VNIVERSITAT DE VALÈNCIA Facultad de Ciencias Biológicas

PHD Thesis

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Mining of new insecticidal protein genes plus determination of the insecticidal spectrum and mode of action of *Bacillus thuringiensis* Vip3Ca protein

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To my family

Dr. Juan Ferré Manzanero, Catedràtic del Departament de Genètica de la Facultat de Ciències Biològiques de la Universitat de València,

Informa:

Que En Joaquín Gomis i Cebolla, Llicenciat en Ciències Biològiques , ha realitzat sota la seva direcció el treball de investigació recollit en la tesi doctoral que porta per títol "Mining of new insecticidal protein genes plus determination of the insecticidal spectrum and mode of action of *Bacillus thuringiensis* Vip3Ca protein" per tal de optar al Grau de Doctor per la Universitat de València.

I per fer constància, d'acord amb la legislació vigent, signa la present a Burjassot, a 7 de Novembre de 2018.

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1. - ABSTRACT/RESUM

Abstract

Bacillus thuringiensis is an entomopathogenic bacterium that belongs to the *Bacillus cereus* group and produces a wide variety of insecticidal proteins along with other virulence factors contributing to its pathogenicity. *Bacillus thuringiensis* has been considered as the most successful bioinsecticidal agent during the last century. Currently, it is widely used as a microbial agent of the major insect pests. In the present doctoral thesis, we performed the screening for new *B. thuringiensis* insecticidal protein genes from selected *B. thuringiensis* isolates based on their gene content. Also, we studied in detail different aspects (insecticidal spectrum, cross-resistance, mode of action and cell death response of *Spodoptera exigua* intoxicated with the Vip3Ca protein) of the new Vip3Ca protein family.

Regarding the mining of new insecticidal proteins, we found one new couple of binary Vip-like proteins (Vip2Ac-like_1-Vip4Aa-like_1), two new Vip-like proteins (Vip2Ac-like_1 and Vip4Aa-like_2), one Sip1A-like protein (Sip1A-like_1) and eight Crystal-like proteins (Cry23A-like, Cry45Aa-like_1, Cry45Aa-like_2, Cry45Aa-like_3, Cry32Ea-like, Cry32Da-like, Cry32Eb-like and Cry73Aa-like). The Vip-like proteins have been detected in the supernatant, marginally expressed, while the Crystal-like proteins have been found in the parasporal crystal (E-SE10.2: 2.5 % - 30 % ; O-V84.2: 7.0 % - 9.8 % Cry45-like proteins, 30.4 % - 30.5 % Cry32-like proteins and 2.8 % - 4.25 % Cry73Aa-like), except the Cry23Aa-like protein, that has also been found in the supernatant at 24 h and 48 h. In addition, the toxicity of the supernatant (E-SE10.2: 24 h and 48 h; O-V84.2: 24 h and 48 h) and parasporal crystal (E-SE10.2 and O-V84.2) has been tested in *S. exigua* and *Spodoptera littoralis*, where the Cry23Aa-like showed some toxicity to both insect species.

In the case of the Vip3Ca protein, we first extended the study of its insecticidal activity to ten lepidopteran pests (*Cydia pomonella, Grapholita molesta, Sesamia nonagrioides, Galleria mellonella, Plutella xylostella, Pectinophora gossypiella, Ephestia kuehniella, Plodia interpunctella* and *Ostrinia furnacalis*), one aphid (*Nezara viridula*) and one model organism (*Drosophila melanogaster*). Several methodologies of biossays were used. For the species tested by surface contamination, *C. pomonella* was the most susceptible one, followed by *G. mellonella*, with percentages of mortality higher than 50%. Regarding the four species tested by diet incorporation, *O. furnacalis* was the most

susceptible one, with an LC_{50} value of 0.31 µg/g. For the other types of bioassays, *Tuta absoluta* showed some susceptibility to high concentrations of Vip3Ca (30 % mortality). Also, several Cry1A, Cry2A, Dipel, Vip3 and Vip3/Cry2Ab resistant insect colonies were tested against the Vip3Ca protein to determine if they presented cross-resistance. The results showed that the insect colonies resistant to Cry1Ac (*Helicoverpa armigera*, *Trichoplusia ni, O. furnacalis* and *P. interpunctella*) or Cry2Ab (*H. armigera* and *T. ni*) were not cross-resistant to Vip3 proteins (Vip3Aa and Vip3Ca). In contrast, the *H. armigera* colonies resistant to Vip3Aa or Vip3Aa/Cry2Ab showed cross-resistance to the Vip3Ca protein.

We should mention that, as a secondary work arising from the search for susceptible species, arose the monographic study on the susceptibility of *G. molesta* to those formulated by *B. thuringiensis*, its toxins (Cry and Vip3) and synergies between the Cry and Vip3 proteins. As a result, we found that *G. molesta* is highly susceptible to *B. thuringiensis*, Dipel® and Xentari® formulates, and to the *B. thuringiensis* toxins Cry1Aa, Cry1Ac, Cry1C, Vip3Aa and Vip3Af. Once determined that *G. molesta* is susceptible to Cry1Aa, Cry1Ac, Cry1C and Vip3Aa toxins, the possible synergies between these proteins were evaluated in dose-response assays. The combinations of Cry1Aa-Vip3Aa and Cry1C-Vip3Aa proteins were antagonistic while the combination of Cry1Ac-Vip3Aa proteins shows an additive effect. It is interesting to mention that the antagonism detected in the Cry1Aa-Vip3Aa and Cry1C-Vip3Aa protein combinations increased as the amount of Vip3A protein in the mixture was higher; this fact suggests that, though these proteins do not compete for the same binding sites, they may interact in events prior to the binding, making more or less accessible the binding to the receptor.

To study in more detail the mode of action of the Vip3Ca protein, we chose *M. brassicae* because of its relatively high susceptibility to this toxin.

(I) Proteolysis of the Vip3Ca protein and oligomerization

The proteolytic pattern of the Vip3Ca protein with trypsin and midgut juice showed that theVip3Ca protein was processed into two main fragments of ~70 kDa and ~20 kDa. The kinetics of Vip3Ca with the midgut juice from insect species with different susceptibility correlated with the susceptibility of the insect species to this toxin. To discard that the differences in the toxicity of the Vip3Ca protein was due to inappropriate activation of the protein, the Vip3Ca protein was processed with midgut juice of *O. nubilalis* or *M.*

brassicae and this did not significantly change its toxicity to either insect species. In an attempt to determine if the Vip3Ca protein makes oligomers, the size of the Vip3Ca digested with trypsin and midgut juice of *M. brassicae* was determined by size exclusion chromatography. As a result, we found that the Vip3Ca protein, either activated with trypsin or midgut juice, forms an oligomer composed of 4 units, that is stable up to two days in solution. Also, the toxin fragments of ~70 kDa and ~20 kDa coeluted together, suggesting that both protein bands are needed to form the oligomer.

(II) Histopathological effects and in vivo / in vitro binding of Vip3Ca

The histopathological effects of Vip3Ca were determined in midgut sections of *M. brassicae* intoxicated for different intervals with the Vip3Ca protein. The results indicated that the Vip3Ca protein was able to disrupt the midgut epithelium of *M. brassicae*. To know if the Vip3Ca protein binds to the midgut apical membrane, the larvae were fed with Vip3Ca and, after 3 h of exposure, the binding of the Vip3Ca protein to the midgut epithelium was detected. With the aim to demonstrate that the Vip3Ca protein bound specifically to the midgut epithelium of *M. brassicae* and compete for the same binding sites than Vip3Aa, binding assays were performed with biotin-labelled Vip3 proteins. The homologous competition showed that the Vip3Aa and Vip3Ca proteins bound specifically to the brush border membrane vesicles (BBMV) because the respective unlabelled Vip3 proteins were able to displace the homolog proteins. The heterologous competition experiments indicated that Vip3Ca and Vip3Aa share binding sites.

(III) Cell death response of *S. exigua* intoxicated with Vip3Ca2

The election of *S. exigua* was due to the fact that a previous publication described a set of 47 *S. exigua* genes that responded to the intoxication by the Vip3A protein. The gene expression analysis indicated that the number of the *S. exigua* genes is dose-dependent. Regarding the up-regulated genes, these were involved in the immune system and hormone modulation, whereas the down-regulated genes were those involved in the digestion process and peritrophic membrane permeability. In addition, the gene encoding a component of the Jak-Stat pathway was found down-regulated after 24 h of exposure at the higher dose tested. The effect of the Vip3Aa and Vip3Ca proteins on the midgut epithelium indicated that the release of aminopeptidase N (APN) is higher when the larvae are exposed to Vip3Aa rather than Vip3Ca, at concentrations that produce growth inhibition > 99 %. Also, the fitness cost associated to the intoxication with the Vip3Ca

and Vip3Aa protein is affected by an increase in the time of pupation and a reduction of the percentage of pupation. To determine if the down-regulation of the *Jak-Stat* pathway is involved in the apoptosis, the expression level of the caspases and TUNEL staining of midgut sections was performed. The results showed the presence of TUNEL-positive cells in the *S. exigua* midgut sections exposed to sublethal concentrations of Vip3Ca protein and the *Se-caspase-4* was up-regulated at all the time intervals, while the *Se-caspase-1* and *Se-caspase-2* were up-regulated at 12 h.

Resum

Bacillus thuringiensis és un bacteri que pertany al grup de *Bacillus cereus* i produeix una gran varietat de proteïnes insecticides juntament amb altres factors de virulència que contribueixen a la seva patogenicitat. *Bacillus thuringiensis* s'ha considerat com el agent bioinsecticida amb més èxit durant el segle passat. Actualment, és àmpliament utilitzat com agent microbià per a les principals plagues d'insectes com a formulat (selecció de aïllats de *B. thuringiensis* escollits per la seua combinació de proteïnes insecticides present en el cristall parasporal) o mitjançant l'expressió de les proteïnes insecticides (cultius Bt) en plantes d'interès agronòmic (dacsa, cotó, soja, etc.).

En quant al seu cicle de vida, *B. thuringiensis* és un bacteri gram-positiu, anaeròbic facultatiu, quimioorganotròfic i amb una distribució cosmopolita. El tret característic de *B. thuringiensis* es la formació de inclusions cristal·lines (proteïnes Cry) que son tòxiques contra insectes, especialment contra plagues que produeixen grans danys en diversos cultius. El mecanisme general de toxicitat de *B. thuringiensis* consisteix en un primer pas en la ingesta de les proteïnes insecticides i solubilització al tracte intestinal. Després de la solubilització, les proteïnes (protoxines) son activades per les proteases intestinals a la seua forma activa (toxines). Les toxines travessen la membrana peritrófica, s'uneixen als receptors específics de la vora en raspall en la membrana apical del intestí d'insectes susceptibles. Seguit de la unió de la proteïna al receptors específics, aquesta s'insereix, de manera immediata e irreversible, en la membrana cel·lular formant porus que provoquen la lisis cel·lular. La lisis massiva de moltes cèl·lules del intestí provoca la disrupció del intestí i la mort del insecte per septicèmia. A mes a més, *B. thuringiensis* també produeix altres factors de virulència que faciliten l'acció de les proteïnes insecticides com son: proteases (destrueixen el pèptids antimicrobians generats per part del insecte com a resposta a la intoxicació amb *B. thuringiensis*), bacteriocines i pèptids antibacterians (competeixen amb la microbiota resident al intestí del insecte), quitinases i "enhancins" (desintegren la membrana peritrófica).

En la present tesi doctoral, s'ha realitzat la detecció de nous gens de proteïnes insecticides de *B. thuringiensis* a partir d'un conjunt d'aïllats de *B. thuringiensis* seleccionats basat en el seu contingut genètic. També vam estudiar detalladament diferents aspectes (espectre insecticida, resistència creuada, mode d'acció i resposta de mort cel·lular de *Spodoptera exigua* intoxicada amb la proteïna Vip3Ca) de la nova família de proteïnes Vip3Ca.

La recerca de nous gens de proteïnes insecticides amb un espectre insecticida i mode d'acció diferent al descrit per a les diferents toxines *B. thuringiensis* es una aproximació utilitzada per incrementar l'espectre insecticida de les toxines de *B. thuringiensis* i retardar l'aparició de poblacions d'insectes resistents. Un mètode utilitzat per a la recerca de nous gens de proteïnes insecticides es el cribat de col·leccions de aïllats de *B. thuringiensis* de diferents localitzacions i hàbitats mitjançant la utilització de la reacció en cadena de la polimerasa (PCR) o, recentment, mitjançant tecnologies d'alt rendiment com son: la seqüenciació massiva de DNA genòmic de bacteris i la identificació de proteïnes mitjançant de cromatografia líquida acoplada a espectrometria de masses.

Pel que fa a la recerca de nous gens de proteïnes insecticides, vam partir d'un conjunt d'aïllats de *B. thuringiensis* possibles portadors de nous gens *vip1* i *vip2*. En un primer pas, vàrem realitzar dues PCR on, en la primera d'aquestes, determinàvem la presència de gens *vip1* i *vip2*, mentre que, en la segona PCR, caracteritzàvem a quin del gens *vip1* i *vip2* descrits eren més semblants les seqüencies obtingudes. Com a resultat obtinguérem la presència de dos nous al·lels de les parelles de gens *vip2Ac1* - *vip1Ca1* i *vip2Bb1* - *vip1Bb1* als aïllats *B. thuringiensis* V-J20.2, V-LE1.1, V-V54.26, V-V54.31, E-TE7.43, E-TE16.5 i E-TE18.40. També es detectaren la presència de dos seqüencies amb baix percentatge d'identitat als gens *vip1Bb1* i *vip1Da1* als aïllats de *B. thuringiensis* E-10.2 i O-V84.2.

En un segon pas, per tal de determinar la seqüencia completa dels gens amb baix percentatge d'identitat a *vip1Bb1* i *vip1Da*, es va realitzar la seqüenciació massiva de DNA genòmic de ambdós bacteris mitjançant la plataforma de seqüenciació Illumina HiSeq-PE150. A partir de les lectures netes, subministrades per Novogene S.L., varen ser ensamblades, anotades, i les seqüències codificants foren predites abans de realitzar una recerca per homologia contra una base de dades específica de toxines de *B. thuringiensis*, i posteriorment es va validar el resultat obtingut amb la recerca per homologia contra la base de dades "Non-Redundant database" del NCBI. Com a resultat obtinguérem la predicció de 24 gens de proteïnes insecticides entre els aïllats de *B. thuringiensis* E-10.2 i O-V84.2. Per tal de determinar quants dels gens insecticides predits son expressats pels aïllats de *B. thuringiensis* E-10.2 i O-V84.2, es va buscar la seua presència al sobrenedant i en la mescla d'espores i cristalls per medi de la identificació de proteïnes amb cromatografia líquida acoblada a espectrometria de masses. Com a resultat, trobàrem una nova parella de proteïnes tipus Vip (Vip2Ac-like_1-Vip4Aa-like_1), dues noves

proteïnes tipus Vip (Vip2Ac-like_1 i Vip4Aa-like_2), una proteïna semblant a Sip1A (Sip1A-like_1) i vuit proteïnes tipus Cry (Cry23Aa-like, Cry45Aa-like_1, Cry45Aa-like_2, Cry45Aa-like_3, Cry32Ea-like, Cry32Da-like, Cry32Eb-like i Cry73Aa-like).

Una vegada determinada el nombre de noves proteïnes insecticides que són produïdes pels aïllats de *B. thuringiensis* E-10.2 i O-V84.2, es va procedir a determinar la seua localització subcel·lular i en quina quantitat eren produïdes per *B. thuringiensis*. Per tal de determinar la localització subcel·lular de les proteïnes tipus Vip i Cry es van detectar les proteïnes presents en el sobrenedant (24h i 48h) i en la mescla d'espores i cristalls per separat, en tres rèpliques independents. El resultat obtingut fou que les proteïnes tipus Vip es van detectar en els sobrenedants, mentre que les proteïnes tipus Cry es van trobar en el cristall parasporal, excepte per la proteïna Cry23Aa-like que també es troba en el sobrenedant del aïllat de *B. thuringiensis* E-SE10.2 al temps 24 h i 48 h.

Pel que respecta a la quantificació de les proteïnes tipus Vip i Cry en tres rèpliques independents, es van utilitzar dues aproximacions diferents: (I) Determinar l'abundància relativa de les proteïnes tipus Vip i Cry en la mateixa rèplica a un temps fixe, i (II) anàlisi lliure de marca dels sobrenedants a 24 h i 48 h dels aïllats de *B. thuringiensis* E-SE10.2 i O-V84.2 per comparar la quantitat de les proteïnes a dos temps concrets. Pel que fa als resultats obtinguts de la abundància relativa, indiquen que les proteïnes tipus Vip s'expressen de manera marginal al sobrenedant a 24 h i 48 h en els aïllats de *B. thuringiensis* E-SE10.2 i O-V84.2. A més a més, en el cas de l'aïllat de *B. thuringiensis* E-SE10.2, la proteïna Cry23Aa-like fou la proteïna més abundant del sobrenedant a 24 h i 48 h, indicant que el cultiu es trobaria en l'inici de la fase d'esporulació. Per a les proteïnes Cry, es van detectar en la mescla d'espores i cristalls, on l'aïllat de *B. thuringiensis* E-SE10.2 expressa únicament la proteïna Cry23Aa-like, amb un percentatge en pes entre el 2.5% - 30%, mentre que l'aïllat de *B. thuringiensis* O-V84.2 expressa set proteïnes diferents amb els següents percentatges en pes: 1.4% - 2.8% Cry45Aa-like_1, 2.0% - 3.3% Cry45Aa-like_1, 1.8% - 5.3% Cry45Aa-like_3, 24.3% - 25.9% Cry32Ea-like, 4.6% - 6.1% Cry32Da-like, 4.4% - 6.2% Cry32Eb-like i 2.8% - 4.2% Cry73Aa-like.

En quant als resultats obtinguts de l'anàlisi lliure de marca dels sobrenedants dels aïllats de *B. thuringiensis* E-SE10.2 i O-V84.2 a 24 h i 48 h, indiquen que les proteïnes insecticides Vip4Aa-like_1 i Vip4Aa-like_2 de l'aïllat de *B. thuringiensis* O-V84.2 mostren diferències significatives entre les 24 h i 48 h. La proteïna Vip4Aa-like_1 es

troba incrementada en la seua quantitat dues vegades a 48 h respecte a les 24 h, mentre que la proteïna Vip4Aa-like_2 es va detectar a 24 h però no es detecta a 48 h. Pel que respecta a la resta de proteïnes tipus Vip, es varen trobar al sobrenedant i no mostraren diferències significatives en la seua producció entre les 24 h i 48 h.

Per finalitzar aquest estudi, es va comprovar la toxicitat de les proteïnes tipus Vip i Cry expressades pels aïllats de *B. thuringiensis* E-SE10.2 and O-V84.2, mitjançant la realització de bioassajos amb neonats de les espècies d'insectes plaga *Spodoptera exigua* i *Spodoptera littoralis* utilitzant el sobrenedant concentrat dels aïllats de *B. thuringiensis* E-SE10.2 and O-V84.2 (autoclavats i no autoclavats) i les proteïnes solubilitzades de la mescla d'espores i cristalls. El resultat dels bioassajos amb les proteïnes solubilitzades de la mescla d'espores i cristalls de l'aïllat de *B. thuringiensis* E-SE10.2 indica que la proteïna Cry23Aa-like mostra certa toxicitat a *S. exigua* i *S. littoralis*, mentre que no s'observa cap efecte en les proteïnes solubilitzades de la mescla d'espores i cristalls de l'aïllat de *B. thuringiensis* O-V84.2. En quant a les proteïnes tipus Vip, el sobrenedant concentrat, autoclavat i no autoclavat, dels aïllats de *B. thuringiensis* E-SE10.2 i O-V84.2 no mostraren toxicitat front a cap de les dues espècies d' insectes provades.

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Les proteïnes Vip3 son proteïnes insecticides produïdes per *B. thuringiensis* durant la fase vegetativa i secretades al medi extracel·lular. En el moment d'escriptura d'aquesta tesi, les proteïnes Vip3 es troben agrupades en tres famílies proteiques: Vip3A, Vip3B i Vip3C. Les proteïnes Vip3A son les més abundants i, per tant, amb les que s'ha dut a terme la majoria dels estudis publicats i sobre les que més informació es troba disponible sobre el seu espectre insecticida i mode de acció. Pel que respecta a les proteïnes Vip3B i Vip3C, són menys abundants i pocs estudis s'han realitzat amb aquestes proteïnes.

La proteïna Vip3A té una mida al voltant de ~85-90 kDa quant és secretada al medi, mentre que al ser activada per les proteases intestinals genera dos fragments, un de 20 kDa (N-terminal) i l'altre de 60 kDa (C-terminal). La toxina activada mostra toxicitat contra un ampli rang d'espècies de lepidòpters, amb alguns d'aquests que fins i tot mostren una baixa susceptibilitat a les proteïnes Cry, com ara pot ser *Agrotis ipsilon, S. exigua* i *Spodoptera frugiperda*. Pel que respecta al mode d'acció de les proteïnes Vip3, principalment Vip3Aa, aquest segueix la mateixa seqüencia d'esdeveniments descrits per a les proteïnes Cry: (I) Ingesta de les proteïnes Vip3A per l'insecte, (II) activació de la

proteïna Vip3A per part de les proteases intestinals i migració a través de la membrana peritròfica, (III) unió de la proteïna Vip3A activada al receptors específics de membrana de la vora en raspall de les cèl·lules intestinals, i (IV) formació del porus i lisi cel·lular.

Pel que respecta a la informació disponible sobre la Vip3Ca, prèvia a la realització de la present tesi doctoral, fou un treball conjunt del grup d'investigació "Control Biotecnològic de Plagues", de la Universitat de València, i el grup d'investigació "Protección de Cultivos", de la "Universidad Pública de Navarra". En aquest treball, publicat en una nota curta, es descriu per primera vegada la proteïna Vip3Ca i demostren que és moderadament tòxica contra *Mamestra brassicae, Trichoplusia ni* i *Crysodeixis chalcites*. Donat que ja teníem informació prèvia sobre l'espectre insecticida de la proteïna Vip3Ca, en un primer pas vam ampliar l'estudi sobre el seu espectre insecticida en deu espècies més de lepidòpters plaga (*Cydia pomonella*, *Grapholita molesta*, *Sesamia nonagrioides*, *Galleria mellonella*, *Plutella xylostella*, *Pectinophora gossypiella*, *Ephestia kuehniella*, *Plodia interpunctella* i *Ostrinia furnacalis*), una espècie d'àfid (*Nezara viridula*) i un organisme model (*Drosophila melanogaster*). Es van utilitzar diverses metodologies de bioassaig. Per a les espècies que es va provar la proteïna per contaminació superficial, *C. pomonella* va ser la més susceptible, seguida de *G. mellonella*, amb percentatges de mortalitat superiors al 50%. Pel que fa a les quatre espècies on la proteïna es va provar incorporada en la dieta, *O. furnacalis* va ser la més susceptible, amb un valor LC_{50} de 0.31 μg/g. Pel que respecta a la resta dels bioassaigs, *Tuta absoluta* va mostrar certa susceptibilitat a altes concentracions de Vip3Ca (30% de mortalitat).

En un segon pas, avaluàrem la proteïna Vip3Ca en colònies d'insectes resistents a les proteïnes Cry1A, Cry2A iVip3. Pel que respecta a la proteïna Vip3A, es troba descrit que les colònies d'insectes resistents a les proteïnes Cry1A o Cry2A no mostren resistència creuada a la Vip3A i *vice versa*. Per tal de dur a terme aquest objectiu, es varen provar diferents colònies d'insectes resistents a les proteïnes Cry1A (*H. armigera*, *T. ni*, *O. furnacalis* i *P. interpunctella*), Cry2A (*H. armigera* i *T. ni*) i Vip3 i Vip3/Cry2Ab (*H. armigera*) per a la seua susceptibilitat front a la proteïna Vip3Ca, per determinar si presentaven resistència creuada. Els resultats van mostrar que les colònies d'insectes resistents a Cry1A (*H. armigera*, *T. ni*, *O. furnacalis* i *P. interpunctella*) o Cry2Ab (*H. armigera* i *T. ni*) no mostraren resistència creuada a les proteïnes Vip3 (Vip3Aa i

Vip3Ca). Per contra, les colònies de *H. armigera* resistents a les proteïnes Vip3Aa o Vip3Aa/Cry2Ab van mostrar resistència creuada a la proteïna Vip3Ca.

Hem de mencionar que, com un treball secundari sorgit a partir de la recerca d'espècies susceptibles, fou l'estudi monogràfic sobre la susceptibilitat de *G. molesta* als formulats de *B. thuringiensis*, les seues toxines i sinergies entre les proteïnes Cry i Vip3. Com a resultat obtinguérem que *G. molesta* és altament susceptible al formulats de *B. thuringiensis*, Dipel® i Xentari®, i a les toxines de *B. thuringiensis* Cry1Aa, Cry1Ac, Cry1C, Vip3Aa i Vip3Af. Una vegada determinat que *G. molesta* és susceptible a les toxines Cry1Aa, Cry1Ac, Cry1C i Vip3Aa, es va determinar les possibles sinergies entre aquestes proteïnes. Les combinacions de proteïnes Cry1Aa-Vip3Aa i Cry1C-Vip3Aa foren antagòniques mentre que la combinació de proteïnes Cry1Ac-Vip3Aa va mostrar un efecte additiu. És interesant mencionar que el antagonisme detectat en les combinacions de proteïnes Cry1Aa-Vip3Aa i Cry1C-Vip3Aa augmentava segons era major la quantitat de proteïna Vip3A en la mescla; aquest fet suggereix que encara que aquestes no competeixen llocs d'unió potser interactuen en esdeveniments anteriors a la unió fent més o menys accessible la unió al receptor.

Per aprofundir en el mode d'acció de la proteïna Vip3Ca i comprovar si és similar al descrit per a la proteïna Vip3A, escollirem *M. brassicae* degut a la seua relativament alta susceptibilitat a la proteïna Vip3C i Vip3A. Els experiments duts a terme per estudiar el mode d'acció de la proteïna Vip3Ca foren els següents: (I) Proteòlisi i oligomerització de la proteïna Vip3Ca, (II) efectes histopatològics i unió *in vivo* / *in vitro* de Vip3Ca, i (III) resposta de la mort cel·lular de *S. exigua* intoxicada amb Vip3Ca2.

(I) Proteòlisi i oligomerització de la proteïna Vip3Ca

En un primer pas volguérem demostrar si la proteïna Vip3Ca mostrava un patró d'activació similar a la proteïna Vip3Aa. S'ha descrit que, per a la proteïna Vip3A, l'activació amb proteases (tripsina o suc intestinal) genera dos fragments de 20 kDa i 60 kDa. Pel que respecta a la proteïna Vip3Ca, primer es va determinar el seu patró proteolític amb tripsina i suc intestinal, mostrant que la proteïna Vip3Ca es processava a dos fragments majoritaris de ~ 70 kDa i ~ 20 kDa, tant si utilitzàvem tripsina com suc intestinal. Hem de fer notar que pel que respecta a la cinètica d'activació de la proteïna Vip3Ca amb el suc intestinal d'espècies d'insectes que mostren diferent susceptibilitat, es correlacionava amb la susceptibilitat d'aquestes espècies d'insectes a la proteïna Vip3Ca.

Donat que la cinètica d'activació de la proteïna Vip3Ca amb el suc intestinal de diferents espècies d'insectes es correlaciona amb la susceptibilitat d'aquestes per a la Vip3Ca, volguérem determinar si això es devia a una incorrecta activació de la proteïna. En el cas de la Vip3Aa s'ha demostrat que la diferent susceptibilitat mostrada per part de les diferents espècies d'insectes no es deu a una inapropiada activació de la proteïna. En quant a la proteïna Vip3Ca, per descartar que les diferències en la toxicitat de la proteïna Vip3Ca es devien a una activació inapropiada de la proteïna, la proteïna Vip3Ca es va processar amb suc intestinal de *O. nubilalis* o *M. Brassicae* i això no va modificar significativament la seva toxicitat a cap espècie d'insecte.

Per finalitzar aquest punt volguérem demostrar si la proteïna Vip3Ca forma oligòmers (tetràmers) com ja s'ha demostrar per a la proteïna Vip3Aa. Per determinar si la proteïna Vip3Ca forma oligòmers, la mida de la Vip3Ca digerida amb tripsina i suc intestinal de *M. brassicae* es va determinar per cromatografia d'exclusió molecular. Com a resultat, vam trobar que la proteïna Vip3Ca activada, tant amb tripsina com amb suc intestinal, forma un oligòmer de 4 unitats que és estable fins a dos dies en solució. A més, els fragments de la toxina de ~ 70 kDa i ~ 20 kDa elueixen junts, el que suggereix que ambdós fragments de proteïnes són necessaris per formar l'oligòmer, com ja s'ha demostrat per a la proteïna Vip3Aa.

(II) Efectes histopatològics i unió *in vivo* / *in vitro* de Vip3Ca

En un primer pas volguérem demostrar si la proteïna Vip3Ca és capaç de causar danys en l'intestí d'espècies d'insectes susceptibles i si s'uneix la proteïna de manera específica a receptors de membrana i competeix pels mateixos llocs d'unió que la Vip3Aa.

Els efectes histopatològics de la proteïna Vip3Ca es van determinar en talls histològics de l'intestí de *M .brassicae* intoxicat a diferents temps amb la proteïna Vip3Ca. Els resultats van indicar que la proteïna Vip3Ca pot causar grans danys en l'epiteli intestinal de *M. brassicae*, però quan es va comparar amb la Vip3Aa (control positiu de toxicitat), es va veure que la proteïna Vip3Ca necessita més temps per a mostrar els mateixos efectes histopatològics que la proteïna Vip3Aa. Per determinar si els danys observat a l'epiteli intestinal de *M. brassicae* es devien a la unió de la proteïna Vip3Ca a les cèl·lules de l'epiteli intestinal, les larves de *M .brassicae* es van alimentar amb Vip3Ca durant 3 h. El resultat va ser que la Vip3Ca s'unia a l'epiteli de *M. brassicae*, observat mitjançant tècniques immunofluorecents.

Amb l'objectiu de demostrar que la unió observada de la proteïna Vip3Ca a l'epiteli intestinal de *M. brassicae* és específica i que la proteïna Vip3Ca competeix pels mateixos llocs d'unió que la proteïna Vip3Aa, es van realitzar assaigs d'unió amb les proteïnes Vip3 marcades amb biotina. La competència homòloga va mostrar que les proteïnes Vip3Aa i Vip3Ca s'uneixen de manera específica a les vesícules de membrana de vora en raspall (BBMV) perquè les respectives proteïnes Vip3 no marcades poden desplaçar les proteïnes homòlogues. Els experiment de competència heteròloga van indicar que Vip3Ca i Vip3Aa comparteixen llocs d'unió indicant per primera vegada que diferents famílies de proteïnes Vip3 competeixen pels mateixos llocs d'unió.

El fet que les proteïnes Vip3Aa i Vip3Ca competeixen pels mateixos llocs d'unió té importants implicacions en la gestió del control de plagues. La combinació de les proteïnes Vip3Aa i Vip3Ca no seria optima per al control d'espècies d'insectes plaga, perquè l'aparició de resistència a la Vip3Aa (per modificació del receptor) provocaria al mateix temps la presencia de resistència creuada cap a la Vip3Ca.

(III) Resposta de la mort cel·lular de *S. exigua* intoxicada amb Vip3Ca2

L'elecció de *S. exigua* es va deure al fet que publicacions anteriors descrivien un conjunt de 47 gens en *S. exigua* que responien a la intoxicació de la proteïna Vip3A. L'anàlisi d'expressió gènica dels 47 gens candidats indica que el nombre de gens de *S. exigua* que responen és dependent de la dosi, on el nombre màxim de gens que responien es trobaren a la dosi de 10.000 ng/cm^2. En quant als gens sobre-expressats a les diferents dosis provades, els resultats indiquen que es troben implicats en el sistema immune i la modulació hormonal, mentre que els gens reprimits participen en el procés de digestió i la permeabilitat a la membrana . A més, el gen que codifica un component de la ruta Jak-Stat es va trobar reprimit després de 24 hores d'exposició a la dosi més alta provada.

Pel que respecta a l'efecte de les proteïnes Vip3Ca i Vip3Aa a les dosis utilitzades en l'epiteli intestinal, els resultats indiquen que la presència de dany (medit com l'alliberament d'aminopeptidasa N) és major quan les larves són exposades a la proteïna Vip3Aa més que quan són exposades a concentracions que produeixen una inhibició del creixement > 99% de la proteïna Vip3Ca. Hem de fer notar que també es mesura el cost biològic associat a la intoxicació amb les proteïna Vip3Ca i Vip3Aa on les larves de *S. exigua* mostraren un augment en el temps de pupació i una reducció del percentatge de pupació.

Per determinar si la repressió de la via Jak-Stat està implicada en l'apoptosi, es va mesurar el nivell d'expressió de les caspases de *S. exigua* (*Se-caspasa-1*, *Se-caspasa-2*, *Se-caspasa-3, Se-caspasa-4*, *Se-caspasa-5* i *Se-caspasa-6*) i es realitzaren talls histològics de l'intestí de *S. exigua* que es tenyiren amb la tècnica del TUNEL. Els resultats obtinguts indicaren que *Se-caspasa-4* estava sobre-expressada en tots els intervals temporals (3h, 6h, 12h i 24h) mentre que la *Se-caspasa-1* i *Se-caspase-2* sols estaven sobre-expressades a les 12 h. En quant als resultats obtinguts en els talls histològics tenyits per la tècnica del TUNEL, mostraren la presència de cèl·lules positives per a la Vip3Ca a la dosi de 10.000 ng/cm^2 mentre que per a la Vip3Aa es detectaren presència de cèl·lules positives per a les dosis entre 1-100 ng/cm^2 però no a la màxima dosi, degut a que el dany fou tan gran que probablement participen altres vies de mort cel·lular que no involucren l'apoptosi.

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En resum, en la present tesi doctoral hem detectat la presència d'una sèrie de nous gens tipus Vip i Cry a partir de dos aïllats de *B. thuringiensis* i, per primera vegada, hem aprofundit en el mode d'acció de la proteïna Vip3Ca. El resultats obtinguts sobre l'espectre insecticida de la proteïna i el mode d'acció de la proteïna Vip3Ca suposen una gran font d'informació sobre el comportament d'aquesta proteïna tant poc estudiada fins al moment de la realització de la present tesi doctoral. Pel que respecta a les conclusions específiques obtingudes a partir del treball realitzat, durant les ultimes cinc anualitats, han sigut les següents:

1.- En els aïllats de *B. thuringiensis* E-SE10.2 i O-V84.2 es van identificar 13 proteïnes insecticides noves a partir de dos aïllaments seleccionats pel seu contingut gènic. Les proteïnes tipus Vip i Sip (Vip2Ac-like_1-Vip4Aa-like_1, Vip2Ac-like_1, Vip4Aa-like_2 i Sip1A-like_1) s'expressaven de manera marginal en el sobrenedant mentre que les proteïnes tipus Cry (Cry23Aa-like, Cry45Aa-like_1, Cry45Aa-like_2, Cry45Aa-like_3, Cry32Ea-like, Cry32Da-like, Cry32Eb-like i Cry73Aa-like) es localitzaven en el cristall parasporal

2.- L'estudi de l'espectre insecticida de la proteïna Vip3Ca es va ampliar a 12 noves espècies d'insectes, on *O. furnacalis* fou la més susceptible. Pel que fa a l'anàlisi de la resistència creuada a les plagues de lepidòpters, indiquen que la resistència deguda a la selecció amb proteïnes Cry no confereix resistència creuada a Vip3Ca, mentre que les

poblacions d'insectes resistents a Vip3A mostren resistència creuada a la proteïna Vip3Ca

3.- *Grapholita molesta* és altament susceptible als formulats de *B. thuringiensis* i les seves toxines individuals (Cry i Vip3). Pel que fa a les interaccions entre les proteïnes Cry i Vip3, es va trobar l'antagonisme en les combinacions Cry1Aa-Vip3Aa i Cry1C-Vip3Aa

4.- La proteïna Vip3Ca es processa en dos fragments de ~ 70 kDa i ~ 20 kDa, tant utilitzant tripsina com suc intestinal. La proteïna Vip3Ca activada amb tripsina i suc de midgut de *M. brassicae* forma un oligòmer de 4 subunitats. La proteïna Vip3Ca és capaç d'unir-se a l'epiteli produint la lisi cel·lular. A més, els assaigs d'unió van mostrar que Vip3Ca s'uneix específicament i competeix pels mateixos llocs d'unió que la proteïna Vip3Aa

5.- L'exposició de les larves de *S. exigua* a les concentracions subletals de la proteïna Vip3Ca activa diferents vies de resposta d'insectes que desencadenen la regulació d'alguns gens pertanyents al grup de resposta a patògens, indueixen l'alliberament d'APN i desencadena l'activació de la via depenent de caspasa que indueix la resposta apoptòtica

2. - INTRODUCTION

2.1. - General aspects of *Bacillus thuringiensis*

The control of insect pests in agriculture is mainly achieved using chemical insecticides. However, the use of these chemical pesticides has led to several problems, including environmental pollution and increase in human health effects, such as cancer and several immune system disorders. The selection of insect resistant populations has also caused significant and major outbreaks of secondary pests (1). The entomopathogenic bacteria, viruses and fungi have been proposed as biological control agents for pest control, due to the fact that these control agents are specific for one or few range of insect pest without affecting the non-target insects and don't produce environmental pollution in field applications (2). However, they show some disadvantages such as the following: [1] A narrow spectrum of activity that enables them to kill only certain insect species, [2] low environmental persistence in the field, [3] they require precise application practices, since many of these pathogens are specific to young insect larval stages or are sensitive to irradiation (1).

Bacillus thuringiensis is an aerobic, spore-forming, gram-positive and entomopathogenic bacterium that belong to the *Bacillus cereus* group and produces a wide variety of insecticidal proteins, such as Cry, Cyt, Mtx-like, Bin-like,Vip and Sip proteins, along with other virulence factors contributing to its pathogenicity (Figure 1).

B. thuringiensis has been considered as the most successful bioinsecticide during the last century. Currently, it is widely used as a microbial agent of the major insect pests (which represents ~2% of the total insecticidal market) (1,5). In the market, there can be found more than 98 formulated sprayable bacterial pesticides (6). Moreover, recent studies confirmed new applications of the *B. thuringiensis* strains. These new features include plant growth promoting bioremediation of heavy metals and other chemicals, anticancer activities, polymer production, and antagonistic effects against plant and animal pathogenic microorganisms (Figure 1) (4).

2.1.1.-Life cycle of *Bacillus thuri*ngiensis

B. thuringiensis is a widespread bacteria with a complex life cycle (Figure 2) which is commonly found in soil, water, plants, stored cereals and dead insects. Due to the economic importance of *B. thuringiensis,* the majority of the research has focused on

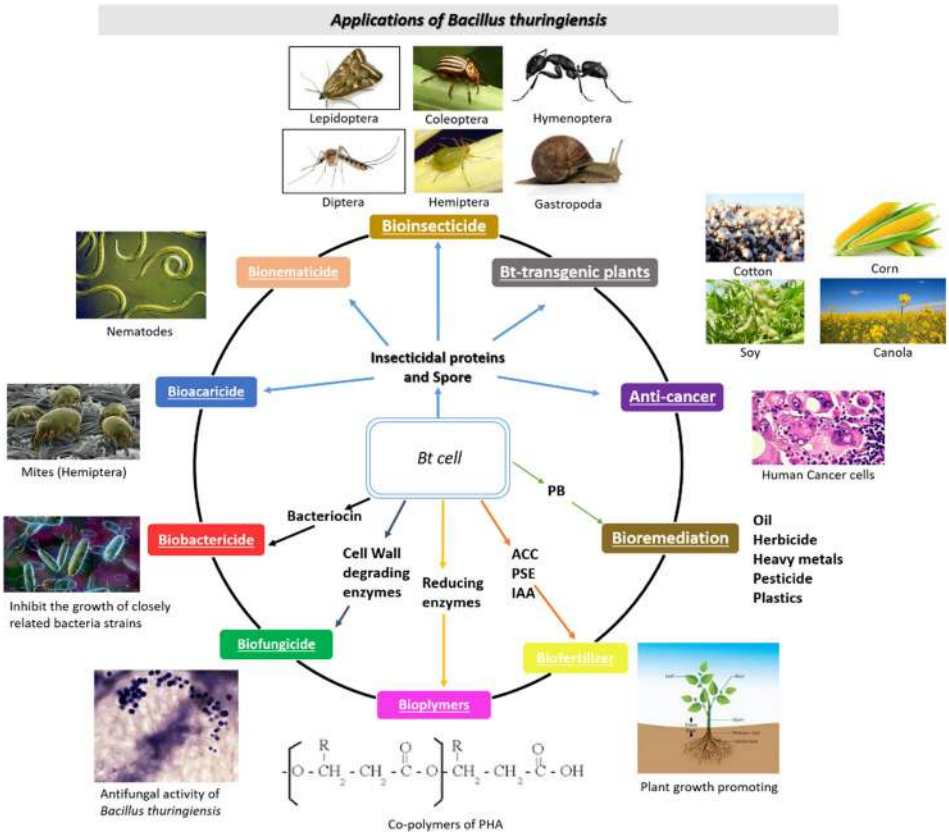

Figure 1. *Bacillus thuringiensis* cell factory potentials. ACC: 1-aminocyclopropane-1-carboxylate (ACC) deaminase, PSE: phosphate solubilization enzymes, IAA: indole-3-acetic acid, PB: proteins involved in bioremediation. This figure has been adapted from Palma et al., 2014 (3) and Jouzani et al., 2017 (4).

direct effects against insect pests relevant to agriculture. However, studies focusing purely on the ecology of *B. thuringiensis*, including its interactions with a diverse range of organisms that occupy the same niches are scarce (7). The ecology and lifestyle of *B. thuringiensis* have been a target of many as yet unanswered questions. Two theories try to explain the life cycle of *B. thuringiensis*: [1] *B. thuringiensis* is a soil-dwelling microorganism that obtains nutrition for its survival and reproduction in nature from decaying organic matter or root exudates, reaching the aerial parts of plants when these germinate and emerge from soil (Figure 2) (7–9). [2] *B. thuringiensis* is a specialized pathogen that, by colonizing and killing its hosts, and multiplying in their cadavers, is then deposited in soil and plants, which thereby become natural reservoirs (Figure 2) (10–13).

2.1.1.1. - Niches occupied by *Bacillus thuringiensis* in the environment

Soil Environment

The soil has been considered the main storage of *B. thuringiensis* spores. The authors that consider *B. thuringiensis* as a soil microorganism propose that *B. thuringiensis* could germinate in the soil and is a natural soil inhabitant. However, the authors that consider a *B. thuringiensis* as a specialized pathogen consider that the soil act as a storage milieu for the spores, as they do not germinate in these places, requiring specific nutrients and pH conditions for that (7).

The researchers who argue that *B. thuringiensis* is a soil microorganism generally believe in its saprophytic lifestyle (14–16). However, recent reports demonstrated that *B. thuringiensis* in the colonization of insect cadavers is in fact necrotrophic, which appears to be an important feature to ensure bacterial proliferation and dispersion in the environment. Naryanan et al. (17) demonstrated that an infected dead insect on a leaf surface caused the death of one third of the larvae around. Dubois et al. (18) showed that the necrotrophic stage is part of a complex life cycle of *B. thuringiensis* that involves the activation and regulation of several genes that alter its metabolism after the host death. Such changes are required for survival and colonization of the host cadaver, involving the production of enzymes (proteases, lipases, esterases and chitinases) that allow the use of host contents and the cuticle breakage for the release of toxins and spores.

Epiphytic Environment - Phylloplane

B. thuringiensis has been isolated from the phylloplane (the surface of a leaf considered as a habitat for the microorganisms) in natural or artificial ways. It has been shown that *B. thuringiensis* can reach this niche by rain splash from the soil to lower leaves, from soil as a result of being carried by germinating seeds, from animal feces such as those from insects or birds, and from dead insects (7). The variation in the *B. thuringiensis* survival rates in the phylloplane seems to be linked to the plant species (9,19). In addition, some reports indicate that *B. thuringiensis* is a poor leaf colonizer, being found mostly as spores in these habitats. Even with nutritionally rich leaf surface that leads to spore germination, vegetative *B. thuringiensis* cells sporulate again after a few rounds of division, which confers a survival ability for long periods, even under stress conditions, such as desiccation (20). In addition, the leaf exudates can also affect *B. thuringiensis* survival negatively; for instance, organic acids that lower surface pH can increase

mortality rates of *B. thuringiensis* in this environment (21,22). So the leaves have been suggested to work as a secondary reservoir that participate in the recycling process of the bacterium by returning cells and spores to the soil (7).

Epiphytic Environment - Rhizosphere

Some reports indicate that *B. thuringiensis* is able to colonize the rhizosphere of the plants, for instance, a *B. thuringiensis* strain highly toxic against *Simulium trifasciatum* has been isolated from the rhizosphere of *Ficus doliaria* (23). Moreover, Hendricksen and Hansen (8) reported that the population of *B. thuringiensis var kurstaki* was found 260 times higher in the rhizosphere than in the phylloplane of the same plants. These results suggest that the *B. thuringiensis* is more effective in the colonization of the rhizosphere and roots than in the phylloplane due to a richer nutrient availability in these environments that favours the microbial growth (7).

Endophytic Environment

Few studies in cotton, soybean, corn, sugar cane, cabbage, ricebean, gahat and lentil have reported that *B. thuringiensis* was successful in endophytic colonization, even with concomitant production of Cry toxins. The efficient *B. thuringiensis* colonization of cabbage seedlings roots suggests that this might be in fact the main route of its penetration in the plant. After this event, vegetative cells, spores and crystals were found in several parts of the seedlings, which characterized a complete *B. thuringiensis* colonization (24–27). Similarly, *B. thuringiensis* was able to colonize the roots of certain legumes, passion fruit and cacao (28,29). *B. thuringiensis* can reach the interior of the plant through the roots, stomata and wounds, or through the action of hydrolytic enzymes (30,31). It is known that Cry toxins are inactivated by UV radiation and can be washed out from the leaves by rain or irrigation; when *B. thuringiensis* grows endophytically, these adverse conditions do not occur (7).

Aquatic Environment

The adaptation of *B. thuringiensis* to the aquatic environment has been assessed by few reports. Ichimatsu et al. (14) isolated a great variety of *B. thuringiensis* serovars from 50% of running (river, stream, and ditch) and still water (pond) samples in Japan. They found that 26.7% of isolates exhibited larvicidal activities against *Culex pipiens* and *Clogmia albipunctata*. Menon and Mestral (32) have shown that *B. thuringiensis var*

kurstaki remains in water for large periods: 40 days in sea water and 70 days in lake water. Konecka et al. (33)isolated a *B. thuringiensis* strain from a forest creek sample that appeared to be 24× more toxic than HD1 against *Cydia pomonella* (Lepidoptera) larvae. Taken together, these data suggest that *B. thuringiensis* is also ubiquitous in aquatic environments. Since most part of water-isolated serovars were also isolated from soil and phylloplane, it is likely that they reach the water bodies through rain, percolation, floodings, wind, animal excrements, etc (7).

Paratenic Behavior

The intake of contaminated nutrients is the main access route of *B. thuringiensis* to invertebrate or vertebrate animals. For the vertebrate animals, some studies report the colonization of the whole or part of the intestinal tract. Wilcks et al. (34) demonstrated that *B. thuringiensis* colonize the intestinal tract of germ-free rats where the cells grew vegetatively for various generations before sporulation and elimination by the feces. Ammons et al. reported the presence of *B. thuringiensis* in rectal samples from milk cows, with indication that multiplication of *B. thuringiensis* cells were done in the digestive tract of the cows. Zhang et al. (35) showed the presence of *B. thuringiensis* in the intestinal tract of chickens, with the duodenum being the main portion colonized and the chickens kept releasing the bacterium through their feces for a certain time, even after removal of *B. thuringiensis* from the diet. Regarding the invertebrate animals, *B. thuringiensis* has also been isolated from fecal pellets of non-susceptible caterpillars from forests of conservation areas (36). In addition, the germination of *B. thuringiensis* in the alimentary tract was demonstrated in different invertebrates with sporulation occurring after defecation (8). If we assume that intestinal colonization does not occur, the fact that the *B. thuringiensis* spores are able to cross the digestive tract of these animals and reach their feces does provide a nutrient-rich environment for their multiplication (9,14,16). This ecological feature of survival, proliferation and sporulation in paratenic hosts warrants to *B. thuringiensis* a wide dispersion in the environment through animals migration and defecation (7).

Pathogenic Behavior

The pathogenic behaviour of *B. thuringiensis* is the aspect most studied because it can infect economically important pest that produce damages in several crops. Following the ingestion by the insect, the proteins (Cry, Vip, Sip, etc.) are dissolved in the intestinal

tract. After solubilisation, the proteins (protoxins) must be activated through proteolytic cleavage by the insect midgut proteases to generate active toxins. The activated toxins pass through the peritrophic membrane, and bind to specific receptors on the apical brush border of the midgut microvilli in susceptible insects. Following binding, the toxin rapidly, and irreversibly, inserts into the cell membrane. Insertion results in the formation of pores, which leads to epithelial cell lysis (Figure 2) (3,13).

Moreover, *B. thuringiensis* produce other virulence factors in the midgut that facilitate the action of the *B. thuringiensis* toxins such as: proteases (destroy insect-produced antimicrobial peptides), bacteriocins and antibacterial peptides target (competing gut microbiota), chitinases and enhancins (disrupt the peritrophic membrane). In addition, when *B. thuringiensis* crosses the midgut epithelium, it produces immune inhibitors and cytolisins (help to evade haemocytes), antibiotics and quorum quenchers (domination of the host), degradative enzymes and iron uptake systems (nutrient acquisition from the host) (Figure 2) (13).

2.1.1.2. - *Bacillus thuringiensis* is a bona fide insect pathogen

Despite the hypothesis that support that *B. thuringiensis* might only be an opportunistic pathogen and might even be incapable of full virulence without the aid of other bacteria, it appears to express a sufficiently wide range of virulence factors to equip it well for a pathogenic lifestyle (13). The ingested spores are able to germinate in the gut and synthesize a modified cell surface that help protect them from antimicrobial peptides (AMPs) synthesized by the host or competing bacteria. To be able to compete with other gut bacteria, *B. thuringiensis* produces its own AMPs, bacteriocins and express a number of drug efflux transporters to combat antibiotics produced by competitors (Figure 2). The viable *B. thuringiensis* cells in the gut environment attack the host producing specific toxins secreted in the vegetative growth phase (Vip, Sip, etc.) and in the sporulation phase as crystalline inclusions (Cry, Cyt, Mtx-like, etc.). These toxins target, and cause the destruction of, the epithelial cells of the midgut. In addition, the germination of the spore allows the expression and production of other virulence factors involved in the destruction of the midgut tissues, which include an array of phospholipases, enterotoxins and proteases (Figure 2) (3,13).

To invade the insect, *B. thuringiensis* must cross the peritrophic matrix, a largely chitinous web that separates the midgut cells from the gut contents. Once the midgut has

been breached by the action of the Cry, Cyt and Vip proteins, the *B. thuringiensis* cells or spores can enter into the haemocoel. Within the haemolymph, the bacteria must avoid destruction by the innate humoral immune system and circulating haemocytes. Production of the metalloproteases might help to protect *B. thuringiensis* from the innate immune system through cleavage of AMPs or by facilitating its escape from haemocytes. The expression of enterotoxins by *B. thuringiensis* during septicaemia and iron acquisition systems has been reported, and these might play an active role in colonization. The increased reproduction of *B. thuringiensis* within the haemolymph results in acute septicaemia. Furthermore, *B. thuringiensis* has been shown to reproduce in insect cadavers at the expense of intestinal enterococci that were otherwise found to rapidly exploit hosts killed in the absence of *B. thuringiensis*. This ability to compete might be facilitated by the production of quorum quenchers and antibiotics (Figure 2) (13).

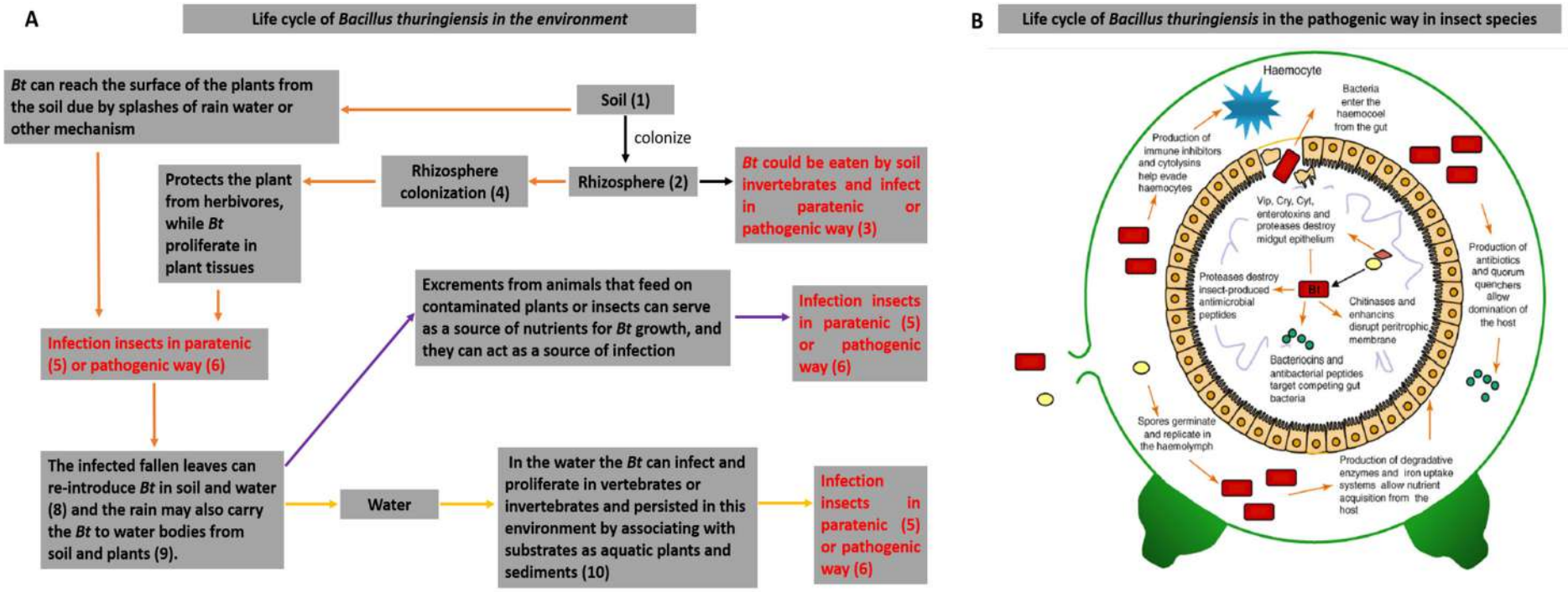

Figure 2 Life cycle *B. thuringiensis*. A) Simplified view of complex lifestyle of *B. thuringiensis* (Argôlo-Filho et al., 2014). The numbers indicate the possible steps followed by *B. thuringiensis* from the soil reservoir to the insect gut. The different colors of the arrows indicate different strategies used by *B. thuringiensis* to occupy different niches and disperse in environment with or without causing diseases. B) Life cycle of *B. thuringiensis* in the pathogenic pathway in insect species (13). This figure has been adapted from Argôlo-Filho et al., 2014 (7) and Raymond et al., 2010 (13).

2.1.1.3. - Transmission of infection

The process of transmission of infection is an important part of the ecology of every pathogen. In the case of *B. thuringiensis*, the horizontal transmission fits with the biology of a highly virulent bacterium that needs to kill its host in order to proliferate. There are two possible routes of horizontal transmission for a *B. thuringiensis* strain active against a leaf-eating insect (13):

1. Indirect transmission via a reservoir in which the spores released from a cadaver are quickly deposited in soil. The bacteria must recolonize plant material to infect hosts at a later date. This could be done by co-migration of *B. thuringiensis* with the growing plant, transfer by biotic or abiotic means or even through endophytic transport

2. Direct transmission, in which new infections are produced by larvae feeding on spores and crystals recently released onto leaf material from a *B. thuringiensis* killed cadaver

The possible routes of transmission above suggested that the toxin production takes place only in two places: (1) within the host or (2) on/within the plants. The contact with the host occurs periodically. The vegetative growth of *B. thuringiensis* on leaves is not only poor but also periodic and might be linked to fluctuation in leaf chemistry or ambient humidity. If transmission occurs indirectly, then Cry toxin production must take place in situ on the leaf surface in a relatively dry environment, protected probably by microsites. Sporulation occurs after replication within host cadavers when limiting resources are exhausted. Again, this is a relatively dry environment, and spores and crystals might be protected from rain and UV radiation when associated with the insect cuticle and other tissue fragments (13).

2.1.2. - Classification and Taxonomy of *Bacillus thuringiensis*

The placement of *B. thuringiensis* as a separate species within the genus Bacillus spp has been controversial since the publication of “The Genus Bacillus” in 1973 and “Bergey’s Manual of Determinative Bacteriology” in 1974. The genus Bacillus spp is one of the most diverse genera in the Bacilli class and includes aerobic and facultative anaerobic, rod-shaped, gram-positive spore-forming bacteria. Based on phylogenetic heterogeneity,

eight genera in the class Bacilli have been proposed: Bacillus, Alicyclobacillus, Paenibacillus, Brevibacillus, Aneurinibacillus, Virgibacillus, Salibacillus and Gracilibacillus. In the "The Genus Bacillus", *B. thuringiensis* is considered a variety of *B. cereus* along with *Bacillus anthracis* and *Bacillus mycoides*. Certainly, *B. thuringiensis*, *B. anthracis* and *B. cereus* share many common phenotypic and genotypic properties to the extent that the three species have been placed under one group called the Bacillus cereus (BC) group (37).

The most widely used method for classification of *B. thuringiensis* isolates has been H serotyping, the immunological reaction to the bacterial flagellar antigen. The *hag* gene encodes flagellin, which is responsible for eliciting the immunological reaction in H serotyping. Specific flagellin amino acid sequences have been correlated to specific *B. thuringiensis* H serotypes and at least 69 H serotypes and 82 serological varieties (serovars) of *B. thuringiensis* have been characterized. H serotyping, however, is limited in its capability to distinguish strains from the same H serotype or from the same serovar (38). With the continuously increasing number of *B. thuringiensis* strains, additional procedures have been implemented to continue with a systematic characterization of novel *B. thuringiensis* strains. Widespread use of DNA sequencing technologies in clinical, public health, and research laboratories has resulted in rapid and accurate molecular diagnostic methods. A bacterial isolate can now be identified more rapidly by 16S rRNA sequence analysis than by conventional methods. In the second edition of Bergey's manual of systematic bacteriology, the phenotypic characteristics and the laborious task of DNA-DNA hybridization procedures have been replaced by 16S rRNA sequence analysis as the basis for taxonomic classification (Ludwig et al., 2001). However, the differentiation between closely related species using 16S rRNA gene sequences can be difficult. For example, in some bacteria, the sequences may appear to be identical, as has been reported for strains of *B. anthracis*, *B. cereus*, and *B. thuringiensis* (39).

Due to the economic importance of *B. thuringiensis*, alternative methods have been developed to classify the members of the BC group, such as multi-locus sequence typing (MLST) (40,41), PCR amplified fragment polymorphisms of flagellin genes (PCR-AFPF) (42), Rep-PCR fingerprinting patterns (43), and plasmid patterns (44). Certainly none of the several methods (H serotyping, 16 RNA gene sequences, MLST, PCR-AFPF, Rep-PCR fingerprinting patterns, and plasmid patterns) developed to characterize the

members of BC group are able to identify, individually, a novel *B. thuringiensis* strain with 100% of confidence. Thus, the need to use combination of some of them to properly characterize a novel *B. thuringiensis* strain.

2.1.3. - Economic impact of the *Bacillus thuringiensis* products present in the market

The use of *B. thuringiensis* in biological control can be done by three strategies: classical, conservative and augmentative. The most common way for pest control is the augmentative-inundative application of *B. thuringiensis*. The success of *B. thuringiensis* as microbial control agent (MCA) is due to the practical similarity to conventional pesticides: *B. thuringiensis*-based products are relatively cheap and easy to formulate, have long shelf life and can be applied using conventional application equipment. In addition, the use of *B. thuringiensis* is compatible with other strategies for crop management such as agricultural practices, biological control using natural enemies and chemical treatments. *B. thuringiensis* formulations are by far the MCA most used at a global scale and account for almost 98% of the bacterial MCA (Figure 3) (6).

Regarding the transgenic Bt crops, the rapid adoption, 1996 to 2016, confirm that biotech crops have delivered substantial agronomic, environmental, economic and health benefits (5). A total of 24 countries, 19 developing and 5 industrialized countries, planted biotech crops in 2017. The top ten countries were led by the USA which grew 75 million hectares (40% of global total), Brazil with 50.2 million hectares (26%), Argentina with 23.6 million hectares (12%), Canada with 13.1 million hectares (7%), India with 11.4 million hectares (6%), Paraguay with 3.0 million hectares (2%), Pakistan with 3 million hectares (2%), China with 2.8 million hectares (1%), South Africa with 2.7 million hectares (1%) and Bolivia with 1.3 million hectares (1%). Regarding the EU countries, Spain and Portugal continued to plant biotech crops in 2017 at 131,535 hectares, indicating a slight decrease of 4% from 136,363 hectares in 2016 (Figure 3) (5).

The four major biotech crops in 2017 are soybeans, maize, cotton, and canola. These crops occupied most (99%) of the global biotech crop area. The adoption trend show alarge increase for soybeans, cotton, and canola and a slight decrease in biotech maize (Figure 3). In addition, the Bt crops suppose an important percentage of the crops seeding market in 2017. For cotton, soybean, maize, and canola, the global adoption rates (%) for principal biotech crops in 2017 are 80%, 77%, 32% and 30%, respectively (5).

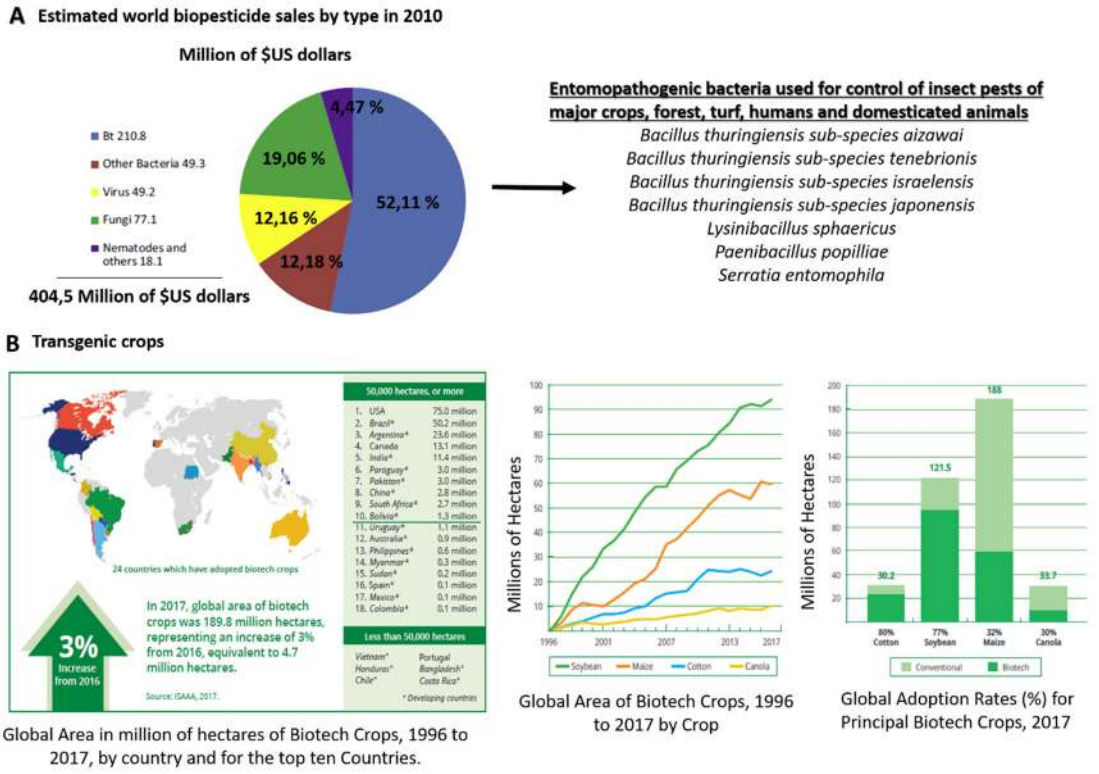

Figure 3 Economic impact and global status of the *Bacillus thuringiensis* products present in the market. A) Estimated world biopesticide sales by type in 2010 and entomopathogenic bacteria used for control of insect pests of major crops, forest, turf, humans and domesticated animals (6). B) Distribution of biotech crops (*B. thuringiensis* crops) by country and crops from 1996 to 2017 (5). This figure has been adapted from Lacey et al., 2015 (6) and ISAA Brief 53 2017 (5).

The economic benefits of the biotech crops of the six principal countries during the first 21 years of commercialization of biotech crops, 1996 to 2016 were, in descending order of magnitude, the USA (US$80.3 billion), Argentina (US$23.7 billion), India (US$21.1 billion, Brazil (US$19.8 billion), China (US$19.6 billion), Canada (US$8 billion), and others (US$13.6 billion) for a total of US$186.1 billion. In 2016, the six countries that gained the most economically from biotech crops were: the USA (US$7.3 billion), Brazil (US$3.8 billion), India (US$1.5 billion), Argentina (US$2.1 billion), China (US$1 billion), Canada (US$0.82 billion), and others (US1.8 billion) for a total of US$18.2 billion. The global economic benefits of US$18.2 billion were divided between the developing countries at US$10 billion and US$8.2 for industrial countries (5).

2.2. - Mining of new insecticidal protein genes of *Bacillus thuringiensis*

Regardless of whether we consider *B. thuringiensis* a soil-dwelling microorganism or a specialized pathogen, *B. thuringiensis* shows a high genetic plasticity. Different series of approaches have been used for isolating novel insecticidal protein genes from *B. thuringiensis*, such as the construction of *B. thuringiensis* DNA libraries, followed by screening by Western Blotting and PCR. Currently, the next generation sequencing (NGS) allows rapid sequencing of entire genomes and the number of *B. thuringiensis* whole genomes that have been sequenced has increased quickly in the recent years (Figure 4). Consequently, the number of putative insecticidal protein genes predicted based on the genome data available have increased exponentially.

2.2.1 - PCR approach used in the mining of new insecticidal protein genes

The mining of new insecticidal proteins is a tool that helps researchers to cope with the problem of the eventual appearance of resistance in the field against currently used *B. thuringiensis* toxins. Thus, it is necessary to explore the potential of new insecticidal proteins in new *B. thuringiensis* isolates for pest control. One of the first approach developed was the construction of *B. thuringiensis* DNA libraries, followed by screening by Western Blotting or a hybridization-based method (45,46). With the invention of the polymerase chain reaction (PCR), specific applications to mining new insecticidal genes quickly started to be developed, such as PCR hybridization, PCR-RFLP, E-PCR, PCR-SSCP, Multiplex-PCR, etc. (47–51). With the combination of the specific PCR applications, there have been identified more than 800 *B. thuringiensis* quaternary rank toxins (52) (Figure 4). Although the PCR approach has demonstrated to be a great technique in the mining of new insecticidal protein genes, it is limited to finding new insecticidal protein genes with enough homology to the primers used. These new insecticidal protein genes should display a small number of synonymous or non-synonymous substitutions and encoded identical or slightly different proteins compared to previously described alleles. In addition, since these techniques yield only a part of the gene sequence, time-consuming PCR walking strategies or construction of genomic libraries and screening are needed to obtain a single full-length coding sequence (53).

2.2.2 - Novel insecticidal protein genes and high-throughput technologies (NGS and LC/MSMS)

Currently, the next-generation sequencing (NGS) technologies are a useful tool to discover new insecticidal protein genes that would otherwise be difficult to identify by the PCR approach. NGS technologies provide a fast and reliable framework to obtain complete genomic sequences, and offer excellent cost-benefit ratios. This cost may be reduced only if raw sequence production is outsourced and the researcher completes the assembly steps. The cleaning, assemble, mapping and annotation of the data generated by the high-throughput technologies can be done using a regular desktop computer or in a cluster of the Spanish Supercomputing Network (RES). Regarding the tools to manage the data generated by the NGS technologies, they can be done with private bioinformatics software, such as CLC Genomics Workbench or Geneious Pro, or using open source softwares that are freely available in the BioLinux distribution, among other alternatives.

Several reports have been published that use the high-throughput technologies to assess the mining of new insecticidal protein genes and can be grouped in the following strategies:

1. Whole genome sequencing, assembly and annotation of the *B. thurinigensis* standards such as *B. thuringiensis* serovar *morrisoni* (BGSC 4AA1), *B. thuringiensis* serovar *kurstaki* (HD1 and HD73), *B. thuringiensis* serovar *galleriae* (HD29), *B. thuringiensis* serovar *tolworthi* (BGSC 4L1-4L3), etc (https://www.ncbi.nlm.nih.gov/genome/microbes/)
2. Whole genome sequencing of a *B. thuringiensis* isolate that shows a wide insecticidal spectrum or insecticidal activity against pests that are difficult to control (54–58)
3. Mining, expression and insecticidal spectrum of new insecticidal protein genes detected by whole genome sequencing (53,55,59,60)
4. Screening of the *B. thuringiensis* toxin gene content in a *B. thuringiensis* collection by pool deconvolution approach (61)

The emergence of mass sequencing programs offers huge potential for the discovery of new toxin sequences and suppose a challenge to the *B. thuringiensis* toxin nomenclature system. Such a challenge include the large numbers of new variant toxins that may

overwhelm the system, as well as nomenclature difficulties within the present system (62).

2.2.3. - *Bacillus thuringiensis* Toxin Nomenclature

Since the identification of the Cry1Aa protein in 1985 (46), the number of insecticidal protein genes and whole genome sequences of *B. thuringiensis* has been growing every year, generating the need to develop a nomenclature system (Figure 4). A first nomenclature system was developed where the names of the Cry toxins included a Roman numeral (primary rank distinction) depending on the insecticidal activity of the crystal protein: CryI for proteins toxic to lepidopterans, CryII for proteins with toxicity against both lepidopterans and dipterans, CryIII for proteins toxic to coleopterans, and CryIV for proteins toxic exclusively for dipterans (63). However, this system exhibited important complications, such as the following: [1] the activity of new toxins had to be assayed against a big set of insects before the toxin could be named, [2] some novel homologous proteins were in fact non-toxic as expected and others exhibited dual toxicity against dipteran and lepidopteran species (64).

To avoid the problems of the old toxin nomenclature, the *B. thuringiensis* Toxin Nomenclature Committee developed a novel system of classification where a novel toxin received a four-rank name depending on its degree of pairwise amino acid identity to previously named toxins (64) as follows (Figure 4):

1. Proteins with % of similarity < 45% are assigned to a different primary rank (protein name and arabic number, eg, Vip1 and Vip3)
2. Proteins with % of similarity < 78% are assigned to a different secondary rank (a capital letter, eg, Vip3A and Vip3C)
3. Proteins with % of similarity < 95 % are assigned to a different terciary rank (a lowercase letter, eg, Vip3Aa and Vip3Ab)
4. Proteins with % of similarity > 95 % are assigned to a different quaternary rank (an arabic number, e.g., Vip3Aa1 and Vip3Aa2)

The advantages of the novel nomenclature system developed by the *B. thuringiensis* Toxin Nomenclature Committee consist in grouping the proteins according to the sequence identity, which does not imply a similar protein structure, host range or even mode of action among the proteins (3).

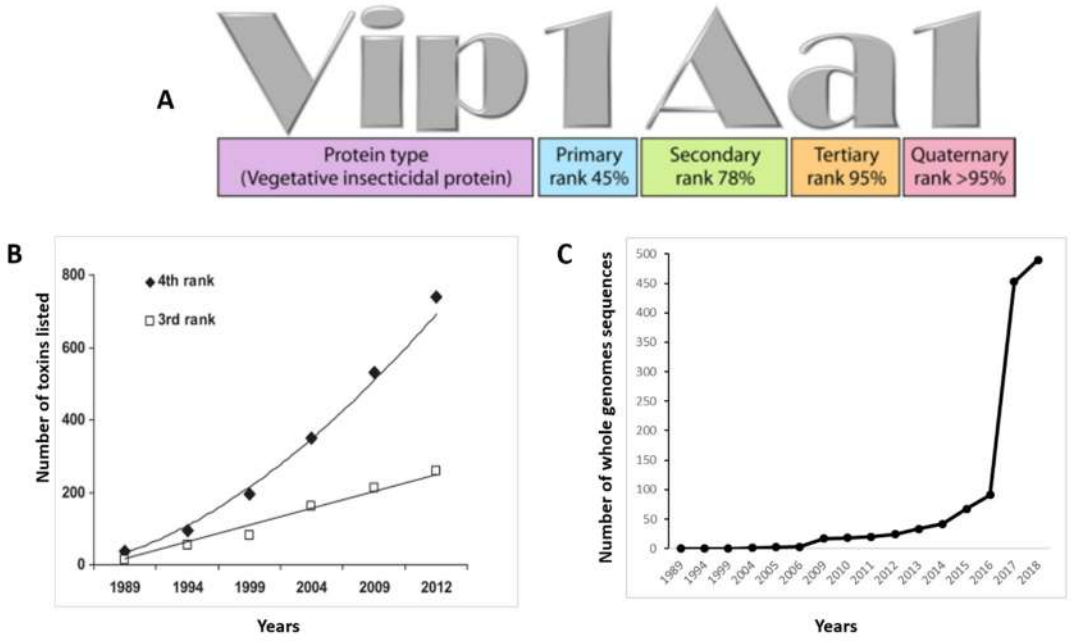

Figure 4 Schematic overview of the current nomenclature system used by the *B. thuringiensis* Toxin Nomenclature Committee. A: The system consists of four ranks based on amino acid sequence identity. The primary, secondary, and tertiary ranks distinguish proteins with less than > 45, 78, and 95 % sequence identities, respectively. The quaternary rank distinguishes proteins sharing > 95 % sequence identity (65). B: Number of Cry, Cyt and Vip from 1989 to 2013, listed in the *B. thuringiensis* Toxin Nomenclature website at either tertiary (holotype) or quaternary (allelic) rank (52). C: Number of whole genome sequences of *Bacillus thuringiensis* from 1989 to 2018, listed in the Microbial Genome website.

2.3. - Insecticidal proteins of *Bacillus thuringiensis*

B. thuringiensis strains synthesize δ-endotoxins (crystal and cytolytic toxins) at the beginning of the stationary growth phase as parasporal crystalline inclusions. Once ingested by the insect, the crystal is solubilized in the midgut and then the toxins are processed by the midgut proteases to a toxic core. After the activation, the toxic core crosses the peritrophic membrane and binds to specific receptors located in the brush border membrane of the midgut epithelium, leading to cell disruption and insect death. Additionally, *B. thuringiensis* isolates can produce other insecticidal proteins during the vegetative growth phase that are secreted into the culture medium, Vip and Sip proteins. These insecticidal proteins show a similar mode of action with the Cry proteins but do not compete for their binding sites (3).

2.3.1 - Crystal Toxins (δ-endotoxins)

Cry toxins are defined as proteins that have significant sequence similarity to existing toxins within the nomenclature or be a *B. thuringiensis* parasporal inclusion protein that exhibits pesticide activity, or toxic effect, to a target organism (64). Currently, the different kinds of Cry proteins (Three-domain proteins, Bin-like proteins, Etx_Mtx-like proteins and Unclassified Cry proteins) constitute the largest group of insecticidal proteins by *B. thuringiensis* and show toxicity against lepidopterans, coleopterans, hemipterans, dipterans, Gastropoda and human-cancer cells of various origins (Table 1 and Figure 5).

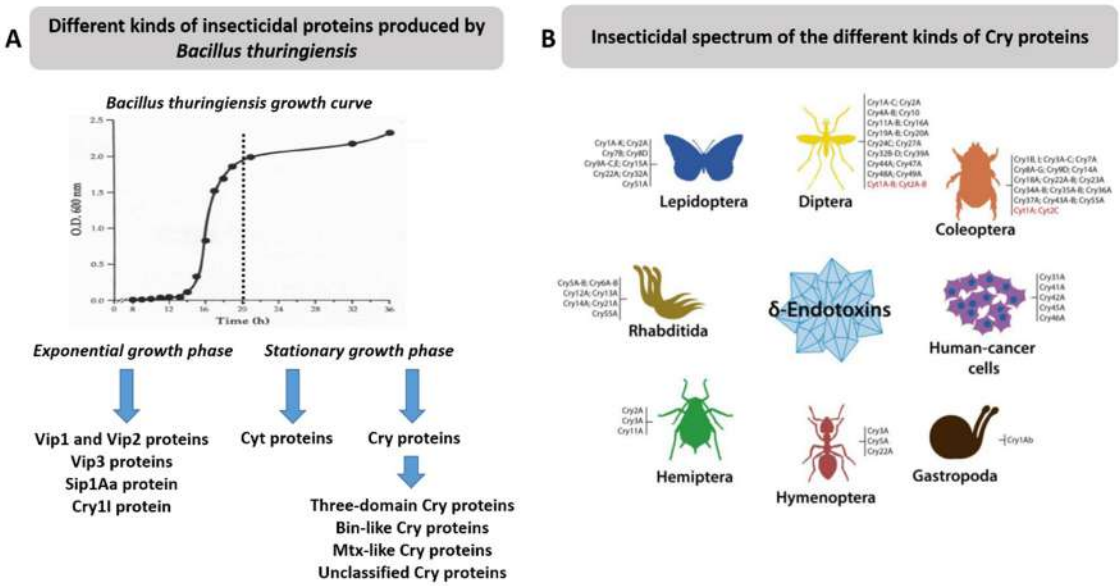

Figure 5 Insecticidal proteins produced by *B. thuringiensis*. A: Schematic representation of the different kinds of insecticidal proteins produced by *B. thuringiensis*. B: Overview of the insecticidal spectrum of the Cry proteins with their target pest in each class. Cry1A-C (separated by hyphen) indicates a group of Cry1A, Cry1B and Cry1C toxins. Cry1B and Cry1I (separated by colon) indicates different Cry1B and Cry1I toxins. Semicolons separate groups or individual toxins. Cyt toxins are in red. This figure has been adapted from Palma et al., 2014 (3) and Chattopadhyay et al., 2018 (66).

Tabla 1. The summary of *B. thuringiensis* toxin proteins, structure and their activities.

Protein family	Number of proteins	Insect order	Protein family	Number of proteins	Insect order	Protein family	Number of proteins	Insect order	Protein family	Number of proteins	Insect order
Cry1(A–N)	275	Lep and Col	Cry23(A)	1	Col	Cry45(A–B)	2	AT	Cry67(A)	2	ND
Cry2(A–B)	82	Dip and Lep	Cry24(A–C)	3	Dip	Cry46(A)	3	AT	Cry68(A)	1	ND
Cry3(A–C)	19	Col	Cry25(A)	1	Dip	Cry47(A)	1	Dip	Cry69(A)	3	ND
Cry4(A–C)	17	Dip	Cry26(A)	1	Col	Cry48(A)	5	Dip	Cry70(A)	3	ND
Cry5(A–E)	13	Rha	Cry27(A)	1	Dip	Cry49(A)	5	Dip	Cry71(A)	1	ND
Cry6(A–C)	4	Rha and Hem	Cry28(A)	2	Col	Cry50(A–B)	3	ND	Cry72(A)	1	ND
Cry7(A–L)	37	Col	Cry29(A–B)	2	Dip	Cry51(A)	2	Lep	Cry73(A)	1	ND
Cry8(A–T)	59	Col	Cry30(A–G)	13	Dip	Cry52(A–B)	2	Dip	Cry74(A)	1	ND
Cry9(A–G)	37	Lep	Cry31(A)	12	ND	Cry53(A)	2	ND	Cry75(A)	1	Col
Cry10(A)	5	Dip	Cry32(A–W)	31	Dip	Cry54(A–B)	5	Lep			
Cry11(A–B)	8	Dip	Cry33(A)	1	ND	Cry55(A)	3	Col and Rha			
Cry12(A)	1	Rha	Cry34(A–B)	11	Col	Cry56(A)	4	ND	Cry78Aa	1	Hem
Cry13(A)	1	Rha	Cry35(A–B)	11	Col	Cry57(A)	2	ND			
Cry14(A)	2	Col and Rha	Cry36(A)	1	Col	Cry58(A)	1	ND			
Cry15(A)	1	Lep	Cry37(A)	1	Col	Cry59(A–B)	2	Lep			
Cry16(A)	1	Dip	Cry38(A)	1	Col	Cry60(A–B)	6	ND			
Cry17(A)	1	Dip	Cry39(A)	1	Dip	Cry61(A)	3	ND			
Cry18(A–C)	3	Col	Cry40(A–D)	4	Dip	Cry62(A)	1	ND			
Cry19(A–C)	3	Dip and Lep	Cry41(A–C)	5	AT	Cry63(A)	1	AT			
Cry20(A–B)	4	Lep	Cry42(A)	1	ND	Cry64(A–C)	3	AT			
Cry21(A–H)	10	Rha and Dip	Cry43(A–C)	7	Col	Cry65(A)	2	AT and AB			
Cry22(A–B)	7	Coleoptera	Cry44(A)	1	Dip	Cry66(A)	2	ND			

Lep: Lepidoptera; Dip: Diptera; Col: Coleoptera; Rha: Rhabditia; Hem: Hemiptera AT: antitumoral activity; AB: antibacterial activity and ND: no known invertebrate target. Blue: Three-domain, Green: Unclassified Cry, Orange: Etx-Mtx-like, Pink: Bin-like, Parasporin: Toxins named with red colour. Adapted from Adang et al., 2014 (67); Jouzani et al., 2017 (4); Liu et al., 2018 (68) and Wang et al., 2018 (60).

2.3.1.1 - Three domain Cry proteins

Sequence alignments of different Cry toxins show that the majority of the Cry proteins contain five typical conserved blocks located in the N-terminal part of the protoxins (domains I, II and III) and three additional conserved blocks are located in the C-terminal end of the protoxins (Figure 6). The C-terminal extension found in the large protoxins is not part of the active core but is believed that participate in the crystal formation and putuative mechanism to generate new specificities in the nature. Also other three-domain protoxins lack the extended C-terminal region and are, instead, synthesized as shorter protoxins of approximately 70-kDa (3,69).

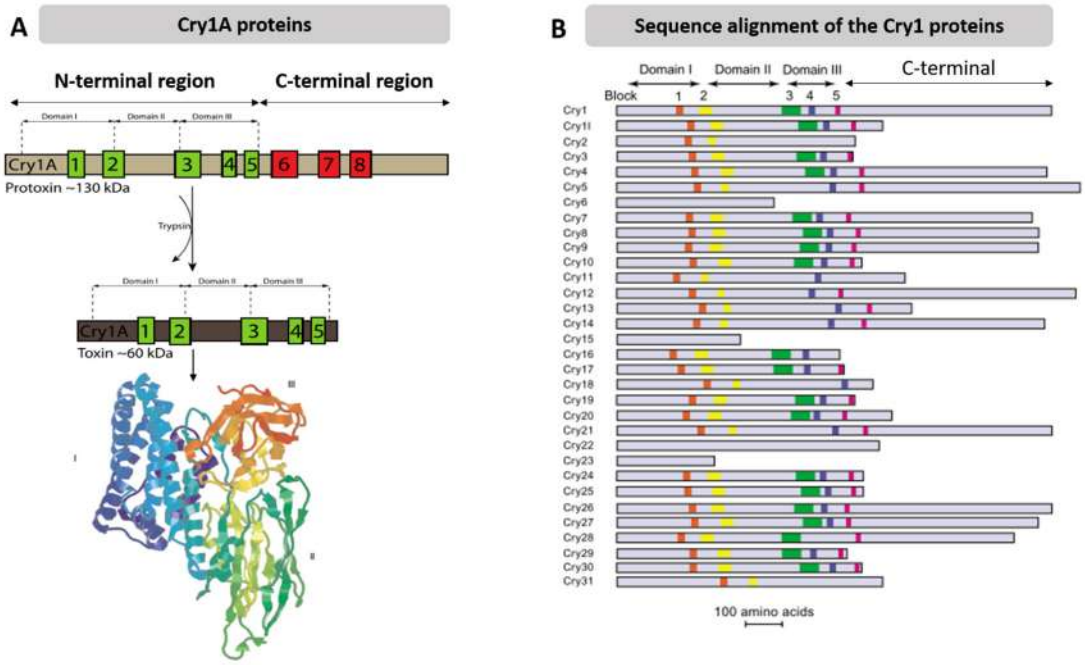

Figure 6 Primary and tertiary structure of the Cry proteins. A: Amino acid conserved blocks (1 to 8) among different three-domain Cry proteins (colored boxes). Green boxes represent the five conserved amino acid blocks of the N-terminal while the red boxes indicate the three additional conserved amino acid blocks of the C-terminal part. Three-dimensional structure of Cry1Ac protoxin with the toxic core comprising the domains I, II and III. B: Relative lengths of Cry protoxins and position of the five conserved blocks and C-terminal region. This figure has been adapted from Palma et al., 2014 (3) and Ruud de Maagd et al., 2001 (69).

The domain I or perforating domain is located in the N-terminal region. This domain consists of seven α-helices, six amphipathic α-helices around a central core helix (Figure 6). The domain I may be involved in the toxin membrane insertion and pore formation. Domain II or β-prism consists of three antiparallel β-sheets and plays an important role in toxin-receptor interactions. The domain III or galactose-binding domain consist in two antiparallel β-sheet sandwich and is involved in receptor binding and pore formation (3,69).

2.3.1.2 - Mode of action of the three domain Cry proteins

The mode of action of the three-domain crystal proteins has been mainly studied in lepidopteran pests. Two different models have been proposed to explain the mode of action of the three-domain Cry proteins: the sequential binding model and the signalling pathway model (Figure 7). The crystal inclusion, after the ingestion by the insect, is dissolved in the alkaline midgut lumen and the native proteins (protoxin) present in the crystal composition are activated to the protease-resistant toxic core, N-terminal region, by the action of the midgut proteases (Figure 6). The toxic core of the Cry proteins crosses the peritrophic membrane and enter in the extracellular media. The sequential binding model suggests that the toxic core of the Cry toxins bind to cadherin-like proteins (transmembrane protein that function as toxin receptors) and undergo a conformational change that facilitate the proteolytic removal of the α-1 helix from domain I and formation of the oligomeric pre-pore structure. Later, binding to a secondary receptor, such as an aminopeptidase or alkaline phosphatase, facilitates the insertion of the pre-pore structure into the membrane, leading to cell death (3,70). In contrast, the signalling-pathway model suggests that the toxic activity of the three-domain Cry proteins is mediated by the specific binding to cadherin receptors, leading to the activation of an Mg^{2+}-dependent signal-transduction pathway which, in subsequent steps, activates the G protein (Gα), adenylyl cyclase (AC), and protein kinase A (PKA). The activation of the Mg^{2+}-dependent signal-transduction pathway increased the exocytosis of the intracellular cadherin receptors into the membrane and the PKA modifies downstream molecules that promote the biochemical activities that destroy the cell (37,70).

