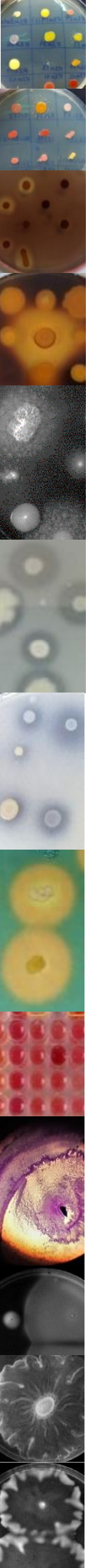


VNIVERSITAT DE VALÈNCIA

BACTERIAL COMMUNITIES ASSOCIATED WITH THE LICHEN *Ramalina farinacea* (L.) Ach.: COMPOSITION, BIODIVERSITY AND BIOTECHNOLOGICAL POTENTIAL

ÀNGELA FIGÀS SEGURA

Supervisor: Dr. Elena González Biosca

November 2017

DOCTORAL THESIS

Department of Microbiology and Ecology

Doctorate Programme of Biomedicine and Biotechnology

Department of Microbiology and Ecology
Doctorate Programme of Biomedicine and Biotechnology

DOCTORAL THESIS

November 2017

BACTERIAL COMMUNITIES ASSOCIATED WITH THE LICHEN *Ramalina farinacea* (L.) Ach.: COMPOSITION, BIODIVERSITY AND BIOTECHNOLOGICAL POTENTIAL

Author:
Àngela Figàs Segura

Supervisor:
Dr. Elena González Biosca

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,

INFORMA QUE

Àngela Figàs Segura, licenciada en Biología por la Universitat de València, ha realizado bajo su dirección el presente trabajo, titulado: "**BACTERIAL COMMUNITIES ASSOCIATED WITH THE LICHEN *Ramalina farinacea* (L.) Ach.: COMPOSITION, BIODIVERSITY AND BIOTECHNOLOGICAL POTENTIAL",** y que hallándose concluido, autoriza su presentación a fin de que pueda ser juzgado por el tribunal correspondiente y optar así a la obtención de grado de Doctor por la Universitat de València, con la Mención de "Doctor Internacional", dentro del Programa de Doctorado en Biomedicina y Biotecnología.

Y para que así conste, en cumplimiento de la legislación, firmo el presente informe en Valencia, en noviembre de 2017:

Dra. Elena González Biosca

Para la realización de la presente Tesis Doctoral, la autora ha sido beneficiaria de una beca predoctoral para la contratación de personal investigador en formación VALi+d concedida por la Conselleria d'Edudació, Cultura i Esport en la Resolución del 1 de julio de 2014, así como una beca BEFPI (BEFPI/2016/032) en la Resolución de 7 de junio de 2016, de la misma entidad, para realizar una estancia predoctoral de 4 meses en el laboratorio del Dr. Fierer en la University of Boulder (CO, USA). Esta Tesis ha sido financiada parcialmente con el proyecto PROMETEO (PROMETEOII/2013/021) y los fondos de Ayuda a la Investigación de la Universitat de València a la línea BACPLANT, así como otros fondos de BACPLANT y del laboratorio del Dr. Fierer.

A mis padres, mi hermana y

los que rodean mi vida.

AGRADECIMIENTOS

Hace más de tres años que empecé mis andaduras con esto del doctorado. A decir verdad, en mi mente nunca había tenido una idea clara de realizar algo así, pero las cosas del futuro, o en este caso ya del pasado, que nunca se sabe lo que guardan para uno, me llevaron a embarcarme en esta tarea. Pero esto no podría haber sido así sino hubiera sido gracias a mi directora de tesis, Elena. Gracias por haber contado conmigo desde un principio para la realización de este trabajo, por enseñarme tanto y motivarme a intentar hacer las cosas con rigor científico y poder descubrir en la ciencia y la investigación una forma de vida capaz de atraparte. Agradecerte también tu paciencia y tu tesón en todo, sobre todo en la última etapa de este proyecto, sabemos que no ha sido fácil. Gracias por todo, de corazón.

Por supuesto, y aunque nombrados en segundo lugar, ni esta Tesis ni nada de lo que he hecho en mi vida hubiese sido posible sin el apoyo incondicional de mi familia, mis padres Jose y Joana, y mi *sistah* Marta, por estar ahí siempre, apoyando y alentando cada uno de mis proyectos, tanto profesionales como personales, porque nada de esto se hubiera hecho sin vosotros. Por tanta paciencia y confianza depositada en mí, y por los siempre buenos momentos que pasamos juntos. A mi familia, que os quiero y sois lo mejor.

No podía faltar, por supuesto, una de las personas más importantes en mi día a día en el laboratorio BACPLANT, Rixar. Desde que entré en el laboratorio hace ya unos cuantos años, no he dejado de aprender de ti, y de reírme también. Gracias por todas las enseñanzas útiles y las no tan útiles, por tu alegría y por ser un ejemplo de esfuerzo y un maestro para mí. OMIS Rixar!!

Tampoco olvidaré los buenos momentos que pasamos en almuerzos y comidas Rixar, Sara, Eva, José Vicente y Joaquín, donde todo eran risas y por unos momentos nos olvidábamos de nuestras obligaciones y problemas. A las chicas de micro, Lucía, porque siempre tienes unas palabras y una historia que contar, y a Vero, que en este último año hemos ido contando meses, días, páginas y palabras a la par. Gracias por transmitir siembre tanta alegría. A Lorena, siempre dando ánimos de buena mañana.

A todas las chicas que han pasado por el laboratorio y que me han hecho compañía en lo que a veces hubiera sido un lugar solitario, Carolina, Bea, Claudia y Belén. Gracias por los buenos ratos pasados y también por transmitirme vuestro apoyo.

Agradecer a mis amigos y amigas por todo el apoyo que me han dado, por escuchar mis problemas, que sé que a veces para uno estos temas pueden quedar muy lejos, por los ánimos y por los momentos que juntos te hacen desconectar y coger fuerza.

I would like to thank all the people at the FiererLab. First of all, to Noah Fierer for giving me the opportunity to stay in your lab, it was a pleasure to meet you and the work you develop there. Secondly, to all the team, Jessica, Matt, Hannah, Angela, John and Manu, for all your help and patience with my inexperience in many fields. You

were really helpful. Thanks to Jill and Mark, for helping and listening to me every day. Thank you too because you made my stay at USA more pleasant.

Gracias a Santi y Carmen, por estar siempre tan pendientes de mí y preocuparos siempre tanto, y a toda la familia Verdoy. Por todos los buenos momentos que hemos pasado juntos, en los que nunca os habéis olvidado de este trabajo, también lo habéis seguido casi de principio a fin.

Agradecer también a todas aquellas personas que de una manera u otra han participado en la consecución de esta Tesis, a los que estuvieron y ya no están y a los que han contribuido en las últimas etapas.

Agradecer también a Aurora Ortí Bueno por su asesoramiento en la revisión del inglés.

Por último, y siempre importante para mí, a Pablo, porque siempre he pensado que contigo empezó todo. Llegaste al principio y has aguantado hasta al final de esta etapa, con todos sus altibajos. Gracias por tu paciencia y darme ánimos a diario, por apoyarme, por distraerme cuando más lo necesitaba, por estar siempre ahí y enseñarme a reírme de casi todo. Porque me has enseñado que al final todo es como una metáfora de una carrera, que requiere gran esfuerzo y sacrificio para llegar a la meta. Espero poder correr muchas más como esta a tu lado. Gracias ROUnner.

INDEX

LIST OF ABBREVIATIONS

ABGM-N: AB medium supplemented with glucose and mannitol without nitrogen source

ABG-N: AB medium supplemented with glucose without nitrogen source

ABL: AB medium enriched with lichen extracts

ABLGM: AB medium enriched with lichen extracts and glucose and mannitol

ACC deaminase: 1-aminocyclopropane-1-carboxylate deaminase

AND: ácido desoxirribonucleico

AMB: antioxidant maceration buffer

CAS: chrome azurol S

CECT: Colección Española de Cultivos Tipo

CFU: colonies forming unit

CLSM: confocal laser scanning microscopy

DAPI: 4',6-diamidino-2-phenylindole

DMSO: dimethyl sulfoxide

DNA: deoxyribonucleic acid

dNTPs: triphosphate deoxynucleotides

***et al.*:** *et altri*

FISH: fluorescent *in situ* hybridization

HDTM: hexadecyltrimethylammonium bromide

IAA: indole acetic acid

KB: King's B medium

ML: maximum likelihood

MM: molecular mass

NNI: nearest neighbour interchange

OTU: operational taxonomic unit

p: p-value

PBS: phosphate buffered saline

PCR: polymerase chain reaction

PVK: Pikovskaya

rRNA: ribosomal ribonucleic acid

RST: Ringer solution with Tween 20

SEM: scanning electron microscopy

SSCP: single-strand conformational polymorphism

TEM: transmission electron microscopy

T20: Tween 20

T80: Tween 80

UFC: unidades formadoras de colonia

w/v: weight per volume

INDEX OF FIGURES

INDEX OF TABLES

RESUMEN

Los líquenes son un ejemplo clásico de asociación simbiótica mutualista autosuficiente, compuesta por, al menos, un hongo filamentoso (micobionte, heterótrofo) y cianobacterias y/o algas verdes unicelulares (fotobiontes, fotosintéticos). Se desarrollan gracias a la combinación adecuada de hongos y algas y/o cianobacterias, generando un nuevo organismo, el talo liquénico (holobionte). Estos simbiontes obligados presentan claros papeles funcionales. El hongo liquenizante produce el talo para el establecimiento de los fotobiontes que proporcionan el carbono fijado, mientras que el micobionte aporta agua, nutrientes minerales y protección frente a diferentes tipos de estrés abiótico. Dicha asociación mutualista les permite colonizar y crecer en ambientes diversos y extremos que no podrían habitar los simbiontes de forma independiente.

Estudios posteriores sobre la composición de los talos liquénicos mediante técnicas dependientes del cultivo han revelado la presencia de bacterias no fotosintéticas en los mismos. Sin embargo, la gran diversidad de bacterias asociadas a los líquenes solo se ha empezado a dilucidar mediante el avance en las técnicas independientes del cultivo. Estos estudios han propuesto a las bacterias como simbiontes adicionales, obligados o facultativos, y a cada uno de los simbiontes liquénicos como organismos multifuncionales dentro del holobionte. Este nuevo conocimiento ha cambiado la visión clásica de la simbiosis liquénica, ampliando la definición de liquen para incluir a las comunidades bacterianas asociadas, compuestas por una gran diversidad de taxones. Estas comunidades bacterianas consideradas ahora como una parte integral de los talos liquénicos, interaccionarían entre ellas y con los fotobiontes y el micobionte, por lo que actualmente los líquenes son considerados como una simbiosis multiespecies.

Mediante los estudios basados en técnicas dependientes del cultivo se han detectado también algunos de los papeles funcionales de estas bacterias liquénicas, como la fijación de nitrógeno y la solubilización de fosfatos, así como la liberación de aminoácidos y/o la producción de fitohormonas que podrían estar relacionados con el aporte de nutrientes en los talos liquénicos. Aun así, la información sobre la composición, diversidad y potencial metabólico de las bacterias asociadas a los líquenes es todavía muy escasa.

En los últimos años, ha habido un interés creciente por las bacterias asociadas a los líquenes, pero debido dificultades en el aislamiento de estas bacterias por la falta de protocolos de análisis bacteriológicos adecuados, así como de medios de cultivo con condiciones nutritivas que reproduzcan las de los talos liquénicos, la mayoría de estudios se han llevado a cabo mediante técnicas independientes del cultivo. Dichas investigaciones han evidenciado la gran diversidad y abundancia de las bacterias que colonizan los talos liquénicos normalmente formando biopelículas multiespecies, con distintos papeles funcionales y potenciales metabólicos, muchos de ellos aun por explorar.

La distribución mundial de los líquenes está bien documentada, pero el conocimiento sobre la influencia de factores geográficos en las comunidades bacterianas asociadas a los talos liquénicos es todavía muy escasa. No obstante, algunos estudios han demostrado que dichas comunidades varían entre diferentes especies liquénicas en diferentes regiones, existiendo

además una compleja relación entre los líquenes y sus bacterias asociadas. Del mismo modo, otros factores intrínsecos del talo liquénico (especie de liquen, edad, tipo de crecimiento, etc.) y factores ambientales (exposición solar y tipo de sustrato que colonizan, entre otros) son también determinantes y modulan a nivel taxonómico las bacterias asociadas a líquenes.

Los líquenes han evolucionado en una gran diversidad que les ha permitido adaptarse a una gran variedad de ambientes, hipotetizándose que las comunidades bacterianas asociadas podrían participar en dicha adaptación. A su vez, estos organismos, a modo de microecosistemas, podrían estar involucrados en la diversificación de las bacterias que los habitan. Por ello, los talos liquénicos son considerados como reservorios de una gran diversidad bacteriana, que a su vez podría estar implicada en la tolerancia de los líquenes a diferentes condiciones ambientales.

En el presente estudio, nos hemos centrado en la caracterización de las comunidades bacterianas asociadas al liquen *Ramalina farinacea* (L.) Ach. por presentar una amplia distribución en zonas de clima mediterráneo, incluyendo la península ibérica y las Islas Canarias. Se trata de un liquen fruticuloso que vive habitualmente como epífito sobre una gran variedad de sustratos y bajo distintas condiciones ambientales. Entre los sustratos que puede colonizar se incluyen arbustos, setos, troncos y ramas de árboles, tanto en zonas de bosques caducifolios como en otras expuestas al sol y al viento en árboles aislados, pero principalmente en bosques de encinas y pinos y, ocasionalmente, en rocas y muros. Este liquen presenta una amplia distribución en la mayoría de zonas templadas y boreales del hemisferio norte, en media y alta montaña, y en el centro y sur de Europa, así como en zonas áridas de clima mediterráneo. También se puede encontrar en zonas de menor estrés ambiental como ecosistemas húmedos, pero también en otras más restrictivas de alta montaña. Esta capacidad de *R. farinacea* de colonizar y sobrevivir en una gran variedad de ambientes sugiere una gran plasticidad ecofisiológica, a la que podrían contribuir sus comunidades bacterianas asociadas. No obstante, apenas existen estudios sobre las comunidades bacterianas asociadas a esta especie liquénica pese a que dicha información es crucial para dilucidar el papel de estos simbiontes y entender mejor las interacciones que conforman esta asociación, así como suponer una nueva fuente de microorganismos con propiedades y actividades de interés.

En base a dichos antecedentes, el objetivo general de esta tesis doctoral ha sido estudiar las comunidades bacterianas asociadas al liquen *R. farinacea* con especial interés en su composición, diversidad y potencial biotecnológico. Este objetivo general se puede desglosar en los siguientes objetivos específicos:

1. Aislar y caracterizar metabólica y fisiológicamente una colección de cepas bacterianas a partir de poblaciones de *R. farinacea* de distintas zonas geográficas españolas, tanto por su posible contribución a la simbiosis liquénica como -con especial interés- por su potencial biotecnológico, así como iniciar la identificación molecular de las cepas bacterianas de mayor interés biotecnológico.
2. Estudiar la composición y diversidad de las comunidades bacterianas asociadas a las distintas poblaciones de *R. farinacea* estudiadas, mediante técnicas dependientes del cultivo, y determinar la influencia del origen geográfico o la localización en el talo liquénico (ecto- o endoliquénica).
3. Analizar la composición y diversidad de las comunidades bacterianas asociadas a las mismas poblaciones de *R. farinacea* mediante técnicas independientes del cultivo, así

como investigar la influencia de la localización geográfica o en el talo (ectoliquénica o endoliquénia, o bien apical, media y basal), así como el efecto de un tratamiento de desinfección.

Para la consecución de estos objetivos, se analizaron talos de poblaciones de *R. farinacea* recolectados en áreas de *Pinus canariensis* de dos zonas geográficas diferentes de la isla de Tenerife (Canarias), en La Guancha y La Esperanza, y en áreas de *Quercus rotundifolia* de dos localizaciones geográficas distintas de la península ibérica, El Toro (Castellón) y Lidón (Teruel), todas ellas con clima mediterráneo.

Con respecto al primer objetivo de la Tesis Doctoral, para aislar las cepas bacterianas de las muestras de *R. farinacea* se siguió el protocolo de análisis bacteriológico desarrollado por Biosca *et al*. (2016), consistente tanto en el lavado de la superficie de los talos liquénicos (bacterias ectoliquénicas) como en la disrupción por machacado de estos mismos talos lavados (bacterias endoliquénicas) con tampón antioxidante, utilizando medios de cultivo mínimos enriquecidos con extractos liquénicos, sin (ABL) o con fuentes de carbono adicionales (glucosa y manitol) (ABLGM) y prolongando la incubación 15 días a 26ºC. Dada la escasa información sobre la abundancia de las comunidades bacterianas asociadas a los líquenes, y en particular en *R. farinacea*, se llevó a cabo un recuento de las bacterias cultivables heterótrofas a partir de los distintos talos liquénicos analizados. Los datos comparativos de los recuentos de las bacterias aerobias heterótrofas mesófilas obtenidos de los talos de las poblaciones de *R. farinacea* de La Guancha y El Toro fueron similares (alrededor de 10^5-10^6 UFC/g) tanto en las muestras ectoliquénicas como en las endoliquénicas, siendo inferiores en las poblaciones liquénicas de La Esperanza y Lidón (alrededor de 10^4-10^5 UFC/g). Los recuentos bacterianos fueron similares en los dos medios de cultivo enriquecidos con extractos liquénicos (ABL y ABLGM), aunque generalmente superiores en el medio oligotrófico ABL, sin fuentes de carbono adicionales.

La caracterización metabólica y fisiológica de las cepas bacterianas de *R. farinacea* se inició con la detección de la producción de pigmentos en el medio KB. Un gran porcentaje de las bacterias recuperadas de los talos liquénicos mostraron producción de pigmentos, en su mayoría de carácter celular, siendo los más abundantes el amarillo (32,3%) y el rosa (18,98%), y en menor medida el naranja (10,41%) y el blanco (10,49%). Un 27,82% de las cepas no produjeron pigmentos en las condiciones ensayadas.

A continuación, la caracterización de las cepas bacterianas se siguió mediante el uso del sistema miniaturizado API ZYM que permite detectar 19 actividades enzimáticas, siguiendo las instrucciones del fabricante, pero optimizando el tiempo de incubación a 48 h a 26ºC. En dichas condiciones, entre el 80% y el 100% de las cepas ensayadas presentaron actividades esterasa, esterasa lipasa, leucina arilamidasa, fosfatasa ácida y naftol-AS-BI-fosfhohidrolasa. El resto de actividades se detectaron en un porcentaje de cepas que varió del 10 al 50%, y un par de actividades no se detectaron en ninguna de las cepas bacterianas ensayadas.

Después, se prosiguió estudiando el potencial hidrolítico de las cepas bacterianas mediante metodología convencional en el medio de cultivo KB suplementado con distintas macromoléculas (almidón, caseína, Tween 20 y Tween 80), o con el uso de otros medios, como el agar gelatina y el DNAsa. Además, se investigó la capacidad de dichas cepas de degradar

celulosa, pectina, quitina y xilano. También se determinó la capacidad de las cepas bacterianas de *R. farinacea* para fijar nitrógeno, solubilizar fosfatos y/o producir sideróforos, mediante el uso de medios de cultivo mínimo libres de nitrógeno, el medio Pikovskaya's y el medio CAS agar, respectivamente. Todas las actividades se ensayaron en experimentos independientes partiendo de cultivos ajustados a la misma concentración, y las placas inoculadas se incubaron a 26ºC durante al menos 7 días realizando lecturas periódicas. Los resultados revelaron que las polisacarasas (amilasa, celulasa, pectinasa, xilanasa y quitinasa) se detectaron en porcentajes diferentes, 43,82%, 31,53%, 51,94%, 61,07% y 79,88% según su actividad, respectivamente. Respecto a la actividad lipasa, el porcentaje de cepas capaces de degradar Tween 20 (53,07%) fue mayor que en el caso de Tween 80 (23,46%). En relación a la actividad proteasa, tanto la gelatina como la caseína fueron utilizadas por un porcentaje similar de cepas, entre un 31,14% y un 30,46%, respectivamente. La cantidad de cepas capaces de degradar ADN fue del 32,38%. Con respecto a la capacidad de las cepas bacterianas de aportar nutrientes limitantes, los resultados revelaron que un gran porcentaje de ellas fueron capaces de fijar nitrógeno (92,24%) y producir sideróforos (84,14%), siendo menor el porcentaje de las solubilizadoras de fosfatos (48,77%).

La detección de actividades relacionadas con la promoción del crecimiento del talo liquénico, se centró en estudiar la producción de fitohormonas, como la auxina ácido indol acético (AIA) que se determinó tras 24 h y 72 h y se cuantificó mediante una curva patrón de AIA. Muchas de las cepas ensayadas produjeron AIA en presencia del precursor triptófano, a concentraciones crecientes conforme aumentó el tiempo de incubación, oscilando entre 0 y 100 µl/ml a las 72 h. A continuación, se determinó la capacidad de las cepas bacterianas para producir el enzima ACC desaminasa (involucrado en la biosíntesis del etileno). Para ello, se llevó a cabo la detección molecular del gen *acdS* que codifica para este enzima mediante el uso del par de cebadores degenerados ACC R y ACC F. Los resultados mostraron que dicho gen sólo se detectó en el 18,54% de las cepas ensayadas.

La producción de biopelículas se determinó en placas de microtitulación de 96 pocillos a 26ºC durante 48 h y 72 h, ensayando cada una de las cepas bacterianas por sextuplicado. Tras el experimento, las cepas se clasificaron en no productoras, productoras débiles, moderadas y fuertes, en función de la media de los valores obtenidos de DO_{600} nm comparados con los del control negativo, consistente en medio de cultivo sin inocular. Tras 48 h de incubación, la mayor parte de las cepas produjeron biopelículas de intensidad fuerte (53,06%), siendo los porcentajes de aquellas que produjeron biopelículas de intensidad moderada y débil más bajos (26,90% y 16,33%, respectivamente). La proporción de cepas productoras de biopelículas de intensidad fuerte y moderada se incrementó con el tiempo de incubación (72 h).

Dado que la movilidad es un factor que contribuye en la formación de biopelículas, se investigó la movilidad tipo swimming y swarming de las cepas bacterianas en medio semisólido con 0,3% y 0,7% de agar, respectivamente, tras 24 y 48 h de incubación en KB a 26ºC. En cuanto a la movilidad tipo swimming, los resultados mostraron como un gran número de cepas fueron capaces de llevar a cabo este tipo movilidad natatoria, siendo un 70,43% de ellas positivas. Respecto a la movilidad tipo swarming, sólo se observó en un 5,6% de las cepas estudiadas.

La adscripción taxonómica inicial de una selección de cepas bacterianas de *R. farinacea* se realizó mediante la amplificación del gen *ARNr 16S* por PCR (cebadores 616V y 699R) y posterior secuenciación de los amplificados purificados. El análisis de las secuencias obtenidas se llevó a cabo con el programa Chromas Lite 2.1.1 y la aplicación BLASTN del NCBI. Las cepas bacterianas estudiadas se adscribieron a géneros tales como *Arthrobacter, Bacillus*, *Burkholderia*, *Curtobacterium*, *Erwinia*, *Kocuria*, *Leifsonia*, *Methylobacterium, Microbacterium*, *Micrococcus*, *Mycobacterium, Nocardioides*, *Pantoea*, *Pseudomonas*, *Sphingomonas*, *Staphylococcus*, etc., muchos de ellos con miembros con una gran diversidad de actividades. Tras el alineamiento se realizó un estudio de la filogenia de la colección de cepas bacterianas identificadas presuntivamente, incluyendo una selección de secuencias de géneros bacterianos obtenidos mediante comparación por BLAST y la realización de un árbol filogenético.

El segundo objetivo de la presente Tesis Doctoral fue estudiar la composición y diversidad de las bacterias heterótrofas cultivables asociadas a las poblaciones de *R. farinacea* estudiadas de la isla de Tenerife (La Guancha y La Esperanza) y la península ibérica (El Toro y Lidón). Para ello, se seleccionaron un total de 286 cepas bacterianas de *R. farinacea*, tanto ectoliquénicas como endoliquénicas, a partir de las diferentes placas de aislamiento entre las que presentaron diferente morfología colonial, aisladas de muestras de talos de las distintas localizaciones geográficas. Para la identificación molecular presuntiva de la selección de cepas bacterianas se siguió la metodología anteriormente mencionda. Después, el estudio de beta y alfa diversidad se realizó mediante un análisis de varianza permutacional (PERMANOVA) con la función "Adonis" del paquete VEGAN de R. Para cuantificar las diferencias en la composición de las comunidades bacterianas se utilizó una matriz de disimilitudes medias basada en Bray-Curtis, calculado mediante el paquete MCTOOLSR de R. La diferencia de diversidad bacteriana entre las diferentes muestras se determinó mediante el uso de los índices de diversidad de Riqueza, Shannon y Simpson.

El análisis de beta diversidad reveló que la estructura de las comunidades bacterianas asociadas a *R. farinacea* está determinada, principalmente, por el factor geográfico, tanto atendiendo al origen insular o peninsular de las cepas (PERMANOVA, $R2=0,29$, $p<0,01$) como al origen geográfico cada una de las poblaciones liquénicas estudiadas (PERMANOVA, $R2=0,65$, $p<0,01$), siendo menor la influencia debida a la localización de estas bacterias en el talo liquénico, en la fracción ectoliquénica o endoliquénica (PERMANOVA, $R2=0,17$, $p<0,05$). Estos factores explicaron el 29%, 65% y el 17% de la composición de la diversidad de las comunidades bacterianas, respectivamente.

Los índices de Riqueza y de diversidad de Shannon y Simpson mostraron resultados similares al comparar la diversidad de las bacterias asociadas a los talos liquénicos de la isla y la península. El índice Shannon mostró valores mayores en las cepas insulares, lo que indica que los taxones presentes en la isla están representados por un número parecido de individuos en comparación con los de la península. El índice Simpson reveló valores similares tanto en las cepas de la isla como en las de la península, indicando una representación equitativa de las diferentes especies bacterianas. Cuando el estudio de diversidad bacteriana se estratificó para cada una de poblaciones de *R. farinacea* de La Guancha, La Esperanza, El Toro y Lidón, se observaron ciertas diferencias entre una de ellas y el resto. El índice de Riqueza mostró valores similares entre las cuatro poblaciones liquénicas, aunque la población de Lidón fue la que

presentó el valor más alto, mostrando que el número de especies bacterianas en esta zona era más alto que en el resto. Lo mismo sucedió con los índices de diversidad Shannon y Simpson, indicando que las especies bacterianas tuvieron una representación más equitativa en Lidón que en el resto de localizaciones geográficas.

Por otra parte, las cepas bacterianas aisladas de los talos de las diferentes poblaciones de *R. farinacea* se asociaron a 3 fila principales, siendo *Proteobacteria* el predominante, seguido de *Actinobacteria* y *Firmicutes.* La presencia de *Proteobacteria* fue mayor en la isla (65,98%) que en la península (47,01%), seguido de *Actinobacteria*, cuya presencia fue más abundante en la península (46,68%) que en la isla (23,65%). *Firmicutes* fue el grupo con menor representación en ambas zonas (10,6% en la isla, 6,32% en la península). En las muestras obtenidas de La Guancha y La Esperanza la mayoría de cepas bacterianas se adscribieron al filo *Proteobacteria* (75,67% y 54,54%, respectivamente), seguido de *Actinobacteria* y *Firmicutes*. La asignación taxonómica de las cepas bacterianas de El Toro mostró menor diferencia en la proporción de *Actinobacteria* y *Proteobacteria* comparado con las otras poblaciones (49,29% y 40,84%, respectivamente). La cantidad de bacterias asignadas a *Firmicutes*, fue igualmente escasa (9,86%). En el caso de Lidón, el grupo principal fue *Actinobacteria* (54,65%).

La diversidad de las bacterias identificadas se asignó a un total de 37 géneros diferentes. De entre ellos, sólo dos resultaron ubicuos, *Bacillus* y *Sphingomonas*. Otros géneros frecuentes entre las cepas bacterianas identificadas fueron *Burkholderia*, *Curtobacterium*, *Erwinia*, *Kocuria* y *Methylobacterium*, y otros menos frecuentes, fueron *Arthrobacter*, *Averyella*, *Rhodococcus*, *Massilia*, *Sanguibacter*, *Subtercola*, etc. Además, cabe destacar, que algunas cepas no se pudieron asignar a ningún grupo taxonómico, indicando la presencia de nuevas especies.

Cuando se estudió la diversidad bacteriana atendiendo a la localización externa o interna en los talos liquénicos, los resultados mostraron que los valores en los índices de Riqueza y diversidad Shannon y Simpson fueron mayores entre las cepas bacterianas endoliquénicas que entre las ectoliquénicas, indicando un mayor número de especies diferentes y más uniformemente representadas en el interior del talo que en su superficie. La identificación taxonómica inicial de las bacterias asociadas a las fracciones ectoliquénica y endoliquénica de *R. farinacea* reveló algunas diferencias remarcables. Aunque *Proteobacteria* fue, en general, el grupo predominante, seguido de *Actinobacteria*, el número de cepas de *Proteobacteria* aisladas fue mayor en la parte ectoliquénica (69,57%) que en la endoliquénica (43,42%), en la que el número de *Actinobacteria* fue ligeramente superior (48,23%). La presencia de este grupo en la parte ectoliquénica fue del 22,83%. Respecto a *Firmicutes,* estuvo igualmente representado en ambas fracciones liquénicas (8,34%).

El tercer objetivo de esta Tesis Doctoral consistió en estudiar la composición y diversidad de las comunidades bacterianas asociadas a *R. farinacea* mediante técnicas independientes del cultivo, con especial interés en determinar la influencia del origen geográfico o liquénico (ectoliquénico o endoliquénico, o bien apical, medio o basal) así como el efecto de una desinfección inicial en dichas comunidades. Como paso previo a la extracción del ADN, se procesaron submuestras de talos de cada una de las poblaciones liquénicas de las cuatro localizaciones geográficas de forma global, incluyendo tanto las comunidades bacterianas ectoliquénicas como las endoliquénicas, así como muestras de talos individuales. Además,

también se analizaron las comunidades bacterianas asociadas a ambas fracciones liquénicas por separado. Asimismo, se analizaron submuestra de talos adicionales dividiéndolos en tres, zonas apical, media y basal, previamente a la extracción de ADN. Adicionalmente, algunas de las muestras se sometieron a un tratamiento de desinfección con etanol al 70%, incluyendo talos sin tratar como control. La extracción de ADN de los diferentes talos de *R. farinacea* se llevó a cabo con un kit comercial y la amplificación del gen *16S ARNr* y secuenciación de las muestras se realizó con los cebadores 515F y 806R. Tras la edición y filtrado de las secuencias, se compararon con las depositadas en la base de datos Greengenes para eliminar aquellas con alto grado de divergencia. A cada uno de los OTU obtenidos se le asignó una clasificación taxonómica usando Greengenes. Finalmente, cada muestra se ajustó a 1929 secuencias totales. Los análisis estadísticos se llevaron a cabo con el programa R. Las secuencias obtenidas se agruparon en un total de 848 OTUs.

El estudio de beta diversidad reveló que las comunidades bacterianas asociadas a *R. farinacea* se vieron determinadas principalmente por el factor geográfico (PERMANOVA, R2= 0,24526, p<0,001 para las muestras agrupadas según fueran de la isla o de la península, PERMANOVA, R2=0,47, p<0,001 o bien atendiendo al origen de cada una las poblaciones liquénicas, La Guancha, La Esperanza, El Toro y Lidón). Este factor geográfico tuvo una influencia en la composición de estas comunidades del 24,53% entre las insulares y las peninsulares, y del 47,05% a nivel de cada localización geográfica individual. Otro factor que influyó, aunque en menor grado, fue la localización ectoliquénica o endoliquénica de las bacterias en el talo, que explicó el 3,03% de esta composición bacteriana (PERMANOVA, R2=0.038, p<0.01).

El análisis de alfa diversidad de las muestras en cada localización geográfica se determinó mediante los índices de Riqueza y los de diversidad Shannon y Simpson. Cuando las muestras se agruparon en función del origen insular o peninsular, la mayor riqueza bacteriana se observó entre las poblaciones peninsulares, estando además más equitativamente representada que en el caso de las muestras insulares. Cuando se analizó la alfa diversidad atendiendo a las cuatro localizaciones geográficas, la que mostró un mayor índice de Riqueza fue El Toro, que presentó un mayor número de especies bacterianas. Los índices de diversidad Shannon y Simpson revelaron que la zona de El Toro también fue la que presentó mayor diversidad bacteriana, mientras que la población de *R. farinacea* de La Esperanza fue en la que se observó menor diversidad.

Los principales OTUs registrados en las muestras se asignaron a los fila *Proteobacteria*, *Acidobacteria* y *Planctomycetes*. Los grupos *Bacteroidetes*, *Planctomycetes* y *Proteobacteria* fueron más abundantes en las localizaciones de la península, mientras que *Acidobacteria*, *Cyanobacteria* y *Firmicutes* tuvieron una mayor representación en las muestras procedentes de la Isla. La proporción de *Proteobacteria* fue muy similar en las cuatro localizaciones geográficas. De entre las *Proteobacteria*, *Alphaproteobacteria* fue la clase predominante en todas las poblaciones seguida por *Acidobacteria*. En esta última, su presencia fue ligeramente superior en las poblaciones insulares (42,21% en La Guancha y 33,03% en La Esperanza) que en las peninsulares (27,3% in El Toro and 30,45% in Lidón). La presencia de *Planctomycetes* fue mucho menor en todas las poblaciones (alrededor de un 1% y un 3% de las secuencias insulares y peninsulares, respectivamente). En el caso de *Cyanobacteria* y *Firmicutes*, su prevalencia fue menor comparada con los grupos previamente citados, pero con ciertas

diferencias. La abundancia de *Cyanobacteria* fue mayor en las muestras de Tenerife (alrededor del 1,5%) que en El Toro y Lidón (alrededor del 0,1-0,2%). Lo mismo ocurrió con el filo *Firmicutes*, cuya prevalencia fue mayor en La Guancha y La Esperanza (alrededor del 1%), que en El Toro y Lidón (alrededor del 0,4%).

La identificación a nivel de género reveló que un gran número de cepas bacterianas asociadas a *R. farinacea* no se pudieron asignar a ninguna especie con secuencia depositada en la base de datos utilizada. Sin embargo, algunos géneros fueron relativamente abundantes entre dichas bacterias tales como *Beijerinckia* (2,48%), *Edaphobacter* (2,42%), *Sphingomonas* (1,94%), *Burkholderia* (1,45%), *Terriglobus* (1,01%), *Pseudomonas* (0,6%) o *Hymenobacter* (0,45%).

En relación al análisis de alfa diversidad en función del origen ectoliquénico o endoliquénico de las cepas bacterianas, los resultados mostraron unos índices de Riqueza y diversidad Shannon y Simpson fueron mayores entre las cepas ectoliquénicas que la endoliquénicas, indicando la presencia de un mayor número de especies bacterianas y más uniformemente representadas en la superficie de *R. farinacea* que en su interior. El análisis de los principales taxones bacterianos asociados a estas fracciones ecto- y endoliquénicas, mostró resultados similares a los observados en los análisis anteriores. Las bacterias asignadas a *Proteobacteria* fueron las mayoritarias en ambas fracciones (59,42% ectolilquénicas y 53,22% endoliquénicas), seguidas de las asignadas a *Acidobacteria* (31,30% ectoliquénicas y 41,42% endoliquénicas) y *Planctomycetes* (2,09% ectoliquénicas y 2,52% endoliquénicas). La presencia de *Cyanobacteria* y *Bacteroidetes* presentó diferencias más notables, siendo más abundantes en la fracción ectoliquénica.

El estudio de diversidad bacteriana en función de la localización en las zonas apical, media y basal del talo de *R. farinacea*, reveló valores similares en los índices de Riqueza y diversidad Shannon y Simpson, aunque la zona apical fue la que mostró valores más bajos, presentando por tanto un menor número de especies y menor diversidad. En relación con la diversidad bacteriana, a nivel de filo, los principales grupos fueron *Proteobacteria*, *Acidobacteria* y *Bacteroidetes.* Sin embargo, los resultados indicaron una distribución preferente de algunos grupos en algunas de estas partes, como en el caso de *Cyanobacteria*, mayoritario en la zona media del talo (2,19%), disminuyendo en las zonas apical (1,30%) y basal (0,09%). Lo mismo sucedió con el grupo *Firmicutes*, con una mayor presencia en la zona apical (2,09%), disminuyendo en las zonas media (1,17%) y basal (0,29%). Por el contrario, el grupo *Planctomycetes* fue más abundante en la zona basal (4,68%), disminuyendo en las zonas media (2,63%) y apical (0,56%).

Cuando se estudió el efecto de un tratamiento de desinfección con etanol, los resultados obtenidos con la identificación taxonómica mostraron como los fila *Proteobacteria*, *Acidobacteria*, *Plantctomyctes* y *Bacteroidetes* continuaron siendo los dominantes, sin diferencias en la abundancia de estos grupos entre las muestras sometidas o no al tratamiento de desinfección.

CONCLUSIONES

A continuación, se resumen las principales conclusiones de este estudio:

1. El aislamiento de bacterias asociadas a poblaciones del liquen *R. farinacea* mediante el análisis bacteriológico y los medios de cultivo enriquecidos con extractos liquénicos (ABL y ABLGM) utilizados en este estudio han evidenciado una gran abundancia de bacterias heterótrofas cultivables (entre 10^4 y 10^6 UFC/g), siendo los recuentos obtenidos generalmente mayores que en estudios previos con otras metodologías y medios de cultivo convencionales que no reproducen las condiciones nutritivas de los talos liquénicos.

2. La abundancia de bacterias cultivables aisladas de los talos de *R. farinacea* ha resultado similar tanto en la fracción ectoliquénica como en la endoliquénica de los talos analizados. No obstante, los recuentos de bacterias cultivables han sido diferentes en función del origen geográfico de los talos, siendo mayor en los de las poblaciones de *R. farinacea* de La Guancha y El Toro que en los de La Esperanza y Lidón. Estos resultados podrían estar relacionados con las diferentes condiciones ambientales en cada ubicación.

3. La caracterización de las cepas bacterianas aisladas de *R. farinacea* confirma la importancia de su presencia en los talos de este liquen, tanto por sus posibles papeles funcionales en el reciclado y/o aporte de nutrientes y/o la promoción del crecimiento a través de la producción de fitohormonas o mediante formación de biopelículas, necesarios para el funcionamiento de esta simbiosis liquénica, como por sus potenciales aplicaciones biotecnológicas, dado que:

i) Un alto porcentaje de ellas produce pigmentos, siendo los más frecuentes el amarillo y el rosa, lo que podría relacionarse, en parte, con la tolerancia frente a diferentes condiciones de estrés ambiental, como la radiación UV o el estrés oxidativo.

ii) Muchas de las cepas bacterianas son capaces de producir enzimas hidrolíticos, tales como amilasas, celulasas, pectinasas, quitinasas y xilanasas, así como lipasas, proteasas y DNAsas, que podrían contribuir al reciclaje de nutrientes en las partes senescentes de los talos de *R. farinacea* suministrando azúcares, ácidos grasos, aminoácidos y nucleótidos a las zonas en crecimiento, ayudando así al mantenimiento de los mismos. Esta versatilidad hidrolítica también resulta de interés por su posible aplicación en distintas industrias biotecnológicas.

iii) Una gran mayoría de las cepas bacterianas son capaces de fijar nitrógeno y producir sideróforos, y muchas de ellas también de solubilizar fosfatos inorgánicos. Dichas actividades también podrían contribuir a cubrir ciertos requerimientos nutricionales limitantes y esenciales para el crecimiento del talo liquénico. Las cepas que las poseen podrían explotarse como biofertilizantes.

iv) Un porcentaje elevado de las cepas bacterianas produce la auxina ácido indolacético, y en algunas de ellas también se detectó el enzima ACC desaminasa, implicado en la síntesis de etileno. Dichas hormonas, capaces de modular el crecimiento de las plantas, también podrían influir en los procesos morfogenéticos de los líquenes y sus simbiontes, siendo estas cepas productoras de fitohormonas de interés como potenciales fitoestimulantes.

v) Casi todas las cepas bacterianas ensayadas son capaces de producir biopelículas, muchas presentando movilidad tipo swimming y algunas de ellas también tipo swarming. La formación de biopelículas podría estar relacionada con la capacidad de colonización de los líquenes en ambientes con condiciones específicas y extremas, pudiendo aumentar la absorción de nutrientes y conferir protección frente a estrés ambiental. Dichas cepas podrían explotarse también en aplicaciones biotecnológicas.

4. La identificación molecular de una selección de cepas bacterianas de *R. farinacea* de acuerdo con sus diferentes potenciales fisiológicos y metabólicos, ha permitido adscribirlas a diferentes taxones bacterianos, algunos todavía poco estudiados y/o potenciales nuevas especies.

5. El estudio de la composición y diversidad de las comunidades bacterianas heterótrofas asociadas a *R. farinacea* mediante técnicas dependientes del cultivo representa una pequeña fracción de la gran variedad de bacterias asociadas a este liquen. No obstante, aporta nueva información sobre estas comunidades cultivables en poblaciones de *R. farinacea* de diferentes zonas insulares y peninsulares de clima mediterráneo, así como sobre la influencia en ellas de la localización geográfica y en talo liquénico, lo que podría estar relacionado con sus diferentes papeles funcionales en esta especie liquénica.

6. Entre las bacterias aisladas de *R. farinacea,* los grupos predominantes son ciertos fila, como *Acidobacteria, Planctomycetes y Proteobacteria* y clases como *Actinobacteria, Alphaproteobacteria, Bacilli, Betaproteobacteria* y *Gammaproteobacteria*. Además, algunas de las cepas aisladas de este liquen podrían ser nuevas especies.

7. El estudio de la composición y diversidad de las comunidades bacterianas asociadas a *R. farinacea* mediante técnicas independientes del cultivo ha revelado que dichas comunidades están determinadas principalmente por la geografía, pero también por la localización en el talo liquénico, puesto que se han obtenido diferencias en las secuencias de bacterias entre las distintas localizaciones geográficas, así como entre las fracciones ectoliquénicas y endoliquénicas, y entre las partes apical, media y basal del talo.

8. Entre los taxones identificados en las comunidades bacterianas de *R. farinacea*, muchos de ellos pertenecen a grupos bacterianos con representantes conocidos por sus actividades enzimáticas y/o su papel potencial en el reciclaje y/o suministro de nutrientes en los talos liquénicos, y que podrían contribuir al mantenimiento de la simbiosis liquénica multiespecies. Muchas de estas especies bacterianas se han aislado, caracterizado e identificado en este estudio, siendo algunas de ellas potencialmente nuevas, como resultado de que los líquenes son ambientes de comunidades bacterianas todavía poco explorados.

9. Los líquenes suponen una nueva fuente de numerosos, diversos y nuevos microorganismos, con diversos potenciales biotecnológicos, muchos de ellos todavía por descubrir.

INTRODUCTION

1. LICHENS

Lichens are symbiotic organisms, usually composed of a fungal partner, the mycobiont, and one or more photoautotrophic partners, the photobionts (Nash, 2008), resulting in a unique entity, the lichen thallus or **holobiont** (Grube *et al*., 2009). The holobiont has been defined as the host organism together with all of its symbiotic microbiota (Gilbert *et al*., 2010). The resulting structure is the phenotype of the lichen-forming fungi. The fungal partner is the responsible of the lichen species name, and these mycobionts barely can exist without the symbiotic partners (Honegger, 1998, 2012). The photobionts can be a green algae (**chlorolichens**) or a cyanobacterium (**cyanolichens**) or, in some cases, both and they have their own scientific name. Most free-living algae and cyanobacteria occur in aquatic or at least very moist terrestrial habitats, but as part of lichens they occur abundantly in habitats that are frequently dry as well. Nevertheless, this bipartite or tripartite definition of the lichen symbiosis implies, in most cases, more partners (Nash, 2008).

The term symbiosis was coined by Heinrich Anton de Bary in 1879 (Bary, 1879), as the association of different species of organisms living together. This is the most accepted definition nowadays and it includes different types of symbiotic associations, namely mutualistic, when all the partners get a benefit, commensalistic, when some of the partners get a benefit but the others result unharmed, and parasitic, when some of them suffer some damage (Dimijian, 2000).

The symbiotic relationship is sometimes difficult to determine since, in some cases, it could be mutualistic in some periods and parasitic in others. Moreover, sometimes, the symbiosis could be established as a facultative relationship, while in others is an obligate state (Hawksworth, 1989). Furthermore, this association could be of a fix duration or could be permanent, when the partners are never separated (Paracer, 2000).

Mutualistic symbiosis are extraordinary in their variety and ubiquity and in these cases, each one of the partners supplies with some benefit to the other members of the association, either with some nutritional or structural contribution. Lichen symbiosis is an example of self-supporting mutualism that has evolved for at least 400 million years (Taylor *et al.,* 1995). Lichens are stable and well-balanced relationships between the autotrophic members, photobionts, and the heterotrophic component, the mycobiont. This symbiosis is obligate for the fungus but the photobionts are able to persist outside the lichen thallus structure (Mukhtar *et al*., 1994; Nash, 2008; O'Brien *et al*., 2005; Sanders, 2005; Wirtz *et al*., 2003). The lichen symbiosis implies a physiological integration and a flow of nutrients among their members (Smith and Douglas, 1987). The photobionts supply with carbon nutrition to the fungus, being polyols in the case of green algal lichens and glucose in the case of cyanolichens (Nash, 2008; Smith and Douglas, 1987). This process of carbon transfer is due to the cell wall permeabilization to carbohydrate in the lichenized photobiont (Hill, 1976). Furthermore, the cyanobionts supply with nitrogen to the mycobiont, due to their ability for fixing nitrogen (Nash, 2008). The mycobiont obtains a great benefit from this relationship, but also provides

protection to the photobiont against different abiotic stresses to the photobiont (Nash, 2008). The interactions that maintain the equilibrium of the lichenization state confers to the mycobiont and photobiont(s) partner(s) the ability to persist in environments, and sometimes to tolerate extreme and stress conditions, such as high irradiations, UV light, extreme temperatures and dehydration, where they wouldn't be able to grow individually (de Vera *et al*., 2008; Grube and Berg, 2009; Kranner and Lutzoni, 1999), suggesting a simultaneous evolution of the lichen partners (del Campo *et al*., 2013; Grube and Berg, 2009).

As a consequence, lichen species are ubiquitously widespread and adapted to extreme ecological conditions, being present from high altitudes to the sea level, in Artic boreal and tropical habitats and close to volcanos (Gadd, 2007; Grube *et al*., 2012b; Nash, 2008; Stocker-Wörgötter, 2008). Even some experiments were performed to assess if lichens could survive to space conditions. The European Space Agency launched two lichen species into space, *Rhizocarpon geographicum* and *Xanthoria elegans*, and exposed them to these conditions for 16 days. Once on Earth conditions again, both lichen specimens recovered their full metabolism and activity within 24 h. That experience showed that lichens could survive to high-vacuum, extreme temperatures, huge levels of UV and cosmic radiation – which is lethal for bacteria and most other microorganisms (Sancho *et al*., 2007).

During the last ten years new insights into the lichen composition have revealed the presence of non-photosynthetic bacterial communities in the lichen thallus which have been considered as active multifunctional partners in the holobiont (Grube *et al*., 2009). The presence of bacteria in lichens was reported many years ago (Cardinale *et al*., 2006; González *et al*., 2005; Henkel and Plotnikova, 1973; Iskina, 1938; Panosyan and Nikogosyan, 1966; Selbmann *et al*., 2010), but the high diversity and number of bacteria associated with lichen thalli has begun to be revealed through advanced culture-independent techniques (Bates *et al*., 2011; Grube and Berg, 2009; Hodkinson and Lutzoni, 2009). Further, non-symbiotic fungi have been found associated with the members of the lichen thallus, making the understanding of the lichen symbiosis even more complex (Aschenbrenner *et al*., 2014; Cernava *et al*., 2016; Grube *et al*., 2015; Grube and Berg, 2009; Muggia *et al*., 2013; Nash, 2008). Therefore, the classical paradigm of the complex symbiotic system based on a myco-centric view is changing to a wider concept. It might include a microbial community, composed of a large diversity of taxa, in addition to the main symbionts, with an effective interaction among them, being lichens now considered as multispecies symbiosis (Aschenbrenner *et al*., 2016; Grube *et al*., 2014).

1.1 LICHEN BIOLOGY

Lichens are long-lived organisms with a slow rating growth that appears in a wide diversity of forms, colours and morphologies. Despite the fact of being resistant to different abiotic stresses, they can be vulnerable to slight changes in environmental conditions that can disrupt the symbiotic association integrity (Erlacher *et al*., 2015).

During the process of development and morphogenesis of the lichen thallus, mycobiont and photobionts suffer an intricate series of changes that affect their morphology, biochemistry and physiology, giving as a result, a completely new organism with new characteristics and properties (Chapman and Margulis, 1999; Honegger, 2008a).

When the lichen-forming fungi establishes the association with the photobionts, the symbiotic phenotype and features of the lichen are expressed. In the majority of cases, lichen algal photobionts are located extracellularly within the lichen thalli (Honegger, 2008a). In the case of cyanolichens, a symbiosis with free-living cyanobacteria is established with bacterial cells being found also on thallus surfaces (Honegger, 2008a).

It is known that a big number of algal and cyanobacterial species are compatible with many lichen-forming fungal species. Usually, most of the genera of lichen-forming fungi establish an association with only one genus of photobionts (Rambold *et al*., 2011). The symbiotic phenotype is expressed only when the lichen-forming fungi meets the compatible photobiont, although when they are in an axenic culture, these lichen-forming fungi can grow and develop a kind of thallus independently of their photobiont (Ahmadjian, 1988).

Growing rates in lichens are very slow. In the case of lichens from extreme climates, they have short periods in which they are full metabolically active and can grow (Kappen, 1993). Thus, very low cell turnover rate and minimal annual size increases could be determined, being the longest age estimates in the range of millennia. In contrast, there are some lichen species with a short life, and they finish their complete development within months or few years. Lichens growing in temperate or subtropic to tropic areas have a growth range of millimeters to a few centimeters per year, respectively (Honegger, 2008a).

1.1.1 Lichen metabolites

Lichens are interesting organisms because of their metabolites. Some of them are primary metabolites and others secondary metabolites. The most common primary metabolites are proteins, amino acids, polyols, carotenoids, polysaccharides, and vitamins. These metabolites are water-soluble and are bound in the cell walls and the protoplasts (Elix and Stocker-Wörgötter, 2008; Fahselt, 1994). Both mycobiont and algal photobiont produce these compounds (Elix and Stocker-Wörgötter, 2008). Secondary metabolites are normally organic compounds produced by the fungus and deposited on the surface of the hyphae. They are water-insoluble and can only be extracted with organic solvents. In the case of carbon sources, lichens are furnished by the photosynthetic activity of the algal partners (Elix and Stocker-Wörgötter, 2008). Different types of carbohydrates produced by the algal photobiont contribute to the carbon cycle in the lichen and supply to the fungus, being a polyol such as ribitiol, erythriol or sorbitol. When the lichens also contain cyanobacteria, the carbohydrate supplied is glucose (Elix and Stocker-Wörgötter, 2008). These carbohydrates are stored in the fungus as mannitol, another sugar alcohol (Brodo *et al*., 2001).

Secondary metabolites have been used in the identification of lichens, for example, at genus level. In lichens growing on exposed surfaces or environments, light-absorbing compounds are located in the upper cortical area of vegetative and generative parts of the thallus. These pigments act as light-screens regulating the solar irradiation that reaches the lichen thallus zone where algal cells are located in order to protect them from an excess of ultraviolet irradiation (Rubio *et al.,* 2002).

Regarding the polysaccharides, the best-known types of polymeric storage products in lichens are lichenan, isolichenan and galactomannan, each one of them having a variety of different chemical structure depending on the ancestor (Elix and Stocker-Wörgötter, 2008). Structural polysaccharides are also important in fungi biochemistry and have maintained features in their evolution, thus being considered taxonomically important at the highest levels of classification. Some examples are the presence of chitin, chitosan or cellulose in the fungal cell wall, characteristics that help to identify fungi at class level (Elix and Stocker-Wörgötter, 2008).

1.1.2 Lichen inorganic nutrients

In lichens, as in other organisms, the need to accumulate and process macro- and micronutrients is necessary and critical for their growth and development. Due to their physiological characteristics, lichens depend on atmospheric sources of nutrients (Nieboer *et al*., 1978) and they have developed mechanisms to concentrate nutrients (Nash, 2008). Atmospheric deposition to lichens could take place through precipitation, fog and dew, in the case of wet deposition, and sedimentation, impaction, and gas absorption, in the case of dry deposition (Knops *et al.,* 1991).

The source of nutrients in lichens may come from the atmosphere but also from the substrate. Many lichens grow on soils or rocks being, therefore, in contact with lithic sources of nutrients. Lichens may participate in wearing out rocks by mechanical and chemical processes (Syers and Iskandar, 1973). Since pH affects nutrients solubility, nutrient availability may be different between limestones and acidic substrates with the consequent differences in lichen communities growing in such substrates (Nash, 2008). Soil particles usually have a high concentration of several metals such as Al, Fe, Sc, Ti and other elements of lithic origin, and could be incorporated into intracellular lichen spaces. The solubilization of these particles suppose a source of nutrients, but sometimes the process is not fast and many of the particles are not available (Nash, 2008).

Something similar occurs in epiphytic lichens growing on the surface of trees. Epiphytic lichens are influenced by changes in nutrient processing that occurs in the canopy and in the bark of trees. Some elements as potassium seep from foliage and may be uptaked by these lichens. In some trees, there are stemflows during precipitation, and this supposes an important source of nutrients (Nash, 2008). As it happens in rocks and soil, the availability of nutrients is different depending on the composition and the pH of the bark tree). There are different lichen communities on trees with acid barks, as conifers, compared to those with more neutral bark as *Fraxinus*, *Tilia*, etc. (Nash, 2008).

Nutrient necessities in lichens are complex to establish due to difficulties in culturing them. Nevertheless, some of these nutritional requirements could be inferred partially due to the substrate specificity in which lichens grow and the nutrient composition of such substrates (Brodo, 1973). When some nutrient is added, the growth and some metabolic processes are stimulated. It is well studied, for example, the effect of nitrogen limitation on lichen growth.

Nitrogen is one of the essential macronutrients and it participates in the synthesis of proteins and nucleic acids. It restricts growth and lichen productivity due to its limited accessibility. The principal source of nitrogen is the atmospheric one, as N_2, but this is a chemically inert form

not readily usable by most of the organisms, with only some prokaryotes being able to fix the atmospheric nitrogen (Seefeldt *et al*., 2009). The inorganic forms of nitrogen that can be assimilated by many organisms are nitrate (NO_3^-) and ammonia (NH_3, or ammonium ions, NH_4^+). Their availability is crucial for growth and survival of green-algal lichens. In these chlorolichens, the nitrogen concentrations should be related to the atmospheric presence of these ions (Hyvärinen and Crittenden, 1998). In the case of cyanolichens, cyanobacteria can take atmospheric N_2 directly. Due to this ability, cyanolichens have higher levels of nitrogen than chlorolichens (Nash, 2008).

Around 10% of lichen species have cyanobacteria as photobionts. The main species of cyanobacteria present in these lichens are *Calothrix*, *Fischerella* (=*Stigonema*), *Gloeocapsa*, *Nostoc* or *Scytonema*, all of them able to fix N_2. In the case of tripartite lichens, when cyanobacteria are the secondary photobionts, they are located in specialized structures named **cephalodia**, where a functional and spatial separation of carbohydrate and nitrogen fixation occurs (Grube *et al*., 2012b, 2014; James and Henssen, 1976).

The nitrogen-fixation process is energy-dependent, and only occurs in some species of bacteria and archaea. It is catalyzed by an enzyme complex called nitrogenase, a metalloprotein that involves a Fe-protein (dinitrogenase reductase) and a Fe-Mo protein (dinitrogenase) (Nordlund and Högbom, 2013). Fe-protein is a homodimerous and it is extremely sensitive to oxygen. Fe-Mo protein has a structure of α_2 β_2 tetramer. Each one of the αβ units, harbour a set of cofactors, being the Fe-Mo cofactor the active site where molecular nitrogen is reduced (Nordlund and Högbom, 2013). Ferredoxin provides the electrons to the dinitrogenase reductase (Fe-protein), which transfers electrons to the dinitrogenase (Fe-Mo protein) which will transfer electrons and protons to N_2, to form two NH_3. Nitrogenase is an enzyme which could be easily inhibited by O_2, therefore it is necessary a separation of nitrogen fixation from the photolysis of water during photosynthesis (Nordlund and Högbom, 2013; Seefeldt *et al*., 2009). Some cyanobacteria, as *Nostoc*, solved the problem forming specialized cells named **heterocysts**, in which the phycocyanin accessory pigments and water-splitting magnesium center of photosystem PS II are lost (Tel-Or and Stewart, 1976). Thus, heterocysts maintains internal anaerobiosis or microaerobic conditions protecting nitrogenase from O_2 inactivation (Tel-Or and Stewart, 1976).

Phosphorus limitation also affects lichen productivity. Unlike nitrogen, phosphorus has no gas phase, and usually it is lost from ecosystems by sedimentation and secondary mineral formation. Some studies were conducted with nitrogen and phosphorus as fertilizers in lichens hypothesizing that the growth at the thallus apices creates a sink for these nutrients, and that remobilization of nitrogen and phosphorus in older thallus regions creates a source of these nutrients (Ellis *et al*., 2005; Hyvärinen and Crittenden, 2000).

1.1.3 Lichen selectivity and specificity

The association between the mycobiont and the photobiont/s is described by the terms **selectivity** and **specificity** (Yahr *et al*., 2006). Thus, the frequency rate of association between compatible lichenic partners defines the selectivity, while the specificity is the taxonomic range of partners that can participate in this association. The specificity is sometimes influenced by

the environmental conditions (DePriest, 2004; Rambold *et al*., 2011). Most of the lichens are developed under *Ascomycetes* lichen-forming fungi, approximately 99% of them, with only few lichenized *Basidiomycetes* (less than 1%) and *Deuteromycetes* (Honegger, 2012; Lutzoni *et al*., 2004; Nash, 2008). Generally, lichens have a high specificity choosing the photobiont (Beck, 2002; Rambold *et al*., 2011), but in the case of the mycobionts, this specificity is not as narrow as it is usually assumed (Nash, 2008).

Regarding the population structure, usually a single primary photobiont is found in a single thallus, but in some cases the photobiont population is composed of multiple algal genotypes (Muggia *et al*., 2008; Nelsen and Gargas, 2008; Yahr *et al*., 2004), meaning a favorable and advantageous situation allowing the lichen to adapt and respond to environmental changes and, thus, to colonize different habitats (Piercey-Normore, 2006).

1.1.4 Thallus internal organization

Lichens do not possess a vascular system and lack waxy-impermeable barriers which allow a continuous exchange of substances with the surrounding environment (Sanders, 2001). Thus, they lack specialized mechanisms for controlling their water content, which is regulated, possibly, by the water potential of the environment they inhabit (Nash, 2008; Sanders, 2001).

Lichen symbiosis classically is considered as an association between two functional partners, although it often involves more organisms, such as cyanobacteria (Grube *et al*., 2014). In fact, the number and type of photobionts associated with the lichenized fungi defines the lichens as bi- or tripartite (Lohtander *et al*., 2003; Magain *et al*., 2012; Magain and Sérusiaux, 2014; Miadlikowska and Lutzoni, 2004). **Bipartite** lichens are referred as chlorolichens when the photobiont population are green algae, and as cyanolichens when the photobionts are cyanobacteria (Henskens *et al*., 2012). In bipartite lichens where cyanobacteria are the primary photobionts, they provide fixed carbon and nitrogen to the lichen. In **tripartite** lichens cyanobacteria suppose part of the thallus as a photobiont member as well as the green algae (Grube *et al*., 2014). These bacteria have the main role fixing nitrogen (Grube *et al*., 2014).

Therefore, the environment has a strong influence on the lichen thallus structure, being the abiotic environmental factors the ones that may shape/modulate its morphology and anatomy. Their construction has to ensure the positive net photosynthetic function for sufficient growth rates (Sanders, 2001). This assumes that photobionts are in the right distribution to receive the amount of light needed, that the diffusion of carbon dioxide to them happens readily and that the loss of water is adapted to the environment, being minimum under dry conditions and maximum in wet environments (Nash, 2008). Thus, environmental conditions at different geographic locations strongly influence the distribution and abundance of lichen species, which are also affected through time (de la Torre *et al*., 2010; Nelsen and Gargas, 2008; Werth, 2011).

Lichens Mediterranean taxa are distributed along regions with Mediterranean climates, including Southern Europe, Northern Africa, Macaronesia and Southern California/Northern Baja California. They include taxa from arid and semi-arid areas (Galloway, 2008).

1.2 LICHEN GROWTH FORMS AND STRUCTURE

1.2.1 Lichen thallus structure and morphology

Internally, the thallus structure of the vegetative body is either **homoiomerous** (without stratification) or **heteromerous** (with stratification) (Aschenbrenner *et al*., 2016). A representative drawing of the structure of homoiomerous and heteromerous thalli is shown in figure 1a and 1b, respectively**.**

Homoiomerous thallus

This lichen structure is characterized by a homogenous (evenly) distribution of the mycobionts and photobionts in the thallus (Aschenbrenner *et al*., 2016). These types of lichens are gelatinous and absorb much more water in relation to their dry weight than non-gelatinous lichens do. Thus, the CO_2 diffusion to the photobiont is committed or even blocked when the thallus is supersaturated, resulting in a limiting factor under these circumstances. Examples of this thallus structure are found in the genera *Caloplaca*, *Pyrenopsis* or *Collema* (Lange and Tenhunen, 1981; Nash, 2008).

Heteromerous thallus (stratified lichens)

The majority of lichens belong to the group of the internally stratified thallus. This stratification includes the following layers with different functions:

-Upper cortex: the main functions are mechanical protection, the modification of energy budgets (Kershaw, 1985), antiherbivore defense (Reutimann and Scheidegger, 1987), and protection of the photobiont against excessive light (Jahns, 1988; Kappen, 1988).

-Medullary layer: it occupies the internal thalline volume and is formed by long-celled, not tightly interwoven hyphae forming a layer with a very high internal airspace. The upper part of the medulla forms the photobiont layer. In fruticose lichens (see below), this layer is often formed as a supporting tissue and consists of conglutinated hyphae. The hyphal cell walls and the medullary layer are often encrusted with crystalline secondary products that make this medullary hypha hydrophobic. Water transport to the photobiont seems to be restricted to mycobiont cell walls. In periods with water-saturated conditions, the cells of the symbionts are turgid, and during dry periods the photobiont cells lose water and collapsed (Büdel and Scheidegger, 2008).

-Lower cortex: it has a great ability for absorbing water directly, however, it may have a major role in retaining extrahalline, capillary water. It is well-developed in typical foliose lichens (see below) of the *Parmeliaceae* and other groups (Jahns, 1984).

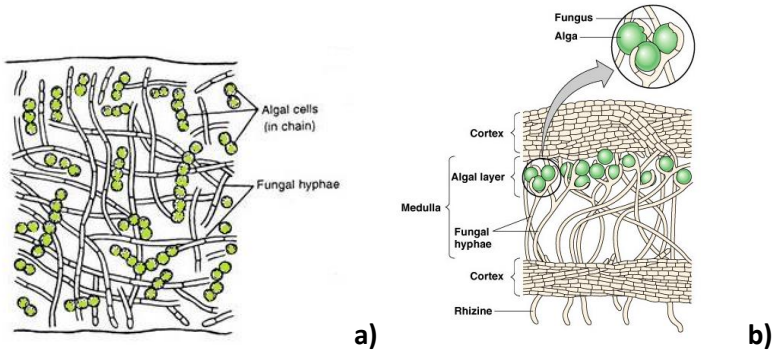


Figure 1. Representative drawing of thallus anatomy. **a)** Homoiomerous thallus; **b)** Heteromerous thallus. Taken from: http://www.biologydiscussion.com and http://classes.midlandstech.edu, respectively.

Morphology

Lichen morphology is traditionally classified into three main groups: **crustose** (crust-like biofilm), **foliose** (leaf-like) and **fruticose** (branched tree-like, shrubby, pendulous) types. Examples of these main morphologies are shown in figure 2. The appearance of the lichen thallus is determined by the mycobiont. There are some special types as the gelatinous lichens that differ from the others due to the lack of aerial hyphae systems, the possession of hydrophobic cell wall surfaces and the lack of air-filled zones (Büdel and Scheidegger, 2008; Hawksworth *et al*., 1995; Honegger, 2012; Nash, 2008).

Figure 2. Representative pictures of different types of lichen thalli. **a, b)** Crustose. **c, d)** Foliose. **e, f)** Fruticose. **e, g)** Gelatinous. Pictures a, b, c, d, e, f, author's personal pictures. Picture g, taken from: http://bobklips.com/.

1.3 LICHEN SYMBIOSIS

1.3.1 The mycobiont

Fungi are heterotrophic organisms that have developed different strategies for acquiring organic carbon. Lichen-forming fungi are a taxonomically heterogeneous group of nutritional specialists that acquire fixed carbon from the small population of photobionts, the green algal and/or cyanobacterial cells (Honegger, 2008b).

The fungal partner in the lichen symbiosis is the one that controls and maintains the photobiont cell population and ensures an optimal illumination and gas exchange to the algae and/or cyanobionts (Honegger, 1991, 1992, 2008b).

Lichenization occurs typically in the classes fungal *Lecanoromycetes* and *Arthoniomycetes* and the order *Verrucariales* within the phylum *Ascomycota*, although in some occasions appears in others as *Dothideomycetes* (Gargas *et al*., 1995; Schoch *et al*., 2009). The members of the class *Lecanoromycetes* are the most lichenized fungi (Grube *et al*., 2014).

1.3.2 The photobiont

Photobionts are essential components in the lichen symbiosis, being responsible of light harvesting under an extreme range of ecological conditions, necessary for the establishment and formation of the lichen thallus (Beck, 2002).

Approximately 85% of lichen-forming ascomycetes have been reported to be associated with green algal as photobionts, around 10% with cyanobacteria (as primary photobiont) and about 3% with both of them (algae as the primary photobiont and cyanobacteria as de secondary one), for the establishment of the lichen symbiosis (Friedl and Büdel, 2008; Tschermak-Woess, 1988). Related to the algae and cyanobacteria, around 120 genera are known to participate as photobionts in lichens, although not all of them have been identified at species level (Honegger, 2012; Piercey-Normore, 2006).

Green algae

Eukaryotic green algae, with a wide variety of morphologies, suppose the main photobionts partners in most lichen symbiosis. They are organized in coccoid, sarcinoid or filamentous forms without flagella (Friedl and Büdel, 2008).

In the family of the lichenized fungi *Lecanorales*, the most frequent algal photobionts belong to the genus *Trebouxia*, while *Trentepholia* is more frequent in lichens of the orders *Arthoniales* (e.g. *Roccella*), *Gyacetales* (e.g. *Coenogonium*) and *Sphaeriales*, which gives an orange color to the lichen thallus (Friedl and Büdel, 2008). Other important phycobionts are the genera *Chlorella* and *Chlorella*-like algae, as well as *Coccomixa*, *Elliptochloris*, *Diplosphaera* and *Nanochloris* (Tschermak-Woess, 1988), although they are more frequent in some crustose lichens of the *Lecanorales* and *Calicales*. The green alga *Coccomyxa* is common in the fungal

families *Baeomycetaceae* and *Peltigeraceae* as well as in lichenized *Basidiomycetes* (Friedl and Büdel, 2008).

The *Trebouxiophyceae* class comprises the majority of the unicellular green algal lichen photobionts (Friedl, 2006). Around 60% of the fungal species among the *Lecanoromycetidae* class is associated with members of the algal genera *Trebouxia* or *Asterochloris* (Honegger, 2012). Morphologically, these algal genera have a central chloroplast with a central pyrenoid (Friedl and Büdel, 2008; Škaloud and Peksa, 2008, 2010; Tschermak-Woess, 1988), with cellulosic walls (Brunner and Honegger, 1985; Honegger, 1984) and are tolerant to desiccation.

Cyanobacteria

Cyanobacteria are prokaryotes with thylakoids distributed freely in the cytoplasm and a circular DNA not associated to histones and concentrated in the cytoplasm in areas called "nucleoid" (Nash, 2008). The orders *Chroococcales, Nostocales, Pleurocapsales* and *Stigonematales* comprise the most representative members of the cyanobacteria lichen photobionts (Friedl and Büdel, 2008; Tschermak-Woess, 1988), with the genera *Nostoc*, *Gloeocapsa* and *Chroococcidiopsis* being the most common (Friedl and Büdel, 2008). The association between the lichen-forming fungi and the cyanobacteria has an exclusive character in some taxa. In other cases, they could be associated with different lichen-forming fungi, as in the case of genotypes of *Nostoc*, which are the most common cyanobacterial lichen photobionts (Honegger, 2012).

The contribution of the cyanobacteria, as *Nostoc* and other prokaryotic **diazotrophic** (able to fix nitrogen) lichen photobionts, is to provide photosynthates and fix nitrogen to the fungal partner (McDonald *et al*., 2012b). Furthermore, some of them possess heterocystes, increasing up to five times the frequency of these structures when cyanobacteria are lichenized compared with the free-living state (Feige and Jensen, 1992).

In tripartite lichens, despite the fact of being cyanobacteria and algae physically separated, cyanobacteria can be present as a layer below the green-algae, or as colonies next to the thallus, a condition which is known as **cyanotrophy**. Both cephalodiate (with cephalodia) and cyanotrophic lichens are indicators of the re-distribution of metabolic functions in lichens with more than two partners (Grube *et al*., 2012a).

1.3.3 The bacteria

Additional partners in the multispecies lichen symbiosis

Bacterial colonies on lichen thalli surfaces were observed by microscopy many years ago (Grube *et al*., 2012b). The first observations, probably, were made with the lichen parasite *Chondromyces lichenicola* (now classified as *Melittangium lichenicola* in *Myxobacteriaceae*) (Thaxter, 1892). Thereafter, Cengia-Sambo (Cengia-Sambo, 1925, 1931) also observed bacteria in lichens and coined the term "polysymbiosis" for the multipartite relationships. Subsequent studies reported other bacteria associated with lichens, some of them being able to degrade

cellulose (Navahradak, 1949). In 1956, *Azotobacter* was detected in lichens (Scott, 1956) and that revealed that part of the fixed-nitrogen in lichens might be provided by non-photosynthetic bacteria. Other common bacterial genera reported were *Clostridium* (Iskina, 1938), *Beijerinckia* (Panosyan and Nikogosyan, 1966), *Bacillus* and *Pseudomonas* (Henkel and Plotnikova, 1973). The presence of *Actinobacteria* in lichens was also described, and in cyanolichens a defensive role was suggested for them (Zook, 1983).

Lichens are able to colonize oligotrophic habitats, such as nude rocks, speculating that their associated bacteria might supply some relevant nutrients as fixed nitrogen, growth factors, etc. (Honegger, 1997). Earlier studies about the role of bacteria in lichens relied on functions found in the culturable fraction of the lichen-associated bacterial community (Grube *et al*., 2012a). Liba *et al.* (2006) found nitrogen-fixing bacteria in lichen species from Brazil. Further analysis of these bacteria showed that some of them were able to excrete amino acids and the hormone indole-3-acetic acid (IAA), while others solubilized phosphate or released ethylene (Liba *et al*., 2006). These data would support a relevant role of bacteria in the nutritional and hormonal amendment in lichens (Grube *et al*., 2012a).

Using sequencing methods, some of the first detected activities in lichenic bacteria were the nitrogen fixation (Hodkinson, 2011; Hodkinson and Lutzoni, 2009) or the mobilization of fungal cell wall components such as water-soluble glucans (Hodkinson, 2011). Through comparative omic studies made with the lung lichen *Lobaria pulmonaria*, it was reported that the bacteria present in this lichen provide vitamin B12, nutrients, growth hormones to the algae and resistance to pathogens (Grube *et al*., 2015). In other lichens, as *Peltigera membranaceae*, the bacterial symbionts are known to participate in phosphate solubilization, which could be involved in algal growth promotion (Sigurbjörnsdóttir *et al*., 2015).

In recent years, **ectolichenic** (associated to the external surface of the lichen thallus) and **endolichenic** (associated with the inner area of the lichen thallus) bacterial communities have been characterized by using different molecular tools (Bates *et al*., 2011; Cardinale *et al*., 2006, 2008; Grube *et al*., 2009; Grube and Berg, 2009; Hodkinson, 2011; Hodkinson and Lutzoni, 2009; Mushegian *et al*., 2011; Selbmann *et al*., 2010). Further studies using fluorescence *in situ* hybridization and confocal laser scanning microscopy (FISH-CLSM) and other molecular and novel microscopic techniques, reported that *Alphaproteobacteria* represented a dominant group in some studied lichen and they appeared, in many cases, forming biofilm-like colonies (Bates *et al*., 2011; Cardinale *et al*., 2006, 2008, 2012b; Grube *et al*., 2009; Grube and Berg, 2009; Hodkinson *et al*., 2012; Hodkinson and Lutzoni, 2009; Mushegian *et al*., 2011; Selbmann *et al*., 2010). However, within culturable isolates, *Alphaproteobacteria* was not the most represented group, with other bacterial taxa being found in significant amounts (Grube *et al*., 2012a). *Rhizobiales* appeared to be the predominant order in the lichen-associated bacteria, although members of *Acidobacteriaceae*, *Acetobacteraceae* and *Brucellaceae* were also present (Ramanan *et al*., 2016).

