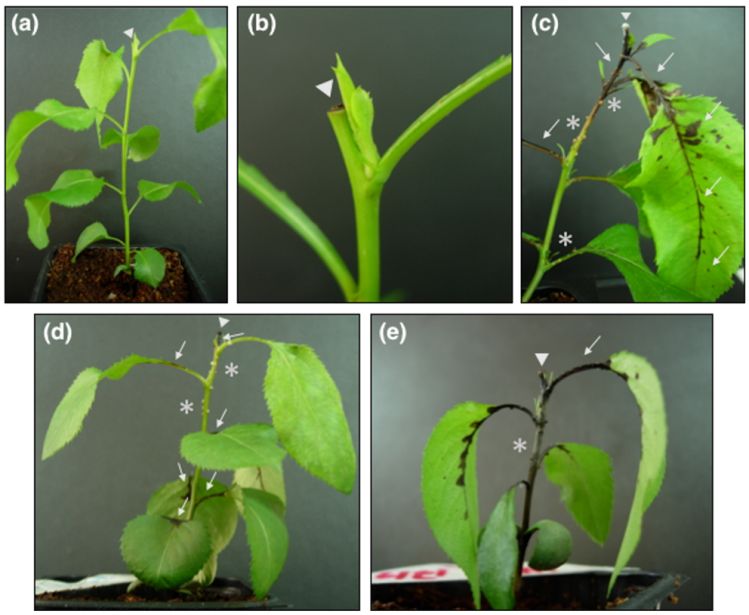


Fig. 2. Representative pictures of one-month-old pear plantlets inoculated with *Erwinia amylovora* CFBP 1430 in the wound resulting from cutting the tip of the stem with scissors. Control plant inoculated with sterile $_{di}H_2O$ (a) and detail of control plant sprouting during the incubation (b). Plant showing typical necrosis and exudates that progress from the inoculation site downwards (c). Characteristic necrosis of petioles occurring earlier than that of the stem (d). Plant showing advanced fire blight symptoms within the entire stem, petioles, raquis and limb of the leaves (e). Asterisks, arrows and triangles indicate the presence of exudates, necrosis and the inoculation site, respectively.

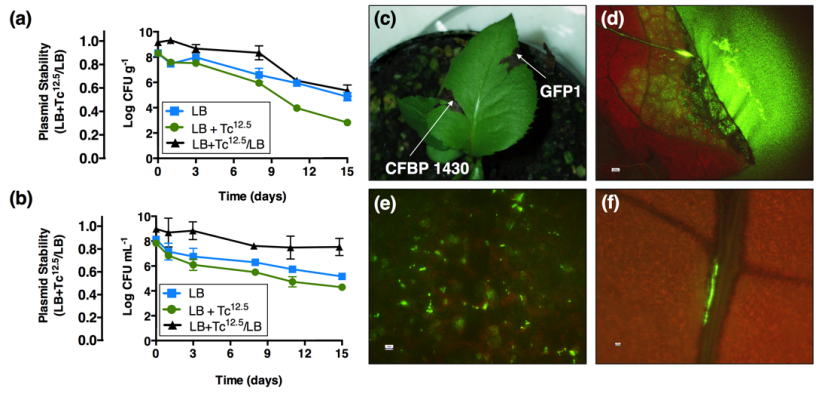


Fig. 3. Characterization of the stability of the plasmid pHC60 in the *Erwinia amylovora* GFP1 strain under different conditions. Graphs representing plasmid stability in soil (a) and water (b) microcosms incubated at 26ºC. Plasmid stability was represented as the ratio of plate counts on LB+Tc$^{12.5}$ (allowing growth of only cells carrying pHC60) with respect to those on LB (allowing growth of *E. amylovora* cells carrying, or not carrying, the plasmid). Represented data are average values of three experiments. Vertical lines denote the SD. Representative picture showing the similar virulence (i.e. necrosis extent) of the *E. amylovora* transformant and parental GFP1 and CFBP 1430 strains, respectively, 3 days after the inoculation of a leaf by cutting with *E. amylovora*-contaminated scissors (c). Representative epifluorescence microscopy images showing cells of the green fluorescent transformant GFP1 advancing throughout leaf tissues after its inoculation with scissors (d). The GFP labeled cells are green and plant cells containing chlorophyll emit red fluorescence when excited with the laser used for discriminating the GFP. Detail of GFP1 cells within the intercellular spaces of the mesophyll of an inoculated leaf (e). *E. amylovora* aggregate of cells in the vascular vessels far from the inoculation site in a leaf (f).

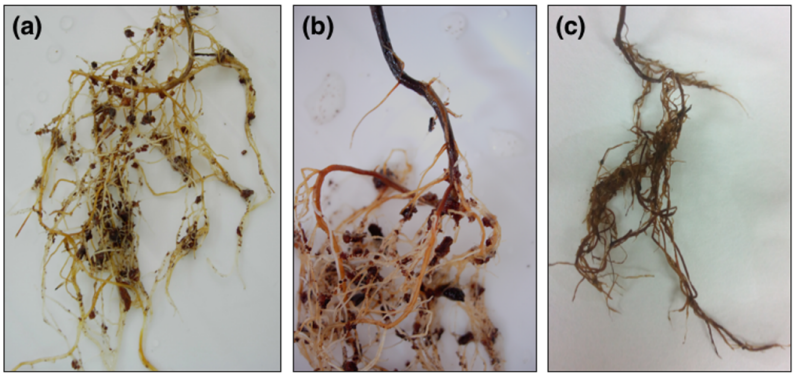
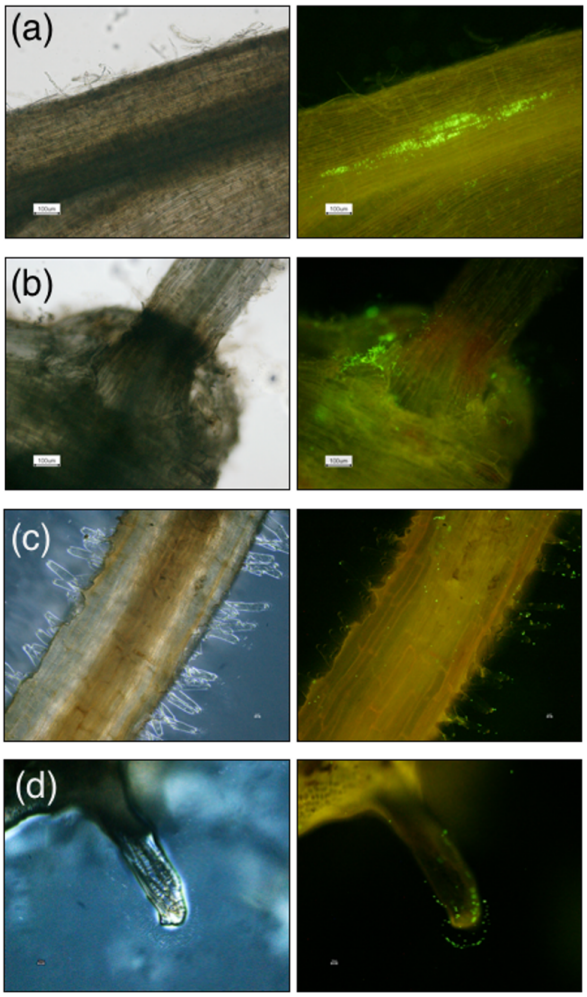


Fig. 4. Representative pictures of the necrosis degrees observed in pear plantlets soil irrigated with water contaminated with *Erwinia amylovora* CFBP 1430. Control plants and infected plants during the first stages of infection show a pale brown colored main root and white lateral roots (a). Plants showing intermediate fire blight symptom development in the aerial part (i.e. partial necrosis of the stem, petioles and/or leaves) usually have a partially necrosed main root, with lateral roots being white and/or reddish (b). Severely infected plants with a total necrosis of stem, petioles and leaves, also used to show a total necrosis of the radicular system, with a clear blackening of the main and lateral roots (c).



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749 **Fig. 5. Representative images showing the root colonization patterns of**
750 **one-month-old pear plantlets inoculated with the *Erwinia amylovora* green**
751 **fluorescent GFP1 strain.** Pictures on the left and right correspond to optical and
752 epifluorescence microscopy images, respectively. *E. amylovora* cell aggregates
753 on the main root surface (a) and around the site of emergence of a secondary root
754 (b). Details of *E. amylovora* individuals or groups of cells on the surface of the
755 mucilaginous layer around root hairs (c, d). All the pictures were taken from plants
756 three days after their inoculation.

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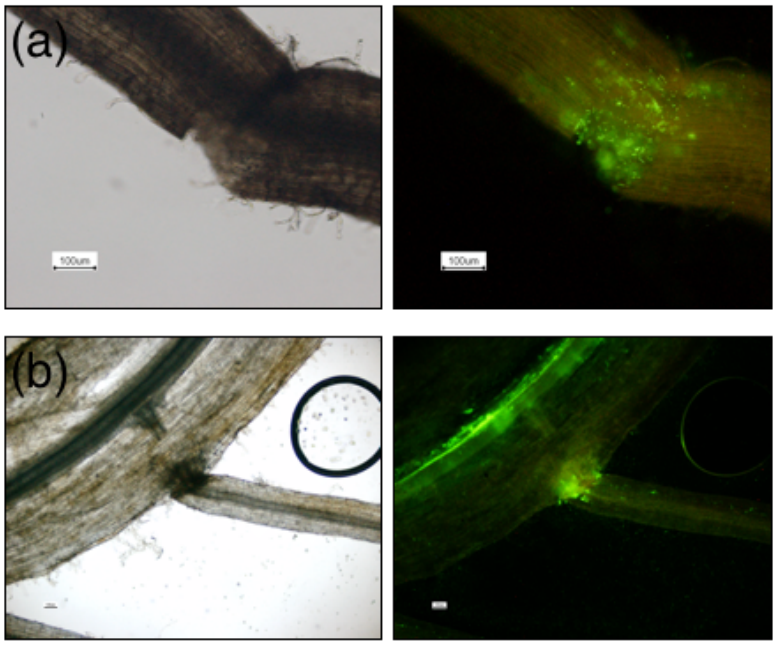

759

760 **Fig. 6. Representative images of the invasion pathways employed by**
761 ***Erwinia amylovora* GFP1 during the infection of one-month-old pear**
762 **plantlets.** Images on the left and on the right correspond to optical and
763 epifluorescence microscopy, respectively. *E. amylovora* cells of the GFP1 strain
764 growing inside a wound of a secondary root three days after the inoculation (a).
765 Accumulation of green fluorescent *E. amylovora* cells in the site of emergence of a
766 lateral root five days after the inoculation (b).

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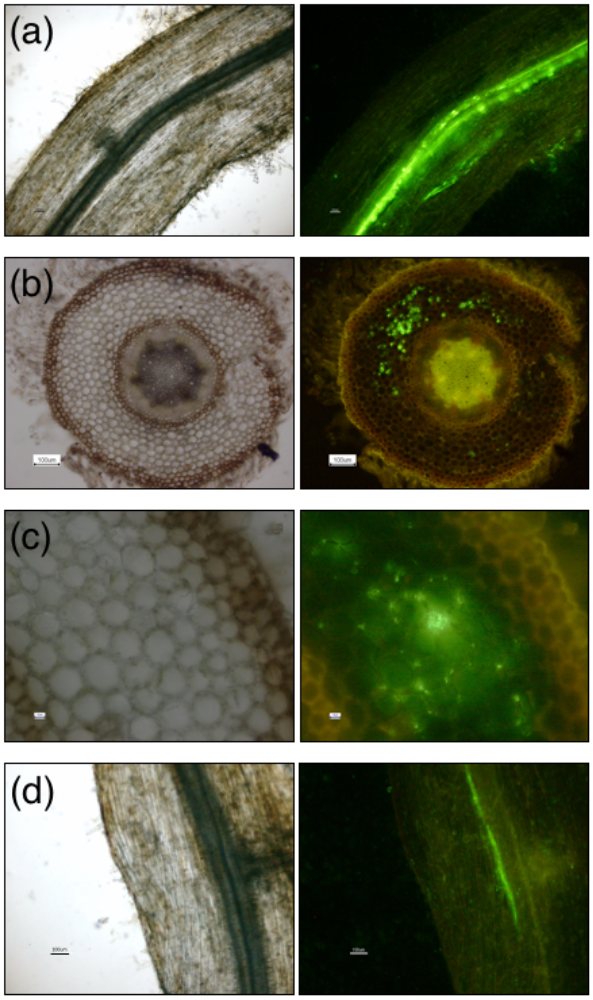
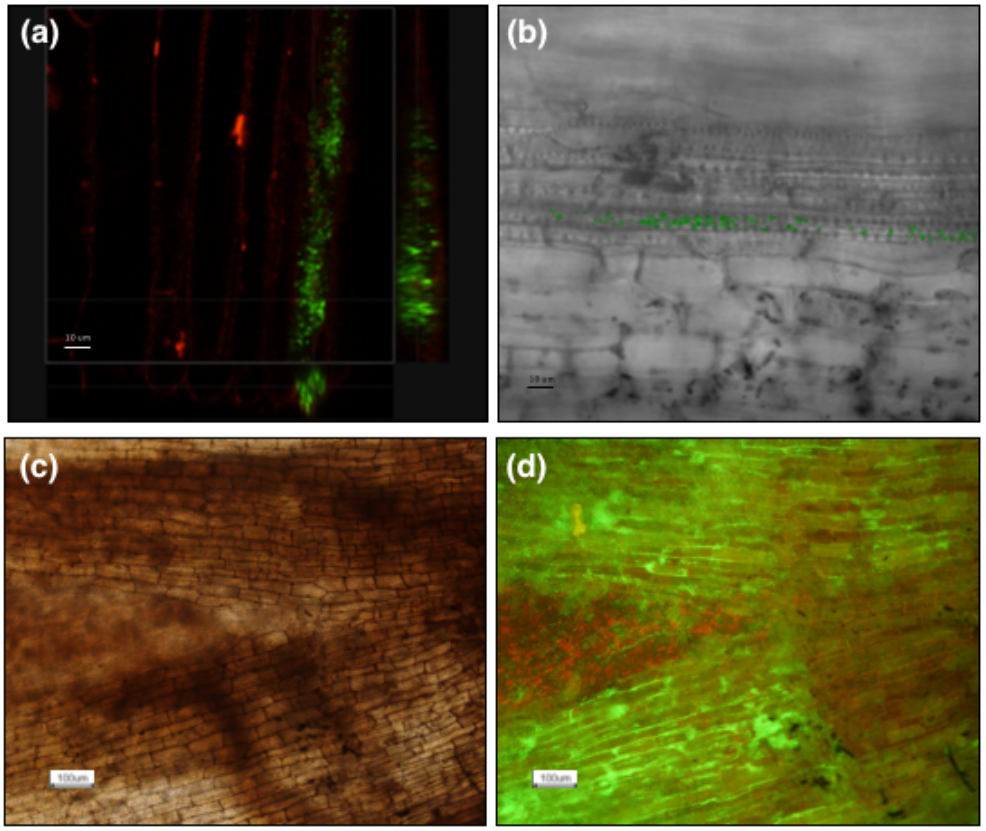


772

773 **Fig. 7. Representative images illustrating the different migration routes**
774 **of *Erwinia amylovora* GFP1 within inoculated roots of one-month-old pear**
775 **plantlets.** Images on the left and right correspond to optical and epifluorescence
776 microscopy, respectively. Abundant *E. amylovora* cells within the vascular cylinder
777 in the main root three days after the inoculation (a). Cross sections of the main
778 root showing *E. amylovora* cells multiplying in the apoplast of the parenchymatic
779 tissues of the cortex of the main root five days after the inoculation (b). Detail of
780 cells in the apoplast of the cortical parenchyma (c). *E. amylovora* cells growing
781 adjacent to the endodermis of the vascular cylinder (d).



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783 **Fig. 8. Laser scanning confocal (a, b), optical (c) and epifluorescence (d)**
784 **microscopy images of stem sections of one-month-old pear plantlets**
785 **inoculated in the roots with the green fluorescent *Erwinia amylovora* GFP1**
786 **strain.** Laser scanning confocal microscopy image of an intermediate section of
787 the stem indicating the presence of green fluorescent *E. amylovora* cells inside a
788 vascular vessel of an asymptomatic plant three days after the inoculation (a).
789 Overlay of GFP fluorescence and a transmitted light image of a section of the
790 stem of the same plant, where *E. amylovora* cells can be seen inside a xylem
791 vessel with the characteristic helicoidal cell wall thickening (b). Optical and
792 epifluorescence microscopy images of a necrosed section of the stem (c) of a
793 pear plantlet seven days after the inoculation, where abundant *Erwinia amylovora*
794 GFP1 cells can be seen occupying the intercellular spaces of the cortical
795 parenchyma (d).

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Annex VI

Santander *et al.,* 2014a

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RESEARCH ARTICLE

Exploring new roles for the *rpoS* gene in the survival and virulence of the fire blight pathogen *Erwinia amylovora*

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Keywords
starvation; cross-protection; motility; exopolysaccharides; loquats; incompatible plant–pathogen interactions.

Abstract

Erwinia amylovora causes fire blight in economically important plants of the family *Rosaceae*. This bacterial pathogen spends part of its life cycle coping with starvation and other fluctuating environmental conditions. In many Gram-negative bacteria, starvation and other stress responses are regulated by the sigma factor RpoS. We obtained an *E. amylovora rpoS* mutant to explore the role of this gene in starvation responses and its potential implication in other processes not yet studied in this pathogen. Results showed that *E. amylovora* needs *rpoS* to develop normal starvation survival and viable but nonculturable (VBNC) responses. Furthermore, this gene contributed to stationary phase cross-protection against oxidative, osmotic, and acid stresses and was essential for cross-protection against heat shock, but nonessential against acid shock. RpoS also mediated regulation of motility, exopolysaccharide synthesis, and virulence in immature loquats, but not in pear plantlets, and contributed to *E. amylovora* survival in nonhost tissues during incompatible interactions. Our results reveal some unique roles for the *rpoS* gene in *E. amylovora* and provide new knowledge on the regulation of different processes related to its ecology, including survival in different environments and virulence in immature fruits.

Introduction

Erwinia amylovora is a nonobligate plant pathogenic bacterium that causes fire blight in most species of the subfamily *Maloideae*, of the family *Rosaceae*. Over the last few centuries, this disease has spread all over the world, causing serious economical losses in places where susceptible hosts are cultivated (Kamber *et al.*, 2012). Fire blight affects fruit trees such as apple (*Malus domestica*), pear (*Pyrus communis*), loquat (*Eriobotrya japonica*), or quince (*Cydonia oblonga*), and also a variety of ornamental species of the genera *Sorbus, Crataegus, Pyracantha*, and *Cotoneaster*, among others. The success of *E. amylovora* dissemination is related to the ability of this enterobacterial phytopathogen to survive under unfavorable conditions and to spread through different means, including insects, birds, rainwater, and contaminated pruning tools (Thomson, 2000; van der Zwet *et al.*, 2012). Accordingly, *E. amylovora* spends part of its life cycle facing starvation until it finds new susceptible hosts where it can multiply again.

As in the case of *E. amylovora*, it is accepted that bacterial pathogens outside their hosts are mostly exposed to the oligotrophic conditions prevailing in natural environments, and rarely finding a nutrient source allowing active growth for prolonged periods (Morita, 1997; Edwards, 2000). Thus, the perdurability of pathogens in nature is related to their ability to survive to nutrient scarcity, a stress that bacteria also experience during the entry into the stationary growth phase in batch culture conditions. In *Escherichia coli* and other bacterial species, the main regulator during the adaptation of cells to starvation is the alternative sigma factor of the RNA polymerase RpoS, encoded by the gene *rpoS* (Ishihama, 1997; Hengge-Aronis, 2002; Navarro Llorens *et al.*, 2010). Genes homologous to the *E. coli rpoS* gene have been identified and characterized in a variety of bacterial species pertaining to the branch gamma of the

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proteobacteria (Dong & Schellhorn, 2010). The sigma factor RpoS controls different physiological processes, and it is induced not only by starvation but also by other stresses and during the entry into the stationary growth phase, acting as the master regulator of the general stress response. As a result, starved and stationary phase cells become protected against multiple stresses to which they were not exposed, in a phenomenon called cross-protection (Hengge-Aronis, 2002). In the case of bacterial pathogens, this cross-protection might not only be important for their survival outside the host, but also during the infectious process, when they are exposed to host defenses and other stresses inside plant tissues. In this regard, the virulence and/or pathogenicity of many bacterial species are also regulated by RpoS, via either the direct control of virulence/pathogenicity factors or indirectly, modulating the expression of virulence-related genes that enhance the biological fitness of the pathogen in the environment (Dong & Schellhorn, 2010).