2.3.1.3 - Non-Three-Domain Cry Toxins

Epsilon toxin_ Metaxin 2 (Etx_Mtx2)-like Cry toxins

The Etx_Mtx2-like Cry proteins such as Cry15, Cry23, Cry33, Cry38, Cry45, Cry46, Cry51, Cry60, Cry64 and Mtx3 and Mtx2 share features of the ETX_MTX2 family that include the Mtx proteins from *Lysinbacillus sphearicus* and the *Clostridium perfringens* epsilon toxin. The latter toxin has an extended beta sheet structure related to aerolysin, a pore-forming toxin produced by the *Aeromonas hydrophila*, which forms beta-barrel pores in target cells. A similar mode of action is also likely for the Etx_Mtx2-like Cry toxins above (3,66).

Binary (Bin)-like Cry toxins

The Bin-like Cry toxins such as Cry34, Cry35, Cry36, Cry37, Cry49, BinA and BinB belong to the Toxin_10 family of proteins and show an aerolysin-like fold that is very similar of the Etx_Mtx2-like Cry proteins. Cry35 contains a QxW motif, which is structurally very similar to carbohydrate-binding domains of ricin and Mtx1. It is interesting to note that some of the above toxins are able to act alone to cause toxicity (e.g., Cry36), whereas others require a second protein to act as binary toxins (e.g., BinA/BinB, Cry23/Cry37, Cry34/Cry35 and Cry48/Cry49). Cry34 is a member of the aerolysin family, where the structure of the protein (PBD accession number 4JOX) shows a single domain protein with a beta-sandwich conformation and a hydrophobic core. Although there is no obvious homology at the level of their amino acid sequences, the Cry34/Cry35 pair show structural similarity to another binary toxin, Cry23/Cry37 (3).

Unclassified Cry toxins

In few years, new crystal proteins have been reported, such as the 42 kDa protein, sphaericolysins, alveolysins, enhancin-like proteins and P19 and P20 helper proteins. The 42-kDa protein exhibited high similarity to BinA and BinB and no toxicity has been reported yet. Sphaericolysin, a protein with a unique N-terminal sequence and cholesterol-dependent cytolysins motif, is very effective to control *Blattella germanica* and *Spodoptera litura*. The enhancin-like proteins show similarity to viral enhancin proteins; for the Bel enhancin, it has been reported to be effective in enhancing the toxicity of Cry1Ac against *Helicoverpa armigera* but not when it is combined with Cry9Ea against *Sopodoptera exigua* and *Trichoplusia ni*. The helper proteins, P19 and

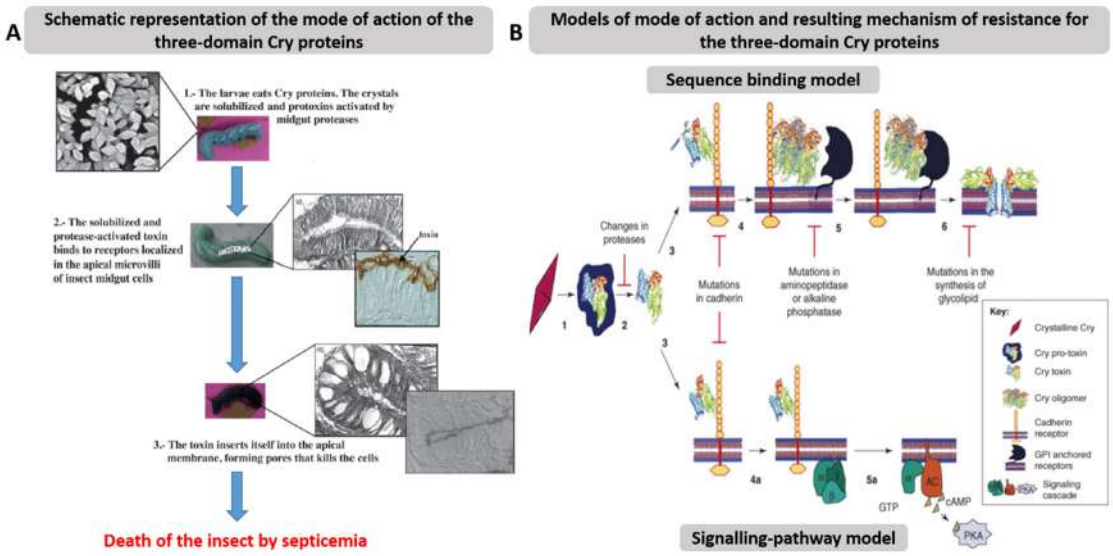

Figure 7 Mode of action of the three-domain Cry proteins. A: Schematic representation of the mechanism of toxicity of the three-domain Cry proteins. B: The two different models that explain the mode of action Cry proteins: the sequence binding model and the signalling-pathway model. Steps of the sequence binding model: (1) Toxin solubilisation in the midgut lumen, (2) Activation by midgut proteases, (3 and 4) Binding to primary receptor (cadherin, ABCC2, etc.) and cleavage of helix α-1 and trigger the toxin oligomerization, (5) The toxin oligomer then binds to a secondary receptor, such as aminopeptidase or alkaline phosphatase, (6) Toxin insertion into the membrane of the insect cells. Regarding the signalling-pathway model proposes that the interaction of the Cry toxin (monomer or oligomer) with the primary receptor activate an intracellular signalling pathway that is mediated by activation of protein G (4a) which, in a subsequent step, activates adenylyl cyclase. This signal then increases the levels of cyclic adenosine monophosphate, which activates protein kinase A and leads to cell death (5a). This figure has been adapted Pardo-López et al., 2013 (71) and Bravo et al., 2011 (1).

P20 have a role in the production of the stable parasporal crystal for those Cry proteins without C-terminal region such as Cry11A. Also the helper proteins improve the production of Cry proteins in acrystalliferous *Bt* strains and *E.coli* strains. P20 also synergizes with the toxicity of Cry11A against third-instar larvae of *Aedes aegypti* (3). In addition, few unclassified *B. thuringiensis* toxins, such as Cry6, Cry22, Cry55 and Cry46, have been reported. The Cry22 is reported to show four cadherin-like domains and a C-terminal region with structural similarities to domain III of the three-domain toxins. No structural data are available for the Cry6 proteins, which show features of the Smc protein

family within their central regions. The Cry55 and Cry46 proteins have no known structural homolog and show no conserved domains (3).

2.3.2 - Secretable proteins

Apart from the production of the δ-endotoxins in the parasporal crystal, screening programs have been performed with the aim to evaluate active insecticidal components in culture supernatants. Different Bacillus spp isolates (*B. cereus* strain AB78, *B. thuringiensis* strain AB88) were identified that produce a proteinaceous active component that causes mortality to *Diabrotica virgifera, Agrotis ipsilon* and other lepidopteran larvae. The proteinaceous active components of the respective Bacillus spp isolates were identified as three proteins of 85, 45 and 88 kDa respectively. The *B. thuringiensis* toxin nomenclature named these active components as Vip1, Vip2 and Vip3 (65). It should be mentioned that another insecticidal protein has been later reported: the Sip1A protein (72). Currently the Sip1A protein is not included in the *B. thuringiensis* toxin nomenclature but can be consider as a vegetative insecticidal protein.

2.3.2.1 - Vip1 and Vip2 proteins

The Vip1 and Vip2 proteins act as a binary toxin. The *vip1* and *vip2* genes are present in *B. cereus, B. thuringiensis, L. sphaericus* and *Brevibacillus laterosporus*. These two genes are located in an operon of ~4-kb with two open reading frames separated by an intergenic space of 4 to 16 bp in the genome sequence or in a megaplasmid (3,65). The Vip1 and Vip2 proteins are expressed concomitantly and encode an approximately ~100 and ~50-kDa proteins, respectively. They are produced during the vegetative growth phase of *B. thuringiensis*, and their levels remain high until after the sporulation stage. Vip1 and Vip2 contain and N-terminal signal peptide sequences, which for the Vip1 protein is processed after secretion into a smaller ~80-kDa mature protein.

The protein pairs Vip1/Vip2 show toxic activity against some coleopteran larvae and *Aphis gossypii*, but they do not show toxicity against lepidopteran larvae (Table 2). Their homology to other bacterial binary toxins suggests that Vip1 and Vip2 form a A+B type of binary toxin, where Vip2 is the cytotoxic A-domain and Vip1 the receptor-binding domain responsible of the translocation of the cytotoxic Vip2 into the host cell. Vip2 exhibits sequence and structural homology with the enzymatic domain of toxin CdtA from *Clostridium difficile* and the iota-toxin domain Ia from *C. perfringens*, both

possessing ADP-ribosyltransferase activity that targets actin, inducing cytoskeletal disorders and cell death (57,65). The mode of action of the Vip1/Vip2 proteins is not totally understood (Figure 8). The intoxication process start with the ingestion of the toxins by the larva, followed by the proteolytic activation in the midgut. The activated Vip1 proteins forms oligomers of seven units of Vip1 molecules. These oligomers recognize specific receptors in the midgut brush border membrane, where the toxin is then inserted into the membrane. The mechanism by which Vip2 enters the cell is still unknown but based on its homology with the C2-I component of the C2 clostridial binary toxin, it seems likely that Vip2 enters the cell via receptor-mediated endocytosis (65). Leuber et al., 2006 (73) proposed a second possibility, in which the proton gradient across the midgut brush border membrane of insect cells could promote the directly delivered into the cytoplasm of the Vip2Ac monomers via the channel formed by Vip1Ac.

Table 2 Insecticidal spectrum of different Vip1/Vip2 protein combinations on different insect species. The number of "+" symbols indicate the activity level.

Protein combination	Insect order	Insect species	Activity	Reference
Vip1Aa+Vip2Aa	Coleoptera	*Diabrotica virgifera* *Diabrotica longicornis* *Dibrotica undecimpunctata*	+++ +++ +	Warren 1997 (74)
Vip1Aa+Vip2Ab	Coleoptera	*Diabrotica virgifera*	+++	Warren 1997 (74)
Vip1Ac1+Vip2Ae3	Homoptera	*Aphis gossypii*	+++	Yu et al.,2011 (75)
Vip1Ae1+Vip2Ae1	Homoptera	*Aphis gossypii*	+++	Sattar et al., 2011 (76)
88% similarity to *vip2Ac* and *vip1Ac*	Coleoptera	*Sitophilus zeamais*	+++	Shingote et al., 2013 (77)
Vip1Ad1-Vip2Ag1	Coleoptera	*Holotrichia oblita* *Anomala corpulenta* *Holotrichia parallela*	+++ +++ +++	Bi et al., 2014 (59)
Vip1Ba1-Vip2Ba1	Coleoptera	*Diabrotica virgifera*	+++	Schnepf et al., 2005 (78)
Vip1Da1-Vip2Ad1	Coleoptera	*Diabrotica virgifera* *Diabrotica undecimopunctata* *Diabrotica barberi* *Leptinotarsa decemlineata* *Anthonomus grandi*	+++ +++ +++ +++ +++	Boets et al., 2004 (79)
Vip1Aa2+Vip2Aa2	Coleoptera	*Diabrotica virgifera*	+++	Feitelson et al., 2003 (80)
Vip1Bb1-Vip2Bb1	Coleoptera	*Diabrotica virgifera*	+++	Feitelson et al., 2003 (80)

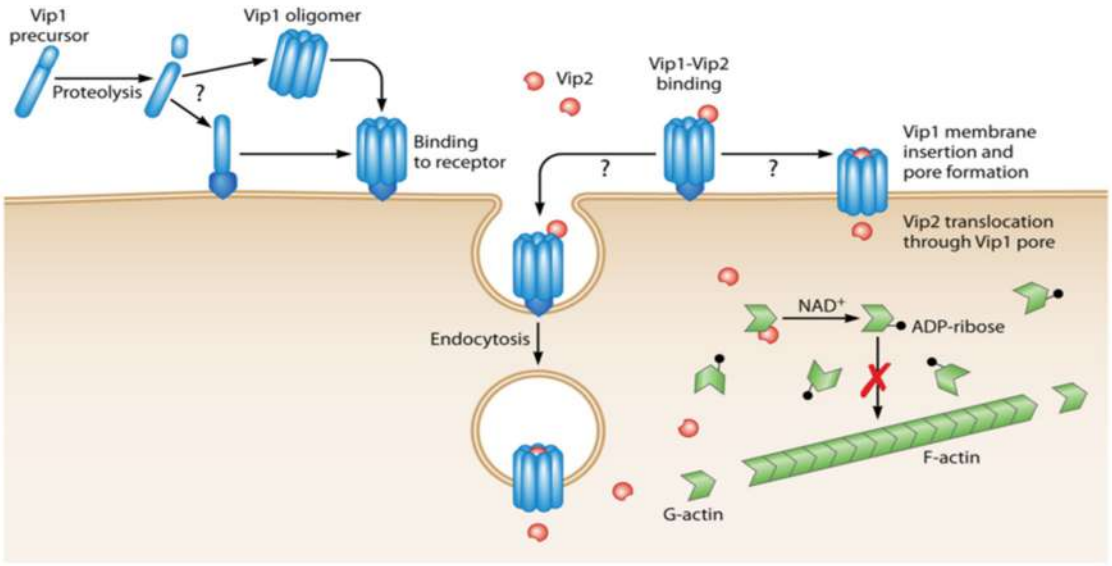

Figure 8 Mode of action of the binary Vip1/Vip2 toxin. The Vip1 protoxin is processed by midgut proteases. The activated toxin (monomer or oligomer) binds to specific receptors. Vip2 then binds to the oligomer Vip1 protein and enters the cell either by endocytosis of the whole complex or directly through the pore formed by Vip1. Once inside the cytosol, Vip2 catalyzes the transfer of the ADP-ribose group from NAD to the actin monomers, preventing their polymerization. Adapted from Chakroun et al. (65).

2.3.2.2 - Sip proteins

The secreted insecticidal protein, Sip protein, shows toxicity against some coleopteran larvae (Table 3). The Sip1Aa protein was initially isolated from the *B. thurnigiensis* strain EG2158, while the Sip1Ab protein was obtained from the *B. thurnigiensis* strain QZL38. These proteins are ~41 kDa proteins with a secretion signal of 30 amino acids. For the Sip1Aa protein, it was found that it was N-terminally processed in the first 43 amino acids by active proteases present in the culture medium to the toxic core (81). Regarding the structure of the Sip proteins, it shows similarity to the 36-kDa Mtx3 mosquitocidal toxin and suggests that the toxicity of the Sip1Aa1 protein toxicity may be caused by pore formation, but the mode of action of Sip1A proteins remains unknown (72,81).

Table 3 Activity spectrum of different Vip1/Vip2 protoxin combinations on different insect species. The number of "+" symbols indicate the activity level.

Protein	Insect order	Insect species	Activity	Reference
Sip1Aa	Coleoptera	*Diabrotica virgifera*	+++	Donovan et al., 2006 (72)
		Dibrotica undecimpunctata	+++	
		Leptinotarsa decemlineata	+++	
Sip1Ab	Coleoptera	*Colaphellus bowringi*	+++	Sha et al., 2018 (81)

2.3.2.3 - Vip3 proteins

The Vip3 proteins (Vip3A, Vip3B and Vip3C) are encoded by the *vip3* genes that are present in strains of *B. thuringiensis* (82–84). Different strategies for screening new *vip3* genes have been applied to several *B. thuringiensis* collections (51,75,76,83–91) and, as a result, 65 *vip3Aa*, 2 *vip3Ab*, 1 *vip3Ac*,4 *vip3Ad*, 1 *vip3Ae*, 4 *vip3Af*, 15 *vip3Ag*, 2 *vip3Ah*, 1 *vip3Ai*, 2 *vip3Aj,* 2 *vip3Ba,*3 *vip3Bb,* 1 *vip3Bc* and 4 *vip3Ca genes* have been reported (http://www.lifesci.sussex.ac.uk/Home/ Neil_Crickmore/Bt/). Interestingly most of the studies carried out on the Vip3 proteins have been performed with the Vip3A proteins, and hence, little information is available about the insecticidal spectrum and mode of action of the Vip3B and Vip3C proteins. The *vip3* genes (*vip3A, vip3B* and *vip3C*) encoded an approximately ~85-90 kDa protein that is secreted to the medium and activated by the insect midgut proteases to two fragments of 20 kDa and 60 kDa (82–84). The activated protein shows toxic activity against a wide range of lepidopteran larvae and certain species with less susceptibility to Cry1A proteins (e.g., *Agrotis ipsilon, S. exigua* and *Spodoptera frugiperda*) (Table 4).

Several reports have focused on interactions among the Vip3 proteins and Cry proteins (mainly Cry1, Cry2 and Cry9). Yu et al. (92) observed synergism between the Vip3Aa and Cyt2Aa proteins against *Chilo suppressalis* and *S. exigua,* but this protein combination was slightly antagonistic against *Chilo quinquefasciatus*. Dong et al. (93) reported synergism between Vip3Aa and Cry9Ca in *Plutella xylostella*. Bergamasco et al. (94) reported synergism between Vip3A and Cry1Ia in *S. frugiperda*, *Spdoptera albula*, and *Spodoptera cosmioides* but slight antagonism in *Spodoptera eridania*. Lemes et al. (95) found antagonism for the combination of Cry1Ca and Vip3Aa, Vip3Ae, or Vip3Af, and for the combination of Vip3Af with either Cry1Aa or Cry1Ac in *Heliothis*

virescens. Also Vip3Aa and Cry1Ca showed antagonism in *S. frugiperda*, whereas the same combination was synergistic in *Diatraea saccharalis*. Crialesi-Legori et al. (96) described that mixtures of Vip3Aa, Vip3Ae and Vip3Af with Cry1Aa, Cry1Ab, Cry1Ac, Cry1Ca and Cry1Ea, tested against *Anticarsia gemmatalis* and *Chrysodeixis includes*, showed synergistic interactions in most of them. Graser et al. (97) reported an additive effect among the Cry1Ab, Vip3Aa20, and Cry1F proteins for *Ostrinia nubilalis* and *S. frugiperda*. Also, interactions between different Bt-crops (Vip3A: COT102, COT202 and COT203, Cry proteins: Cotton lines 02A, 67B, 69D and MON 15985) and their offspring demonstrated that the combined activity of different Bt crops expressing different kinds of *B. thuringiensis* proteins produce and additive effect (98,99). The mechanisms underlying synergism and antagonism are unknown. For the antagonism between the Vip3A and Cry1C proteins, Lemes et al. (95) hypothesized that a physical interaction of the two proteins impairs the access of the binding epitopes to the membrane receptor. For the synergism, the formation of and hetero-oligomer that increased the ability for membrane insertion or pore formation was proposed to explain the synergism between Cry1Ac and Cry1Aa (100). In this sense, a recent report demonstrated that the Cry9Aa and Vip3A proteins show specific binding between them and that this is a primary and fundamental step for the synergistic activity of Cry9Aa and Vip3Aa (101).

Table 4 Insecticidal spectrum of the Vip3 proteins in lepidopteran insect species reported in the bibliography. Adapted from Chakroun et al. (65) and Zack et al. (102).

Protein family	Susceptible insect species		Protein family	Susceptible insect species
Vip3Aa Vip3Ab Vip3Ac Vip3Ac Vip3Ae Vip3Af Vip3Ag Vip3Ah Vip3Ai Vip3Aj	*Agrotis ípsilon*	*Chilo suppresalis*	Vip3Ba Vip3Bb Vip3Bc	*Chrysodeixis includes* *Ostrinia nubilalis* *Helicoverpa armigera* *Helicoverpa punctigera*
	Agrotis segetum	*Phthorimaea operculella*		
	Spodoptera frugiperda	*Chrysodeixis chalcites*		
	Spodoptera exigua	*Earias vitella*		
	Spodoptera albula	*Pieris brassicae*		
	Spodoptera eridania	*Prays oleae*		
	Spodoptera cosmoides	*Tuta absoluta*		
	Spodoptera littoralis	*Ephestia kuehniella*		
	Spodoptera littura	*Ectomyelois ceratoniae*		
	Helicoverpa armigera	*Chironomus tepperi*		
	Helicoverpa punctigera	*Anticarsia gemmatalis*	Vip3Ca	*Agrotis ipsilon* *Chrysodeixis chalcites* *Helicoverpa armigera* *Mamestra brassicae* *Spodoptera exigua* *Spodoptera frugiperda* *Spodoptera littoralis* *Trichoplusia ni*
	Helicoverpa zea	*Cydia pomonella*		
	Heliothis virescens	*Dendrolimus pini*		
	Danaus plexippus	*Lobestria botrana*		
	Manduca sexta	*Mamestra brassicae*		
	Scirpophaga incertulas	*Plutella xylostella*		
	Bombix mori	*Chilo partellus*		
		Chilo quinquefasciatus		

Regarding the mode of action of the Vip3 proteins (mainly Vip3Aa), although the Vip3 proteins do not share structural homology with the Cry proteins, the toxic action follow the same sequence of events (3,65): [1] Ingestion of the Vip3 proteins by the susceptible insects. [2] Activation by the midgut proteases and crossing the peritrophic membrane. [3] Binding to the specific receptors in the apical membrane of the epithelial midgut cells. [4] Pore formation and cell lysis (Figure 9).

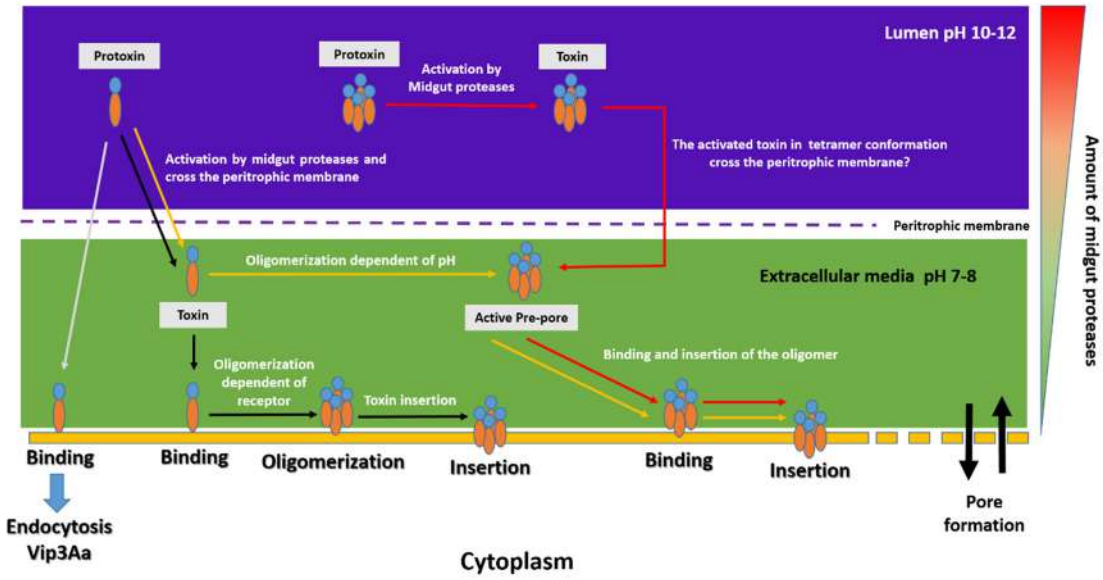

Figure 9. Proposed mode of action of the Vip3A proteins. The full-length protein is proteolytically processed by the midgut juice proteases in the lumen. The activated Vip3 protein (monomer or tetrameric conformation) crosses the peritrophic membrane and enter the extracellular media. The activated monomeric Vip3 protein could oligomerize in the extracellular media, binding to the receptor and insert in the cell membrane. Alternatively, the activated monomer of the Vip3A protein could cross the peritrophic membrane and interact with a binding molecule that trigger the oligomerization and insertion of the Vip3Aa in the membrane. Pores are then formed, which lead to the death of the cell. The arrows with different colours indicate possible modes of action of the Vip3 proteins. This figure has been adapted from Chakroun et al. (65); Kunthic et al. (103,104), Bel et al. (105), Palma et al. (106), Zack et al. (102), Jian et al. (107).

(1) Ingestion of the Vip3 proteins by susceptible insects

The behavioural effects of the insect after the ingestion of the Vip3A protein show similarities for the ones described for the Cry proteins: feeding cessation, loss of gut peristalsis and paralysis of the insect. In addition the analysis of the histopathological effects in midgut sections show the lysis of the epithelial cells, release of the cellular components to the lumen and finally the disruption of the midgut structure (65).

(2) Activation by the midgut proteases and crossing the peritrophic membrane

The activation of the Vip3 proteins by the midgut juice produces four main fragments: 62-66, 45, 33 and 22 kDa (108–111). The 22-kDa fragment is the N-terminal part of the protein (from amino acids 1 to 198) while the 62-66 kDa fragment corresponds to the C-terminal part of the protein (from amino acid 199 to the end of protein). The 45- and 33-kDa fragments derived from the proteolysis of the C-terminal part of the Vip3 protein (82,102,105). The Vip3 proteins, as opposite to the Cry proteins, do not contain a minimal fragment that retained the insecticidal activity, as does the N-terminal region of the Cry proteins (domains I, II and III). Several studies indicate that the 62-66-kDa fragment is the Vip3Aa active fragment, though the incubation of with commercial serine proteases or insect midgut juice showed the unstable nature of the 62-66 kDa fragment, which started to break down into the 45-, 33-kDa and other small fragments (108,110–112). Interestingly, the Vip3 proteins, after the activation by the midgut juice or commercial proteases, oligomerize in a tetrameric conformation in which the structure of the protein is pH sensitive: at high pH the structure of the tetramer is affected (aggregation in large particle size) but not at neutral or acidic pH (the tetrameric conformation is stable) (104). Also the 20-kDa fragment produced upon proteolytic processing co-purifies with the 62-66 kDa fragment, suggesting that, after activation of the full-length protein, the two fragments remain together in the tetramer (102–106). In addition, the 22 kDa fragment is needed for the formation of the tetramer since the expression of the C-terminal part of the Vip3Ab1 and Vip3Bc1 oligomerize in a dimer conformation instead of the tetrameric form of the full length protein (102).

The proteolytic activation is not a critical step in the Vip3A insect toxicity and specificity since *O. nubilalis* (non-susceptible insect) midgut juice could process the Vip3 protein into the C-terminal and the activated Vip3 protein was toxic when fed to *Spodoptera frugiperda* (susceptible insect). However, some studies in Spodoptera species showed that

differences in mortality disappeared when the trypsin-activated protein was used instead of the full- length protein (113–115), whereas this phenomenon has not been observed in other insect species (85).

(3) Binding to the specific receptors in the apical membrane of the epithelial midgut cells

The Vip3A binds to the apical microvilli of the midgut epithelial cells as demonstrated by the *in vivo* immunolocalization studies (111). To determine if the binding of the Vip3A protein was specific, binding assays were performed with brush border membrane vesicles (BBMVs) of susceptible and non-susceptible insects. The specific binding of the Vip3 proteins to the BBMVs from susceptible insects was first shown using biotin-labelled Vip3A proteins (94,108,109,112,116–119). The quantitative binding analysis showed that the binding of the Vip3Aa protein was found to be saturable, mostly irreversible, differentially affected by the presence of divalent cations and showed slightly lower affinities but higher concentrations of binding sites than the Cry1A and Cry2A proteins (111). Regarding the binding of the Vip3A protein to the BBMVs, the homologous competition of Vip3A showed that the 62-kDa and the 20-kDa fragments of Vip3Aa16 bound to BBMVs, and both fragments were displaced by the addition of non-labelled protein (111). However, Liu et al. 2011 report that the 62-kDa fragment was able to bind to *Helicoverpa armigera* BBMVs and that the 20-kDa fragment was found in the supernatant of the binding reaction mixture. Competition binding assays between the Vip3 and Cry proteins demonstrate the absence of shared binding sites. This has been shown for Vip3Aa with Cry1Ac, Cry1Ab, Cry1Fa, Cry2Ae, and Cry2Ab and for Vip3Af with Cry1Ab and Cry1F (108,110–112,116–118,120). However, a partial competition of CryIa for the Vip3Aa binding sites was reported for *S. eridiana* and for the Cry9Aa and Vip3A proteins, which show interaction between them that improve the binding to the binding sites of both proteins (94,100). Regarding the competition among proteins of the Vip3A family, this has been tested in *S. frugiperda* where the Vip3Ae, Vip3Af and Vip3Ad proteins competed for the Vip3Aa binding sites with no significant differences in their binding parameters (111). Interestingly, Pan et al., 2017 (121) reported differences in the dissociation constants between the Vip3Aa60 and Vip3Ad5 to *S. exigua* midguts. This fact could be due that both Vip3 proteins share a 79.1 % similarity in the C-terminus.

With the aim to identify the BBMVs proteins that interact with the Vip3 proteins, ligand blot analysis was performed with different Vip3 proteins in different insect species: [1] Vip3Aa recognized 80- and 110-kDa proteins in *Manduca sexta*. [2] Vip3A bound to a

65 kDa protein in *Prays oleae* and *Agrotis segetum*. [3] Vip3A bound to proteins of 55 and 100 kDa proteins in *Spodoptera littoralis*. [4] Vip3Aa bound to 65 kDa proteins in *Ephestia kuehniell*a*, S. frugiperda, S. albula, S. cosmioides* and *S. eridania*. [5] Vip3A recongized 65 and 130 kDa proteins in *Diatraea flavipennella* and *Elasmopalpus lignosellus*; and [6] Vip3A bound to 55 and 100 kDa proteins, Vip3Af and Vip3Ae bound to 65 kDa in *S. littoralis* (94,108–110,118,119,122,123). Regarding the identification of the receptor of the Vip3Aa protein in the midgut cells, very few studies have been performed. Two studies proposed two binding molecules as possible Vip3 receptors: a 48 kDa protein with homology to tenascins, and the S2 ribosomal protein (124,125). Recently Jian et al. (107) described that the Scavenger receptor-C (SR-C) act as a receptor *in vitro* and *ex vivo* in Sf9 cells. In addition, they showed that downregulation of the SR-C expression in Sf9 cells and in *S. exigua* larvae midgut reduced the toxicity of Vip3Aa to both systems. Also, the heterologous expression of SR-C in transgenic *Drosophila melagonaster* midgut enhanced the virulence of Vip3Aa to *D. melanogaster* larvae.

(4) Pore formation and cell lysis

The Vip3 proteins activated with trypsin or midgut juice have a pore forming activity in dissected midguts of *M. sexta* and planar lipid bilayers, while the full-length Vip3Aa protein was unable to form pores (104,122). In addition, the pore-forming ability of activated Vip3Aa was also demonstrated by fluorescence quenching using *H. armigera* BBMVs (117). The ion channels formed by the Vip3 proteins were able to destroy the transmembrane potential and they were voltage independent and cation selective (122). A recent study demonstrated a pH dependence of the activity of the activated Vip3Aa protein in the permeabilization of calcein-entrapped liposomes and pore formation. For the permeabilization of calcein-entrapped liposomes, the maximum of the calcein release was observed at neutral pH. Furthermore, the calcein-release decreased at acidic pH and the liposome permeability was lost at pH 10. These results suggest that the membrane permeability caused by the activated Vip3A proteins is highly efficient at neutral pH, but not at acidic or alkaline pH (104). Regarding the pore formation, this was detected in the range of pH 5-8 but not at pH 10. At the range of pH 5-8, the current-voltage signal suggests the formation of stable pores, while at acidic pH the pores become unstable. The pore size changed as a function of the pH; while at pH 5-6 the size of the pore was 0.7-1.0 mm, at pH 7.4-8.0 it was about 1.3-1.4 mm (104).

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2.4. - Insect resistance to Vip3 proteins and cross-resistance analysis against populations selected for resistance to Cry and Vip3 proteins

Few cases of laboratory selection in lepidopteran pests to Vip3 proteins have been reported. Pickett et al. (126) reported a *H. virescens* colony that reached a 2,040-fold resistance level (Vip3-Sel) to theVip3Aa protein compared to the susceptible population (Vip3-Unsel). Resistance ranged from completely autosomal recessive to incompletely autosomal dominant depending on the dose, and the results of the bioassays on the offspring from the backcrosses of the F_1 progeny with the Vip3-Sel indicated that the resistance was due to more than one locus (126). Regarding the fitness costs, survival of resistant insects was temperature dependent, where the Vip3-Sel colony showed reduced survival to adult, hatching, egg viability, mating success and lower intrinsic rate of population increase (127). Also the BBMVs of the Vip3-Unsel and Vip3-Sel showed similar binding of Vip3A, indicating that the binding alteration is not the basis of resistance (128). Barkhade and Thakare (129) reported a *S. littura* colony that, after 12 generations, reached a resistant ratio of 285-fold compared to a susceptible colony. This Vip3Aa resistant colony showed the lack of two casein-degrading bands (in native electrophoresis) and a reduced proteolytic activity (~2- fold) with several protease substrates.

Regarding the presence of resistance alleles in field populations, this was addressed with four different populations using the F_2 screen method (130–132): *H. armigera* and *Helicoverpa punctigera* in Australia, *S. frugiperda* in Brazil, and *S. frugiperda* in USA. For the *H. armigera* and *H. punctigera* populations, the results indicate that the resistance alleles were present in the populations as natural polymorphisms. The frequency of the resistance alleles (0.027 and 0.008, respectively) is above the mutation rates normally encountered (130). The resistance for both insect populations was due to alleles at the same locus, and resistance was found autosomal recessive, most probably conferred by a single gene. The Vip3 resistance in the populations of *H. armigera* and *H. puntigera* did not result in cross- resistance to Cry1Ac or Cry2Ab (Mahon et al., 2012). The Vip3 *H. armigera* resistant colony showed a reduction in the proteolytic activity of the midgut proteases and similar binding of Vip3A (Chakroun et al., 2016b), indicating that the binding alteration is not the basis of this resistance as described above for the *H. virescens* laboratory colony. For the Vip3 *S. frugiperda* resistant colony isolated in Brazil, the

frequency of resistance alleles (0.0009) was relatively low. The resistance ratio was >3200-fold compared to the susceptible strain; the inheritance of Vip3Aa20 resistance was autosomal recessive and monogenic. Regarding the fitness cost, the Vip3-resistant strain showed an 11% reduction in the survival rate until adult stage and a ~50% lower reproductive rate compared with susceptible strain (Bernandi et al., 2015). For the Vip3 *S. frugiperda* resistant colony isolated in the USA, the frequency of resistance alleles (0.0048) was relatively low. The resistance ratio in diet and whole-plate bioassays was >632-fold relative to the susceptible population. The resistance was found monogenic, autosomal, recessive and it did not confer cross-resistance to Cry1F, Cry2Ab2 or Cry2Ae proteins. Moreover, Chen et al. (133) reported, for the Vip3 *S. frugiperda* resistant colony isolated in the USA, the presence of recessive fitness cost (reduced pupal weight and growth rate), at the individual level, on sorghum, but not in non *B. thuringiensis* corn, cotton and artificial diet. However, at the population level, not fitness costs were detected in the different non *B. thuringiensis* hosts.

With the aim to improve the pest control and insect resistant management, the evaluation of cross-resistance between Cry and Vip3A proteins has been evaluated in several Cry and Vip3resistant strains. So far, no significant cross-resistance between these two classes of proteins has been described in the Cry or Vip3 resistant strains. Regarding the Cry resistant strains, several studies indicated the lack of cross-resistance of the Cry-resistant insect populations to the Vip3A proteins. Jackson et al. (134) described the lack of cross-resistance in three Cry-resistant *H. virescens* strains (YHD2, resistant to Cry1Ac and CXC and KCBhyb, both resistant to Cry1Ac and Cry2Aa2) to the Vip3Aa protein. Fang et al. (135) showed the lack of cross-resistance, in a *T. ni* Cry1Ac-resitant colony, to Vip3Ac1 and Vip3Aa1. Anilkumar et al. (136) demonstrated, in a *Helicoverpa zea* Cry1Ac-resistant colony, the lack of cross-resistance against the Vip3Aa protein. Vélez et al. (137) showed, in a *S. frugiperda* Cry1F-resistant colony, the absence of cross-resistance to Vip3Aa. Welch et al. showed the lack of cross-resistance to Vip3A in the *H. zea* selected against CryAc/MVP II. Qian et al. (138), in a *H. armigera* Cry1Ac-resistant colony, showed the lack of cross-resistance to Vip3A. Horikoshi et al. (139) demonstrated, in two colonies of *S. frugiperda* (Cry1A.105/Cry2Ab2- and Cry1A.105/Cry2Ab2/Cry1F-resistant colonies), the lack of cross-resistance to Vip3Aa.. Wei et al. showed the lack of cross-resistance in four *H. armigera* resistant colonies (two colonies resistant to Cry1Ac and two colonies resistant to Cry2Ab) to the Vip3Aa protein.

In the case of the Vip3A resistant strains, some recent studies show the lack of cross-resistance of the Vip3-resistant insect populations to the Cry proteins. Mahon et al. showed the lack of cross-resistance in two Vip3-resistant *H. armigera* colonies (SP85 and SP477) and one Vip3-resistant *H. punctigera* colony to the Cry1Ac and Cry2Ab proteins. Chakroun et al. demonstrated the absence of cross-resistance to the Cry1Ac and Cry2Ab proteins in isofemale lines of a Vip3-resistant *H. armigera* colony derived from the SP85 colony (Mahon et al., 2012). Horikoshi et al. (139) showed the lack of cross-resistance, of two Vip3-resistant *S. frugiperda* colonies (Vip3A- and Vip3A/Cry1Ab), to several Cry-resistant Bt crops (Cry1F, Cry1A.105/Cry2Ab2, Cry1A.105/Cry2Ab2/Cry1F, Cry2Ab/Cry1Ac, Cry1Ac/CryF, Cry1Ab/Cry2Ae). Yang et al. (132) showed, in a *S. frugiperda* Vip3-resistant insect colony, the lack of cross-resistance to Cry1F, Cry2Ab and Cry2Ae.

2.5. - Host-response to *Bacillus thuringiensis* proteins in non-model insects.

The ingestion of *B. thuringiensis*, or its toxins, activates different mechanisms to reduce the cellular and tissue damage produced by the *B. thuringiensis* or its toxins. Little is known about the pathways and the genes involved in the response to *B. thuringiensis* intoxication. Recent reports identified a set of genes involved in the host response to *B. thuringiensis* and its virulence factors (Figure 10):

(1) Serine proteinases

These proteins are the main digestive enzymes in the gut of the insects. The down-regulation of several serine proteinases has been observed after the ingestion of *B. thuringiensis* commercial products, as well as individual toxins, in *S. exigua* and *S. frugiperda* (140–142). Also, in other lepidopteran pests, down-regulation of serine proteinases has been reported: Sparks et al. (143) found down-regulation of the chymotrypsin-like protease after the infection of *Lymantria dispar* with *B. thuringiensis*. Vellichirammal et al. (144) found, in *O. nubilalis*, five transcripts (two coding for trypsin-like serine proteases and three coding for chymotrypsin- like serine proteases) that were down-regulated after Cry1F ingestion. Zhang et al. (145) found, in a Cry1Ab-resitant *Ostrinia furncalis* strain, down-regulation of the trypsin-like and chymotrypsin-like proteases. Yao et al. (146) found, in a Cry1Ab-resistant *O. nubilalis* strain, the down-regulation of the trypsin-like and chymotrypsin-like proteases. On the contrary, Lei et al. (147) found, in two Cry1Ac-resistant *P. xylostella* strains, the up-regulation of the catalytic genes and Song et al. (148) found, in *S. litura*, that most of the serine proteases were up-regulated when larvae were fed with the Vip3Aa toxin.

(2) Antimicrobial peptides

Antimicrobial peptides (AMPs) are small molecular weight proteins able to kill or block bacterial growth and have been grouped in four families based on their structures or unique sequences: the α-helical peptides (cecropin and moricin), cysteine-rich peptides (insect defensin and drosomycin), proline-rich peptides (apidaecin, drosocin, and lebocin), and glycine-rich peptides/proteins (attacin and gloverin) (149,150). Two studies showed a strong up-regulation of AMPs in the midgut of *S. exigua* larvae exposed to Cry1Ca or Vip3Aa toxins (142,151), where for *S. exigua* more than 30 AMPs were found

up-regulated. Because these two toxins have different modes of action, the results suggest that up-regulation of AMPs was probably associated to the cell damage produced by the toxins. In agreement with the role of AMPs in defense against *B. thuringiensis*, Hwang and Kim (152) reported that silencing of gloverin by RNAi in *S. exigua* increased larval susceptibility to *B. thuringiensis*. Regarding the role of the AMPs in other insect species, it has been reported the up-regulation of the AMPs in *Galleria mellonella* and *Tribolium castaneum* after the intoxication with *B. thuringiensis* (153–155).

(3) REPAT proteins

REPAT proteins are midgut specific proteins that participate in the midgut response to *B. thuringiensis* and baculoviruses in Spodoptera spp (156,157). The first members (REPAT1 to REPAT4) were found to be strongly up-regulated in response to different *B. thuringiensis* toxins and baculoviruses (156). To date, more than 40 different members have been described in *S. exigua* (140,156,158–160) and many of them have been found up-regulated after larval treatment with Cry1Ca, Vip3Aa and *B. thuringiensis* formulations (140,142,159–162). The functions of REPAT proteins are still unknown, but their ability to translocate into the cell nucleus has suggested their possible role as transcription factors, regulating the response to cellular damage and/or cell differentiation (159).

(4) α-arylphorin

The α-arylphorin has been considered a storage protein produced by the fat body, but recent reports show that it can stimulate midgut stem cell proliferation (163–165) and its production in the lepidopteran midgut epithelium has also been established (166,167). In addition, a new role of the α-arylphorin has been proposed in insect immunity (168,169). The increase of arylphorin levels results in midgut hyperplasia (164,170). Arylphorin was found to be up-regulated in response to *B. thuringiensis* formulations and Cry1Ca toxin in *S. exigua* (140), suggesting its possible effect in promoting repair of the midgut epithelium after damage produced by *B. thuringiensis* toxins. Increased levels of arylphorin were proposed to sequester Cry1Ac toxin in the gut lumen in *H. armigera* (171). Interestingly, reduction of the ABCC2 expression by RNAi also induced the up-regulation of arylphorin in the absence of exposure to *B. thuringiensis* (161). In contrast, reduced levels of arylphorin transcripts were detected upon exposure of *S. exigua* to Vip3Aa or upon exposure of *L. dispar* to *B. thuringiensis var. kurstaki* (142,143). The

reported discrepancies in the regulation of arylphorin may represent differences in the mode of action of Cry versus Vip3Aa toxins and the effect of *B. thuringiensis* spores versus purified proteins on midgut cells.

(5) Midgut differentiation factor (MDP)

The midgut differentiation factors (MDF1, MDF2, MDF3 and MDF4) are 30 amino acid peptides with high identity to the C-terminus of fetuin, isolated from conditioned media *H. virescens* culture cells (MDF1 and MDF2) and chymotryptic digestion of *L. dispar* hemolymph (172–175). MDF1 and MDF2 promoted the development of stem cells to columnar, goblet cells and newly differentiated cells to become cuboidal in shape, respectively. MDF3 and MDF4 were active in the differentiation of the stem cells to columnar and goblet cells (175). However, 100% differentiation of Lepidoptera stem cell cultures has never been observed with these MDFs, suggesting the existence of additional differentiation factors, including ecdysone, α-arylphorin and insulin-related bombyxin (163,164,176). The MDF1 peptide has been involved in the midgut healing response to Cry1Ac *in vitro* (177). The treatment of *H. virescens* cell culture with Cry1Ac increase in the number of immunopositive cells and the insect species treated with *B. thuringiensis* show an up-regulation of the synthesis of an MDF1-like factor and ostensibly directing increased stem cell differentiation.

(6) Chitin deacetylase

The peritrophic matrix is a dynamic, complex, structure that participates in the immobilization of digestive enzymes, actively protecting the gut from parasite invasion and intercepting toxins. The major proteins of the peritrophic matrix are chitin deacetylase-like and mucin-like proteins, the latter with multiple chitin-binding domains that may cross-link chitin fibrils to provide a barrier against abrasive food particles and parasites, one of the major functions of the matrix (178). The chitin deacetylase genes were found to be among the strongest down-regulated genes after intoxication of *S. exigua* larvae with the Vip3A toxin, baculoviruses and after feeding *H. armigera* larvae with a mixture of spores and crystals from *B. thuringiensis* (142,179,180). The down-regulation of the chitin deacetylase could be explained as a mechanism to decrease peritrophic matrix permeability and reduce the amount of toxin passing through the peritrophic membrane and binding to the midgut epithelial cells (142,179).

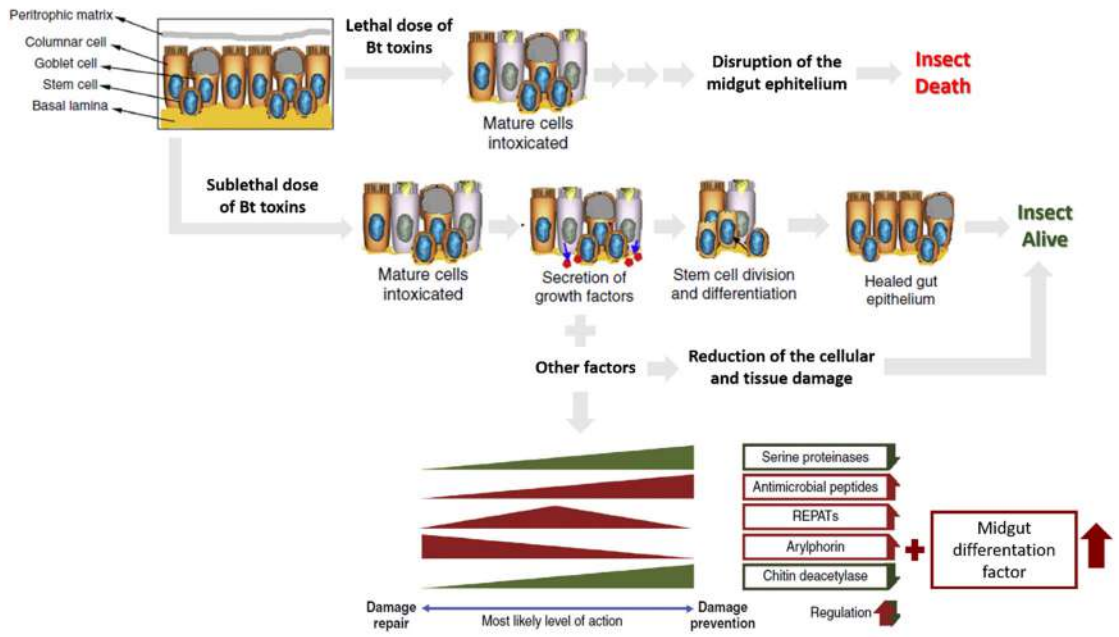

Figure 10 Model of the response of insect epithelial cells to *Bacillus thuringiensis* and/or its toxins. In the lethal condition, the epithelial cells swell and burst and the midgut tissues collapse dealing to the insect death. In the nonlethal condition, the epithelial cells growth factors and other factors contribute to the renewal of the columnar cell layer and the reduction of the cellular and tissue damage, respectively. This figure has been adapted from Tanaka et al. (181), Catagnola et al. (182) and Herrero et al. (183).

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3. - OBJECTIVES

Objectives:

In order to carry out this Doctoral Thesis, we focussed the study mainly on the new protein family Vip3Ca: insecticidal spectrum, cross-resistance analysis, mode of action and cell death response of *S. exigua* intoxicated with the Vip3Ca protein. In addition, we performed the mining of new *B. thuringiensis* insecticidal protein genes from selected *B. thuringiensis* isolates based on their gene content.

The study of these general objectives was performed by performing the more specific objectives described below:

1. Mining of new insecticidal protein genes and quantification of the new toxins in the supernatant and the crystal based in a set of *B. thuringiensis* isolates selected for their gene content

2. Increase the knowledge on the insecticidal spectrum of the Vip3Ca protein and analysis of cross-resistance in lepidopteran pests selected for resistance against Cry1, Cry2 and Vip3A

3. Determine the mode of action of the Vip3Ca protein in *Mamestra brassicae* as a model susceptible insect

4. Characterization of the gene expression and cell death response in *S. exigua* intoxicated with Vip3Ca

4. - CONTRIBUTIONS

4.1. - Contributions made by the PhD student in the publications.

In the present doctoral thesis, five research studies have been published in journals indexed in the main databases, three of them as first author, and the rest as a second author. The research studies published are the following:

1. - Gomis-Cebolla, J.; Ricietto, A.P.S.; Ferré, J. 2018. A Genomic and Proteomic Approach to Identify and Quantify the Expressed *Bacillus thuringiensis* Proteins in the Supernatant and Parasporal Crystal. Toxins. Vol 10, Issue 5, p. 193 - 211. doi:10.3390/toxins10050193

2. - Gomis-Cebolla, J.; Wang, Y.; Quan, Y.; He, K.; Walsh, T.; James, B.; Downes, S.; Kain, W.; Wang, P.; Leonard, K.; Morgan, T.; Oppert, B.; Ferré, J. 2018. Analysis of cross-resistance to Vip3 proteins in eight insect colonies, from four insect species, selected for resistance to *Bacillus thuringiensis* insecticidal proteins. Journal of Invertebrate Pathology. Vol 155, p. 64 - 70, doi.org/10.1016/j.jip.2018.05.004

3. - Hernández-Martínez, P.; Gomis-Cebolla, J.; Ferré, J.; Escriche, B. 2017. Changes in gene expression and apoptotic response in *Spodoptera exigua* larvae exposed to sublethal concentrations of Vip3 insecticidal proteins. Scientific Reports. Vol 7, Article number 16245, doi:10.1038/s41598-017-16406-1

4. - Gomis-Cebolla, J.; Ruiz de Escudero, I.; Vera-Velasco, N.M.; Hernández-Martínez, P.; Hernández-Rodríguez, C.S.; Ceballos, T.; Palma, L.; Escriche, B.; Caballero, P.; Ferré, J. 2017. Insecticidal spectrum and mode of action of the *Bacillus thuringiensis* Vip3Ca insecticidal protein. Journal of Invertebrate Pathology. Vol 142, p. 60 - 67, doi.org/10.1016/j.jip.2016.10.001

5. - Ricietto, A.P.S.; Gomis-Cebolla, J.; Ferré, J. 2016. Susceptibility of *Grapholita molesta* (Busck, 1916) to formulations of *Bacillus thuringiensis*, individual toxins and their mixtures. Journal of Invertebrate Pathology. Vol 141, p. 1 - 5, doi.org/10.1016/j.jip.2016.09.006

Regarding the research studies included in the doctoral thesis, one of them was a collaboration with a Spanish group from the "Universidad Pública de Navarra" (Gomis-Cebolla et al., 2017). Regarding the rest of the articles (Ricietto et al., 2016; Hérnandez-Martínez et al., 2017; Gomis-Cebolla et al., 2017 and Gomis-Cebolla et al., 2018) were international collaborations with the following institutions: Universidade Estadual de Londrina, Chinese Academy of Agricultural Sciences, Cornell University, Commonwealth Scientific and Industrial Research Organisation, and United States Department of Agriculture.

Since the PhD student does not apper as the first author in all the published articles, a technical report is provided about the research work performed by the PhD student in the articles included in the doctoral thesis.

Chapter 1. Identification and quantification of the insecticidal protein genes in *Bacillus thuringiensis* isolates

Article		Authors		
		Gomis-Cebolla, J	Ricietto, A.P.S	Ferré, J
A Genomic and proteomic Approach to identify and Quantify the Expressed *Bacillus thuringiensis* Proteins in the Supernatant and Parasporal Crystal	**Experimental design**	**X**	**X**	**X**
	Experiments			
	1.-Bacterial Strains and Growth Conditions for DNA Analysis	**X**	**X**	**-**
	2.-Genomic DNA Preparation	**X**	**X**	**-**
	3.-Identification of Vip1- and Vip2-Type Genes	**X**	**X**	**-**
	4.-Genome Sequencing, Assembly and Annotation Analysis	**X**	**-**	**-**
	5.-Sample Preparation for in Gel Digestion LC/MSMS Analysis and Insecticidal Activity of Bt Isolates	**X**	**-**	**-**
	6.-In Gel Digestion LC/MSMS Analysis	**X**	**-**	**-**
	Protein Identification of the in Gel Digestion LC/MSMS Analysis with Paragon Algorithm and Mascot	**X**	**-**	**-**
	7.-Label Free Analysis of the Concentrated Supernatant 24 h vs. 48 h in Both Bt Isolates	**X**	**-**	**-**
	Data analysis	**X**	**X**	**X**
	Writing the article	**X**	**X**	**X**

Chapter 2. Toxicity and Cross-Resistance of the Vip3 proteins to lepidopteran insect species

Article		Authors			
		Ricietto, A.P.S	Gomis-Cebolla, J	Vilas-Boas, G.T	Ferré, J
Susceptibility of *Grapholita molesta* (Busck, 1916) to formulations of *Bacillus thuringiensis*, individual toxins and their mixtures	**Experimental design**	**X**	**X**	**-**	**X**
	Experiments				
	1.-Insect rearing and bioassays optimization	**X**	**X**	**-**	**-**
	2.-Expression of Cry and Vip3 proteins	**X**	**X**	**-**	**-**
	3.-Susceptibility of *Grapholita molesta* to Cry and Vip proteins and *Bacillus thuringiensis* commercial products	**X**	**-**	**-**	**-**
	4.-Interactions of Vip3Aa with Cry1 proteins in *Grapholita molesta*	**X**	**X**	**-**	**-**
	5.-Statistical analysis	**X**	**X**	**-**	**-**
	Data analysis	**X**	**X**	**-**	**X**
	Writing the article		**X**	**X**	**X**

Chapter 2. Toxicity and Cross-Resistance of the Vip3 proteins to lepidopteran insect species

Article		Authors												
		Gomis-Cebolla, J	Wang, Y	Quan, Y	He, K	Walsh, T	James, B	Downes, S	Kain, W	Wang, P	Leonard, K	Morgan, T	Oppert, B	Ferré, J
Analysis of cross-resistance in eight insect colonies, from four insect species, selected for the resistance to *Bacillus thuringiensis* insecticidal proteins	**Experimental design**	**X**	-	-	-	-	-	-	-	-	-	-	-	**X**
	Experiments													
	1.-Insect rearing of the respective pests	-	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	-
	2.-Expression and purification of Vip3A and Vip3Ca proteins	**X**	-	-	-	-	-	-	-	-	-	-	-	-
	3.-Dose-response assays of the respective susceptible and resistant insect populations	-	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	-
	4.-Stadistical analysis	**X**	**X**	**X**	**X**	-	-	-	**X**	**X**	**X**	**X**	**X**	-
	Data analysis	**X**	-	-	-	-	-	-	-	-	-	-	-	-
	Writing the article	**X**	-	-	-	-	-	-	-	-	-	-	-	**X**

Chapter 3. Mode of action of the Vip3Ca insecticidal protein from *Bacillus thuringiensis*

Article		Authors									
		Gomis-Cebolla, J	Ruiz de Escudero, I	Vera-Velasco, N.M	Hernández-Martínez, P	Hernández-Rodríguez, C.S	Ceballos, T	Palma, L	Escriche, B	Caballero, P	Ferré, J
Insecticidal spectrum and mode of action of the *Bacillus thuringiensis* Vip3Ca insecticidal protein	**Experimental design**	**X**	-	-	**X**	**X**	-	-	**X**	-	**X**
	Experiments										
	1.- Insect colonies	**X**	**X**	**X**	**X**	-	-	-	-	-	-
	2.- Expression and purification of Vip3 proteins	**X**	**X**	**X**	**X**	-	-	-	-	-	-
	3.-Insect toxicity assays	**X**	-	-	-	-	**X**	-	-	-	-
	4.-Midgut juice preparation and Vip3 proteins processing	**X**	**X**	**X**	**X**	-	-	-	-	-	-
	5.-Insect toxicity assays of the activated Vip3 proteins with midgut juice from *Mamestra brassicae* and *Ostrinia nubilalis*	-	**X**	-	-	-	-	-	-	-	-
	6.-Histopathological effects and in vivo binding of Vip3 proteins in *Mamestra brassicae*	**X**	-	-	-	-	-	-	-	-	-
	7.-Vip3 toxins labeling, preparation of *Mamestra brassicae* BBMV, and binding analyses	**X**	-	-	-	-	-	-	-	-	-
	Data analysis	**X**	-	-	-	**X**	-	**X**	**X**	**X**	-
	Writing the article	**X**	-	-	-	-	-	-	-	-	**X**

Chapter 3. Mode of action of the Vip3Ca insecticidal protein from *Bacillus thuringiensis*

Article		Authors			
		Hernández-Martínez, P	Gomis-Cebolla, J	Escriche, B	Ferré, J
Changes in gene expression and apoptotic response in *Spodoptera exigua* larvae exposed to sublethal concentrations of Vip3 insecticidal proteins	**Experimental design**	**X**	**-**	**X**	**X**
	Experiments				
	1.-Insects rearing	**X**	**X**	**-**	**-**
	2.-Expression and purification of Vip3Aa and Vip3Ca proteins	**X**	**X**	**-**	**-**
	3.-Growth inhibition assays.	**-**	**-**	**-**	**-**
	4.-Effects on larva development after Vip3 protein exposure	**X**	**X**	**-**	**-**
	5.-Gene expression analysis	**X**	**-**	**-**	**-**
	6.-Measurement of aminopeptidase activity in the midgut lumen	**X**	**-**	**-**	**-**
	7.-Sectioning of insect tissues and TUNEL staining.	**X**	**X**	**-**	**-**
	Data analysis	**X**	**X**	**X**	**X**
	Writing the article	**X**	**-**	**-**	**X**

4.2. - Timeline and workflow of the research work carried out by the PhD student.

4.2.1. - Timeline of the research work developed during the doctoral thesis

The present doctoral thesis has been financed by two different projects of the Ministry of Economy and Competitiveness. In the first two annuities, the objectives of the BABIGEN-CYCIT project were fulfilled (Figure 11), with the following objectives:

1. - Insecticidal spectrum and mode of action of Vip3Ca form *B. thuringiensis*
2. - Susceptibility of *Grapholita molesta* to *B. thuringiensis* toxins and formulations
3. - Identification of the *vip1* and *vip2* genes in selected *B. thuringiensis* isolates based on their gene content

On the other hand, during the last two years, the objectives of the DAMBT project were fulfilled (Figure 11) with the following works:

1. - Characterization of the cell death in *S. exigua* intoxicated with Vip3Aa and Vip3Ca proteins
2. - Analysis of the cross-resistance to Vip3Ca protein in resistant insect colonies selected with Cry1, Cry2 and Vip3 proteins
3. - Mining of new insecticidal proteins from a selected *B. thuringiensis* based on their gene content

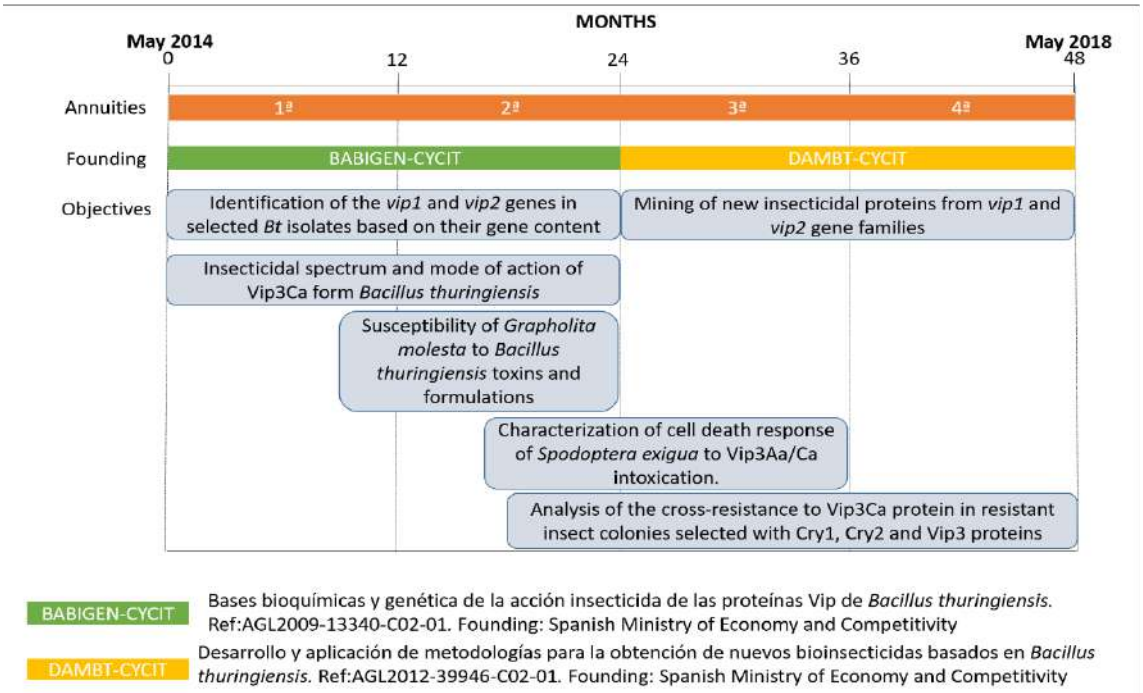

Figure 11 Chronology of the research work carried out during the four-year duration of the doctoral thesis funded by two different projects of the Ministry of Economy and Competitively of the Spanish Government.

4.2.2. - Workflow of the research work developed during the doctoral thesis

The starting point of the present doctoral thesis were the articles from Hernández-Rodríguez et al. (1) and Palma et al. (2). In Hernández-Rodríguez et al. (1), from a Spanish *B. thuringiensis* collection, a subset of isolates were selected based on their *vip1* and *vip2* gene content. In Palma et al. (2), a new Vip3 protein family, Vip3Ca, was discovered in four isolates independently and was active against *M. brassicae*, *A. ipsilon*, *T. ni*, etc. (2). Based on the results obtained in the previous studies, the workflow of the present doctoral thesis used three different approaches: (1) Mining of new insecticidal protein genes, (2) toxicity of the Vip3Ca protein and analysis of cross-resistance, (3) mode of action of Vip3Ca and host-response of *S. exigua* intoxicated with Vip3Ca (Figure 12). Regarding the structure of the present thesis, it is based on three different approaches that constitute the three chapters of the doctoral thesis (Figure 12):

1. **Chapter 1: Identification and quantification of the insecticidal protein genes in *Bacillus thuringiensis* isolates**

   This chapter is focused in the mining of new *vip1* and *vip2* protein genes in a selected set of *B. thuringiensis*, selected based on their gene content. A set of new *vip1* and *vip2* alleles was discovered and the sequences were deposited in the GenBank. In the case of the mining of new insecticidal protein genes, two *B. thuringiensis* isolates were sequenced and their gene content was predicted with the Rast Server. Also the protein detection and quantification of the predicted insecticidal protein genes was done in the supernatant and crystal by LC-ESI-MSMS (3).

2. **Chapter 2: Toxicity and Cross-Resistance of the Vip3 proteins to the lepidopteran insect species**

   This chapter is focused on enlarging the insecticidal spectrum of Vip3Ca, after the report of Palma et al. in 2012, and the analysis of cross-resistance of insect populations selected against the Cry1, Cry2 and Vip3 proteins, respectively. The insecticidal spectrum of Vip3Ca was increased in 12 new insect species, among which *O. furnacalis* is a very susceptible target for the Vip3Ca protein. Regarding the analysis of cross-resistance of insect populations selected with the Cry1, Cry2 and Vip3 proteins, to the Vip3Ca protein, they were performed in collaboration with several laboratories (4–6). For the insect species *G. molesta*, due the lack of data and worldwide distribution of the pest, we decided to write a monographic article about the susceptibility to *B. thuringiensis*. This study was done in collaboration with a PhD student from Brazil, in 2015-2016 (4).

3. **Chapter 3: Mode of action of the Vip3Ca protein from Bacillus thuringiensis**

   This chapter is focused on the determination of the mode of action of the Vip3Ca protein and the host-response of *S. exigu*a intoxicated with Vip3Ca. The study of the mode of action of the Vip3Ca protein was determined using *M. brassicae* as a susceptible species based on publish data to date. Regarding the host response to the Vip3Ca protein, we chose *S. exigua* based on previous works where *S. exigua*

susceptible species based on publish data to date. Regarding the host response to the Vip3Ca protein, we chose *S. exigua* based on previous works where *S. exigua* was intoxicated with Vip3Aa and a set of genes had been already established that responded to the intoxication by the Vip3 proteins (7–9). In addition, midgut sections were stained to determine the presence of apoptosis as a possible mechanism that regulate the cell death response (10).

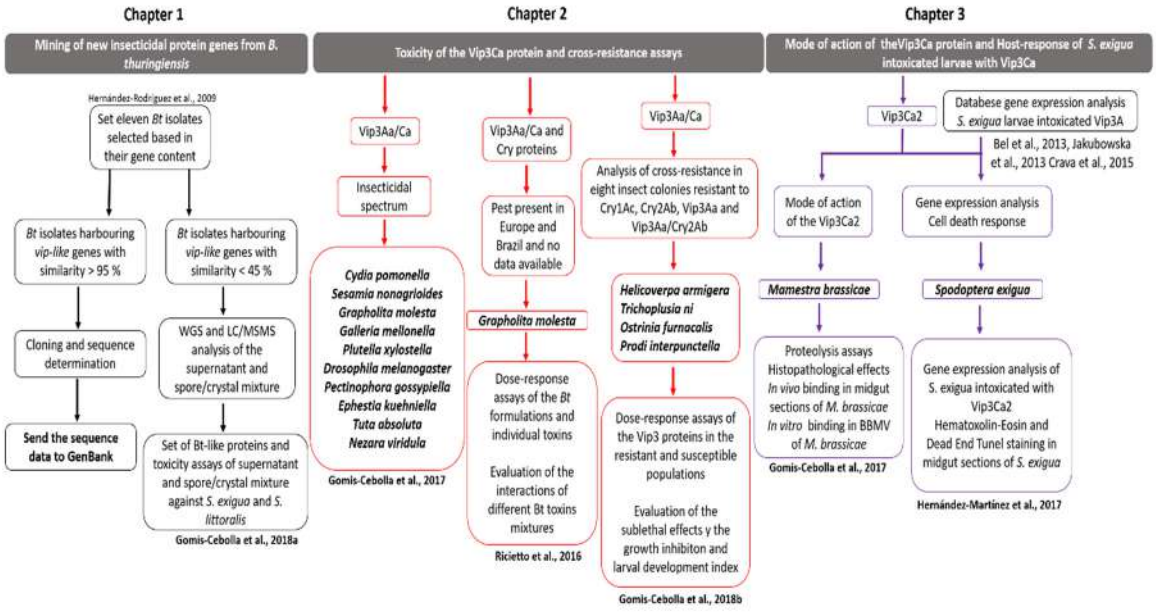

Figure 12 Workflow of the research work carried out during the four-year duration of the PhD period in the research projects BABIGEN-CYCIT and DAMBT-CYCIT.

References:

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5. - CHAPTER 1:

Identification and quantification of the insecticidal protein genes in *Bacillus thuringiensis* isolates

These results are included in:

Gomis-Cebolla, J.; Ricietto, A.P.S.; Ferré, J. 2018. A Genomic and Proteomic Approach to Identify and Quantify the Expressed *Bacillus thuringiensis* Proteins in the Supernatant and Parasporal Crystal. Toxins. Vol 10, Issue 5, p. 193 - 211. doi:10.3390/toxins10050193

Special Issue Toxins: **Insecticidal Toxins from *Bacillus thuringiensis***

toxins

MDPI

Article

A Genomic and Proteomic Approach to Identify and Quantify the Expressed *Bacillus thuringiensis* Proteins in the Supernatant and Parasporal Crystal

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Abstract: The combined analysis of genomic and proteomic data allowed us to determine which *cry* and *vip* genes are present in a *Bacillus thuringiensis* (*Bt*) isolate and which ones are being expressed. Nine *Bt* isolates were selected from Spanish collections of *Bt* based on their *vip1* and *vip2* gene content. As a first step, nine isolates were analyzed by PCR to select those *Bt* isolates that contained genes with the lowest similarity to already described *vip1* and *vip2* genes (isolates E-SE10.2 and O-V84.2). Two selected isolates were subjected to a combined genomic and proteomic analysis. The results showed that the *Bt* isolate E-SE10.2 codifies for two new vegetative proteins, Vip2Ac-like_1 and Sip1Aa-like_1, that do not show expression differences at 24 h vs. 48 h and are expressed in a low amount. The *Bt* isolate O-V84.2 codifies for three new vegetative proteins, Vip4Aa-like_1, Vip4Aa-like_2, and Vip2Ac-like_2, that are marginally expressed. The Vip4Aa-like_1 protein was two-fold more abundant at 24 h vs. 48 h, while the Vip4Aa-like_2 was detected only at 24 h. For Vip2Ac-like_2, no differences in expression were found at 24 h vs. 48 h. Moreover, the parasporal crystal of the E-SE10.2 isolate contains a single type of crystal protein, Cry23Aa-like, while the parasporal crystal from O-V84.2 contains three kinds of crystal proteins: 7.0–9.8% weight of Cry45Aa-like proteins, 35–37% weight of Cry32-like proteins and 2.8–4.3% weight of Cry73-like protein.

Keywords: insect pest control; crop protection; vip proteins; cry proteins

Key Contribution:

- Genomic and proteomic analysis can identify insecticidal proteins genes and quantify their expressed products.
- The *Bt* isolates E-SE10.2 and O-V84.2 produce five new Vip-like and Sip-like proteins and eight new Cry-like proteins

1. Introduction

Bacillus thuringiensis (*Bt*) is an entomopathogenic bacterium that produces several types of insecticidal proteins, such as Cry, Cyt, Vip, Sip, Mtx-like, and Bin-like proteins, along with other virulence factors contributing to its pathogenicity [1,2]. The Vip proteins are a family of proteins that are secreted during the vegetative growth phase and that have been classified into four groups according to their sequence homology: Vip1, Vip2, Vip3, and Vip4 [2]. Because of repeated applications of *Bt* sprays and the widespread adoption of *Bt*-crops (transgenic crops protected from insects by the expression of *cry* and/or *vip3* genes), some insect populations have developed resistance to *Bt*

toxins [3–6]. Therefore, in this arms race against insects, it is necessary to explore the potential of new insecticidal proteins for pest control. A series of approaches have been used for isolating novel insecticidal protein genes from *Bt*, such as PCR, which has further evolved into specific applications to mining new insecticidal genes, such as PCR hybridization, PCR-RFLP, E-PCR and PCRSSCP [7–11]. In addition, the construction of *Bt* DNA libraries, followed by screening by Western Blotting or a hybridization-based method, has also been used to detect novel insecticidal protein genes [12–14]. The PCR approaches being used to detect *vip* genes are based on the presence of conserved blocks in the DNA sequence of these genes [11,15] and most of the studies have focused on genes from the *vip3* family. Therefore, the PCR approach is limited to finding *vip* genes with enough homology to the primers used. An additional problem with the PCR approach is that it does not provide the full length of the new *vip* genes. On the other hand, the library-based methods are time-consuming and laborious. The next generation sequencing (NGS) allows rapid sequencing of entire genomes at a low cost-effective ratio [16,17]. The number of *Bt* whole genomes that have been sequenced has increased quickly in the past decade. To date, 459 *Bt* strains have been sequenced, with a mean genome size ranging from 5.3 MB to 6.7 MB and a mean guanine-cytosine content (GC content) between 34% and 35% (https://www.ncbi.nlm.nih.gov/genome/?term=Bacillus+thuringiensis). The combination of the low cost NGS with the development of many freeware tools has enabled the rapid detection of insecticidal protein genes at the genome level. On the other hand, the development of the mass spectrometry (MS)-based proteomics has enabled the detection of proteins from complex mixtures from different stages of a microorganism [18]. The combination of the genomic and proteomic approaches is a very successful approach for validation and correction of predicted genomic coding information in a wide variety of organisms [18–22].

In this study, the identity of *vip* genes has been determined in nine *Bt* isolates which were candidates to harboring new *vip1* and *vip2* genes (Vip1 and Vip2 constitute a binary toxin and their genes are normally located in an operon). Two of these isolates, which were found to carry *vip*-type genes with a similarity lower than 45% to already reported genes, were subjected to whole genome sequencing and to different kinds of proteomic analysis to determine and estimate the relative abundance of the expressed insecticidal protein genes.

2. Results

2.1. Identification of Vip1-, Vip2-, and Vip4-Type Genes

To identify the specific genes within the *vip1* and *vip2* gene families, a strategy based on PCR-Sanger Sequencing was used. A first PCR with "screening primers" was performed to confirm the presence of *vip* genes. The results showed that the nine isolates were positive for the presence of a *vip1-type* gene, and that seven were positive for the presence of a *vip2-type* gene (Table 1). Those samples that gave positive for a determined gene type were subjected to a second PCR with "typing primers" to narrow down the identity of the gene. The results allowed us to classify the isolates into two types of isolates containing a *vip1–vip2* gene pair: those with a gene pair with high similarity (>95%) to *vip2Bb* (KR065728)–*vip1Bb* (KR065727) (V-J20.2 and V-LE1.1), and those with a gene pair with high similarity to *vip2Ac* (KR065726)–*vip1Ca* (KR065725) (V-V54.26, V-V54.31, E-TE7.43, E-TE16.5 and E-TE18.40). In addition to these two categories, two isolates were identified to contain just a single *vip* gene with low similarity to all reported ones; one had the highest similarity to *vip1Bb* (E-SE10.2) and the other had the highest similarity to *vip1Da* (O-V84.2), which was later shown to belong to the *vip4Aa* family (Table 1).

Table 1. Identification of *vip1*, *vip2* and *vip4* genes in selected isolates of *Bacillus thuringiensis*.

Name of Isolate	Identified with *vip1* Primers			Identified with *vip2* Primers		
	Similarity (%) ‡	Coverage (%) *	Closest Homolog	Similarity (%) ‡	Coverage (%) *	Closest Homolog
V-J20.2	100	44	*vip1Bb1*	97	70	*vip2Bb1*
V-LE1.1	100	40	*vip1Bb1*	99	72	*vip2Bb1*
V-V54.26	99	49	*vip1Ca1*	99	71	*vip2Ac1*
V-V54.31	100	49	*vip1Ca1*	98	73	*vip2Ac1*
E-SE10.2	62	30	*vip1Bb3*		No DNA amplification	
E-TE7.43	100	49	*vip1Ca1*	99	64	*vip2Ac1*
E-TE16.5	98	43	*vip1Ca1*	98	73	*vip2Ac1*
E-TE18.40	100	30	*vip1Ca1*	100	45	*vip2Ac1*
O-V84.2	40	40	*vip4Aa1*		No DNA amplification	

* The coverage values represent the mean of two replicates of the typing or screening PCR products to the full sequence length of the respective *vip1*, *vip2* and *vip4* genes deposited in GenBank. ‡ The similarity values provided by BlastX (NCBI) represent the mean value of the in silico translation of two replicates of the PCR products.

2.2. Genome Sequencing of the Bt Isolates E-SE10.2 and O-V84.2, Contig Assembly and Gene Annotation

Whole genome sequencing of the *Bt* isolates E-SE10.2 and O-V84.2 resulted in 10,401,436 high quality reads for the *Bt* isolate E-SE10.2 and 9,210,116 high quality reads for the *Bt* isolate O-V84.2, with an average length of 150 bp for both *Bt* isolates. For the E-SE10.2 isolate, the 97.4% of the reads were assembled in 222 scaffolds while for the O-V84.2 isolate the 98.2% of the reads were assembled in 249 scaffolds. For the E-SE10.2 isolate, the results of the assembled paired reads were as follows: genome size of 6.1 Mb, N50 was 71 kb, the GC content 36%, and the longest scaffold length was 258 kb. For the O-V84.2 isolate, the genome size was 6.3 Mb, N50 was 123 kb, the GC content 36%, and the longest scaffold length was 336 kb. Coding sequence prediction of the assembled reads showed that the 222 scaffolds of the E-SE10.2 isolate defined 6156 coding sequences (CDS) and that the 249 scaffolds of the O-V84.2 isolate defined 6457 CDS. For both isolates, the CDS represented the 79% of the length of the bacterial genome, and contained 71.5% of annotated genes, 28.5% of hypothetical genes, and 60–68 tRNAs (Table 2). In addition, for both isolates, 60% of the CDS could be associated to a subsystem category, being more abundant the ones associated with amino acids and derivatives, carbohydrates, protein metabolism, and cofactors, prostetic groups and pigments, in decreasing order (Figure S1).

Table 2. Summary of the automated genome annotation of the *Bt* isolates E-SE10.2 and O-V84.2 by the Rast server.

Features	E-SE10.2		O-V84.2	
	Gene Content	Length (Mb)	Gene Content	Length (Mb)
Genome Content *	6216	6.1	6525	6.3
Coding sequences ‡	6156 (99%)	4.8	6457 (98.9%)	5
Annotated genes	4398 (70%)	4.05	4615 (71.4%)	4.21
Hypothetical genes	1758 (28.2%)	0.75	1842 (28.2%)	0.79
Predicted insecticidal genes §	6	0.002	18	0.4
tRNAs	60	0.004	68	0.005

* The gene content values refer the total of predicted sequences (Coding sequences and tRNAs) predicted by the Rast server. ‡ The coding sequences values refer to the total predicted sequences (protein encoding genes and rRNA). The annotated genes refer to the predicted sequences that were included in subsystem category, while the hypothetical genes refer to the predicted sequences that were not included in any subsystem category. The percentage of coding sequences was calculated by dividing the values of the coding sequences, annotated genes and hypothetical genes, by the value of genome content. § The predicted insecticidal genes refer to the coding sequences that report similarity to the homemade *Bt* database at amino acid level (BlastX).

Regarding the insecticidal protein genes present in the *Bt* isolates E-SE10.2 and O-V84.2, the results indicated a total of 24 coding sequences (6 in E-SE10.2 and 18 in O-V84.2) (Table 3). For the *Bt* isolate E-SE10.2, four out of the six sequences showed homology to a *vip* gene, one to a *sip* gene and one to a *cry* gene (Table 3). In the case of the *Bt* isolate O-V84.2, 10 out of the 18 sequences showed homology to a *vip* gene, one to a *sip* gene and seven to a *cry* gene (Table 3).

Table 3. Insecticidal genes of the E-SE10.2 and O-V84.2 isolates predicted by Glimmer v2 software and filtered against a customized *Bt* protein database and then with the non-redundant database (NCBI).

Sample	Gene Identity ‡	Closest Homolog *	Similarity (%)	Coverage (%)
E-SE10.2	*vip1Ad-like_1*	AGC08395.1	55	24
	vip1Bb-like_1	AAR40282.1	61	99
	vip2Aa-like	1QS1_A	41	23
	vip2Ac-like_1	AAO86513.1	30	30
	sip1Aa-like_1	ABC71340.1	75	98
	cry23Aa-like	AAF76375.1	75	98
O-V84.2	*vip1Ad-like_2*	AGC08395.1	26	34
	vip1Ba-like	AAR40886.1	28	30
	vip1Da-like	CAI40767.1	37	12
	vip2Ac-like_2	AAO86513.1	33	47
	vip2Ac-like_3	AAO86513.1	37	41
	vip2Bb-like	AKI69695.1	30	43
	vip4Aa1-like_1	AEB52299.1	40	80
	vip4Aa1-like_2	AEB52299.1	40	83
	vip4Aa-like_3	AEB52299.1	49	94
	vip4Aa-like_4	AEB52299.1	52	97
	sip1Aa-like_3	ABC71340.1	32	33
	cry45Aa-like_1	BAD22577.1	61	100
	cry45Aa-like_2	BAD22577.1	69	99
	cry45Aa-like_3	BAD22577.1	68	85
	cry32Ea-like	ADK66923.1	47	98
	cry32Eb-like	AGU13828.1	51	41
	cry32Da-like	BAB78603.1	40	98
	cry73Aa-like	AEH76822.1	88	80

‡ The genes predicted by the gene prediction software were named based on the homologous gene in the database that showed more identity and coverage in the BlastX. * Access number of the gene that showed the highest identity in the protein database considered in the analysis.

2.3. Global Analysis of the Proteins Identified by in Gel Digestion LC/MSMS Analysis of the Bt Isolates E-SE10.2 and O-V84.2

To determine the proteins that are being expressed in the *Bt* isolates E-SE10.2 and O-V84.2, an LC/MSMS analysis was done. By this method, we first screened the protein content in the concentrated supernatants and in the parasporal crystals at three growth phases, two during the log phase of growth (Phase T1 at 24 h and Phase T2 at 48 h), and one in the stationary phase when the crystal is formed (Phase T3 at 72 h). In the concentrated supernatant at Phase T1, 627 and 225 proteins were identified for E-SE10.2 and O-V84.2, respectively, while, at Phase T2, the number of proteins identified were 637 and 530, respectively. In the case of the proteins identified in the solubilized crystal (Phase T3), the numbers were 512 and 185, respectively. A total of 1791 and 940, respectively, were identified considering the three growth phases together and this represents about the 29.03% and 14.55% of the respective predicted proteins from the genomic data for the *Bt* isolates E-SE10.2 and O-V84.2. The pairwise comparison of the identified proteins at the T1, T2 and T3 growth phases showed that the shared expressed proteins of the T1-T2, T2-T3, and T1-T3 phases were 406 and 160, 221 and 73, 219 and 33, respectively, for the *Bt* isolates E-SE10.2 and O-V84.2. The identified proteins of *Bt* isolates E-SE10.2 and O-V84.2 at the three growth phases were classified according to their gene ontology (GO) terms (Figure S2).

2.4. Protein Identification of the Expressed Predicted Putative Insecticidal Protein Genes

To determine if the predicted insecticidal protein genes are being expressed, the protein expression was assessed by proteomic analysis. We considered a positive identification only those proteins that were identified with both Protein Pilot v4.5 and Mascot algorithms in at least two of the replicates. Considering the two isolates together, we found a total of five secretable proteins (Vip-like and Sip-like)

and eight crystal proteins (Cry-like and Mtx-like) (Table 4 and Tables S1–S3). For the E-SE10.2 isolate, only three out of the six putative insecticidal protein genes automatically annotated were found to be expressed (Table 3), and 10 out of 18, in the case of the O-V84.2 isolate (the seven Cry proteins, the Vip2Ac-like_2 protein, and the two Vip4-like proteins). Regarding the Vip2Ac-like_3 protein, it was detected just in one replicate with Mascot (Table 4 and Table S3) and the Sip1Aa-like_2 protein was detected in two replicates, but in one of them only with Mascot (Table 4, Tables S1 and S3) and, therefore, the Vip2Ac-like_3 and Sip1Aa-like_2 proteins were not considered a positive identification.

According to the similarity to the closest homolog, the Vip2Ac-like, Vip4Aa-like, Cry32Aa-like and Sip1Aa-like_2 proteins could be considered new *Bt*-like proteins (different to Cry, Vip or Sip because of a similarity lower than 45%). Regarding the Cry45Aa-like, Cry73Aa-like, Cry23Aa-like and Sip1Aa-like-1 proteins, according to the similarity to the closest homolog (between 45% and 75%), they could be considered new protein families of their respective reference proteins (e.g., with a different number) (Table 3). Regarding the subcellular localization of the putative insecticidal proteins, we performed an LC/MSMS analysis with the concentrated culture supernatants and with the solubilized crystal proteins (Table 4). In the supernatant of the culture broth, we could detect at 24 h and 48 h the Vip2Ac-like_1, Sip1Aa-like_1 and Cry23Aa-like proteins in the *Bt* isolate E-SE10.2. For the *Bt* isolate O-V84.2, we detected Vip4Aa-like_1, Vip4Aa-like_2 and Vip2Ac-like_2 proteins at 24 h, whereas, at 48 h, we detected Vip4Aa-like_1 and Vip2Ac-like_2 proteins (Table 4). In the fraction of solubilized crystal proteins, we detected the Cry23Aa-like protein in the *Bt* isolate E-SE10.2, while, for the *Bt* isolate O-V84.2, we detected the Cry45Aa-like_1, Cry45Aa-like_2, Cry45Aa-like_3, Cry32Ea-like, Cry32Eb-like, Cry32Da-like and Cry73Aa-like proteins. The putative insecticidal proteins identified in the supernatant and solubilized crystal agree with the prediction of the SignalIP server 4.0, except for the Cry23Aa-like protein which has been found in the supernatant and in the crystal even though it does not contain signal peptide to be exported out of the cell (Table 4).

Table 4. Identification of expressed proteins from the identified putative insecticidal protein genes in the concentrated supernatant and in the solubilized proteins from the spore/crystal mixture of the *Bt* isolates E-SE10.2 and O-V84.2 by in gel digestion LC/MSMS analysis *.

Sample	Protein Identity	Mass Protein (kDa)	SignalIP Server 4.1	Supernatant (LB)						Spore/Crystal Mixture (CCY)		
				24 h			48 h			72 h		
				Rep. 1	Rep. 2	Rep. 3	Rep. 1	Rep. 2	Rep. 3	Rep. 1	Rep. 2	Rep. 3
E-SE10.2	Vip2Ac-like_1	51.6	Yes	+/+	+/+	+/+	+/+	+/+	+/+	−/−	−/−	−/−
	Sip1Aa-like_1	40.7	Yes	+/+	+/+	+/+	+/+	+/+	+/+	−/−	−/−	−/−
	Cry23Aa-like	29.3	No	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
O-V84.2	Vip4Aa-like_1	97.5	Yes	+/+	+/+	+/+	+/+	+/+	+/+	−/−	−/−	−/−
	Vip2Ac-like_2	80.79	Yes	+/+	+/+	+/+	−/+	+/+	+/+	−/−	−/−	−/−
	Vip4Aa-like_2	87.5	Yes	+/+	+/+	−/+	−/−	−/−	−/−	−/−	−/−	−/−
	Vip2Ac-like_3	23.2	Yes	−/−	−/+	−/−	−/−	−/−	−/−	−/−	−/−	−/−
	Sip1Aa-like_2	38.7	Yes	−/−	−/+	+/+	−/−	−/−	−/−	−/−	−/−	−/−
	Cry45Aa-like_1	30.6	No	−/−	−/−	−/−	−/−	−/−	−/−	+/+	+/+	+/+
	Cry45Aa-like_2	29.3	No	−/−	−/−	−/−	−/−	−/−	−/−	+/+	+/+	+/+
	Cry45Aa-like_3	25.6	No	−/−	−/−	−/−	−/−	−/−	−/−	+/+	+/+	+/+
	Cry32Ea-like	151.2	No	−/−	−/−	−/−	−/−	−/−	−/−	+/+	+/+	+/+
	Cry32Da-like	153.7	No	−/−	−/−	−/−	−/−	−/−	−/−	+/+	+/+	+/+
	Cry32Eb-like	76.8	No	−/−	−/−	−/−	−/−	−/−	−/−	+/+	+/+	+/+
	Cry73Aa-like	72.2	No	−/−	−/−	−/−	−/−	−/−	−/−	+/+	+/+	+/+

* +/+, the insecticidal protein genes were identified with Protein Pilot (Paragon algorithm) and Mascot; +/−, the insecticidal protein genes were identified with Protein Pilot (Paragon algorithm) but not with Mascot; −/+, the insecticidal protein genes were identified with Mascot but not Protein Pilot (Paragon algorithm); −/−, the insecticidal protein genes were not identified with either Protein Pilot (Paragon algorithm) or Mascot.