As a result of these studies, the bacteria associated with lichens and their functional roles were recognized conferring a new insight to the lichen concept (Hodkinson *et al*., 2012; Hodkinson and Lutzoni, 2009). Lichens are known as one of the oldest type of symbiotic relationship, with their structure being influenced by the nature of their symbionts. Algae and bacteria share a

symbiotic relationship that affect their physiology and existence, affecting the lichen survival as well, and showing the ecological significance of these interactions (Hodkinson *et al*., 2012; Lutzoni *et al*., 2001; Ramanan *et al*., 2016).

Many lichen species are distributed in a wide range of geographical locations, being found in all continents and both hemispheres. Whether the bacterial community composition associated with lichens follows or not the lichen geographical distribution, is the object of a new line in the lichen microbiome research (Hodkinson *et al*., 2012).

Analyses of bacteria associated with lichens

Culture-dependent techniques

Culture media used for the isolation of lichen-associated bacteria have been chosen or designed, usually, depending on the environmental origin of the lichen thalli, attending at characteristics such as the substrate (soil, tree barks, etc.) but also focusing on the isolation of some bacterial groups interesting for their potential biotechnological properties (González *et al*., 2005), such as diazotrophic bacteria (Cardinale *et al*., 2006; Liba *et al*., 2006). In the last decade these studies have centered on the isolation of lichen-associated culturable bacteria with different aims: to focus on different bacterial targets, either individual isolates or specific species, or either with taxonomic or biotechnological purposes (Suzuki *et al*., 2016). A summary of such studies including the isolation culture media and the aim of these studies, is shown in tables 1 and 2.

Table 1. A list of studies in which different culture media were used for isolation of certain bacterial groups associated with lichens with different purposes. Adapted from Suzuki *et al*., (2016).

Lichen species	Isolation medium	Purpose of isolation	Reference
Not identified	Soil extract agar, humic acid agar, and glycerol asparagine agar	Biotechnology	Gonzalez *et al*., 2005
Cladonia digitata, C. rangiferina, C. coniocracea, C. pyxidata, C. coccifera, Pseudevernia furfuracea, Hypogymnia physodes, Roccella phycopsis, R. fuciformis	TYE (tryptone yeast extract), sugar-rich N free	Diazotrophs	Cardinale *et al*., 2006
Canoparmelia caroliniana, C. crozalsiana, C. texana, Parmotrema sancti-angeli, P. tinctorum	N-free NFb	Diazotrophs	Liba *et al*., 2006
C. arbuscula, Lecanora polytropa, Umbilicaria cylindrica	R2A, for aquatic marine bacteria	Untargeted	Grube *et al*., 2009
L. fuscobrunnea, U. decussata, Usnea antarctica, Xanthoria elegans	TYE	Psychrophiles	Selbmann *et al*., 2010
Cladonia sp., *C. rangiferina*	Lichen extract, M3	Acidobacteria	Pankratov, 2012
Ochrolechia sp.	MY/R2A	Antimicrobial Antioxidants	Kim *et al*., 2014
Usnea sp., C. borealis, Psoroma sp., *Stereocaulon* sp., *Umbilicaria* sp., *Cetraria* sp., *Ochrolechia* sp.	R2A, ISP4, MY	Biotechnology	Lee *et al*., 2014
L. helicopis, Verrucaria ceuthocarpa, Hydropunctaria maura, Caloplaca verruculifera	Marine agar	Untargeted	Sigurbjornsdottir *et al*. 2014
L. pulmonaria	R2A, SCA, ISP2	Biotechnology	Cernava *et al*. 2015a, b
Lichina confinis, L. pygmaea, Roccella fuciformis, Collema auriformis	Marine agar, AIA, ISP2 with Nalidixic acid	Littoral lichens; actinobacteria	Parrot *et al*. 2015
P. furfuracea, P. pseudotinctorum, R. farinacea	ABL, ABLG, ABLM, ABLGM, With or without lichen extract	Biotechnology and diversity	Biosca *et al*., 2016

Starting with the first studies about the diversity of lichen-associated bacteria, the isolated strains were assigned to the genera *Azotobacter, Bacillus, Beijerinckia, Clostridium* and *Pseudomonas* (Henkel and Plotnikova, 1973; Iskina, 1938; Panosyan and Nikogosyan, 1966). Thereafter (Table 1 and 2), a study focused on the isolation of *Actinobacteria* because of their biotechnological potential was conducted by González *et al*. (2005) using lichens collected in three different environments, in Alaska and in tropical areas. More than 300 lichenic bacterial strains were isolated, identified and assigned through DNA fingerprinting and fatty acid analyses to the families *Geodermatophilaceae, Micromonosporaceae*, *Nocardiaceae, Pseudonocardiaceae, Streptomycetaceae*, *Streptosporangiaceae* and *Thermomonosporaceae* (González *et al*., 2005).

Cardinale *et al*. (2006) isolated ecto- and endolichenic bacteria from nine lichen species of *Cladonia* sp., *Pseudevernia* sp., *Hypogymnia* sp. and *Rocella* sp. collected in Austria and France. They only isolated 34 morphologically distinct bacterial colonies from the external and internal surfaces of all lichen thalli analyzed, with interest in diazotrophic bacteria.

Liba *et al.* (2006) focused on the isolation of bacteria associated with cyanolichens from Brazil, working with lichen species of *Canoparmelia*. and *Parmotrema*, obtaining several nitrogen-fixing positive isolates belonging to different genera of *Gammaproteobacteria*.

Selbmann *et al*. (2010) analyzed the bacterial communities associated with 16 epilithic Antartic lichens belonging to the species *Acarospora*, *Buellia*, *Lecanora*, *Lecidea*, *Rhyzocarpon*, *Umbilicaria*, *Usnea*. and *Xanthoria*. As a result, they got 30 bacterial isolates, being some of them psychrotolerants. A new species of *Deinococcus–Thermus* was reported and other strains represented new potential taxa.

Pankratov (2012) isolated bacteria from lichens collected in bog and tundra areas in Northern Russia. The author found a high abundance of *Alphaproteobacteria* and *Actinobacteria* in all the lichen samples studied. Other groups like *Gammaproteobacteria*, *Betaproteobacteria* and *Firmicutes* were in low numbers or even absent.

Lee *et al*. (2014) studied the culturable bacteria associated with Antarctic and Arctic lichens. The isolates recovered were assigned to the taxa *Actinobacteria*, *Alphaproteobacteria, Bacteroidetes*, *Betaproteobacteria, Deinococcus–Thermus*, *Firmicutes* and *Gammaproteobacteria*. They found *Alphaproteobacteria* as the predominant group, with most of the isolates being assigned to *Aurantimonas, Burkholderia, Deinococcus, Frondihabitans, Hymenobacter, Methyloferula, Nakamurella, Paenibacillus, Pseudomonas, Psychrobacter, Rhodanobacter, Sphingomonas* and *Streptomyces.*

Lichen species of *Calopalca*, *Hydropunctaria*, *Lecanora*. and *Verrucaria*, were collected at a rocky promontory in Iceland by Sigurbjörnsdóttir *et al*. (2014). A total of 93 bacterial strains were isolated and identified as belonging to the groups *Actinobacteria, Alphaproteobacteria*, *Bacilli*, *Cytophagia*, *Flavobacteria*, *Gammaproteobacteria* and *Sphingobacteria.*

Kim *et al*. (2014) studied a crustose lichen, *Ochrolechia* sp., isolating strains that belonged to the genera *Burkholderia* and *Sphingomonas.*

Parrot *et al*. (2015) in an attempt to isolate potentially bioactive bacteria, used marine and littoral lichens as a novel source of *Actinobacteria*. At family level, the following taxa were identified: *Brevibacteriaceae*, *Cellulomonadaceae, Gordoniaceae, Microbacteriaceae, Mycobacteriaceae, Nocardioidaceae*, *Promicromonosporaceae, Pseudonocardiaceae* and *Streptomycetaceae.*

More recently, Biosca *et al*. (2016) developed new lichen enriched media based on the use of novel lichen extracts to increase the recovery of lichen-associated culturable bacteria, initially using the lichen *P. furfuracea*. The authors designed different culture media mimicking the lichen nutritional conditions. They also evaluated different isolation procedures and the effects of different disinfection methods of thalli samples on the recovery of lichen-associated bacteria, concluding that disinfection not only is unnecessary after an extensive washing of thalli samples but also negatively affects culturability of endolichenic bacteria. Their methodology and the lichen enriched media enhanced the recovery of a higher number of bacterial isolates (Biosca *et al*., 2016). A scheme of the isolating bacteria protocol developed by these authors is shown in figure 3.

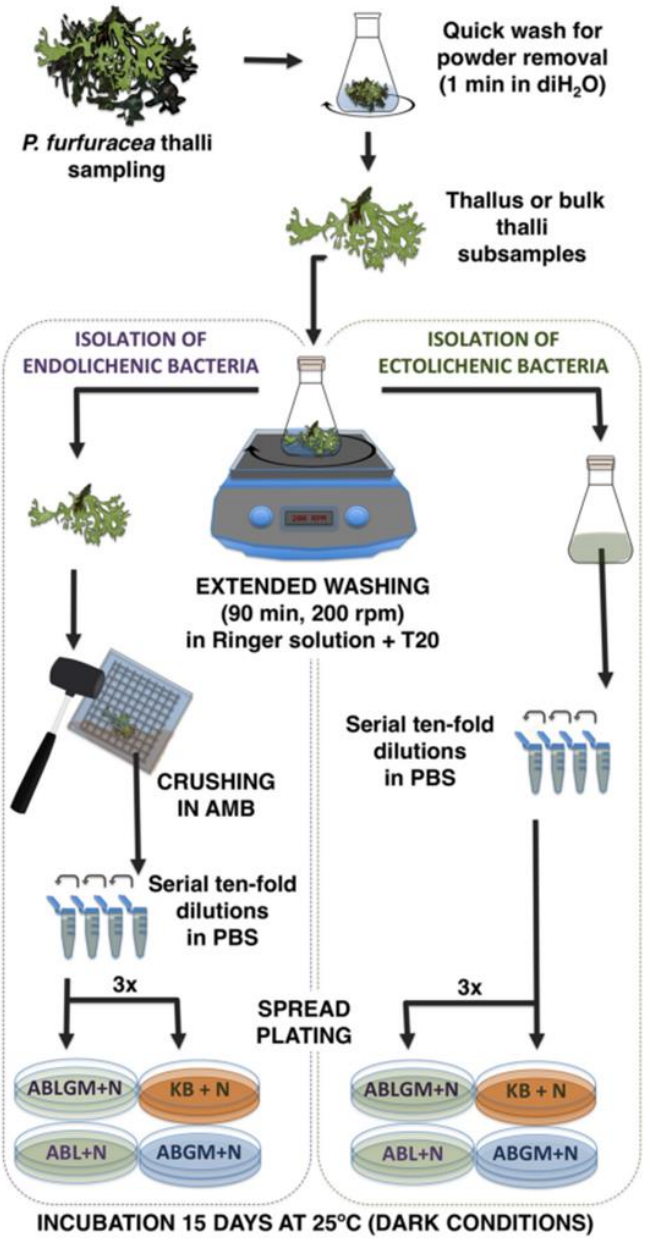


Figure 3. A representative scheme showing the isolation protocol of culturable bacteria associated with *P. furfuracea*. Fresh thalli samples were collected and washed to remove environmental powder. Samples were processed within an extended washing in Ringer solution supplemented with Tween 20 (T20) for the recovery of ectolichenic bacteria. Washed thalli were crushed in AMB to isolate endolichenic bacteria. Ecto- and endolichenic culturable bacteria were estimated in triplicate on lichen enriched media with or without carbon source (ABLGM and ABL), KB and ABGM with natamycin. Spread inoculated plates were incubated at 25°C for 15 days under dark conditions. Taken from Biosca *et al*. (2016).

Table 2. Some of the relevant studies about lichen-associated bacteria and the taxa described.

Lichen species	Isolation medium	Disinfection treatment	Taxa described	Reference
Not specified	Embley,1992 (actinomycetes), with cycloheximide and nalidixic acid (to prevent fungal and not-actinomycetes growth)	-	*Actinobacteria*	González *et al.*, 2005
Cladonia digitata, *C. rangiferina*, *C. coniocraea*, *C. pyxidata*, *C. coccifera*, *P. furfuracea*, *Hypogymnia physodes*, *Rocella phycopsis* and *R. fuciformis*	TYE. Sugar-rich/nitrogen-free media	H_2O_2 (9%), 4 min	*Alphaproteobacteria (Rhodospirillaceae). Betaproteobacteria (Burkholderiaceae). Gammaproteobacteria (Xanthomonadaceae). Actinobacteria (Cellulomonadaceae, Microbacteriaceae, Micromonosporaceae, Streptomycetaceae, Streptosporangiaceae). Firmicutes (Bacillaceae, Paenibacilllaceae, Staphylococcaceae)*	Cardinale *et al.*, 2006
Canoparmelia caroliniana, *C. crozalsiana*, *C. texana*, *P. sanctiangeli* and *P. tinctorum*	NFb (nitrogen-free medium) with nystatin (to prevent fungal growth)	-	*Gammaprotebacteria*	Liba *et al.*, 2006
Acarospora sp., *A. flavocordia*, *Buellia frigida* Darb., *Lecanora fuscobrunnea*, *Lecanora* sp., *Lecidea cancriformis*, *Rhyzocarpon* sp., *Umbilicaria aprina*, *U. decusata*, *Usnea antarctica*, *Xanthoria elegans* and *Lecidea* sp.	TYE with napagin (to prevent fungal and algal growth)	H_2O_2, 5 min	*Gammaproteobacteria (Pseudomonadaceae). Actinobacteria (Intrasporangiaceae, Micrococcaceae, Mycobacteriaceae). Deinococcus-Thermus. Firmicutes (Paenibacillaceae, Bacillaceae)*	Selbmann *et al.*, 2010
Cladonia sp., *C. rangiferina* and *Sphaerophorus globosa*	LE1 (with extract of Cladonia and Cetraria) and M3 media (Pankratov, 2012)	-	*Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Actinobacteria and Firmicutes*	Pankratov, 2012
Usnea sp., *Cladonia borealis*, *Psoroma* sp., *Stereocaulon* sp., *Cladonia borealis*, *Umbilicaria* sp., *Cetraria sp.*, *Cladonia* sp., *Ochrolechia* sp.	R2A (Reasoner and Geldreich, 1985). ISP4 (Shirling and Gottlieb, 1966). Malt extract-Yest extract (Haley and Callaway, 1978)	-	*Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Actinobacteria, Bacteroidetes, Deinococcus–Thermus, Firmicutes*	Lee *et al.*, 2014
L. helicopis, *Verrucaria ceuthocarpa*, *Hydropunctaria maura*, and *Calopalca verruculifera*	Marine agar (MA)	H_2O_2 (3%) or not sterilized	*Alphaproteobacteria, Gammaproteobacteria, Actinobacteria, Bacilli, Cytophagia, Flavobacteria and Sphingobacteria*	Sigurbjörnsdóttir *et al.*, 2014.
Ochrolechia sp.	MY agar media. R2A agar	-	*Sphingomonas* sp. and *Burkholderia* sp.	Kim *et al.*, 2014
Lichina pygmaea, *L. confinis*, *Roccella fuciformis* and *Collema auriforme*	MA agar media. Actinomycete Isolation Agar (AIA). With nalidixic acid and cycloheximide	-	*Actinobacyeria (Brevibacteriaceae, Cellulomonadaceae, Gordoniaceae Microbacteriaceae, Mycobacteriaceae, Nocardioidaceae, Promicromonosporaceae, Pseudonocardiaceae and Streptomycetaceae*	Parrot *et al.*, 2015
P. furfuracea, *P. pseudotinctorum* and *R. farinacea*	AB (Ordax et al., 2009) with or without lichen extract and with or without glucose and/or manitol and natamycine (to prevent fungal growth)	Ethanol 70%, 60 and 30 seconds. H_2O_2 (8%), 5 min. Or not sterilized	Not specified	Biosca *et al.*, 2016

There are some taxonomic groups which seem to be common among the isolated bacteria in different lichen species, such as *Actinobacteria*, *Alphaproteobacteria*, *Firmicutes*, and *Gammaproteobacteria* (Cardinale *et al*., 2008; Lee *et al*., 2014; Liba *et al*., 2006; Suzuki *et al*., 2016), and others less commonly present as *Bacteroidetes*, *Betaproteobacteria*, *Deinococcus-Thermus*, and *Rhizobiales* (Lee *et al*., 2014; Mushegian *et al*., 2011). These common bacterial lineages isolated support the idea about the existence of ubiquitous taxa across different lichen species (Lee *et al*., 2014). Many of these bacteria belong to well-known groups with metabolic activities that may provide a benefit to the lichen symbiosis (Grube *et al*., 2009). Furthermore, some groups have received special attention since they represent a source of novel molecules, such as *Actinomycetes* (González *et al*., 2005). *Gammaproteobacteria* represents a relevant group with non-photosynthetic nitrogen-fixing bacteria (Liba *et al*., 2006). Focusing on cyanobacteria, *Nostoc* is the main genus found in lichens with cyanobionts (Cardinale *et al*., 2012a). Many of these studies have centered on the isolation of individual species for taxonomic purposes or full genomic sequencing.

The most common retrieved bacterial families from lichens are *Bacillaceae*, *Burkholderiaceae*, *Micromonosporaceae*, *Nocardioidaceae, Paenibacillaceae, Pseudomonadaceae, Pseudonocardiaceae* and *Streptomycetaceae* (Suzuki *et al*., 2016), also important for the production of enzymes and bioactive molecules of biotechnological interest such as lipases and proteases and antagonistic molecules. Some genera considered ubiquitous are *Acinetobacter*, *Bacillus*, *Burkholderia* and *Paenibacillus*, others quite common are *Leifsonia*, *Microbacterium*, *Micrococcus*, *Pseudomonas* and *Sphingomonas*, whereas other less detected in the culturable fraction are *Frondicola*, *Luteibacter* and *Methylobacterium* (Grube *et al*., 2009). Interestingly, *Burkholderia* is present in the culturable fraction but hardly detected by *in situ* hybridization in many cases (Cardinale *et al*., 2006, 2008) or other culture-independent techniques.

New bacterial species have been discovered through the study of culturable lichen-associated bacterial communities during the last years (An *et al*., 2008, 2009; Cardinale *et al*., 2011; Li *et al*., 2007; Selbmann *et al*., 2010), as it is summarized in table 3, revealing the high prevalence of bacteria in the lichen thallus. At least 14 novel culturable bacterial strains associated with lichens were thus identified and gave new opportunities to discover bioactive metabolites of interest (Parrot *et al*., 2016), showing that simple culture methods can be favorable for the isolation of undescribed taxa (Biosca *et al*., 2016; Lee *et al*., 2014).

Table 3. Some new bacterial strains isolated from different lichen species and the culture media employed. They are officially described and/or have a fully sequenced genome and/or produce molecules that have been identified. Adapted from Suzuki *et al*. (2016).

Bacterial strain	Isolation medium	Reference
Corallococcus sp. ATCC 25944	Not referenced	McCurdy, 1971
Actinoplanes sp. ATCC 55532	ISP3	Singh *et al.*, 1997
Streptomyces uncialis "ex Julian Davis *et al*. 2005"	Not referenced	Davies *et al*., 2005
Herminiimonas saxobsidens NS11	Potassium oxalate	Lang *et al*., 2007
Nocardioides exalbidus RC825	IAM-A1	Li *et al*., 2007
Schumannella luteola KHIA	Modified Dettmer	An *et al*., 2008
Leifsonia lichenia 2Sb	Modified Dettmer	An *et al.*, 2009
Mucilaginibacter lappiensis ANJLI2	Lichenin	Mannisto *et al*., 2010
Streptomyces sp. RI104-LiC106	HV (Humic Acid-Vitamin) agar	Motohashi *et al*., 2010
Streptomyces sp. RI104-LiB101	HV agar	Motohashi *et al*., 2010
Streptomyces sp. L-91-3	Not referenced	Lavallee, 2011
Frondihabitans cladoniiphilus CafT13	TYE	Cardinale *et al*. 2011
Actinomycetospora iriomotensis IR73-Li102	Humic acid vitamin agar	Yamamura *et al*., 2011a
Actinomycetospora rishiriensis RI109-Li102	Humic acid vitamin agar	Yamamura *et al*., 2011b
Sphingomonas sp. PAMC26605	Not referenced	Shin *et al*., 2012
Sphingomonas sp. PAMC26621	Not referenced	Lee *et al.*, 2012a
Sphingomonas sp. PAMC26617	Not referenced	Lee *et al*., 2012b
Sphingobacterium cladoniae No.6	LB (Luria Bertani)	Lee *et al.*, 2013
Luteimicrobium album RI148-Li105	Humic acid vitamin agar	Hamada *et al.*, 2012
Streptomyces cyaneofuscatus T178	Tryptone soy agar (TSA)	Brana *et al.*, 2015
Streptomyces cyaneofuscatus T35	TSA	Brana *et al.*, 2015
Streptomyces cyaneofuscatus T140	TSA	Brana *et al*., 2015
Streptomyces cyaneofuscatus T163	TSA	Brana *et al*., 2015

The traditional culture methods used for lichen-associated bacteria imply some limitations that don't allow the complete elucidation of the specific species associated with lichens, but advances in molecular and sequencing techniques supposed a huge progress, revealing the high diversity of the lichen bacterial communities (Lee *et al*., 2014; Molins *et al*., 2013). Some of the molecular approaches, although based on culture techniques, suggest that the diversity of bacteria associated with lichens might be much higher than previously thought (Cardinale *et al*., 2006; González *et al*., 2005; Lenova and Blum, 1983; Liba *et al*., 2006), with up to millions of bacterial cells being present per gram of thallus (Grube *et al*., 2009). In fact, studies as the one conducted by Biosca *et al*. (2016) got a great improvement in the number of bacteria recovered from lichens, increasing them up to the order of 10^4 CFU/g, a number much higher than those obtained by other authors.

Identification of lichen bacterial isolates was based, in most of the studies, on sequence similarities and phylogenetic analyses of their *16S rRNA* gene partial sequences using different universal primers for its amplification by the polymerase chain reaction (PCR). PCR products were purified and sequenced with the same primers used for amplification and the sequences were compared with those of the strains available in databases such as EzTaxon-e (Kim *et al*., 2012) or National Center for Biotechnology Information (NCBI) to find closely related species and to choose reference sequences for the phylogenetic analyses (Cardinale *et al*., 2004; Lee *et*

al., 2014). Other techniques used for the identification were those based on DNA fingerprinting and fatty acid analysis (González *et al*., 2005).

The identification of culturable bacterial strains is usually carried out through Sanger sequencing methodology (Figure 4), known as the chain termination method. It is based on the selective incorporation of chain-terminating dideoxynucleotides (ddNTPs) (Zhou and Li, 2015). The chain-terminating nucleotides lack a 3'-OH group needed for the formation of the phosphodiester bond between two nucleotides, which causes the stop of the DNA polymerase in the extension of the DNA. When a primer and template are incubated with DNA polymerase in combination with a mixture of ddTTP and dTTP (2',3'-dideoxythymidine triphosphate) and with other deoxyribonucleoside triphosphates, being one of them labeled with ^{32}P, the modified nucleotides (ddNTPs) terminated the DNA stranded elongation and the final resulting products is a mixture of fragments with the same 5' and with ddT residues at 3' ends (Sanger *et al*., 1977; Sanger and Coulson, 1976; Zhou and Li, 2015).

The sample of DNA is split in four individual sequencing reactions in which the standard dNTPs (dATP, dGTP, dCTP and dTTP) are added, as well as the DNA polymerase, and only one of the four ddNTPs (ddATP, ddGTP, ddCTP or ddTTP). The DNA is extended in different rounds, and the fragments produced are denatured and separated according to different sizes by gel electrophoresis. The DNA bands can be visualized by UV light. Alternatively, the ddNTPs may be labelled by fluorescence and could be detected automatically in sequencing machines (Sanger *et al*., 1977; Sanger and Coulson, 1976; Zhou and Li, 2015).

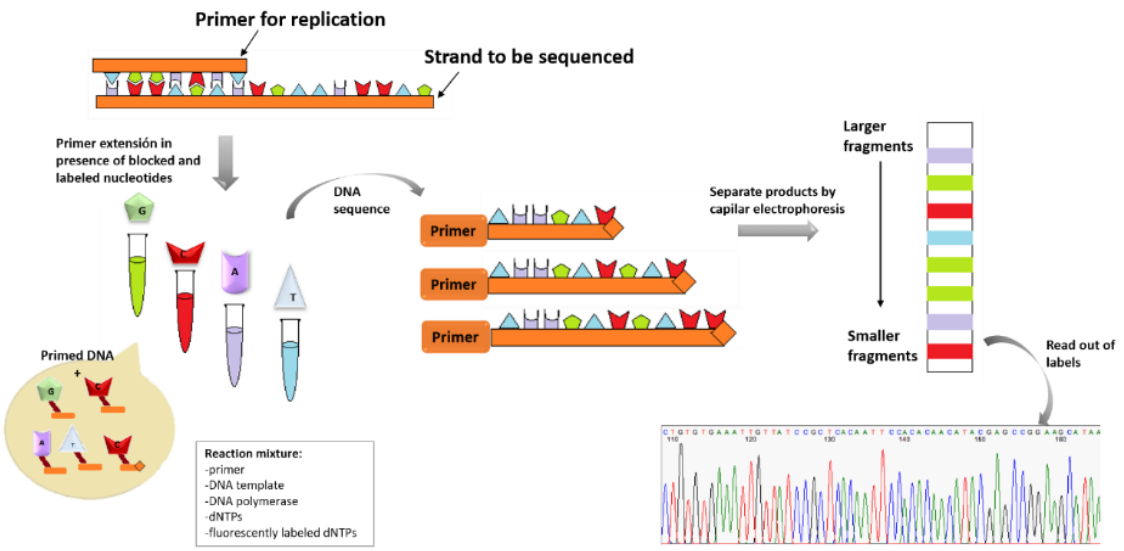


Figure 4. Scheme of Sanger sequencing methodology. Adapted from www.tes.com.

Culture-independent techniques

During the last decade, the study of lichen-associated bacteria has experienced an increasing development of culture-independent microscopic and/or molecular techniques, such as fluorescence *in situ* hybridization (FISH) and confocal laser scanning microscopy (CLSM), sequencing of microbial populations (Cardinale *et al*., 2006, 2008; Grube *et al*., 2009; Suzuki *et al*., 2016), and other approaches as fingerprinting of *RNA gene*, denaturing gradient gel

electrophoresis (DGGE), single-stranded conformation polymorphism (SSCP), and more recently sequencing technologies (Suzuki *et al*., 2016).

Techniques as FISH and CLSM have permitted the visualization of the abundance and location of lichen bacterial communities (Cardinale *et al*., 2008), showing that they generally colonize hydrophilic lichen surfaces, appearing either as individual colonies and/or forming biofilms. Among these bacterial communities, *Alphaproteobacteria* is usually the predominant taxonomic group, being lower than the presence of other bacterial groups (Bates *et al*., 2011; Cardinale *et al*., 2012b; Grube *et al*., 2009; Schneider *et al*., 2011). Further, FISH has revealed the succession of active lichen bacterial communities as well as the effects of habitat variation (Cardinale *et al*., 2012b). Besides, CLSM has been used to complement diversity studies based on other techniques, like fingerprinting or sequencing methods, providing localized information on microbial diversity (Bent and Forney, 2008).

Using FISH with bacterial group-specific probes (Grube *et al*., 2009) showed, in some lichen species (*Cladonia arbuscula*, *Lecanora polytropa* and *Umbilicaria cylindrica)*, the prevalence of *Alphaproteobacteria* in a range between 45-75%, being other bacterial groups present in lower abundance. This research group determined, as well, the location within the thallus of the bacterial communities, showing that they appeared colonizing both the surface of the extracellular polysaccharides and immersed in the intercellular gelatinous matrix (Grube *et al*., 2009). Using PCR-SSCP fingerprints, bacterial communities of the former lichen species were compared for both universal bacterial and group-specific fingerprints for *Alphaproteobacteria*, *Burkholderia* and *Pseudomonas*, obtaining differences in diversity results in the communities of both genera compared with the class (Grube *et al*., 2009).

In fingerprinting techniques, where PCR products yield analyzable banding patterns (Portillo *et al.,* 2011), different bacterial primers were able to be used, either universal or group specific, giving bands as a result that could be purified, characterized and sequenced. This methodology was able to reveal taxonomic diversity within lichen bacterial communities, being inexpensive, fast and allowing the comparison of banding patterns between samples. However, it didn't result as resolute as sequencing techniques required for phylogenetic inference (Grube and Berg, 2009). To overcome these handicaps, the analyses of *16S rRNA* libraries were commonly used to identify total bacterial communities in lichens (Aschenbrenner *et al*., 2014; Grube *et al*., 2012b, 2015; Sigurbjörnsdóttir *et al*., 2016). New generation sequencing (NGS), such as 454 pyrosequencing or Illumina, have allowed a massive sequencing of samples.

Sequencing-based on Illumina technology (Figure 5) have allowed a rapid and large-scale sequencing. In this case, templates used for sequencing are immobilized on a flow-cell surface where the DNA is available and easily accessible to enzymes. This type of solid-phase amplification produces up to 1.000 identical copies of each one of the template molecules, creating densities of around ten million single-molecule clusters per square centimeter (Illumina, 2010). This technology based on sequencing by synthesis employs four nucleotides labelled with fluorescence used to sequence the huge number of millions of clusters generated on the flow cell surface. Many sequencing cycles are performed and in each one of them, a labeled deoxynucleoside triphosphate (dNTP) is added to the nucleic acid chain. These nucleotides labels are a signal of termination for the polymerization. When a dNTP is

incorporated, a fluorescence is emitted, and this is a reflection of the base which is enzymatically split, allowing the incorporation of the next nucleotide (Illumina, 2010). All dNTPs (A, C, T, G) terminators bounded are present as individual molecules and the base calls are made from signal intensities measured in each cycle (Illumina, 2010).

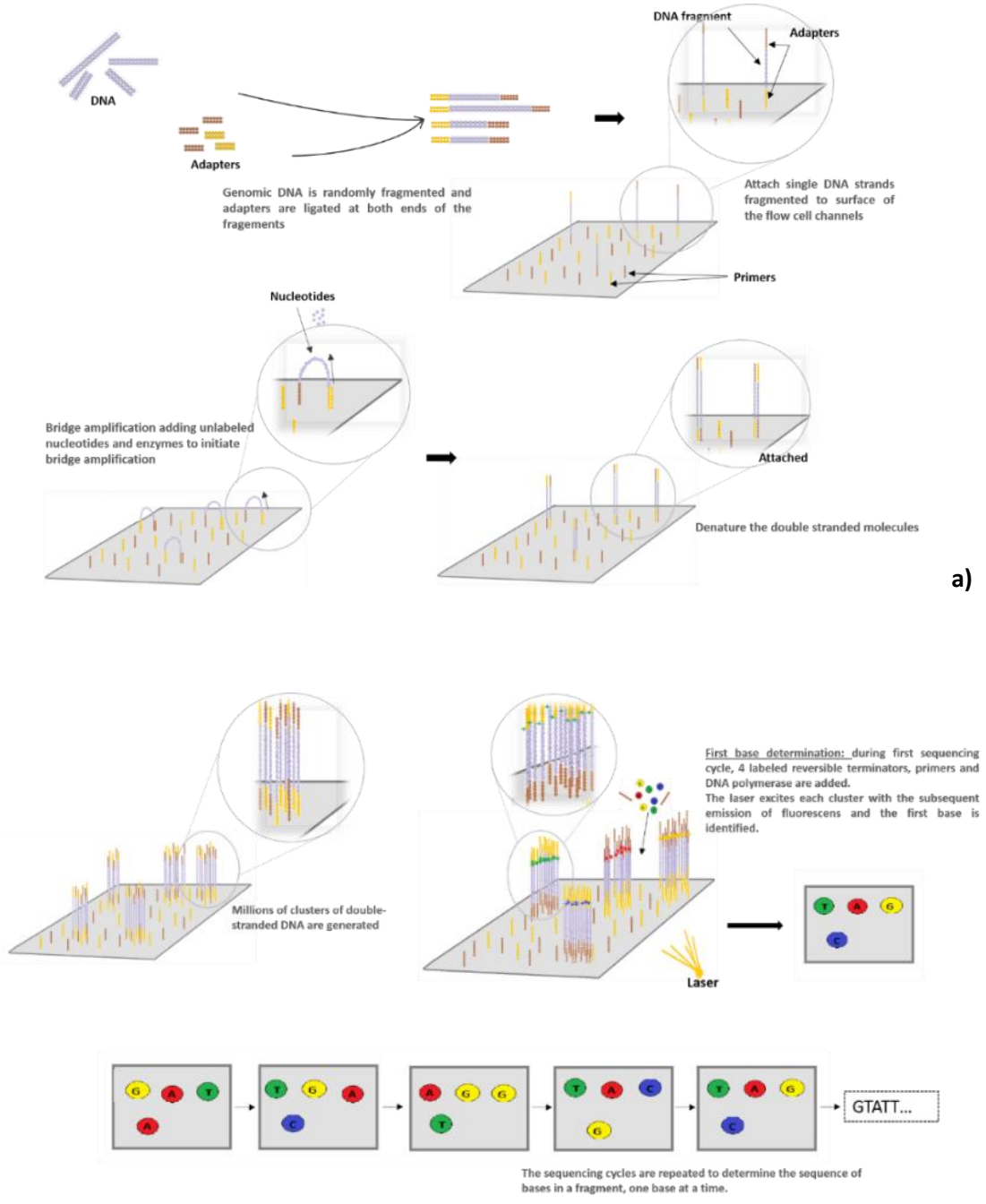


Figure 5. A representative scheme of Illumina sequencing technology. **a)** Sample preparation. Genomic DNA is fragmented and ligated to the adapters. Then, these fragments are attached to the surface of the flow-cell channels. Nucleotides and enzymes are added, and the amplification process starts. Afterwards, the double-stranded molecules are denaturated. **b)** Sequencing procedure. Several sequencing cycles are carried out. During the first cycle, four labelled reversible terminators, primers and DNA polymerase are added. A laser excites the sample, and the excitation emitted is captured as a fluorescence corresponding to the first base in each cluster. Then, new cycles of sequencing are initiated to determine the sequence of bases in a fragment. Taken from Illumina (2010).

Through these culture-independent techniques, differences were revealed in the abundance and diversity of lichen bacterial communities compared to the results achieved with culture-dependent methods. The bacterial communities of most lichens studied were dominated by *Proteobacteria*, being *Alphaproteobacteria* the dominant class, followed by other common taxa such as *Betaproteobacteria*, *Gammaproteobacteria* and *Deltaproteobacteria*. The phyla *Actinobacteria, Bacteroidetes*, *Firmicutes* and *Verrucomicrobia* were also frequently present (Grube *et al*., 2015). These studies revealed, in some cases, the presence of interesting taxonomic groups, as the order *Myxococcales* which is in relative abundance and, sometimes, in higher presence than those of *Actinobacteria*. Members of this group of *Myxococcales* are attractive due to their large genomes and the production of interesting bioactive compounds (Dworkin 2001).

The above-mentioned findings support the fact that using molecular techniques such as pyrosequencing and FISH analyses overcomes the limits imposed by traditional culture-based methods (Bates *et al*., 2011; Cardinale *et al*., 2008; Lee *et al*., 2014).

Metagenomics, as a method to detect lichen-associated bacteria of biotechnological interest, has shown that, in some cases, novel interesting molecules and biosynthetic pathways could be discovered and used as a guide for developing strategies for the culture of microorganisms of interest (Kampa *et al*., 2013).

The description of a community of organisms is usually completed with studies of their biodiversity. The biodiversity represents the variety and heterogeneity of organisms or traits at all levels of the hierarchy of life, from molecules to ecosystems (Morris *et al*., 2014). The diversity studies of the biological communities are carried out using indices of diversity that provide a quantitative estimation of the variability that composes the communities to compare biological entities (Heip *et al*., 1998). The different diversity indices try to describe the properties of the communities of organisms allowing the comparison between geographical areas, taxa and trophic levels (Morris *et al*., 2014). The species diversity indices commonly used are richness (S), Shannon's diversity (H'), Simpson's diversity (D1), Simpson's dominance (D2) and Simpson's evenness (E). Richness (S), is defined as the number of species or attributes present in a natural system and it is the simplest index metric used to represent diversity (Morris *et al*., 2014; Whittaker, 1972). Other indices combine measures of richness and abundance, as Shannon's (H') and Simpson's (D1) diversity indices. Shannon's diversity index (H') defines the uncertainty of an unknown individual. That means that in a system with a high diversity uniformly distributed, an unknown individual could belong to any species. When the system has a very low diversity, being formed by one or few species, the prediction of the identity of the unknown individuals is easier, with less uncertainty in the system (Morris *et al*., 2014; Shannon, 1948). In the Simpson's original diversity index, D_1 is the complement that expresses the idea that taking two samples randomly from a given community, they would belong to the same species. Then, the less diversity the community, the higher this probability (Heip *et al*., 1998; Morris *et al*., 2014). D_2 is the inverse of Simpson's original index, and it is used to convert Simpson's dominance index to a diversity statistic (Heip *et al*., 1998; Morris *et al*., 2014). Regarding Simpson's evenness index, it expresses how uniformly distributed are the individuals in the community over the different species. Low values of evenness indicate that

one or few species dominate, while high values mean that a relatively equal number of individuals belonging to each species (Heip *et al*., 1998; Morris *et al*., 2014).

As a summary of most studies of lichen bacterial communities based on culture-independent approaches, it could be established the general relationship between microbiomes, photobionts and mycobionts in the context of the holobiont, which indicates that most lichens harbor symbiotic *Alphaproteobacteria* as predominant class of the *Proteobacteria*, with other taxa such as *Betaproteobacteria*, *Deltaproteobacteria, Gammaproteobacteria* and the phyla *Actinobacteria, Bacteroidetes*, *Firmicutes*, and *Verrucomicrobia* being also frequently present. Deeper studies have revealed that at lower taxonomic levels there are some patterns that bring light to the importance of some groups, as *Acetobacteriaceae* (*Rhodospirillales*), *Acidobacteria*, and *Actinobacteria* in lichens growing on substrates such as acid rocks and soils as well as intertidal (the foreshore and seashore and sometimes referred to as the littoral zone) lichens (Aschenbrenner *et al*., 2014; Grube *et al*., 2015). Therefore, these studies have shown differences in abundance and diversity of lichen-associated bacterial communities when comparing culture-dependent and culture-independent techniques (Grube *et al*., 2015). A summary of some of these culture-independent studies using different techniques is shown in table 4.

Table 4. Summary of different studies through culture-independent approaches targeting the diversity of lichen-associated bacteria. Adapted from Suzuki *et al*. (2016).

Lichen species	Techniques applied	Phyla/classes described	Reference
Cladonia digitata, C. rangiferina, C. coniocracea, C. pyxidata, C. coccifera, P. furfuracea, Hypogymnia physodes, Roccella phycopsisj, R. fuciformisj	ITS fingerprinting, sequencing of bands	*Gammaproteobacteria, Actinobacteria, Betaproteobacteria*	Cardinale *et al.*, 2006
C. arbuscula	FISH, SSCP	*Alphaproteobacteria, Actinobacteria, Betaproteobacteria*	Cardinale *et al.*, 2008
C. ristatella, C. cryptochlorophaea, C. cf. sobolescens, C. peziziformis, C. subtenuis, Flavoparmelia caperata, P. perforatum, Peltigera phyllidiosa, Lasallia pensylvanica, Umbilicaria mammulata	Sanger sequencing of PCR products using universal primers	*Alphaproteobacteria, Acidobacteria, Gammaproteobacteria*	Hodkinson and Lutzoni, 2009
C. arbuscula, Lecanora polytropa, U. cylindrica	FISH, SSCP	*Alphaproteobacteria, Actinobacteria, Betaproteobacteria*	Grube *et al.*, 2009
Xanthoparmelia plittii, X. somloënsis	Pyrosequencing of *16S rRNA* genes	*Alphaproteobacteria, Acidobacteria, Bacteroidetes, Gammaproteobacteria, Deltaproteobacteria, Fibrobacteres*	Mushegian *et al.*, 2011
Parmelia sulcata, Rhizoplaca chrysoleuca, U. americana, U. phaea	Pyrosequencing of *16S rRNA* genes	*Alphaproteobacteria, Acidobacteria, Gammaproteobacteria, Firmicutes, Verrucomicrobia, Planctomycetes, Actinobacteria, Betaproteobacteria, Bacteroidetes, Deltaproteobacteria*	Bates *et al.*, 2011
Hydropunctaria maura, Ophioparma ventosa, Pertusaria corallina, Rhizocarpon geographicum	DGGE, Clone libraries	*Alphaproteobacteria, Betaproteobacteria, Actinobacteria, Acidobacteria, Cyanobacteria, Firmicutes, Chloroflexi, Bacteroidetes*	Bjelland *et al.*, 2011
L. pulmonaria	FISH, SSCP	*Alphaproteobacteria*	Cardinale *et al.*, 2012a
Cetraria islandica, L. pulmonaria, Lecanora polytropa, C. arbuscula, U. cylindrica, C. coccifera	FISH, SSCP	*Alphaproteobacteria, Betaproteobacteriae, Gammaproteobacteriae, Actinobacteriae*	Cardinale *et al.*, 2012b
Solorina crocea	Pyrosequencing of *16S rRNA* genes	*Acidobacteria, Proteobacteria, Planctomycetes, Actinobacteria*	Grube *et al.*, 2012
Cladonia sp., *Flavocetraria* sp., *Ophioparma* sp., *Umbilicaria* sp., *Usnea* sp., *Dictyonema* sp., *Leptogium* sp., *Peltigera* sp., *Sticta* sp.	Pyrosequencing of *16S rRNA* genes	*Alphaproteobacteria, Acidobacteria, Betaproteobacteria, Gammaproteobacteria, Actinobacteria, Verrucomicrobia, Planctomycetes, Bacteroidetes, Deltaproteobacteria, Firmicutes*	Hodkinson *et al.*, 2012
Arthrorhaphis citrinella, Baeomyces placophyllus, B. rufus, Icmadophila ericetorum, Psora decipiens, Trapeliopsis granulosa	FISH	*Alphaproteobacteria, Acidobacteria*	Muggia *et al.*, 2013
Rhizocarpon spp.	DGGE, 20 sequenced bands	*Alphaproteobacteria, Acidobacteria, Bacteroidetes, Firmicutes, Cyanobacteria, Actinobacteria*	Esposito *et al.*, 2013
L. pulmonaria	Pyrosequencing of *16S rRNA* genes, FISH	*Alphaproteobacteria, Bacteroidetes, Verrucomicrobia, Deltaproteobacteria, Actinobacteria, Betaproteobacteria, Gammaproteobacteria, Acidobacteria, Planctomycetes*	Aschenbrenner *et al.*, 2014
Peltigera membranacea	Shotgun pyrosequencing and *16S rRNA* genes analysis	*Alphaproteobacteria, Bacteroidetes, Actinobacteria, Betaproteobacteria, Verrucomicrobia, Gammaproteobacteria, Deltaproteobacteria*	Sigurbjornsdottir *et al.*, 2015
Cladonia symphycarpa, Diploschistes muscorum	Pyrosequencing of *16S rRNA* genes	*Proteobacteria, Bacteroidetes, Acidobacteria, Actinobacteria, Verrucomicrobia, Chloroflexi, Planctomycetes, Armatimonadetes, Cyanobacteria*	Wedin *et al.*, 2016

Bacterial abundance and diversity across different parts of the lichen thallus

Lichens are considered as self-contained ecosystems (Farrar, 1985), sheltering bacterial communities which are subjected to the influence of ecological processes operating on small scales and therefore they are not static communities (Mushegian *et al.,* 2011).

Bacterial communities are shaped across the lichen thallus since they are affected by different abiotic and biotic factors in the different thallus parts. The younger growing parts of lichens, as apices, are usually dominated by *Alphaproteobacteria*, and might act therefore as anabolic centers; the oldest senescing parts might be the catabolic areas in the lichen ecosystem. It is hypothesized that bacteria of the old parts convert this biomass into more simple molecules which could be used by the growing parts of lichens or released to the substrate (Aschenbrenner *et al*., 2016; Ellis *et al*., 2005).

Mushegian *et al*. (2011) studied the bacterial community associated with different thallus parts (central, intermediate and edges) of 9 lichens of the genus *Xanthoparmelia*. In these lichens, the center of the thallus represents the oldest part and its growth is due to a combination of ancient and regenerating tissues. By contrast, the edges of the thallus are tissues of recent growth. The center of the thalli has been exposed to bacterial colonization for a longer period than those of the edge parts and also, to environmental factors. Furthermore, in the central parts, there is a huge number of reproductive structures, such as **isidia**, a column-shaped reproductive structure, not present in the edges parts.

The bacterial communities of single lichen thallus of the different *Xanthoparmelia* species studied by Mushegian *et al*. (2011), were dominated by *Proteobacteria* and *Acidobacteria*. When bacterial diversity was studied across different thallus parts, it was found that *Acidobacteria* had a significantly higher abundance in the center than in the edges of the lichen thallus. In the case of *Proteobacteria*, the *Alphaproteobacteria* was the dominating group, being the main orders *Rhizobiales, Rhodospirillales* and *Sphingomonadales*. Other bacterial groups that appeared in large proportion were unclassified.

When all lichens species were analyzed as a pool, the data obtained showed that the centers of the lichens harbour a higher number of bacterial species with well-established communities, whereas those present in the edges seemed to be less diverse and more variable. In addition, lichen centers suffered a bacterial dispersion during longer periods than edges. Furthermore, there was a similarity in richness and composition between the center and intermediate parts of the lichens, suggesting that the isidiated lichen center may have an important role determining the bacterial community composition compared with the non-isidiated lichen edges parts (Mushegian *et al*., 2011).

Another study that tried to elucidate the bacterial communities associated with the different parts of the lichen thallus was the one conducted by Pankratov (2012). This author focused on lichens of *Cladonia* and *Sphaerophorus* genera collected from the bog of Lake Verkhnee near the White Sea Biological Station, a tundra region. In this case, the lichen thallus was divided into three parts: the upper living part of the thallus, the intermediate decaying part and the underlying peat. This study revealed that *Alphaproteobacteria*, *Acidobacteria* and *Actinobacteria* were the more abundant groups. Furthermore, the highest number of

Alphaproteobacteria was found in the living parts of the lichen thalli. In the samples from bog area, *Actinobacteria* was the dominant group in the peat and living thallus, being in lower number in the decaying zone. *Betaproteobacteria* appeared in low number in all analyzed lichens, being the highest number in the intermediate decaying part of tundra lichens, and the lowest in the *Sphagnum* bog samples. Members of *Firmicutes* and *Gammaproteobacteria* were minor in all lichen samples. *Acidobacteria* showed the highest number in the living zone of the *Sphagnum* bog lichens (Pankratov, 2012).

Besides, the presence of lichen-associated bacteria on the surface of the lichen thallus or in intercellular spaces have been previously revealed (de los Ríos *et al*., 2005b), usually developing biofilm-like structures (Grube *et al*., 2009). The ectolichenic and endolichenic bacterial community fractions only have been studied in some lichen species, such as *C. arbuscula*, *U. cylindrica* or *L. polytropa*, being usually more specific in the endolichenic fraction than in the ectolichenic one. However, in many of these studies, the lichen surface was subjected to disinfection (Grube *et al*., 2009) which could bias the results.

In some lichen species it was visible a continuous layer of bacterial colonies (Honegger, 2012). An example of the presence of ectolichenic bacteria is shown in figure 6. In *R. farinacea* transmission electron microscopy (TEM) allowed the visualization of cyanobacterial aggregates associated externally to the cortex (García-Breijo *et al.,* 2010).

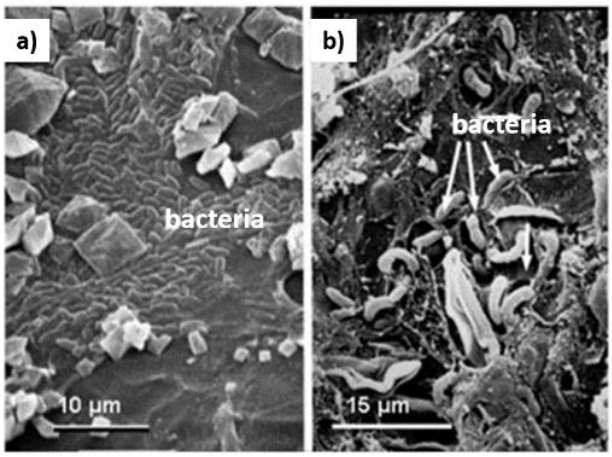


Figure 6. Representative pictures of ectolichenic bacteria associated with lichens visualized with scanning electron microscopy (SEM) micrography. **a)** Bacterial biofilm on the upper cortex of lichen thallus. **b)** Bacteria within the tubular thallus of a reindeer lichen. Taken from Honegger (2012).

In the lung lichen *L. pulmonaria*, Aschenbrenner *et al*. (2014) revealed that the bacterial colonization on symbiotic propagules shared the microbiome composition at class level with the rest of the thallus. With FISH-CLS these authors were able to determine the localization of these bacterial communities on the surface of these propagules, which are young structures produced on the upper surface of the lichen thallus, lacking bacterial taxa common on the lower surface of the thallus. The upper surface of thalli might be composed of bacterial taxa that tolerate desiccation and could come from rain, wind and some animals, whereas the lower surface, usually in the shadow, presents other ecological conditions for its colonization (Aschenbrenner *et al*., 2014).

In another study, Grube *et al*. (2015) showed that bacteria colonizing the lichen surface are well-adapted to abiotic stress, such as osmotic and oxidative stress, thus suggesting that bacterial communities on long-living lichen thallus surface are quite constant over seasons. Then, bacteria sensitive to oxidative stress and other extreme and selective conditions hardly survive. These non-adapted bacteria that will be degraded by oxidation, would suppose a source of additional nutrients, an important factor in lichens living in oligotrophic environments (Paungfoo-Lonhienne *et al*., 2010; White *et al*., 2012). Therefore, the authors hypothesized that periods of hydration may act as a selective condition for the enrichment and acquisition of stress-tolerant and more specific bacterial communities, which could be involved in enhancing the longevity and persistence of lichens under extreme ecological conditions (Grube *et al*., 2015).

Bacterial diversity shaped by geography in lichens

Lichens are interesting for their ubiquity and particular tolerance to different environmental conditions. Most of the lichen bacterial communities are correlated with differences in large-scale geography (Hodkinson *et al*., 2012).

The distribution of lichens around the world is well documented (Galloway, 2008), but the knowledge about the geographical patterns of their associated bacteria is scarce (Cardinale *et al*., 2012a). Some studies were conducted to elucidate the influence of geography in the structure of the bacterial communities associated with lichens. It was demonstrated that these communities vary between different types of lichens in different regions, and that exists a complex relationship between lichens and their associated bacteria (Hodkinson *et al*., 2012). These authors found that lichen-associated bacteria may be modulated by geographical differences in accordance with dispersal patterns. Also, that geography was not significant on a small spatial scale, in which the dispersion of lichens occurs quite frequently. By contrast, it was significant on a large spatial scale, in which the host dispersal could be a limiting factor (Hodkinson *et al*., 2012). Another study focused on the influence of geography as a factor modulating the taxonomical structure of the lichen-associated bacteria, was the one conducted by Cardinale *et al*. (2012a). These authors studied the lung lichen *L. pulmonaria* and selected *Alphaproteobacteria* and *Burkholderia* for a fingerprinting analysis of their geographically correlated structure. They assumed that the lichen offers a similar habitat independently of the growing region and the distance among these locations, since *L. pulmonaria* has strict requirements for growing and it offers stable environmental parameters to the associated bacteria. However, their results showed a high diversity, suggesting that site-specific environmental factors were affecting the taxonomic structure of the studied bacterial taxa, concluding that the bacterial groups investigated were shaped by geography and habitat. In the case of *Alphaproteobacteria*, it was shown that this group was the dominant among the lichen-associated bacteria, as it was demonstrated in other studies, and that it was maintained across space, showing a good correlation with geography. *Burkholderia* genus was present in lower abundance and didn't show this geographical correlation. These results suggested an ecological significance of different bacterial groups of the lichen microbiome and their implication in the lichen symbiosis (Cardinale *et al*., 2012a).

A different study with the lichen *Cetraria aculeate*, commonly growing at high latitudes, from various habitats in different countries (Antarctic, Iceland, Germany and Spain) also revealed that the dominant bacterial group was *Alphaproteobacteria*, and among them, the *Acetobacteriaceae* (Printzen *et al*., 2012). Further, alphaproteobacterial communities of lichen samples from high latitudes were impoverished and more related among them than those of extrapolar habitats, suggesting that their composition in this lichen species might be influenced by environmental parameters (Printzen *et al*., 2012).

Another interesting study that addressed the influence of the environment on lichen-associated bacteria was the one conducted by Aschenbrenner *et al*. (2017). They compared the microbiome and the intermicrobiome relationship of three organisms, the lung lichen *L. pulmonaria*, the co-occurring moss *Pterygynandrum filiforme*, and the bark of the maple tree, *Acer pseudoplatanus*, on which the two epiphytes grow. Their results showed an overlapping in the microbial communities at all taxonomic levels. They found a quantitative distribution of generalist microorganisms in all habitats, while the specialist ones were different in their taxonomic affiliation and abundances in each ecosystem (Aschenbrenner *et al*., 2017). The ecological facilitation may explain some of the shared bacterial taxa among the three organisms. This ecological concept refers to positive outcomes of encounters (Bruno *et al*., 2003), as when one organism offers a more favourable local environment for another. The results of Aschenbrenner *et al*. (2017) suggested an ecological facilitation among the studied species

The influence of geography as well as lichen intrinsic traits (species, thallus age, growing type) and environmental factors (sun exposure and substrate type) as determinant factors shaping the taxonomical structure of lichen-associated bacteria has been studied in several lichen species (Aschenbrenner *et al*., 2014; Cardinale *et al*., 2006, 2012b; Lee *et al*., 2014). For instance, sunlight exposure had a significant effect on lichenic bacterial communities, with a higher number of bacteria in lichens growing under shaded conditions than in the ones exposed to the sun. Aging appeared to have a significant influence on the bacterial community structure as well, with the old senescing parts of the lichen thallus harboring a higher number and more variable community of bacteria than the younger parts (Cardinale *et al*., 2012b). The older parts of the lichen harboured diverse bacterial taxa, whereas in the younger and vital parts, the spectrum of growing bacteria was more limited, and these bacteria were the most adapted ones, mostly *Alphaproteobacteria*. *Betaproteobaceria* were more abundant in the older parts of the thallus, with other predominant groups such as *Actinobacteria* and *Gammaproteobacteria* (Cardinale *et al*., 2012b). The substrate type is also an important factor that modulates the bacterial communities. In the case of lichens growing on rocks, they shelter fewer bacteria than those growing in other substrate types as soil or bark of tree. Other factors considered relevant, as thallus forms and growth types did not have a clear effect on the taxonomic structure of lichenic bacterial community (Cardinale *et al*., 2012b).

In summary, this kind of studies exemplify the ecology of lichen-associated bacterial communities and give evidence of the influence of the environment in their composition. (Cardinale *et al*., 2012b).

Interestingly, it has been suggested that lichen-forming fungi are able to adapt to variable environmental conditions modifying their bacterial community. Nevertheless, there is still scarce information about how this variation of species in the bacterial communities associated with lichens is influenced by geographical ranges and other intrinsic factors (Cardinale *et al*., 2012a, 2012b; Hodkinson *et al*., 2012; Printzen *et al*., 2012).

Contribution of bacteria to lichen symbiosis

Lichens have evolved into a wide diversity that allowed them the adaptation to a huge variety of environments, being hypothesized that bacterial communities might participate in the adaptation of lichens to different ecological conditions (Farrar, 1985). Lichens, as self-contained ecosystem, may be involved in the promotion of bacterial diversification (Kirkelund *et al.,* 2007). Therefore, they are considered as a nursery of bacterial diversity which might be implied in the lichen tolerance to adverse conditions as extreme climates, salt, radionuclides, etc. (Grube *et al.,* 2009).

The potential functional roles and metabolic activities of lichen-associated bacteria remains still practically unknown in most of lichen species. However, some important eco-physiological roles have been proposed based mainly on culture-independent approaches (Cardinale *et al*., 2012b; Grube *et al*., 2009, 2015; Muggia *et al*., 2013; Navarro-Noya *et al*., 2014; Printzen *et al*., 2012; Schneider *et al*., 2011; Sigurbjörnsdóttir *et al*., 2014, 2016):

- Nutrient supply of nitrogen, phosphorus, sulphur and iron.
- Production of bioactive metabolites conferring resistance against biotic stresses.
- Resistance to abiotic factors.
- Vitamins supply, like vitamin B12, giving support to photosynthesis.
- Hormones supply for fungal and algal growth.
- Detoxification of metabolites.
- Degradation and nutrient recycling of senescent parts of the lichen thallus

In the lichen symbiosis, the supply of essential nutrients could be covered by bacterial partners which possess extracellular enzymes that could contribute to the mobilization and recycling of nutrients compounds in the lichen thallus. In tripartite lichens cyanobacteria provide nitrogen, but non-photosynthetic bacteria might also fix and supply nitrogen.

Functional and metagenomic studies revealed many of the lytic activities in which lichen-associated bacteria are involved, as chitinolysis, proteolysis and glucanolysis (Grube *et al*., 2009, 2015; Lee *et al*., 2014; Schneider *et al*., 2011; Sigurbjörnsdóttir *et al*., 2016).

Phosphate solubilizing bacteria can release phosphorus making it available to plants (Chhabra *et al*., 2013; Zhao *et al*., 2014). Phosphate solubilization involves several enzymes as alkaline and acid phosphatases, phytases and some organic acids, as gluconic acid, which are necessary for acidification, an essential step for the solubilization of mineral phosphates (Rodríguez *et al*., 2006; Sharma *et al*., 2013; Sigurbjörnsdóttir *et al*., 2016).

Siderophores are organic compounds able to chelate ferric iron (Fe(III)) from the environment. Under iron-limited conditions, many Gram-negative and Gram-positive bacteria synthesize

these low molecular weight iron-chelating compounds (Krewulak and Vogel, 2008). Metagenomic analyses have shown the presence of Ton and Tol transport systems involved in the iron uptake in the microbiome of some lichens. Metaproteomic approaches revealed the presence of at least four different types of TonB receptors in *Proteobacteria* and *Bacteroidetes* phyla (Grube *et al*., 2015).

Algal and fungal cells that form the holobiont contain polysaccharides and proteins, nutrient sources that lichenic bacteria might be able to degrade and exploit through different lytic activities, when the fungal and algal partners become metabolically inactive, thus recycling the older parts of the thallus (Grube *et al*., 2009; Sigurbjörnsdóttir *et al*., 2016).

Phytohormones play an important role regulating plant growth and development. One of the most physiologically active phytohormones is the auxin indole acetic acid (IAA), which has been reported to be produced by several bacterial strains associated with some lichen species (Grube *et al*., 2009; Hayat *et al*., 2010; Liba *et al*., 2006). It has been proposed that IAA can influence morphogenetic processes in both mycobiont and photobiont partners (Grube and Berg, 2009).

The presence of bacteria and their metabolites, even if minute, could be involved in ecological and biological roles in the lichen symbiosis (Parrot *et al*., 2016). The mechanisms that regulate the abundance and the diversity of lichen-associated bacteria are still unknown, but it is hypothesized that they also might protect lichens against invasion by pathogenic bacteria, being secondary metabolites with antibacterial activities involved in this function (Boustie and Grube, 2005; (Grube *et al*., 2009). A diagram summarizing the different potential contributions of symbiotic partners in the lichen symbiosis is shown in figure 7.

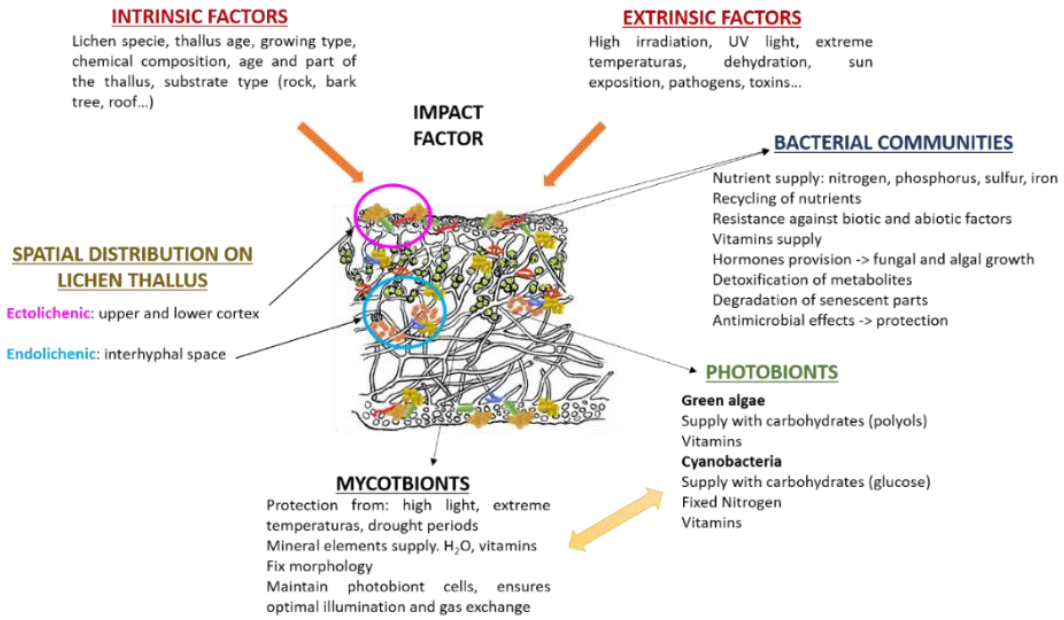


Figure 7. Representative diagram showing the relationships among the different partners in lichen symbiosis, with their potential respective roles: photobionts, mycobionts and bacterial symbionts are summarized, as well as intrinsic and extrinsic factors affecting the lichen ecosystem. Adapted from Parrot *et al*. (2016).

1.3.4 Other microorganisms associated with lichens

There is a diversity of eukaryotic organisms that may also be associated with lichen thallus. Lichens can host fungal species different from the mycobiont, such as lichenicolous fungi (highly specialized and successful group of organisms that develop on lichens) (Lawrey *et al*., 2007; Lawrey and Diederich, 2003) and endolichenic fungi, which are endophytes, living in the lichen thalli without causing damage and without producing sporulating structures at the surface of the lichen host (Girlanda *et al*., 1997; Suryanarayanan *et al*., 2005; U 'Ren *et al*., 2010).

Tardigrades are one of the eukaryotes known to inhabit lichens (Bartels and Nelson, 2007). In 2012, Bates *et al*. (2012) studied the eukaryote organisms associated with the surface of foliose lichens through pyrosequencing, using primers targeting the *18S rRNA* gene. They found around 50 eukaryotic phylotypes belonging to nine phyla within the main clades of Eukarya: *Alveolata* (*Ciliophora*), *Fungi* (Ascomycota, *Basidiomycota*, *Blastocladiomycota*, *Chytridiomycota*), *Metazoa* (*Rotifera*, *Tardigrada*), *Rhizaria* (*Cercozoa*), and *Viridiplantae* (*Chlorophyta*).

In 2016, Spribille *et al*. (2016) provided new insights about potential new members in the lichen symbiosis. They discovered ubiquitous yeasts embedded in the cortex, hypothesizing the idea that more than one fungus may participate in the construction of the lichen structure.

As a result of these studies, the concept of lichens as symbiotic associations of only two or three partners have changed, being now considered as minute ecosystems where numerous symbiotic partners may interact, thus being considered as multispecies symbiosis (Aschenbrenner *et al*., 2016).

1.4 LICHEN PROPERTIES AND BIOTECHNOLOGICAL APPLICATIONS

Humans have exploited lichens for many purposes during hundreds of years, for human and animal nutrition, as dyers, in the perfume and alcohol production, in medicine and cosmetic purposes and as bioindicators, among others (Kosanić *et al*., 2012; Kumar *et al*., 2010; Srivastava *et al*., 2013).

During XIX century lichens were recognized and increasingly used as bioindicators, since their vital functions are related to environmental effects, either from natural or human origin. Thus, these organisms have been used to detect the presence of some pollutants (Hawksworth *et al*., 2005), allowing an immediate measure of contamination levels in big areas (Hawksworth *et al*., 2005). Many contaminants have been detected and monitored, as sulphur dioxide, ammonia, fluorides, alkaline dust, radioactive metals and heavy metals, chlorinated hydrocarbons, as well as eutrophication and acid rain.

Derivative products from lichens could be found among different food industry applications as well. Some of them, including cyanobacterial lichen species, as *Nephroma arcticum*, contain antifreeze proteins (AFPs) that could be applied to some food products, as ice creams (Oksanen, 2006).

Lichens are pigment-rich organisms (Elix and Stocker-Wörgötter, 2008; Friedl and Büdel, 2008; Kappen, 2000; Nybakken *et al*., 2004; Vráblíková *et al*., 2006). Natural pigments have important functions as screening of UVB for melanins and parietins and can be used also as dyes (Cohen and Towers, 1995; Nybakken *et al*., 2004; Oksanen, 2006; Vráblíková *et al*., 2006), as well as catalysts in wood pulp production and in the paper industry (Oksanen, 2006). Carotenoids are important natural pigments also found in cyanobacterial species (Lee and Schmidt-Dannert, 2002).

Lichen secondary metabolites have diverse biological actions, as antibiotic, antimycotic, enzyme inhibitory, allergenic, antiviral, anti-inflammatory, analgesic antipyretic, antiproliferative and cytotoxic effects (Huneck, 1999; Manojlovic *et al*., 2010; Manojlović *et al*., 2010; Molnár and Farkas, 2010; Shukla *et al*., 2010; Srivastava *et al*., 2013). Lichens chemistry have been studied for more than a hundred years and numerous compounds with biotechnological interest have been found (Elix and Stocker-Wörgötter, 2008). Of the total lichen secondary metabolites, relatively few of them have been analyzed deeply for their biological and therapeutic activities, mostly, due to difficulties in obtaining them in relatively enough quantities and purity (Kumar *et al*., 2010). Among these lichen products, are remarkable the usnic acid, acetone, methanol, light petroleum, phenolic compounds, anthraquinones, dibenzofurans, depsides, depsidones, depsones, gamma-lactones and pulvinic acid derivatives (Kumar *et al*., 2010; Srivastava *et al*., 2013).

Lichens, as well-adapted structures to a wide variety of ecological niches, some of them with extreme conditions, have mechanisms for surviving. These are, in some cases, of chemical nature, such as UV screens, osmolytes, etc. with some potential applications in biotechnology. Other compounds, like the allelopathic, produced by the holobiont could have an important functional role in lichen existence and maintenance.

Biotechnological applications of lichen-associated bacteria

The World Health Organization defines bioprospecting as the systematic search for and development of new sources of chemical compounds, genes, microorganisms, macroorganisms, and other valuable products from nature (Timmermans, 2001).

Lichen-associated bacteria represent an important source of bioactive molecules (Cardinale *et al*., 2006, 2012b; Parrot *et al*., 2015). Some of them were reported to be potent antibiotics at very low concentrations (Davis *et al*., 2005). In the same way, lichens are a source for antagonistic bacteria which could be used for biological control to protect plants against biotic and abiotic stress (Cernava *et al*., 2015b).

The isolation of culturable bacteria from lichens have shown particular groups commonly recovered such as *Gammaproteobacteria*, *Firmicutes* and *Actinobacteria* (Cardinale *et al*., 2006; Grube *et al*., 2009; Selbmann *et al*., 2010), well known for their production of enzymes and bioactive compounds with biotechnological applications, such as secondary metabolites with antibacterial properties against human pathogens and cytotoxic properties (Parrot *et al*., 2015; Schroeckh *et al*., 2009). In some studies, these activities were demonstrated experimentally (Grube *et al*., 2009; Lee *et al*., 2014), while in others they were concluded

through the detection of genes involved in the biosynthesis of secondary metabolites (Parrot *et al*., 2015). Cernava *et al*. (2015a) used the lichen *L. pulmonaria* for screening the antagonistic potential of lichen-associated bacterial communities, mainly dominated by *Stenotrophomonas*, *Pseudomonas* and *Burkholderia*, through multi-omics and high-resolution mass spectrometry techniques.

Volatile organic compounds (VOCs) can affect the growth, antibiotic production, and gene expression of soil bacteria. They are compounds with high vapor pressure at room temperature, and they are produced by most of organisms acting as communication molecules (Effmert *et al*., 2012). In some cases, they are related to either the growing promotion or the growth reduction in *Arabidopsis thaliana* (Ryu *et al.,* 2003) and could be involved in the growth suppression of soil-borne pathogenic fungi (Garbeva *et al*., 2014; Kai *et al*., 2007).

A number of bacterial colonies retrieved from the lichens *C. arbuscula*, *L. polytropa* and *U. cylindrica* had the ability to grow in nitrogen-free medium, indicating their role as nitrogen-fixing bacteria (Grube and Berg, 2009). Furthermore, many of them displayed different lytic activities and were able to solubilize phosphate or to produce growth-promoting hormones as IAA (Aschenbrenner *et al*., 2014; Sigurbjörnsdóttir *et al*., 2016).

Some of these interesting bacteria and cyanobacteria isolates belonged to the genera *Bacillus*, *Burkholderia, Nostoc, Paenibacillus*, and *Pseudomonas.* Even the cyanobionts presented bioactive molecules with some potential biotechnological interest (Suzuki *et al*., 2016). Despite representing a minor percentage of the total of bacteria associated with lichens, they should be taken into account when their role in the lichen symbiosis is evaluated (Suzuki *et al*., 2016).

2. THE LICHEN *Ramalina farinacea*

2.1 BIOLOGY

R. farinacea is an epiphytic fruticose lichen species (Figure 8) with pendant greenish thallus with an asexual reproduction (del Campo *et al*., 2010; Del Hoyo *et al*., 2011). This lichen lives preferably as an epiphyte and unusually as saxicolous (growth in a rock) (García-Breijo *et al.,* 2010). It has a wide distribution in Mediterranean areas, including the Iberian Peninsula, the Canary Islands and California. However, *R. farinacea* can also be found in boreal forests in the north part of Europe and in the middle and high mountains, in the central and southern Europe as well as in xeric Mediterranean areas. This lichen species can colonize different substrates on a diversity of shrubs, hedgerows and trunks and twigs within shaded deciduous woodlands to sunny wind-exposed isolated trees and barely on rocks and walls, but especially present in oak forests and also on pinus trees (del Campo *et al*., 2010; Del Hoyo *et al*., 2011). It can tolerate very extreme ecological conditions suffering desiccation during long periods in summer and rehydration by dew in the nights and/or periods of rain that take place during spring and autumn seasons. It could be also found in less stressful environments such as humid ecosystems but also in more restricted areas as in high mountains. This suggests an ecophysiological plasticity of *R. farinacea* to adapt and survive in a great variety of environments (del Campo *et al*., 2010; Del Hoyo *et al*., 2011).

It is speculated that *R. farinacea* was originated in the Macaronesian-Mediterranean region and continued colonizing gradually more temperate and boreal regions of the Northern hemisphere, being the Canary Islands, most probably, the Southernmost limit in the Atlantic region (del Campo *et al*., 2013).

R. farinacea is a special case in which the mycobiont associates specifically with two genetically and morphologically different phycobionts, *Trebouxia* microalgae (*T. jamesii* (TR1) and *Trebouxia* sp. TR9) that coexist within the lichen thallus (Casano *et al*., 2015; del Campo *et al*., 2013; Del Hoyo *et al*., 2011; García-Breijo *et al*., 2010). This lichen species exemplifies the type of lichen symbiosis that is maintained through the propagation of the mycobiont and the two phycobiont taxa. The presence, association and interaction of these two photobionts might allow the lichen to proliferate in different habitats and the distinct and complementary ecophysiological responses of each phycobionts might help to the preservation of this pattern of lichen symbiosis (Casano *et al*., 2011; García-Breijo *et al*., 2010). In fact, these two phycobionts show different physiological responses to some environmental conditions (Casano *et al*., 2011), as the oxidative stress (Del Hoyo *et al*., 2011).

Figure 8. Picture of the fruticose lichen *R. farinacea*. Source: author's personal pictures.

2.2 STRUCTURE

Morphologically, *R. farinacea* presents a heteromerous thallus. The biochemical composition of the cell walls of the *Trebouxia* microalgae revealed a low presence of cellulose (König and Peveling, 1984) and some uncommon polymers in green microalgae, such as β-galactofuranans (Cordeiro *et al*., 2005, 2007, 2008). In other lichen species, these galactofuranose-rich heteropolysaccharides have a structural function, as in *R. gracilis* and *Cladina confusa* (Cordeiro *et al*., 2007, 2008).