In a previous work, Santander *et al.* (2014) monitored the regulation of the *rpoS* gene during *E. amylovora* exposure to starvation and characterized two physiological mechanisms developed by this pathogen to deal with this stress, the starvation survival and the viable but nonculturable (VBNC) responses. Both are characterized by cell growth arrest, but the latter implies the inability of viable cells to form colonies on solid media. With the purpose of investigating the role of *rpoS* on these survival strategies, an *rpoS* mutant of the European reference *E. amylovora* strain CFBP 1430 and a complemented strain were obtained, and the effects of the *rpoS* impairment on culturability, viability, and integrity of starved cells over time were evaluated. We additionally explored other functions of the sigma factor RpoS in *E. amylovora,* some of which have not yet even been studied in other pathogens. First, we determined the contribution of *rpoS* to stationary phase cross-protection against different stresses. Given that RpoS controls virulence/pathogenicity in many pathogens, we also evaluated its role in the control of *E. amylovora* virulence and pathogenicity factors, such as motility or the synthesis of the two main exopolysaccharides, levan and amylovoran. Finally, we assessed the effect of the *rpoS* mutation on *E. amylovora* virulence in different types of susceptible host plant material, as well as its potential role during incompatible plant–pathogen interactions.

Materials and methods

Bacterial strains, vectors, and culture conditions

The bacterial strains and plasmids used in this study are shown in Table 1. Bacterial cells were cryopreserved in 25% (v/v) glycerol at −80 °C. For most experiments, *E. amylovora* and *E. coli* strains were grown on Luria–Bertani (LB) agar plates or broth with shaking (200 r.p.m.) at 28 °C or at 37 °C, respectively. Late stationary phase ($OD_{600\ nm} > 2$) and mid-log phase ($OD_{600\ nm} = 0.5$) cultures were obtained as follows. Cells were grown overnight in LB, diluted 1/100 into fresh medium, and incubated at 28 °C with shaking for 16 h in the case of stationary phase cultures, or *c.* for 6 h in the case of mid-log phase cultures. In some experiments, *E. amylovora* cells were grown in modified basal medium A (MBMA) (per L, 3 g KH_2PO_4, 7 g K_2HPO_4, 1 g $(NH_4)_2SO_4$, 2 mL glycerol, 0.5 g citric acid, 0.03 g $MgSO_4$) (Torriani, 1960) supplemented with 100 μg L^{-1} nicotinic acid (Starr & Mandel, 1950) (MBMANic). When necessary, antibiotics were added to the media at the following final concentrations: kanamycin 20 μg mL^{-1} (Km^{20}), ampicillin 100 μg mL^{-1} (Ap^{100}), and chloramphenicol 10 μg mL^{-1} (Cm^{10}).

Obtaining and complementation of an *E. amylovora* mutant in the *rpoS* gene

Primers used in this study (Table 2) were designed based on the genome sequence of the European reference *E. amylovora* strain CFBP 1430 (Smits *et al.*, 2010) (WT1, Table 1), available in the ASAP database, https://asap.ahabs.wisc.edu (Glasner *et al.*, 2003). To obtain an *rpoS* mutant, a DNA fragment containing the *E. amylovora rpoS* gene was amplified by PCR, cloned into the pGEM-T Easy Vector System I (Promega, Madison, WI), digested with BamHI, and interrupted with the kanamycin resistance interposon Ω-Km from pHP45Ω-Km (Table 1). The constructed vector pGEM*rpoS*::Ω-Km was introduced into strain CFBP 1430 by electroporation, and marker exchange mutagenesis was carried out using the Roeder & Collmer (1985) low-phosphate medium. Given that *rpoS* mutants of many bacterial species are sensitive to hydrogen peroxide (H_2O_2), *rpoS* mutations were phenotypically confirmed by a growth inhibition halo assay. Briefly, overnight cultures in LB were washed with SS and plated onto MBMANic agar. Then, a sterile paper filter disk was placed on seeded plates, 5 μL of 33% (v/v) H_2O_2 loaded onto each disk, and the plates were incubated for 48 h at 28 °C. Afterward, the correct disruption of the *rpoS* gene was verified by PCR (Table 2) and DNA sequencing.

To obtain a complemented strain, a functional copy of *rpoS* was amplified by PCR (Table 2), cloned into the XmaI–SpeI site in the broad-host-range plasmid pBBR1MCS-4 (Table 1), and the resulting construct electroporated into the mutant strain *rpoS*$^{-}$. The mutant complementation was confirmed phenotypically by the growth inhibition halo assay, as previously described. Then, both the inactivation and complementation of the

Table 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
E. amylovora		
CFBP 1430	Wild-type strain 1, WT1; isolated from *Crataegus* sp. in France	Paulin & Samson (1973)
rpoS$^-$	Mutant strain derived from CFBP 1430; *rpoS*::ΩKm; Km^r	This study
rpoS$^+$	Complemented mutant strain containing a functional copy of *rpoS* in pBBR4::*rpoS;* Km^r, Ap^r	This study
Ea 1/79	Wild-type strain 2, WT2; isolated from *Malus sylvestris* in Germany	Falkenstein *et al.* (1988)
Ea 1/79Sm-del100	Mutant strain derived from Ea 1/79 containing a deletion from *ams*A to *ams*F; deficient in amylovoran synthesis; Cm^r; Amy$^-$	Bugert & Geider (1995)
Ea 1/79-18M	Mutant strain derived from Ea 1/79 lacking levansucrase activity; *rls*B::pfdA8; Km^r; Lev$^-$	Du & Geider (2002)
E. coli		
DH5α	*Sup*E44Ω*lac*U169 (80*lacZ*ΩM15) *hsdR*17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*-A1	Hanahan (1983)
Plasmids		
pGEM-T	SP6; T7; lacZ; Ap^r	Promega
pGEM*rpoS*	pGEM-T containing a 1047-bp PCR product with the wild-type *E. amylovora* (CFBP 1430) *rpoS* gene; Ap^r	This study
pGEM*rpoS*::Ω-Km	pGEM*rpoS* containing the Ω-Km interposon from pHP45Ω-Km in the unique BamHI site inside the *rpoS* gene; Ap^r; Km^r	This study
pHP45Ω-Km	Plasmid containing the interposon Ω-Km; Ap^r, Km^r	Fellay *et al.* (1987)
pBBR1MCS-4	Broad-host-range expression vector; RK2; lacZ; Ap^r	Kovach *et al.* (1995)
pBBR4::*rpoS*	pBBR1MCS-4 containing a functional copy of the *E. amylovora* (CFBP 1430) *rpoS* gene in the XmaI–SpeI restriction site	This study

Table 2. Primers used in this study

Primers	Sequence
rpoS amplification	
*rpoS*5′	ACCTTGCGGTTCTGCCTTG
*rpoS*3′	GTTCTTCGCGCTATCATTCACGGAAG
Mutagenesis confirmation by PCR	
*rpoS*_EXT.F	GAAGGTGGCAATAAAGGTATCG
*rpoS*_EXT.R	GTAGTGCTGCCAGTACCAAA
T4-Ω	AGCTTGCTCAATCAATCACCG
Mutagenesis confirmation by RT-PCR	
RT-*rpoS*.F	AAGATTGCCCGACGTTACAG
RT-*rpoS*.R	AGACGAATGGTACGGGTTTG
Mutant complementation	
*rpoS*_XmaI.F*	AAATTAA**CCCGGG**ACCTTGCGGTTCTGCCTTG
*rpoS*_SpeI.R†	CCAAGC**ACTAGT**TTCGCGCTATCATTCACGG

*Bold nucleotides indicate the restriction site for XmaI; underlined nucleotides are a clamp sequence.

†Bold nucleotides indicate the restriction site for SpeI; underlined nucleotides are a clamp sequence.

rpoS gene were additionally corroborated by endpoint reverse transcriptase (RT)-PCR (Table 2).

To characterize the mutant and complemented strains, a biochemical profile was determined using the API 20E and API 50CH systems (BioMérieux). Gelatin hydrolysis was additionally tested by a gelatin plate method, according to Smith & Goodner (1958), using 4% (w/v) gelatin as the substrate and an incubation period of 7–14 days at 28 °C.

Oligotrophic microcosms preparation and analysis of population cell dynamics

To determine the role of *rpoS* in *E. amylovora* responses to starvation, natural water microcosms were prepared similar to Santander *et al.* (2012, 2014). Briefly, *E. amylovora* cultures of strains CFBP 1430, *rpoS*$^-$, and *rpoS*$^+$ were grown overnight in LB and washed twice with sterile natural water. Thereafter, bacterial suspensions were adjusted to an $OD_{600\ nm}$ of 1.0 (about 10^9 CFU mL^{-1}) and diluted 1/100 into natural water microcosms, reaching *c.* 10^7 CFU mL^{-1}. Microcosms were incubated at 28 °C. Population dynamics were monitored for 12 days, estimating the number of culturable, viable, and total cells throughout this period according to Santander *et al.* (2012, 2014). This experiment was repeated twice, with three technical replicates in each independent assay.

Stationary phase cross-protection assays

To study the contribution of the *rpoS* gene to stationary phase cross-protection against oxidative, osmotic, and acid stresses, and also against heat and acid shock, late stationary phase and mid-log phase cultures of *E. amylovora* strains CFBP 1430, *rpoS*$^-$, and *rpoS*$^+$ were exposed to each stress and the behavior of culturable cells compared. For this purpose, late or mid-log phase cultures were washed thrice in sterile saline (SS), diluted to

10^6 CFU mL^{-1}, and subjected to the above-mentioned stresses in 1.5 mL microcentrifuge tubes placed in a dry bath preheated to the assay temperature. Unless otherwise indicated, stresses were assayed at 28 °C. All the incubation periods and conditions employed for each stress were previously optimized in initial assays (data not shown). Changes in the culturability of *E. amylovora*-challenged cells were monitored throughout time by drop plate on LB agar.

The assayed stresses were selected based on other publications with *rpoS* mutants and/or some aspects related to the ecology of *E. amylovora*. Oxidative stress was assayed for 9 min in SS containing 25 mM H_2O_2. Heat shock was assessed at 42 °C in SS for 25 min. Osmotic stress was evaluated for 60 min using artificial nectar (25.5% free sugars) composed of a mixture 5.5 : 4.5 of fructose and glucose in 10 mM phosphate buffer (PB) pH 7.0. The selected sugar composition was based on that present in pear nectar, and the sugar concentration can be present both in pear and in apple nectar (Farkas *et al.*, 2004; Konarska *et al.*, 2005; Spinelli *et al.*, 2005). Acid stress and acid shock were analyzed using PB pH 5.5 for 48 h and PB pH 3.5 for 30 min, respectively. All the assays were performed in at least three independent experiments.

Swimming motility assays

Swimming motility was determined on semisolid LB medium containing 0.3% (w/v) agar, supplemented with antibiotics when required. Overnight cultures of *E. amylovora* strains CFBP 1430, *rpoS*$^-$, and *rpoS*$^+$ were pelleted by centrifugation, and these pellets were used to inoculate plates with a sterile toothpick. Motility plates were incubated at 22 °C (Raymundo & Ries, 1981) for 48 h and photographed with a digital camera (Spectracore Inc., Ontario, NY). Motility areas were determined with IMAGEJ software (Schneider *et al.*, 2012). Swimming motility was assessed in three independent experiments with 10 technical replicates in each case.

Quantification of amylovoran and levansucrase activity

Amylovoran was measured by the cetylpyridinium chloride (CPC) method, according to Edmunds *et al.* (2013). Briefly, *E. amylovora* strains CFBP 1430, *rpoS*$^-$, *rpoS*$^+$ as well as the positive and negative control strains Ea 1/79 (WT2) and Amy$^-$ (Table 1), respectively, were grown overnight in MBMANic plus 1% (w/v) sorbitol, 1/100 diluted in the same medium, and incubated for 40 h at 28 °C with shaking (200 r.p.m.). Then, the $OD_{600\ nm}$ of bacterial suspensions was measured, cells pelleted by centrifugation, and supernatants filtered-sterilized through a pore size of 0.2 μm. Amylovoran was quantified after mixing 0.8 mL of supernatant with 40 μL of 50 mg mL^{-1} CPC. The turbidity of amylovoran was measured at an $OD_{600\ nm}$ after an incubation period of 10 min at room temperature. Amylovoran production was determined in three independent experiments with, at least, six technical replicates in each one.

The secreted levansucrase activity was measured according Ordax *et al.* (2010). Briefly, overnight cultures in Standard I Nutrient Broth (Merck) of *E. amylovora* strains CFBP 1430, *rpoS*$^-$, *rpoS*$^+$, and the positive and negative control strains Ea 1/79 and Lev$^-$ (Table 1), respectively, were 1/100 diluted into fresh medium, incubated at 28 °C (200 r.p.m.) for 20 h, and the $OD_{600\ nm}$ of cells measured (see below). Afterwards, cells were removed by centrifugation, supernatants mixed 1 : 1 with assay buffer (50 mM Na_2HPO_4, 2 M sucrose, 0.05%, w/v, sodium azide), and the turbidity caused by levan formation measured at $OD_{580\ nm}$, after 24 h of incubation at 28 °C. Secreted levansucrase activity was determined in three independent experiments performed in triplicate. Levan production was also qualitatively determined by comparison of colony sizes on Sucrose Nutrient Agar (SNA) plates (EPPO, 2013).

Quantitative data were normalized dividing the $OD_{600\ nm}$ or $OD_{580\ nm}$, respectively, corresponding to amylovoran or levan quantification, by the $OD_{600\ nm}$ of the corresponding analyzed culture.

Virulence assays in immature loquats and pear plantlets

The role of *rpoS* on the *E. amylovora* virulence was evaluated in immature loquats (*E. japonica* cv. Tanaka) and in 4-week-old pear plantlets (*P. communis* cv. Passe Crassane) similar to Santander *et al.* (2014). Briefly, fruits were surface-disinfected for 5 min with 2% (w/v) NaOCl and thoroughly washed with sterile distilled water. Disinfected loquats were inoculated in a single wound (0.5 cm deep) performed with a sterile 100-μL pipette tip. Pear plantlets were inoculated in the wound resulting from the removal of the upper part (about 1 cm) of the stem. To prepare bacterial inocula, 1 mL overnight cultures were washed thrice in SS and their $OD_{600\ nm}$ adjusted to 1.0. Then, cell suspensions were serially 10-fold-diluted in SS and fruits and plantlets inoculated with 2 μL of 10^7, 10^6, or 10^5 CFU mL^{-1}, corresponding to about 10^4, 10^3, and 10^2 CFUs per wound. Each inoculum dose was assayed in 11 fruits and 10 plantlets, in two independent assays. Negative controls were inoculated with SS. Immature fruits and pear plantlets were incubated under controlled conditions and monitored for fire blight symptom

development. To confirm results, *E. amylovora* was re-isolated from fruits and plants showing fire blight symptoms and identified by molecular procedures, following the protocols of the European and Mediterranean Plant Protection Organization (EPPO, 2013).

Hypersensitive response (HR) elicitation and survival assays in nonhost tissues

To determine the contribution of RpoS to the elicitation of the HR and the survival of *E. amylovora* inside nonhosts during incompatible interactions, tobacco (*Nicotiana tabacum* L. cv. Xanthi) plants grown under greenhouse conditions were employed. Overnight cultures of *E. amylovora* strains CFBP 1430, *rpoS*$^{-}$, and *rpoS*$^{+}$ in LB broth were washed thrice in SS and cell densities adjusted to an $OD_{600\ nm}$ of 1.0. Tobacco leaves were infiltrated with about 25 μL of bacterial suspension, using a needleless syringe. Each strain was inoculated twice per leaf, in two leaves per plant, in a total of six plants. Negative controls were inoculated with SS. Infiltrated plants were incubated in the laboratory for 8 days at room temperature, inside a biosafety cabinet. To determine the survivability of inoculated strains in nonhost tissues, two inoculated leaves were processed at times 0, 1, 2, 3, 5, and 8 days as follows. Inoculated leaves were rinsed with sterile distilled water. Then, challenged tissues were cut with sterile scissors and photographed, and infiltrated areas were measured with IMAGEJ software (Schneider *et al.*, 2012). Afterward, each leaf section was homogenized with a sterile micropistil in 0.2 mL antioxidant maceration buffer (AMB) (Gorris *et al.*, 1996) and serially 10-fold-diluted in SS. The number of culturable cells was determined by drop plate on CCT semi-selective medium (Ishimaru & Klos, 1984). Results were represented as CFUs cm^{-2} of infiltrated area analyzed. This experiment was performed twice, in two independent repeats. The ability of *rpoS*$^{-}$ and *rpoS*$^{+}$ to grow on CCT agar plates and to survive in AMB was confirmed in preliminary experiments (data not shown), and no differences were observed when compared to the wild-type strain during the assayed periods.

Statistical analysis

For the statistical analysis, data of *E. amylovora* culturable, viable, and total cell counts from independent experiments were normalized by logarithmic transformation. In the case of percentages, data were normalized by arcsine transformation prior to the analysis. Null data from culturable counts below the detection limit were excluded from statistical analysis. Statistical significance of differences was determined by two-way ANOVA followed by Bonferroni or Dunnett's post *hoc* tests. $P < 0.05$ were considered significant.

Results

Construction and characterization of the *E. amylovora rpoS* mutant and the complemented strain

The mutation of the *E. amylovora rpoS* gene was carried out by homologous recombination with a copy of the wild-type gene interrupted with the Ω-Km interposon (Supplementary Information, Fig. S1a), as described in the materials and methods section. Double recombinants were confirmed by PCR (Fig. S1b) and DNA sequencing. The obtained PCR amplicons (Fig. S1b) coincided with those expected (Fig. S1a) in each strain, and the sequencing data confirmed the interruption of the *rpoS* gene with the Ω-Km interposon in the *rpoS* mutant (data not shown). The complemented strain was obtained transforming the mutant *rpoS*$^{-}$ with a plasmid containing a wild-type copy of the *E. amylovora rpoS* gene. The RT-PCR analysis revealed the presence of *rpoS* transcripts in both the wild-type and the complemented strains, but not in the mutant strain, confirming the interruption of *rpoS* also at the transcription level (Fig. S1c). The *E. amylovora rpoS* mutation and complementation were phenotypically confirmed by the growth inhibition halo assay in the presence of H_2O_2 (Fig. S2). After 48 h of incubation at 28 °C the wild-type strain developed halos of about 3 cm^2, while the *rpoS* mutant showed an increased sensitivity to oxidative stress, with halos of 12 cm^2. The restoration of the parental phenotype in the complemented strain was evidenced by halos of about 1.2 cm^2, slightly smaller than those observed in the wild-type strain.

The *E. amylovora* strains CFBP 1430, *rpoS*$^{-}$, and *rpoS*$^{+}$ showed nearly the same biochemical profiles in API 20E and API 50CH systems, except for a reduced gelatinase activity of the *rpoS* mutant in the gelatinase test of the API 20E system (data not shown), which was confirmed in the gelatin plate method (Fig. S3).

Erwinia amylovora rpoS mutant fails to develop normal starvation responses

Population dynamics of *E. amylovora* strains CFBP 1430, *rpoS*$^{-}$, and *rpoS*$^{+}$ exposed to nutrient limitation in natural water at 28 °C are shown in Fig. 1. During the first 7 days, the wild-type strain (Fig. 1a) slightly decreased culturable cell numbers from 10^7 to 10^6 CFU mL^{-1}, developing a typical starvation survival response. In the following 5 days, a more pronounced entry of culturable cells into the VBNC state was observed, with a drop from

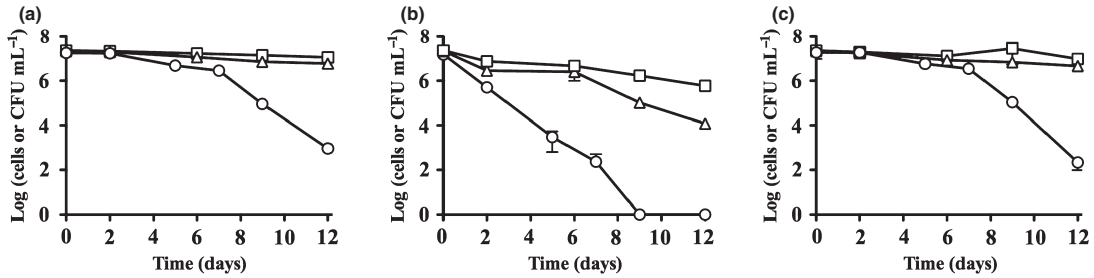

Fig. 1. Role of *rpoS* on *Erwinia amylovora* starvation responses in natural water microcosms at 28 °C. Strains represented are CFBP 1430 (a), *rpoS*$^{-}$ (b), and *rpoS*$^{+}$ (c). Total, viable, and culturable cell counts are indicated with squares, triangles, and circles, respectively. Data shown correspond to the average value of two independent experiments with three technical replicates. Bars represent the SD.