2.5. Gene synteny, Conserved Domains and Phylogenetic Analysis of the Expressed Putative Insecticidal Protein Genes

In the E-SE10.2 isolate, the *vip2Ac-like_1* gene was found in an operon together with a non-expressed *vip1Bb-like* gene, with the peculiarity that the *vip1Bb-like* gene was upstream of the *vip2Ac-like_1* (Figure 1), contrary to the general relative location of *vip1* and *vip2* genes in operons. The *cry32Aa-like* gene was found in an operon with a predicted truncated *cry37-like* gene. In the O-V84.2 isolate, the genes for the Vip2Ac-like_2, Vip4Aa-like_1, and Vip4Aa-like_2 proteins were found in operons containing the pairs *vip2Ac-like_2–vip4Aa-like_1* and *vip2Ac-like_3–vip4Aa-like_2* (Figure 1). Regarding the *cry* genes of this isolate, they were found in different scaffolds with different transcription origins (Figure 1).

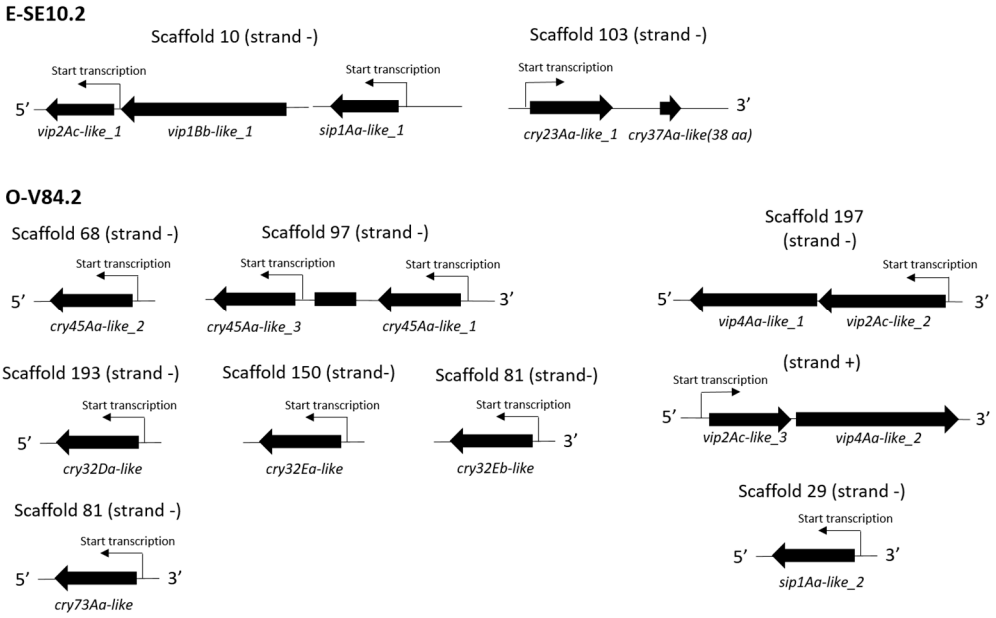

Figure 1. Gene synteny of the putative insecticidal protein genes of the E-SE10.2 and O-V84.2 isolates detected by LC/MSMS analysis. Strand + indicate that the respective genes are in the positive DNA strand. Strand—means that the respective genes are in the negative DNA strand.

The phylogenetic analysis of the Vip-like and Sip1-like proteins indicate that the Vip4-like, Vip2-like, and Sip1Aa-like_2 create a basal branch in their respective families (Figure 2). Regarding the Cry proteins, the Cry32E-like, Cry45Aa-like, Cry73Aa-like, and Cry23Aa-like proteins fall into their respective clusters, whereas the Cry32Da-like protein falls into the Cry66A cluster (Figure 3). The analysis of the sequences revealed that the *vip4Aa-like_1* and *vip4Aa-like_2* showed the predicted conserved domains of the PA14 superfamily and the clostridial binary toxin B/anthrax toxin PA, which are present in the Vip1 proteins. The *vip2Ac-like_1*, *vip2Ac-like_2* and *vip2Ac-like_3* genes showed the predicted conserved domain Vip2 superfamily, which is present in the Vip2 proteins. Moreover, *vip2Ac-like_2* showed the predicted conserved domain anthrax toxin lethal factor (ATLF) and *vip2Ac-like_3* showed one of the two Vip2 superfamily conserved domains present in the Vip2 proteins. The *sip1Aa-like* genes (*sip1Aa-like_1* and *sip1Aa-like_2*) showed the predicted conserved domain of the MTX superfamily which is present in the *Lysinibacillus sphearicus* and *Clostridium perfringens*. Regarding the Cry-like proteins, the analysis of the sequences revealed that the *cry23Aa-like* gene and the *cry45Aa-like* genes (*cry45Aa-like_1*, *cry45Aa-like_2*, and *cry45Aa-like_3* have a similarity of 75% to each other) showed the predicted conserved domain MTX. Among the *cry32-like* genes, *cry32Ea-like* and *cry32Da-like* showed a similarity of 88% to each other (at the amino acid level) and both carried the predicted conserved domains Endotoxin_N, Endotoxin_M, and Delta_Endotoxin_C,

which are typical of the three-domain Cry proteins. The *cry32Eb-like* gene showed low similarity to the other two *cry32-like* genes (61% amino acid similarity to *cry32Ea-like* and 64% amino acid similarity to *cry32Da-like*) and did not show any conserved domains. The *cry73Aa-like* gene also showed the predicted conserved domains Endotoxin_N, Endotoxin_M, and Delta_Endotoxin_C.

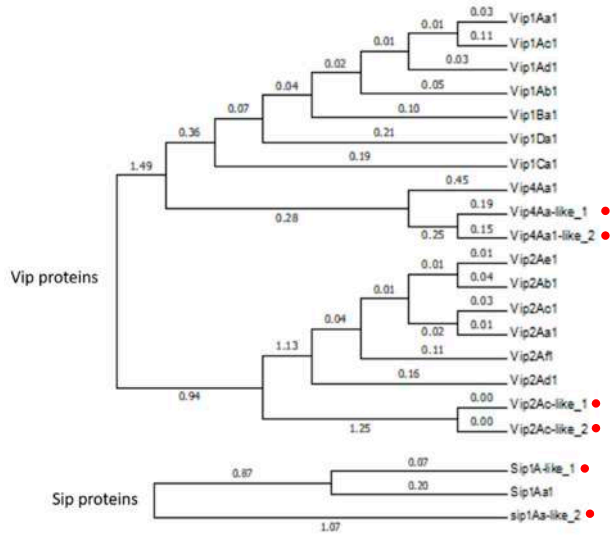

Figure 2. Phylogenetic analysis of the Vip1/Vip2- and Sip1A-type proteins detected in the *Bt* isolates E-SE10.2 and O-V84.2. The red dots indicate the position of the new putative proteins in the phylogenetic tree. Branch lengths represent the number of substitutions per site of the multiple-sequence alignment as a measure of divergence (Mega v6 software).

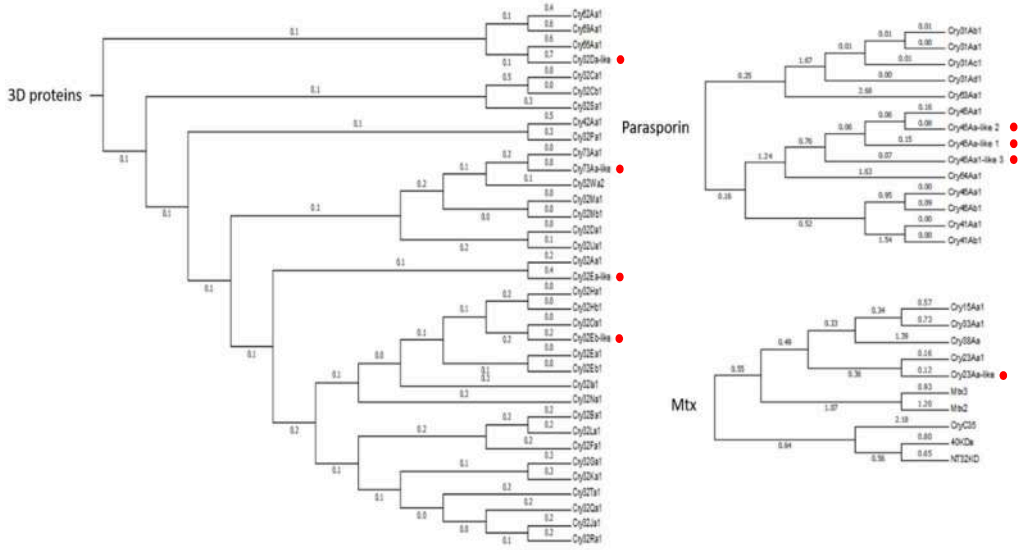

Figure 3. Phylogenetic analysis of the Cry-type proteins detected in the *Bt* isolates E-SE10.2 and O-V84.2. The red dots indicate the position of the new putative proteins in the phylogenetic tree. Branch lengths represent the number of substitutions per site of the multiple-sequence alignment as a measure of divergence (Mega v6 software).

2.6. Relative Abundance of the Putative Insecticidal Proteins in the Supernatant and in the Crystal of the Bt Isolates E-SE10.2 and O-V84.2

To determine the relative abundance of the putative insecticidal protein genes in the supernatants and crystals of the *Bt* isolates E-SE10.2 and O-V84.2, we performed two types of analyses: first, an emPAI analysis to determine the relative abundance within a same replicate at a given time (Table 5

and Table S3); and, second, a label free analysis to compare between different times in the log phase (T1 vs. T2) (Table 6 and Table S4). The results showed that the putative vegetative insecticidal proteins were minimally expressed in the supernatant of both *Bt* isolates, being the most abundant protein flagellin FlaA (Table 5). In the *Bt* isolate E-SE10.2, among the putative insecticidal proteins found in the supernatant, the most abundant in all replicates was the Cry23Aa-like protein. In contrast, for O-V84.2, all secretable proteins were similarly represented (Table 5). Regarding the relative abundance of the proteins in the solubilized crystals, the crystal of E-SE10.2 contained only the Cry23Aa-like protein. In the case of O-V84.2, the percent weight corresponding to Cry proteins was close to the 50% of the solubilized proteins from the crystal (Table 5), being the most abundant, by far, the Cry32Ea-like protein.

To be able to compare the expression level of the proteins between 24 h and 48 h, a label free analysis was performed (Table 6 and Table S4). Only the putative insecticidal proteins Vip4Aa-like_1 and Vip4Aa-like_2, from the *Bt* isolate O-V84.2, showed significant differences at the two growth phases (Tables 5 and 6). The former increased two-fold at 48 h compared to 24 h (Table 6), and the latter was only found at 24 h but not at 48 h (Table 5). The other proteins found in the supernatant did not show statistical differences in their production at 24 h vs. 48 h.

Table 5. Estimation of the relative production expressed as weight percentage of the insecticidal protein genes in the supernatant and solubilized proteins from the spore and crystal mixtures of the *Bt* isolates E-SE10.2 and O-V84.2 identified with Mascot.

Supernatant	24 h (% Weight)			48 h (% Weight)		
	R1	R2	R3	R1	R2	R3
E-SE10.2						
Non Secretable toxins	98.56	99.06	99.36	97.87	99.26	99.63
Flagellin protein FlaA	55.21	73.82	87.19	39.75	36.04	43.19
Secretable toxins	1.44	0.94	0.64	2.13	0.74	0.37
Vip2Ac-like_1	0.09	0.06	0.03	0.09	0.10	0.06
Sip1A-like_1	0.09	0.03	0.02	0.06	0.08	0.02
Cry23Aa-like *	1.26	0.85	0.59	1.98	0.56	0.29
O-V84.2						
Non Secretable toxins	99.99	99.99	99.99	99.99	99.99	99.99
Flagellin protein FlaA	99.20	99.37	98.35	84.14	99.15	88.35
Secretable toxins	0.0032	0.0012	0.0030	0.0111	0.00007	0.0075
Vip4Aa-like_1	0.0014	0.0008	0.0016	0.0019	0.00004	0.0029
Vip4Aa-like_2	0.0005	0.0001	0.0002	-	-	-
Vip2Ac-like_2	0.0013	0.0002	0.0010	0.0092	0.00003	0.0046
Crystal	**72 h (% Weight)**					
	R1	**R2**	**R3**			
E-SE10.2						
Non-crystal toxins	69.52	95.26	97.51			
Crystal toxins	30.48	4.74	2.49			
Cry23A-like	30.48	4.74	2.49			
O-V84.2						
Non-crystal toxins	52.86	51.12	53.25			
Crystal toxins	47.14	48.88	46.75			
Cry45Aa-like_1	2.82	2.46	1.41			
Cry45Aa-like_2	3.05	2.03	3.38			
Cry45Aa-like_3	1.88	5.29	2.20			
Cry32Ea-like	24.30	25.06	25.89			
Cry32Da-like	6.10	5.40	4.60			
Cry32Eb-like	6.20	4.46	5.02			
Cry73Aa-like	2.79	4.18	4.25			

* The Cry23Aa-like protein was detected in the supernatant and the crystal of the *Bt* isolate E-SE10.2, but, according to the prediction of the SignalIP server 4.1, it is most likely not secretable.

Table 6. Label free analysis of the putative insecticidal protein genes of the *Bt* isolates E-SE102 and O-V84.2 in the concentrated supernatant at 24 h versus 48 h, identified with Protein Pilot v4.5.

***Bt* Isolate**	**Proteins**	***t*-Value †**	***p*-Value Φ**	**Mean Peaks Area**		**Standard Deviation Peaks Area**		**Fold Change 24/48 §**	**Status**
				24 h	**48 h**	**24 h**	**48 h**		
E-SE10.2	Vip2Ac-like_1	0.71	0.52	307,838	266,602	54,005	84,577	1.15	No differences
	Sip1Aa-like_1	0.89	0.42	110,172	76,938	47,032	44,040	1.43	No differences
	Cry23Aa-like *	0.32	0.77	5,796,029	4,858,544	4,951,202	1,257,383	1.19	No differences
O-V84.2	Vip4Aa-like_1	4.07	0.04	134,357	68,825	26,801	7636	1.95	Increased
	Vip2Ac-like_2	0.56	0.61	32,544	27,573	13,419	7512	1.18	No differences

† Student's *t*-test statistical analysis was performed between the concentrated supernatant at 24 h versus 48 h. Φ With a p value lower than 0.05, it was considered that the differences observed between the concentrated supernatant at 24 h versus 48 h were statistically significant. § The fold change was calculated by dividing the mean value at 24 h by the mean values at 48 h. * The Cry23Aa-like protein was detected in the supernatant and the crystal of the *Bt* isolate E-SE10.2, but, according to the prediction of the SignalIP server 4.1, is most likely not secretable.

3. Discussion

A screening of Spanish collections of *Bt* isolates was undertaken to search for novel members of the Vip family. As a result, nine *Bt* isolates were selected for harboring new binary insecticidal protein genes of the *vip1/vip2* family [11]. As a first step, the PCR-Sanger Sequencing approach revealed new alleles of already described *vip1* and *vip2* genes (*vip2Ac2-vip1Ca2* and *vip2Bb4-vip1Bb3*) and two sequences with low similarity to the *vip1Bb1* (from the *Bt* isolate E-SE10.2) and *vip4Aa1* (from the *Bt* isolate O-V84.2) genes. In a second step, the *Bt* isolates E-SE10.2 and O-V84.2 were subjected to whole genome sequencing with the Illumina HiSeq-PE150 sequencing platform. Then, the genomes of E-SE10.2 and O-V84.2 were assembled in 222 and 249 scaffolds codifying for 6156 CDS and 6457 CDS, respectively. The CDS predicted for both genomes represented close to the 99% of the total number of genes predicted in the genomes. In addition, from this 99% of the predicted CDS, 28% belonged to hypothetical genes and 72% to annotated genes by the Rast server. Moreover, the results obtained from the automated annotation indicated that both *Bt* genomes had a similar subsystems category distribution (Figure S1).

The supernatants at 24 h (growth Phase T1) and 48 h (growth Phase T2) and the crystal proteins (growth Phase T3) of both *Bt* isolates were also analyzed and annotated with GO terms (Figure S2). The quantity of the proteins expressed at the three different growth phases for the *Bt* isolates E-SE10.2 and O-V84.2 were 10.2–3.5% and 10.4–8.2%, 8.3–2.8%, respectively, of their genome encoded sequences. This low percentage of expressed proteins detected indicates that, in our experimental conditions, we only detect a small part of the predicted proteins by the genome data prediction, a phenomenon that has also been found in other studies [21,22]. The low percentage of detected expressed proteins should not be interpreted as that the rest of the proteins cannot be expressed, since they could do it under different growth conditions. Considering both isolates together, the number of annotated proteins in each growth phase, T1, T2 and T3, was 42.3%, 49.8% and 56.9%, respectively. The distribution of the GO terms over the different growth phases is similar in the *Bt* isolates E-SE10.2 and O-V84.2 (Figure S2). The most common and abundant GO terms in all the phases (cellular biosynthetic process, organic substance biosynthetic process, cellular nitrogen compound metabolic process, and organonitrogen compound metabolic process) indicate that both *Bt* isolates metabolize the carbon and nitrogen in the media to produce all the organic and organonitrogen compounds that they need (Figure S2). The specific GO term macromolecule metabolic process of the T3 growth phase indicates that both *Bt* isolates express proteins of a relatively high molecular mass, such as the Cry-like proteins detected (Figure S2).

Regarding the predicted insecticidal protein genes in both *Bt* genomes, we were able to find some of the predicted gene products: one new couple of binary Vip-like proteins (Vip2Ac-like_1-Vip4Aa-like_1), two new Vip-like proteins (Vip2Ac-like_1 and Vip4Aa-like_2), one Sip1A-like protein (Sip1A-like_1), and eight Crystal-like proteins (Cry23A-like, Cry45Aa-like_1, Cry45Aa-like_2, Cry45Aa-like_3, Cry32Ea-like, Cry32eDa-like, Cry32Eb-like and Cry73Aa-like) (Table 4). The discrepancies of the protein identification between the replicates can be attributed to metabolic flow changes in cells during development, resulting from enzyme-related changes or that some proteins exist with extremely low abundances such that they cannot be detected by MS. To determine if the detected *Bt-like* proteins are being secreted or that they form inclusion bodies, we performed an LC/MSMS analysis with the supernatant (24 h and 48 h) and solubilized crystal proteins. In the supernatant of both *Bt* isolates at 24 h, the Vip4Aa-like_1, Vip4Aa-like_2, Vip2Ac-like_1, Vip2Ac-like_2, and Sip1Aa-like_1 proteins were detected, while at 48 h only Vip4Aa-like_1, Vip2Ac-like_1, Vip2Ac-like_2, and Sip1Aa-like_1 were detected. Again, the extremely low abundance of these proteins might be responsible for the differences found at 24 h and 48 h. Regarding the Vip4Aa-like proteins, this is the first time that there has been demonstrated that they are expressed and secreted to the medium in the log phase. Regarding the crystal proteins, they were found in the crystal of both *Bt* isolates, except for the Cry23Aa-like, which was also found in the supernatant at 24 h and 48 h. The detection of the Cry23Aa-like protein and sporulation factors (Stage

V sporulation protein, spore coat protein B, spore coat polysaccharide biosynthesis protein spsB and spore coat polysaccharide synthesis) in the supernatant at 24 h (and also at 48 h) of the *Bt* isolate E-SE10.2 indicates that the cells already started the sporulation process.

The relative abundance of the *Bt-like* proteins was estimated in the supernatant and the parasporal crystal in both *Bt* isolates. In the supernatant (24 h and 48 h) of both *Bt* isolates, the Vip-like, Sip1-like and Cry23Aa-like were marginally expressed. Regarding the crystal proteins in the *Bt* isolate O-V84.2, the Cry-like proteins represent around the half of the total crystal weight, while for the *Bt* isolate E-SE10.2, the Cry23Aa-like protein represents between 2.5% and 30% of the crystal weight. The high variability observed in the amount of Cry23Aa-like could be due to the different replicates are not in the same time point of the sporulation process. The crystal composition of the *Bt* isolate O-V84.2 was also determined for those proteins with a percentage of similarity lower than 45%. The crystal was composed by four kinds of proteins: 7.0–9.8% Cry45-like proteins (Cry45Aa-like_1, Cry45Aa-like_2 and Cry45Aa-like_3), 30.4–30.5% Cry32-like proteins (Cry32Ea-like and Cry32Da-like), 5.0–6.2% Cry32Eb-like, and 2.8–4.25% Cry73Aa-like, while the *Bt* isolate E-SE10.2 only produced the Cry23Aa-like protein.

The expression levels of the Vip-like, Sip1-like and Cry23Aa-like proteins were compared between 24 h and 48 h. The amount of Vip4Aa-like_1 protein was increased two-fold at 24 h vs. 48 h, while the Vip4Aa-like_2 was only detected at 24 h. As regard to the rest of the proteins (Vip2Ac-like, Sip1A-like, and Cry23A-like proteins), no differences in expression were observed. These results suggest that the Vip4Aa-like_2, Vip2A-like and Sip1A-like proteins were expressed at the 24 h while the Vip4Aa-like_1 was expressed later at the end of the 24 h and the beginning of the 48 h periods.

4. Conclusions

In summary, the combined use of the genomic and proteomic data allowed us to determine which of the identified insecticidal protein genes, present in the *Bt* isolates E-SE10.2 and O-V84.2, are being expressed and, if so, at which relative abundance. Considering the two *Bt* isolates together, we were able to identify five new insecticidal protein genes that are expressed within the first 24 h, except for *vip4Aa-like_1*, which is expressed after the 24 h. In the parasporal crystals, we found nine new crystal proteins. The spore/crystal mixture of the *Bt* isolate E-SE10.2 contains solely the Cry23Aa-like protein, while the crystal of the *Bt* isolate O-V84.2 contains four kinds of Cry proteins: Cry45-like, Cry32-like, Cry32Eb-like, and Cry73Aa-like.

5. Materials and Methods

5.1. Bacterial Strains and Growth Conditions for DNA Analysis

Nine *Bt* isolates from a Spanish collection, known to carry *vip1* and *vip2* genes, were selected for this study [11]. For further gene identification of the *vip1* and *vip2* genes, the *Bt* isolates were grown in 4 mL LB medium overnight (ON) at 29 °C and 200 rpm. For the whole genome sequencing, only those *Bt* isolates with *vip1* and *vip2* genes with less than 60% similarity to already described *vip1* and *vip2* genes were chosen. The isolates were grown in 10 mL LB medium until OD of 0.6 at 29 °C and 200 rpm.

5.2. Genomic DNA Preparation

Total genomic DNA used for gene identification (GI) was isolated from a single colony of the *Bt* isolates. Cells were collected at 9000× *g* for 10 min at 4 °C and the pellet was washed in 2 mL of TE buffer (1 M Tris-HCl, 10 mM EDTA, pH 8.0). The pellet was dissolved in 200 µL of TEL buffer (TE buffer + 4 mg/mL lysozyme) and further incubated at 37 °C for 30 min. Then, 400 µL of lysis solution (0.2 M NaOH, 1% SDS) was added. After gentle mixing, 300 µL of the neutralization buffer (3 M KAc, pH 5.5) was added and the mixture incubated for 5 min on ice. The mixture was centrifuged at 14,000× *g* for 15 min at 4 °C and the supernatant was transferred to a new tube. One volume of cold

100% ethanol was added and the samples kept at −20 °C for 16 h. The samples were centrifuged at 14,000× *g* for 15 min at 4 °C and the supernatant was transferred to a new tube and the pellet washed with 1 mL of cold 70% ethanol. The pellet was dried with the Eppendorf concentrator 5301 for 5 min at 42 °C and solubilized in 50 µL of TE buffer. Total genomic DNA, used for whole genome sequencing (WGS), was purified as described in the manufacturer instructions of the DNeasy Blood & Tissue Kit Qiagen. The DNA for GI was quantified using Nanodrop 2000 (Thermo Scientific, Waltham, MA, USA), while for WGS, the DNA was measured with a Qubit Fluorimetrer. In addition, the integrity of the DNA for GI and WGS was evaluated by agarose gel electrophoresis (1% agarose).

5.3. Identification of Vip1- and Vip2-Type Genes

Identification of *vip1* and *vip2* genes was performed with primer pairs designed from conserved regions within the *vip1* and *vip2* gene families, respectively. A first PCR with "screening primers" [11] was performed to confirm the presence of *vip* genes. With the positive samples, a second PCR was performed with the "typing primers" [11,23] for the identification of the *vip1* and *vip2* genes. PCR reactions contained, in a final volume of 25 µL, 100 ng of the DNA template, 0.25 U of Biotools polymerase (Biotools), 2.5 µL of 10-fold reaction buffer, 10 mM of each dNTPs, and 0.3 µM of the corresponding primers (*vip1sc*, *vip2sc*, *vip2 typing* [11] or *vip1 typing* [23]). PCR amplifications were carried out in an Eppendorf Mastercycler thermal cycler as follows: 5 min denaturation at 95 °C, 35 cycles of amplification (1 min denaturation at 94 °C, 1 min of annealing at 45 °C, and 2 min of extension at 72 °C), and an extra extension step of 10 min at 72 °C. To determine the similarity of the amplified sequences to already described *vip1* and *vip2* genes, the PCR products obtained with the "typing primers" (or with the "screening primers" for those samples that did not give amplification with the "typing primers") were ligated into the pGEM®-T Easy plasmid (Promega), cloned in *Escherichia coli* DH10β, and sequenced. DNA sequence analysis and contig assembly was performed using DNAstar v5 and NCBI BLAST tools (Blastx) [24].

5.4. Genome Sequencing, Assembly and Annotation Analysis

Genome Sequencing for the *Bt* isolates E-SE10.2 and O-V84.2 was performed with the Illumina HiSeq-PE150 sequencing platform (Novogene S.L Hong Kong, China). From the clean reads (without adapters, low quality, N and duplication) provided by Novogene S.L., first we evaluated the quality of the data with FastQC software (0.11.5, Babraham Bioinformatics Institute, Cambridge, Cambridgeshire, United Kingdom, 2016). Then, the reads were assembled with SoapdeNovo2 (kmer size 35 and genome size 5,600,000 bp) and the gaps were closed with GapCloser (maximum read length 150, overlap 25 bp and thread number 1) [25]. The assembled reads were annotated with Rast server (Figure S1) and the coding sequence (CDS) prediction was performed with the Glimmer v2 [26]. First, the predicted genes were filtered against a customized *Bt* protein database (https://sourceforge.net/projects/bt-proteindatabase/files/Btdatabase/) with Blastx (genetic code bacteria and archaea, e-value 0.001 and word size 6) to select those CDS with homology to the *Bt* toxins [24]. Next, the putative insecticidal protein genes were compared against the Non-Redundant database and only the concordant results along the customized *Bt* protein database and Non-Redundant database were selected as true positive. Moreover, for the selected putative insecticidal genes, prediction of conserved domains was carried out with CD-search [27] and the gene sinteny was determined in the assembled sequences.

5.5. Sample Preparation for in Gel Digestion LC/MSMS Analysis and Insecticidal Activity of Bt Isolates

A single colony of *Bt* was grown in 100 mL of LB at 29 °C for 24 h and 48 h for detection of the secretable proteins, while for the detection of proteins in the parasporal crystal the culture was grown in 100 mL of CCY at 29 °C until culture sporulation (72 h). The supernatant of *Bt* was concentrated by trichloroacetic acid (TCA) precipitation. Briefly, the cells were collected at 6000× *g* for 15 min at 4 °C and filtered through sterile 0.45 µm cellulose acetate filters (GE Healthcare Life Sciences). The sample was incubated with 10% TCA (final concentration) and kept at 4 °C for 24 h. Then, the

sample was centrifuged at 16,000× *g* for 20 min at 4 °C. The pellet was washed with 100 mL of cold acetone (−18 °C), centrifuged at 16,000× *g* for 20 min at 4 °C, and let dry at room temperature for 5 min. The precipitated proteins were solubilized in 50 mM carbonate buffer containing 10 mM dithiothreitol (pH 11.3) for 48 h, with two buffer changes (Figure S3). Crystals (together with spores) were separated by centrifugation at 6000× *g* for 12 min at 4 °C. The pellet containing the parasporal crystals was washed three times with ice cold solution A (1 M NaCl, 5 mM EDTA, 10 mM PMSF, 1% Triton X-100) and centrifuged at 17,000× *g* for 12 min at 4 °C between washes. The pellet was then washed three times with ice cold solution B (10 mM KCl) and centrifuged at 24,000× *g* for 15 min at 4 °C. The crystals in the final pellet were solubilized in 20 mL of 50 mM carbonate buffer containing 10 mM dithiothreitol (pH 11.3) by incubation at room temperature for 2 h with continuous shaking (Figure S3). Concentration of the proteins in the supernatant and in the solubilized crystals was estimated with the Bradford method [28]. The purity of the expressed proteins in the supernatant and the crystal was analyzed by SDS-PAGE and stained with Coomassie brilliant blue R-250 (Sigma-Aldrich, St. Louis, MO, USA) (Figure S3).

5.6. In Gel Digestion LC/MSMS Analysis

The detection of the expression of the putative insecticidal proteins was done by LC/MSMS at the proteomics facility of the SCSIE (Servei Central de Suport a la Investigació Experimental), at the University of Valencia, Spain. First, a 1D SDS-PAGE (without resolving gel) was performed with 30 µg of total protein in three replicates of the concentrated supernatant (24 h and 48 h) and solubilized crystal proteins. The bands were cut out and in gel digested with 500 ng sequencing grade trypsin (Promega). The digestion was stopped with trifluoroacetic acid (TFA, 1% final concentration). After subjecting the samples to a double extraction with acetonitrile (ACN), all the peptide solutions were dried in a rotatory evaporator. Samples were solubilized with 50 µL of 2% ACN, 0.1% TFA. A sample aliquot of 5 µL was loaded onto a trap column (NanoLC Column, 3 µ C18-CL, 350 µm × 0.5 mm, Eksigent) and desalted with 0.1% TFA at 3 µL/min for 5 min. The peptides were then loaded onto an analytical column (LC Column, 3 µ C18-CL, 75 µm × 12 cm, Nikkyo) equilibrated in 5% ACN 0.1% formic acid (FA). The elution was carried out with a linear gradient of 5–35% B in A for 30 min (A: 0.1% FA; B: ACN, 0.1% FA) at a flow rate of 0.3 µL/min. Peptides were analyzed in a nanoESI qQTOF (5600 TripleTOF, ABSCIEX) mass spectrometer. Eluted peptides were ionized applying 2.8 kV to the spray emitter. Analysis was carried out in a data-dependent mode (DDA). Survey MS1 scans were acquired from 350 to 1250 m/z for 250 ms. The quadrupole resolution was set to "UNIT" for MS2 experiments, which were acquired 100–1500 m/z for 50 ms in "high sensitivity" mode. The following switch criteria were used: charge: 2+ to 5+; minimum intensity; 70 counts per second (cps). Up to 25 ions were selected for fragmentation after each survey scan and the collision energy was automatically selected by the instrument according to the following equation: $|CE| = (\text{slope}) \times (m/z) + (\text{intercept})$; Charge (Unknown, 1, 2, 3, 4, 5), Slope (0.0575, 0.0575, 0.0625, 0.0625, 0.0625, 0.0625), Intercept (9, 9, −3, −3, −6, −6)

5.7. Protein Identification of the in Gel Digestion LC/MSMS Analysis with Paragon Algorithm and Mascot

The MS/MS information of three replicates of the concentrated supernatant (24 h and 48 h) and solubilized crystal proteins were sent to Paragon algorithm [29] via the Protein Pilot v 4.5 (ABSciex). Protein Pilot v 4.5 default parameters were used to generate peak list directly from the 5600 TripleTof Sciex. The Paragon algorithm of Protein Pilot v 4.5 was used to search in a homemade database that was created combining all the coding sequences predicted by Glimmer v2 software for the *Bt* isolates E-SE10.2 and O-V84.2; the new database was named *Bt*_combined (https://sourceforge.net/projects/bt-combined/files/Bt_combined/). The search in the respective protein database was done with the following parameters: trypsin specificity, cys-alkylation, and the search effort set to through. To avoid using the same spectral evidence in more than one protein, the identified proteins were grouped based on MS/MS spectra (proteins sharing MS/MS spectra are grouped, regardless of the peptide

sequence assigned) by the Protein-Pilot Progroup algorithm. A protein group in a Progroup Report is a set of proteins that share some physical evidence, the formation of protein groups in Pro Group was guided entirely by observed peptides only and the unobserved regions of protein sequence play no role in explaining the data (Tables S1 and S2). The protein within each group which can explain more spectral data is that protein shown as the primary protein of the group. Only the proteins of the group for which there is individual evidence (unique peptides with enough confidence) are also listed (Tables S1 and S2). In addition, to support the identification of the Protein Pilot v 4.5 (ABSciex) and estimate the relative production of the insecticidal proteins in the three replicates of the concentrated supernatant and solubilized crystal proteins, a series of Mascot MS/MS ion searches with the output of the 5600 TripleTof Sciex were done with the *Bt*_combined protein database. The following parameters were used: MS/MS "ion search", enzyme "trypsin", fixed modifications "carbamidomethyl (C)", variable modifications "deamidated (NQ) and oxidation (M)", mass values "monoisotopic", protein mass "unrestricted", peptide mass tolerance "50 ppm", fragment mass tolerance "0.6 Da", max miss cleavages "1", instrument type "ESI-QUAD-TOF", number of queries for E-SE10.2 "(Supernatant 24 h: R1 7468, R2 8755, R3 7682; Supernatant 48 h: R1 9, 243, R2 8602, R38,286; Crystal: R1 6779, R2 7173 R3 7790)" and for O-V84.2 "(Supernatant 24 h: R1 3708, R2 4459, R3 3536 Supernatant 48 h: R1 6016, R2 6654, R3 6123; Crystal: R1 5206 R2 5206 R3 4476)", significance threshold "p-value < 0,05", max number of families "auto", ions score or expect cut-off "20", and preferred taxonomy "all entries". The Exponentially Modified Protein Abundance Index (emPAI) was expressed as molar and weight percentage [30] (Table 5 and Table S3).

We defined as a true positive all those proteins with homology to the *Bt* toxins higher than 100 aa that had been identified with Protein Pilot v4.5 and Mascot in at least two of the replicates. In addition, for the identified proteins, the functional annotation was performed with the SwissProt Database using the Blast2GO v5.0 software (Figure S2) [31].

5.8. Label Free Analysis of the Concentrated Supernatant 24 h vs. 48 h in Both Bt Isolates

The data obtained from the 5600 TripleTof Sciex of the concentrated supernatant and solubilized proteins from the crystal were analyzed by Peak View 1.1 following the parameters: Unused $\geq$ 1.3, confidence > 95% and with maximum 50 peptides for protein. For the protein library construction of the global analysis, a joint search with the *Bt*_combined protein database was performed with the three replicates of the concentrated supernatant (24 h and 48 h) and solubilized crystal proteins (Table S5). In the case of the specific conditions analysis (Supernatant: E-SE10.2 24 h vs. 48 h, and O-V84.2 24 h vs. 48 h), a joint search with the *Bt*_combined protein database was performed with the three replicates of the concentrated supernatant (Table S4). The search in the respective analysis was done with the following parameters: trypsin specificity, cys-alkylation, and the search effort set to through. First, a global analysis was done to study grouped data analysis and samples distribution. A joined search with all the samples was performed with the Peak View 1.1 that identified 1816 proteins and the quantitative data obtained was analyzed with Marker View 1.3. Briefly, for the grouped data analysis, a PCA analysis was done with the non-normalized area of the peaks and with the area peaks corrected by the total areas sum. In the case of the samples distribution, a PCA analysis was done with the area of the peaks corrected by the total areas sum (Table S5). For the specific conditions analysis, a specific search with Peak View 1.1 was done with the respective samples to study the statistical significant differences. The quantitative data was analyzed with Marker View 1.3. Prior to data analysis of the E-SE10.2 24 h vs. 48 h, and O-V84.2 24 h vs. 48 h, we applied a normalization by total areas sum, and then a grouped data analysis with PCA analysis was done. A student's t-test statistical analysis with the concentrated supernatant (E-SE10.2 24 h vs. 48 h, and O-V84.2 24 h vs. 48 h) was performed to determine the differentially expressed proteins between two experimental conditions with the Marker View 3.1 software (Table S4).

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6651/10/5/193/s1, Figure S1: Subsystem category distribution of the *Bt* isolates E-SE10.2 and O-V84.2 by the genome annotation based on the Rast server. The right pie chart indicates the percentage or predicted encoding genes associated to at least one subsystem; Figure S2: Functional annotation of the protein identification from the concentrated supernatants at 24 h and 48 h in LB medium, and of the solubilized proteins from the crystal at 72 h in CCY medium. The number of sequences that belong to the group are indicated in brackets; Figure S3: SDS-PAGE of the three replicates of the concentrated supernatants and solubilized proteins from the spore/crystal mixtures and growth curve of the *Bt* isolates E-SE10.2 and O-V84.2; Table S1: Protein summary of three replicates of concentrated supernatants and solubilized proteins from spore/crystal mixtures with Protein Pilot v4.5; Table S2: Peptide summary of three replicates of concentrated supernatants and solubilized proteins from spore/crystal mixtures identified with Protein Pilot v4.5; Table S3: Result of the Mascot search in the three replicates of the concentrated supernatants and solubilized proteins form spore/crystal mixtures; Table S4: Label free analysis at 24 h vs. 48 h of concentrated supernatants of the proteins identified with Protein Pilot v4; Table S5: Protein library construction and PCA analysis of the joint search with the concentrated supernatants and solubilized proteins from spore/crystal mixtures identified with Protein Pilot v4.5.

Availability of Data and Material: The clean reads of the *Bt* isolates have been deposited at Sequence Read Archive (SRA). E-SE10.2 SRR5115619 and O-V84.2 SRR5121303. The sequences of the genes have been deposited in the GenBank. V-J20.2: *vip1Bb3* (KR065727) and *vip2Bb4* (KR065728). V-V54.26: *vip1Ca2* (KR065725) and *vip2Ac2* (KR065726). E-SE10.2: vip2Ac-like_1 (KY420183), sip1Aa-like_1 (KY420184), cry23Aa-like (KY420185). O-V84.2: *vip4Aa-like_1* (KY420182), *vip4Aa-like_2* (KY420193), *vip2Ac-like_2* (KY420195), *vip2Ac-like_3* (KY420197), *cry45Aa-like_1* (KY420188), *cry45Aa-like_2* (KY420189), *cry32Ea-like* (KY420190), *cry32Da-like* (KY420191), *cry45Aa1-like_3* (KY420192), *cry73Aa-like* (KY420194), *cry32Eb-like* (KY420196).

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6. - CHAPTER 2:

Toxicity and Cross-Resistance of the Vip3 proteins to lepidopteran insect species

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Susceptibility of *Grapholita molesta* (Busck, 1916) to formulations of *Bacillus thuringiensis*, individual toxins and their mixtures

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ABSTRACT

The Oriental fruit moth, *Grapholita molesta* (Lepidoptera: Tortricidae), is a major pest of fruit trees worldwide, such as peach and apple. *Bacillus thuringiensis* has been shown to be an efficient alternative to synthetic insecticides in the control of many agricultural pests. The objective of this study was to evaluate the effectiveness of *B. thuringiensis* individual toxins and their mixtures for the control of *G. molesta*. Bioassays were performed with Cry1Aa, Cry1Ac, Cry1Ca, Vip3Aa, Vip3Af and Vip3Ca, as well as with the commercial products DiPel® and XenTari®. The most active proteins were Vip3Aa and Cry1Aa, with LC_{50} values of 1.8 and 7.5 ng/cm^2, respectively. Vip3Ca was nontoxic to this insect species. Among the commercial products, DiPel® was slightly, but significantly, more toxic than XenTari®, with LC_{50} values of 13 and 33 ng commercial product/cm^2, respectively. Since Vip3A and Cry1 proteins are expressed together in some insect-resistant crops, we evaluated possible synergistic or antagonistic interactions among them. The results showed moderate to high antagonism in the combinations of Vip3Aa with Cry1Aa and Cry1Ca.

1. Introduction

Bacillus thuringiensis is an entomopathogenic bacterium that produces several types of insecticidal proteins, such as Cry, Cyt, Vip, and Sip proteins, along with other virulence factors contributing to its pathogenicity. Among them, Cry and Vip3 proteins are the ones that have been best characterized (Vilas-Bôas et al., 2012; Palma et al., 2014). Cry proteins are produced during the sporulation phase as parasporal crystals and some of them exhibit a specific toxic effect to insects belonging to different orders, mainly Lepidoptera, Coleoptera and Diptera (Schnepf et al., 1998; Ricieto et al., 2013; Palma et al., 2014). Vip3 proteins are produced during the vegetative growth and are not concentrated in crystals since they are secreted to the environment or the culture medium (Estruch et al., 1996; Chakroun et al., 2016). Vip3 proteins are very toxic to Lepidoptera (Chakroun et al., 2012; Hernández-Martínez et al., 2013). The mode of action of Cry proteins involves solubilization and activation by gut proteases, recognition and binding to midgut receptors, pore formation, and cell lysis, finally causing the death of the insect (Bravo et al., 2007). Vip3 proteins are also cleaved by midgut proteases and bind to specific receptors in the insect midgut, which are different from those of Cry proteins (Chakroun et al., 2016).

Biopesticides based on *B. thuringiensis* have been used since the middle of the last century. They are widely used in organic farming and, compared to chemical insecticides, they have some advantages since they lack safety periods and are harmless to non-target organisms, including insect predators and other beneficial insects. On the other hand, due to the low persistence of the active ingredient in the environment, repeated applications is common practice. This has led to some outbreaks of resistance to *B. thuringiensis* commercial products (Ferré & Van Rie, 2002) and more recently, to Bt-crops (transgenic crops protected from insects by the expression of *cry* or/and *vip3* genes) expressing a single Cry protein (Tabashnik et al., 2009).

Grapholita molesta (Busck, 1916) (Lepidoptera: Tortricidae) is native from Asia, although it is present in all temperate zones of Europe, America, Africa and Australia. It is considered a pest of economic importance around the world since it causes damage to production of fruits like peaches, nectarines, apricots and apples, and can be associated with attacks in others crops (Myers et al., 2007; Piñero and Dorn, 2009; Kirk et al., 2013). Data on the insecticidal activity of *B. thuringiensis* Cry proteins for the control of other Tortricidae pests, such as *Cydia pomonella* and *Lobesia botrana*, have been reported (Boncheva et al., 2006; Ruiz de Escudero et al., 2007). However, despite the fact that formulations

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of *B. thuringiensis* have been tested in the field and laboratory for the control of *G. molesta* (Rama et al., 2001; Monteiro and Souza, 2010), there are no reports on the activity of individual *B. thuringiensis* proteins on this pest.

In the present study, we show the effectiveness of *B. thuringiensis* bioinsecticides for the control of *G. molesta*, test the insecticidal activity of some individual Cry1A and Vip3 proteins, and show an antagonistic effect in some of their combinations.

2. Materials and methods

2.1. Insects

A colony of *G. molesta* was established and maintained at the University of Valencia (Spain), originally obtained from Entomos AG (Switzerland). Insects were reared on semi-artificial diet (Guennelon et al., 1981) under controlled conditions of temperature (25 ± 2 °C), humidity (RH 70 ± 10%) and photoperiod (16:8 h light:dark) (Arioli et al., 2007). The same diet and rearing conditions were used in the bioassays.

2.2. Cry and Vip3 proteins

Escherichia coli clones carrying plasmids with *cry1Aa*, *cry1Ac* and *cry1Ca* genes were kindly provided by Ruud de Maagd (Plant Research International, Wageningen, Netherlands). The *vip3Aa16* gene was kindly provided by Slim Tounsi (Centre de Biotecnologie de Sfax) and *vip3Af1* by Jeroen Van Rie (Bayer CropScience, Ghent, Belgium). The *vip3Ca2* gene was isolated from an autochthonous *B. thuringiensis* strain (Palma et al., 2012).

Cry proteins were expressed, solubilized and trypsin activated as described elsewhere (Hernández-Martínez et al., 2008) and stored frozen in 20 mM Tris, 150 mM NaCl, pH 8.0. IPTG was used to induce expression of Vip3A proteins from *E. coli* BL21 cells and Vip3Ca from *E. coli* WK6 cells. The cells were then centrifuged and resuspended in lysis buffer (20 mM phosphate saline buffer, pH 8.0 with 3 mg/mL lysozyme, 10 µg/mL DNAse and 100 µM PMSF) and incubated with shaking for 30 min at 37 °C. The cells were lysed by sonication and the supernatant recovered by centrifugation at 27,000*g* and filtered through sterile 0.45 µm and 0.22 µm cellulose acetate filters. The Vip3 proteins were purified by isoelectric point precipitation (IPP) (Chakroun et al., 2012) with 0.1 M acetic acid to reach pH 5.5 (Vip3Aa), pH 5.4 (Vip3Af) or pH 5.95 (Vip3Ca). The partially purified Vip3 proteins were solubilized and stored frozen in 20 mM Tris, 150 mM NaCl, pH 9.0.

The concentration of Cry1 and Vip3 proteins was estimated by the Bradford method (Bradford, 1976). The quality of the expressed proteins was checked by 12% SDS-PAGE with Coomassie brilliant blue R-250 (Sigma-Aldrich) staining (Fig. 1). Cry1A activated proteins were observed as bands of around 62 kDa and Vip3 protoxins as bands of approximately 89 kDa.

2.3. Bacillus thuringiensis commercial products

DiPel DF® (*B. thuringiensis* subsp. *kurstaki*) and XenTari GD® (*B. thuringiensis* subsp. *aizawai*) (formulations as wettable granules) were kindly provided by Kenogard S.A. (Barcelona, Spain).

2.4. Bioassays

Different concentrations of formulations and protein solutions were dispensed on the diet surface. Prior to the sample application, the surface of the diet was sterilized under UV light for 10 min. A volume of 50 µL of each concentration was applied on the surface

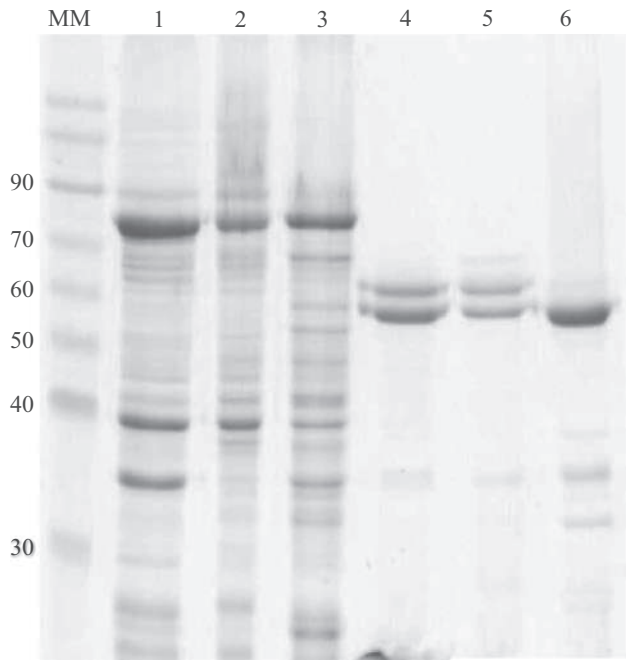

Fig. 1. SDS-PAGE of *Escherichia coli* lysates. MM, Molecular Mass Markers "PINK Plus Prestained Protein Ladder" (Genedirex); lane 1, Vip3Aa; lane 2, Vip3Af; lane 3 Vip3Ca; lane 4, Cry1Aa; lane 5, Cry1Ac; lane 6 Cry1Ca.

of solidified diet (2 cm^2 multiwell plates, Bio-Cv-16, C-D International) and let dry in a flow hood. Once dried, one larva was transferred to each well using 16 neonates per replicate, with two replicates per concentration. Preliminary assays were done at 100 and 1000 ng/cm^2 for DiPel®, XenTari® and Cry1 proteins, and at 2500 ng/cm^2 for Vip3 proteins. The larvae mortality was scored after 7 days.

Dose-response bioassays were performed only for those proteins causing a mortality higher to 90% in the preliminary assays. At least seven serial dilutions and a control with just buffer were tested for each protein. Bioassays were carried out in triplicate with sixteen neonate larvae per replicate (n = 48). Mortality was scored after 7 days. Only bioassays for which the mortality in the controls was lower than 12% were considered.

2.5. Statistical analyses

Estimates of LC_{50} and LC_{90} were obtained using the POLO-PC software (LeOra software, Berkeley, CA). LC_{50} and LC_{90} values were considered significantly different if their 95% fiducial limits (FL_{95}) did not overlap.

Tests for possible synergistic/antagonistic interactions between Vip3Aa and Cry1 proteins were initially performed at a single concentration of each protein. The expected mortality was estimated, assuming simple independent action, by the formula:

$$P = 1 - (1 - P_1)(1 - P_2)$$

which is equivalent to equation 11.33 of Finney (1971). P_1 and P_2 represent the proportions of dead larvae for toxins 1 and 2, respectively. Significance of deviations between the observed and expected mortality values was determined using Fisher's exact test and Chi-square test. A second type of experiment to test for interactions between Vip3Aa and Cry1 proteins was carried out with dose-response assays in which the proportions of two proteins in the mixture were close to the ratio between their LC_{50} values. The expected LC_{50} value of the mixture was estimated assuming simple

similar action (Finney, 1971) according to the formula of Tabashnik (1992), which derives from equation 11.8 from Finney (1971):

$$LC_{50(m)} = \left[\frac{r_a}{LC_{50(a)}} + \frac{r_b}{LC_{50(b)}}\right]^{-1}$$

where a and b refer to the components the mixture, r_a and r_b are the relative proportions of a and b. The ratio of the observed LC_{50} of the mixture over the expected $LC_{50(m)}$ is a measure of the antagonism. An antagonism factor (AF) greater than 1 indicates an antagonistic interaction. If the ratio is equal to 1 indicates an additive toxicity, and a value lower than 1 indicates that the interaction is a synergistic.

3. Results

3.1. Susceptibility of G. molesta to Cry and Vip proteins and B. thuringiensis commercial products

A first approach to determine whether *G. molesta* was susceptible to *B. thuringiensis* commercial products and their individual insecticidal proteins consisted on performing bioassays at a high concentration of the insecticidal samples (Table 1). The two commercial products (DiPel® and XenTari®) produced high mortality to *G. molesta* neonate larvae. Among the Cry1 and Vip3 proteins, Cry1Aa, Cry1Ac, Cry1Ca, Vip3Aa and Vip3Af showed high toxicity to *G. molesta*. In contrast, Vip3Ca was nontoxic.

Response-dose assays were carried out for those samples that showed highest insecticidal activity. DiPel® was slightly more toxic than XenTari®, with significantly lower LC_{50} and LC_{90} values (Table 2). Among the individual proteins, all except Vip3Ca were very active, with LC_{50} values between 1.8 ng/cm^2 (Vip3Aa) and 57 ng/cm^2 (Cry1Ca). Despite significant differences at the LC_{50} level, no significant differences of activity were found at the LC_{90} level among the active proteins.

Table 1
Susceptibility of *G. molesta* neonates to Cry1 activated toxins, Vip3A protoxins, and *B. thuringiensis* commercial products.[a]

		Mortality (%)		
		100 ng[b]/cm^2	1000 ng/cm^2	2500 ng/cm^2
Commercial product	DiPel®	100.00 ± 0.00	100.00 ± 0.00	
	XenTari®	80.00 ± 0.11	100.00 ± 0.00	
Toxin	Cry1Aa	97.00 ± 0.03	100.00 ± 0.00	
	Cry1Ac	93.00 ± 0.07	96.87 ± 0.03	
	Cry1Ca	60.00 ± 0.07	100.00 ± 0.00	
Protoxin	Vip3Aa			100.00 ± 0.00
	Vip3Af			94.00 ± 0.00
	Vip3Ca			23.00 ± 0.03

[a] Bioassays were performed with 16 larvae per replicate, with two replicates (n = 32), and the mortality was scored at 7 days.
[b] Nanograms of formulated product in the case of the commercial products, and of solubilized protein in the case of individual proteins.

Table 2
Susceptibility of *G. molesta* neonates to Cry1 activated toxins and Vip3A protoxins in dose-response assays[a].

Protein	Slope ± SE[b]	LC_{50} (FL_{95})[c]	LC_{90} (FL_{95})[c]
Cry1Aa	0.59 ± 0.08	7.5 (2.7–29)a,b	1120 (160–154,000)a
Cry1Ac	1.21 ± 0.12	24 (11–64)b,c	252 (86–2700)a
Cry1Ca	1.13 ± 0.11	57 (30–116)c	780 (310–4000)a
Vip3Aa	0.49 ± 0.77	1.8 (0.5–4.5)a	700 (130–37,000) a
Vip3Af	0.81 ± 0.10	27 (13–86) b, c	1000 (240–20,000) a
Vip3Ca		>3600	
DiPel®	1.75 ± 0.22	13 (9–17)	69 (46–127)
XenTari®	1.40 ± 0.14	33 (18–56)	269 (137–818)

[a] Bioassays were performed with 16 larvae per replicate, with three replicates (n = 48), and the mortality was scored at 7 days.
[b] Standard error.
[c] Values expressed in ng/cm^2 with 95% fiducial limits. LC values followed by the same letter (in the same column) are not significantly different based on the overlap of FL.

3.2. Interactions of Vip3Aa with Cry1 proteins

Combinations between Vip3Aa and the active Cry1 proteins were tested to check for synergistic or antagonistic interactions. The concentrations to use in the mixture were chosen close to their respective LC_{50} values. The observed mortality was compared to the expected mortality assuming no interaction (Table 3). Significant antagonistic interactions were found for Vip3Aa-Cry1Aa and Vip3Aa-Cry1Ca ($P < 0.001$). No significant interaction was found for Vip3Aa-Cry1Ac.

The interaction Vip3Aa-Cry1Aa and Vip3Aa-Cry1Ca was investigated in dose-response assays at the 1:1 ratio and at ratios close to their respective LC_{50} ratios (LC_{50} Cry1 to LC_{50} Vip3 and *vice versa*). The results, at the LC_{50} level, confirmed the antagonism for both Vip-Cry combinations in the different ratios tested (Table 4).

Table 3
Susceptibility of *G. molesta* to combinations of Vip3Aa and Cry1 proteins[a].

Protein	Concentration (ng/cm^2)	Mortality (%)		P values	
		Observed[b]	Expected	Fischer's test	Chi-square
Cry1Aa	5	32	45.8[c]		
Cry1Ac	24	63	50[c]		
Cry1Ca	57	64	50[c]		
Vip3Aa	1.2	37	46.5[c]		
Vip3Aa+Cry1Aa	1.2 + 5	12	57[d]	<0.0001	<0.0001
Vip3Aa+Cry1Ac	1.2 + 24	69	76[d]	0.80	0.61
Vip3Aa+Cry1Ca	1.2 + 57	42	77[d]	0.0018	0.0013

[a] Bioassays were performed with 16 larvae per replicate, with three replicates (n = 48), and the mortality was scored at 7 days.
[b] Percentage of death insects due the action the individual toxins and their mixtures at the dose tested.
[c] Expected mortality according to the Probit linear regression analysis (Table 2).
[d] Expected mortality assuming simple independent action calculated with the 11.33 equation (Finney, 1971) using the observed mortality values for each individual protein.

Table 4
Evaluation antagonism, at the LC_{50} level, of different mixtures of Vip3Aa and Cry1 proteins to *G. molesta* larvae.[a]

Proteins	Ratios[b]	b ± SE[c]	LC_{50} (ng/cm^2)		AF[f]
			Observed (FL_{95})[d]	Expected[e]	
Cry1Aa	0:100	0.59 ± 0.08	7.5 (2.7–29.4)		
Vip3Aa+Cry1Aa	25:75	1.39 ± 0.14	31 (19–47)	4.2	7.4
	50:50	1.86 ± 0.20	71 (52–97)	2.9	24.5
	75:25	1.73 ± 0.22	60 (42–85)	2.2	27.0
Vip3Aa	100:0	0.49 ± 0.77	1.8 (0.5–4.5)		
Vip3Aa+Cry1Ca	97:3	1.38 ± 0.19	120 (48–288)	1.86	64.5
	50:50	1.47 ± 0.15	62 (33–116)	3.49	17.8
	3:97	1.57 ± 0.15	364 (250–536)	29.1	12.5
Cry1Ca	0:100	1.13 ± 0.11	57 (30–116)		

[a] Bioassays were performed with 16 larvae per replicate, with three replicates (n = 48), and the mortality was scored at 7 days.
[b] The ratios were expressed as percentage between Vip3 and Cry1 proteins in the mixture.
[c] Slope ± standard error.
[d] LC values with 95% fiducial limits.
[e] Expected mortality considering simple similar action.
[f] Antagonism factor (calculated as the ratio of the observed LC_{50} to expected LC_{50}).

Moreover, the data indicate that the degree of antagonism varied depending on the Vip3 to Cry ratio. For both Cry1 proteins, the antagonism was higher when the proportion of Vip3 in the mixture was higher. This phenomenon is much more evident with Cry1Ca and Vip3Aa: just a 3% of Cry1Ca in the mixture led to an AF = 64.5, which represents a reduction in the potency of Vip3Aa of 67-fold (LC_{50} of the mixture divided by the LC_{50} of Vip3Aa pure).

4. Discussion

Despite of *B. thuringiensis* being the most common biological agent used in pest control, little has been reported about the control of *G. molesta* and only some commercial formulations have been tested in field and under laboratory conditions. Some authors have reported contradictory data in relation to field assays. Rama et al. (2001) tested preparations of *B. thuringiensis* in Italian farms and no toxic effect against *G. molesta* was observed. The authors justified this result by the small short life of the tested formulations. In contrast, Monteiro and Souza (2010) reported that formulations of *B. thuringiensis* were able to control this pest to a level not significantly different to the chemical insecticides. The results obtained by us clearly show that DiPel® and XenTari® are very effective for the control of *G. molesta* under laboratory conditions.

The results with individual *B. thuringiensis* proteins showed that, with the exception of Vip3Ca, the rest were highly active against this pest. For Cry1 proteins, Cry1Aa was not significantly different to Cry1Ac at the LC_{50} level, but when compared to Cry1Ca, Cry1Aa is 7.6-fold more toxic. At LC_{90} level, no significant difference was found among the Cry1 proteins. For Vip3A proteins, Vip3Aa was 15-fold more toxic than Vip3Af at LC_{50} level, though this difference was not observed at the LC_{90} level.

Toxicity of Cry and Vip3 proteins against other pest species of the order Tortricidae has already been reported by other authors (Herrero et al., 2001; Boncheva et al., 2006; Sauka et al., 2007; Ruiz de Escudero et al., 2007; Li and Bouwer, 2012; Ruiz de Escudero et al., 2014; Baranek et al., 2015). In those cases, Cry1Aa, Cry1Ac and Cry1C were toxic, as they are against *G. molesta*. For Vip3A proteins, Vip3Aa and Vip3Af are toxic to *C. pomonella* and *L. botrana*, as they are for *G. molesta*.

Studies on synergism/antagonism between insecticidal proteins are potentially important in providing evidence of possible unexpected effects, not only on the target pest, but on non-target organisms. This has even become a requirement by some regulatory agencies for safety assessment of GM crops combining different insecticidal proteins with the same or different mode of action (Levine et al., 2015). With *G. molesta*, we have found antagonistic interactions between Vip3Aa and Cry1Aa or Cry1Ca, though not between Vip3Aa and Cry1Ac. We have considered evidence of antagonism when the expected LC_{50} value did not fall within the fiducial limits of the observed LC_{50} value of the mixture. The antagonistic effect became more evident as the proportion of Vip3Aa in the mixture increased and this effect was especially drastic in the 97:3 Vip3Aa:Cry1Ca mixture, with an AF of 64.5 (Table 4). Interactions between Vip3Aa and Cry1 proteins had also been reported in other insect species. Combinations of Vip3Aa, Vip3Ae and Vip3Af with Cry1Aa, Cry1Ac and Cry1Ca were found antagonistic in *Heliothis virescens*. Furthermore, the combination Vip3Aa-Cry1Ca showed different results depending on the species tested. For *H. virescens* and *Spodoptera frugiperda*, the interaction was antagonistic, whereas, the same combination in *Diatraea saccharalis* was synergistic (Lemes et al., 2014). Mixtures of Vip3Aa, Vip3Ae and Vip3Af with Cry1Aa, Cry1Ab, Cry1Ac, Cry1Ca and Cry1Ea were tested against *Anticarsia gemmatalis* and *Chrysodeixis includens* and synergistic interactions were found in most of them, especially the strong synergisim of the combination Vip3Af-Cry1Ab in both insect species (Crialesi-Legori et al., 2014). A Vip3Aa and Cry1Ia mixture resulted in a slight antagonism in *Spodoptera eridania* while presenting synergism in other three *Spodoptera* species (Bergamasco et al., 2013). In trying to explain antagonistic interactions, some authors suggested that the two proteins could form a complex that would lead to their inactivation (Lemes et al., 2014). Another speculative explanation would be that antagonism results from the interaction of both proteins with the same membrane protein, even if they bind to different epitopes (Lemes et al., 2014). In our study, being that Vip3Aa is much more toxic than Cry1Ca to *G. molesta* and as little as 3% of the latter is enough to decrease the potency of Vip3Aa so dramatically (67-fold), it seems clear that the Cry protein is blocking some key factor for the toxicity of Vip3Aa. Despite the fact that the phenomenon of the antagonistic/synergistic interaction between some Vip3A and Cry1 proteins seems quite general, the biochemical/physiological basis have not been searched for yet. Future work is needed to find out at which level the interactions are taking place (in the lumen, previously to binding to the membrane, at the binding step, or further down the toxic process) and, ideally, to pinpoint the determinants responsible for such interactions.

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Analysis of cross-resistance to Vip3 proteins in eight insect colonies, from four insect species, selected for resistance to *Bacillus thuringiensis* insecticidal proteins

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ABSTRACT

Bacillus thuringiensis Vip3 proteins are synthesized and secreted during the vegetative growth phase. They are activated by gut proteases, recognize and bind to midgut receptors, form pores and lyse cells. We tested the susceptibility to Vip3Aa and Vip3Ca of Cry1A-, Cry2A-, Dipel- and Vip3-resistant insect colonies from different species to determine whether resistance to other insecticidal proteins confers cross-resistance to Vip3 proteins. As expected, the colonies resistant to Cry1A proteins, Dipel (*Helicoverpa armigera*, *Trichoplusia ni*, *Ostrinia furnacalis* and *Plodia interpunctella*) or Cry2Ab (*H. armigera* and *T. ni*) were not cross-resistant to Vip3 proteins. In contrast, *H. armigera* colonies resistant to Vip3Aa or Vip3Aa/Cry2Ab showed cross-resistance to the Vip3Ca protein. Moreover, the Vip3Ca protein was highly toxic to *O. furnacalis* (LC_{50} not significantly different from that of Cry1Ab), whereas the Vip3Aa protein only showed moderate growth inhibition at the highest concentration tested (100 μg/g of diet). These results extend the cross-resistance studies between Vip3 and Cry proteins, show for the first time cross-resistance between proteins within the Vip3 subfamily, and points to *O. furnacalis* as a target for the Vip3Ca protein.

1. Introduction

Vip3 insecticidal proteins are synthesized by *Bacillus thuringiensis* (Bt) during the vegetative growth phase and are active against lepidopteran insects (Chakroun et al., 2016a; Estruch et al., 1996). Vip3 proteins are classified into three protein subfamilies based on their amino acid sequence identity: Vip3A, Vip3B, and Vip3C (Crickmore et al., 2013). Most studies on the insecticidal activity of Vip3 proteins have been performed on the Vip3A protein subfamily, in particular, with the Vip3Aa protein. Vip3Ca was discovered more recently and show some toxic effect against some lepidopteran species (Palma et al., 2012; Gomis-Cebolla et al., 2017). In contrast to Vip3Ca, Vip3A proteins have a broad insecticidal spectrum against lepidopteran pests (Chakroun et al., 2016a). The fact that the insecticidal spectrum and the mode of action of the Vip3 proteins differ from that of the Cry1 and Cry2 proteins, makes Vip3 proteins good candidates to be used in combinations with Cry proteins in Insect Resistance Management (IRM) programs.

The mode of action of Vip3 proteins (Vip3A and Vip3C) shares some similarities to that of the Cry proteins in that they are synthesized in the form of protoxins, which are further processed by midgut proteases rendering the active toxin (Estruch et al., 1996; Yu et al., 1997; Lee et al., 2003; Chakroun et al., 2012; Caccia et al., 2014; Gomis-Cebolla et al., 2017). The activated toxins bind to specific receptors in the midgut membrane leading to the disruption of the midgut epithelial cells and eventual death of the larva. The Vip3 receptors are not shared by Cry proteins (Lee et al., 2006; Abdelkefi-Mesrati et al., 2009; Sena et al., 2009; Gouffon et al., 2011; Chakroun et al., 2014; Gomis-Cebolla et al., 2017). However, it has been recently shown that the Vip3Aa and Vip3Ca proteins compete for shared binding sites (Gomis-Cebolla et al.,

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2017).

In agriculture worldwide, repeated applications of *Bt* sprays and widespread adoption of *Bt*-crops (transgenic crops protected from insects by the expression of *cry* or/and *vip3* genes) have led to resistance (Ferré and Van Rie, 2002; Ferré et al., 2008, Tabashnik, 2015; Tabashnik et al., 2009). Therefore, in this arms race against insects, it is necessary to keep exploring the potential of new insecticidal proteins for pest control and, at the same time, to test for their compatibility in combinations with other proteins in terms of cross-resistance. Although cross-resistance studies have been performed with Cry1- and Cry2-resistant colonies against Vip3Aa (Jackson et al., 2007; Fang et al., 2007; Anilkumar et al., 2008; Vélez et al., 2013; Huang et al., 2014; Qian et al., 2015; Welch et al., 2015; Horikoshi et al., 2016), and with Vip3Aa-resistant colonies against Cry1A proteins (Mahon et al., 2012; Chackroun et al., 2016b; Pickett et al., 2017), cross-resistance to Vip3Ca has never been tested.

In this study, we tested the susceptibility of Cry1-, Cry2- and Dipel-resistant colonies from four insect species (*Trichoplusia ni*, *Plodia interpunctella*, *Helicoverpa armigera* and *Ostrinia furnacalis)* to the Vip3Aa and Vip3Ca proteins and compared the results to the non-selected controls. In addition, we tested two Vip3Aa-resistant colonies from *H. armigera* for cross-resistance to Vip3Ca.

2. Materials and methods

2.1. Insect colonies

2.1.1. Insect rearing of T. ni strains

Three *T. ni* strains were used to examine their response to Vip3Aa and Vip3Ca. The *T. ni* Cornell laboratory strain (Wang et al., 2007) was used as the susceptible control strain. The two resistant strains were a Cry1Ac-resistant strain, GLEN-Cry1Ac-BCS (Wang et al., 2007), and a Cry2Ab-resistant strain, GLEN-Cry2Ab-BCS (Song et al., 2015). Both of the resistant *T. ni* strains were near-isogenic to the susceptible Cornell strain and the resistance is fixed (i.e., they were homozygous for the resistance genes). The *T. ni* colonies were maintained on artificial diet without exposure to Bt toxins (Bell et al., 1981).

2.1.2. Insect rearing of H. armigera strains

Five *H. armigera* strains were used to determine their response to Vip3Ca. The *H. armigera* susceptible colony, GR, was used as a control (Mahon et al., 2007). The *H. armigera* homozygous resistant colonies ISOC8, (Cry1Ac), Sp15 (Cry2Ab resistant) and Sp85 (Vip3A resistant) were established from lab selection (ISOC8) and positive F_2 tests in 2002 (Sp15) and 2010, (Sp85) respectively. The *H. armigera* Cry2Ab/Vip3A resistant strain was established by reciprocal crosses placing male pupae from one strain with female pupae from the other in cages (Walsh et al., 2014). All of the resistant strains were repeatedly outcrossed to a susceptible colony and reselected with the appropriate toxin(s). The Cry2Ab resistant line Sp15 carries an ABCA2 transporter mutation that confers the phenotype (Tay et al., 2015), but the mechanism of resistance is unknown for ISOC8 (Cry1Ac) and Sp85 (Vip3A). The rearing methods used to maintain *H. armigera* were modified from those described by Teakle and Jensen (1985).

2.1.3. Insect rearing of P. interpunctella strains

Two *P. interpunctella* strains were used to characterize their response to Vip3Aa. The *P. interpunctella* susceptible colony, EP, was obtained from a grain storage bin and has been maintained in the laboratory on cracked wheat diet (Oppert et al., 2010). The resistant colony EP-Dpl500 was selected from the parental EP, with 500 mg Dipel (Bt subspecies *kurstaki*, strain HD-1) per kg diet, gradually increasing the dose to 10,000 mg/kg, the maintenance dose for this resistant colony.

2.1.4. Insect rearing of O. furnacalis strains

Two strains of *O. furnacalis*, a Bt susceptible strain and a Cry1Ab-resistant strain were established in the laboratory. The Bt susceptible strain was collected from the field and had been reared using standard rearing techniques without exposure to any insecticide before bioassays were conducted (Song et al., 1999). The Cry1Ab-resistant strain was selected from the Bt susceptible strain by exposure to trypsin-activated Cry1Ab. The Cry1Ab-resistant strain was initially exposed throughout larval development to Cry1Ab in the artificial diet (2.5 ng of toxin/g diet). The toxin concentration was increased in succeeding generations to target 40–70% mortality in the exposed insects. After 51 generations, larvae were reared on diet containing 400 ng of toxin/g diet. The Cry1Ab-selected colony had developed > 100-fold resistance to Cry1Ab after 35 generations (Xu et al., 2010).

2.2. Source and expression of Vip3 proteins for cross-resistance assays

Vip3Aa (NCBI accession No AAW65132) was overexpressed in recombinant *Escherichia coli* BL21 carrying the *vip3Aa16* gene (Abdelkefi-Mersati et al., 2009). The Vip3Ca protein (NCBI accession No AEE98106) was prepared from recombinant *E. coli* WK6 carrying the *vip3Ca2* gene (Palma et al., 2012).

The Vip3Aa protein was expressed following the conditions described by Chakroun et al., 2012. For the Vip3Ca protein, a single colony was inoculated in 7 ml of LB medium containing 100 µg/ml ampicillin and grown overnight at 37 °C and 180 rpm. A 1/100 dilution of the culture in 700 ml LB medium containing 100 µg/ml ampicillin was further incubated at 37 °C and 180 rpm. The culture was induced with 1 mM IPTG at an OD of 0.7 and it was grown overnight at 37 °C and 200 rpm. Cells were collected at 6000 g for 15 min at 4 °C. The pellet was weighed and suspended in 3 ml lysis buffer (PBS, pH 8.0, containing 3 mg/ml lysozyme, 10 µg/ml DNase, and 100 µM PMSF) per gram of pellet. The sample was incubated at 37 °C for 30 min and then sonicated on ice applying two 1 min pulses at 70 W at a constant duty cycle, separated by a 10-s cooling period on ice. Then, the insoluble material was separated by centrifugation at 16,000 g for 15 min at 4 °C and the soluble cellular fraction sequentially filtered through sterile 0.45 µm and 0.22 µm cellulose acetate filters.

2.3. Purification of Vip3 proteins for cross-resistance assays

Vip3 proteins used for dose-response assays for the *T. ni*, *H. armigera* and *O. furnacalis* colonies were purified by isoelectric point precipitation (IPP) (Chackroun et al., 2012; Gomis-Cebolla et al., 2017). The pH of the lysate was lowered with acetic acid to pH 5.5 for Vip3Aa and pH 5.95 for Vip3Ca. The pellets were recovered by centrifugation at 16,000 g for 10 min and then dissolved in 20 mM Tris, 150 mM NaCl, pH 9, and dialyzed against the same buffer overnight. The Vip3Aa protein used for dose-response assays for the *P. interpunctella* colonies was purified by immobilized metal ion absorption chromatography (IMAC) on a Hi-Trap chelating HP column (GE Healthcare) charged with Ni^{2+} (Fig. 1A) (Chackroun et al., 2012). The purified proteins were frozen at −80 °C and then lyophilized. The concentration of the Vip3 proteins purified by IPP was estimated by densitometry after SDS-PAGE separation. The concentration of the Vip3Aa purified by Hi-Trap chelating HP column was measured by the method of Bradford (Bradford et al., 1976). In both methods, bovine serum albumin (BSA) was used as standard. The purity of the Vip3 proteins was analyzed by SDS-PAGE (Fig. 1).

2.4. Insect toxicity assays

2.4.1. Dose-response assays for the susceptible and resistant T. ni strains

Examination of *T. ni* strains for their susceptibilities to Vip3Aa and Vip3Ca were conducted using the surface contamination method (Kain et al., 2004). Briefly, an aliquot of 200 µl of Vip3Aa or Vip3Ca solution was spread on the surface of diet in 30-ml cups (surface area is approximately 7 cm^2) and 10 neonate larvae were placed in each cup that

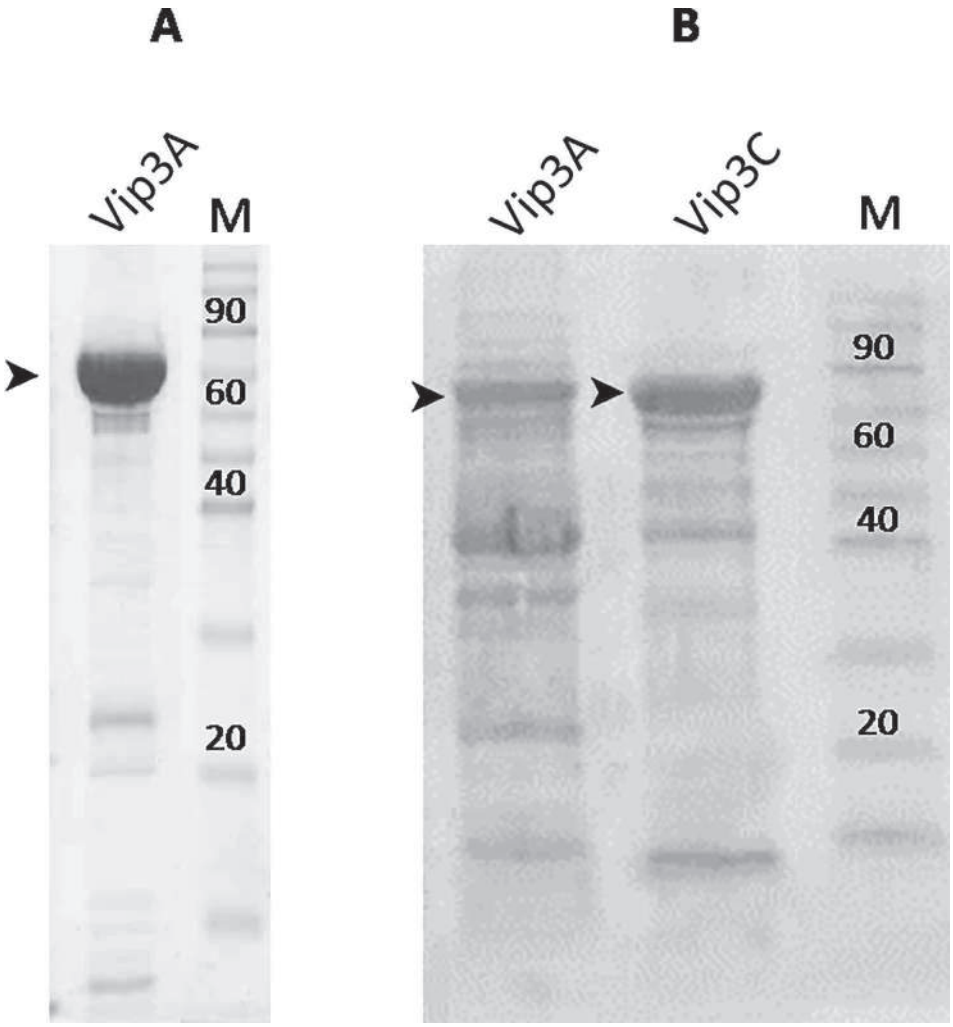

Fig. 1. SDS-PAGE of Vip3Aa and Vip3Ca protoxins after partial purification from *E. coli* extracts. (a) Vip3Aa purified by Hi-Trap chelating HP column charged with Ni^{+2}. (b) Vip3Aa and Vip3Ca purified by isoelectric point precipitation. M: Molecular Weight Markers ("PINK PlusPrestained Protein Ladder", from Genedirex). The arrowhead indicates the band corresponding to the Vip3 protein.

was covered with a lid and placed at 27 °C. Eight concentrations of Vip3Aa or Vip3Ca in 2-fold serial dilutions from 800 to 6.25 µg/ml and a non-toxin control were used for each bioassay. Five replicate cups of larvae (50 larvae in total for each dose) were included for each concentration of toxin. Larval mortality and growth inhibition (larval developmental stage remaining in the first instar) were recorded after 4 days of rearing on diet. The bioassay data were subjected to Probit analysis using the software POLO, LeOra Software to obtain the LC_{50} and IC_{50} of the Vip proteins (Russell et al., 1977).

2.4.2. Dose-response assays for the susceptible and resistant H. armigera strains

The susceptibility of *H. armigera* strains to Vip3Aa and Vip3Ca was conducted using the surface contamination method as follows. Approximately 300 µl of standard diet was added to straight sided 96 well trays providing 0.567 cm^2 of surface area. Once the diet had cooled, 20 µl of a solution containing an appropriate concentration of toxin was added and allowed to air dry. One neonate was placed in each well before it was sealed with a perforated heat-sensitive lid. Trays were incubated at 25 °C, 60% RH, and 14:10 h light: dark for 8 days. Six concentrations of Vip3Ca in 2-fold serial dilutions from 20 to 0.625 µg/cm^2 and a non-toxin control were used for each bioassay. A minimum of three replicate bioassays of larvae (16 larvae in total for each dose) were included for each concentration of toxin. The numbers of dead and alive larvae were counted and the data were subjected to Probit analysis using the software POLO, LeOra Software to obtain the LC_{50} of the Vip proteins (Russell et al., 1977). Moreover, the instars of surviving larvae were recorded to obtain the larval development index (LDI) of surviving larvae.

$$LDI = \frac{[(nL1 \times 1) + (nL2 \times 2) + (nL3 \times 3) + (nL4 \times 4) + (nL5 \times 5)]}{N}$$

where nL1, nL2, nL3, nL4 and nL5 are the numbers of alive larvae in the respective larvae development stage and N refer the total number of alive larvae. We consider as statistically significant all those LDI values where the 95% confidence intervals of the mean did not overlap with another LDI value for the susceptible and resistant population (Cry1Ac-, Cry2Ab-, Vip3A- and Vip3/Cry2Ab-resistant) of *H. armigera*. Confirmation of resistance in the Cry1-, Cry2-, Vip3-, and Vip3/Cry2Ab-resistant insects at the time of Vip3 bioassays was obtained from single dose assays at a discriminant dose (Table S1).

2.4.3. Dose-response assays for the susceptible and resistant P. interpunctella strains

The *P. interpunctella* strains used in the current study are EP (Dipel-susceptible) from which EP-Dpl500 was selected with Dipel, and the parent strain for the Dipel-resistant strain which was selected and maintained on 10,000 mg/kg Dipel (the resistant strain used in this study). The Dpl500 strain was moderately resistant to Cry1F (18-fold) and Cry1Ab (over 100-fold), and very resistant to Cry1Ac (> 3000-

fold) and Dipel (with no mortality observed at the highest dose) (Table S2). The bioassays of *P. interpunctella* strains with Vip3 toxins were conducted using 4 mm round diet disks punched out from a flattened cereal mixture (Herrero et al., 2001). Briefly, 5 µl of 11 different doses of Vip3Aa (from 0.1 to 100 µg/4 mm diet disk for Dipel-susceptible insects, and from 2 to 100 µg/4 mm diet disk for Dipel-resistant insects) and Vip3Ca (from 2 to 100 µg/4 mm diet disk for Dipel-susceptible insects, and from 2 to 100 µg/4 mm diet disk for Dipel-resistant insects) were added to diet disks using a micropipettor, with controls of 5 µl water only for each replicate. Treated diet disks were placed in 16-well black assay trays (Frontier Agricultural Sciences, Newark, DE, USA), and eggs were added to each well (n = 16 individuals per dose, three independent biological replicates for each toxin and *P. interpunctella* strain). Trays were covered with perforated adhesive plastic sheets and incubated at 28 °C and 75% relative humidity (R.H.) in darkness. Mortality was recorded at approximately 21 d. Data was analyzed by Probit analysis using the software POLO, LeOra Software (Russell et al., 1977) to obtain the LC_{50} of the Vip proteins, reported in mg of toxin per 4 mm (15 mg) diet disk.

2.4.4. Dose-response assays for the susceptible and resistant O. furnacalis strains

The susceptibility of *O. furnacalis* neonates to Vip3 proteins (Vip3A and Vip3Ca) was determined in dose-response assays in agar-free semi-artificial diet (He et al., 2005). Briefly, a single neonate was randomly transferred into each well of 48-well tray and then covered with a piece of paper and the lid. Trays were held in a growth chamber for seven days at 27 °C, 80% RH and a 16:8 h photoperiod. Number of dead larvae and the weight of larvae surviving per tray were recorded after seven days of exposure. If a larva had not developed beyond the first instar and weighed ± 0.1 mg, it would be counted as dead for calculating practical mortality. Average larval weight of survivors would be used to determine the larval growth inhibition rate as a function of toxin concentration. Bioassays were repeated on two dates with total of 96 larvae per concentration and included 6–10 concentrations of purified toxin. Dilutions of Vip3 toxins were prepared in 20 mM Tris 150 mM NaCl, pH 9. The same buffer was used as a negative control. Bioassay data were subjected to Probit analysis using the software POLO, LeOra Software 1977 to obtain the LC_{50} of the Vip proteins (Russell et al., 1977). Confirmation of resistance in the Cry1Ab-resistant insects at the time of Vip3 bioassays was obtained from dose-response bioassays with Cry1Ab (Table S3).

3. Results and discussion

3.1. Cry1A and Dipel-resistant colonies

We first tested the vulnerability of susceptible and Cry1A and Dipel-resistant colonies (*P. interpunctella, T. ni, H. armigera* and *O. furnacalis*) against Vip3 proteins (Table 1). Previously it was demonstrated in *T. ni* that Cry1Ac-resistant strains were not cross-resistant to Vip3A proteins (Fang et al., 2007), but Vip3Ca had never been tested. Our results show that Vip3Ca is 5–10-fold less active than Vip3Aa to this insect species and that Cry1Ac-resistance does not confer cross-resistance to either of the two Vip3 proteins. However, growth inhibition assays showed that Cry1Ac-resistant insects were slightly less affected by both Vip3Aa and Vip3Ca (IC_{50} around 2-fold higher) compared to the susceptible insects (Table 2).

P. interpunctella had never been tested for cross-resistance to Vip3 proteins. Although the LC_{50} for Vip3Aa in the Dipel-resistant strain was approximately 5-fold higher than that of the Dipel-susceptible strain, the fiduciary limits were overlapping. Conversely, the Dipel-susceptible strain was approximately 5-fold less susceptible to Vip3Ca than the Dipel-resistant strain, but again with overlapping fiduciary limits. Therefore, cross-resistance to Vip3 proteins was not observed in the two *P. interpunctella* strains. In comparing responses of the two Vip3 toxins to Cry toxins, Dipel-susceptible insects were less susceptible to Vip3 than Cry toxins, whereas Dipel-resistant insects were more susceptible to Vip3 toxins than Dipel, Cry1F, and Cry1Ac (Table S2).

Lack of cross-resistance to Vip3Aa in Cry1Ac-resistant *H. armigera* colony has been previously shown (Mahon et al., 2012; Chackroun et al., 2016b) and, therefore, only Vip3Ca was used in our study. The LC_{50} values for the Cry1Ac-resistant versus susceptible colonies were statistically different, indicating that resistant insects were 3.3-fold more susceptible to Vip3Ca. This difference could reflect a cost in the Cry1Ac-resistant insects which makes them less fit to withstand the same doses of Vip3Ca. As with *P. interpuntella,* fitness costs in Cry1Ac-resistant *H. armigera* may induce increased susceptibility to other toxins, such as Vip3Ca. An assay with sublethal doses of Vip3Ca was performed to test whether the slightly higher mortality produced by this protein on Cry1Ac-resistant larvae was reflected in the development time. As is indicated in Table 3, no significant differences in the larval development index (LDI) were detected between susceptible and Cry1Ac-resistant population.

Regarding *O. furnacalis*, this insect species has never been tested before against Vip3 proteins. The results of *O. furnacalis* (both susceptible and Cry1Ab-resistant strains) with Vip3Aa show that this species is not susceptible to this protein (Table 1). Only at 100 µg/g diet about 50% growth inhibition was observed in the two strains. In contrast, Vip3Ca was highly active to *O. furnacalis* larvae from both strains. The LC_{50} value of Vip3Ca for the susceptible strain was not significantly different from that of Cry1Ab (LC_{50} = 0.23 µg/g, FL = 0.17–0.30) (Table S3). However, because of the different slope values of their respective regression lines (Tables 1 and S2), Vip3Ca has an LC_{90} value (LC_{90} = 0.98 µg/g, FL = 0.81–1.31) lower than that of Cry1Ab (LC_{90} = 2.94 µg/g, FL = 1.94–2.53), suggesting that the former is more effective to control this pest than the latter. The small difference (LC_{50} 3.12-fold and LC_{90} 3 fold) between Vip3Ca LC values of the susceptible and Cry1Ab-resistant insects is statistically different, suggesting that Cry1Ab-resistance in this strain confers minimum cross-resistance to Vip3Ca.

3.2. Cry2Ab-resistant colonies

Prior to this study, cross-resistance to Vip3 proteins was not found in two Cry2A-resistant colonies from *Heliothis virescens* (Jackson et al., 2007). In the present study, Cry2Ab-resistant colonies of *T. ni* and *H. armigera* were tested against Vip3 proteins, and the results were compared to their susceptible controls (Table 1). Cry2Ab-resistant *T. ni* was not cross-resistant to either of the two Vip3 proteins as measured by mortality (Table 1) or growth inhibition (Table 2).

Cry2Ab-resistant *H. armigera* had never been tested for cross-resistance to Vip3 proteins, though lack of cross resistance or genetic linkage with Cry2Ab-resistance loci had been reported for Vip3Aa-resistant colonies (Mahon et al., 2007; Mahon et al., 2012; Chackroun et al., 2016b). Similarly to Cry1Ac-resistant *H. armigera* colony, the Cry2Ab-resistant insects were slightly more vulnerable (2.3-fold) than susceptible ones (Table 1). As with Cry1Ac-resistant insects, sublethal doses of Vip3Ca did not drive differences in larvae development (Table 3).

3.3. Vip3Aa-resistant colonies

Cross-resistance within the Vip3 subfamily of proteins has not yet been established. We tested the susceptibility of two Vip3-resistant *H. armigera* colonies (one resistant to Vip3Aa alone and the other resistant toVip3Aa and Cry2Ab) against the Vip3Ca protein (Table 1). The highest Vip3Ca doses tested (20 µg/cm^2 for the Vip3Aa-resistant insects and 10 µg/cm^2 for the Vip3Aa/Cry2Ab-resistant insects) only caused a mortality of 4.7% and 6.2%, respectively. Compared to the mortality observed in the susceptible control insects and the Cry1- and Cry2-resistant insects, these results clearly indicate that resistance to Vip3Aa

Table 1
Evaluation of the susceptibility to Vip3 proteins of susceptible and Cry1-, Cry2-, Dipel- and Vip3-resistant insect colonies from different insect species.