2.3 SYMBIOTIC PARTNERS

As above mentioned, a previous study performed on *R. farinacea* showed the co-occurrence of at least two different species of *Trebouxia* genera algae (TR1 and TR9) (Figure 9), in different lichen populations collected in Spain, thus suggesting a selective association of the lichen *R.*

farinacea with their symbionts (del Campo *et al*., 2010). TR1 could be *T. jamesii* in a 100% identity, while TR9 belongs to a closely related *T. jamesii* undescribed species (92% identity of *nrITS* sequences), being TR1 predominant in the populations in the Iberian Peninsula and California whereas TR9 is more abundant in those in the Canary Islands (Casano *et al*., 2011; del Campo *et al*., 2010, 2013).

Regarding the genetic diversity of *R. farinacea*, it has been described a higher diversity in both mycobiont and phycobionts in the Canary Islands populations than in the ones from the Iberian Peninsula due to a differential specialization of the symbionts. This could be explained due to the opportunities for speciation have a broadly predictable relationship to the life cycle of oceanic islands, and in the Canary Islands there is an immaturity-speciation pulse model of island evolution (Whittaker *et al*., 2007).

During the last years, the studies conducted through molecular approaches have shown the high diversity of lichen-associated bacterial communities, composed of millions of bacterial cells per gram of lichen thallus (Bates *et al*., 2011; Biosca *et al*., 2016; Cardinale *et al*., 2006, 2008, 2012a; González *et al*., 2005; Grube *et al*., 2009; Grube and Berg, 2009; Hodkinson and Lutzoni, 2009; Liba *et al*., 2006). These bacteria associated form stable communities and are considered as a third partner of the lichen symbiosis (Grube *et al*., 2015). During the last decade, some studies have shown the presence of numerous bacterial aggregates associated to the hyphae of the cortex. These bacterial communities seem to be part of the complexity of lichen symbiosis (Aschenbrenner *et al*., 2016; Grube *et al*., 2009; Liba *et al*., 2006). They might be implied in the recycling of nutrients and mineral elements in the lichen thallus.

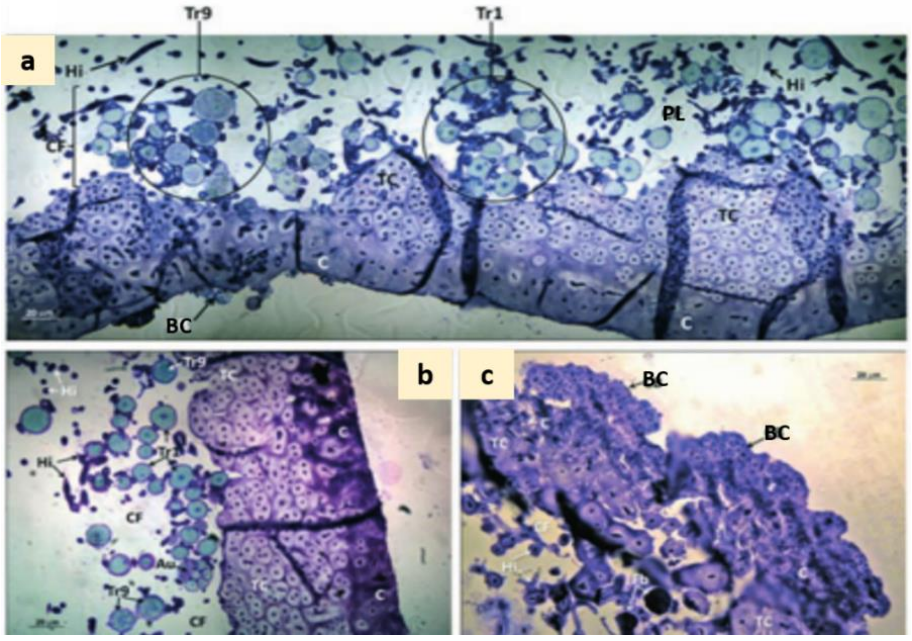


Figure 9. Semifine sections of *R. farinacea* thalli. **a)** Location of the two photobionts, *Tr1* and *Tr9* in the photobionts layer (PL). A bacterial colony is visible in the external cortex. **b)** Transversal section of *R. farinacea* thallus whit the two photobionts, *Tr1* and *Tr9*. **c)** Transversal section where it is possible to appreciate bacterial colonies associated with the external cortex. Taken and adapted from García-Breijo *et al*. (2010).

Besides, through TEM some aggregates of cyanobacteria were found associated externally to the cortex (García-Breijo *et al*., 2010). However, bacteria associated with *R. farinacea* have not been explored yet and their role in this lichen symbiosis is still unelucidated.

OBJECTIVES

Based on the background exposed in the introduction and the so far scarce information on the diversity, functional roles and biotechnological potential of bacterial communities associated with lichens, and particularly on the lichen species *R. farinacea*, it is ncessecary to to advance in the knowledge of these communities stil little explored. Therefore, the main objective of this Doctoral Thesis has been the study of the bacterial communities associated with the lichen *R. farinacea* with special interest in their composition, diversity and biotechnological potential. This main research objective (of this Doctoral Thesis) can be divided in the following partial objectives:

1. To isolate and characterize physiologically and metabolically a collection of bacterial strains from populations of *R. farinacea* from different geographical Spanish locations, either for their possible contribution to the lichenic symbiosis and -with special interest- for their biotechnological potential, as well as to initiate a molecular identification of the isolated strains of most biotechnological interest.
2. To study the composition and diversity of the bacterial communities associated with the different populations of *R. farinacea* studied, through culture-dependent techniques, and to determine the influence of the geographical origin or the location in the lichen thallus (ecto- or endolichenic).
3. To analyze the composition and diversity of the bacterial communities associated with the same populations of *R. farinacea* through culture-independent techniques, as well as to investigate the influence of geography, the location in the lichen thallus (ectolichenic or endolichenic, or apical, middle and basal) and the effect of a disinfection treatment.

3. *R. farinacea* LICHEN SAMPLES, SITE DESCRIPTION AND SAMPLING PROCEDURE

Lichen thalli of *R. farinacea* from four non-polluted different locations (Figure 10) in Spain, two in Tenerife (Canary Islands) and two in the Iberian Peninsula were analyzed. Those from Tenerife were sampled from two *Pinus canariensis* Chr. Sm. Ex DC. forests at La Guancha (28º23'23''N 16º37'47'' W) and La Esperanza (28º26'23''N 16º22'29''W), respectively. Those from the Iberian Peninsula were collected from two *Quercus rotundifolia* Lam. forests at El Toro (Castellón) (39º57'39.8''N 0º46'15.2''W) and Lidón (Teruel) (40º43'38.1''N 1º04'29.9''W). Environmental data of each one of the four locations at the moment of the sampling are summarized in table 5. Ten lichen thalli appearing healthy were collected from tree bark from at least five randomly selected trees within an area of about 50 m^2, at each location. Thalli samples were collected under aseptic conditions, separately transferred into sterile plastic Petri plates and transported and stored under refrigeration until processing within one day after sampling.

Figure 10. Map of Spain indicating the geographical locations where the thalli samples of *R. farinacea* were collected. On the left, the island of Tenerife, with the two locations of La Guancha and La Esperanza. On the right, the two locations in the Iberian Peninsula, El Toro (Castellón) and Lidón (Teruel).

Table 5. Environmental data of the locations where *R. farinacea* thalli samples were collected.

Location	Temperature	Humidity	Altitude (m)
La Guancha	18ºC	70%	370
La Esperanza	16.5ºC	80%	632
El Toro	16ºC	65%	1011
Lidón	11ºC	61%	1211

4. ANALYSES OF CULTURABLE BACTERIA ASSOCIATED WITH *R. farinacea*

4.1 ISOLATION OF *R. farinacea* CULTURABLE BACTERIA

Five lichen thalli from each location were used for the bacteriological analysis of bulk thalli samples, using part of the remaining thalli to prepare lichen enriched media (Biosca *et al*., 2016). Isolation of bacteria associated with *R. farinacea* from bulk thalli samples was made following the method described by Biosca *et al.* (2016) on lichen enriched media (Figure 11). Briefly, bulk samples of 1 g of *R. farinacea* (0.2 g subsamples of five thalli from five different trees) were analyzed for the isolation of both ectolichenic and endolichenic bacteria after a 1 min wash in sterile distilled water to remove the environmental powder. An extended washing (90 min) in sterile Ringer solution plus 0.05% of Tween 20 (T20) (RST) was performed at 200 r.p.m. at room temperature to isolate ectolichenic bacteria. Thereafter, the washed thalli were crushed in sterile antioxidant maceration buffer (AMB) to isolate endolichenic bacteria. To estimate ecto- and endolichenic bacterial populations associated with *R. farinacea,* aliquots from thalli washings and crushed washed thalli suspensions were serially tenfold diluted in 10 mM phosphate buffered saline (PBS) (pH 7.0) and plated (0.1 ml) in triplicate on AB minimal medium (Chilton *et al*., 1974) enriched with 0.5% fresh *R. farinacea* extracts (ABL) with or without 0.5% defined carbon sources, glucose and mannitol (ABLGM) supplemented with 21.6 mg/L of the fungicide natamycin, according to Biosca *et al.* (2016). Plates were incubated for two weeks at 26ºC under dark conditions and bacterial counts (colony forming units per gram (CFU/g) of thalli) were determined periodically. Bacterial colonies showing different morphologies on each medium were purified and cryopreserved at -80ºC in 25% (v/v) glycerol, for subsequent characterization (see below).

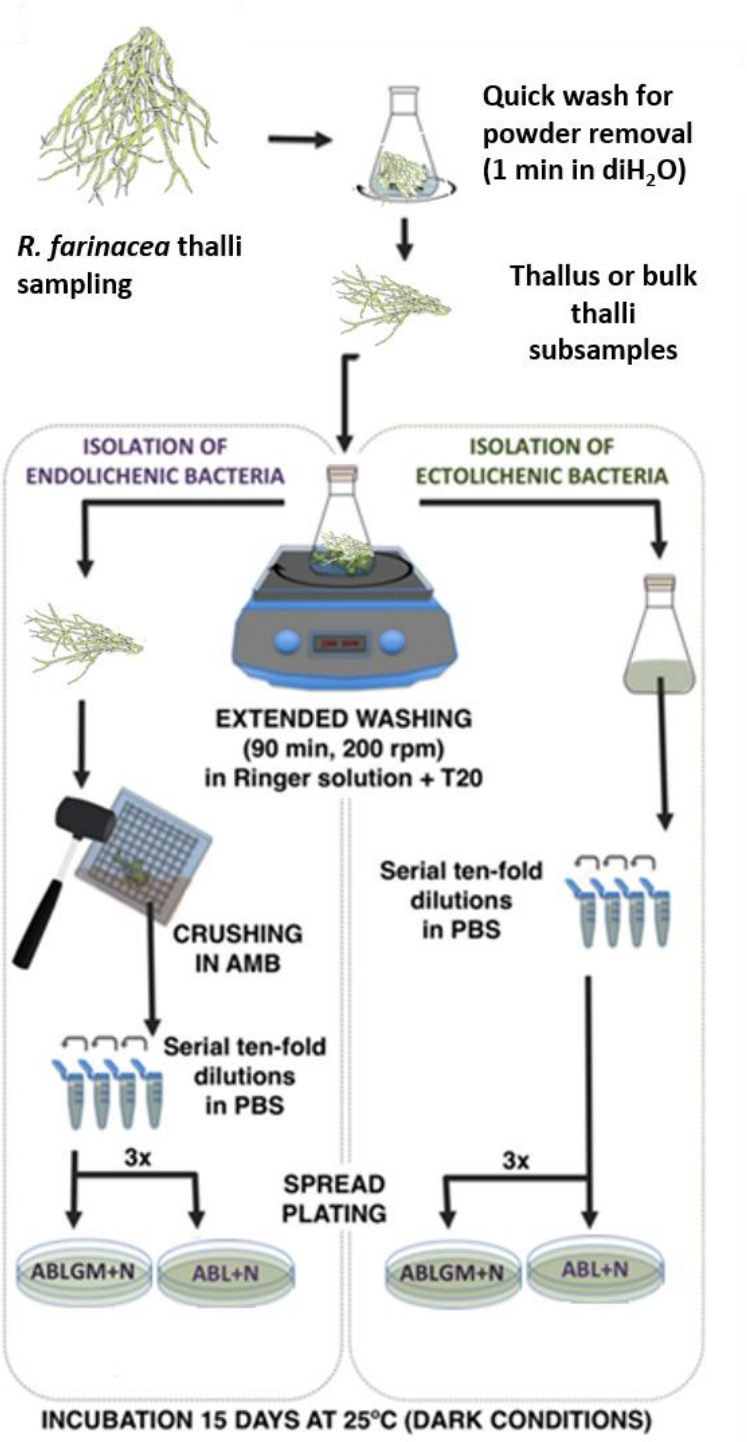


Figure 11. A representative scheme showing the protocol for the isolation of culturable bacteria associated with *R. farinacea*. Fresh lichen thalli samples were collected and washed to remove the environmental powder. Afterwards, samples were processed by an extended washing in Ringer solution supplemented with Tween 20 (T20) to recover ectolichenic bacteria. Washed thalli were crushed in AMB buffer to isolate endolichenic bacteria. Ecto- and endolichenic culturable bacteria were estimated in triplicate on lichen enriched media supplemented or not with defined carbon sources (ABLGM and ABL, respectively) and the fungicide natamycin (N). Spread inoculated plates were incubated at 25°C during 15 days under dark conditions. Adapted from Biosca *et al*. (2016).

4.2 CHARACTERIZATION OF FUNCTIONAL AND/OR BIOTECHNOLOGICAL ACTIVITIES OF *R. farinacea* CULTURABLE BACTERIA

A collection of *R. farinacea* representative bacterial isolates of the four geographical locations sampled were screened for different functional and/or biotechnological activities using bacterial cultures incubated during 24-48 h at 26ºC on the general King's B (KB) medium (King *et al*., 1954) unless otherwise indicated. Bacterial suspensions of each isolate were prepared in 10 mM sterile PBS pH 7.0, adjusted to an optical density at 600nm ($OD_{600\ nm}$) of 0.1 (about 10^8 CFU/ml). Then, they were washed twice with PBS (13.000 r.p.m., 2 min) to remove residual media, and pellets were re-suspended in PBS to use them for the inoculation of the different media (see below). Plates were spot inoculated with a multipoint inoculator (Denley Instruments Ltd, UK). Some reference bacterial strains from the Spanish Type Culture Collection (CECT), such as *Aeromonas hydrophila* CECT 5173, *Azotobacter vinelandii* CECT 204, *Bacillus cereus* CECT 495, *Enterobacter cloacae* CECT 194, *Escherichia coli* CECT 101, and *Pseudomonas fluorescens* CECT 378 were included as negative or positive controls for some of the activities tested. All tests were performed, at least, in duplicate. Plates were incubated during 7 days at 26°C after inoculation, performing periodic readings. For those activities requiring the addition of a reagent for the reading, it was carried out after 7 days of incubation.

4.2.1 Initial characterization: pigments and enzymatic activities

Pigments detection

KB medium widely used for the detection of bacterial pigments (Lamichhane and Varvaro, 2013) was used to investigate the pigment production of bacterial strains, either cellular or diffusible.

General enzymatic activities

As an initial approach for the detection of general enzymatic activities in *R. farinacea* associated bacteria, a collection of 40 selected bacterial isolates were analyzed using the API ZYM® multi-test system (BioMerieux, France). This system consists in the use of one gallery that allows the detection of 19 different enzymatic activities: alkaline phosphatase, esterase, esterase lipase, lipase, leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, chymotrypsine, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, ß-galactosidase, ß-glucuronidase, α-glucosidase, ß-glucosidase, N-acetyl-ß-glucosaminidase, α-mannosidase and α-fucosidase. Prior to the inoculation of the galleries, incubation chambers were sterilized under UV light during 20 min and filled with sterile water to provide a humid atmosphere. Bacterial suspensions, prepared as described above, were used for the inoculation of the API ZYM® galleries following the instructions of the manufacturer, except for the incubation period that was extended up to 48 hours at 26°C. This modification was done to allow the detection of potential enzymatic activities even in those strains with slow growth

rates. After incubation, the reading of the galleries was performed by applying the reagents and conditions specified in the kit.

4.2.2 Hydrolytic activities

Based on the results obtained with the API ZYM® system several hydrolytic activities were assayed with the collection of bacterial strains by conventional methodology. Some of the macromolecules were chosen for their use for the detection of general hydrolytic activities, whereas others were included due to they are part of the lichen thallus and may be important for nutrient recycling.

Polysaccharase activities

The potential of bacterial strains to hydrolyze different polysaccharides such as starch, cellulose, xylan, pectin, and chitin was determined. Initially, a general polysaccharase activity assay was made to detect amylase activity, inoculating bacterial strains in the culture medium Starch agar (KB medium supplemented with 0.2% (w/v) starch), according to Biosca and Amaro (1996). For this activity, a final time (7 days) reading was made, staining the plates with an iodine solution (0.3% (w/v) I_2 and 0.7% (w/v) IK). The iodine present in the solution reacts with the polysaccharides included in the culture medium, giving a purple colour to the medium. Amylase activity was considered positive when not-stained halos appeared surrounding the bacterial growth zone, indicating the hydrolysis of this polysaccharide.

Thereafter, other polysaccharides were included as substrates according to the composition of the lichen thallus, such as cellulose, chitin, pectin, and xylan. The ability to hydrolyze cellulose was studied using the Cellulose Agar medium (Gupta *et al.*, 2012). This medium contains 0.2% (w/v) carboxymethylcellulose (CMC), an organic component derived from cellulose. According to the authors, adding Congo Red to the medium allows the visualization of the cellulase activity. In our case, cellulase activity was detected directly due to the appearance of clearance halos surrounding the bacterial growth area, without the need of adding any colourant.

Regarding chitinase, pectinase and xylanase activities, the minimal medium described by Nagpure and Gupta (2013) was used. This medium was designed as broth for the detection of chitinase activity. A modification by adding agar was made in order to optimize the detection and reading time of this hydrolytic activity. Furthermore, pectin and xylan were also assayed as substrates in this medium after adjusting their concentrations to properly detect their hydrolysis. The final concentration assayed of each one of the substrates in the different media was 1% (w/v) colloidal chitin, 1% (w/v) pectin and 0.5% (w/v) xylan. The reading of these activities was made following the same methodology used for the amylase activity, by staining the plates (7 days post-inoculation) with the same iodine solution, instead of the Congo Red reagent suggested by the authors.

Positive and negative controls used for the detection of these activities were the strains of *B. cereus* CECT 495 and *E. coli* CECT 101, respectively.

Lipase activities

To evaluate the ability of the bacterial strains to hydrolyze lipids, KB medium supplemented with one of the two synthetic lipids, Tween 20 (lauric acid) or Tween 80 (oleic acid) at 1% (w/v), and amended with 0.015% (w/v) $CaCl_2$ was used, as described in Biosca and Amaro (1996).

Periodic readings were made during 7 days, considering as a positive activity the appearance of a halo of precipitate surrounding the growth area of the tested strains. This activity halo is produced as a result of the release of fatty acids, as a consequence of the lipase activity. These released fatty acids join to the calcium ions present in the culture medium and precipitate.

As positive and negative controls, the strains of *A. hydrophila* CECT 5173 and *E. coli* CECT 101 were used, respectively.

Protease activities

The detection of proteases was tested using two different proteins as substrate, casein, and gelatin. For detecting casein hydrolases, KB medium was supplemented with 10% (v/v) casein (skim milk) according to Biosca and Amaro (1996). The gelatinase detection was assayed on the medium described by Smith and Goodner (1958) supplemented with 1.2% (w/v) bacteriological gelatin (Pronadisa). Positive results were directly detected by the appearance of a clear halo around the bacterial growth area, carrying out periodic readings for 7 days.

Positive and negative controls were the strains of *B. cereus* CECT 495 and *E. coli* CECT 101, respectively.

DNAse activity

To analyze the ability of the studied bacterial strains to hydrolyze DNA, the commercial DNAse Agar medium (Pronadisa) was employed. The reading was made 7 days post-inoculation by adding 1 M (v/v) hydrochloric acid. This acid produces the precipitation of the DNA present in the culture medium giving opacity to the areas where it is present. Bacterial strains able to degrade the DNA to nucleotides were detected by the presence of a clear halo around the bacterial growth zone.

As positive and negative controls the strains of *B. cereus* CECT 495 and *E. coli* CECT 101 were used, respectively.

4.2.3 Nutrient supplying activities

The ability of bacterial strains to provide different nutrients such as nitrogen, phosphate, and iron to the lichen thallus was determined.

Nitrogen fixation

The ability of bacterial strains to fix nitrogen was studied on different culture media designed for this purpose. One of them was the Norris medium (Atlas, 2004). Another one was the sugar-rich/N-free medium described by Cardinale *et al*.(2006) for lichenic bacteria. The last one was the AB minimal medium (Chilton *et al*., 1974) without the nitrogen source but supplemented with 0.5% (w/v) glucose (ABG-N) or 0.5% (w/v) glucose and mannitol (ABGM-N), according to Biosca *et al*. (2016). The ability to fix nitrogen was monitored for 7 days, being detected by the ability of the tested bacterial strains to grow on the nitrogen-free culture media.

As positive and negative controls the strains of *A. vinelandii* CECT 204 and *E. coli* CECT 101 were used, respectively.

Phosphate solubilization

The solubilization of phosphate by bacterial strains was screened on Pikovskaya's medium (PVK) (Pikovskaya, 1948; Sundara-Rao and Sinha, 1963), which contains 0.5% (w/v) calcium phosphate ($Ca_3(PO_4)_2$) which confers opacity to the medium. Periodic readings were made during 7 days and the ability of bacterial strains to solubilize phosphate was determined by the appearance of a clear halo around bacterial growth.

Positive and negative controls used for the detection of this activity were the strains of *P. fluorescens* CECT 378 and *B. cereus* CECT 495, respectively.

Siderophores production

Siderophores were detected on CAS agar (Schwyn and Neilands, 1987), based on the utilization of chrome azurol S (CAS) and hexadecyltrimethylammonium bromide (HDTMA) as indicators. The complex CAS/HDTMA joins ferric ion (Fe^{3+}) resulting in a blue colouration. Positive reactions, visualized by a colour change of the CAS reagent from blue to orange (Schwyn and Neilands, 1987), were detected by periodic readings for 7 days. To favour the growth of some strains unable to grow on the regular CAS agar due to the toxicity of HDTMA, this medium was modified as follows. Firstly, some assays were made with the modified culture medium suggested by Milagres *et al*. (1999), but adapted to the growth conditions *of R. farinacea* bacterial strains. In this case, half of the medium in the plate was regular CAS agar, and a half was KB agar (Figure 11). Secondly, a modification of the former medium was made by supplementing the CAS agar with an upper layer of KB medium, avoiding the direct contact of the bacterial strains with the CAS agar layer (Figure 11).

Positive and negative controls used for the detection of these activities were the strains of *P. fluorescens* CECT 378 and *B. cereus* CECT 495, respectively.

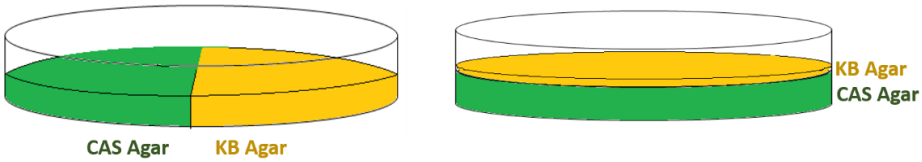


Figure 12. Representative images of the optimized culture medium employed for the detection of siderophores in *R. farinacea* bacterial strains. On the left, the modified media proposed by Milagres *et al*. (1999), in which half of the media on the plate was CAS agar (green part) and a half was KB agar (yellow part). On the right, the modification performed in this study with the CAS agar (green) at the bottom and the KB agar (yellow) on the top.

4.2.4 Growth promoting activities

The potential ability of bacterial strains to stimulate the growth of the lichen thallus was initiated through direct or indirect detection of phytohormones such as auxins or ethylene.

Auxins detection

Semiquantitative detection of indole acetic acid (IAA)

Production of IAA was determined by a colourimetric method described by Patten and Glick (2002) in a microtiter assay with some modifications. Briefly, overnight bacterial cultures were diluted and adjusted to an OD_{600} nm of 0.2 into KB broth supplemented with 0.1 % of tryptophan. The concentration of bacteria was adjusted after previous assays conducted to enable a better determination of the IAA production by those strains. From overnight cultures, 100 µl aliquots were transferred to polystyrene 96-well microplates (Nunc™ MicroWell™, Thermo Scientific), using 4 wells per bacterial strain and plate. Inoculated plates were incubated at 26ºC for 24 h, 48 h and 72 h in a humid chamber. The incubation period was extended up to 72 h because of the slow-growing strains. Thereafter, 200 µl of the Salkowski reagent ($FeCl_3H_2SO_4$) (Acuña *et al*., 2011) were added to each well. After 30 min at room temperature, OD_{530} nm was measured using a Fluostar Optima (BMG Labtech) plate reader. The bacterial strains *P. fluorescens* CECT 378 and *E. coli* CECT 101, and *E. cloacae* CECT 194 were included as negative and positive controls, respectively.

Quantification of IAA

IAA was quantified in a selection of bacterial strains based on the results of the previous assay using the method of Patten and Glick (2002) with some modifications. For this purpose, 5 mL of bacterial cultures in KB broth with 0.1% tryptophan were incubated for 24 h and 72 h, centrifuged at 8000 r.p.m. for 15 min to obtain bacterial supernatants that were mixed with Salkowski reagent in a proportion of 1:2, respectively. After 30 min of incubation at room temperature, OD_{530} nm was measured. The quantification of IAA produced by each bacterial strain was carried out using a standard curve with known IAA concentrations. This standard curve was prepared using KB broth, employed for the growth of the bacterial isolates, and making dilutions of IAA from 25 µg/ml to 0 µg/ml.

ACC deaminase detection

As a first approximation to determine the ability of selected bacterial strains to produce the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase which is involved in the ethylene biosynthesis, a molecular detection of the *acdS* gene that codifies for ACC deaminase was carried out. The primers used are the degenerated pair ACC R and ACC F (Table 6).

Table 6. Primers used for the molecular detection of the *acdS* gene.

Primers	Sequence	Gene	Reference
ACC R	5'-TTDCCHKYRTANACBGGRTC-3'	*acdS*	(Nikolic *et al*., 2011)
ACC F	5'GGBGGVAAYAARMYVMGSAAGCTYGA-3'		

DNA extraction of bacterial strains was made by cell lysis, through 10 min thermal shock at 95ºC in an SBH130D block heater (Stuart), followed by a spinning at 13.000 r.p.m. for 10 min to obtain total DNA in the supernatant fraction. Supernatants were transferred to new sterile tubes and used as DNA samples.

The *acdS* gene amplification by PCR was made, initially, in a reaction volume of 50 µl with the following mix: 1 µl of DNA sample, 33.75 µL of sterile MiliQ water, 5 µL of 10X buffer, 5 µL of 2 mM dNTPs, 2.5 µL of each primer and 0.25 µL of DreamTaq DNA polymerase (Thermo Scientific). Amplification was performed in a 2720 Thermocycler (Applied Biosystems). Amplification conditions recommended by Nikolic *et al.* (2011) are described in table 7.

Table 7. PCR conditions for the molecular detection of the *ACC deaminase* gene provided by Nikolic *et al*. (2011).

Number of cycles	Time per cycle	Temperature	Stage
1	3 min	95ºC	Initial denaturing
30-35	30 sec	95ºC	Denaturing
	1 min	46ºC	Annealing
	1 min	72ºC	Extending
1	5 min	72ºC	Final extension

The uses of this reaction mix and amplification conditions conducted to the appearance of unspecific bands apart from the one expected, a 750 bp fragment (see results section 7.4.2). Therefore, some modifications were assayed to optimize the PCR, such as: i) the variation of the annealing temperature; ii) the modification of the number of cycles; iii) the addition of DMSO, since it reduces the temperature of annealing of the primers, and iv) a combination of the annealing temperature and/or the modification of the number of cycles and/or the addition of DMSO.

Aliquots of 10 µl of PCR products were separated in agarose gels (1% (w/v) by electrophoresis (35 min at 100 volts) in 1X Tris-acetate-EDTA (TAE) buffer, using 1 µl of loading buffer (Pilot Gel Loading Dye, 10X) and 10 µl of Mass Ruler DNA ladder mix (Thermo Scientific) as molecular weight pattern. This molecular ladder allows the determination of bands from 100 bp to 5000

bp. Gels were stained during 15 min with 0.1% ethidium bromide (Sigma) and visualized under UV light using a Gel Printer Plus (Tecnología para Diagnóstico e Investigación S.A.).

4.2.5 Biofilm formation activities

Biofilm production

The production of biofilm was determined using a microtiter assay following the method of Chelvam *et al*. (2014) and Santander and Biosca, (2017) with some modifications. Briefly, overnight bacterial cultures were diluted and adjusted to an OD_{600} nm of 0.2 into 0.5 x KB broth. The modification of the concentrations of the bacterial cultures and the culture medium are justified after some initial assays, conducted to determine the best growing conditions to detect the biofilm production by the lichenic bacterial strains. From these bacterial cultures, aliquots of 160 µl were transferred to polystyrene 96-well microplates (Nunc™ MicroWell™, Thermo Scientific). In each assay, each one of the strains was inoculated in 6 wells per plate and incubated at 26ºC for 48 h and 72 h in a humid chamber. Thereafter, unbound cells and growth medium were removed by inversion of the plate for 5 min and biofilms heat-fixed at 80ºC for 30 min. Afterwards, biofilms were stained with 1% (w/v) crystal violet (Brown *et al*., 2013) and incubated at room temperature for 15 min. The staining solution was removed by inversion of the plates, followed by an extensive washing with distilled water and then, dried upside down. For biofilm quantification, a decouloring solution (80 % absolute ethanol, 20 % acetone) was added to the wells, incubated for 15 min and the OD_{600} nm determined using a Fluostar Optima (BMG Labtech) plate reader. Bacterial strains were classified as follows according to the mean OD_{600} nm values recorded and compared with the values of the negative control (ODc): OD ≤ ODc = no biofilm producer, ODc < OD ≤ (2 × ODc) = weak biofilm producer, (2 × ODc) < OD≤ (4 × ODc) = moderate biofilm producer and (4 × ODc) < OD = strong biofilm producer. Negative control wells contained uninoculated KB broth and remained negative. Some reference bacterial strains such as *E. coli* CECT 101, *P. fluorescens* CECT 378, and *E. cloacae* CECT 194 were included as positive controls.

Swimming and swarming motility

Since motility is important for biofilm formation (Houry *et al*., 2010) swimming and swarming motility of a selection of bacterial strains were also investigated. Motility was assessed on semisolid agar according to Santander *et al*. (2014) with some modifications with respect to the culture medium. Instead of Luria-Bertani (LB) agar, the medium used was as follows: 1% tryptone, 0.5% glucose and 0.3% or 0.7% agar for swimming or swarming motility assay, respectively. The procedure consisted in the inoculation of the different strains in both media by stinging, starting from a 24-48 h bacterial culture in KB medium at 26ºC. Incubation conditions were at 26ºC for 48 h. The diameter of motility halos was determined at 24 and 48 h of incubation.

4.3 STATISTICAL ANALYSES

All statistical analyses performed in the present study were conducted in GraphPad programme. The statistical test applied was Two-way ANOVA performing multiple comparisons with Tukey's correction, unless otherwise indicated.

4.4 MOLECULAR IDENTIFICATION

Bacterial strains of *R. farinaceae* showing the highest number of activities or some characteristic of interest were selected for a presumptive identification by the partial amplification of the *16S rRNA* gene by PCR and subsequent sequencing of the purified PCR products, as described below.

5. DIVERSITY OF CULTURABLE BACTERIA ASSOCIATED WITH *R. farinacea* THROUGH CONVENTIONAL *16S rRNA* GENE SEQUENCING

A study of the diversity of culturable bacteria associated with *R. farinacea* was made by PCR partial amplification of the *16S rRNA* gene followed by Sanger sequencing and a presumptive molecular identification of the bacterial isolates. This same methodology was applied for the identification of *R. farinacea* bacterial isolates selected in the previous section (4.4) by their physiological and/or biotechnological interest.

5.1 DNA EXTRACTION

R. farinacea bacterial isolates were prepared for PCR amplification using 24 h cultures on KB plates, taking 1 or 2 colonies from each one of the strains and resuspending them in 300 µl of sterile Mili Q water. These bacterial suspensions were denatured by heat-shock at 95°C during 10 min in an SBH130D thermoblock (Stuart) to produce cell lysis. Afterwards, they were centrifuged at 13.000 r.p.m. for 10 min to obtain total DNA in the supernatant.

5.2 DNA AMPLIFICATION BY PCR

Supernatants were transferred to new sterile tubes and used for the partial *16S rRNA* gene amplification which was performed by PCR using the pair of primers: 616V and 699R (Arahal *et al*., 2008) (Table 8) amplifying a 1000 bp region of the *16S rRNA* gene. These primers bind the positions 8–25 and 1099–1113, respectively (*Escherichia coli* numbering). PCR amplification was carried out with these primers at a concentration of 5 mM, using 0.025 U/µl DreamTaq polymerase (Thermo Scientific) and 0.2 mM dNTPs in a final volume of 100 µl in a 2720 thermocycler (Applied Biosystems).

Table 8. Primers used for the amplification of the partial sequence of the *16S rRNA* gene.

Primers	Sequence (5'-3')	Gene	Reference
616V	5'-AGAGTTTGATYMTGGCTCAG-3'	*16S rRNA*	Arahal *et al.*, 2008
699R	5'-GCGRGGGCTCGTTTT-3'		

PCR conditions recommended by Arahal *et al.* (2008) are shown in table 9. The application of these conditions turned out with the appearance of unspecific bands. To reduce the number of these bands some modifications were introduced to optimize the amplification conditions. These modifications consisted in increasing the annealing temperature and reducing the number of cycles.

Table 9. PCR conditions for the amplification of the partial sequence of the *16S rRNA* gene described by Arahal *et al*. (2008).

Number of cycles	Time per cycle	Temperature	Stage
1	10 min	94ºC	Initial denaturing
40	1 min	94ºC	Denaturing
	1 min	55ºC	Annealing
	1 min	72ºC	Extending
1	10 min	72ºC	Final extension

The visualization of the PCR products was made following the protocol described in section 4.2.4.

5.3 PURIFICATION OF PCR PRODUCTS

The amplified products were purified with the FavorPrep GEL purification/PCR Purification Mini Kit (Favorgen, FAGCK 001-1) following the manufacturer's instructions. The quality, purity and concentration of the PCR products were determined with a NanoDrop2000c spectrophotometer (Thermo Scientific) from the Central Service for Experimental Research (SCSIE) of the Universitat de València. The concentration of all samples was adjusted to a minimum of 20 ng/µl. Samples were frozen at -20ºC until their use.

5.4 SANGER SEQUENCING

Sequencing of the purified PCR products was made with the forward strand as a template, unless difficulties in the sequencing process were found in which case both strands were sequenced. The sequencing of the samples was made by the method of Sanger mostly at StabVida (Lisboa) service.

5.5 BIOINFORMATIC ANALYSIS

The analysis of the quality and edition of the sequences obtained was made with the Chromas Lite 2.1.1 programme. Sequences were manually checked and cleaned. Those sequences with errors in the assignment of the nucleotide bases, excessive background noise and/or low resolution were rejected, proceeding again with their PCR amplification, purification and sequencing. The criteria followed in this study for the analyses of the quality of the sequences were the ones described by the *Instituto de Conservación y Mejora de la Agrodiversidad Valenciana* (COMAV).

Once edited, the sequences were aligned with the Molecular Evolutionary Genetics Analysis (MEGA) software v.6.0 (MEGA 6) (Tamura *et al*., 2013) using the Clustal W alignment application. Once sequences were aligned, as a consequence of the differences in their length, not-overlapping ends were deleted to equalize the length of these sequences.

To select the best evolutionary model of nucleotide substitution, an application built in MEGA 6 called *Find best DNA model*, was used. After selecting the most suitable evolutionary model of nucleotide substitution, a phylogenetic tree was made based on the Maximum Likelihood (ML) method using the the Kimura 2- (Kimura, 1980) or Tamura 3-parameter models of evolution (Tamura, 1992). In this ML method, an initial tree is built using a fast but suboptimal method such as Neighbour-Joining, and its branch lengths are adjusted to maximize the likelihood of the data set for that tree topology under the desired model of evolution. Then variants of the topology are created using the NNI (nearest neighbour Interchange) method to search for topologies that fit the data better. Maximum-Likelihood branch lengths are computed for these variant tree topologies and the greatest likelihood retained as the best choice so far. This search continues until no greater likelihoods are found. To construct the phylogenetic tree, the application *Phylogeny* of MEGA 6 was employed. The robustness and confidence levels of the tree branch nodes of the resulting tree were determined with the *bootstraps* test, with 1000 replicates (Hillis and Bull, 1993) with MEGA 6 software.

5.6 TAXONOMIC IDENTIFICATION AND PHYLOGENETIC ANALYSIS

The taxonomic identification of *R. farinacea* bacterial strains was made using the BLASTn (http://blast.ncbi.nlm.nih.gov) programme, with the database available at the National Center for Biotechnology Information (NCBI). According to the results obtained with this identification, the partial sequences of the *16S rRNA* gene of the closest strains obtained from the Genebank were included for the construction of the phylogenetic tree.

5.7 DETERMINATION OF BACTERIAL COMMUNITY COMPOSITION

Differences in the community composition (beta diversity) were assessed using a permutational multivariate analyses of variance (PERMANOVA) with the 'Adonis' function in the VEGAN package in R (Version 3.2.2). Bray–Curtis dissimilarity matrices were used to quantify differences in bacterial community composition, as calculated using the R package MCTOOLSR (Version 0.3.2).

5.8 DETERMINATION OF BACTERIAL DIVERSITY AND ABUNDANCE

The diversity differences among distinct samples were determined by using Richness, Shannon and Simpson indexes, representing them in box plots. In the case of the Richness index, the data were previously normalized.

6. MOLECULAR ANALYSES OF BACTERIAL COMMUNITIES ASSOCIATED WITH *R. farinacea* THROUGH MULTIPLEX SEQUENCING OF *16S rRNA* GENE

6.1 *R. farinacea* THALLI SAMPLES

For the molecular analyses of *R. farinaceae* bacterial communities, prior to DNA extraction, thalli subsamples from each geographical location were processed as a whole, including both ectolichenic and endolichenic bacterial communities, as well as analyzing the ectolichenic and endolichenic fractions separately as described by Biosca *et al.* (2016). Individual thallus or bulk thalli (0.2 g subsamples of five thalli from five different trees) samples of 1 g of *R. farinacea* were analyzed for the isolation of both ectolichenic and endolichenic bacteria as described in section 4.1. Washing thalli solutions for ectolichenic bacteria and washed and crushed thalli in AMB buffer for endolichenic ones were kept frozen for subsequent DNA extraction. In thalli samples processed as a whole, the same conditions and proportions above-mentioned for crushing the samples were used. Further, additional thalli subsamples were also analyzed after subdividing them into basal, middle and apical parts, prior to DNA extraction.

In additional experiments, some samples were subjected to a disinfection treatment with 70% ethanol during 1 min under stirring conditions, followed by two washes with sterile distilled water during 5 min (Biosca *et al*., 2016), using untreated samples as control. The final number of samples used in this study was 60.

The thalli samples that were used for DNA extractions and thereafter sequenced were the following:

- Five individual thalli per each one of the four geographical locations from which ectolichenic and endolichenic bacterial fractions were obtained.

- Thalli from each one of the four geographical locations were also subdivided in basal, middle and apical parts, and processed as a whole, without separating the ectolichenic and the endolichenic bacterial fractions.

- Bulk samples from each one of the four geographical locations, with and without disinfection treatment, from which ectolichenic and endolichenic bacterial fractions were obtained.

6.2 DNA EXTRACTION

The DNA extraction of the different *R. farinaceae* thalli samples was performed using the PowerSoil DNA extraction kit (MoBio Laboratories Inc., Carlsbad, CA, USA) following the instructions of the manufacturer. We included some negative controls without lichen samples for the extraction plate to verify the lack of possible contaminations.

6.3 DNA AMPLIFICATION BY PCR

Aliquots of 1 µl of the DNA extraction of each sample were used for *16S rRNA* gene PCR amplification and sequencing using the 515f/806r primer set (Caporaso *et al*., 2011), whose sequence is specified in table 10. This primer pair amplifies the region V4-V5 of the *16S rRNA*

gene of Bacteria and Archaea (Caporaso *et al*., 2011, 2012), which correspond to the region 533–786 in the *E. coli* strain 83972 sequence (GreenGenes accession no. prokMSA_id:470367). These primers include Illumina sequencing adapters and 12 bp barcode to allow multiplexed sequencing.

Table 10. Primers used for the amplification of the partial sequence of the *16S rRNA* gene and subsequent sequencing by Illumina technology.

Primers	Sequence (5'-3')	Target gene	Reference
515f	5'-GTGYCAGCMGCCGCGGTAA -3'	*16S rRNA*	(Caporaso *et al*., 2011)
806r	5'- GGACTACNVGGGTWTCTAAT-3'		

PCR reactions contained 10.5 µL of sterile PCR water, 12.5 µL of Master Mix Promega (Promega), 1 µL of each of one of the forward and reverse primers (10 µM final concentration) and 1.0 µL of genomic DNA. Reactions were held with the conditions described in table 11 (Caporaso *et al*., 2011). The expected band must have a size of about 200 bp. All samples were PCR tested, at least, by duplicate.

Table 11. PCR conditions recommended by Caporaso *et al*. (2011) for the amplification of the partial sequence of the *16S rRNA* gene and Illumina sequencing.

Number of cycles	Time per cycle	Temperature	Stage
1	3 min	94ºC	Initial denaturing
35	45 sec	94ºC	Denaturing
	1 min	60ºC	Annealing
	90 sec	72ºC	Extending
1	10 min	72ºC	Final extension

6.4 PYROSEQUENCING OF PCR PRODUCTS

After PCR reactions, all the amplicons obtained were normalized in their concentrations. Thereafter, they were pooled in equimolar concentrations and sequenced on the Illumina MiSeq instrument. All sequencing runs were conducted at the University of Colorado BioFrontiers Institute Next-Gene Sequencing Core Facility.

6.5 BIOINFORMATIC ANALYSES

As the fragments were sequenced through the above-mentioned adapters, the program *cutadapt* was used to trim these adapters at sequences of 200 bp. Thereafter, the sequences were demultiplexed using a custom Python script ('prep_fastq_for_uparse_paired.py', at https://github.com/leffj/helper-code-for-uparse) (Edgar, 2013). Then, we merged the paired-end reads using USEARCH (Version 7) (Edgar, 2010), checked the quality of the sequences (usearch8 -fastq_stats) and conduct a quality filtering at a "maxee" rate of 0.005 (maximum per sequence expected error frequency value). The sequences were dereplicated and the

singleton sequences (phylotypes represented by only a single read) were removed. Filtered sequences were clustered to create a *de novo* database at 97% similarity threshold (sequences that share ≥97% sequence similarity). Then, the *de novo* database was filtered against an existing public database to remove highly divergent sequences. In this case, the database was Greengenes (McDonald *et al*., 2012a) with a confidence threshold of 0.75.

6.6 BACTERIAL TAXONOMIC IDENTIFICATION

The OTU table mapping of the raw/demultiplexed sequences was built to *de novo* database at a 97% similarity generating phylotype counts. A taxonomic classification was added to each OTU using the Ribosomal Database Project (RDP) classifier (Wang *et al*., 2007) with the Greengenes database (McDonald *et al*., 2012a) with a confidence threshold of 0.5. Finally, chloroplasts and mitochondria sequences were removed. All the samples of this study were rarefied at 1929 sequences per sample, to avoid overrepresentation of some of them (using the R package 'MCTOOLSR', at https://github.com/leffj/mctoolsr).

6.7 DETERMINATION OF BACTERIAL COMMUNITY COMPOSITION, BACTERIAL DIVERSITY AND ABUNDANCE

These analyses were made following the methodology and bioinformatic programmes explained and mentioned in the previous section for the study of the diversity of culturable bacteria.

6.8 STATISTICAL ANALYSES

All statistical analyses performed for molecular analyses of *R. farinaceae* bacterial communities, either using Sanger or Illumina sequencing, were conducted in R (R Development Core Team, 2013), unless otherwise indicated.

For the determination of bacterial community composition, significant differences in the relative abundances of individual bacterial taxa across sample types were determined using Kruskal–Wallis tests and Bonferroni corrections. Univariate analyses and principal coordinate analysis were performed using R software (R Development Core Team, 2013).

For the determination of bacterial diversity and abundance, a Student's t-test was used for pairwise comparisons among the sample types.

7. ANALYSES OF CULTURABLE BACTERIA ASSOCIATED WITH *R. farinacea*

7.1 ISOLATION OF LICHEN-ASSOCIATED BACTERIA

Culturable cell counts of ectolichenic and endolichenic heterotrophic bacteria recovered from *R. farinacea* populations from different Spanish geographical locations in the Canary island of Tenerife (La Guancha and La Esperanza) and the Iberian Peninsula (El Toro and Lidón) are represented in figure 13. Bacterial counts were recorded regularly until the 15th day when a stabilization of the number of bacterial colonies appearing on culture media was observed. Thus, an extended period of incubation increased the recovery of culturable bacteria from *R. farinacea* thalli. As shown in figure 13, in general, the number of colony forming units (CFU)/g was higher in the lichen enriched culture medium without added carbon sources (ABL) than in the ABL medium amended with glucose and mannitol (ABLGM), both for ectolichenic and endolichenic bacteria. One exception was the results corresponding to the ectolichenic bacteria recovered from *R. farinacea* thalli samples from La Esperanza which showed a higher number of colonies in ABLGM than in ABL isolation plates (Figure 13b).

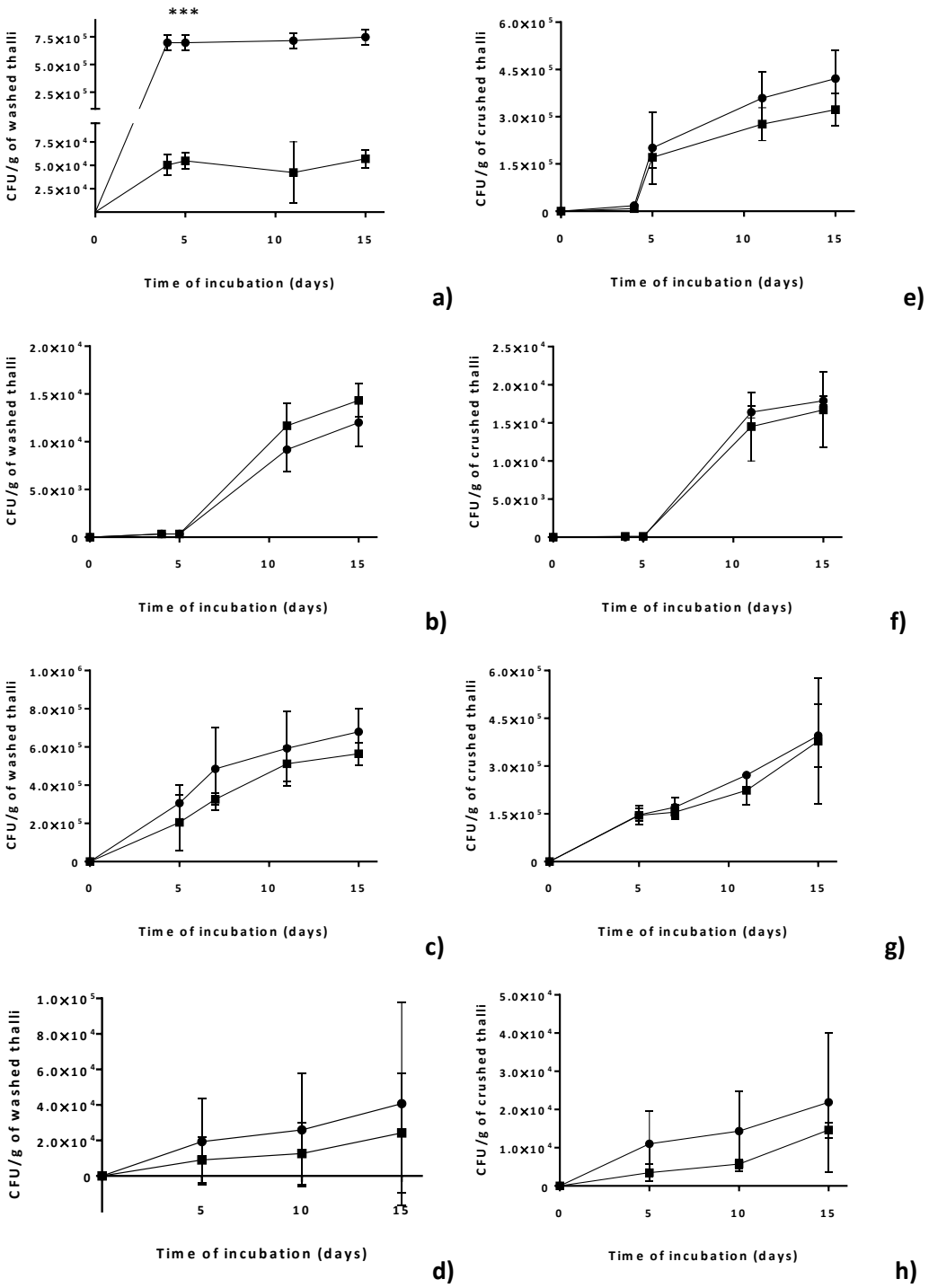


Figure 13. Culturable cell counts (CFU/g) of ectolichenic (whased thalli; **a,b,c,d**) and endolichenic (crushed whashed thalli; **e,f,g,h**) heterotrophic bacteria from *R. farinacea* populations from different Spanish geographical locations in the Canary island of Tenerife (La Guancha (**a, e**) and La Esperanza (**b, f**)) and the Iberian Peninsula (El Toro (**c, g**) and Lidón (**d, h**)), during 15 days of incubation in lichen enriched ABL (●, without carbon source added) and ABLGM culture media (■, with glucose and mannitol) at 26ºC. Each symbol represents the average value of bacterial counts made by triplicate on each culture medium for the same bulk thalli sample (composed by five different lichen thalli) from each *R. farinacea* population. The standard deviation of the data is shown by vertical lines. Significant differences are indicated by asterisks ($p<0.001$ (***)).

The comparative data of bacterial counts of the four populations of *R. farinacea* from different geographical areas at final time of incubation (15 days) are shown in figure 14. Culturable heterotrophic bacterial numbers recorded from the lichen population from the area of La Guancha were very similar to those obtained from thalli sampled in the temperate area of El Toro, being around 10^5-10^6 CFU/g, both for ectolichenic and endolichenic bacteria. Regarding the bacterial counts recorded from *R. farinacea* populations from the zone of La Esperanza and the geographical location of Lidón, both with more extreme conditions, similar results were obtained, being about 10^4-10^5 CFU/g, and thus lower to the ones recorded in the other two locations. The highest number of culturable bacteria was obtained from the lichen thalli collected from El Toro, followed by the ones from the thalli sampled in La Guancha and, thereafter, by the other two sampling locations where bacterial counts were similar, but slightly lower in the ones recovered from the lichen population from La Esperanza. As mentioned above, in general, culturable bacterial counts were higher in ABL than in ABLGM plates, regardless the geographical or lichenic origin of the samples.

When comparing between the culturable counts of ectolichenic and endolichenic heterotrophic bacteria from the *R. farinacea* populations from the four sampling locations in ABL and ABLGM plates at final incubation time, in general, not relevant differences were observed. However, some significant differences were found when comparing the results obtained for both media between some populations, which might depend on the bacteria associated with each lichen population. In this sense, in the *R. farinacea* population from La Guancha, significant differences were found when the counts of ectolichenic isolates were compared between ABL and ABLGM media ($p<0.0001$).

In general, the recovery of *R. farinacea* ectolichenic and endolichenic bacteria was higher on ABL medium than on ABLGM medium. Besides, a higher number of bacterial isolates were obtained in the ectolichenic fraction than in the endolichenic one, but without significant differences.

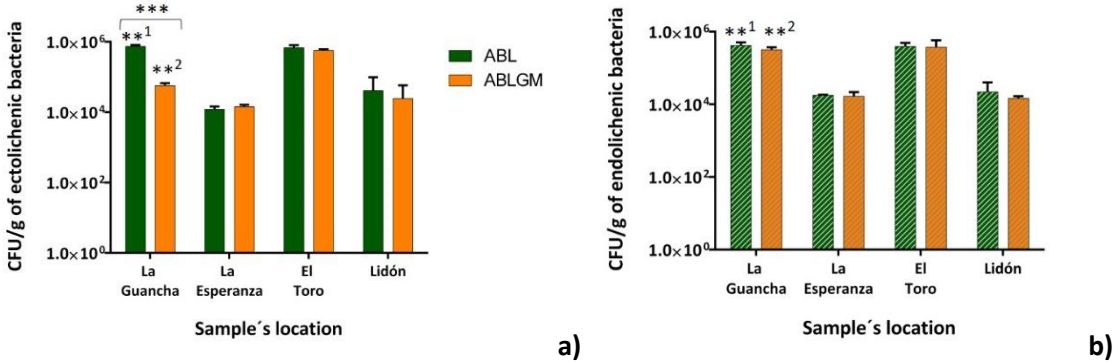


Figure 14. Culturable cell counts (CFU/g) of ectolichenic (**a**) and endolichenic (**b**) heterotrophic bacteria isolated from *R. farinacea* populations from different Spanish geographical locations, in the Canary island of Tenerife (La Guancha and La Esperanza) and the Iberian Peninsula (El Toro and Lidón), after 15 days of incubation at 26ºC. ABL (lichen enriched culture medium without carbon source added). ABLGM (lichen enriched culture medium supplemented with glucose and mannitol). Each bar represents the mean value of bacterial counts made by triplicate on each culture medium for the same bulk thalli sample (composed by five different thalli from the same *R. farinacea* population). The standard deviation of the data is indicated by vertical lines. Significant differences are marked with asterisks ($p<0.01$ (**), $p<0.001$ (***)). 1: significant differences found when compared the results of the counts from La Guancha in ABL medium for the ectolichenic and the endolichenic bacteria. 2: significant differences found when compared the results of the counts from La Guancha in ABLGM medium for the ectolichenic and the endolichenic bacteria.

7.2 FUNCTIONAL AND BIOTECHNOLOGICAL CHARACTERIZATION OF CULTURABLE BACTERIA

Once bacterial strains were purified and cryopreserved they were characterized as follows:

7.2.1 Bacterial pigments

Bacterial strains were grown on KB medium to determine their ability to produce pigments, either cellular or diffusible. In figure 15 are represented the percentages of the pigmented bacterial strains of *R. farinacea* studied, grouping them according to the geographical origin of the lichen populations analyzed and their ecto- or endolichelic location in the lichen thallus. Accordingly, a high percentage of the recovered bacteria were pigmented. When comparing the results obtained between the bacterial strains from *R. farinacea* populations from the Island and those from the Peninsula, the peninsular ones presented a higher percentage of pigmented strains (81.56%) than those from the Island (58.12%). The main colors among the pigmented bacteria from the Island were yellow (28.63%) and white (15.81%), while in those from the Peninsula were yellow (31.84%) and pink (28.77%). With regards to the lichen populations considering the four geographical locations (Figure 15), the one with the highest percentage of pigmented bacteria was El Toro, with a 94.02% (being a 97.22% of them ectolichenic, and 90.32% endolichenic), followed by La Esperanza with a 75.76% (89.65% of the ectolichenic and 64.75% of the endolichenic), Lidón with a 65.61% (82.43% of the ectolichenic and 50.60% of endolichenic) and La Guancha with a 51.19% (48.19% of ectolichenic and 54.12% of endolichenic). In general, the percentage of pigmented bacterial strains was higher for the ectolichenic fraction than the endolichenic one. Nevertheless, these differences were

not significant when the number of pigmented bacteria was compared among the locations, as well as when considering their ecto- or endolichenic position in the lichen thallus.

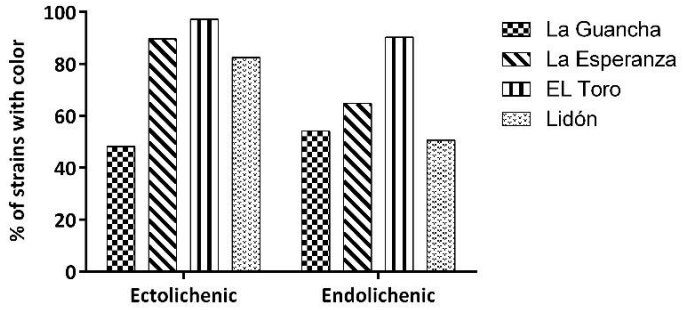


Figure 15. Percentages of pigmented bacterial strains of *R. farinacea* from the four different sampling geographical locations in Spain, two from the Canary Island of Tenerife (La Guancha and La Esperanza) and two from the Iberian Peninsula (El Toro and Lidón), and according to their ectolichenic or endolichenic location in the lichen thallus. Each bar represents the result of the percentage value of the pigmented strains made by duplicate in two independent experiments.

In figure 16a are represented the percentages of pigmented bacterial strains of *R. farinacea* according to the colour of the pigments produced, grouping them by their geographical and lichenic origin for each lichen population studied. A representative picture of the variety of pigments produced by *R. farinacea* bacterial strains is shown in figure 16b. The most frequent pigment among the studied bacterial strains was yellow (32.3%), followed by pink (18.98%), orange (10.41%) and white (10.49%). A 27.82% of the tested bacteria appeared to be uncolored under the assayed conditions. Most of the pigments observed were cellular, with only a minority being yellow diffusible. In some cases, as in the case of the strains from thalli collected at La Esperanza, El Toro and Lidón, the percentage of yellowish strains was more abundant in the ectolichenic fraction (22.73%, 16.41% and 24.84%, respectively) than in the endolichenic one. The same could be observed with the orange-type pigments of the strains from the locations of El Toro and Lidón (17.41% and 4.46%, respectively). Pink pigments were, in general, more abundant in the endolichenic fraction than in the ectolichenic one, with percentages of pink colonies of 1.19% in La Guancha, 15.15% in La Esperanza and 30.48% in El Toro. Despite these differences, the statistical analyses of data revealed that they were not significant when compared the ectolichenic and endolichenic bacteria for each one of the pigments for each geographical location.

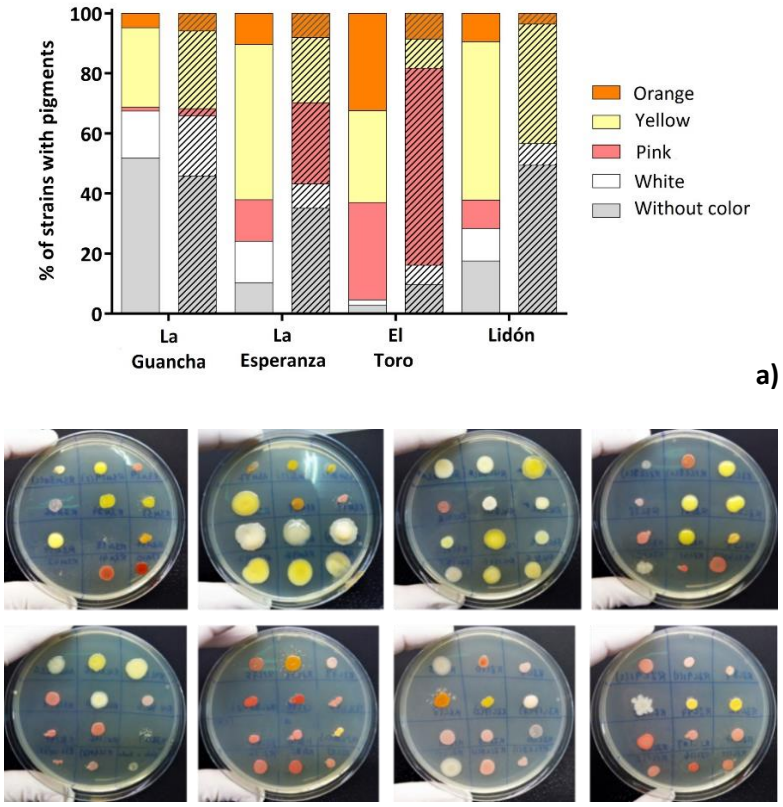


Figure 16. Pigments produced by *R. farinacea* bacterial strains. Percentages of pigmented bacterial strains for each one of the four geographical locations in Spain, at the Canary Island of Tenerife (La Guancha and La Esperanza) and at the Iberian Peninsula (El Toro and Lidón), and according to their ectolichenic (plain bars) or endolichenic (striped bars) location in the lichen thallus (**a**). In each bar, each one of the colors represents the percentage of bacteria producing this pigment. The colors of the bars correspond to the color of the pigment: orange, yellow, pink, white and uncolored (grey). Each bar represents the result of the percentage values of the pigmented strains made by duplicate in two independent experiments. Representative pictures of the variety of pigments produced by *R. farinacea* bacterial strains (**b**).

7.2.2 General enzymatic activities: API ZYM system

A first approach to characterize a selection of *R. farinacea* bacterial strains isolated from lichen populations collected from the four different geographical areas, was made through the detection of general enzymatic activities with the miniaturized system APY ZYM® (Biomerieux). The incubation time of the galleries inoculated with the bacterial strains was established after performing initial assays with readings at different periods of time (24 h, 48 h and 72 h), fixing the final reading time at 48 h (data not shown). In general, *R. farinacea* bacterial strains displayed different and varied enzymatic profiles (Table 12, Figure 17), although some of the activities detected were common in a high percentage of them, such as esterase (80%), lipase esterase (85%), leucine arylamidase (80%), acid phosphatase (80%) and naphthol-AS-BI-phosphohydrolase (100%) activities. The valine arylamidase activity was present in 55% of the tested bacteria. The rest of the activities were detected in a percentage ranging from 10% to 50%. Two activities were not detected in any of the tested bacterial strains, the N-acetyl-β-glucosaminidase and the α-fucosidase. Not significant differences were found when the results obtained were compared between ectolichenic and endolichenic bacterial strains (Figure 17). However, in some cases, the ectolichenic strains were more active than the endolichenic ones,

as it was observed in the case of the alkaline phosphatase, esterase lipase, valine arylamidase, cystine arylamidase, trypsine, β-galactosidase, α- and β-galactosidase and α-manosidase activities. In other cases, the endolichenic strains were more active than the ectolichenic ones, for the activities esterase, lipase, α-galactosidase and β-glucoronidase. The differences observed between bacterial strains from the ectolichenic and endolichenic fractions for each one of the activities tested were not significant.

Table 12. Enzymatic profiles detected in a selection of *R. farinacea* bacterial strains by using the API ZYM system.

Bacterial profiles	Control	Alkaline phosphatase	Esterase (C4)	Esterase lipase (C8)	Lipase (C14)	Leucine arylamidase	Valine arylamidase	Cystine arylamidase	Trypsin	α-chymotrypsin	Acid phosphatase	Naphthol-AS-BI-phosphohydrolase	α-galactosidase	β-galactosidase	β-glucoronidase	α-glucosidase	β-glucosidase	N-acetyl-β-glucosaminidase	α-manosidase	α-fucosidase
R1EL[a]. Profile 1	-[b]	+	+	+	-	+	+	-	-	-	+	+	-	-	-	-	+	-	-	-
R1EL. Profile 2	-	+	+	+	-	+	+	-	-	-	+	+	-	+	-	-	+	-	-	-
R2EL. Profile 3	-	-	-	-	-	+	+	-	-	-	+	+	-	-	-	-	-	-	-	-
R3EL. Profile 4	-	-	-	+w	-	-	-	-	-	-	+w	+	-	-	-	+	-	-	-	-
R3EL. Profile 5	-	-	+w	+w	-	-	-	+w	+w	-	-	+	-	-	-	+	+	-	-	-
R4EL. Profile 6	-	-	+	+	+ +	+ + +	+	+w	-	-	-	+	-	+w	-	-	-	-	-	-
R4EL. Profile 7	-	-	+ +	+ +	+ +	+ + +	+ +	+	-	-	+ + +	+	-	-	-	-	-	-	-	-
R4EL. Profile 8	-	-	+w	+ +	+ +	+ ++	+ +	+	+	+	+ +	+ +	-	+ + +	+	+ + +	+ + +	-	+	-
R1EN. Profile 9	-	-	+	+	-	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-
R1EN. Profile 10	-	-	+	+	-	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-
R1EN. Profile 11	-	-	-	-	-	+	-	-	-	+	+	+	-	-	-	-	-	-	-	-
R1EN. Profile 12	-	-	+	+	+	+	+	-	+	-	-	+	-	-	-	-	-	-	-	-
R1EN. Profile 13	-	-	+	+	-	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-
R2EN. Profile 14	-	-	+	+	+	+	+	-	-	-	+	+	+	+	-	+	+	-	-	-
R2EN. Profile 15	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-
R3EN. Profile 16	-	+ + +	+ +	+ +	+w	+ + +	+ +	+w	-	-	+ + +	+ + +	-	-	-	-	+	-	-	-
R3EN. Profile 17	-	-	+ +	+ +	-	-	-	-	-	-	+	+	-	-	+w	-	-	-	-	-
R3EN. Profile 18	-	-	+ +	+ +	+ +	+ + +	+ +	+	-	-	+w	+	+w	+ + +	-	+ + +	+w	-	+	-
R4EN. Profile 19	-	-	+ +	+ +	+ +	+ + +	+ +	+	+w	+w	+	+	+w	+ + +	+w	+ + +	+ + +	-	+	-
R4EN. Profile 20	-	+w	+w	+w	-	+w	-	-	-	-	-	+w	+ + +	+ +	-	-	+w	-	-	-

a: Bacterial strains from *R. farinacea* thalli samples from La Guancha (R1), La Esperanza (R2), El Toro (R3) and Lidón (R4). EL: ectolichenic bacteria, EN: endolichenic bacteria. **b**: -: negative activity +w: weak positive activity; +: positive activity; ++; intermediate positive activity; +++: strong positive activity.

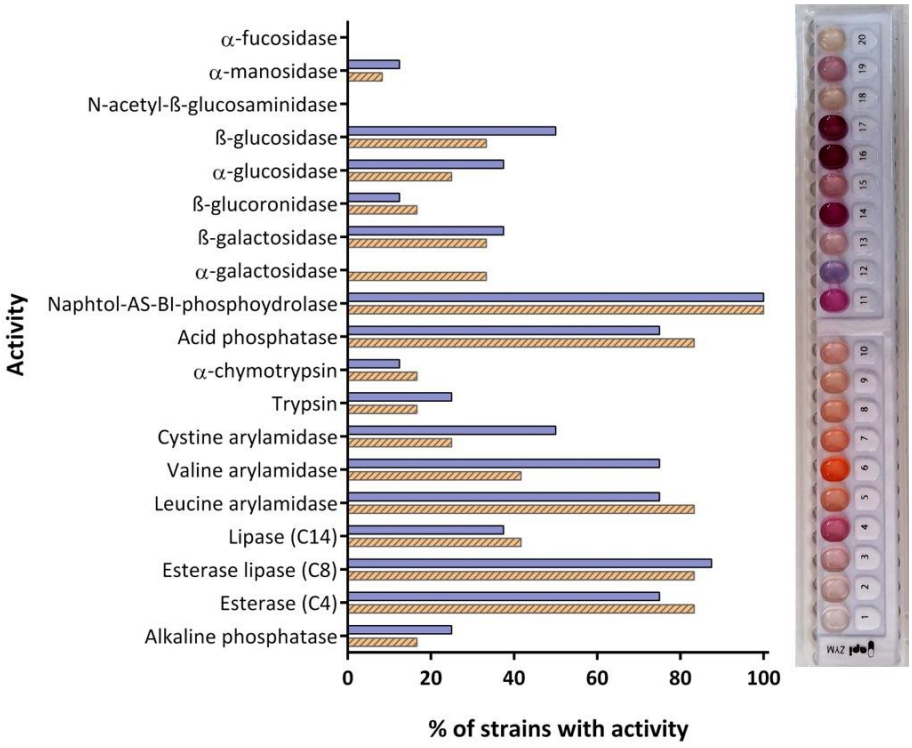


Figure 17. Percentages of bacterial strains of *R. farinacea* with general enzymatic activities detected with the miniaturized system API ZYM®. Blue bars: ectolichenic bacterial strains. Orange striped bars: endolichenic bacterial strains. Each bar represents the result of the percentage value of the tested strains made by duplicate in two independent experiments. A representative picture of an API ZYM® gallery is shown at the right of the graphic.

7.2.3 Hydrolytic activities

Polysaccharase activities

In figure 18 are shown the results obtained for the polysaccharase activities tested (amylase, cellulase, xylanase, pectinase and chitinase) with the *R. farinacea* bacterial strains, grouping them according to their geographical location and ectolichenic or endolichenic origin. A 61.8% of the tested bacteria showed one or more of the polysaccharase activities assayed. Among them, a high proportion of bacteria showed chitinase activity, with a 79.88% of the strains, followed by xylanase (61.07%), pectinase (51.94%), amylase (43.82%) and cellulase (31.53%) activities. Regarding the geographical location of the lichen populations studied, it was observed that in the populations of the Peninsula there was a higher percentage of bacteria showing one or more of these hydrolytic activities (57.1%) than in those from the Island (47.7%). Peninsular strains were more active in cellulase and amylase activities than those of insular origin, while insular strains were more active in pectinase, chitinase and xylanase than those from the Peninsula. Taking into consideration the four geographical locations (Figure 18), the highest percentage of bacterial strains with polysaccharase activities was detected in the lichen samples from El Toro, with a 59.31% of strains, followed by those from the

populations of La Esperanza (57.5%), Lidón (53.13%) and La Guancha (42.82%). When analyzing the results with some more detail, we found that the *R. farinacea* population from La Esperanza was the one with the highest proportion of bacterial strains producing amylase (47.92%), La Guancha where more strains produced cellulase (37.41%) and El Toro with more strains producing xylanase (71.6%). The highest number of bacterial strains producing pectinase and chitinase activities where isolated from *R. farinacea* populations from Lidón and El Toro (60.05% and 90.32%, respectively). Although in some locations and for some activities, the endolichenic bacteria seemed to be more active than the ectolichenic ones, as in the case of those from lichen thalli from El Toro for the cellulase activity. The differences observed among bacterial strains from different geographical and lichenic origins were not significant.

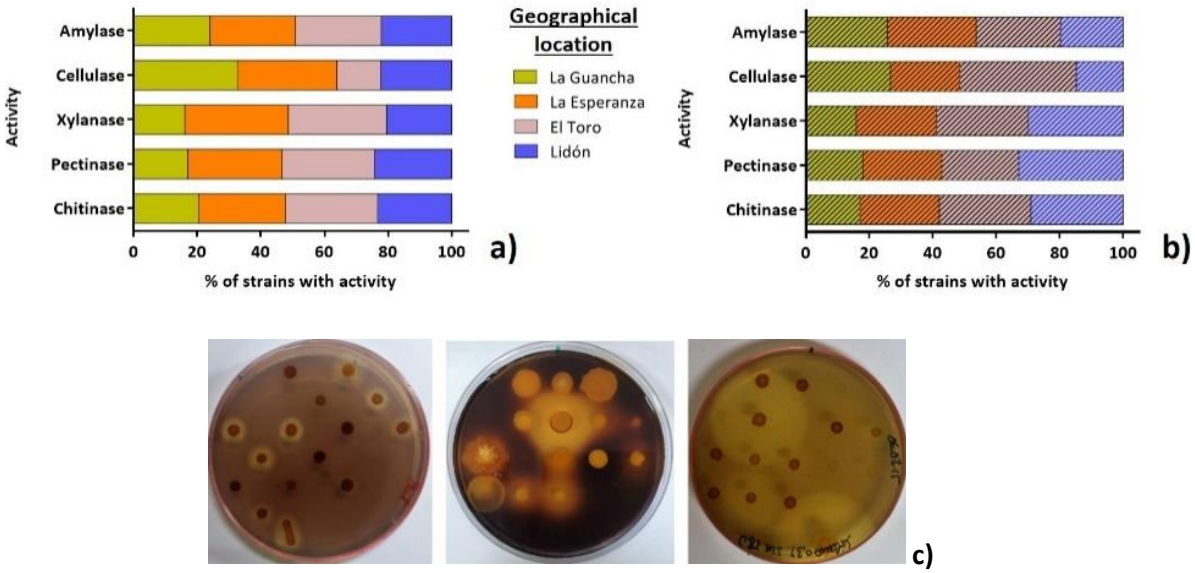


Figure 18. Percentages of ectolichenic (plain bars) (**a**) and endolichenic (striped bars) (**b**) bacterial strains of *R. farinacea* populations from each one of the four Spanish geographical locations studied, with different polysaccharases activities: amylase, cellulase, xylanase, pectinase and chitinase. Each bar represents the percentages of positive strains tested by duplicate in two independent experiments. Representative pictures of the detection of pectinase, amylase and xylanase activities (from left to right) with a selection of *R. farinacea* bacterial strains (**c**).