10^6 to 10^3 CFU mL^{-1}, while viable and total cell counts were maintained at numbers similar to the initial ones, about 10^7 cells mL^{-1}. The mutant *rpoS*$^{-}$ (Fig. 1b) did not develop the starvation survival response observed in the parental strain (Fig. 1a). By contrast, it showed a continuous loss of culturability throughout time ($P < 0.001$), with colony counts below the detection limit (1 CFU mL^{-1}) 9 days after the inoculation. Unlike the parental strain (Fig. 1a), the *rpoS*$^{-}$ strain also showed a drop in viable (from about 10^7 to 10^4 cells mL^{-1}) ($P < 0.001$) and total cell numbers (from about 10^7 to 10^5 cells mL^{-1}) ($P < 0.01$) throughout the experiment, these being higher than culturable cell counts (Fig. 1b). Thus, the *E. amylovora rpoS* mutant (Fig. 1b) entered the VBNC state faster than the wild-type strain during the exposure to starvation. Furthermore, the faster adoption of this strategy was accompanied by a loss of viability and cell integrity not observed in the parental strain. In the case of the complemented strain *rpoS*$^{+}$ (Fig. 1c), culturable, viable, and total cell population dynamics were very similar to those observed in the parental strain (Fig. 1a).

RpoS is necessary for full *E. amylovora* stationary phase cross -protection against several stresses

Results corresponding to the stationary phase cross-protection assays in *E. amylovora* strains CFBP 1430, *rpoS*$^{-}$, and *rpoS*$^{+}$ are represented in Fig. 2. The *rpoS* mutant was more sensitive ($P \leq 0.01$) than the parental strain to oxidative stress (Fig. 2a), heat shock (Fig. 2b), osmotic stress (Fig. 2c), and acid stress at pH 5.5 (Fig. 2d), regardless of the growth phase analyzed. By contrast, no differences among strains were observed when exposed to acid shock (Fig. 2e). The *rpoS*-mediated protection of log phase cells was especially significant ($P < 0.0001$) for osmotic (Fig. 2c) and acid stress (Fig. 2d). In the case of stationary phase cells, the protection due to *rpoS* was similar for all the stresses ($P < 0.0001$) (Fig. 2a, b, c and d). Furthermore, significant differences between log and stationary phase cells of the three assayed *E. amylovora* strains were detected in most of the stresses assayed ($P \leq 0.01$), stationary phase cells being more stress resistant than log phase cells (Fig. 2a, c, d and e). However, in the case of heat shock (Fig. 2b), differences between log and stationary phase cells were only significant in the parental strain ($P \leq 0.05$), but not in the *rpoS* mutant. The complemented strain *rpoS*$^{+}$ behaved similarly or survived better than the parental strain when exposed to the stresses analyzed (Fig. 2).

Erwinia amylovora rpoS mutant fails to develop a normal swimming motility

Results of swimming motility assays are summarized in Fig. 3. After an incubation period of 48 h at 22 °C, the three assayed *E. amylovora* strains showed different sizes of swimming areas. The wild-type strain developed swimming halos of 7.1 ± 1.0 cm^2 SD, while the *rpoS* mutant reached 4.7 ± 0.8 cm^2 SD ($P < 0.0001$). The complemented strain *rpoS*$^{+}$, with a swimming halo of 8.1 ± 0.8 cm^2 SD, showed increased motility with respect to the mutant *rpoS*$^{-}$ ($P < 0.0001$), and also to the parental strain ($P < 0.001$). These results reveal the necessity of *rpoS* for *E. amylovora* to develop normal swimming motility.

Erwinia amylovora rpoS mutant shows altered exopolysaccharides production

The relative quantification of amylovoran and levansucrase activity in *E. amylovora* strains CFBP 1430, *rpoS*$^{-}$, and *rpoS*$^{+}$, as well in the positive (Ea 1/79) and negative (Amy^{-}, Lev^{-}) controls, is shown in Fig. 4. The *rpoS* mutant strain produced 1.2 times less amylovoran than the parental strain, this small difference being statistically significant ($P < 0.05$) (Fig. 4a). The complemented strain showed amylovoran levels 1.2 times above those observed

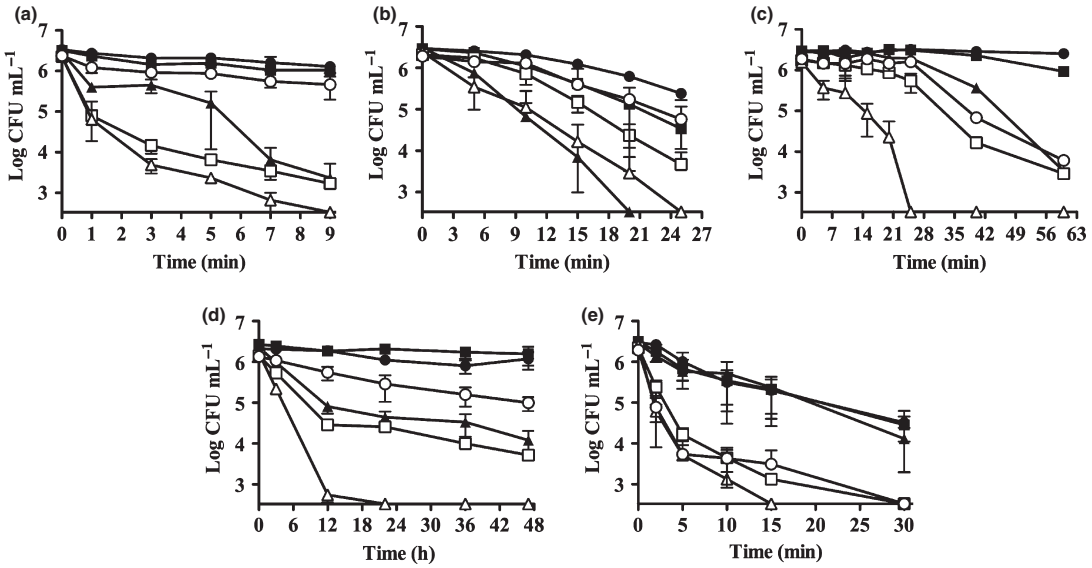

Fig. 2. Role of *rpoS* on the *Erwinia amylovora* stationary phase cross-protection against oxidative stress (a), heat shock (b), osmotic stress (c), acid stress (d), and acid shock (e), using strains CFBP 1430 (squares), *rpoS*$^-$ (triangles), and *rpoS*$^+$ (circles). Stationary and log phase cells are represented with filled and empty symbols, respectively. Represented data corresponds to the mean of at least three independent experiments. Bars show the SD.

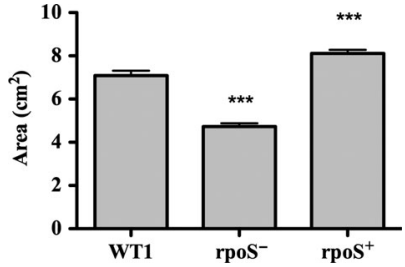

Fig. 3. Effect of *rpoS* mutation on the *Erwinia amylovora* swimming motility. Data shown correspond to the average value of three independent assays after 48 h of incubation at 22 °C and with, at least, 10 technical replicates. Bars represent the SD. The three asterisks indicate statistically significant differences with the wild-type strain, with a *P* value below 0.001. WT1, *E. amylovora* strain CFBP 1430.

in the parental strain ($P < 0.01$). The positive control strain Ea 1/79 showed levels of amylovoran similar to CFBP 1430, and the amount of amylovoran in the negative control Amy$^-$ was near 0.

The secreted levansucrase activity in the *rpoS* mutant was about 2.1 times higher than that observed in the parental strain CFBP 1430 ($P < 0.0001$) (Fig. 4b). Levansucrase activity in the complemented strain was 2.2 times lower ($P < 0.0001$) than that in the parental strain, confirming the repression of levan production exerted by *rpoS*. The positive (Ea 1/79) and negative (Lev$^-$) control strains displayed levansucrase levels slightly lower than the wild-type strain CFBP 1430 and near 0, respectively. These phenotypes were additionally confirmed on SNA plates, where colonies of *rpoS*$^-$ produced more levan than those of the parental strain (data not shown).

rpoS mutation affects *E. amylovora* virulence in immature fruits but not in pear plantlets

Results from the virulence analysis of the *E. amylovora* strains CFBP 1430, *rpoS*$^-$, and *rpoS*$^+$ in immature loquats and in pear plantlets are shown in Figs. 5 and 6. In immature fruits, the three assayed strains showed similar time lapses between their inoculation and the onset of fire blight symptoms. However, the *rpoS* mutant was able to cause necrotic lesions in a greater percentage of fruits throughout time, differences with the parental strain being statistically significant at 2 dpi ($P < 0.01$) in fruits inoculated with 10^4 CFUs (Fig. 5a), or at 3 dpi ($P < 0.01$) in those challenged with 10^3 CFUs (Fig. 5b). In fruits inoculated with 10^2 CFUs (Fig. 5c), results were more variable and no significant differences were detected among the assayed strains.

The analysis of the necrotic areas over time in immature loquats inoculated with 10^3 CFUs per wound

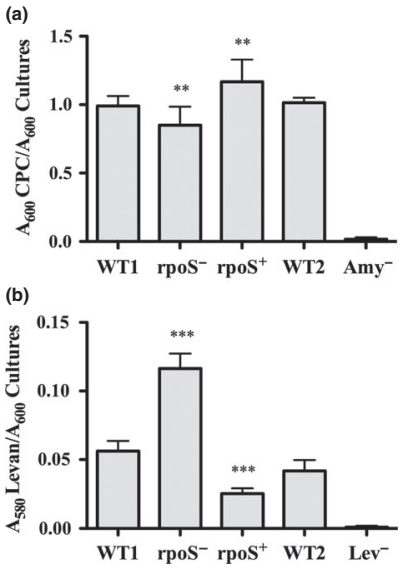

Fig. 4. Effect of *rpoS* mutation on amylovoran production (a) and levansucrase activity (b). In the case of amylovoran quantification, represented data correspond to the mean of three independent repeats, with at least six replicates. Levansucrase activity was measured on three occasions, in triplicate. Bars correspond to the SD. Asterisks denote statistically significant differences with the wild-type strain (**$P < 0.01$; ***$P < 0.001$). WT1 and WT2, *Erwinia amylovora* strains CFBP 1430 and Ea 1/79, respectively.

(Fig. 6a and b) revealed a greater ability of *rpoS*$^-$ to necrose tissues with respect to the parental strain. Necrotic areas of the *rpoS* mutant were about 2.2 times larger than those of the wild type at 5 dpi ($P < 0.001$), and 1.7 times at 7 dpi ($P < 0.001$). No statistically significant differences were observed between the parental and the complemented strain in any of the postinoculation periods analyzed (Fig. 6a and b).

The virulence analysis using pear plantlets and different bacterial doses revealed no differences among strains, either in the onset of symptoms, the number of plants showing fire blight symptoms at a given time (data not shown), or in the intensity of exudates and/or necrosis in inoculated plants (Fig. 6c). Negative controls did not show any of the above-mentioned symptoms in any of the periods assayed (Fig. 6c).

Erwinia amylovora survival during incompatible plant–pathogen interactions depends on the *rpoS* gene

Tobacco leaf sections inoculated with either of the assayed *E. amylovora* strains (CFBP 1430, *rpoS*, and *rpoS*$^+$) developed a typical HR within the following 20 h

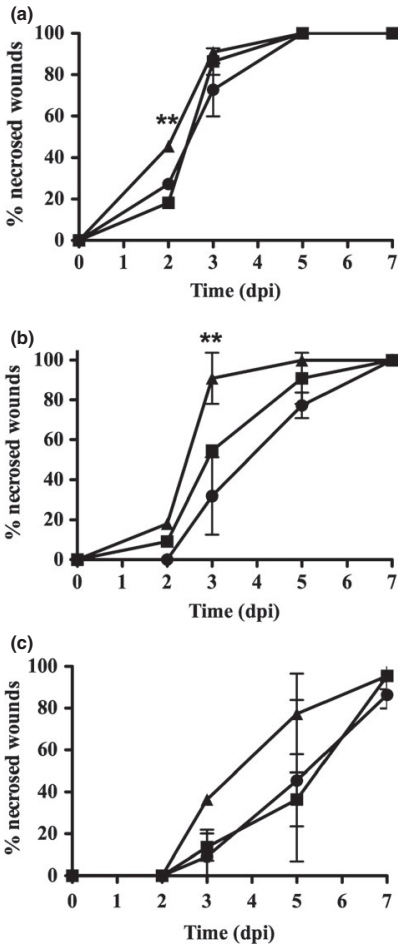

Fig. 5. Evaluation of virulence of the *Erwinia amylovora* strains CFBP 1430 (squares), *rpoS*$^-$ (triangles), and *rpoS*$^+$ (circles) in immature loquats at different inoculum doses. Fruits were inoculated in parallel with 10^4 (a), 10^3 (b), and 10^2 (c) CFU per wound. Represented data are the average values of two independent experiments with 11 replicates. Bars correspond to the SD. Asterisks denote statistically significant differences with the wild-type strain (**$P < 0.01$).

postinoculation (hpi), with no differences observed among strains. Negative controls did not elicit any response. With regard to survival inside tobacco leaf tissues (Fig. 7), all the strains behaved similarly during the first 24 hpi, showing a sharp loss of culturability from about 10^6 to 10^5 CFU cm^{-2}. During the following days, the wild-type strain first experienced a slight increase of culturability, followed by a slow drop of culturable cell numbers, which were about 10^5 CFU cm^{-2} at 8 dpi. This behavior was not observed in the mutant *rpoS*$^-$, which continued decreasing in culturable cell

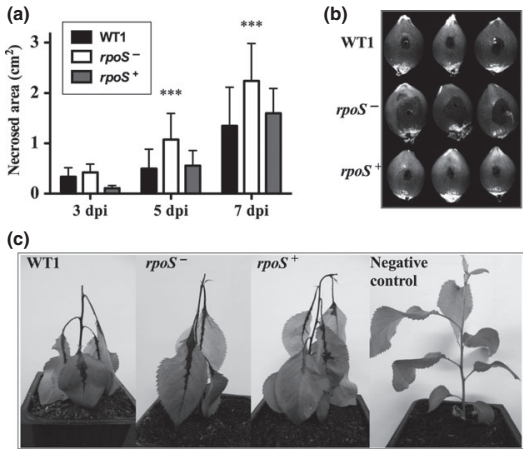

Fig. 6. Evaluation of the *Erwinia amylovora* virulence in immature fruits (a, b) and in pear plantlets (c) after their inoculation with 10^3 CFU per wound. Virulence was measured as the average necrosed areas in fruits or plants throughout time. Data in the graph are the average values of two independent experiments with 11 replicates. Bars correspond to the SD. Pictures represent characteristic fire blight symptoms in fruits and plantlets 5 days after their inoculation. WT1, *E. amylovora* strain CFBP 1430. Asterisks denote statistically significant differences with the wild-type strain (***$P < 0.001$).

numbers throughout the experimental period, reaching 6.5×10^3 CFU cm^{-2} at 8 dpi. The complemented strain behaved in a very similar way to the parental strain throughout the entire experiment. Differences between the mutant and the wild type (or the complemented strain) were statistically significant from time 2 ($P < 0.05$) to 8 dpi ($P < 0.001$).

Discussion

Microorganisms in nature are predominantly facing starvation and adapting to environmental fluctuations through complex developmental mechanisms tightly regulated at the genetic level (Ishihama, 1997; Morita, 1997; Edwards, 2000; Navarro Llorens *et al.*, 2010). In *E. coli* and other bacterial species, the alternative sigma factor RpoS plays an important role in the adaptation of the cell physiology to starvation, controlling genes related to stress responses (Edwards, 2000; Hengge-Aronis, 2000), DNA protection and repair (Vijayakumar *et al.*, 2004), nutrient recycling, and correct protein folding (Dong & Schellhorn, 2009), among others. In the present study, an *rpoS* mutant of the reference European *E. amylovora* strain CFBP 1430 was obtained, and the role of *rpoS* on starvation responses and other functions related to this gene hitherto unexplored in the fire blight pathogen were investigated.

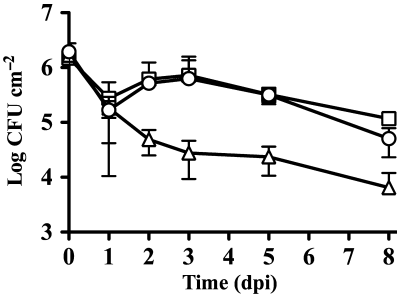

Fig. 7. Survival curves of *Erwinia amylovora* strains CFBP 1430 (squares), *rpoS*$^-$ (triangles), and *rpoS*$^+$ (circles) in tobacco leaf tissues. Represented data are the average values of two independent experiments performed in duplicate. Bars correspond to the SD.

Natural water microcosms were employed to analyze the role of the *rpoS* gene in *E. amylovora* responses to starvation, the main factor limiting bacterial survival in nature (Morita, 1997). The rapid entry of the *rpoS* mutant into the VBNC state accompanied by a loss of viability and cell integrity revealed the importance of this gene for the development of normal starvation responses in the fire blight pathogen, and hence for the survival and the adaptation of *E. amylovora* to natural oligotrophic conditions. These results were similar to those found in *E. coli* and *Salmonella enterica* mutants in the same gene (Boaretti *et al.*, 2003; Kusumoto *et al.*, 2012), indicating common functions of *rpoS* in human and plant pathogenic bacterial species. Regarding this, both survival under nutrient scarcity and the ability to form colonies on solid-rich media, a feature that is lost during entry into the VBNC state, have been related to the ability of bacterial cells to face the internal and external oxidative stresses generated by starvation-induced growth arrest and by growth on solid media, respectively (McDougald *et al.*, 2002; Boaretti *et al.*, 2003; Kong *et al.*, 2004). Interestingly, the sigma factor RpoS controls the expression of genes encoding catalases and many other proteins related to protection against oxidative stress (Eisenstark *et al.*, 1996; Hengge-Aronis, 2000, 2002). Accordingly, the altered regulation of oxidative stress-related proteins in the *rpoS* mutant might explain the altered starvation responses observed in this strain.

The alternative sigma factor RpoS controls genes related to stress resistance during the entry into stationary phase, and as a consequence, stationary phase cells become cross-protected against multiple stresses to which exponentially growing cells are sensitive (Hengge-Aronis, 2000; Lacour & Landini, 2004; Navarro Llorens *et al.*, 2010; Battesti *et al.*, 2011). In our work, we analyzed the role of *rpoS* on *E. amylovora* stationary phase cross-protection against oxidative and osmotic stresses, directly

related to the infectious process in the fire blight pathogen (Pusey, 2000; Venisse *et al.*, 2001), and also against acid stress, a condition to which *E. amylovora* and other phytopathogens have to adapt to colonize the plant apoplast (Grignon & Sentenac, 1991). The significant differences between the survivability of log and stationary phase cells of the parental and the *rpoS* mutant strains demonstrated the *rpoS* gene requirement for complete *E. amylovora* stationary phase cross-protection against oxidative, osmotic, and acid stresses. This highlights the contribution of *rpoS* to *E. amylovora* protection against these stresses during host infections and the apoplast colonization of nectaries or leaves. We additionally reported, for the first time, the essential role of the *rpoS* gene for *E. amylovora* stationary phase cell protection against heat shock, to which bacteria have to cope with environmental temperature fluctuations. This stress depends on the expression of heat shock proteins, which have been involved in the adaptation of bacterial cells to the environment (Feder & Hofmann, 1999). *E. amylovora,* usually detected in temperate regions, has, in recent years, been spreading to warmer countries which have more pronounced temperature variations (EPPO, 2013). Moreover, heat-shock proteins have also been implicated in infectious process in both animal and plant pathogens (Mantis & Winans, 1991; Feder & Hofmann, 1999), including *E. amylovora* during infection of immature fruits (Zhao *et al.*, 2005).