Insect species	Protein tested	Replicates	Colony	Slope ± SE[1]	LC_{50} (FL_{95})[2]	Resistance[3] ratio
T. ni					µg/cm^2	
	Vip3Aa	R1[4]	Susceptible	4.0 ± 0.4	0.95 (0.84–1.09) a	–
			Cry1Ac-resistant	5.0 ± 0.6	1.41 (1.07–1.91) a	1.48
			Cry2Ab-resistant	2.7 ± 0.3	1.10 (0.51–1.77) a	1.15
		R2[4]	Susceptible	4.3 ± 0.5	1.05 (0.65–1.80)[5] a	–
			Cry1Ac-resistant	4.2 ± 0.4	2.16 (1.30–3.83)[5] a	2.05
			Cry2Ab-resistant	3.4 ± 0.4	1.63 (1.36–1.90) a	1.55
	Vip3Ca	R1[4,§]	Susceptible	2.5 ± 0.2	8.53 (5.42–15.09) [b]	–
			Cry1Ac-resistant	2.5 ± 0.3	10.02 (6.07–18.3) b	1.17
			Cry2Ab-resistant	1.6 ± 0.2	16.12 (8.33–82.09) b	1.88
		R2[4,§]	Susceptible	3.3 ± 0.4	3.02 (2.53–3.54) c	–
			Cry1Ac-resistant	2.9 ± 0.3	4.95 (2.82–8.33) c	1.63
			Cry2Ab-resistant	3.0 ± 0.3	4.05 (3.40–4.78) c	1.33
P. interpunctella					µg/15 mg diet disk	
	Vip3Aa		Susceptible	0.43 ± 0.09	15.9 (2.25 – 151) d	–
			Dipel-resistant	0.65 ± 0.16	78.7 (27.2–792) d	4.95
	Vip3Ca		Susceptible	0.58 ± 0.20	67.5 (5.87–394) e	–
			Dipel-resistant	0.42 ± 0.12	13.7 (1.04–76.0) e	0.20
H. armigera					µg/cm^2	
	Vip3Ca		Susceptible	2.1 ± 0.20	5.34 (4.34–6.65) f	–
			Cry1Ac-resistant	2.1 ± 0.30	1.60 (1.11–2.11) g	0.30
			Cry2Ab-resistant	1.9 ± 0.27	2.36 (1.70–3.14) g	0.44
			Vip3Aa-resistant	–	NA[6]	0
			Vip3Aa/Cry2Ab-resistant	–	NA[6]	0
O. furnacalis					µg/g	
	Vip3Aa		Susceptible	–	UD[7]	–
			Cry1Ab-resistant	–	UD[7]	0
	Vip3Ca		Susceptible	2.52 ± 0.37	0.31 (0.22–0.38)h	–
			Cry1Ab-resistant	1.67 ± 0.15	0.97 (0.74–1.22)i	3.12

[1] SE: Standard error of the slope.
[2] LC_{50} values followed by the same letter are not significantly different from their corresponding susceptible strain based on the overlap of fiducial limits (FL).
[3] Resistance Ratio was calculated dividing the LC_{50} value of the resistant strain by the LC_{50} value of the susceptible strain.
[4] The dose-response assays, R1 and R2, are biological replications. Within each biological replication, three *T. ni* strains were assayed using the same Vip solutions at the same time.
[5] FL_{90}, instead of FL_{95}, was calculated and presented, as the heterogeneity of the data was above the default threshold ($g > 0.5$) by POLO for adequate FL_{95} calculation.
[6] NA: Non active. The highest dose tested (20 µg/cm^2 for the Vip3-resistant and 10 µg/cm^2 for theVip3Aa/Cry2Ab-resistant) caused a mortality of 4.7% and 6.2%, respectively.
[7] UD: Unable to determine. The highest dose tested (100 µg/g for the susceptible and resistant *O. furnacalis* strains) there was not significant mortality observed. However, it showed about 50% growth inhibition.
[§] The differences observed in the Vip3 proteins were considered as variations between replicates.

Table 2
Evaluation of the growth inhibition to Vip3Aa and Vip3Ca of susceptible and Cry1-, Cry2-resistant *T. ni* insect colonies.

Insect species	Protein tested	Replication	Colony	Growth inhibition		Resistance ratio[4]
				Slope ± SE[1]	IC_{50} (FL_{95})[2]	
T. ni					µg/cm^2	
	Vip3Aa	R1[3]	Susceptible	5.5 ± 0.7	0.33 (0.30–0.37) a	–
			Cry1Ac-resistant	4.3 ± 0.5	0.64 (0.44 0.86) b	1.93
			Cry2Ab-resistant	4.0 ± 0.7	0.24 (0.18–0.29) a	0.72
		R2[3]	Susceptible	4.0 ± 0.5	0.30 (0.26–0.35) a	–
			Cry1Ac-resistant	3.6 ± 0.4	0.64 (0.55–0.73) b	2.10
			Cry2Ab-resistant	5.0 ± 0.9	0.27 (0.22–0.31) a	0.88
	Vip3Ca	R1[3]	Susceptible	3.4 ± 0.3	1.17 (1.00–1.35) c	–
			Cry1Ac-resistant	4.3 ± 0.5	2.20 (1.34–3.42) c	1.88
			Cry2Ab-resistant	4.2 ± 0.5	1.38 (1.21–1.57) c	1.18
		R2[3]	Susceptible	4.2 ± 0.7	0.41 (0.33–0.47) d	–
			Cry1Ac-resistant	3.8 ± 0.6	0.93 (0.75–1.09) c	2.28
			Cry2Ab-resistant	4.8 ± 0.7	0.54 (0.45–0.62) d	1.33

[1] SE: Standard error of the slope.
[2] IC values followed by the same letter are not significantly different based on the overlap of FL.
[3] The dose-response assays, R1 and R2, are biological replications. Within each biological replication, three *T. ni* strains were assayed using the same Vip solutions at the same time.
[4] Resistance Ratio was calculated dividing the LC_{50} value of the resistant strain by the LC_{50} value of the susceptible strain.

Table 3
Evaluation of the larval development index to Vip3Ca of susceptible and Cry1-, Cry2-, Vip3-, and Vip3/Cry2Ab-resistant *H. armigera* insect colonies.

Dose (μg/cm^2)	LDI[1] ± CI[2]				
	Susceptible	Cry1Ac-resistant	Cry2Ab-resistant	Vip3A-resistant	Vip3A/Cry2Ab-resistant
20	1.06 ± 1.25	0.62 ± 2.00	0.00 ± 0.00	3.58 ± 2.86	–
10	1.00 ± 1.24	0.83 ± 3.59	1.09 ± 4.69	3.73 ± ND*	3.56 ± 5.53
5	2.00 ± 1.17	1.72 ± 3.96	2.10 ± 2.24	3.9 ± ND*	3.60 ± 5.15
2.50	2.70 ± 0.61	2.27 ± 1.87	2.66 ± 2.58	3.9 ± ND*	3.60 ± 5.10
1.25	2.54 ± 0.56	2.83 ± 1.84	2.87 ± 2.93	3.9 ± ND*	3.62 ± 4.77
0.60	3.05 ± 0.56	3.05 ± 1.80	3.12 ± 1.83	3.9 ± ND*	3.80 ± 2.80
Controls (μg/cm^2)					
0	3.60 ± 0.63	3.50 ± 1.29	3.78 ± 1.28	3.20 ± 2.48	3.65 ± 4.44
Cry1Ac[3]	0.16 ± 0.43	2.97 ± 0.54	0.00 ± 0.00	0.0 ± 0.0	0.00 ± 0.00
Cry2Ab[3]	0.17 ± 0.43	1.00 ± 0.00	3.80 ± 0.94	0.0 ± 0.0	3.60 ± 5.10
Vip3A[3]	0.67 ± 0.54	0.66 ± 2.87	1.00 ± 0.00	2.90 ± 1.20	3.40 ± 6.35

[1] Larval development index calculated as: LDI = [1 × (Number of L1 larvae) + 2 × (Number of L2 larvae) + 3 × (Number of L3 larvae) + 4 × (Number of L4 larvae) + 5 × (Number of L5 larvae)]/Total of alive larvae. Differences between LDI values from the susceptible and resistant populations were considered statistically significant if the 95% confidence intervals (CI) of the mean did not overlap.

[2] CI: Confidence interval of the mean.

[3] Discriminant dose for Cry1Ac (0.25 μg/cm^2), Cry2Ab (0.25 μg/cm^2), and Vip3Aa (20 μg/cm^2), respectively.

* ND: Not possible to calculate the 95% CI of the Vip3A-resistant *H. armigera* colony because the assay was done with one replicate.

conferred cross-resistance to Vip3Ca. This conclusion is supported by the results in Table 3, where Vip3Ca did not cause any delay in the development of the two Vip3Aa-resistant colonies.

The results obtained regarding cross-resistance are in agreement with the differences in the mode of action of Cry and Vip3 proteins. Several studies demonstrated that Vip3Aa does not share binding sites with Cry1 or Cry2 proteins (Lee et al., 2006; Abdelkefi-Mesrati et al., 2009; Sena et al., 2009; Gouffon et al., 2011; Chakroun et al., 2014) and recent work showed that Vip3Aa and Vip3Ca share binding sites in *Mamestra brassicae* (Gomis-Cebolla et al., 2017). Some of the Cry-resistant insect colonies evaluated in this study had previously been examined for alteration in Cry protein binding and reduced binding was found for the Cry proteins used as the selective agent. For example, Cry1Ac toxins did not bind to gut membrane proteins in the Cry1Ac-resistant *T. ni* colony (Wang et al.; 2007), gut proteins from a Dipel-resistant *P. interpunctella* colony had drastically reduced binding of Cry1Ab (Herrero et al., 2001), and gut proteins from the Cry2Ab-resistant *H. armigera* colony had a greatly reduced binding of Cry2Ab (Caccia et al., 2010). In the case of *O. furnacalis*, no binding assays have been reported with resistant strains. Although binding is not the sole mechanism of resistance to Bt toxins, it is the one that confers more specific and higher levels of resistance (Ferré and Van Rie, 2002; Ferré et al., 2008).

Despite the fact that Vip3Aa and Vip3Ca have been shown to share binding sites in *M. brassicae* (Gomis-Cebolla et al., 2017), our cross-resistance result is not straightforward to explain, Vip3Aa binding apparently was not affected in the Vip3Aa-resistant *H. armigera* colony (Chackroun et al., 2016b). We can think of several scenarios to explain this apparent paradox. First, the binding of Vip3Aa to a "functional" receptor (by this we mean a membrane molecule that triggers the subsequent steps to kill the cell) can be masked by binding to other molecules in the membrane. Examples have been reported for Cry proteins, such as the case of Cry1Ac in *H. virescens*, which binds to three binding sites while only binding site A is responsible for toxicity and, thus, reduced Cry1Ac binding is not detected in resistant insects (Lee et al., 1995; Jakka et al., 2015). Second, assuming that no brush border epitope has been altered in the Vip3Aa-resistant insects, the mechanism of resistance may lay in successive steps, such as membrane insertion, pore formation, or any other post-binding event such as a signal transduction leading to cell death. If this was the case, our results would indicate that Vip3Aa and Vip3Ca, besides sharing a binding epitope in the brush border membrane, share a post-binding step which would be impaired in resistant insects. Whichever the case, our cross-resistance results indicate these two Vip3 proteins share common steps in the mode of action.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jip.2018.05.004.

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7. - CHAPTER 3:

Mode of action of the Vip3Ca insecticidal protein from *Bacillus thuringiensis*

These results are included in:

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Insecticidal spectrum and mode of action of the *Bacillus thuringiensis* Vip3Ca insecticidal protein

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ABSTRACT

The Vip3Ca protein, discovered in a screening of Spanish collections of *Bacillus thuringiensis*, was known to be toxic to *Chrysodeixis chalcites*, *Mamestra brassicae* and *Trichoplusia ni*. In the present study, its activity has been tested with additional insect species and we found that *Cydia pomonella* is moderately susceptible to this protein. Vip3Ca (of approximately 90 kDa) was processed to an approximately 70 kDa protein when incubated with midgut juice in all tested species. The kinetics of proteolysis correlated with the susceptibility of the insect species to Vip3Ca. The activation was faster to slower in the following order: *M. brassicae* (susceptible), *Spodoptera littoralis* (moderately susceptible), *Agrotis ipsilon* and *Ostrinia nubilalis* (slightly susceptible). Processing Vip3Ca by *O. nubilalis* or *M. brassicae* midgut juice did not significantly changed its toxicity to either insect species, indicating that the low susceptibility of *O. nubilalis* is not due to a problem in the midgut processing of the toxin. *M. brassicae* larvae fed with Vip3Ca showed binding of this toxin to the apical membrane of the midgut epithelial cells. Histopathological inspection showed sloughing of the epithelial cells with further disruption, which suggests that the mode of action of Vip3Ca is similar to that described for Vip3Aa. Biotin-labeled Vip3Ca and Vip3Aa bound specifically to *M. brassicae* brush border membrane vesicles and both toxins competed for binding sites. This result suggests that insects resistant to Vip3A may also be cross-resistant to Vip3C, which has implications for Insect Resistance Management (IRM).

1. Introduction

Bacillus thuringiensis is a gram positive bacterium that during the vegetative growth phase produces proteins (Vip, phospholipases, proteases, chitinases, etc.) which are secreted to the medium, contributing to the pathogenicity of this bacterium against insects (Schnepf et al., 1998). Vip proteins are a class of secreted insecticidal proteins which were first described in 1996 and which share no sequence homology with the Cry proteins (Estruch et al., 1996). To date, more than 100 *vip3* genes have been identified (http://www.btnomenclature.info/). Vip3Aa was the first member of the family of Vip3 proteins ever described and most studies dealing with the insecticidal activity and mode of action Vip3 proteins have been carried out with this protein (Chakroun et al., 2016). The fact that Vip3A proteins differ from the Cry1 and Cry2 proteins in terms of insecticidal spectra and because they target different midgut binding sites (Lee et al., 2006; Sena et al., 2009; Gouffon et al., 2011; Chakroun and Ferré, 2014) makes them ideal candidates to be used in combination with Cry proteins in IRM programs.

The mode of action of the Vip3Aa protein resembles that of the Cry proteins in that they are synthesized in the form of protoxins which are processed by proteases in the larva midgut rendering the active toxin (Yu et al., 1997; Lee et al., 2003). The activated toxin binds to specific receptors in the midgut membrane (Lee et al., 2003, 2006; Abdelkefi-Mesrati et al., 2009; Sena et al., 2009; Gouffon et al., 2011; Chakroun and Ferré, 2014). Finally,

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the bound toxin provokes the disruption of the midgut epithelial cells (Yu et al., 1997; Abdelkefi-Mesrati et al., 2011a,b) by the formation of pores in the apical membrane (Lee et al., 2003; Liu et al., 2011).

A screening of Spanish collections of *Bacillus thuringiensis* was undertaken to search for novel members of the Vip3 family. As a result, new *vip3* genes were discovered constituting a novel family which was classified as *vip3Ca* (Palma et al., 2012). All Vip3Ca proteins discovered so far contain 803 amino acids (molecular mass of approximately 90 kDa) and they have insecticidal activity to some lepidopteran species. The aim of the present paper was to explore additional insect species potentially susceptible to Vip3Ca and, at the same time, to study its mode of action in *Mamestra brassicae*, an insect species that have shown susceptibility to this protein.

2. Materials and methods

2.1. Insect colonies

Insects were grown in the insectaries of the Public University of Navarra and of the University of Valencia at 25 ± 2 °C, 60 ± 5% RH, and L16: D8 h. *Sesamia nonagrioides*, *Grapholita molesta*, *Cydia pomonella*, *Mamestra brassicae* and *Pectinophora gossypiella* were reared in artificial diet (Poitout and Bues, 1974; Greene et al., 1976; Guennelon et al., 1981; Eizaguirre and Albajes, 1992; Pérez-Guerrero et al., 2004). *Galleria mellonella* was reared in a honey based diet (10.5% glycerine, 22% honey, 14% cornmeal, 5.5% brewer's yeast, 14% wheat flour, 14% infant cereals, 9.5% milk powder and 10.5% water). *Plutella xylostella* and *Tuta absoluta* were reared on leaves of *Brassica oleracea* var. *capitata* and *Solanum lycopersicum*, respectively. *Ephestia kuehniella* larvae were reared in wheat flour. *Nezara viridula* nymphs and adults were reared in *Phaseolus vulgaris* pods supplemented with peanuts (*Arachis hypogaea*). *Drosophila melanogaster* was reared on laboratory in cornmeal/yeast medium.

2.2. Source and expression of Vip3 proteins

Vip3Aa (NCBI accession No. AAW65132) was overexpressed in recombinant *Escherichia coli* BL21 carrying the *vip3Aa16* gene (Abdelkefi-Mesrati et al., 2009). The Vip3Ad (NCBI accession No. CAI43276) protein was expressed from *E. coli* WK6 carrying the *vip3Ad2* gene (kindly supplied by Bayer CropScience N.V., Ghent, Belgium). Vip3Ca (NCBI accession No. AEE98106) protein was prepared from recombinant *E. coli* WK6 carrying the *vip3Ca2* gene (Palma et al., 2012).

The Vip3Aa protein was expressed following the conditions described by Chakroun et al. (2012). In the case of the Vip3Ca and Vip3Ad, a single colony was inoculated in 4 ml of LB medium containing 100 μg/ml ampicillin and grown overnight at 37 °C and 180 rpm. A 1/100 dilution of the culture in 700 ml LB medium containing 100 μg/ml ampicillin was further incubated at 37 °C and 180 rpm. When the OD was in the range value 0.7–0.8, 1 mM IPTG (final concentration) was added for induction. The culture was growth overnight at 37 °C and 200 rpm. Cells were collected at 6000*g* for 15 min at 4 °C and the pellet was weighed and resuspended in 3 ml lysis buffer (PBS, pH 8.0, containing 3 mg/ml lysozyme, 10 μg/ml DNase, and 100 μM PMSF) per gram of pellet. The sample was incubated at 37 °C for 30 min and then kept in the freezer at least 30 min. Then the sample was sonicated on ice applying two 1 min pulses at 70 W and at a constant duty cycle, separated by a 10 s cooling period on ice. Insoluble material was separated by centrifugation at 16,000*g* for 15 min at 4 °C and the soluble cellular fraction sequentially filtered through sterile 0.45 μm and 0.22 μm cellulose acetate filters (GE Healthcare Life Sciences).

2.3. Purification of the Vip3 proteins

Vip3 proteins used for bioassays or proteolysis assays were purified by immobilized metal ion absorption chromatography (IMAC) on a Hi-Trap chelating HP column (GE Healthcare) charged with Ni^{2+}. The eluted Vip3 protein was collected on PBS containing 0.1 mM EDTA (pH 8.0) to avoid inactivation of the protein. Buffer exchange was performed immediately with storage buffer (20 mM Tris, 150 mM NaCl, pH 9.0) with a PD-10 desalting column (GE Healthcare) to prevent protein precipitation and aggregation. Concentration of the purified protein was estimated with the Bradford method (Bradford, 1976). The purity of the expressed protein was then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie brilliant blue R-250 (Sigma-Aldrich).

For histopathological studies, the Vip3Aa, Vip3Ad, and Vip3Ca, proteins were purified by isoelectric point precipitation (Chakroun et al., 2012). The pH of the lysate was lowered with acetic acid to pH 5.5 for Vip3Aa, pH 4.95 for Vip3Ad, and pH 5.95 for Vip3Ca. The pellets were recovered by centrifugation at 16,000*g* for 10 min and then resuspended in storage buffer and dialysed against the same buffer overnight. The purified proteins were stored at −20 °C.

For binding assays, Vip3 proteins were purified by isoelectric point precipitation as described above. Solubilized Vip3 proteins were incubated with 1% trypsin (wt/wt) for 2 h at 37 °C. Activated Vip3Ca and Vip3Aa were used as competitors in binding assays. For biotin labeling, Vip3Aa was further purified by anion-exchange chromatography. After overnight dialysis against 20 mM Tris-HCl (pH 9), the Vip3Aa activated protein was purified on a HiTrap Q HP (5-ml bed volume) column equilibrated in the same dialysis buffer, using an ÄKTA explorer 100 chromatography system (GE Healthcare, United Kingdom). Proteins were eluted with a 100 ml linear gradient (0–80%) of 1 M NaCl.

2.4. Insect toxicity assays

Several methodologies were used in the bioassays depending on the insect species tested. For *M. brassicae*, *S. nonagrioides*, *C. pomonella*, and *G. molesta*, bioassays were performed on neonates using surface contamination assays. Surface contamination assays were also employed with L3 larvae for *G. mellonella* and *P. xylostella*. Bioassays with *P. gossypiella* and *E. kuehniella* were conducted with neonates and L3 larvae, respectively, using toxins in diet incorporation assays. Bioassays with *T. absoluta* were performed on L2 larvae in leaf disks. With the homopteran *N. viridula*, bioassays were conducted on N1 using toxins in feeding assays (Palma et al., 2014). With the dipteran *D. melanogaster*, bioassays were conducted on L1 using surface contamination assays.

For a preliminary screening of the activity of the Vip3Ca protein, two concentrations (0.4 and 4 μg/cm^2) were tested when the assay was done on artificial diet in surface contamination assays. In diet incorporation assays, the Vip3Ca protein was tested at a single concentration of 60 μg/g and 100 μg/g, for *P. gossypiella* and *E. kuehniella*, respectively. For the leaf disk and feeding assays the Vip3Ca was tested also at a single concentration in both bioassays (200 mg/ml and 1 mg/ml, respectively). Sixteen larvae were used for each protein concentration and the bioassay was repeated at least twice. Bioassays were conducted at 25 °C, 60 ± 5% RH, and a 16:8 (light/dark) photoperiod. Mortality and effective mortality (dead larvae plus number of larvae that remained in the initial instar) were scored after 21 days for *P. gossypiella*, 7 days for *S. nonagrioides*, *M. brassicae*, *G. molesta*, *C. pomonella* and *G. mellonella*,

4 days for *P. xylostella*, *E. kuehniella* and *D. melanogaster*, and 3 days for *T. absoluta* and *N. viridula*. Determination of the LC_{50} (concentration of protein killing 50% of tested individuals) for Vip3Ca was done for the most susceptible species (*M. brassicae*, *C. pomonella* and *E. kuehniella*) as reported elsewhere (Ruiz de Escudero et al., 2014). The LC_{50} of Vip3Aa for *C. pomonella* was calculated in the same way as for Vip3Ca. The storage buffer was use to dilute Vip3 proteins and as the negative control. Only the bioassays with less than 10% control mortality were considered.

2.5. Midgut juice preparation and Vip3 proteins processing

Midgut juice preparation was obtained from last instar larvae of *M. brassicae*, *Agrotis ipsilon*, *Spodoptera littoralis* and *Ostrinia nubilalis*. Midguts were dissected and cut open to eliminate food clumps. The midgut with the remaining contents was centrifuged at 16,000*g* for 10 min and the supernatant, which constituted the midgut juice, was recovered. The protein concentration was measured with the Bradford method (Bradford, 1976).

For the time course assays, the Vip3Ca protein was mixed with several dilutions of midgut juice in a final volume of 25 µl and incubated at 30 °C. The reaction was stopped at different time intervals by adding the electrophoresis loading buffer and heating at 99 °C for 10 min. Reaction products were separated by 12% SDS-PAGE and the gels stained with Coomassie brilliant blue R-250 (Sigma-Aldrich).

For the insect toxicity assays, Vip3Aa and Vip3Ca were processed with midgut juice from *M. brassicae* and *O. nubilalis*, and with commercial bovine trypsin (Sigma-Aldrich). Both proteins where incubated with midgut juices for 24 h at 30 °C, at the ratio 100:0.36 and 100:16 (protein:midgut juice of *M. brassicae* and *O. nubilalis*, respectively). Trypsin activation was performed as described above.

2.6. Insect toxicity assays of the activated Vip3 proteins with midgut juice from M. brassicae and O. nubilalis and with trypsin

As for *M. brassicae*, bioassays with *O. nubilalis* were performed on neonates using surface contamination assays and mortality was scored after 7 days. *M. brassicae* and *O. nubilalis* larvae (three replicates of 24 larvae) were fed with the native and the activated forms of Vip3Aa and Vip3Ca. Larvae groups received 0.74 µg/cm^2 and 4.5 µg/cm^2 of each protein form, which corresponded to the LC_{30} and an LC_{90} values, respectively, of Vip3Ca for *M. brassicae*. Bioassays were repeated three times.

2.7. Histopathological effects and in vivo binding of Vip3 proteins in M. brassicae

Vip3Aa, Vip3Ad, and Vip3Ca (6 µg each) were used to intoxicate L2/L3 instar larvae of *M. brassicae*. Briefly, after 1 h, 3 h and 6 h of exposure, larvae were cut at the two ends and immediately fixed with 4% paraformaldehyde (PFA) for two days and then transferred to 30% sucrose in PBS for two additional days. Larvae were keep at −80 °C until they were fixed to a support and coated with Tissue Tech gel (Sakura, Japan). Sections of 30 µm were prepared using the cryostat microtome Leica CM 1510S. Slides with the tissue sections were stored at −20 °C until used (Chakroun and Ferré, 2014).

Histopathological effects were observed in midgut sections after staining with hematoxylin-eosin (Ruiz et al., 2004). Binding of the Vip3Ca protein to the midgut epithelial membrane was performed as described by Chakroun and Ferré (2014) using an anti-Vip3Aa polyclonal antibody coupled to anti-Ig conjugated with FITC.

2.8. Vip3 toxins labeling, preparation of M. brassicae BBMV, and binding analyses

Vip3Aa and Vip3Ca proteins were labeled with biotin using the ECL Protein Biotinylation Module kit (GE Healthcare), according to the manufacturer's instructions. Brush border membrane vesicles (BBMV) were prepared by the differential magnesium precipitation method from last instar larvae (Wolfersberger et al., 1987). Protein concentration in BBMV was estimated by the Bradford's method (Bradford, 1976).

For competition experiments, 20 ng of biotinylated toxin was incubated with 20 µg of BBMV in 0.1 ml of binding buffer (PBS pH 7.4, 0.1% BSA) at different concentrations of competitor (unlabeled toxin) at room temperature for 90 min. The reaction was stopped by changing to a new microcentrifuge tube and centrifugation at 16,000*g* for 10 min. The pellet, containing the toxin bound to BBMV, was washed with 500 µl of the binding buffer. The final pellet was resuspended in 10 µl of the binding buffer without BSA and then transferred to a new microcentrifuge tube containing 5 µl of electrophoresis loading buffer (5×). The mixture was heated at 99 °C for 5 min. Samples were run on 10% SDS-PAGE and proteins blotted into a nitrocellulose filter. The filter was blocked with 3 mg/ml of blocking reagent (GE Healtcare) for 1 h. The retained labeled toxins were revealed by probing with streptavidin-conjugated peroxidase (GE Healtcare) and then the Amersham™ ECL™ Western Blotting Detection Reagents luminescence kit.

3. Results

3.1. Host range and insecticidal activity of Vip3Ca

In an attempt to expand the range of susceptible insect species, Vip3Ca was tested with ten new insect species at high protein concentrations (Table 1). Of the six species tested by surface contamination, *C. pomonella* was the most susceptible, followed by *G. mellonella*, with percentages of mortality higher than 50%. From the other types of bioassays, *E. kuehniella* and *T. absoluta* showed some susceptibility to high concentrations of Vip3Ca (between 30% and 50% mortality). The rest of species (*G. molesta*, *P. gossypiella*, *P. xylostella*, *S. nonagrioides*, *D. melanogaster* and *N. viridula*) can be considered non-susceptible, with percentages of mortality lower than 30%.

The LC_{50} of the Vip3Ca protein was calculated for *C. pomonella* (6 µg/cm^2), *E. kuehniella* (180 µg/g), and *M. brassicae* (1.3 µg/cm^2), which was shown to be one of the most susceptible insect species in a previous work (Palma et al., 2012). In addition, the LC_{50} of Vip3Aa to *C. pomonella* was also calculated (0.014 µg/cm^2) for comparison (Table 2).

3.2. Proteolysis of the Vip3Ca protein by midgut juice from different insect species

To determine whether species with different susceptibility to Vip3Ca showed different efficiencies in the activation process of the Vip3Ca protoxin, the time course of the conversion of protoxin (of approximately 90 kDa) into toxin (of approximately 70 kDa) was measured using midgut juice from four lepidopteran species, which, from less to more susceptible to Vip3Ca, were: *O. nubilalis*, *A. ipsilon*, *S. littoralis*, and *M. brassicae* (Palma et al., 2012). The results showed a correlation between susceptibility and speed of protoxin processing (Fig. 1). In the same experimental conditions, the midgut juice of *O. nubilalis* hardly converted the protoxin to the 70 kDa form even at 60 min using the highest concentration of midgut juice. The *A. ipsilon* midgut juice was able to convert

Table 1
Absolute and functional mortality produced by Vip3Ca on ten insect species.

Bioassay method	Insect species	Larval instar	Vip3Ca concentration	Absolute mortality	Functional mortality	Days
Surface contamination	*Cydia pomonella*	Neonate	0.4 μg/cm^2	28.6 ± 0.2	28.6 ± 0.2	7
			4 μg/cm^2	81.20 ± 0.10	100.0 ± 0.0	7
	Grapholita molesta	Neonate	0.4 μg/cm^2	0.10 ± 0.10	0.10 ± 0.10	7
			4 μg/cm^2	22.30 ± 0.10	22.30 ± 0.10	7
	Sesamia nonagrioides	Neonate	0.4 μg/cm^2	6.7 ± 0.7	6.7 ± 0.7	7
			4 μg/cm^2	29.6 ± 0.7	29.6 ± 0.7	7
	Galleria mellonella	L3	0.4 μg/cm^2	36 ± 6	36 ± 6	4
			4 μg/cm^2	54 ± 4	54 ± 4	4
	Plutella xylostella	L3	0.4 μg/cm^2	15.80 ± 0.10	15.80 ± 0.10	7
			4 μg/cm^2	18.80 ± 0.10	18.80 ± 0.10	7
	Drosophila melanogaster	Neonate	0.4 μg/cm^2	0.0 ± 0.0	0.0 ± 0.0	4
			4 μg/cm^2	0.0 ± 0.0	0.0 ± 0.0	4
Diet incorporation	*Pectinophora gossypiella*	Neonate	60 μg/g	9.00 ± 0.10	9.00 ± 0.10	21
	Ephestia kuehniella	L3	100 μg/g	42 ± 13	44 ± 2	4
Leaf disk	*Tuta absoluta*	L2	200 mg/ml	34 ± 10	37 ± 7	3
Feeding assay	*Nezara viridula*	Neonate	1 mg/ml	10 ± 7	10 ± 7	3

Table 2
Quantitative parameters from concentration–mortality responses of some susceptible species to the Vip3Ca protein.

Treatment	Regression line		LC_{50}	Goodness of fit value		95% FL[a]	
	Slope ± SE	a[b] ± SE		χ^2	df[c]	Lower	Upper
E. kuehniella			μg/g				
Vip3Ca[d]	1.6 ± 0.3	1.3 ± 0.6	180	0.55	2	140	270
M. brassicae			μg/cm^2				
Vip3Ca[e]	2.5 ± 0.3	4.70 ± 0.10	1.3	1.83	3	1.1	1.6
Vip3Aa[f]	1.50 ± 0.10	3.30 ± 0.10	0.014	0.9	3	0.011	0.018
C. pomonella			μg/cm^2				
Vip3Ca[e]	0.6 ± 1.0	4.62 ± 0.06	6	4.88	6	3	19
Vip3Aa[e]	0.64 ± 0.12	1.21 ± 0.19	0.013	1.20	2	0.004	0.029

[a] Fiducial limits.
[b] Intercept.
[c] Degrees of freedom.
[d] The LC_{50} was calculated at 3 days.
[e] The LC_{50} was calculated at 7 days.
[f] Data adapted from Ruiz de Escudero et al. (2014).

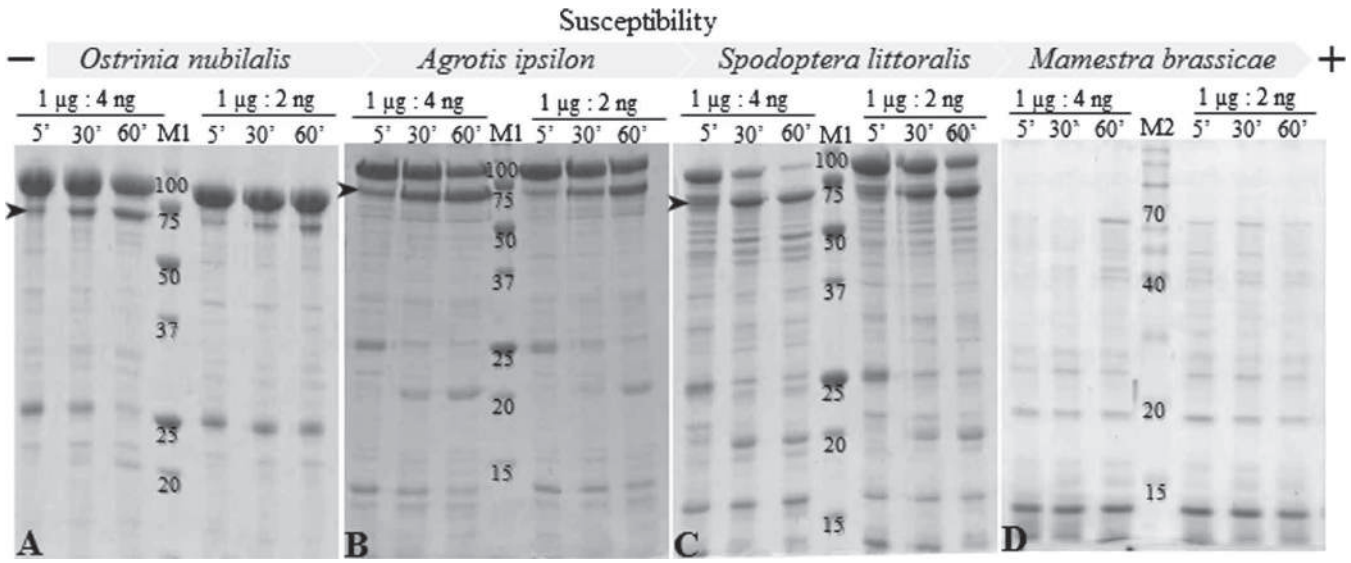

Fig. 1. Time course of the Vip3Ca processing by midgut juice from lepidopteran insects with different susceptibility to the protein. The assay was performed with 10 μg of Vip3Ca in a final volume of 25 μl. Vip3Ca was incubated with 20 ng and 40 ng of total midgut juice protein for 5 min, 30 min and 60 min at 30 °C. Molecular markers (M1 and M2, in kDa) are indicated in the gels. Arrowheads show the position of the activated toxin.

approximately 50% of the protoxin into the 70 kDa form after 60 min. The midgut juice from *S. littoralis* processed most of the protoxin, especially at the highest concentration. The midgut juice of the most susceptible species, *M. brassicae*, not only converted the protoxin to the 70 kDa form but even processed further this form to smaller products.

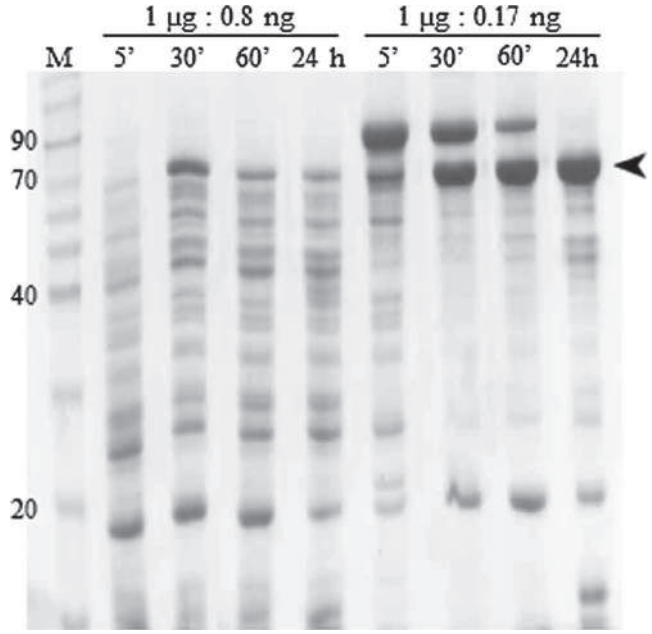

Fig. 2. Time course of the Vip3Ca processing by diluted midgut juice of *M. brassicae*. The assay conditions are as in Fig. 1 except for the ratio Vip3Ca/midgut juice protein and that the assay was extended up to 24 h.

To show that all four species were able to fully convert the protoxin to the activated 70 kDa form given the appropriate conditions, the Vip3Ca protoxin was incubated with different concentrations of midgut juice. Using 20 ng of midgut juice from both *O. nubilalis* and *A. ipsilon* to 1 µg of Vip3Ca, the protoxin was completely converted to the 70 kDa form (data not shown). For *M. brassicae*, the Vip3Ca protoxin was incubated with more diluted midgut juice (0.8 and 0.17 ng of midgut juice total protein for every 1 µg of the Vip3Ca protein). With the lowest dilution, after 24 h the Vip3Ca was completely converted to the 70 kDa form without practically further processing (Fig. 2).

3.3. Insecticidal activity of the Vip3Ca activated with midgut juice of *M. brassicae* and *O. nubilalis*, and with trypsin

To determine whether the low toxicity of Vip3Ca to *O. nubilalis* is related with an inappropriate activation in the larva midgut, *O. nubilalis* neonate larvae were exposed to Vip3Ca activated by midgut juice from *M. brassicae* (a more susceptible species) and, reciprocally, *M. brassicae* neonate larvae were exposed to Vip3Ca activated by midgut juice from *O. nubilalis*. Vip3Aa was used for comparison. The results showed that Vip3Ca activated by midgut juice from *O. nubilalis* was as active, against *M. brassicae*, as the protoxin and the trypsin-activated forms (Fig. 3A). Similar results were obtained for Vip3Aa treated in the same way (Fig. 3B). Similarly, Vip3Ca activated by midgut juice from *M. brassicae* was practically as active against *O. nubilalis* as the trypsin-activated or the non-activated protoxin (Fig. 3A). In the case of Vip3Aa, neither the protoxin nor the activated toxins, by either trypsin or *M. brassicae* midgut juice, were toxic to *O. nubilalis* (Fig. 3B).

3.4. Comparative histopathological effects of Vip3 proteins to *M. brassicae* midgut sections

The histopathological effects of Vip3Aa, Vip3Ca and Vip3Ad to *M. brassicae* were studied in midgut sections of second or third instar larvae which had been fed with a solution of Vip3 protein with sucrose. No tissue damage could be observed after one hour

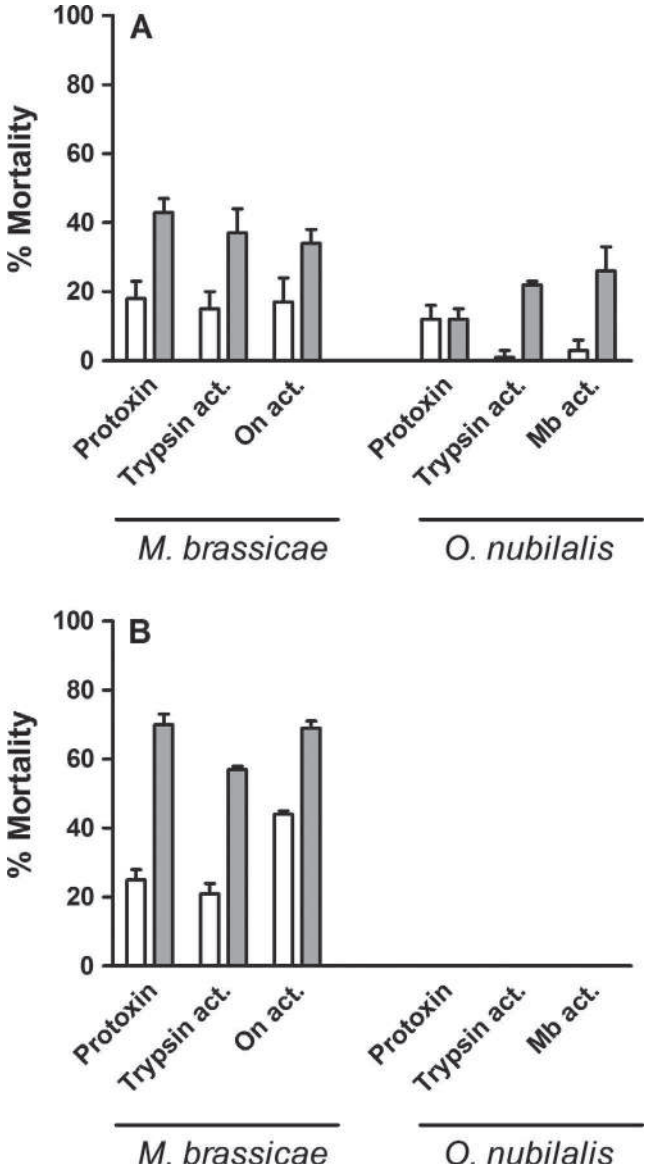

Fig. 3. Toxicity of protoxin, trypsin activated, and midgut juice activated Vip3Ca (A) and Vip3Aa (B) against *M. brassicae* and *O. nubilalis* neonate larvae. Bars represent mean mortality (with SE) of three replicates (of 24 larvae each) after 7 days, at a toxin concentration of 0.74 µg/cm^2 (white bars) and 4.5 µg/cm^2 (grey bars).

of intoxication with any of the Vip3 proteins. After 3 h, visible damage was observed along the whole midgut only in the case of Vip3Aa, the most toxic protein. After 6 h, the midgut was highly damage in larvae that had ingested Vip3Aa. For Vip3Ca, the midgut showed signs of damage but they were not as drastic as for Vip3Aa. For the non-toxic Vip3Ad protein, no damage was evident at any time (Fig. 4).

3.5. In vivo binding of Vip3Ca to *M. brassicae* midgut epithelium

To know if that Vip3Ca binds to the midgut apical membrane after ingestion, larvae were fed with Vip3Ca for different times, dissected and the Vip3Ca protein revealed with a polyclonal Vip3 antibody. Hardly any binding could be detected in larvae that had been dissected one hour after the start of the ingestion. However, after 3 h, binding of Vip3Ca to the brush border membrane was evident (green signal) (Fig. 5). No bound Vip3Ca could be observed after 6 h due to disruption of the apical membrane (as revealed by the loss of the red signal).

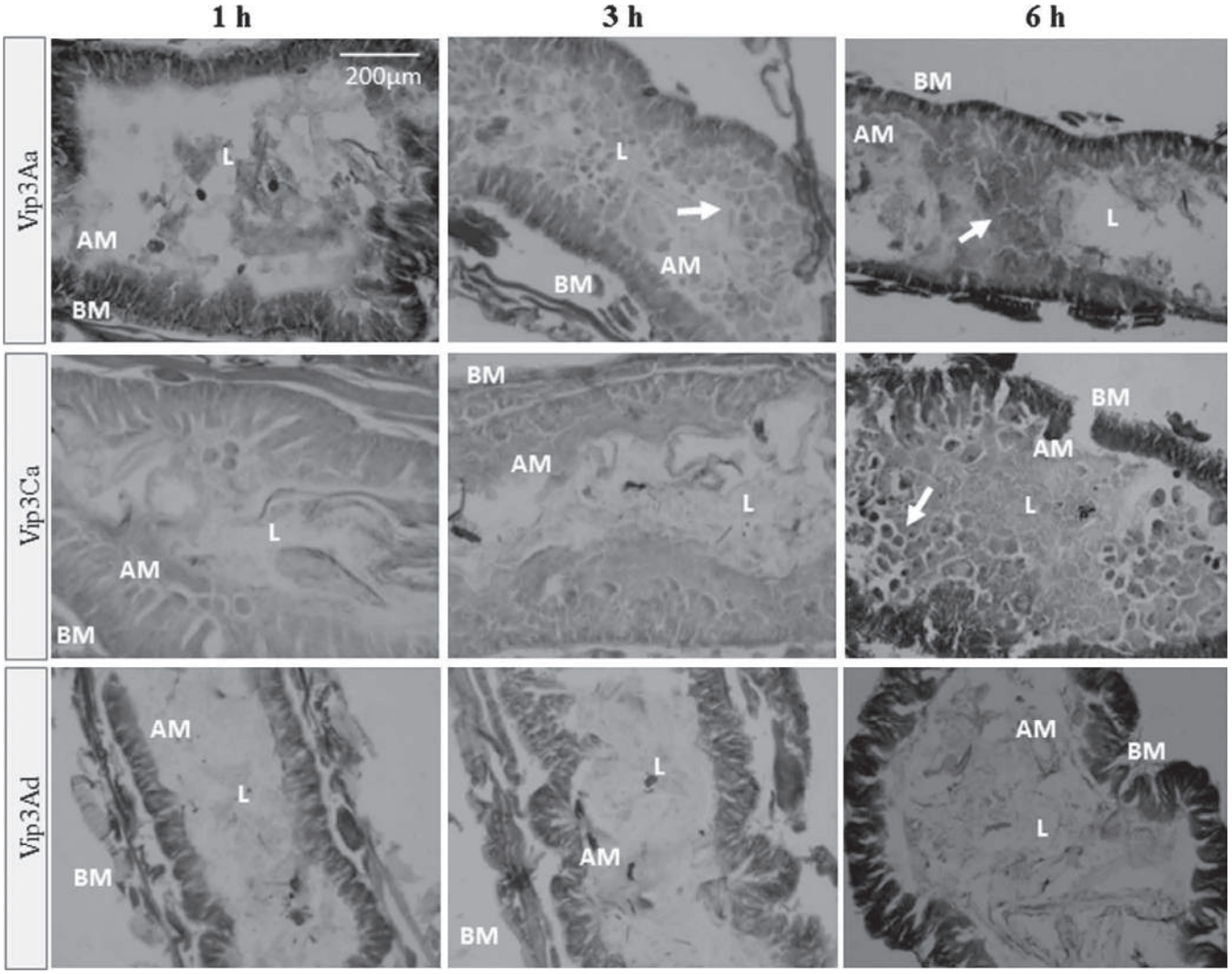

Fig. 4. Histopathological effects of Vip3Ca to the *M. brassicae* midgut after oral ingestion. The Vip3Aa protein was included as a positive control and Vip3Ad protein as a negative control. Midgut sections (30 µm) were stained with hematoxylin-eosin (Ruiz et al., 2004). Magnification 100×. BM, basal membrane; AM, apical membrane; L, gut lumen. White arrows point at excised cells.

3.6. In vitro binding of Vip3 proteins to M. brassicae BBMV

To show that Vip3Aa and Vip3Ca bound specifically to *M. brassicae* BBMV, competition experiments were performed with biotin-labeled toxins. Both toxins bound specifically, since the unlabeled homolog (the same unlabeled toxin as the labeled one) competed for binding with the labeled toxin (Fig. 6A and B). To determine whether the two Vip3 proteins recognized common binding sites, heterologous competition (the competitor is a different toxin than the labeled one) was performed with labeled Vip3Ca and unlabeled Vip3Aa and *vice versa*. The results showed that both toxins competed heterologously with the labeled toxin, indicating that Vip3Aa and Vip3Ca share binding sites (Fig. 6A and C).

4. Discussion

The Vip3Ca protein is the first member of the most recent new family of Vip3 proteins, discovered in a screening program of Spanish collections of *Bacillus thuringiensis*, and it was shown to be toxic to some lepidopteran species, *M. brassicae* among them (Palma et al., 2012). In order to further characterize the insecticidal spectrum of the Vip3Ca protein, we tested ten additional insect species, (eight lepidopterans, one dipteran and one homopteran) (Table 1). LC_{50} values were obtained from the two most susceptible species and also from *E. kuehniella* because of its importance for storage grain in Spain. Compared to Vip3Aa, the LC_{50} values of Vip3Ca for *C. pomonella* and *M. brassicae* were two orders of magnitude higher (Table 2), indicating that Vip3Ca although toxic, is much less toxic than the Vip3Aa protein for these two species.

Since the mode of action of Vip3Ca proteins has never been approached before, we set out to determine the role of proteolysis and binding in the toxicity of this protein. We first studied the processing of the Vip3Ca protein by the midgut juice of *M. brassicae*, *S. littoralis*, *A. ipsilon* and *O. nubilalis* (Figs. 1 and 2). In all cases, the Vip3Ca protoxin was processed to a 70 kDa protein, in contrast to the 62 kDa protein generated from Vip3Aa when incubated with midgut juice (Lee et al., 2003; Chakroun et al., 2012; Caccia et al., 2014). The kinetics of the proteolysis positively correlated with the susceptibility of the species. The conversion of the protoxin into the activated toxin proceeded much faster with the midgut juice of the most susceptible species. This result suggests that the speed of conversion of the protoxin into the 70 kDa toxin might contribute to the susceptibility of the species to the Vip3Ca protein.

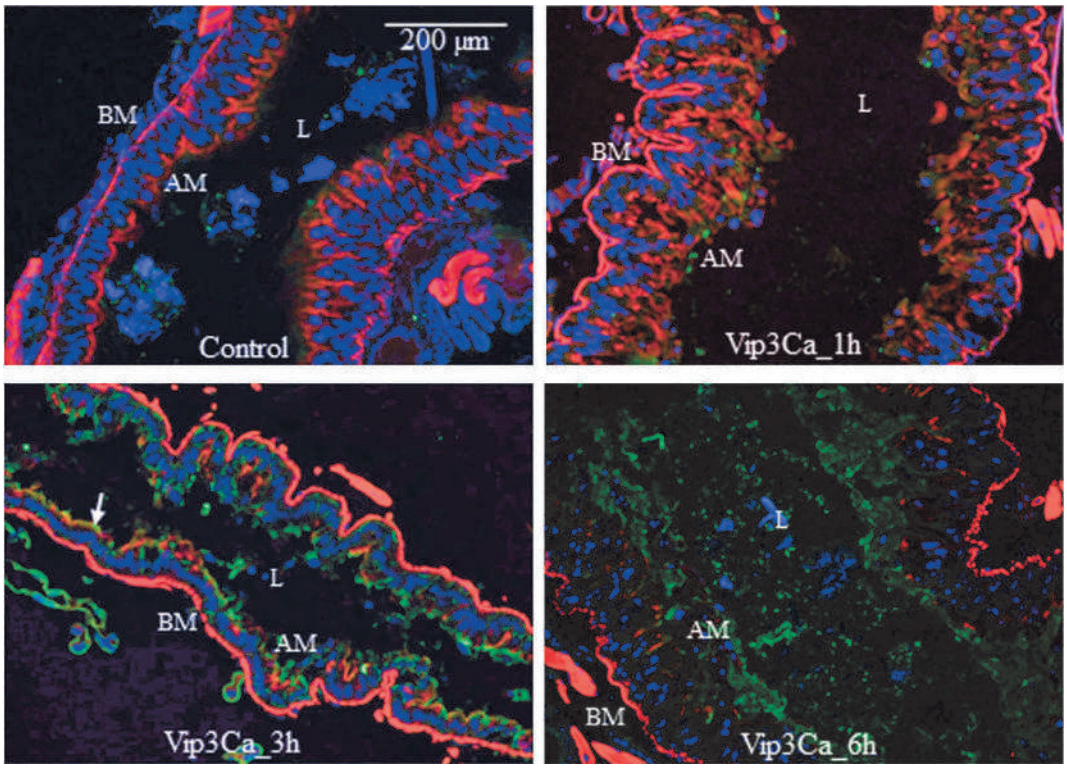

Fig. 5. *In vivo* binding of Vip3Ca in *M. brassicae* after oral ingestion. Larvae fed with just a sucrose solution were used like as negative controls. Binding of the Vip3Ca protein was detected in midgut sections (30 µm) with an anti-Vip3Aa polyclonal antibody and was revealed with anti-IgG coupled with FITC (green fluorescence). Nuclei and actin were stained with DAPI (blue fluorescence) and phalloidin (red fluorescence), respectively. Magnification 100×. BM, basal membrane; AM, apical membrane; L, gut lumen. The white arrow shows binding to the apical membrane.

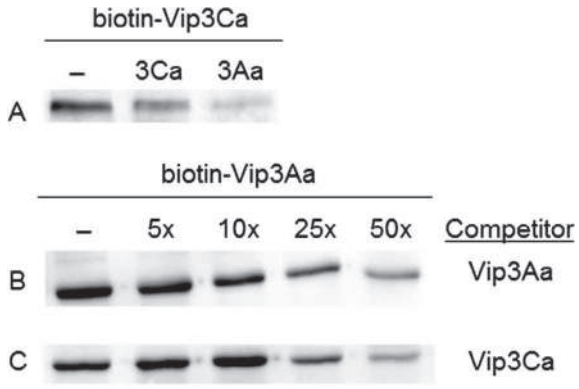

Fig. 6. Binding of biotinylated Vip3Ca and Vip3Aa toxins to *M. brassicae* BBMV. For biotinylated Vip3Ca (A), a 100-fold excess of unlabeled competitor was used (–, no competitor; 3Ca, Vip3Ca; 3Aa, Vip3Aa). For biotinylated Vip3Aa, binding was tested without competitor (–) and in the presence of an excess (5-fold to 50-fold) of unlabeled Vip3Aa (B) and Vip3Ca (C), as indicated on the top of the figure.

In contrast, this type of correlation has not been found for Vip3Aa (Yu et al., 1997).

Yu et al. (1997), showed that the processing of Vip3Aa by midgut juice of different lepidopteran species, including *O. nubilalis*, did not have an effect on its toxicity against them, indicating that midgut processing was not responsible for the very low susceptibility of *O. nubilalis* to this toxin. We have found the same result with Vip3Aa and Vip3Ca using *M. brassicae* and *O. nubilalis* (Fig. 3). Therefore, other factors different to the protoxin activation must be critical in the tolerance of *O. nubilalis* to these two Vip3 toxins.

Several studies have dealt with the histopathological effects of Vip3Aa to larvae from different species (Yu et al., 1997; Abdelkefi-Mesrati et al., 2011a,b; Sellami et al., 2015). We have obtained similar results with *M. brassicae* larvae fed with Vip3Ca, for which disruption of the midgut epithelium could be observed (Fig. 4). However, compared with the most toxic protein Vip3Aa, the damage caused by Vip3Ca took longer to be visible, in agreement with its lower toxicity. Vip3Ad was used as a negative control since it has no toxicity to *M. brassicae* (Ruiz de Escudero et al., 2014). As expected, the Vip3Ad caused no visible damage to the midgut epithelium.

The in vivo binding of Vip3Ca was shown by fluorescence microscopy of the midgut sections stained with specific fluorophores (Fig. 5). The ingested Vip3Ca protein bound to the brush border membrane of the midgut epithelial cells, similarly as it has been reported for Vip3Aa (Yu et al., 1997; Chakroun and Ferré, 2014). To show that binding of Vip3Ca to the brush border membrane is specific, BBMV from *M. brassicae* were prepared and used for *in vitro* binding assays with biotin-labeled Vip3Ca and Vip3Aa. Binding was shown to be specific since homologous competition of Vip3Ca and Vip3Aa was observed (Fig. 6). Heterologous competition experiments also showed that Vip3Aa competed for Vip3Ca binding sites and *vice versa*, indicating that they bind to shared binding sites. Although the overall similarity of the full length Vip3Aa and Vip3Ca proteins is just of 70%, the two proteins share highly conserved regions (92% similarity of the N-terminal region up to amino acid 195 and 66% similarity in a carbohydrate binding domain spanning from amino acid position 544–662) that might be involved in interaction with membrane binding sites. According to our results, at least some of the binding sites would be recognized by both Vip3 proteins. This result might have implications for IRM since, in the event that insects would become resistant to one of these Vip3 proteins due to a change in one of the shared membrane receptors, they could also develop cross-resistance to the other Vip3 protein.

In summary, the Vip3Ca protein (the first representative of this new family of Vip3C proteins) seems to follow a mode of action similar to that of the Vip3Aa protein: it is processed by gut proteases to a smaller molecular weight protein and binds to the brush border of the midgut epithelial membrane, with eventual lysis of midgut cells. Vip3Ca shares binding sites with Vip3Aa, which might have implications for IRM. It seems that there is a difference compared to Vip3Aa mode of action, since the kinetics of the activation of Vip3Ca may play a role in the degree of susceptibility of susceptible insects. With this study, we aimed to shed some light on the activity spectrum and mode of action of the recent new family of Vip3C proteins.

Acknowledgments

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7.2 - Stability of the Vip3Ca protein to the serine proteases and midgut juice proteases of *Mamestra brassicae*.

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Objective:

Determination of the oligomer formation of the Vip3Ca protein digested with trypsin and midgut juice proteases from *Mamestra brassicae.*

Materials and Methodology:

1. - Vip3Ca expression and purification

The Vip3Ca protein (NCBI accession No AEE98106) was prepared from recombinant *Escherichia coli* WK6 carrying the *vip3Ca2* gene (1). A single colony of Vip3Ca was inoculated in 4 ml of LB medium containing 100 µg/ml ampicillin and grown overnight at 37°C and 180 rpm. A 1/100 dilution of the culture in 700 ml LB medium containing 100 µg/ml ampicillin was further incubated at 37°C and 180 rpm, until and OD value of 0.7-0.8. For the induction, 1mM IPTG was added. The culture was growth overnight at 37°C and 200 rpm. The cells were collected and resuspended in lysis buffer (20 mM phosphate saline buffer, pH 8.0, with 3 mg/mL lysozyme, 10 µg/mL DNAse and 100 µM PMSF) and incubated with shaking for 30 min at 37°C. The cells were lysed by sonication and the supernatant recovered by centrifugation at 27,000 x g and filtered through sterile 0.45 µm and 0.22 µm cellulose acetate filters. The Vip3Ca protein was purified by ion metal affinity chromatography as described previously (2).

2. - Vip3Ca processing, trypsin and midgut juice treatment

The Vip3Ca protein was subjected to different proteolysis treatments. In all of them, the reaction was stopped with SDS-PAGE loading buffer, heated at 99°C for 10 min and frozen in liquid nitrogen. The samples were stored at -20°C until use.

Trypsin treatment

The Vip3Ca was incubated with commercial trypsin (trypsin from bovine pancreas, SIGMA T8003) with the storage buffer (20 mM Tris 150 mM NaCl, pH 9). For the time course assays, the Vip3Ca was activated at different time intervals (0, 0.5, 1, 2, 6, 24, 48, 76 hours) with at the following ratio 1µg of Vip3Ca protein: 0.24 µg of trypsin in a final volume of 25 µl at 30°C.

Midgut juice treatment

The midgut juice (MJ) was obtained from the last instar larvae of *M. brassicae*. Midguts were dissected and centrifuged at 16,000 g for 10 min and the supernatant was recovered. The protein concentration was measured with the Bradford method (3). For the time course assays, the Vip3Ca protein was mixed at the ratio of 1 µg of protein : 0.8 µg of MJ for 30 minutes, in a final volume of 25 µl of storage buffer at 30°C. The proteolysis reactions were stopped adding 5 µl of loading buffer and boiling the samples for 10 min at 99ºC.

3. - Gel filtration chromatography

Gel filtration chromatography was performed with an ÄKTA explorer 100 chromatography system (GE Healthcare) in a Superdex-200 10/300 GL column (GE Healthcare Life sciences, USA) equilibrated and eluted with the storage buffer, to a flow rate of 0.5 ml/min. The Superdex-200 10/300 GL column was calibrated with the gel filtration calibration kit HMW (Ge Healthcare Ref: 28-4038-42) with the following standard proteins: 250 µl of 10 mg/ml thyroglobulin, 50 µl of 3 mg/ ml ferritin, 200 µl of 10 mg/ml aldolase, 150 µl of 10 mg/ml conalbumin and 200 µl of 200 µl of ovalbumin, at a flow rate of 0.5 ml/min. Blue Dextran (SIGMA D-5751) was used to determine the void volume of the column. The elution time, expressed as milliliters, was calculated from the middle of the elution peak. The expected size of the processed Vip3Ca (24 % w:w

trypsin, for 0.5 h and 48 h; 1 µg Vip3Ca : 0.8 µg MJ) was estimated with the linear regression of the standard line (r^2 0.98, y=-0.3075x + 1.9102, where x is the log of the molecular weight in Da and y is the $k_{Av)}$**.** The eluted proteins were collected in fractions of 1 ml and aliquots were loaded on an SDS-PAGE gel to check the presence of the oligomer.

Results:

1. - Time course of trypsin processing of Vip3Ca

Trypsin treatment of the Vip3Ca protein in Tris-HCl buffer (pH 9.0), at 24:100 trypsin Vip3Ca (w:w), for 30 min, rendered a major band of around 70 kDa, as well as other smaller bands (Figure 1). Whereas the concentration of the smaller bands decreased with the incubation time, the ~70 kDa band seemed to become more intense as the incubation proceeded. The controls at time 0 and 76 h show that the Vip3Ca2 protoxin is stable at 30ºC and the smaller bands do not come from protein degradation.

2. - Size exclusion chromatography of the Vip3Ca2 activated with trypsin at 24 % (w:w) protoxin:toxin for 30 min and 48 h

In an attempt to know if the processed Vip3Ca with trypsin form oligomers, the processed Vip3Ca was analysed under non denaturing conditions in a gel filtration chromatography column. For this purpose, Vip3Ca, treated with trypsin at 0.5 h and 48 h, was loaded on the Superdex-200 10/300 GL column (exclusion limit of the 1300 kDa). For the Vip3Ca processed with trypsin for 30 min, a main peak was eluted at 11.6 ml, while if the Vip3Ca is processed for 48 h, a main peak was eluted at 11.4 ml (Figure 2b and 2d). This peak correspond to the toxin bands of ~70 kDa and ~20 kDa (Figure 2a and 2c) with a size of 451 kDa for at 0.5 h and 512 kDa at 48 h (Table 1). The toxin band of ~20 kDa was only detected in the output fractions at 0.5 h and not at 48 h due to the few amount of the sample injected (Figure 2c). Moreover, other minor peaks were also detected at different retention times but none of them were validated by SDS-PAGE.

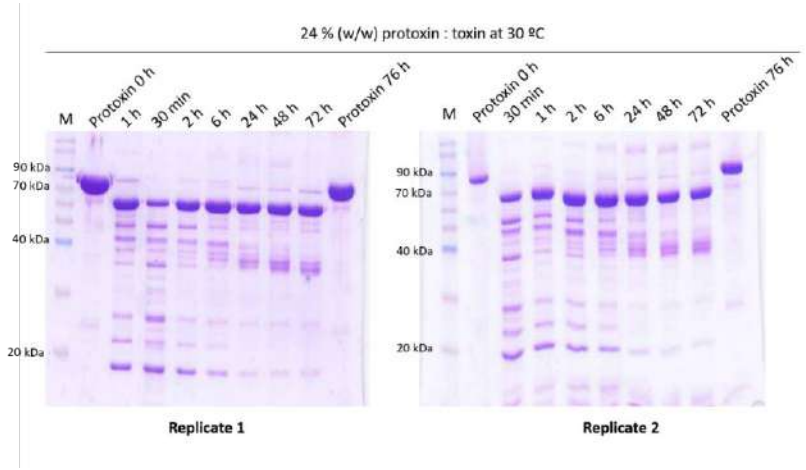

Figure 1. Time course of trypsin processing of Vip3Ca. Reactions were performed at 30°C at different concentrations of trypsin in Tris-HCl buffer. The ratios of trypsin: Vip3Ca (w:w) was 24:100. Aliquots were withdrawn at different times, as shown at the top of each lane. Molecular weight marker (M) Pink Plus Prestained Protein Ladder, Genedirex.

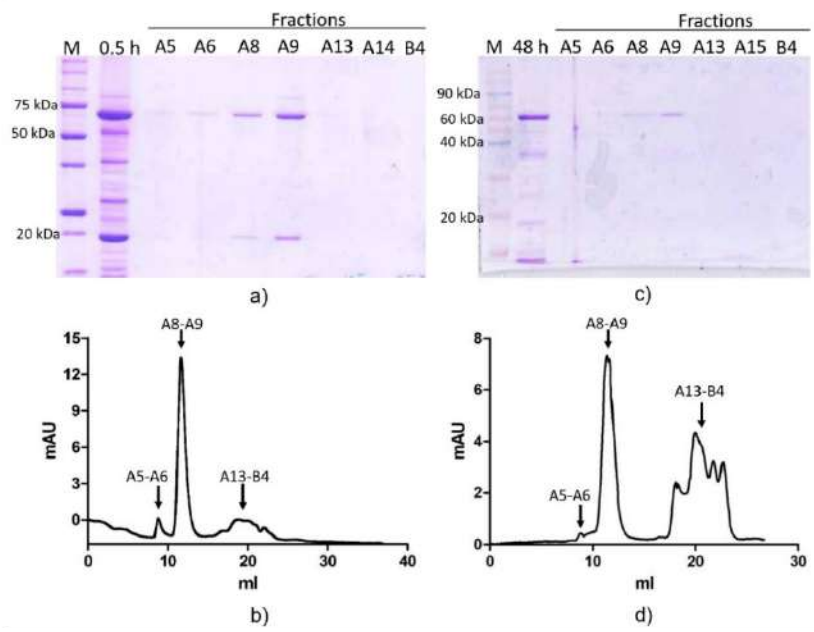

Figure 2. Gel filtration chromatography of Vip3Ca processed with trypsin. a) SDS-PAGE of the processed Vip3Ca protein incubated for 0.5 h. b) Gel filtration chromatography of the Vip3Ca processed with 24 % w:w trypsin: protoxin for 30 min. c) SDS-PAGE of the processed Vip3Ca protein with at 24 % w:w of trypsin : protoxin for 48 h. d) Gel filtration chromatography of the Vip3Ca processed with 24 % w:w of trypsin : protoxin for 48 h. Molecular weight marker: M, Pink Plus Prestained Protein Ladder, Genedirex and Precision Plus Protein™ Unstained Protein Standards, BioRad.

3. - Stability of Vip3Ca protoxin to M. brassicae midgut juice

When the Vip3Ca protein, apparently degraded after treatment with a very high concentration of MJ (Figure 5, lane of 0.5 h), was subjected to gel filtration chromatography, the Vip3Ca protein eluted as a single main peak at 11 ml with an approximately size of 395 kDa (Table 1), along with two peaks associated with the MJ (Figure 5b). The size of the SDS-PAGE of the fractions of the peak at 11 ml showed three bands, one of 66 kDa, 35 kDa and 17 kDa (Figure 3, lane A10 - A12). This result confirmed the existence of a core of 66 kDa extremely stable to MJ proteases, and showed that the apparent degradation of Vip3Ca at high concentrations of MJ, as observed by SDS-PAGE, was an artefact.

Table 1. Estimation of the molecular weight of the processed Vip3Ca (30 min trypsin-treated, 48h trypsin treated, and 30 min midgut juice-treated)

Protein	**Molecular Weight estimation**		**Conformation**
	ET (ml)	MW (kDa)	
Trypsin_0.5 h	11.6	451.85	Tetramer
Trypsin_48 h	11.4	512.86	Tetramer
Midgut juice_1µg of protein: 0.8 µg of midgut juice	11.9	395.36	Tetramer

[1] Elution time expressed as milliliters

[2] Molecular weight expressed in kDa.

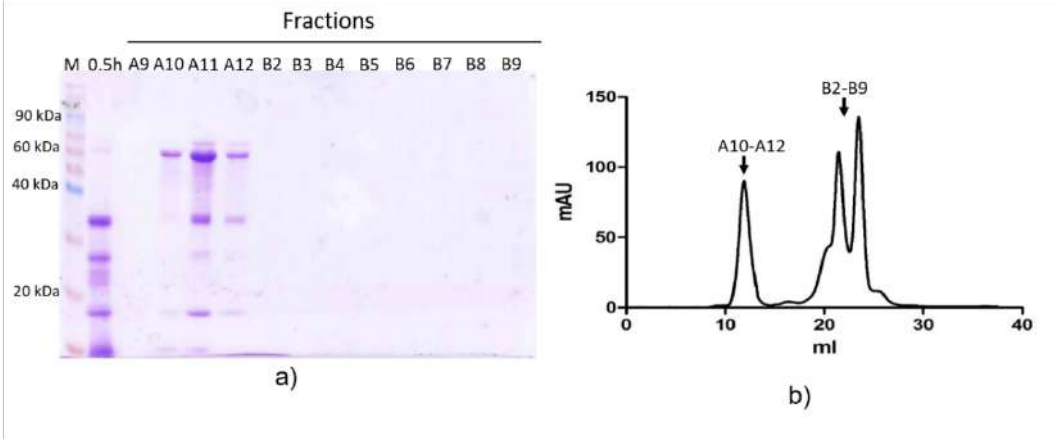

Figure 3. Gel filtration chromatography of Vip3Ca processed with midgut juice of *M. brassicae*. a) SDS-PAGE of the processed Vip3Ca protein incubated midgut juice of *M. brassicae* at a ratio of 1µg of protein : 0.8 µg of midgut juice for 5 min. b) Gel filtration chromatography of the Vip3Ca protein incubated midgut juice of *M. brassicae* at ratio of 1 µg of protein : 0.8 µg of midgut juice for 5 min. M: Pink Plus Prestained Protein Ladder, Genedirex.

Discussion

The mode of action of the Vip3A has been assimilated to that of the Cry proteins. Recent reports suggest that the main steps of the mode of actions of the Vip3 proteins are not so similar to the ones described for Cry proteins. The full length of the Vip3 protein, protoxin, form oligomers of 4 units that is activated by the is activated in the insect midgut to the active form of the Vip3 proteins that is also an oligomer of 4 units (4–9). This observation indicate that the 60 kDa and 20 kDa bands remain together and are needed for the protein structure and insect toxicity (9,10). So far it has been proposed that the Vip3A monomer (60 kDa and 20 kDa) cross the peritrophic membrane and binding to specific sites in the epithelial membrane. However, recent studies suggest that the activated form of the Vip3 proteins (tetrameric conformation) could make pores in a planar lipids bilayer (7), thus further work is needed to demonstrate if the oligomer or monomer bind to the midgut epithelium.

Regarding the instability of the 60 kDa band after the proteolysis with trypsin and midgut juice (11–14) is an artefact produced by an inadequate inactivation of the proteases. When the samples is heated in the presence of SDS andß-mercaptoetanol, the protein unfolds and become available hidden cut sites for the trypsin or midgut juice proteases (15).

Our results suggest that, when exposed to trypsin the Vip3Ca protoxin render two fragments of 17 kDa and 66 kDa approximately. The increase of the 66 kDa band with time at high concentrations of trypsin (Figure 1) can be explained as follows: since trypsin autodigests, the shorter the incubation time of the Vip3Ca protein with trypsin, then the higher the trypsin concentration still present in the sample, and thus, the more efficient processing of the 66 kDa peptide by trypsin under denaturing conditions. Regarding the apparent degradation of the 66 kDa fragment at high concentrations of trypsin or MJ (Figure 2) may due to the action of the proteases upon addition of SDS with the loading buffer. This is inferred from the results when the sample is subjected to gel filtration chromatography and trypsin is separated from the activated Vip3Ca prior to SDS-PAGE analysis.

Gel filtration chromatography shows that the proteolytically processed Vip3Ca protein elutes as a high molecular mass protein, 395 - 512 kDa (Table 1). The SDS-PAGE analysis of the elution peak shows two bands of 66 kDa and 17 kDa, which indicates that these two molecules remain associated under native conditions in an oligomer (tetramer), as was previously reported for the Vip3Aa (4,9,15). The elution of the processed Vip3Ca protein from the gel filtration column, as a high molecular mass protein, is in agreement with a previous studies (4,6,7,9,15) that show that the trypsin-activated Vip3Aa protein aggregates in solution to form an oligomer.

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Changes in gene expression and apoptotic response in *Spodoptera exigua* larvae exposed to sublethal concentrations of Vip3 insecticidal proteins

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The insecticidal Vip3 proteins from *Bacillus thuringiensis* (Bt), along with the classical Bt Cry proteins, are currently used in Bt-crops to control insect pests, since they do not share the same mode of action. Here we characterized the response of *Spodoptera exigua* larvae after Vip3 challenge. The expression profile of 47 genes was analyzed in larvae challenged with three concentrations of Vip3Ca. Results showed that the up-regulated genes were mainly involved in immune response, whereas the down-regulated genes were mainly involved in the digestion process. Other mechanisms of cellular response to the damage such as apoptosis were analyzed. For this analysis, sections from the midguts were examined by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining. The nuclei of the midgut epithelial cells were stained at the highest concentration of the Vip3Ca protein and at lower concentrations of Vip3Aa in agreement with the different potency of the two proteins. In addition, apoptosis was also examined by the analysis of the expression of five *caspase* genes. The present study shows that exposure of *S. exigua* larvae to sublethal concentrations of Vip3 proteins activates different insect response pathways which trigger the regulation of some genes, APN shedding, and apoptotic cell death.

The concern over the excessive use of chemical insecticides has increased in recent years, due to the ecological impact, as well as to the selection for resistance in field insect populations[1]. Among the different biological alternatives for pest control, one of the most popular is the use of bioinsecticies based on *Bacillus thuringiensis*[2]. This bacterium produces a wide range of insecticidal proteins which are active against a number of agricultural pest species[3]. Insecticidal crystal proteins (Cry proteins) are being used to control insect pests in formulated sprays since 1938[4] and, since 1996, they have been expressed in transgenic crops to protect them from insect attack[5]. More recently, a novel class of insecticidal proteins (Vip3 proteins) have also been introduced in transgenic crops to complement the toxic action of the Cry proteins, as well as to reduce the risk of insect resistance development in the field[6] (ISAAA GM Approval Database. http://www.isaaa.org/gmapprovaldatabase).

Cry proteins are highly specific against their target insects and are generally recognized as pore-forming toxins (PFTs)[7]. The mode of action of these proteins has been extensively studied for more than 20 years, especially for Cry1A proteins[8,9]. In general, it is accepted that these toxins are solubilized in the insect gut and then activated by the action of digestive enzymes. The active forms bind to specific receptors in the brush border of epithelial midgut cells and induce pores in the membrane which eventually lead to septicemia and insect death[9]. Nowadays, different models have been proposed to explain how these proteins exert their cytotoxicity, however some aspects remain unclear[8,10]. Much less is known about the mode of action of Vip3 proteins. The Vip3Aa protein was the first member of the family of Vip3 proteins being described[11] and most studies dealing with the mode of action of Vip3 proteins have been performed with this protein[12]. The available information supports that these proteins act by forming pores in the midgut epithelial cells[13,14]. As for Cry proteins, Vip3 proteins are synthesized

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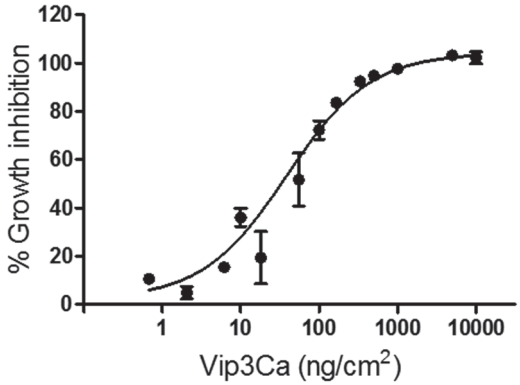

Figure 1. Growth inhibition dose-response curve of newly molted 4th instar *S. exigua* larvae after exposure to Vip3Ca for 24 h. Each value represents the mean of at least three independent experiments (±SEM).

as protoxins which are processed by proteases in the larva midgut rendering the active form, which then binds to specific receptors in the brush border of epithelial midgut cells[15–18]. After binding, the Vip3 protein induces disruption of the midgut epithelial cells[18–21] by its pore forming activity[13,14]. One very interesting feature of the Vip3 proteins mode of action is that they do not share membrane binding sites with Cry proteins, a property which does not only complement the spectrum of activity of Cry proteins, but also decreases the chances of cross-resistance[13,14,17,22,23].

The insect gut is not only an organ of digestion but also constitutes the first physical barrier that protects the host against penetration of both pathogenic and commensal microorganisms[24,25]. In mammals, some studies have identified the mechanisms that regulate gut mucosal immunity, revealing a central role of innate immunity in these processes[26]. Despite the fact only few studies have been conducted in insects, the available data suggest that midgut epithelial tissue of the insects challenged with either pathogenic or nonpathogenic bacteria[27–29] is able to trigger an immune response to reduce the cellular and tissue damage. Therefore, the insecticidal activity of Cry and Vip proteins might be affected by the host defense response, since they exert their toxic action in the midgut of the target insects.

Transcriptomic and proteomic approaches are being helpful to elucidate which mechanisms are involved in the host responses to *B. thuringiensis* proteins in non-model insects of agricultural importance[30–37]. In general, these analyses point out that, after protein exposure, the insects usually increase their immune function in addition to reduce their digestive activity[38,39]. Some reports have shown that the expression of some components of the mitogen-activated protein kinase (MAPK) cascade were up-regulated in response to Cry proteins in Coleoptera, Diptera, and Lepidoptera[40–43]. Additionally, other gene families have been described to be transcriptionally regulated in response to Cry and Vip3 proteins in *Spodoptera exigua* larvae[30,31,44–46], such as the *response to pathogens* (*REPAT*) genes, though their specific role in host response still remains unclear[38,45].

Apoptosis has also been described as a mechanism of cellular response after the exposure of cultured cells with different PFTs[47–50]. *In vitro* experiments with midgut primary culture cells from *Heliothis virescens* showed that Cry toxins induced apoptosis in epithelial cells[51]. More recently, similar results were observed when CF1 or Sf9 cultured cells were exposed to Cry1A or Vip3Aa proteins, respectively[52,53]. *In vivo* assays also showed that apoptosis could be observed in insect midgut epithelial cells when Cry proteins were administered to both *Culex pipiens* and *Bombyx mori* larvae[54,55].

The activation of different mechanisms of response in *S. exigua* larvae after the exposure to different *B. thuringiensis* proteins (Cry and Vip3 proteins) has been reported[30,31,38,56]. These mechanisms of response might contribute to reduce the damage produced by *B. thuringiensis* proteins to the insect. To date, the genes identified in the *S. exigua* response to *B. thuringiensis* proteins have been found to be involved in many different aspects of the insect biology such as: metabolism, immune-response, detoxification, and epithelial renewal, among others[38]. The present work extends previous studies carried out on *S. exigua* larvae with the analysis of the expression profile of 47 genes after Vip3Ca exposure. These selected genes were previously found differentially expressed after the exposure to other *B. thuringiensis* proteins and other pathogens[31,56,57]. Thus, the data obtained in the present study could help understand whether the *S. exigua* response to Vip3Ca is specific or, on the contrary, it is a conserved feature independent of the toxic agent to which the insects are exposed to. Furthermore, the damage produced by Vip3Ca and Vip3Aa proteins has been characterized by measuring the *in vivo* response of the midgut epithelial cells (APN shedding and apoptosis).

Results

Growth inhibition assays. Susceptibility of *S. exigua* 4th instar larvae against the Vip3Ca protein was determined in terms of the effect on larval growth inhibition. The results showed a dose-response relationship, with an EC_{50} of 38.4 ng/cm^2 (Fig. 1). Therefore, despite the fact that Vip3Ca has negligible activity, in terms of mortality, against *S. exigua*[58], it has a clear effect on larval growth inhibition.

Analysis of the gene expression upon Vip3Ca challenge. The expression profile of 47 genes was analyzed in *S. exigua* midguts by qRT-PCR, after 24 h exposure to Vip3Ca. To be able to compare gene expression

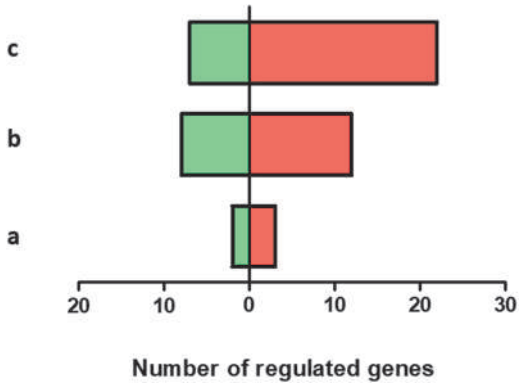

Figure 2. Overview of the regulated genes in *S. exigua* midgut after Vip3Ca challenged at 100 (**a**), 1000 (**b**), and 10000 (**c**) ng/cm^2. A total of 47 genes were analyzed. The number of up- and down-regulated genes are indicated in red and green bars, respectively.

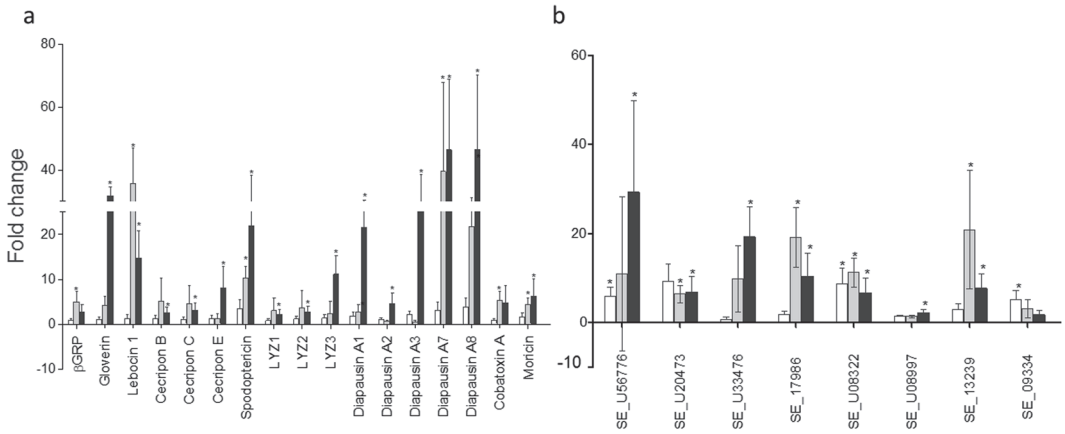

Figure 3. Analysis of the genes whose expression is up-regulated after 24 h exposure at three concentrations of Vip3Ca. (**a**) Immuno-related genes (**b**) Non immuno-related genes. White, grey, and black bars represent the gene expression of each transcript after Vip3Ca challenged at 100, 1000, and 10000 ng/cm^2, respectively. Abbreviations: βGRP: beta-1,3-glucan recognition protein. The expression of each gene in the gut of Vip3Ca exposed larvae was compared to its control in the gut of control larvae (exposed to WK6). Fold-changes were determined by using the REST MCS software. Each bar represents the mean of three independent experiments (±SD). Significant differences were indicated by an asterisk.

results with those previously reported after Vip3Aa challenge[31,56], the concentration of Vip3Ca to cause 99% growth inhibition on 4th instar *S. exigua* larvae (1000 ng/cm^2) was used. In order to test whether a lower or higher concentration of Vip3Ca could alter the regulatory effect on these genes, their expression levels were also determined after a challenge with either 100 or 10000 ng/cm^2 of Vip3Ca.

The results showed that the number of genes whose expression was altered was different depending on the exposure condition (concentration of Vip3Ca) (Fig. 2). At the lowest concentration of Vip3Ca (100 ng/cm^2), only 5 genes (about 11%) were regulated (Fig. 2 and Supplementary Table S1), whereas at the other two concentrations tested, 1000 and 10000 ng/cm^2, the number of regulated genes was 20 (about 43%) and 29 (around 62%), respectively. The distribution of up- and down-regulated genes, according to the concentration of Vip3Ca used in each treatment, is summarized in Supplementary Fig. S1. Almost all the regulated genes at 100 and 1000 ng/cm^2 were also found regulated at 10000 ng/cm^2 of Vip3Ca, suggesting that the response can be dose-dependent. The ratio of up-regulated and down-regulated genes at 100 and 1000 ng/cm^2 was similar (3 up- and 2 down-regulated, and 12 up- and 8 down-regulated, respectively). However, at 10000 ng/cm^2, the ratio of up-regulated genes and down-regulated genes was higher (22 vs. 7, respectively). The levels of transcriptional activation ranged from 2.8-fold to 46.5-fold, whereas the levels of transcriptional repression ranged from 2.5-fold to 653-fold (Figs 3, 4 and Supplementary Table S1).

At 100 ng/cm^2, the up-regulated genes included a member of the *REPAT* family (*REPAT2*), a gene coding for a juvenile hormone binding protein (SE_U56776), and one for for a pancreatic lipase (SE_U08322). Of the two down-regulated genes, one had homology with a lipase gene, whereas the other two had no homology to known genes from public sequence databases (SE_U12696).

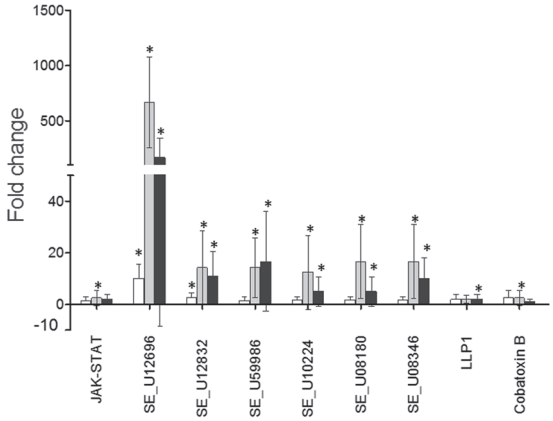

Figure 4. Analysis of the genes whose expression is down-regulated after exposure 24 h at three concentrations of Vip3Ca. White, grey, and black bars represent the gene expression of each transcript after Vip3Ca challenged at 100, 1000, and 10000 ng/cm^2, respectively. The expression of each gene in the gut of Vip3Ca exposed larvae was compared to its control in the gut of control larvae (exposed to WK6). Fold-changes were determined by using the REST MCS software. Each bar represents the mean of three independent experiments (±SD). Significant differences were indicated by an asterisk.

At the concentration of Vip3Ca to provoke 99% growth inhibition on 4th instar *S. exigua* larvae (1000 ng/cm^2) most of the up-regulated genes (7 out of 12) encoded antimicrobial peptides (AMPs), being the most up-regulated ones *Diapausin A6* and *Lebocin 1*. The gene coding for the beta-1,3-glucan recognition protein (β-GRP) was only slightly overexpressed. Additionally, the other up-regulated genes showed homology with genes that encoded juvenile hormone binding proteins (SE_U17986 and SE_U13239) and for pancreatic lipases (SE_U20473 and SE_U08322). Around 88% of the down-regulated genes showed homology to known genes from public sequence databases, including those coding for lipases, proteases and chitin deacetylases (Supplementary Table S1). The biggest repression (653-fold) was observed for one gene which no homology to known gene (SE_U12696). The *Cobatoxin B* gene, as well as the immune signaling pathway *JAK-STAT* gene, were also found slightly down-regulated.

At the highest concentration used (10000 ng/cm^2) the scenario observed was similar to the one described for the larvae exposed to 1000 ng/cm^2. Fifteen of the twenty-two up-regulated genes encoded AMPs (Fig. 3). These 15 AMPs belong to different groups such as the cysteine-rich peptides (Diapausin A1-A3, A6 and A7, and Spodoptericin), glycine- and proline-rich peptides (Gloverin), amphipatic peptides (Cecropin B, C, and E, and Moricin) and lysozymes (LYZ1, 2, and 3). Other up-regulated genes showed homology with genes that encoded juvenile hormone binding proteins (SE_56776, SE_U17986, and SE_U13239), for a Diapausin precursor (SE_U33476), for pancreatic lipases (SE_U20473 and SE_U08322), and for a gene with no homology to any known gene (SE_U08997). The down-regulated genes showed homology with those coding for lipases, proteases and chitin deacetylases (Supplementary Table S1). Again, the biggest repression (167-fold) was observed for the gene SE_U12696. The gene encoding for the AMP LLP1 was also found slightly down-regulated.