Lipase activities

A 39.7% of *R. farinacea* bacterial strains showed lipase activity when two different lipids were used, Tween 20 (T20) and Tween 80 (T80). There was a higher percentage of strains able to hydrolize T20 than T80 as substrate, with percentages that arise 53.07% and 23.46%, respectively. The *R. farinacea* populations sampled from the Peninsula showed a higher percentage of positive bacteria (40.71%) for lipase activities than those from the Island (37.07%). As shown in figure 19 the lichen population with the highest proportion of bacteria able to produce lipases was the one collected from El Toro, with a 41.62% of the strains, followed by those from La Guancha (39.39%), Lidón (39.06%) and La Esperanza (32.29%). The results corresponding to the T80 lipid showed that the highest number of positive strains with hydrolytic activity for this lipid was found among those from La Esperanza (30.69 %), while in the case of the T20 it was found among those isolated from Lidón (70.95 %). With regards to

the positive bacterial strains taking into consideration their lichenic origin, for T20 there was, in general, a higher percentage of positive ectolichenic strains than endolichenics. The highest differences were detected among bacterial strains from the lichen population of El Toro with a 71.60% of ectolichenics. In the case of T80, both ectolichenic and endolichenic bacteria showed similar percentages of activity, although relevant differences were found in the lichen populations from La Esperanza (13.39% of ectolichenics, 44% of endolichenics) and Lidón (11.62% of ectolichenics and 3.77% of endolichenics). The statistical analyses of the results only revealed significant differences with the endolichenic bacteria from *R. farinacea* populations from La Esperanza and Lidón for T80 ($p<0.05$), with a higher proportion of strains with this hydroltic activity from La Esperanza than from Lidón.

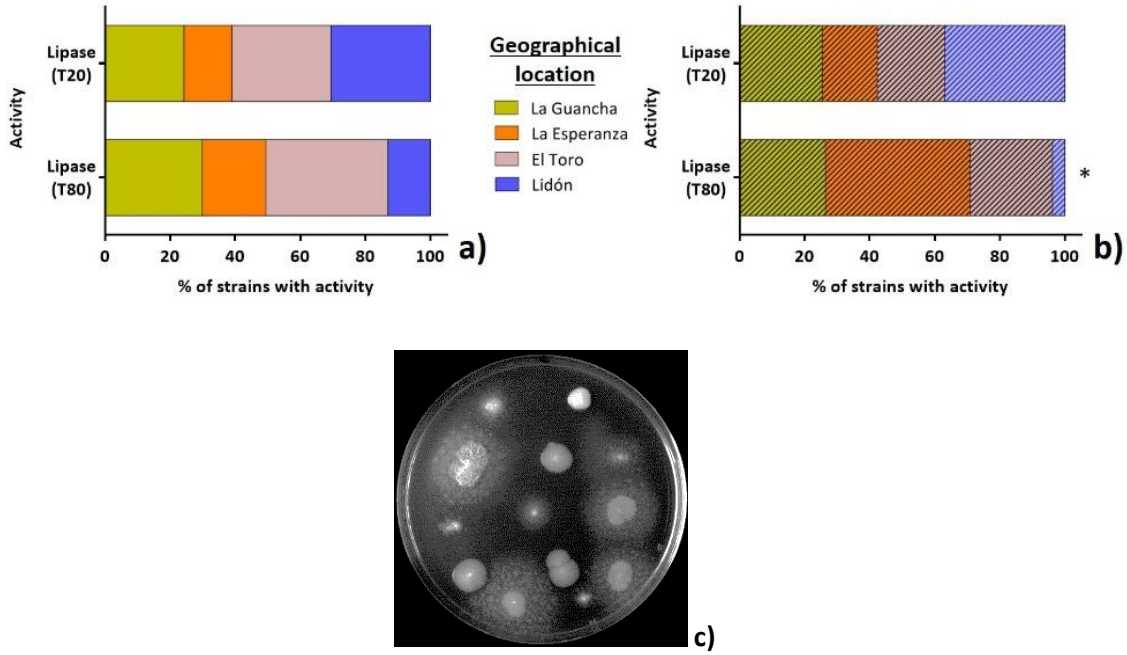


Figure 19. Percentages of ectolichenic (plain bars) (**a**) and endolichenic (striped bars) (**b**) bacterial strains of *R. farinacea* populations from each one of the four Spanish geographical locations, with lipase activities using the synthetic lipids Tween 20 (T20) and Tween 80 (T80). Each bar represents the percentages of positive strains tested by duplicate in two independent experiments. Significant results are marked with an asterisk ($p<0.05$). A representative picture of the detection of lipase activity using T20 in a selection of *R. farinacea* bacterial strains (**c**).

Protease activities

Two different substrates were used to detect protease activity, casein and gelatine. From the tested strains, 29.93% had protease activity, with a 31.14% of them showing hydrolytic activity for the gelatine substrate, and 30.46% for casein. When contrasting the results according to the insular or peninsular location of *R. farinacea* populations, bacterial strains from the Island were more active than those from the Peninsula (32.31% and 28.62%, respectively), although with slight differences. In the Island more postive strains were found for caseinase activity, while in the Peninsula, gelatinase activity was the predominant one among the tested strains. When the results were analyzed considering the four geographical locations (Figure 20), the highest percentage of bacteria with gelatinase activity was found in the lichen population from

Lidón, with a 36.45% of the tested strains, while in the case of caseinase activity the most active bacteria were detected in the *R farinacea* population from La Esperanza, with a 37.5% of bacterial strains consdiering both ecto- and endolichenic. However, the percentage of positive bacteria for protease activity from the four locations showed very similar proportions. *R. farinacea* populations from La Guancha, Lidón and La Esperanza had a 32.83%, 32.28% and 31.79% of positive bacterial strains, respectively, while El Toro was the location with less proportion of strains with protease activity, with a 26.58%. When comparing the general protease activity between the ectolichenic and the endolichenic bacterial strains, 32.73% and 26.79% were positive, respectively. In fact, for both gelatine and caseine, a higher percentage of endolichenic bacteria (34.09% and 31.36%, respectively) than ectolichenic ones (28.57% and 25%, respectively) showed protease activity. Significant differences were not observed when protease activities were compared between ectolichenic and endolichenic strains or among strains from different geographical locations.

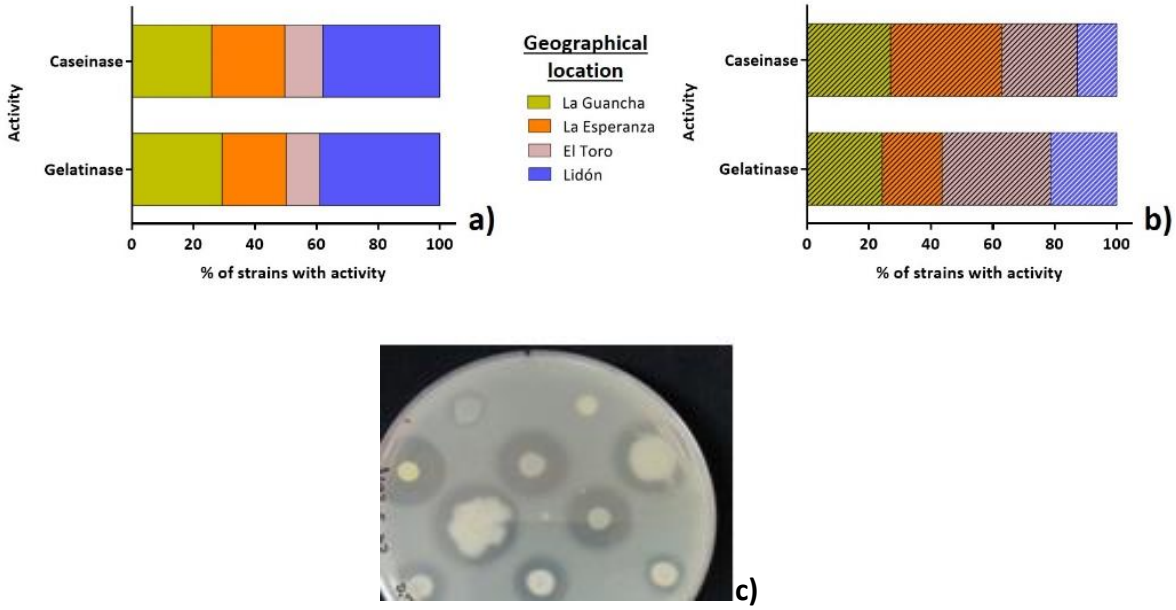


Figure 20. Percentages of ectolichenic (plain bars) (**a**) and endolichenic (stripped bars) (**b**) bacterial strains of *R. farinacea* populations from each one of the four Spanish geographical locations showing protease activities using the proteins casein and gelatin. Each bar represents the percentages of the positive strains tested by duplicate in two independent experiments. Representative picture of the detection of protease activity (with casein as substrate) in a selection of *R. farinacea* bacterial strains (**c**).

DNAse activities

A total of 32.38% of the *R. farinacea* bacterial strains showed nuclease activity, being those from the lichen populations from the Peninsula more active than those from the Island (34.94% and 27.21%, respectively). When the results were analyzed according to the four populations of *R. farinacea* sampled (Figure 21), in those from Lidón was found the greatest proportion of active strains for this activity (46.87%). The bacterial strains from the thalli from the rest of geographical locations had less proportion of positives for the DNAse activity, with a 27.08%, 27.27% and 28.32% for La Esperanza, La Guancha and El Toro, respectively (Figure 21). Besides, the number of endolichenic bacteria from lichen thalli from Lidón showing positive

results was higher than the ectolichenic ones, with percentages of 56.60% and 34.88%, respectively (Figure 21). Regarding the samples from the locations of La Guancha and El Toro, the ectolichenic bacterial isolates were more active (32.65% and 35.80%, respectively), than the endolichenic ones (22% and 28%, respectively). In La Esperanza, these differences were less notable, with a 28% of the positive bacteria being endolichenic and a 26.09% ectolichenics (Figure 21). No significant differences were obtained when comparing the activities according to the ectolichenic or endolichenic origin or the geographical origin of the strains.

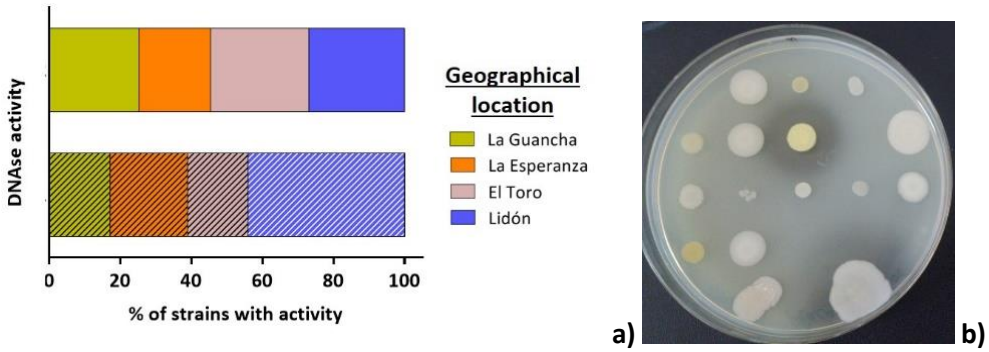


Figure 21. Percentages of ectolichenic (plain bars) and endolichenic (striped bars) bacterial strains of *R. farinacea* populations from each one of the four Spanish geographical locations with DNAse activity. Each bar represents the percentages of positive strains tested by duplicate in two independent experiments (**a**). A representative picture of the detection of DNAse activity in a selection of *R. farinacea* bacterial strains (**b**).

As a summary of the hydrolytic potential of the *R. farinacea* bacterial strains studied, regardless their geographical and lichenic origin, a total of 40.95% of them had one or more hydrolytic activities. A 61.8% of them were positive for polysaccharases activities, a 39.7% for lipase activities, a 32.38% for the DNAse activity and a 29.93% for protease activities.

7.2.4 Nutrient supplying activities

Nitrogen fixation

The ability of *R. farinacea* bacterial strains to fix nitrogen was tested using different media designed for this purpose, as Norris medium, the one described by Cardinale *et al.* (2006) and two different minimal media, both with glucose and one of them also with mannitol, but without a nitrogen source (ABG-N and ABGM-N, respectively). A higher number of bacteria able to fix nitrogen were detected in the ABG-N and ABGM-N media than in the others (data not shown). Therefore, ABG-N and ABGM-N were the culure media employed in this assay. These two media were used according to initial results performed with a selection of strains, in which some differences were found in their ability to detect bacteria able to fix nitrogen. However, subsequent results revealed no significant differences between these two nitrogen free media since only in the case of the bacterial strains from El Toro, there were two of them able to grow in ABG-N media and not in ABGM-N. Thus, almost all tested bacterial strains had the ability to fix nitrogen in both media. In the case of the bacterial strains from lichen thalli from the Island, an 87.07% of them were able to fix nitrogen in both media, while in those from the Peninsula, this percentage was slightly higher, with a 97.4% of positive strains

(97.77% for ABG-N and 97.03% for ABGM-N). According to each geographical origin (Figure 22), 100% of bacterial strains from lichen thalli from Lidón were able to fix nitrogen, followed by the strains from El Toro with a 95.95%, La Guancha with an 88.89% and La Esperanza with an 83.65%. The ability to fix nitrogen was also high and very similar between ectolichenic (94.90%) and endolichenic (92.73%) bacteria, regardless the geographical origin or the culture medium used. A 92.73% of endolichenic bacterial strains fixed nitrogen in both ABG-N and ABGM-N media, with small differences in the ectolichenic strains (95.41% in ABG-N and 94.39% in ABGM-N). The differences found when comparing among all these results were not significant.

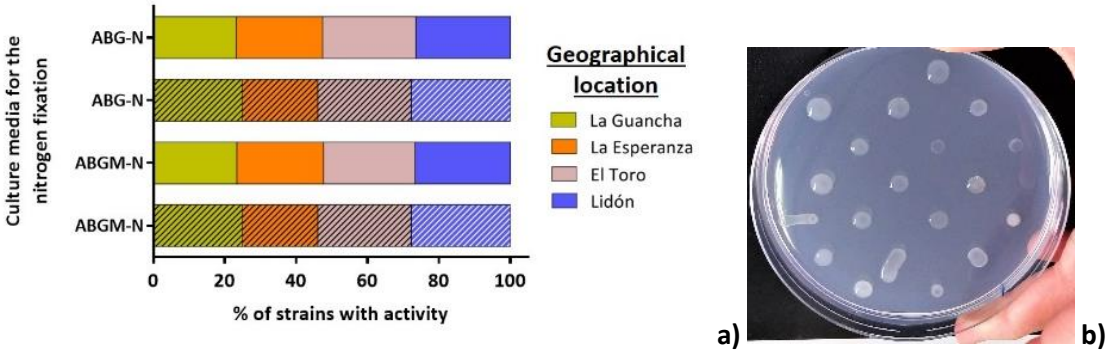


Figure 22. Percentages of ectolichenic (plain bars) and endolichenic (striped bars) bacterial strains of *R. farinacea* populations from each one of the four Spanish geographical locations with the ability of fix nitrogen on two minimal culture media (both with glucose and one of them with mannitol) without nitrogen (ABG-N and ABGM-N). Each bar represents the percentages of the strains tested by duplicate in two independent experiments (**a**). A representative picture of the activity of nitrogen fixation in a selection of *R. farinacea* bacterial strains (**b**).

Phosphate solubilization

The ability of the *R. farinacea* associated bacteria to solubilize phosphate was determined by adding $Ca_3(PO_4)_2$ to the Pikovskaya culture media. The results showed that around 50% of the tested bacterial strains were able to transform the $Ca_3(PO_4)_2$ in a soluble form. However, some differences were found between the results obtained with the strains from lichen thalli from the Island, with a higher number of positive strains being able to solubilize phosphates (62.59%), than in those from the Peninsula (34.94%). Furthermore, differences were also observed when the results were analyzed according to the lichen populations from the four geographical locations (Figure 23). Bacterial strains from thalli samples from La Guancha and Lidón showed very similar percentages for phosphate solubilizing bacteria, with a 75.74% and 75.89%, respectively. This percentage decreased among the strains from thalli from La Esperanza up to a 35.57%, and even more for those from El Toro, with only a 9.91% of tested strains (Figure 23). The proportion of ectolichenic and endolichenic bacterial strains that were positive was very similar, with percentages of 49.53% and 49.02%, respectively. Interestingly, significant differences were found between the results of the endolichenic bacteria from lichen thalli from La Guancha and El Toro ($p<0.05$), with percentages of positive bacteria of 78% and 8.70%, respectively, as well as between the endolichenic bacteria from Lidón and El Toro ($p<0.05$), with percentages of 77.36% and 8.70%, respectively.

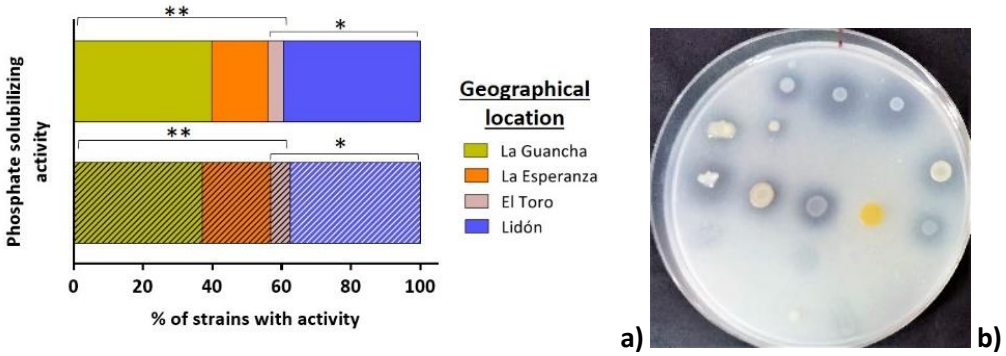


Figure 23. Percentages of ectolichenic (plain bars) and endolichenic (striped bars) bacterial strains of *R. farinacea* populations from each one of the four Spanish geographical locations with phosphatase activity. Each bar represents the combination of the resulting percentages of the strains tested by duplicate in two independent experiments. Significant results are marked with asterisks ($p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***)) (**a**). A representative picture of the detection of phospahte activity in a selection of *R. farinacea* bacterial strains (**b**).

Siderophores production

The original CAS agar medium widely used for the detection of bacteria able to produce siderophores to uptake iron, was modified to improve the growth of *R. farinacea* bacterial strains unable to grow on this medium (Figure 24a). To this end, a second culture medium was added to these CAS agar plates, KB since it was the one routinely used for the growth of lichenic bacteria. A first improvement was made dividing the plate in two halves, one containing the KB medium and the other one the CAS agar (Figure 24b). The inoculation of bacteria was made from the side of the KB medium to the side of the CAS agar. Thus, some of the bacteria were able to grow in the CAS agar, probably because they took some nutrients from the KB medium that helped them growing in the CAS agar. The second modification was the addition of a thin second layer of KB medium on the layer of CAS agar (Figure 24c). This modification avoids the bacteria to be in direct contact with the CAS agar, with the KB medium layer being thin enough to allow the bacteria to uptake iron from CAS agar at the bottom of the plates. This improvement allowed the growth of many lichenic bacteria that were unable to grow in direct contact with the original CAS agar. Therefore, a higher number of *R. farinacea* bacterial strains were able to show their ability to produce siderophores. The progress of the results to improve CAS agar is shown in figure 24.

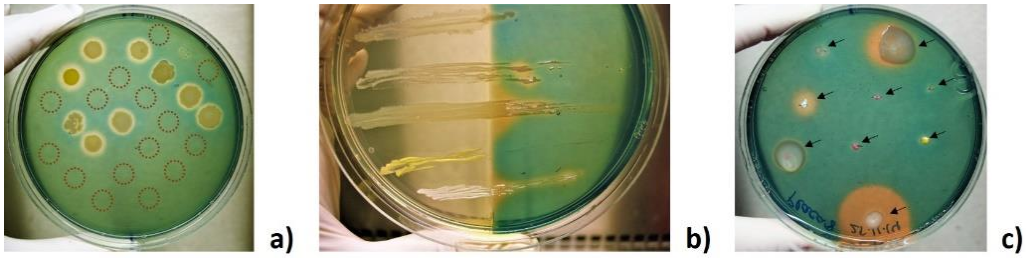


Figure 24. Representative pictures of the modifications of the CAS agar to improve the detection of siderophores with *R. farinaceae* bacterial strains. Circles represent inoculated bacterial strains unable to grow on regular CAS agar (**a**). A plate with KB agar in the left half, and CAS agar in the right half showing strains growing on this modified medium (**b**). A plate with CAS agar at the bottom and KB on the upper layer allowing the growth of all inoculated bacterial strains regardless their ability or not to produce siderophores (**c**).

The detection of siderophores in the *R. farinacea* bacterial strains was determined on the optimized CAS agar, with the results showing that a high percentage of them produced these iron-chelating compounds. From all the tested bacterial strains, 84.14% were positive for this activity, regardless their insular (86.39%) or peninsular (82.89%) origin. Considering the four geographical origins of the *R. farinacea* strains (Figure 25), the ones isolated from lichen thalli from Lidón were the most active with a 97.16% of them being able to produce siderophores. They were followed by the bacterial strains from La Guancha, with a 90.91%, La Esperanza with a 77.08% and El Toro with a 75.14%. Not differences were found regardless the endolichenic (84.09%) or ectolichenic (84.18%) origin of the bacterial strains (Figure 25). The small differences observed among the results obtained either with strains from different geographical locations and/or origin in the thallus were not significant.

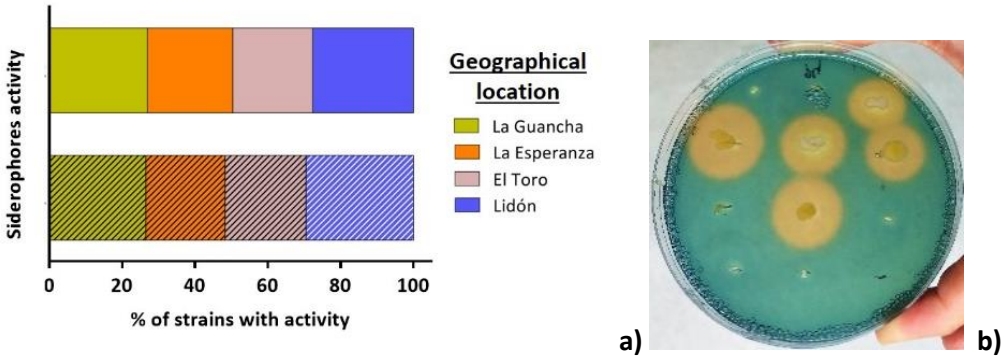


Figure 25. Percentages of ectolichenic (plain bars) and endolichenic (striped bars) bacterial strains of *R. farinacea* populations from each one of the four Spanish geographical locations with the ability of produce siderophores according to their ectolichenic (plain color bars) or endolichenic (striped bars) location in the thallus (**a**). Each bar represents the percentage of the strains tested by duplicate in two independent experiments. A representative picture of the result of siderophore production in a collection of *R. farinacea* bacterial strains (**b**).

Overall**,** the results of the nutrient supplying activities studies have shown that a high percentage of *R. farinacea* bacterial strains had the ability to fix nitrogen and produce siderophores, regardless their geographical or lichenic origin, with values ranging between 70% and 100%. Related to the phosphate solubilizing activity, a higher percentage of positive strains for this activity was detected among those isolated from thalli samples from La Guancha and Lidón (around 70%) than those from El Toro (around 30%) and Lidón (10%). The bacterial strains isolated from lichen populations from La Guancha and Lidón, were in general, more active than the ones from the other two locations for the activities studied.

7.2.5 PHYTOHORMES DETECTION

Auxin production

Many of the bacterial strains of *R. farinacea* tested in this study showed the ability to synthesize the auxin IAA in the presence of the precursor L-tryptophan, despite their production of IAA was variable (Figure 26 and 27). For the detection of auxins, a first approach was carried out through a non-quantitative method, although based on the differences of color intensity produced after the transformation of the L-tryptophan in IAA and the

measurement of the consequent color at A_{530} nm at, 24, 48 and 72 h, after the addition of Salkowskis's reagent. As shown in figure 26a, the bacterial strains that showed the highest IAA values at the three reading times were those from lichen thalli from the location of La Guancha, followed by those from Lidón and El Toro. The strains from La Esperanza were the ones less active in the production of this hormone. In all cases, the results obtained showed that the ectolichenic bacterial strains from the four geographical locations produced more IAA than the endolichenic ones. As expected, the levels of this hormone were increasing within time, reaching maximum values at 72 h.

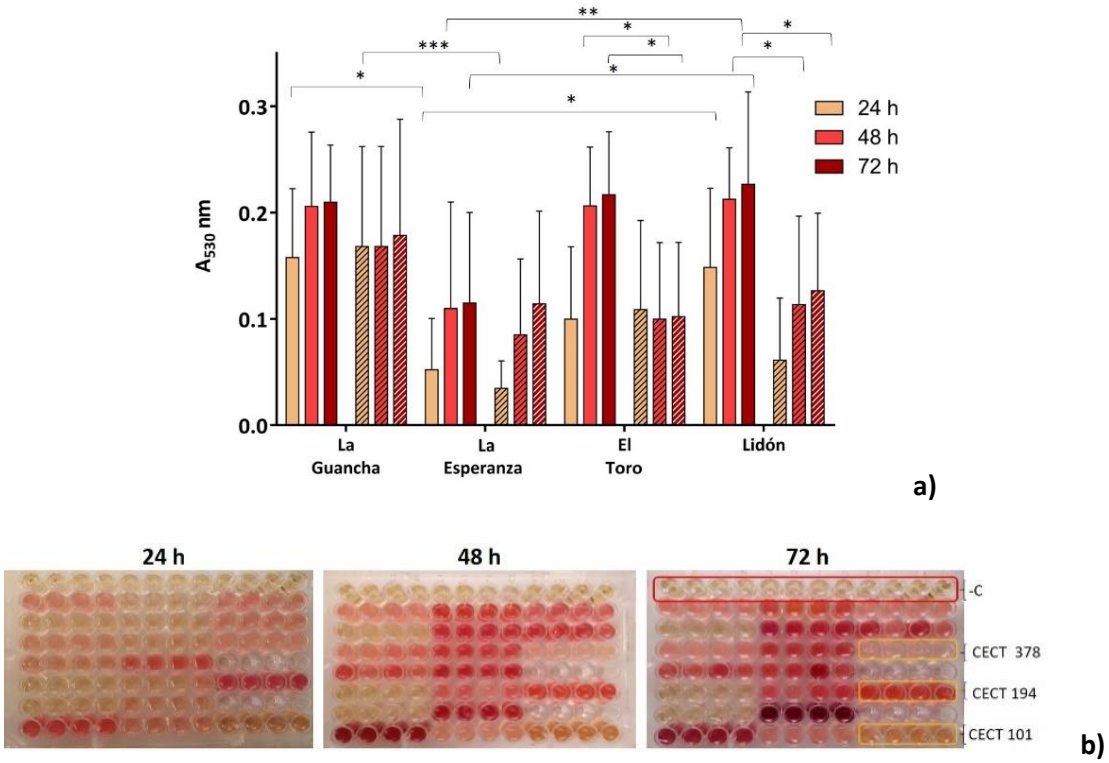


Figure 26. IAA phytohormone production by bacterial strains of *R. farinacea* populations from the four geographical locations in Spain. Average values of measures at A_{530} nm for the ectolichenic (plain bars) and endolichenic (striped bars) bacterial strains from each location, after 24 h, 48 h and 72 h of incubation at 26ºC. Each bar represents the average values for all strains from the same geographical location (each strain was tested by quadruplicate). The standard deviation of the data is indicated by the vertical lines. Significant results are marked with asterisks ($p<0.05$ (*); $p<0.01$ (**), $p<0.001$ (***) (**a**). Representative pictures showing the colored reaction by the production of IAA after adding the Salkowski's reagent in microtiter plates at different incubation times (24 h, 48 h and 72 h). First row of wells was filled with KB broth without bacterial inoculum as negative control (-C, wells framed in red). Three bacterial strains were used as positive controls (*P. fluorescens*: CECT 378; *E. cloacae*: CECT 194; *E. coli*: CECT 101. Wells framed in yellow) (**b**).

Afterwards, and according to the abovementioned results, the procedure continued with the quantification of the concentration of IAA produced by the bacterial strains based on the results of a standard curve of IAA. A selection of those strains positive for IAA from the previous test was made for this assay. In this case, the readings were performed at 24 h and 72 h, since the differences between 48 h and 72 h were not significant, and some of the assayed bacteria showed a slow growth rate. Thus, an extended period of incubation helped to visualize the hormone production in those cases. In figure 27a are represented the mean values of IAA production for the tested bacterial strains of each one of the four populations of

R. farinacea at 24 h and 72 h. Auxin concentrations ranged from 0.0012 to 41.42 µg/ml at 24 h and 0.0027 to 100.62 µl/ml at 72 h. The most active producers of IAA were the bacterial strains isolated from *R. farinacea* populations from La Guancha and Lidón with averages values of 7.4 and 5.25 µg/ml at 24 h, respectively, and 19.38 and 16.7 µg/ml at 72 h, respectively. The bacterial strains from La Esperanza and El Toro showed less hormone production than the ones from the other locations, with values of 1.91 and 0.78 µg/ml at 24 h respectively, and 6.55 and 2.55 µg/ml at 72 h, respectively. Some significant differences were found as shown in figure 27a.

In figure 27b are shown the percentages of bacterial strains according their IAA production at different concentrations at 24 h and 72 h. At 24 h, most of the assayed bacterial strains showed a low hormone production, in the range from 0 to 5 µg/ml. Taking into account the insular or peninsular origin of *R. farinacea* populations, a higher percentage of bacterial strains producing more than 5 µg/ml of hormone after 24 h was detected in the Island than in the Peninsula (37.31% and 16.92%, respectively). These percentages increased after 72 h (62.71% in the Island and 41.53% in the Peninsula). When considering each one of the four geographical locations (Figure 27b), between 90% and 100% of bacterial strains from lichen populations from La Esperanza and El Toro were in the range mentioned above. By contrast, in the case of strains from La Guancha and El Toro, there were different proportions of bacteria that produced this hormone at different concentrations. Around 80% of them produced IAA between 0 and 10 µg/ml, and around 20% of them showed a hormone production between 10 and more than 15 µg/ml, in both cases. After 72 h of incubation, only in the case of the bacteria isolated from *R. farinacea* thalli from El Toro there wasn't a remarkable increasing production of IAA. Related to the bacteria from lichen thalli from La Esperanza, around 50% increased their production from 5 µg/ml or more than 15 µg/ml. In the case of La Guancha, around 25% of the bacteria increased their production between 5 to 15 µg/ml, with more than 50% producing more than 15 µg/ml. Regarding the bacteria isolated from Lidón, the two main groups of production were high (> 15 µg/ml) and low (from 0 to 5 µg/ml) hormone concentration.

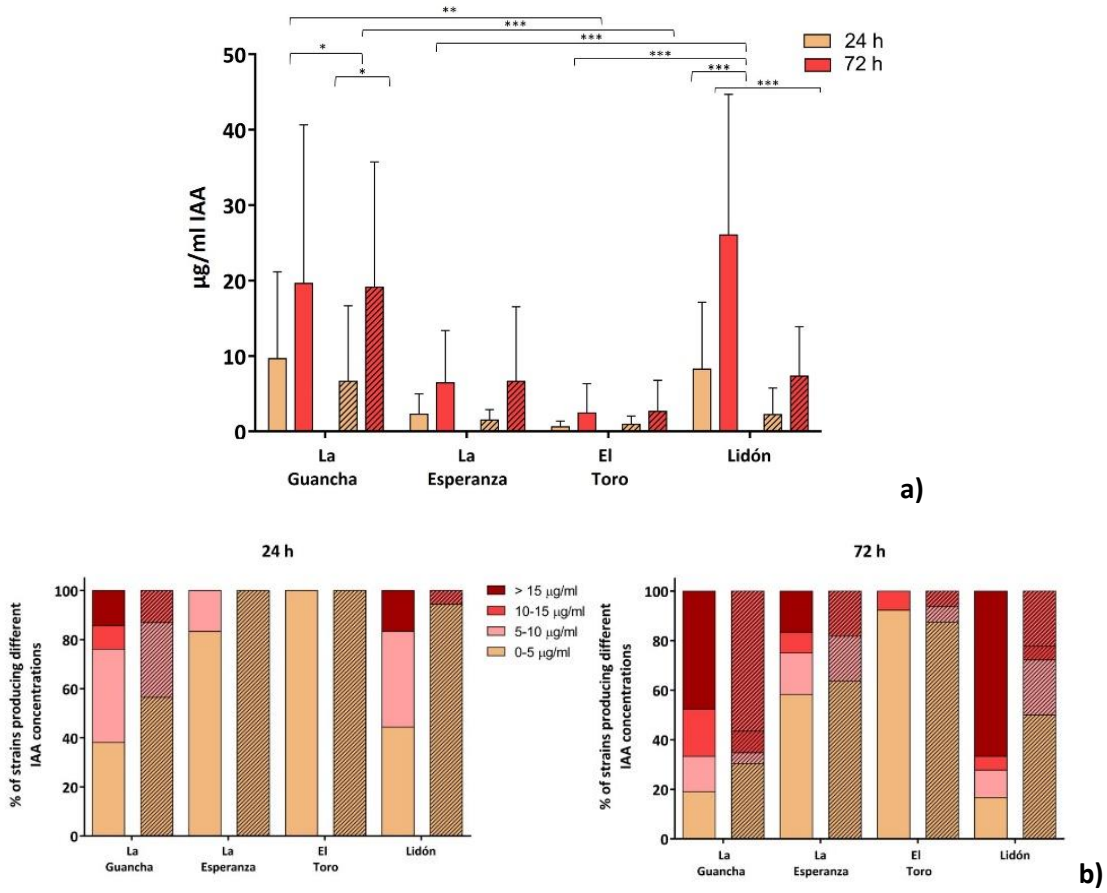


Figure 27. Quantification of the IAA production by the bacterial strains isolated from *R. farinacea* populations from the four geographical locations in Spain. Each bar represents the average concentration value produced by the bacterial strains from each geographical location grouped by their ectolichenic (plain bars) and endolichenic (striped bars) origin. Significant results are marked with asterisks ($p<0.05$ (*); $p<0.01$ (**), $p<0.001$ (***)) (**a**). Percentages of bacterial strains able to produce IAA at different concentrations and times (24 h and 72 h), where each bar represents the percentages of ectolichenic and endolichenic bacterial strains from each geographical location (**b**).

Detection of ACC deaminase gene

The PCR detection of the *acdS* gene in the *R. farinacea* bacterial strains was made as previously described (Nikolic *et al*., 2011). However, by using the original conditions for the PCR reaction some unspecific bands were obtained. Therefore, this reaction and amplification conditions were modified in order to improve the detection of the *acdS* gene. In figure 28 are shown representative pictures of the improvement of the amplification conditions, visualized after the separation of PCR products by gel electrophoresis.

The final reaction mix for a volume of 50 µL the PCR was: 1 µl DNA sample, 33.5 µL of sterile MiliQ water, 5 µL of 10X buffer, 5 µL 2 mM dNTPs, 2.5 µL of each primer, 0.25 µL of DMSO (dimethyl sulfoxide) (0.5%) and 0.25 µL of Taq polymerase (DreamTaq DNA polymerase, Thermo Scientific). DMSO was added as an additional optimization to reduce more the appearance of unspecific bands. Regarding the amplification conditions, the number of cycles and the temperature of annealing were modified, being the final conditions those shown in table 13.

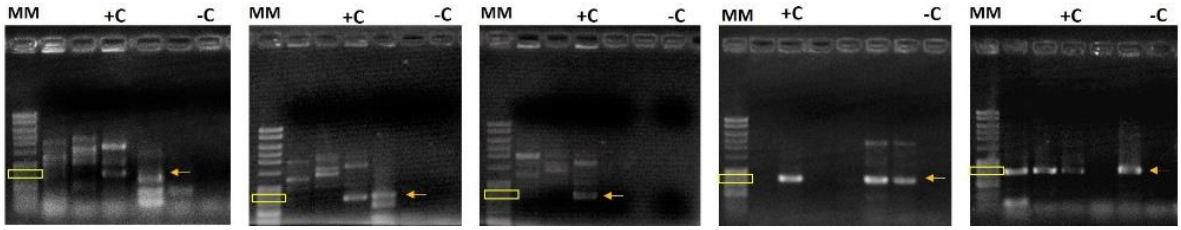


Figure 28. Representative pictures showing the progressive improvement of the PCR detection of *acdS* gene by modifying the amplification conditions to reduce unspecific bands. From the left to the right it is shown the reduction of these bands and the visualization of the band of interest of 750 bp (yellow rectangle in MM and arrow in the bacterial strains tested). MM: molecular weight marker. +C: positive control (*E. cloacae*: CECT 194). -C, negative control.

Table 13. Final PCR conditions for the molecular detection of the *acdS* gene.

Number of cycles	Time per cycle	Temperature	Stages
1	3 minutes	95ºC	Initial denaturing
27	30 seconds	95ºC	Denaturing
	1 minute	50ºC	Annealing
	1 minute	72ºC	Extending
1	5 minutes	72ºC	Final extension

The molecular detection of the *acdS* gene in the *R. farinacea* bacterial strains trough PCR showed that only an 18.54% of tested strains had the gene that codifies for the ACC deaminase, being a 17.85% of them from the Island and a 19.30% from the Peninsula. When the results were analyzed considering each one of the four geographical locations from where thalli samples were collected, we found that the bacterial strains from *R. farinacea* populations from La Guancha and Lidón presented the highest numbers of positive detections for this gene (23.53% and 30.30%, respectively), while those from La Esperanza and El Toro showed the lowest ones (9.09% and 4.17%, respectively).

When contrasting the PCR results between ectolichenic and endolichenic bacterial strains for each geographical location (Figure 29), some differences were found. In the lichen population from La Guancha, a 14.71% of the positive results were detected among the endolichenic strains and a 8.82% among the ectolichenic ones. By contrast, in the strains from La Esperanza a similar proportion of positives was observed (about 4.55%), regardless the lichenic origin. Interestingly, in the bacterial strains from El Toro, the *acdS* gene was detected only among the ectolichenic ones (4.17%), while in those from Lidón a higher percentage of positive results was recorded among those ectolichenic (21.24%) than endolicenic (6.06%).

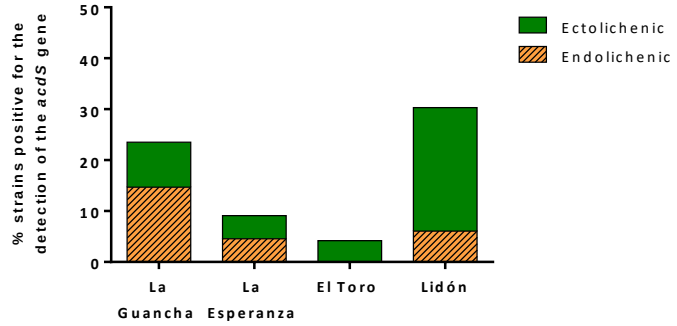


Figure 29. Percentages of bacterial strains of *R. farinacea* positive for the detection of the *acdS* gene. Each bar represents the percentage of ectolichenic and endolichenic positive bacterial strains for each one of the four geographical locations from where *R. farinacea* thalli were sampled in Spain.

7.2.6 Biofilm formation

Biofilm production

The ability to produce biofilms by the bacterial strains isolated from *R. farinacea* populations from different Spanish geographical locations was also evaluated at different times of incubation, taking also into consideration their origin in the lichen thallus. The results were finally recorded after 48 h and 72 h of incubation at 26ºC. In figure 30 are shown the average values at A_{600} nm, after the staining of bacterial biofilms with crystal violet for each one of the geographical locations and according to the ectolichenic or endolichenic origin of the strains. In all cases, the differences observed between readings at 48 h and 72 h were not remarkable, although mean values were higher at 72 h than at 48 h. The groups of bacterial strains that produced more biofilms were the ones isolated from lichen thalli from La Guancha and Lidón, with average values higher than A_{600}=0.50, either at 48 h and 72 h, regardless the origin in the thallus. They were followed by the group of bacterial strains isolated from thalli populations from La Esperanza and El Toro, with mean values around A_{600}=0.40 for both incubation times, regardless their lichenic origin. These tiny differences were not significant, but gave an idea about the intensity of the biofilms produce by these lichenic bacteria.

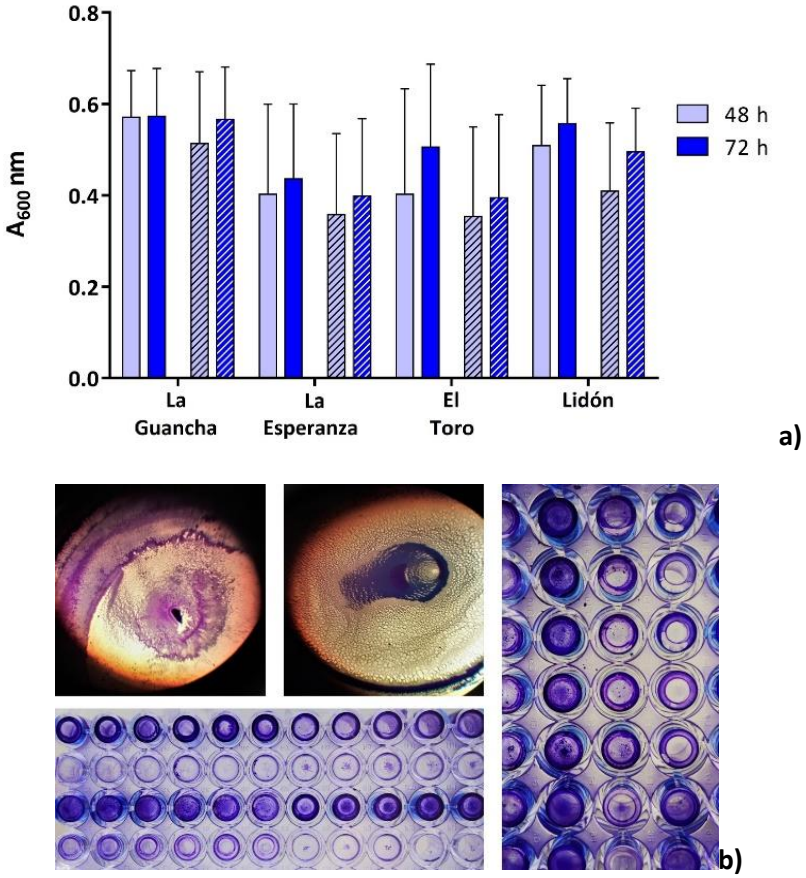


Figure 30. Biofilm production by bacterial strains of *R. farinacea* from lichen populations from four geographical locations in Spain. Each bar represents the average values at A_{600} nm of bacterial strains grouped by their geographical location and ectolichenic (plain bars) and endolichenic (striped bars) origin after 48 h and 72 h. Six replicates were made for each bacterial strain. The standard deviation of the data is indicated by the vertical lines (**a**). Representative pictures showing the production of biofilms after dying with crystal violet. The two small pictures in the upper part on the left and center were taken through a light microscope (40x). The pictures in the lower part on the left and the one on the right are representative images of the different intensities of biofilms produced. The wells with darker colours correspond to strong biofilms, and the ones with lighter colours to medium or weak biofilm production (**b**).

With the aim to cluster the bacterial strains according to the amount of biofilm produced, they were classified in four categories: strong, moderate, weak or not biofilm producers. At 48 h, most of the bacterial strains produced moderate and strong biofilms, with a higher proportion in the case of those from lichen population from the Island than in those from the Peninsula (91.31% and 67.74%, respectively). These percentages increased after 72 h of incubation (97.1% in insular strains and 93.56% in those peninsular ones). When considering bacterial strains grouping them according to the geographical and lichen thallus origin (Figure 31), it was found that an 82.93% of the strains from the lichen populations of La Guancha produced strong biofilms at 48 h (89.47% ectolichenic and 77.27% endolichenic), while the rest of bacterial strains produced moderate biofilms (10.53% of ectolichenics and 22.73% of endolichenics). In the lichen population of La Esperanza, a 42.86% of the tested bacteria produced strong biofilms (46.67% ectolichenics and 38.46% endolichenics), a 35.71% moderate (33.33% ectolichenics and 38.46% endolichenics), with less proportion of weak biofilm producers (14.29%). Some of them were unable to produce this structure. In the case

of bacterial strains from El Toro, the main categories of biofilms produced were strong (40.74% with 53.85 % ectolichenic and 28.57%, endolichenic) and weak (37.04% of the bacteria, 46.15% ectolichenic and 28.6% endolichenic). Only a 14.81% produced moderate biofilms and a 7.40% were unable to form them. All the bacteria isolated from lichen thalli from Lidón produced biofilms after 48 h, a 45.71% formed strong biofilms (58.82% ectolichenics and 33.33% endolichenics), 40% moderate ones (23.53% ectolichenics and 55.56% endolichenics), and 14% weak biofilms (17.65% ectolichenics and 11.11% endolichenics). At 72 h there was an increase in the number of bacteria able to produce strong and moderate biofilms in all *R. farinacea* bacterial strains (Figure 31). Significant differences in the percentages of bacterial strains able to produce different categories of biofilms were observed between ecto- and endolichenic fractions, and in some cases at different times of incubation (Figure 31).

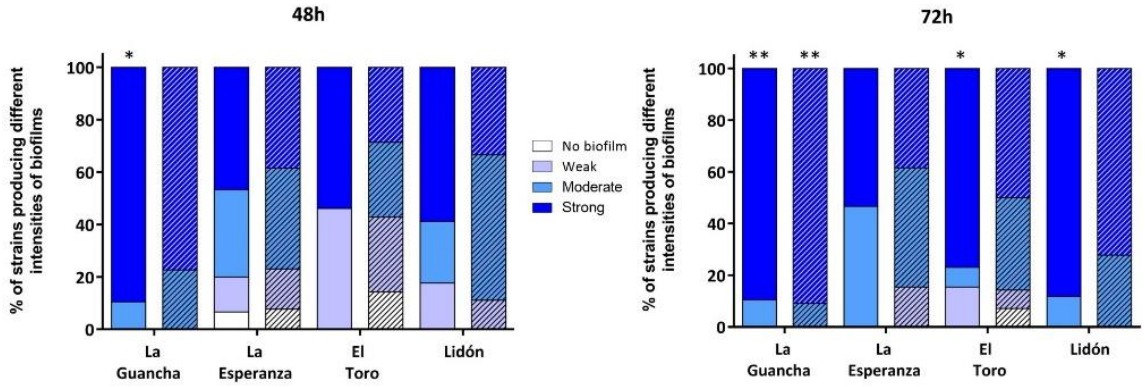


Figure 31. Percentages of bacterial strains of *R. farinacea* populations from four geographical locations in Spain, producing biofilm at different intensities after 48 h (left) and 72 h (right) of incubation. Each bar represents the percentages of bacterial strains grouped by their ectolichenic (plain bars) or endolichenic (striped bars) origin for each geographical location. Six replicates were made for each bacterial strain. Significant results are marked with asterisks ($p<0.05$ (*); $p<0.01$ (**)).

Swimming and swarming motility

Swimming

The results of swimming motility with *R. farinacea* bacterial strains showed that a high number of them (70.43%) presented this type of motility, being very similar between the strains from insular *R. farinacea* populations (71.43%) and those peninsular (69.49%). With regards to the results according to the the four geographical locations (Figure 32a), the highest number of bacterial strains positive for swimming motility were those from *R. farinacea* thalli from La Guancha (86.49%), followed by those from lichen thalli from Lidón (84.34%), El Toro (51.85%) and La Esperanza (42.11%). When comparing between ectolichenic and endolichenic bacterial strains from La Guancha, percentages of 94.44% and 78.95% were recorded, respectively (Figure 32a). The average of the distance travelled by these bacteria was 4.15 cm and 3.89 cm, respectively (Figure 32b). In the case of the bacterial strains from thalli from La Esperanza, there was an important difference between the ectolichenic (77.77%) and the endolichenic (10%) ones, with average distance reaching 3.01 cm and 1.65 cm, respectively (Figure 32b). For those bacterial strains isolated from lichen thalli from El Toro, there was a 60% of the ectolichenic bacteria with swimming motility, with an average distance of 0.44 cm, and a

41.67% of endolichenic bacteria, with an average of 0.39 cm (Figure 32 a and b). With regards to bacterial strains from thalli collected from Lidón, the percentages of motile bacteria were very similar for both ectolichenic (86.67%) and endolichenic (82.35%) strains, with average distance reached of 3.83 cm and 2.73 cm, respectively (Figure 32 a and b). All swimming motility data and the significant differences among them are represented in graphics in figure 32 (a and b), as well as some representative pictures of the swimming motility (Figure 32c).

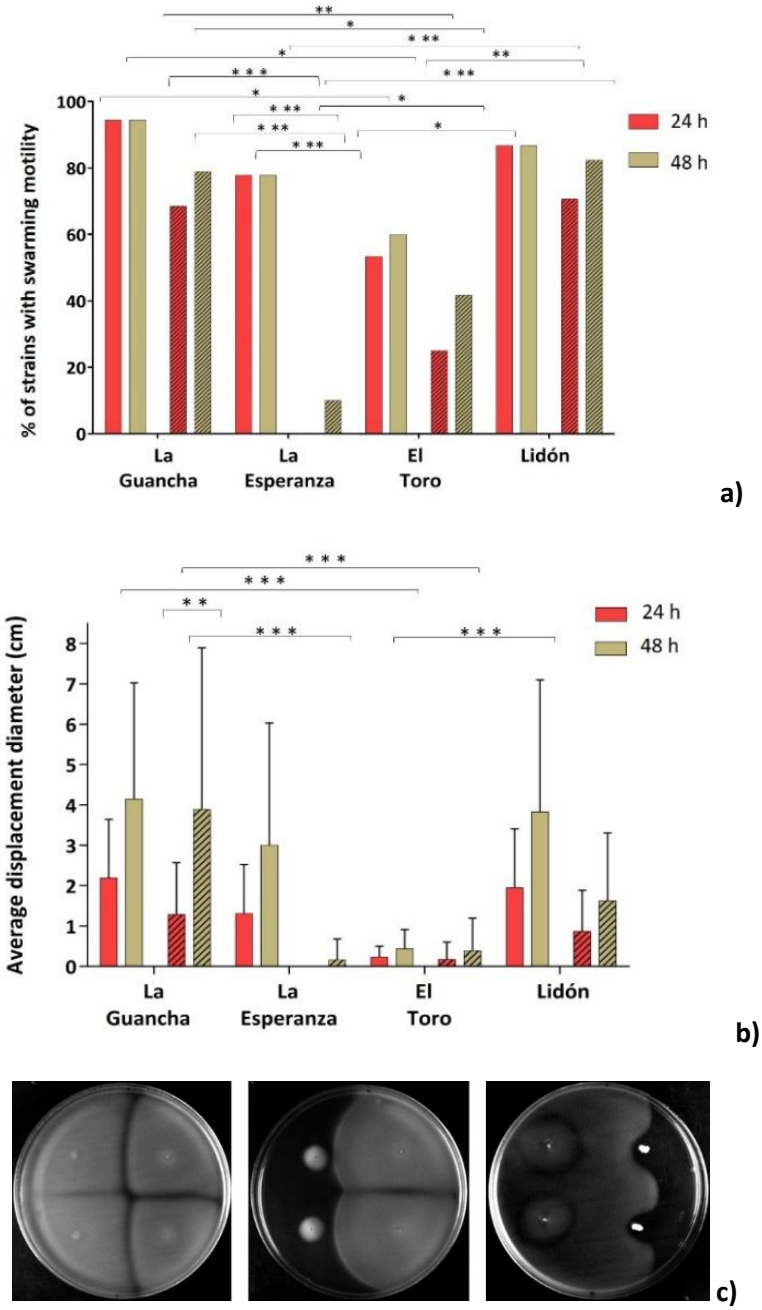
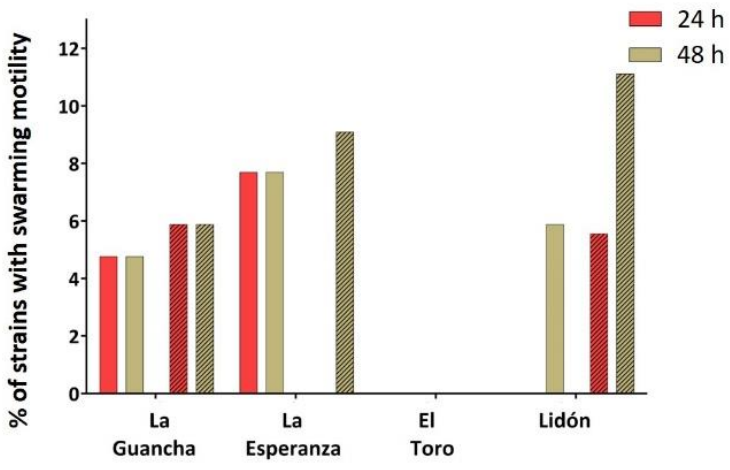


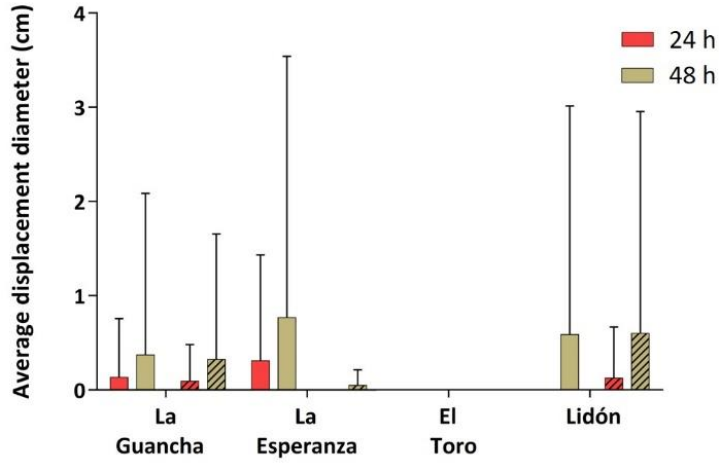
Figure 32. Swimming motility of bacterial strains isolated from *R. farinacea* populations from four geographical locations in Spain. Percentages of bacterial strains showing swimming motility at 24 h and 48 h of incubation at 26ºC. Each bar represents the percentage of these strains according to their ectolichenic (plain bars) and endolichenic (striped bars) origin for each geographical location. Significant results are marked with asterisks ($p<0.05$ (*); $p<0.01$ (**)), $p<0.001$ (***)) (**a**). Average value of the distance travelled by the tested strains at 24 h and 48 h. Each bar represents the average distance for strains according to their ectolichenic (plain bars) and endolichenic (striped bars) origin for each geographical location. The standard deviation of the data is indicated by the vertical lines (**b**). Representative pictures of different strains showing swimming motility as well as one strain negative for this motility (last picture, strain on the right) (**c**).

Swarming

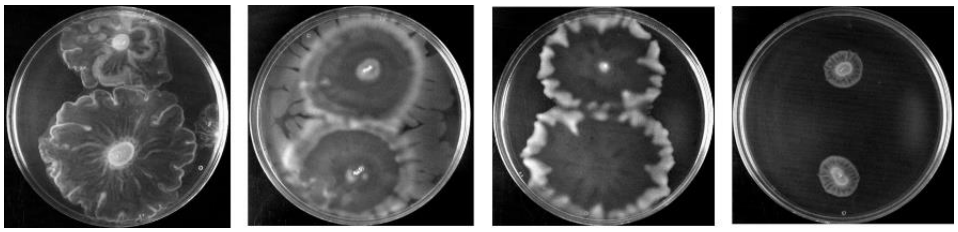
Swarming motility was studied as well with *R. farinacea* strains. The percentages of bacteria able to move with swarming motility was low compared with those of swimming motility. Only a 5.6% of them were positive for swarming, with a 6.45% of the strains being from *R. farinacea* thalli from the Island and a 4.76% from the Peninsula. The results of swarming motility of *R. farinacea* according to each geographical location and their lichenic origin are shown in figure 33a. The strains from thalli from La Esperanza and Lidón were the ones with higher percentage of positives with an 8.33% and 8.57%, respectively, followed by those from La Guancha with a 5.26%. None of the tested bacterial strains from lichen thalli from from El Toro showed this motility. With regards to the lichenic origin of the strains for each geographical location, in the lichen population from La Guancha, the maximum diameter of movement reached 7.85 cm. In the endolichenic fraction, the maximum distance reached by swarming were 5.5 cm. In the location of La Esperanza a 7.69% of the ectolichenic strains were positive and a 9.09% of the endolichenics. In this case, the range of the diameter reached was between 0.55 cm and the invasion of the surface of the plate. Related the location of Lidón, there was a 5.88% of ectolichenic positive bacteria and 11.11% endolichenic. The maximuum distance reached the edge of the plate. Representative pictures of different shapes produced by swarming motility are shown in figure 33c.



a)



b)



c)

Figure 33. Swarming motility of bacterial strains isolated from *R. farinacea* populations from four geographical locations in Spain (**a**). Percentages of bacterial strains showing swarming motility at 24 h and 48 h. Each bar represents the percentage of these strains according to their ectolichenic (plain bars) and endolichenic (striped bars) origin for each geographical location. Average value of the distance travelled by the tested strains at 24 h and 48 h. Each bar represents the average distances for strains according to their ectolichenic (plain bars) and endolichenic (striped bars) origin for each geographical location. The standard deviation of the data is indicated by the vertical lines (**b**). Representative pictures of different strains showing swarming (**c**).

When compared the global results obtained with the two motility assays (figure 34), there was a notable difference between both type of motilities, being swimming the most frequent one among the bacterial strains associated with *R. farinacea* populations from the four different Spanish geographical locations (Figure 34a). Bacterial strains from thalli samples from El Toro were the only ones without swarming motility. In all cases, the percentages of motile strains and the average of the distance travelled, was higher for the swimmers than for the swarmers (Figure 34 a and b). Despite these notable differences, they were not significant.

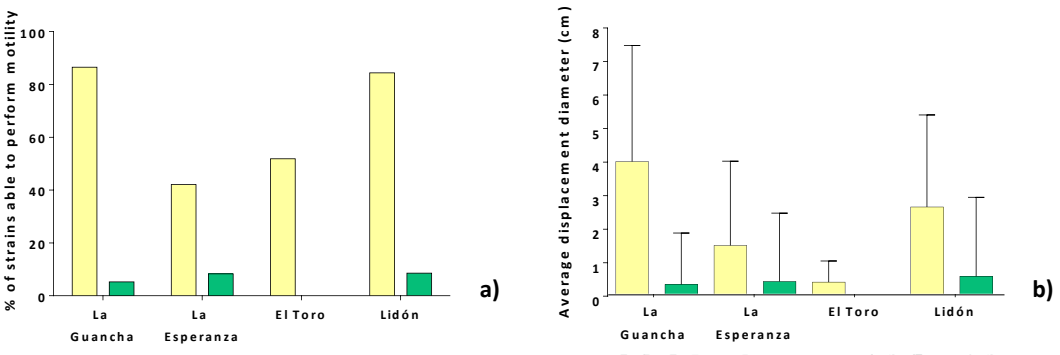


Figure 34. Percentages of bacterial strains of *R. farinacea* able to perform swimming (yellow) and/or swarming (green) motility from lichen populations from four geographical locations in Spain (**a**). Average distance travelled by the tested strains by swimming (yellow) or swarming (green) motility. Each bar represents the average distances for each location. The standard deviation of the data is indicated by vertical lines (**b**).

7.3 MOLECULAR IDENTIFICATION

A group of 65 *R. farinacea* bacterial strains from the four geographical Spanish locations among the most active was selected for their presumptive identification through partial amplification and sequencing of the *16S rRNA* gene. Due to the appearance of unspecific bands by using the PCR conditions described by Arahal *et al*. (2008) using the primers 616V and 699R, the amplification conditions were modified. The final conditions used for this PCR were those shown in table 14.

Table 14. Final PCR conditions for the amplification of the partial sequence of the *16S rRNA* gene.

Number of cycles	Time per cycle	Temperature	Stage
1	5 minutes	94ºC	Initial denaturing
25	30 seconds	94ºC	Denaturing
	45 seconds	56ºC	Annealing
	45 seconds	72ºC	Extending
1	10 minutes	72ºC	Final extension

Once obtained the sequences of the selected bacterial strains, and after their cleaning, analyses and BLAST contrasting, the results showed that these strains belonged to the phyla *Actinobacteria* (47.69% of the strains), *Proteobacteria* (40%) and *Firmicutes* (12.31%).

The main genera to which these bacteria were assigned were *Bacillus*, *Burkholderia, Curtobacterium*, *Erwinia, Frondihabitans, Kocuria, Leifsonia, Methylobacterium, Microbacteria, Pseudomonas*, *Sphingomonas* and *Streptomyces,* among others.

All the selected bacteria that were taxonomically identified had a wide variety of activities (Table 15). The most active genera were *Burkholderia*, *Curtobacterium*, *Erwinia*, *Leifsonia*, *Nocardioides, Pseudomonas* and *Staphylococcus*. In general, bacterial strains assigned to these

genera presented different hydrolytic activities as polysaccharases, lipases, proteases and nucleases, as well as activities related with the supply of limited nutrients (nitrogen, phosphate and/or iron), also producing auxins and biofilms. Other active strains but unable to solubilize phosphates were those assigned to the genera *Arthrobacter*, *Bacillus*, *Kocuria*, *Methylobacterium*, *Microbacterium*, *Micrococcus*, *Mycobacterium*, *Pantoea*, *Sphingomkonas*, *Stenotrophomonas* and *Streptomyces*.

Table 15. Presumptive taxonomical identification of *R. farinacea* bacterial strains according to their functional and biotechnological characterization.

STRAIN	Pigments production	Polysaccharase					Lipase		Protease		Nuclease	Nutrient supplying				Growth stimulation		Biofilm formation			Nearest bacterial species
		Amylase	Cellulase	Xylanase	Pectinase	Chitinase	Tween 20	Tween 80	Caseinase	Gelatinase	DNAse	Nitrogen		Phosphate solubilization	Siderophores production	IAA	ACC-deaminase	Biofilms	Motility		
												ABGM-N	ABG-N						Swimming	Swarming	
R1L2[a]	-	+[b]	+	+	-	+	+	-	+	+	+	+	+	-	+	-	+	-	-	-	*Bacillus megaterium*
R1L7	+	-	+	-	-	-	+	-	+	+	+	+	+	+	+	+	-	+	+	-	*Erwinia* sp.
R1L17	-	-	+	+	-	+	+	-	-	+	+	+	+	-	+	-	-	-	-	-	*Microbacterium* sp.
R1L19	-	+	+	+	+	+	+	-	-	-	-	+	+	+	+	+	-	+	+	-	*Pseudomonas rhizosphaerae*
R1L20	-	+	+	+	-	+	+	+	-	-	+	+	+	+	+	+	+	-	-	-	*P. rhizosphaerae*
R1L21	+	-	+	+	+	+	-	-	-	+	+	+	+	-	+	-	-	-	-	-	*Microbacterium* sp.
R1L23	-	+	-	+	+	+	+	-	-	+	+	+	+	+	+	+	-	+	+	-	*P. rhizosphaerae*
R1L26	+	+	+	+	-	+	+	-	-	+	+	+	+	+	+	-	-	-	-	-	*P. rhizosphaerae*
R1L32	+	-	-	+	+	+	-	-	+	+	-	+	+	+	+	+	+	+	+	-	*Pseudomonas koreensis*
R1L33	+	+	-	-	+	-	+	+	-	-	+	+	+	+	+	+	-	+	-	-	*Burkholderia* sp.
R1M1	-	+	-	+	+	+	+	-	-	-	-	+	+	+	+	+	-	+	-	-	*Burkholderia* sp.
R1M12	-	+	+	-	+	+	+	+	-	-	-	+	+	+	+	+	-	+	-	-	*Averyella dalhaousiensis*
R1M13	-	+	-	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-	-	-	*B. sordidicola*
R1M14	-	+	-	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	-	*B. sordidicola*
R1M18	+	+	+	+	+	+	+	+	-	+	-	+	+	-	-	-	-	-	-	-	*C. flaccumfaciens*
R1M22	+	+	+	+	+	+	+	+	-	+	+	+	+	-	+	-	-	-	-	-	*C. flaccumfaciens*
R1M24	-	+	+	+	-	-	-	-	+	+	-	+	+	+	+	-	-	-	+	-	*Erwinia* sp.
R1M25	-	-	+	-	-	-	-	+	-	-	+	+	+	+	+	+	+	+	+	-	*Erwinia billingiae*
R2L10	+	-	-	+	-	+	-	-	-	-	-	+	+	-	-	-	-	-	+	-	*Micrococcus luteus*
R2L21	-	+	+	+	-	+	+	-	-	-	-	+	+	+	+	+	+	+	-	-	*Pseudomonas alcaligenes*
R2L22	+	-	+	-	-	+	+	+	+	-	+	+	+	-	+	-	-	-	-	-	*Bacillus simplex*
R2M3	-	-	-	+	+	+	-	-	+	+	-	-	-	+	+	-	-	-	-	-	*Frondihabitans* sp.
R2M10	+	+	-	+	+	+	-	-	-	-	-	+	+	-	+	-	-	-	-	-	*Kocuria palustris*
R2M14	+	-	+	+	+	-	-	+	+	-	+	+	+	-	+	+	-	-	-	-	*Kocuria rhizophila*
R2M15	+	-	-	+	+	+	-	-	-	-	-	+	+	-	+	+	-	+	-	-	*Sphingomonas* sp.
R2M19	-	+	+	+	+	+	+	-	+	+	+	+	+	-	+	+	-	+	-	-	*Arthrobacter rhombi*
R2M20	+	+	-	+	+	+	-	-	+	-	-	+	+	-	+	-	-	+	-	-	*Frondihabitans sucicola*
R2M24	+	-	-	-	-	-	+	+	+	-	+	-	-	+	+	-	-	+	-	-	*Staphylococcus pasteuri*
R3L1	+	-	+	-	-	-	+	-	+	+	+	+	+	-	+	-	-	-	-	-	*Stenotrophomonas rhizophila*
R3L2	+	+	-	+	+	+	+	-	-	-	-	+	+	-	+	+	-	+	-	-	*Methylobacterium* sp.
R3L7	+	+	-	+	+	+	+	-	-	-	+	+	+	-	-	-	-	-	-	-	*Methylobacterium* sp.
R3L17	+	+	-	+	+	+	-	+	-	-	+	+	+	-	+	-	-	-	-	-	*Sphingomonas* sp.
R3L21	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	-	-	-	-	*Bacillus subtilis*
R3L22	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	-	-	-	-	*B. subtilis*
R3L24	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	-	-	-	-	*B. subtilis*
R3L28	-	+	-	+	+	+	+	+	-	-	-	+	+	-	+	-	-	-	-	-	*Methylobacterium* sp.

Table 15. Continued.

STRAIN	Pigments production	Polysaccharase					Lipase		Protease		Nuclease	Nutrient supplying				Growth stimulation		Biofilm formation			Nearest bacterial species
		Amylase	Cellulase	Xylanase	Pectinase	Chitinase	Tween 20	Tween 80	Caseinase	Gelatinase	DNAse	Nitrogen		Phosphate solubilization	Siderophores production	IAA	ACC-deaminase	Biofilms	Motility		
												ABGM-N	ABG-N						Swimming	Swarming	
R3L30	+	-	-	+	+	+	+	+	-	-	+	+	+	-	+	-	-	-	-	-	*Mycobacterium hodleri*
R3M1	+	+	+	+	-	+	+	-	+	+	+	+	+	-	+	-	-	-	-	-	*Streptomyces anulatus*
R3M3	+	-	+	+	+	+	-	-	+	+	-	+	+	-	+	-	-	-	-	-	*C. flaccumfaciens*
R3M6	-	-	-	-	-	-	-	-	+	+	-	-	-	+	+	-	-	+	-	-	*Staphylococcus* sp.
R3M9	+	-	+	+	+	+	-	-	+	+	-	+	+	-	+	-	-	-	-	-	*C. flaccumfaciens*
R3M16	+	+	-	+	+	+	-	-	-	-	-	+	+	-	+	-	-	-	-	-	*M. hodleri*
R3M19	+	-	+	-	-	-	+	+	+	+	-	+	+	-	+	+	-	+	-	-	*Stenotrophomonas* sp.
R3M22	+	+	+	+	+	+	+	+	-	-	+	+	+	-	+	-	-	-	-	-	*Kocuria rosea*
R3M31	+	-	+	+	+	+	-	+	+	+	-	+	+	-	+	-	-	-	-	-	*C. flaccumfaciens*
R3M34	+	-	+	+	+	+	-	-	+	+	-	+	+	-	+	-	-	-	-	-	*Curtobacterium* sp.
R3M35	+	-	+	+	+	+	-	+	+	+	-	+	+	-	+	-	-	-	-	-	*Curtobacterium* sp.
R3M36	+	-	+	+	+	+	-	+	+	+	-	+	+	-	+	-	-	-	-	-	*Curtobacterium* sp.
R4L7	+	+	-	-	-	+	+	+	+	+	-	+	+	+	+	+	+	-	-	-	*Pseudomonas fluorescens*
R4L12	+	+	+	+	+	+	-	-	-	+	+	+	+	-	+	-	-	-	-	-	*Burkholderia* sp.
R4L13	+	-	-	-	-	+	+	+	+	+	-	+	+	+	+	-	-	-	-	-	*Pseudomonas* sp.
R4L27	-	+	+	+	+	+	+	-	-	+	-	+	+	-	+	-	-	-	-	-	*Streptomyces puniceus*
R4L31	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	-	+	-	-	*Leifsonia poae*
R4L34	-	+	+	+	+	+	+	-	+	+	+	+	+	-	+	-	-	-	-	-	*Pantoea agglomerans*
R4L39	+	+	+	+	+	+	+	+	+	-	+	+	+	-	+	-	-	-	-	-	*L. poae*
R4L40	+	+	+	+	+	+	+	-	-	+	-	+	+	+	+	+	-	+	-	-	*Streptomyces* sp.
R4M3	-	+	+	-	+	+	+	-	-	+	-	+	+	+	+	-	-	+	-	-	*Streptomyces coeruleofuscus*
R4M5	-	+	-	+	+	+	+	-	-	-	-	+	+	+	+	+	-	+	+	-	*Burkholderia* sp.
R4M11	-	-	-	+	+	+	+	-	-	-	+	+	+	+	+	+	-	+	+	-	*L. poae*
R4M17	+	-	-	+	+	+	+	-	-	-	+	+	+	+	+	-	-	-	-	-	*L. poae*
R4M23	+	-	+	+	+	+	-	-	+	+	+	+	+	+	+	+	-	+	-	-	*C. flaccumfaciens*
R4M25	+	-	-	+	+	+	+	-	-	-	+	+	+	+	+	-	-	-	-	-	*L. poae*
R4M31	-	-	-	+	+	+	+	-	-	-	+	+	+	+	+	+	-	+	-	-	*Nocardioides* sp.
R4M33	+	+	-	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-	-	-	*Burkholderia* sp.
R4M34	-	+	-	+	+	+	+	-	-	+	+	+	+	-	+	-	-	-	-	-	*L. poae*

a: Bacterial strains from *R. farinacea* thalli samples from La Guancha (R1), La Esperanza (R2), El Toro (R3) and Lidón (R4). L: ectolichenic bacteria, M: endolichenic bacteria.

b:-, negative activity; +, positive activity

After the alignment of the sequences, a phylogenetic study of the bacterial strains of *R. farinacea* was made. A selection of the nearest bacterial species, obtained through the comparison by BLAST, were included and represented in a phylogenetic tree following the method of Maximum Likelihood with the MEGA6 program. In figure 35 is represented this phylogenetic tree.

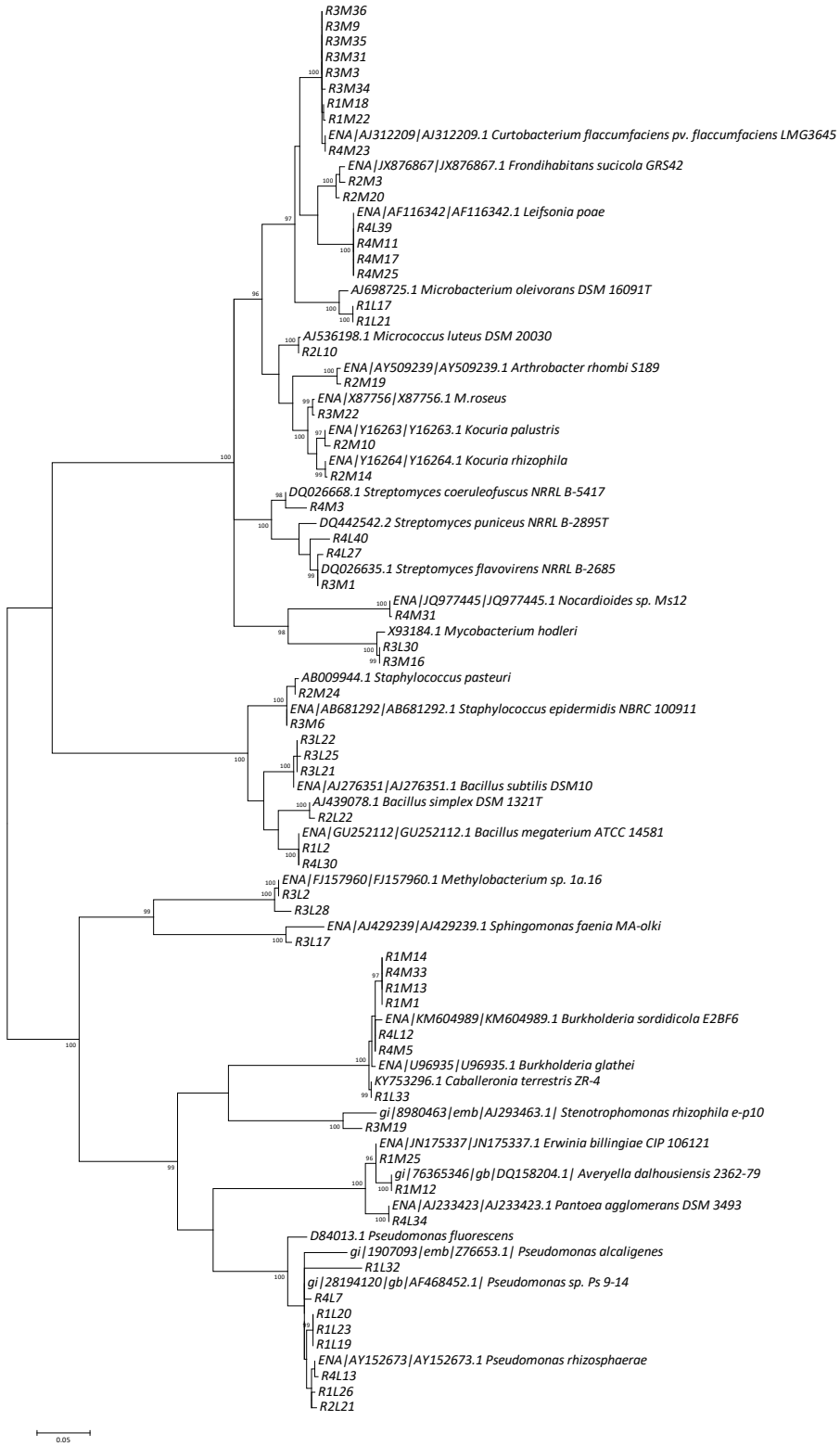


Figure 35. Phylogenetic analyses based on the partial sequence of the *16S rRNA* gene of culturable bacteria associated with the *R. farinacea* populations from the four geographical locations in Spain studied. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. R1: bacterial strains from La Guanha. R2: bacterial strains from La Esperanza. R3: bacterial strains from El Toro. R4: bacterial strains from Lidón. L: ectolichenic bacteria. M: endolichenic bacteria. The percentage of replicate trees in which the associated taxa clustered together is shown next to the branches, with 1000 of bootstrap replications. The sequences of some reference type strains were included in the tree.