It is noteworthy that *rpoS* was apparently unnecessary for stationary phase cross-protection against acid shock. *E. amylovora* cells are subjected to this stress during fruit wound colonization, because of the release of tissue cell vacuolar compounds to the apoplast during the wounding, reaching pHs as low as 3.5 or 4. The colonization of wounds is a natural infection route frequently used by the fire blight pathogen (Vanneste & Eden-Green, 2000). The *rpoS*-independent protection of stationary phase cells against some stresses has been previously reported in *Vibrio vulnificus* (Rosche *et al.*, 2005) and *Pseudomonas aeruginosa* (Jørgensen *et al.*, 1999), and it has been attributed to other factors different to RpoS acting also during the entry into stationary phase.

The alternative sigma factor RpoS participates in the regulation of virulence in several pathogens (Dong & Schellhorn, 2010), including phytopathogenic bacteria (Flavier *et al.*, 1998; Andersson *et al.*, 1999; Wilf & Salmond, 2012). Consistently, we analyzed the participation of *rpoS* in the control of different virulence/pathogenicity factors such as motility and amylovoran and levan production. Motility has been reported as important during the early *E. amylovora* infection process, allowing cells to colonize apple blossoms, or to reach other natural infection sites such as stomata on the leaves (van der Zwet *et al.*, 2012). Amylovoran and levan are the two main exopolysaccharides of the fire blight pathogen. The first one is considered a pathogenicity factor, necessary for fire blight symptom development, and the second one is a virulence factor (Geider, 2000; van der Zwet *et al.*, 2012; Vrancken *et al.*, 2013). Both exopolysaccharides play additional roles, protecting *E. amylovora* cells against environmental challenges such as oxidative stress, desiccation, or starvation (Venisse *et al.*, 2001; Ordax *et al.*, 2010; Vrancken *et al.*, 2013). Our results revealed that *rpoS* contributes to the regulation of motility and the production of exopolysaccharides. In the case of motility, the *E. amylovora rpoS* mutant resulted significantly less motile than the parental strain, indicating the role of *rpoS* in enhancing cell motility during the colonization of nectaries and/or leave stomata under environmental conditions. Similar results were observed in *V. vulnificus* or *P. aeruginosa* (Dong & Schellhorn, 2010), although the control exerted by RpoS on motility-related genes may vary depending on the bacterial species analyzed (Dong & Schellhorn, 2010; Wilf & Salmond, 2012). With respect to the observed alteration of exopolysaccharide production in the *E. amylovora rpoS* mutant, mainly in levan secretion, a similar phenomenon has also been observed in *E. coli* (Ionescu & Belkin, 2009), *P. aeruginosa* (Suh *et al.*, 1999), and in the phytopathogenic bacterium *Ralstonia solanacearum* (Flavier *et al.*, 1998), but, as far as we know, this is the first report in the fire blight pathogen.

Given that the *E. amylovora rpoS* mutant showed an increased sensitivity to different stresses and an altered production of exopolysaccharides, the expected phenotype derived from the *rpoS* mutation would be an altered virulence, either diminished because of the sensitivity to stress inside the plant (Andersson *et al.*, 1999) or increased due to the overproduction of levan (Bereswill & Geider, 1997; Geider, 2000). According to our results, the *rpoS* mutation in the *E. amylovora* strain CFBP 1430 had different effects depending on the host model employed. In this respect, the mutant *rpoS*$^{-}$ was more virulent in immature loquats, but apparently as virulent as the parental strain in pear plantlets, indicating *rpoS* dispensability for fire blight symptom development, according to Anderson *et al.* (1998). However, there might be some kind of regulation of genes related to necrosis, at least in immature loquats, as the strain *rpoS*$^{-}$ caused more intense necrosis than the parental strain. Divergences in virulence results depending on the host assayed have also been reported in *rpoS* mutants of *Pectobacterium carotovorum* ssp. *carotovorum* (Andersson *et al.*, 1999) and *Serratia* sp. (Wilf & Salmond, 2012) and might be due to the distinct organs exposed to the pathogen, the inoculation procedure, and/or the different chemical composition or defenses elicited in each type of organ/host.

To explore the role of *rpoS* during *E. amylovora* incompatible interactions, we investigated the ability of the *rpoS* mutant to elicit a normal HR response and survive in tobacco plant tissues. The similar onset and characteristics of the necrosis observed in tobacco leaves inoculated either with the parental strain or the *rpoS* mutant revealed an apparently unaltered HR-inducing system. However, while the parental strain was able to slightly increase its numbers a few days after infiltration into tobacco leaves, the *rpoS* mutant showed a continuous drop of culturability over time. Differences among strains could be related to the inability of the mutant to face up to the stress conditions of the tobacco leaves' apoplast during the HR. This agrees with results of stationary phase cross-protection assays, in which *rpoS* was important for the protection of *E. amylovora* cells against oxidative stress. Interestingly, unlike other plant pathogens, *E. amylovora* also elicits an HR during compatible interactions (Venisse *et al.*, 2001), but the survival of the *rpoS* mutant was not compromised when artificially inoculated into immature loquats or pear plantlets. This apparent contradiction might be explained by the protective effect of the *E. amylovora* amylovoran capsule, whose production is induced by sorbitol and other environmental signals present in susceptible hosts (Geider, 2000), but not in non-*Rosaceae* plants (Deguchi *et al.*, 2006) during incompatible interactions.

In summary, this study reports new roles for the sigma factor RpoS in different ecological aspects of the fire blight pathogen life cycle. First, our results demonstrate the important contribution of the *rpoS* gene to the maintenance of culturability during the starvation survival response, as well as viability and cell integrity during entry into the VBNC state, revealing new aspects of these survival strategies not yet reported in any phytopathogenic bacteria. Second, *rpoS* is necessary for full stationary phase cross-protection against oxidative, osmotic, and acid stresses, and also against heat shock, which are directly related to the *E. amylovora* infectious process and/or survival in host and nonhost environments. Third, we provide evidence of the control exerted by the regulator RpoS on swimming motility and exopolysaccharide synthesis, as well as on fire blight symptom development in immature fruits, a trait not yet described in *E. amylovora*. Finally, we show, for the first time, that this sigma factor is essential for survival in the face of plant defense mechanisms during incompatible plant–pathogen interactions.

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Statement

We describe new roles for the *rpoS* gene in *E. amylovora*, including its contribution to: (1) the starvation survival and VBNC responses; (2) the stationary phase cross-protection; (3) the control of virulence factors such as motility and exopolysaccharide synthesis; (4) virulence; and (5) survival during incompatible plant–pathogen interactions.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. PCR and RT-PCR confirmation of the *rpoS* mutation and complementation in *Erwinia amylovora.*

Fig. S2. Phenotypic confirmation of the *Erwinia amylovora rpoS* mutation and complementation, by the growth inhibition halo assay, using 25 mM H_2O_2.

Fig. S3. Gelatin hydrolysis assay using nutrient broth agar containing 4 % (w/v) gelatin, after an incubation period of 11 days at 28 °C. Gelatin hydrolysis appears as a whitish halo surrounding the bacterial colonies.

Annex VII

Santander *et al.,* 2016, unpublished

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Title

***Erwinia amylovora* catalases KatA and KatG are virulence factors and delay the viable but nonculturable (VBNC) response**

Running title

Functional roles of *Erwinia amylovora* catalases

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Abstract

The life cycle of the plant pathogen *Erwinia amylovora* comprises periods inside and outside the host in which it faces oxidative stress caused by hydrogen peroxide (H_2O_2) and other compounds. Sources of this stress are plant defences, other microorganisms and/or exposure to starvation or other environmental challenges. However, the functional roles of H_2O_2 neutralizing enzymes like catalases during plant-pathogen interactions and/or under starvation conditions in phytopathogens of the family *Enterobacteriaceae* have not been yet investigated. In this work, the contribution of *E. amylovora* catalases KatA and KatG to virulence and survival in non-host environments was determined using catalase mutants and expression and activity analyses. The participation of *E. amylovora* exopolysaccharides (EPS) in oxidative stress protection was also investigated. Our study revealed: a different growth phase regulation of each catalase, with an induction by H_2O_2 and host tissues; the significant role of *E. amylovora* catalases as virulence and survival factors during plant-pathogen interactions; the EPSs induction by H_2O_2 despite the fact that apparently they do not contribute to protection against this compound; and the participation of both catalases in the detoxification of the starvation-induced intracellular oxidative stress, favoring the maintenance of culturability and, hence, delaying the development of the VBNC response.

Keywords

Fire blight; oxidative stress; gene expression; EPS; virulence factor; starvation; mass spectrometry

Introduction

Bacteria and other organisms inhabiting oxic environments possess mechanisms to detoxify reactive oxygen species (ROS). These ROS can be intracellularly produced by their own metabolism (Cabiscol *et al*., 2000; Kashmiri & Mankar, 2014), and also by abiotic and biotic processes outside the cell, such as during growth on solid rich media, competing microbiota or during host defences (Klotz & Loewen, 2003; Kong *et al*., 2004; Torres, 2010; Trias *et al.,* 2008). Among ROS, hydrogen peroxide (H_2O_2) is an especially toxic compound, due to its high stability, its ability to penetrate cell walls and membranes, and its high reactivity with proteins, lipids and nucleic acids (Klotz & Loewen, 2003; Linley *et al*., 2012). Accordingly, bacterial species, and particularly the pathogenic ones, have evolved specific enzymes, generally named hydroperoxidases, to neutralize H_2O_2 allowing their survival in diverse environments. These enzymes, usually containing a heme group, are termed catalases or/and peroxidases depending on the reaction pathway employed to decompose H_2O_2. Catalases cleave two hydrogen peroxide molecules in a two-step reaction cycle, releasing water and molecular oxygen. Peroxidases, on the other hand, use hydrogen peroxide to oxidize the heme iron, which is reduced by other electron donors such as NADH or ascorbate (Chelikani *et al*., 2004).

Erwinia amylovora is an economically important phytopathogenic bacterium that affects plants mainly pertaining to *Spiraeoideae,* a subfamily of *Rosaceae*. According to the annotated genomes, this pathogen possesses a monofunctional heme-containing catalase (KatA) and a bifunctional heme-containing catalase/peroxidase (KatG). As a non-obligate pathogen, the *E. amylovora* life cycle comprises of periods inside and outside the host, during which it needs to overcome oxidative stress. When inside a plant, *E. amylovora* has to cope with the H_2O_2 released by host cells, either during compatible or incompatible plant-pathogen interactions (Abdollahi *et al*., 2015; Venisse, 2001; 2003). During incompatible interactions, *E. amylovora* behaves similar to other pathogens. Bacteria are detected by plant cells, which respond with an oxidative burst followed by a hypersensitive response (HR), usually reducing the pathogen populations and stopping tissue colonization (Venisse *et al.,* 2001). In compatible plant-pathogen interactions, however, while many phytopathogenic bacteria use a type three-secretion system (TTSS) to avoid or reduce both the release of ROS and the HR (Berger *et al*., 2007; Fones & Preston, 2012), *E. amylovora* uses it to promote the opposite reaction. Both the oxidative burst and the HR are favored and employed to kill plant cells and progress within host tissues, causing the characteristic necrosis of fire blight disease

(Abdollahi *et al.*, 2015; Iakimova *et al.*, 2013; Venisse *et al.*, 2001; 2003). Siderophores, the lipopolysaccharide and exopolysaccharides (EPSs) seem to participate in *E. amylovora* protection against H_2O_2 (Berry *et al.,* 2009; Dellagi *et al.,* 1997; Király *et al.*, 1997; Venisse *et al.*, 2001; 2003), but the functional roles of catalases during *E. amylovora* interactions with plants have not yet been explored.

The *E. amylovora* exposure to biotic and abiotic sources of oxidative stress can also occur outside plants. For example, some of the microbiota inhabiting plant surfaces can exert antagonistic activity by the production of H_2O_2 (Trias *et al.,* 2008). Moreover, some preventive and/or control treatments of fire blight and other plant diseases consist of the application of H_2O_2 or other compounds causing oxidative stress (Adaskaveg *et al.,* 2006; Linley *et al.,* 2012; Teviotdale *et al.,* 1991). Besides, starvation and other stresses causing growth arrest are experienced by non-obligate phytopathogens during epiphytic stages, in soil, rainwater, vectors, etc., leading to the intracellular accumulation of H_2O_2 (McDougald *et al.*, 2002). Related to this, starvation conditions induce in *E. amylovora* and other bacteria the viable but nonculturable (VBNC) response (Oliver, 2010; Ordax *et al.*, 2012; 2015; Santander *et al.*, 2012; 2014a, b), characterized by the inability of viable cells to grow on routine solid media (Oliver, 2010; Ramamurthy *et al.,* 2014). Although mechanisms controlling this physiological response are not well known, some studies suggest the ability to detoxify H_2O_2 as a crucial factor enabling bacterial cells to grow on solid media (Flores-Cruz & Allen, 2011; Imamura *et al.,* 2015; Kong *et al.*, 2004; Kong *et al.,* 2014; Zhong *et al.,* 2009). However, the exact contribution of catalases to this process in *E. amylovora* and other pathogenic bacteria has not yet been determined.

In this work, the regulation, activity and functional roles of the *E. amylovora* catalases KatA and KatG in virulence and survival in non-host environments were analyzed by means of expression studies and insertional mutants in *katA* and/or *katG* genes. The potential contribution of *E. amylovora* EPSs in protection against oxidative stress was also investigated. In this study for the first time we provide valuable data on the contribution of *E. amylovora* catalases to plant-pathogen interactions and survival responses.

Results

Catalase gene expression is growth phase dependent, and induced by H_2O_2 and host tissues

The modulation of catalase gene expression in the *E. amylovora* wild-type strain CFBP 1430 under different conditions is represented in Fig. 1. The expression of *katA* gene in stationary phase cells was approximately 1.7 times higher than in log phase cells ($p < 0.05$) (Fig. 1a). The regulation of *katG* under the same conditions was the opposite, with expression levels in log phase cultures being about 3.4 times higher than in stationary phase cells ($p < 0.05$) (Fig. 1b). Moreover, exposure of *E. amylovora* log phase cells to a sub-lethal dose of H_2O_2 induced an increase in *katA* (Fig. 1a) and *katG* (Fig. 1b) gene expression 1.5 ($p < 0.05$) and 4.3 ($p < 0.01$) times above the levels observed in untreated cells, respectively. Furthermore, cells inoculated on pear slices, compared to stationary phase cells, raised the catalase mRNA levels 1.5 times in the case of *katA* ($p < 0.05$) (Fig. 1a), and 7 times in the case of *katG* ($p < 0.05$) (Fig. 1b).

The lambda Red recombinase system is useful in order to obtain mutants of the *E. amylovora* strain CFBP 1430

A summary of all the strains and plasmids used and obtained in this work is shown in Table 1. The electroporation of competent cells of the *E. amylovora* wild-type strain CFBP 1430 expressing the lambda Red recombinase genes with the linearized mutagenic constructs *katA*::Ω-Km or *katG*::Tc rendered 158 and 15 colonies, respectively, resistant to ampicillin (Ap) (due to the plasmid pKD46 carrying the lambda Red recombinase system) and kanamycin (Km) or tetracycline (Tc) (due to marker exchange mutagenesis), respectively. The mutation of *katA* and *katG* genes in a random selection of colonies was confirmed by PCR (Table S1; Fig. S1), and two mutants (*katA*$^-$ and *katG*$^-$) were selected for further experiments. The electroporation of the mutant *katA*$^-$ with the mutagenic construct *katG*::Tc rendered 27 colonies resistant to Ap^{100}, Km^{20} and $Tc^{12.5}$. The mutation of the two catalase genes in randomly selected colonies was also confirmed by PCR (Fig S1), and one of the double mutants (*katAG*$^-$) was selected for further experiments. Plasmid pKD46 curing after growth at 37ºC in medium without ampicillin was confirmed in the three mutant strains *katA*$^-$, *katG*$^-$ and *katAG*$^-$ by their inability to grow on ampicillin-amended medium, and by negative plasmid detection after plasmid isolation and agarose gel electrophoresis. The electroporation of the mutants *katA*$^-$ and *katG*$^-$ with plasmids pBBR4*KatA* and pBBR4*katG,* respectively, rendered about 10^4 Ap^{100} resistant transformants in each case, of which one was selected (*katA*$^+$ and *katG*$^+$, respectively) for further experiments.

KatA and KatG are the only sources of catalase activity in *E. amylovora,* with KatA being responsible for most of the activity detected

For the initial characterization of mutants, the specific catalase activity was measured in total protein extracts of overnight cultures in LB (Fig. 2). The wild-type strain showed an activity of 15821 ± 377 U mg protein^{-1} SD. This value was 13.9 times higher than that of mutant *katA*$^{-}$ ($p < 0.001$), but statistically indistinguishable from that of the *katG*$^{-}$ strain. These results indicate that under the assayed conditions, KatA is responsible for most of the catalase activity in *E. amylovora* and is able to compensate KatG catalase activity in the case of a strain defective in the corresponding gene (Fig. 2). No activity was detected in the double mutant *katAG*$^{-}$ ($p < 0.001$). Complemented *katA*$^{+}$ and *katG*$^{+}$ strains showed a specific catalase activity higher than that of the parental strain ($p < 0.001$ and $p < 0.05$, respectively) (Fig. 2).

Additionally, the specific catalase activity of an *E. amylovora* mutant in *rpoS* (Table1), highly sensitive to H_2O_2, was very similar to that of the mutant *katA*- ($p > 0.05$). Similarly, the complemented strain *rpoS*$^{+}$ (Table1) showed a specific catalase activity comparable to that of the complemented strain overexpressing *katA* ($p > 0.05$) (Fig. S2).

Only KatA is detected by native gel zymography and mass spectroscopy

When sufficient amounts of *E. amylovora* protein extracts were analyzed, catalase activity staining revealed two catalase isozymes (Fig. 3a). However, these two activity bands were only detectable in protein extracts of strains with a functional copy of the *katA* gene, such as the wild-type strain (Fig. 3a). The liquid chromatography - tandem mass spectrometry (LC-MS/MS) analysis of the proteins present in zymographic activity bands confirmed that peptides corresponding to KatA, but not to any other catalase, were abundant in the lower band and, to a lesser extent, in the upper band (Tables S2 and S3). These data suggest that the upper band was probably an aggregated form of the lower one. Catalase activity zymograms of *E. amylovora* wild-type cell extracts revealed a clear increase in KatA catalase activity in the stationary phase with respect to log phase cells (Fig. 3b). However, the induction of KatA activity in cells exposed to 0.35 mM H_2O_2 on LBA was not evident (Fig. 3c). Catalase staining also revealed a higher abundance of KatA in the soluble fraction of crude lysates than in total membrane proteins (Fig. 3c). Interestingly, heat treatment of crude lysates prior to nondenaturing polyacrylamide gel electrophoresis (native PAGE) did not fully

inactivate KatA, which was detectable by catalase staining after native PAGE of 1 μg total proteins (data not shown).

The zymographic staining of peroxidase activity also allowed the detection of a peroxidase band in the *E. amylovora* wild-type strain (Fig. 3d). However, in this case, this band only was detectable in protein extracts of 30 to 50-day-old cultures on LB agar (LBA) (Fig. 3d), but undetectable in total proteins (even using 150 μg) of stationary or log-phase cultures in LB or overnight cultures on LBA (data not shown). The parallel staining of catalase and peroxidase activities revealed similar sizes for peroxidase and catalase (KatA) bands. As expected, band protein identification revealed KatA as one of the most abundant proteins in samples showing peroxidase activity; however, a protein annotated as a peroxiredoxin (osmotically-inducible protein C) (GenBank ID CBJ45751.1) (Table S4) was also present in lower amounts.

It was not possible to detect activity bands corresponding to KatG in stationary or log phase cultures in LB, overnight plate cultures on LBA containing, or not, 0.35 mM H_2O_2, or 30 to 50-day-old cultures on LBA, regardless of the amount of total proteins loaded onto the gel (from 1 to 150 μg), or the performance of native PAGE at 4ºC to reduce protein degradation. Mass spectroscopy identification of proteins coming from either zymogram activity bands, or portions of gels above activity bands corresponding to KatA (the theoretical lengths of KatA and KatG are 490 and 730 aminoacids, respectively), did not allow the detection of KatG in any of the samples analyzed. Given the detection of catalase activity in crude lysates of the *E. amylovora* mutant *katA*$^-$ (Fig. 2), which presumably corresponds to KatG, and the *in vivo* detection of catalase activity associated with this enzyme in different assays (see results below), it is probable that KatG was not produced in the conditions assayed, degraded after cell lysis and/or during native PAGE conditions and/or it was below the detection limit.