Determination of epithelial damage by APN shedding assays. Shedding of membrane-bound APN to the lumen, as a marker for epithelial damage, was measured after 24 h exposure to Vip3 proteins at the concentration of 100 ng/cm^2 of Vip3Aa and at 3 different concentrations of Vip3Ca (100, 1000, and 10000 ng/cm^2). The results showed that, in larvae exposed to Vip3Ca, the APN activity in the luminal fluid increased *ca*. 3-fold and 6-fold at 1000 or 10000 ng/cm^2, respectively, though no significant change was observed at 100 ng/cm^2 (Fig. 5). A correlation between growth inhibition and the amount of APN released into the luminal fluid produced by Vip3Ca protein was observed (Supplementary Fig. S2).

The luminal APN activity of those larvae exposed to 100 ng/cm^2 of Vip3Aa increased *ca*. 5-fold. Interestingly, the APN activity in the lumen fluid of larvae exposed to a concentration that produces a 99% of growth inhibition for each respective Vip3 protein (100 ng/cm^2 for Vip3Aa and 1000 ng/cm^2 for Vip3Ca) was significantly higher for Vip3Aa.

Determination of epithelial cell damage by the TUNEL assay. To test whether exposure to sub-lethal concentrations of Vip3Ca could trigger a signaling pathway leading to the death of the epithelial cells by apoptosis, midguts of larvae exposed for 24 h to Vip3Ca were sectioned and stained with the DeadEnd™ Fluorimentric TUNEL system. Midguts of larvae exposed to Vip3Aa were used for comparative purposes. The results showed that in control larvae (exposed to WK6 proteins) and in starving larvae, no TUNEL-positive cells were observed. No TUNEL-positive cells were observed either after Vip3Ca treatment at the two lowest concentrations used (10 and 100 ng/cm^2). However, a few TUNEL-positive cells were observed in the gut of larvae intoxicated with 1000 ng/cm^2 of Vip3Ca and, at the highest concentration used (10000 ng/cm^2), almost all the cells were TUNEL-positive (Fig. 6). In the case of Vip3Aa challenge, TUNEL-positive cells were observed in the gut of the larvae intoxicated with the three lowest concentrations (1, 10 and 100 ng/cm^2) and no TUNEL-positive cells were found at the highest concentration (1000 ng/cm^2) (Fig. 6).

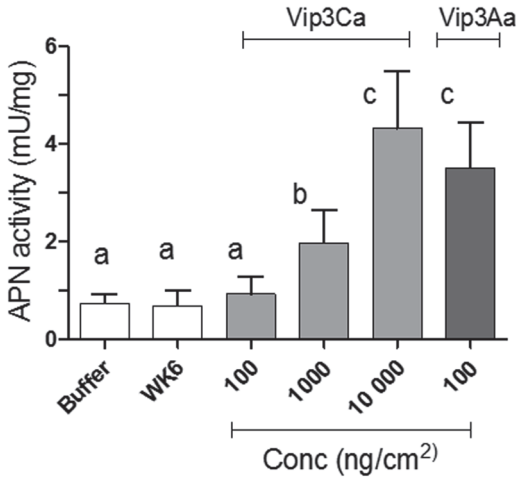

Figure 5. Activity of the APN protein in the midgut lumen of larvae exposed for 24 h to WK6 proteins (control larvae), Vip3Ca (three concentrations) or Vip3Aa (one concentration). Means were compared by one-way ANOVA, followed by Dunnett's comparison test ($P < 0.001$). Significant differences between control larvae and treated larvae were indicated by different letters.

Analysis of the expression levels of apoptosis-related genes in *S. exigua* larval midgut challenged with Vip3Ca. The induction of the apoptotic process was analyzed in the midgut epithelial cells, at the molecular level, by measuring the change in transcription levels of 5 *caspase* genes by qRT-PCR. The transcription levels of the gene encoding a component of the JAK-STAT pathway was also analyzed since this pathway has been related with the renewal of the midgut tissue[59,60]. To analyze the time course of apoptosis, the expression level of the six genes were monitored at 4 different time points: 3 h, 6 h, 12 h, and 24 h after Vip3Ca challenge at 10000 ng/cm^2. After either 3 h or 6 h of exposure, only *Se-Caspase-4* was found up-regulated, whereas after 12 h of exposure four of the 5 *caspase* genes studied were found up-regulated (Fig. 7). In contrast, none of the 5 *caspase* genes studied were found regulated after 24 h exposure. These data suggest that the main transcriptional induction of apoptotic machinery occurs after 12 h exposure. The gene coding a component of the JAK-STAT pathway was found down-regulated after 3 h, 6 h, and 12 h of Vip3Ca challenge (Fig. 7).

Fitness cost analysis. Since the exposure to Vip3 proteins affected the transcriptional pattern of *S. exigua* larvae and caused epithelial and cellular damage, we wanted to determine whether the exposure also had an associated fitness cost. The results showed significant differences in the time to pupation for those larvae exposed to higher concentrations of either Vip3Aa (10, 100, and 10000 ng/cm^2) or Vip3Ca (100, 1000, and 10000 ng/cm^2), as compared to control larvae and with larvae exposed to lower concentrations (Fig. 8a). Interestingly, the time to pupation was also found significantly longer in starving larvae. The percentage of pupation was found significantly lower in those larvae exposed to the highest concentration of either Vip3Aa or Vip3Ca (Fig. 8b).

Discussion

A better knowledge of the different mechanisms of insect response after the exposure to different *B. thuringiensis* proteins will broaden our understanding of how larvae response might help to reduce the damage produced by these insecticidal proteins. In the present study, the expression profile of 47 selected genes was analyzed in *S. exigua* larvae challenged with three concentrations of Vip3Ca. These genes were previously found differentially expressed after the exposure to Vip3Aa, Cry1Ca, and other pathogens[31,56,57]. In order to compare our results with those obtained previously by Bel *et al.*[31] and by Crava *et al.*[56], a concentration of Vip3Ca which caused a 99% of growth inhibition was used as a starting point. Thus, the data obtained could help understand whether the *S. exigua* response to Vip3Ca is specific or, on the contrary, it is a conserved feature independent of the toxic agent to which they are exposed to.

The gene expression results showed that at the lowest concentration of Vip3Ca tested (100 ng/cm^2) the number of regulated genes was lower (about 11%) than when larvae were exposed to 1000 or 10000 ng/cm^2 of Vip3Ca (around 43% and 62%, respectively). Moreover, almost all the regulated genes at 100 and 1000 ng/cm^2 were also found regulated at 10000 ng/cm^2 of Vip3Ca, suggesting that the host response is dose-dependent. In general, our results agree with previous gene expression studies which showed the up-regulation of genes involved in immune system and hormone modulation (e.g. JH binding protein), and the down-regulation of genes involved in the digestion process (e.g. serine proteases) and peritrophic membrane permeability (e.g. chitin deacetylases), upon exposure to different *B. thuringiensis* proteins[28,30,31,35,36,61–65]. The highest value of down-regulation (653-fold) found in this study was obtained for one gene with unknown function when larvae were exposed to 1000 ng/cm^2 of Vip3Ca. Similar results were observed by Bel *et al.*[31] when *S. exigua* larvae were exposed to Vip3Aa. This gene encodes a putative protein called REVIP because it was detected in Response-to-Vip intoxication.

In our study we have included different *S. exigua* immune-related genes which were classified in three categories by Pascual *et al.*[66]: (a) pathogen recognition, (b) immune signaling pathways and melanization process, and (c) antimicrobial effectors (Supplementary Table S1). A general upregulation of the immune-related genes was

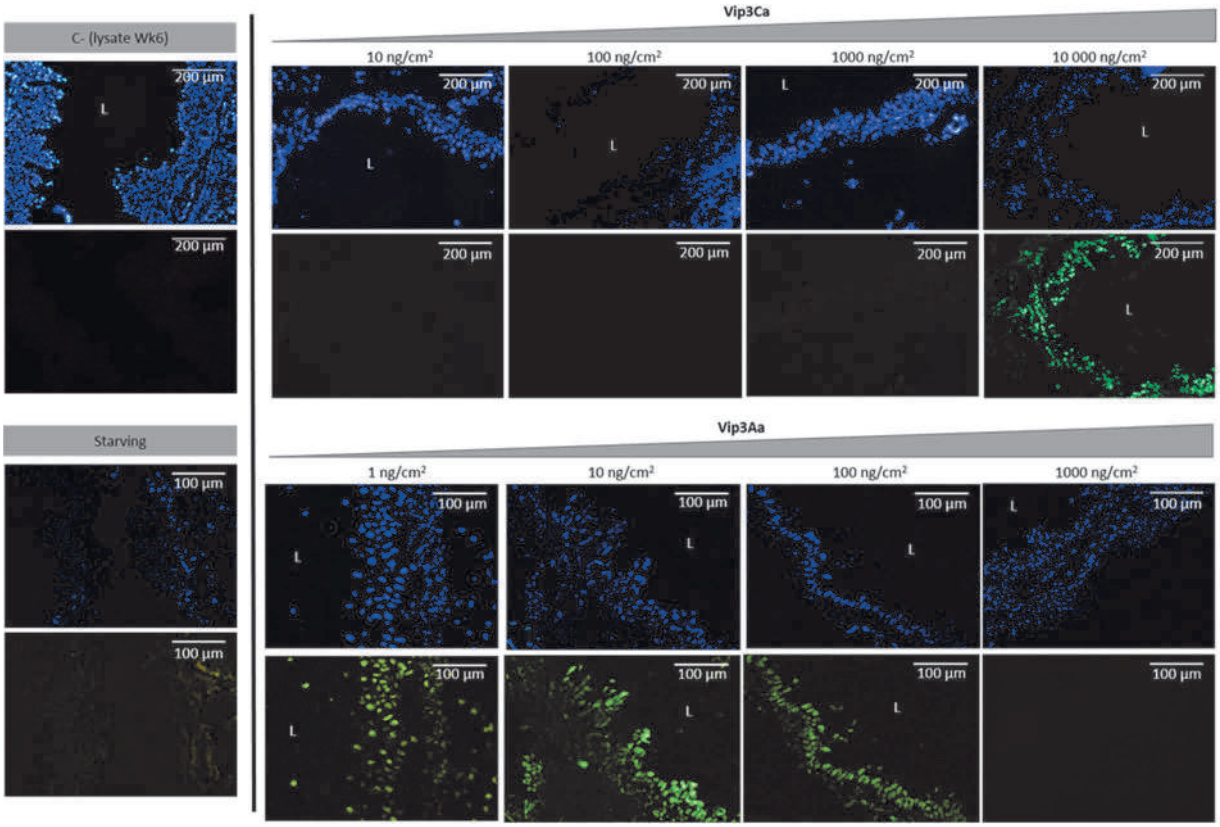

Figure 6. Effect of sublethal concentrations of Vip3Ca and Vip3Aa proteins to *Spodoptera exigua* midgut epithelial cells. L4 newly molted larvae were intoxicated with Vip3 proteins for 24 h and then midgut tissue sections were prepared and stained with TUNEL (green signal) and DAPI (blue signal). As controls, larvae fed with the empty vector (WK6) and 24 h starving larvae were used. Magnification was 100× for the Vip3Ca protein and 200× for the Vip3Aa protein. L, gut lumen.

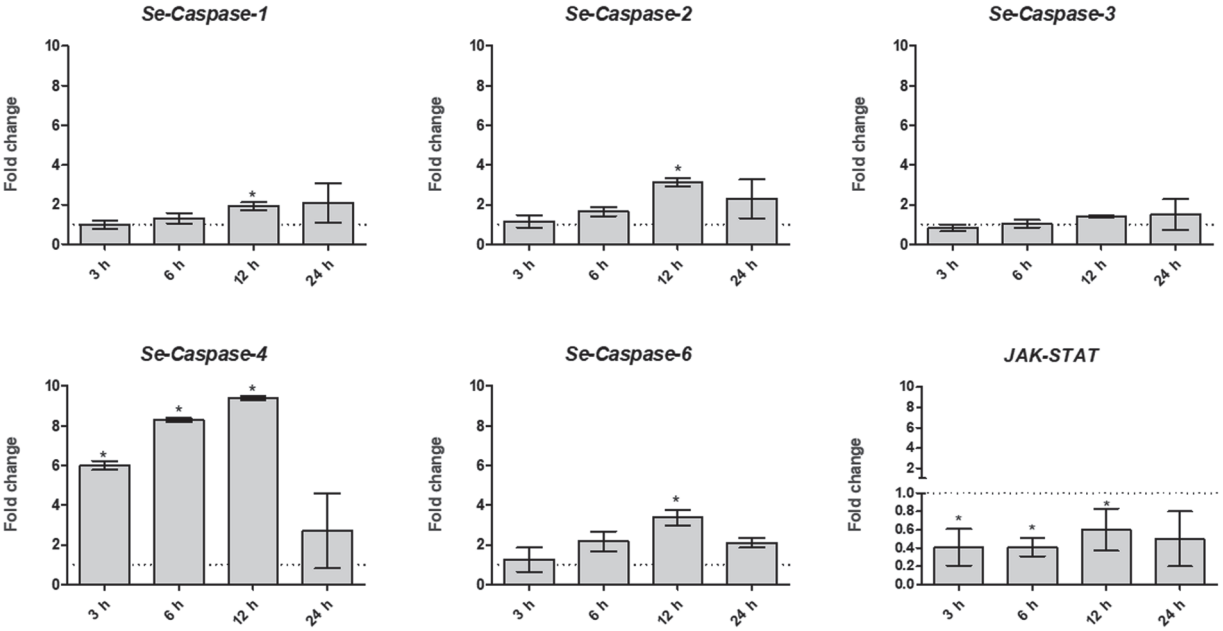

Figure 7. Analysis of the transcriptional induction of apoptotic related genes at 10000 ng/cm² of Vip3Ca for 3 h, 6 h, 12 h, and 24 h. The expression of each gene in the gut of larvae exposed to Vip3Ca was compared to its control in the gut of control larvae (exposed to WK6 empty vector). Fold-changes were determined by using the REST MCS software. Each bar represents the mean of three independent experiments (±SD). Significant differences were indicated by an asterisk.

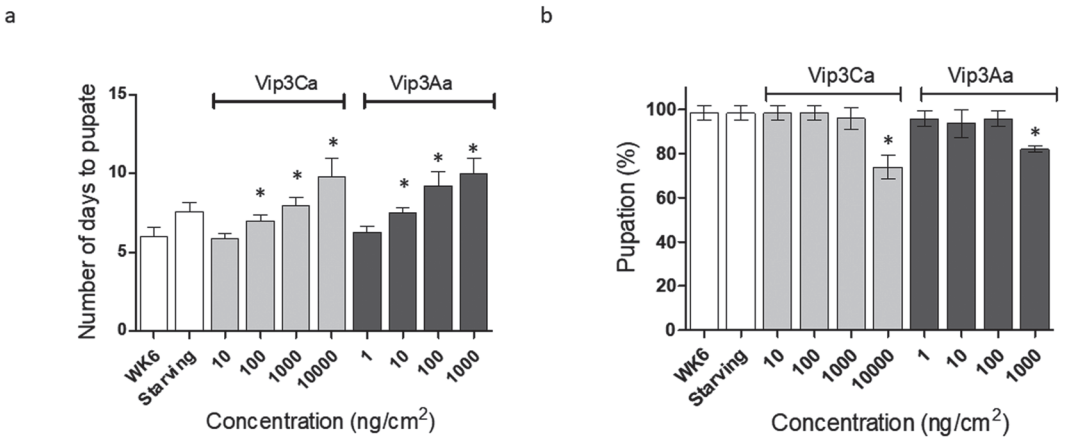

Figure 8. Analysis of the associated fitness cost on *S. exigua* 4th instar larvae after Vip3Ca or Vip3Aa exposure. Two parameters were measured: (**a**) number of days to pupation, and (**b**) % of pupation. Means were compared by one-way ANOVA, followed by Dunnett's comparison test ($P < 0.001$). Significant differences between control larvae and treated larvae were indicated by an asterisk.

observed after Vip3Ca exposure at 1000 and 10000 ng/cm^2, though it is worth to note that the genes belonging to the antimicrobial effectors were more clearly regulated after Vip3Ca exposure than the other genes. These antimicrobial effectors are produced to act as barriers against the progress of bacterial infections[67]. Here we analyzed the transcriptional response of 24 genes coding for 21 AMPs and 3 lysozymes. These genes were described in a detailed study performed by Crava *et al.*[56] where most of the 24 genes were found up-regulated after Vip3Aa exposure at a concentration to provoke 99% growth inhibition on 4th instar *S. exigua* larvae. Our results showed that none of these genes were found regulated in response to the lowest concentration of Vip3Ca used. Conversely, when *S. exigua* larvae were exposed at 1000 or 10000 ng/cm^2 of Vip3Ca, the number of regulated AMPs genes was 8 and 15, respectively. Our data agree with previous data obtained from Crava *et al.*[56] in that the response of the *S. exigua* larvae might be associated to the level of cell damage produced by the different *B. thuringiensis* proteins used and not to the mode of action of these proteins.

Genes from the three main immune signaling pathways (*Toll, Imd*, and *JAK-STAT*) were also represented in our study. The results showed that neither the Toll-like receptor gene nor the *Imd* gene were found to be regulated at any of the 3 concentrations tested. In contrast, the gene encoding a component of the JAK-STAT pathway was down-regulated after 24 h exposure to 1000 ng/cm^2 of Vip3Ca. Additionally, the expression of this gene was also found down-regulated when larvae were exposed at 10000 ng/cm^2 of Vip3Ca for lower times (3 h, 6 h, and 12 h). The JAK-STAT pathway has been shown to be involved in the activation of the midgut renewal by the proliferation and differentiation of the stem cells[59,60,68]. Thus, we speculate that the negative regulation of this pathway might be affecting the renewal of the midgut cells that have been damaged by the action of the Vip3Ca protein. This negative regulation might be a host defense response for gut healing processes after entomopathogen exposure[39]. The lack of regulation of the Toll and Imd immune signaling pathways was also described when S. *exigua* larvae were exposed to Vip3Aa protein[31]. It is worth to note that the activation of most of the immune signaling pathways relies mainly on post-translational modifications (e.g. phosphorylation) and to a lesser extent on transcriptional regulations[40,69].

The effect of Vip3Aa and Vip3Ca proteins on the midgut epithelial cells was measured by determining the APN activity into the luminal fluid. The results showed that both Vip3 proteins cause APN shedding into the luminal fluid in *S. exigua* larvae. The ability of some *B. thuringiensis* proteins and other pore forming toxins (PFTs) to cause shedding of cell surface proteins has already been reported[70–72]. In agreement with our results, Valaitis *et al.*[70] found that exposure to Vip3Aa induced shedding of APN in *Lymantria dispar* larvae. However, they hypothesized that the APN shedding would not be involved in host defense to *B. thuringiensis* proteins, since the inhibition of the APN shedding by cyclic AMP did not affect their toxicity[70]. Here we observed a clear dose-response relationship between the growth inhibition produced by increasing concentrations of Vip3Ca and the APN activity in the lumen of the larvae. This result supports that the depletion of the APN is stronger when the damage produced to the epithelial cells is larger. Moreover, when comparing the APN shedding observed after exposure using a concentration of either Vip3Aa or Vip3Ca proteins which produces 99% growth inhibition (100 and 1000 ng/cm^2, respectively), the results showed that the luminal APN activity is higher in those larvae exposed to Vip3Aa than in those exposed to Vip3Ca. This result suggests that the cell damage produced by the Vip3Aa protein might be larger than the one produced by the Vip3Ca protein. Moreover, this result is in agreement with the results obtained in the gene expression analysis, since the exposure to Vip3Aa seems to regulate the expression of more genes that the exposure to Vip3Ca, at the same dose, in *S. exigua* larvae.

The analysis of the *S. exigua* midguts exposed to sublethal concentrations of Vip3Aa and Vip3Ca proteins by TUNEL assays showed the presence of TUNEL-positive cells at different concentrations of each protein (Fig. 6). TUNEL-positive cells were clearly observed at the highest concentration (10000 ng/cm^2) of the Vip3Ca protein. In contrast, TUNEL-positive cells were detected at the lowest and intermediate concentrations of Vip3Aa (1, 10, and 100 ng/cm^2). No TUNEL-positive cells were observed at the highest concentration of Vip3Aa (1000 ng/cm^2),

probably due to the fact that, at this concentration, epithelial cells might be responding by other mechanisms and/or by other signal-transduction pathways to respond to such an attack[73,74] or it might happen that most of the cell membrane was disrupted. The histopathological effects produced in larvae from *Mamestra brassicae* after exposure to either Vip3Aa or Vip3Ca has been described by Gomis-Cebolla *et al.* (2016)[18]. The results showed that feeding with both Vip3 proteins produced disruption of the midgut epithelium, though the damage caused by Vip3Aa protein was detected earlier, in agreement with its higher toxicity. Consistent with our data, Tanaka *et al.*[55] reported the presence of TUNEL-positive midgut cells in *B. mori* larvae when treated with sublethal concentrations of Cry1Aa and few TUNEL-positive cells when larvae were exposed to lethal concentrations. On the basis of these observations, we can speculate that disruption of midgut cell membranes, by the pore formation activity, is a main event that occurs when the insect species are exposed to a high dose of an active *B. thuringiensis* protein. Nevertheless, other events, such as apoptosis, may happen when larvae are exposed to sublethal doses. Thus, apoptotic events might involve a host defense response to the damage produced by the toxic agent, leading to renewal of the epithelial layer[39,73].

Although the mode of action of Vip3 proteins is still not completely resolved, it is commonly accepted that, similarly to Cry proteins, they bind to specific receptors and form pores in the brush border of the epithelial cells[13,15,17,18]. Additionally, some studies have reported that Cry proteins can activate different intracellular cascade pathways, leading to apoptotic cell death[10,51,52,54,55]. The ability to produce pores in their target cells and also to activate different intracellular cascade pathways has also been described for other toxins (e.g. aerolysin and alpha-toxin) produced by other bacteria[75,76]. Apoptosis is a special and highly regulated type of programmed cell death that can be induced by different factors. Apoptosis has a fundamental role in biological process such as: development, tissue homeostasis, DNA damage response, and immune response[77]. In mammalian cells, PFTs kill cells by two different mechanisms: (1) apoptosis, characterized by the activation of initiator caspases that trigger effector- caspases to cleave cellular substrates, and (2) inflammatory responses by the activation of inflammatory caspases[78]. Caspases (cysteine-dependent aspartate-specific proteases) are a family of evolutionary conserved proteins that have been described for playing a key role in apoptosis[79]. In addition to the TUNEL assays, apoptosis was monitored by the analysis of the expression levels of five *caspase* genes. The results showed that expression level of the *Se-Caspase-4* was highly up-regulated after 3 h, 6 h, and 12 h. This gene has special sequence features and its function has not been assessed yet[79]. Nevertheless, as this gene is regulated after 3 h of exposure we can hypothesized that it might play a role in the pro-apoptotic proteolytic cascade as an initiator caspase. The expression levels of the initiator *Se-Caspase-6* and the effector *Se-Caspases-1* and *-2* genes were found up-regulated after 12 h of Vip3Ca exposure. The expression levels of the gene coding *Se-Caspase*-3 was not regulated at the different times tested, indicating that maybe it would not be involved in the host response to Vip3 exposure.

Here we show for the first time that Vip3Aa and Vip3Ca trigger apoptosis in *S. exigua* midgut epithelial cells *in vivo*. However, further research will be required to define the apoptotic signal transduction pathway induced by both Vip3 proteins in *S. exigua* larvae. It was recently reported that Vip3Aa can induce apoptosis in Sf9 cultured cells and that this is mediated by the mitochondrial and caspase dependent pathways[53]. Portugal *et al.*[52] showed that Cry1A proteins induced apoptosis in CF1 cultured cells. Interestingly, the authors observed that the pore formation activity of the Cry1A proteins is necessary to induce apoptosis. It is possible that, with Vip3 proteins, pore formation is also a necessary step for cells to enter the apoptotic pathway.

In summary, the results from the present study show that exposure of *S. exigua* larvae to sublethal concentrations of Vip3Ca (a protein with low activity against this insect) activates different insect response pathways which trigger the regulation of some genes (such as the antimicrobial effectors, *caspases*), induces APN shedding, and triggers other signals that lead to apoptotic cell death. Understanding the host response process to the *B. thuringiensis* proteins currently used in insect control will help to shed light on insect defensive mechanisms to toxic agents.

Materials and Methods

Insects. Larvae from the *S. exigua* FRA colony were kindly supplied by M. López-Ferber, INRA (St Christol les Alés, France). The insects were reared on artificial diet at 25 °C with a relative humidity of 70% RH and a photoperiod of 16 h/8 h (light/dark). The FRA colony had been maintained for more than 10 years without exposure to pathogens[80].

Expression and purification of Vip3Aa and Vip3Ca proteins. The Vip3Aa protein (NCBI accession No. AAW65132) was overexpressed in recombinant *Escherichia coli* BL21 carrying the *vip3Aa*16 gene. The Vip3Ca protein (NCBI accession No. AEE98106) was overexpressed in *E. coli* WK6 carrying the expression vector pMaab 10 (kindly supplied by Bayer CropScience N.V., Ghent, Belgium). Protein expression and lysis of Vip3Aa and Vip3Ca was carried out following the conditions described by Abdelkefi-Mesrati *et al.*[81] and Gomis-Cebolla *et al.* (2016)[18], respectively.

Vip3Aa and Vip3Ca proteins in the cell lysates were further purified by isoelectric point precipitation (Ipp) as described by Chakroun *et al.*[82]. The pH of the lysates was lowered with acetic acid to pH 5.5 for Vip3Aa, and to pH 5.9 for Vip3Ca. After centrifugation, the precipitated proteins were resuspended in solubilization buffer (20 mM Tris-HCl, 150 mM NaCl, pH 9) and dialyzed against the same buffer overnight. The amount of Vip3 proteins was quantified by densitometry after SDS-PAGE electrophoresis using the TotalLab 1D *v*13.01 software. As a control, the empty *E. coli* WK6 strain was cultured and processed in the same way as described above for the Vip3Ca producing strain.

Growth inhibition assays. Susceptibility of newly molted 4th instar *S. exigua* larvae to Vip3Ca was determined by growth inhibition assays using surface contamination method as previously described[80]. Sixteen larvae were individually exposed to each concentration for 24 h. As a control, 16 larvae were exposed to a protein

preparation from the empty *E. coli* WK6 strain. For this, the pellet obtained after the Ipp of the empty *E. coli* WK6 was prepared in the same way as with the Vip3Ca protein. Five independent biological replicates were performed. The percentage of growth inhibition (% GI) was calculated as described Herrero *et al.*[83], using the formula: % $GI = [1 - (RG_t/RG_c)] \times 100$, where RG_t and RG_c represent the relative growth of larvae after exposure to either Vip3Ca or the empty *E. coli* WK6 strain preparation (control larvae), respectively. Relative growth was calculated as $RG = [(W_1 - W_0)/W_0]$, where W_0 and W_1 are the initial and final weight of the larva, respectively. The effective concentrations which produced a reduction in the larval growth of 50% and 99% were calculated using the GraphPad Prism *v*. 5.1 (GraphPad Inc., La Jolla, CA, US) using a non-linear logistic model (Hill equation).

Effects on larva development after Vip3 protein exposure. The effect of Vip3Ca and Vip3Aa proteins on two parameters of fitness was determined: time to pupation and percentage of pupation. Sixteen 4th instar larvae were individually exposed to 10, 100, 1000, and 10000 ng/cm^2 of Vip3Ca or to 1, 10, 100, and 1000 ng/cm^2 of Vip3Aa. As a control, 16 larvae were exposed to a protein preparation from the empty *E. coli* WK6 strain. After 24 h exposure, larvae were transferred to non-intoxicated diet to complete their development. Three independent biological replicates were performed. The developmental time from 4th instar larvae to pupa and the number of pupae was daily recorded.

Gene expression analysis. The expression profile of 47*S. exigua* genes, which were previously described as responding to a Vip3Aa challenge or other pathogens[31,56,57] (Supplementary Table S1), was analyzed under two different conditions: WK6 exposed (used as a control) and Vip3Ca exposed. For this purpose, sixteen newly molted 4th instar larvae were individually exposed to 100, 1000, and 10000 ng/cm^2 of Vip3Ca for 24 h. For the analysis of the expression levels of apoptosis-related genes, five *S. exigua caspase* genes were selected[79] (Supplementary Table S2). Six newly molted 4th instar larvae were individually exposed to 10000 ng/cm^2 of Vip3Ca for 3 h, 6 h, 12 h and 24 h. Then, larvae were dissected and midguts pooled and stored at −80 °C until used. Three independent biological replicates were performed.

RNA was purified from larval midguts using the RNAzol RT reagent (Sigma-Aldrich, St. Louis, MO, US) according to the manufacturer's instructions. Purified RNA (0.5 μg) was then treated with DNase I (Invitrogen, Carlsbad, CA, US) and subsequently reverse-transcribed to cDNA using oligo-(dT) primers and the SuperScript II Reverse Transcriptase (Invitrogen), following the manufacturer's protocol. Quantitative real-time PCR (qRT-PCR) was carried out using EvaGreen® (Biotis, Vilnius, Lithuania) following standard protocols and measured in an ABI Prism 7700 Sequence Detection System (Applied Biosystems). Each reaction was performed in a final volume of 20 μl, which contained 4 μl of cDNA (300 ng). Forward and reverse primers were added to a final concentration of 300 pM. Primers used in this study are described in Supplementary Tables S1 and S2. The 47 selected genes analyzed in this study, including the house-keeping gene, were previously designed and their efficiency tested by other authors[31,56,57]. Specific primers for the five *S. exigua caspase* genes used in this study were designed on the basis of their sequences from the NCBI database (HQ328953, HQ328958, HQ328966, HQ328975, and HQ328993) using Primer Express Software from Applied Biosystems (Carlsbad, CA, US) (Supplementary Table S2). Prior to quantifying differential expression among different treatments, the efficiency of each pair of primers was evaluated by performing 3-fold dilution series experiments. The specific amplification of transcripts was verified by dissociation curve analysis. The Rest MCS software (version 2) was used to obtain the expression ratios (-fold change)[84].

Measurement of aminopeptidase activity in the midgut lumen. The APN activity in the midgut lumen from *S. exigua* larva treated with either Vip3Ca or Vip3Aa proteins was measured as a marker to evaluate the damage produced by the proteins after 24 h exposure. At least three independent replicates were performed for each condition. In each replicate, sixteen 4th instar newly molted larvae were exposed to four different concentrations of Vip3Ca (10 100, 1000, and 10000 ng/cm^2) and to one single concentration of Vip3Aa (100 ng/cm^2). The concentration of Vip3Aa used produced a 99% of growth inhibition according to a previous study[31]. As a control, WK6 proteins prepared as for the Vip3Ca sample were used. The contents from at least 10 midguts (for each condition) were obtained and transferred into 100 μl of 50 mM Tris-HCl, pH 7.5, 1 mM PMSF. Then, midgut contents were vortexed for 30 s, centrifuged at 21 000 x *g* for 5 min at 4 °C, and the supernatant was used for activity assays. Protein concentration was determined by Bradford[85]. APN activity was determined using 4 mM L-leucyl-p-nitroanilide in 50 mM Tris-HCl (pH 7.5) buffer as substrate. The released of p-nitroanilide was monitored at 405 nm for 2 min using a Tecan Infinite 200 plate reader (Switzerland). An extinction coefficient of 9.9 mM^{-1} cm^{-1} for p-nitroanilide was used.

Sectioning of insect tissues and TUNEL staining. Fragmentation of the DNA in the midgut epithelial cells from *S. exigua* larva exposed to either Vip3Ca or Vip3Aa proteins was measured as a marker of apoptosis. DNA fragmentation was measured using the principle of TUNEL (TdT-mediated dUTP Nick-End Labeling), which consists on the catalytical incorporation of a labelled dUTP (2′-deoxyuridine 5′-triphosphate) at the 3′- hydroxyl (-OH) group of the DNA end using a terminal deoxynucleotidyl transferase. For TUNEL staining assays, 4th instar newly molted larvae were treated for 24 h under four different conditions: WK6 exposed (used as a control), Vip3Ca exposed, Vip3Aa exposed, and starving (larvae kept without food for 24 h). In each assay, 16 larvae were exposed to four different concentrations of Vip3Ca (10, 100, 1000, and 10000 ng/cm^2) or Vip3Aa (1, 10, 100, and 1000 ng/cm^2). Three independent biological replicates for each treatment were performed. After 24 h exposure, only larvae actively eating (as determined by observing the food bites) were selected, flash frozen and kept at −80 °C until used. Sections of 10 μm were prepared by the microscopy facilities at the Universitat de València using the cryostat microtome Leica CM 1510 S. Slides with the tissue sections were stored at −20 °C until used. Tissue sections were treated with the DeadEnd™ Fluorimentric TUNEL system (Promega) following the

manufacturer's instructions. Nuclear DNA was stained with DAPI (4′,6-diamidino-2-phenylindole) as described by Chazotte[86]. Coverslips were mounted using mounting medium from Sigma. Tissue sections were then examined using Leica DMI2500 microscope equipped with a digital color camera (Leica DFC300 FX). Tissue sections were stained with hematoxylin and eosin as was described elsewhere[87] to check the quality of the midgut sections (Supplementary Fig. S3).

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Author Contributions

P.H.M., J.F., and B.E., contributed to the design of the study. P.H.M., and J.G.C., performed the experiments. P.H.M., J.F., J.G.C., and B.E., analyzed the data. P.H.M., and J.F., wrote the manuscript. All authors reviewed the manuscript.

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8. - DISCUSSION

8. - General Discussion

B. thuringiensis is one of the entomopathogenic bacteria used for the control of the plant eating insect species. *B. thuringiensis* shows high specificity to lepidopteran pests due the presence of parasporal crystals that contain the insecticidal proteins such as Cry proteins. An approach to improve the effect of these insecticidal proteins is combining them either in a *B. thuringiensis* formulation (*B. thuringiensis* isolates selected by their combination of insecticidal proteins in the parasporal crystal) or by plant expression (Bt crops). The use of Bt crops has increased quickly during the last decade and, despite its high efficacy as a biological control agent, there are some concerns over the narrow spectrum of activity of the individual toxins and also the threat of emergence of resistance in insects populations.

An way to address these concerns is the search for novel toxins with new insecticidal spectra or new mode of action. One way to discover new insecticidal protein genes is the screening of *B. thuringiensis* collections (PCR or NGS approach) from different locations and environments. Several studies have been performed with this aim which have rendered a high number of new insecticidal protein genes (http://www.lifesci.sussex.ac.uk/Home/Neil_Crickmore/Bt/). As an example, the *B. thuringiensis* vegetative insecticidal proteins (Vip1, Vip2, Vip3 and Sip) are a class of new proteins discovered in a screening of different *B. thuringiensis* collections (1–3). Interestingly this class of new insecticidal proteins has shown to be significantly more active against several agronomically important insects, specifically the Vip3 proteins.

Knowing the importance of these proteins for the biological control, this thesis has been dedicated to determine the toxicity and mode of action of Vip3Ca (a member of a new Vip3 protein family) and mining of new insecticidal protein genes in *B. thuringiensis* from selected *B. thuringiensis* isolates based on their gene content.

8.1 - Characterization of the mode of action of Vip1 and Vip2 proteins and mining of new *vip1* and *vip2* protein genes

Our first observation starts with the identification of *vip1*-, *vip2*- and *vip4*-type genes in the eleven *B. thuringiensis* isolates selected based on their content in *vip1* and *vip2* genes (4). As a first step, the PCR-Sanger Sequencing approach revealed, in 7 out 9 *B. thuringiensis* isolates, two new alleles of the binary toxins Vip2Ac-Vip1Ca and Vip2Bb-Vip1Bb and, in 2 out 9 *B. thuringiensis* isolates, the presence of two sequences with low similarity to *vip1Bb1* (from the *B. thuringiensis* isolate E-SE10.2) and *vip4Aa1* (from the *B. thuringiensis* isolate O-V84.2). In a second step, the genes from the *B. thuringiensis* isolates harbouring the new alleles of the binary toxins Vip2Ac-Vip1Ca and Vip2Bb-Vip1Bb were cloned to determine the full length of the *vip1* and *vip2* gene pairs. Also the *Bt* isolates E-SE10.2 and O-V84.2 were subjected to whole genome sequencing and in gel digestion LC/MSMS analysis of the supernatant and spore/crystal mixture.

The sequence of the two new alleles of the binary toxins Vip2Ac-Vip1Ca and Vip2Bb-Vip1Bb was determined by genome walking and deposited in the GenBanK and in the *Bacillus thuringiensis* toxin nomenclature database: *vip2Bb3* (KR065728) - *vip1Bb2* (KR065727), *vip2Ac2* (KR065726) - *vip1Ca2* (KR0065725) (See Annex 10.1). Regarding the mining of new *vip1* and *vip2* genes in the *B. thuringinsis* isolates E-SE10.2 and O-V84.2, these were subjected to whole genome sequencing and in gel digestion LC/MSMS analysis to identify and quantify the expressed *B. thuringiensis* proteins. The genomes of both *B. thuringiensis* isolates contained 6156 coding sequences (CDS) and 6457 CDS, respectively. This result indicated that the CDS represent the 80 % of the genome length in both *B. thuringiensis* isolates. Regarding the number of expressed of *B. thuringiensis* proteins in three different growth phases (supernatant at 24 h and 48 h, and crystal 72 h), these were determined by in gel digestion LC/MSMS analysis. The total proteins encountered in the different growth phases for the *B. thuringiensis* isolates E-SE10.2 and O-V84.2 were 10.2/3.5 %, 10.4/8.2 %, and 8.3/2.8 %, respectively, of their respective genome CDS in each growth phase.

The supernatants (at 24 h [growth phase T1] and 48 h [growth phase T2]) and the crystal proteins [growth phase T3] of both *B.thuringiensis* isolates were also analyzed and annotated with GO terms. In each growth phase (T1, T2 and T3), the number of annotated proteins were 42.3 %, 49.8 % and 56.9 %, respectively. The distribution of the GO terms

along the different growth phases showed that the most common and abundant GO terms in all the phases are the following: “cellular biosynthetic process”, “organic substance biosynthetic process”, “cellular nitrogen compound metabolic process” and “organonitrogen compound metabolic process”. Regarding the presence of an specific GO term in each growth phase, there was only present one, in the T3, with the annotation of the term “macromolecule metabolic process”.

Related to the predicted insecticidal protein genes in both *B. thuringiensis* genomes, we found part of the predicted genes by the CDS prediction software. Specifically, one new couple of binary Vip-like proteins (Vip2Ac-like_1-Vip4Aa-like_1), two new Vip-like proteins (Vip2Ac-like_1 and Vip4Aa-like_2); one Sip1A-like protein (Sip1A-like_1) and eight Crystal-like proteins (Cry23A-like, Cry45Aa-like_1, Cry45Aa-like_2, Cry45Aa-like_3, Cry32Ea-like, Cry32eDa-like, Cry32Eb-like and Cry73Aa-like). To determine the subcellular localization of the *B. thuringiensis*-like proteins, the supernatant and solubilized crystal proteins were analyzed by LC/MSMS. In the supernatant of both *B. thuringiensis* isolates, we detected the Vip4Aa-like_1, Vip4Aa-like_2, Vip2Ac-like_1, Vip2Ac-like_2, Sip1Aa-like_1 proteins. As regard the crystal proteins, they were found in the crystal of both *B. thuringiensis* isolates except for the Cry23Aa-like protein, that was also found in the supernatant at 24 h and 48 h.

The relative abundance of the *B. thuringiensis*-like proteins was estimated in the supernatant and in the solubilized crystal proteins of both *B. thuringiensis* isolates. In the supernatant (24h and 48h) of both *B. thuringiensis* isolates, the Vip-like, Sip1-like and Cry23Aa-like proteins were marginally expressed. The expression level of the Vip-like, Sip1-like and Cry23Aa-like was compared between 24 h and 48h. The Vip4Aa-like_1 protein was increased 2-fold at 24h vs. 48 h, while the Vip4Aa-like_2 only was expressed at 24 h. As regard to the rest of the Vip2Ac-like, Sip1A-like and Cry23A-like proteins, no expression differences were observed between the 24 h and 48 h. Regarding the crystal proteins in the *B. thuringiensis* isolate O-V84.2, the Cry-like proteins represented the half of the total weight percentage, while for the *B. thuringiensis* isolate E-SE10.2 the Cry23Aa-like protein represent a weight in the range of 2.5 % - 30 %. In addition, the crystal composition of the *B. thuringiensis* isolate O-V84.2 was determined for those proteins with a percentage of similarity lower than 45%. The crystal was composed by four kinds of proteins: 7.0 % - 9.8 % Cry45-like proteins, 30.4 % - 30.5 % Cry32-like

proteins, and 2.8 % - 4.25 % Cry73Aa-like proteins, while the *B. thuringiensis* isolate E-SE10.2 only expressed a Cry23Aa-like protein.

To evaluate the toxicity of the concentrated supernatant and crystal proteins of the *B. thuringiensis* isolates E-SE10.2 and O-V84.2, a single dose assay was performed against *S. exigua* and *S. littoralis*. The result of the autoclaved and non-autoclaved concentrated supernatants showed that *S. exigua* and *S. littoralis* were not susceptible to the Vip-like and Sip-like proteins detected in the supernatant. Regarding the solubilized crystal proteins, Cry23Aa-like showed some toxicity to the *S. exigua* (30 % mortality at 7 days) and *S. littoralis* (50 % mortality at7 days), while the for the *B. thuringiensis* isolate O-V84.2, there were none active against *S. exigua* and *S. littoralis*. Although the solubilized crystal proteins of the *B. thuringiensis* isolate E-SE10.2 are toxic against Spodoptera species, we cannot confirm that the observed mortality was due only to Cry23Aa-like, because other proteins present in the mixture could synergize with Cry23Aa-like or contribute to the observed mortality. Further experiments are needed to confirm this hypothesis (See Annex 10.2).

8.2 - Toxicity of the Vip3Ca protein and analysis of cross-resistance to Vip3Ca in insect populations selected for resistance against Cry1Ac, Cry2Ab, Vip3Aa and Vip3Aa/Cry2Ab

Our second observation starts with the increment of the insecticidal spectrum of the Vip3Ca protein and analysis of cross-resistance to the Vip3Ca protein in insect populations selected for resistance against Cry1Ac, Cry2Ab, Vip3Aa and Vip3Aa/Cry2Ab. The Vip3Ca protein is a new Vip3 protein family from *B. thuringiensis*, moderately active against *Chrysodeixis chalcites, H. armigera, M. brassicae, T. ni* and *S. littoralis*. Strong growth inhibition was observed in *S. exigua* and *S. frugiperda*, whereas *O. nubilalis* and *Lobestria botrana* showed very low susceptibility to the Vip3Ca protein (5).

The insecticidal spectrum of the Vip3Ca protein was broaden, adding ten new lepidopteran pests (*Cydia pomonella, Grapholita molesta, Sesamia nonagrioides, Galleria mellonella, P. xylostella, Pectinophora gossypiella, E. kuehniella, Plodia interpunctella* and *O. furnacalis*), one aphid pest (*Nezara viridula*) and one model

organism (*Drosophila melanogaster*) (6–8). Of the six species tested by surface contamination, *C. pomonella* was the most susceptible to the Vip3Ca protein, followed by *Trichoplusia ni* and *G. mellonella*, with percentages of mortality higher than 50% (7,8). Regarding the four species tested by diet incorporation, *O. furnacalis* was the most susceptible to the Vip3Ca protein with an LC_{50} value (0.31 µg/g) in the range of the positive control, Cry1Ab (0.97 µg/g) (8). From the other types of bioassays, *Tuta absoluta* showed some susceptibility to high concentrations of Vip3Ca (up to 30 % mortality). The rest of species (*G. molesta, P. gossypiella, P. xylostella, P. interpunctella, E. kuehniella, S. nonagrioides, Drosophila melanogaster* and *N. viridula*) can be considered non-susceptible (6–8). Interestingly, for most of the new insect species tested, the Vip3Aa protein (positive control of toxicity) is more active than the Vip3Ca protein. As an example, for both *C. pomonella* and *M. brassicae* the Vip3Aa protein is 100-fold more active than the Vip3Ca protein. In the case of *O. furnacalis* is the other way round: the Vip3Aa is marginally toxic whereas the Vip3Ca protein is very active. Therefore, this points out at *O. furnacalis* as a good target of the Vip3Ca protein (7,8).

In an attempt to determine if the resistance to Cry1A-, Cry2A-, Dipel-, Vip3- and Vip3/Cry2Ab conferred cross-resistance to the Vip3 proteins, resistant insect colonies were tested against the Vip3Ca protein and the Vip3Aa protein (along with their respective susceptible colonies). The results indicated that the insect colonies resistant to Cry1Ac, Dipel (*H. armigera, T. ni, O. furnacalis* and *P. interpunctella*) or Cry2Ab (*H. armigera* and *T. ni*) were not cross-resistant to the Vip3 proteins (Vip3Aa and Vip3Ca). Regarding the *H. armigera* colonies resistant to Vip3Aa or Vip3Aa/Cry2Ab, they showed cross-resistance to the Vip3Ca protein (8). These results complement the published cross-resistance studies between Vip3 and Cry proteins and show, for the first time, cross-resistance between proteins within the Vip3 subfamily (9–19).

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The monographic study about the susceptibility of *G. molesta* to *B. thuringiensis* (*B. thuringiensis* formulations, individual toxins and their mixtures) was performed due the lack of toxicity data of this insect species. As a first step, to determine the susceptibility of *G. molesta* to Dipel ®, Xentari ®, active Cry1 proteins (Cry1Aa, Cry1Ac and Cry1C) and Vip3 protoxins (Vip3Aa, Vip3Af and Vip3Ca), the test consisted on performing bioassays at a high concentration of the insecticidal toxins. The results obtained indicated that DiPel® and XenTari® are very effective for the control of *G. molesta* under

laboratory conditions, that the *B. thuringiensis* toxins Cry1Aa, Cry1Ac, Cry1Ca, Vip3Aa and Vip3Af showed high toxicity to *G. molesta*, and that the Vip3Ca was nontoxic (6).

Dose-response assays were carried out for the *B. thuringiensis* formulations (Dipel ® and Xentari ®) and for the individual toxins (Cry1Aa, Cry1Ac, Cry1Ca, Vip3Aa and Vip3Af). The *B. thuringiensis* formulation DiPel® was slightly more toxic than XenTari®, with significantly lower LC_{50} and LC_{90} values. The individual proteins were very active, with LC_{50} values from 1.8 ng/cm^2 (Vip3Aa) to 57 ng/cm^2 (Cry1Ca). For Cry1 proteins, Cry1Aa was not significantly different to Cry1Ac at the LC_{50} level, but when compared to Cry1Ca, Cry1Aa is 7.6-fold more toxic. In the case of the Vip3 proteins, Vip3Aa was 15-fold more toxic than Vip3Af at the LC_{50} level. At the LC_{90} level, no significant differences were found among the Cry1 and Vip3 proteins. To evaluate the synergistic or antagonistic interactions of the Vip3Aa protein and the active Cry1 proteins, different combinations (at a concentration close to their LC_{50} values) were chosen for the single dose assays. Significant antagonistic interactions were found for Vip3Aa - Cry1Aa and Vip3Aa - Cry1Ca. No significant interaction was found for Vip3Aa - Cry1Ac. The interactions of Vip3Aa - Cry1Aa and Vip3Aa-Cry1Ca were investigated in dose-response assays at different ratios where the antagonistic effect became more evident as the proportion of Vip3Aa in the mixture increased, and this effect was especially drastic in the Vip3Aa - Cry1Ca mixture, with an antagonism factor (AF) of 64.5.

8.3 - Determination of the mode of action of the Vip3Ca protein in *Mamestra brassicae* and characterization of the cell death response in *Spodoptera exigua* after Vip3Ca intoxication

Our third observation starts with the study of the mode of action the Vip3Ca protein in *M. brassicae* and the characterization of the cell death response of *S. exigua* intoxicated with Vip3Ca2. For the study of the mode of action of the Vip3Ca protein, we were based on the previous work of Palma et al. (5), and regarding the cell death response of larvae intoxicated with the Vip3Ca protein, we were based on the published articles by Bel et al., Jakubowska et al., and Crava et al. (20–22). The study of the mode of action of the Vip3Ca protein was done in the following order: [1] proteolysis of Vip3Ca with trypsin

and midgut juice, [2] oligomerization of the Vip3Ca, [3] histopathological effects of Vip3Ca in the midgut epithelium and [4] *in vivo* and *in vitro* binding assays.

In a first step, the proteolytic pattern of the Vip3Ca protein was determined digesting the toxin with trypsin and midgut juice from insect species with different susceptibility to Vip3Ca. The result obtained indicated that the Vip3Ca protein (molecular weight ~90 kDa) was processed to protein bands of ~70 kDa and ~20 kDa when the protein was incubated with trypsin (See Annex 10.3) or the midgut juice from all tested species. Regarding the kinetics of Vip3Ca with the midgut juice from insect species with different susceptibility to Vip3Ca, it correlated with the susceptibility of the insect species to Vip3Ca. The activation was from faster to slower in the following order: *M. brassicae* (susceptible), *S. littoralis* (moderately susceptible), *A. ipsilon* (slightly susceptible) and *O. nubilalis* (no susceptible). To discard that the differences in the toxicity of the Vip3Ca protein was due to inappropriate activation of the protein, the Vip3Ca protein was processed with midgut juice of *O. nubilalis* or *M. brassicae* and fed to each other species; there was no significant change in its toxicity to either insect species. This result suggested that the low susceptibility of *O. nubilalis* and the other insect species was not due to a problem in the midgut processing of the toxin. The kinetics of Vip3Ca with trypsin showed, after 0.5 h, major bands of around ~70 kDa and ~20 kDa, as well as other smaller bands. Instead, the amount of the smaller bands decreased with the incubation time and the ~70 kDa band seemed to become more intense (See Subchapter 7.2).

In a second step, the oligomerization of the Vip3Ca digested with trypsin and midgut juice of *M. brassicae* was determined by size exclusion chromatography (See Annex 10.3). As a result, the activated Vip3Ca protein (at the ratio of 1µg of protein : 0.24 µg of trypsin) for 0.5 h and 48 h eluted in one peak at 11 ml approximately. This peak correspond to the toxins bands of ~70 kDa and ~20 kDa, with a apparent size from 395 - 512 kDa. Regarding the activated Vip3Ca protein at the ratio of 1µg of protein : 0.80 µg of midgut juice for 0.5 h, although the input showed a fully degraded protein, a main peak was eluted at 11 ml. This peak correspond to the toxins bands of ~70 kDa and ~20 kDa, with an approximately size of 395 kDa, and a ~35 kDa band that belonged to the midgut juice (See Subchapter 10.3). These results suggest that the ~70 kDa and ~20 kDa fragments remain together after the activation with trypsin or midgut juice of *M. brassicae* and that they form an oligomer of 4 to 6 units that which is stable up to two days. The same behaviour has been described for the Vip3A proteins (23–27), for which the trypsin-

activated Vip3Aa protein aggregates in solution to form a tetramer. In addition, the proteolysis of the Vip3Ca protein with midgut juice can produce artefactual band patterns probably due to the partial inactivation, at the end of the incubation and prior to SDS-PAGE, of the proteases present in the midgut juice as reported Bet et al. and Banyuls et al. for the Vip3Aa and Vip3Af proteins (25,28).

In a third step, the histopathological effects of Vip3Ca with the Vip3Aa (positive control of toxicity) and Vip3Ad (negative control of toxicity) were determined in midgut sections of *M .brassicae* intoxicated at different intervals with the respective proteins. The results obtained show that the Vip3Ca protein was able to disrupt the midgut epithelium of *M. brassicae.* Nevertheless, compared with the Vip3Aa protein, the damage produced by Vip3Ca took longer to be visible. To determine if the damage caused by the Vip3Ca protein in the midgut epithelium was due to the binding of Vip3Ca to the midgut apical membrane, the larvae were fed with Vip3Ca at different intervals, then dissected and the toxin localization revealed with an anti-Vip3Aa polyclonal antibody. After 3 h of exposure, the binding of the Vip3Ca protein to the midgut epithelium became more intense while at 1h and 6h hardly any binding could be detected in the midgut sections.

Finally, to show that the Vip3Ca protein bound specifically to the BBMV of *M. brassicae* and competed for the same binding sites of Vip3Aa, competition experiments were performed with biotin-labelled Vip3 proteins. The homologous competition showed that the Vip3Aa and the Vip3Ca proteins bound specifically to the BBMV since both Vip3 proteins were able to displace the respective unlabelled homolog proteins. The heterologous competition experiments indicated that Vip3Ca and Vip3Aa share binding sites. This result has implications in the Insect Resistance Management since the Vip3Ca protein could develop cross-resistance to other Vip3A proteins, as it has been reported in this doctoral thesis for the *H. armigera* insect colony resistant to the Vip3Aa protein that showed cross-resistance to the Vip3Ca protein.

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Regarding the characterization of the cell death response of *S. exigua* intoxicated with Vip3Ca, this was performed in 4^{th} instar larvae. First, we determined the growth inhibition dose-response curve for Vip3Ca in newly molted *S. exigua* larvae with and EC_{50} of 38.4 ng/cm^2, indicating that the Vip3Ca show a clear effect in growth inhibition of the larvae despite the fact that the activity of the protein is marginal. Therefore, *S. exigua* show

different responses to the Vip3 proteins: Vip3Aa is highly toxic with negligible effect in the in growth inhibition, while Vip3Ca shows a strong growth inhibition with marginal toxicity (5,29). The study of the gene expression analysis upon the Vip3Ca challenge at three concentrations was done for 47 *S. exigua* genes that had been described as responding to the Vip3Aa challenge (20–22). The gene expression analysis indicated that the number of the *S. exigua* genes is dose-dependent where most of the *S. exigua* genes were regulated at the three concentrations (100, 1000 and 10000 ng/cm^2). Regarding the different kinds of *S. exigua* regulated genes, the results showed the up-regulation of genes involved in the immune system and hormone modulation, and the down-regulation of genes involved in the digestion process and peritrophic permeability. In addition, the regulation of the immune signalling pathways (*Toll, Imd* and *Jak-Stat*) was assessed in the current study. The gene encoding a component of the *Jak-Stat* pathway was found down-regulated after 24 h exposure to 10000 ng/cm^2, thus we suggest that this gene is involved in the renewal of the damaged cells of the midgut and in the activation of the apoptosis pathway to eliminate the damaged cells.

The effect of the Vip3Aa and Vip3Ca proteins on the midgut epithelium was determined by the release of the APN activity into the luminal fluid. The result obtained supports that release APN is higher when the larvae were exposed to concentrations of Vip3Aa (100 ng/cm^2) and Vip3Ca (1000 ng/cm^2) that produce growth inhibition > 99 %. In addition, the fitness cost produced by the intoxication with the Vip3 proteins show an increase in the time of pupation and a reduction of the percentage of pupation, especially for those larvae exposed to the highest concentrations of Vip3Aa and Vip3Ca. Since the exposure to Vip3 proteins affected the transcriptional pattern, caused epithelial and cellular damage, plus a fitness cost, we would like to determine if the down-regulation of the *Jak-Stat* pathway involves the apoptosis as a host defence response to damage produced by the Vip3 proteins. The result showed the presence of TUNEL-positive cells in the *S. exigua* midgut sections exposed to sublethal concentrations of Vip3Aa and Vip3Ca proteins. In the case of the Vip3Ca protein, the TUNEL-positive cells were observed at 10000 ng/cm^2, while for the Vip3Aa protein, the TUNEL-positive cells were observed at 1, 10, 100 ng/cm^2, but not at 1000 ng/cm^2. The lack of TUNEL-positive cells was probably due to the fact that the cells respond by other mechanisms or that the cells of the midgut epithelium were disrupted. In addition, the expression level of the *S. exigua* caspases was evaluated after 3 h, 6 h and 12 h. As a result, the *Se-caspase-4* was up-

regulated at all the time intervals while the *Se-caspase-1*, *Se-caspase-1* and *Se-caspase-2* were up-regulated at 12 h.

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9. - CONCLUSIONS

Conclusions:

I. Thirteen new insecticidal proteins were identified from two selected (E-SE10.2 and O-V84.2) *B. thuringiensis* isolates. The secretable proteins (Vip-like and Sip-like) were marginally expressed in the supernatant. Regarding the crystal proteins (Cry-like), these were found in the parasporal crystal; the isolate E-SE10.2 produces a pure crystal, while the isolate O-V84.2 produces a crystal with a mixture of four proteins

II. The insecticidal spectrum of the Vip3Ca protein was increased in 12 new insect species, among which *O. furnacalis* was the most susceptible. Regarding the analysis of cross-resistance in lepidopteran pests, the results indicate that the resistance due to selection with Cry proteins does not confer cross-resistance to Vip3Ca. In contrast, the insect populations resistant to Vip3A show cross-resistance to Vip3Ca

III. *Grapholita molesta* is highly susceptible to *B. thuringiensis* formulations and their individual toxins (Cry and Vip3 proteins). Regarding the interactions between the Cry and Vip3 proteins, antagonism was found and this was higher as the amount of Vip3A protein increased

IV. The Vip3Ca protein was processed into two protein fragments of ~70 kDa and ~20 kDa, both by trypsin and midgut juice from insect species with different susceptibility to Vip3Ca protein. The Vip3Ca protein activated with trypsin and midgut juice of *M. brassicae* form an oligomer of 4 subunits. The Vip3Ca protein was able to bind to the midgut epithelium producing the cellular lysis and the disruption of the midgut epithelium. Also, the binding assays showed that Vip3Ca bound specifically and competed for the same binding sites than the Vip3Aa protein

V. The exposure of *S. exigua* larvae to sublethal concentrations of the Vip3Ca protein activates different insect response pathways which trigger the regulation of some genes belonging to the pathogen response group, induces APN shedding, and triggers the caspase dependent pathway that induce the apoptotic response

10.- ANNEXES

(unpublished data)

Chapter 1

10.1 - Identification of the *vip1* and *vip2* gene pairs, *vip2Ac2 - vip1Ca2* and *vip2Bb4 - vip1Bb3* from *Bacillus thuringiensis.*

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Objective: Identification of the *vip1* and *vip2* genes pairs, *vip2Ac2 - vip1Ca2* and *vip2Bb4 - vip1Bb3*

Materials and Methodology:

1. - Cloning of the genes pairs vip2Ac2 - vip1Ca2 and vip2Bb4 - vip1Bb3 from the respective Bacillus thuringiensis isolates

From the results of the identification of *vip1*- and *vip2*- type genes reported by Gomis-Cebolla et al., 2018 a set degenerative primers were designed to determine the full length of the genes pairs *vip2Ac2 - vip1Ca2* and *vip2Bb4 - vip1Bb3* in the *Bacillus thuringiensis isolates* V-V54.26 and V-J20.2 (Table 1). The PCR reactions were performed with the Kapa Hifi PCR Kit (KR0368 – v11.17) according to the manufacturer's instructions. The PC amplification were carried out in an Eppendorf Mastercycler thermal cycler as follows: PCR_1: 5 min denaturation at 95 ºC, 35 cycles of amplification (20 sec at 98 ºC, 15 sec of annealing at 47 ºC, and 1 min at 72 ºC), and an extra extension step of 10 min at 72 ºC. PCR_2: 5 min denaturation at 95 ºC, 35 cycles of amplification (20 sec at 98 ºC, 15 sec of annealing at 43 ºC, and 90 sec at 72 ºC), and an extra extension step of 10 min at 72 ºC. PCR_3 and 4: 5 min denaturation at 95 ºC, 35 cycles of amplification (20 sec at 98 ºC, 15 sec of annealing at 45 ºC, and 90 sec at 72 ºC), and an extra extension step of 10 min at 72 ºC.

Table 1. Set of degenerative primers used to determine the full length of the genes pairs *vip2Ac2* - *vip1Ca2* and *vip2Bb4* - *vip1Bb3*.

Reactions	Primers	Sequence (5' → 3')*	Length (nt)	Primer parameters	
				% GC	Tm (ºC)
PCR_1	Vip2-Vip1_f	ccsatmccagaaaatattac	20	35-40	47
	Vip2-Vip1_r	gctgtttgttgatcataaataag	23	30	
PCR_2	Vip1-TAA_f	atggatgattttaactccc	19	36	
	Vip1Ca1-TAA_r	ttatctaagatttgttaggtc	21	29	45
	Vip1Bb1-TAA_r	ttatagatctgaaatatttaaatg	24	17	44
PCR_3	Vip2Ba2_f	gctatgaaagtgaaaaattan	21	29	45
	Vip2Ba2_r	ttattttgttaataatgtagcatc	24	21	
PCR_4	Vip2Ac1_f	atgaaaagaatggagggaaa	20	33	46
	Vip2Ac1_r	ttaatttgttaataatgttgcatc	20	21	

*IUPAC nucleotide code: S: G or C; M: A or C; N: any base.

To determine the sequence of the gene pairs *vip2Ac2* - *vip1Ca2* and *vip2Bb4* - *vip1Bb3*, the amplified sequences were ligated into the pGEM®-T Easy plasmid (Promega), cloned in Escherichia coli DH10ß, and sequenced. DNA sequence analysis and contig assembly was performed using DNAstar v5 and NCBI BLAST tools (Blastx) (1).

Results:

The gene pairs *vip2Ac2* - *vip1Ca2* and *vip2Bb4* - *vip1Bb3* were organized in operon in the genomic or plasmid DNA of the *Bacillus thuringiensis* isolates V-J20.2 and V-V54.26, respectively. The *vip2* genes (*vip2Ac2* and *vip2Bb4*) were located before the *vip1*genes (*vip1Ca2* and *vip1Bb3*) in the DNA sequence by an intergenic region of 4 bp (lowercase letter). Regarding the conserved domains in the *vip1* and *vip2* genes are the following:

1. Signal peptide of the gram - positive bacteria in the first 30 - 50 nucleotides of the DNA sequences (underlined)
2. Vip2 superfamily: A family of actin-of ADP-ribosylating toxin. A member of the Bacillus sp produced vegetative insecticidal proteins (VIPs) possesses high specificity against the major insect pest, corn rootworms, and belongs to a classs of binary toxins and regulators of biological pathways distinct from classical A-B toxins. VIP2 shares significant sequence similarity with enzymatic components

of other binary toxins, *Clostridium botulinum* C2 toxin, *C. perfringens* iota toxin, *C. piroforme* toxin and *C. difficile* toxin (bold purple)

3. PA14 domain: This domain forms an insert in bacterial beta-glucosidases and is found in other glycosidases, glycosyltransferases, proteases, amidases, yeast adhesins, and bacterial toxins, including anthrax protective antigen (PA). The crystal structure of PA shows that this domain has a beta-barrel structure. The PA14 domain sequence suggests a binding function, rather than a catalytic role. The PA14 domain distribution is compatible with carbohydrate binding (bold green)
4. Clostridial binary toxin B/anthrax toxin PA: The N-terminal region of this family contains a calcium-binding motif that may be an EF-hand (bold orange)

Sequence:

vip2Ac2 - *vip1Ca2*

ATGAAAAGAATGGAGGGAAAATTGTTTATGGTGTCCACAAAATTACAAGCAGTTACTAAAGCTG
TATTACTTAGTACAGTTTTATCTATATCTTTATTAAATAATGAAGTGATAAAAGCTGAACAATT
AAATATGAATTCTCAAAATAAATATACTAATTTTGAAAATCTAAAAATCACTGACAAGGTAGAG
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CAGCAACAATTGGATTTAATAAATCTTTAACAGAAGGTAATACAATTAATTCTGATGCAATGGC
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TAATGGGTATATGGTCCATGTAGATAAGGTGTCAAAAGTGGTAAAAAAAGGGGTGGAGTGCTTA
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GCTGGGGTATGAAGAATTATGAAGAGTGGGCTAAAGATTTAACCGATTTACAAAGGGAAGCTTT
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TAAAGATAAATACTGATCAAACTGATGGTAGATATGGAATAATTGGAGTGGATGGTAAGGCTGA
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TCTAGATAA

vip2Bb4 - vip1Bb3

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GTTAATGAAGATGGAAGTGTTAAAGTCAACATGACAGAATATAATGAAGGATATCCACTTAGAA
TTGAATCCGCCTACCATTTAAATATTTCAGATCTATAA

References:

1. Altschul, S.F.; Gish, W.; Miller, W.; Myers, E.W.; Lipman DJ. Basic local alignment search tool. J Mol Biol. 1990;215:403–10.

10.2 - Insecticidal activity of the concentrated supernatant and the spore/crystal mixture of *Bacillus thuringiensis* isolates E-SE10.2 and O-V84.2 in *Spodoptera exigua* and *Spodoptera littoralis*

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Objective: Determine the insecticidal activity of the supernatant and the solubilized crystal proteins of the *Bacillus thuringiensis* isolates E-SE10.2 and O-V84.2 in *Spodoptera exigua* and *Spodoptera littoralis*

Materials and Methodology:

1. - Sample preparation for insecticidal activity of Bacillus thuringiensis isolates

A single colony of *Bacillus thuringiensis* was grown in 100 ml of LB at 29 °C for 24 h and 48 h for detection of the secretable proteins, while for the detection of proteins in the parasporal crystal the culture was grown in 100 ml of CCY at 29 °C until culture sporulation (72 hours). The supernatant of *B. thuringiensis* was concentrated by trichloroacetic acid (TCA) precipitation. Briefly, the cells were collected at 6,000 g for 15 min at 4 °C and filtered through sterile 0.45 μm cellulose acetate filters (GE Healthcare Life Sciences). The sample was incubated with 10% TCA (final concentration) and kept at 4 °C for 24 h. Then, the sample was centrifuged at 16000 g for 20 min at 4 °C. The pellet was washed with 100 ml of cold acetone (-18 °C), centrifuged at 16000 g for 20 min at 4 °C, and let dry at room temperature for 5 min. The precipitated proteins were solubilized in 50 mM carbonate buffer containing 10 mM dithiothreitol (pH 11.3) for 48 h, with two buffer changes.

Crystals (together with spores) were separated by centrifugation at 6000 × g for 12 min at 4 °C. The pellet containing the parasporal crystals was washed three times with ice cold solution A (1 M NaCl, 5 mM EDTA, 10 mM PMSF, 1% Triton X-100) and centrifuged

at 17,000 x g for 12 min at 4 °C between washes. The pellet was then washed three times with ice cold solution B (10 mM KCl) and centrifuged at 24,000 x g for 15 min at 4 °C. The crystals in the final pellet were solubilized in 20 ml of 50 mM carbonate buffer containing 10 mM dithiothreitol (pH 11.3) by incubation at room temperature for 2 h with continuous shaking. Concentration of the proteins in the supernatant and in the solubilized crystals were estimated with the Bradford method (1). The purity of the expressed proteins in the supernatant and the crystal was analyzed by SDS-PAGE and stained with Coomassie brilliant blue R-250 (Sigma-Aldrich).

2. - Insecticidal activity of the Bacillus thuringiensis isolates E-SE10.2 and O-V84.2

Spodoptera littoralis and *Spodoptera exigua* were grown in the insectaries of the University of Valencia at 25 ± 2 °C, 70 ± 5% RH, and L16: D8 h and reared in artificial diet (2). The autoclaved (121 °C, 1 bar, 20 min) and non-autoclaved concentrated supernatant (E-SE10.2 10-fold and O-V84.2 20-fold concentrated), and the solubilized crystal proteins were dispensed on the diet surface. Prior to the sample application, the surface of the diet was sterilized under UV light for 5 min. A volume of 50 µl of the autoclaved and non-autoclaved concentrated supernatant was applied on the surface of the solidified diet and let dry at room temperature. Once dried, one larva was transferred to each well using 16 neonates per replicate, with two replicates per concentration. Larvae mortality was scored after 7 days.

Results:

To determine whether any of the Vip, Sip, and Cry proteins expressed in the *B. thuringiensis isolates* E-SE10.2 and O-V84.2, we performed bioassays with *Spodoptera exigua* and *Spodoptera littoralis* neonate larvae with the autoclaved and non-autoclaved concentrated supernatant, and the solubilized proteins from the spore/crystal mixture (Table 1). The analysis of the solubilized proteins from the crystal of E-SE10.2 indicated that they showed some toxicity to both *S. exigua* and *S. littoralis*, whereas no toxicity was found for the solubilized crystals of O-V84.2 (Table 1). Neither the autoclaved nor the non-autoclaved concentrated supernatants showed any toxicity to these two insect species (Table 1).

Table 1. Insecticidal activity of the autoclaved supernatants, non-autoclaved supernatants and crystal proteins of the *Bacillus thuringiensis* isolates E-SE102 and O-V84.

Sample	Replicate[†]	Amount (µg (ng))[‡]	% Mortality*	
			S. exigua	***S. littoralis***
E-SE10.2				
24 h Non autoclaved	R1	17.5 (250)	0 ± 0	0 ± 0
24 h Autoclaved	R1	-	0 ± 0	6.2 ± 6.2
48 h Non-autoclaved	R1	51.0 (1,100)	0 ± 0	0 ± 0
48 h Autoclaved	R1	-	0 ± 0	0 ± 0
Crystal solubilized	R1	7.5 (2,280)	31.2 ± 5.2	50 ± 6.7
O-V84.2				
24 h Non-autoclaved	R1	4.1 (0,13)	0 ± 0	13.1 ± 6.8
24 h Autoclaved	R1	-	5.7 ± 5.7	6.2 ± 6.2
48 h Non-autoclaved	R1	20.5 (227)	13.8 ± 4.9	0 ± 0
48 h Autoclaved	R1	-	0 ± 0	6.2 ± 6.2
Crystal solubilized	R1	13 (6,871)	3.54 ± 2.7	0 ± 0

[†] In the single dose assays, we used the first replicate (R1) at 24h, 48h and 72h that is shown in the Chapter 1, Table 5.

[‡] The amount indicated correspond to the total amount of protein in the sample measured by Bradford. Inside of the brackets is indicated the inferred amount of the putative *Bacillus thuringiensis* toxins from the weight percentage of the first replicate (Chapter 1, Table 5).

[*] Bioassays were performed with 16 larvae per sample, with two replicates (n=32), and the mortality was scored at 7 days and corrected subtracting the mortality of the negative control (50 mM Na_2CO_3 pH 11.3).

References:

1. Bradford M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976;72:248–54.
2. Greene, G.L.; Leppla, N.C.; Dickerson W. Velvetbean caterpillar: a rearing procedure and artificial medium. J Econ Entomol. 1976;69:487–8.

11. - SUPPLEMENTARY INFORMATION

Chapter 1

Supplementary Materials: A Genomic and Proteomic Approach to Identify and Quantify the Expressed *Bacillus thuringiensis* Proteins in the Supernatant and Parasporal Crystal

Joaquín Gomis-Cebolla, Ana Paula Scaramal Ricietto and Juan Ferré

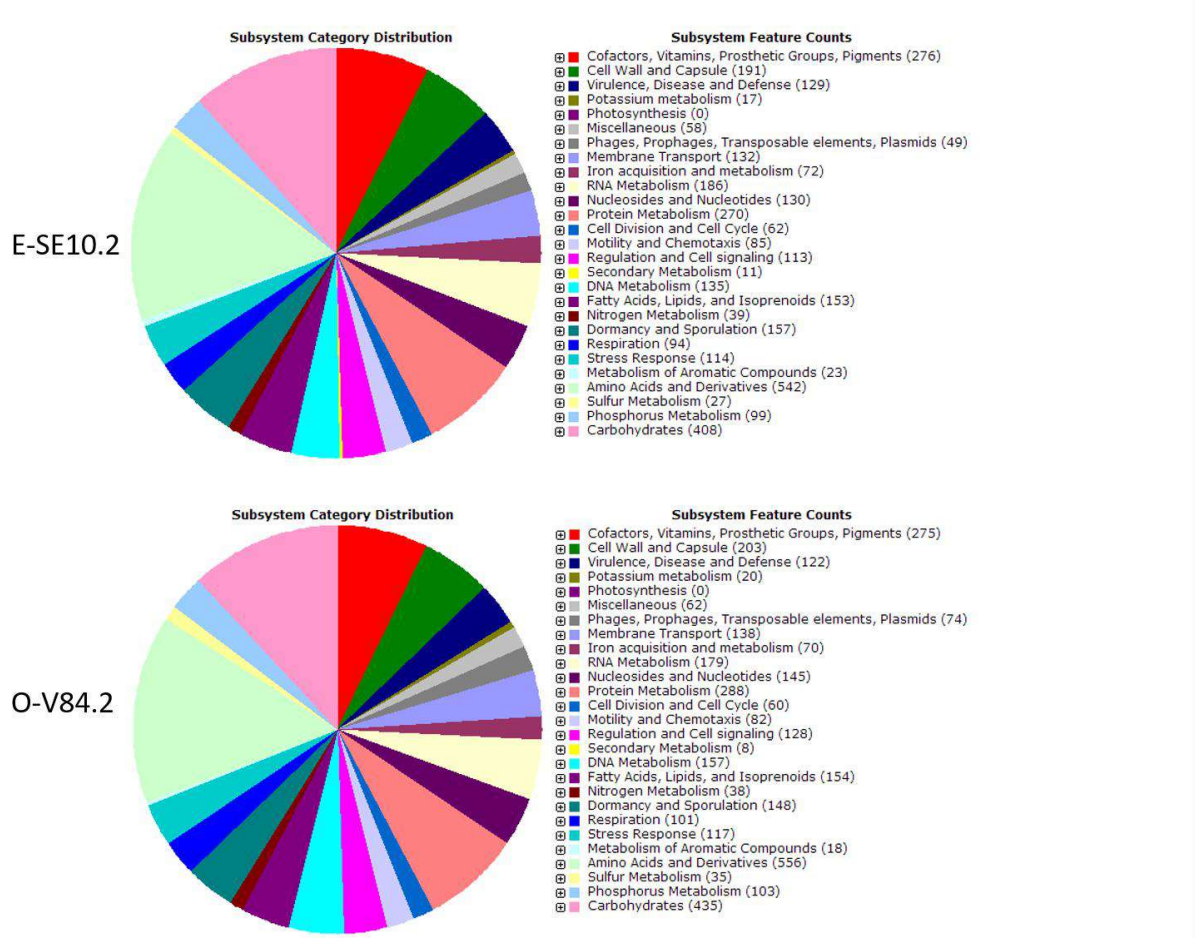

Figure S1. Subsystem category distribution of the *Bt* isolates E-SE10.2 and O-V84.2 by the genome annotation based on the Rast server. The right pie chart indicates the percentage or predicted encoding genes associated to at least one subsystem.

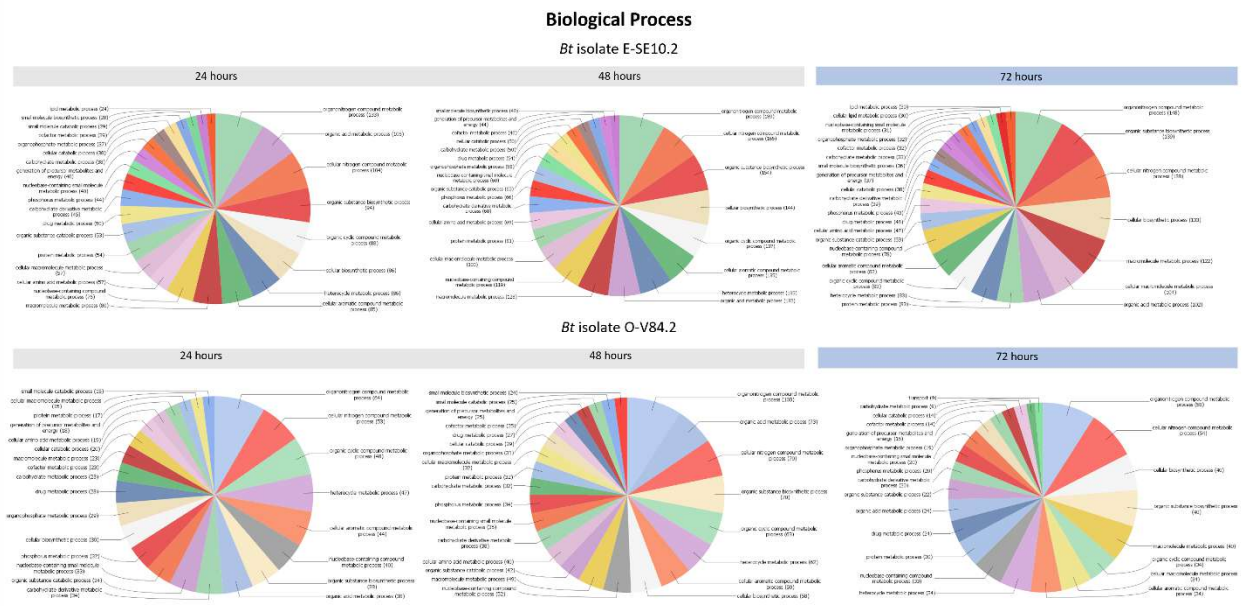

Figure S2. Functional annotation of the protein identification from the concentrated supernatants at 24 h and 48 h in LB medium, and of the solubilized proteins from the crystal at 72 h in CCY medium. The number of sequences that belong to the group are indicated in brackets.

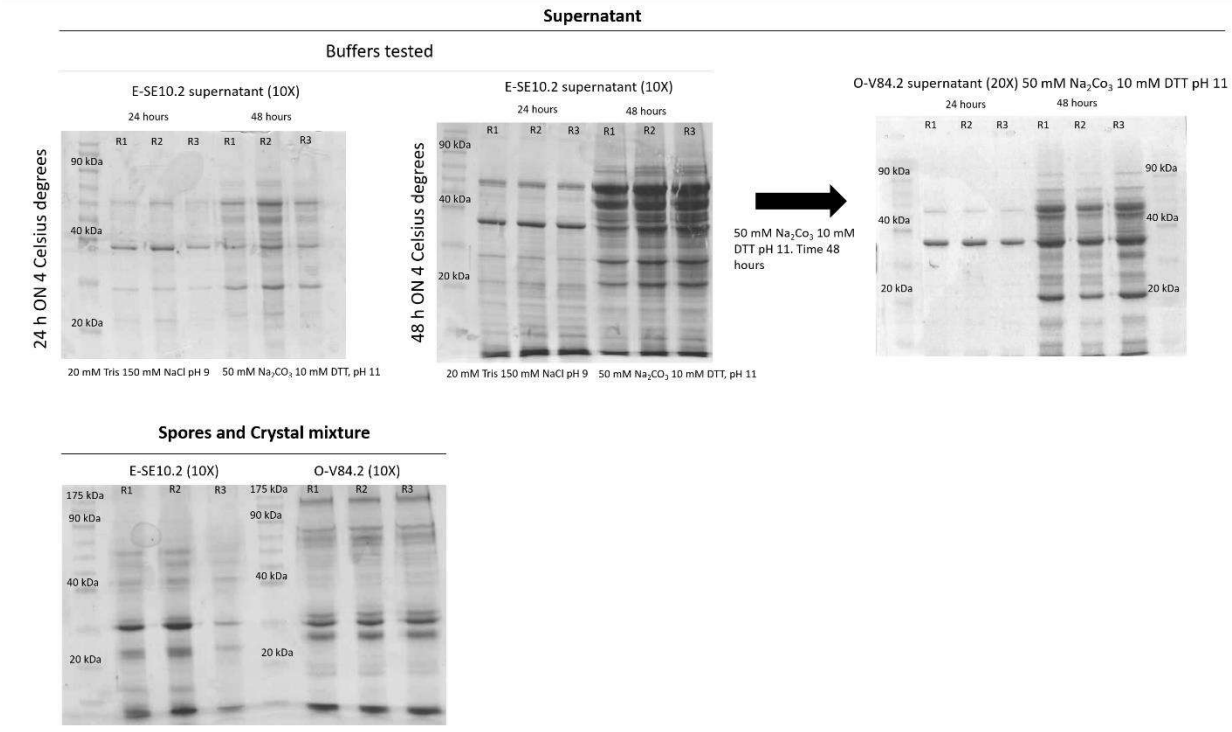

Figure S3. SDS-PAGE of the three replicates of the concentrated supernatants and solubilized proteins from the spore/crystal mixtures and growth curve of the *Bt* isolates E-SE10.2 and O-V84.2.

Supplementary Materials: A Genomic and Proteomic Approach to Identify and Quantify the Expressed *Bacillus thuringiensis* Proteins in the Supernatant and Parasporal Crystal

Joaquín Gomis-Cebolla, Ana Paula Scaramal Ricietto and Juan Ferré

The supplementary material tables (Table S1 - S5) can be download from the following link: http://www.mdpi.com/2072-6651/10/5/193/s1.