8. DIVERSITY AND COMPOSITION ANALYSES OF CULTURABLE BACTERIA ASSOCIATED *WITH R. farinacea* THROUGH CONVENTIONAL *16S rRNA* GENE SEQUENCING

The study of the diversity of culturable bacteria associated with populations of the lichen *R. farinacea* from four geographical locations in Spain, two from the Island of Tenerife (La Guancha and La Esperanza), and two from the Iberian Peninsula (El Toro and Lidón) was carried out with a total number of 286 bacterial strains. Figure 36 shows a representative Venn diagram illustrating the OTUs overlapping among the bacterial strains isolated from the samples of *R. farinacea* from the four Spanish locations at a threshold of 0.005. Some of these OTUs were ubiquitous for all the geographical locations analyzed, while others were only present in some of them.

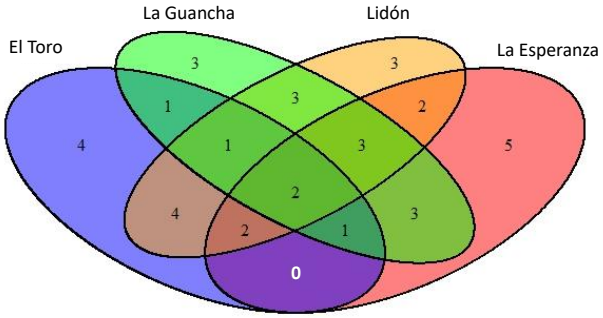


Figure 36. Venn diagram showing the OTUs shared among the culturable bacterial strains associated with *R. farinacea* populations from different geographical locations in the Canary island of Tenerife (La Guancha and La Esperanza) and the Iberian Peninsula (El Toro and Lidón).

8.1 BETA DIVERSITY

The analysis of the beta diversity (changes in species composition among different geographic areas (Anderson *et al*., 2011)) revealed that the structure of the culturable bacterial community associated with *R. farinacea* was mainly determined by the geographical factor, either by the two main areas, the Island and the Peninsula (PERMANOVA, R2=0.29, p<0.01) and by the four Spanish locations where *R. farinacea* thalli samples were collected (PERMANOVA, R2=0.65, p<0.01). The ectolichenic or endolichenic location of bacteria in the thallus had less influence than the geographical location (PERMANOVA, R2=0.17, p<0.05). These factors explained the 29%, 65%, and 17% of the composition of the diversity of the culturable bacterial communities, respectively, which were well differentiated among them, in the Island and the Peninsula as well as in the four geographical locations, as shown in figure 37a and b. By contrast, bacterial communities in the ecto- and endolichenic fractions were less differentiated, as shown in figure 37c, with both overlapping communities in some areas.

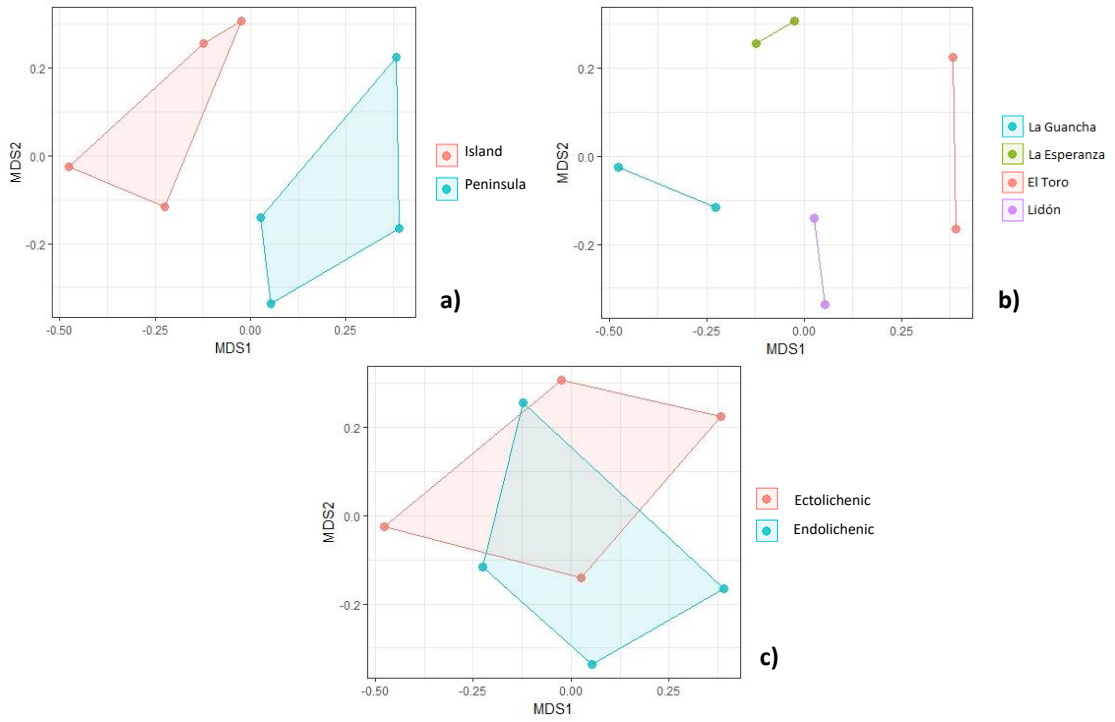


Figure 37. Multidimensional scaling (MDS) plot of the Bray–Curtis based dissimilarity matrix of culturable bacterial strains associated with populations of the lichen *R. farinacea* from the two main sampling areas, the Canary Island (Tenerife) and Iberian Peninsula (**a**) and from the four different sampling geographical locations in Spain (La Guancha and La Esperanza in Tenerife, and El Toro and Lidón in the Peninsula) (**b**), as well as according to their ectolichenic or endolichenic position in the thallus (**c**).

8.2 ALPHA DIVERSITY

8.2.1 Influence of geographical location

The study of the alpha diversity (diversity at local scale), showed that the values of the Richness, Shannon and Simpson diversity indices in the Island and the Peninsula were similar. Richness index values were similar, although with more bacterial species in the Island than in the Peninsula. By contrast, Shannon diversity index showed higher values in the Island than in the Peninsula, meaning that the insular bacterial species were represented by a more similar number of individuals. Furthermore, Simpson diversity index values were high and close to 1, although slightly higher in the Island than in the Peninsula, which indicated that a relatively similar number of individuals was assigned to each bacterial species. Differences among both indices and areas were not significant ($p>0.05$) (Figure 38).

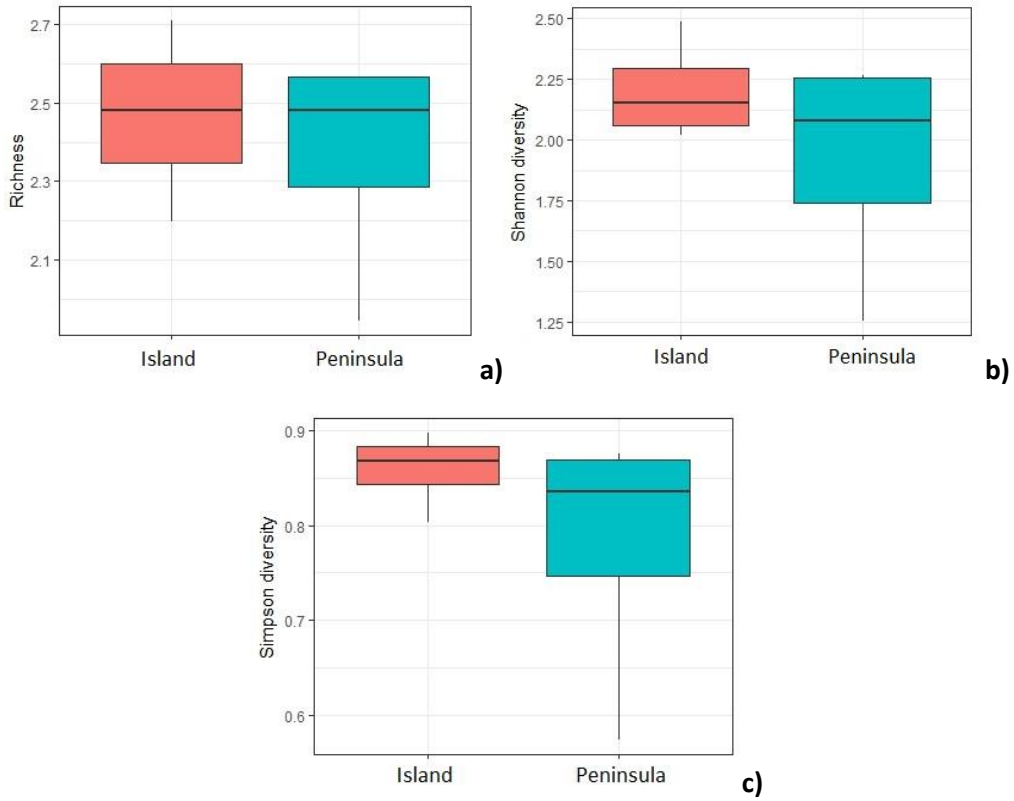


Figure 38. Box plots showing the diversity indices of the culturable bacterial strains associated with *R. farinacea* populations from the two main sampling areas, the Island and the Peninsula. Richness (**a**), Shannon (**b**) and Simpson (**c**) diversity indices.

The study of the alpha diversity of the culturable bacteria associated with the populations of *R. farinacea* from the four geographical locations where thalli were collected also showed differences among them (Figure 39). Richness index showed that the *R. farinacea* populations from the location of Lidón was the one with the highest diversity values, meaning that the number of different bacterial species found in this location was higher than the ones present in the other locations. Similar results were obtained with the Shannon index, meaning that the bacterial species from Lidón were numerous and quite evenly represented. The bacterial strains from the *R. farinacea* population from La Guancha showed higher Richness values than the ones from La Esperanza and El Toro. The latest was the location from where *R. farinacea* presented the lowest number of bacterial species and they were not as equally represented in the other three locations. Shannon diversity index revealed that *R. farinacea* thalli from the locations of La Esperanza and Lidón where the ones with most diverse bacteria, followed by those from the location of La Guancha, being the thalli from El Toro the ones with the lowest bacterial diversity, as shown by the values reached by this index, being around 2 (of a maximum of 5) in all cases, indicating that a relatively representative number of species were recovered from these lichen populations. Besides, Simpson index showed that all these bacterial species were composed of a quite equal number of individuals. Only in the cases of La Esperanza, the value of this index was slightly lower, meaning that some species were more represented than others. The differences observed among the indices and the four geographical locations were not significant ($p > 0.05$).

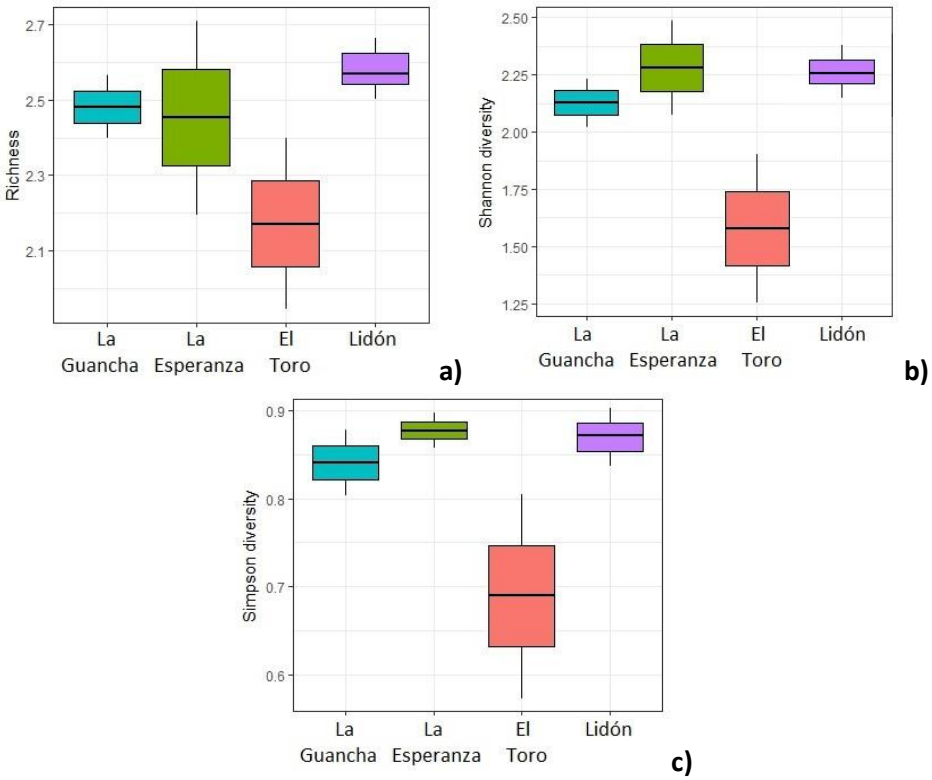


Figure 39. Box plots showing the diversity indices of the culturable bacterial strains associated with *R. farinacea* thalli from the four Spanish locations, two from the Canary Island of Tenerife (La Guancha and La Esperanza) and two located at the Iberian Peninsula (El Toro and Lidón). Richness (**a**), Shannon (**b**) and Simpson (**c**) diversity indices.

All bacterial strains analyzed through the partial amplification of the *16S rRNA* gene were presumptively assigned to 37 different taxa belonging to 3 main phyla, being *Proteobacteria* the predominant one, followed by *Actinobacteria* and *Firmicutes.*

The results obtained when grouping the bacterial strains into two main groups attending at their insular or peninsular origin (Figure 40a), showed that at phylum level, *Proteobacteria* was the main group, with a higher presence in the Island (65.98%) than in the Peninsula (47.01%), followed by the *Actinobacteria*, more abundant in the Peninsula (46.68%) than in the Island (23.65%). *Firmicutes* was the group with less representation in both areas (10.6% in the Island, 6.32% in the Peninsula) (Figure 40a). The representation of the classes belonging to *Proteobacteria* was different in the Island with respect to the Peninsula, where the main groups were *Gammaproteobacteria* (35.65%) and *Alphaproteobacteria* (30.57%), respectively. In the Island, the predominant orders were *Enterobacteriales* (21.73%), *Micrococcales* (20.03%), *Pseudomonadales* (13.11%), *Burkholderiales* (11.47%) and *Sphingomonadales* (10.99%), while in the Peninsula, the most abundant ones were *Rhizobiales* (26.53%), *Micrococcales* (25.95%), *Propionibacteriales* (10.09%), *Enterobacteriales* (6.87%), *Streptomycetales* (6.47%) and *Burkholderiales* (6.32%). With regard to the families (Figure 40b), the Island was represented mostly by *Microbacteriaceae* (15.17%), *Enterobacteriaceae* (11.76%), *Sphingomonadaceae* (10.99%), *Burkholderiaceae* (10.54%) and *Erwiniaceae* (9.97%), while in the Peninsula by *Methylobacteriaceae* (26.53%), *Microbacteriaceae* (23.50%), *Streptomycetaceae* (6.48%), *Erwiniaceae* (5.74%), *Bacillaceae* (5.68%) and *Burkholderiaceae*

(5.30%). No significant differences were observed in the different bacterial taxa levels when bacterial strains from the Island were compared with those from the Peninsula.

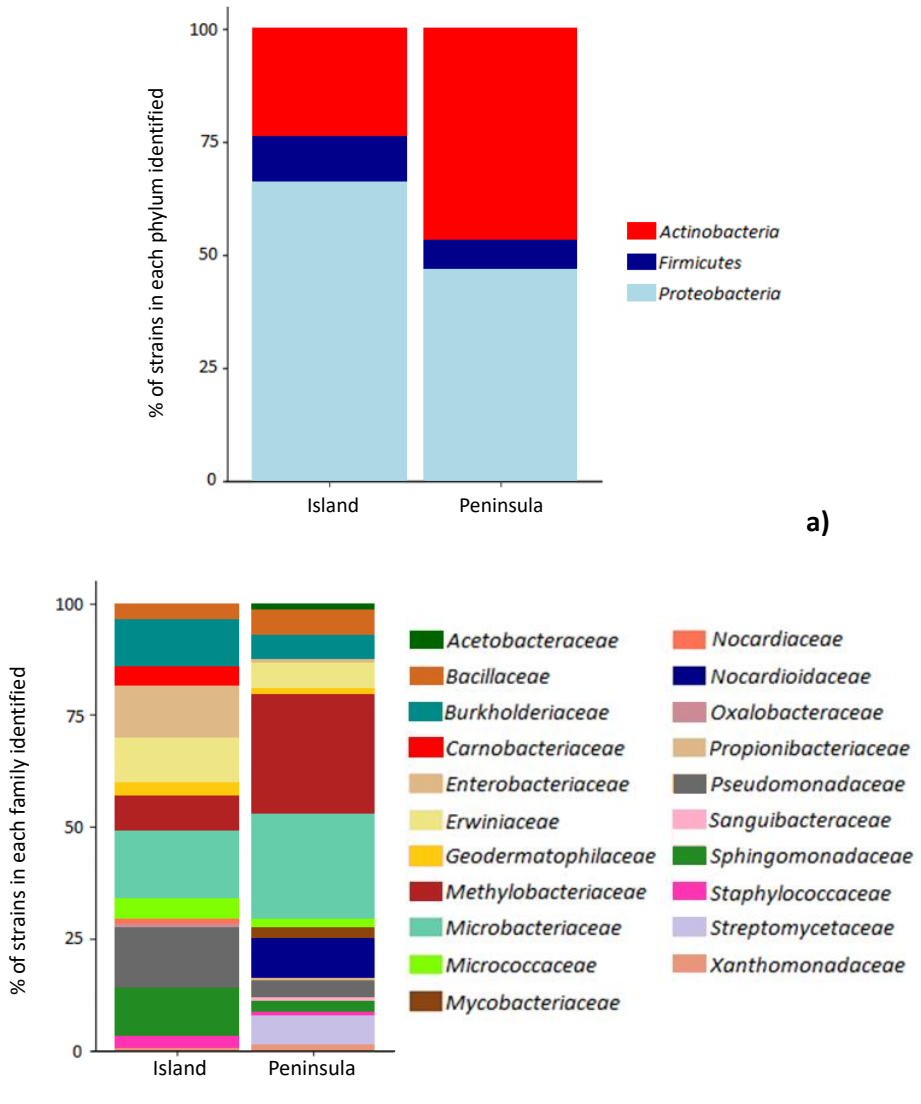


Figure 40. Taxonomical identification at phylum (**a**) and family (**b**) level of the culturable bacterial strains associated with *R. farinacea*. The percentages of strains assigned to each phylum and family are represented attending the main sampling areas, the Island and the Peninsula.

The results of the taxonomical identification at phylum level when bacterial strains were grouped according to each one of the four geographical origins are shown in figure 41. In the case of bacterial strains from *R. farinacea* populations from La Guancha, they were assigned mostly to the *Proteobacteria* (75.67%), followed by the *Actinobacteria* (12.16%), and the *Firmicutes* (9.46%). Regarding those of La Esperanza, again *Proteobacteria* was the main group among the identified bacteria, with the 54.54% of the strains. *Actinobacteria,* with a 30.91%, had more abundance compared with the previous location and *Firmicutes,* in minor proportion, with a 7.27% of the strains. Unclassified bacteria were detected in strains from *R. farinacea* thalli from La Guancha and La Esperanza (2.70% and 7.27% of the strains, respectively). In the case of the samples collected from the *R. farinacea* population of El Toro, the differences between the *Actinobacteria* and *Proteobacteria* were less notable (49.29% of *Proteobacteria* and 40.84% of *Actinobacteria*). The proportion of *Firmicutes* was similar to that

of the locations of the Island (9.86%). In the *R. farinacea* population from Lidón, the main group of bacteria were assigned to the *Actinobacteria* with a 54.65%, followed by *Proteobacteria* with a 43.02% of the strains. The phylum *Firmicutes* was underrepresented in Lidón with a 2.32% of the identified bacteria (Figure 41).

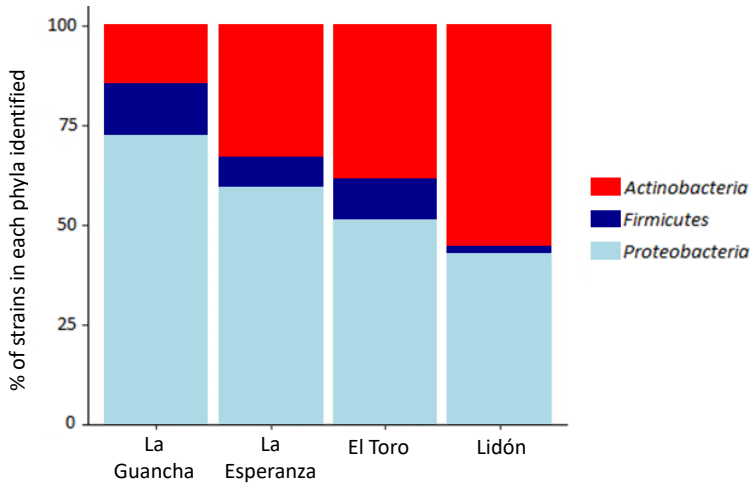


Figure 41. Taxonomical identification of the culturable bacterial strains associated with *R. farinacea* at phylum level. The percentages of strains assigned to each phylum are represented for each one of the four geographical locations in Spain.

The classes to which the isolated bacteria were assigned were *Actinobacteria* (12.16%), *Alphaproteobacteria* (22.03%), *Bacilli* (9.46%), *Betaproteobacteria* (7.69%) and *Gammaproteobacteria* (25.52%). In the case of the bacterial strains isolated from thalli from La Guancha, the main group was *Gammaproteobacteria* (63.51%) and then *Actinobacteria* (12.16%), *Betaproteobacteria* (10.81%) and *Bacilli* (9.46%). In the lichen populations of La Esperanza and El Toro, bacteria were assigned mostly to the *Alphaproteobacteria* (32.73% and 46.48%, respectively). *Betaproteobacteria* and *Gammaproteobacteria* numbers were very similar in La Esperanza (9.10% and 12.73%, respectively). In El Toro, *Bacilli* were represented in a 9.86% and the number of *Gammaproteobacteria* detected was the lowest among the four populations with a 2.82%. None *Betaproteobacteria* was identified among the isolated bacteria in this population. In the case of Lidón, the main group was *Actinobacteria* (54.64%), followed by *Gammaproteobacteria* (19.76%), *Alphaproteobacteria* (12.79%) and *Betaproteobacteria* (10.46%).

The main orders within the bacteria isolated from *R. farinacea* were *Micrococcales* (22.73%), *Enterobacteriales* (16.08%), *Rhizobiales* (16.08%), *Pseudomonadales* (8.39%), *Burkholderiales* (7.7%) and, in minor proportions, *Sphingomonadales*, *Propionibacteriales* and *Bacillales*.

The most representative bacterial families found in this study (Figure 42) were *Microbacteriaceae* (19.58%), *Methylobacteriaceae* (16.08%), *Erwiniaceae* (9.44%), *Pseudomonadaceae* (8.39%), *Burkholderiaceae* (7.34%), *Enterobacteriaceae* (6.64%), *Sphingomonadaceae* (5.24%) and, in less proportion, *Bacillaceae*, *Nocardioidaceae* and *Streptomycetaceae*, among others. These families were differently represented within the four lichen populations. The one from La Guancha was mainly represented by the families *Erwiniaceae* (21.62%), *Pseudomonadaceae* (21.62%) and *Enterobacteriaceae* (14.54%). In the case of La Esperanza, the main groups were *Sphingomonadaceae* (18.18%), *Microbacteriaceae*

(16.36%) and *Methylobacteriaceae* (14.54%). Regarding the main groups represented in El Toro, *Methylobacteriaceae* was the predominant one (40.85%), followed by *Microbacteriaceae* (15.50%) and *Nocardioidaceae* (12.67%). In Lidón, the main family groups were *Microbacteriaceae* (32.55%), *Erwiniaceae* (11.63%), *Burkholderiaceae* (10.47%), *Methylobacteriaceae* (10.47%) and *Streptomycetaceae* (10.47%), among others.

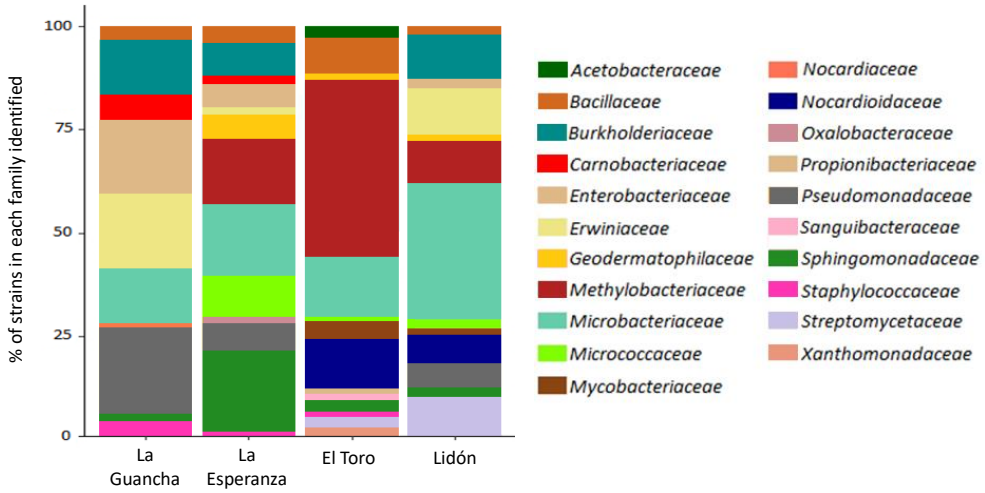


Figure 42. Taxonomical identification at family level of the culturable bacterial strains associated with *R. farinacea*. The percentages of strains assigned to each family are represented for each one of the four geographical locations in Spain.

8.2.2 Influence of location in the lichen thallus

In order to evaluate the potential effect on bacterial diversity of the location in the lichen thallus on bacterial diversity, a study of alpha diversity was performed grouping bacterial strains by their ectolichenic or endolichenic origin. The results showed that, although both ectolichenic and endolichenic fractions had very similar values of Richness and Shannon diversity indices, the composition of the endolichenic fraction had higher values of both indices than the ectolichenic one, revealing a slightly higher number of different species in the inner part of the lichen thallus and more evenly representation of them than those in the outer part (Figure 43). Similarly, Simpson diversity index showed in both cases high index values close to 1, indicating that a relatively equal number of individuals belonged to each one of the bacterial species retrieved (Figure 43). The differences among Richness and Shannon diversity indices and the two bacterial fractions were not significant ($p>0.05$). However, Simpson diversity index results gave significant differences between ectolichenic and endolichenic fractions ($p<0.05$).

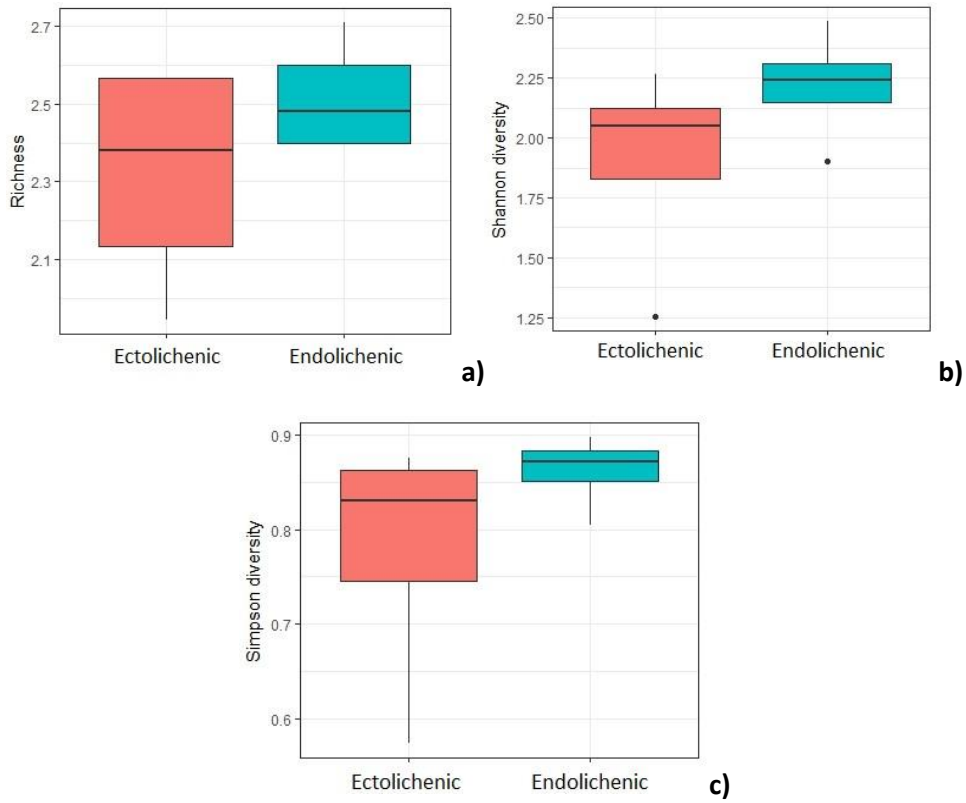


Figure 43. Box plots showing the diversity indices of the culturable bacterial strains associated with *R. farinacea* attending at their location in the lichen thallus, ectolichenic or endolichenic. Richness (**a**), Shannon (**b**) and Simpson (**c**) diversity indices.

Some differences were found within the ectolichenic and endolichenic bacterial strains identified. At phylum level (Figure 44a), in general, *Proteobacteria* was the predominant group, followed by *Actinobacteria* and *Firmicutes.* Nevertheless, the number of *Proteobacteria* strains was higher in the ectolichenic fraction (69.57%) than in the endolichenic one (43.42%), while it was opposite for the *Actinobacteria* with higher percentages for the endolichenic bacteria (48.23%) than for the ectolichenic ones (22.83%). *Firmicutes* was equally represented in ectolichenic and endolichenic bacteria (8.34%). At class level, in the case of the ectolichenic bacteria, *Alphaproteobacteria* and *Gammaproteobacteria* were the predominant groups (32.15% and 31.76%, respectively), with fewer representation of *Betaproteobacteria* (5.66%). Related to the endolichenic bacteria, the percentages of these three classes were lower and very similar among them (17.28%, 15.03% and 11.11%, respectively).

The most representative orders in the ectolichenic bacterial fraction were *Rhizobiales* (24.34%), *Enterobacteriales* (15.26%), *Pseudomonadales* (15.11%), *Micrococcales* (11.72%), *Bacillales* (8.35%) and *Sphingomonadales* (7.81%). While in the endolichenic one they were *Micrococcales* (34.26%), *Enterobacteriales* (13.34%), *Burkholderiales* (11.11%) and *Propionibacteriales* (8.75%). When bacterial strains were assigned at family level (Figure 44b), ectolichenic bacteria were represented mainly by *Methylobacteriaceae* (24.34%), *Pseudomonadaceae* (15.11%), *Erwiniaceae* (9.42%), *Microbacteriaceae* (9.07%), *Sphingomonadaceae* (7.81%) and *Bacillaceae* (7.74%), being the endolichenic ones represented by the families *Microbacteriaceae* (29.6%), *Burkholderiaceae* (10.16%),

Methylobacteriaceae (10.07%), *Nocardioidaceae* (8.75%) and *Erwiniaceae* (6.28%). The differences observed in the different bacterial taxonomic levels between the ectolichenic and the endolichenic fractions were not significant.

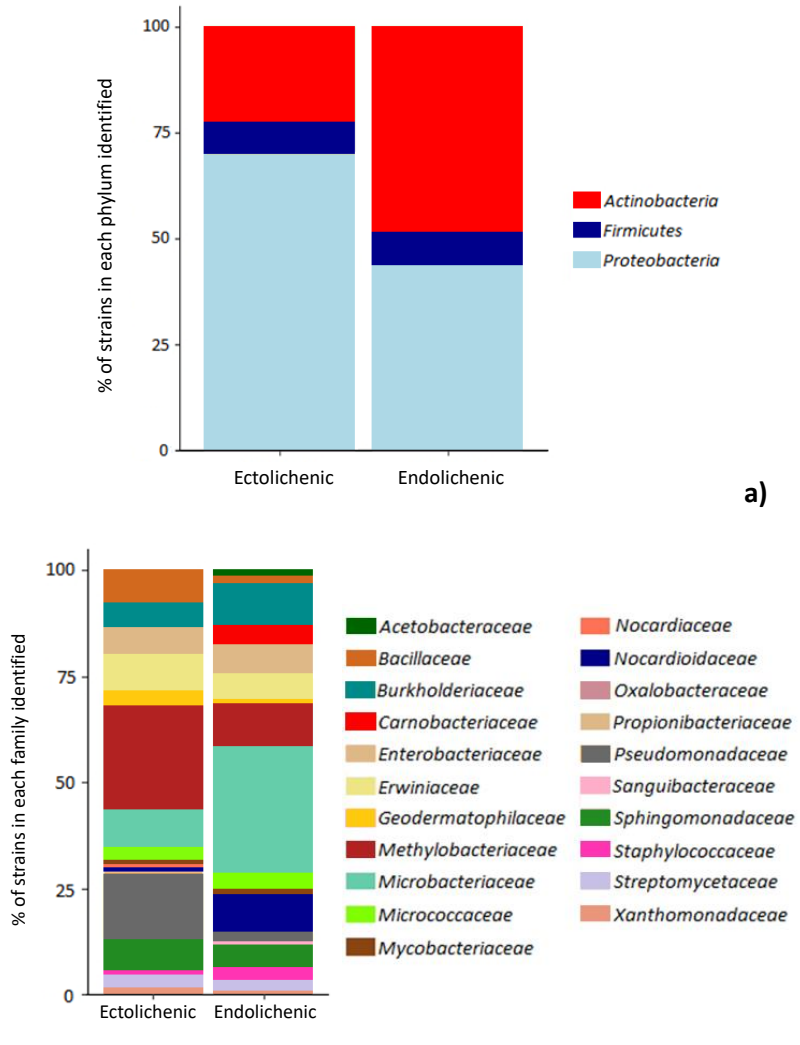


Figure 44. Taxonomical identification at phylum (**a**) and family (**b**) level of the culturable bacterial strains associated with *R. farinacea*. The percentages of strains assigned to each phylum and family are represented attending the location within the lichen thallus, ectolichenic or endolichenic.

The diversity of the studied bacteria was composed of 37 different genera which are summarized in table 16 according to their presence or absence among the different geographical locations where *R. farinacea* populations were sampled and taking into consideration their location in the outer and inner part of the lichen thallus. Among these genera, only two of them seemed to be ubiquitous, since they were identified in the four *R. farinacea* populations studied, *Bacillus* and *Sphingomonas.* Further, different species were potentially assigned to these bacteria (considering a minimum of 98% of similarity in the partial sequence of the *16S rRNA* gene), as *B. frigotolerans, B. campisalis, B. megaterium, B. cereus, B. nealsonii and S. polyaromaticivorans*, among others. Other bacterial taxa were present in three of the four populations, and included species of the genera *Burkholderia* and *Erwinia* (absent in El Toro), *Curtobacterium* (absent in La Esperanza), *Kocuria* and *Methylobacterium* (absent in La Guancha). Furthermore, some of them were exclusive of one lichen population. Related to this, in the *R. farinacea* population from La Guancha some unique

genera detected were *Averyella, Enterobacter* and *Rhodococcus.* In La Esperanza, *Arthrobacter, Frondicola, Massilia, Micrococcus, Modestobacter* and *Paraburkholderia*. With regard to El Toro, four genera were only detected there, *Microlunatus, Roseomonas, Sanguibacter* and *Xanthomonas.* In the lichen population of Lidón, those unique genera were *Gibbsiella, Nocardioides* and *Subtercola*. Some interesting differences in the distribution of these genera were found. It was due to the fact that some of them were present only in the two locations from the Island, while others were located only in those from the Peninsula. This is the case of the genera *Arthrobacter, Averyella, Carnobacterium, Enterobacter, Frondicola, Frondihabitans, Klebsiella, Massilia, Micrococcus, Microlunatus, Modestobacter, Paraburkholderia* and *Rhodococcus,* identified from lichen population from the Island. Furthermore, in the lichen populations of La Guancha and La Esperanza, there were some bacteria not assigned to any of the taxonomic groups present on the database of the GenBank in the NCBI when the BLAST was performed, that could be indicative of new species. In the case of the lichen populations from the Peninsula, the bacterial genera found were *Blastococcus, Friedmaniella, Gibbsiella, Microlunatus, Mycobacterium, Nocardioides, Roseomonas, Sanguibacter, Streptomyces, Subtercola* and *Xanthomonas.*

Interestingly, when the ectolichenic and endolichenic bacterial fractions were compared some differences were detected related to presence or absence of some genera. In this sense, the genera found in the ectolichenic fraction of the lichen thallus were *Averyella, Blastococcus, Gibbsiella, Micrococcus, Microlunatus, Rhodococcus* and *Stenotrophomonas,* while in the endolichenic one, some bacterial strains were assigned to the genera *Arthrobacter, Frondicola, Kocuria, Massilia, Nocardioides, Roseomonas, Sanguibacter, Subtercola* and *Xanthomonas.* Other genera were present in both lichenic fractions, such as *Bacillus, Burkholderia, Caballeronia, Carnobacterium, Curtobacterium, Erwinia, Frondihabitans, Klebsiella, Leifsonia, Methylobacterium, Microbacterium, Modestobacter, Mycobacterium, Pantoea, Paraburkholderia, Pseudomonas, Sphingomonas* and *Staphylococcus.*

The differences observed among the four populations of *R. farinacea* at the different bacterial taxonomic levels were not significant.

Table 16. Classification of bacterial strains isolated from populations of *R. farinacea* sampled in four Spanish geographical locations, according to the nearest taxa at genus level. The presence or absence of these genera in each geographical location and ectolichenic or endolichenic position in the thallus is also provided.

Genus (Phylum)	Geographical origin							
	Tenerife				Iberian Peninsula			
	La Guancha		La Esperanza		El Toro		Lidón	
	EctoL[a]	EndoL[a]	EctoL	EndoL	EctoL	EndoL	EctoL	EndoL
Arthrobacter (Actinobacteria)	-[b]	-	-	+	-	-	-	-
Averyella (γ-Proteobacteria)	+[b]	-	-	-	-	-	-	-
Bacillus (Firmicutes)	+	+	+	-	+	+	+	-
Blastococcus (Actinobacteria)	-	-	-	-	+	-	+	-
Burkholderia (β-Proteobacteria)	+	+	-	+	-	-	+	+
Caballeronia (β-Proteobacteria)	+	-	-	-	-	-	-	+
Carnobacterium (Firmicutes)	+	-	-	+	-	-	-	-
Curtobacterium (Actinobacteria)	-	+	-	-	-	+	+	+
Enterobacter (γ-Proteobacteria)	+	+	-	-	-	-	-	-
Erwinia (γ-Proteobacteria)	+	+	-	+	-	-	+	-
Friedmaniella (Actinobacteria)	-	-	-	-	-	+	+	-
Frondicola (Actinobacteria)	-	-	-	+	-	-	-	-
Frondihabitans (Actinobacteria)	+	-	+	+	-	-	-	-
Gibbsiella (γ-Proteobacteria)	-	-	-	-	-	-	+	-
Klebsiella (γ-Proteobacteria)	+	+	+	+	-	-	-	-
Kocuria (Actinobacteria)	-	-	-	+	-	+	-	+
Leifsonia (Actinobacteria)	-	-	-	+	-	-	+	+
Massilia (β-Proteobacteria)	-	-	-	+	-	-	-	-
Methylobacterium (α-Proteobacteria)	-	-	+	+	+	+	+	+
Microbacterium (Actinobacteria)	+	-	-	-	-	-	+	+
Micrococcus (Actinobacteria)	-	-	+	-	-	-	-	-
Microlunatus (Actinobacteria)	-	-	-	-	+	-	-	-
Modestobater (Actinobacteria)	-	-	+	+	-	-	-	-
Mycobacterium (Actinobacteria)	-	-	-	-	+	+	-	+
Nocardioides (Actinobacteria)	-	-	-	-	-	-	-	+
Pantoea (γ-Proteobacteria)	+	+	-	-	-	-	+	+
Paraburkholderia (β-Proteobacteria)	-	-	+	+	-	-	-	-
Pseudomonas (γ-Proteobacteria)	+	+	+	-	-	-	+	-
Rhodococcus (Actinobacteria)	+	-	-	-	-	-	-	-
Roseomonas (α-Proteobacteria)	-	-	-	-	-	+	-	-
Sanguibacter (Actinobacteria)	-	-	-	-	-	+	-	-
Sphingomonas (α-Proteobacteria)	-	+	+	+	+	-	-	+
Staphylococcus (Firmicutes)	+	+	-	+	-	+	-	-
Stenotrophomonas (γ-Proteobacteria)	+	-	-	-	+	-	-	-
Streptomyces (Actinobacteria)	-	-	-	-	-	+	+	+
Subtercola (Actinobacteria)	-	-	-	-	-	-	-	+
Xanthomonas (γ-Proteobacteria)	-	-	-	-	-	+	-	-
Unclassified	+	+	+	+	-	-	-	-

[a] EctoL (ectolichenic); EndoL (endolichenic). [b] +, presence; -, absence.

A phylogenetic study of the bacterial strains isolated from each *R. farinacea* population from each one of the four geographical origins was performed, as well as a global analysis including all bacterial strains, regardless their geographical origin. The resulting phylogenetic trees are shown in figures 45, 46, 47 and 48 for bacterial strains isolated from each one of the four lichen populations, and in figure 49 for all bacterial strains, regardless their geographical or lichenic origin. Trees are divided in colours by families. Some of the closest species to which the bacterial strains were assigned were *Bacillus megaterium, B. subtilis, Curtobacterium citreum, C. flaccumfaciens, Friedmaniella spumicola, Frondihabitas cladoniiphilus, F. sucicola, Klebsiella aerogenes, Leifsonia poae, Methylobacterium cerastii, M. phyllostachios, Microbacterium oxydans, M. pygmaeum, Modestobacter versicolor, Pantoea agglomerans, Pseudomonas fluorescens, Roseomonas aerilata* and *Subtercola boreus* among others.

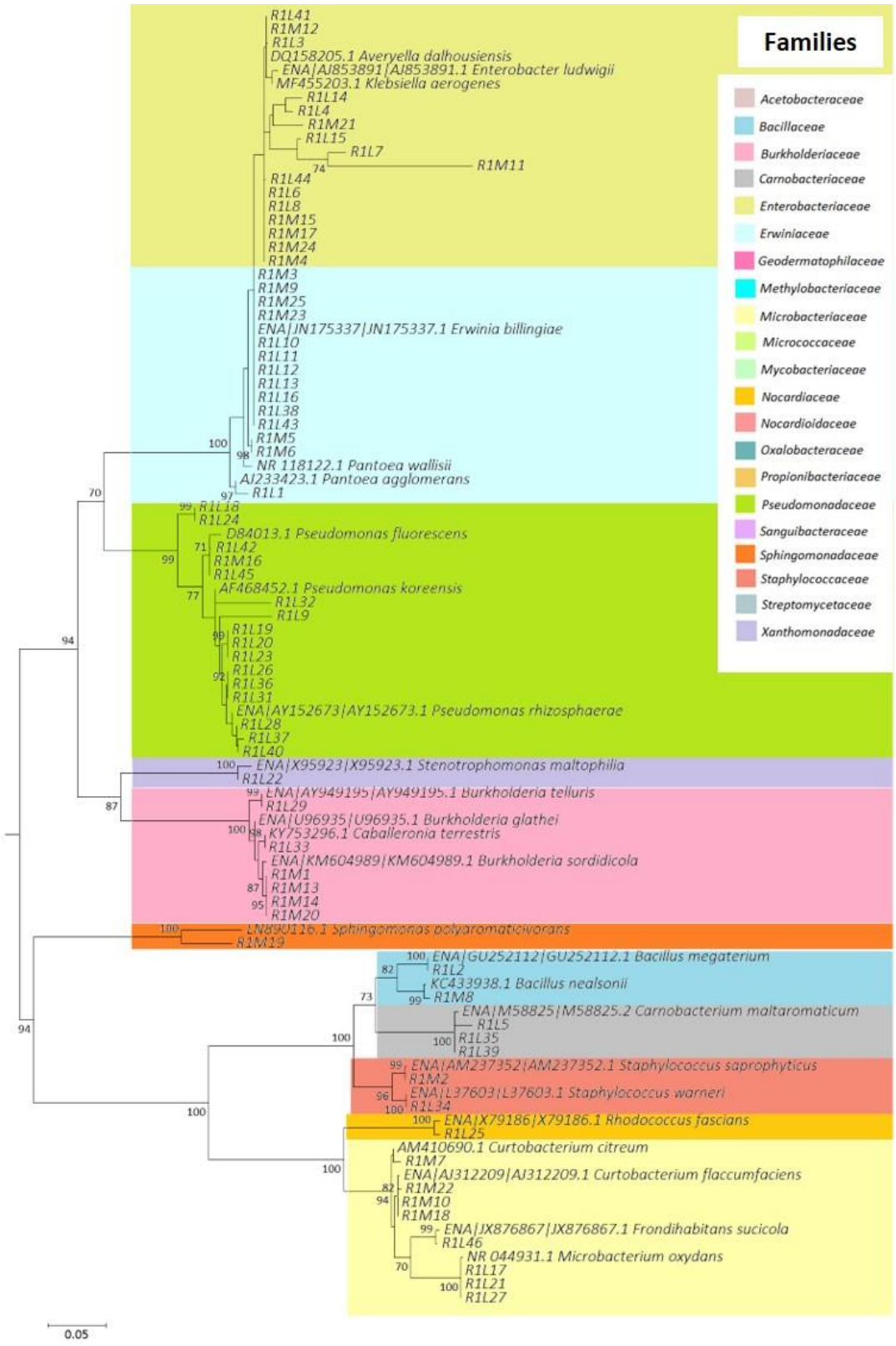


Figure 45. Phylogenetic tree based on the partial sequence of the *16S rRNA* gene of culturable bacterial strains isolated from the *R. farinacea* population from La Guancha (Tenerife). The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model. Each colour represents a different family. R1: bacterial strains from La Guancha. L: ectolichenic bacteria. M: endolichenic bacteria. The percentage of replicate trees in which the associated taxa clustered together is shown next to the branches, with 1000 of bootstrap replications. Some reference type strains were included in the phylogenetic tree.

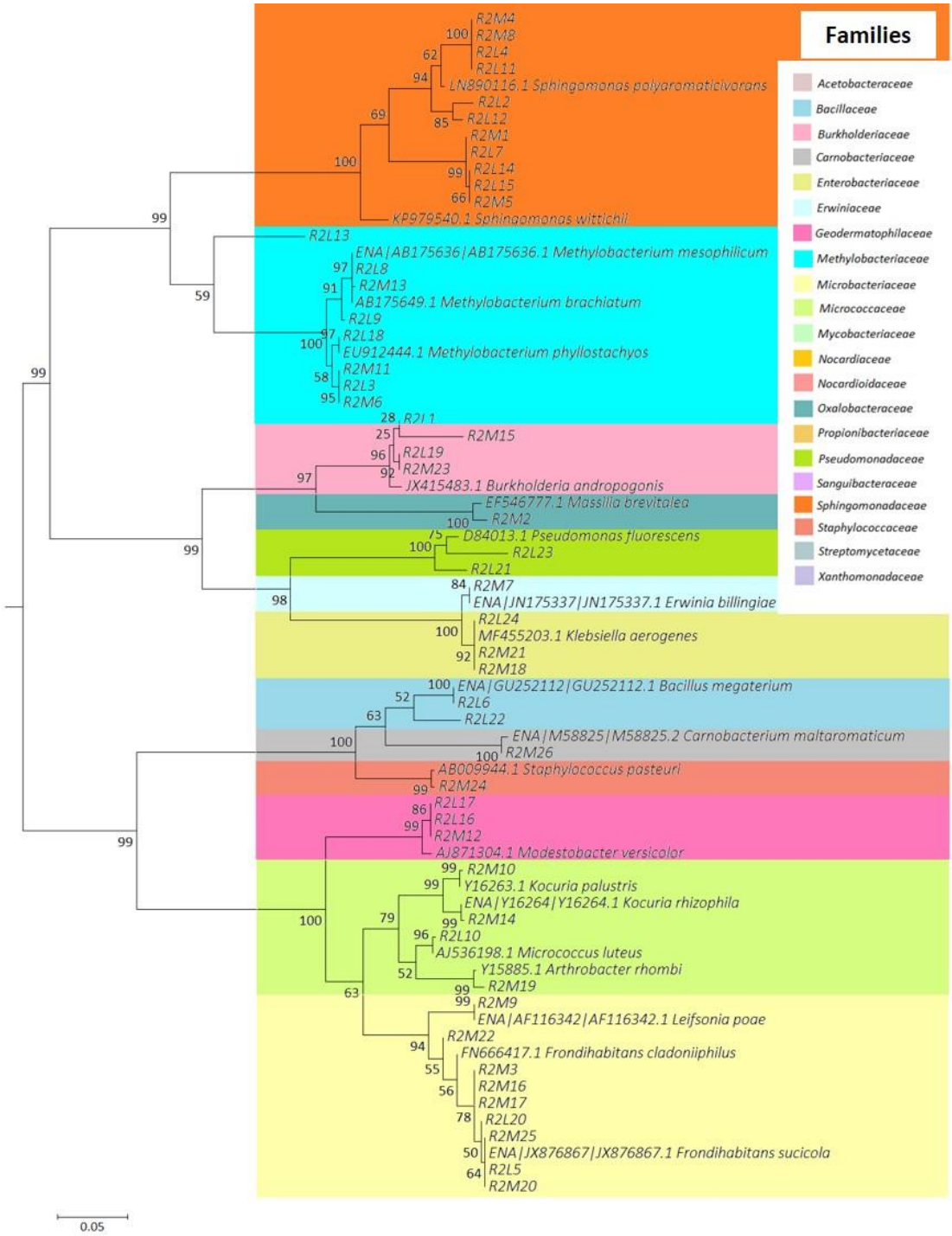


Figure 46. Phylogenetic tree based on the partial sequence of the *16S rRNA* gene of culturable bacterial strains isolated from the *R. farinacea* population from La Esperanza (Tenerife). The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura 3-parameter model. Each colour represents a different family. R2: bacterial strains from La Esperanza. L: ectolichenic bacteria. M: endolichenic bacteria. The percentage of replicate trees in which the associated taxa clustered together is shown next to the branches, with 1000 of bootstrap replications. Some reference type strains were included in the phylogenetic tree.

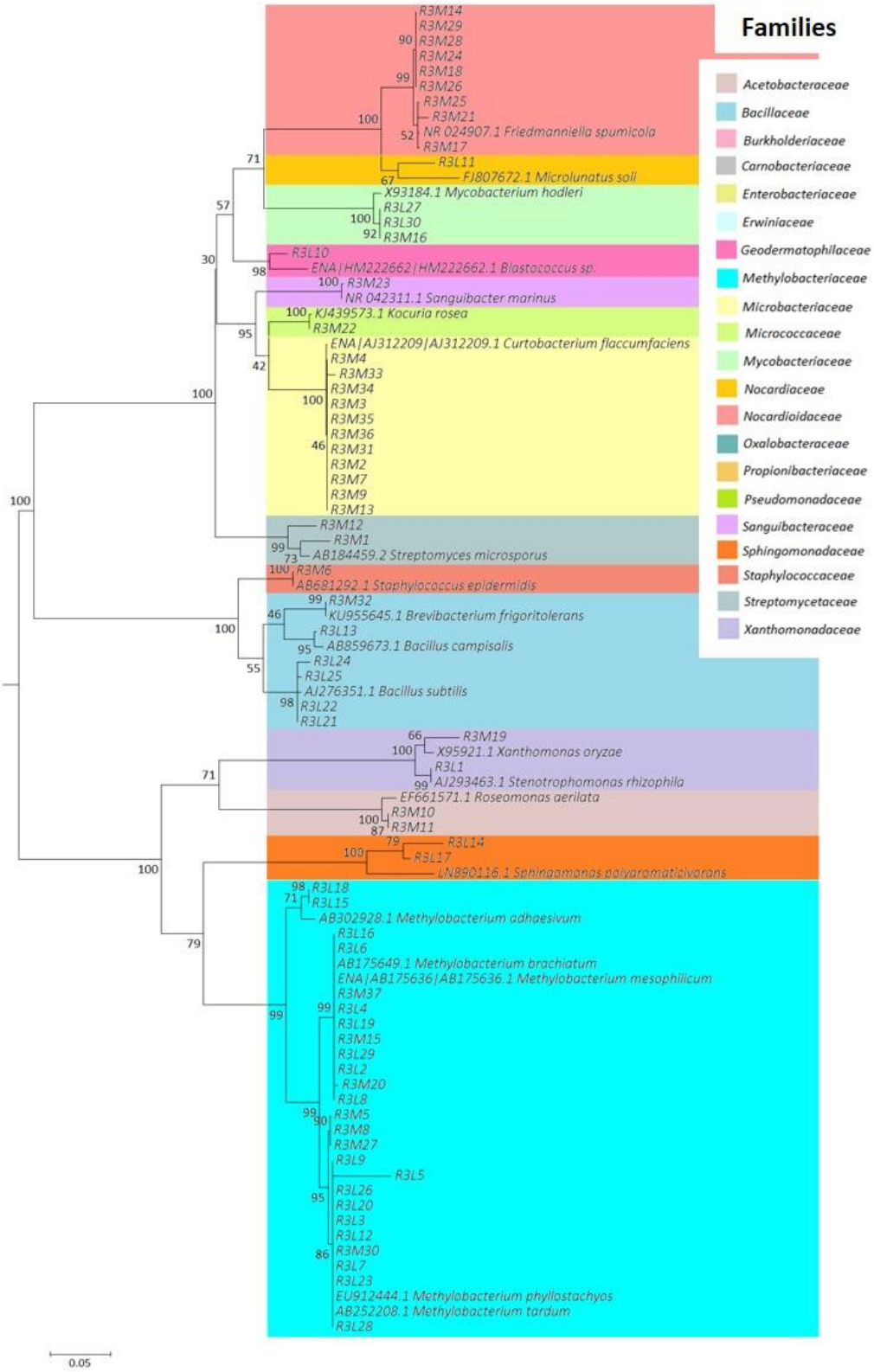


Figure 47. Phylogenetic tree based on the partial sequence of the *16S rRNA* gene of culturable bacterial strains isolated from the *R. farinacea* population from El Toro (Peninsula). The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura 3-parameter model. Each colour represents a different family. R3: bacterial strains El Toro. L: ectolichenic bacteria. M: endolichenic bacteria. The percentage of replicate trees in which the associated taxa clustered together is shown next to the branches, with 1000 of bootstrap replications. Some reference type strains were included in the phylogenetic tree.

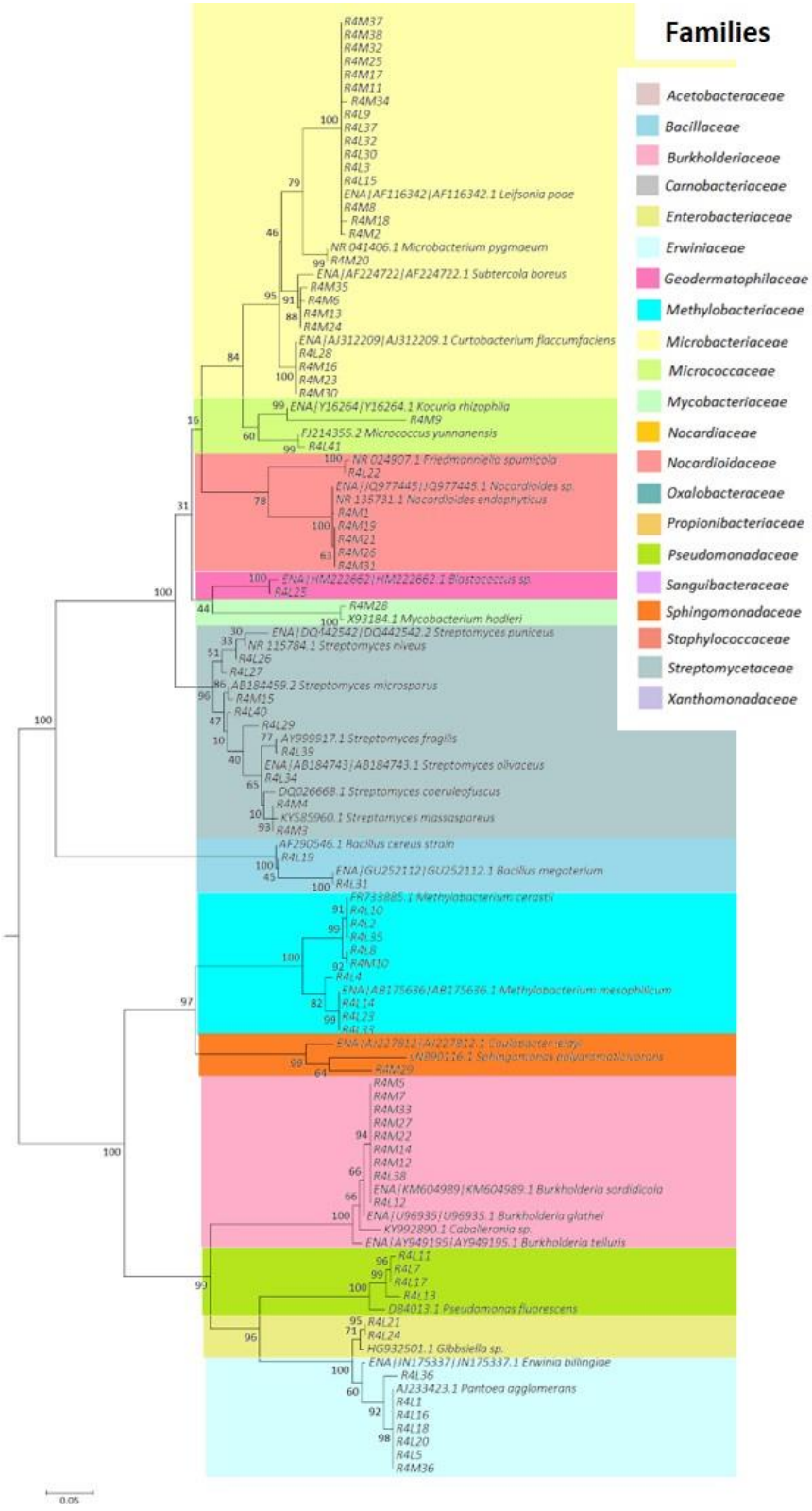
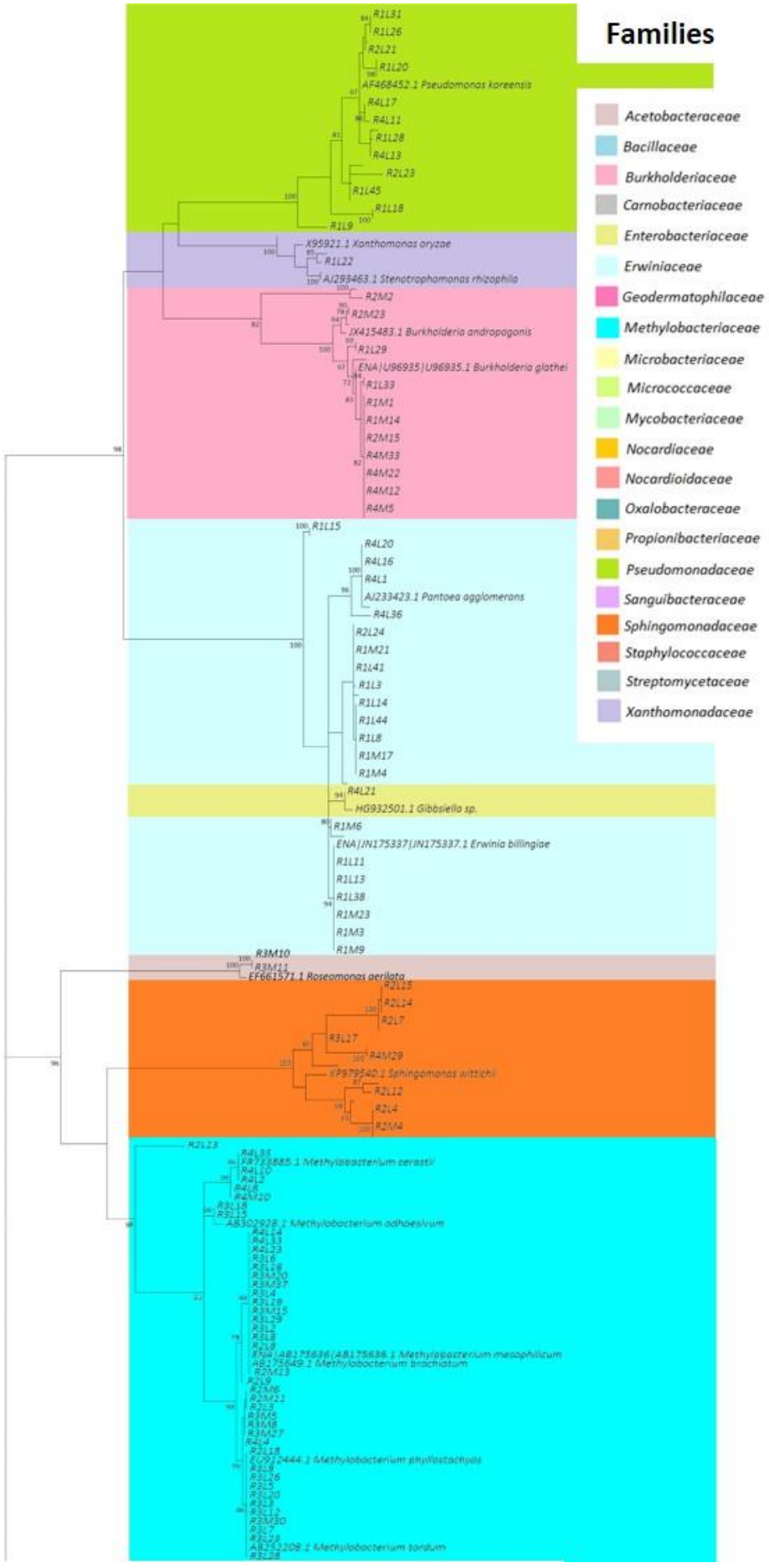


Figure 48. Phylogenetic tree based on the partial sequence of the *16S rRNA* gene of culturable bacterial strains isolated from the *R. farinacea* population from Lidón (Peninsula). The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura 3-parameter model. Each colour represents a different family. R4: bacterial strains from Lidón. L: ectolichenic bacteria. M: endolichenic bacteria. The percentage of replicate trees in which the associated taxa clustered together is shown next to the branches, with 1000 of bootstrap replications. Some reference type strains were included in the phylogenetic tree.


Families
Acetobacteraceae
Bacillaceae
Burkholderiaceae
Carnobacteriaceae
Enterobacteriaceae
Erwiniaceae
Geodermatophilaceae
Methylobacteriaceae
Microbacteriaceae
Micrococcaceae
Mycobacteriaceae
Nocardiaceae
Nocardioidaceae
Oxalobacteraceae
Propionibacteriaceae
Pseudomonadaceae
Sanguibacteraceae
Sphingomonadaceae
Staphylococcaceae
Streptomycetaceae
Xanthomonadaceae
R1L31
R1L26
R2L21
R1L20
AF468452.1 Pseudomonas koreensis
R4L17
R4L11
R1L28
R4L13
R2L23
R1L45
R1L18
R1L9
X95921.1 Xanthomonas oryzae
R1L22
AJ293463.1 Stenotrophomonas rhizophila
R2M2
R2M23
JX415483.1 Burkholderia andropogonis
R1L29
ENA|U96935|U96935.1 Burkholderia glathei
R1L33
R1M1
R1M14
R2M15
R4M33
R4M22
R4M12
R4M5
R1L15
R4L20
R4L16
R4L1
AJ233423.1 Pantoea agglomerans
R4L36
R2L24
R1M21
R1L41
R1L3
R1L14
R1L44
R1L8
R1M17
R1M4
R4L21
HG932501.1 Gibbsiella sp.
R1M6
ENA|JN175337|JN175337.1 Erwinia billingiae
R1L11
R1L13
R1L38
R1M23
R1M3
R1M9
R3M10
R3M11
EF661571.1 Roseomonas aerilata
R2L15
R2L14
R2L7
R3L17
R4M29
KP879540.1 Sphingomonas wittichii
R2L12
R2L4
R2M4
R2L23
R4L35
KP733865.1 Methylobacterium zeraisii
R4L10
R4L2
R4L8
R4M20
R3L16
R3L15
AB302928.1 Methylobacterium adhaesivum
R4L14
R4L11
R4L23
R3L6
R3L18
R3M30
R3M37
R3L4
R3L19
R3M35
R3L29
R3L2
R3L8
R3L9
R3M1|AB175636|AB175636.1 Methylobacterium mesophilicum
AB175649.1 Methylobacterium brachiatum
R2M23
R2L9
R2M6
R2M22
R2L5
R3M5
R3M8
R3M27
R4L4
R2L18
EU912444.1 Methylobacterium phyllostachyos
R3L8
R3L26
R2L5
R3L20
R3L3
R3L12
R3M30
R3L7
R3L28
AB732208.1 Methylobacterium soli-dum
R3L28

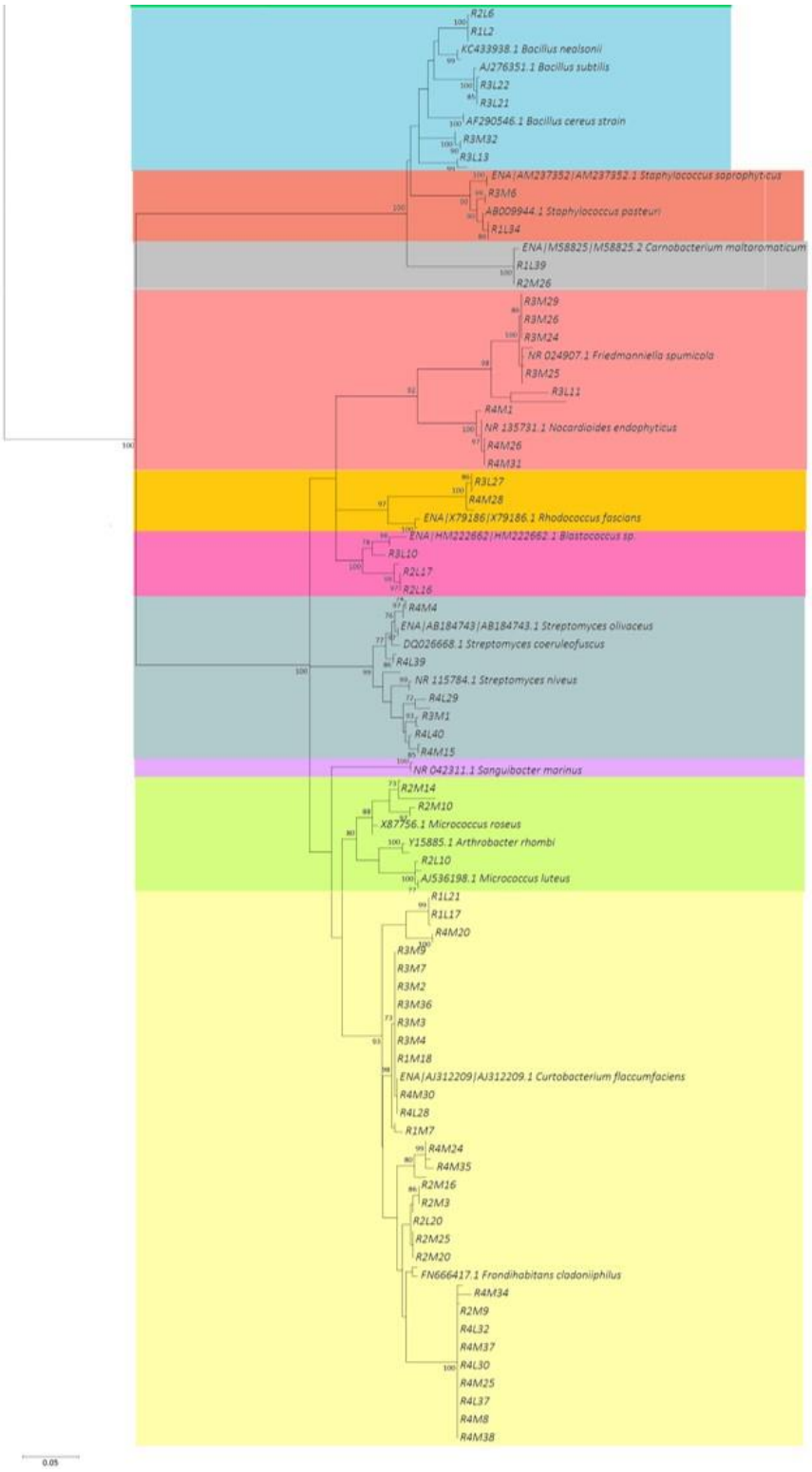


Figure 49. Phylogenetic analyses based on the partial sequence of the *16S rRNA* gene of culturable bacteria isolated bacteria associated with all four *R. farinacea* populations. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter. Each colour represents a different family. R1: bacterials strains from La Guancha. R2: bacterial strains from La Esperanza. R3: bacterial strains from El Toro. R4: bacterial from Lidón. L: ectolichenic bacteria. M: endolichenic bacteria. The percentage of replicate trees in which the associated taxa clustered together is shown next to the branches, with 1000 of bootstrap replications. Some reference type strains were included in the phylogenetic tree.

9. DIVERSITY AND COMPOSITION ANALYSES OF BACTERIAL COMMUNITIES ASSOCIATED WITH *R. farinacea* THROUGH MULTIPLEX SEQUENCING OF *16S rRNA* GENE

A total of 60 lichen thallus samples of *R. farinacea* from four geographical locations in Spain, two from the Canary island of Tenerife (La Guancha and La Esperanza) and two from the Iberian Peninsula (El Toro and Lidón) were analyzed and the *16S rRNA* gene sequences obtained were clustered in a total of 848 OTUs. Although the primers set were designed for amplifying both bacterial and archaeal *16S rRNA* genes, none archaeal sequences were detected. Figure 50 shows a representative Venn diagram illustrating the OTU overlapping among bacterial communities of the bulk thalli samples of *R. farinacea* from different Spanish geographical locations at a treshold of 0.005. Some of the OTUs were ubiquous for all the locations analyzed, while others were exclusively present in some of them.

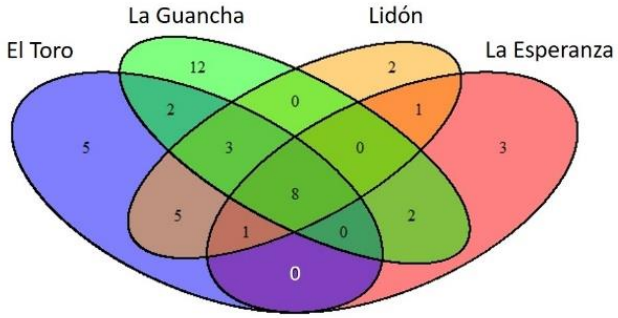


Figure 50. Venn diagram showing the OTUs shared among the bacterial communities associated with *R. farinacea* populations from different geographical locations in the Canary island of Tenerife (La Guancha and La Esperanza) and the Iberian Peninsula (El Toro and Lidón).