Catalases, particularly KatA, are necessary for effective *in vivo* detoxification of H_2O_2 and menadione

Both the mutation of catalase genes and the complementation of *E. amylovora* impaired strains were phenotypically characterized by a growth inhibition halo assay using H_2O_2 and menadione, a compound which generates intracellular oxidative stress (Fig. 4). Catalase mutants showed increased sensitivity to the two H_2O_2 concentrations assayed with respect to the wild-type strain ($p < 0.001$), with the mutant *katA*$^-$ being more sensitive to the lowest concentration assayed (0.5M) compared to the mutant defective in *katG* ($p < 0.001$) (Fig. 4a). Both mutants behaved similarly when exposed

to 1 M H_2O_2 ($p > 0.05$), showing growth inhibition halos about 2.7 times larger than those observed in the wild-type strain (Fig. 4a). The largest growth inhibitory effect of H_2O_2 on bacterial growth was detected in the double mutant *katAG*$^-$, which developed halos about 6 times larger than those observed in the wild-type strain ($p < 0.001$) regardless of the concentration assayed (Fig. 4a). The complementation of *katA*$^-$ and *katG*$^-$ mutants with functional copies of the wild-type catalase genes totally or partially restored the mutant phenotypes (Fig. 4a).

With respect to menadione (Fig. 4b), only mutants *katA*$^-$ and *katAG*$^-$ showed significant sensitivity to this compound with respect to the parental strain. In the case of *katA*$^-$, the effects of menadione on bacterial growth were only detectable with the lower concentration assayed ($p < 0.05$), growth inhibition areas of this mutant being 1.4 times larger than those observed in the wild-type strain (Fig. 4b). The complementation of the catalase gene mutation restored the parental phenotype, with differences between strains *katA*$^-$ and *katA*$^+$ being small, but significant ($p < 0.01$) (Fig. 4b). The double mutant showed high sensitivity to menadione, with growth inhibition areas 1.7 and 3.3 times larger ($p < 0.001$) than those observed in the parental strain after treatment with 0.5 and 1 M menadione, respectively (Fig. 4b).

EPSs are induced by H_2O_2, but do not increase *E. amylovora* protection against this compound.

The study of the H_2O_2 minimal inhibitory concentration (MIC) (Table 2) revealed different H_2O_2 detoxifying capacities in each of the *E. amylovora* strains assayed. The wild-type strain grew in the presence of 20 mM H_2O_2 (Table 2), but not 40 mM H_2O_2 (data not shown). Catalase mutants showed increased sensitivity to H_2O_2 with respect to the parental strain, with mutant *katA*$^-$ unable to grow at 0.4 mM H_2O_2, and the growth of mutant *katG*$^-$ seriously impaired at 6 - 10 mM H_2O_2 and negative at 20 mM H_2O_2 (Table 2). The complementation of mutant *katA*$^-$ completely restored the parental phenotype. In the case of the *katG*$^+$ strain, however, the complementation resulted in an improvement of growth just at 6 – 10 mM H_2O_2, but not at 20 mM H_2O_2 (Table 2). The double mutant *katAG*$^-$ grew only at 0.06 mM H_2O_2 (Table 2).

The use of 1 % sorbitol or sucrose as carbon sources induced approximately 6-fold or 2-fold amylovoran and levan levels, respectively, with respect to glucose (data not shown). However, results corresponding to the H_2O_2 MIC assays did not vary regardless of the assayed carbon source, indicating an apparent null protective effect of *E. amylovora* EPSs against H_2O_2 under the assayed conditions (Table 2). Similarly,

248 the repeat of the H_2O_2 growth inhibition halo assay on minimal solid medium containing
249 sorbitol, sucrose or glucose as the carbon source resulted in undetectable differences
250 among media, regardless of the carbon source used, with all the strains showing
251 similar growth inhibition halos to those summarized in Fig. 4a.

252 Interestingly, the presence of H_2O_2 in culture media during *E. amylovora* growth
253 induced EPS synthesis (Fig. 5). In the case of amylovoran, the effects of H_2O_2 were
254 more visible in assays with medium containing a low concentration of sorbitol (0.2 %
255 w/v), with a 5-fold induction of EPS synthesis in the presence of 0.1 mM H_2O_2 with
256 respect to control medium without the oxidizing compound ($p < 0.001$). In medium
257 containing 1 % sorbitol, amylovoran production was 1.2 ($p < 0.05$) and 1.4 times ($p <$
258 0.001) higher in cells treated with 0.1 and 10 mM H_2O_2, respectively, than in control
259 cells (Fig. 5a). Levan production increased 1.4 times in cells exposed to 0.1 mM H_2O_2
260 ($p < 0.01$) and 3.6 times in those challenged with 10 mM H_2O_2 ($p < 0.001$) (Fig. 5b).

261 **Catalases KatA and KatG are virulence factors in *E. amylovora***

262 Virulence differences among *E. amylovora* strains were evaluated over seven days,
263 monitoring both the onset of fire blight symptoms and the necrosis progression in
264 immature fruits. The percentage of symptomatic pears over time was similar for most of
265 the assayed strains, except the ones inoculated with the catalase mutants *katG*$^-$ and
266 *katAG*$^-$ (Fig. 6a). These mutants were slower than the other strains in developing fire
267 blight symptoms throughout the assayed period; differences with the parental strain
268 being significant at time 4 dpi ($p < 0.01$) in both mutants, and at time 5 dpi ($p < 0.05$)
269 also in the case of the double mutant *katAG*$^-$ (Fig. 6a). When the extent of fire blight
270 lesions at 5 dpi was determined (Fig. 6b), significant differences ($p < 0.001$) were
271 detected in the size of necrotic areas compared to the wild-type strain, despite no
272 differences in growth rates were observed (Fig. S3). Smaller necrotic areas in mutants
273 *KatAG*$^-$ and *katG*$^-$ were probably due to the delay of these strains in the onset of
274 symptoms. The mutant *KatA*$^-$, however, which showed a similar onset of symptoms to
275 the wild type strain, also caused reduced necrotic lesions compared to the parental
276 strain ($p < 0.05$). The complementation of mutants restored the parental phenotype
277 (Fig. 6b).

278 **Catalases, especially KatG, favor survival in non-host tissues**

279 Catalase mutants inoculated into *Nicotiana benthamiana* leaf apoplast generated an
280 HR indistinguishable from that of the wild-type or the complemented *E. amylovora*
281 strains. However, the quantification of CFUs cm^{-2} of infiltrated tissue revealed

differences in the survival percentages among strains (Fig. 7). The parental strain experienced an increase to 124.8 ± 18.4 % SD with respect to time 0 during the first 3 dpi (Fig. 7), something which was not observed in the catalase mutants over the same time period. On the contrary, mutants *katA*$^-$*,* *katG*$^-$*,* and *katAG*$^-$ experienced a significant reduction in survival to 63.4 ± 35 ($p < 0.05$), 45.8 ± 44.1 ($p < 0.01$) and 30.0 ± 37.9 % SD, respectively. At 8 dpi the survival of the wild-type strain was 25.4 ± 7.7 % SD with respect to time 0 (Fig. 7), while for mutants *katA*$^-$*,* *katG*$^-$ and *katAG*$^-$ survivability was reduced to 17.7 ± 3.9 , 7.2 ± 0.4 and 5.9 ± 0.7 % SD, respectively, with statistically significant differences only in the case of *katG*$^-$ ($p < 0.05$) and *katAG*$^-$ ($p < 0.001$). The complemented strain *katA*$^+$ behaved in a similar way to the parental strain. In the case of *katG*$^+$ a significant increase in survival with respect to the wild-type strain was observed at time 8 dpi ($p < 0.05$) (Fig. 7).

Catalases favor the maintenance of culturability in starved cells, delaying the induction of the VBNC state

Prior to starvation survival studies, the effect of the mutation of catalase genes on the ability of *E. amylovora* to grow on LBA plates was assessed. No growth impairment was observed after drop-plating diluted overnight cultures on LBA, regardless of the assayed strain (Fig. S4). However, some differences were observed when responses to starvation were examined in *E. amylovora* catalase mutants (Fig. 8). The exposure of the wild-type strain to low nutrient conditions at 28ºC for 33 days caused a drop in culturable cell counts similar to that observed in single catalase mutants (Fig. 8a). Nevertheless, the double mutant *katAG*$^-$, showed a marked decrease in culturability with respect to the above-mentioned strains, which was significant from time 12 to 33 dpi ($p < 0.001$) (Fig. 8a). Complemented strains *katA*$^+$ and *katG*$^+$; however, experienced an improved maintenance of culturability with respect to the wild-type and catalase mutants, especially significant in *katA*$^+$ (Fig. 8a). In the case of *katG*$^+$, a small but significant improvement in culturability was observed, but only at the end of the experiment ($p < 0.001$) (Fig. 8a).

Total cell numbers, determined by flow cytometry, were maintained over time in about 8.20 log units, regardless of the assayed strain (Fig. 8b). Viability decreased about 0.2 log units from time 0 to time 33 dpi, but no differences were observed among strains (Fig. 8b). VBNC cell counts (viable cells minus culturable cells) at time 33 dpi were similar in the wild-type and the single mutants *katA*$^-$ and *katG*$^-$ (Fig. 8b). However, complementation with *katA* and *katG* genes reduced the number of VBNC cells,

316 although differences with the parental strain were only significant in the case of *katA*$^{+}$
317 ($p < 0.001$). The highest number of VBNC cells was recorded in the double mutant
318 *katAG*$^{-}$, with almost all the viable cells being non-culturable at 33 dpi ($p < 0.001$) (Fig.
319 8b). Interestingly, the addition of catalase to LB improved the culturability of *katAG*$^{-}$
320 cells from time 0 to time 26 dpi (data not shown), but did not avoid entry into the VBNC
321 state (Fig. 8b). At final time (33 dpi), the medium LB + CAT allowed the recovery of
322 about 2.3, 28.0, 1.4, 6.25 and 5.2 times more culturable cells of wild-type, *katA*$^{-}$, *katA*$^{+}$,
323 *katG*$^{-}$ and *katG*$^{+}$ strains, respectively, than on regular LB (Fig. 8b). The addition of
324 catalase to LBA plates did not enhance the recovery of culturability of the mutant
325 *katAG*$^{-}$ at this time period (Fig. 8b), but it did enhance recoverability from time 0 to 6 dpi
326 (data not shown).

327 **Discussion**

328 Catalases play a crucial role in preventing the formation of the highly reactive
329 hydroxyl radicals by neutralizing the H_2O_2 produced by metabolism, competing
330 microbiota and/or host defenses (Guo *et al*., 2012; Torres, 2010; Trias *et al.,* 2008).
331 However, despite the presumable key role of these enzymes in the survival and/or
332 proliferation of *E. amylovora* inside the host, or under conditions favoring the
333 intracellular accumulation of H_2O_2, such as nutrient starvation (McDougald *et al.,* 2002),
334 their regulation and physiological functions in this bacterial species had not yet been
335 investigated. In our study, differences in the growth phase regulation of *E. amylovora*
336 catalase genes together with their induction by H_2O_2 and host tissues may reflect the
337 contribution of catalases to cell homeostasis during ROS exposure within the host, but
338 also in the stationary growth phase, a physiological state resembling that of
339 microorganisms under the starvation conditions prevailing in nature (Navarro-Llorens *et*
340 *al.,* 2010).

341 The number and functions of catalases in plant pathogens can vary greatly
342 depending on the species analyzed (Jittawuttipoka *et al.,* 2009; Klotz & Hutchenson,
343 1992; Lee *et al*., 2005; Xu *et al.,* 2001). In the case of *E. amylovora*, only two genes
344 annotated as catalases are present on its genome. In fact, in our study we
345 demonstrated the absence of catalase activity in the double mutant *katAG*$^{-}$, confirming
346 that KatA and KatG are the only sources of catalase activity in the fire blight pathogen.
347 Interestingly, this information contrasts with the higher number of catalase isozymes
348 described in other plant associated bacteria which do not use oxidative stress to kill
349 host cells (Guo *et al*., 2012; Jamet *et al*., 2003; Tondo *et al*., 2010). However, the *E.*

350 *amylovora* specific catalase activity determined in this work was about 8 times higher
351 than that of the plant pathogen *Pseudomonas aeruginosa* PAO1 (harboring two
352 catalases) under similar conditions (Ma *et al.*, 1999). Likewise, Venisse *et al.* (2003)
353 identified that *E. amylovora* tolerance to H_2O_2 was 10 times greater than that of *P.*
354 *syringae* pv. tabaci*,* which possesses three catalase isozymes (Elkins *et al.*, 1999;
355 Klotz & Hutchenson, 1992). These data suggest the low number of catalases in *E.*
356 *amylovora* is compensated for by greater total catalase activity compared to other
357 phytopathogens.

358 It is worth mentioning that despite our efforts to detect the *E. amylovora* KatG
359 catalase-peroxidase by zymography and LC-MS/MS, we only succeeded in detecting
360 KatA. Similarly, in a previous work on the visualization of *E. amylovora* antioxidant
361 enzymes (Keck *et al.*, 1997), only one catalase band was detected in four different *E.*
362 *amylovora* strains, indicating the probable degradation of KatG under standard
363 experimental conditions.

364 Zymograms revealed major KatA activity in stationary compared to log phase cells,
365 which correlates well with expression analysis. However, although an induction of the
366 *katA* gene was observed after exposure to H_2O_2, the increase in KatA activity under the
367 same conditions was not evident in zymograms. Ma *et al* (1999) made a similar
368 observation while analyzing expression and activity of an orthologous catalase gene in
369 *P. aeruginosa.* These results suggest other regulation mechanisms of KatA in addition
370 to expression modulation.

371 Another purpose of our study was to investigate the contribution of *E. amylovora*
372 EPSs to oxidative stress resistance. Although the role of these macromolecules against
373 H_2O_2 has been demonstrated in some bacteria (Chen *et al.*, 2004; Davies & Walker,
374 2007; Fones & Preston, 2012), the case of *E. amylovora* has been discussed due to
375 contradictory results in different works (Király *et al.*, 1997; Venisse *et al.*, 2003). In our
376 study, two different assays indicated a similar growth inhibitory effect of H_2O_2
377 regardless of the induction of amylovoran or levan by medium composition. This
378 occurred in all the assayed strains, including the double mutant *KatAG*$^-$, which was
379 highly sensitive to oxidative stress. This supports the hypothesis of Venisse *et al* (2003)
380 that these macromolecules do not directly participate in *E. amylovora* protection
381 against H_2O_2. However, it is noteworthy that we observed an induction of amylovoran
382 and levan biosynthesis by H_2O_2, hitherto undescribed. This oxidizing compound is
383 synthetized during the oxidative burst, which is one of the first plant responses against

pathogens. It works as a bactericidal agent, favors the stiffening of plant cell walls and also acts as a signal molecule for plant cells (De Gara *et al*., 2003). Given the important role of *E. amylovora* EPSs during host tissue colonization (Geider, 2000; van der Zwet *et al.,* 2012), H_2O_2 might also be a good signaling molecule for *E. amylovora,* contributing to a rapid induction of EPSs and maybe other virulence/pathogenicity factors. This H_2O_2-mediated EPS induction might also explain the mucous phenotype developed by *E. amylovora* cells grown on media containing copper (Bereswill *et al*., 1998; Ordax *et al*., 2012), which increases ROS levels via the Fenton and Haber-Weiss reactions (Espírito Santo *et al*., 2011).

In this work, we also demonstrated that catalases are important virulence factors in *E. amylovora*, this being the first report of the role of catalases as virulence determinants in a phytopathogen of the family *Enterobacteriaceae*. The reduced virulence of catalase mutants in immature fruits and their lower survival in non-host tissues probably reflects an inefficient adaptive response to H_2O_2 upon exposure to oxidative stress during interactions with plants. Related to this, it is worth mentioning that, although several assays revealed KatA as the major catalase in the fire blight pathogen, the KatG deficient mutant was more affected than the one impaired in KatA during plant-pathogen interactions. This correlates well with expression studies, showing a 4.7-fold induction of *katG* gene expression with respect to that of *katA* during the infection of immature fruits. Our results might also be explained by an increase in KatG activity during interactions with plants, and/or other functional roles for this enzyme different from H_2O_2 dismutation during these interactions.

Regarding the role of catalases during *E. amylovora* responses to starvation, experiments with oligotrophic water microcosms revealed a faster entry of the mutant *katAG*$^-$ into the VBNC state, compared to the remaining strains. However, and similar to menadione assays, the presence of just one of the two catalases (either KatA or KatG) in single catalase mutants was enough for *E. amylovora* to develop a regular response to starvation. Additionally, a significant increase in culturable cell numbers (i.e. a diminished number of cells entering the VBNC state) was also observed either when catalase genes, especially *katA*, were overexpressed in complemented strains, or when an exogenous source of catalase was added to culture media. These data suggest a connection between catalase activity and the maintenance of culturability under starvation conditions, the opposite response to that occurring in the VBNC state. In fact, different studies have also provided mostly indirect evidence of the correlation between catalase activity and the VBNC response. For example, in *Vibrio vulnificus* a

decrease of catalase activity occurs in parallel with the loss of culturability of viable cells in response to cold under starvation conditions (Kong *et al.,* 2004). Moreover, oxidative-stress (H_2O_2) sensitive mutants impaired in the genes encoding the sigma factor RpoS or the regulator of H_2O_2-inducible gene OxyR develop an enhanced entry into the VBNC state in response to starvation, and/or show defective growth on solid medium unless it is amended with catalase (Flores-Cruz & Allen, 2011; Kong *et al.*, 2004; Kusumoto *et al.,* 2012; Miguel *et al.*, 2000; Santander *et al.,* 2014a; Shuxian *et al.,* 2012). However, these mutants are affected in the regulation of multiple genes, apart from catalases. As far as we know, we have demonstrated for the first time the contribution of catalases to the maintenance of culturability, hence delaying the starvation-induced VBNC response in a bacterial species.

Interestingly, contrary to that described in an *oxyR* mutant of *V. vulnificus* (Kong *et al.,* 2004), the regular growth of the *E. amylovora* mutant completely lacking catalase activity (*katAG*$^-$) on LBA plates evidenced that catalases are not an essential requirement to fight the H_2O_2 formed during solid media preparation (Kong *et al.,* 2004). On the other hand, the stronger induction of the VBNC response in an *E. amylovora* mutant *rpoS*$^-$ (Santander *et al.,* 2014a), not observed in the mutant *katA*$^-$, together with the similar specific catalase activities in these two mutants indicate that a loss of catalase activity is not enough to trigger the VBNC response, with other genes regulated by *rpoS* probably also being involved.

In summary, we have characterized, for the first time, the regulation of *E. amylovora* catalase genes *katA and katG*, and have also explored the contribution of catalases to exogenous and endogenous H_2O_2 detoxification, showing their outstanding role as virulence and survival factors during plant-pathogen interactions. Furthermore, we have identified a previously nondescribed induction of *E. amylovora* EPSs in the presence of H_2O_2, which might contribute to a rapid bacterial response to plant defenses. Finally, the demonstration of the role of catalases in enhancing the maintenance of culturability (i.e. delaying the VBNC response) under natural starvation conditions highlights the link between oxidative stress and the VBNC response, and demonstrates the contribution of catalases to the persistence of the pathogen in the environment in a culturable state.

Experimental procedures

Bacterial strains and culture conditions

The bacterial strains used in this study are summarized in Table 1. Unless otherwise specified *E. amylovora* and *E. coli* strains were grown at 28ºC and 37ºC, respectively, either on LBA plates or in liquid LB with shaking (150 rpm). When necessary, media were amended with kanamycin (Km^{20}; 20 µg mL^{-1}), ampicillin (Ap^{100}; 100 µg mL^{-1}), and/or tetracycline ($Tc^{12.5}$; 12.5 µg mL^{-1}).

Analysis of *katA* and *katG* gene expression

Sample preparation and RNA protection. Catalase gene expression was analyzed in *i) E. amylovora* cells from stationary (OD_{600} nm > 1) and log (OD_{600} nm 0.3) phase cultures in modified basal medium A (MBMA) (Edmunds *et al.*, 2013) containing 100 µg L^{-1} nicotinic acid and 0.2 % (wt/vol) glucose (MBMANic + 0.2 %Glc) as the carbon source; *ii)* log phase cultures in the same medium treated for 10 min with 1 mM H_2O_2, a sublethal dose of the oxidizing agent previously determined by a minimal inhibitory concentration (MIC) assay; *iii)* cells during infection of susceptible plant material. In the latter case, immature pears (*Pyrus communis* cv. Devoe) were surface disinfected with 2% (w/vol) sodium hypochlorite (NaOCl) as previously described (Santander *et al.*, 2014a; b), sliced into 1-cm-thick pieces and surface inoculated with 0.2 mL of *E. amylovora* overnight cultures in MBMANic + 0.2 % Glc. Fruits were incubated for 72 h at 28ºC inside a wet chamber, and bacterial cells recovered from the inoculated slice surface were re-suspended in fresh medium (MBMANic + 0.2 % Glc) and RNA protected as explained below.