A simplified version of the supplementary tables have been attached to the doctoral thesis. The simplified version of the supplementary tables are the following:

Table S1: Protein summary of three replicates of concentrated supernatants and solubilized proteins from spore/crystal mixtures with Protein Pilot v4.5

Table S2: Peptide summary of three replicates of concentrated supernatants and solubilized proteins from spore/crystal mixtures identified with Protein Pilot v4.5

Table S3: Result of the Mascot search in the three replicates of the concentrated supernatants and solubilized proteins form spore/crystal mixtures

Regarding the label free analysis of concentrated supernatant at 24 h vs 48 h of the *Bacillus thueingiensis* isolates E-SE10.2 and O-V84.2 a report with the main results have been attached to the doctoral thesis

Table S1. Protein summary of three replicates of concentrated supernatants and solubilized proteins from spore/crystal mixtures with Protein Pilot v4.5

Sample	Replicate	N	Unused	%Cov	Homology	Peptides(95%)
E-SE10.2						
Supernatant 24 h	1	58	19.33	44.89	Vip2Ac-like_1	10
		65	17.79	45.60	Sip1Aa-like_1	9
		24	30.88	70.90	Cry23Aa-like	30
	2	86	16.85	38.67	Vip2Ac-like_1	8
		198	8.70	31.32	Sip1Aa-like_1	7
		21	35.08	70.90	Cry23Aa-like	34
	3	52	22.63	43.33	Vip2Ac-like_1	12
		83	16.00	38.19	Sip1Aa-like_1	9
		18	40.59	76.49	Cry23Aa-like	39
E-SE10.2						
Supernatant 48 h	1	96	16.84	35.56	Vip2Ac-like_1	9
		118	14.29	35.44	Sip1Aa-like_1	8
		34	30.69	72.39	Cry23Aa-like	34
	2	98	16.41	40.00	Vip2Ac-like_1	8
		175	10.11	28.30	Sip1Aa-like_1	7
		59	21.53	45.15	Cry23Aa-like	21
	3	138	14.95	34.22	Vip2Ac-like_1	7
		275	6.80	25.55	Sip1Aa-like_1	5
		81	21.56	47.39	Cry23A-like	21
E-SE10.2 Crystal 72 h	1	1	67.44	94.78	Cry23A-like	68
	2	2	60.41	91.04	Cry23Aa-like_1	61
	3	9	39.33	71.27	Cry23Aa-like	31
O-V84.2 Supernatant						
24 h	1	46	12.71	22.34	Vip4Aa-like_1	7
		74	6.02	15.69	Vip4Aa-like_2	4
		63	8.94	13.03	Vip2Ac-like_2	5
	2	58	12.18	22.91	Vip4Aa-like_1	6
		178	1.53	4.59	Vip4Aa-like_2	1
		176	1.73	5.32	Vip2Ac-like_2	1
	3	54	10.01	8.13	Vip4Aa-like_1	7
		95	4.02	10.22	Vip2Ac-like_2	2
		173	1.57	7.65	Sip1Aa-like_2	1
O-V84.2 Supernatant						
48 h	1	289	2.74	8.02	Vip4Aa-like_1	1
	2	142	8.41	11.91	Vip4Aa-like_1	6
		277	3.09	11.20	Vip2Ac-like_2	2
	3	241	4.02	14.78	Vip4Aa-like_1	2
		227	4.74	11.48	Vip2Ac-like_2	3
O- V84.2 Crystal 72 h	1	8	29.44	59.71	Cry45Aa-like_1	17
		9	26.00	59.11	Cry45Aa-like_2	18
		13	23.10	83.40	Cry45Aa-like_3	17

Table S1. Protein summary of three replicates of concentrated supernatants and solubilized proteins from spore/crystal mixtures with Protein Pilot v4.5

Sample	Replicate	N	Unused	%Cov	Homology	Peptides(95%)
O- V84.2 Crystal 72 h		1	163.9	70.94	Cry32Ea-like	48
		2	80.18	49.56	Cry32Da-like	121
		4	55.05	53.35	Cry32Eb-like	38
		5	54.18	60	Cry73Aa-like	35
	2	9	39.49	90.48	Cry45Aa-like_1	29
		17	23.59	72.86	Cry45Aa-like_2	21
		10	33.54	89.36	Cry45Aa-like_3	27
		1	180.55	82.06	Cry32Ea-like	163
		2	101.10	67.87	Cry32Da-like	64
		7	57.44	67.66	Cry32Eb-like	49
		3	79.34	76.28	Cry73Aa-like	62
	3	8	27.66	58.24	Cry45Aa-like_1	17
		13	23.45	65.06	Cry45Aa-like_2	19
		11	23.59	83.4	Cry45Aa-like_3	17
		1	172.42	73.31	Cry32Ea-like	136
		5	47.1	54.25	Cry32Da-like	32
		2	74.45	52.95	Cry32Eb-like	45
		3	59.08	61.24	Cry73Aa-like	40

Table S2. Peptide summary of three replicates of concentrated supernatants and solubilized proteins from spore/crystal mixtures identified with Protein Pilot v4.5

Sample	Replicate	Homology	Contrib	Conf	Sequence
E-SE10.2 Supernatant 24 h	1	Vip2Ac-like_1	2	99	AKEWAESNFKDWKK
			2	99	FIKDLINSLN
			2	99	IDNLYASASIDR
			2	99	KNITIEPYIKK
			2	99	LKEMNDKIDRYDQISGK
			2	99	NLGIEITR
			2	99	QLVSNVPNNLLLNIMK
			2	99	SAPILLSLTLAK
			1.19382	96.38	EMNDKIDRYDQISGK
			0.879426	92.29	NITIEPYIKK
			0.732828	88.92	GTSIGHLNEDHIITER
			0.383	93.68	TLFIQIDHYGHK
			0.051098	18.54	SLTSEQQK
		Sip1Aa-like_1	2	99	AETTSSHAVTNQQPITQR
			2	99	AIDLSNIR
					FISWFKDNLASPGGYDSIAEQM
			2	99	GLK
			2	99	FTVPSQEVTLAPGHK
			2	99	NYIKNGDTLYIETPAK
			2	99	SINMQEQIIDGWFLAR
			2	99	TGDTIYNGVSELK
			2	99	VAALNDMNISNVNYTSK
			1.481486	98.26	AESYIDIVHNR
			0.238072	58.2	AIDLSNIRK
			0.055517	20.62	NITGTTQK
					DGALIAAAYVSITDLADYNPNLG
		Cry23Aa-like	2	99	LTNEGNGVAHFK
			2	99	FKGEGYIEGAQGLR
			2	99	GEGYIEGAQGLR
			2	99	GIINIQDEINDYMK
					GIINIQDEINDYMKGMYGATSV
			2	99	K
			2	99	GMYGATSVK
			2	99	GMYGATSVKSTYDSSFK
			2	99	MGIINIQDEINDYMK
			2	99	SYIQVTEYPMDDNGRR
			2	99	TDTRTWTDTTTVK
			2	99	TWTDTTTVK
			2	99	TWTDTTTVKAPPR
					VFNESVTPQYDVIPTEPVNNHIT
			2	99	TK
					VIDNPGTSEVTSTVTFTWTETDT
			2	99	VTSAVTK

Table S2. Peptide summary of three replicates of concentrated supernatants and solubilized proteins from spore/crystal mixtures identified with Protein Pilot v4.5

Sample	Replicate	Homology	Contrib	Conf	Sequence
			0.8041	99	SYIQVTEYPMDDNGR
			0.20621	45.73	STYDSSFK
			0.116339	95.21	SYIQVTEYPMDDNGRRPASN
			0.0846	93.42	VIPTEPVNNHITTK
			0.071092	65.84	IEGAQGLR
			0.000435	52.11	LGLTNEGNGVAHFK
	2	Vip2Ac-like_1	2	99	DSTEFFAGVFNYLYSPK
			2	99	GTSIGHLNEDHIITER
			2	99	IDNLYASASIDR
			2	99	IDNLYASASIDRTETIIK
			2	99	NMNDKTLFTITDK
			2	99	SEDLGYADKYFYAANDSR
			1.69897	98.93	TSIVVVNGR
			1.408936	97.97	YFYAANDSR
			0.924453	93.48	AKEWAESNFKDWKK
			0.50307	80.89	TLFIQIDHYGHK
			0.195861	52.41	NLGIEITR
			0.110698	35.89	NITIEPYIK
		Sip1Aa-like_1	2	99	AIDLSNIR
			2	99	TGDTIYNGVSELK
			2	99	VAALNDMNISNVNYTSK
			1.251812	98.27	AESYIDIVHNR
			0.694649	99	ETTSSHAVTNQQPITQR
			0.568636	97.46	NYIKNGDTLYIETPAK
			0.134304	54.85	FTVPSQEVTLAPGHK
			0.02365	15.77	MVYSGTHDLK
					DGALIAAAYVSITDLADYNPNLG
		Cry23Aa-like	2	99	LTNEGNGVAHFK
			2	99	ESVTPQYDVIPTEPVNNHITTK
			2	99	GEGYIEGAQGLR
			2	99	GIINIQDEINDYMK
			2	99	GMYGATSVK
			2	99	GMYGATSVKSTYDSSFK
			2	99	MGIINIQDEINDYMK
					RPASNTYLIQGSLAPNVTLINDR
			2	99	K
			2	99	SYIQVTEYPMDDNGR
			2	99	SYIQVTEYPMDDNGRR
					SYIQVTEYPMDDNGRRPASNTY
			2	99	LIQGSLAPNVTLINDR

Table S2. Peptide summary of three replicates of concentrated supernatants and solubilized proteins from spore/crystal mixtures identified with Protein Pilot v4.5

Sample	Replicate	Homology	Contrib	Conf	Sequence
					SYIQVTEYPMDDNGRRPASNTY
			2	99	LIQGSLAPNVTLINDRK
			2	99	TDTRTWTDTTTVK
					VFNESVTPQYDVIPTEPVNNHIT
			2	99	TK
					VIDNPGTSEVTSTVTFTWTETDT
			2	99	VTSAVTK
			1.376751	96.68	TWTDTTTVKAPPR
			1.275724	99	TWTDTTTVK
			1.229148	99	LGLTNEGNGVAHFK
			0.545155	99	FKGEGYIEGAQGLR
			0.449772	70.09	STYDSSFK
			0.111259	95.47	SYIQVTEYPMDDNGRRPASN
			0.047692	67.6	GVAHFKGEGYIEGAQGLR
			0.032452	74.44	SVTPQYDVIPTEPVNNHITTK
			0.009661	72.63	SYIQVTEYPMDDNGRRPA
			0.001305	24.69	GYIEGAQGLR
	3	Vip2Ac-like_1	2	99	AKEWAESNFKDWKK
			2	99	DSTEFFAGVFNYLYSPK
			2	99	FIKDLINSLN
			2	99	IDNLYASASIDR
			2	99	IDNLYASASIDRTETIIK
			2	99	LKEMNDKIDRYDQISGK
			2	99	NMNDKTLFTITDK
			2	99	QLVSNVPNNLLLNIMK
			2	99	SAPILLSLTLAK
			2	99	TSIVVVNGREVIK
			1.080922	95.23	NITIEPYIK
			0.521434	80.67	NLGIEITR
			0.440093	75.94	EMNDKIDRYDQISGK
			0.421361	74.7	GTSIGHLNEDHIITER
			0.101275	32.11	NLLQLK
		Sip1Aa-like_1	2	99	AESYIDIVHNR
			2	99	AETTSSHAVTNQQPITQR
					FISWFKDNLASPGGYDSIAEQM
			2	99	GLK
			2	99	FTVPSQEVTLAPGHK
			2	99	NYIKNGDTLYIETPAK
			2	99	SINMQEQIIDGWFLAR
			2	99	TGDTIYNGVSELK
			2	99	VAALNDMNISNVNYTSK
					DGALIAAAYVSITDLADYNPNLG
		Cry23Aa-like	2	99	LTNEGNGVAHFK

Table S2. Peptide summary of three replicates of concentrated supernatants and solubilized proteins from spore/crystal mixtures identified with Protein Pilot v4.5

Sample	Replicate	Homology	Contrib	Conf	Sequence
			2	99	ESVTPQYDVIPTEPVNNHITTK
			2	99	FKGEGYIEGAQGLR
			2	99	GEGYIEGAQGLR
			2	99	GIINIQDEINDYMK
					GIINIQDEINDYMKGMYGATSV
			2	99	K
			2	99	GMYGATSVK
			2	99	GMYGATSVKSTYDSSFK
			2	99	GVAHFKGEGYIEGAQGLR
			2	99	IINIQDEINDYMK
			2	99	MGIINIQDEINDYMK
					RPASNTYLIQGSLAPNVTLINDR
			2	99	K
			2	99	SYIQVTEYPMDDNGR
			2	99	SYIQVTEYPMDDNGRR
					SYIQVTEYPMDDNGRRPASNTY
			2	99	LIQGSLAPNVTLINDRK
			2	99	TDTRTWTDTTTVK
			2	99	TWTDTTTVKAPPR
					VFNESVTPQYDVIPTEPVNNHIT
			2	99	TK
					VIDNPGTSEVTSTVTFTWTETDT
			2	99	VTSAVTK
			1.167491	99	TWTETDTVTSAVTK
			0.551294	99	SYIQVTEYPMDDNGRRPASN
			0.465974	99	TWTDTTTVK
			0.284833	55.57	STYDSSFK
			0.071604	90.42	GYRDGALIAAAY
			0.048662	93.47	SYIQVTEYPMDDNGRRPA
			0.000435	15.63	IQDEINDYMK
E-SE10.2 Supernatant 48 h	1	Vip2Ac-like_1	2	99	DSTEFFAGVFNYLYSPK
			2	99	EGVLGYYDTISK
			2	99	IDNLYASASIDR
			2	99	IDNLYASASIDRTETIIK
			2	99	NLGIEITR
			2	99	SEDLGYADKYFYAANDSR
			2	99	TSIVVVNGR
			1.200659	96.88	LKEMNDKIDRYDQISGK
			0.701147	89.44	NMNDKTLFTITDK
			0.307153	68.34	TLFIQIDHYGHK
			0.296709	67.32	NLLQLK

Table S2. Peptide summary of three replicates of concentrated supernatants and solubilized proteins from spore/crystal mixtures identified with Protein Pilot v4.5

Sample	Replicate	Homology	Contrib	Conf	Sequence
			0.197911	54.81	LKEMNDKIDR
			0.121478	40.45	SLDFEKDKEK
		Sip1Aa-like_1	2	99	AIDLSNIR
			2	99	ETTSSHAVTNQQPITQR
			2	99	FTVPSQEVTLAPGHK
			2	99	NYIKNGDTLYIETPAK
			2	99	SQSTSITNGLQLGFK
			2	99	TGDTIYNGVSELK
			2	99	VAALNDMNISNVNYTSK
			0.208309	59.34	FISWFK
			0.078314	87.71	AETTSSHAVTNQQPITQR
					DGALIAAAYVSITDLADYNPNLG
		Cry23Aa-like	2	99	LTNEGNGVAHFK
			2	99	GEGYIEGAQGLR
			2	99	GIINIQDEINDYMK
					GYRDGALIAAAYVSITDLADYNP
			2	99	NLGLTNEGNGVAHFK
			2	99	MGIINIQDEINDYMK
			2	99	RPASNTYLIQGSLAPNVTLINDR
					RPASNTYLIQGSLAPNVTLINDR
			2	99	K
					RPASNTYLIQGSLAPNVTLINDR
			2	99	KE
			2	99	SYIQVTEYPMDDNGR
			2	99	SYIQVTEYPMDDNGRR
					SYIQVTEYPMDDNGRRPASNTY
			2	99	LIQGSLAPNVTLINDR
					SYIQVTEYPMDDNGRRPASNTY
			2	99	LIQGSLAPNVTLINDRK
					VFNESVTPQYDVIPTEPVNNHIT
			2	99	TK
					VIDNPGTSEVTSTVTFTWTETDT
			2	99	VTSAVTK
			1.161151	95.08	STYDSSFK
			1.148742	94.89	GMYGATSVKSTYDSSFK
			0.325139	97.21	GSLAPNVTLINDR
			0.018181	22.21	GMYGATSVK
			0.009661	77.35	TWTDTTTVK
	2	Vip2Ac-like_1	2	99	AKEWAESNFKDWKK
			2	99	EGVLGYYDTISK
			2	99	GTSIGHLNEDHIITER
			2	99	IDNLYASASIDR
			2	99	IDNLYASASIDRTETIIK
			2	99	LKEMNDKIDRYDQISGK

Table S2. Peptide summary of three replicates of concentrated supernatants and solubilized proteins from spore/crystal mixtures identified with Protein Pilot v4.5

Sample	Replicate	Homology	Contrib	Conf	Sequence
			2	99	QLVSNVPNNLLLNIMK
			2	99	SEDLGYADKYFYAANDSR
			0.269218	62.75	YFYAANDSR
			0.104025	69.29	TSIVVVNGR
		Sip1Aa-like_1	2	99	AIDLSNIR
			2	99	FTVPSQEVTLAPGHK
			2	99	NYIKNGDTLYIETPAK
			2	99	TGDTIYNGVSELK
			2	99	VAALNDMNISNVNYTSK
			0.089909	91.82	ETTSSHAVTNQQPITQR
		Cry23Aa-like	2	99	GEGYIEGAQGLR
			2	99	GIINIQDEINDYMK
			2	99	GMYGATSVK
			2	99	GMYGATSVKSTYDSSFK
			2	99	MGIINIQDEINDYMK
					RPASNTYLIQGSLAPNVTLINDR
			2	99	K
			2	99	SYIQVTEYPMDDNGR
					SYIQVTEYPMDDNGRRPASNTY
			2	99	LIQGSLAPNVTLINDR
			2	99	TWTDTTTVKAPPR
					VFNESVTPQYDVIPTEPVNNHIT
			2	99	TK
			1.29243	99	SYIQVTEYPMDDNGRR
			0.130182	35.72	STYDSSFK
			0.101824	97.88	TWTDTTTVK
			0.001305	19.89	ESVTPQYDVIPTEPVNNHITTK
	3	Vip2Ac-like_1	2	99	AKEWAESNFKDWKK
			2	99	DSTEFFAGVFNYLYSPK
			2	99	GTSIGHLNEDHIITER
			2	99	IDNLYASASIDR
			2	99	LKEMNDKIDRYDQISGK
			2	99	QLVSNVPNNLLLNIMK
			2	99	SAPILLSLTLAK
			0.454693	82.52	TLFIQIDHYGHK
			0.441291	81.86	TSIVVVNGR
			0.056011	26.1	FKEITGLRK
		Sip1Aa-like_1	2	99	AETTSSHAVTNQQPITQR
					FISWFKDNLASPGGYDSIAEQM
			2	99	GLK
			2	99	TGDTIYNGVSELK
			0.767004	99	QRAESYIDIVHNR
			0.015473	75.26	ETTSSHAVTNQQPITQR

Table S2. Peptide summary of three replicates of concentrated supernatants and solubilized proteins from spore/crystal mixtures identified with Protein Pilot v4.5

Sample	Replicate	Homology	Contrib	Conf	Sequence
		Cry23Aa-like		2	99 GEGYIEGAQGLR
				2	99 GIINIQDEINDYMK
					GIINIQDEINDYMKGMYGATSV
				2	99 K
				2	99 GMYGATSVKSTYDSSFK
				2	99 MGIINIQDEINDYMK
				2	99 PASNTYLIQGSLAPNVTLINDR
					SYIQVTEYPMDDNGRRPASNTY
				2	99 LIQGSLAPNVTLINDRK
				2	99 TWTDTTTVK
				2	99 TWTDTTTVKAPPR
					VFNESVTPQYDVIPTEPVNNHIT
				2	99 TK
			1.552842		99 FKGEGYIEGAQGLR
E-SE10.2					DGALIAAAYVSITDLADYNPNLG
Crystal 72 h	1	Cry23Aa-like		2	99 LTNEGNGVAHFK
					DGALIAAAYVSITDLADYNPNLG
					LTNEGNGVAHFKGEGYIEGAQG
				2	99 LR
				2	99 ESVTPQYDVIPTEPVNNHITTK
				2	99 FKGEGYIEGAQGLR
				2	99 GEGYIEGAQGLR
				2	99 GIINIQDEINDYMK
					GIINIQDEINDYMKGMYGATSV
				2	99 K
				2	99 GMYGATSVK
				2	99 GMYGATSVKSTYDSSFK
					GYRDGALIAAAYVSITDLADYNP
				2	99 NLGLTNEGNGVAHFK
				2	99 IQDEINDYMK
				2	99 MGIINIQDEINDYMK
				2	99 PASNTYLIQGSLAPNVTLINDRK
				2	99 PNLGLTNEGNGVAHFK
				2	99 RPASNTYLIQGSLAPNVTLINDR
					RPASNTYLIQGSLAPNVTLINDR
				2	99 K
					STYDSSFKVFNESVTPQYDVIPTE
				2	99 PVNNHITTK
				2	99 SYIQVTEYPMDDNGR
				2	99 SYIQVTEYPMDDNGRR
				2	99 SYIQVTEYPMDDNGRRPASN

Table S2. Peptide summary of three replicates of concentrated supernatants and solubilized proteins from spore/crystal mixtures identified with Protein Pilot v4.5

Sample	Replicate	Homology	Contrib	Conf	Sequence
					SYIQVTEYPMDDNGRRPASNTY
				2 99	LIQGSLAPNVTLINDR
					SYIQVTEYPMDDNGRRPASNTY
				2 99	LIQGSLAPNVTLINDRK
				2 99	TDTRTWTDTTTVK
				2 99	TDTRTWTDTTTVKAPPR
					TNVEVAYIIQTGNYNVPVNVESD
				2 99	MTGTLFCR
				2 99	TWTDTTTVK
				2 99	TWTDTTTVKAPPR
				2 99	TWTETDTVTSAVTK
					VFNESVTPQYDVIPTEPVNNHIT
				2 99	TK
				2 99	VGGSVSSKATF
					VIDNPGTSEVTSTVTFTWTETDT
				2 99	VTSAVTK
				2 99	VSAEYNYSTTETTTK
			1.537602	97.06	STYDSSFK
			0.928118	99	GSLAPNVTLINDRK
			0.422508	62.16	GYKVGGSVSSK
			0.350665	97.94	SLAPNVTLINDRK
			0.067019	94.14	SYIQVTEYPMDDNGRRPA
			0.047208	85.59	VIPTEPVNNHITTK
			0.030118	68.52	SVKSTYDSSFK
			0.004804	63.06	GVAHFKGEGYIEGAQGLR
			0.003051	62.61	TDTTTVK
					MGIINIQDEINDYMKGMYGATS
			0.000869	98.53	VKSTYDSSFK
			0.000435	29.24	WTETDTVTSAVTK
	2	Cry23Aa-like		2 99	DGALIAAAYVSITDLADYN
					DGALIAAAYVSITDLADYNPNLG
				2 99	LTNEGNGVAHFK
				2 99	ESVTPQYDVIPTEPVNNHITTK
				2 99	FKGEGYIEGAQGLR
				2 99	GEGYIEGAQGLR
				2 99	GIINIQDEINDYMK
				2 99	GMYGATSVK
				2 99	GMYGATSVKSTYDSSFK
				2 99	IEGAQGLR
				2 99	LGLTNEGNGVAHFK
				2 99	MGIINIQDEINDYMK
				2 99	PNLGLTNEGNGVAHFK
				2 99	RPASNTYLIQGSLAPNVTLINDR

Table S2. Peptide summary of three replicates of concentrated supernatants and solubilized proteins from spore/crystal mixtures identified with Protein Pilot v4.5

Sample	Replicate	Homology	Contrib	Conf	Sequence
				2	99 SVKSTYDSSFK
				2	99 SVTPQYDVIPTEPVNNHITTK
				2	99 SYIQVTEYPMDDNGR
				2	99 SYIQVTEYPMDDNGRR
				2	99 SYIQVTEYPMDDNGRRPASN
					SYIQVTEYPMDDNGRRPASNTY
				2	99 LIQGSLAPNVTLINDRK
				2	99 TDTRTWTDTTTVK
					TNVEVAYIIQTGNYNVPVNVESD
				2	99 MTGTLFCR
				2	99 TWTDTTTVK
				2	99 TWTDTTTVKAPPR
				2	99 TWTETDTVTSAVTK
					VFNESVTPQYDVIPTEPVNNHIT
				2	99 TK
				2	99 VGGSVSSKATF
					VIDNPGTSEVTSTVTFTWTETDT
				2	99 VTSAVTK
			1.657577		99 IQDEINDYMK
			1.244125		99 TSVKSTYDSSFK
			1.017729		99 SYIQVTEYPMDDNGRRPA
			0.68403		99 GYIEGAQGLR
			0.617983	76.35	STYDSSFK
			0.614394	76.1	VGGSVSSK
			0.341989	98.54	EGYIEGAQGLR
			0.143876	90.01	YSTTETTTK
			0.066007	86.77	SLAPNVTLINDRK
			0.014574	73.15	GLTNEGNGVAHFK
			0.008331	29.39	NHITTK
					MGIINIQDEINDYMKGMYGATS
			0.000435	94.95	VKSTYDSSFK
					DGALIAAAYVSITDLADYNPNLG
	3	Cry23Aa-like		2	99 LTNEGNGVAHFK
					DGALIAAAYVSITDLADYNPNLG
					LTNEGNGVAHFKGEGYIEGAQG
				2	99 LR
				2	99 GEGYIEGAQGLR
				2	99 GIINIQDEINDYMK
					GIINIQDEINDYMKGMYGATSV
				2	99 K
				2	99 GMYGATSVK
				2	99 GMYGATSVKSTYDSSFK
				2	99 MGIINIQDEINDYMK
				2	99 SVTPQYDVIPTEPVNNHITTK

Table S2. Peptide summary of three replicates of concentrated supernatants and solubilized proteins from spore/crystal mixtures identified with Protein Pilot v4.5

Sample	Replicate	Homology	Contrib	Conf	Sequence
			2	99	SYIQVTEYPMDDNGR
			2	99	SYIQVTEYPMDDNGRR
			2	99	TDTRTWTDTTTVK
			2	99	TDTRTWTDTTTVKAPPR
			2	99	TWTDTTTVKAPPR
					VFNESVTPQYDVIPTEPVNNHIT
			2	99	TK
					VIDNPGTSEVTSTVTFTWTETDT
			2	99	VTSAVTK
			1.744727	98.4	TWTDTTTVK
			1.69897	99	IQDEINDYMK
			1.124939	93.26	VGGSVSSK
			1.022276	99	SYIQVTEYPMDDNGRRPA
			0.847712	98.73	TWTETDTVTSAVTK
			0.428291	98.33	FKGEGYIEGAQGLR
			0.278189	50.22	STYDSSFK
			0.134304	95.14	SYIQVTEYPMDDNGRRPASN
			0.045275	73.33	YSTTETTTK
O-V84.2 Supernatant 24 h	1	Vip4Aa-like_1	2	99	AAGHMDTAIKQEAR
			2	99	AFYYYGGSSSYR
					DYRDPEDLTPEVTLGDAIAMAF
			2	99	DTTEKDGELYYK
			2	99	GMNIMLQK
			2	99	GQGIKGDAGHFNGK
			2	99	YVSDPYNSHTVRDPYTDWEK
			0.394695	85.85	DPYTDWEK
			0.264401	77.46	YVSDPYNSHTVR
			0.047208	66.58	FNGIASNGTYQG
		Vip4Aa-like_2	2	99	GMNIMIK
			2	99	HLKPNTIYQLSASVK
					QHNWANTTLATGEGVTGNAG
			2	99	K
			0.01055	96.99	AKENQLGDVLK
		Vip2Ac-like_2	2	99	HIGSNANISDDPK
			2	99	LFSETFTNTTR
			2	99	NTGLEINNVTIINQK
			2	99	SIEELKSPAKPGEYDINR
			0.69897	95.87	NVVEYMVEK
			0.120904	65.05	WTNSLSSTHIK
			0.115771	63.91	FTNIYFK

Table S2. Peptide summary of three replicates of concentrated supernatants and solubilized proteins from spore/crystal mixtures identified with Protein Pilot v4.5

Sample	Replicate	Homology	Contrib	Conf	Sequence
	2	Vip4Aa-like_1	2	99	AFYYYGGSSSYR
			2	99	GDSIVTVK
			2	99	LNLQTTQASGLYGVIK
			2	99	LPQYGPETTLNASQGYQR
					TFVNLNEYGGFKPNTAYLFSVYT
			2	99	K
			1.455932	99	YYNTGTAPIYNVKPTTNFVLK
					NPLVAAYPSVGVHMENLIISNN
			0.554396	93.1	R
			0.17134	71.6	YVSDPYNSHTVR
		Vip4Aa-like_2	1.508638	99	LTLETTQASGLYGR
			0.020907	89.21	IAFDTTEQNGK
			0.003926	26.75	AKENQLGDVLK
		Vip2Ac-like_2	1.721247	99	HIGSNANISDDPK
			0.007889	40.47	NVVEYMVEK
			0.004365	27.83	FTNIYFK
	3	Vip4Aa-like_1	2	99	AAGHMDTAIK
			2	99	AAGHMDTAIKQEAR
			2	99	AFYYYGGSSSYR
			2	99	ESVTGIVFDQK
			2	99	YVSDPYNSHTVR
			0.014574	98.17	GDSIVTVK
		Vip2Ac-like_2	2	99	HIGSNANISDDPK
			2	99	WTNSLSSTHIK
			0.013676	23.82	EFHQLTPYAK
		Sip1Aa-like_2	1.508638	99	AIVTYTLQK
			0.063989	83.65	YLVEVK
O-V84.2 Supernatant 48 h	1	Vip4Aa-like_1	2	99	LPQYGPETTLNASQGYQR
			0.243364	91.05	AAGHMDTAIKQEAR
			0.21467	89.64	DPYTDWEK
			0.133713	82.98	KYVSDPYNSHTVR
			0.132533	82.88	AAGHMDTAIK
			0.012781	28.57	YVSDPYNSHTVRDPYTDWEK
	2	Vip4Aa-like_1	2	99	ESVTGIVFDQK
			2	99	LPQYGPETTLNASQGYQR
			1.744727	99	AAGHMDTAIKQEAR
			1.568636	99	AKENQIGNVLQAGSTYPDK
			1.080922	97.95	YVSDPYNSHTVRDPYTDWEK
			0.012334	34.43	YVSDPYNSHTVR
		Vip2Ac-like_2	2	99	HIGSNANISDDPK
			0.345823	93.38	NVVEYMVEK

Table S2. Peptide summary of three replicates of concentrated supernatants and solubilized proteins from spore/crystal mixtures identified with Protein Pilot v4.5

Sample	Replicate	Homology	Contrib	Conf	Sequence
			0.340084	93.24	FTNIYFK
			0.331614	98.26	SINGDAGMSSGPLK
			0.074172	68.43	WTNSLSSTHIK
	3	Vip4Aa-like_1	2	99	ESVTGIVFDQK
			2	99	LPQYGPETTLNASQGYQR
			0.019088	27.37	AKENQIGNVLQAGSTYPDK
		Vip2Ac-like_2	2	99	HIGSNANISDDPK
			2	99	NVVEYMVEK
			0.742321	97.16	FTNIYFK
O-V84.2 Crystal					
72 h	1	Cry45Aa-like_1	2	99	AGQTREYQIPVTR
			2	99	GVTPNPTSQPIVR
			2	99	ILHEMGYTDSTHIYVAHFIGSGK
					LYNLNVQVPDPIVTDNPTNSAV
			2	99	AR
			2	99	MGLQSYVNVVETPLPGR
			2	99	PIVTDNPTNSAVAR
			2	99	RSGLDIPIFDPIVSR
			2	99	RSGLDIPIFDPIVSRQ
			2	99	SEAGFLFAK
			2	99	SGLDIPIFDPIVSR
			2	99	SGLDIPIFDPIVSRQ
			2	99	TFIVQTGPFDK
			1.886056	99	IVQTGPFDK
			1.200659	96.05	TYTTEISR
			0.777284	89	KLTEDYWR
			0.744727	88.1	YVNHSMAR
			0.628932	84.1	AILDLKK
			0.102373	90.25	VVETPLPGR
			0.025028	70.12	VPDPIVTDN
			0.014125	27.23	EYQIPVTR
		Cry45Aa-like_2	2	99	DPGNIYNYNMK
			2	99	GTTPNPTSQPIVR
			2	99	MGLQSFVNVVERPLLGR
			2	99	PGNIYNYNMK
			2	99	PIVTDNPTNSAYAK
			2	99	QHNTTLKSSGYLGR
			2	99	SEAGLLFAK
			2	99	VPDPIVTDNPTNSAYAK
			2	99	YNPNPYTAHFTGSGK
			2	99	YNPNPYTAHFTGSGKLTGK
			1.958608	99	SGLDIPIFDPVISR
			1.537602	98.26	AIIDLLK
			0.966576	93.34	EYQIPVTGR

Table S2. Peptide summary of three replicates of concentrated supernatants and solubilized proteins from spore/crystal mixtures identified with Protein Pilot v4.5

Sample	Replicate	Homology	Contrib	Conf	Sequence
			0.896196	95.2	QHNTTLK
			0.60206	83.55	SSGYLGR
			0.03292	74.69	DPIVTDNPTNSAYAK
			0.003926	25.5	PYTAHFTGSGK
			0.002614	21.44	GNIYNYNMK
		Cry45Aa-like_3	2	99	AGQTRKYQIPLTGR
			2	99	GLDIPIFDPVVSR
			2	99	GLDIPIFDPVVSRQ
			2	99	GYTPNPTSQPIVR
			2	99	MGLQSYVDVVETPLSGR
					MTGYNGGVISEILDMQGVANIT
			2	99	YPQFGIAHFTGSGK
			2	99	NVVLECDITGTTTCVYR
			2	99	PVVIDNPTNSALAR
			2	99	RGLDIPIFDPVVSR
			2	99	SEAGFVFAK
			1.346787	99	YPGNSVDR
			0.752027	90.06	KYQIPLTGRR
			0.640165	86.76	YQIPLTGRR
			0.293282	99	KYQIPLTGR
			0.031984	81.5	PTNSALAR
			0.014125	68.8	YTTSNTYTTETSR
		Cry32Ea-like	2	99	AQAITELQGIQAAIR
			2	99	AVQAAQSAIQALFTDQNQTR
			2	99	DAACNWEK
			2	99	DAACNWEKNPNNPQLK
			2	99	DAACNWEKNPNNPQLKETVR
			2	99	DLIIDGGYQGER
			2	99	EGAGEGYVTISDGTEDNTETVK
					ERNMTNQPSEVSGTNAYPGNN
			2	99	NR
			2	99	ETLPVTLPVGSEDR
			2	99	FTAGVETPR
			2	99	GFVGNSKELEVFVQR
			2	99	GMEPDLLSAIPVFWDIN
			2	99	GMEPDLLSAIPVFWDINSSYN
					GMEPDLLSAIPVFWDINSSYNSS
			2	99	SGLPCR
			2	99	GPGSTGGDLVK
			2	99	GPGSTGGDLVKLPVPEGR
			2	99	GSETSLLTAYVEAATLHLGLLK

Table S2. Peptide summary of three replicates of concentrated supernatants and solubilized proteins from spore/crystal mixtures identified with Protein Pilot v4.5

Sample	Replicate	Homology	Contrib	Conf	Sequence
				2	99 GYITYGNASVVK
				2	99 IDESKLKPYTR
					IGNASVLVLSNWSANLSQNVCV
				2	99 NPAHGYILR
					IGTTDGVANIDNLEVIEANPLTG
				2	99 EALAR
				2	99 IHIYNEGGGDLILDK
					IHIYNEGGGDLILDKIEFIPIEGTLE
				2	99 EYQANQDLEK
					IPYVYHPFLQGSLPAVPGETYDIF
				2	99 QQLSNAIGK
				2	99 ITQIPAVK
				2	99 KAVNALFKSDAK
				2	99 KEGAGEGYVTISDGTEDN
				2	99 KEGAGEGYVTISDGTEDNTETVK
				2	99 KETLPVTLPVGSEDR
				2	99 LPVPEGR
				2	99 LRPDITLNHILHAETLVQK
				2	99 LSYVGLGMLNSDYR
				2	99 LSYVGLGMLNSDYRDFVSSPTR
				2	99 LVECMSDEIYPQEK
				2	99 LYEVPTKSQLTR
				2	99 LYRDAACNWEKNPNNPQLK
				2	99 MDQYYNNNEYEILDHGR
				2	99 MDQYYNNNEYEILDHGRR
					MDSYVSTSLMTAQNGSPLSGYV
				2	99 TK
				2	99 MMTAAEQITNK
				2	99 MMTAAEQITNKAIAEQAR
				2	99 NMNVPYGIPPTNPCDRDYQR
				2	99 NMTNQPSEVSGTNAYPGNNNR
				2	99 NPNNPQLKETVR
				2	99 NTLQLNVTDYLVDQAAK
					NVLRNGDFSAGLSNWQGAEGA
				2	99 LVQK
				2	99 PGTPQAQISWDK
				2	99 SDYRDFVSSPTR
				2	99 SPIQEGYK
					SPIQEGYKERNMTNQPSEVSGT
				2	99 NAYPGNNNR
				2	99 SSYNSSSGLPCR

Table S2. Peptide summary of three replicates of concentrated supernatants and solubilized proteins from spore/crystal mixtures identified with Protein Pilot v4.5

Sample	Replicate	Homology	Contrib	Conf	Sequence
				2	99 TAALGIPFTHPFNDTSLIK
				2	99 TGLQQQYNTGDWNK
				2	99 TLQLNVTDYLVDQAAK
				2	99 TQFVATNTILYNR
				2	99 TSSNVSVR
					TVYTPLEGNVGGNWLSPEPVPSI
				2	99 DVIEGNIVDTPGLFK
					TYPVLTNQTIPQDMACDSCDAG
				2	99 TVMR
					VTARKEGAGEGYVTISDGTEDN
				2	99 TETVK
				2	99 VTDYLVDQAAK
				2	99 WLHNISFEAIDGGVNTSVIK
				2	99 WQGAEGALVQK
				2	99 WYNTGLQQQYNTGDWNK
				2	99 YASNADVNVFCTLQK
				2	99 YDKEIHK
					YDKEIHKNMNVPYGIPPTNPCD
				2	99 RDYQR
				2	99 YGDFDSDNWSGENGWK
					YLHMPGANIPQFSDTVFPTYAY
				2	99 QK
				2	99 YPLAQAPGSVLQNMDYK
					YPLAQAPGSVLQNMDYKELMN
				2	99 K
			1.958608		98.92 LESIELIR
			1.920818		98.75 LTQSDMR
			1.886056		98.66 AKQTLVK
			1.853872		98.56 AIAEQAR
			1.823909		98.46 LYEVPTK
			1.795881		98.44 MCLLDLVK
			1.552842		97.19 KAVNALFK
			1.468521		99 AVNALFK
			1.408936		96.09 AVNALFKSDAK
			1.229148		94.09 DLIIDGGYQGERGTKK
			1.031517		90.74 LTQSDMRSPIQEGYKER
			0.943095		98.4 SKELEVFVQR
			0.863279		86.3 ADDPIFK
			0.847712		85.81 NPNNPQLK
			0.716699		80.77 DLIIDGGYQGERGTK
			0.701147		80.12 MPYFSAR
			0.681937		98.91 NEYEILDHGR
			0.336299		95.98 NNEYEILDHGR
			0.28735		48.43 SLYGQRNVLR

Table S2. Peptide summary of three replicates of concentrated supernatants and solubilized proteins from spore/crystal mixtures identified with Protein Pilot v4.5

Sample	Replicate	Homology	Contrib	Conf	Sequence
			0.208309	38.06	LYRDAACNWEK
			0.185752	69.41	GFVGNSK
			0.095826	99	ADDPIFKGR
			0.078834	75.44	GPSNNYEASAYR
			0.014125	22.94	MITQIPAVK
			0.001741	97.95	THPFNDTSLIK
			0.001741	16.83	TLEEYQANQDLEK
			0.001305	18.92	NPLTGEALAR
		Cry32Da-like	2	99	ASGGTDYQVIK
			2	99	ATFDNLEVIEANPLTGEALAR
			2	99	AVNALFTSDAK
			2	99	DNATTFVDPK
			2	99	EGSGEGYVTISDGTEDNTETLK
			2	99	FEPVRDNATTFVDPK
			2	99	FTVGEVATR
			2	99	GPGSTGGDLVSLPTYAK
			2	99	GQALIGLK
			2	99	GTTYRSDPAQNTMSAIEAR
			2	99	GYETILLPLYAQAANIHLIVLR
			2	99	IEIPLTSSAGQLYTIR
			2	99	INDYQIDYK
					IPYVYNAFLQGALPPVPGETSDIF
			2	99	QQLSSAVAIAR
			2	99	IWETNPTPGNTSQLLDAFR
			2	99	KEGSGEGYVTISDGTEDNTETLK
			2	99	LKPDITLNHILQAETEVQK
			2	99	LVECMSDKIYPQEK
			2	99	NLALDHIDR
			2	99	NLALDHIDRINDYQIDYK
			2	99	NLLNHGDFEFPDWSGENGWK
					QIGATIFGWTHTSVDPNNTIDTT
			2	99	K
			2	99	SCQDAMPHLDTR
			2	99	SDPAQNTMSAIEAR
			2	99	TDSYPMTAHSSSLSGYVTK
			2	99	TFEIFPETNR
					THTNNKTDSYPMTAHSSSLSGY
			2	99	VTK
			2	99	TKQWFEPR
					TLDTNMITALQGLTDEYEYYHIP
			2	99	LDVSTGQK
			2	99	TSNHVSVMSGNPIFK

Table S2. Peptide summary of three replicates of concentrated supernatants and solubilized proteins from spore/crystal mixtures identified with Protein Pilot v4.5

Sample	Replicate	Homology	Contrib	Conf	Sequence
			2	99	VESIELIR
			2	99	VWESMIGYAQALIK
			2	99	VYTAAVKTEFTR
					YLHMPGADNPQLSDQVYPTYA
			2	99	YQK
			2	99	YSNGEYKDVEAWNLR
			2	99	YTWVGNSWTK
			1.886056	98.9	DLEVFITR
			1.721247	99	PAQNTMSAIEAR
			1.60206	99	FEPVRDNATTFVD
			0.570248	93.81	MCLLDQVK
			0.457175	69.03	INDYQIDYKNK
			0.406714	88.75	KAVNALFTSDAK
			0.390406	63.49	VQQTFVK
			0.267606	50.47	IFPETNR
			0.216811	92.01	ALDHIDR
			0.154282	33.72	GQALIGLKTESTR
			0.136677	30.63	YRDMQWR
			0.114639	26.57	EIYTSIR
			0.100727	23.76	EFIFYR
			0.074688	18.35	YTGEEQAGGLIR
			0.000869	22.74	SQNGFNPSFAGNAR
		Cry32Eb-like	2	99	ALPVVPGETYDIFLQLSDAINIAR
			2	99	ALYDQRNALVNGDFSAR
			2	99	ATSPVSSNVFPTLR
			2	99	DAIQMLFTDSNQNR
			2	99	EAVNTLFLNDALQLK
			2	99	EGSGEGYVTISDGTEENTETLK
			2	99	FIADEKATSPVSSN
			2	99	FIADEKATSPVSSNVFPTLR
			2	99	GAVQIAR
			2	99	IKGAVQIAR
			2	99	IPYVYNQFLQR
			2	99	ISNHVYVTSDNPIFK
			2	99	KEGSGEGYVTISDGTEEN
			2	99	KEGSGEGYVTISDGTEENTETLK
			2	99	LDHVEVVEVGPLTGDALTR
			2	99	LVECISDPIHTK
			2	99	LVECISDPIHTKEK
			2	99	MCLLEQVK
			2	99	NALVNGDFSAR
			2	99	NLLHHGDFESPDWSGENR

Table S2. Peptide summary of three replicates of concentrated supernatants and solubilized proteins from spore/crystal mixtures identified with Protein Pilot v4.5

Sample	Replicate	Homology	Contrib	Conf	Sequence
			2	99	NLLHHGDFESPDWSGENRWK
			2	99	QILEAETWVQK
			2	99	SKDLELLVAR
			2	99	VTDYQVDQAAK
			2	99	YLNMPNANTPQFSDK
			1.29243	96.08	MSYASNHYK
			1.221849	95.4	LQSAITLK
			1.070581	93.44	DLELLVAR
			0.735182	85.49	MNQDVTR
			0.413413	67.9	FIADEK
			0.09691	73.14	MPNANTPQFSDK
			0.063486	17.24	IGTTDGMAK
					VTARKEGSGEGYVTISDGTEENT
			0.057992	15.9	ETLK
			0.026872	67.1	ISNHVYVTSDN
			0.004804	66.22	ECISDPIHTK
		Cry73Aa-like	2	99	EVYSTVYGR
			2	99	FGAGWGLPSTEVEDNYTR
			2	99	FGTDPQAQWK
					GPGSTGGDLIQLASGYLQYSFPS
			2	99	TDNR
			2	99	IYNNPYGVQIQLSR
					MNQNCNHNEYEILDSGNVSCQ
			2	99	PK
			2	99	NLYEAETVPIAFR
			2	99	NLYEAETVPIAFRK
			2	99	NQNCNHNEYEILDSGNVSCQPK
			2	99	QAYDLAPNPTDYNK
					QAYDLAPNPTDYNKYPYLDPYS
			2	99	KDPIYGK
			2	99	QFMDAVEYLVSQK
			2	99	QFTSTNTVIEYAMPSFR
			2	99	RFGTDPQAQWK
			2	99	SLTSTNTIATDKTTQIPAVK
			2	99	SPHLVTELTK
			2	99	SSVDAIESTLVR
			2	99	TAEYTNHCTNTYDK
					TVHNVSATDIGNLSLSLGAIPFGF
			2	99	SFYNK
					VGGFEVPLLTTFAQAANLHLLLL
			2	99	R
			2	99	YASTNGSTLTLK
			2	99	YASTNGSTLTLKK

Table S2. Peptide summary of three replicates of concentrated supernatants and solubilized proteins from spore/crystal mixtures identified with Protein Pilot v4.5

Sample	Replicate	Homology	Contrib	Conf	Sequence
			2	99	YYTAPVDWNLFNDFRR
			1.886056	99	PYSKDPIYGK
			1.823909	98.92	TTQIPAVK
			1.408936	97.21	LEGVQR
			0.995679	92.62	SEAVSRLEGVQR
			0.879426	99	YQGVMNTSIGR
			0.44855	95.7	SLTSTNTIATDK
			0.295849	98.79	HLVTELTK
			0.280669	91.68	TIATDKTTQIPAVK
			0.051098	91.99	ITNTLHYVGS
			0.036212	34.19	LTPYNSNTKVIK
			0.01055	45.01	TVHNVSATDIGN
			0.003488	21.79	NNQHITTVGY
	2	Cry45Aa-like_1	2	99	GVTPNPTSQPIVR
			2	99	ILHEMGYTDSTHIYVAHFIGSGK
					LYNLNVQVPDPIVTDNPTNSAV
			2	99	AR
			2	99	MGLQSYVNVVETPLPGR
			2	99	NKYEFVNAEMSWLNGQYPVR
			2	99	NVVLECDIDGYTMCYYR
			2	99	PIVTDNPTNSAVAR
			2	99	RSGLDIPIFDPIVSR
			2	99	RSGLDIPIFDPIVSRQ
			2	99	SEAGFLFAK
			2	99	SGLDIPIFDPIVSR
			2	99	SGLDIPIFDPIVSRQ
			2	99	TFIVQTGPFDK
			2	99	TYTTEISR
			2	99	VQVPDPIVTDNPTNSAVAR
			1.795881	99	EYQIPVTR
			1.744727	99	SGLDIPIFD
			1.537602	99	ILHEMGYTDSTHIYVAH
					TVGYTETLTDSQSTTTEHGVTAG
			1.161151	95.67	ASVTVK
			1.040959	94.26	YVNHSMAR
			0.931814	92.52	AILDLKK
			0.59176	99	THTFIVQTGPFDK
			0.563837	81.34	KLTEDYWR
			0.045757	81.24	VVETPLPGR
			0.025488	74.42	ILHEMGYTDSTHIY
			0.014125	77.7	AILDLK
			0.007889	25.14	LYNLNVQVPD
		Cry45Aa-like_2	2	99	DPGNIYNYNMK

Table S2. Peptide summary of three replicates of concentrated supernatants and solubilized proteins from spore/crystal mixtures identified with Protein Pilot v4.5

Sample	Replicate	Homology	Contrib	Conf	Sequence
			2	99	GTTPNPTSQPIVR
			2	99	GYWVAISQILMENGVPGIR
			2	99	MGLQSFVNVVER
			2	99	MGLQSFVNVVERPLLGR
			2	99	PGNIYNYNMK
			2	99	PIVTDNPTNSAYAK
			2	99	SEAGLLFAK
			2	99	VPDPIVTDNPTNSAYAK
			2	99	YNPNPYTAHFTGSGK
					DPGNIYNYNMKVPDPIVTDNPT
			1.187087	99	NSAYAK
			1.080922	99	EYQIPVTGR
			0.617983	86.94	AIIDLLK
			0.47237	97.78	SFIVQTGPFNK
			0.122053	40.57	SSGYLGR
			0.102373	92.51	PYTAHFTGSGK
			0.005243	29.41	IVQTGPFNK
		Cry45Aa-like_3	2	99	GLDIPIFDPVVSR
			2	99	GLDIPIFDPVVSRQ
			2	99	GYTPNPTSQPIVR
			2	99	ITYPQFGIAHFTGSGK
			2	99	LTGKMGLQSYVDVVETPLSGR
			2	99	MGLQSYVDVVETPLSGR
			2	99	MSVPDPVVIDNPTNSALAR
					MTGYNGGVISEILDMQGVANIT
			2	99	YPQFGIAHFTGSGK
			2	99	NVVLECDITGTTTCVYR
			2	99	PVVIDNPTNSALAR
			2	99	RGLDIPIFDPVVSR
			2	99	RGLDIPIFDPVVSRQ
			2	99	SVPDPVVIDNPTNSALAR
					SWEDSLQITVPPGYQTTHTFIVQ
			2	99	TGPFDK
					TITFNETQSDSQSTTTEHGITAG
			2	99	VSATVK
			2	99	YPGNSVDR
			1.180456	96.02	KYQIPLTGR
			0.219683	53.11	YQIPLTGR
			0.092589	28.92	YQIPLTGRR
		Cry32Ea-like	2	99	AIAEQAR
			2	99	AQAITELQGIQAAIR
			2	99	AVNALFK
			2	99	AVQAAQSAIQAL

Table S2. Peptide summary of three replicates of concentrated supernatants and solubilized proteins from spore/crystal mixtures identified with Protein Pilot v4.5

Sample	Replicate	Homology	Contrib	Conf	Sequence
				2	99 AVQAAQSAIQALFTDQNQTR
				2	99 CEWGESGELFADELGPVR
				2	99 DAACNWEKNPNNPQLK
				2	99 DLIIDGGYQGER
				2	99 DQYYNNNEYEILDHGR
				2	99 DQYYNNNEYEILDHGRR
				2	99 EGAGEGYVTISDGTEDNTETVK
				2	99 ELEVFVQR
					ERNMTNQPSEVSGTNAYPGNN
				2	99 NR
				2	99 ETVRTQFVATNTILYNR
				2	99 FTAGVETPR
				2	99 GFVGNSKELEVFVQR
					GMEPDLLSAIPVFWDINSSYNSS
				2	99 SGLPCR
					GMNSVCETCDPNCMPEIPSGG
				2	99 SICDRPSQYGNR
				2	99 GPGSTGGDLVK
				2	99 GSETSLLTAYVEAATLHLGLLK
				2	99 GYITYGNASVVK
				2	99 IEFIPIEGTLEEYQANQDLEK
					IGNASVLVLSNWSANLSQNVCV
				2	99 NPAHGYILR
					IGTTDGVANIDNLEVIEANPLTG
				2	99 EALAR
				2	99 IHIYNEGGGDLILDK
					IHIYNEGGGDLILDKIEFIPIEGTLE
				2	99 EYQANQDLEK
					IPYVYHPFLQGSLPAVPGETYDIF
				2	99 QQLSNAIGK
				2	99 KEGAGEGYVTISDGTEDN
				2	99 KEGAGEGYVTISDGTEDNTETVK
					KEGAGEGYVTISDGTEDNTETVK
				2	99 FTAGVETPR
				2	99 KETLPVTLPVGSEDR
				2	99 LESIELIR
					LQGSLPAVPGETYDIFQQLSNAI
				2	99 GK
				2	99 LRPDITLNHILHAETLVQK
				2	99 LSYVGLGMLNSDYR
				2	99 LSYVGLGMLNSDYRDFVSSPTR

Table S2. Peptide summary of three replicates of concentrated supernatants and solubilized proteins from spore/crystal mixtures identified with Protein Pilot v4.5

Sample	Replicate	Homology	Contrib	Conf	Sequence
				2	99 LVECMSDEIYPQEK
				2	99 LYEVPTKSQLTR
				2	99 MCLLDLVK
				2	99 MDQYYNNNEYEILDHGR
				2	99 MDQYYNNNEYEILDHGRR
					MDSYVSTSLMTAQNGSPLSGYV
				2	99 TK
				2	99 MITQIPAVK
				2	99 MMTAAEQITNK
				2	99 MMTAAEQITNKAIAEQAR
				2	99 NGDFSAGLSNWQGAEGALVQK
				2	99 NLGIWVGFK
				2	99 NMNVPYGIPPTNPCDR
				2	99 NMNVPYGIPPTNPCDRDYQR
				2	99 NMTMAVLDIVAVWPTFDIK
				2	99 NMTNQPSEVSGTNAYPGNNNR
				2	99 NPNNPQLK
				2	99 NPNNPQLKETVR
				2	99 NTLQLNVTDYLVDQAAK
					NVLRNGDFSAGLSNWQGAEGA
				2	99 LVQK
					NYQSESFGITPYSDETTMMNGP
				2	99 SNNYEASAYR
				2	99 PVFWDINSSYNSSSGLPCR
				2	99 SANLSQNVCVNPAHGYILR
				2	99 SDAKNTLQLNVTDYLVDQAAK
				2	99 SPIQEGYK
				2	99 SPIQEGYKER
				2	99 TAALGIPFTHPFNDTSLIK
				2	99 TGLQQQYNTGDWNK
				2	99 THPFNDTSLIK
				2	99 TQFVATNTILYNR
				2	99 TVEIFPETNHVCIEIGETAGTFK
				2	99 TVYTPLEGNVGGN
					TVYTPLEGNVGGNWLSPEPVPSI
				2	99 DVIEGNIVDTPGLFK
					TYPVLTNQTIPQDMACDSCDAG
				2	99 TVMR
				2	99 VTDYLVDQAAK
				2	99 WLHNISFEAIDGGVNTSVIK

Table S2. Peptide summary of three replicates of concentrated supernatants and solubilized proteins from spore/crystal mixtures identified with Protein Pilot v4.5

Sample	Replicate	Homology	Contrib	Conf	Sequence
				2	99 WYNTGLQQQYN
				2	99 WYNTGLQQQYNTGDWNK
					YAAYIGYGWTHVSADENNQIYP
				2	99 NMITQIPAVK
				2	99 YASNADVNVFCTLQK
				2	99 YGDFDSDNWSGENGWK
					YGDFDSDNWSGENGWKTSSNV
				2	99 SVR
					YLHMPGANIPQFSDTVFPTYAY
				2	99 QK
				2	99 YLPVGNVWHVTN
					YLPVGNVWHVTNQLFSSGGAS
				2	99 VVTGWLFR
				2	99 YPLAQAPGSVLQNMDYK
					YPLAQAPGSVLQNMDYKELMN
				2	99 K
			1.67778	97.87	ITQIPAVK
			1.420217	99	VATNTILYNR
			1.327902	95.34	AVNALFKSDAK
			1.180456	99	SKELEVFVQR
			0.906578	99	ADVNVFCTLQK
			0.906578	87.59	LYEVPTK
			0.89279	98.49	MTAAEQITNK
			0.876148	86.67	ETLPVTLPVGSEDR
			0.838632	85.51	KAVNALFK
			0.823909	84.98	LTQSDMR
			0.764472	82.84	ADDPIFK
			0.735182	81.55	DFVSSPTR
			0.707744	98.93	GPSNNYEASAYR
			0.634512	99	SICDRPSQYGNR
					FGLQWGMDPSIVNVYYGYLTN
			0.496209	99	NIATYTNHCTR
			0.477556	66.67	LPVPEGR
					SPIQEGYKERNMTNQPSEVSGT
			0.419075	99	NAYPGNNNR
			0.276544	47.14	MPYFSAR
			0.235077	92.67	IATYTNHCTR
			0.226945	96.3	IGNASVLVLSN
					YAAYIGYGWTHVSADENNQIYP
			0.217527	82.03	N
			0.153663	29.83	GFVGNSK
			0.078314	16.45	SLYGQRNVLR
			0.055517	88.54	SSYNSSSGLPCR
			0.047692	98.74	ADDPIFKGR
			0.03292	66.37	LRPDITLN

Table S2. Peptide summary of three replicates of concentrated supernatants and solubilized proteins from spore/crystal mixtures identified with Protein Pilot v4.5

Sample	Replicate	Homology	Contrib	Conf	Sequence
			0.030584	80.1	WYNTGLQQQY
			0.025488	38.3	IPYVYHPF
			0.013676	42.31	NIATYTNHCTR
			0.013228	48.89	HILHAETLVQK
			0.008774	32.6	TVEIFPETN
			0.004804	18.82	IMPGYER
			0.003488	50.79	IQEGYKER
			0.003488	18.94	PLTGEALAR
			0.001741	49.44	TVEIFPETNHV
		Cry32Da-like	2	99	ASGGTDYQVIK
			2	99	ATFDNLEVIEANPLTGEALAR
			2	99	AVQTAQGAIQALFTDSNQNR
			2	99	EGSGEGYVTISDGTEDNTETLK
			2	99	ELGTSQGVLELSIER
			2	99	FEPVRDNATTFVDPK
			2	99	GPGSTGGDLVSLPTYAK
			2	99	GQALIGLK
			2	99	GSTGGDLVSLPTYAK
			2	99	GTTYRSDPAQNTMSAIEAR
			2	99	GYETILLPLYAQAANIHLIVLR
			2	99	ICIEIGEISGTFK
			2	99	IDPFDEPIIIDK
					IDPFDEPIIIDKIEFIPIQGSVETYE
			2	99	ADQDLEK
			2	99	IEFIPIQGSVETYEADQDLEK
			2	99	IEIPLTSSAGQLYTIR
			2	99	INDYQIDYK
					IPYVYNAFLQGALPPVPGETSDIF
			2	99	QQLSSAVAIAR
			2	99	IWETNPTPGNTSQLLDAFR
			2	99	KEGSGEGYVTISDGTEDNTETLK
			2	99	LKPDITLNHILQAETEVQK
			2	99	LVECMSDKIYPQEK
					MIRPPHLFEWPQTMTFFFEDVN
			2	99	VR
					NEFISSMTIQVLDTVALWPTYDP
			2	99	K
			2	99	NLALDHIDRINDYQIDYK
			2	99	NLLNHGDFEFPDWSGENGWK
			2	99	NNPSNENGSDYGCGCR
			2	99	NVLRNGDFMAGLSNWR

Table S2. Peptide summary of three replicates of concentrated supernatants and solubilized proteins from spore/crystal mixtures identified with Protein Pilot v4.5

Sample	Replicate	Homology	Contrib	Conf	Sequence
					QASLTNEYTQSQNGFNPSFAGN
			2	99	AR
					QIGATIFGWTHTSVDPNNTIDTT
			2	99	K
			2	99	QNTFPPIEVPEGTPPPSHR
			2	99	SCQDAMPHLDTR
			2	99	SDPAQNTMSAIEAR
			2	99	TFEIFPETNR
					TLDTNMITALQGLTDEYEYYHIP
			2	99	LDVSTGQK
			2	99	TSNHVSVMSGNPIFK
			2	99	VWESMIGYAQALIK
					YLHMPGADNPQLSDQVYPTYA
			2	99	YQK
			2	99	YNQDEFYSGFFGPENSCK
			2	99	YRELGTSQGVLELSIER
			2	99	YTDYDVPSTPYSGGNLTYNAFR
			2	99	YTGEEQAGGLIR
			1.958608	99	DLEVFITR
			1.744727	99	FEPVRDNATTFVD
			1.67778	98.21	TDSYPMTAHSSSLSGYVTK
			1.552842	97.57	AVNALFTSDAK
			1.49485	99	KAVNALFTSDAK
			1.468521	97.1	FTVGEVATR
			1.376751	99	PQLSDQVYPTYAYQK
			0.832683	87.25	DVEAWNLR
			0.661543	80.85	IFPETNR
			0.614394	78.58	VESIELIR
			0.501689	92.29	MCLLDQVK
			0.488117	70.93	NLALDHIDR
			0.471083	69.75	EIYTSIR
			0.367543	61.06	VYTAAVK
			0.335358	57.79	YTWVGNSWTK
			0.314258	94.74	ALDHIDR
			0.252588	48.16	VQQTFVK
			0.199971	74.56	INDYQIDYKNK
			0.188425	38.97	VYTAAVKTEFTR
			0.140261	30.99	LVECMSDK
			0.109579	25.27	QWFEPR
			0.101824	23.69	LLYEQR
			0.088842	21.13	ELTETVR
			0.071604	17.38	DNATTFVDPK

Table S2. Peptide summary of three replicates of concentrated supernatants and solubilized proteins from spore/crystal mixtures identified with Protein Pilot v4.5

Sample	Replicate	Homology	Contrib	Conf	Sequence
					NMANAPLEAYGTNGYTNNPM
			0.002614	99	TNYPSDNAGANAYPGNHNR
		Cry32Eb-like	2	99	ALPVVPGETYDIFLQLSDAINIAR
			2	99	ALYDQRNALVNGDFSAR
			2	99	ATSPVSSNVFPTLR
			2	99	DAIQMLFTDSNQNR
			2	99	EAVNTLFLNDALQLK
			2	99	EGSGEGYVTISDGTEENTETLK
			2	99	FIADEKATSPVSSNVFPTLR
			2	99	GFVGNSKDLELLVAR
					GNAYPGATNMNYQSESFGFTPY
			2	99	SEENSK
			2	99	IKGAVQIAR
			2	99	IPYVYNQFLQR
			2	99	ISNHVYVTSDN
			2	99	ISNHVYVTSDNPIFK
			2	99	KEGSGEGYVTISDGTEENTETLK
			2	99	LDHVEVVEVGPLTGDALTR
			2	99	LVECISDPIHTK
			2	99	MCLLEQVK
			2	99	MGAYPDKINMPVNQGLDCR
			2	99	NALVNGDFSAR
			2	99	NLLHHGDFESPDWSGENR
					NMQMPEAYGTSEYASNQNIM
			2	99	NYPSESYR
			2	99	QILEAETWVQK
			2	99	VTDYQVDQAAK
			2	99	YLNMPNANTPQFSDK
			1.853872	99	LQSAITLK
			1.67778	98.55	GAVQIAR
			1.420217	99	SKDLELLVAR
			1.387216	97.22	DLELLVAR
			1.113509	99	ISNHVYVTSDNPIFKGR
			1.060481	93.94	MNQDVTR
			0.514279	77.04	MSYASNHYK
			0.241088	52.36	NLLHHGDFESPDWSGENRWK
			0.080922	23.3	IGTTDGMAK
			0.018634	56	KEGSGEGYVTISDGTEEN

Table S2. Peptide summary of three replicates of concentrated supernatants and solubilized proteins from spore/crystal mixtures identified with Protein Pilot v4.5

Sample	Replicate	Homology	Contrib	Conf	Sequence
			0.003488	85.89	LVECISDPIHTKEK
					ALELYQEAAADWNMNPNDAD
		Cry73Aa-like	2	99	AKER
			2	99	CNHNEYEILDSGNVSCQPK
			2	99	DMTLMVLDIVAVWPTYNPR
			2	99	FGAGWGLPSTEVEDNYTR
			2	99	FGTDPQAQWK
			2	99	GPGSTGGDLIQLASGYLQY
					GPGSTGGDLIQLASGYLQYSFPS
			2	99	TDNR
			2	99	GWTNNSSVDAIESTLVR
					ITNTLHYVGSSTTWEQSFSATPI
			2	99	GSIK
			2	99	IYNNPYGVQIQLSR
					KITNTLHYVGSSTTWEQSFSATPI
			2	99	GSIK
					MNQNCNHNEYEILDSGNVSCQ
			2	99	PK
			2	99	NLYEAETVPIAFR
			2	99	NLYEAETVPIAFRK
			2	99	NNQHITTVGYSGGWWNGIPK
			2	99	NQNCNHNEYEILDSGNVSCQPK
			2	99	PTDYNKYPYLDPYSKDPIYGK
			2	99	QAYDLAPNPTDYNK
					QAYDLAPNPTDYNKYPYLDPYS
			2	99	K
					QAYDLAPNPTDYNKYPYLDPYS
			2	99	KDPIYGK
			2	99	QFMDAVEYLVSQK
			2	99	QFTSTNTVIEYAMPSFR
			2	99	RQFTSTNTVIEYAMPSFR
			2	99	SEAVSRLEGVQR
			2	99	SLTSTNTIATDK
			2	99	SPHLVTELTK
			2	99	SSVDAIESTLVR
			2	99	TAEYTNHCTNTYDK
					TVHNVSATDIGNLSLSLGAIPFGF
			2	99	SFYNK
					VGGFEVPLLTTFAQAANLHLLLL
			2	99	R
			2	99	YASTNGSTLTLK
			2	99	YASTNGSTLTLKK

Table S2. Peptide summary of three replicates of concentrated supernatants and solubilized proteins from spore/crystal mixtures identified with Protein Pilot v4.5

Sample	Replicate	Homology	Contrib	Conf	Sequence
					YPLAQAPGFELPSMNYQGVMN
			2	99	TSIGR
			2	99	YPYLDPYSKDPIYGK
			2	99	YYTAPVDWNLFNDFR
			2	99	YYTAPVDWNLFNDFRR
			1.721247	98.48	EVYSTVYGR
			1.327902	99	HLVTELTK
			0.906578	89.87	TTQIPAVK
			0.692504	83.13	RFGTDPQAQWK
			0.640165	97.88	PYSKDPIYGK
			0.632644	80.45	LEGVQR
			0.463442	96.12	TVIEYAMPSFR
			0.28735	97.32	NNQHITTVGY
			0.185087	86.84	TFAIFDTGR
			0.172631	96.86	NTFAIFDTGR
			0.134896	31.27	LTFDER
			0.069051	94.36	TVHNVSATDIGN
			0.02826	68.82	PYGVQIQLSR
					ALELYQEAAADWNMNPNDAD
			0.014574	97.01	AK
	3	Cry45Aa-like_1	2	99	GVTPNPTSQPIVR
			2	99	ILHEMGYTDSTHIYVAHFIGSGK
					LYNLNVQVPDPIVTDNPTNSAV
			2	99	AR
			2	99	MGLQSYVNVVETPLPGR
			2	99	RSGLDIPIFDPIVSR
			2	99	RSGLDIPIFDPIVSRQ
			2	99	SEAGFLFAK
			2	99	SGLDIPIFDPIVSR
			2	99	SGLDIPIFDPIVSRQ
			2	99	TFIVQTGPFDK
			2	99	YVNHSMAR
			1.823909	99	VPDPIVTDN
			1.769551	99	PIVTDNPTNSAVAR
			0.896196	98.78	VPDPIVTDNPTN
			0.58336	81.3	AILDLKK
			0.38405	68.51	KLTEDYWR
			0.119758	32.76	AGQTREYQIPVTR
			0.018181	47.23	IVQTGPFDK
			0.012334	23.76	EYQIPVTR
			0.007889	38.77	TPNPTSQPIVR
			0.002177	50.48	LTEDYWR
		Cry45Aa-like_2	2	99	DPGNIYNYNMK
			2	99	GTTPNPTSQPIVR

Table S2. Peptide summary of three replicates of concentrated supernatants and solubilized proteins from spore/crystal mixtures identified with Protein Pilot v4.5

Sample	Replicate	Homology	Contrib	Conf	Sequence
			2	99	MGLQSFVNVVERPLLGR
			2	99	PIVTDNPTNSAYAK
			2	99	QHNTTLKSSGYLGR
			2	99	SEAGLLFAK
			2	99	SSGYLGRDPGNIYNYNMK
			2	99	VPDPIVTDNPTNSAYAK
			2	99	YNPNPYTAHFTGSGK
			1.920818	99	SGLDIPIFDPVISR
			1.522879	98.27	EYQIPVTGR
			0.946922	93.35	AIIDLLK
			0.882729	99	PGNIYNYNMK
			0.170696	46.24	SSGYLGR
		Cry45Aa-like_3	2	99	GLDIPIFDPVVSR
			2	99	GLDIPIFDPVVSRQ
			2	99	GYTPNPTSQPIVR
			2	99	KYQIPLTGR
			2	99	MGLQSYVDVVETPLSGR
					MTGYNGGVISEILDMQGVANIT
			2	99	YPQFGIAHFTGSGK
			2	99	NVVLECDITGTTTCVYR
			2	99	PVVIDNPTNSALAR
			2	99	SEAGFVFAK
					TITFNETQSDSQSTTTEHGITAG
			2	99	VSATVK
			1.366532	97.35	YQIPLTGR
			1.30103	99	YPGNSVDR
			0.853872	91.07	KYQIPLTGRR
			0.050122	85.77	YTTSNTYTTETSR
		Cry32Ea-like	2	99	AIAEQAR
			2	99	AQAITELQGIQAAIR
			2	99	AVNALFKSDAK
			2	99	AVQAAQSAIQALFTDQNQTR
			2	99	DAACNWEKNPNNPQLK
			2	99	DAACNWEKNPNNPQLKETVR
			2	99	DLIIDGGYQGER
			2	99	EGAGEGYVTISDGTEDNTETVK
			2	99	ETLPVTLPVGSEDR
					ETLPVTLPVGSEDRYLPVGNVW
			2	99	HVTNQLFSSGGASVVTGWLFR
			2	99	FTAGVETPR
			2	99	GFVGNSKELEVFVQR

Table S2. Peptide summary of three replicates of concentrated supernatants and solubilized proteins from spore/crystal mixtures identified with Protein Pilot v4.5

Sample	Replicate	Homology	Contrib	Conf	Sequence
				2	99 GMEPDLLSAIPVFWDIN
				2	99 GMEPDLLSAIPVFWDINSSYN
					GMEPDLLSAIPVFWDINSSYNSS
				2	99 SGLPCR
				2	99 GPGSTGGDLVK
				2	99 GPGSTGGDLVKLPVPEGR
				2	99 GSETSLLTAYVEAATLHLGLLK
				2	99 GYITYGNASVVK
					GYITYGNASVVKGPGSTGGDLV
				2	99 K
				2	99 IDESKLKPYTR
				2	99 IEFIPIEGTLEEYQANQDLEK
					IGTTDGVANIDNLEVIEANPLTG
				2	99 EALAR
				2	99 IHIYNEGGGDLILDK
					IHIYNEGGGDLILDKIEFIPIEGTLE
				2	99 EYQANQDLEK
					IPYVYHPFLQGSLPAVPGETYDIF
				2	99 QQLSNAIGK
				2	99 KAVNALFK
				2	99 KAVNALFKSDAK
				2	99 KEGAGEGYVTISDGTEDN
				2	99 KEGAGEGYVTISDGTEDNTETVK
				2	99 KETLPVTLPVGSEDR
				2	99 LESIELIR
				2	99 LRPDITLNHILHAETLVQK
				2	99 LSYVGLGMLNSDYR
				2	99 LSYVGLGMLNSDYRDFVSSPTR
				2	99 LTQSDMR
				2	99 LVECMSDEIYPQEK
				2	99 LYEVPTKSQLTR
				2	99 MDQYYNNNEYEILDHGR
				2	99 MDQYYNNNEYEILDHGRR
					MDSYVSTSLMTAQNGSPLSGYV
				2	99 TK
				2	99 MMTAAEQITNK
				2	99 NGDFSAGLSNWQGAEGALVQK
				2	99 NLGIWVGFK
				2	99 NMNVPYGIPPTNPCDRDYQR

Table S2. Peptide summary of three replicates of concentrated supernatants and solubilized proteins from spore/crystal mixtures identified with Protein Pilot v4.5

Sample	Replicate	Homology	Contrib	Conf	Sequence
				2	99 NMTMAVLDIVAVWPTFDIK
				2	99 NMTNQPSEVSGTNAYPGNNNR
				2	99 NTLQLNVTDYLVDQAAK
					NVLRNGDFSAGLSNWQGAEGA
				2	99 LVQK
				2	99 PGTPQAQISWDK
				2	99 PVFWDINSSYNSSSGLPCR
				2	99 SPIQEGYK
				2	99 SPIQEGYKER
					SPIQEGYKERNMTNQPSEVSGT
				2	99 NAYPGNNNR
				2	99 SSYNSSSGLPCR
				2	99 TAALGIPFTHPFNDTSLIK
				2	99 TGLQQQYNTGDWNK
				2	99 TQFVATNTILYNR
					TVYTPLEGNVGGNWLSPEPVPSI
				2	99 DVIEGNIVDTPGLFK
					TYPVLTNQTIPQDMACDSCDAG
				2	99 TVMR
					VTARKEGAGEGYVTISDGTEDN
				2	99 TETVK
				2	99 VTDYLVDQAAK
				2	99 WLHNISFEAIDGGVNTSVIK
				2	99 WQGAEGALVQK
				2	99 WYNTGLQQQYNTGDWNK
					YAAYIGYGWTHVSADENNQIYP
				2	99 NMITQIPAVK
				2	99 YASNADVNVFCTLQK
				2	99 YDKEIHK
					YDKEIHKNMNVPYGIPPTNPCD
				2	99 RDYQR
				2	99 YGDFDSDNWSGENGWK
					YLHMPGANIPQFSDTVFPTYAY
				2	99 QK
					YLPVGNVWHVTNQLFSSGGAS
				2	99 VVTGWLFR
				2	99 YPLAQAPGSVLQNMDYK
					YPLAQAPGSVLQNMDYKELMN
				2	99 K
			1.958608		98.88 AKQTLVK
			1.920818		99 AVNALFK
			1.920818		98.75 ITQIPAVK
			1.795881		98.4 MCLLDLVK

Table S2. Peptide summary of three replicates of concentrated supernatants and solubilized proteins from spore/crystal mixtures identified with Protein Pilot v4.5

Sample	Replicate	Homology	Contrib	Conf	Sequence
			1.508638	96.9	NPNNPQLKETVR
			1.508638	96.9	TSSNVSVR
			1.366532	99	SKELEVFVQR
			1.356547	99	HILHAETLVQK
			1.275724	94.69	NPNNPQLK
			1.244125	98.61	MMTAAEQITNKAIAEQAR
			1.026872	98.91	MITQIPAVK
			0.966576	89.16	LYEVPTK
			0.931814	88.27	DLIIDGGYQGERGTK
			0.806875	84.36	ADDPIFK
					ERNMTNQPSEVSGTNAYPGNN
			0.785156	99	NR
			0.705534	80.29	MPYFSAR
			0.686133	79.42	LYRDAACNWEK
			0.552842	98.11	NEYEILDHGR
			0.536107	70.92	GFVGNSK
			0.531653	70.56	DLIIDGGYQGERGTKK
			0.464706	99	ADDPIFKGR
			0.185752	34.81	LYRDAACNWEKNPNNPQLK
			0.096367	19.92	SLYGQRNVLR
			0.082494	79.28	GPSNNYEASAYR
			0.064997	54.84	SPIQEGYKERN
			0.027797	21.87	DQYYNNNEYEILDHGRR
			0.008774	33.96	LRPDITLN
			0.006123	67.23	LPVPEGR
			0.002614	42.41	IQEGYKER
		Cry32Da-like	2	99	ASGGTDYQVIK
			2	99	ATFDNLEVIEANPLTGEALAR
			2	99	AVNALFTSDAK
			2	99	AVQTAQGAIQALFTDSNQNR
			2	99	DLEVFITR
			2	99	DNATTFVDPK
			2	99	EGSGEGYVTISDGTEDNTETLK
			2	99	FEPVRDNATTFVDPK
			2	99	FTVGEVATR
			2	99	GPGSTGGDLVSLPTYAK
			2	99	GQALIGLK
			2	99	GYETILLPLYAQAANIHLIVLR
			2	99	IDPFDEPIIIDK
			2	99	IEIPLTSSAGQLYTIR
			2	99	INDYQIDYK

Table S2. Peptide summary of three replicates of concentrated supernatants and solubilized proteins from spore/crystal mixtures identified with Protein Pilot v4.5

Sample	Replicate	Homology	Contrib	Conf	Sequence
					IPYVYNAFLQGALPPVPGETSDIF
				2 99	QQLSSAVAIAR
				2 99	IWETNPTPGNTSQLLDAFR
				2 99	KAVNALFTSDAK
				2 99	KEGSGEGYVTISDGTEDNTETLK
				2 99	LVECMSDKIYPQEK
				2 99	NLALDHIDRINDYQIDYK
				2 99	NLLNHGDFEFPDWSGENGWK
					QIGATIFGWTHTSVDPNNTIDTT
				2 99	K
				2 99	QNTFPPIEVPEGTPPPSHR
				2 99	SCQDAMPHLDTR
				2 99	SDPAQNTMSAIEAR
				2 99	TFEIFPETNR
					TLDTNMITALQGLTDEYEYYHIP
				2 99	LDVSTGQK
				2 99	TSNHVSVMSGNPIFK
				2 99	VESIELIR
					YLHMPGADNPQLSDQVYPTYA
				2 99	YQK
				2 99	YSNGEYKDVEAWNLR
				2 99	YTDYDVPSTPYSGGNLTYNAFR
				2 99	YTWVGNSWTK
			1.508638	97.38	VQQTFVK
			1.337242	96.13	TKQWFEPR
			0.899629	89.21	LAQSDMR
			0.764472	85.11	VYTAAVKTEFTR
			0.716699	83.37	NLALDHIDR
			0.402305	64.45	IFPETNR
			0.231362	45.61	YPWVR
			0.221849	78.72	MCLLDQVK
			0.131356	29.57	YTGEEQAGGLIR
			0.077794	49.87	INDYQIDYKNK
			0.033858	89.99	PAQNTMSAIEAR
		Cry32Eb-like		2 99	ALPVVPGETYDIFLQLSDAINIAR
				2 99	ALYDQRNALVNGDFSAR
				2 99	ATSPVSSNVFPTLR
				2 99	DAIQMLFTDSNQNR
				2 99	EAVNTLFLNDALQLK

Table S2. Peptide summary of three replicates of concentrated supernatants and solubilized proteins from spore/crystal mixtures identified with Protein Pilot v4.5

Sample	Replicate	Homology	Contrib	Conf	Sequence
			2	99	EGSGEGYVTISDGTEENTETLK
			2	99	FIADEKATSPVSSNVFPTLR
			2	99	GAVQIAR
			2	99	GFVGNSKDLELLVAR
			2	99	IKGAVQIAR
			2	99	IPYVYNQFLQR
			2	99	ISNHVYVTSDNPIFK
			2	99	KEGSGEGYVTISDGTEENTETLK
			2	99	LDHVEVVEVGPLTGDALTR
			2	99	LVECISDPIHTK
			2	99	MCLLEQVK
			2	99	NALVNGDFSAR
			2	99	NLLHHGDFESPDWSGENR
			2	99	NLLHHGDFESPDWSGENRWK
			2	99	QILEAETWVQK
			2	99	VTDYQVDQAAK
			2	99	YLNMPNANTPQFSDK
			1.21467	95.5	LQSAITLK
			1.207608	95.47	DLELLVAR
			0.449772	94.61	SKDLELLVAR
			0.106238	27.82	MSYASNHYK
			0.066007	90.42	ISNHVYVTSDN
			0.007005	17.97	KEGSGEGYVTISDGTEEN
		Cry73Aa-like	2	99	FGAGWGLPSTEVEDNYTR
			2	99	FGTDPQAQWK
					GPGSTGGDLIQLASGYLQYSFPS
			2	99	TDNR
			2	99	GWTNNSSVDAIESTLVR
			2	99	IRYASTNGSTLTLKK
					ITNTLHYVGSSTTWEQSFSATPI
			2	99	GSIK
			2	99	IYNNPYGVQIQLSR
			2	99	LQARTAEYTNHCTNTYDK
					MNQNCNHNEYEILDSGNVSCQ
			2	99	PK
			2	99	NLYEAETVPIAFR
			2	99	NLYEAETVPIAFRK
			2	99	NQNCNHNEYEILDSGNVSCQPK
			2	99	QAYDLAPNPTDYNK

Table S2. Peptide summary of three replicates of concentrated supernatants and solubilized proteins from spore/crystal mixtures identified with Protein Pilot v4.5

Sample	Replicate	Homology	Contrib	Conf	Sequence
					QAYDLAPNPTDYNKYPYLDPYS
			2	99	KDPIYGK
			2	99	QFMDAVEYLVSQK
			2	99	QFTSTNTVIEYAMPSFR
			2	99	RFGTDPQAQWK
			2	99	SPHLVTELTK
			2	99	SSVDAIESTLVR
			2	99	TAEYTNHCTNTYDK
			2	99	TTQIPAVK
					TVHNVSATDIGNLSLSLGAIPFGF
			2	99	SFYNK
					VGGFEVPLLTTFAQAANLHLLLL
			2	99	R
			2	99	YASTNGSTLTLK
			2	99	YASTNGSTLTLKK
			2	99	YYTAPVDWNLFNDFRR
			1.823909	99	PYSKDPIYGK
			1.30103	95.95	EVYSTVYGR
			1.259637	95.57	LEGVQR
			0.879426	98.37	SLTSTNTIATDK
			0.552842	99	ITNTLHYVGS
			0.530178	74.92	SEAVSRLEGVQR
			0.255707	50	ITDAVRSEAVSR
			0.234331	96.03	TVHNVSATDIGN
			0.071604	18.29	LTFDER
			0.071092	59.16	SLTSTNTIATDKTTQIPAVK
			0.057496	85	SFSATPIGSIK
			0.009217	51.8	PHLVTELTK

Table S3. Result of the Mascot search in the three replicates of the concentrated supernatants and solubilized proteins form spore/crystal mixtures.

Sample	Replicate	Protein	Mass protein (Da)	% mol emPAI	% weight emPAI
E-SE10.2 Supernatant_24h	1	Cry23Aa-like	29,277	1.74	1.26
		Vip2Ac-like_1	51,622	0.07	0.09
		Sip1Aa-like_1	40,792	0.09	0.09
	2	Cry23Aa-like	29,277	1.16	0.86
		Vip2Ac-like_1	51,622	0.05	0.06
		Sip1Aa-like_1	40,792	0.03	0.03
	3	Cry23Aa-like	29,277	0.81	0.60
		Vip2Ac-like_1	51,622	0.02	0.03
		Sip1Aa-like_1	40,792	0.02	0.02
E-SE10.2 Supernatant_48h	1	Cry23Aa-like	29,277	2.66	1.99
		Vip2Ac-like_1	51,622	0.07	0.09
		Sip1Aa-like_1	40,792	0.06	0.06
	2	Cry23Aa-like	29,277	0.77	0.42
		Vip2Ac-like_1	51,622	0.08	0.01
		Sip1Aa-like_1	40,792	0.08	0.01
	3	Cry23Aa-like	29,277	0.42	0.30
		Vip2Ac-like_1	51,622	0.05	0.06
		Sip1Aa-like_1	40,792	0.02	0.02
E-SE10.2 Crystal_72h	1	Cry23Aa-like	29,277	33.84	30.48
	2	Cry23Aa-like	29,277	5.60	4.74
	3	Cry23Aa-like	29,277	2.79	2.49
O-V84.2 Supernatant_24h	1	Vip4Aa-like_1	97,552	5.68E-04	1.08E-05
		Vip4Aa-like_2	87,546	2.60E-04	3.19E-06
		Vip2Ac-like_2	80,797	6.00E-04	1.13E-05
	2	Vip4Aa-like_1	97,552	3.49E-04	8.67E-04
		Vip4Aa-like_2	87,546	8.31E-05	1.85E-04
		Vip2Ac-like_2	80,797	1.41E-04	2.91E-04
		Vip2Ac-like_3	23,282	1.66E-04	9.86E-05
		Sip1Aa-like_2	38,717	9.14E-05	9.02E-05
	3	Vip4Aa-like_1	97,552	6.48E-04	1.61E-03
		Vip4Aa-like_2	87,546	1.08E-04	2.41E-04
		Vip2Ac-like_2	80,797	4.97E-04	1.02E-03
		Sip1Aa-like_2	38,717	2.38E-04	2.34E-04
O-V84.2 Supernatant_48h	1	Vip4Aa-like_1	97,552	7.89E-03	1.96E-02
		Vip4Aa-like_2	80,797	4.47E-03	9.22E-03
	2	Vip4Aa-like_1	97,552	2.68E-03	6.64E-03
		Vip4Aa-like_2	80,797	2.75E-03	5.66E-03
	3	Vip4Aa-like_1	97,552	1.19E-03	2.99E-03
		Vip4Aa-like_2	80,797	2.26E-03	4.68E-03

Table S3. Result of the Mascot search in the three replicates of the concentrated supernatants and solubilized proteins form spore/crystal mixtures.

Sample	Replicate	Protein	Mass protein (Da)	% mol emPAI	% weight emPAI
O-V84.2 Crystal_72h	1	Cry45Aa-like_1	30,698	3.72	2.83
		Cry45Aa-like_2	29,334	4.22	3.06
		Cry45Aa-like_3	25,605	2.98	1.88
		Cry32Ea-like	151,178	6.50	24.30
		Cry32Da-like	153,302	1.61	6.11
		Cry32Eb-like	76,802	3.27	6.20
		Cry73Aa-like	72,173	1.57	2.80
	2	Cry45Aa-like_1	30,698	3.04	2.47
		Cry45Aa-like_2	29,334	2.62	2.03
		Cry45Aa-like_3	25,605	7.82	5.29
		Cry32Ea-like	151,178	6.27	25.07
		Cry32Da-like	153,302	1.33	5.40
		Cry32Eb-like	76,802	2.20	4.46
		Cry73Aa-like	72,173	2.19	4.19
	3	Cry45Aa-like_1	30,698	1.81	2.82
		Cry45Aa-like_2	29,334	4.48	6.27
		Cry45Aa-like_3	25,605	3.34	5.28
		Cry32Ea-like	151,178	6.65	25.89
		Cry32Da-like	153,302	1.17	6.21
		Cry32Eb-like	76,802	2.54	7.23
		Cry73Aa-like	72,173	2.29	6.60

Label free analysis of the concentrated supernatant of the *Bacillus thuringiensis* isolates E-SE10.2 and O-V84.2.

SAMPLE INFORMATION:

Number of sample: 18. Six experimental conditions, three samples in each group.

Bt isolate E-SE10.2 concentrated supernatant at 24 h (E24h) and 48 h (E48h).
Bt isolate O-V84.2 concentrated supernatant at 24 h (O24h) and 48 h (O48h).
*S*olubilized proteins form the crystal of *Bt* isolate E-SE10.2 (EC) and *Bt* isolate O-V84.2 (OC).

SAMPLES ANALISYS:

30 µg of each sample was introduced in one dimension (1D) SDS-PAGE electrophoresis gel without resolving to clean sample for in gel digestion sample preparation.

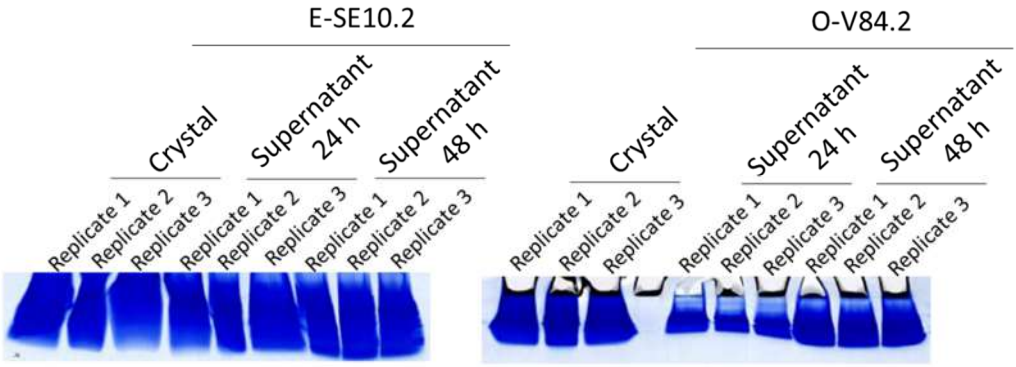

Figure 1. Samples (30 µg of each) loaded into the (1D) SDS-PAGE electrophoresis gel without resolving

IN GEL DIGESTION

The bands were cut out and the samples were digested with sequencing grade trypsin (Promega) as described elsewhere (Shevchenko A, Jensen ON, Podtelejnikov AV, Sagliocco F, Wilm M, Vorm O, Mortensen P, Boucherie H, Mann M. Linking genome and proteome by mass spectrometry: large-scale identification of yeast proteins from two dimensional gels. Proceedings of the National Academy of Sciences of the United States of America 1996; 93:14440-14445). The digestion mixture were dried in a vacuum centrifuge, resuspended in 50 µL of 2% acetonitrile (ACN), 0.1% trifluoroacetic acid (TFA).

LC/MSMS ANALYSIS

5 µl of each sample were loaded onto a trap column (NanoLC Column, 3µ C18-CL, 75 µm x 15 cm; Eksigent) and desalted with 0.1 % TFA at 3µl/min during 5 min. The peptides were loaded onto an analytical column (LC Column, 3 µ C18-CL, 75umx12cm,

Nikkyo) equilibrated in 5 % acetonitrile 0.1 % FA (formic acid). Peptide elution was carried out with a linear gradient of 5 to 35 % B in 45 min (A: 0.1% FA; B: ACN, 0.1% FA) for at a flow rate of 300 nl/min (Figure 1 and 2).

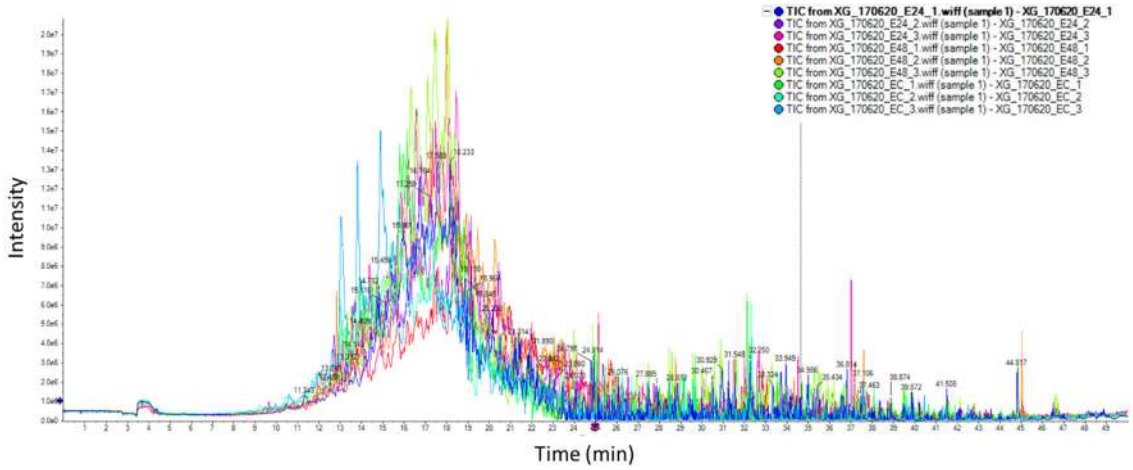

Figure 2. Chromatogram of the concentrated supernatant (24 h and 48 h) and solubilized proteins from the crystal form the *Bacillus thuringiensis* isolate E-SE10.2

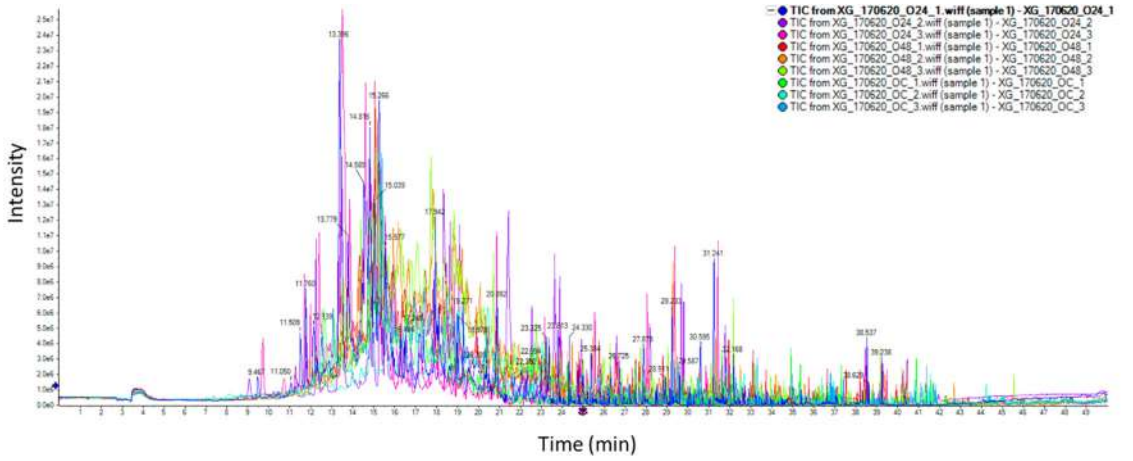

Figure 3. Chromatogram of the concentrated supernatant (24 h and 48 h) and solubilized proteins from the crystal form the *Bacillus thuringiensis* isolate O-V84.2

Peptides were analysed in a mass spectrometer nanoESI qQTOF (5600 TripleTOF, ABSCIEX). Eluted peptides were ionized applying 2.8 kV to the spray emitter. Analysis were carried out in a data-dependent mode (DDA). Survey MS1 scans were acquired from 350–1250 m/z for 250 ms. The quadrupole resolution was set to 'UNIT' for MS2 experiments, which were acquired 100–1500 m/z for 50 ms in 'high sensitivity' mode. Following switch criteria were used: charge: 2+ to 5+; minimum intensity; 70 counts per second (cps). Up to 25 ions were selected for fragmentation after each survey scan. Dynamic exclusion was set to 15 s.