9.1 BETA DIVERSITY

The analyses of the changes in bacterial species composition among geographical areas (beta diversity) of the different *R. farinacea* samples composed of a bulk-thalli are shown in figure 51. The multidimensional scaling plot (MDS) of the Bray-Curtis dissimilarity matrix revealed that the bacterial communities associated with the studied lichen species were mainly determined by the geographical location factor (PERMANOVA, $R2= 0.24526$, $p<0.001$ grouping them according to their insular or peninsular origin, and $R2=0.47$, $p<0.001$, according to each one of the four geographical locations (La Guancha, La Esperanza, El Toro and Lidón)). The bacterial communities of *R. farinacea* from populations of the two main geographical areas, the Island and the Peninsula, were well differentiated among them (Figure 51a), with and influence of the 24.53% of this geographical factor, and the same happened when the analyses were performed taking into consideration the four geographical areas (Figure 51b), having an influence of the 47.05% on the bacterial composition. Other factor that explained the diversity of the community composition, although with less weight than the geographical location, was the location ecto- or endolichenic in the lichen thallus (Figure 51c), a factor that explained the 3.03% of this composition (PERMANOVA, $R2=0.038$, $p<0.01$). By contrast, the effect of the location in different parts of the thallus (apical, middle and basal) as well as the influence of

the disinfection treatment were negligible (p>0.05). There were not interactions among the variables studied that could affect *R. farinacea* associated bacteria (p>0.05).

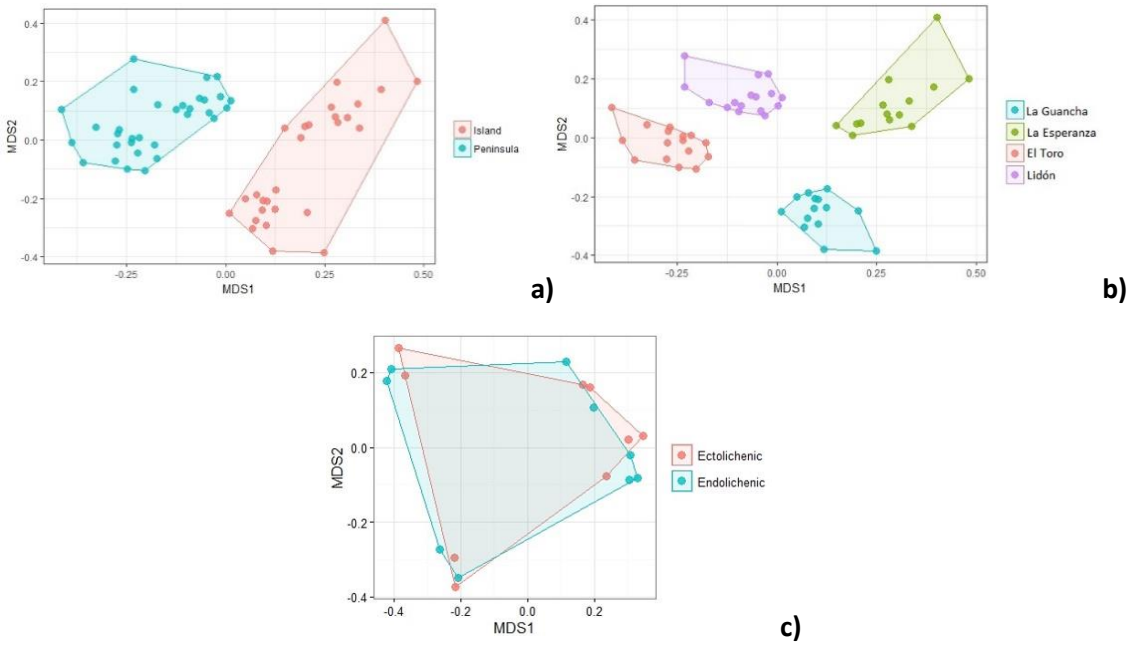


Figure 51. Multidimensional scaling (MDS) plot of the Bray–Curtis based dissimilarity matrix of bacterial communities associated with populations of the lichen *R. farinacea* from the two main sampling areas, the Canary Island (Tenerife) and the Iberian Peninsula (**a**) and from the four-different sampling geographical locations in Spain (La Guancha and La Esperanza in Tenerife, and El Toro and Lidón in the Iberian Peninsula) (**b**), as well as according to their ectolichenic or endolichenic position (**c**).

9.2 ALPHA DIVERSITY

9.2.1 Influence of geographical location

The study of the intrinsic diversity at local scale through the alpha diversity of the bulk thalli samples of *R. farinacea* from each location in the Island and the Peninsula was determined by the Richness, Shannon and Simpson diversity indices. Thus, when the lichen samples were clustered into two groups attending to the insular or peninsular location, the highest Richness and Shannon diversity appeared in the Peninsula (Figure 52 a and b, respectively), revealing a higher number of bacterial species in peninsular thalli. Further, these species were more equally represented than those of the thalli collected in the Island. This diversity results were supported by Simpson index values, which in both cases were around 0.9, indicating a quite relatively equal number of individuals belonging to each one of the species in each lichen population (Figure 52c). Significant differences (p<0.01) were recorded between insular and peninsular bacterial communities, when the values of Richness, Shannon and Simpson were compared.

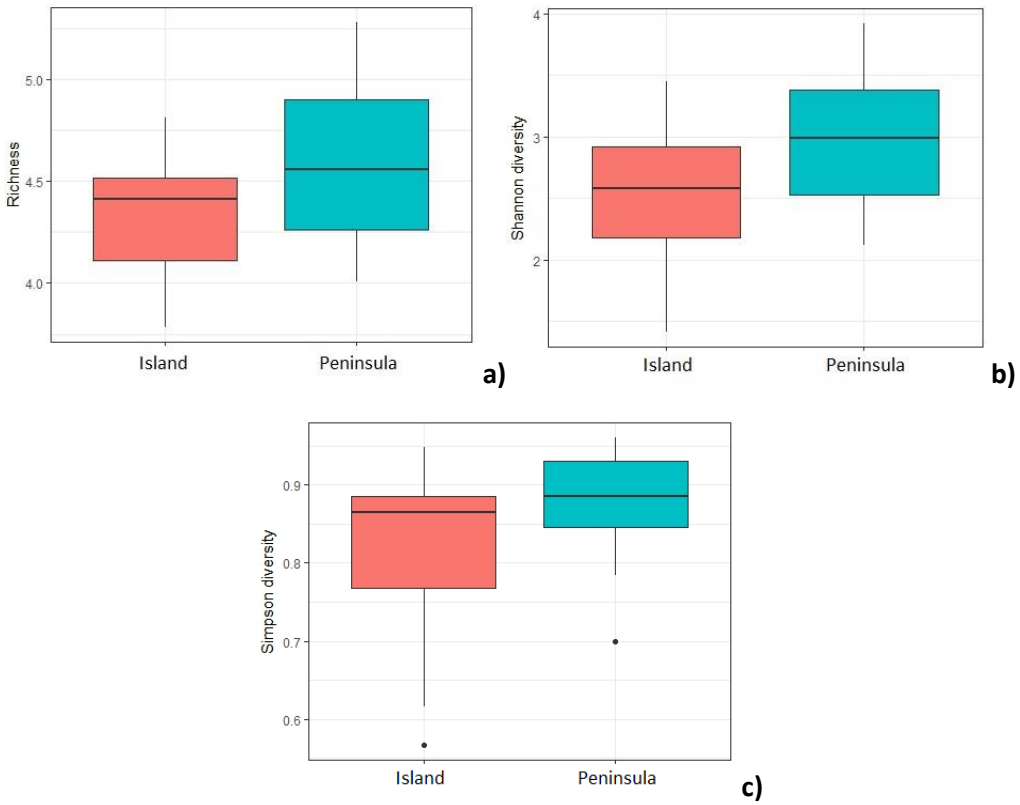


Figure 52. Box plots showing the diversity indices of the bacterial communities associated with *R. farinacea* populations from the two main sampling areas, the Island and the Peninsula. Richness (**a**), Shannon (**b**) and Simpson (**c**) diversity indices.

Moreover, when the same diversity indices were applied to compare among bacterial communities of *R. farinacea* thalli populations from four different Spanish geographical locations, using bulk thalli samples, the results showed that the samples from El Toro, in the Iberian Peninsula, were the ones with the highest Richness (Figure 53a) and, therefore with a higher number of different bacterial species. With regards to the *R. farinacea* populations from La Esperanza, La Guancha and Lidón, they showed similar values of Richness among them, but lower than in the case of El Toro. Shannon diversity index (Figure 53b) indicated that the *R. farinacea* population from El Toro was the one with the highest bacterial diversity, followed by the ones from La Guancha and Lidón, being the lichen population from La Esperanza the one with the lowest bacterial diversity. Simpson index (Figure 53c) showed that each one of the bacterial species was represented by a very similar number of individuals, mostly in the case of La Guancha, El Toro and Lidón, with indices values around 0.9. In the case of the results obtained for the population from La Esperanza, the index value was lower (around 0.75), meaning that some of the species were more represented than others. Richness different results between *R. farinacea* from El Toro and the rest of lichen populations were significant with p-values lower than 0.01. With Shannon index results, there were also significant differences when comparing the values of El Toro with those of La Guancha ($p<0.05$), La Esperanza ($p<0.001$) and Lidón ($p<0.01$), as well as between La Guancha and La Esperanza ($p<0.001$) and between La Esperanza and Lidón ($p<0.01$). Simpson diversity analyses gave significant differences when comparing lichen samples from La Esperanza with those from El Toro ($p<0.001$), La Guancha ($p<0.001$) and Lidón ($p<0.01$).

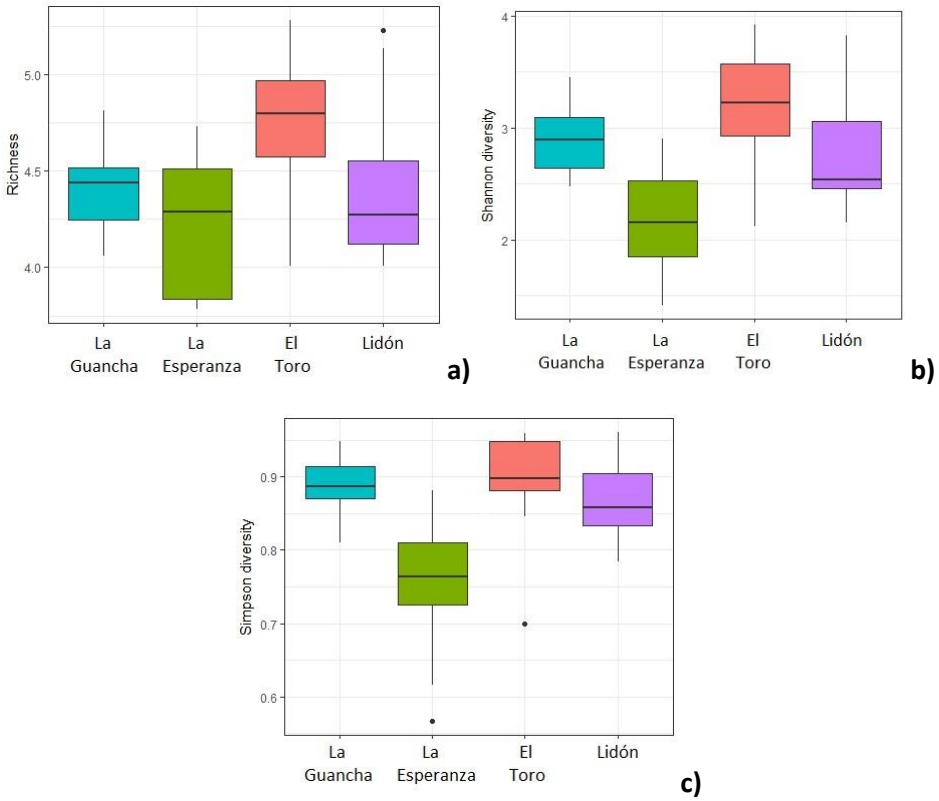


Figure 53. Box plots showing the diversity indices of the bacterial communities associated with *R. farinacea* populations from the four Spanish locations, two located at the Iberian Peninsula (El Toro and Lidón), and two from the Canary Island of Tenerife (La Guancha and La Esperanza). Richness (**a**), Shannon (**b**) and Simpson (**c**) diversity indices.

The main bacterial OTUs registered in the four lichen populations were the ones related to the phyla *Proteobacteria, Acidobacteria* and *Planctomycetes* (Figure 54). The groups of *Bacteroidetes*, *Planctomycetes* and *Proteobacteria* were more numerous in the areas of the Peninsula ($p<0.001$, $p<0.001$ and $p<0.05$, respectively), while the groups *Acidobacteria*, *Cyanobacteria* and *Firmicutes* ($p<0.001$, $p<0.001$ and $p<0.01$, respectively), were more abundant in the Island (Figure 54a).

The proportion of *Proteobacteria* was similar in the four lichen populations analyzed (52.69% in La Guancha, 61.86% in La Esperanza, 60.73% in El Toro and 62.97% in Lidón), but with significant differences among them($p<0.05$) (Figure 54b). In the case of *Acidobacteria*, their presence was slightly higher in the insular populations (42.21% in La Guancha and 33.03% in La Esperanza) than in the peninsular ones (27.3% in El Toro and 30.45% in Lidón), being these differences significant ($p<0.001$). The presence of *Planctomycetes* was much lower in all locations (around 1% in both insular populations, and an average of 3% in the peninsular ones, ($p<0.001$)). In the case of the phyla *Cyanobacteria* and *Firmicutes,* their prevalence was lower than in the case of the three previous phyla, but there were some significant differences among *R. farinacea* populations from the different locations ($p<0.001$ and $p<0.05$, respectively). Related to *Cyanobacteria,* their abundance was higher in the thalli samples from the Canary Island (La Guancha (1.55%) and La Esperanza (1.46%)) than in those from the Peninsula (El Toro (0.20%) and Lidón (0.14%)). The same happened with the phylum *Firmicutes*, which prevalence was higher in La Guancha and La Esperanza (1.01% and 1.31%,

respectively), than in El Toro (0.43%) and Lidón (0.35%) (Figure 54b). These differences were visible too when the thalli samples were grouped into two main groups, the Island and the Peninsula (Figure 54a).

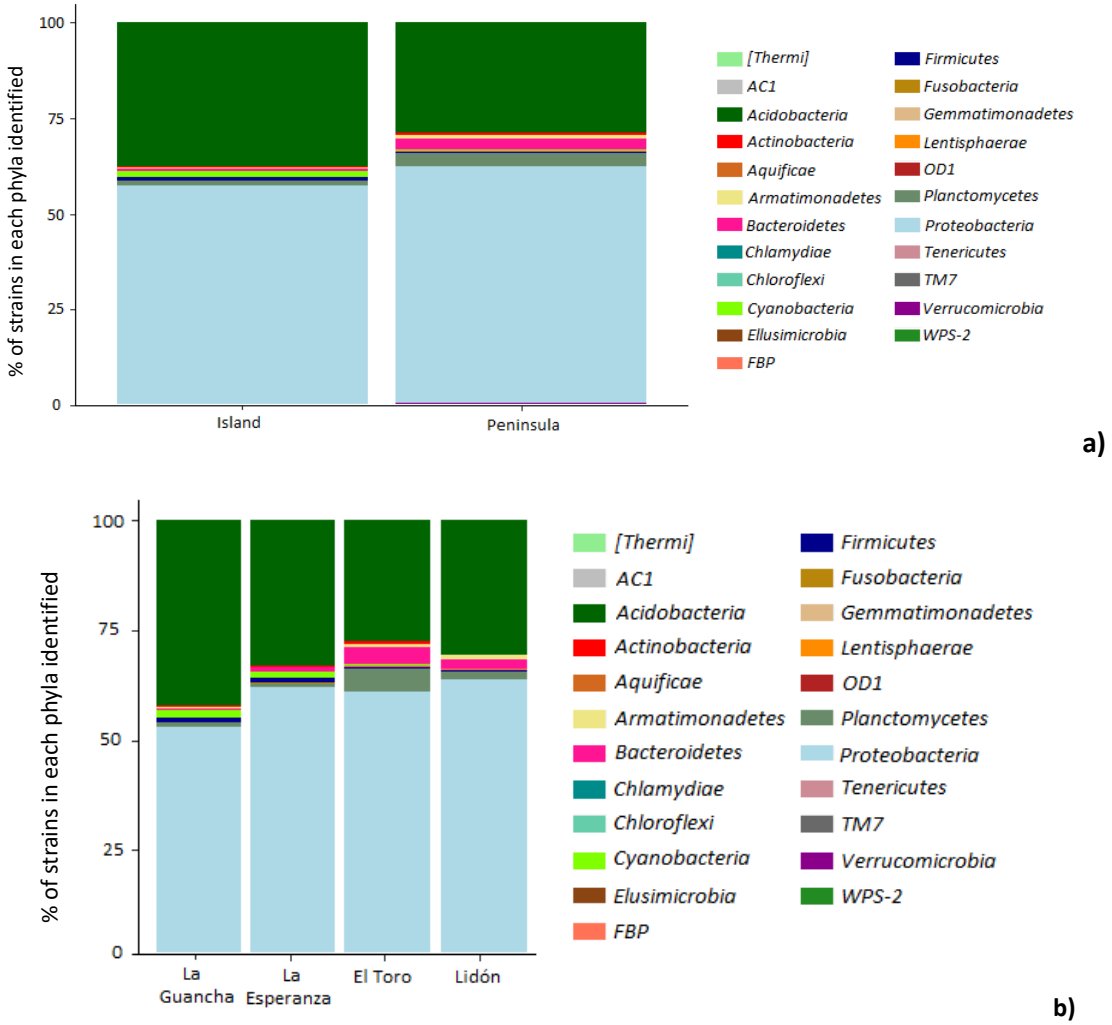


Figure 54. Taxonomical identification of the sequences of bacteria associated with *R. farinacea* populations at phylum level. The percentages of bacterial sequences assigned to each phylum are represented for bulk thalli samples attending either to the main sampling areas, the Island and the Peninsula (**a**) or for each one of the four geographical locations in Spain (**b**).

Among *Proteobacteria*, *Alphaproteobacteria* class was the predominant one in all *R. farinacea* populations (48.99% in La Guancha, 54.96% in La Esperanza, 58.64% in El Toro, and 61.38% in Lidón), with significant differences ($p<0.05$). *Acidobacteria* was the second predominant class, more abundant in the lichen populations from the Island (42.85% in La Guancha and 33.47% in La Esperanza) than in those from the Peninsula (27.02% in El Toro and 30.44% in Lidón) ($p<0.001$). Other important classes but less abundant were *Betaproteobacteria* ($p>0.05$), *Planctomycetia* ($p<0.001$), *Gammaproteobacteria* ($p<0.001$), *Bacilli* ($p<0.05$), *Sphingobacteria* ($p<0.001$) and *Cytophagia* ($p<0.001$). The main orders among the cited classes were: i) *Acidobacteriales,* more abundant in the lichen populations from Tenerife than in those from the Peninsula (42.06% in La Guancha, 33.49% in La Esperanza, 27.03% in El Toro and 30.45% in Lidón); ii) *Rhodospirillales*, with very similar proportions in the Island and Peninsula (25.48% in La Guancha, 47.65% in La Esperanza, 29.85% in El Toro and 37.45% in Lidón); iii) *Rhizobiales*,

more abundant in the *R. farinacea* populations from the Peninsula (around 18% in both locations) than in those from the Island (7.75% in La Guancha and 2.13% in La Esperanza). Other relevant orders but in lower proportions were *Caulobacterales*, *Sphingomonadales*, *Gemmatales* and *Pseudomonadales.* The presence of the abovementioned orders was significantly different among the four *R. farinacea* populations analyzed ($p<0.001$).

Acetobacteraceae, Acidobacteriaceae, Methlylocystaceae and *Caulobacteraceae* were the dominating families in the bacterial communities associated with *R. farinacea* (Figure 55). The former was the predominant one in the lichen population from La Esperanza (47.62%), followed by the populations from Lidón (37.38%), El Toro (29.79%) and La Guancha (25.38%), being these differences significant ($p<0.001$). When the comparison was performed attending at the insular or peninsular origin of the lichen populations, the proportion was very similar in both of them (36.50% and 33.60%, respectively ($p<0.001$)). These differences were slightly higher in the *Acidobacteriaceae* family, with a 38.18% of members of this group being present in the lichen population from the Island (42.87% in La Guancha and 33.50% in La Esperanza) and a 28.74% in those from the Peninsula (27.04% in El Toro and 37.38% in Lidón), ($p<0.001$). *Methylocystaceae* was predominant in the lichen populations collected in the areas of the Peninsula with a 15.21%, but with a lower presence in those from the Island (2.81%) ($p<0.001$). *Caulobacteraceae* was the family with less presence in the samples studied, with an 8.95% of members in the Island and a 4.72% in the Peninsula ($p<0.05$).

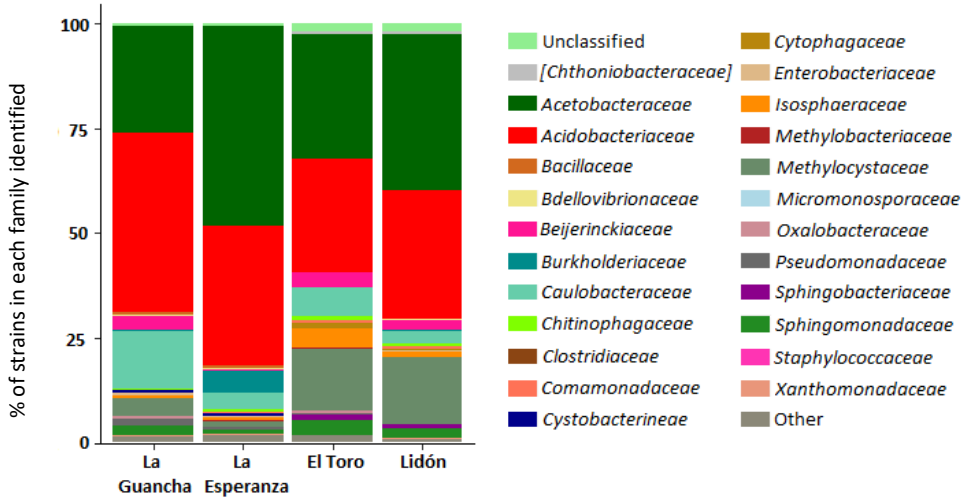


Figure 55. Taxonomical identification of the sequences of bacteria associated with *R. farinacea* at family level. The percentages of bacterial sequences assigned to each family are represented for bulk thalli samples for each one of the four geographical locations in Spain.

Another approximation in this study was to explore the taxonomical composition of bacteria associated to individual thallus from each one of the *R. farinacea* populations from the different locations. In general, the results showed the same main phyla in all the analyzed thalli: *Proteobacteria, Actinobacteria, Acidobacteria, Bacteroidetes* and *Planctomycetes* (Figure 56). Nevertheless, it was remarkable that some of these main phyla were present, mainly or exclusively, in the thalli samples collected in the Island or the Peninsula, as happened with the *Cyanobacteria*, mostly visible in three individual thalli of La Guancha and La Esperanza, *Chlamidyae*, found in one thallus of La Guancha, *Actinobacteria* and *Gemmatimonadetes*, in three thalli and one thallus from El Toro, respectively, or *Armatimonadetes* in a thallus from

Lidón. The differences among the taxonomical groups or their presence in each individual thallus were not significant.

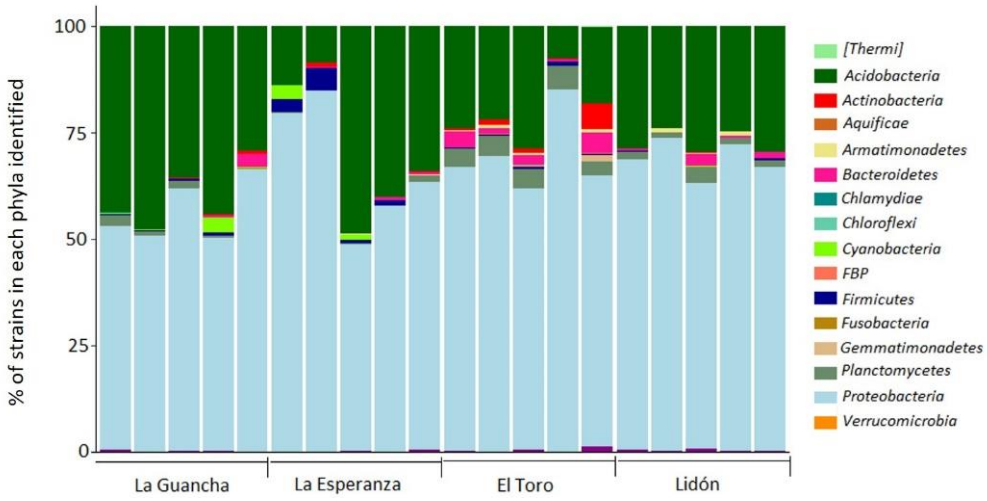


Figure 56. Taxonomical identification of the sequences of bacteria associated with *R. farinacea* at phylum level. The percentages of bacterial sequences assigned to each phylum are represented for individual thallus for each one of the four geographical locations in Spain.

The prevalence of the main group of bacterial families mentioned above for bulk thalli samples (Figure 55) was more visible in detail when the results were compared for individual thallus (Figure 57). In general, these families were *Acetobacteraceae*, *Acidobacteriaceae*, *Caulobacteraceae* and *Methylocystaceae*. However, some differences were detected, as a higher presence of the family *Pseudomonadaceae* in one single thallus from La Guancha was observed when compared with the other thalli, or the high presence of *Burkholderiaceae* in one thallus of La Esperanza. Moreover, an increasing or decreasing presence of some of these groups was observed when changing from one geographical location to another, as happened with the *Caulobacteraceae*, *Isosphaeraceae*, *Methylocystaceae* or *Sphingomonadaceae*. Differences among the taxonomical groups or those related to their presence in each individual thallus were not significant.

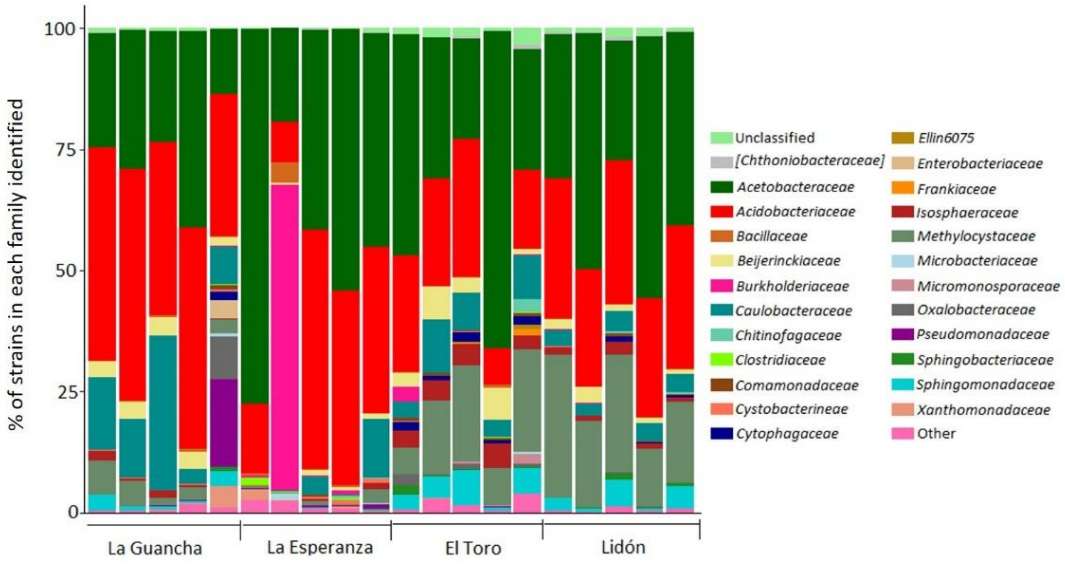
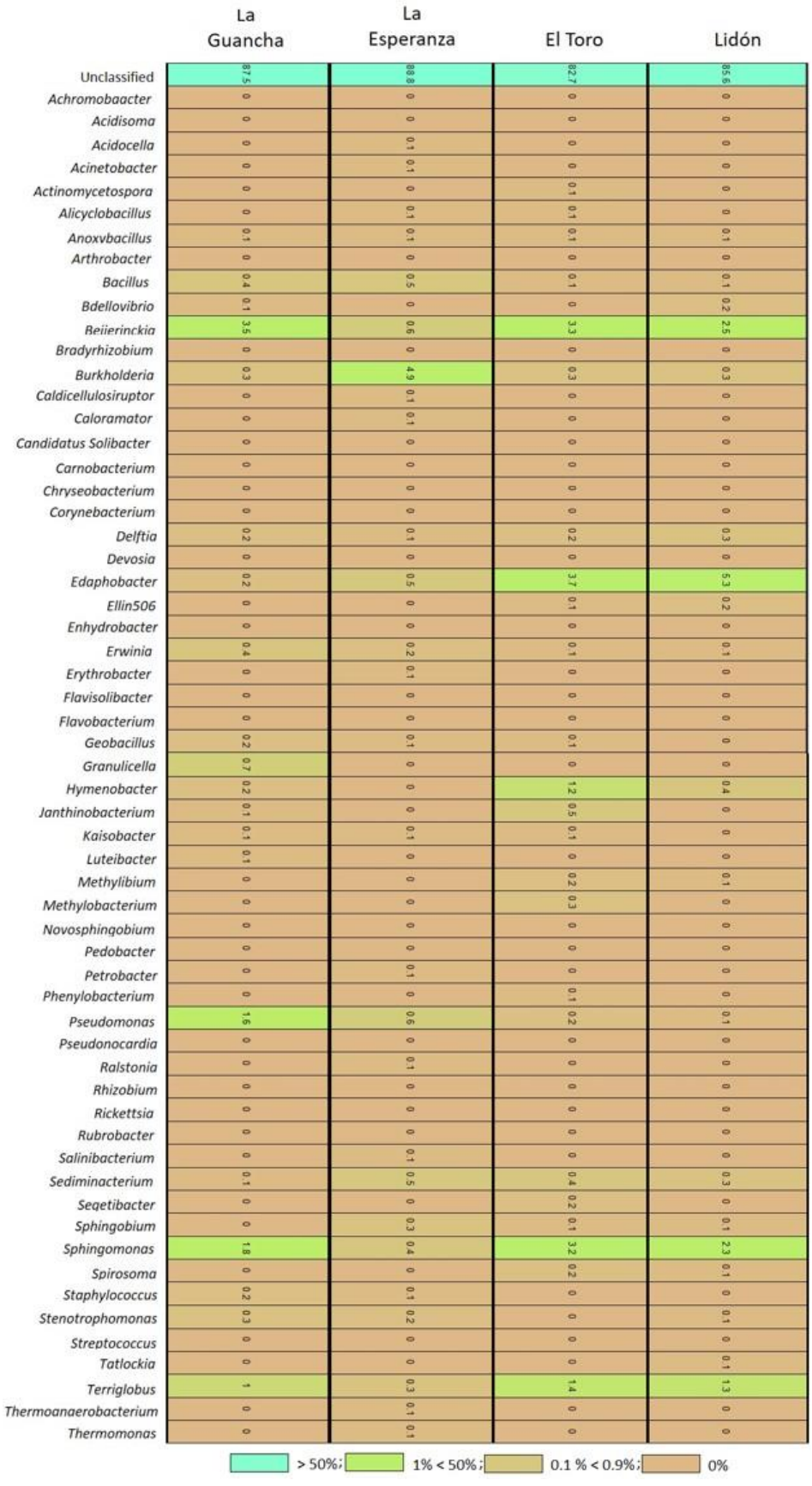


Figure 57. Taxonomical identification of the sequences of bacteria associated with *R. farinacea* at family level. The percentages of bacterial sequences assigned to each family are represented for individual thallus of each one of the four geographical locations in Spain.

Identification at genera level of bacterial sequences of the bulk thalli samples of *R. farinacea* brought back a big proportion of bacteria associated with lichens not closely related to the sequences present in the data base used (Greengenes, McDonald *et al*., 2012a), meaning that many of them could be new bacterial genera and/or species. In fact, more than the 80% of these bacterial sequences analyzed from each *R. farinacea* population were included in unclassified genera. However, some common genera were *Beijerinckia* (2.48%), *Edaphobacter* (2.42%), *Sphingomonas* (1.94%), *Burkholderia* (1.45%), *Terriglobus* (1.01%), *Pseudomonas* (0.6%) or *Hymenobacter* (0.45%). Some of the genera were identified in all populations, as *Anoxybacillus*, *Bacillus*, *Beijerinckia*, *Burkholderia*, *Delftia*, *Edaphobacter*, *Pseudomonas*, *Sphingomonas*, *Terriglobus*, etc. By contrast, other genera, were detected in only one of the four lichen populations, as *Acidocella*, *Acinetobacter*, *Caldicellulosiruptor*, *Caloramator*, *Erythrobacter* or *Salinibacter* in La Esperanza, or *Actynomycetospora*, *Devosia, Methylobacterium* or *Segetibacter* in El Toro, *Tatlockia* in Lidón, etc. Table 17 shows some of the bacterial genera identified and their proportion in each one of the *R. farinacea* populations from different geographical locations.

Table 17. Heatmap showing the relative abundance of some of the bacterial genera identified in the four populations of *R. farinacea* from La Guancha, La Esperanza, El Toro, and Lidón.



9.2.2 Influence of location in the lichen thallus

At the ectolichenic or endolichenic fraction

The analyses of the alpha diversity of the bacteria associated to the ectolichenic and endolichenic fractions of the *R. farinacea* thalli studied, revealed that, the ectolichenic fraction had higher values of Richness, Shannon and Simpson diversity indices (Figure 58 a and b) than the endolichenic one. These results indicating a higher number of different species and more equally represented in the bacteria associated with the thalli surface than those inside of it. Moreover, Simpson diversity index values reaffirm these results, indicating that the species identified in each one of the lichenic fractions are composed of relatively equal number of individuals, with values of the index being around 0.9 (Figure 58c). The differences observed in the different indices of diversity studied, were not significant.

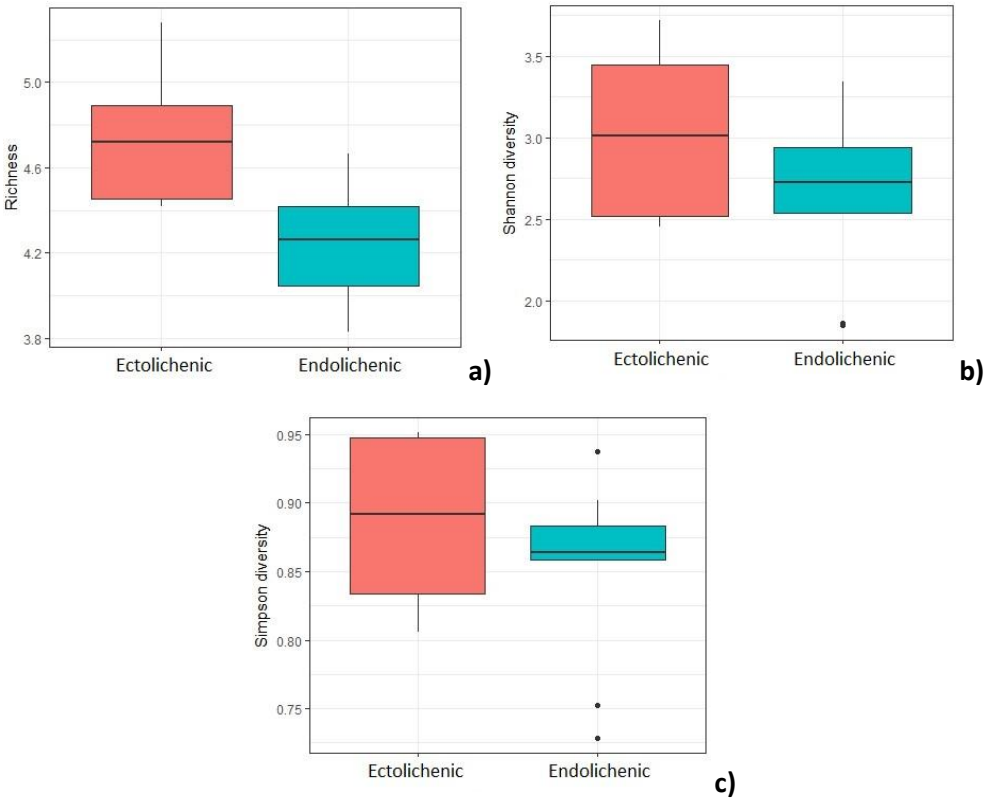


Figure 58. Box plots showing the diversity indices of the bacterial communities associated with *R. farinacea* populations attending at their location in the lichen thallus, ectolichenic or endolichenic. Richness (**a**), Shannon (**b**) and Simpson (**c**) diversity indices.

The analyses of the main taxa of bacteria associated with the ectolichenic and endolichenic fractions of the *R. farinacea* thalli, showed similar results to those observed in the bacterial diversity analyses were the effect of geographical location was studied. *Proteobacteria* were the most abundant members in both fractions (59.42% in ectolichenic and 53.22% in endolichenic), followed by *Acidobacteria* (31.30% in ectolichenic and 41.42% in endolichenic) and *Planctomycetes* (2.09% in ectolichenic and 2.52% in endolichenics). *Cyanobacteria* and *Bacteroidetes* showed more notably differences when compared both sides of the thallus,

being more abundant in the ectolichenic side. *Cyanobacteria* were present in a 1.06% in the ectolichenic part and in a 0.16% in the endolichenic one. *Bacteroidetes* were present in a 3.61% in the ectolichenic fraction and in a 0.88% in the endolichenic one. However, the differences observed were not significant.

At order level, the main groups were *Acidobacteriales* (31.38% in ectolichenic and 41.25% in endolichenic fractions), *Rhodospirillales* (28.47% in ectolichenic and 32.75% in endolichenic sides), *Rhizobiales* (16.82% in ectolichenics and 11.52% in endolichenic parts) and *Caulobacterales* (7.69% in ectolichenics and 6.5% in endolichenic fractions). These percentages were very similar in both thalli sides, without significant differences between them. There were two groups present although in less proportion but with significant differences ($p<0.05$), *Saprospirales* (1.93% in ectolichenics and 0.25% in endolichenic fractions) and *Sphingomonadales* (3.74% in ectolichenics and 1.12% in endolichenic sides).

Results at family level (Figure 59) showed as predominant taxa *Acidobacteriaceae* (31.39% in the ectolichenic and 41.25% in the endolichenic parts), *Acetobacteriaceae* (28.34% in the ectolichenic and 32.71% in the endolichenic fractions) and *Methylocystaceae* (13.77% in ectolichenic and 9.67% in endolichenic side). In general, the differences found in these families between the two lichenic fractions were not significant. However, the presence of the family *Chitinophagaceae* was significantly higher in the ectolichenic side (1.93%, $p<0.05$) than in the endolichenic one (0.25%).

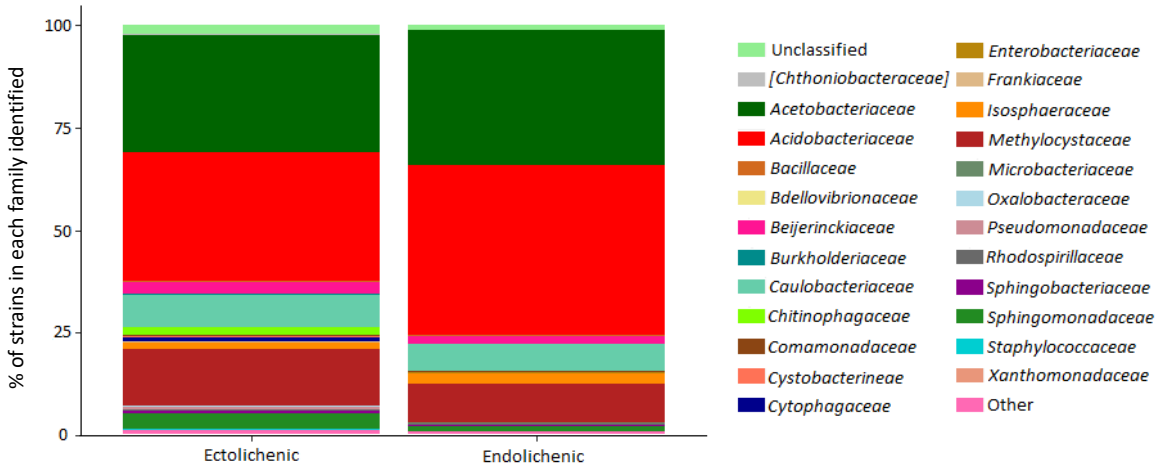
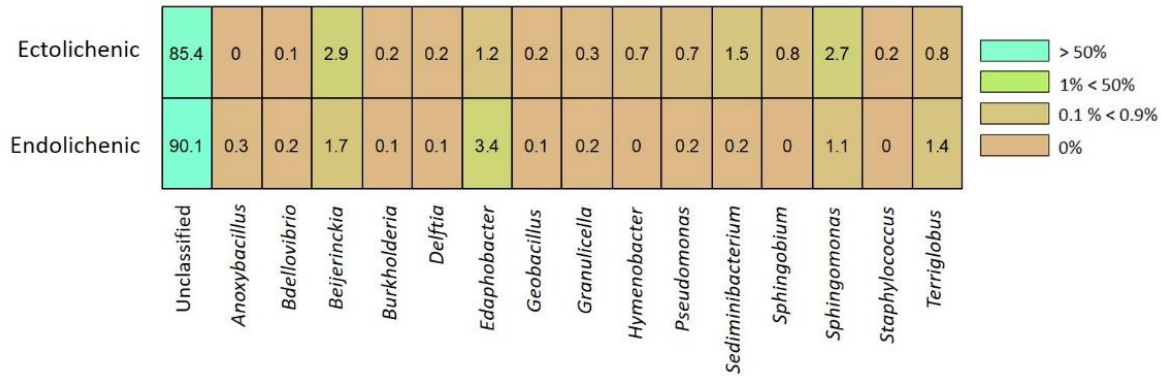


Figure 59. Taxonomical identification of the sequences of bacteria associated with *R. farinacea* at family level. The percentages of bacterial sequences assigned to each family are represented at the ecto- or endolichenic fraction in the thallus.

It is noteworthy that around 85% of the bacteria were unclassified at genera level. Besides, among the identified ones, there were some genera present only in the ectolichenic part, as *Hymenobacter*, *Sphingobium* and *Staphylococcus*, and others only in the endolichenic one, as *Anoxybacillus*. Other genera were common at both sides, as *Bdellovibrio*, *Delftia*, *Geobacillus* or *Sediminibacterium*, among others. In table 18 are shown some of the identified genera and their proportion at each fraction of the lichen thalli.

Table 18. Heatmap showing the relative abundance of some of the bacterial genera identified at the ectolichenic and endolichenic fractions of the *R. farinacea* thalli.



At apical, middle or basal parts

The study of diversity of bacteria associated with *R. farinacea* at the different thallus parts (apical, middle and basal) showed similar values in Richness (Figure 60a), Shannon (Figure 60b) and Simpson (Figure 60c) diversity indices, although the apical part was the one with the lowest values in the number of species and diversity. Simpson index results showed that in the apical part lower percentages of species dominated the bacterial diversity. The differences observed were not significant.

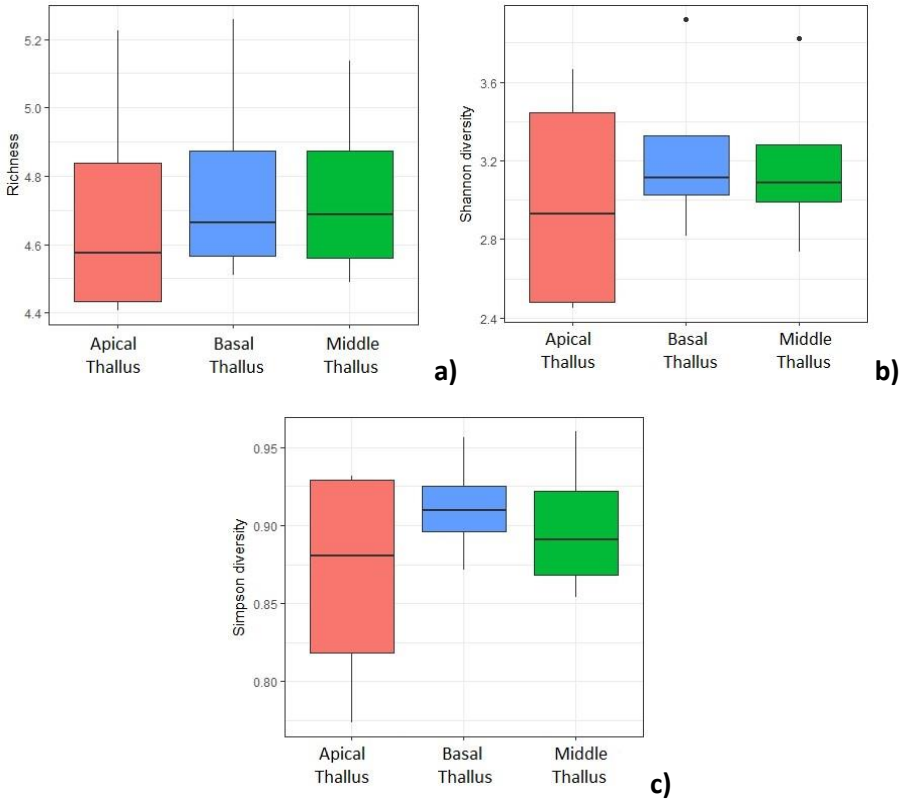


Figure 60. Box plots showing the diversity indices of the bacterial communities associated with *R. farinacea* populations attending at their location in the different parts of the lichen thallus, apical, middle and basal. Richness (**a**), Shannon (**b**) and Simpson (**c**) diversity indices.

Regarding the bacterial diversity along the different parts of the thallus (apical, middle and basal), at phylum level the main groups were *Proteobacteria*, *Acidobacteria* and *Bacteroidetes,* with very different proportions among the three studied thallus parts (around 55%, 35% and 3%, respectively) (Figure 61). Furthermore, the results showed a preferential distribution of some bacterial groups in some of these thallus parts. The biggest differences at phylum level were observed in the case of *Cyanobacteria, Firmicutes* and *Planctomycetes* (Figure 61). *Cyanobacteria* were mainly present (2.19%) at the middle part of the thallus, decreasing in the apical part (1.30%) and even more in the basal part (0.09%). By contrast, *Firmicutes* had the highest percentage in the apical part (2.09%), also decreasing along the middle (1.17%) and basal parts (0.29%). Regarding *Planctomycetes,* they were mainly detectedat the basal part (4.68%), decreasing in the middle (2.63%) and apical parts (0.56%). Other groups present were *Armatimonadetes*, *Verrucomicrobia* or *Actinobacteria* (Figure 61).

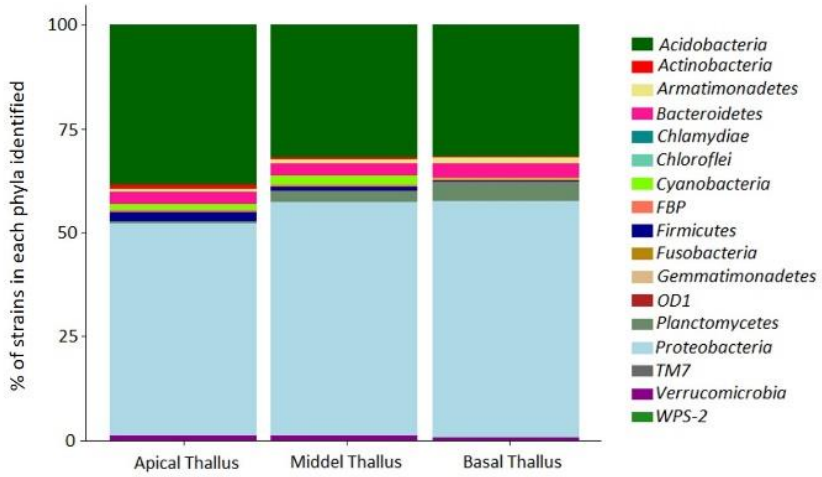


Figure 61. Taxonomical identification of the sequences of bacteria associated with *R. farinacea* at phylum level. The percentages of bacterial sequences assigned to each phylum are represented at the apical, middle and basal parts in the lichen thallus.

At class level, the main groups were *Alphaproteobacteria* (around 50% in the apical, middle and basal part), *Acidobacteria* (around 30% in the three parts) and *Betaproteobacteria* (around 1.5% along the thallus), and in less proportion *Sphingobacteria* and *Bacilli*.

Some orders showed different proportion along the different parts of the lichen thallus, with a preference distribution in some of them, as it happened with *Gemmatales*, with the main percentage at the basal parts (4.41%), decreasing in the middle (2.40%) and apical parts (0.46%). With regards to the abundance of *Bacillales*, *Actinomycetales* and *Myxococcales* it was higher in the apical part, decreasing along the middle and basal parts. Something similar happened at family level in some groups. For instance, there were less abundance of members of the family *Isosphaeraceae* at the apical part than in the middle and basal parts (0.46%, 2.39% and 4.41%, respectively). By contrast, in the case of the family *Cystobacterineae* its members decreased from the apical to the basal part (1.16%, 0.24% and 0.065%, respectively). The same happened with the family *Bacillaceae* whose abundance was bigger at the apical part (1.48%) than in the middle (0.64%) and basal (0.21%) part of the thallus.

Despite the differences observed at different bacterial taxonomical levels along the different parts of the lichen thallus they were not significant. However, it is worth to mention that in

various taxa, they were significant. These results suggesting possible different roles along these thallus parts.

9.2.3 Influence of disinfection treatment

When the effect of a disinfection treatment (ethanol at 70% for 1 min) on the diversity of bacterial communities associated with *R. farinacea* was studied, the results obtained showed that the dominant phyla still were *Proteobacteria*, *Acidobacteria*, *Plantctomyctes* and *Bacteroidetes*, without significant differences between the disinfected and undisinfected samples. The same happened at class level, being the main groups *Alphaproteobacteria*, *Acidobacteria*, *Betaproteobacteria*, *Planctomycetia* and *Gammaproteobacteria,* regardless the disinfection treatment. However, some small differences were observed at family level (Figure 62). For instance, with the *Chitinophagaceae*, which presence was twice in the disinfected samples than in the non-disinfected ones (1.09% and 0.55%, respectively). The opposite was observed in the case of the family *Burkholderiaceae*, with a higher abundance in the non-disinfected samples than in the disinfected ones (1.61% and 0.39%, respectively). However, the main families in these disinfected and non-disinfected samples were *Acetobacteraceae*, *Acidobacteriaceae*, *Methylocystaceae* and *Caulobacteraceae*. The differences found in the taxonomical groups were not significant.

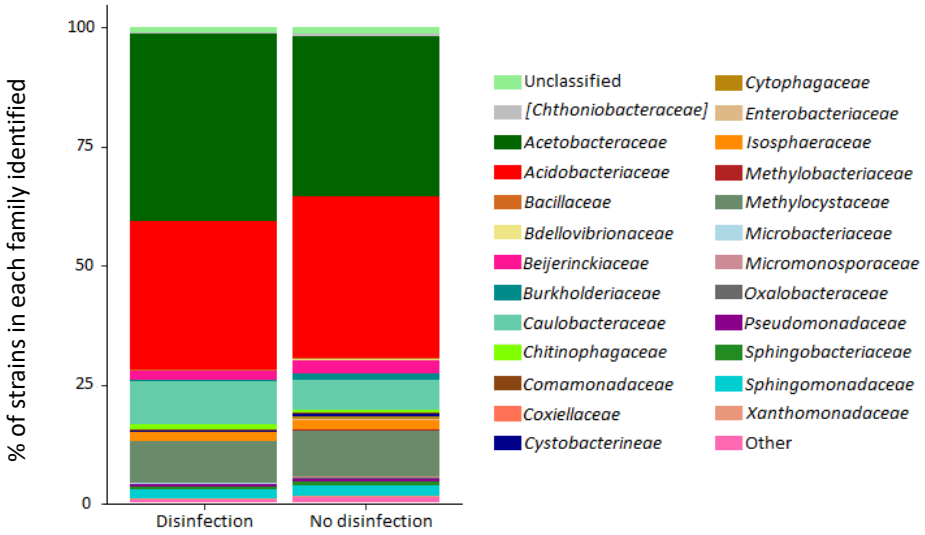


Figure 62. Taxonomical identification of the sequences of bacteria associated with *R. farinacea* at family level. The percentages of bacterial sequences assigned to each family with or without disinfection treatment (ethanol at 70% per 1 min) are represented.

DISCUSSION

Classically lichens have been defined as a self-supporting mutualistic symbiosis between a main fungal partner (mycobiont), that provides refuge for one or more photosynthetic partners, such as green algae and/or cyanobacteria (photobionts), forming a unique symbiotic structure or holobiont, the lichen thallus (Grube and Berg, 2009). The morphological, physiological and adaptive integration of the symbionts allow them to adopt new properties that represent evolutionary innovations, which permit them to colonize habitats with extreme environmental conditions that could not colonize independently (Cardinale *et al*., 2006; Grube *et al*., 2015). In fact, lichens are one of the oldest examples of symbiosis and of greater diversification, with more than 18,000 lichen species (Kirmizigül *et al*., 2003; Nash, 2008). These unique symbiotic associations can also harbour other microorganisms such as non-photosynthetic bacterial partners that could be facultative symbionts (Aschenbrenner *et al*., 2014; Grube *et al*., 2015; Hodkinson and Lutzoni, 2009; Selbmann *et al*., 2010).

The first investigations on heterotrophic bacteria associated with lichens were initiated by culture dependent techniques, which allowed in some cases the isolation of bacteria able to fix nitrogen and to solubilize phosphates (Cardinale *et al*., 2006; González *et al*., 2005; Lenova and Blum, 1983; Liba *et al*., 2006). The contribution of these bacteria to the lichen symbiosis could be by providing nutrients to cover certain lichens requirements. Subsequent studies, mostly using culture independent approaches, have shown the great abundance and diversity of bacterial communities associated with lichens (Grube *et al*., 2009, 2015), revealing that lichens constitute an unexplored environment of bacterial communities. These studies have also begun to elucidate some of the functional roles that these bacteria could play, such as increasing the tolerance of lichens to different types of stress, as well as contributing to their longevity and persistence in extreme environmental conditions (Cernava *et al*., 2017; Grube *et al*., 2015; Grube and Berg, 2009; Parrot *et al*., 2016; Selbmann *et al*., 2010). In fact, lichen-associated bacteria are now recognized as an integral part of the lichens, these being in turn considered as a multispecies symbiosis (Aschenbrenner *et al*., 2016; Cernava *et al*., 2016).

However, the knowledge about the composition, diversity and metabolic and/or physiological potential of bacteria associated with lichens is still very scarce but essential to understand both the microbial interactions in the lichen thallus and their biotechnological potential. In the present study, we provide for the first time new knowledge on the abundance of culturable bacteria associated with the Mediterranean lichen *R. farinacea* and the metabolic and physiological potential of these bacteria, as well as on the composition and diversity of the bacterial communities associated with this lichen species, both by culture-dependent and independent techniques.

Culturable bacterial populations associated with *R. farinacea*

The number of studies conducted to isolate bacteria associated with lichens is not numerous, with the added inconvenient of the lack of a standarized methodology for the bacteriological analyses of lichen thalli. However, Biosca *et al*. (2016) developed recently a new methodology to improve the recovery of bacteria from both the external (ectolichenic) and the internal

(endolichenic) fractions of the lichen thallus, as well as novel culture media enriched with lichen extracts to mimic lichen nutritional conditions. This novel methodology together with the lichen enriched media proved to dramatically increase the number of both ectolichenic and endolichenic bacteria (10^4 to 10^6 CFU/g) recovered from lichen samples from different lichen species, particularly when compared to other methodologies and culture media previously used by other authors (Cardinale *et al*., 2006, 2008, Cernava *et al*., 2015a, 2015b; Grube *et al*., 2009; Liba *et al*., 2006; Parrot *et al*., 2015; Selbmann *et al*., 2010; Sigurbjörnsdóttir *et al*., 2014; Suzuki *et al*., 2016).

In the present work, the culture media enriched with *R. farinacea* extracts and the methodology developed by the group of Biosca *et al*. (2016) was applied, which allowed providing new data on the abundance of the culturable ectolichenic and endolichenic bacteria associated with *R. farinacea*. Thus, the bacteriological analysis of lichen thalli from *R. farinacea* populations from different geographical locations in Spain, yield ecto- and endolichenic culturable bacterial counts that ranged from 10^4 to 10^6 CFU/g, after 15 days of incubation at 26ºC, with culturable counts increasing along the time. It is worth to mention that the bacterial densities recovered from bulk thalli samples of the populations of *R. farinacea* from different geographical origins were variable. Related to this, a higher number of bacterial colonies were obtained from the lichen thalli collected from La Guancha and El Toro (around 10^6 CFU/g) than those from La Esperanza and Lidón (around 10^5 CFU/g). This result could be related to the different environmental conditions in different geographical locations, since in the locations of La Esperanza and Lidón these conditions were more extreme than in the other two locations. In this sense, temperatures were more extreme in Lidón, while environmental humidity was higher in La Esperanza than in the other locations. These results agree with previous ones suggesting a selective colonization of lichen thalli by bacteria according to environmental, geographical and climate conditions (Cardinale *et al*., 2012b; Selbmann *et al*., 2010). Furthermore, bacteria exhibit different sensitivity to desiccation and high humidity, being some of them affected negatively by water or high humidity, as it happens in *Deinococcus radiodurans* and the survival of this bacterium under starvation (Yang *et al*., 2009). The same was found in other studies with bacterial species as *Bacillus subtilis, Escherichia coli, Salmonella pullorum, Serratia marcescens, Staphylococcus albus, S. pneumoniae, Streptococcus haemolyticus, S. derby, Proteus vulgaris* and *Pseudomonas aeruginosa* (type 1), in which an increase in death rates at intermediate (approx. 50–70%) to high (approx. 70–90%) relative humidity environments was found (Tang, 2009).

When the numbers of *R. farinacea* bacteria isolated from the surface (ectolichenic fraction) and the inner part (endolichenic fraction) of the thalli were compared, in general, similar abundances were recorded at final incubation time, regardless the geographical location. However, a slightly higher number of isolates were recovered from the lichen surface, although without significant differences. In other lichen species, it was found that culturable bacterial counts from internal thalli, were slightly higher than those from the thalli surface (Biosca *et al*., 2016; Cardinale *et al*., 2008). These differences were justified due to the differences in nutrient availability on thallus surface compared with the inner thallus tissue, as well as because the lower protection of the bacteria on lichen surfaces against abiotic stresses, such as UV radiation or lower water availability, as well as meteorological events (Biosca *et al*., 2016; Cardinale *et al*., 2012b). However, other studies have reported the influence of

additional factors such as the lichen age, the type of inhabiting substrate, as highly affecting the number of microorganisms found in lichens (Cardinale *et al*., 2012b).

Interestingly, additional differences were observed in bacterial culturable counts between the two lichen-enriched culture media employed, independently of the geographical origin of the *R. farinacea* population analyzed. In general, the highest bacterial numbers were obtained on nutrient-poor ABL medium, where bacterial colonies also developed faster than on ABLGM medium supplemented with glucose and mannitol. These results agree with the characteristic oligotrophic nature of lichens and with a previous study of our group (Biosca *et al*., 2016). However, in some cases, bacterial counts were very similar in both media, being higher on ABLGM medium only in one case. Based on the present results both media can be used to the recovery of lichen-associated bacteria, which also could increase the diversity of isolated bacteria, according to Biosca *et al*. (2016).

The variability in the methodologies and culture media used as well as in the incubation periods and conditions reported for the isolation of lichen-associated bacteria make very difficult to compare the results obtained in this study with those reported in other investigations. However, the bacterial densities recovered from *R. farinacea* thalli samples were higher than those obtained in other lichen species where CFU/g values ranged from 10^2 to 10^4 (Cardinale *et al*., 2006; Jian *et al*., 2017). Our better results could be due, at least in part, to the use of lichen enriched media with lichen extracts of the *R. farinacea* populations analyzed that may provide unique nutrients and/or growth factors, absent in synthetic growth media, as pointed out by Biosca *et al*. (2016). Another variable to take into consideration is that the target of isolation in the present study was the maximum variability among the heterotrophic mesophilic aerobic bacteria. Therefore, avoiding the biases due to the use of culture media specific for some bacterial groups, as the ones used for *Actinobacteria*, or to isolate bacteria able to fix nitrogen, solubilize phosphate or produce phytohormones (Cardinale *et al*., 2006, 2008, Cernava *et al*., 2015a, 2015b; Grube *et al*., 2009; Jian *et al*., 2017; Parrot *et al*., 2015; Sigurbjörnsdóttir *et al*., 2014).

When comparing the bacterial culturable densities of the *R. farinacea* populations analyzed in this study (10^5 – 10^6 UFC/g) with those found by other authors using culture-independent methods, we found that in some cases the values were similar o higher than the ones obtained in this study by using culture-dependent methods. In this sense, Grube *et al*. (2009) showed a similar abundance of bacteria associated with the lichens *Cladonia arbuscula*, *Lecanora polytropa* and *Umbilicaria cylindrica* through a semi-automated quantification of CLSM images, as the ones obtained through culturable isolation in *R. farinacea* in this study, with values that ranged from 10^4 to 10^6 bacteria per cubic milliliter of lichen volume. Later on, Pankratov (2012) through DAPI staining and hybridization with group-specific probes for *Alphaproteobacteria*, *Acidobacteria, Actinobacteria* and *Betaproteobacteria*, found abundances of about 10^8 cells/g in the lichens studied. Thus, the methodology and culture media employed allowed the reduction of the differences between the culturable and non-culturable bacterial fractions associated with the *R. farinacea* populations studied.

Overall, in this work, a high number of bacteria were isolated from both the ectolichenic and endolichenic fractions of *R. farinacea* thalli, also revealing the importance of lichens as new relevant sources of numerous and diverse microorganisms, potentially new species. These

results agree with those of other studies pointing out the importance and potential contribution of lichen-associated bacteria to the lichen symbiotic sustainability through diverse functional roles (Grube *et al*., 2015). However, some functional activities assigned to lichenic bacteria were proposed based on omic approaches, being the studies conducted with culturable bacteria still very scarce (Aschenbrenner *et al*., 2016; Parrot *et al*., 2015; Sigurbjörnsdóttir *et al*., 2016). On the other hand, the potential functional roles and/or biotechnological potential of bacterial communities associated with *R. farinacea* has not yet been explored.

Characterization of bacteria associated with the lichen *R. farinacea*

The present work supposes the first characterization of a collection of *R. farinacea* associated bacteria, isolated from the surface (ectolichenic) and the inner part (endolichenic) of lichen thalli of *R. farinacea* populations from two geographical locations in the island of Tenerife and other two in the Mediterranean slope in the Iberian Peninsula, the four locations with common characteristics of Mediterranean climate. The characterization was focused on the study of some functional roles of *R. farinacea* associated bacteria, because their pigments may be involved in the pigmentation of this lichen species and/or have some beneficial roles that could influence the lichen symbiosis; their hydrolytic potential since hydrolases can be a key factor in the nutrient recycling with the thallus; their potential ability to supply nitrogen, phosphate and iron which are limiting nutrients for the lichen growth; their potential production of phytohormones for lichen growth stimulation and their ability to produce biofilms with different functional roles as well. Furthermore, it was considered particularly interesting to explore the biotechnological potential of the *R. farinacea* associated bacteria in all these activities, still barely studied in other lichenic bacteria.

Lichens are organisms with a great diversity of colours due to the presence of secondary metabolites produced by the mycobiont (Shukla *et al*., 2010). Some lichenic pigments have been used traditionally as dyes (Parrot *et al*., 2015), and more recently as food colourants, antioxidants, antimicrobial and anticancer agents, and as bioindicators (Rao *et al*., 2017). Lichen pigments may be a consequence of chemical reactions between biomass compounds and several cations, conducting to the production of different mineral complex and compounds (Gayathri *et al*., 2014). However, lichen-associated bacteria could also contribute to lichen pigmentation. In this study, a high percentage of *R. farinacea* bacterial strains produced pigments, ranging from 50% to almost 100%, according to the geographical origin of the lichen thalli samples, since a higher percentage of pigmented bacteria was found in El Toro (94.03%) and a lower one in La Esperanza (75.75%), Lidón (65.61%) and La Guancha (51.19%). The former could be related to the environmental conditions where the thalli were grown because in the population of El Toro bacteria were more exposed to sunlight due to the distribution of the trees in that area. In the other lichen populations, the environmental conditions might be different, as for example in the case of Lidón, where trees were more abundant and leafier and lichens were less exposed to solar radiation. The most frequent pigments were yellow and pink, which were mostly present among ectolichenic and endolichenic bacterial strains, respectively. This could be related to the role of bacterial pigments in the tolerance to environmental stress since yellow pigments in ectolichenic bacteria might be involved in their protection from sun exposure, among other abiotic stresses

to which they are more exposed than endolichenic ones. Related to this, it has been reported that under some conditions, as UV radiation, some bacterial strains can enhance their pigment production (El-Bialy and Abou El-Nour, 2015). The former could be related to the high percentage of pigmented bacteria found in *R. farinacea* in the environmental conditions of the different geographical locations where they were sampled. The potential functional roles of bacterial pigments in lichens might also be involved in the protection of the photobionts from the excess of irradiation, acting as sun-screen (de la Torre *et al*., 2010). In this sense, *R. farinacea* thalli were collected from the bark of trees suffering sun exposure during many hours per day for long periods of the year. With regards to the prevalence of pink pigments among endolichenic bacteria, they could have other antibiotic and/or anti-freezing activities, among others still to be explored. Different pigments have been used traditionally as colourants in the textile industry, and the use of bacterial pigments is eco-friendly compared with chemical ones (Chadni *et al*., 2017). Thus, *R. farinacea* bacterial pigments could be exploited as antimicrobial, antioxidants, anticancer agents, as well as food colourants.

Recent studies based on metagenomic and culture techniques have demonstrated that bacterial communities may help to the lichen thallus maintenance, in part due to their glucanolytic, chitinolytic and proteolytic activities (Cernava *et al*., 2017; Grube *et al*., 2009, 2015; Lee *et al*., 2014; Schneider *et al*., 2011; Sigurbjörnsdóttir *et al*., 2016). Thus, *R. farinacea* associated bacteria could contribute to the recycling of nutrients in the senescent parts of lichen thalli through the supply of nutrients such as sugars, fatty acids, amino acids, and nucleotides. For this reason, the characterization of *R. farinacea* bacterial strains continued with the study of their hydrolytic potential. It was initiated using a selection of bacterial strains and the API-ZYM system that allows the detection of general enzymatic activities such as aminopeptidases, esterases, phosphatases, glycosidases, and proteases. Most of the strains exhibited various and diverse enzymatic activities, such as esterase, lipase esterase, leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, valine arylamidase, N-acetyl-β-glucosaminidase and the α-fucosidase. Therefore, some of these activities and additional ones were then evaluated by conventional systems using a wide range of substrates, some of them related to the lichen composition, in order to detect different polysaccharases, lipases, proteases, and nucleases. In general, a high percentage of bacterial strains inhabiting *R. farinacea* thalli showed one or more hydrolyses.

More than 60% of *R. farinacea* bacterial strains showed a remarkable polysaccharase activity, with several strains being able to degrade diverse heteropolysaccharides such as cellulose, chitin, pectin, starch, and xylan. Chitin is one of the major fungal cell wall components (García-Fraile *et al*., 2015) and cellulose and xylan are components of the cell wall of *R. farinacea* photobionts (Casano *et al*., 2015; König and Peveling, 1984; Olafsdottir and Ingólfsdóttir, 2001). The production of cellulases, chitinases, xylanases and other polysaccharases, could allow the degradation of these compounds in the older thallus parts, which could provide nutrients to the younger and growing parts of the thallus (Grube *et al*., 2015; Grube and Berg, 2009; Sigurbjörnsdóttir *et al*., 2016). Besides, growth areas in foliose and fruticose lichens as *R. farinacea*, are surrounded by cellulose and hemicellulose compounds, tree bark and another type of vegetation as mosses. Moreover, pectin is a component of plant tissues and could be exploited by bacteria associated with the *R. farinecae* populations studied, living on the bark of pines or oaks. In these cases, cellulases, pectinases, and xylanases might play an important role

in the lichen symbiosis, especially when a saprophytic activity could benefit this symbiotic state (Beckett *et al*., 2013; Palmqvist *et al*., 2008). Some of these polysaccharases have been also detected in bacterial strains in other lichen species trough functional metagenomic or culturable studies (Cardinale *et al*., 2006; Grube *et al*., 2009, 2015; Sigurbjörnsdóttir *et al*., 2014).

On the other hand, this hydrolytic potential might have different biotechnological applications. Amylases are important enzymes in paper, textile, detergent, and pharmaceutical industries, (de Souza and Magalhães, 2010; Rajagopalan and Krishnan, 2008; Reddy *et al*., 2003), as well as in fine-chemical and analytical chemistry industries, clinical, food, drinks, brewing, and distilling industries (Gupta *et al*., 2004; Kandra, 2003; Kumar, 2015; Pandey *et al*., 2000). In the literature, different bacterial species have been found to produce amylases, but in the industry, the main genera used is *Bacillus* (de Souza and Magalhães, 2010). Cellulases are also of relevant biotechnological interest for the production of bioethanol, biofuel and other types of sustainable energy derivatives from biomass and agricultural wastes (Koeck *et al*., 2014; Kumar *et al*., 2008; Vallejos, 2013). Some bacterial strains isolated from lichens and associated mostly to the phyla *Actinobacteria, Bacteroidetes, Firmicutes* and *Proteobacteria* were found as able to degrade cellulose, paper, xylan, and lignin (Cernava *et al*., 2017; Pankratov, 2012; Sigurbjörnsdóttir *et al*., 2014, 2015). Some examples are strains from the genera *Bacillus, Pseudomonas,* and *Microbacterium* (Himmel *et al*., 2010; Větrovský *et al*., 2014; Yang *et al*., 2014). Besides, xylanases are interesting as additives in livestock feed, as well as for the production of flours at industrial scale. Pectinases are also necessary for textile and food industry, as stabilizers and emulsifiers (Cerreti *et al*., 2016; Kumar *et al*., 2008), as well as in juice and wine production for improving organoleptic properties, increasing clarification, maceration and colour stabilizing during storage, or also for the conversion of pectin of waste materials to soluble sugars, ethanol and biogas (Kumar *et al*., 2008). Chitinases have also many applications in different fields, as in the waste management, treating chitinous derived products from the seafood industry, in the preparation of cell proteins and in the conversion of this chitinous waste in biofertilizers (Rathore *et al*., 2015; Sakai *et al*., 1998). As well as in the biocontrol of phytopathogenic fungi or as biopesticides against insects and pest, as an alternative to chemical control (Melchers and Stuiver, 2000). Finally, more recent uses are related to the chitinoligomers produced by chitinases as antitumor and antihypertensive agents (Rathore *et al*., 2015). Some bacterial genera with members with chitinolytic activity are *Bacillus, Chromobacterium, Erwinia, Pseudomonas, Serratia, Streptomyces* and *Vibrio* (Sigurbjörnsdóttir and Vilhelmsson, 2016; Stoykov *et al*., 2015). Among the bacterial strains isolated from *R. farinacea* in this study, many of them presented different hydrolytic polysaccharase activities, being assigned to the genera *Arthrobacter, Bacillus, Burkholderia, Curtobacterium, Erwinia, Frondihabitans, Kocuria, Microbacterium, Micrococcus, Methylobacterium, Mycobacterium, Pantoea, Pseudomonas, Sphingomonas, Stenotrophomonas, Streptomyces,* among others.

Moreover, around 30% of the *R. farinacea* bacterial strains analyzed produced lipases, which could also contribute to lichen symbiosis through lipid recycling, as described in other lichen species (Lee *et al*., 2014). The percentage of strains showing lipase activities was higher using Tween 20 than Tween 80, probably because the latest is a lipid derived from oleic acid, more complex and difficult to degrade than the lauric acid of Tween 20. In other lichen species,

some of the bacterial genera showing lipase activities were *Burkholderia, Deinococcus, Frondihabitans, Pseudomonas, Rhodanobacter, Sphingomonas* and *Subtercola* (Lee *et al*., 2014), some of them also detected in our study with lipase activities, as the ones assigned to the genera *Burkholderia* and *Pseudomonas,* or others as *Arthrobacter, Averyella, Bacillus, Curtobacterium, Kocuria, Methylobacterium, Microbacterium, Staphylococcus, Stenotrophomonas, Streptomyces,* etc. Lipases are very versatile and widely used enzymes in biotechnology as well, in a wide variety of food products, for instance, in cheese and cream production, in the acceleration of lipolysis and in fat and oil hydrolysis too (Navarro-González and Periago, 2012).

Lichenic bacteria could also provide nutrients by releasing amino acids from the proteins present in lichen thallus, reducing the cost of synthesizing new amino acids (Liba *et al*., 2006). Around 30% of the *R. farinacea* bacterial strains produced proteases, with a 31.14% of them being able to use gelatine as a substrate, and a 30.46% casein. Proteases represent an important group of industrial enzymes, being around the 60% of the total enzyme market (Sawant and Nagendran, 2014). Main uses are as detergent, in leather tanning, in food and pharmaceutical industries (Gupta and Khare, 2007; Kalpana *et al*., 2008). In addition, microbial proteases are increasingly used in the treatment of various human disorders as cancer, inflammation, cardiovascular disorders, necrotic wounds, etc. (Chanalia *et al*., 2011; Jisha *et al*., 2013). Furthermore, proteases can be used as immune–stimulatory agents (Biziulevičius, 2006) but also in several bioremediation processes (Sawant and Nagendran, 2014).