RNA isolation and semi-quantitative reverse transcription (RT)-PCR conditions. The analysis of *katA* and *katG* gene expression was carried out by semi-quantitative RT-PCR, as previously described (Santander *et al.,* 2014b). RNA isolation, DNase treatments and RT-PCRs were performed with the RNeasy Mini Kit (Qiagen), DNA-free™ DNA Removal kit (Thermo Fisher Scientific) and one-step Access RT-PCR System (Promega), respectively, following the manufacturer's instructions. The number of thermal cycles for cDNA amplification was previously optimized to ensure they fell within the exponential phase of the amplification reaction (25 in the case of *katA* and 28 in that of *katG*), as described elsewhere (Rezzonico and Duffy, 2007; Santander *et al.*, 2014b). Relative expression levels were calculated based on band densities. For each of the assayed conditions, the expression level of each target gene was normalized to that of the constitutively expressed *rrs* gene, which was used as control. The fold-increase of expression compared to that in control cultures was then determined. Gene

485 expression analyses were performed in triplicate in, at least, two independent
486 experiments.

487 **Construction of catalase mutants and complemented strains**

488 According to the available *E. amylovora* genomes, the only proteins annotated as
489 catalases in this pathogen are KatA (GenBank ID CBA20721.1) and KatG (GenBank ID
490 CBA21169.1). Catalase mutants *katA*$^-$, *katG*$^-$ and *katAG*$^-$ were constructed using the λ
491 Red recombinase system (Datsenko & Wanner, 2000). This strategy allows site-
492 directed insertional mutagenesis after transformation of cells expressing recombinase
493 genes with a PCR amplicon carrying an antibiotic cassette flanked by DNA sequences
494 homologous to the target locus. In our case, mutagenic PCR products were obtained
495 using plasmids pGEM*katA*::Ω-Km or pGEM*katG*::Tc (Table 1) as templates (Santander
496 and Biosca, unpublished data), and the primers U-21 and Rev-22 (Table S1). After the
497 PCR, methylated plasmid DNA was removed by digestion with *Dpn*I, and the PCR
498 products were purified. *E. amylovora* CFBP 1430 cells carrying plasmid pKD46 (Table
499 1) were treated with 10 mM L-arabinose to induce recombinase genes and transformed
500 with purified PCR products. Double recombinants were selected based on antibiotic
501 resistances, and marker - exchange mutagenesis was confirmed by PCR (see
502 supporting information, Fig. S4) and DNA sequencing. Plasmid pKD46 was cured by
503 incubation at 37ºC (Datsenko & Wanner, 2000), which was confirmed by loss of
504 plasmid-derived ampicillin resistance, and by the absence of the corresponding
505 plasmid band after plasmid DNA isolation and gel electrophoresis.
506 To complement the mutation of *katA* and *katG*, DNA fragments containing the
507 coding region of each gene, together with their corresponding putative promoters, were
508 amplified by PCR and cloned into the broad-host-range and low-copy number vector
509 pBBR1MCS-4 (Table 1). Putative promoters were predicted using the software
510 BPROM (Solovyev & Salamov, 2011). Complementation plasmids were transferred to
511 mutant strains by electroporation.

512 **Total protein extraction, fractionation, heat treatment and quantification**

513 *E. amylovora* cultures were washed and re-suspended in 0.5 mL sterile 50 mM
514 potassium phosphate buffer (K-PB) pH 7.4 plus 0.05 % tween 20 (T20) and 1 mM of
515 the protease inhibitor phenylmethylsulfonyl fluoride (PMSF). Cell suspensions were
516 mechanically disrupted in tubes containing 0.5 g of glass beads (0.1 mm diameter),
517 using a Precellys 24 homogenizer (Bertin Technologies, France). Cell debris were

removed by centrifugation at 17000 *x g* for 10 min at 4ºC, and supernatants containing total proteins transferred to new tubes and preserved on ice.

For some assays fractionated crude lysates were employed. To this aim, a centrifugation step of 17000 *x g* for 90 min at 4ºC was performed (Biosca *et al*., 1993). The supernatant, corresponding to the cytoplasmic fraction was transferred to a new tube. The pellet containing total membrane proteins was re-suspended in ice-cold 200 μL 50 mM K-PB pH 7.0 containing 0.05 % T20 and 1 mM PMSF.

To determine the heat resistance of *E. amylovora* catalases, 400 μL total protein extracts were subjected to 55ºC for 25 min using a dry bath.

Total or fractionated protein contents were quantitated with the Pierce™ BCA Protein Assay Kit (Thermo Scientific) following the manufacturer's instructions, and using bovine serum albumin as the standard.

Specific catalase activity quantification

In this assay, the specific catalase activity of the *E. amylovora* wild-type, catalase mutants and complemented strains was measured. Additionally, in order to determine the catalase activity in a mutant in the alternative sigma factor RpoS obtained in a previous work (Santander *et al.,* 2014a), which was very sensitive to H_2O_2, the *E. amylovora* strains *rpoS*$^-$ and *rpoS*$^+$ (Table 1) were also included in this assay. Catalase activity in whole protein extracts was measured spectrophotometrically at A_{520} nm, using a colorimetric Catalase Assay Kit (Sigma Aldrich), according to the manufacturer's instructions. A catalase unit (U) was defined as the amount of enzyme necessary to decompose 1 μmole of H_2O_2 to oxygen and water per minute at pH 7.0, 25ºC and a substrate concentration of 50 mM H_2O_2. The specific catalase activity was calculated as the measured catalase U per mg of total protein. Cell lysates were assayed in triplicate.

Zymographic detection of catalase and peroxidase activities

To visualize catalase and peroxidase isozymes in *E. amylovora,* crude lysates or fractionated soluble or total membrane protein extracts were first subjected to non-denaturing PAGE. To this aim, 1 – 150 μg total protein extracts were mixed with 4x loading buffer (20 % saccharose, 0.05 % bromophenol blue), loaded onto a 10 % polyacrylamide mini gel and run under native conditions at 30 mA for 1 h, using Tris-Glycine running buffer (25 mM Tris, 192 mM glycine, pH 8.3).

550 Catalase and peroxidase zymograms were carried out based on Manchenko (2003).
551 For the zymographic detection of catalases protein extracts of *E. amylovora* stationary
552 (OD_{600} nm > 2) and log phase (OD_{600} nm 0.5) cultures in LB, or overnight plate cultures
553 on LBA or LBA plus 0.35 mM H_2O_2 were used. After native PAGE, the gel was rinsed
554 with distilled water and immersed in 50 mM K-PB pH 7.4 containing 0.03 % H_2O_2 for 15
555 min at room temperature, under gentle agitation. Then, the gel was rinsed again with
556 distilled water and submerged in a freshly prepared 1:1 aqueous mixture of 2 %
557 potassium ferricyanide and 2 % ferric chloride, until white catalase bands appeared on
558 a blue-green background. The reaction was stopped with abundant distilled water.

559 For peroxidase zymograms total protein extracts from *i)* 30 to 50-day-old cultures on
560 LBA, similar to Keck *et al* (1997); and *ii)* overnight plate cultures on LBA or *iii)* LBA plus
561 0.35 mM H_2O_2 were used. In this case, after completion of native PAGE, the gel was
562 rinsed with distilled water, immersed in 50 mM sodium acetate buffer pH 5.0 containing
563 0.003 % H_2O_2 and 0.05 % 3-amino-9-methylcarbazole or 3,3'-diaminobenzidine, and
564 incubated at room temperature with gentle shaking until peroxidase activity bands
565 appeared (Manchenko, 2003). The reaction was stopped using 50 % glycerol, after
566 rinsing the gel with distilled water.

567 **Protein identification by mass spectrometry (MS).**

568 For protein identification, activity bands from native polyacrylamide gels were cut
569 just after their zymographic detection and fixed with 40 % ethanol and 10 % glacial
570 acetic acid until use. The subsequent analyses were performed by the Proteomics
571 Support Unit of the Central Service for Experimental Research (SCSIE), at the
572 Universitat de València. For liquid chromatography and tandem mass spectrometry
573 (LC–MS/MS), samples were prepared by trypsinization as described elsewhere
574 (Shevchenko *et al*., 1996). Digestion reactions were stopped with trifluoroacetic acid (1
575 % final concentration) and peptides were extracted twice with acetonitrile, dried in a
576 rotatory evaporator and re-suspended in 10 μL of a solution composed of 2 %
577 acetonitrile and 0.1 % trifluoroacetic acid.

578 For LC–MS/MS, 5 μL of individual samples were loaded onto a trap column
579 (NanoLC Column, 3μC18-CL, 350 mm x 0.5 mm; Eksigent), desalted for 5 min with 0.1
580 % trifluoroacetic acid at 3 Ml min^{-1} and the peptides loaded onto an analytical column
581 (LC Column, 3μ C18-CL, 75 umx12cm, Nikkyo) equilibrated in 5% acetonitrile plus 0.1
582 % formic acid. A linear gradient elution of 5 to 35% acetonitrile, 0.1 % formic acid in 0.1
583 % formic acid was performed for 45 min at a flow rate of 300 nL min^{-1}. Peptides were

analyzed in a mass spectrometer nanoESI qQTOF (5600 TripleTOF, ABSCIEX). Sample ionization was carried out applying 2.8 kV to the spray emitter. Analysis was performed in a data dependent mode. Survey MS1 scans were acquired from 350–1250 m z^{-1} for 250 ms. A control of the system sensitivity was conducted with 2 fmol of 6 proteins (LC Packings).

Proteins were identified using the Protein-Pilot v4.5 (Sciex) search software. A peak list from 5600 TripleTof wiff files was directly generated using ProteinPilot default parameters. The ProteinPilot v 4.5 Paragon algorithm (Shilov *et al.*, 2007) was employed to search either the NCBI complete database, or a homemade database. The search parameters were trypsin specificity, cys-alkylation, no taxonomy restriction, and the search effort set to through. Sets of proteins sharing some physical evidence were grouped using the Pro group algorithm.

Growth inhibition halo assay

For this assay, 50 µL of overnight cultures in MBMANic + 0.2 % Glc adjusted to an OD_{600} nm of 1.0 were mixed with 10 mL of melted top agar (0.65 %), poured onto a plate containing 20 mL of solid medium (1.5 % agar), and let to dry under the hood. Afterwards, a sterile 0.5-cm-diameter filter paper disk was placed on the inoculated top agar surface and 10 µL of H_2O_2 or menadione at 0.5 or 1 M were loaded onto the disk. H_2O_2 is an important defense and signaling compound employed by plants and other organisms against pathogens. Menadione causes intracellular oxidative stress generating superoxide anions by redox cycling, which are dismutated inside the cell to H_2O_2 and other ROS by different mechanisms (Greenberg & Demple, 1989). Plates were incubated for 24 h at 28ºC and the growth inhibition area measured.

In a different set of experiments, in order to determine the potential role of the *E. amylovora* EPSs in protection against H_2O_2, the same assay was repeated amending media with 1 % glucose (control conditions), 1 % sorbitol (to induce amylovoran biosynthesis) or 1 % sucrose (to induce levan production) (Geider, 2000).

These assays were performed, in triplicate, in two independent experiments.

H_2O_2 MIC determination under EPS inducing conditions and effect of H_2O_2 on EPS production

The possible role of *E. amylovora* EPSs against H_2O_2 was also evaluated with a H_2O_2 MIC assay under control and EPS inducing conditions. To this aim, MBMANic + 1 % glucose (Glc) was used as the control medium, and media containing either 1 % sorbitol (Sor) or 1 % sucrose (Suc), were used to induce amylovoran and levan synthesis, respectively (Geider, 2000), as mentioned above. Prior to perform this

assay, the enhanced EPS production in these media was confirmed by EPS quantification as follows. Amylovoran was quantified by the CPC turbidimetric assay (A_{600} nm) as described elsewhere (Edmunds *et al.,* 2013; Santander *et al.,* 2014a). Levan production was measured comparing the turbidity of culture supernatants (A_{580} nm) of cells grown in medium containing Glc and Suc as the sole carbon source, respectively. Cells were inoculated at ca. 10^6 CFU mL^{-1} in tubes with 3 mL of medium containing 0 to 40 mM H_2O_2. Tubes were incubated at 28ºC with shaking (150 rpm), and the medium turbidity measured 24 and 48 h after the inoculation. This assay was carried out in two independent experiments in duplicate.

To investigate the effects of H_2O_2 on EPS biosynthesis, relative amylovoran and levan levels in culture supernatants of *E. amylovora* wild-type cells grown overnight in the absence or presence of H_2O_2 (0.1 or 10 mM) were measured. Amylovoran was induced in minimal basal medium A (Edmunds *et al*., 2013) containing either 0.2 or 1 % sorbitol as the carbon source and quantified as described above. Levan production was determined turbidimetrically (A_{580} nm) in LB, after mixing overnight culture supernatants with levansucrase activity buffer (50 mM K-PB pH 7.0, 2 M sucrose, 0.05%, w/v, sodium azide) in a ratio of 1:1, according to Bereswill *et al* (1998). In both cases, EPS levels were expressed relative to the A_{600} nm of the analyzed culture, as previously described (Santander *et al.,* 2014a). This experiment was performed in triplicate, in two independent assays.

Virulence/pathogenicity determination

Prior to virulence assays, growth curves in LB were performed to discard possible differences in virulence related to impaired growth. The virulence/pathogenicity of the *E. amylovora* catalase mutants, and the complemented and parental strains, was evaluated in immature pears (*P. communis* cv. Devoe). Fruits were surface-disinfected and inoculated with 10^3 CFUs in a wound using a sterile 100-μL pipette tip, as previously described (Santander *et al.,* 2014a). Each strain was assayed in 10 fruits, in two independent assays. Negative controls were inoculated with sterile saline (SS) (0.9 % NaCl). Challenged fruits were incubated at 28ºC in a wet chamber for one week under quarantine conditions. Fire blight symptom development was monitored over time, and the progression of necrosed areas analyzed with IMAGEJ software (Schneider *et al*., 2012), according to Santander *et al* (2014a). To confirm results, *E. amylovora* was re-isolated from symptomatic fruits and identified by specific PCR, as described elsewhere (EPPO, 2013; Santander *et al.,* 2014a; b).

HR elicitation and survival assays in non-host tissues

The potential role of the *E. amylovora katA* and *katG* genes in HR elicitation and/or survival in non-host tissues was evaluated according to Santander *et al* (2014a), employing *N. benthamiana* plants. Briefly, overnight cultures were washed, adjusted to an OD_{600} nm of 1.0 in SS, and infiltrated into different leaf sections with a needle-less syringe. Each leaf was inoculated in separate sections with each of the tested *E. amylovora* strains and a negative control (SS). A total of three plants and three leaves per plant were infiltrated with all the strains and incubated for 8 days at room temperature, under quarantine conditions. The survivability of each strain, inoculated in parallel in three leaves, was determined at times 0, 3 and 8 days post-inoculation (dpi) on semi-selective CCT agar medium (Ishimaru & Klos, 1984), after homogenizing and tenfold serially diluting infiltrated leaf sections according to Santander *et al* (2014a). Plates were incubated at 28ºC for 48-72 h, and bacterial colonies counted after this period. The survival percentage was calculated with respect to the CFUs cm^{-2} at time 0. This experiment was performed twice, in two independent repeats. The ability of mutants and complemented strains to grow on CCT similar to the wild-type strain was confirmed in previous experiments.

Microcosm preparation and monitoring of cell population dynamics

Prior to starvation-survival experiments and given the described production of H_2O_2 during solid media preparation (Kong *et al.,* 2004), the ability of the *E. amylovora* catalase mutant and complemented strains to grow on LBA was compared to that of the wild-type strain. To this aim overnight cultures in LB were adjusted to an OD_{600} nm of 0.1 (ca. 10^8 CFU mL^{-1}), serially tenfold diluted in SS and 5-μL drops of dilutions from 10^0 to 10^{-5} plated on LBA, and the growth of the different strains at the different dilutions after a 72-h period were compared.

Oligotrophic water microcosms were prepared and inoculated at a final cell density of ca. $2x10^7$ CFU mL^{-1} according to Santander *et al* (2014a), using overnight cultures washed twice in SS. Inoculated microcosms were incubated at 28ºC, under static, dark conditions. Culturable cell population dynamics were then determined on LBA and LBA containing 2000 U/plate bovine liver catalase (Sigma) (LB+Cat). Bacterial viability and total cell counts were measured by flow cytometry. For this purpose, 0.1 mL microcosm aliquots were taken at times 0 and 33 dpi, stained for 15 min with 0.1 mL of a mixture 1:1 of 3.34 mM SYTO9 and 20 mM propidium iodide following the Baclight Live/Dead viability kit (Life Technologies) manufacturer's instructions. Afterwards, 0.3 mL

FACSFlow solution (10 mM phosphate-buffered saline, 150 mM NaCl, pH 7.4; Becton-Dickinson) was added to the tube and live and dead bacterial cells were counted with a BD FACSVerse™ flow cytometer (BD Biosciences), with 488 nm excitation. Red and green fluorescence were measured with 700/54 and 527/32 filters, respectively. The trigger was set on FSC. Analysis of data was performed using BD FACSuite™ software (BD Biosciences). This experiment was conducted in triplicate, in two independent assays.

Statistical analysis

Data without a normal distribution such as percentages, or culturable, viable and/or total cell counts, were normalized by arcsine or log transformation, respectively, prior the statistical analysis. Comparisons between two means were carried out by a Student's t test. When the analysis of differences between more than two means was required, a one-way analysis of variance was performed (one-way ANOVA). When needed, the study of the effects and possible interactions of more than one factor on the analyzed means was performed using a two-way ANOVA followed by Bonferroni or Tukey post hoc tests. A $p < 0.05$ was considered significant.

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977 **Table and figure legends**

978 **Table 1.** Bacterial **s**trains and plasmids used in this study.

979 **Table 2.** H_2O_2 MIC in MBMANic containing different carbon sources to
980 induce *Erwinia amylovora* EPSs [a].

981 **Figure 1. Semi-quantitative RT-PCR analysis of *katA* (a) and *katG* (b) genes in**
982 ***Erwinia amylovora* wild type strain.** ST, stationary phase cultures; L, log phase
983 cultures; L + H_2O_2, log phase cells exposed to 1 mM H_2O_2 for 10 min; PEAR, *E.*
984 *amylovora* cells recovered from pear slices 72 h after their inoculation. Data are
985 average values of, at least, two experiments performed in triplicate. Vertical lines
986 correspond to the SD. Asterisks denote statistically significant differences between two
987 conditions (*$p < 0.05$; **$p < 0.01$).

988 **Figure 2. Specific catalase activity quantification.** Total crude lysates were
989 mechanically extracted from 2 mL overnight cultures in LB, and proteins quantified by
990 the BCA procedure. The specific catalase activity was measured with the Catalase
991 Assay Kit (Sigma-Aldrich), as the U of catalase per mg of protein. One catalase unit
992 was defined as the amount of enzyme necessary to decompose 1 μmole of H_2O_2 per
993 minute at pH 7.0, 25ºC and a substrate concentration of 50 mM H_2O_2. Data in the
994 graph correspond to the average values of an experiment performed in triplicate.
995 Vertical lines correspond to the SD. Asterisks denote statistically significant differences
996 with the wild-type strain, or between mutants and complemented strains (*$p < 0.05$; **p
997 < 0.01; ***$p < 0.001$).

998 **Figure 3. Zymographic detection of catalase and peroxidase activities in**
999 ***Erwinia amylovora*.** Zymograms were performed based on Manchenko (2003), as
1000 explained in the experimental procedures section. Zymographic identification of
1001 catalase isozymes (designated with Roman numerals) after native PAGE of 20, 10, 5
1002 and 2 μg total protein extracts (lanes 1, 2, 3 and 4, respectively) from overnight cultures
1003 in LB of the *E. amylovora* wild-type strain (a). Catalase staining of 5 μg total protein
1004 extracts from stationary and log phase cultures in LB. Lanes: 1, wild type; 2, *katA*$^-$; 3,
1005 *katA*$^+$; 4, *katG*$^-$; 5, *katG*$^+$; 6, *katAG*$^-$ (b). Catalase zymography of fractionated protein
1006 extracts of wild-type strain cultures on LBA supplemented, or not, with 0.35 mM H_2O_2.
1007 Lanes: 1, total membrane proteins; 2, soluble proteins (c). Peroxidase activity staining
1008 after native PAGE of 30 μg of 50-day-old plate cultures on LB of *E. amylovora*. Lanes:
1009 1, wild-type; 2, mutant *katA*$^-$ (d).