The collision energy was automatically selected by the instrument according to the following equations: |CE| = (slope) x (m/z) + (intercept).

Table 1. Parameters used for the calculation of the collision energy of the concentrated supernatant and solubilized crystal proteins from the *Bt* isolates E-SE10.2 and O-V84.2.

Charge	Slope	Intercept
Unknown	0.0575	9
1	0.0575	9
2	0.0625	-3
3	0.0625	-3
4	0.0625	-6
5	0.0625	-6

DATA ANALYSIS

The MS/MS information (TripleTof wiff files) were analyzed with Protein Pilot v 4.5 (ABSciex). ProteinPilot default parameters were used to generate peak list directly from 5600 TripleTof wiff files. The Paragon algorithm of ProteinPilot was used to search in a homemade *Bacillus thuringiensis* protein database, called *Bt_combined* (https://sourceforge.net/projects/bt-combined/files/Bt_combined/) with the following parameters: trypsin specificity, cys-alkylation, no taxonomy restriction, and the search effort through. To avoid using the same spectral evidence in more than one protein, the identified proteins are grouped based on MS/MS spectra by the Protein-Pilot Progroup algorithm. Thus, proteins sharing MS/MS spectra are grouped, regardless of the peptide sequence assigned. The protein within each group that can explain more spectral data with confidence is shown as the primary protein of the group. Only the proteins of the group for which there is individual evidence (unique peptides with enough confidence) are also listed, usually toward the end of the protein list.
For protein library construction a joint search were performed with all samples for global analysis and with the supernatant at 24 h and 48 h for the *Bt* isolates E-SE10.2 and O-V84.2.

RESULT INTERPRETATION

The MSMS output files (wiff files) obtained from the 5600 TripleTof were analysed by Peak View 1.1 with proteins identified by Unused ≥1.3, confidence > 95% with maximum 50 peptides for protein. The quantitative data obtained by Peak View were visualized with Marker View 1.3

1. - GLOBAL ANALYSIS:

A joint search with the *Bt_combined* protein database was performed with the three replicates of the concentrated supernatant (24h and 48h) and solubilized crystal proteins. The search in the respective analysis were done with the following parameters: trypsin specificity, cys-alkylation, and the search effort set to through.

The joined search with all the samples identified 1816 proteins and the quantitative data obtained was analyzed with Marker View 1.3.

1.1. - GROUPED DATA ANALYSIS

A PCA analysis was done with the non-normalized area of the peaks (Figure 4) and with the area peaks corrected by the total areas sum (Figure 5).

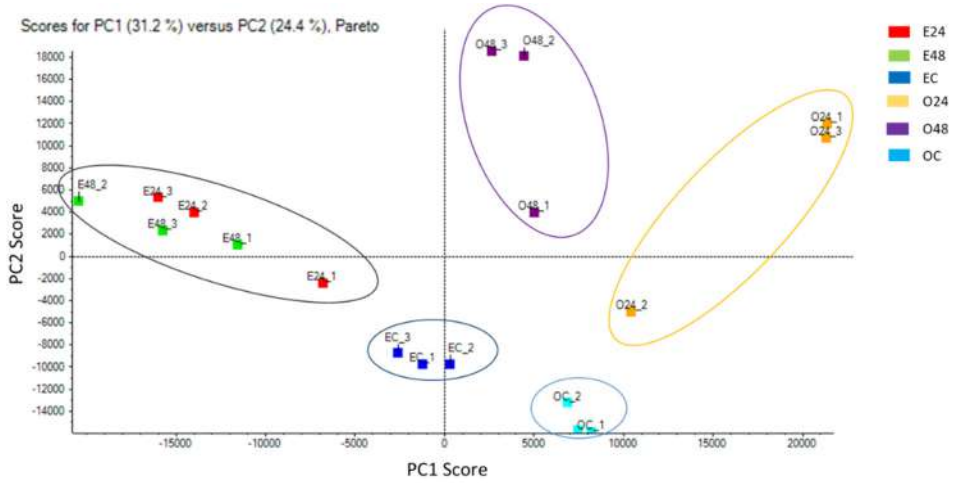

Figure 4. PCA analysis with the non-normalized area of the concentrated supernatant (24 h and 48 h) and solubilized crystal proteins from the *Bt* isolates E-SE10.2 and O-V84.2.

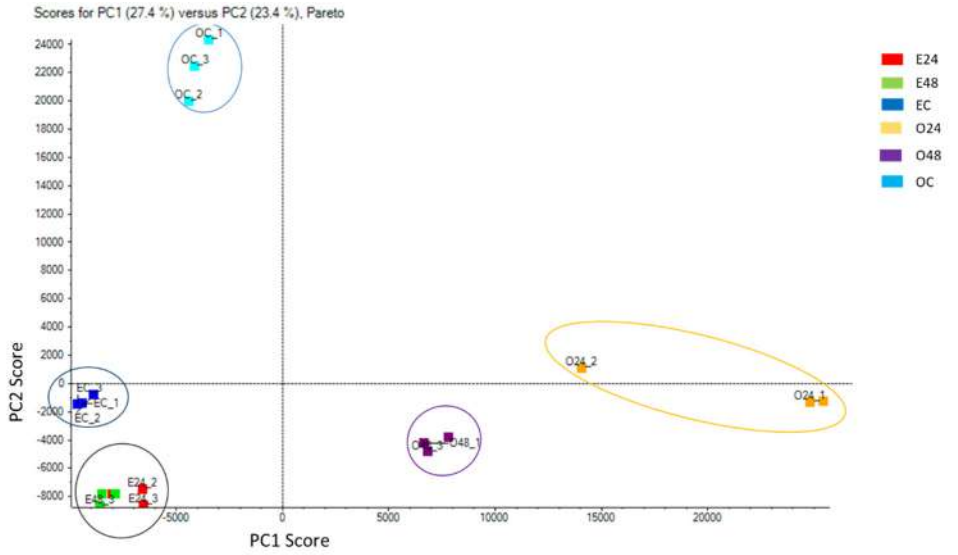

Figure 5. PCA analysis with the area peaks corrected by the total areas sum of the concentrated supernatant (24 h and 48 h) and solubilized crystal proteins from the *Bt* isolates E-SE10.2 and O-V84.2.

To evaluate the samples distribution inside of each grouped data, a PCA analysis was done with the distribution of the peaks area corrected by the total areas sum.

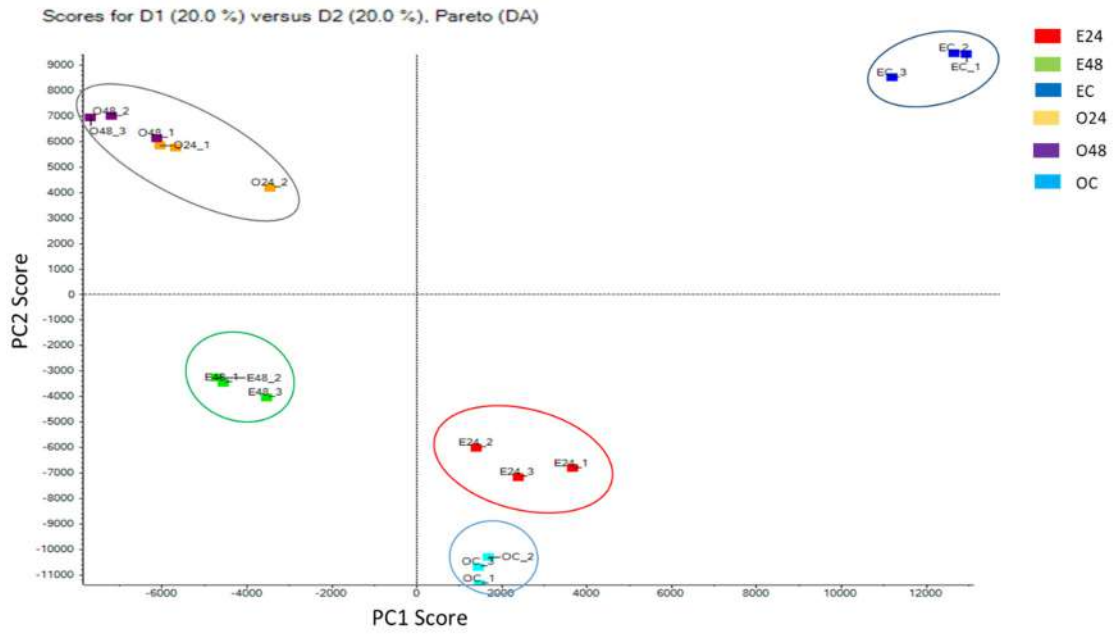

Figure 6. PCA analysis with the distribution of the peaks area corrected by the total areas sum of the concentrated supernatant (24 h and 48 h) and solubilized crystal proteins from the *Bt* isolates E-SE10.2 and O-V84.2.

RESULT

The data obtained from the global analysis of the concentrated supernatant (24 h and 48 h) and solubilized proteins form the crystal indicate the follow results:

1. The solubilized crystal of the *Bt* isolates E-SE10.2 and O-V84.2 have been grouped in two clusters (Figure 4 and 5). The distribution of the peaks area of the solubilized proteins from the crystal, suggest that the variability of the protein amount are not statistically significant (Figure 6).
2. The supernatant of the *Bt* isolates E-SE10.2 at 24 h and 48 h have been grouped in the same cluster, suggesting that the protein composition of the supernatant is similar at 24 h and 48 h (Figure 4 and 5). The distribution of the peaks area of the supernatant at 24 h and 48 h, suggest that the variability of the protein amount between 24 h and 48 h are statistically significant (Figure 6).
3. The supernatant of the *Bt* isolates O-V84.2 at 24 h and 48 h have been grouped in two cluster, suggesting that the protein composition of the supernatant between the 24 h and 48 h statistically different (Figure 4 and 5). The distribution of the peaks area of the supernatant at 24 h and 48 h, suggest that the variability between 24 h and 48 h are not statistically significant (Figure 6).

2. - SPECIFIC ANALYSIS:

A joint search with the *Bt_combined* protein database was performed with the three replicates of the concentrated supernatant of the *Bt* isolate at 24h and 48h. The search in the respective analysis were done with the following parameters: trypsin specificity, cys-alkylation, and the search effort set to through.

The joined search with the supernatant at 24 h and 48 h identified 875 proteins and the quantitative data obtained was analyzed with Marker View 1.3.

2.1. - GROUPED DATA ANALYSIS E-SE10.2

A PCA analysis was done with the non-normalized area of the peaks (Figure 7) and with the area peaks corrected by the total areas sum (Figure 8) of the concentrated supernatant at 24 h and 48 h.

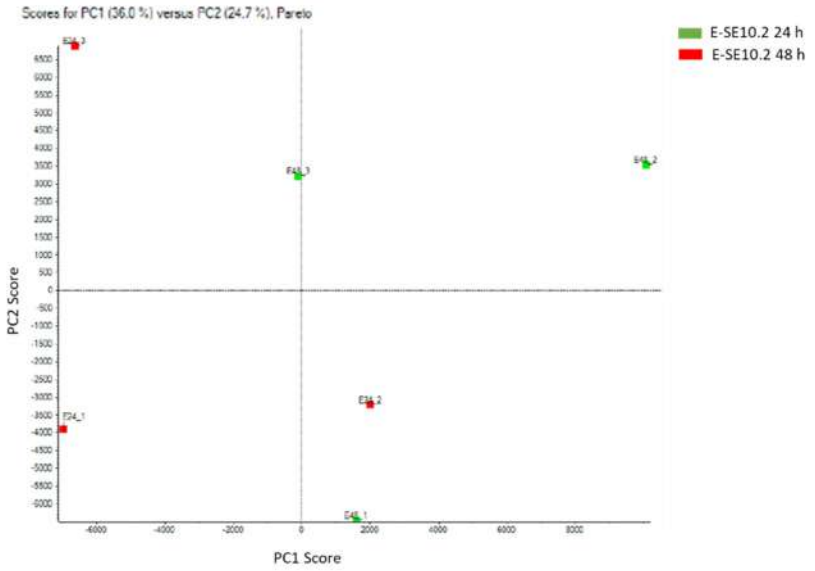

Figure 7. PCA analysis with the non-normalized area of the concentrated supernatant of the *Bt* isolate 24 h and 48 h.

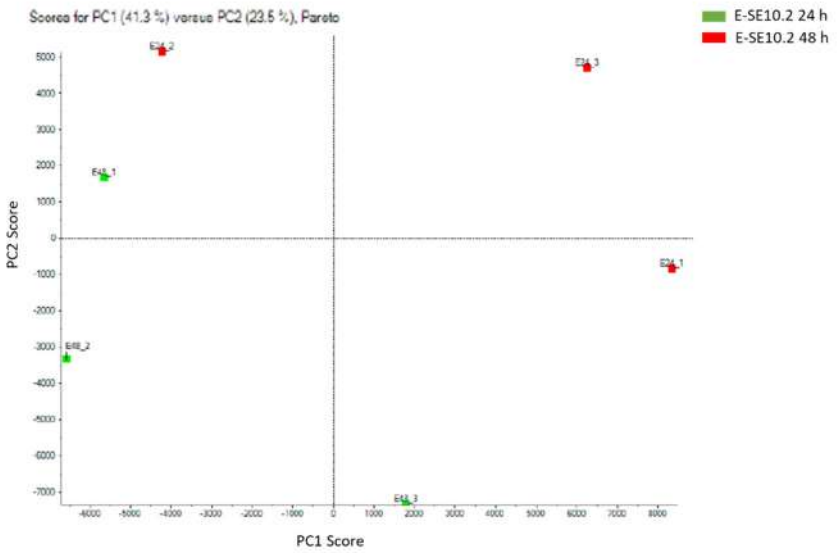

Figure 8. PCA analysis with the area peaks corrected by the total areas sum of the concentrated supernatant 24 h and 48 h of the *Bt* isolate E-SE10.2

2.1. - DIFFERENTAL EXPRESION ANALYSIS E-SE10.2

A Student t-test analysis was performed with the supernatant of the *Bt* isolate E-SE10.2 at 24 h and 48 h in the Marker View 1.3. As a result, 39 proteins were differential expressed between two groups, 11 up- regulated (red) and 28 down-regulated (green).

Specific proteins of the Bt isolate E-SE10.2

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
Alcohol dehydrogenase (EC 1.1.1.1)	1.10E-04	6.00E+05	1.84E+05	2.33E+04	1.18E+04	3.26E+00	5.13E-01
Multimodular transpeptidase-transglycosylase (EC 2.4.1.129) (EC 3.4.-.-)	3.00E-04	2.71E+04	6.16E+04	3.73E+03	3.16E+03	4.40E-01	-3.57E-01
beta-lactamase II	1.11E-03	5.78E+04	2.12E+04	2.81E+03	4.87E+03	2.72E+00	4.35E-01
Siderophore biosynthesis non-ribosomal peptide synthetase modules @ Bacillibactin synthetase component F (EC 2.7.7.-)	2.25E-03	1.18E+05	2.83E+05	2.85E+04	2.97E+04	4.16E-01	-3.81E-01
Succinate dehydrogenase flavoprotein subunit (EC 1.3.99.1)	2.86E-03	8.52E+04	3.34E+05	3.93E+04	4.93E+04	2.55E-01	-5.93E-01
Malonyl CoA-acyl carrier protein transacylase (EC 2.3.1.39)	3.72E-03	6.72E+04	1.37E+05	1.42E+04	9.00E+03	4.92E-01	-3.08E-01
3-deoxy-manno-octulosonate cytidylyltransferase (EC 2.7.7.38)	3.74E-03	2.29E+04	1.30E+04	7.52E+02	1.70E+03	1.76E+00	2.46E-01
putative cytochrome P450 hydroxylase	4.23E-03	7.31E+04	2.08E+05	2.23E+04	2.98E+04	3.51E-01	-4.54E-01
FIG01226668: hypothetical protein	6.40E-03	9.66E+04	1.79E+05	1.93E+04	1.95E+04	5.38E-01	-2.69E-01
Isocitrate lyase (EC 4.1.3.1)	8.10E-03	2.06E+05	1.39E+05	1.78E+04	1.48E+04	1.48E+00	1.70E-01
S-layer protein, putative	1.02E-02	6.20E+04	1.84E+05	2.99E+04	1.25E+04	3.36E-01	-4.74E-01

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
Translation elongation factor Tu	1.77E-02	5.12E+05	1.27E+06	1.37E+05	2.49E+05	4.04E-01	-3.94E-01
Possible response regulator aspartate phosphatase	1.84E-02	1.91E+05	3.65E+05	4.76E+04	5.96E+04	5.23E-01	-2.81E-01
spore coat polysaccharide synthesis	2.03E-02	7.44E+04	2.99E+04	1.59E+04	1.15E+04	2.49E+00	3.96E-01
Dihydrolipoamide acetyltransferase component (E2) of acetoin dehydrogenase complex (EC 2.3.1.-)	2.79E-02	1.55E+05	1.15E+06	5.99E+04	3.13E+05	1.35E-01	-8.70E-01
N-acetyltransferase family protein	3.08E-02	2.49E+05	6.20E+05	1.15E+05	1.51E+05	4.02E-01	-3.96E-01
Exosporium protein K	3.32E-02	2.03E+04	5.53E+04	5.83E+03	1.38E+04	3.66E-01	-4.36E-01
polyketide synthase type I	3.53E-02	1.09E+05	1.73E+05	2.02E+04	2.71E+04	6.33E-01	-1.98E-01
Possible response regulator aspartate phosphatase	3.56E-02	6.74E+04	2.18E+05	1.61E+04	5.65E+04	3.09E-01	-5.10E-01
NADP-dependent malic enzyme (EC 1.1.1.40)	3.74E-02	5.38E+05	3.21E+05	8.14E+03	7.61E+04	1.67E+00	2.24E-01
FIG01234973: hypothetical protein	3.75E-02	5.98E+05	1.64E+05	1.75E+05	6.40E+04	3.64E+00	5.61E-01
Pyruvate formate-lyase (EC 2.3.1.54)	3.82E-02	5.14E+05	1.94E+05	1.13E+05	1.09E+04	2.64E+00	4.22E-01
O-antigen biosynthesis protein RfbC	3.93E-02	1.20E+05	3.42E+04	3.57E+04	1.39E+04	3.51E+00	5.45E-01
L-serine dehydratase, beta subunit (EC 4.3.1.17)	4.28E-02	1.05E+04	2.20E+04	5.28E+03	3.45E+03	4.79E-01	-3.20E-01
Beta-lysine acetyltransferase (EC 2.3.1.-)	4.28E-02	1.17E+05	1.72E+05	7.54E+03	2.30E+04	6.79E-01	-1.68E-01
hypothetical protein	4.31E-02	1.83E+04	4.45E+04	9.70E+03	1.18E+04	4.11E-01	-3.86E-01

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
zwittermicin A resistance protein	4.32E-02	5.35E+04	1.46E+05	3.17E+04	4.24E+04	3.67E-01	-4.35E-01
Topoisomerase IV subunit A (EC 5.99.1.-)	4.37E-02	4.13E+04	2.18E+04	3.47E+03	8.55E+03	1.89E+00	2.77E-01
PTS system, diacetylchitobiose-specific IIC component (EC 2.7.1.69)	4.73E-02	1.51E+04	5.37E+04	3.99E+03	1.62E+04	2.82E-01	-5.50E-01
FIG01227334: hypothetical protein	4.75E-02	1.37E+03	8.22E+03	1.24E+03	3.12E+03	1.66E-01	-7.80E-01
Porphobilinogen synthase (EC 4.2.1.24)	4.94E-02	1.50E+04	3.72E+04	9.50E+03	2.20E+03	4.02E-01	-3.96E-01
Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein OppA (TC 3.A.1.5.1)	5.03E-02	6.17E+06	3.46E+06	1.31E+06	1.01E+06	1.78E+00	2.51E-01
Unspecified monosaccharide ABC transport system, ATP-binding protein	5.34E-02	3.52E+04	8.19E+04	2.24E+04	9.01E+03	4.30E-01	-3.66E-01
Flagellin protein FlaA	5.52E-02	1.75E+07	8.16E+06	4.33E+06	1.22E+06	2.15E+00	3.32E-01
Butyryl-CoA dehydrogenase (EC 1.3.99.2)	5.53E-02	3.70E+04	1.68E+05	1.00E+04	5.77E+04	2.20E-01	-6.57E-01
Pyrrolidone-carboxylate peptidase (EC 3.4.19.3)	5.78E-02	2.95E+04	6.74E+04	1.36E+04	1.95E+04	4.39E-01	-3.58E-01
Putative stomatin/prohibitin-family membrane protease subunit YbbK	6.09E-02	1.17E+05	2.86E+05	8.78E+03	7.71E+04	4.08E-01	-3.90E-01
Homoserine O-acetyltransferase (EC 2.3.1.31)	6.21E-02	3.81E+04	8.27E+04	1.17E+04	2.35E+04	4.61E-01	-3.37E-01
FIG01226051: hypothetical protein	6.29E-02	8.65E+04	1.67E+05	2.59E+04	4.30E+04	5.19E-01	-2.85E-01

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
FIG01116163: hypothetical protein	6.46E-02	1.35E+04	4.75E+04	6.83E+03	1.77E+04	2.83E-01	-5.48E-01
4-hydroxybenzoyl-CoA thioesterase family active site	6.50E-02	1.01E+05	1.95E+05	1.71E+04	4.83E+04	5.18E-01	-2.86E-01
D-alanyl-D-alanine carboxypeptidase (EC 3.4.16.4)	6.50E-02	2.68E+05	1.52E+05	5.11E+04	6.01E+04	1.76E+00	2.46E-01
Polypeptide composition of the spore coat protein CotJC	6.59E-02	4.29E+03	1.19E+04	1.81E+03	4.08E+03	3.59E-01	-4.44E-01
hypothetical protein	6.75E-02	3.03E+04	1.31E+04	9.27E+03	7.10E+03	2.31E+00	3.63E-01
hypothetical protein	6.75E-02	2.21E+05	7.43E+04	7.25E+04	7.21E+04	2.98E+00	4.74E-01
hypothetical protein	6.81E-02	1.15E+03	4.67E+03	1.39E+03	1.93E+03	2.46E-01	-6.09E-01
FIG01227037: hypothetical protein	6.84E-02	8.21E+04	3.45E+05	2.08E+04	1.29E+05	2.38E-01	-6.23E-01
acetyltransferase, GNAT family	7.15E-02	1.32E+04	3.08E+04	4.82E+03	9.84E+03	4.28E-01	-3.68E-01
Thioredoxin reductase (EC 1.8.1.9)	7.58E-02	1.48E+05	2.79E+05	2.95E+04	7.37E+04	5.31E-01	-2.75E-01
Diaminohydroxyphosphoribosylaminopyrimidine deaminase (EC 3.5.4.26) / 5-amino-6-(5-phosphoribosylamino)uracil reductase (EC 1.1.1.193)	7.65E-02	1.34E+04	4.96E+03	4.43E+03	4.27E+03	2.70E+00	4.31E-01
FIG01115625: hypothetical protein	7.72E-02	2.18E+04	3.38E+04	6.53E+03	1.77E+03	6.45E-01	-1.91E-01
6-phosphogluconolactonase (EC 3.1.1.31)	7.78E-02	1.38E+04	4.89E+03	5.11E+03	3.87E+03	2.83E+00	4.51E-01
Heat shock protein 60 family co-chaperone GroES	8.17E-02	1.22E+05	2.87E+04	5.46E+04	2.34E+04	4.24E+00	6.27E-01

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex (EC 2.3.1.12)	8.20E-02	5.75E+05	1.53E+06	8.77E+04	5.19E+05	3.76E-01	-4.25E-01
hypothetical protein	8.22E-02	2.26E+03	9.79E+03	1.04E+03	4.20E+03	2.30E-01	-6.37E-01
Methyltransferase (EC 2.1.1.-)	8.29E-02	9.69E+04	2.29E+04	3.99E+04	4.51E+03	4.24E+00	6.27E-01
Pyruvate dehydrogenase E1 component beta subunit (EC 1.2.4.1)	8.36E-02	1.37E+05	3.17E+05	5.27E+04	1.08E+05	4.31E-01	-3.66E-01
Spore coat protein B	8.36E-02	3.25E+03	1.09E+04	5.71E+02	4.15E+03	2.99E-01	-5.24E-01
endonuclease/exonuclease/phosphatase family	8.39E-02	5.23E+04	1.83E+04	2.00E+04	7.61E+03	2.86E+00	4.56E-01
Chitin binding protein	8.41E-02	1.01E+06	3.35E+05	3.96E+05	1.54E+05	3.01E+00	4.78E-01
hypothetical protein	8.56E-02	1.00E+05	4.50E+05	4.14E+04	1.97E+05	2.22E-01	-6.53E-01
FIG01227780: hypothetical protein	8.62E-02	1.06E+05	6.00E+04	2.61E+04	5.68E+03	1.77E+00	2.47E-01
Glycine dehydrogenase [decarboxylating] (glycine cleavage system P1 protein) (EC 1.4.4.2)	8.79E-02	1.94E+05	1.36E+05	3.58E+04	2.32E+04	1.43E+00	1.55E-01
Dihydrolipoamide dehydrogenase of branched-chain alpha-keto acid dehydrogenase (EC 1.8.1.4)	8.92E-02	3.98E+05	4.93E+05	4.27E+04	5.78E+04	8.07E-01	-9.31E-02
Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein OppA (TC 3.A.1.5.1)	9.34E-02	4.90E+05	3.01E+05	1.09E+05	1.01E+05	1.63E+00	2.11E-01

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
Tellurium resistance protein TerD	9.38E-02	6.82E+04	1.34E+05	3.99E+04	1.15E+04	5.09E-01	-2.94E-01
SSU ribosomal protein S11p (S14e)	9.44E-02	1.63E+05	8.02E+04	2.28E+04	5.21E+04	2.03E+00	3.08E-01
Asparagine synthetase [glutamine-hydrolyzing] (EC 6.3.5.4)	9.64E-02	1.79E+04	5.09E+04	8.80E+03	2.09E+04	3.52E-01	-4.53E-01
Phosphonate ABC transporter phosphate-binding periplasmic component (TC 3.A.1.9.1)	9.68E-02	1.40E+03	4.93E+02	5.81E+02	3.75E+02	2.84E+00	4.53E-01
UDP-N-acetylglucosamine 4,6-dehydratase (EC 4.2.1.-)	9.72E-02	1.71E+04	8.10E+03	5.64E+03	2.05E+03	2.11E+00	3.23E-01
DNA-binding response regulator	1.02E-01	2.43E+05	1.46E+05	6.41E+04	2.97E+04	1.67E+00	2.23E-01
Imidazolonepropionase (EC 3.5.2.7)	1.06E-01	5.25E+05	2.37E+05	1.92E+05	1.17E+05	2.21E+00	3.45E-01
FIG01225201: hypothetical protein	1.07E-01	1.13E+03	4.39E+03	4.58E+02	2.08E+03	2.58E-01	-5.88E-01
Homoserine dehydrogenase (EC 1.1.1.3)	1.08E-01	6.87E+05	1.11E+05	3.60E+05	3.95E+04	6.18E+00	7.91E-01
SSU ribosomal protein S13p (S18e)	1.09E-01	1.50E+05	1.00E+05	2.06E+04	3.37E+04	1.50E+00	1.76E-01
DNA polymerase X family	1.12E-01	5.20E+04	8.70E+04	1.55E+04	2.39E+04	5.97E-01	-2.24E-01
Alanyl-tRNA synthetase (EC 6.1.1.7)	1.12E-01	1.60E+05	1.26E+05	2.36E+04	1.61E+04	1.28E+00	1.06E-01
LSU ribosomal protein L7/L12 (P1/P2)	1.12E-01	1.29E+05	9.43E+04	9.71E+03	2.38E+04	1.37E+00	1.37E-01

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein OppA (TC 3.A.1.5.1)	1.15E-01	2.56E+04	1.20E+04	4.49E+03	9.46E+03	2.13E+00	3.28E-01
Non-specific DNA-binding protein Dps / Iron-binding ferritin-like antioxidant protein / Ferroxidase (EC 1.16.3.1)	1.16E-01	1.09E+06	1.59E+06	3.52E+05	1.41E+05	6.82E-01	-1.66E-01
Exodeoxyribonuclease III (EC 3.1.11.2)	1.17E-01	9.52E+03	4.12E+04	6.42E+03	2.17E+04	2.31E-01	-6.36E-01
LSU ribosomal protein L13p (L13Ae)	1.18E-01	8.82E+04	1.53E+05	2.47E+04	4.60E+04	5.76E-01	-2.40E-01
CGEA protein	1.19E-01	9.40E+03	5.93E+04	2.03E+03	3.29E+04	1.58E-01	-8.00E-01
Methylcrotonyl-CoA carboxylase carboxyl transferase subunit (EC 6.4.1.4)	1.20E-01	3.50E+05	1.48E+05	1.42E+05	9.63E+04	2.36E+00	3.74E-01
DNA topoisomerase I (EC 5.99.1.2)	1.24E-01	4.16E+04	1.20E+05	1.04E+04	5.42E+04	3.46E-01	-4.60E-01
Protein-export membrane protein SecD (TC 3.A.5.1.1) / Protein-export membrane protein SecF (TC 3.A.5.1.1)	1.25E-01	2.66E+04	1.00E+05	2.30E+04	5.33E+04	2.66E-01	-5.76E-01
Glucose 1-dehydrogenase (EC 1.1.1.47)	1.27E-01	8.77E+04	4.84E+04	1.25E+04	2.87E+04	1.81E+00	2.59E-01
Collagen adhesion protein	1.28E-01	4.24E+05	1.60E+05	1.91E+05	6.25E+04	2.66E+00	4.24E-01
Malonyl CoA-acyl carrier protein transacylase (EC 2.3.1.39)	1.28E-01	1.25E+05	2.24E+05	6.15E+04	6.49E+04	5.59E-01	-2.52E-01

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
Lipoteichoic acid synthase LtaS Type Ia	1.30E-01	5.44E+04	8.24E+04	6.75E+03	2.04E+04	6.61E-01	-1.80E-01
cytosolic long-chain acyl-CoA thioester hydrolase family protein	1.31E-01	3.40E+04	5.76E+04	1.60E+04	1.45E+04	5.90E-01	-2.29E-01
NADH-dependent butanol dehydrogenase A (EC 1.1.1.-)	1.32E-01	4.07E+04	5.04E+04	2.45E+03	7.12E+03	8.08E-01	-9.25E-02
Thioredoxin reductase (EC 1.8.1.9)	1.42E-01	1.41E+05	2.18E+05	5.89E+04	3.90E+04	6.46E-01	-1.90E-01
Dihydroxyacetone kinase, ATP-dependent (EC 2.7.1.29)	1.45E-01	1.42E+05	2.31E+05	4.54E+04	6.86E+04	6.15E-01	-2.11E-01
Asparagine synthetase [glutamine-hydrolyzing] (EC 6.3.5.4) AsnH	1.45E-01	6.75E+03	3.68E+03	2.38E+03	1.53E+03	1.84E+00	2.64E-01
Tellurium resistance protein TerD	1.46E-01	2.29E+04	4.20E+04	9.67E+03	1.48E+04	5.44E-01	-2.64E-01
hypothetical protein	1.49E-01	2.68E+03	8.45E+03	1.53E+03	4.55E+03	3.17E-01	-4.99E-01
Flagellar hook-associated protein FliD	1.50E-01	7.90E+04	3.32E+04	3.51E+04	4.50E+03	2.38E+00	3.77E-01
CTP synthase (EC 6.3.4.2)	1.53E-01	5.21E+05	2.91E+05	1.84E+05	1.16E+05	1.79E+00	2.53E-01
hypothetical protein	1.55E-01	2.04E+05	2.57E+05	3.04E+04	4.19E+04	7.92E-01	-1.01E-01
Aminopeptidase YpdF (MP-, MA-, MS-, AP-, NP- specific)	1.58E-01	7.67E+04	1.24E+05	3.74E+04	2.67E+04	6.20E-01	-2.08E-01
Hypothetical protein perhaps functionally coupled to transcription elongation factor GreA	1.62E-01	1.28E+04	1.84E+04	1.81E+03	4.70E+03	6.94E-01	-1.59E-01
Methylthioribose-1-phosphate isomerase (EC 5.3.1.23)	1.63E-01	3.03E+04	5.95E+03	1.96E+04	2.33E+03	5.09E+00	7.06E-01
DegV family protein	1.65E-01	1.47E+04	7.32E+03	5.55E+03	5.11E+03	2.01E+00	3.04E-01

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
Glycerate kinase (EC 2.7.1.31)	1.65E-01	7.21E+04	1.88E+04	4.31E+04	2.94E+03	3.83E+00	5.83E-01
Hydroxyethylthiazole kinase (EC 2.7.1.50)	1.67E-01	6.51E+03	3.07E+04	2.10E+03	1.97E+04	2.12E-01	-6.73E-01
ATP synthase F0 sector subunit b	1.67E-01	1.51E+05	4.63E+05	3.72E+04	2.56E+05	3.27E-01	-4.86E-01
NLP/P60 family protein	1.69E-01	2.86E+05	4.23E+05	7.80E+04	1.13E+05	6.77E-01	-1.69E-01
Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein OppA (TC 3.A.1.5.1)	1.69E-01	1.34E+06	7.43E+05	4.18E+05	4.55E+05	1.81E+00	2.57E-01
Penicillin-binding protein	1.71E-01	3.71E+04	2.03E+04	4.02E+03	1.43E+04	1.83E+00	2.62E-01
Two-component response regulator SA14-24	1.71E-01	4.75E+05	3.34E+05	1.19E+05	2.01E+04	1.42E+00	1.54E-01
Isovaleryl-CoA dehydrogenase (EC 1.3.8.4)	1.71E-01	4.27E+05	2.26E+05	1.72E+05	9.43E+04	1.89E+00	2.77E-01
Valyl-tRNA synthetase (EC 6.1.1.9)	1.76E-01	8.27E+05	1.04E+06	1.08E+05	1.86E+05	7.94E-01	-1.00E-01
Acetoin dehydrogenase E1 component alpha-subunit (EC 1.2.4.-)	1.77E-01	3.76E+04	7.97E+04	3.08E+04	3.22E+04	4.72E-01	-3.26E-01
Isoleucyl-tRNA synthetase (EC 6.1.1.5)	1.77E-01	1.63E+04	2.99E+04	6.10E+03	1.19E+04	5.45E-01	-2.64E-01
polyhydroxyalkanoate synthesis repressor PhaR	1.80E-01	3.38E+03	1.18E+04	1.97E+03	7.36E+03	2.86E-01	-5.43E-01
Hydroxyacylglutathione hydrolase (EC 3.1.2.6)	1.82E-01	2.80E+04	4.62E+04	1.33E+04	1.43E+04	6.05E-01	-2.18E-01
hypothetical protein	1.82E-01	4.75E+04	2.37E+04	1.96E+04	1.62E+04	2.01E+00	3.03E-01
Phage N-acetylmuramoyl-L-alanine amidase (EC 3.5.1.28)	1.82E-01	2.88E+05	1.87E+05	6.31E+04	8.61E+04	1.54E+00	1.88E-01

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
Fe-bacillibactin uptake system FeuA, Fe-bacillibactin binding	1.82E-01	1.51E+05	6.11E+04	7.95E+04	2.09E+04	2.47E+00	3.94E-01
Pyridoxamine 5'-phosphate oxidase (EC 1.4.3.5)	1.85E-01	2.63E+04	5.15E+04	1.10E+04	2.26E+04	5.11E-01	-2.91E-01
N-acetylmuramoyl-L-alanine amidase	1.86E-01	2.21E+05	3.68E+05	3.75E+04	1.32E+05	6.00E-01	-2.22E-01
Protein export cytoplasm protein SecA ATPase RNA helicase (TC 3.A.5.1.1)	1.87E-01	4.95E+04	8.89E+04	2.42E+04	3.43E+04	5.56E-01	-2.55E-01
acetyltransferase, GNAT family	1.87E-01	4.16E+04	2.48E+04	1.45E+04	1.07E+04	1.68E+00	2.26E-01
Arsenic efflux pump protein	1.88E-01	7.24E+03	4.49E+03	2.50E+03	1.21E+03	1.61E+00	2.08E-01
SAM-dependent methyltransferases	1.89E-01	1.23E+05	6.23E+04	5.42E+04	3.36E+04	1.97E+00	2.95E-01
Chitosanase	1.90E-01	6.74E+05	4.63E+05	1.04E+05	1.92E+05	1.46E+00	1.63E-01
hypothetical protein	1.91E-01	3.36E+03	1.22E+04	6.86E+02	7.91E+03	2.74E-01	-5.62E-01
Acetoin dehydrogenase E1 component beta-subunit (EC 1.2.4.-)	1.93E-01	1.05E+05	6.66E+05	8.23E+04	5.08E+05	1.57E-01	-8.04E-01
FIG01245734: hypothetical protein	1.96E-01	8.90E+04	5.75E+04	2.54E+04	2.44E+04	1.55E+00	1.90E-01
dTDP-glucose 4,6-dehydratase (EC 4.2.1.46)	1.97E-01	3.48E+05	2.12E+05	1.05E+05	1.10E+05	1.64E+00	2.15E-01
Late competence protein ComER, proline oxidase (EC 1.5.1.2)	1.98E-01	6.65E+04	1.12E+05	2.21E+04	4.29E+04	5.91E-01	-2.28E-01
SpoIISA like protein	1.99E-01	2.09E+03	1.06E+04	1.47E+03	7.85E+03	1.98E-01	-7.03E-01

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein OppA (TC 3.A.1.5.1)	2.02E-01	5.11E+06	3.16E+06	1.73E+06	1.35E+06	1.62E+00	2.09E-01
FIG002379: metal-dependent hydrolase	2.03E-01	3.27E+04	4.58E+04	7.86E+03	1.21E+04	7.14E-01	-1.46E-01
Streptomycin biosynthesis StrF domain protein	2.05E-01	2.04E+04	9.28E+03	4.71E+03	1.07E+04	2.20E+00	3.42E-01
Phospho-N-acetylmuramoyl-pentapeptide-transferase (EC 2.7.8.13)	2.07E-01	6.70E+04	3.97E+04	1.66E+04	2.55E+04	1.69E+00	2.27E-01
DUF124 domain-containing protein	2.07E-01	3.18E+04	5.00E+04	1.75E+04	9.03E+03	6.35E-01	-1.97E-01
S-adenosylmethionine synthetase (EC 2.5.1.6)	2.07E-01	2.24E+05	1.25E+05	9.53E+04	2.76E+04	1.80E+00	2.54E-01
Spore coat protein B	2.09E-01	2.05E+03	9.18E+02	1.09E+03	2.64E+02	2.23E+00	3.49E-01
N-acetylglucosamine-6-phosphate deacetylase (EC 3.5.1.25)	2.10E-01	8.10E+04	4.31E+04	3.51E+04	2.51E+04	1.88E+00	2.74E-01
FIG01226217: hypothetical protein	2.12E-01	1.69E+05	1.21E+05	3.82E+04	4.06E+04	1.39E+00	1.44E-01
EPSX protein	2.12E-01	7.21E+04	4.30E+04	2.65E+04	2.07E+04	1.68E+00	2.25E-01
Succinate dehydrogenase iron-sulfur protein (EC 1.3.99.1)	2.13E-01	1.44E+04	3.15E+04	8.59E+03	1.68E+04	4.56E-01	-3.41E-01
Phage-related protein	2.16E-01	5.22E+05	4.71E+05	1.49E+04	4.99E+04	1.11E+00	4.43E-02
alkaline serine protease, subtilase family	2.16E-01	4.13E+05	1.65E+05	2.28E+05	1.79E+05	2.50E+00	3.99E-01
LSU ribosomal protein L1p (L10Ae)	2.17E-01	1.28E+05	7.53E+04	4.34E+04	4.50E+04	1.70E+00	2.31E-01
hypothetical protein	2.18E-01	2.97E+05	4.15E+05	9.90E+04	9.89E+04	7.16E-01	-1.45E-01

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
Toxic anion resistance protein TelA	2.19E-01	1.67E+04	3.14E+04	2.69E+03	1.45E+04	5.32E-01	-2.74E-01
S-layer homology domain protein	2.20E-01	3.13E+05	2.14E+05	6.32E+04	9.54E+04	1.46E+00	1.64E-01
FIG01226904: hypothetical protein	2.22E-01	2.68E+03	1.33E+04	2.30E+03	1.07E+04	2.01E-01	-6.96E-01
NADH dehydrogenase (EC 1.6.99.3)	2.23E-01	7.37E+02	3.63E+03	4.11E+02	2.89E+03	2.03E-01	-6.93E-01
Glucosamine--fructose-6-phosphate aminotransferase [isomerizing] (EC 2.6.1.16)	2.23E-01	6.36E+05	4.92E+05	1.19E+05	1.24E+05	1.29E+00	1.11E-01
FIG01225131: hypothetical protein	2.23E-01	2.22E+04	1.11E+04	1.12E+04	3.26E+03	2.00E+00	3.00E-01
Penicillin-binding protein	2.23E-01	6.37E+04	1.07E+05	2.47E+04	4.32E+04	5.95E-01	-2.26E-01
ATP synthase delta chain (EC 3.6.3.14)	2.26E-01	3.36E+04	1.35E+05	2.20E+04	1.03E+05	2.49E-01	-6.04E-01
Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein OppA (TC 3.A.1.5.1)	2.26E-01	4.45E+05	6.96E+05	1.33E+04	2.51E+05	6.40E-01	-1.94E-01
phaP protein	2.26E-01	3.12E+04	5.42E+04	7.00E+03	2.34E+04	5.76E-01	-2.39E-01
hypothetical protein	2.26E-01	4.20E+05	1.53E+05	2.71E+05	5.80E+04	2.75E+00	4.39E-01
Polyribonucleotide nucleotidyltransferase (EC 2.7.7.8)	2.27E-01	9.44E+05	1.27E+06	3.35E+05	1.16E+05	7.42E-01	-1.30E-01
Cysteine synthase (EC 2.5.1.47)	2.27E-01	3.19E+05	4.77E+05	1.36E+05	1.35E+05	6.70E-01	-1.74E-01
hypothetical protein	2.29E-01	4.29E+06	5.15E+05	3.86E+06	8.28E+05	8.33E+00	9.20E-01
Metal-dependent hydrolases of the beta-lactamase superfamily I; PhnP protein	2.33E-01	3.72E+04	4.92E+04	1.01E+04	1.07E+04	7.58E-01	-1.20E-01

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
Dihydrolipoamide acyltransferase component of branched-chain alpha-keto acid dehydrogenase complex (EC 2.3.1.168)	2.33E-01	2.09E+05	1.48E+05	5.92E+04	4.50E+04	1.41E+00	1.50E-01
sensor histidine kinase, putative	2.34E-01	3.74E+04	5.10E+04	1.24E+04	1.13E+04	7.33E-01	-1.35E-01
Putative uncharacterized protein YndJ	2.36E-01	1.92E+06	2.68E+06	3.31E+05	7.99E+05	7.17E-01	-1.45E-01
hypothetical protein	2.37E-01	3.19E+04	1.74E+04	1.35E+04	1.19E+04	1.83E+00	2.63E-01
ABC transporter, ATP-binding protein	2.39E-01	1.06E+07	1.68E+07	3.34E+06	6.48E+06	6.33E-01	-1.99E-01
hypothetical protein	2.39E-01	8.28E+04	1.84E+05	1.06E+04	1.06E+05	4.49E-01	-3.47E-01
ABC transporter, ATP-binding protein	2.41E-01	2.91E+04	3.82E+04	4.90E+03	9.59E+03	7.62E-01	-1.18E-01
Asparagine synthetase [glutamine-hydrolyzing] (EC 6.3.5.4) YisO	2.41E-01	1.66E+05	7.04E+04	1.02E+05	4.56E+04	2.36E+00	3.73E-01
Short-chain dehydrogenase/reductase SDR	2.41E-01	1.81E+05	1.06E+05	4.19E+04	7.95E+04	1.71E+00	2.33E-01
Fructose-bisphosphate aldolase class II (EC 4.1.2.13)	2.43E-01	3.77E+04	1.11E+04	2.86E+04	1.18E+04	3.40E+00	5.31E-01
3-ketoacyl-CoA thiolase [isoleucine degradation] (EC 2.3.1.16)	2.44E-01	7.97E+05	5.55E+05	1.79E+05	2.44E+05	1.44E+00	1.57E-01
Inner spore coat protein D	2.45E-01	2.76E+03	1.26E+04	1.50E+03	1.05E+04	2.20E-01	-6.58E-01
SSU ribosomal protein S10p (S20e)	2.49E-01	1.87E+03	6.56E+03	2.06E+03	5.10E+03	2.86E-01	-5.44E-01
hypothetical protein	2.51E-01	5.67E+05	8.00E+05	2.53E+05	1.28E+05	7.09E-01	-1.50E-01

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
FIG01114171: hypothetical protein	2.54E-01	9.16E+03	2.43E+04	1.04E+04	1.61E+04	3.77E-01	-4.24E-01
Malate dehydrogenase (EC 1.1.1.37)	2.56E-01	5.24E+05	3.16E+05	1.53E+05	2.19E+05	1.66E+00	2.20E-01
Glycerophosphoryl diester phosphodiesterase, periplasmic (EC 3.1.4.46)	2.57E-01	1.77E+05	1.20E+05	4.54E+04	5.89E+04	1.48E+00	1.70E-01
Deblocking aminopeptidase (EC 3.4.11.-)	2.58E-01	5.83E+04	1.48E+05	3.50E+02	9.97E+04	3.93E-01	-4.06E-01
Glutamate-1-semialdehyde aminotransferase (EC 5.4.3.8)	2.58E-01	3.61E+04	1.60E+04	2.08E+04	1.58E+04	2.26E+00	3.55E-01
membrane protein, putative	2.58E-01	2.31E+05	1.00E+05	1.46E+05	3.19E+04	2.30E+00	3.63E-01
Catalase (EC 1.11.1.6)	2.59E-01	1.20E+06	1.03E+06	1.81E+05	1.31E+05	1.17E+00	6.71E-02
Dipicolinate synthase subunit B	2.61E-01	1.48E+05	2.73E+05	4.24E+04	1.41E+05	5.43E-01	-2.65E-01
FIG01235645: hypothetical protein	2.63E-01	4.67E+05	3.13E+05	1.12E+05	1.66E+05	1.49E+00	1.74E-01
Cell envelope-associated transcriptional attenuator LytR-CpsA-Psr, subfamily F2 (as in PMID19099556)	2.63E-01	1.33E+05	6.93E+04	7.02E+04	4.11E+04	1.92E+00	2.83E-01
Manganese-dependent inorganic pyrophosphatase (EC 3.6.1.1)	2.65E-01	6.03E+05	3.63E+05	2.13E+05	2.39E+05	1.66E+00	2.21E-01
FIG01226521: hypothetical protein	2.65E-01	2.88E+03	1.69E+03	7.75E+02	1.32E+03	1.70E+00	2.32E-01
L-serine dehydratase, alpha subunit (EC 4.3.1.17)	2.69E-01	1.60E+06	3.73E+05	1.42E+06	4.21E+05	4.29E+00	6.33E-01
hypothetical protein	2.69E-01	9.97E+04	1.32E+05	2.63E+04	3.37E+04	7.58E-01	-1.21E-01
hypothetical protein	2.71E-01	6.68E+02	2.07E+03	8.37E+02	1.61E+03	3.22E-01	-4.92E-01

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
DNA-binding response regulator	2.73E-01	1.57E+03	2.68E+04	2.32E+03	2.92E+04	5.86E-02	-1.23E+00
hypothetical protein	2.76E-01	2.34E+04	1.49E+04	8.39E+03	8.07E+03	1.57E+00	1.96E-01
hypothetical protein	2.76E-01	8.17E+03	3.63E+03	5.21E+03	2.96E+03	2.25E+00	3.52E-01
hypothetical protein	2.76E-01	3.12E+05	5.69E+05	3.77E+04	3.01E+05	5.48E-01	-2.61E-01
Serine hydroxymethyltransferase (EC 2.1.2.1)	2.77E-01	5.28E+05	3.25E+05	2.40E+05	7.14E+04	1.63E+00	2.11E-01
Purine nucleoside phosphorylase (EC 2.4.2.1); N-Ribosylnicotinamide phosphorylase (EC 2.4.2.1) ## possible	2.79E-01	3.81E+05	2.31E+05	7.58E+04	1.77E+05	1.65E+00	2.17E-01
XpaF1 protein	2.79E-01	5.16E+03	2.45E+04	1.06E+03	2.28E+04	2.10E-01	-6.77E-01
SSU ribosomal protein S18p @ SSU ribosomal protein S18p, zinc-independent	2.80E-01	7.03E+03	4.48E+03	2.98E+03	1.59E+03	1.57E+00	1.96E-01
ATP synthase beta chain (EC 3.6.3.14)	2.83E-01	2.69E+05	4.80E+05	5.61E+04	2.53E+05	5.59E-01	-2.52E-01
FIG01225294: hypothetical protein	2.83E-01	2.47E+05	1.73E+05	6.51E+04	7.83E+04	1.42E+00	1.53E-01
Phage shock protein A	2.84E-01	5.78E+04	1.36E+05	3.99E+04	9.32E+04	4.26E-01	-3.70E-01
LSU ribosomal protein L5p (L11e)	2.88E-01	3.99E+04	2.34E+04	1.93E+04	1.17E+04	1.71E+00	2.32E-01
Preprotein translocase subunit YajC (TC 3.A.5.1.1)	2.89E-01	8.14E+04	2.94E+05	6.66E+04	2.59E+05	2.77E-01	-5.58E-01
Stage V sporulation protein involved in spore cortex synthesis (SpoVR)	2.89E-01	1.24E+05	6.02E+04	7.78E+04	6.21E+03	2.07E+00	3.15E-01
FIG01229848: hypothetical protein	2.92E-01	5.49E+04	1.02E+05	4.25E+04	5.20E+04	5.37E-01	-2.70E-01

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
Manganese superoxide dismutase (EC 1.15.1.1)	2.93E-01	9.67E+05	2.34E+06	1.09E+06	1.59E+06	4.14E-01	-3.83E-01
2-hydroxy-3-oxopropionate reductase (EC 1.1.1.60)	2.94E-01	6.92E+04	1.22E+05	5.00E+04	5.68E+04	5.67E-01	-2.47E-01
Amidohydrolase AmhX	2.94E-01	2.37E+04	3.99E+04	4.94E+03	2.00E+04	5.94E-01	-2.26E-01
Glycosyl transferase, group 2 family protein	2.94E-01	3.84E+04	2.60E+04	1.53E+04	4.67E+03	1.48E+00	1.69E-01
Transcriptional regulator, IclR family	2.94E-01	2.78E+04	3.62E+04	5.66E+03	1.02E+04	7.67E-01	-1.15E-01
Secreted and spore coat-associated protein 1, similar to biofilm matrix component TasA and to camelysin	2.95E-01	6.73E+04	1.68E+04	6.22E+04	3.63E+03	4.00E+00	6.02E-01
Phenylalanyl-tRNA synthetase beta chain (EC 6.1.1.20)	2.95E-01	4.79E+04	2.94E+04	2.30E+04	5.96E+03	1.63E+00	2.12E-01
Alanine dehydrogenase (EC 1.4.1.1)	2.97E-01	2.30E+06	1.69E+06	5.09E+05	6.99E+05	1.36E+00	1.33E-01
Oligoendopeptidase F (EC 3.4.24.-)	3.00E-01	1.77E+06	1.44E+06	3.96E+05	2.49E+05	1.23E+00	8.96E-02
Exoenzymes regulatory protein AepA in lipid-linked oligosaccharide synthesis cluster	3.00E-01	6.23E+03	1.59E+04	6.58E+03	1.18E+04	3.92E-01	-4.07E-01
RND multidrug efflux transporter; Acriflavin resistance protein	3.01E-01	4.60E+03	8.59E+03	1.91E+03	5.00E+03	5.36E-01	-2.71E-01
Oligopeptide transport ATP-binding protein OppF (TC 3.A.1.5.1)	3.01E-01	7.26E+04	1.17E+05	1.87E+04	5.58E+04	6.21E-01	-2.07E-01

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
Manganese superoxide dismutase (EC 1.15.1.1)	3.02E-01	6.59E+03	1.28E+04	1.81E+03	7.82E+03	5.15E-01	-2.88E-01
FIG017263: hypothetical protein	3.03E-01	1.31E+05	1.82E+05	2.68E+04	6.41E+04	7.19E-01	-1.43E-01
Chaperone protein DnaK	3.03E-01	1.66E+05	1.11E+05	6.85E+04	3.71E+04	1.50E+00	1.76E-01
Fructose-1,6-bisphosphatase, GlpX type (EC 3.1.3.11)	3.05E-01	2.17E+05	1.44E+05	6.79E+04	8.17E+04	1.50E+00	1.77E-01
Ferrichrome-binding periplasmic protein precursor (TC 3.A.1.14.3)	3.06E-01	9.81E+04	7.18E+04	2.44E+04	2.99E+04	1.37E+00	1.35E-01
Dihydrolipoamide dehydrogenase of acetoin dehydrogenase (EC 1.8.1.4)	3.07E-01	1.25E+06	2.01E+06	7.26E+05	8.41E+05	6.25E-01	-2.04E-01
Metallo-dependent hydrolases, subgroup C	3.08E-01	2.38E+04	3.69E+04	7.30E+03	1.67E+04	6.45E-01	-1.91E-01
FIG01251828: hypothetical protein	3.08E-01	5.14E+05	3.14E+05	2.56E+05	8.66E+04	1.64E+00	2.14E-01
Nicotinate-nucleotide adenylyltransferase (EC 2.7.7.18)	3.10E-01	7.75E+04	1.19E+05	3.00E+04	5.12E+04	6.53E-01	-1.85E-01
hypothetical protein	3.11E-01	8.65E+04	1.42E+05	4.36E+04	6.83E+04	6.09E-01	-2.15E-01
Electron transfer flavoprotein, alpha subunit	3.13E-01	1.38E+06	1.02E+06	4.54E+05	2.50E+05	1.35E+00	1.31E-01
Tellurite resistance protein	3.14E-01	4.41E+03	1.25E+04	1.95E+03	1.06E+04	3.52E-01	-4.54E-01
Manganese transport protein MntH	3.16E-01	3.93E+02	2.19E+03	3.58E+02	2.35E+03	1.80E-01	-7.46E-01
Phage lysin; N-acetylmuramoyl-L-alanine amidase, family 3 (EC:3.5.1.28)	3.18E-01	4.43E+04	1.51E+05	1.35E+04	1.40E+05	2.93E-01	-5.32E-01

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
Seryl-tRNA synthetase (EC 6.1.1.11)	3.20E-01	6.08E+05	7.80E+05	2.27E+05	4.62E+04	7.80E-01	-1.08E-01
ATP/GTP-binding protein, SA1392 homolog	3.22E-01	6.58E+04	2.57E+04	5.34E+04	1.47E+04	2.56E+00	4.09E-01
Aconitate hydratase (EC 4.2.1.3) @ 2-methylisocitrate dehydratase (EC 4.2.1.99)	3.27E-01	9.65E+05	6.02E+05	4.89E+05	6.32E+04	1.60E+00	2.05E-01
hypothetical protein	3.27E-01	2.33E+06	3.05E+06	6.92E+05	8.78E+05	7.62E-01	-1.18E-01
Iron-sulfur cluster assembly protein SufD	3.29E-01	6.72E+03	1.08E+04	6.45E+02	5.50E+03	6.23E-01	-2.05E-01
heat shock protein, Hsp20 family	3.29E-01	7.46E+03	3.85E+04	8.52E+03	4.20E+04	1.94E-01	-7.12E-01
Citrate synthase (si) (EC 2.3.3.1)	3.29E-01	6.93E+04	1.61E+05	7.70E+04	1.17E+05	4.30E-01	-3.66E-01
enterotoxin / cell-wall binding protein	3.29E-01	6.71E+04	4.31E+04	2.57E+04	2.70E+04	1.55E+00	1.92E-01
response regulator aspartate phosphatase	3.30E-01	1.75E+04	3.40E+04	3.31E+03	2.24E+04	5.15E-01	-2.88E-01
Cytochrome c oxidase polypeptide I (EC 1.9.3.1)	3.30E-01	5.90E+03	1.55E+04	3.42E+03	1.31E+04	3.81E-01	-4.19E-01
Glycine dehydrogenase [decarboxylating] (glycine cleavage system P2 protein) (EC 1.4.4.2)	3.31E-01	1.04E+06	1.58E+06	6.95E+05	4.67E+05	6.56E-01	-1.83E-01
ABC transporter, ATP-binding protein	3.32E-01	1.48E+04	2.91E+04	1.29E+04	1.81E+04	5.07E-01	-2.95E-01
oxidoreductase, short chain dehydrogenase/reductase family	3.34E-01	8.05E+03	2.21E+04	5.61E+03	1.92E+04	3.65E-01	-4.38E-01
Hemolysin BL lytic component L2	3.35E-01	2.04E+04	1.05E+04	1.32E+04	7.12E+03	1.94E+00	2.88E-01

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
PXO1-87	3.37E-01	2.52E+04	1.20E+04	1.81E+04	7.11E+03	2.10E+00	3.22E-01
NAD-dependent malic enzyme (EC 1.1.1.38)	3.38E-01	1.04E+05	2.56E+04	1.09E+05	6.41E+03	4.08E+00	6.10E-01
alternate gene name: yznA	3.39E-01	8.96E+03	6.63E+02	1.15E+04	2.35E+02	1.35E+01	1.13E+00
DUF1696 domain-containing protein	3.39E-01	1.75E+04	2.63E+04	8.41E+03	1.10E+04	6.67E-01	-1.76E-01
Thermostable carboxypeptidase 1 (EC 3.4.17.19)	3.40E-01	2.49E+05	3.26E+05	4.28E+04	1.07E+05	7.63E-01	-1.17E-01
metallo-beta-lactamase family protein	3.42E-01	1.52E+03	2.91E+03	8.23E+02	1.91E+03	5.24E-01	-2.80E-01
3-oxoacyl-[acyl-carrier-protein] synthase, KASII (EC 2.3.1.41)	3.44E-01	2.10E+05	1.58E+05	7.35E+04	2.67E+04	1.33E+00	1.25E-01
D-Glucosaminate-6-phosphate ammonia-lyase (EC 4.3.1.-)	3.45E-01	8.01E+04	1.16E+05	4.49E+04	3.66E+04	6.90E-01	-1.61E-01
Respiratory nitrate reductase alpha chain (EC 1.7.99.4)	3.49E-01	1.84E+04	3.65E+04	7.24E+03	2.59E+04	5.04E-01	-2.97E-01
FIG01231863: hypothetical protein	3.52E-01	1.09E+05	1.69E+05	8.32E+03	8.65E+04	6.45E-01	-1.90E-01
FIG01226765: hypothetical protein	3.52E-01	1.64E+03	6.24E+03	1.49E+03	6.63E+03	2.63E-01	-5.80E-01
Butyryl-CoA dehydrogenase (EC 1.3.99.2)	3.53E-01	3.55E+04	2.46E+04	1.28E+04	1.25E+04	1.44E+00	1.59E-01
Hypothetical ATP-binding protein UPF0042, contains P-loop	3.53E-01	4.08E+04	2.55E+04	2.14E+04	1.15E+04	1.60E+00	2.04E-01
hypothetical protein	3.54E-01	1.98E+05	2.90E+05	8.00E+04	1.24E+05	6.85E-01	-1.65E-01

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
Zinc ABC transporter, periplasmic-binding protein ZnuA	3.58E-01	2.61E+03	4.01E+03	1.14E+03	1.95E+03	6.52E-01	-1.86E-01
Undecaprenyl-diphosphatase (EC 3.6.1.27)	3.58E-01	5.27E+04	1.15E+05	1.91E+04	9.17E+04	4.57E-01	-3.40E-01
Enoyl-CoA hydratase [isoleucine degradation] (EC 4.2.1.17) / 3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35)	3.59E-01	2.57E+05	1.56E+05	1.46E+05	5.30E+04	1.64E+00	2.16E-01
Translation elongation factor G	3.59E-01	3.80E+06	3.31E+06	4.61E+05	6.64E+05	1.15E+00	5.99E-02
hypothetical protein	3.61E-01	3.01E+04	6.23E+04	1.88E+04	4.71E+04	4.82E-01	-3.17E-01
Porphobilinogen deaminase (EC 2.5.1.61)	3.64E-01	2.30E+05	1.18E+05	1.64E+05	6.90E+04	1.95E+00	2.90E-01
SMS protein	3.64E-01	1.65E+02	1.58E+03	2.21E+01	2.10E+03	1.05E-01	-9.81E-01
thermonuclease family protein, (pXO1-141)	3.65E-01	5.58E+04	7.92E+04	3.38E+04	1.75E+04	7.04E-01	-1.52E-01
acetyltransferase, CYSE/LACA/LPXA/NODL family	3.65E-01	9.53E+03	2.41E+04	8.33E+03	2.15E+04	3.96E-01	-4.02E-01
Uncharacterized N-acetyltransferase BT9727_3663 (EC 2.3.1.-)	3.66E-01	1.11E+03	2.54E+03	1.45E+03	1.92E+03	4.37E-01	-3.60E-01
FIG01227508: hypothetical protein	3.69E-01	1.18E+04	2.40E+04	1.34E+04	1.60E+04	4.91E-01	-3.09E-01
Transketolase (EC 2.2.1.1)	3.71E-01	7.96E+05	1.15E+06	1.52E+05	5.28E+05	6.95E-01	-1.58E-01
membrane protein, putative	3.74E-01	2.19E+04	4.20E+04	2.09E+03	3.07E+04	5.21E-01	-2.83E-01
Aminomethyltransferase (glycine cleavage system T protein) (EC 2.1.2.10)	3.75E-01	2.45E+05	3.70E+05	1.91E+05	1.35E+04	6.63E-01	-1.78E-01

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
Acetylornithine deacetylase (EC 3.5.1.16)	3.79E-01	4.17E+05	5.28E+05	1.51E+05	1.21E+05	7.90E-01	-1.03E-01
hypothetical protein	3.79E-01	2.99E+05	4.49E+05	1.30E+05	2.21E+05	6.65E-01	-1.77E-01
hypothetical protein	3.80E-01	1.28E+05	6.32E+04	1.01E+05	7.88E+03	2.03E+00	3.08E-01
Xaa-Pro aminopeptidase (EC 3.4.11.9)	3.80E-01	4.31E+04	3.31E+04	1.50E+04	8.09E+03	1.30E+00	1.15E-01
response regulator aspartate phosphatase	3.81E-01	8.09E+04	1.18E+05	5.49E+04	3.31E+04	6.84E-01	-1.65E-01
Ribosomal-protein-L7p-serine acetyltransferase	3.83E-01	1.27E+04	3.29E+04	1.18E+04	3.12E+04	3.85E-01	-4.14E-01
hypothetical protein	3.84E-01	1.03E+05	6.27E+04	6.34E+04	1.59E+04	1.65E+00	2.17E-01
PTS system, cellobiose-specific IIB component (EC 2.7.1.69)	3.84E-01	3.22E+03	1.37E+03	2.88E+03	9.12E+02	2.35E+00	3.70E-01
DNA-directed RNA polymerase beta' subunit (EC 2.7.7.6)	3.85E-01	1.96E+05	2.18E+05	2.28E+04	3.11E+04	8.99E-01	-4.60E-02
hypothetical protein	3.85E-01	1.15E+05	8.56E+04	4.61E+04	1.13E+04	1.34E+00	1.28E-01
Malate synthase (EC 2.3.3.9)	3.86E-01	3.73E+04	1.87E+04	2.49E+04	2.18E+04	1.99E+00	3.00E-01
FIG01231899: hypothetical protein	3.86E-01	7.52E+04	1.22E+05	6.70E+03	7.40E+04	6.15E-01	-2.11E-01
2,4-dienoyl-CoA reductase [NADPH] (EC 1.3.1.34)	3.86E-01	3.47E+03	7.59E+03	2.08E+03	6.45E+03	4.57E-01	-3.40E-01
Phosphoglucosamine mutase (EC 5.4.2.10)	3.87E-01	1.98E+05	1.47E+05	7.71E+04	4.08E+04	1.35E+00	1.29E-01
response regulator aspartate phosphatase	3.88E-01	1.68E+05	2.72E+05	6.06E+04	1.63E+05	6.18E-01	-2.09E-01
Oligopeptide transport ATP-binding protein OppD (TC 3.A.1.5.1)	3.89E-01	6.19E+04	9.90E+04	4.79E+04	4.61E+04	6.26E-01	-2.04E-01

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
ABC transporter, periplasmic spermidine putrescine-binding protein PotD (TC 3.A.1.11.1)	3.91E-01	6.78E+04	1.02E+05	2.31E+04	5.34E+04	6.65E-01	-1.77E-01
Aminopeptidase Y (Arg, Lys, Leu preference) (EC 3.4.11.15)	3.92E-01	6.70E+05	9.04E+05	2.45E+05	3.38E+05	7.41E-01	-1.30E-01
Phage major capsid protein #Fam0019	3.92E-01	1.03E+04	5.25E+03	7.14E+03	5.64E+03	1.97E+00	2.93E-01
hypothetical protein	3.93E-01	9.96E+04	1.18E+05	2.28E+04	2.39E+04	8.45E-01	-7.31E-02
Acyl-coenzyme A synthetases/AMP-(fatty) acid ligases, YtcI homolog	3.93E-01	2.47E+04	5.43E+04	1.20E+04	4.73E+04	4.55E-01	-3.42E-01
Similar to CDP-glucose 4,6-dehydratase (EC 4.2.1.45)	3.95E-01	5.21E+03	2.79E+04	5.15E+03	3.65E+04	1.87E-01	-7.29E-01
response regulator, putative	3.95E-01	5.27E+05	1.21E+06	3.21E+05	1.09E+06	4.36E-01	-3.61E-01
Shikimate 5-dehydrogenase I alpha (EC 1.1.1.25)	3.96E-01	2.75E+04	3.69E+04	1.08E+04	1.32E+04	7.45E-01	-1.28E-01
Cell surface protein	3.97E-01	4.68E+04	3.20E+04	1.57E+04	2.16E+04	1.46E+00	1.65E-01
Methionine aminopeptidase (EC 3.4.11.18)	3.97E-01	8.84E+03	3.44E+03	8.49E+03	4.12E+03	2.57E+00	4.10E-01
Carbonic anhydrase (EC 4.2.1.1)	3.99E-01	1.26E+04	1.92E+04	4.04E+03	1.05E+04	6.60E-01	-1.81E-01
Acetylornithine deacetylase/Succinyl-diaminopimelate desuccinylase and related deacylases	4.02E-01	2.47E+05	3.06E+05	8.10E+04	7.37E+04	8.06E-01	-9.36E-02

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein OppA (TC 3.A.1.5.1)	4.05E-01	1.23E+03	3.38E+03	8.63E+02	3.55E+03	3.64E-01	-4.39E-01
Fumarylacetoacetate hydrolase family protein	4.05E-01	2.33E+03	3.43E+03	5.35E+02	1.81E+03	6.80E-01	-1.68E-01
hypothetical protein	4.07E-01	2.15E+03	4.97E+03	2.97E+03	4.27E+03	4.32E-01	-3.65E-01
Spore coat polysaccharide biosynthesis protein SpsF	4.09E-01	6.19E+03	4.05E+03	8.01E+02	3.58E+03	1.53E+00	1.85E-01
Bll5861 protein	4.10E-01	1.79E+05	2.49E+05	1.03E+05	8.08E+04	7.19E-01	-1.43E-01
nitroreductase family protein	4.11E-01	4.20E+04	2.98E+04	6.67E+03	2.04E+04	1.41E+00	1.50E-01
Urocanate hydratase (EC 4.2.1.49)	4.14E-01	1.14E+06	8.73E+05	4.43E+05	1.32E+05	1.30E+00	1.14E-01
Cysteine synthase (EC 2.5.1.47)	4.15E-01	3.80E+04	7.05E+04	4.11E+04	4.64E+04	5.38E-01	-2.69E-01
Lead, cadmium, zinc and mercury transporting ATPase (EC 3.6.3.3) (EC 3.6.3.5); Copper-translocating P-type ATPase (EC 3.6.3.4)	4.16E-01	5.25E+04	8.10E+04	2.79E+04	4.52E+04	6.48E-01	-1.88E-01
Peptide synthetase	4.16E-01	1.73E+04	3.24E+04	7.57E+03	2.56E+04	5.32E-01	-2.74E-01
oxidoreductase of aldo/keto reductase family, subgroup 1	4.17E-01	1.94E+04	1.08E+04	1.48E+04	2.01E+03	1.80E+00	2.56E-01
3-oxoacyl-[acyl-carrier protein] reductase (EC 1.1.1.100)	4.18E-01	1.94E+04	2.82E+04	6.85E+03	1.45E+04	6.90E-01	-1.61E-01
Ribonuclease J1 (endonuclease and 5' exonuclease)	4.20E-01	2.21E+05	2.66E+05	7.67E+04	2.82E+04	8.30E-01	-8.11E-02

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
Inosine-5'-monophosphate dehydrogenase (EC 1.1.1.205) / CBS domain	4.21E-01	7.89E+05	1.21E+06	4.65E+05	6.55E+05	6.52E-01	-1.86E-01
Dihydroxyacetone kinase family protein	4.22E-01	2.82E+04	3.95E+04	1.72E+04	1.30E+04	7.15E-01	-1.45E-01
Sip1Aa-like_1	4.22E-01	1.10E+05	7.69E+04	4.70E+04	4.40E+04	1.43E+00	1.56E-01
Catalase (EC 1.11.1.6)	4.24E-01	3.73E+03	9.34E+03	3.52E+03	9.59E+03	4.00E-01	-3.98E-01
Ribonuclease J2 (endoribonuclease in RNA processing)	4.24E-01	2.85E+05	3.88E+05	2.00E+04	1.77E+05	7.36E-01	-1.33E-01
Ferrochelatase, protoheme ferro-lyase (EC 4.99.1.1)	4.25E-01	1.49E+04	3.12E+04	9.30E+03	2.82E+04	4.77E-01	-3.21E-01
Two-component response regulator, controling glutamine utilization	4.28E-01	5.96E+04	1.15E+05	4.56E+04	9.33E+04	5.19E-01	-2.85E-01
FIG01233992: hypothetical protein	4.28E-01	5.11E+02	1.93E+03	3.97E+02	2.49E+03	2.65E-01	-5.77E-01
Fumarate hydratase class II (EC 4.2.1.2)	4.29E-01	8.96E+04	5.63E+04	5.71E+04	2.57E+04	1.59E+00	2.02E-01
Thioredoxin	4.31E-01	6.19E+04	7.83E+04	1.83E+04	2.61E+04	7.92E-01	-1.02E-01
Hemoprotein HemQ, essential component of heme biosynthetic pathway in Gram-positive bacteria	4.32E-01	7.94E+04	8.76E+04	9.66E+03	1.29E+04	9.06E-01	-4.27E-02
Glucose-1-phosphate cytidylyltransferase (EC 2.7.7.33)	4.32E-01	8.02E+04	4.98E+04	5.33E+04	1.89E+04	1.61E+00	2.07E-01
Glucose-6-phosphate isomerase (EC 5.3.1.9)	4.34E-01	1.95E+05	2.52E+05	4.97E+04	9.76E+04	7.73E-01	-1.12E-01
Acetoacetyl-CoA reductase (EC 1.1.1.36)	4.34E-01	8.16E+03	1.04E+04	3.47E+03	2.93E+03	7.81E-01	-1.07E-01

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
phage infection protein	4.35E-01	3.62E+03	5.20E+03	1.62E+03	2.63E+03	6.96E-01	-1.57E-01
forespore-specific protein, putative	4.47E-01	3.68E+03	8.60E+03	2.88E+03	8.97E+03	4.28E-01	-3.69E-01
hypothetical protein	4.49E-01	2.95E+05	2.55E+05	1.50E+04	7.38E+04	1.16E+00	6.32E-02
hypothetical protein	4.50E-01	6.94E+04	1.11E+04	1.08E+05	4.28E+03	6.27E+00	7.97E-01
Heat shock protein 60 family chaperone GroEL	4.51E-01	1.03E+07	1.24E+07	3.70E+06	1.98E+06	8.32E-01	-7.99E-02
FIG01229538: hypothetical protein	4.52E-01	4.64E+04	3.33E+04	1.79E+04	2.04E+04	1.39E+00	1.44E-01
Uncharacterized secreted protein associated with spyDAC	4.54E-01	1.13E+04	7.56E+03	4.00E+03	6.46E+03	1.49E+00	1.73E-01
Translation initiation factor 3	4.55E-01	1.65E+03	7.14E+03	1.02E+03	1.03E+04	2.31E-01	-6.36E-01
Phosphoenolpyruvate-protein phosphotransferase of PTS system (EC 2.7.3.9)	4.55E-01	2.00E+05	2.35E+05	6.37E+04	2.74E+04	8.52E-01	-6.95E-02
3-ketoacyl-CoA thiolase (EC 2.3.1.16) @ Acetyl-CoA acetyltransferase (EC 2.3.1.9)	4.56E-01	2.78E+04	1.47E+04	2.40E+04	1.01E+04	1.89E+00	2.76E-01
Flagellar M-ring protein FliF	4.59E-01	3.71E+02	6.90E+02	1.62E+02	6.03E+02	5.37E-01	-2.70E-01
hypothetical protein	4.59E-01	6.50E+05	4.43E+05	3.27E+05	2.92E+05	1.47E+00	1.66E-01
Fe-S oxidoreductase	4.60E-01	8.24E+04	1.09E+05	4.37E+04	3.68E+04	7.53E-01	-1.23E-01
Transcription-repair coupling factor	4.60E-01	6.44E+03	1.09E+04	7.93E+03	4.90E+03	5.89E-01	-2.30E-01
Cystine-binding periplasmic protein precursor	4.60E-01	1.18E+06	6.38E+05	9.03E+05	6.93E+05	1.85E+00	2.67E-01
hypothetical protein	4.61E-01	1.94E+06	1.61E+06	3.70E+05	5.95E+05	1.21E+00	8.24E-02
Glyoxalase family protein	4.61E-01	6.21E+04	8.73E+04	2.47E+04	4.58E+04	7.11E-01	-1.48E-01
surface antigen gene	4.64E-01	5.22E+03	9.11E+03	1.20E+03	7.51E+03	5.72E-01	-2.42E-01
Streptomycin biosynthesis StrF domain protein	4.67E-01	3.04E+04	5.05E+04	2.34E+04	3.56E+04	6.03E-01	-2.20E-01

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
N-acetylglucosamine-1-phosphate uridyltransferase (EC 2.7.7.23) / Glucosamine-1-phosphate N-acetyltransferase (EC 2.3.1.157)	4.67E-01	1.26E+05	1.74E+05	6.31E+04	7.98E+04	7.27E-01	-1.38E-01
PTS system, N-acetylglucosamine-specific IIB component (EC 2.7.1.69) / PTS system, N-acetylglucosamine-specific IIC component (EC 2.7.1.69)	4.70E-01	5.72E+04	1.24E+05	4.32E+04	1.29E+05	4.60E-01	-3.37E-01
Chaperone protein DnaJ	4.70E-01	7.84E+04	1.08E+05	2.25E+04	5.57E+04	7.29E-01	-1.37E-01
Spore coat polysaccharide biosynthesis protein spsB	4.71E-01	1.85E+05	9.98E+04	1.66E+05	5.17E+04	1.86E+00	2.69E-01
Asparaginyl-tRNA synthetase (EC 6.1.1.22)	4.71E-01	1.30E+05	1.05E+05	4.60E+04	2.73E+04	1.24E+00	9.32E-02
Biotin carboxyl carrier protein of acetyl-CoA carboxylase	4.78E-01	6.95E+04	8.87E+04	3.64E+04	1.90E+04	7.84E-01	-1.06E-01
ATP-dependent RNA helicase YfmL	4.79E-01	2.75E+04	3.86E+04	2.07E+04	1.27E+04	7.11E-01	-1.48E-01
Cytosol aminopeptidase PepA (EC 3.4.11.1)	4.81E-01	7.60E+05	8.91E+05	1.75E+05	2.30E+05	8.54E-01	-6.88E-02
DNA-binding protein HBsu	4.83E-01	2.86E+04	1.52E+04	2.53E+04	1.47E+04	1.88E+00	2.74E-01
Membrane-associated zinc metalloprotease	4.84E-01	6.00E+04	7.21E+04	2.45E+04	5.26E+03	8.32E-01	-7.99E-02
hypothetical protein	4.84E-01	2.57E+05	1.54E+05	2.04E+05	8.24E+04	1.67E+00	2.22E-01
Isoleucyl-tRNA synthetase (EC 6.1.1.5)	4.86E-01	5.08E+05	4.18E+05	1.19E+05	1.64E+05	1.22E+00	8.54E-02

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
Xaa-Pro aminopeptidase (EC 3.4.11.9)	4.86E-01	3.40E+05	5.14E+05	2.40E+05	3.06E+05	6.63E-01	-1.79E-01
Beta-lactamase class A	4.87E-01	7.11E+03	1.05E+04	1.47E+03	6.91E+03	6.77E-01	-1.69E-01
Lead, cadmium, zinc and mercury transporting ATPase (EC 3.6.3.3) (EC 3.6.3.5); Copper-translocating P-type ATPase (EC 3.6.3.4)	4.88E-01	1.56E+04	2.14E+04	9.05E+03	9.78E+03	7.26E-01	-1.39E-01
ATP-dependent Clp protease, ATP-binding subunit ClpC / Negative regulator of genetic competence clcC/mecB	4.91E-01	2.81E+05	3.71E+05	1.34E+05	1.55E+05	7.58E-01	-1.20E-01
Nitroreductase family protein	4.91E-01	5.84E+04	8.66E+04	4.75E+04	4.35E+04	6.75E-01	-1.71E-01
Biotin carboxylase of methylcrotonyl-CoA carboxylase (EC 6.3.4.14)	4.93E-01	1.74E+05	1.11E+05	1.31E+05	2.80E+04	1.57E+00	1.96E-01
Cytochrome c oxidase polypeptide II (EC 1.9.3.1)	4.94E-01	1.30E+05	2.18E+05	1.46E+05	1.42E+05	5.95E-01	-2.25E-01
FIG01225916: hypothetical protein	4.96E-01	4.84E+03	9.67E+03	6.12E+03	9.19E+03	5.00E-01	-3.01E-01
Methylglutaconyl-CoA hydratase (EC 4.2.1.18)	5.06E-01	4.02E+04	2.38E+04	3.53E+04	4.98E+03	1.69E+00	2.28E-01
Thiaminase II (EC 3.5.99.2)	5.06E-01	8.22E+04	1.11E+05	6.14E+04	9.74E+03	7.42E-01	-1.30E-01
Malonyl CoA-acyl carrier protein transacylase (EC 2.3.1.39)	5.06E-01	5.12E+03	8.88E+03	5.23E+03	7.17E+03	5.76E-01	-2.40E-01
Histidine ammonia-lyase (EC 4.3.1.3)	5.13E-01	4.03E+05	3.25E+05	1.61E+05	8.50E+04	1.24E+00	9.31E-02

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
Choloylglycine hydrolase (EC 3.5.1.24)	5.14E-01	3.38E+04	3.95E+04	5.41E+03	1.21E+04	8.56E-01	-6.76E-02
hypothetical protein	5.17E-01	1.75E+04	3.20E+04	1.39E+04	3.12E+04	5.45E-01	-2.64E-01
Aldehyde dehydrogenase (EC 1.2.1.3)	5.21E-01	4.44E+05	6.42E+05	4.04E+05	2.58E+05	6.92E-01	-1.60E-01
Vip2Ac-like_1	5.22E-01	3.08E+05	2.67E+05	5.40E+04	8.46E+04	1.15E+00	6.25E-02
FIG006988: Lipase/Acylhydrolase with GDSL-like motif	5.24E-01	2.51E+03	5.63E+03	2.33E+03	6.93E+03	4.46E-01	-3.51E-01
hypothetical protein	5.25E-01	1.42E+03	2.32E+03	1.52E+03	1.65E+03	6.11E-01	-2.14E-01
Naphthoate synthase (EC 4.1.3.36)	5.26E-01	5.14E+05	3.82E+05	2.58E+05	2.06E+05	1.35E+00	1.30E-01
hypothetical protein	5.30E-01	7.98E+03	9.31E+03	1.09E+03	3.00E+03	8.57E-01	-6.72E-02
Nitroreductase family protein	5.31E-01	7.03E+03	2.58E+03	1.00E+04	3.73E+03	2.73E+00	4.36E-01
Dihydrolipoamide succinyltransferase component (E2) of 2-oxoglutarate dehydrogenase complex (EC 2.3.1.61)	5.33E-01	6.89E+04	1.09E+05	4.07E+04	8.86E+04	6.33E-01	-1.98E-01
hypothetical Membrane Spanning Protein	5.33E-01	1.45E+05	1.90E+05	5.36E+04	9.63E+04	7.66E-01	-1.16E-01
metallo-beta-lactamase family protein	5.33E-01	1.80E+05	3.28E+05	1.32E+05	3.33E+05	5.48E-01	-2.61E-01
Oxalate decarboxylase (EC 4.1.1.2)	5.36E-01	4.27E+04	2.83E+04	3.04E+04	1.97E+04	1.51E+00	1.78E-01
Beta-lactamase (EC 3.5.2.6)	5.37E-01	1.24E+06	9.69E+05	1.81E+05	6.18E+05	1.28E+00	1.06E-01
Putative ATP:guanido phosphotransferase YacI (EC 2.7.3.-)	5.37E-01	2.14E+04	1.41E+04	1.64E+04	7.76E+03	1.52E+00	1.82E-01

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
SSU ribosomal protein S4p (S9e)	5.40E-01	3.54E+04	5.83E+04	3.53E+04	4.72E+04	6.07E-01	-2.17E-01
Glucose 1-dehydrogenase (EC 1.1.1.47)	5.42E-01	1.01E+04	1.39E+04	5.09E+03	8.06E+03	7.31E-01	-1.36E-01
hypothetical protein	5.42E-01	3.64E+04	5.70E+04	4.50E+04	2.70E+04	6.39E-01	-1.95E-01
hypothetical protein	5.45E-01	6.06E+03	4.14E+03	4.46E+03	1.77E+03	1.46E+00	1.66E-01
Leucyl-tRNA synthetase (EC 6.1.1.4)	5.45E-01	2.34E+05	1.73E+05	1.34E+05	8.18E+04	1.35E+00	1.31E-01
outer membrane protein CC2294	5.46E-01	1.19E+03	1.98E+03	1.32E+03	1.58E+03	6.02E-01	-2.20E-01
oxidoreductase of aldo/keto reductase family, subgroup 1	5.46E-01	1.27E+04	9.10E+03	8.47E+03	2.53E+03	1.39E+00	1.44E-01
D-alanyl-D-alanine carboxypeptidase (EC 3.4.16.4)	5.48E-01	7.82E+04	1.14E+05	1.15E+04	8.68E+04	6.85E-01	-1.64E-01
hypothetical protein	5.48E-01	1.32E+04	1.59E+04	6.31E+03	2.89E+03	8.29E-01	-8.16E-02
FIG01225143: hypothetical protein	5.49E-01	9.43E+03	1.48E+04	5.72E+03	1.25E+04	6.37E-01	-1.96E-01
Methyltransferase (EC 2.1.1.-)	5.50E-01	7.48E+04	5.58E+04	4.13E+04	2.79E+04	1.34E+00	1.27E-01
Peptide deformylase (EC 3.5.1.88)	5.50E-01	5.15E+04	6.22E+04	2.25E+04	1.70E+04	8.28E-01	-8.18E-02
Orotidine 5'-phosphate decarboxylase (EC 4.1.1.23)	5.52E-01	2.79E+04	2.30E+04	4.80E+03	1.13E+04	1.21E+00	8.25E-02
6-phosphogluconate dehydrogenase, decarboxylating (EC 1.1.1.44)	5.53E-01	3.08E+04	3.71E+04	5.38E+03	1.52E+04	8.29E-01	-8.14E-02
Butyryl-CoA dehydrogenase (EC 1.3.99.2)	5.58E-01	2.17E+05	1.79E+05	9.04E+04	3.15E+04	1.21E+00	8.19E-02
Amino acid ABC transporter, amino acid-binding protein	5.59E-01	4.44E+04	2.81E+04	3.16E+04	3.11E+04	1.58E+00	1.99E-01

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
conserved hypothetical protein	5.60E-01	5.01E+03	2.08E+03	7.06E+03	2.99E+03	2.41E+00	3.82E-01
metallo-beta-lactamase family protein	5.61E-01	1.42E+04	2.15E+04	1.35E+04	1.47E+04	6.60E-01	-1.81E-01
Lysine 2,3-aminomutase (EC 5.4.3.2)	5.63E-01	8.02E+05	1.08E+06	3.29E+05	6.73E+05	7.40E-01	-1.31E-01
Uracil-DNA glycosylase, family 1	5.63E-01	1.32E+04	1.77E+04	3.75E+03	1.12E+04	7.44E-01	-1.29E-01
Prespore specific transcriptional activator RsfA	5.67E-01	1.41E+05	2.59E+05	1.85E+05	2.69E+05	5.42E-01	-2.66E-01
FIG01227891: hypothetical protein	5.70E-01	1.80E+04	2.52E+04	6.05E+03	1.82E+04	7.13E-01	-1.47E-01
Fe-bacillibactin uptake system FeuA, Fe-bacillibactin binding	5.72E-01	1.39E+04	2.24E+04	1.71E+04	1.65E+04	6.23E-01	-2.05E-01
FIG01228827: hypothetical protein	5.73E-01	4.63E+03	7.27E+03	3.47E+03	6.38E+03	6.37E-01	-1.96E-01
3-oxoacyl-[acyl-carrier protein] reductase (EC 1.1.1.100)	5.74E-01	1.41E+04	8.56E+03	1.08E+04	1.15E+04	1.65E+00	2.18E-01
2-oxoglutarate oxidoreductase, alpha subunit (EC 1.2.7.3)	5.74E-01	2.06E+05	1.41E+05	1.55E+05	9.58E+04	1.47E+00	1.66E-01
Enoyl-CoA hydratase (EC 4.2.1.17)	5.76E-01	6.60E+04	4.51E+04	5.40E+04	1.57E+04	1.47E+00	1.66E-01
Ribulose-phosphate 3-epimerase (EC 5.1.3.1)	5.76E-01	1.09E+05	6.91E+04	9.59E+04	5.84E+04	1.58E+00	1.99E-01
Flagellar hook-associated protein FlgK	5.76E-01	1.11E+04	1.53E+04	1.64E+03	1.10E+04	7.25E-01	-1.40E-01
ECF-type sigma factor negative effector	5.77E-01	6.26E+03	4.77E+03	1.88E+03	3.68E+03	1.31E+00	1.18E-01