Extracellular DNA is a ubiquitous biopolymer in aquatic and terrestrial ecosystems which represents an important and convenient component that can be enzymatically modulated and utilized by bacteria for multiple purposes, such as in biofilm formation, as nutrient source, antimicrobial and in horizontal gene transfer (Dang *et al*., 2016; Seper *et al*., 2011; Vorkapic *et al*., 2016). In the *R. farinacea* bacterial strains tested, around 32% of them showed nuclease activity, which could be of application, for example, in the treatment of viral infections (Chen *et al*., 2014; Matousek *et al*., 1995) and in some types of cancer (Alcázar *et al*., 1995). Also for some biomedical processes as in the disruption, addition, and edition of genes (Pan *et al*., 2013).

Thereafter, the study was focused on the evaluation of the ability of the *R. farinacea* bacterial strains to fix nitrogen, solubilize phosphate and/or produce siderophores. These activities were selected because they have been related to the nutrient supply, being some of these elements essential for thallus growth but very limited (Palmqvist *et al*., 2008). Some studies have shown that in the case of large foliose and fruticose lichens as *R. farinacea*, their growth may be limited by nitrogen (Kurina and Vitousek, 1999; Palmqvist and Dahlman, 2006). Related to this, Liba *et al*. (2006) suggested that lichens lacking cyanobacteria could take profit of non-photosynthetic bacteria able to fix nitrogen. Grube and Berg (2009) shared this hypothesis, arguing that if non-photosynthetic bacteria were able to fix nitrogen and release nitrogenated compounds as amino acids, they could contribute to the nitrogen input of lichens. This might allow the lichen thallus to become independent of other external sources, where nitrogen availability could be limited, as in biogeochemical balanced forest systems (Makkonen *et al*., 2007), where this activity is of vital importance. In this work, most of the studied bacteria were able to fix nitrogen, around 93%, regardless of their geographical or lichenic origin.

Furthermore, some of the tested bacteria presented a characteristic mucous appearance due to the production of exopolysaccharides, which is related to the protection of the nitrogenase system from oxidation by oxygen (Sabra *et al*., 2000; Shrimant, 2012). The ability to fix this element has a relevant influence on the ecology of lichens, promoting the colonization of niches with special ecological features, as those oligotrophic in nitrogen (Büdel and Scheidegger, 2008). Furthermore, bacteria able to fix nitrogen could be used as plant-growth promoting bacteria to improve the productivity of crops such as some cereals, as maize and wheat (Oldroyd and Dixon, 2014; Souza *et al*., 2014), as environmentally friendly biofertilizers compared with chemical ones routinely used with a high impact.

On the other hand, around 50% of bacterial strains were able to solubilize inorganic phosphates. In nature, only few microorganisms are able to mobilize phosphate through its solubilization often by the production of organic acids (Ahemad and Kibret, 2014; Liba *et al*., 2006). Phosphate supposes an essential nutrient, and phosphate solubilizing bacteria could contribute to meet the requirements of this element in the lichen thallus. In a study by Hyvärinen and Crittenden (2000) with the lichen *C. portentosa,* it was shown that phosphorus was recycled from the senescing parts of the thalli to the growing apices. These authors suggested that it might exist a kind of source-sink of this element within the thallus, being an adaptation of this lichen species to places with phosphorus availability limitation. Furthermore, several strains isolated from various lichen species have been reported as phosphate-solubilizers (Grube *et al*., 2009, 2015; Liba *et al*., 2006; Sigurbjörnsdóttir *et al*., 2014), as in the lichen *Peltigera membranaceae*, where bacterial symbionts are known to participate in phosphate solubilization, which could be involved in algal growth promotion (Sigurbjörnsdóttir *et al*., 2015). Phosphatases could be used as biocatalyzers in the removal of toxic heavy metals (Chaudhuri *et al*., 2015) and play an important role in the bioremediation of industrial, municipal and nuclear wastewater (Chaudhuri *et al*., 2017).

Another growth limiting factor in lichens is iron, which usually is present in nature as insoluble hydroxide or oxyhydroxides forms. Some bacteria can produce and secrete high-affinity iron chelators, known as siderophores, when growing under low iron conditions (Ahemad and Kibret, 2014), thus making iron available for microorganisms able to use these siderophores. Furthermore, these iron chelators could create a stable complex with other elements as heavy metals such as aluminum, cadmium, lead, and zinc, among others (Ahemad and Kibret, 2014) which could reduce the toxicity of these metals to lichens. In our study, around 84% of *R. farinacea* bacterial strains were able to produce siderophores. Although strains able to acquire iron have been detected in some lichen species through metagenomic approaches (Cernava *et al*., 2017; Grube *et al*., 2015), this is the first time that the production of siderophores has been described in lichen-associated bacteria through culturable methods. Siderophores could be applied in a wide variety of fields, as for example, they could be used in agriculture to enhance plant growth and the weathering of soil minerals and in strategies of biocontrol (de Serrano, 2017). Also in bioremediation, as chelating agents in the mobilization of heavy metals and radionuclides, as well as in oil-contaminated environments due to the emulsifying effects of siderophores (de Serrano, 2017). Moreover, they have an important application in medicine for the treatment of iron overload which could become toxic by increasing oxidative stress (Ahmed and Holmström, 2014; Sampaio *et al*., 2014).

The use of combined metagenomic and metaproteomic approaches has allowed determine some potential functional roles of some bacterial orders associated to lichens. In this sense, Cernava *et al*., (2017) found genes related to nitrogen metabolism in the orders *Chthoniobacterales* and *Rhodospirillales* in the lichen *L. pulmonaria.* Functional genes related to the phosphate metabolism were also assigned to *Proteobacteria*, mainly to *Myxococcales,* while those involved in iron metabolism were detected in *Sphingobacteriales* and *Sphingomonadales* (Cernava *et al*., 2017).

In general, most of the bacterial strains isolated from *R. farinacea* were able to fix and/or solubilize nutrients, regardless their geographical or lichenic origin. Thus, suggesting that they could favour and/or allow the growth of this lichen species under nutrient-limited conditions prevailing in the environment where they grow, as the bark of trees, by supplying these nutritional requirements.

Based on the results of the different activities of *R. farinacea* bacterial strains studied related to the nutrient recycling and supply, a selection of the most active ones was made to continue with their characterization through the study of their potential ability to stimulate lichen growth by producing phytohormones and/or to form biofilms, two features barely studied of lichen-associated bacteria but very relevant for lichen symbiosis.

Recent studies have reported that plant hormones could stimulate lichens growth (Erlacher *et al*., 2015; Grube *et al*., 2015; Sigurbjörnsdóttir *et al*., 2016). In this study, the production of the phytohormone auxin, indole acetic acid (IAA), was investigated among the *R. farinacea* bacterial strains. The results revealed that most of the bacterial strains tested were able to produce IAA in different concentrations, reaching values after 72 h from 0.0027 µg/ml to 100.62 µg/ml. Since some of the tested strains showed a slow growth rate, the number of bacteria able to produce this auxin increased over time. Several microorganisms have been found to synthesize IAA, an auxin identical to that one found in plants, as in *Pseudomonas* (Costacurta and Vanderleyden, 1995), *Pantoea* (Beattie and Lindow, 1999), *Acinetobacter* (Huddedar *et al*., 2002), *Stenotrophomonas maltophilia* (Park *et al*., 2005), *Serratia* (Liba *et al*., 2006), and members of the order *Rhizobiales* (Erlacher *et al*., 2015; Grube *et al*., 2015). In the lichen *L. pulmonaria, Proteobacteria* was the main group with members identified as potential producers of auxin (Cernava *et al*., 2017). In this study, IAA was detected in strains assigned to genera as *Arthrobacter, Bacillus, Burkholderia, Curtobacterium, Erwinia, Kocuria, Leifsonia, Methylobacterium, Micrococcus, Nocardioides, Pseudomonas, Stenotrophomonas* and *Streptomyces,* among others.

Ethylene is an essential hormone for growth and plant development (Ahemad and Kibret, 2014). Thus, an assay was conducted to detect the ACC deaminase enzyme that deaminates the ethylene precursor, 1-aminocyclopropane-1-carboxylate acid (ACC). There is a relation between the ACC deaminase and the auxin IAA. Bacteria producing these two hormones can promote plant growth increasing plant height, biomass and root length. Furthermore, they may exert a protective effect against some abiotic stresses, such as desiccation, salinity, metals and other pollutants (Esquivel-Cote *et al*., 2013; Glick, 2014; Shahzad *et al*., 2013). In our case, despite most of the tested bacterial strains produced auxins, the molecular detection of the ACC deaminase was not as expected, with only an 18.54% of the strains being positive, mostly ectolichenic. Interestingly, this is the first study reporting this activity in lichenic bacteria.

Besides, despite the gene codifying for the ACC deaminase enzyme was detected in a low percentage of the *R. farinacea* bacterial strains tested, this is not the only way bacteria can stimulate plant growth. Different activities act synergistically, as the uptake and nutrient mobilization and phytohormones production (Ahemad and Kibret, 2014; Glick, 2014). Therefore, these strains still could be interesting for plant growth promotion. Ethylene can be produced by some microorganisms and has influence in many plant processes as seed germination, in senescent organs and in plants responses to some stresses (Liba *et al*., 2006). Some bacteria able to produce ethylene are *S. maltophilia*, *S. marcescens* and some *Pseudomonas* species (Berner *et al*., 1999). In this study, some bacterial strains detected with the gene for the ACC deaminase were assigned to the genera *Erwinia* and *Pseudomonas.*

Bacterial phytohormones can influence plant growth but the production of some of them as IAA (Grube *et al*., 2009; Liba *et al*., 2006), ethylene and others acting as signaling molecules could also influence the morphogenetic processes in lichens and their symbionts (Grube and Berg, 2009). The detection of *R. farianacea* bacterial strains able to produce phytohormones gives support to the potential relationship between these bacteria and the holobiont growth (Grube and Berg, 2009). Previously, some bacterial species belonging to the taxa *Acinetobacter calcoaceticus, Pantoea sp., P. stutzeri, S. maltophilia* and *S. marcescens,* associated with the lichens *Canoparmelia caroliniana*, *C. crozalsiana*, *C. texana*, *Parmotrema sanctiangeli* and *P. tinctorum*, were reported to produce IAA, and some of them producing ethylene as well (Liba *et al*., 2006), also in the lichen species *C. arbuscula, L. polytropa and U. cylindrica*, where a 21% of the bacterial strains isolated showed the ability to produce IAA (Grube *et al*., 2009). Besides, these strains could be used as biofertilizers to replace synthetic agrochemicals, which have a relevant negative environmental impact on soils, waters and different ecological systems. Therefore, the use of biofertilizers or a combination of both approaches could contribute reducing the impacts of agrochemical compounds (Bhardwaj *et al*., 2014; García-Fraile *et al*., 2015; Shahzad *et al*., 2013).

By the use of microscopic techniques, it has been observed that lichens are colonized by bacterial communities growing in aggregates and/or biofilms (Cardinale *et al*., 2008; Erlacher *et al*., 2015; Grube *et al*., 2009). However, the ability of lichen culturable bacteria to form biofilms has not been yet explored. Therefore, biofilm formation was determined with the *R. farianacea* bacterial strains. Almost a 100% of the tested strains were able to produce biofilms, most of them in a strong way (69.9% with a strong biofilm production, 24.6% moderate, 4.6% weak). These results are in accordance with the observation of bacterial aggregates in *R. farinacea* thalli collected in different points of the north of Spain (García-Breijo *et al*., 2010). This could be related to the ability of lichens to colonize environments with specific and extreme conditions, as well as to increase nutrients uptake (Grube *et al*., 2015; Grube and Berg, 2009; Koczan *et al*., 2009). In this sense, in the lichen *Xanthoparmelia mexicana* microscopic studies described that bacterial cells able to fix nitrogen appeared in aggregates forming biofilms, which increases the nitrogen supply with respect to single bacterial cells (Gayathri *et al*., 2014).

In some lichens, bacterial biofilms are thought to be involved in their attachment to surfaces and, in some cases, these biofilms seem microhabitats with a high diversity of microorganisms interacting among them (de los Ríos *et al*., 2002). Biofilms may play different functional roles in

microbial communities, as protection against environmental stresses, restricting the diffusion of some molecules and compounds from the surrounding area into the biofilm (Donlan and Costerton, 2002; Koczan *et al*., 2011); allowing nutrient availability via water channels that provide nutrient and metabolites exchange and the removal of potentially toxic metabolites (Koczan *et al*., 2011). Furthermore, they provide an environment for syntrophic relationships and permits the horizontal gene transfer, very important for the evolution of microbial communities (Kokare *et al*., 2009). All of these functions might be important in the maintenance of the lichen bacteria association, as well as in the stabilization of the lichen thallus. Bacteria able to form biofilms in nature are embedded in an extracellular matrix of exopolymers, extracellular polymeric substances, consisting of polysaccharides, proteins, nucleic acids and lipids (Burmølle *et al*., 2014; Hori and Matsumoto, 2010). In this study, it was observed that several *R. farinacea* strains were able to produce exopolysaccharides (some levane-type, data not shown). The exopolysaccharides forming the biofilm have different applications and they could be exploited in some industrial sectors. For instance, in the pharmaceutical field, for tissue engineering or as new anticancer drugs or additives, in the food industry, as additives or prebiotics as well as in environmental protection as emulsifiers for oil pollution recovery or as chelators for toxic metals removal, etc. (Berlanga and Guerrero, 2016; Di Donato *et al*., 2016).

Biofilm formation is a process with distinct phases, including planktonic (free-swimming), attachment, mature biofilm and detachment (Berlanga and Guerrero, 2016; Sauer *et al*., 2002). Motility of bacterial cells is necessary for the first stages of biofilm formation, to reach surfaces suitable for the biofilm establishment but also to move and expand with the biofilm (Bak *et al*., 2015a; Flemming and Wingender, 2010; Houry *et al*., 2010; Ryder *et al*., 2007). Thus, the motility of *R. farinacea* bacterial strains was also investigated. Some examples of motile bacteria in lichens are *Chtoniobacterales, Myxococclaes, Rhodospirillales* and *Sphingobacteriales* (Cernava *et al*., 2017), and some of the species are *B. subtilis, E. coli, P. aeruginosa, Rhizobium etli* and *S. liquefaciens,* among others (Kearns, 2010; Kearns and Losick, 2003; Verstraeten *et al*., 2008). Many of the bacterial strains associated with *R. farinacea* biofilm producers were taxonomically assigned to members of the genera *Arthrobacter, Averyella, Burkholderia, Erwinia, Frondihabitans, Methylobacteria, Pseudomonas, Sphingomonas* and *Staphylococcus,* among others. The results showed that around 70% of the tested bacterial strains presented swimming motility, while the percentage of strains with swarming motility was not as high, but still some of them (5.6%) presented this type of motility. Swarming depends on the interaction of several parameters, such as agar concentration, incubation temperature, cell density, and nutrient-rich medium, being some of them critical for surface migration (Tambalo *et al*., 2010). Thus, it cannot rule out that by modifying the conditions we had detected more positive strains or that they express this motility in nature. In fact, swimming and swarming are two types of motility important for surface colonization in natural habitats, allowing bacteria to reach open surfaces in animal or plant tissues. Nonetheless, the production of biofilms was not limited to the strains with motility. In fact, other surface structures such as pilis or fimbria could also be involved in biofilm formation (Bak *et al*., 2015b; Koczan *et al*., 2011), but their role remains to be elucidated in lichenic bacteria. Interestingly, many of the bacteria isolated from the different populations of *R. farincea* produced exopolysaccharides, which are also involved in biofilm

formation (Balsanelli *et al*., 2014; Koczan *et al*., 2011; Limoli *et al*., 2015). The ability of these bacteria to produce biofilms could have several applications as in wastewater treatments (Miura *et al*., 2007), groundwater bioremediation (Castro and Tufenkji, 2007), and even in biomedicine (Tenke *et al*., 2006). It could be also of interest in some plant growth promoting bacteria, known as biofilmed biofertilizers (BFBFs), since plant-associated nitrogenase activity, rhizoremediation, plant and soil carbon sequestration, and plant growth-promoting activities are enhanced by these biofilms (Malusá *et al*., 2012; Seneviratne *et al*., 2009).

The methodology used for the molecular identification of a selection of bacterial strains associated with *R. farinacea* was made according to their different physiologic and metabolic characteristics and because of their biotechnological interest. The identification gave a presumptive assignment of the selected bacterial strains and revealed that these were related to different bacterial taxa. Based on the similarity of the partial sequences of *16S rRNA* gene through a BLASTnt, different genera and species were initially assigned, as for example *B. megaterium, B. subtilis, Burkholderia sordidicola, Curtobacterium flaccumfaciens, Erwinia* sp., *Kocuria rhizophila, Leifsonia poae, Nocardioides* sp., *Pantoea agglomerans, P. koreensis, P. rhizosphaerae, S. pasteuri, Stenotrophomonas* sp., *Streptomyces* sp., etc., some of them could be new species.

Overall, these results of the extensive characterization of culturable bacterial strains of *R. farinacea* from different geographical and lichenic origins carried out in this study, are an example of the complex and functionally diverse bacteria associated with lichens. These bacteria may play numerous and diverse functional roles, providing nutrients to the host or growth regulating factors, as well as protection by forming biofilms, thus showing the importance of these bacterial activities for the lichen symbiosis, as pointed out for bacteria from other lichen species but mainly by culture-independent methods.

Finally, as shown by Grube *et al*. (2015), despite the culturable bacterial fraction represents a minor percentage of all bacteria associated with lichens, which makes necessary the use of omic techniques in combination with bioinformatics to carry out complete characterization of these bacterial communities, working with culturable bacteria have several advantages since it allows the study of microbial interactions within the lichen thallus, as well as the exploration of the diverse biotechnological potentials of these bacteria and the description of new bacterial taxa. Although undoubtedly a holistic approach combining dependent and independent culture techniques is the most accurate way to approach the analyses of these abundant and diverse lichenic bacterial communities with a wide spectrum of biotechnological applications, still to explore.

Diversity and composition of culturable bacteria associated with *R. farinacea*

In this study, the diversity and composition of culturable bacteria associated with the different populations of *R. farinacea* analyzed, as well as the potential influence of factors as geography and/or the location in the lichen thallus on the structure of these bacteria (beta diversity), was investigated. The results have revealed new data on the diversity and composition of bacteria associated with *R. farinacea*, as well as that the geography was the main factor determining the bacterial communities on the four lichen populations studied. In addition, the location ecto- or endolichenic in the lichen thallus also had an influence on the bacterial structure,

which could be related to their functional roles in the lichen symbiosis. In this sense, these results obtained in this work suggest that bacteria present in lichens are not an extension of the microbiota, but they are specifically associated with each lichen species and/or lichen population. The bacterial diversity found in the four populations of *R. farinacea* as well as in their inner or outer thallus fractions indicates that geographical and lichenic origin shape these bacterial communities. Biotic and abiotic factors may have an additional influence on them, as well as the functional roles that those bacteria may play under the conditions imposed by these factors. In other studies, in which the environment nearby lichens (soil, bark trees, etc.) was considered, it was found that the specific structure of lichenic bacterial communities was different to that one found around them (Aschenbrenner *et al*., 2017; Bates *et al*., 2011; Bjelland *et al*., 2011).

As a first approach, the alpha diversity of culturable bacteria associated with *R. farinacea* populations from different geographical origins was analyzed. When this bacterial diversity was investigated by grouping lichen populations by their insular or peninsular origin, the highest diversity indices (although with small differences) were found in the Island, with a higher number of bacterial species and more evenly represented. At a small geographical scale, when bacterial diversity was studied taking into consideration the origin of the four populations of *R. farinacea* studied, the ones with the highest diversity indices were Lidón (from the Peninsula) and La Esperanza (from the Island), followed by those from La Guancha (from the Island). The *R. farinacea* population showing the lowest indices' values was the one from El Toro (from the Peninsula). Despite the differences observed, the bacterial diversity values of the lichen populations from the Island (La Guancha and La Esperanza) were very similar. The opposite happened with the lichen populations from the Peninsula (El Toro and Lidón), where the differences in these same indices' values were more notable.

R. farinacea bacterial strains were assigned to different genera and species, all of them classified in one of the three phyla identified. The main phylum was *Proteobacteria* (76%), followed by *Actinobacteria* (12.2%) and *Firmicutes* (9.5%). The proportion of these phyla was maintained in the Island and the Peninsula, as well as when studied the diversity composition in lichens populations from La Guancha, La Esperanza, and El Toro. However, in the case of Lidón, *Actinobacteria* predominated over *Proteobacteria*. In other lichen species from the genera *Caloplaca, Cetraria*, *Cladonia*, *Hyrdopunctaria, Lecanora, Lichinia*, *Ochrolechia*, *Psoroma*, *Roccella*, *Stereocaulon*, *Umbilicaria*, *Usnea*, *Verrucaria* or *Xanthoria*, these three phyla were found as the main ones among culturable bacteria, being present also the phyla *Deinococcus-Thermus* and *Bacteroidetes* in some of them (Lee *et al*., 2014; Liba *et al*., 2006; Parrot *et al*., 2015; Selbmann *et al*., 2010; Sigurbjörnsdóttir *et al*., 2014), that were absent in our culturable fraction.

Based on molecular studies performed in other lichen species, *Alphaproteobacteria* was expected to be the predominant class among the bacteria associated with lichens (Bates *et al*., 2011; Bjelland *et al*., 2011; Cardinale *et al*., 2008; Schoch *et al*., 2009). In the culturable bacteria obtained from *R. farinacea*, the main class in the lichen populations from the Island was *Gammaproteobacteria*. However, when compared between the two insular populations, in the case of La Esperanza, the main class was *Alphaproteobacteria.* In the Peninsula, the main class was *Alphaproteobacteria,* although again, in one of the peninsular *R. farinacea*

populations the predominant class was *Actinobacteria*. Other classes identified, although in less proportion, were *Bacilli* and *Betaproteobacteria*. In other lichen species from other latitudes, different bacterial classes were obtained among the isolates, as *Flavobacteria*, *Cytophagia* or *Sphingobacteria* (Sigurbjörnsdóttir *et al*., 2014). Members of the *Alphaproteobacteria* are known to participate in symbiotic relationships in lichens (Bates *et al*., 2011; Cardinale *et al*., 2008; Hodkinson and Lutzoni, 2009). Among these bacterial group, nitrogenases are known to be ubiquitous, suggesting their participation in the nitrogen fixation (Grube and Berg, 2009). In other cases, members of the *Gammaproteobacteria* were found in other lichen species as *C. caroliniana, C. crozalsiana, C. texana, P. sanctiangeli* and *P. tinctorum,* being able to fix nitrogen (Liba *et al*., 2006).

The main bacterial orders identified in this study were *Rhizobiales, Micrococcales, Enterobacteriales, Pseudomonadales, Propionibacteriales, Burkholderiales, Bacillales* or *Streptomycetales*. Among these, *Rhizobiales* has been proposed as a group that contributes to the lichen symbiosis by providing nitrogen (Hodkinson and Lutzoni, 2009). It was the predominant order in the *R. farinacea* population from El Toro, and one of the main ones in the population from Lidón (both being peninsular). This order was found to be the most ubiquitous in other lichen species as *U. esculenta, Parmelia omphalodes* and *L. retigera* (Jian *et al*., 2017). Moreover, members of this group were reported to be particularly abundant in lichens, with potential symbiotic functions, as in nutrient cycling or by providing some secondary metabolites (Erlacher *et al*., 2015; Grube *et al*., 2015). *Micrococcales* was the main order in the *R. farinacea* populations from La Esperanzan and Lidón, while in La Guancha was *Enterobacteriales*. Another relevant order was *Sphingomonadales,* the second main group in La Esperanza. Members of this group are heterotrophic bacteria quite abundant and commonly present in nature, usually in soils and aquatic environments (Aschenbrenner *et al*., 2017; Cavicchioli *et al*., 2003; Kersters *et al*., 2006; Notomista *et al*., 2011). These bacteria are characterized by their ability to grow in oligotrophic environments (Cavicchioli *et al*., 2003) as well as to degrade diverse carbon sources (Sigurbjörnsdóttir *et al*., 2014). Further, through metagenome studies, it has been suggested that members of this group may promote the growth of their hosts by the production of hormones, with other potential functions such as phosphate solubilization, ammonia assimilation and oxidative stress responses (Aschenbrenner *et al*., 2017).

Bacterial families were dominated by *Microbacteriaceae, Methylobacteriaceae, Erwiniaceae, Pseudomonadaceae, Burkholderiaceae, Enterobacteriaceae*, *Sphingomonadaceae* and others as *Bacillaceae, Nocardioidaceae* or *Streptomycetaceae.* Some of these families are recognized to be of biotechnological interest due to their production of bioactive molecules (Suzuki *et al*., 2016). In this study, the final proportion of some of these families in the culturable fraction ranged from 4% to 20%, while in other studies the families as *Burkholderiacea* might represent at a maximum 1% of the lichen bacterial communities, being others about 10 to 100 times less abundant (Suzuki *et al*., 2016). These differences could be due to the fact that in our study lichen enriched media was used for the isolation of bacteria from *R. farinacea*, while nutrient-rich media has been reported to reduce the number of lichenic bacteria recovered (Biosca *et al*., 2016).

Some of the genera found among the bacterial strains recovered from the four populations of *R. farinacea* were *Arthrobacter, Averyella, Bacillus, Burkholderia, Curtobacterium, Enterobacter, Erwinia, Frondicola, Kocuria, Micrococcus, Methylobacterium, Nocardiodes, Rhodococcus, Roseomonas, Sphingomonas, etc.* Species of *Arthrobacter* have been found in other lichen species from the Antarctica, as *U. antarctica* (Cardinale *et al*., 2006). Others, as *Burkholderia*, with species such as *B. sordidicola*, seem to be common in the bacterial culturable fraction of other lichen species (Grube and Berg, 2009), although members of this genus appeared in the groups of bacteria associated with non-lichenized fungi as well (Bertaux *et al*., 2005; Bianciotto *et al*., 2000; Lim *et al*., 2003; Yara *et al*., 2006). However, *Burkholderia* was not found in Antarctic lichens, which had more abundance of psychrotolerant bacterial species (Selbmann *et al*., 2010). In the populations of *R. farinacea* studied, this genus was absent in the case of El Toro, where a predominance of species of *Methylobacterium* was found, which participate in nitrogen fixation and oxidative stress tolerance, carbon metabolism, etc. (Erlacher *et al*., 2015), some activities tested in this work.

Overall, many of the bacterial genera found in the populations of *R. farinacea* studied were common to those found in other investigations based on culturable methods with other lichen species, as *C. arbuscula, Collema auriforme, L. polytropa, Lichina confinis, L. pygmaea, Roccella fuciformis* and *U. cylindrica,* with some bacterial genera known to produce bioactive compounds as *Acinetobacter, Bacillus, Burkholderia, Curtobacter, Frondicola, Leifsonia, Luteibacter, Methylobacterium, Microbacterium, Micrococcus, Mycobacterium, Nocardioides, Paenibacillus, Pseudomonas, Sphingomonas and Streptomyces* (Grube and Berg, 2009; Parrot *et al*., 2015). In the populations of *R. farinacea* studied, some of the bacterial genera appeared to be ubiquitous, as *Bacillus* and *Sphingomonas*, while others were present in three of the four populations, as *Burkholderia, Erwinia, Curtobacterium, Kocuria* or *Methylobacterium*. Others were found from only one of the lichen populations, as *Arthrobacter, Averyella, Enterobacter, Frondicola, Massilia, Micrococcus, Microlunatus, Nocardiodides, Rhodococcus, Roseomonas, Sanghibacter,* etc. This tendency was observed in other studies, were some genera were found to be common on other lichen species, as *C. arbuscula, L. polytropa* and *U. cylindrica*, as *Acinetobacter, Bacillus, Burkholderia* and *Paenibacillus*, while others were less abundant, as *Frondicola, Luteibacter* or *Methylobacterium*, or identified only once, as *Pseudomonas* and *Leifsonia* (Grube *et al*., 2009).

The presence of diverse culturable bacteria in *R. farinacea*, together with few previous similar studies in other lichen species, have revealed lichens as a novel source of bacterial strains with different biotechnological potentials, as *Bacillus, Pseudomonas*, *Burkholderia* (Suzuki *et al*., 2016), or *Sphingomonas*, which is known to be involved in different pathways of aromatic compounds metabolism, as well as to have genes related to nitrogen fixation and iron acquisition (Aschenbrenner *et al*., 2017), as also shown in this work. Among the *Actinobacteria*, different strains were recovered in this study, belonging to the genera *Curtobacterium, Friedmaniella, Frondihabitans, Leifsonia, Microbacterium, Micrococcus, Nocardioides* and *Streptomyces,* among others. In other lichen species, the most abundant genera were *Micromonospora* and *Streptomyces* (González *et al*., 2005), or *Curtobacterium, Microbacterium, Micrococcus, Mycobacterium*, *Nocardioides* and *Streptomyces*, all of them with strains known to produce bioactive compounds, including exopolysaccharides, anthracyclines or enediyne, among others (Parrot *et al*., 2015). Lichen colonization by

heterotrophic bacteria that produce enzymes able to degrade macromolecules may benefit lichens exposed to oligotrophic environmental conditions (Lee *et al*., 2014; Liba *et al*., 2006).

Among bacterial strains isolated from *R. farinacea*, many of them were able to produce bioactive compounds and enzymes of biotechnological interest, such as lipases and proteases. However, in other studies, these hydrolytic activities were only detected through molecular techniques. In the present study, a wide variety of *R. farinacea* bacterial strains showed different hydrolytic activities and others related to the nutrient supply and growth promotion.

Further, our data revealed that culturable bacteria isolated from *R. farinacea* populations from four different geographical locations in Spain presented differences in their composition, which might be influenced by the environmental conditions as climate, UV radiation, growth substrate (i.e. bark tree), near vegetation and others abiotic and biotic factors. Similar differences have been reported in other lichen species, as in *Cetraria aculeata*, in which differences were reported between the alphaproteobacterial communities of high latitudes (more depauperated and closely related to each other) than in those of extrapolar habitats (Printzen *et al*., 2012). This is in agreement with findings for the fungal and algal partners as well (Fernández-Mendoza *et al*., 2011). Moreover, in *C. aculeata*, the two polar bacterial communities were more similar to each other than to the two temperate ones (Printzen *et al*., 2012). Something similar was reported by Cardinale *et al*. (2012a) with populations of the lichen *L. pulmonaria* collected from four different locations around Europe when their bacterial communities were studied through FISH-CLSM and PCR-SSCP. They found that the bacterial groups were differentially shaped by the geography and the habitat (Cardinale *et al*., 2012a).

Besides, Lee *et al*. (2014) proposed that lichen-forming fungi produce diverse secondary metabolites that provide a selective environment which determines the phylotypes of bacterial partners so as to promote survival in different environmental conditions. These results could agree with some hypotheses about that symbiotic lifestyle may increase the evolutionary potential of the symbiotic holobiont by the adaptation of the other symbionts to ecological variations and conditions, in which the lichen host transfers parts of its stress response to microbial partners and is able to better adapt to environmental changes by a habitat-adapted symbiont association (Gilbert *et al*., 2010; Rodriguez *et al*., 2008). In other symbiotic systems bacterial communities are known to vary in response to environmental factors as well, as it occurs in corals due to heat stress, or in insects due to variations in the diet (Feldhaar, 2011; Glasl *et al*., 2016; Littman *et al*., 2010).

When the diversity of culturable bacteria associated with the ecto- and endolichenic fractions of *R. farinacea* was studied, the results showed that the main phyla were *Proteobacteria, Actinobacteria,* and *Firmicutes*. For the ectolichenic fraction, *Proteobacteria* was the predominant group (69.57%), but in the endolichenic one, both *Proteobacteria* and *Actinobacteria* showed similar abundances (43.21% and 48.23%, respectively). The main class in the ectolichenic fraction was *Alphaproteobacteria* while *Actinobacteria* was in the endolichenic one. The orders were mainly represented by *Rhizobiales, Enterobacteriales, Pseudomonadales, Micrococcales, Bacillales,* and *Sphingomonadales* in the ectolichenic fraction, while *Micrococcales, Enterobacteriales, Burkholderiales* and *Propiobibacteriales* were the most frequent in the endolichenic one. With regard to bacterial families, the *R. farinacea*

bacterial strains belonged to families such as *Methylobacteriaceae, Pseudomonadaceae, Erwiniaceae, Burkholderiaceae, Sphingomonadaceae, Nocardioidaceae, Bacillaceae,* etc. without significant differences between their presence in the ectolichenic and endolichenic fraction, although some differences were found in some cases, as for example, *Burkholderiaceae* and *Micrococcaceae* were more abundant in the endolichenic fraction, and *Methylobacteriaceae* were more predominant in the ectolichenic one. Although many genera were present both inside and outside of the lichen thallus, some others were differentially distributed, many of them belonging to *Actinobacteria*. De Los Ríos *et al*. (2005) using samples of the lichen *Lecidea*, found similar results, while in other studies, as one conducted with Antarctic lichens, no differences were found among the bacterial genotypes from either the surface or the inner part of the lichen thallus, finding distinct bacterial phenotypes only from different lichen thallus (Selbmann *et al*., 2010). In other cases, as with littoral lichens (Parrot *et al*., 2015), members of *Pseudonocardiaceae* were isolated only from the ectolichenic fraction. These authors proposed that bacterial communities associated with lichens might show a specific distribution in the lichen thallus. In a recent study conducted with *L. pulmonaria*, some differences were found among the upper and lower surface of the lichen thallus, suggesting that each surface might facilitate the colonization of distinct bacteria due to dissimilar microclimatic conditions in both sides (Aschenbrenner *et al*., 2017) which partially agree with our results with *R. farinacea* bacterial strains.

In summary, despite the diversity study of *R. farinacea* culturable bacteria represents a small fraction of the wide variety of bacteria associated with this lichen species, the results obtained provided for the first time data on the diverse and abundant culturable bacteria recovered from this lichen by using culture media enriched with *R. farinacea* extracts. Moreover, the differences found among the culturable bacteria associated with *R. farinacea* populations from four different geographical locations might suggest that these bacteria are shaped by geography, despite members of *Proteobacteria (Gammaproteobacteria* and *Alphaproteobacteria)* and *Actinobacteria* were the most common phyla found among the bacterial isolates of *R. farinacea*. In addition, the differences observed in the bacterial taxa regarding their ecto- or endolichenic origin, also suggests different microclimatic conditions in different fractions of the *R. farinacea* thallus. Furthermore, some of the isolated bacterial strains associated with this lichen could be new species or genera.

Diversity and composition of bacteria associated with *R. faraincea* through non-culturable techniques

To complete the study of the diversity and composition of the bacterial communities associated with *R. farinacea,* thalli samples from the four different geographical locations in Spain were further analyzed through a non-culturable approach. Moreover, we also investigated if such diversity could be different between the ecto- and endolichenic fractions of the lichen thallus, or along the different parts of the thallus (apical, middle and basal), or even change by applying a disinfection treatment often used in this kind of studies.

Firstly, a study of the beta diversity, which indicates the influence of geography and/or location factors, among others, on the bacterial structure in lichen thallus, was carried out. The

main factor that appeared to influence the bacterial composition was the geographical location, either by grouping *R. farinacea* thalli samples according to their insular or peninsular origin or considering their four different geographical locations. In fact, the bacterial composition of the populations of *R. farinacea* was significantly different among the four different Spanish geographical locations with a Mediterranean climate but with different conditions, as the bark trees where lichen thalli were growing, the percentage of relative humidity at the moment of sampling or the temperature. The second main factor that influenced the bacterial composition was the ecto- or endolichenic location of these bacteria in the lichen thallus. These results were similar to those obtained in the beta diversity study of the bacterial culturable fraction of *R. farinacea* performed in this work. Nonetheless, the composition of the bacterial communities was apparently not affected by their position along the different parts of the lichen thallus, either apical, middle or basal, neither by the disinfection treatment applied.

The study of the alpha diversity, which indicates the intrinsic biodiversity in each one of the lichen populations, in bulk thalli samples, indicated that bacterial communities associated with *R. farinacea* populations from the Peninsula were more diverse than those from the Island, with a higher number of different species and more evenly represented. When this diversity was studied considering the four different populations of *R. farinacea*, it was found that the lichen population from El Toro was the one with the highest values in the diversity indices studied (Richness, Shannon and Simpson), followed by the populations from Lidón and La Guancha. The population from La Esperanza was the one with the lowest diversity. These results were different from those obtained in the bacterial culturable fraction isolated from *R. farinacea*, since thalli samples from both the Island and the Peninsula showed very similar diversity indices, only being slightly higher in the Island. When considering the four lichen populations analyzed from the different geographical locations in Spain, the one with the highest diversity indices was La Esperanza, while the one with the lowest indices values was El Toro. These results were opposite to the ones obtained using culturable techniques. An appreciation about these results should be made, since in the case of the study of the alpha diversity using culture independent techniques, Richness index showed values ranging between 4 and 5, and Shannon index between 2 and 4. The values obtained with the bacterial culturable fraction were ranged between 2 and 3, and 1.50 and 2.50, respectively. These differences mean that, as expected, the diversity results obtained using molecular approaches was higher than using culturable ones, as well as that these *R. farinacea* populations showed a high bacterial diversity. In this sense, the values of diversity for the bacterial culturable fraction were, in a rough way, around the half of those obtained by using culture-independent techniques, which suppose a good approximation of the diversity considering the difficulties in culturing bacteria.

Despite the influence of the abovementioned factors on the bacterial composition of the populations of *R. farinacea* studied and the differences in the diversity indices, the lichen populations from the Island (La Guancha and La Esperanza) and the Peninsula (El Toro and Lidón) shared the main taxonomic groups, which were *Proteobacteria*, *Acidobacteria* and *Planctomycetes*, appearing in less abundance other taxa such as *Bacteroidetes*, *Cyanobacteria*, *Firmicutes* or *Verrucomicrobia*. When comparing with the results obtained with the bacterial culturable fraction among the four populations of *R. farinacea*, the main phyla were

Proteobacteria, Actinobacteria and *Firmicutes.* These results also confirm previous studies in other lichen species reporting that these organisms can harbour a wide variety of bacterial taxa (Bates *et al*., 2011; Cardinale *et al*., 2008; Grube *et al*., 2015; Suzuki *et al*., 2016). Besides, it has been reported that *R. farinacea* could have some ecophysiological plasticity thanks to some of the photobionts partners that could allow them to adapt to ecologically diverse environments. Furthermore, several studies have reported the combined influence of other symbionts, as bacteria, which may also contribute to lichen adaptive response to different environmental contexts by improving its survival under different biotic and abiotic stresses along the lichen adaptation (Aschenbrenner *et al*., 2014; Cernava *et al*., 2015a, 2017; del Campo *et al*., 2013; Eymann *et al*., 2017; Parrot *et al*., 2016; Schneider *et al*., 2011). These could be related to the differences observed in bacterial diversity among the populations of *R. farinacea* from geographical origins and the given conditions in those locations.

Some of the minority bacterial phyla found associated with the *R. farinacea* populations under study, as *Verrucomicrobia*, were also reported in the lichen *L. pulmonaria*, that harboured bacterial members involved in: i) the degradation of various complex polysaccharides as cellulose and xylan (Grube *et al*., 2015; Herlemann *et al*., 2013); ii) the metabolism of aromatic compounds; iii) the production of vitamins; and iv) the defense against antibiotics and oxidative stress (Cernava *et al*., 2017).

Interestingly, significant differences in all taxonomic levels were observed when comparing the bacterial communities from each one of the four populations of *R. farinacea* studied, being particularly remarkable the different abundance of *Cyanobacteria* and *Firmicutes*. Members of both taxa were more numerous in the lichen thalli collected in the island of Tenerife than in those from the Iberian Peninsula. Cyanobacterial photobionts, that in some cases are coexisting in tripartite lichens together with the green-algal photobionts, might share with algae the functions of fixing nitrogen and carbon (Grube *et al*., 2015; Hodkinson *et al*., 2012). The lower number of cyanobacterial members in lichen thalli samples from the Iberian Peninsula could be compensated by non-photosynthetic bacteria able to fix nitrogen, as reported in other lichen species (Bates *et al*., 2011; Liba *et al*., 2006). In fact, Grube *et al*. (2015) proposed that nitrogen fixing bacteria play a role in the delivery of nitrogen to the main symbiotic partners (mycobiont and photobiont), and that this may be particularly important in lichens without nitrogen-delivering cyanobacteria. One of the classes that harbors a wider diversity of bacteria able to fix nitrogen is *Alphaproteobacteria*, as previously found in other lichen species such as *L. pulmonaria, P. sulcata*, *Rhizoplaca chrysoleuca*, *U. americana* and *U. phaea* (Bates *et al*., 2011; Cardinale *et al*., 2012a; Erlacher *et al*., 2015; Eymann *et al*., 2017).

Results through metaomic and sequencing techniques, which allowed the identification of the bacterial microbiota associated with lichens, suggested that *Alphaproteobacteria* dominated these microbial communities (Aschenbrenner *et al*., 2014; Eymann *et al*., 2017; Grube *et al*., 2015; Hodkinson, 2011; Hodkinson *et al*., 2012; Schneider *et al*., 2011). This class was also found in this study as the most abundant one among *Proteobacteria* in *R. farinacea.* Other classes found in high proportion in the lichen populations studied were *Acidobacteria* (more abundant in the *R. farinacea* populations from the Island than in those from the Peninsula), *Betaproteobacteria*, *Planctomycetia, Gammaproteobacteria, Bacilli, Sphingobacteria* and *Citophagia.* By contrast, the main classes identified among the culturable bacteria isolated

from *R. farinacea* were *Gammaproteobacteria, Alphaproteobacteria, Actinobacteria, Bacilli* and *Betaproteobacteria*, being the main class in the Island, *Gammaproteobacteria* and in the Peninsula, *Alphaproteobacteria.* Other bacterial classes identified through culturable independent techniques in the populations of *R. farinacea* were *Cytophagia* and *Sphingobacteria*, found as well in other lichen species as *Caloplaca marina*, *C. verruculifera* and *L. helicopis*, through culturable methods (Sigurbjörnsdóttir *et al*., 2014). *Cytophagales* and *Sphingobacteria* are interesting due to their ability to degrade different macromolecules, as diverse polysaccharides as starch, cellulose, β-glucan and pectin (Reichenbach, 2006; Sigurbjörnsdóttir *et al*., 2014).

The main orders found among the bacterial sequences obtained from *R. farinacea* thalli belonged to *Acidobacteriales* (more abundant in insular lichen populations), *Rhodospirillales*, *Rhizobiales* (more widely present in peninsular populations) and in less proportion *Caulobacterales, Gemmatales, Myxococcales, Pseudomonadales,* and *Sphingomonadales.* These orders were common in other lichen species as *L. pulmonaria* (Cernava *et al*., 2017) studied using metagenomic techniques. These authors reported that bacterial members associated to these orders such as *Rhodospirillales* and *Sphingomonadales* had genes related to important functional roles, as involved in potassium and nitrogen metabolism. Furthermore, genes related to iron metabolism seemed to be present in *Sphingomonadales* members. *Rhodospirillales* were found to be involved in the production of some antibiotics as phenazines which inhibits bacterial and fungal growth (Mavrodi *et al*., 2010), thus helping to the growth control of bacterial and fungal pathogens (Cernava *et al*., 2017). Genes of the metabolism of phosphate were assigned to members of *Proteobacteria,* as *Myxococcales,* and the synthesis of the hormone auxin was attributed mostly to proteobacterial members (Cernava *et al*., 2017). In the same lichen *L. pulmonaria, Rhizobiales* were found as a group that harbors also interesting bacterial members well-known by their ability to fix nitrogen (Hodkinson and Lutzoni, 2009), as the ones belonging to *Beijerinckiaceae, Bradyrhizobiaceae* or *Rhizobiaceae,* or to utilize methanol, as *Methylocystaceae, Methylobacteriaceae,* or members of the active *Actinobacteria* as *Nocardiaceae* and *Streptomycetaceae* (Eymann *et al*., 2017). Some of these groups have been identified in this study as associated with the populations of *R. farinacea* studied as well, as the families *Beijerinckiaceae, Methylobacteriaceae, Methylocystaceae, Nocardiaceae* and *Streptomycetaceae,* either by the direct sequence of the bacterial isolates or by culture-independent techniques.

Another aim of this study was to determine the composition of bacteria associated with *R. farinacea* in single thallus. The results obtained showed that the main bacterial groups were the same detected in bulk thalli samples, as mentioned above. An interesting appreciation that could be made is that some taxonomical groups were present exclusively in one single thallus of these *R. farinacea* populations, sometimes appearing in higher proportion than in the rest of the bacterial groups in this thallus. For example, the phyla *Chlamydiae,* present in one thallus from La Guancha, *Gemmatimonadetes* in one thallus from El Toro, or the family *Clostridiaceae* in three thalli from La Esperanza and one from El Toro, or *Frankiaceae* in one thallus from El Toro. All these taxonomical groups appeared as small minorities when samples were composed by bulk thalli, being these taxa masked by those present in a higher proportion. The presence of these bacterial taxa in some individual thallus might be related to some specific

functional roles in particular thallus due to their particular geographical location and the surrounding environmental conditions.

The second main factor that influenced the composition of the bacterial communities associated with the lichen populations of *R. farinacea* was the external and internal location in the lichen thallus. The study of the alpha diversity of the bacteria associated to the ecto- and endolichenic fractions of this lichen species showed that the ectolichenic one presented higher values in the diversity indices studied (Rhichness, Shannon and Simpson) than the endolichenic one. These results were opposite to the ones obtained with the study of the alpha diversity in the culturable fraction, where the endolichenic fraction showed higher indices values. The diversity level of the culturable fraction was around the half of the one appeared using molecular techniques not based on culture methods. Regarding the taxonomical composition of the bacteria associated to the external and the internal fractions of *R. farinacea* thalli*,* the main phyla were again, in order of predominance, *Proteobacteria, Acidobacteria* and *Planctomycetes*, with the presence of *Cyanobacteria* and *Firmicutes* in a higher level in the ectolichenic fraction than in the endolichenic one. No significant differences were found, except at the order level with the *Saprospirales* (*Bacteroidetes*) and *Sphingomonadales* (*Alphaproteobacteria*). These results agree with previous studies with other lichen species, such as *C. arbuscula, L. polytropa* and *U. cylindrica* (Grube *et al*., 2009), where a similar abundance of bacteria in the outer and the inner fractions of the lichen thallus was reported. When compared with the culturable fraction the main orders found were *Rhizobiales, Enterobacteriales, Micrococcales, Bacillales* and *Pseudomonadales,* among others.

Since it has been previously reported that bacterial communities could vary in some specific parts of the lichen thallus (Grube *et al*., 2009; Mushegian *et al*., 2011), we also tried to determine if this was also the case in *R. farinacea*. Taking into consideration the bacterial location along the lichen thallus, no relevant differences were observed in the alpha diversity among apical, middle and basal parts, with very similar values of Richness, Shannon and Simpson diversity indices. However, when the composition of the bacterial communities was analyzed, some differences were found among the apical, the middle or the basal parts of the thallus. These results agree with the fact that the bacterial communities within the lichen thallus are not static and instead are subjected to the effects of ecological processes operating on small scales, as it was reported in *Xanthoparmelia* lichens (Mushegian *et al*., 2011). This structure of the bacterial communities in lichens could be related to the role that these bacteria may have in the lichen symbiosis, being some of the taxa common and highly spread in different lichen species. In our study, a higher prevalence of *Cyanobacteria* and *Firmicutes* in the apical and middle part than in the basal one, and the *Planctomycetes* in the middle and basal parts than in the apical one was found, which suggests that these taxa could have a preferential site and/or functions associated to this location in the lichen thallus.

Regarding the bacterial functional roles, our previous studies in this work have demonstrated that some *R. farinacea* culturable bacteria belonging to these taxa have enzymatic activities that could be related to nutrient recycling of senescent parts of the lichen thallus as well as with the supply of essential nutrients to the holobiont, which agree with studies with other lichen species (Davies *et al*., 2005; González *et al*., 2005; Grube and Berg, 2009). Moreover, it has been suggested that *Alphaproteobacteria* was the most common taxa in the growing parts

of lichens, but some differences could be found, with a higher diversity in older parts (Cardinale *et al*., 2008; Hodkinson *et al*., 2012; Liba *et al*., 2006).

Besides, it should be mentioned that the diversity of bacteria associated with *R. farinacea* thalli was apparently not altered by the disinfection treatment applied, although it is known that in most cases, few seconds are enough to kill a wide diversity of bacterial species (Moorer, 2003). These results are not visible when the amplification of the bacterial DNA present in samples is made, as shown in other studies using omic techniques in which metagenome results also comprised a fraction of inactive or dead bacteria (Cernava *et al*., 2016). Nonetheless, the effects of disinfection treatments are tangible when they are applied in culturable bacteria studies, as shown by Biosca *et al*. (2016), that proved that these treatments reduced the diversity and number of bacteria recovered from lichens. This practice has been performed by many authors whose results were probably biased by those disinfection treatments (Cardinale *et al*., 2006; Grube *et al*., 2009; Selbmann *et al*., 2010; Sigurbjörnsdóttir *et al*., 2014).

Finally, although the primers set used were designed for amplifying both bacterial and archaeal *16S rRNA* genes (Caporaso *et al*., 2011), none archaeal sequences were detected in the analyzed samples, as reported in other studies using these same primers with other lichen species as *C. arbuscula* (Cardinale *et al*., 2008), *P. sulcata, Rhizoplaca chrysoleuca, U. americana,* and *U. phaea* (Bates *et al*., 2011). However, the presence of *Archaea* has been described in other lichens as *L. pulmonaria, Ophioparma ventosa* and *Hydropunctaria maura* (Bjelland *et al*., 2011; Eymann *et al*., 2017; Schneider *et al*., 2011), using other primers set or metaproteomic techniques.

Overall, the results from this study provide new insights into the diversity of bacterial communities associated with *R. farinacea* populations from different Mediterranean-climate areas, showing that certain phyla, as *Acidobacteria*, *Planctomycetes* and *Proteobacteria* and classes as *Actinobacteria*, *Alphaproteobacteria*, *Bacilli*, *Betaproteobacteria,* and *Gammaproteobacteria* are the predominant groups in this lichen species, similarly to other lichen species (Cardinale *et al*., 2006; Grube *et al*., 2009; Selbmann *et al*., 2010). Furthermore, the bacterial community composition of *R. farinacea* is mainly determined by geography since some phyla were more abundant in the lichen populations from the Island than in those from the Peninsula. Other authors supported this fact, arguing that bacterial community trends are correlated with differences in large-scale geography, among other factors (Hodkinson *et al*., 2012). Also by thalli location because some differences were also found in the bacterial communities between ectolichenic and endolichenic fractions and among apical, middle and basal part of the lichen thallus. These differences were observed both by culture dependent and culture independent methods. Finally, it is worth to mention that among all the identified taxa, many of them belonged to bacterial groups with culturable representative strains that are well known because of their enzymatic activities and/or their potential role in the nutrient supply and/or recycling of lichen senescent parts, which could contribute to *R. farinacea* multispecies symbiosis. Many of these bacteria were isolated, extensively characterized and identified in this work.

CONCLUSIONS

Finally, the results obtained in this Doctoral Thesis provide new knowledge on the bacterial communities associated with *R. farinacea*. Below are summarized the main conclusions obtained in this study:

1. The isolation of bacteria associated with the populations of *R. farinacea* through the bacteriological analyses and the lichen enriched culture media (ABL and ABLGM) used in this study has evidenced the high abundance of culturable heterotrophic bacteria (between 10^4 and10^6 CFU/g). The bacterial counts obtained were, generally, higher than those reported in other studies using other methods and convencional synthetic culture media.
2. The abundance of culturable heterotrophic bacteria isolated from thalli of *R. farinacea* were similar both in the ectolichenic and the endolichenic fraction of the analyzed thalli. Nevertheless, culturable bacterial counts were different according to the different geographical origins, being higher in *R. farinacea* thalli from La Guancha and El Toro, than those from La Esperanza and Lidón. These results could be related to the different environmental conditions at each location.
3. The characterization of the bacterial strains isolated from *R. farinacea* confirms the importance of their presence in this lichen, either for their possible functional roles in the recycling and/or supply of nutrients and/or growth promotion by phytohormones production or through the formation of biofilms, contributing to the functioning of this lichen symbiosis, and because of their biotechnological potential applications, due to:

   i) A high percentage of them produce pigments, being yellow and pink the most frequents ones, which could be related, partly, with the tolerance to different environmental conditions, as UV radiation or oxidative stress.

   Ii) Many of the bacterial strains are able to produce hydrolytic enzymes, as amylases, cellulases, pectinases, chitinases and xylanases, as well as lipases, proteases and DNAses, which could contribute to the recycling of nutrients in the senescent parts of *R. farinacea* thalli, supplying with sugars, fatty acids, amino acids and nucleotides to the growing areas, therefore helping to lichen maintenance. This hydrolytic versatility is interesting because of its potential applications in different biotechnological industries.

   iii) Most of the strains are able to fix nitrogen and produce siderophores, and many of them can solubilize inorganic phosphates as well. These activities could contribute to cover certain limiting and essential nutritional requirements for the growth of the lichen thalli. Strains with these abilities could be exploited as biofertilizers.

   iv) A high percentage of the bacterial strains produce the auxin indole acetic acid, and in some of them, it was detected the ACC deaminase enzyme as well. These hormones, which can modulate the growth in plants, could have an influence in the morphogenetic processes of lichens and their symbionts, being these strains producers of phytohormones of interest as potential phytostimulants.

   v) Almost all bacterial strains assayed can produce biofilms, and many of them have swimming motility and some of them swarming motility as well. Biofilm formation could be related to the colonizing ability of lichens in environments with specific and

extreme conditions, being able to increase nutrients uptake. These bacteria could be exploited biotechnologically too.

4. Molecular identification of a selection of bacterial strains from *R. farinacea* according to their physiological and metabolic potentials allowed their assignment to different bacterial taxa, some of them barely studied and/or potentially new species.
5. The study of the diversity and composition of the heterotrophic bacterial communities associated with *R. farinacea* through culture-dependent techniques represents a small fraction of the high variety of bacteria associated with this lichen. However, this study provides new information about these culturable bacteria in *R. farinacea* populations from different insular and peninsular locations with a Mediterranean climate, as well as about the influence of geographical location and location within the lichen thallus on these bacterial communities, which could be related to different functional roles in this lichen species. Furthermore, some of the bacterial strains isolated from this lichen could be new genera and/or species.
6. Among bacterial isolates from *R. farinacea*, the predominant groups are certain phyla, as *Acidobacteria, Proteobacteria* and *Planctomycetes* and classes as *Actinobacteria, Gammaproteobacteria, Alphaproteobacteria, Betaproteobacteria* and *Bacilli.*
7. The study of the diversity and composition of bacterial communities associated with *R. farinacea* through culture-independent techniques, has revealed that these communities are determined, mainly, by geography, but also by the location within the lichen thallus since some differences were found among the bacterial sequences from different geographical locations, and also between the ectolichenic and endolichenic fraction, and among the apical, middle and basal parts of the thallus.
8. Among the identified taxa in the bacterial communities associated with *R. farinacea*, many of them belong to bacterial groups with well-known representatives because of their enzymatic activities and/or because of their potential role in the recycling and/or supply of nutrients in the lichen thalli, and that could contribute to the maintenance of the multispecies lichenic symbiosis. Many of these bacterial strains have been isolated, characterized and identified in this study, being some of them potentially new species, as a result that lichens are environments with bacterial communities still scarcely studied.
9. Lichens suppose a new source of numerous, diverse and new microorganisms with different biotechnological potentials, many of them still to explore.

The results obtained in this research work provide new knowledge about the bacterial communities associated with *R. farinacea* on their composition, diversity and potential functional roles in this lichen, as well as on their potential biotechnological interest. Furthermore, evidence is supplied on the different factors affecting the diversity of the bacteria associated with this lichen, bringing up the importance of factors as geography and the location of these bacteria in *R. farinacea* thalli, allowing a better understanding of the important roles of these bacteria in this multispecies symbiosis.

REFERENCES

Acuña, J. J., Jorquera, M. A., Martínez, O. A., and Blackburn, D. M. (2011). Indole acetic acid and phytase activity produced by rhizosphere bacilli as affected by pH and metals. *J. Soil Sci. Plant Nutr.* 11, 1–12.

Ahemad, M., and Kibret, M. (2014). Mechanisms and applications of plant growth promoting rhizobacteria: current perspective. *J. King Saud Univ. - Sci.* 26, 1–20. doi:10.1016/j.jksus.2013.05.001.

Ahmadjian, V. (1988). The lichen alga *Trebouxia*: does it occur free-living? *Plant Syst. Evol.* 158, 243–247.

Ahmed, E., and Holmström, S. J. M. (2014). Siderophores in environmental research: roles and applications. *Microb. Biotechnol.* 7, 196–208. doi:10.1111/1751-7915.12117.

Alcázar, H. M., Alcázar, S. L., Rivera, R. M. L., and Benítez, M. T. R. (1995). Perspectivas terapéuticas de las nucleasas en el cáncer. *Investig. Biomédica Int.* 22.

An, S.Y., Xiao, T., and Yokota, A. (2008). *Schumannella luteola* gen. nov., sp. nov., a novel genus of the family *Microbacteriaceae*. *J. Gen. Appl. Microbiol.* 54, 253–258. doi:10.2323/jgam.54.253.

An, S.Y., Xiao, T., and Yokota, A. (2009). *Leifsonia lichenia* sp. nov., isolated from lichen in Japan. *J. Gen. Appl. Microbiol.* 55, 339–343. doi:10.2323/jgam.55.339.

Anderson, M. J., Crist, T. O., Chase, J. M., Vellend, M., Inouye, B. D., Freestone, A. L., *et al*. (2011). Navigating the multiple meanings of β diversity: a roadmap for the practicing ecologist. *Ecol. Lett.* 14, 19–28. doi:10.1111/j.1461-0248.2010.01552.x.

Arahal, D. R., Sánchez, E., Macián, M. C., and Garay, E. (2008). Value of *recN* sequences for species identification and as a phylogenetic marker within the family '*Leuconostocaceae*'. *Int. Microbiol.* 11, 33–39. doi:10.2436/20.1501.01.42.

Aschenbrenner, I. A., Cardinale, M., Berg, G., and Grube, M. (2014). Microbial cargo: do bacteria on symbiotic propagules reinforce the microbiome of lichens? *Environ. Microbiol.* 16, 3743–3752. doi:10.1111/1462-2920.12658.

Aschenbrenner, I. A., Cernava, T., Berg, G., and Grube, M. (2016). Understanding microbial multi-species symbioses. *Front. Microbiol.* 7, 1–9. doi:10.3389/fmicb.2016.00180.

Aschenbrenner, I. A., Cernava, T., Erlacher, A., Berg, G., and Grube, M. (2017). Differential sharing and distinct co-occurrence networks among spatially close bacterial microbiota of bark, mosses and lichens. *Mol. Ecol.* doi:10.1111/mec.14070.

Atlas, R. M. (2004). *Handbook of microbiological media*. 3rd ed. Washington DC: CRC Press.

Bak, F., Bonnichsen, L., Jørgensen, N. O. G., Nicolaisen, M. H., and Nybroe, O. (2015a). The biosurfactant viscosin transiently stimulates n-hexadecane mineralization by a bacterial consortium. *Environ. Biotechnol.* 99, 1475–1483. doi:10.1007/s00253-014-6054-3.

Bak, G., Lee, J., Suk, S., Kim, D., Lee, J. Y., Kim, K.S., *et al*. (2015b). Identification of novel sRNAs involved in biofilm formation, motility, and fimbriae formation in *Escherichia coli*. *Sci. Rep.* 5, 3–19. doi:10.1038/srep15287.

Balsanelli, E., De Baura, V. A., De Oliveira, F. P., Maltempi De Souza, E., and Monteiro, R. A. (2014). Exopolysaccharide biosynthesis enables mature biofilm formation on abiotic surfaces by *Herbaspirillum seropedicae*. *PLoS One* 9. doi:10.1371/journal.pone.0110392.

Bartels, P. J., and Nelson, D. R. (2007). An evaluation of species richness estimators for tardigrades of the great smoky mountains National Park, Tennessee and North Carolina, USA. *J. Limnol.* 66, 104–110. doi:10.4081/jlimnol.2007.s1.104.

Bates, S. T., Berg-Lyons, D., Lauber, C. L., Walters, W. A., Knight, R., and Fierer, N. (2012). A preliminary survey of lichen-associated eukaryotes using pyrosequencing. *Lichenol.* 44, 137–146. doi:10.1017/S0024282911000648.

Bates, S. T., Cropsey, G. W. G., Caporaso, J. G., Knight, R., and Fierer, N. (2011). Bacterial communities associated with the lichen symbiosis. *Appl. Environ. Microbiol.* 77, 1309–1314. doi:10.1128/AEM.02257-10.

Beattie, G. A., and Lindow, S. E. (1999). Bacterial colonization of leaves: a spectrum of strategies. *Amercian Phytopathol. Soc.* 89, 353–359.

Beck, A. (2002). Photobionts: diversity and selectivity in lichen symbioses. *Int. Lichenol. Newsl.* 35, 18–25.

Beckett, R. P., Zavarzina, A. G., and Liers, C. (2013). Oxidoreductases and cellulases in lichens: possible roles in lichen biology and soil organic matter turnover. *Fungal Biol.* 117, 431–438. doi:10.1016/j.funbio.2013.04.007.

Bent, S. J., and Forney, L. J. (2008). The tragedy of the uncommon: understanding limitations in the analysis of microbial diversity. *ISME J.* 244, 689–695. doi:10.1038/ismej.2008.44.

Berlanga, M., and Guerrero, R. (2016). Living together in biofilms: the microbial cell factory and its biotechnological implications. *Microb. Cell Fact.* 15. doi:10.1186/s12934-016-0569-5.

Berner, D. K., Schaad, N. W., and Völksch, B. (1999). Use of ethylene-producing bacteria for stimulation of *Striga* spp. seed germination. *Biol. Control* 15, 274–282. doi:10.1006/bcon.1999.0718.

Bertaux, J., Schmid, M., Hutzler, P., Hartmann, A., Garbaye, J., and Frey-Klett, P. (2005). Occurrence and distribution of endobacteria in the plant-associated mycelium of the ectomycorrhizal fungus *Laccaria bicolor* S238N. *Environ. Microbiol.* 7, 1786–1795. doi:10.1111/j.1462-2920.2005.00867.x.

Bhardwaj, D., Ansari, M. W., Sahoo, R. K., and Tuteja, N. (2014). Biofertilizers function as key player in sustainable agriculture by improving soil fertility, plant tolerance and crop productivity. *Microb. Cell Fact.* 13, 1–10.

Bianciotto, V., Lumini, E., Lanfranco, L., Minerdi, D., Bonfante, P., and Perotto, S. (2000). Detection and identification of bacterial endosymbionts in arbuscular mycorrhizal fungi belonging to the family *Gigasporaceae*. *Appl. Environ. Microbiol.* 66, 4503–9. doi:10.1128/AEM.66.10.4503-4509.2000.

Biosca, E. G., and Amaro, C. (1996). Toxic and enzymatic activities of *Vibrio vulnificus* biotype 2 with respect to host specificity. *Appl. Environ. Microbiol.* 62, 2331–2337.

Biosca, E. G., Flores, R., Santander, R. D., Díez-Gil, J. L., and Barreno, E. (2016). Innovative approaches using lichen enriched media to improve isolation and culturability of lichen-

associated bacteria. *PLoS One*, 1–22. doi:10.1371/journal.pone.0160328.

Biziulevičius, G. A. (2006). Where do the immunostimulatory effects of oral proteolytic enzymes ('systemic enzyme therapy') come from? Microbial proteolysis as a possible starting point. *Med. Hypotheses* 67, 1386–1388. doi:10.1016/j.mehy.2006.05.051.

Bjelland, T., Grube, M., Hoem, S., Jorgensen, S. L., Daae, F. L., Thorseth, I. H., *et al*. (2011). Microbial metacommunities in the lichen-rock habitat. *Environ. Microbiol. Rep.* 3, 434–442. doi:10.1111/j.1758-2229.2010.00206.x.

Braña, A. F., Fiedler, H., Nava, H., and Blanco, G. (2015). Two *Streptomyces* species producing antibiotic, antitumor, and anti-inflammatory compounds are widespread among intertidal macroalgae and deep-sea coral reef invertebrates from the central Cantabrian Sea. *Environ. Microbiol.* 69, 512–524. doi:10.1007/s00248-014-0508-0.

Brodo, I. M. (1973). 'Substrate ecology', in *The Lichens*, eds. V. Ahmadjian and M. E. Hale (New York: Academic Press), 401–441. doi:10.1016/B978-0-12-044950-7.50017-9.

Brodo, I. M., Duran, S. S., and Sharnoff, S. (2001). 'How lichens live: physiology and growth', in *Lichens of North America*, ed. Y. U. Press, 36–41.

Brown, H. L., van Vliet, A. H. M., Betts, R. P., and Reuter, M. (2013). Tetrazolium reduction allows assessment of biofilm formation by *Campylobacter jejuni* in a food matrix model. *J. Appl. Microbiol.* 115, 1212–1221. doi:10.1111/jam.12316.

Brunner, U., and Honegger, R. (1985). Chemical and ultrastructural studies on the distribution of sporopollenin-like biopolymers in six genera of lichen phycobionts. *Can. J. Bot.* 63, 2221–2230.

Bruno, J. F., Stachowicz, J. J., and Bertness, M. D. (2003). Inclusion of facilitation into ecological theory. *Trends Ecol. Evol.* 18, 119–125. doi:10.1016/S0169-5347(02)00045-9.

Büdel, B., and Scheidegger, C. (2008). 'Thallus morphology and anatomy', in *Lichen Biology*, ed. T. H. Nash (Cambridge University Press), 40–68.

Burmølle, M., Ren, D., Bjarnsholt, T., and Sørensen, S. J. (2014). Interactions in multispecies biofilms: do they actually matter? *Trends Microbiol.* 22, 84–91. doi:10.1016/j.tim.2013.12.004.

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Huntley, J., Fierer, N., *et al*. (2012). Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME J.* 6, 1621–1624. doi:10.1038/ismej.2012.8.

Caporaso, J. G., Lauber, C. L., Walters, W. a, Berg-Lyons, D., Lozupone, C. a, Turnbaugh, P. J., *et al*. (2011). Global patterns of *16S rRNA* diversity at a depth of millions of sequences per sample. *Proc. Natl. Acad. Sci. U. S. A.* 108 Suppl, 4516–4522. doi:10.1073/pnas.1000080107.

Cardinale, M., Brusetti, L., Quatrini, P., Borin, S., Puglia, A. M., Rizzi, A., *et al*. (2004). Comparison of different primer sets for use in automated ribosomal intergenic spacer analysis of complex bacterial communities. *Appl. Environ. Microbiol.* 70, 6147–6156. doi:10.1128/AEM.70.10.6147.

Cardinale, M., de Castro Jr, J. V., Müller, H., Berg, G., and Grube, M. (2008). *In situ* analysis of the bacterial community associated with the reindeer lichen *Cladonia arbuscula* reveals

predominance of *Alphaproteobacteria*. *FEMS Microbiol. Ecol.* 66, 63–71. doi:10.1111/j.1574-6941.2008.00546.x.

Cardinale, M., Grube, M., and Berg, G. (2011). *Frondihabitans cladoniiphilus* sp. nov., an actinobacterium of the family *Microbacteriaceae* isolated from lichen, and emended description of the genus *Frondihabitans*. *Int. J. Syst. Evol. Microbiol.* 61, 3033–3038. doi:10.1099/ijs.0.028324-0.

Cardinale, M., Grube, M., Castro, J. V., Müller, H., and Berg, G. (2012a). Bacterial taxa associated with the lung lichen *Lobaria pulmonaria* are differentially shaped by geography and habitat. *FEMS Microbiol. Lett.* 329, 111–115. doi:10.1111/j.1574-6968.2012.02508.x.

Cardinale, M., Puglia, A. M., and Grube, M. (2006). Molecular analysis of lichen-associated bacterial communities. *FEMS Microbiol. Ecol.* 57, 484–495. doi:10.1111/j.1574-6941.2006.00133.x.

Cardinale, M., Steinová, J., Rabensteiner, J., Berg, G., and Grube, M. (2012b). Age, sun and substrate: triggers of bacterial communities in lichens. *Environ. Microbiol. Rep.* 4, 23–28. doi:10.1111/j.1758-2229.2011.00272.x.

Casano, L. M., Braga, M. R., Álvarez, R., del Campo, E. M., and Barreno, E. (2015). Differences in the cell walls and extracellular polymers of the two *Trebouxia* microalgae coexisting in the lichen *Ramalina farinacea* are consistent with their distinct capacity to immobilize extracellular Pb. *Plant Sci.* 236, 195–204. doi:10.1016/j.plantsci.2015.04.003.

Casano, L. M., Del Campo, E. M., García-Breijo, F. J., Reig-Armiñana, J., Gasulla, F., Del Hoyo, A., *et al*. (2011). Two *Trebouxia* algae with different physiological performances are ever-present in lichen thalli of *Ramalina farinacea*. Coexistence versus competition? *Environ. Microbiol.* 13, 806–818. doi:10.1111/j.1462-2920.2010.02386.x.

Castro, F. D., and Tufenkji, N. (2007). Relevance of nontoxigenic strains as surrogates for *Escherichia coli* O157:H7 in groundwater contamination potential: role of temperature and cell acclimation time. *Environ. Sci. Technol.* 41, 4332–4338. doi:10.1021/es0701558.

Cavicchioli, R., Ostrowski, M., Fegatella, F., Goodchild, A., and Guixa-Boixereu, N. (2003). Life under nutrient limitation in oligotrophic marine environments: an eco/physiological perspective of *Sphingopyxis alaskensis* (formerly *Sphingomonas alaskensis*). *Microb. Ecol.* 45, 203–217. doi:10.1007/s00248-002-3008-6.

Cengia-Sambo, M. (1925). Ancora della polisimbiosi nei licheni ad alghe cianoficee. I batteri simbionti. *Atti della Soc. Ital. di Sci. Nat.* 18, 6.

Cengia-Sambo, M. (1931). Biologie des lichens. Les substances carbohydratées dans les lichens et la fonction de fixation de l'azote des céphalodies. *Boll. della Sez. Ital. Soc. internazionale di Microbiol.* 11, 1–8.

Cernava, T., Aschenbrenner, I. a., Grube, M., Liebminger, S., and Berg, G. (2015a). A novel assay for the detection of bioactive volatiles evaluated by screening of lichen-associated bacteria. *Front. Microbiol.* 6, 1–9. doi:10.3389/fmicb.2015.00398.

Cernava, T., Berg, G., and Grube, M. (2016). High life expectancy of bacteria on lichens. *Microb. Ecol.* 72, 510–513. doi:10.1007/s00248-016-0818-5.

Cernava, T., Erlacher, A., Aschenbrenner, I. A., Krug, L., Lassek, C., Riedel, K., *et al*. (2017).

Deciphering functional diversification within the lichen microbiota by meta-omics. *Microbiome* 5. doi:10.1186/s40168-017-0303-5.

Cernava, T., Müller, H., Aschenbrenner, I. A., Grube, M., and Berg, G. (2015b). Analyzing the antagonistic potential of the lichen microbiome against pathogens by bridging metagenomic with culture studies. *Front. Microbiol.* 6. doi:10.3389/fmicb.2015.00620.

Cerreti, M., Liburdi, K., Benucci, I., and Esti, M. (2016). The effect of pectinase and protease treatment on turbidity and on haze active molecules in pomegranate juice. *LWT - Food Sci. Technol.* 73, 326–333. doi:10.1016/j.lwt.2016.06.030.

Chadni, Z., Rahaman, M. H., Jerin, I., Hoque, K. M., and Reza, M. A. (2017). Extraction and optimisation of red pigment production as secondary metabolites from *Talaromyces verruculosus* and its potential use in textile industries. *Mycology* 8, 48–57. doi:10.1080/21501203.2017.1302013.

Chanalia, P., Gandhi, D., Jodha, D., and Singh, J. (2011). Applications of microbial proteases in pharmaceutical industry. *Rev. Med. Microbiol.* 22, 96–101. doi:10.1097/MRM.0b013e3283494749.

Chapman, M., and Margulis, L. (1999). Morphogenesis by symbiogenesis. *Int. Microbiol.* 1, 319–326.

Chaudhuri, G., Selvaraj, U., Babu, V., and Thilagaraj, R. W. (2017). 'Recent trends in phosphatase-mediated bioremediation', in *Phosphoric Acid Industry-Problems and Solutions*, eds. M. S. Wiener and B. B. T. Valdez (Rijeka: InTech), 75–100. doi:10.5772/711.

Chaudhuri, G., Venu-Babu, P., Dalal, D., and Thilagaraj, W. R. (2015). Application of alkaline phosphatase for heavy metals precipitation using ascorbic acid 2-phosphate as an effective natural substrate. *Int. J. Environ. Sci. Technol.* 12, 3877–3886. doi:10.1007/s13762-014-0749-y.

Chelvam, K. K., Chai, L. C., and Thong, K. L. (2014). Variations in motility and biofilm formation of *Salmonella enterica* serovar Typhi. *Gut Pathog.* 6, 2. doi:10.1186/1757-4749-6-2.

Chen, J., Zhang, W., Lin, J., Wang, F., Wu, M., Chen, C., *et al*. (2014). An efficient antiviral strategy for targeting hepatitis B virus genome using transcription activator-like effector nucleases. *Mol. Ther.* 22, 303–311. doi:10.1038/mt.2013.212.