1010 **Figure 4. H_2O_2 (a) and menadione (b) growth inhibition halo assays with**
1011 ***Erwinia amylovora* wild-type, catalase mutants and complemented strains.** The

1012 growth inhibitory effect of H_2O_2 and menadione were determined by a disk assay on
1013 double layered agar plates of MBMA + 0.2 % Glc, as described in the experimental
1014 procedures section. Data correspond to average values of two independent
1015 experiments performed in triplicate. Vertical lines represent the SD. Statistically
1016 significant differences with the wild-type are represented with asterisks above the result
1017 bars. Differences between mutants and the corresponding complemented strains are
1018 labeled with asterisks above brackets encompassing the two strains (*$p < 0.05$; **$p <$
1019 0.01; ***$p < 0.001$).

1020 **Figure 5. H_2O_2-mediated induction of the *Erwinia amylovora* EPSs**
1021 **biosynthesis.** Relative amylovoran (a) and levan (b) levels were measured as
1022 previously described (Santander *et al.,* 2014a), in supernatants of wild- type cells
1023 grown overnight in media containing 0, 0.1 or 10 mM H_2O_2. Amylovoran production was
1024 induced in minimal basal medium A (Edmunds *et al*., 2013) either with 0.2 (light bars)
1025 or 1 % (dark bars) sorbitol. Levan synthesis was determined by measuring the relative
1026 levansucrase activity of wild-type overnight culture supernatants in LB (Bereswill *et al*.,
1027 1998). This assay was performed in triplicate. Vertical lines correspond to the SD.
1028 Asterisks denote statistically significant differences with untreated cells (* $p < 0.05$; **
1029 $p < 0.01$; *** $p < 0.001$).

1030 **Figure 6. Effect of the mutation of the *Erwinia amylovora katA* and *katG***
1031 **catalase genes on fruit blight symptom development.** Immature pears were
1032 inoculated with 10^3 CFU per wound, and virulence was measured as the percentage of
1033 symptomatic fruits over time (a), and the average surface of necrosed areas in
1034 challenged fruits at time 5 dpi (b), according to Santander *et al* (2014a). Data in graphs
1035 correspond to the average values of two independent experiments with 10 replicates.
1036 Vertical lines designate the SD. Asterisks indicate statistically significant differences
1037 with the wild-type strain (*$p < 0.05$; ****$p < 0.0001$).

1038 **Figure 7. Survival of *Erwinia amylovora* wild type, catalase mutants and**
1039 **complemented strains in *Nicotiana benthamiana* leaf tissues**. The survival
1040 percentage of each strain was calculated as the number of isolated CFU cm^{-2} at a
1041 given time with respect to time 0, according to Santander *et al* (2014a). Data are the
1042 average values of two independent experiments performed in triplicate. Vertical lines
1043 correspond to the SD. Asterisks denote statistically significant differences with the wild-
1044 type strain (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

1045 **Figure 8. Survival of *Erwinia amylovora* wild type, catalase mutants and**
1046 **complemented strains in oligotrophic natural water microcosms at 28ºC.**

Microcosms preparation and culturable, viable and total cell population dynamics were monitored as previously described (Santander *et al.*, 2014a; b). Culturable population dynamics on LB (a). Viable and total cell populations, and culturable cell counts on LB and LB+CAT at times 0 and 33 dpi (b). Data are average values of two independent experiments performed in triplicate. Vertical lines correspond to the SD. The dashed line indicates the detection limit for culturable cell counts, and the arrow plateable cell counts below the detection limit. Asterisks show statistically significant differences between total, viable or culturable cell populations with respect to the wild-type. Asterisks above the word VBNC denote differences only in the number of VBNC cells, not in viable cell counts (***$p < 0.001$).

1061 **Table 1.** Strains and plasmids used in this study.

1062

Strain or plasmid		Genotype or description	Source or reference
Strains			
E. amylovora			
	CFBP 1430	Wild type strain 1; isolated from *Crataegus* sp. in France	Paulin & Samson (1973)
	katA$^-$	Mutant strain derived from CFBP 1430; *katA*::ΩKm; Km^r	This study
	katA$^+$	Complemented mutant strain containing a functional copy of *katA* in pBBR4::*katA;* Km^r, Ap^r	This study
	katG$^-$	Mutant strain derived from CFBP 1430; *katG*::Tc; Tc^r	This study
	katG$^+$	Complemented mutant strain containing a functional copy of *katG* in pBBR4::*katG;* Tc^r, Ap^r	This study
	katAG$^-$	Double mutant strain derived from CFBP 1430; *katA::*Ω*Km, katG*::Tc; Km^r, Tc^r	This study
	rpoS$^-$	Mutant strain derived from CFBP 1430; *rpoS*::ΩKm; Km^r	Santander *et al* (2014a)
	rpoS$^+$	Complemented mutant strain containing a functional copy of *rpoS* in pBBR4::*rpoS;* Km^r, Ap^r	Santander *et al* (2014a)
E. coli			
	DH5α	*Sup*E44Ω*lac*U169 (80*lacZ*ΩM15) *hsdR*17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*-A1	Hanahan (1983)
Plasmids			
	pBBR1MCS-4	Broad-host-range and low-copy number expression vector; RK2; lacZ; Ap^r	Kovach *et al* (1995)
	pKD46	Plasmid containing the L-arabinose inducible lambda-red recombinase, Ap^r	Datsenko & Wanner (2000)
	pGEM*katA*::Ω-Km	pGEM*katA* containing the Ω-Km interposon from pHP45Ω-Km in the unique BamHI site inside the *katA*S gene; Ap^r; Km^r	Santander and Biosca, unpublished
	pGEM*katG*::Tc	pGEM*katG* containing the tetracycline resistance from pHC60 in the unique BamHI site inside the *katG* gene; Ap^r; Tc^r	Santander and Biosca, unpublished
	pBBR4::*katA*	pBBR1MCS-4 containing a functional copy of the *E. amylovora* (CFBP 1430) *katA* gene in the HindIII-*Spe*I restriction site	This study
	pBBR4::*katG*	pBBR1MCS-4 containing a functional copy of the *E. amylovora* (CFBP 1430) *katG* gene in the SalI-SpeI restriction site	This study

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Table 2. H_2O_2 MIC in MBMANic containing different carbon sources to induce *Erwinia amylovora* EPSs [a].

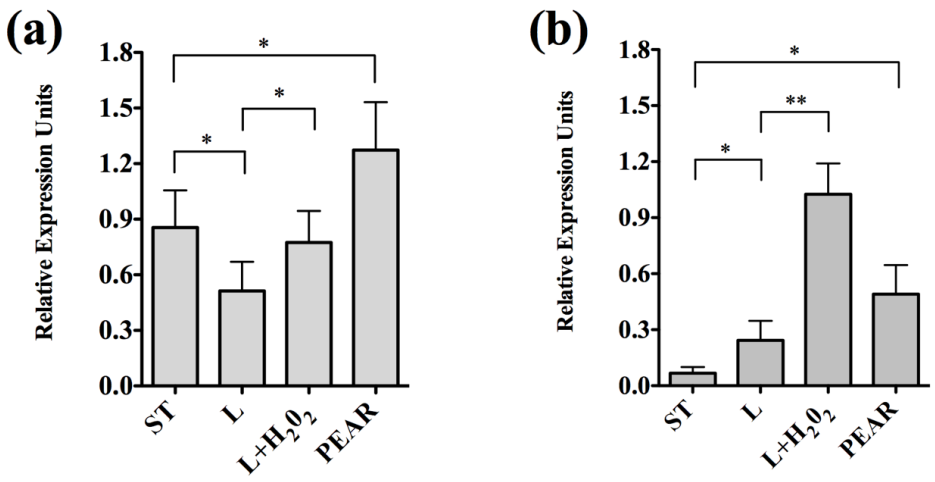
	Growth (after 24h / after 48 h)																	
[H_2O_2]	**WT**			***katA*⁻**			***katA*⁺**			***katG*⁻**			***katG*⁺**			***katAG*⁻**		
(mM)	**Glc**	**Sor**	**Suc**	**Glc**	**Sor**	**Suc**	**Glc**	**Sor**	**Suc**	**Glc**	**Sor**	**Suc**	**Glc**	**Sor**	**Suc**	**Glc**	**Sor**	**Suc**
0.06	**ND**[b]	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	+ / +	+ / +	+ / +
0.1	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	- / -	- / -	- / -
0.2	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	- / -	- / -	- / -
0.4	+ / +	+ / +	+ / +	- / +	- / +	- / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	- / -	- / -	- / -
0.6	+ / +	+ / +	+ / +	- / -	- / -	- / -	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	- / -	- / -	- / -
0.8	+ / +	+ / +	+ / +	- / -	- / -	- / -	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	- / -	- / -	- / -
1	+ / +	+ / +	+ / +	- / -	- / -	- / -	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	- / -	- / -	- / -
2	+ / +	+ / +	+ / +	- / -	- / -	- / -	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	- / -	- / -	- / -
4	+ / +	+ / +	+ / +	- / -	- / -	- / -	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	- / -	- / -	- / -
6	+ / +	+ / +	+ / +	- / -	- / -	- / -	+ / +	+ / +	+ / +	- / **W**[c]	- / **W**	- / **W**	- / +	- / +	- / +	- / -	- / -	- / -
8	+ / +	+ / +	+ / +	- / -	- / -	- / -	+ / +	+ / +	+ / +	- / **W**	- / **W**	- / **W**	- / +	- / +	- / +	- / -	- / -	- / -
10	+ / +	+ / +	+ / +	- / -	- / -	- / -	+ / +	+ / +	+ / +	- / **W**	- / **W**	- / **W**	- / +	- / +	- / +	- / -	- / -	- / -
20	- / +	- / +	- / +	- / -	- / -	- / -	+ / +	+ / +	+ / +	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -

[a] The carbon sources employed were: 1 % glucose (Glc, control), 1 % sorbitol (Sor, induces amylovoran) and 1 % sucrose (Suc, induces levan)

[b] ND, not determined

[c] W, weak growth

1073 **Figures**



1074

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1078 *amylovora* cells recovered from pear slices 72 h after their inoculation. Data are
1079 average values of, at least, two experiments performed in triplicate. Vertical lines
1080 correspond to the SD. Asterisks denote statistically significant differences between two
1081 conditions (*$p < 0.05$; **$p < 0.01$).

1082

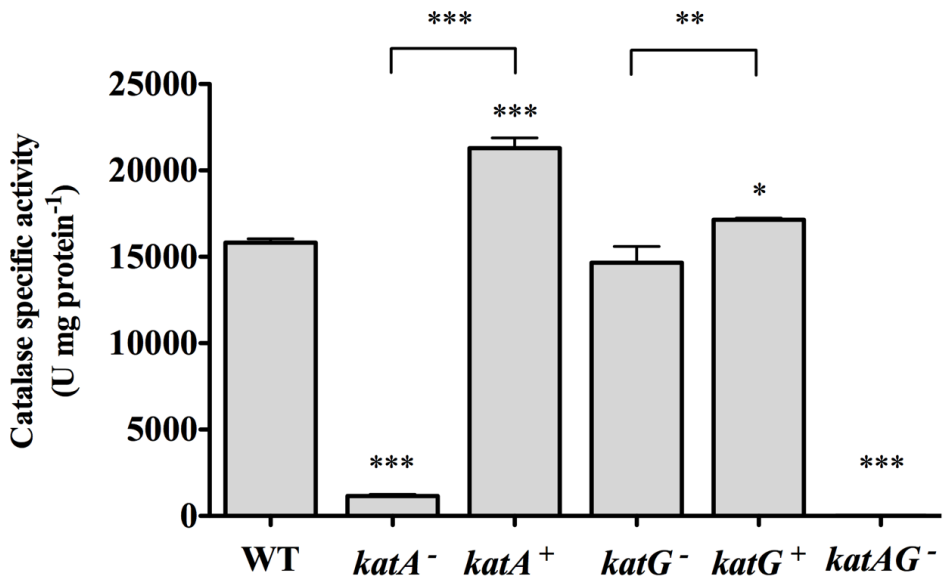
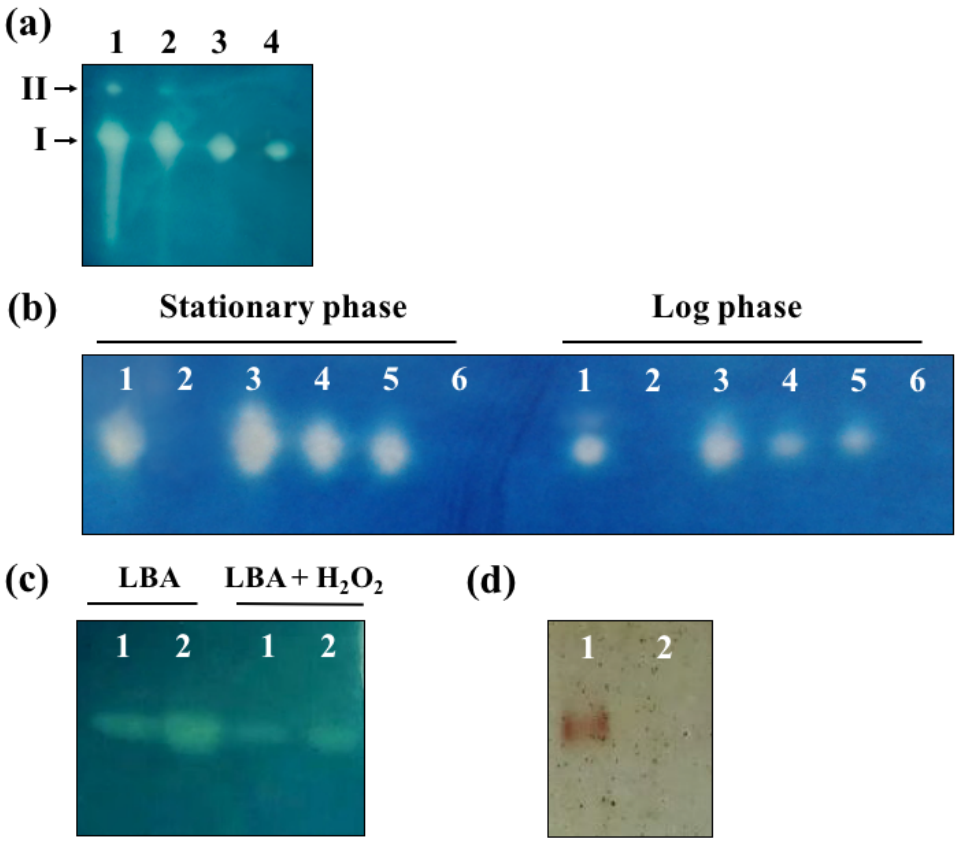


Figure 2. Specific catalase activity quantification. Total crude lysates were mechanically extracted from 2 mL overnight cultures in LB, and proteins quantified by the BCA procedure. The specific catalase activity was measured with the Catalase Assay Kit (Sigma-Aldrich), as the U of catalase per mg of protein. One catalase unit was defined as the amount of enzyme necessary to decompose 1 μmole of H_2O_2 per minute at pH 7.0, 25ºC and a substrate concentration of 50 mM H_2O_2. Data in the graph correspond to the average values of an experiment performed in triplicate. Vertical lines correspond to the SD. Asterisks denote statistically significant differences with the wild-type strain, or between mutants and complemented strains (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).



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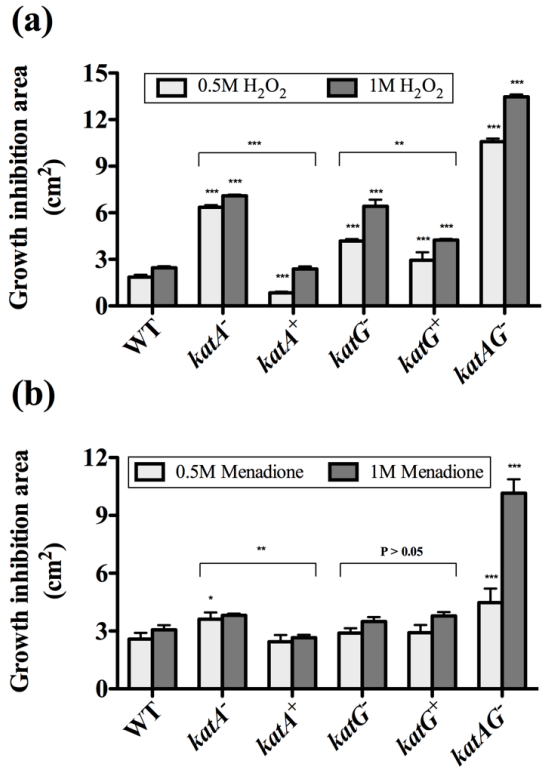
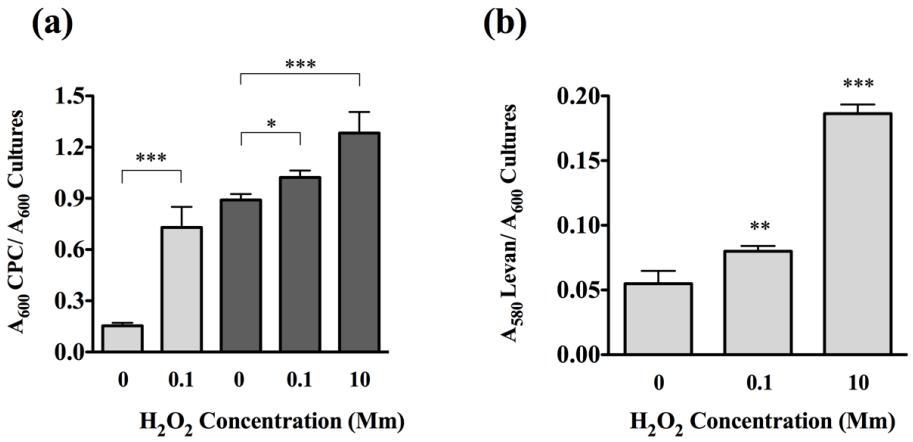


Figure 4. H_2O_2 (a) and menadione (b) growth inhibition halo assays with *Erwinia amylovora* wild-type, catalase mutants and complemented strains. The growth inhibitory effect of H_2O_2 and menadione were determined by a disk assay on double layered agar plates of MBMA + 0.2 % Glc, as described in the experimental procedures section. Data correspond to average values of two independent experiments performed in triplicate. Vertical lines represent the SD. Statistically significant differences with the wild-type are represented with asterisks above the result bars. Differences between mutants and the corresponding complemented strains are labeled with asterisks above brackets encompassing the two strains (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).



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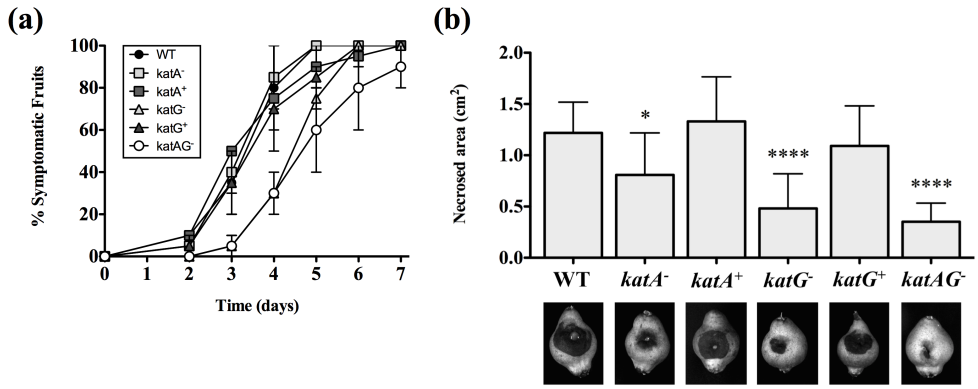

Figure 6. Effect of the mutation of the *Erwinia amylovora katA* and *katG* catalase genes on fruit blight symptom development. Immature pears were inoculated with 10^3 CFU per wound, and virulence was measured as the percentage of symptomatic fruits over time (a), and the average surface of necrosed areas in challenged fruits at time 5 dpi (b), according to Santander *et al* (2014a). Data in graphs correspond to the average values of two independent experiments with 10 replicates. Vertical lines designate the SD. Asterisks indicate statistically significant differences with the wild-type strain (*$p < 0.05$; ****$p < 0.0001$).