Chhabra, S., Brazil, D., Morrissey, J., Burke, J. I., O 'Gara, F., and Dowling, D. N. (2013). Characterization of mineral phosphate solubilization traits from a barley rhizosphere soil functional metagenome. *Microbiologyopen* 2, 717–724. doi:10.1002/mbo3.110.

Chilton, M. D., Currier, T. C., Farrand, S. K., Bendicht, A. J., Gordont, M. P., and Nester, E. W. (1974). *Agrobacterium tumefaciens* DNA and PS8 Bacteriophage DNA not detected in crown gall tumors (nucleic acid hybridization/DNA reassociation kinetics/transformation of plant cells). *Proc. Natl. Acad. Sci. U. S. A.* 71, 3672–3676.

Cohen, P. A., and Towers, G. H. N. (1995). The anthraquinones of *Heterodermia obscurata*. *Phytochemistry* 40, 911–915.

Cordeiro, L. M. C., Carbonero, E. R., Sassaki, G. L., Reis, R. A., Stocker-Wö Rgö Tter, E., Gorin, P. A. J., *et al*. (2005). A fungus-type B-galactofuranan in the cultivated *Trebouxia* photobiont of the lichen *Ramalina gracilis*. *FEMS Microbiol. Lett.* 244, 193–198. doi:10.1016/j.femsle.2005.01.040.

Cordeiro, L. M. C., De Oliveira, S. M., Buchi, D. F., and Iacomini, M. (2008). Galactofuranose-rich heteropolysaccharide from *Trebouxia* sp., photobiont of the lichen *Ramalina gracilis* and its effect on macrophage activation. *Int. J. Biol. Macromol.* 42, 436–440. doi:10.1016/j.ijbiomac.2008.02.002.

Cordeiro, L. M. C., Sassaki, G. L., and Iacomini, M. (2007). First report on polysaccharides of *Asterochloris* and their potential role in the lichen symbiosis. *Int. J. Biol. Macromol.* 41, 193–197. doi:10.1016/j.ijbiomac.2007.02.006.

Costacurta, A., and Vanderleyden, J. (1995). Synthesis of phytohormones by plant-associated bacteria. *Crit. Rev. Microbioloy* 21, 1–18.

Dang, G., Cao, J., Cui, Y., Song, N., Chen, L., Pang, H., *et al*. (2016). Characterization of Rv0888, a novel extracellular nuclease from *Mycobacterium tuberculosis*. *Sci. Rep.* 6. doi:10.1038/srep19033.

Davies, J., Wang, H., Taylor, T., Warabi, K., Huang, X.H., and Andersen, R. J. (2005). Uncialamycin, a new enediyne antibiotic. *Org. Lett.* 7, 5233–5236. doi:10.1021/ol052081f.

Davis, K. E. R., Joseph, S. J., and Janssen, P. H. (2005). Effects of growth medium, inoculum size, and incubation time on culturability and isolation of soil bacteria. *Appl. Environ. Microbiol.* 71, 826–834. doi:10.1128/AEM.71.2.826–834.2005.

de Bary, A. (1879). *Die Erscheinung der Symbiose. Vortrag gehalten auf der Versammlung Deutscher Naturforscher und Aerzte zu Cassel.* Strassburg: K.J. Trübner.

de la Torre, R., Sancho, L. G., Horneck, G., Ríos, A. de los, Wierzchos, J., Olsson-Francis, K., *et al*. (2010). Survival of lichens and bacteria exposed to outer space conditions – Results of the Lithopanspermia experiments. *Icarus* 208, 735–748. doi:10.1016/j.icarus.2010.03.010.

de los Ríos, A., Sancho, L. G., Grube, M., Wierzchos, J., and Ascaso, C. (2005a). Endolithic growth of two *Lecidea lichens* in granite from continental Antarctica detected by molecular and microscopy techniques. *New Phytol.* 165, 181–190. doi:10.1111/j.1469-8137.2004.01199.x.

de los Ríos, A., Wierzchos, J., and Ascaso, C. (2002). Microhabitats and chemical microenvironments under saxicolous lichens growing on granite. *Microb. Ecol.* 43, 181–188. doi:10.1007/s00248-001-1028-2.

de los Ríos, A., Wierzchos, J., Sancho, L. G., Green, T. G. A., and Ascaso, C. (2005b). Ecology of endolithic lichens colonizing granite in continental Antarctica. *Lichenol. Br. Lichen Soc.* 37, 383–395. doi:10.1017/S0024282905014969.

de Serrano, L. O. (2017). Biotechnology of siderophores in high-impact scientific fields. *Biomol. Concepts* 8, 169–178. doi:10.1515/bmc-2017-0016.

de Souza, P. M., and de Oliveira, P. M. (2010). Applications of microbial α-amylase industry. A review. *Brazilian J. Microbiol.* 41, 850–861.

de Vera, J.-P. P., Rettberg, P., and Ott, S. (2008). Life at the limits: capacities of isolated and cultured lichen symbionts to resist extreme environmental stresses. *Orig. Life Evol. Biosph.* 38, 457–468. doi:10.1007/s11084-008-9140-7.

del Campo, E. M., Catalá, S., Gimeno, J., del Hoyo, A., Martínez-Alberola, F., Casano, L. M., *et*

al. (2013). The genetic structure of the cosmopolitan three-partner lichen *Ramalina farinacea* evidences the concerted diversification of symbionts. *FEMS Microbiol. Ecol.* 83, 310–323. doi:10.1111/j.1574-6941.2012.01474.x.

del Campo, E. M., Gimeno, J., De Nova, J. P. G., Casano, L. M., Gasulla, F., García-breijo, F., *et al*. (2010). 'South European populations *of Ramalina farinacea* (L.) Ach. share different *Trebouxia* algae', in *Biology of Lichens – Symbiosis, Ecology, Environmental Monitoring, Systematics, Cyber Applications*, ed. T. H. Nash (Bibliotheca Lichenologica), 247–256.

Del Hoyo, A., Álvarez, R., Del Campo, E. M., Gasulla, F., Barreno, E., and Casano, L. M. (2011). Oxidative stress induces distinct physiological responses in the two *Trebouxia* phycobionts of the lichen *Ramalina farinacea*. *Ann. Bot.* 107, 109–118. doi:10.1093/aob/mcq206.

DePriest, P. T. (2004). Early molecular investigations of lichen-forming symbionts: 1986-2001. *Annu. Rev. Microbiol.* 58, 273–301. doi:10.1146/annurev.micro.58.030603.123730.

Di Donato, P., Poli, A., Taurisano, V., Abbamondi, G., Nicolaus, B., and Tommonaro, G. (2016). Recent advances in the study of marine microbial biofilm: from the involvement of quorum sensing in its production up to biotechnological application of the polysaccharide fractions. *J. Mar. Sci. Eng.* 4, 34. doi:10.3390/jmse4020034.

Dimijian, G. G. (2000). Evolving together: the biology of symbiosis, part 2. *Proc. (Bayl. Univ. Med. Cent).* 13, 217–26.

Donlan, R. M., and Costerton, J. W. (2002). Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin. Microbiol. Rev.* 15, 167–193. doi:10.1128/CMR.15.2.167–193.2002.

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26, 2460–2461. doi:10.1093/bioinformatics/btq461.

Edgar, R. C. (2013). UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat. Methods* 10, 996–998.

Effmert, U., Kalderás, J., Warnke, R., and Piechulla, B. (2012). Volatile mediated interactions between bacteria and fungi in the soil. *J. Chem. Ecol.* 38, 665–703. doi:10.1007/s10886-012-0135-5.

El-Bialy, H. A., and Abou El-Nour, S. A. (2015). Physical and chemical stress on *Serratia marcescens* and studies on prodigiosin pigment production. *Ann. Microbiol.* 65, 59–68. doi:10.1007/s13213-014-0837-8.

Elix, J. A., and Stocker-Wörgötter, E. (2008). 'Biochemistry and secondary metabolites', in *Lichen Biology*, ed. T. H. Nash (Cambridge: Cambridge University Press), 104–133.

Ellis, C. J., Crittenden, P. D., Scrimgeour, C. M., and Ashcroft, C. J. (2005). Translocation of ^{15}N indicates nitrogen recycling in the mat-forming lichen *Cladonia portentosa*. *New Phytol.* 168, 423–434. doi:10.1111/j.1469-8137.2005.01524.x.

Erlacher, A., Cernava, T., Cardinale, M., Soh, J., Sensen, C. W., Grube, M., *et al*. (2015). *Rhizobiales* as functional and endosymbiontic members in the lichen symbiosis of *Lobaria pulmonaria* L. *Front. Microbiol.* 6, 1–9. doi:10.3389/fmicb.2015.00053.

Esquivel-Cote, R., Gavilanes-Ruiz, M., Cruz-Ortega, R., and Huante, P. (2013). Importancia agrobiotecnológica de la enzima ACC desaminasa en Rizobacterias. Una revisión. *Rev.*

Fitotec. Mex. 36, 251–258.

Eymann, C., Lassek, C., Wegner, U., Bernhardt, J., Fritsch, O. A., Fuchs, S., *et al*. (2017). Symbiotic interplay of fungi, algae, and bacteria within the lung lichen *Lobaria pulmonaria* L. Hoffm. as assessed by state-of-the-art metaproteomics. *J. Proteome Res.* 16, 2160–2173. doi:10.1021/acs.jproteome.6b00974.

Fahselt, D. (1994). Secondary biochemistry of lichens. *Symbiosis* 16, 117–165.

Farrar, J. F. (1985). 'The lichen as an ecosystem: observation and experiment', in *Lichenology: Progress and Problems*, eds. D. H. Brown, D. L. Hawksworth, and R. . Bailey (London: Academic Press), 385–406.

Feige, G. B., and Jensen, M. (1992). 'Basic carbon and nitrogen metabolism of lichens', in *Algae and Symbiosis*, ed. W. Reisser (Bristol: Biopress Limites), 277–299.

Feldhaar, H. (2011). Bacterial symbionts as mediators of ecologically important traits of insect hosts. *Ecol. Entomol.* 36, 533–543. doi:10.1111/j.1365-2311.2011.01318.x.

Fernández-Mendoza, F., Domaschke, S., García, M. A., Jordan, P., Martín, M. P., and Printzen, C. (2011). Population structure of mycobionts and photobionts of the widespread lichen *Cetraria aculeata*. *Mol. Ecol.* 20, 1208–1232. doi:10.1111/j.1365-294X.2010.04993.x.

Flemming, H. C., and Wingender, J. (2010). The biofilm matrix. *Nat. Rev. Microbiol.* 8, 623–633. doi:10.1080/08927010310000721 90.

Friedl, T. (2006). Inferring taxonomic positions and testing genus level assignments in coccoid green lichen algae - a phylogenetic analysis of 18S ribosomal RNA sequences from *Dictyochloropsis reticulata* and from members of the genus *Myrmecia* (Chlorophyta, Trebouxiohyceae). *J. Phycol.* 31, 632–639.

Friedl, T., and Büdel, B. (2008). 'Photobionts', in *Lichen Biology*, ed. T. H. Nash (Cambridge University Press), 9–26.

Gadd, G. M. (2007). Geomycology: biogeochemical transformations of rocks, minerals, metals and radionuclides by fungi, bioweathering and bioremediation. *Mycol. Res.* 111, 3–49. doi:10.1016/j.mycres.2006.12.001.

Galloway, D. J. (2008). 'Lichen Biogeography', in *Lichen Biology*, ed. T. H. Nash (Cambridge: Cambridge University Press), 315–335.

Garbeva, P., Hordijk, C., Gerards, S., De Boer, W., and Gillor, O. (2014). Volatile-mediated interactions between phylogenetically different soil bacteria. *Front. Microbiol.* 5. doi:10.3389/fmicb.2014.00289.

García-Breijo, F., Reig-Armiñana, J., Salvá, G., Vázquez, V. M., and Barreno, E. (2010). El liquen *Ramalina farinacea* (L.) Ach. en Asturias. Estructura de talos e identificación molecular de los dos ficobiontes de *Trebouxia* que coexisten. *Boletín Ciencias Nat. R.I.D.E.A* 51, 325–336.

García-Fraile, P., Menéndez, E., and Rivas, R. (2015). Role of bacterial biofertilizers in agriculture and forestry. *AIMS Bioeng.* 2, 183–205. doi:10.3934/bioeng.2015.3.183.

Gargas, A., DePriest, P., Grube, M., and Tehler, A. (1995). Multiple origins of lichen symbioses in fungi suggested by SSU rDNA phylogeny. *Science (80-.).* 268, 1492–1495.

Gayathri, D., Swamy, C., and Devaraja, T. (2014). Role of cyanolichens in biological nitrogen fixation. *Angew. Biol. Forsch.* 2, 17–23.

Gilbert, S. F., Mcdonald, E., Boyle, N., Buttino, N., Gyi, L., Mai, M., *et al*. (2010). Symbiosis as a source of selectable epigenetic variation: taking the heat for the big guy. *Philos. Trans. R. Soc.* 365, 671–678. doi:10.1098/rstb.2009.0245.

Girlanda, M., Isocrono, D., Bianco, C., and Luppi-Mosca, A. M. (1997). Two foliose lichens as microfungal ecological niches. *Mycologia* 89, 531–536. doi:10.2307/3760987.

Glasl, B., Herndl, G. J., and Frade, P. R. (2016). The microbiome of coral surface mucus has a key role in mediating holobiont health and survival upon disturbance. *ISME J* 10, 1–13. doi:10.1038/ismej.2016.9.

Glick, B. R. (2014). Bacteria with ACC deaminase can promote plant growth and help to feed the world. *Microbiol. Res.* 169, 30–39. doi:10.1016/j.micres.2013.09.009.

González, I., Ayuso-Sacido, A., Anderson, A., and Genilloud, O. (2005). Actinomycetes isolated from lichens: evaluation of their diversity and detection of biosynthetic gene sequences. *FEMS Microbiol. Ecol.* 54, 401–415. doi:10.1016/j.femsec.2005.05.004.

Grube, M., and Berg, G. (2009). Microbial consortia of bacteria and fungi with focus on the lichen symbiosis. *Fungal Biol. Rev.* 23, 72–85. doi:10.1016/j.fbr.2009.10.001.

Grube, M., Berg, G., Andrésson, Ó. S., Vilhelmsson, O., Dyer, P. S., and Miao, V. P. W. (2014). 'Lichen genomics: prospects and progress', in *The Ecological Genomics of Fungi*, ed. F. Martin (John Wiley & Sons, Inc.), 191–212.

Grube, M., Cardinale, M., and Berg, G. (2012a). 'Bacteria and the Lichen Symbiosis', in *The Mycota IX*, ed. B. Hock (Springer-Verlag Berlin Heidelberg), 363–372.

Grube, M., Cardinale, M., Castro, J. V. de, Müller, H., and Berg, G. (2009). Species-specific structural and functional diversity of bacterial communities in lichen symbioses. *ISME J.* 3, 1105–1115. doi:10.1038/ismej.2009.63.

Grube, M., Cernava, T., Soh, J., Fuchs, S., Aschenbrenner, I., Lassek, C., *et al*. (2015). Exploring functional contexts of symbiotic sustain within lichen-associated bacteria by comparative omics. *ISME J.* 9, 412–424. doi:10.1038/ismej.2014.138.

Grube, M., Köberl, M., Lackner, S., Berg, C., and Berg, G. (2012b). Host-parasite interaction and microbiome response: effects of fungal infections on the bacterial community of the Alpine lichen *Solorina crocea*. *FEMS Microbiol. Ecol.* 82, 472–481. doi:10.1111/j.1574-6941.2012.01425.x.

Gupta, A., and Khare, S. K. (2007). Enhanced production and characterization of a solvent stable protease from solvent tolerant *Pseudomonas aeruginosa* PseA. *Enzyme Microb. Technol.* 42, 11–16. doi:10.1016/j.enzmictec.2007.07.019.

Gupta, P., Samant, K., and Sahu, A. (2012). Isolation of cellulose-degrading bacteria and determination of their cellulolytic potential. *Int. J. Microbiol.* 2012. doi:10.1155/2012/578925.

Gupta, R., Gupta, N., and Rathi, P. (2004). Bacterial lipases: an overview of production, purification and biochemical properties. *Appl. Microbiol. Biotechnol.* 64, 763–781. doi:10.1007/s00253-004-1568-8.

Hamada, M., Yamamura, H., Komukai, C., Tamura, T., Suzuki, K.I., and Hayakawa, M. (2012). *Luteimicrobium album* sp. nov., a novel actinobacterium isolated from a lichen collected in Japan, and emended description of the genus *Luteimicrobium*. *J. Antibiot. (Tokyo).* 65, 427–431. doi:10.1038/ja.2012.45.

Hawksworth, D. L. (1989). Interacciones hongo-alga en la simbiosis liquénica. *An. Jardín Botánico Madrid* 46, 235–247.

Hawksworth, D. L., Iturriaga, T., and Crespo, A. (2005). Líquenes como bioindicadores inmediatos de contaminación y cambios medioambientales en los trópicos. *Rev. Iberoam. Micol.* 22, 71–82.

Hawksworth, D. L., Kirk, P. M., Sutton, B. C., and Pegler, D. N. (1995). *Dictionary of the fungi*. CAB, Wallingford.

Hayat, R., Ali, S., Amara, U., Khalid, R., and Ahmed, I. (2010). Soil beneficial bacteria and their role in plant growth promotion: a review. *Ann. Microbiol.* 60, 579–598. doi:10.1007/s13213-010-0117-1.

Heip, C. H. R., Herman, P. M. J., and Soetaert, K. (1998). Indices of diversity and evenness. *Océanis* 24, 61–87.

Henkel, P. A., and Plotnikova, T. T. (1973). Nitrogen-fixing bacteria in lichens. *Proc. USSR Acad. Sci.* 6, 807–813.

Henskens, F. L., Green, A. T. G., and Wilkins, A. (2012). Cyanolichens can have both cyanobacteria and green algae in a common layer as major contributors to photosynthesis. *Ann. Bot.* 110, 555–563. doi:10.1093/aob/mcs108.

Herlemann, D. P. R., Lundin, D., Labrenz, M., Jürgens, K., Zheng, Z., Aspeborg, H., *et al*. (2013). Metagenomic *de novo* assembly of an aquatic representative of the verrucomicrobial class *Spartobacteria*. *MBio* 4. doi:10.1128/mBio.00569-12.Editor.

Hill, D. J. (1976). 'The physiology of lichen symbiosis', in *Lichenology: Progress and Problems*, eds. D. Brown, D. L. Hawksworth, and R. . Bailey (London: Academic Press), 457–496.

Hillis, D. M., and Bull, J. J. (1993). An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. *Syst. Biol.* 42, 182–192. doi:10.1093/sysbio/42.2.182.

Himmel, M. E., Xu, Q., Luo, Y., Ding, S.Y., Lamed, R., and Bayer, E. A. (2010). Microbial enzyme systems for biomass conversion: emerging paradigms. *Biofuels* 1, 323–341. doi:10.4155/bfs.09.25.

Hodkinson, B. P. (2011). *A phylogenetic, ecological, and functional characterization of non-photoautotrophic bacteria in the lichen microbiome.* PhD Thesis. Duke University, Durham.

Hodkinson, B. P., Gottel, N. R., Schadt, C. W., and Lutzoni, F. (2012). Photoautotrophic symbiont and geography are major factors affecting highly structured and diverse bacterial communities in the lichen microbiome. *Environ. Microbiol.* 14, 147–161. doi:10.1111/j.1462-2920.2011.02560.x.

Hodkinson, B. P., and Lutzoni, F. (2009). A microbiotic survey of lichen-associated bacteria reveals a new lineage from the *Rhizobiales*. *Symbiosis* 49, 163–180.

Honegger, R. (1984). Cytological aspects of the mycobiont–phycobiont relationship in lichens: haustorial types, phycobiont cell wall types, and the ultrastructure of the cell surface layers in some cultured and symbiotic myco-and phycobionts. *Lichenol.* 16, 111–127.

Honegger, R. (1991). Functional aspects of the lichen symbiosis. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 42, 553–578. doi:10.1146/annurev.pp.42.060191.003005.

Honegger, R. (1992). 'Lichens: mycobiont-photobiont relationships', in *Algae and Symbiosis. Plants, Animals, Fungi, Viruses, Interactions Explored.*, ed. W. Reisser (Bristol: Biopress), 255–275.

Honegger, R. (1997). 'Metabolic interactions at the mycobiont-photobiont interface in lichens', in *Plant Relationships, The Mycota. Vol. V. Part A*, eds. G. Carroll and P. Tudzynski (Berlin: Springer), 209–221.

Honegger, R. (1998). The lichen symbiosis—What Is so spectacular about it? *Lichenol.t* 30, 193–212. doi:10.1006/lich.1998.0140.

Honegger, R. (2008a). 'Morphogenesis', in *Lichen Biology*, ed. T. H. Nash (Cambridge: Cambridge University Press), 69–93.

Honegger, R. (2008b). 'Mycobionts', in *Lichen Biology*, ed. T. H. Nash (Cambridge: Cambridge University Press), 27–39.

Honegger, R. (2012). 'The symbiotic phenotype of lichen-forming *Ascomycetes* and their endo- and epibionts', in *Fungal Associations. The Mycota (A Comprehensive Treatise on Fungi as Experimental Systems for Basic and Applied Research)*, ed. B. Hock (Springer-Verlag, Berlin, Heidelberg), 287–339. doi:doi: 10.1007/978-3-642-30826-0_6.

Hori, K., and Matsumoto, S. (2010). Bacterial adhesion: from mechanism to control. *Biochem. Eng. J.* 48, 424–434. doi:10.1016/j.bej.2009.11.014.

Houry, A., Briandet, R., Aymerlch, S., and Gohar, M. (2010). Involvement of motility and flagella in *Bacillus cereus* biofilm formation. *Microbiology* 156, 1009–1018. doi:10.1099/mic.0.034827-0.

Huddedar, S. B., Shete, A. M., Tilekar, J. N., Gore, S. D., Dhavale, D. D., and Chopade, B. A. (2002). Isolation, characterization, and Plasmid pUPI126-mediated indole-3-acetic acid production in *Acinetobacter* strains from rhizosphere of wheat. *Appl. Biochem. Biotechnol.* 102, 21–39.

Huneck, S. (1999). The significance of lichens and their metabolites. *Naturwissenschaften* 86, 559–570.

Hyvärinen, M., and Crittenden, P. D. (1998). Relationships between atmospheric nitrogen inputs and the vertical nitrogen and phosphorus concentration gradients in the lichen *Cladonia portentosa*. *New Phytol.* 140, 519–530. doi:10.1046/j.1469-8137.1998.00292.x.

Hyvärinen, M., and Crittenden, P. D. (2000). ^{33}P translocation in the thallus of the mat-forming lichen *Cladonia portentosa*. *New Phytol.* 145, 281–288. doi:10.1046/j.1469-8137.2000.00581.x.

Illumina, I. (2010). Illumina Sequencing Technology.

Iskina, R. Y. (1938). On nitrogen fixing bacteria in lichens. *Bull Perm Biol Res Inst* 11, 133–139.

Jahns, H. M. (1984). Morphology, reproduction and water relations - a system of morphogenetic interactions in *Parmelia saxalitis*. *Nov. Hedwigia* 79, 715–737.

Jahns, H. M. (1988). 'The lichen thallus', in *CRC Handbook of Lichenology, Vol. 1*, ed. M. Galun (Boca Raton: CRC Press), 95–143.

James, P. W., and Henssen, A. (1976). 'The morpholical and taxonomic significance of cephalodia', in *Lichenology: Progress and Problems*, eds. D. H. Brown, D. L. Hawksworth, and R. H. Bailey (London: Academic Press), 27–77.

Jian, D.F., Wang, H.Y., Si, H.L., Zhao, L., Liu, C.P., and Zhang, H. (2017). Isolation and culture of lichen bacteriobionts. *Lichenol.* 49, 175–181.

Jisha, V. N., Smitha, R. B., Pradeep, S., Sreedevi, S., Unni, K. N., Sajith, S., *et al*. (2013). Versatility of microbial proteases. *Adv. Enzym. Res.* 1, 39–51. doi:10.4236/aer.2013.13005.

Kai, M., Evmert, U., Berg, G., and Piechulla, B. (2007). Volatiles of bacterial antagonists inhibit mycelial growth of the plant pathogen *Rhizoctonia solani*. *Arch. Microbiol.* 187, 351–360. doi:10.1007/s00203-006-0199-0.

Kalpana, M. D., Rasheedha, A. B., Gnanaprabhal, G. R., Pradeep, B. V., and Palaniswamy, M. (2008). Purification, characterization of alkaline protease enzyme from native isolate *Aspergillus niger* and its compatibility with commercial detergents. *Indian J. Sci. Technol.* 1, 1–6. doi:10.17485/ijst/2008/v1i7/29599.

Kampa, A., Gagunashvili, A. N., Gulder, T. A. M., Morinaka, B. I., Daolio, C., Godejohann, M., *et al*. (2013). Metagenomic natural product discovery in lichen provides evidence for a family of biosynthetic pathways in diverse symbioses. *Proc. Natl. Acad. Sci.* 110, 129–137. doi:10.1073/pnas.1305867110.

Kandra, L. (2003). α-Amylases of medical and industrial importance. *J. Mol. Struct.* 666–667, 487–498. doi:10.1016/j.theochem.2003.08.073.

Kappen, L. (1988). 'Ecophysiological relationships in different in different climatic regions', in *CRC Handbook of Lichenology, Vol. 2*, ed. M. Galun (Boca Raton: CRC Press), 37–99.

Kappen, L. (1993). 'Lichens in the Antarctic region', in *Antarctic Microbiology*, ed. E. Friedmann (New York: Wiley-Liss), 443–490.

Kappen, L. (2000). Some aspects of the great success of lichens in Antarctica. *Antarct. Sci.* 12, 314–324. doi:10.1017/S0954102000000377.

Kearns, D. B. (2010). A field guide to bacterial swarming motility. *Nat. Rev. Microbiol.* 8, 634–644. doi:10.1038/nrmicro2405.

Kearns, D. B., and Losick, R. (2003). Swarming motility in undomesticated *Bacillus subtilis*. *Mol. Microbiol.* 49, 581–590. doi:10.1046/j.1365-2958.2003.03584.x.

Kershaw, K. A. (1985). *Physiological ecology of lichens*., ed. K. A. Kershaw New York: Cambridge University Press.

Kersters, K., De Vos, P., Gillis, M., Swings, J., Vandamme, P., and Stakebrandt, E. (2006). Introduction to the *Proteobacteria*. *Prokaryotes* 5, 3–37. doi:10.1007/0-387-30745-1_1 noi.

Kim, M.K., Park, H., and Oh, T.J. (2014). Antibacterial and antioxidant capacity of Polar microorganisms isolated from Artic lichen *Ochrolechia* sp. *Polish J. Microbiol.* 63, 317–322.

Kim, O.S., Cho, Y.J., Lee, K., Yoon, S.H., Kim, M., Na, H., *et al*. (2012). Introducing EzTaxon-e: a prokaryotic *16S rRNA* gene sequence database with phylotypes that represent uncultured species. *Int. J. Syst. Evol. Microbiol.* 62, 716–721. doi:10.1099/ijs.0.038075-0.

Kimura, M. (1980). A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* 16, 111–120.

King, E. O., Ward, M. K., and Raney, D. E. (1954). Two simple media for the demonstration of pyocyanin and fluorescin. *J. Lab. Clin. Med.* 44, 301–307.

Kirkelund, H. S., Rainey, P. B., Haagensen, J. A. J., and Molin, S. (2007). Evolution of species interactions in a biofilm community. *Nature* 445, 533–536. doi:10.1038/nature05514.

Kirmizigül, S., Koz, Ö., Anil, H., and Içli, S. (2003). Isolation and structure elucidation of novel natural products from Turkish lichens. *Turkish J. Chem.* 27, 493–500.

Knops, J. M. H., Nash Iii, T. H., Boucher, V. L., and Schlesinger, W. H. (1991). Mineral cycling and epiphytic lichens: implications at the ecosystem level. *Lichenol.* 23, 309–321. doi:10.1017/S0024282991000452.

Koczan, J. M., Lenneman, B. R., Mcgrath, M. J., and Sundin, G. W. (2011). Cell surface attachment structures contribute to biofilm formation and xylem colonization by *Erwinia amylovora*. *Appl. Environ. Microbiol.* 77, 7031–7039. doi:10.1128/AEM.05138-11.

Koczan, J. M., Mcgrath, M. J., Zhao, Y., and Sundin, G. W. (2009). Contribution of *Erwinia amylovora* exopolysaccharides amylovoran and levan to biofilm formation: implications in pathogenicity. *Bacteriology* 99, 1237–1244.

Koeck, D. E., Pechtl, A., Zverlov, V. V., and Schwarz, W. H. (2014). Genomics of cellulolytic bacteria. *Curr. Opin. Biotechnol.* 29, 171–183. doi:10.1016/j.copbio.2014.07.002.

Kokare, C. R., Chakraborty, S., Khopade, A. N., and Mahadik, K. R. (2009). Biofilm: importance and applications. *Indian J. Biotechnol.* 8, 159–168.

König, J., and Peveling, E. (1984). Cell walls of the phycobionts *Trebouxia* and *Pseudotrebouxia*: constituents and their localization. *Lichenol.* 16, 129–144. doi:10.1016/j.plantsci.2015.04.003.

Kosanić, M., Ranković, B., and Stanojković, T. (2012). Antioxidant, antimicrobial, and anticancer activity of 3 *Umbilicaria* species. *J. Food Sci.* 71, 25. doi:10.1111/j.1750-3841.2011.02459.x.

Kranner, I., and Lutzoni, F. (1999). 'Evolutionary consequences of transition to a lichen symbiotic state and physiological adaptation to oxidative damage associated with poikilohydry.', in *Plant Responses to Environmental Stresses: From Phytohormones to Genome Reorganization*, ed. Lerner HR (M. Dekker, New York), 591–628.

Krewulak, K. D., and Vogel, H. J. (2008). Structural biology of bacterial iron uptake. *Biochim. Biophys. Acta* 1778, 1781–1804.

Kumar, R., Singh, S., and Singh, O. V (2008). Bioconversion of lignocellulosic biomass:

biochemical and molecular perspectives. *J. Ind. Microbiol. Biotechnol.* 35, 377–391. doi:10.1007/s10295-008-0327-8.

Kumar, S. (2015). Role of enzymes in fruit juice processing and its quality enhancement. *Pelagia Res. Libr.* 6, 114–124.

Kumar, S. V. P., Kekuda, T. R. P., Vinayaka, K. S., Mallikarjun, N., and Swathi, D. (2010). Studies on antibacterial, anthelmintic and antioxidant activities of a macrolichen *Parmotrema pseudotinctorum* (des. Abb.) Hale (*Parmeliaceae*) from Bhadra wildlife sanctuary, Karnataka. *Int. J. PharmTech Res.* 2, 1207–1214.

Kurina, L. M., and Vitousek, P. M. (1999). Controls over the accumulation and decline of a nitrogen-fixing lichen, *Stereocaulon vulcani*, on young Hawaiian lava fows. *J. Ecol.* 87, 784–799.

Lamichhane, J. R., and Varvaro, L. (2013). A new medium for the detection of fluorescent pigment production by pseudomonads. *Plant Pathol.* 62, 624–632. doi:10.1111/j.1365-3059.2012.02670.x.

Lang, E., Swiderski, J., Stackebrandt, E., Schumann, P., Spröer, C., and Sahin, N. (2007). *Herminiimonas saxobsidens* sp. nov., isolated from a lichen-colonized rock. *Int. J. Syst. Evol. Microbiol.* 57, 2618–2622. doi:10.1099/ijs.0.65163-0.

Lange, O., and Tenhunen, J. (1981). Moisture content and CO_2 exchange of lichens. II. Depression of net photosynthesis in *Ramalina maciformis* at high water content is caused by increased thallus carbon dioxide diffusion resistance. *Oecologia* 51, 426–429.

Lavallee, V. (2011). Antipain and its analogues, natural product inhibitors of cathepsin K isolated from *Streptomyces*. PhD Thesis. The University of British Columbia.

Lawrey, J. D., Binder, M., Diederich, P., Molina, M. C., Sikaroodi, M., and Ertz, D. (2007). Phylogenetic diversity of lichen-associated homobasidiomycetes. *Mol. Phylogenet. Evol.* 44, 778–789. doi:10.1016/j.ympev.2006.12.023.

Lawrey, J. D., and Diederich, P. (2003). Lichenicolous fungi: interactions, evolution, and biodiversity. *Bryologist* 106, 80–120.

Lee, D.H., Seoun Hur, J., and Kahng, H.Y. (2013). *Sphingobacterium cladoniae* sp. nov., isolated from lichen, *Cladonia* sp., and emended description of *Sphingobacterium siyangense*. *Int. J. Syst. Evol. Microbiol.* 63, 755–760. doi:10.1099/ijs.0.038844-0.

Lee, H., Chul Shin, S., Lee, J., Jin Kim, S., Kim, B.K., Gyu Hong, S., *et al*. (2012a). Genome sequence of *Sphingomonas* sp. strain PAMC 26621, an Arctic-lichen-associated bacterium isolated from a *Cetraria* sp. *J. Bacteriol.* 194, 3030. doi:10.1128/JB.00395-12.

Lee, J., Chul Shin, S., Jin Kim, S., Kim, B.K., Gyu Hong, S., Hye Kim, E., *et al*. (2012b). Draft genome sequence of a *Sphingomonas* sp., an endosymbiotic bacterium isolated from an Arctic lichen *Umbilicaria* sp. *J. Bacteriol.* 194, 3010–3011. doi:10.1128/JB.00360-12.

Lee, P. C., and Schmidt-Dannert, C. (2002). Metabolic engineering towards biotechnological production of carotenoids in microorganisms. *Appl. Microbiol. Biotechnol.* 60, 1–11. doi:10.1007/s00253-002-1101-x.

Lee, Y. M., Kim, E. H., Lee, H. K., and Hong, S. G. (2014). Biodiversity and physiological characteristics of Antarctic and Arctic lichens-associated bacteria. *World J. Microbiol.*

Biotechnol. 30, 2711–2721. doi:10.1007/s11274-014-1695-z.

Lenova, L. I., and Blum, O. (1983). To the question on the third component of lichens. *Bot. J.* 68, 21–28.

Li, B., Xie, C.H., and Yokota, A. (2007). *Nocardioides exalbidus* sp. nov., a novel actinomycete isolated from lichen in Izu-Oshima Island, Japan. *Actinomycetologica* 21, 22–26.

Liba, C. M., Ferrara, F. I. S., Manfio, G. P., Fantinatti-Garboggini, F., Albuquerque, R. C., Pavan, C., *et al*. (2006). Nitrogen-fixing chemo-organotrophic bacteria isolated from cyanobacteria-deprived lichens and their ability to solubilize phosphate and to release amino acids and phytohormones. *J. Appl. Microbiol.* 101, 1076–1086. doi:10.1111/j.1365-2672.2006.03010.x.

Lim, Y. W., Baik, K. S., Han, S. K., Kim, S. B., and Bae, K. S. (2003). *Burkholderia sordidicola* sp. nov., isolated from the white-rot fungus *Phanerochaete sordida*. *Int. J. Syst. Evol. Microbiol.* 53, 1631–1636. doi:10.1099/ijs.0.02456-0.

Limoli, D. H., Jones, C. J., and Wozniak, D. J. (2015). Bacterial extracellular polysaccharides in biofilm formation and function. *Microbiol. Spectr.* 3. doi:10.1128/microbiolspec.MB-0011-2014.

Littman, R. A., Bourne, D. G., and Willis, B. L. (2010). Responses of coral-associated bacterial communities to heat stress differ with *Symbiodinium* type on the same coral host. *Mol. Ecol.* 19, 1978–1990. doi:10.1111/j.1365-294X.2010.04620.x.

Lohtander, K., Oksanen, I., and Rikkinen, J. (2003). Genetic diversity of green algal and cyanobacterial photobionts in *Nephroma* (*Peltigerales*). *Lichenol.* 35, 325–339. doi:10.1016/S0024-2829(03)00051-3.

Lutzoni, F., Kauff, F., Cox, C. J., McLaughlin, D., Celio, G., Dentinger, B., *et al*. (2004). Assembling the fungal tree of life: progress, classification, and evolution of subcellular traits. *Am. J. Bot.* 91, 1446–80. doi:10.3732/ajb.91.10.1446.

Lutzoni, F., Pagel, M., and Reeb, V. (2001). Major fungal lineages are derived from lichen symbiotic ancestors. *Nature* 411, 937–940. doi:10.1038/35082053.

Magain, N., Goffinet, B., and Sérusiaux, E. (2012). Further photomorphs in the lichen family *Lobariaceae* from Reunion (Mascarene archipelago) with notes on the phylogeny of *Dendriscocaulon* cyanomorphs. *Bryologist* 115, 243–254. doi:10.1639/0007-2745-115.2.243.

Magain, N., and Sérusiaux, E. (2014). Do photobiont switch and cephalodia emancipation act as evolutionary drivers in the lichen symbiosis? A sase study in the *Pannariaceae* (*Peltigerales*). *PLoS One* 9, e89876. doi:10.1371/journal.pone.0089876.

Makkonen, S., Hurri, R. S. K., Rko, M. A., and Rinen, H. V (2007). Differential responses of lichen symbionts to enhanced nitrogen and phosphorus availability: an experiment with *Cladina stellaris*. *Ann. Bot.* 99, 877–884. doi:10.1093/aob/mcm042.

Malusá, E., Sas-Paszt, L., and Ciesielska, J. (2012). Technologies for beneficial microorganisms inocula used as biofertilizers. *Sci. World J.* doi:10.1100/2012/491206.

Männistö, M. K., Tiirola, M., McConnell, J., Häggblom, M. M., and Minna Mä nnistö, C. K. (2010). *Mucilaginibacter frigoritolerans* sp. nov*., Mucilaginibacter lappiensis sp.* nov. and

Mucilaginibacter mallensis sp. nov., isolated from soil and lichen samples. *Int. J. Syst. Evol. Microbiol.* 60, 2849–2856. doi:10.1099/ijs.0.019364-0.

Manojlovic, N. T., Vasiljevic, P. J., Gritsanapan, W., Supabphol, R., and Manojlovic, I. (2010). Phytochemical and antioxidant studies of *Laurera benguelensis* growing in Thailand. *Biol. Res.* 43, 169–176.

Manojlović, N. T., Vasiljević, P., Jusković, M., Slobodan, S. N., and Milenković-Andjelović, A. (2010). HPLC analysis and cytotoxic potential of extracts from the lichen, *Thamnolia vermicularis* var. *subuliformis*. *J. Med. Plants Res.* 4, 817–823. doi:10.5897/JMPR10.104.

Matousek, J., Trnena, L., Svoboda, P., Oriniakova, P., and Lichtenstein, C. P. (1995). The gradual reduction of viroid levels in hop mericlones following heat therapy: a possible role for a nuclease degrading dsRNA. *Biol. Chem. Hoppe. Seyler.* 376, 715–722. doi:10.1515/bchm3.1995.376.12.715.

Mavrodi, D. V., Peever, T. L., Mavrodi, O. V., Parejko, J. A., Raaijmakers, J. M., Lemanceau, P., *et al*. (2010). Diversity and evolution of the phenazine biosynthesis pathways. *Appl. Environ. Microbiol.* 76, 866–879. doi:10.1128/AEM.02009-09.

McCurdy, H. D. (1971). Studies on the taxonomy of the *Myxobacterales*. IV. Melittangium. *Int. J. Syst. Bacteriol.* 21, 50–54.

McDonald, D., Price, M. N., Goodrich, J., Nawrocki, E. P., Desantis, T. Z., Probst, A., *et al*. (2012a). An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. *ISME J.* 6, 610–618. doi:10.1038/ismej.2011.139.

McDonald, T. R., Dietrich, F. S., and Lutzoni, F. (2012b). Multiple horizontal gene transfers of ammonium transporters/ammonia permeases from prokaryotes to eukaryotes: toward a new functional and evolutionary classification. *Mol. Biol. Evol.* 29, 51–60. doi:10.1093/molbev/msr123.

Melchers, L. S., and Stuiver, M. H. (2000). Novel genes for disease-resistance breeding. *Curr. Opin. Plant Biol.* 3, 147–152. doi:10.1016/S1369-5266(99)00055-2.

Miadlikowska, J., and Lutzoni, F. (2004). Phylogenetic classification of peltigeralean fungi (*Peltigerales*, *Ascomycota*) based on ribosomal RNA small and large subunits. *Am. J. Bot.* 91, 449–464. doi:10.3732/ajb.91.3.449.

Milagres, A. M. F., Machuca, A., and Napoleão, D. (1999). Detection of siderophore production from several fungi and bacteria by a modification of chrome azurol S (CAS) agar plate assay. *J. Microbiol. Methods* 37, 1–6. doi:10.1016/S0167-7012(99)00028-7.

Miura, Y., Watanabe, Y., and Okabe, S. (2007). Membrane biofouling in pilot-scale membrane bioreactors (MBRs) treating municipal wastewater: impact of biofilm formation. *Environ. Sci. Technol.* 41, 632–638. doi:10.1021/es0615371.

Molins, A., García-Breijo, F. J., Reig-Armiñana, J., del Campo, E. M., Casano, L. M., and Barreno, E. (2013). Coexistence of different intrathalline symbiotic algae and bacterial biofilms in the foliose Canarian lichen *Parmotrema pseudotinctorum*. *Vieraea* 41, 349–370.

Molnár, K., and Farkas, E. (2010). Current results on biological activities of lichen secondary metabolites: a review. *Verlag der Zeitschrift für Naturforsch.* 65, 157–173. doi:10.1515/znc-2010-3-401.

Moorer, W. R. (2003). Antiviral activity of alcohol for surface disinfection. *Int. J. Dent. Hyg.* 1, 138–142. doi:10.1034/j.1601-5037.2003.00032.x.

Morris, E. K., Caruso, T., Buscot, F., Fischer, M., Hancock, C., Maier, T. S., *et al*. (2014). Choosing and using diversity indices: insights for ecological applications from the German biodiversity exploratories. *Ecol. Evol.* 4, 3514–3524. doi:10.1002/ece3.1155.

Motohashi, K., Takagi, M., Yamamura, H., Hayakawa, M., and Shin-Ya, K. (2010). A new angucycline and a new butenolide isolated from lichen-derived *Streptomyces* spp. *J. Antibiot.* 63, 545–548. doi:10.1038/ja.2010.94.

Muggia, L., Grube, M., and Tretiach, M. (2008). Genetic diversity and photobiont associations in selected taxa of the *Tephromela* atra group (*Lecanorales*, lichenised *Ascomycota*). *Mycol Prog.* 7, 147–160. doi:10.1007/s11557-008-0560-6.

Muggia, L., Klug, B., Berg, G., and Grube, M. (2013). Localization of bacteria in lichens from Alpine soil crusts by fluorescence *in situ* hybridization. *Appl. Soil Ecol.* 68, 20–25. doi:10.1016/j.apsoil.2013.03.008.

Mukhtar, A., Garty, J., and Galun, M. (1994). Does the liquen alga *Trebouxia* occur free-living in nature - Further immunological evidence. *Symbiosis* 17, 247–253.

Mushegian, A. A., Peterson, C. N., Baker, C. C. M., and Pringle, A. (2011). Bacterial diversity across individual lichens. *Appl. Environ. Microbiol.* 77, 4249–4252. doi:10.1128/AEM.02850-10.

Nagpure, A., and Gupta, R. K. (2013). Purification and characterization of an extracellular chitinase from antagonistic *Streptomyces violaceusniger*. *J. Basic Microbiol.* 53, 429–439. doi:10.1002/jobm.201100648.

Nash, T. H. (2008). *Lichen biology*. Cambridge University Press.

Navahradak, D. M. (1949). Lichens and cellulose degrading microorganisms. *Microbiology* 18, 6.

Navarro-González, I., and Periago, M. J. (2012). Enzimas lipolíticas bacterianas: propiedades, clasificación, estructura, aplicaciones tecnológicas y aspectos legales. *An. Vet. Murcia* 28, 45–65.

Navarro-Noya, Y. E., Jiménez-Aguilar, A., Valenzuela-Encinas, C., Alcántara-Hernández, R. J., Ruíz-Valdiviezo, V. M., Ponce-Mendoza, A., *et al*. (2014). Bacterial communities in soil under moss and lichen-moss crusts. *Geomicrobiol. J.* 31, 152–160. doi:10.1080/01490451.2013.820236.

Nelsen, M. P., and Gargas, A. (2008). Dissociation and horizontal transmission of codispersing lichen symbionts in the genus *Lepraria* (*Lecanorales*: *Stereocaulaceae*). *New Phytol.* 177, 264–275. doi:10.1111/j.1469-8137.2007.02241.x.

Nieboer, E., Richardson, D. H. S., and Tomassini, F. D. (1978). Mineral uptake and release by lichens: an overview. *Bryologist* 81, 226–246. doi:10.2307/3242185.

Nikolic, B., Schwab, H., and Sessitsch, A. (2011). Metagenomic analysis of the 1-aminocyclopropane-1-carboxylate deaminase gene (*acdS*) operon of an uncultured bacterial endophyte colonizing *Solanum tuberosum* L. *Arch. Microbiol.* 193, 665–676. doi:10.1007/s00203-011-0703-z.

Nordlund, S., and Högbom, M. (2013). ADP-ribosylation, a mechanism regulating nitrogenase activity. *FEBS J.* 280, 3484–3490. doi:10.1111/febs.12279.

Notomista, E., Pennacchio, F., Cafaro, V., Smaldone, G., Izzo, V., Troncone, L., *et al*. (2011). The marine isolate *Novosphingobium* sp. PP1Y shows specific adaptation to use the aromatic fraction of fuels as the sole carbon and energy source. *Microb. Ecol.* 61, 582–594. doi:10.1007/s00248-010-9786-3.

Nybakken, L., Solhaug, K. A., Bilger, W., and Gauslaa, Y. (2004). The lichens *Xanthoria elegans* and *Cetraria islandica* maintain a high protection against UV-B radiation in Arctic habitats. *Oecologia* 140, 211–216. doi:10.1007/s00442-004-1583-6.

O'Brien, H. E., Miadlikowska, J., and Lutzoni, F. (2005). Assessing host specialization in symbiotic cyanobacteria associated with four closely related species of the lichen fungus *Peltigera*. *Eur. J. Phycol.* 40, 363–378. doi:10.1080/09670260500342647.

Oksanen, I. (2006). Ecological and biotechnological aspects of lichens. *Appl. Microbiol. Biotechnol.* 73, 723–734. doi:10.1007/s00253-006-0611-3.

Olafsdottir, E. S., and Ingólfsdóttir, K. (2001). Polysaccharides from lichens: structural characteristics and biological activity. *Planta Med.* 67, 199–208. doi:10.1055/s-2001-12012.

Oldroyd, G. E., and Dixon, R. (2014). Biotechnological solutions to the nitrogen problem biotechnology. *Curr. Opin. Biotechnol.* 26, 19–24. doi:10.1016/j.copbio.2013.08.006.

Palmqvist, K., and Dahlman, L. (2006). Responses of the green algal foliose lichen *Platismatia glauca* to increased nitrogen supply. *New Phytol.* 171, 343–356.

Palmqvist, K., Dahlman, L., Jonsson, A., and Nash, T. (2008). 'The carbon economy of lichens', in *Lichen Biology*, ed. T. H. Nash (New York: Cambridge University Press), 182–2015.

Pan, Y., Xiao, L., Li, A. S. S., Zhang, X., Sirois, P., Zhang, J., *et al*. (2013). Biological and biomedical applications of engineered nucleases. *Mol. Biotechnol.* 55, 54–62. doi:10.1007/s12033-012-9613-9.

Pandey, A., Nigam, P., Soccol, C. R., Soccol, V. T., Singh, D., and Mohan, R. (2000). Advances in microbial amylases. *Biotechnol. Appl. Biochem.* 31, 135–52. doi:10.1042/BA19990073.

Pankratov, T. A. (2012). *Acidobacteria* in microbial communities of the bog and tundra lichens. *Mikrobiologiya* 81, 51–58. doi:10.1134/S0026261711060166.

Panosyan, A. K., and Nikogosyan, V. G. (1966). The presence of *Azotobacter* in lichens. *Akad Nauk Armian SSR, Biol Zhurn Armen* 19, 3–11.

Paracer, S. (2000). *Symbiosis: an introduction to biological associations*. 2nd ed. New York : Oxford University Press.

Park, M., Kim, C., Yang, J., Lee, H., Shin, W., Kim, S., *et al*. (2005). Isolation and characterization of diazotrophic growth promoting bacteria from rhizosphere of agricultural crops of Korea. *Microbiol. Res.* 160, 127–133. doi:10.1016/j.micres.2004.10.003.

Parrot, D., Antony-Babu, S., Intertaglia, L., Grube, M., Tomasi, S., and Suzuki, M. T. (2015). Littoral lichens as a novel source of potentially bioactive *Actinobacteria*. *Sci. Rep.* 5, 1–14. doi:10.1038/srep15839.

Parrot, D., Legrave, N., Delmail, D., Grube, M., Suzuki, M., and Tomasi, S. (2016). Lichen-associated bacteria as a hot spot of chemodiversity: focus on uncialamycin, a promising compound for future medicinal applications. *Plant Med.* 82, 1143–1152. doi:10.1055/s-0042-105571.

Patten, C. L., and Glick, B. R. (2002). Role of *Pseudomonas putida* indoleacetic acid in development of the host plant root system. *Appl. Environ. Microbiol.* 68, 3795–3801. doi:10.1128/AEM.68.8.3795–3801.2002.

Paungfoo-Lonhienne, C., Rentsch, D., Robatzek, S., Webb, R. I., Sagulenko, E., Nä Sholm, T., *et al*. (2010). Turning the table: plants consume microbes as a source of nutrients. *PLoS One* 5. doi:10.1371/journal.pone.0011915.

Piercey-Normore, M. D. (2006). The lichen-forming ascomycete *Evernia mesomorpha* associates with multiple genotypes of *Trebouxia jamesii*. *New Phytol.* 169, 331–344. doi:10.1111/j.1469-8137.2005.01576.x.

Pikovskaya, R. I. (1948). Mobilization of phosphorous in soil connection with the vital activity of some microbial species. *Mikrobiologiya* 17, 362–370.

Portillo, M. C., Villahermosa, D., Corzo, A., and Gonzalez, J. M. (2011). Microbial community fingerprinting by differential display-denaturing gradient gel electrophoresis standard DGGE analyses resulted in molecular fingerprints. *Appl. Environ. Microbiol.* 77, 351–354. doi:10.1128/AEM.01316-10.

Printzen, C., Fernández-Mendoza, F., Muggia, L., Berg, G., and Grube, M. (2012). Alphaproteobacterial communities in geographically distant populations of the lichen *Cetraria aculeata*. *FEMS Microbiol. Ecol.* 82, 316–25. doi:10.1111/j.1574-6941.2012.01358.x.

R Development Core Team (2013). *R: A language and environment for statistical computing*. , ed. The R Foundation for Statistical Vienna, Austria: R Foundation for Statistical Computing.

Rajagopalan, G., and Krishnan, C. (2008). α-Amylase production from catabolite derepressed *Bacillus subtilis* KCC103 utilizing sugarcane bagasse hydrolysate. *Bioresour. Technol.* 99, 3044–3050. doi:10.1016/j.biortech.2007.06.001.

Ramanan, R., Kim, B. H., Cho, D. H., Oh, H. M., and Kim, H. S. (2016). Algae-bacteria interactions: evolution, ecology and emerging applications. *Biotechnol. Adv.* 34, 14–29. doi:10.1016/j.biotechadv.2015.12.003.

Rambold, G., Friedl, T., and Beck, A. (2011). Photobionts in lichens: possible indicators of phylogenetic relationships? *Am. Bryol. Lichenol. Soc.* 101, 392–397.

Rao, M. P. N., Xiao, M., and Li, W.J. (2017). Fungal and bacterial pigments: secondary metabolites with wide applications. *Front. Microbiol.* 8. doi:10.3389/fmicb.2017.01113.

Rathore, A. S., Gupta, R. D., Rathore, A. S., and Gupta, R. D. (2015). Chitinases from bacteria to human: properties, applications, and future perspectives. *Enzyme Res.* 2015, 1–8. doi:10.1155/2015/791907.

Reddy, N. S., Nimmagadda, A., and Sambasiva Rao, K. R. S. (2003). An overview of the microbial α-amylase family. *African J. Biotechnol.* 2, 645–648.

Reichenbach, H. (2006). The order *Cytophagales*. *Prokaryotes* 7, 549–590. doi:10.1007/978-1-4757-2191-1_37.

Reutimann, P., and Scheidegger, C. (1987). Importance of lichen secondary products in food choice of two oribatid mites (*Acari*) in an alpine meadow ecosystem. *J. Chem. Ecol.* 13, 363–369. doi:10.1007/BF01025896.

Rodríguez, H., Fraga, R., Gonzalez, T., and Bashan, Y. (2006). Genetics of phosphate solubilization and its potential applications for improving plant growth-promoting bacteria. *Plant Soil* 287, 15–21.

Rodriguez, R. J., Henson, J., Volkenburgh, E. Van, Hoy, M., Wright, L., Beckwith, F., *et al*. (2008). Stress tolerance in plants via habitat-adapted symbiosis. *ISME J.* 2, 404–416. doi:10.1038/ismej.2007.106.

Rubio, C., Fernández, E., Hidalgo, M. E., and Quilhot, W. (2002). Effects of solar UV-B radiation in the accumulation of rhizocarpic acid in a lichen species from alpine zones of Chile. *Bol. la Soc. Chil. Quim.* 47, 67–72. doi:10.4067/S0366-16442002000100012.

Ryder, C., Byrd, M., and Wozniak, D. J. (2007). Role of polysaccharides in *Pseudomonas aeruginosa* biofilm development. *Curr. Opin. Microbiol.* 10, 644–648.

Ryu, C.M., Farag, M. A., Hu, C.H., Reddy, M. S., Wei, H.X., Paré, P. W., *et al*. (2003). Bacterial volatiles promote growth in *Arabidopsis*. *Proc. Natl. Acad. Sci.* 100, 4927–4932.

Sabra, W., Zeng, A., Nsdorf, H. L., and Deckwer, W.D. (2000). Effect of oxygen on formation and structure of *Azotobacter vinelandii* alginate and its role in protecting nitrogenase. *Appl. Environ. Microbiol.* 66, 4037–4044.

Sakai, K., Yokota, A., Kurokawa, H., Wakayama, M., and Moriguchi, M. (1998). Purification and characterization of three thermostable endochitinases of a noble bacillus strain, MH-1, isolated from chitin-containing compost. *Appl. Environ. Microbiol.* 64, 3397–3402.

Sampaio, A. F. S., Silva, M., Waleska, D. C., Costa, D. C., Silva, M. E., dos Santos, R. C., *et al*. (2014). Iron toxicity mediated by oxidative stress enhances tissue damage in an animal model of diabetes. *Biometals* 27, 349–361. doi:10.1007/s10534-014-9717-8.

Sancho, L. G., de la Torre, R., Horneck, G., Ascaso, C., de los Rios, A., Pintado, A., *et al*. (2007). Lichens survive in space: results from the 2005 LICHENS experiment. *Astrobiology* 7, 443–454. doi:10.1089/ast.2006.0046.

Sanders, W. B. (2001). Lichens: the interface between Mycology and Plant Morphology. *Bioscience* 51, 1025–1036. doi:10.1641/0006.

Sanders, W. B. (2005). Observing microscopic phases of lichen life cycles on transparent substrata placed *in situ*. *Lichenol.* 37, 373–382. doi:10.1017/S0024282905015070.

Sanger, F., and Coulson, A. R. (1976). A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase. *J. Mol. Biol.* 94, 441–448.

Sanger, F., Nicklen, S., and Coulson, A. R. (1977). Biochemistry DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* 74, 5463–5467.

Santander, R. D., and Biosca, E. G. (2017). *Erwinia amylovora* psychrotrophic adaptations: evidence of pathogenic potential and survival at temperate and low environmental

temperatures. *PeerJ* 5, e3931. doi:10.7717/peerj.3931.

Santander, R. D., Monte-Serrano, M., Rodríguez-Herva, J. J., López-Solanilla, E., Rodríguez-Palenzuela, P., and Biosca, E. G. (2014). Exploring new roles for the *rpoS* gene in the survival and virulence of the fire blight pathogen *Erwinia amylovora*. *FEMS Microbiol. Ecol.* 90, 895–907. doi:10.1111/1574-6941.12444.

Sauer, K., Camper, A. K., Ehrlich, G. D., Costerton, J. W., and Davies, D. G. (2002). *Pseudomonas aeruginosa* displays multiple phenotypes during development as a biofilm. *J. Bacteriol.* 184, 1140–1154. doi:10.1128/JB.184.4.1140.

Sawant, R., and Nagendran, S. (2014). Protease: an enzyme with multiple industrial applications. *World J. Pharm. Pharm. Sci.* 3, 568–579.

Schneider, T., Schmid, E., de Castro, J. V., Cardinale, M., Eberl, L., Grube, M., *et al*. (2011). Structure and function of the symbiosis partners of the lung lichen (*Lobaria pulmonaria* L. Hoffm.) analyzed by metaproteomics. *Proteomics* 11, 2752–2756. doi:10.1002/pmic.201000679.

Schoch, C. L., Sung, G., Pez-gir Ldez, F. L., Townsend, J. P., Miadlikowska, J., Rie Hofstetter, V., *et al*. (2009). The *Ascomycota* tree of life: a phylum-wide phylogeny clarifies the origin and evolution of fundamental reproductive and ecological traits. *Syst. Biol.* 58, 224–239. doi:10.1093/sysbio/syp020.

Schroeckh, V., Scherlach, K., Nützmann, H.W., Shelest, E., Schmidt-Heck, W., Schuemann, J., *et al*. (2009). Intimate bacterial–fungal interaction triggers biosynthesis of archetypal polyketides in *Aspergillus nidulans*. *Proc. Natl. Acad. Sci.* 106, 14558–14563.

Schwyn, B., and Neilands, J. B. (1987). Universal chemical assay for the detection determination of siderophores. *Anal. Biochem.* 160, 47–56.

Scott, G. (1956). Further investigation of some lichens for fixation of nitrogen. *New Phytol.* 55, 111–116. doi:10.1111/j.1469-8137.1956.tb05269.x.

Seefeldt, L. C., Hoffman, B. M., and Dean, D. R. (2009). Mechanism of Mo-dependent nitrogenase. *Annu. Rev. Biochem.* 2, 701–722. doi:10.1146/annurev.biochem.78.070907.103812.

Selbmann, L., Zucconi, L., Ruisi, S., Grube, M., Cardinale, M., and Onofri, S. (2010). Culturable bacteria associated with Antarctic lichens: affiliation and psychrotolerance. *Polar Biol.* 33, 71–83. doi:10.1007/s00300-009-0686-2.

Seneviratne, G., Thilakaratne, R., Jayasekara, A., Seneviratne, K., Padmathilake, K., and De Silva, M. (2009). 'Developing beneficial microbial biofilms on root of non legumes: a novel biofertilizing technique', in *Microbial Strategies for Crop Improvement*, eds. M. Khan, A. Zaidi, and J. Musarrat (Berlin, Heidelberg), 1–358. doi:10.1007/978-3-642-01979-1.

Seper, A., Fengler, V. H. I., Roier, S., Wolinski, H., Kohlwein, S. D., Bishop, A. L., *et al*. (2011). Extracellular nucleases and extracellular DNA play important roles in *Vibrio cholerae* biofilm formationm. *Mol. Microbiol.* 82, 1015–1037. doi:10.1111/j.1365-2958.2011.07867.x.

Shahzad, S. M., Arif, M. S., Riaz, M., Iqbal, Z., and Ashraf, M. (2013). PGPR with varied ACC-deaminase activity induced different growth and yield response in maize (*Zea mays* L.) under fertilized conditions. *Eur. J. Soil Biol.* 57, 27–34. doi:10.1016/j.ejsobi.2013.04.002.

Shannon, C. E. (1948). A mathematical theory of communication. *Bell Syst. Tech. J.* 27, 379–423.

Sharma, S. B., Sayyed, R. Z., Trivedi, M. H., and Gobi, T. A. (2013). Phosphate solubilizing microbes: sustainable approach for managing phosphorus deficiency in agricultural soils. *Springerplus* 2, 587–601. doi:10.1186/2193-1801-2-587.

Shin, S. C., Ahn, D. H., Lee, J. K., Kim, S. J., Hong, S. G., Kim, E. H., *et al*. (2012). Genome sequence of *Sphingomonas* sp. strain PAMC 26605, isolated from Arctic lichen (*Ochrolechia* sp.). *J. Bacteriol.* 194, 1607. doi:10.1128/JB.00004-12.

Shrimant, B. S. (2012). Review: nitrogen fixing microorganisms. *Int. J. Microbiol. Res.* 3, 46–52. doi:10.5829/idosi.ijmr.2012.3.1.61103.

Shukla, V., Pant, G. J., and Rawat, M. S. M. (2010). Lichens as a potential natural source of bioactive compounds: a review. *Phytochem. Rev.* 9, 303–314. doi:10.1007/s11101-010-9189-6.

Sigurbjörnsdóttir, M. A., Andrésson, Ó. S., and Vilhelmsson, O. (2015). Analysis of the *Peltigera membranacea* metagenome indicates that lichen-associated bacteria are involved in phosphate solubilization. *Microbiology* 161, 989–996. doi:10.1099/mic.0.000069.

Sigurbjörnsdóttir, M. A., Andrésson, Ó. S., and Vilhelmsson, O. (2016). Nutrient scavenging activity and antagonistic factors of non-photobiont lichen-associated bacteria: a review. *World J. Microbiol. Biotechnol.* 32, 1–11. doi:10.1007/s11274-016-2019-2.

Sigurbjörnsdóttir, M. A., Heiðmarsson, S., Jónsdóttir, A. R., and Vilhelmsson, O. (2014). Novel bacteria associated with Arctic seashore lichens have potential roles in nutrient scavenging. *Can. J. Microbiol.* 60, 307–317. doi:10.1139/cjm-2013-0888.

Sigurbjörnsdóttir, M. A., and Vilhelmsson, O. (2016). Selective isolation of potentially phosphate-mobilizing, biosurfactant-producing and biodegradative bacteria associated with a sub-Arctic, terricolous lichen, *Peltigera membranacea*. *FEMS Microbiol. Ecol.* 92. doi:10.1093/femsec/fiw090.

Singh, S., Garrity, G., Genillourd, O., Lingham, R., Martin, I., Nallin-Omstead, M., *et al*. (1997). Inhibitor compounds of farnesyl-protein transferase and chemotherapeutic compositions containing the same, produced by strain ATCC 55532. *US5627057.*

Škaloud, P., and Peksa, O. (2008). Comparative study of chloroplast morphology and ontogeny in *Asterochloris* (*Trebouxiophyceae*, *Chlorophyta*). *Biologia.* 3, 869–876. doi:10.2478/s11756-008-0115-y.

Škaloud, P., and Peksa, O. (2010). Evolutionary inferences based on ITS rDNA and actin sequences reveal extensive diversity of the common lichen alga *Asterochloris* (*Trebouxiophyceae*, *Chlorophyta*). *Mol. Phylogenet. Evol.* 54, 36–46. doi:10.1016/j.ympev.2009.09.035.

Smith, D. C., and Douglas, A. (1987). *The Biology of Symbiosis*. , ed. E. Arnold London.

Smith, H. L., and Goodner, K. (1958). Detection of bacterial gelatinases by gelatin-agar plate methods. *J. Bacteriol.* 76, 662–665.

Souza, E. M., Chubatsu, L. S., Huergo, L. F., Monteiro, R., Camilios-Neto, D., Wassem, R., *et al*. (2014). Use of nitrogen-fixing bacteria to improve agricultural productivity. *BMC Proc.* 8,

O23. doi:10.1186/1753-6561-8-S4-O23.

Spribille, T., Tuovinen, V., Resl, P., Vanderpool, D., Wolinski, H., Aime, M. C., *et al*. (2016). *Basidiomycete* yeasts in the cortex of *Ascomycete* macrolichens. *Science (80).* 353, 488–492. doi:10.1126/science.aaf8287.

Srivastava, P., Upreti, D. K., Dhole, T. N., Srivastava, A. K., and Nayak, M. T. (2013). Antimicrobial property of extracts of indian lichen against human pathogenic bacteria. *Interdiscip. Perspect. Infect. Dis.* 2013. doi:10.1155/2013/709348.

Stocker-Wörgötter, E. (2008). Metabolic diversity of lichen-forming ascomycetous fungi: culturing, polyketide and shikimate metabolite production, and *PKS* genes. *Nat. Prod. Rep.* 25, 188–200. doi:10.1039/b606983p.

Stoykov, Y. M., Pavlov, A. I., and Krastanov, A. I. (2015). Chitinase biotechnology: production, purification, and application. *Eng. Life Sci.* 15, 30–38. doi:10.1002/elsc.201400173.

Sundara-Rao, W., and Sinha, M. (1963). Phosphate dissolving microorganisms in the soil and rhizosphere. *Indian J. Agric. Sci.* 33, 272–278.

Suryanarayanan, T. S., Thirunavukkarasu, N., Hariharan, G. N., and Balajr, P. (2005). Occurrence of non-obligate microfungi inside lichen thalli. *Sydowia* 57, 119–129.

Suzuki, M. T., Parrot, D., Berg, G., Grube, M., and Tomasi, S. (2016). Lichens as natural sources of biotechnologically relevant bacteria. *Appl. Microbiol. Biotechnol.* 100, 583–595. doi:10.1007/s00253-015-7114-z.

Syers, J. K., and Iskandar, I. K. (1973). 'Pedogenetic significance of lichens', in *The Lichens*, eds. V. Ahmadjian and M. E. Hale (London: Academic Press, Inc), 225–248. doi:10.1016/B978-0-12-044950-7.50012-X.

Tambalo, D. D., Yost, C. K., and Hynes, M. F. (2010). Characterization of swarming motility in *Rhizobium leguminosarum* bv. *viciae*. *FEMS Microbiol. Lett.* 307, 165–174. doi:10.1111/j.1574-6968.2010.01982.x.

Tamura, K. (1992). Estimation of the number of nucleotide substitutions when there are strong transition-transversion and G + C-content biases. *Mol. Biol. Evol.* 9, 678–687.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. (2013). MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.* 30, 2725–2729.

Tang, J. W. (2009). The effect of environmental parameters on the survival of airborne infectious agents. *J. R. Soc. Interface* 6, 737–746. doi:10.1098/rsif.2009.0227.focus.

Taylor, T. N., Hass, H., Remy, W., and Kerp, H. (1995). The oldest fossil lichen. *Nature* 378, 244. doi:10.1038/378244a0.

Tel-Or, E., and Stewart, W. D. (1976). Photosynthetic electron transport, ATP synthesis and nitrogenase activity in isolated heterocysts of *Anabaena cylindrica*. *Biochim. Biophys. Acta* 423, 189–195.

Tenke, P., Kovacs, B., Jäckel, M., and Nagy, E. (2006). The role of biofilm infection in urology. *World J. Urol.* 24, 13–20. doi:10.1007/s00345-005-0050-2.

Thaxter, R. (1892). On the *Myxobacteriaceae*, a new order of *Schizomycetes*. *Bot. Gaz.* 17, 389–406.

Timmermans, K. (2001). *Trips, CBD and traditional medicines: concepts and questions. Report of an ASEAN workshop on the TRIPS agreement and traditional medicine*., ed. N. A. for D. and F. Control Jakarta.

Tschermak-Woess, E. (1988). 'The algal partner', in *Handbook of lichenology*, ed. M. Galun (CRC, Boca Raton), 39–92.

U 'Ren, J. M., Lutzoni, F., Miadlikowska, J., and Arnold, A. E. (2010). Community analysis reveals close affinities between endophytic and endolichenic fungi in mosses and lichens. *Microbiol. Ecol.* 60, 340–353.

Vallejos, N. G. (2013). Degradación de residuos vegetales mediante inoculación con cepas microbianas. *Enfoque UTE* 4, 1–13.

Verstraeten, N., Braeken, K., Debkumari, B., Fauvart, M., Fransaer, J., Vermant, J., *et al*. (2008). Living on a surface: swarming and biofilm formation. *Trends Microbiol.* 16, 496–506. doi:10.1016/j.tim.2008.07.004.

Větrovský, T. Š., Steffen, K. T., and Baldrian, P. (2014). Potential of cometabolic transformation of polysaccharides and lignin in lignocellulose by soil *Actinobacteria*. *PLoS One* 9. doi:10.1371/journal.pone.0089108.

Vorkapic, D., Pressler, K., and Schild, S. (2016). Multifaceted roles of extracellular DNA in bacterial physiology. *Curr. Genet.* 62, 71–79. doi:10.1007/s00294-015-0514-x.

Vráblíková, H., McEvoy, M., Solhaug, K. A., Barták, M., and Gauslaa, Y. (2006). Annual variation in photoacclimation and photoprotection of the photobiont in the foliose lichen *Xanthoria parietina*. *J. Photochem. Photobiol.* 83, 151–162. doi:10.1016/j.jphotobiol.2005.12.019.

Wang, Q., Garrity, G. M., Tiedje, J. M., and Cole, J. R. (2007). Nave bayesian classifier for rapid assignment of *rRNA* sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.* 73, 5261–5267. doi:10.1128/AEM.00062-07.

Werth, S. (2011). 'Biogeography and phylogeography of lichen fungi and their photobionts', in *Biogeography of microscopic organisms: is everything small everywhere?*, ed. D. Fontaneto (Cambridge University Press), 191–208. doi:10.1017/CBO9780511974878.011.

White, J. F., Crawford, H., Torres, M. S., Mattera, R., Irizarry, I., and Bergen, M. (2012). A proposed mechanism for nitrogen acquisition by grass seedlings through oxidation of symbiotic bacteria. *Symbiosis* 57, 161–171. doi:10.1007/s13199-012-0189-8.

Whittaker, R. H. (1972). Evolution and measurement of species diversity. *Taxon* 213, 213–251.

Whittaker, R. J., Ladle, R. J., Arau, M. B., Delgado, J. D., and Arévalo, J. R. (2007). The island immaturity-speciation pulse model of island evolution: an alternative to the 'diversity begets diversity' model. *Ecography (Cop.).* 30, 321–327. doi:10.1111/j.2007.0906-7590.04761.x.

Wirtz, N., Lumbsch, H. T., Green, T. G. A., Türk, R., Pintado, A., Sancho, L., *et al*. (2003). Lichen fungi have low cyanobiont selectivity in maritime Antarctica. *New Phytol.* 160, 177–183. doi:10.1046/j.1469-8137.2003.00859.x.

Yahr, R., Vilgalys, R., and DePriest, P. T. (2004). Strong fungal specificity and selectivity for algal symbionts in Florida scrub *Cladonia* lichens. *Mol. Ecol.* 13, 3367–3378.

doi:10.1111/j.1365-294X.2004.02350.x.

Yahr, R., Vilgalys, R., and DePriest, P. T. (2006). Geographic variation in algal partners of *Cladonia subtenuis* (*Cladoniaceae*) highlights the dynamic nature of a lichen symbiosis. *New Phytol.* 171, 847–860. doi:10.1111/j.1469-8137.2006.01792.x.

Yamamura, H., Ashizawa, H., Nakagawa, Y., Hamada, M., Ishida, Y., Otoguro, M., *et al*. (2011a). *Actinomycetospora iriomotensis* sp. nov., a novel actinomycete isolated from a lichen sample. *J. Antibiot. (Tokyo).* 64, 289–292. doi:10.1038/ja.2011.15.

Yamamura, H., Ashizawa, H., Nakagawa, Y., Hamada, M., Ishida, Y., Otoguro, M., *et al*. (2011b). *Actinomycetospora rishiriensis* sp. nov., isolated from a lichen. *Int. J. Syst. Evol. Microbiol.* 61, 2621–2625. doi:10.1099/ijs.0.028753-0.

Yang, J. K., Zhang, J. J., Yu, H. Y., Cheng, J. W., and Miao, L. H. (2014). Community composition and cellulase activity of cellulolytic bacteria from forest soils planted with broad-leaved deciduous and evergreen trees. *Appl. Microbiol. Biotechnol.* 98, 1449–1458. doi:10.1007/s00253-013-5130-4.

Yang, Y., Yokobori, S. ichi, and Yamagishi, A. (2009). Bacterial survival in response to desiccation and high humidity at above zero and subzero temperatures. *Adv. Sp. Res.* 43, 1285–1290. doi:10.1016/j.asr.2009.01.018.

Yara, R., Maccheroni, W., Horii, J., and Azevedo, J. L. (2006). A bacterium belonging to the *Burkholderia cepacia* complex associated with *Pleurotus ostreatus*. *J. Microbiol.* 44, 263–268.

Zhao, K., Penttinen, P., Zhang, X., Ao, X., Liu, M., Yu, X., *et al*. (2014). Maize rhizosphere in Sichuan, China, hosts plant growth promoting *Burkholderia cepacia* with phosphate solubilizing and antifungal abilities. *Microbiol. Res.* 169, 76–82.

Zhou, X., and Li, Y. (2015). 'Techniques for Oral Microbiology', in *Atlas of Oral Microbiology*, eds. X. Zhou and Y. Li (Zhejiang University Press: Elsevier Inc.), 15–40. doi:10.1016/B978-0-12-802234-4.00002-1.

Zook, P. D. (1983). A study of the role of bacteria in lichens. *MA thesis, Clark Univ. Worcester, USA*.