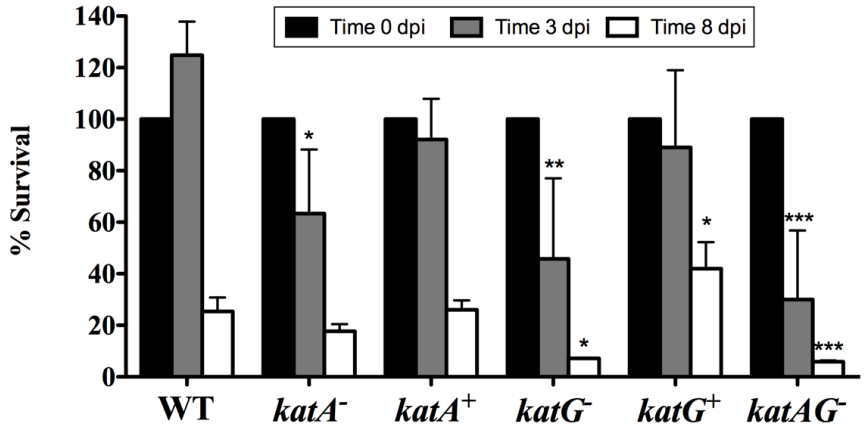

Figure 7. Survival of *Erwinia amylovora* wild type, catalase mutants and complemented strains in *Nicotiana benthamiana* leaf tissues. The survival percentage of each strain was calculated as the number of isolated CFU cm^{-2} at a given time with respect to time 0, according to Santander *et al* (2014a). Data are the average values of two independent experiments performed in triplicate. Vertical lines correspond to the SD. Asterisks denote statistically significant differences with the wild-type strain (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

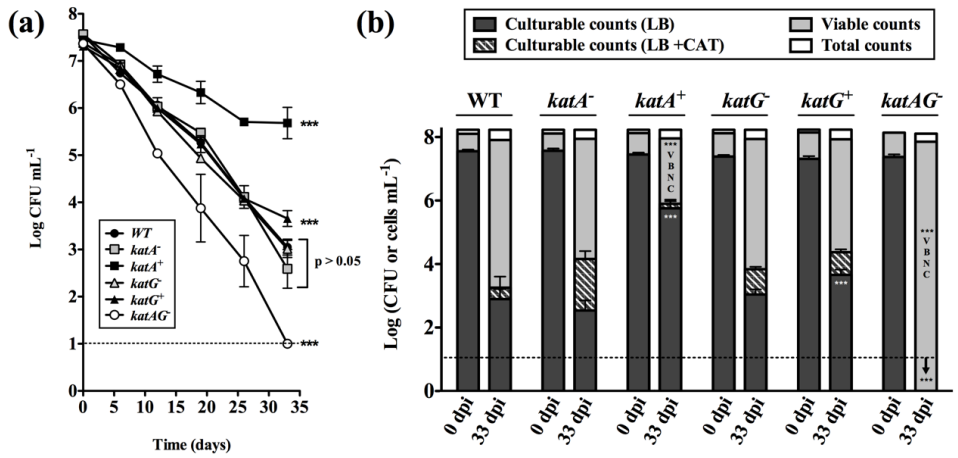

Figure 8. Survival of *Erwinia amylovora* wild type, catalase mutants and complemented strains in oligotrophic natural water microcosms at 28ºC. Microcosms preparation and culturable, viable and total cell population dynamics were monitored as previously described (Santander *et al*., 2014a; b). Culturable population dynamics on LB (a). Viable and total cell populations, and culturable cell counts on LB and LB+CAT at times 0 and 33 dpi (b). Data are average values of two independent experiments performed in triplicate. Vertical lines correspond to the SD. The dashed line indicates the detection limit for culturable cell counts, and the arrow plateable cell counts below the detection limit. Asterisks show statistically significant differences between total, viable or culturable cell populations with respect to the wild-type. Asterisks above the word VBNC denote differences only in the number of VBNC cells, not in viable cell counts (***$p < 0.001$).

Supplementary information

Supplementary tables

Table S1. Primers used in this study.

Primers*	Sequence
Mutagenesis constructs amplification	
U-21	GTTGTAAAACGACGGCCAGTG
Rev-22	CACACAGGAAACAGCTATGACC
Mutagenesis confirmation by PCR	
***katA*_EXT.F**	GTTACGGGCGGTAGTAAAGTT
***katA*_EXT.R**	CCAATGCCATGACTACACTC
T4-Ω	AGCTTGCTCAATCAATCACCG
***katG*_EXT.F**	ACCAGTTTAAGGGAAGCAGATG
***katG*_EXT.R**	CAGGCTATATCCCGTTGTGAAT
***tetA*1**	GGCGTCGAAGCCTGTAAAG
***tetA*2**	ACTGGCACTTCAGGAACAAG
Mutant complementation	
***katA*_HindIII.F**	GAACCTAA**AAGCTT**CCACATGCTGTCAGGTGG
***katA*_SpeI.R**	CCTTGGA**ACTAGT**GACACAGAAGCCTGGAAAG
***katG*_SalI.F**	GTCATC**GTCGAC**CCCTGAACTGTTGGTTCA
***katG*_SpeI.R**	GTCCCT**ACTAGT**GAGCAATAAACTTGCGAACGGTG
RT-PCR	
***katA*_RT-PCR.F**	AGTGGGTAAACTGGTGCTGGA
***katA*_RT-PCR.R**	GGTGAGCATCCGCATAGGC
***katG*_RT-PCR.F**	CCTCAAGTGGGCTTTCAGTC
***katG*_RT-PCR.R**	ATCCGCTAACGACATTTTGCCG
***rrs*.F**	CAGCCACACTGGAACTGAGA
rrs.R	GTGCTTCTTCTGCGGGTAAC

* Bold nucleotides indicate the restriction site for the enzyme indicated in the primer's name; underlined nucleotides are a clamp sequence.

Table S2. Proteins detected by LC–MS/MS analysis in the lower catalase activity band obtained by zymographic staining (according to Manchenko, 2003) of *E. amylovora* protein extracts after native PAGE. Proteins were identified using the Protein-Pilot v4.5 (Sciex) search software (see material and methods for further details). This table lists the winner protein for each group, sorted by Unused ProtScore. Parameters shown in this table and their definitions are: N, rank of the specified protein relative to all other proteins in the list of detected proteins; unused (ProtScore), measure of the protein confidence for a detected protein, calculated from the peptide confidence for peptides from spectra that are not already completely "used" by higher scoring winning proteins; total (ProtScore), measures the total amount of evidence for a detected protein and is calculated using all of the peptides detected for the protein; % Cov (Coverage), is the percentage of matching amino acids from identified peptides having confidence greater than 0 divided by the total number of amino acids in the sequence; % Cov (50), is the percentage of matching amino acids from identified peptides having confidence greater than or equal to 50% divided by the total number of amino acids in the sequence; % Cov (95), is the percentage of matching amino acids from identified peptides having confidence greater than or equal to 95% divided by the total number of amino acids in the sequence; accession #, is the accession number for the protein; name, name of the protein; species, the species for this protein (not all FASTA files have species, so this column may be blank). If "(contaminant)" is present, this protein came from the ABSciex_ContaminantDB FASTA file; peptides (95%), number of distinct peptides having at least 95% confidence. Multiple modified and cleaved states of the same underlying peptide sequence are considered distinct peptides because they have different molecular formulas. Multiple spectra of the same peptide, due to replicate acquisition or different charge states, only count once.

Due to its size, this table is in a separate ".xlsx" supplementary document.

Table S3. Proteins detected by LC–MS/MS analysis in the upper catalase activity band obtained by zymographic staining (according to Manchenko, 2003) of *E. amylovora* protein extracts after native PAGE. Proteins were identified using the Protein-Pilot v4.5 (Sciex) search software (see material and methods for further

details). This table lists the winner protein for each group, sorted by Unused ProtScore. Parameters shown in this table and their definitions are explained in the previous table description.

Due to its size, this table is in a separate ".xlsx" supplementary document.

Table S4. Proteins detected by LC–MS/MS analysis in the peroxidase activity band obtained by zymographic staining (according to Manchenko, 2003) of *E. amylovora* (40-day old cultures on LBA) protein extracts after native PAGE. Like in the previous cases, proteins were identified using the Protein-Pilot v4.5 (Sciex) search software, however, in order to ease interpretation of results, the ProteinPilot v 4.5 Paragon algorithm was employed to search in a homemade database composed of all the sequenced genomes of the genus *Erwinia.* Only proteins related to oxidative stress resistance are shown. This table is also in a separate ".xlsx" supplementary document.

Supplementary figures

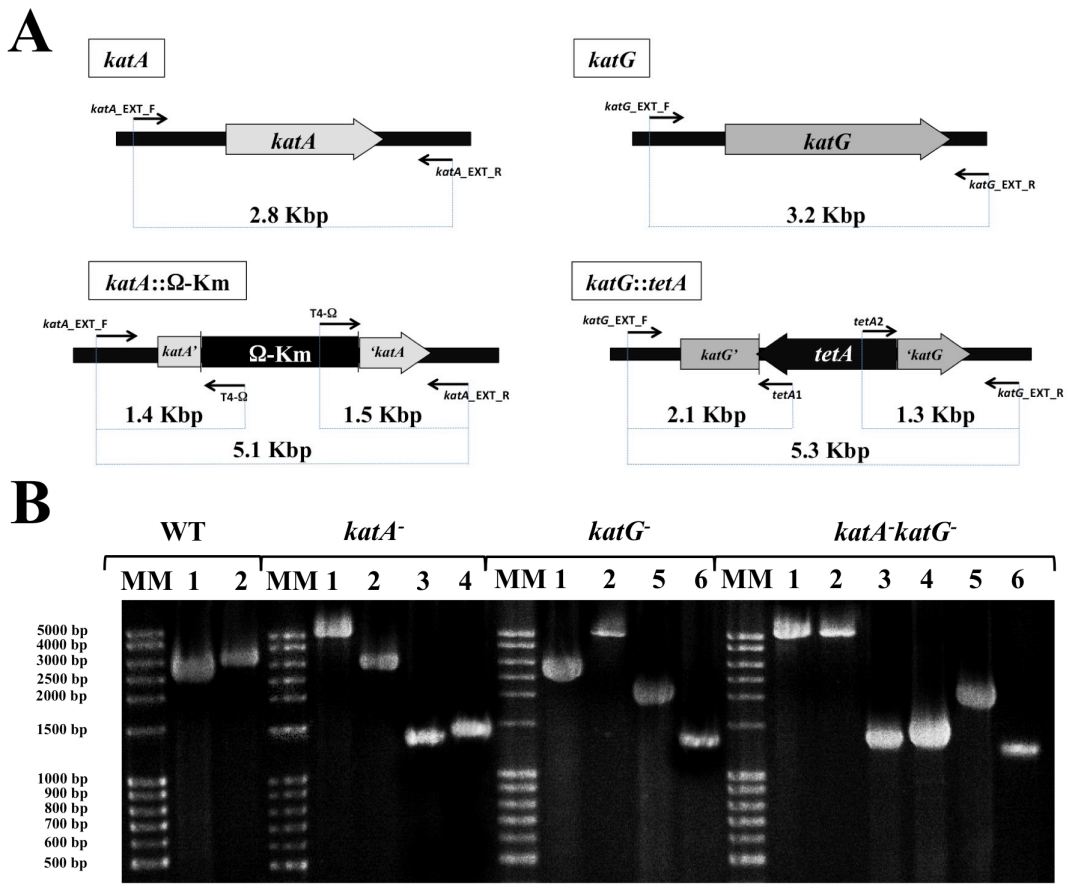

Figure S1. PCR confirmation of *katA* and/or *katG* mutagenesis in *Erwinia amylovora.* Scheme of the wild-type and mutated *katA* and *katG* genes, as well as the theoretical sizes of PCR amplicons in the wild-type (WT) as well as in the mutant strains *katA*$^-$, *katG*$^-$ and *katA*$^-$*katG*$^-$ (*katAG*$^-$), using different combinations of primers (A). PCR confirmation of the marker exchange mutagenesis in mutants *katA*$^-$, *katG*$^-$, and *katAG*$^-$. MM, molecular marker exACTGene Mid Range Plus DNA Ladder (Fisher Scientific); 1, PCR with primers *katA*_EXT.F and *katA*_EXT.R; 2, PCR with primers *katG*_EXT.F and *katG*_EXT_R; 3, PCR with primers *katA*_EXT_F and T4-Ω; 4, PCR with primers *katA*_EXT.R and T4-Ω; 5, PCR with primers *katG*_EXT_F and *tetA*1; 6, PCR with primers *katG*_EXT_R and *tetA*2 (B).

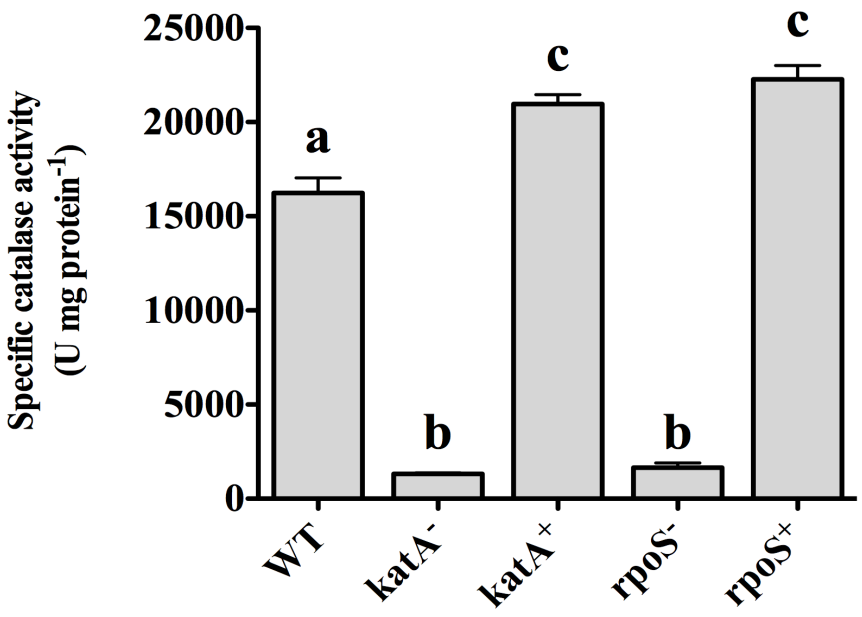

Figure S2. Comparison of the *Erwinia amylovora* KatA and RpoS defective strains specific catalase activity. For comparative purposes the wild-type (WT) as well as the corresponding complemented strains are also included. The specific catalase activity was determined with the Catalase Assay Kit (Sigma-Aldrich), using total proteins extracts from 2 mL overnight cultures in LB. Strains *rpoS*- and *rpoS+* were obtained in a previous work (Santander *et al*., 2014a). Data in the graph correspond to the average values of an experiment performed in triplicate. Bars correspond to the SD. Different letters denote statistically significant differences among strains ($p < 0.01$).

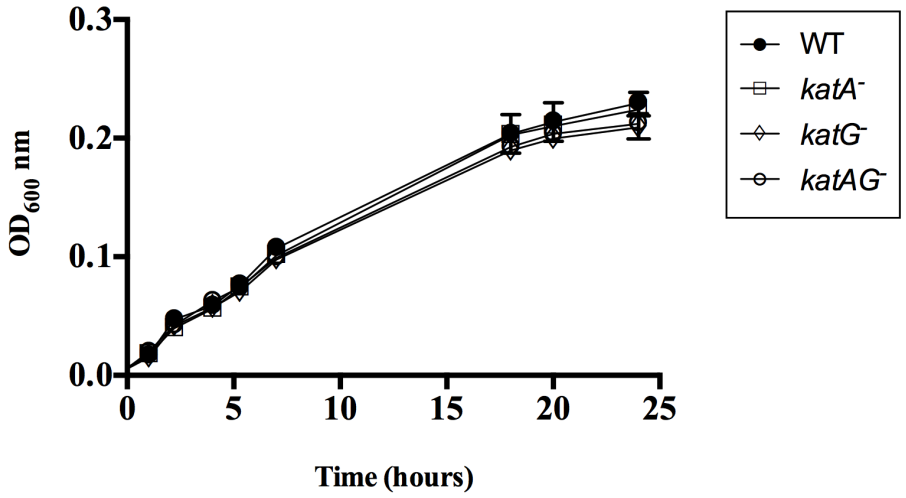

Figure S3. Growth curves of the *Erwinia amylovora* wild-type and catalase mutant strains in LB under static conditions at 28ºC. Data in the graph correspond to the average values of an experiment performed in triplicate. Vertical lines correspond to the SD. No statistically significant differences were observed among strains ($p > 0.05$).

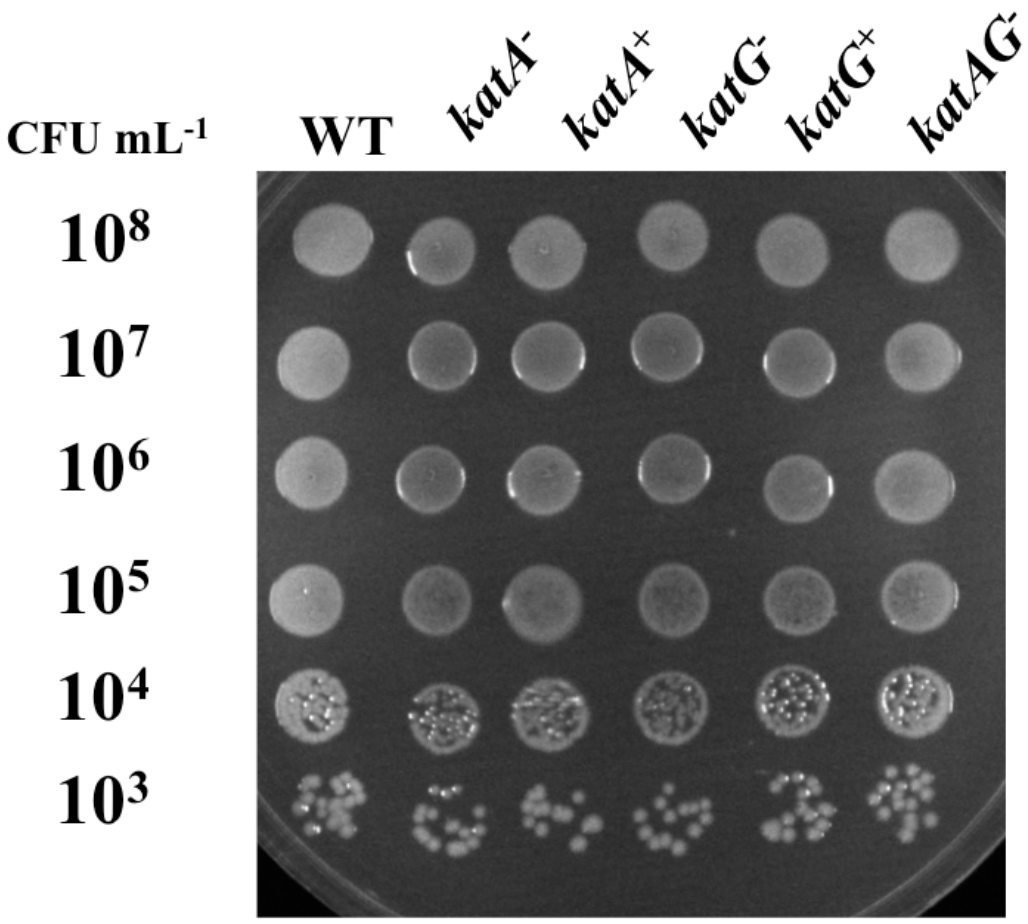

Figure S4. Effect of catalase gene mutation in *E. amylovora* growth on solid medium. Given the described formation of H_2O_2 during solid rich media preparation (Kong *et al.,* 2004), we aimed to compare the growth of the *E. amylovora* wild-type, catalase mutant and complemented strains on LBA plates. For this purpose, overnight cultures were adjusted to an OD_{600} nm of 0.1 (about 10^8 CFU mL^{-1}), and 5 µL drops of serial tenfold dilutions in SS (from 100 to 10^{-5}) were plated on LBA. The growth of each strain was compared after a 72-h incubation period at 28ºC under dark conditions.