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
Phosphoglycerate mutase (EC 5.4.2.1)	5.78E-01	1.20E+05	1.52E+05	5.06E+04	7.24E+04	7.94E-01	-1.00E-01
Oligoendopeptidase F (EC 3.4.24.-)	5.78E-01	1.56E+05	1.85E+05	7.56E+04	2.60E+04	8.41E-01	-7.50E-02
ABC transporter, ATP-binding protein, putative	5.78E-01	4.92E+02	9.64E+02	5.72E+02	1.18E+03	5.11E-01	-2.92E-01
FIG01227559: hypothetical protein	5.79E-01	7.29E+04	6.18E+04	2.73E+04	1.49E+04	1.18E+00	7.16E-02
Translation initiation factor 2	5.81E-01	1.08E+05	1.53E+05	5.96E+04	1.10E+05	7.09E-01	-1.50E-01
Branched-chain amino acid ABC transporter, amino acid-binding protein (TC 3.A.1.4.1)	5.82E-01	1.62E+04	1.93E+04	8.41E+03	9.63E+02	8.36E-01	-7.76E-02
acetyltransferase, GNAT family	5.86E-01	8.20E+04	9.92E+04	1.87E+04	4.45E+04	8.27E-01	-8.26E-02
D-Ribose 1,5-phosphomutase (EC 5.4.2.7)	5.88E-01	4.02E+05	4.80E+05	1.93E+05	1.16E+05	8.38E-01	-7.67E-02
Phenylalanine-4-hydroxylase (EC 1.14.16.1) - Long	5.88E-01	1.53E+05	1.34E+05	4.68E+04	2.44E+04	1.14E+00	5.58E-02
3-oxoacyl-[acyl-carrier-protein] synthase, KASIII (EC 2.3.1.41)	5.89E-01	2.02E+04	2.55E+04	4.68E+03	1.42E+04	7.91E-01	-1.02E-01
FIG01226514: hypothetical protein	5.90E-01	3.14E+04	3.56E+04	1.05E+04	5.48E+03	8.84E-01	-5.36E-02
NADPH dehydrogenase (EC 1.6.99.1)	5.92E-01	2.76E+03	5.95E+03	2.46E+03	8.60E+03	4.64E-01	-3.33E-01
SSU ribosomal protein S3p (S3e)	5.93E-01	2.01E+05	2.41E+05	9.05E+04	7.53E+04	8.36E-01	-7.79E-02

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
Bacillosamine/Legionaminic acid biosynthesis aminotransferase PglE; 4-keto-6-deoxy-N-Acetyl-D-hexosaminyl-(Lipid carrier) aminotransferase	5.94E-01	3.04E+04	2.09E+04	2.56E+04	8.37E+03	1.46E+00	1.63E-01
5-Enolpyruvylshikimate-3-phosphate synthase (EC 2.5.1.19)	5.94E-01	1.75E+04	2.14E+04	3.19E+03	1.06E+04	8.17E-01	-8.78E-02
hypothetical protein	5.94E-01	1.43E+05	1.22E+05	1.44E+04	5.83E+04	1.18E+00	7.02E-02
Chitinase (EC 3.2.1.14)	5.96E-01	1.97E+05	2.35E+05	3.26E+04	1.04E+05	8.37E-01	-7.71E-02
Non-ribosomal peptide synthase (EC 6.3.2.26) (EC 2.3.1.38)	5.97E-01	7.36E+04	8.28E+04	2.49E+04	9.14E+03	8.89E-01	-5.12E-02
Methylmalonate-semialdehyde dehydrogenase (EC 1.2.1.27)	5.98E-01	2.09E+06	1.86E+06	3.04E+05	6.08E+05	1.12E+00	5.09E-02
hypothetical protein	5.98E-01	2.79E+04	2.35E+04	7.57E+03	1.09E+04	1.19E+00	7.50E-02
Alpha/beta hydrolase	5.99E-01	1.10E+04	7.24E+03	1.05E+04	2.01E+03	1.52E+00	1.83E-01
Acetoacetyl-CoA synthetase (EC 6.2.1.16) / Long-chain-fatty-acid--CoA ligase (EC 6.2.1.3)	6.00E-01	5.63E+05	7.94E+05	1.99E+05	6.36E+05	7.09E-01	-1.49E-01
Inosine-uridine preferring nucleoside hydrolase (EC 3.2.2.1)	6.00E-01	1.00E+04	1.87E+04	3.22E+03	2.42E+04	5.38E-01	-2.70E-01
Signal recognition particle receptor protein FtsY (=alpha subunit) (TC 3.A.5.1.1)	6.03E-01	5.92E+03	8.19E+03	4.40E+03	5.38E+03	7.23E-01	-1.41E-01
Formiminoglutamase (EC 3.5.3.8)	6.04E-01	7.51E+04	9.43E+04	3.42E+04	4.76E+04	7.97E-01	-9.87E-02

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
hypothetical protein	6.05E-01	9.90E+04	1.23E+05	6.20E+04	3.73E+04	8.06E-01	-9.37E-02
Similar to eukaryotic Peptidyl prolyl 4-hydroxylase, alpha subunit (EC 1.14.11.2)	6.09E-01	1.34E+03	9.29E+02	1.01E+03	8.05E+02	1.45E+00	1.61E-01
Transcription termination protein NusA	6.10E-01	1.32E+04	2.02E+04	1.24E+04	1.79E+04	6.52E-01	-1.86E-01
hypothetical protein	6.12E-01	1.42E+03	9.03E+02	1.26E+03	9.89E+02	1.57E+00	1.95E-01
hypothetical protein	6.12E-01	8.23E+03	1.09E+04	7.77E+03	1.34E+03	7.54E-01	-1.23E-01
FIG01225390: hypothetical protein	6.12E-01	1.50E+04	2.08E+04	1.17E+04	1.43E+04	7.19E-01	-1.43E-01
Protein YicC	6.14E-01	1.39E+04	1.92E+04	1.39E+04	8.82E+03	7.25E-01	-1.40E-01
Pyruvate dehydrogenase E1 component alpha subunit (EC 1.2.4.1)	6.15E-01	4.77E+05	5.67E+05	2.48E+05	1.26E+05	8.42E-01	-7.49E-02
Magnesium and cobalt transport protein CorA	6.16E-01	5.23E+04	4.68E+04	1.16E+04	1.30E+04	1.12E+00	4.80E-02
2-amino-3-ketobutyrate coenzyme A ligase (EC 2.3.1.29)	6.17E-01	7.60E+05	6.55E+05	2.52E+05	2.20E+05	1.16E+00	6.45E-02
oxidoreductase, aldo/keto reductase family	6.17E-01	1.59E+04	2.16E+04	1.58E+04	8.12E+03	7.36E-01	-1.33E-01
hypothetical protein	6.20E-01	3.34E+04	2.47E+04	1.18E+04	2.45E+04	1.35E+00	1.31E-01
hypothetical protein	6.22E-01	2.18E+05	2.72E+05	1.25E+05	1.24E+05	8.01E-01	-9.64E-02
Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein OppA (TC 3.A.1.5.1)	6.22E-01	5.85E+05	6.38E+05	1.21E+05	1.25E+05	9.16E-01	-3.80E-02
Peptidyl-prolyl cis-trans isomerase (EC 5.2.1.8)	6.22E-01	1.30E+05	1.72E+05	4.77E+04	1.21E+05	7.58E-01	-1.21E-01

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
Arsenate reductase (EC 1.20.4.1)	6.24E-01	4.26E+03	6.44E+03	4.34E+03	5.60E+03	6.62E-01	-1.79E-01
hypothetical protein	6.27E-01	8.32E+04	5.48E+04	6.59E+04	6.62E+04	1.52E+00	1.81E-01
Outer surface protein of unknown function, cellobiose operon	6.28E-01	2.08E+05	1.86E+05	6.32E+04	3.52E+04	1.12E+00	4.94E-02
1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate synthase (EC 1.17.7.1)	6.29E-01	1.71E+05	1.50E+05	4.25E+04	5.28E+04	1.14E+00	5.56E-02
Methionyl-tRNA synthetase (EC 6.1.1.10)	6.34E-01	2.03E+05	1.87E+05	1.58E+04	5.00E+04	1.09E+00	3.66E-02
Cell envelope-associated transcriptional attenuator LytR-CpsA-Psr, subfamily F2 (as in PMID19099556)	6.34E-01	4.60E+04	3.72E+04	2.25E+04	1.89E+04	1.24E+00	9.19E-02
hypothetical protein	6.34E-01	8.55E+05	9.42E+05	4.10E+04	2.72E+05	9.07E-01	-4.24E-02
Putative acyl-coa dehydrogenase	6.35E-01	4.38E+04	3.39E+04	2.82E+04	1.62E+04	1.29E+00	1.10E-01
Prolyl-tRNA synthetase (EC 6.1.1.15), archaeal/eukaryal type	6.36E-01	1.96E+05	1.60E+05	7.52E+04	9.56E+04	1.23E+00	8.86E-02
hypothetical protein	6.37E-01	1.68E+06	1.42E+06	7.26E+05	5.17E+05	1.19E+00	7.43E-02
Hypothetical protein YfkI	6.38E-01	2.83E+04	2.09E+04	1.84E+04	1.70E+04	1.35E+00	1.31E-01
hypothetical protein	6.39E-01	1.45E+05	1.04E+05	1.18E+05	7.00E+04	1.39E+00	1.44E-01
Ribulose-5-phosphate 4-epimerase and related epimerases and aldolases	6.40E-01	1.02E+05	9.07E+04	3.25E+04	1.99E+04	1.12E+00	5.10E-02
Cell division protein FtsX	6.46E-01	5.48E+03	7.73E+03	4.31E+03	6.44E+03	7.10E-01	-1.49E-01
Carboxylesterase (EC 3.1.1.1)	6.48E-01	1.56E+03	9.01E+02	2.01E+03	1.07E+03	1.74E+00	2.40E-01
Pyridoxamine 5'-phosphate oxidase (EC 1.4.3.5)	6.48E-01	2.91E+04	3.63E+04	1.02E+04	2.23E+04	8.01E-01	-9.62E-02

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
Protein ecsC	6.52E-01	2.91E+04	4.13E+04	2.47E+04	3.51E+04	7.06E-01	-1.51E-01
hypothetical protein	6.53E-01	5.37E+04	7.34E+04	4.90E+04	5.02E+04	7.32E-01	-1.36E-01
Hydroxymethylpyrimidine ABC transporter, substrate-binding component	6.55E-01	1.12E+05	9.30E+04	1.26E+04	6.37E+04	1.21E+00	8.17E-02
Penicillin-binding protein 3	6.56E-01	1.09E+05	1.28E+05	2.64E+04	6.25E+04	8.48E-01	-7.17E-02
Malonyl CoA-acyl carrier protein transacylase (EC 2.3.1.39)	6.58E-01	9.67E+04	1.64E+05	1.15E+05	2.11E+05	5.88E-01	-2.30E-01
Beta-lysine acetyltransferase (EC 2.3.1.-)	6.59E-01	3.78E+05	2.92E+05	2.77E+05	1.16E+05	1.29E+00	1.12E-01
BNR repeat domain protein	6.59E-01	1.34E+04	1.71E+04	9.21E+03	1.01E+04	7.81E-01	-1.07E-01
Preprotein translocase secY subunit (TC 3.A.5.1.1)	6.60E-01	4.83E+04	7.36E+04	2.67E+04	8.36E+04	6.56E-01	-1.83E-01
6,7-dimethyl-8-ribityllumazine synthase (EC 2.5.1.78)	6.61E-01	2.48E+05	1.98E+05	1.63E+05	7.21E+04	1.25E+00	9.86E-02
UDP-glucose 4-epimerase (EC 5.1.3.2)	6.63E-01	7.10E+04	5.94E+04	6.52E+03	3.93E+04	1.19E+00	7.72E-02
Chloramphenicol acetyltransferase (EC 2.3.1.28)	6.63E-01	4.72E+04	5.83E+04	1.60E+04	3.64E+04	8.09E-01	-9.21E-02
Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein OppA (TC 3.A.1.5.1)	6.64E-01	4.82E+05	6.90E+05	5.82E+05	5.05E+05	6.98E-01	-1.56E-01
hypothetical protein	6.64E-01	4.27E+04	5.89E+04	3.10E+04	5.01E+04	7.25E-01	-1.39E-01
Enterochelin esterase	6.64E-01	5.64E+04	4.62E+04	6.91E+03	3.47E+04	1.22E+00	8.66E-02
Chitin binding protein	6.68E-01	2.90E+05	2.54E+05	1.24E+05	3.23E+04	1.14E+00	5.81E-02

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
Iron(III) dicitrate transport system, periplasmic iron-binding protein FecB (TC 3.A.1.14.1)	6.70E-01	3.67E+03	6.73E+03	4.43E+03	1.02E+04	5.45E-01	-2.63E-01
Spore coat protein F	6.75E-01	4.29E+03	3.40E+03	2.49E+03	2.36E+03	1.26E+00	1.01E-01
Methionine ABC transporter substrate-binding protein	6.75E-01	1.27E+04	1.95E+04	1.03E+04	2.33E+04	6.49E-01	-1.87E-01
FIG007959: peptidase, M16 family	6.76E-01	2.64E+05	2.80E+05	4.80E+04	3.48E+04	9.44E-01	-2.48E-02
FIG01225599: hypothetical protein	6.79E-01	1.69E+04	1.07E+04	1.86E+04	1.50E+04	1.58E+00	1.98E-01
Stage II sporulation protein related to metaloproteases (SpoIIQ)	6.80E-01	1.13E+05	1.52E+05	3.53E+04	1.41E+05	7.42E-01	-1.30E-01
Anti-sigma F factor antagonist (spoIIAA-2); Anti-sigma B factor antagonist RsbV	6.84E-01	9.79E+03	6.43E+03	7.95E+03	1.05E+04	1.52E+00	1.83E-01
hypothetical protein	6.86E-01	4.16E+05	4.47E+05	1.06E+05	5.82E+04	9.31E-01	-3.12E-02
enterotoxin / cell-wall binding protein	6.86E-01	1.57E+05	1.25E+05	1.05E+05	7.37E+04	1.26E+00	1.00E-01
response regulator, putative	6.87E-01	2.54E+05	3.14E+05	1.22E+05	2.02E+05	8.08E-01	-9.24E-02
Pyruvate kinase (EC 2.7.1.40)	6.87E-01	3.93E+05	3.10E+05	3.03E+05	6.94E+04	1.27E+00	1.02E-01
hypothetical protein	6.91E-01	7.85E+03	1.09E+04	9.06E+03	8.46E+03	7.19E-01	-1.43E-01
hypothetical protein	6.94E-01	4.37E+04	6.03E+04	6.46E+03	6.29E+04	7.26E-01	-1.39E-01
Succinyl-CoA ligase [ADP-forming] alpha chain (EC 6.2.1.5)	6.95E-01	8.79E+03	7.07E+03	6.54E+03	1.05E+03	1.24E+00	9.46E-02
Nitrilotriacetate monooxygenase component B (EC 1.14.13.-)	6.95E-01	7.40E+04	9.27E+04	3.75E+04	6.53E+04	7.99E-01	-9.76E-02

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
Fibronectin/fibrinogen-binding protein	6.96E-01	1.17E+04	9.25E+03	3.46E+03	9.03E+03	1.26E+00	1.02E-01
O-acetylhomoserine sulfhydrylase (EC 2.5.1.49) / O-succinylhomoserine sulfhydrylase (EC 2.5.1.48)	6.97E-01	2.05E+05	1.82E+05	4.49E+04	8.53E+04	1.13E+00	5.36E-02
Cytosol aminopeptidase PepA (EC 3.4.11.1)	6.99E-01	2.66E+05	2.24E+05	1.24E+05	1.23E+05	1.19E+00	7.48E-02
FIG001621: Zinc protease	7.02E-01	4.55E+05	4.15E+05	1.54E+05	7.80E+03	1.09E+00	3.94E-02
Foldase protein PrsA precursor (EC 5.2.1.8) @ Foldase clustered with pyrimidine conversion	7.05E-01	6.21E+04	8.09E+04	5.18E+04	6.06E+04	7.68E-01	-1.15E-01
Phosphatase NagD predicted to act in N-acetylglucosamine utilization subsystem	7.05E-01	6.63E+03	7.80E+03	4.19E+03	2.62E+03	8.49E-01	-7.10E-02
ATP-dependent Clp protease proteolytic subunit (EC 3.4.21.92)	7.07E-01	1.90E+05	2.39E+05	1.78E+05	1.03E+05	7.95E-01	-9.96E-02
2-dehydropantoate 2-reductase (EC 1.1.1.169)	7.10E-01	1.83E+04	2.50E+04	2.39E+04	1.62E+04	7.32E-01	-1.35E-01
Cell division trigger factor (EC 5.2.1.8)	7.12E-01	8.47E+03	1.16E+04	1.03E+04	9.23E+03	7.28E-01	-1.38E-01
Cystathionine gamma-lyase (EC 4.4.1.1)	7.12E-01	1.05E+05	1.21E+05	1.38E+04	6.32E+04	8.70E-01	-6.03E-02
FIG01226713: hypothetical protein	7.14E-01	3.51E+03	4.53E+03	2.62E+03	3.59E+03	7.75E-01	-1.11E-01
FIG002344: Hydrolase (HAD superfamily)	7.14E-01	6.85E+04	7.87E+04	4.16E+04	5.59E+03	8.71E-01	-6.02E-02
NAD synthetase (EC 6.3.1.5)	7.15E-01	3.76E+05	3.35E+05	1.65E+05	6.67E+04	1.12E+00	5.10E-02

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
Phosphoenolpyruvate carboxykinase [ATP] (EC 4.1.1.49)	7.16E-01	5.41E+05	6.29E+05	3.07E+05	2.36E+05	8.60E-01	-6.53E-02
FIG01227012: hypothetical protein	7.17E-01	3.03E+04	4.30E+04	4.69E+04	3.05E+04	7.04E-01	-1.52E-01
Molybdopterin biosynthesis protein MoeA	7.18E-01	1.88E+04	1.54E+04	8.22E+03	1.29E+04	1.23E+00	8.86E-02
Dolichol-phosphate mannosyltransferase (EC 2.4.1.83) in lipid-linked oligosaccharide synthesis cluster	7.19E-01	1.63E+05	2.08E+05	7.81E+04	1.80E+05	7.82E-01	-1.07E-01
Stage II sporulation serine phosphatase for sigma-F activation (SpoIIE)	7.21E-01	8.94E+02	1.29E+03	6.06E+02	1.59E+03	6.96E-01	-1.57E-01
Branched-chain alpha-keto acid dehydrogenase, E1 component, alpha subunit (EC 1.2.4.4)	7.22E-01	7.07E+03	5.76E+03	3.71E+03	4.62E+03	1.23E+00	8.90E-02
hypothetical Membrane Spanning Protein	7.24E-01	2.78E+03	3.72E+03	2.56E+03	3.40E+03	7.48E-01	-1.26E-01
Acetyl-CoA:acetoacetyl-CoA transferase, alpha subunit (EC 2.8.3.8)	7.24E-01	1.29E+05	1.55E+05	9.89E+04	6.37E+04	8.32E-01	-7.97E-02
FIG01225487: hypothetical protein	7.24E-01	4.15E+04	5.01E+04	2.79E+04	2.81E+04	8.27E-01	-8.23E-02
Ribonuclease PH (EC 2.7.7.56)	7.26E-01	1.14E+04	1.36E+04	6.05E+03	7.73E+03	8.42E-01	-7.46E-02
FIG01225999: hypothetical protein	7.28E-01	7.86E+04	6.41E+04	5.45E+04	3.91E+04	1.23E+00	8.90E-02
hypothetical protein	7.33E-01	3.16E+05	2.27E+05	3.33E+05	2.59E+05	1.40E+00	1.45E-01

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
Zwa5A	7.33E-01	2.54E+04	3.08E+04	1.84E+04	1.80E+04	8.24E-01	-8.42E-02
putative cytochrome P450 hydroxylase	7.36E-01	4.42E+04	3.81E+04	1.17E+04	2.62E+04	1.16E+00	6.53E-02
Butyryl-CoA dehydrogenase (EC 1.3.99.2)	7.37E-01	4.32E+04	5.26E+04	3.13E+04	3.28E+04	8.21E-01	-8.55E-02
FIG01230264: hypothetical protein	7.38E-01	3.35E+04	2.74E+04	2.59E+04	1.19E+04	1.22E+00	8.72E-02
Adenylate kinase (EC 2.7.4.3)	7.39E-01	3.26E+04	2.50E+04	3.17E+04	1.65E+04	1.30E+00	1.14E-01
FIG01226004: hypothetical protein	7.40E-01	7.00E+03	9.08E+03	4.26E+03	8.86E+03	7.71E-01	-1.13E-01
ClpB protein	7.43E-01	1.33E+05	1.25E+05	2.66E+04	2.35E+04	1.06E+00	2.43E-02
Chorismate mutase I (EC 5.4.99.5) / 2-keto-3-deoxy-D-arabino-heptulosonate-7-phosphate synthase I beta (EC 2.5.1.54)	7.48E-01	7.38E+04	6.76E+04	1.69E+04	2.58E+04	1.09E+00	3.82E-02
Pyridoxine biosynthesis glutamine amidotransferase, synthase subunit (EC 2.4.2.-)	7.48E-01	6.87E+05	6.01E+05	1.09E+05	3.95E+05	1.14E+00	5.78E-02
Cation-transporting ATPase	7.52E-01	6.54E+03	7.96E+03	5.81E+03	4.23E+03	8.22E-01	-8.51E-02
Acetoacetyl-CoA synthetase [leucine] (EC 6.2.1.16)	7.52E-01	1.33E+05	1.44E+05	4.26E+04	3.65E+04	9.24E-01	-3.45E-02
Phosphate acetyltransferase (EC 2.3.1.8)	7.52E-01	2.00E+05	2.25E+05	3.95E+04	1.15E+05	8.90E-01	-5.05E-02
N-acetylmuramic acid 6-phosphate etherase	7.55E-01	4.56E+04	4.87E+04	3.74E+03	1.52E+04	9.35E-01	-2.93E-02
5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase (EC 2.1.1.14)	7.57E-01	2.28E+06	2.35E+06	3.50E+05	1.44E+05	9.68E-01	-1.41E-02

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
ATP-dependent protease HslV (EC 3.4.25.-)	7.61E-01	3.16E+05	2.70E+05	1.73E+05	1.68E+05	1.17E+00	6.74E-02
L-fuco-beta-pyranose dehydrogenase (EC 1.1.1.122)	7.62E-01	3.75E+05	3.37E+05	1.84E+05	6.54E+04	1.11E+00	4.65E-02
Oligoendopeptidase F (EC 3.4.24.-)	7.63E-01	8.18E+04	9.31E+04	5.25E+04	2.67E+04	8.79E-01	-5.59E-02
FIG01226746: hypothetical protein	7.65E-01	4.73E+04	5.96E+04	3.62E+04	5.47E+04	7.94E-01	-1.00E-01
Phage major tail protein phi13	7.66E-01	1.10E+04	1.43E+04	9.01E+03	1.53E+04	7.69E-01	-1.14E-01
hypothetical protein	7.66E-01	6.37E+04	5.76E+04	1.27E+04	2.98E+04	1.11E+00	4.40E-02
Transcriptional regulator, AsnC family	7.69E-01	3.66E+03	2.88E+03	2.76E+03	3.33E+03	1.27E+00	1.05E-01
FIG042921: similarity to aminoacyl-tRNA editing enzymes YbaK, ProX	7.69E-01	9.29E+03	6.42E+03	1.46E+04	3.46E+03	1.45E+00	1.60E-01
Glycosyl transferase ,group 2 family, anthrose biosynthesis	7.69E-01	9.16E+03	1.05E+04	6.24E+03	4.38E+03	8.68E-01	-6.15E-02
NAD(P)H-flavin oxidoreductase	7.70E-01	8.81E+03	1.16E+04	6.41E+03	1.35E+04	7.60E-01	-1.19E-01
2',3'-cyclic-nucleotide 2'-phosphodiesterase (EC 3.1.4.16)	7.71E-01	7.78E+04	9.49E+04	3.39E+04	8.49E+04	8.20E-01	-8.60E-02
Cell wall surface anchor family protein	7.71E-01	8.60E+05	9.53E+05	2.98E+05	4.24E+05	9.02E-01	-4.50E-02
Competence/damage-inducible protein CinA	7.73E-01	2.09E+04	2.52E+04	1.42E+04	1.93E+04	8.29E-01	-8.12E-02
Phage protein	7.74E-01	7.46E+04	8.27E+04	2.27E+04	3.83E+04	9.03E-01	-4.43E-02
hypothetical protein	7.76E-01	1.12E+06	1.34E+06	1.16E+06	4.71E+05	8.30E-01	-8.09E-02

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
hypothetical protein	7.78E-01	5.80E+06	4.86E+06	4.95E+06	1.26E+06	1.19E+00	7.66E-02
Spermidine synthase (EC 2.5.1.16)	7.80E-01	3.79E+05	4.47E+05	2.90E+05	2.67E+05	8.48E-01	-7.15E-02
Zinc metalloproteinase precursor (EC 3.4.24.29) / aureolysin	7.81E-01	1.40E+05	1.23E+05	7.50E+04	6.86E+04	1.14E+00	5.78E-02
FIG01228082: hypothetical protein	7.84E-01	6.79E+04	7.48E+04	2.61E+04	3.10E+04	9.08E-01	-4.19E-02
Leucine dehydrogenase (EC 1.4.1.9)	7.86E-01	6.15E+06	6.47E+06	1.67E+06	9.18E+05	9.50E-01	-2.24E-02
N-acetylmuramoyl-L-alanine amidase (EC 3.5.1.28)	7.92E-01	2.04E+05	1.80E+05	1.08E+05	9.83E+04	1.13E+00	5.40E-02
FIG01227456: hypothetical protein	7.92E-01	1.00E+05	8.58E+04	7.26E+04	5.18E+04	1.17E+00	6.82E-02
Methionine gamma-lyase (EC 4.4.1.11)	7.97E-01	8.75E+03	1.06E+04	7.75E+03	8.41E+03	8.28E-01	-8.18E-02
Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein OppA (TC 3.A.1.5.1)	7.99E-01	2.17E+04	2.91E+04	2.43E+04	3.92E+04	7.47E-01	-1.27E-01
COG1649 predicted glycoside hydrolase	8.01E-01	2.44E+04	3.11E+04	3.44E+04	2.46E+04	7.87E-01	-1.04E-01
Tripeptide aminopeptidase (EC 3.4.11.4)	8.01E-01	7.75E+04	7.14E+04	2.21E+04	3.18E+04	1.09E+00	3.55E-02
2-methylcitrate dehydratase (EC 4.2.1.79)	8.01E-01	3.26E+06	3.78E+06	2.55E+06	2.18E+06	8.62E-01	-6.44E-02
FIG146262: hypothetical protein	8.04E-01	2.08E+05	2.56E+05	1.70E+05	2.54E+05	8.15E-01	-8.89E-02

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein OppA (TC 3.A.1.5.1)	8.04E-01	3.29E+06	3.46E+06	9.21E+05	5.48E+05	9.52E-01	-2.15E-02
FIG01225424: hypothetical protein	8.05E-01	6.93E+04	6.39E+04	3.05E+04	1.64E+04	1.08E+00	3.51E-02
Transaldolase (EC 2.2.1.2)	8.09E-01	1.33E+05	1.21E+05	4.74E+04	6.87E+04	1.10E+00	4.31E-02
Adenosylmethionine-8-amino-7-oxononanoate aminotransferase (EC 2.6.1.62)	8.09E-01	6.42E+05	6.85E+05	2.26E+05	1.82E+05	9.37E-01	-2.84E-02
Archaeal S-adenosylmethionine synthetase (EC 2.5.1.6)	8.11E-01	1.38E+06	1.46E+06	5.02E+05	2.47E+05	9.42E-01	-2.59E-02
Lysyl-tRNA synthetase (class II) (EC 6.1.1.6)	8.11E-01	3.80E+05	3.92E+05	5.59E+04	5.71E+04	9.70E-01	-1.33E-02
FIG009210: peptidase, M16 family	8.13E-01	4.57E+05	5.07E+05	1.83E+05	2.84E+05	9.02E-01	-4.50E-02
4-hydroxy-tetrahydrodipicolinate synthase (EC 4.3.3.7)	8.14E-01	1.58E+05	1.70E+05	7.28E+04	4.23E+04	9.27E-01	-3.30E-02
FIG013761: LmbE family protein	8.17E-01	1.31E+04	1.54E+04	1.15E+04	1.17E+04	8.48E-01	-7.14E-02
Glutamate-1-semialdehyde aminotransferase (EC 5.4.3.8)	8.18E-01	1.15E+05	1.03E+05	5.46E+04	6.34E+04	1.12E+00	4.75E-02
Glyoxalase family protein	8.19E-01	6.01E+03	5.26E+03	4.07E+03	3.36E+03	1.14E+00	5.78E-02
hypothetical protein	8.19E-01	1.44E+05	1.33E+05	2.47E+04	6.93E+04	1.08E+00	3.39E-02
dTDP-glucose 4,6-dehydratase (EC 4.2.1.46)	8.23E-01	3.90E+04	3.51E+04	1.86E+04	2.11E+04	1.11E+00	4.56E-02

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
Phosphonate ABC transporter phosphate-binding periplasmic component (TC 3.A.1.9.1)	8.24E-01	8.14E+04	7.53E+04	2.80E+04	3.36E+04	1.08E+00	3.34E-02
Macrocin O-methyltransferase (EC 2.1.1.101)	8.25E-01	1.58E+03	1.25E+03	2.13E+03	9.24E+02	1.26E+00	1.00E-01
3'->5' exoribonuclease Bsu YhaM	8.25E-01	7.70E+04	8.24E+04	3.14E+04	2.37E+04	9.35E-01	-2.93E-02
ThiJ/PfpI family protein	8.31E-01	1.49E+05	1.16E+05	2.08E+05	1.32E+05	1.28E+00	1.08E-01
Spore cortex-lytic enzyme, N-acetylglucosaminidase SleL (EC 3.2.1.-)	8.31E-01	2.42E+05	2.67E+05	7.46E+04	1.69E+05	9.07E-01	-4.26E-02
DNA gyrase subunit B (EC 5.99.1.3)	8.32E-01	2.40E+04	2.79E+04	2.26E+04	1.87E+04	8.62E-01	-6.46E-02
Glutamine ABC transporter, periplasmic glutamine-binding protein (TC 3.A.1.3.2)	8.35E-01	1.86E+05	2.00E+05	7.14E+04	7.75E+04	9.32E-01	-3.05E-02
Phosphate butyryltransferase (EC 2.3.1.19)	8.38E-01	2.46E+05	2.36E+05	5.88E+04	5.47E+04	1.04E+00	1.82E-02
6-phospho-beta-glucosidase (EC 3.2.1.86)	8.38E-01	5.61E+05	6.20E+05	8.15E+04	4.39E+05	9.04E-01	-4.37E-02
Ornithine aminotransferase (EC 2.6.1.13)	8.38E-01	1.19E+05	1.34E+05	3.77E+04	1.05E+05	8.91E-01	-4.99E-02
ABC transporter, substrate-binding protein, putative	8.40E-01	4.79E+04	5.77E+04	3.22E+04	7.02E+04	8.29E-01	-8.13E-02
transcriptional regulator/TPR domain protein	8.41E-01	9.33E+04	8.83E+04	2.28E+04	3.30E+04	1.06E+00	2.39E-02

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
Glutamyl aminopeptidase (EC 3.4.11.7); Deblocking aminopeptidase	8.42E-01	2.02E+05	2.25E+05	9.62E+04	1.57E+05	8.98E-01	-4.68E-02
Threonyl-tRNA synthetase (EC 6.1.1.3)	8.42E-01	3.61E+04	2.97E+04	3.83E+04	3.50E+04	1.21E+00	8.42E-02
Cysteine desulfurase (EC 2.8.1.7), SufS subfamily	8.43E-01	2.19E+05	2.02E+05	1.02E+05	9.68E+04	1.09E+00	3.55E-02
3,4-dihydroxy-2-butanone 4-phosphate synthase (EC 4.1.99.12) / GTP cyclohydrolase II (EC 3.5.4.25)	8.45E-01	8.51E+03	7.08E+03	1.04E+04	5.17E+03	1.20E+00	8.02E-02
hypothetical protein	8.47E-01	5.06E+04	4.86E+04	6.52E+03	1.50E+04	1.04E+00	1.75E-02
sensory box/GGDEF family protein, putative	8.52E-01	4.70E+04	5.29E+04	7.73E+03	4.75E+04	8.89E-01	-5.09E-02
aminopeptidase	8.53E-01	3.19E+05	3.25E+05	1.76E+04	4.73E+04	9.82E-01	-8.02E-03
Translation elongation factor Ts	8.54E-01	4.05E+05	4.58E+05	3.26E+05	3.37E+05	8.84E-01	-5.36E-02
penicillin-binding protein, putative	8.54E-01	1.65E+05	1.74E+05	5.66E+04	5.82E+04	9.47E-01	-2.36E-02
4-hydroxyphenylacetate 3-monooxygenase (EC 1.14.13.3)	8.64E-01	9.72E+04	1.02E+05	2.14E+04	3.88E+04	9.53E-01	-2.08E-02
Carboxylesterase (EC 3.1.1.1)	8.66E-01	2.14E+04	1.79E+04	2.36E+04	2.38E+04	1.19E+00	7.69E-02
Ketol-acid reductoisomerase (EC 1.1.1.86)	8.68E-01	3.06E+04	2.81E+04	1.23E+04	2.14E+04	1.09E+00	3.81E-02
Uridine monophosphate kinase (EC 2.7.4.22)	8.69E-01	2.41E+04	2.58E+04	1.38E+04	7.97E+03	9.36E-01	-2.86E-02
hypothetical protein	8.69E-01	4.32E+05	3.88E+05	3.25E+05	2.77E+05	1.11E+00	4.61E-02
hypothetical protein	8.71E-01	2.37E+04	2.15E+04	1.57E+04	1.44E+04	1.10E+00	4.09E-02

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
Serine acetyltransferase (EC 2.3.1.30)	8.72E-01	3.73E+04	3.55E+04	1.50E+04	8.24E+03	1.05E+00	2.07E-02
D-alanine aminotransferase (EC 2.6.1.21)	8.72E-01	2.67E+05	2.45E+05	1.86E+05	1.23E+05	1.09E+00	3.79E-02
Trehalose-6-phosphate hydrolase (EC 3.2.1.93)	8.72E-01	3.30E+05	3.11E+05	1.13E+05	1.46E+05	1.06E+00	2.48E-02
DinB family protein	8.73E-01	2.89E+03	3.11E+03	1.98E+03	8.36E+02	9.30E-01	-3.17E-02
FIG01229492: hypothetical protein	8.73E-01	5.14E+03	5.81E+03	4.29E+03	5.27E+03	8.85E-01	-5.31E-02
Aspartyl-tRNA synthetase (EC 6.1.1.12)	8.78E-01	6.87E+04	6.32E+04	3.83E+04	4.34E+04	1.09E+00	3.62E-02
Endonuclease IV (EC 3.1.21.2)	8.78E-01	6.33E+04	5.85E+04	2.05E+04	4.45E+04	1.08E+00	3.39E-02
GTP-binding protein TypA/BipA	8.81E-01	2.31E+05	2.11E+05	1.15E+05	1.80E+05	1.09E+00	3.91E-02
2-oxoglutarate oxidoreductase, beta subunit (EC 1.2.7.3)	8.84E-01	1.37E+05	1.30E+05	4.98E+04	5.43E+04	1.05E+00	2.16E-02
hypothetical protein	8.85E-01	3.50E+04	3.85E+04	1.88E+04	3.32E+04	9.10E-01	-4.09E-02
D-alanine aminotransferase (EC 2.6.1.21)	8.85E-01	3.85E+04	3.46E+04	2.55E+04	3.48E+04	1.11E+00	4.59E-02
Transaldolase (EC 2.2.1.2)	8.87E-01	3.88E+05	4.21E+05	2.65E+05	2.79E+05	9.20E-01	-3.62E-02
Ornithine carbamoyltransferase (EC 2.1.3.3)	8.87E-01	6.60E+04	7.27E+04	6.03E+04	4.57E+04	9.09E-01	-4.16E-02
Glutamyl-tRNA synthetase (EC 6.1.1.17) @ Glutamyl-tRNA(Gln) synthetase (EC 6.1.1.24)	8.91E-01	1.61E+05	1.53E+05	7.54E+04	5.96E+04	1.05E+00	2.25E-02
Succinyl-CoA synthetase, alpha subunit-related enzymes	8.98E-01	4.56E+04	4.83E+04	6.42E+03	3.23E+04	9.43E-01	-2.54E-02

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
Cell division protein FtsH (EC 3.4.24.-)	9.01E-01	3.62E+05	3.89E+05	2.29E+05	2.54E+05	9.33E-01	-3.03E-02
Methylisocitrate lyase (EC 4.1.3.30)	9.03E-01	4.31E+05	4.06E+05	2.09E+05	2.60E+05	1.06E+00	2.59E-02
Hypothetical protein ywlG	9.05E-01	1.93E+04	2.01E+04	4.88E+03	9.14E+03	9.62E-01	-1.70E-02
2-methylcitrate synthase (EC 2.3.3.5)	9.07E-01	1.62E+05	1.74E+05	9.83E+04	1.32E+05	9.32E-01	-3.05E-02
Methionine ABC transporter substrate-binding protein	9.08E-01	2.04E+05	2.28E+05	2.68E+05	2.13E+05	8.93E-01	-4.92E-02
L-alanine-DL-glutamate epimerase	9.09E-01	1.51E+05	1.64E+05	4.28E+04	1.70E+05	9.21E-01	-3.58E-02
CBS domain containing protein	9.12E-01	2.02E+04	1.81E+04	1.88E+04	2.49E+04	1.12E+00	4.86E-02
Pyrimidine-nucleoside phosphorylase (EC 2.4.2.2)	9.12E-01	1.06E+05	1.02E+05	5.30E+04	1.19E+04	1.04E+00	1.62E-02
Ornithine cyclodeaminase (EC 4.3.1.12) / Siderophore staphylobactin biosynthesis protein SbnB	9.12E-01	3.34E+05	3.15E+05	1.16E+05	2.49E+05	1.06E+00	2.56E-02
Glucokinase (EC 2.7.1.2)	9.14E-01	1.99E+04	2.20E+04	2.51E+04	2.06E+04	9.02E-01	-4.46E-02
Nicotinate phosphoribosyltransferase (EC 2.4.2.11)	9.14E-01	8.24E+04	7.90E+04	4.46E+04	2.42E+04	1.04E+00	1.84E-02
acetyltransferase, GNAT family	9.16E-01	1.61E+05	1.59E+05	1.52E+04	3.17E+04	1.01E+00	6.34E-03
5,10-methylenetetrahydrofolate reductase (EC 1.5.1.20) / Homolog of homocysteine-binding domain	9.19E-01	3.27E+05	3.40E+05	1.39E+05	1.64E+05	9.61E-01	-1.74E-02

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
Zn-dependent hydroxyacylglutathione hydrolase / Polysulfide binding protein	9.22E-01	1.34E+03	1.19E+03	1.82E+03	1.72E+03	1.13E+00	5.21E-02
Phenylalanyl-tRNA synthetase alpha subunit	9.23E-01	4.67E+05	4.85E+05	2.00E+05	2.28E+05	9.63E-01	-1.64E-02
Formate--tetrahydrofolate ligase (EC 6.3.4.3)	9.25E-01	4.69E+05	4.75E+05	6.71E+04	6.54E+04	9.89E-01	-5.02E-03
3-ketoacyl-CoA thiolase (EC 2.3.1.16) @ Acetyl-CoA acetyltransferase (EC 2.3.1.9)	9.27E-01	1.55E+06	1.58E+06	2.02E+05	5.08E+05	9.80E-01	-8.83E-03
Butyryl-CoA dehydrogenase (EC 1.3.99.2)	9.28E-01	1.04E+06	1.01E+06	3.98E+05	3.33E+04	1.02E+00	9.90E-03
HMP-PP hydrolase (pyridoxal phosphatase) Cof, detected in genetic screen for thiamin metabolic genes (PMID:15292217)	9.39E-01	1.87E+04	1.96E+04	7.21E+03	1.55E+04	9.57E-01	-1.89E-02
hypothetical protein	9.43E-01	1.66E+04	1.82E+04	2.11E+04	2.93E+04	9.12E-01	-3.99E-02
Phosphopentomutase (EC 5.4.2.7)	9.43E-01	1.80E+05	1.83E+05	7.97E+04	1.15E+04	9.80E-01	-8.95E-03
Glycerophosphoryl diester phosphodiesterase (EC 3.1.4.46)	9.45E-01	1.54E+04	1.60E+04	1.27E+04	6.56E+03	9.61E-01	-1.71E-02
Coenzyme F420-0:L-glutamate ligase @ Coenzyme F420-1:L-	9.45E-01	1.11E+05	1.13E+05	3.53E+04	3.28E+04	9.82E-01	-7.89E-03
UDP-glucose/GDP-mannose dehydrogenase family	9.46E-01	5.97E+04	6.15E+04	1.45E+04	3.84E+04	9.71E-01	-1.27E-02
Macrolide glycosyltransferase (EC 2.4.1.-)	9.53E-01	8.39E+04	8.64E+04	2.64E+04	6.08E+04	9.71E-01	-1.27E-02

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
N-acetylmuramoyl-L-alanine amidase	9.54E-01	5.00E+04	5.21E+04	2.30E+04	5.08E+04	9.61E-01	-1.73E-02
Cell division protein FtsI [Peptidoglycan synthetase] (EC 2.4.1.129) / Transpeptidase, Penicillin binding protein transpeptidase domain	9.57E-01	2.00E+03	1.92E+03	2.32E+03	5.61E+02	1.04E+00	1.81E-02
HPr kinase/phosphorylase (EC 2.7.1.-) (EC 2.7.4.-)	9.58E-01	7.38E+04	7.25E+04	3.16E+04	2.56E+04	1.02E+00	7.90E-03
Methionine ABC transporter ATP-binding protein	9.58E-01	3.14E+04	3.23E+04	2.62E+04	7.00E+03	9.71E-01	-1.26E-02
Unspecified monosaccharide ABC transport system, substrate-binding component	9.59E-01	1.21E+05	1.19E+05	4.25E+04	5.77E+04	1.02E+00	8.33E-03
3-hydroxyisobutyrate dehydrogenase (EC 1.1.1.31)	9.59E-01	2.73E+05	2.77E+05	1.06E+05	7.38E+04	9.85E-01	-6.59E-03
DNA-directed RNA polymerase beta subunit (EC 2.7.7.6)	9.61E-01	8.00E+05	8.11E+05	3.13E+05	2.27E+05	9.85E-01	-6.35E-03
N-succinyl arginine/lysine racemase	9.64E-01	1.15E+04	1.11E+04	1.17E+04	8.85E+03	1.04E+00	1.57E-02
enterotoxin / cell-wall binding protein	9.67E-01	5.68E+02	5.89E+02	3.44E+02	7.26E+02	9.64E-01	-1.57E-02
hypothetical protein	9.67E-01	1.62E+04	1.59E+04	1.71E+03	1.07E+04	1.02E+00	7.90E-03
FIG01225386: hypothetical protein	9.68E-01	1.19E+04	1.22E+04	6.53E+03	9.64E+03	9.76E-01	-1.05E-02
DNA polymerase, DinB family	9.68E-01	4.95E+04	4.84E+04	1.86E+04	3.89E+04	1.02E+00	9.68E-03

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
Threonine synthase (EC 4.2.3.1)	9.68E-01	6.30E+04	6.15E+04	2.80E+04	5.40E+04	1.02E+00	1.05E-02
dTDP-4-dehydrorhamnose 3,5-epimerase (EC 5.1.3.13)	9.69E-01	1.80E+05	1.83E+05	8.99E+04	7.76E+04	9.84E-01	-6.82E-03
DNA gyrase subunit A (EC 5.99.1.3)	9.69E-01	1.52E+05	1.54E+05	8.30E+04	5.07E+04	9.85E-01	-6.67E-03
Molybdenum ABC transporter, periplasmic molybdenum-binding protein ModA (TC 3.A.1.8.1)	9.71E-01	2.34E+04	2.30E+04	1.55E+04	9.14E+03	1.02E+00	7.69E-03
FIG01229000: hypothetical protein	9.72E-01	2.69E+04	2.59E+04	2.27E+04	3.75E+04	1.04E+00	1.59E-02
Choloylglycine hydrolase (EC 3.5.1.24)	9.72E-01	5.20E+04	5.33E+04	4.75E+04	3.49E+04	9.76E-01	-1.06E-02
ATP synthase epsilon chain (EC 3.6.3.14)	9.72E-01	1.15E+04	1.12E+04	5.89E+03	1.00E+04	1.02E+00	9.66E-03
Pyruvate carboxylase (EC 6.4.1.1)	9.82E-01	1.18E+06	1.19E+06	4.68E+05	4.06E+05	9.93E-01	-3.08E-03
2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N-acetyltransferase (EC 2.3.1.89)	9.83E-01	1.64E+05	1.62E+05	1.55E+05	2.80E+04	1.01E+00	5.73E-03
Non-heme chloroperoxidase (EC 1.11.1.10)	9.89E-01	1.43E+05	1.44E+05	3.74E+04	6.80E+04	9.95E-01	-1.99E-03
3-hydroxybutyryl-CoA dehydrogenase (EC 1.1.1.157)	9.90E-01	4.79E+05	4.77E+05	2.24E+05	3.59E+04	1.00E+00	1.73E-03
Flagellar hook protein FlgE	9.92E-01	8.57E+04	8.54E+04	3.75E+04	2.95E+04	1.00E+00	1.55E-03
Patatin-like phospholipase family	9.93E-01	7.17E+04	7.14E+04	2.53E+04	3.36E+04	1.00E+00	1.37E-03
Heat shock protein GrpE	9.93E-01	1.27E+04	1.27E+04	9.30E+03	5.79E+03	1.00E+00	1.99E-03

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
5'-methylthioadenosine nucleosidase (EC 3.2.2.16) / S-adenosylhomocysteine nucleosidase (EC 3.2.2.9)	9.94E-01	1.84E+05	1.83E+05	5.74E+04	1.39E+04	1.00E+00	6.67E-04
Membrane-associated protein containing RNA-binding TRAM domain and ribonuclease PIN-domain, YacL B.subtilis ortholog	9.96E-01	1.49E+04	1.49E+04	8.01E+03	1.44E+03	1.00E+00	8.62E-04
UDP-N-acetylglucosamine 2-epimerase (EC 5.1.3.14)	9.96E-01	2.44E+05	2.45E+05	1.33E+05	1.38E+05	9.97E-01	-1.16E-03
FIG01226109: hypothetical protein	9.96E-01	2.39E+04	2.39E+04	1.63E+04	1.23E+04	9.97E-01	-1.17E-03
hypothetical protein	9.96E-01	2.63E+05	2.63E+05	5.96E+04	1.02E+05	9.99E-01	-5.40E-04
Arginyl-tRNA synthetase (EC 6.1.1.19)	9.97E-01	2.08E+05	2.08E+05	6.01E+04	6.26E+04	9.99E-01	-4.44E-04
hypothetical protein	9.97E-01	2.42E+06	2.42E+06	5.17E+05	5.74E+05	9.99E-01	-3.08E-04
NLP/P60 family protein	9.97E-01	6.35E+04	6.36E+04	3.95E+04	5.70E+04	9.98E-01	-9.58E-04

Shared proteins of the Bt isolate E-SE10.2 with the Bt isolate O-V84.2

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
Uroporphyrinogen III decarboxylase (EC 4.1.1.37)	1.46E-02	1.12E+05	2.04E+05	2.92E+04	2.48E+04	5.47E-01	-2.62E-01
Membrane protease family protein BA0301	1.67E-02	2.29E+04	1.06E+05	1.22E+04	2.58E+04	2.15E-01	-6.68E-01
ATP synthase alpha chain (EC 3.6.3.14)	2.30E-02	5.19E+05	1.21E+06	2.44E+05	2.28E+05	4.28E-01	-3.69E-01
Electron transfer flavoprotein, beta subunit	2.52E-02	6.60E+05	2.57E+05	1.13E+05	1.54E+05	2.57E+00	4.09E-01
ATP synthase gamma chain (EC 3.6.3.14)	2.83E-02	1.31E+05	4.28E+05	1.51E+04	9.25E+04	3.07E-01	-5.13E-01
FIG01229881: hypothetical protein	3.35E-02	1.17E+03	3.72E+03	1.01E+03	9.49E+02	3.13E-01	-5.04E-01
Cell division protein FtsZ (EC 3.4.24.-)	3.66E-02	1.49E+04	3.22E+04	6.88E+03	6.84E+03	4.63E-01	-3.35E-01
probable metal-dependent peptidase	4.26E-02	6.47E+03	1.68E+04	2.14E+02	3.83E+03	3.86E-01	-4.14E-01
Triosephosphate isomerase (EC 5.3.1.1)	5.96E-02	5.72E+05	3.68E+05	9.42E+04	9.78E+04	1.56E+00	1.92E-01
FIG01225442: hypothetical protein	6.15E-02	5.56E+03	2.20E+04	8.71E+03	4.97E+03	2.53E-01	-5.97E-01
Tryptophan 2-monooxygenase (EC 1.13.12.3)	6.37E-02	3.89E+04	4.75E+04	4.04E+03	4.26E+03	8.18E-01	-8.71E-02
2H phosphoesterase superfamily protein Bsu1186 (yjcG)	6.97E-02	4.59E+03	1.84E+04	2.99E+03	7.46E+03	2.50E-01	-6.03E-01
6-phosphofructokinase (EC 2.7.1.11)	7.02E-02	4.49E+05	3.01E+05	7.92E+04	2.86E+04	1.49E+00	1.74E-01
CBS domain protein AcuB	8.01E-02	3.28E+04	1.05E+05	1.89E+04	4.20E+04	3.13E-01	-5.04E-01
Azoreductase	8.08E-02	1.54E+05	3.64E+05	1.85E+04	1.13E+05	4.24E-01	-3.72E-01

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
UDP-N-acetylglucosamine 1-carboxyvinyltransferase (EC 2.5.1.7)	8.91E-02	1.39E+04	1.70E+03	7.17E+03	2.02E+03	8.18E+00	9.13E-01
hypothetical Membrane Spanning Protein	8.91E-02	7.11E+03	2.22E+04	4.26E+03	9.29E+03	3.20E-01	-4.94E-01
LSU ribosomal protein L19p	9.20E-02	1.21E+05	3.26E+05	5.12E+04	1.27E+05	3.72E-01	-4.29E-01
GTP-sensing transcriptional pleiotropic repressor codY	9.86E-02	9.28E+04	4.19E+04	9.85E+03	3.19E+04	2.22E+00	3.46E-01
Menaquinone-cytochrome c reductase, cytochrome B subunit	1.00E-01	2.86E+03	1.32E+04	1.34E+03	6.34E+03	2.17E-01	-6.63E-01
Arginine deiminase (EC 3.5.3.6)	1.14E-01	3.43E+05	1.02E+05	1.61E+05	4.35E+04	3.35E+00	5.25E-01
Chaperone protein DnaK	1.14E-01	2.18E+05	1.34E+05	5.80E+04	3.72E+04	1.63E+00	2.11E-01
acetyltransferase, GNAT family	1.47E-01	2.29E+03	8.01E+03	2.06E+03	4.53E+03	2.86E-01	-5.44E-01
Butyrate kinase (EC 2.7.2.7)	1.55E-01	3.86E+04	4.94E+04	6.33E+03	8.31E+03	7.82E-01	-1.07E-01
Acetyl-CoA:acetoacetyl-CoA transferase, beta subunit (EC 2.8.3.8)	1.55E-01	9.29E+04	5.04E+04	1.95E+04	3.44E+04	1.84E+00	2.65E-01
UDP-N-acetylglucosamine 1-carboxyvinyltransferase (EC 2.5.1.7)	1.60E-01	7.54E+03	1.64E+04	7.30E+03	4.07E+03	4.60E-01	-3.37E-01
Cystathionine beta-synthase (EC 4.2.1.22)	1.61E-01	4.65E+03	1.44E+03	2.62E+03	6.91E+02	3.22E+00	5.08E-01
FIG01225730: hypothetical protein	1.75E-01	5.53E+03	1.59E+04	1.50E+03	8.80E+03	3.47E-01	-4.59E-01
FIG01225993: hypothetical protein	1.76E-01	1.10E+04	3.69E+04	6.21E+03	2.24E+04	2.98E-01	-5.25E-01
Formamidase amiF (EC 3.5.1.49)	1.77E-01	2.17E+03	6.01E+03	1.42E+03	3.37E+03	3.61E-01	-4.43E-01

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
RNA-binding protein Hfq	1.77E-01	1.06E+05	2.85E+04	6.76E+04	3.73E+04	3.71E+00	5.70E-01
SSU ribosomal protein S5p (S2e)	1.85E-01	6.62E+03	1.11E+04	2.20E+03	3.95E+03	5.99E-01	-2.22E-01
PTS system, cellobiose-specific IIA component (EC 2.7.1.69)	1.88E-01	9.26E+03	1.89E+04	7.72E+03	7.18E+03	4.90E-01	-3.10E-01
Isocitrate dehydrogenase [NADP] (EC 1.1.1.42)	1.91E-01	2.04E+05	3.09E+05	9.17E+04	6.92E+04	6.58E-01	-1.82E-01
Spore coat protein Z	2.02E-01	1.09E+04	1.74E+05	9.00E+03	1.51E+05	6.27E-02	-1.20E+00
FIG01228325: hypothetical protein	2.03E-01	4.47E+02	2.96E+03	9.56E+01	2.33E+03	1.51E-01	-8.21E-01
Branched-chain alpha-keto acid dehydrogenase, E1 component, beta subunit (EC 1.2.4.4)	2.04E-01	2.54E+04	1.77E+04	5.36E+03	6.82E+03	1.43E+00	1.57E-01
SSU ribosomal protein S2p (SAe)	2.04E-01	3.05E+05	1.80E+05	1.15E+05	8.09E+04	1.70E+00	2.30E-01
Enoyl-[acyl-carrier-protein] reductase [NADH] (EC 1.3.1.9)	2.05E-01	3.79E+03	1.41E+04	9.03E+02	9.65E+03	2.69E-01	-5.71E-01
Unspecified monosaccharide ABC transport system, permease component Ia (FIG025991) / Unspecified monosaccharide ABC transport system, permease component Ib (FIG143636)	2.09E-01	2.12E+04	3.81E+04	1.43E+04	1.33E+04	5.57E-01	-2.54E-01
FIG006542: Phosphoesterase	2.17E-01	1.88E+04	8.85E+03	9.23E+03	6.98E+03	2.12E+00	3.27E-01
Methionine aminopeptidase (EC 3.4.11.18)	2.20E-01	3.81E+05	2.07E+05	4.26E+04	1.74E+05	1.84E+00	2.66E-01

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
LSU ribosomal protein L4p (L1e)	2.20E-01	4.01E+04	7.11E+04	2.67E+04	2.54E+04	5.65E-01	-2.48E-01
LSU ribosomal protein L16p (L10e)	2.21E-01	4.52E+04	1.66E+04	2.83E+04	1.63E+04	2.72E+00	4.35E-01
Menaquinone-cytochrome C reductase iron-sulfur subunit	2.24E-01	1.21E+05	2.14E+05	6.32E+04	9.02E+04	5.65E-01	-2.48E-01
NAD-specific glutamate dehydrogenase (EC 1.4.1.2)	2.25E-01	6.04E+05	1.33E+06	4.38E+05	7.22E+05	4.54E-01	-3.43E-01
hypothetical Membrane Spanning Protein	2.31E-01	3.62E+03	1.75E+04	2.71E+03	1.43E+04	2.06E-01	-6.85E-01
Rod shape-determining protein MreB	2.35E-01	1.05E+04	3.10E+04	6.18E+03	2.15E+04	3.37E-01	-4.72E-01
Nucleoside-diphosphate-sugar epimerases	2.39E-01	6.37E+03	3.85E+03	1.63E+03	2.59E+03	1.66E+00	2.19E-01
NADH-ubiquinone oxidoreductase chain H (EC 1.6.5.3)	2.39E-01	1.61E+03	1.28E+04	3.09E+02	1.17E+04	1.25E-01	-9.03E-01
Transcriptional regulator, ArsR family	2.42E-01	1.17E+03	3.72E+03	8.52E+02	2.73E+03	3.14E-01	-5.03E-01
LSU ribosomal protein L35p	2.49E-01	8.29E+03	1.67E+04	4.08E+03	9.15E+03	4.97E-01	-3.04E-01
LSU ribosomal protein L36p	2.51E-01	1.79E+05	2.96E+05	9.69E+04	1.14E+05	6.06E-01	-2.17E-01
Translation initiation factor 1	2.58E-01	8.92E+03	1.74E+04	7.71E+03	8.07E+03	5.12E-01	-2.91E-01
Cytochrome c-type biogenesis protein Ccs1/ResB	2.58E-01	5.99E+04	1.44E+04	5.03E+04	1.21E+03	4.15E+00	6.18E-01
Dihydroxyacetone kinase, ATP-dependent (EC 2.7.1.29)	2.59E-01	5.97E+04	9.47E+04	1.63E+04	3.93E+04	6.30E-01	-2.01E-01
Hypothetical protein YggS, proline synthase co-transcribed bacterial homolog PROSC	2.60E-01	5.09E+03	1.35E+04	4.74E+02	9.37E+03	3.77E-01	-4.24E-01

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
Proline dipeptidase (EC 3.4.13.9)	2.60E-01	2.44E+05	2.99E+05	5.61E+04	4.55E+04	8.16E-01	-8.83E-02
Fe-S oxidoreductase	2.67E-01	1.61E+04	2.71E+04	1.44E+03	1.26E+04	5.93E-01	-2.27E-01
Molybdenum cofactor biosynthesis protein MoaC	2.73E-01	2.74E+04	1.83E+04	9.43E+03	8.06E+03	1.50E+00	1.76E-01
Ferrichrome-binding periplasmic protein precursor (TC 3.A.1.14.3)	2.74E-01	2.58E+04	4.70E+04	1.76E+04	2.28E+04	5.48E-01	-2.61E-01
ATP-dependent hsl protease ATP-binding subunit HslU	2.75E-01	2.64E+05	1.42E+05	1.42E+05	2.47E+04	1.86E+00	2.69E-01
Tellurium resistance protein TerD	2.76E-01	6.01E+04	8.90E+04	1.55E+04	3.37E+04	6.75E-01	-1.70E-01
Ribosomal subunit interface protein	2.78E-01	2.80E+04	1.04E+05	2.94E+04	9.03E+04	2.68E-01	-5.71E-01
Large-conductance mechanosensitive channel	2.79E-01	2.25E+02	7.55E+02	4.56E+01	6.26E+02	2.97E-01	-5.27E-01
Cysteinyl-tRNA synthetase (EC 6.1.1.16)	2.83E-01	5.56E+04	1.08E+05	3.35E+04	6.09E+04	5.17E-01	-2.86E-01
Cytochrome c oxidase polypeptide III (EC 1.9.3.1)	2.84E-01	3.89E+04	1.06E+05	2.32E+03	7.98E+04	3.68E-01	-4.34E-01
Spore cortex-lytic enzyme CwlJ	2.85E-01	1.13E+05	1.51E+05	1.78E+04	4.64E+04	7.46E-01	-1.27E-01
ATP synthase beta chain (EC 3.6.3.14)	2.86E-01	2.95E+05	5.65E+05	6.61E+04	3.26E+05	5.22E-01	-2.83E-01
Sporulation protein, YTFJ Bacillus subtilis ortholog	3.03E-01	6.07E+03	1.25E+04	6.23E+03	6.97E+03	4.87E-01	-3.12E-01
Adenine phosphoribosyltransferase (EC 2.4.2.7)	3.06E-01	1.25E+04	7.19E+04	5.56E+03	7.56E+04	1.73E-01	-7.61E-01
Extracellular protein	3.11E-01	8.05E+03	1.56E+04	8.05E+03	7.85E+03	5.17E-01	-2.87E-01
Ribosome recycling factor	3.11E-01	5.34E+04	7.02E+04	1.60E+04	1.92E+04	7.61E-01	-1.19E-01

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
Ribose-phosphate pyrophosphokinase (EC 2.7.6.1)	3.20E-01	8.96E+03	1.87E+04	7.68E+03	1.23E+04	4.80E-01	-3.19E-01
Phosphocarrier protein of PTS system	3.35E-01	2.14E+04	5.49E+04	2.82E+04	4.37E+04	3.89E-01	-4.10E-01
Membrane-attached cytochrome c550	3.37E-01	5.60E+04	1.71E+05	2.79E+04	1.59E+05	3.28E-01	-4.84E-01
Transcription termination factor Rho	3.42E-01	3.05E+04	1.87E+06	7.18E+03	2.57E+06	1.63E-02	-1.79E+00
LSU ribosomal protein L21p	3.45E-01	2.18E+04	4.23E+04	3.33E+03	2.90E+04	5.15E-01	-2.88E-01
Aspartate-semialdehyde dehydrogenase (EC 1.2.1.11)	3.45E-01	1.66E+04	7.66E+03	1.26E+04	3.85E+03	2.17E+00	3.36E-01
SSU ribosomal protein S12p (S23e)	3.51E-01	1.43E+04	3.40E+04	4.11E+03	2.83E+04	4.20E-01	-3.77E-01
DNA-binding protein HBsu	3.57E-01	6.19E+04	3.67E+04	3.63E+04	1.47E+04	1.69E+00	2.27E-01
SSU ribosomal protein S9p (S16e)	3.76E-01	2.03E+03	3.91E+03	1.22E+03	2.84E+03	5.19E-01	-2.84E-01
Glyoxalase family protein	3.89E-01	3.38E+02	1.41E+03	1.02E+02	1.71E+03	2.39E-01	-6.21E-01
Ferredoxin	3.91E-01	5.28E+02	3.15E+03	3.79E+02	4.19E+03	1.67E-01	-7.76E-01
Hydroxymethylpyrimidine ABC transporter, substrate-binding component	3.94E-01	6.79E+02	9.90E+02	4.85E+02	2.39E+02	6.86E-01	-1.64E-01
comA operon protein, putative	3.95E-01	1.92E+02	1.32E+03	4.10E+01	1.82E+03	1.45E-01	-8.37E-01
Ferrichrome-binding periplasmic protein precursor (TC 3.A.1.14.3)	3.98E-01	9.34E+03	1.76E+04	8.47E+03	1.23E+04	5.30E-01	-2.76E-01
Phosphatidylinositol-specific phospholipase C (EC 4.6.1.13)	4.05E-01	9.22E+02	1.74E+02	1.24E+03	4.39E+01	5.31E+00	7.25E-01

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
Lipid A export ATP-binding/permease protein MsbA	4.14E-01	6.18E+03	9.45E+03	2.85E+03	5.28E+03	6.54E-01	-1.84E-01
Peptide chain release factor 2; programmed frameshift-containing	4.21E-01	1.93E+04	6.51E+03	2.21E+04	1.91E+03	2.97E+00	4.73E-01
Oligopeptide transport system permease protein OppB (TC 3.A.1.5.1)	4.27E-01	3.56E+03	1.15E+04	2.00E+03	1.40E+04	3.08E-01	-5.11E-01
DNA-binding protein HBsu	4.34E-01	6.72E+04	1.45E+04	9.39E+04	1.67E+04	4.65E+00	6.67E-01
hypothetical fig|282458.1.peg.581 homolog	4.43E-01	2.37E+02	1.51E+03	1.31E+02	2.32E+03	1.57E-01	-8.04E-01
3-hydroxyacyl-[acyl-carrier-protein] dehydratase, FabZ form (EC 4.2.1.59)	4.43E-01	4.37E+04	7.50E+04	2.53E+04	5.54E+04	5.83E-01	-2.35E-01
LSU ribosomal protein L20p	4.44E-01	8.02E+04	5.47E+04	4.68E+04	5.83E+03	1.47E+00	1.67E-01
Acetyl-coenzyme A synthetase (EC 6.2.1.1)	4.64E-01	1.89E+03	3.61E+03	1.24E+03	3.24E+03	5.24E-01	-2.81E-01
Thiol peroxidase, Tpx-type (EC 1.11.1.15)	4.65E-01	6.16E+04	8.88E+04	1.37E+04	5.21E+04	6.94E-01	-1.58E-01
hypothetical Membrane Spanning Protein	4.66E-01	1.60E+04	2.14E+04	1.02E+04	3.94E+03	7.49E-01	-1.26E-01
ATP synthase F0 sector subunit a	4.76E-01	5.66E+02	1.02E+03	6.56E+02	7.42E+02	5.57E-01	-2.54E-01
UDP-N-acetylmuramoylalanine--D-glutamate ligase (EC 6.3.2.9)	4.76E-01	2.91E+04	1.55E+04	2.37E+04	1.78E+04	1.87E+00	2.72E-01
Stage III sporulation protein D	4.80E-01	2.44E+03	4.23E+03	1.41E+03	3.52E+03	5.77E-01	-2.39E-01

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
FIG009439: Cytosolic protein containing multiple CBS domains	4.98E-01	1.01E+04	5.70E+03	8.15E+03	5.87E+03	1.76E+00	2.47E-01
FIG01226376: hypothetical protein	4.99E-01	4.41E+02	1.38E+03	5.50E+02	1.96E+03	3.20E-01	-4.95E-01
Nucleoside 5-triphosphatase RdgB (dHAPTP, dITP, XTP-specific) (EC 3.6.1.15)	5.01E-01	8.82E+02	2.85E+03	8.69E+02	4.16E+03	3.10E-01	-5.09E-01
Beta-glucosidase (EC 3.2.1.21)	5.24E-01	2.94E+04	5.04E+04	3.69E+04	3.68E+04	5.84E-01	-2.34E-01
Signal recognition particle, subunit Ffh SRP54 (TC 3.A.5.1.1)	5.31E-01	8.70E+03	2.81E+03	1.35E+04	2.07E+03	3.10E+00	4.91E-01
Protein-glutamine gamma-glutamyltransferase (EC 2.3.2.13)	5.41E-01	7.08E+03	4.59E+03	4.43E+03	4.70E+03	1.54E+00	1.88E-01
DEAD-box ATP-dependent RNA helicase CshA (EC 3.6.4.13)	5.44E-01	3.33E+04	4.70E+04	3.09E+04	1.56E+04	7.09E-01	-1.49E-01
Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein OppA (TC 3.A.1.5.1)	5.44E-01	3.35E+04	2.39E+04	1.34E+04	2.09E+04	1.40E+00	1.47E-01
ATP-dependent Clp protease ATP-binding subunit ClpX	5.47E-01	5.55E+02	1.00E+03	3.84E+02	1.04E+03	5.55E-01	-2.55E-01
NAD kinase (EC 2.7.1.23)	5.48E-01	1.01E+04	1.33E+04	7.64E+03	1.26E+03	7.61E-01	-1.19E-01
Glycine betaine ABC transport system, ATP-binding protein OpuAA (EC 3.6.3.32)	5.50E-01	1.02E+04	1.27E+04	1.60E+03	5.91E+03	8.06E-01	-9.35E-02
Lipoate synthase	5.63E-01	6.38E+04	3.65E+04	6.64E+04	2.66E+04	1.74E+00	2.42E-01

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
Cold shock protein CspD	5.65E-01	1.75E+04	1.27E+04	5.08E+03	1.18E+04	1.38E+00	1.40E-01
Stage V sporulation protein required for dehydratation of the spore core and assembly of the coat (SpoVS)	5.69E-01	4.26E+03	2.93E+03	3.39E+03	4.11E+02	1.45E+00	1.62E-01
Serine protease (EC 3.4.21.-)	5.79E-01	5.50E+03	9.48E+03	3.69E+03	1.03E+04	5.80E-01	-2.37E-01
Stage VI sporulation protein D	5.84E-01	4.24E+03	5.08E+03	1.78E+03	1.68E+03	8.35E-01	-7.84E-02
Autoinducer 2 (AI-2) ABC transport system, periplasmic AI-2 binding protein LsrB	5.85E-01	3.25E+04	2.11E+04	2.88E+04	1.44E+04	1.54E+00	1.87E-01
Acyl carrier protein	5.86E-01	1.02E+04	1.53E+04	5.56E+03	1.33E+04	6.65E-01	-1.77E-01
Tryptophan 2,3-dioxygenase (EC 1.13.11.11)	5.89E-01	1.13E+03	7.66E+02	7.85E+02	7.37E+02	1.48E+00	1.69E-01
hypothetical protein	6.13E-01	3.08E+04	3.88E+04	2.32E+04	3.29E+03	7.94E-01	-1.00E-01
NADPH-dependent glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.13)	6.19E-01	3.81E+04	4.24E+04	2.07E+03	1.28E+04	8.98E-01	-4.68E-02
Catabolite control protein A	6.20E-01	3.34E+04	4.48E+04	2.40E+04	2.79E+04	7.45E-01	-1.28E-01
Methionine ABC transporter substrate-binding protein	6.27E-01	2.69E+03	3.30E+03	7.25E+02	1.77E+03	8.16E-01	-8.83E-02
Single-stranded DNA-binding protein	6.45E-01	1.22E+05	1.60E+05	5.97E+04	1.16E+05	7.60E-01	-1.19E-01
Agmatinase (EC 3.5.3.11)	6.46E-01	1.45E+03	2.08E+03	1.27E+03	1.75E+03	6.99E-01	-1.56E-01
Tryptophanyl-tRNA synthetase (EC 6.1.1.2)	6.50E-01	1.08E+05	1.38E+05	9.27E+04	4.89E+04	7.80E-01	-1.08E-01

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
GMP synthase [glutamine-hydrolyzing], amidotransferase subunit (EC 6.3.5.2) / GMP synthase [glutamine-hydrolyzing], ATP pyrophosphatase subunit (EC 6.3.5.2)	6.58E-01	7.81E+04	6.35E+04	4.25E+04	3.10E+04	1.23E+00	8.99E-02
Universal stress protein family	6.60E-01	2.16E+04	2.78E+04	9.13E+03	1.99E+04	7.77E-01	-1.09E-01
Prolipoprotein diacylglyceryl transferase (EC 2.4.99.-)	6.64E-01	6.35E+02	1.02E+03	4.56E+02	1.30E+03	6.20E-01	-2.08E-01
acetyltransferase, GNAT family	6.64E-01	7.10E+03	9.36E+03	4.65E+03	6.84E+03	7.59E-01	-1.20E-01
LSU ribosomal protein L2p (L8e)	6.66E-01	2.65E+05	3.19E+05	1.60E+05	1.15E+05	8.32E-01	-7.98E-02
PTS system, glucose-specific IIA component (EC 2.7.1.69)	6.73E-01	7.08E+03	5.61E+03	4.64E+03	2.94E+03	1.26E+00	1.01E-01
hypothetical Membrane Spanning Protein	6.76E-01	1.46E+04	1.73E+04	8.45E+03	6.00E+03	8.43E-01	-7.43E-02
Alkaline shock protein	6.80E-01	3.13E+03	1.70E+03	5.18E+03	6.97E+02	1.84E+00	2.65E-01
N-acetyl-L,L-diaminopimelate deacetylase (EC 3.5.1.47)	6.81E-01	1.18E+04	1.49E+04	7.79E+03	9.46E+03	7.89E-01	-1.03E-01
FIG146085: 3'-to-5' oligoribonuclease A, Bacillus type	6.92E-01	1.12E+04	1.53E+04	1.23E+04	1.12E+04	7.31E-01	-1.36E-01
Peptidase, M42 family	6.98E-01	3.55E+05	4.01E+05	5.90E+04	1.71E+05	8.86E-01	-5.26E-02
RND multidrug efflux transporter; Acriflavin resistance protein	6.99E-01	1.68E+04	2.23E+04	1.63E+04	1.62E+04	7.53E-01	-1.23E-01

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
N-acetylmuramoyl-L-alanine amidase, family 2 (EC 3.5.1.28)	7.02E-01	2.35E+04	1.55E+04	3.00E+04	1.28E+04	1.52E+00	1.81E-01
DNA-directed RNA polymerase alpha subunit (EC 2.7.7.6)	7.10E-01	2.35E+05	2.17E+05	5.87E+03	7.10E+04	1.08E+00	3.39E-02
Two-component response regulator YvcP	7.10E-01	9.82E+04	1.21E+05	7.40E+04	6.82E+04	8.09E-01	-9.23E-02
alternate gene name: ipa-62r	7.14E-01	2.65E+05	2.28E+05	7.96E+04	1.43E+05	1.17E+00	6.67E-02
L-lactate dehydrogenase (EC 1.1.1.27)	7.20E-01	7.85E+04	6.76E+04	4.29E+04	2.03E+04	1.16E+00	6.46E-02
Protease production regulatory protein Hpr (ScoC)	7.20E-01	1.32E+04	1.12E+04	8.23E+03	7.49E+02	1.18E+00	7.01E-02
Histidyl-tRNA synthetase (EC 6.1.1.21)	7.24E-01	2.66E+04	3.72E+04	8.90E+03	4.44E+04	7.17E-01	-1.44E-01
Metal-dependent hydrolase	7.32E-01	1.34E+04	1.80E+04	7.27E+03	1.97E+04	7.43E-01	-1.29E-01
hypothetical Membrane Spanning Protein	7.32E-01	1.97E+04	1.43E+04	1.61E+04	1.95E+04	1.38E+00	1.39E-01
Peptidoglycan N-acetylglucosamine deacetylase (EC 3.5.1.-)	7.35E-01	3.07E+03	4.65E+03	1.73E+03	6.91E+03	6.60E-01	-1.80E-01
Translocation-enhancing protein TepA	7.39E-01	1.21E+04	1.62E+04	7.51E+03	1.77E+04	7.47E-01	-1.27E-01
Acetyl-coenzyme A carboxyl transferase alpha chain (EC 6.4.1.2)	7.40E-01	2.47E+04	2.06E+04	1.48E+04	1.27E+04	1.19E+00	7.72E-02
Phage major capsid protein	7.44E-01	1.39E+05	1.84E+05	1.18E+05	1.83E+05	7.57E-01	-1.21E-01
FIG01226061: hypothetical protein	7.51E-01	2.35E+04	2.91E+04	1.29E+04	2.44E+04	8.09E-01	-9.19E-02
Outer spore coat protein E	7.51E-01	5.01E+05	5.97E+05	3.77E+05	3.11E+05	8.39E-01	-7.62E-02
tRNA:m(5)U-54 MTase gid	7.56E-01	1.22E+05	1.94E+05	1.88E+05	3.18E+05	6.28E-01	-2.02E-01

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
transcriptional regulator, XRE family	7.64E-01	5.18E+06	4.26E+06	4.16E+06	2.52E+06	1.22E+00	8.47E-02
SSU ribosomal protein S17p (S11e)	7.68E-01	2.20E+04	1.88E+04	9.98E+03	1.43E+04	1.17E+00	6.84E-02
Iron-sulfur cluster assembly protein SufB	7.75E-01	3.69E+04	4.39E+04	8.23E+03	3.68E+04	8.40E-01	-7.58E-02
RNA binding protein, contains ribosomal protein S1 domain	7.85E-01	2.23E+04	2.57E+04	1.84E+04	4.64E+03	8.70E-01	-6.07E-02
UDP-N-acetylmuramate--alanine ligase (EC 6.3.2.8)	7.86E-01	5.27E+03	6.54E+03	2.60E+03	6.87E+03	8.05E-01	-9.43E-02
Glycine cleavage system H protein	8.02E-01	6.28E+04	5.34E+04	6.83E+03	5.69E+04	1.18E+00	7.04E-02
Predicted ATPase related to phosphate starvation-inducible protein PhoH	8.03E-01	2.71E+03	2.10E+03	2.62E+03	3.04E+03	1.30E+00	1.12E-01
Alkyl hydroperoxide reductase protein F (EC 1.6.4.-)	8.04E-01	2.83E+05	3.24E+05	2.06E+05	1.75E+05	8.72E-01	-5.95E-02
Fumarate hydratase class I, aerobic (EC 4.2.1.2)	8.07E-01	2.41E+04	2.60E+04	9.06E+03	8.81E+03	9.27E-01	-3.31E-02
Non-specific DNA-binding protein Dps / Iron-binding ferritin-like antioxidant protein / Ferroxidase (EC 1.16.3.1)	8.41E-01	4.61E+04	5.01E+04	2.71E+04	1.67E+04	9.20E-01	-3.61E-02
Peptidase T (EC 3.4.11.4)	8.42E-01	3.52E+05	3.29E+05	1.39E+05	1.23E+05	1.07E+00	2.89E-02
general stress protein 26	8.49E-01	2.53E+04	2.20E+04	1.39E+04	2.44E+04	1.15E+00	6.18E-02
Excinuclease ABC subunit A	8.54E-01	1.36E+04	1.46E+04	4.36E+03	7.40E+03	9.32E-01	-3.04E-02

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
S-ribosylhomocysteine lyase (EC 4.4.1.21) / Autoinducer-2 production protein LuxS	8.58E-01	5.25E+04	6.09E+04	5.01E+04	5.82E+04	8.61E-01	-6.48E-02
NAD(P)H oxidoreductase YRKL (EC 1.6.99.-) @ Putative NADPH-quinone reductase (modulator of drug activity B) @ Flavodoxin 2	8.73E-01	1.26E+03	1.46E+03	1.07E+03	1.70E+03	8.62E-01	-6.43E-02
hypothetical protein	8.79E-01	3.57E+04	3.34E+04	1.56E+04	1.93E+04	1.07E+00	2.93E-02
D-alanyl-D-alanine carboxypeptidase (EC 3.4.16.4)	8.82E-01	8.02E+03	7.55E+03	3.91E+03	3.32E+03	1.06E+00	2.61E-02
LSU ribosomal protein L17p	8.88E-01	2.15E+04	2.46E+04	3.17E+04	1.37E+04	8.74E-01	-5.83E-02
Alkyl hydroperoxide reductase protein C (EC 1.6.4.-)	8.91E-01	8.18E+05	7.92E+05	2.45E+05	1.90E+05	1.03E+00	1.41E-02
Purine nucleoside phosphorylase (EC 2.4.2.1)	8.94E-01	3.90E+04	4.19E+04	2.78E+04	2.24E+04	9.30E-01	-3.15E-02
Two-component response regulator SA14-24	9.01E-01	3.32E+04	3.43E+04	1.25E+04	6.20E+03	9.68E-01	-1.40E-02
SSU ribosomal protein S8p (S15Ae)	9.09E-01	1.73E+04	1.95E+04	2.67E+04	1.60E+04	8.87E-01	-5.23E-02
FIG01228193: hypothetical protein	9.11E-01	2.65E+03	2.94E+03	1.07E+03	3.90E+03	9.01E-01	-4.53E-02
Menaquinone-cytochrome C oxidoreductase, cytochrome C subunit	9.12E-01	4.31E+04	4.81E+04	4.20E+04	5.97E+04	8.97E-01	-4.74E-02
lysophospholipase-like family protein	9.14E-01	4.84E+03	5.46E+03	7.75E+03	4.85E+03	8.87E-01	-5.21E-02
Hypothetical protein SAV1839	9.18E-01	4.47E+04	4.76E+04	4.12E+04	1.93E+04	9.38E-01	-2.77E-02

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
LSU ribosomal protein L3p (L3e)	9.22E-01	2.68E+04	2.87E+04	2.56E+04	1.54E+04	9.36E-01	-2.87E-02
GTP-binding protein Obg	9.44E-01	1.32E+03	1.39E+03	8.87E+02	1.34E+03	9.50E-01	-2.23E-02
ATPase, AAA family	9.46E-01	3.41E+04	3.10E+04	5.74E+04	4.80E+04	1.10E+00	4.18E-02
Alanine dehydrogenase (EC 1.4.1.1)	9.52E-01	4.58E+05	4.65E+05	1.55E+05	9.24E+04	9.85E-01	-6.43E-03
CBS domain protein, lmo1865 homolog	9.66E-01	1.13E+03	1.10E+03	6.49E+02	9.07E+02	1.03E+00	1.15E-02
Dihydrolipoamide dehydrogenase of pyruvate dehydrogenase complex (EC 1.8.1.4)	9.73E-01	2.00E+06	2.02E+06	6.76E+05	7.23E+05	9.90E-01	-4.43E-03
Biotin carboxyl carrier protein of methylcrotonyl-CoA carboxylase	9.74E-01	6.69E+03	6.87E+03	5.05E+03	6.99E+03	9.74E-01	-1.12E-02
Inner membrane protein translocase component YidC, short form OxaI-like	9.81E-01	4.01E+04	3.98E+04	1.31E+04	1.68E+04	1.01E+00	3.41E-03

2.2. - GROUPED DATA ANALYSIS O-V84.2

A PCA analysis was done with the non-normalized area of the peaks (Figure 9) and with the area peaks corrected by the total areas sum (Figure 10) of the concentrated supernatant at 24 h and 48 h.

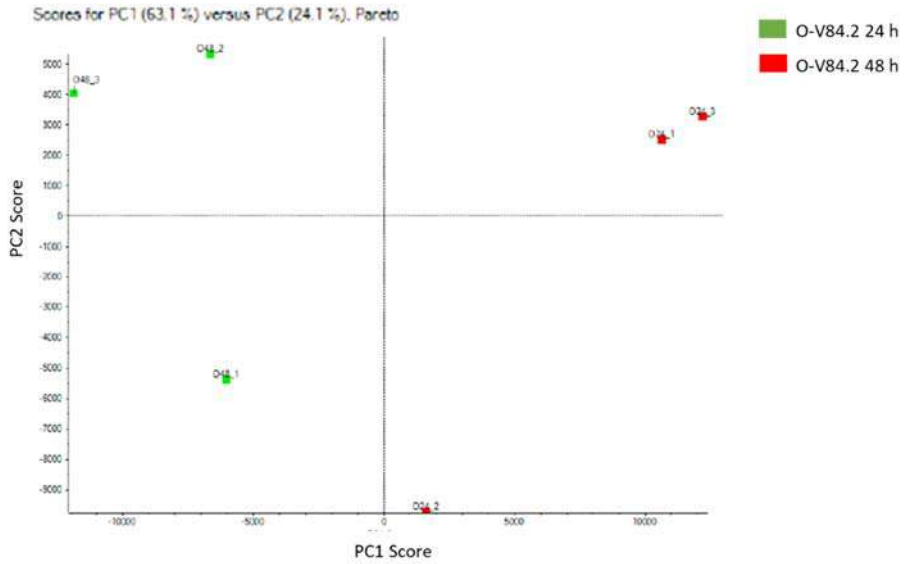

Figure 9. PCA analysis with the non-normalized area of the concentrated supernatant of the *Bt* isolate 24 h and 48 h.

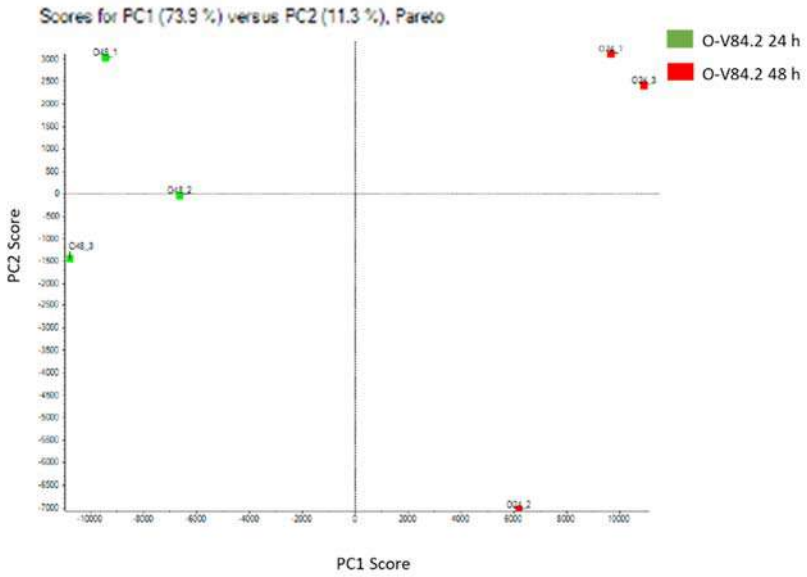

Figure 10. PCA analysis with the area peaks corrected by the total areas sum of the concentrated supernatant 24 h and 48 h of the *Bt* isolate E-SE10.2

2.1. - DIFFERENTAL EXPRESION ANALYSIS O-V84.2

A Student t-test analysis was performed with the supernatant of the *Bt* isolate O-V84.2 at 24 h and 48 h in the Marker View 1.3. As a result, 159 proteins were differential expressed between two groups, 15 up- regulated (dark grey) and 133 down-regulated (light grey).

Specific proteins of the Bt isolate O-V84.2

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
Alcohol dehydrogenase (EC 1.1.1.1)	1.08E-05	2.94E+04	4.14E+05	1.59E+04	1.16E+04	7.12E-02	-1.15E+00
Dihydrolipoamide dehydrogenase of branched-chain alpha-keto acid dehydrogenase (EC 1.8.1.4)	2.53E-05	6.69E+04	6.28E+05	2.98E+04	2.21E+04	1.07E-01	-9.72E-01
Prephenate dehydratase (EC 4.2.1.51)	8.54E-05	2.02E+03	2.38E+04	1.55E+03	1.70E+03	8.49E-02	-1.07E+00
hypothetical protein	2.00E-04	4.17E+04	3.27E+05	2.00E+04	2.69E+04	1.28E-01	-8.94E-01
O-methyltransferase (EC 2.1.1.-)	4.20E-04	2.04E+03	3.17E+04	1.01E+03	2.27E+03	6.44E-02	-1.19E+00
Adenylosuccinate lyase (EC 4.3.2.2)	4.60E-04	1.03E+04	8.62E+04	8.67E+03	8.99E+03	1.19E-01	-9.23E-01
Spermidine synthase (EC 2.5.1.16)	6.50E-04	4.66E+04	1.93E+05	7.75E+03	1.51E+04	2.41E-01	-6.18E-01
NADPH-dependent glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.13)	6.70E-04	6.20E+03	3.93E+04	4.39E+03	3.96E+03	1.58E-01	-8.02E-01
ATP-dependent protease HslV (EC 3.4.25.-)	7.30E-04	1.89E+04	3.42E+05	2.27E+04	3.84E+04	5.53E-02	-1.26E+00
L-alanine-DL-glutamate epimerase	7.40E-04	8.11E+02	1.35E+04	8.29E+02	2.21E+02	5.99E-02	-1.22E+00
Translation elongation factor G	7.80E-04	3.91E+04	3.38E+05	1.75E+04	3.32E+04	1.16E-01	-9.37E-01
Preprotein translocase subunit YajC (TC 3.A.5.1.1)	8.50E-04	3.48E+03	4.17E+04	2.26E+03	4.33E+03	8.36E-02	-1.08E+00
LSU ribosomal protein L1p (L10Ae)	9.00E-04	3.22E+03	1.81E+04	1.50E+03	2.09E+03	1.78E-01	-7.50E-01
hypothetical Membrane Spanning Protein	1.86E-03	3.59E+05	8.09E+04	8.85E+03	2.91E+04	4.44E+00	6.47E-01
Acetylornithine deacetylase (EC 3.5.1.16)	1.87E-03	3.58E+04	4.35E+05	1.05E+04	3.94E+04	8.21E-02	-1.09E+00
Chitinase (EC 3.2.1.14)	2.04E-03	2.14E+05	7.88E+04	7.36E+03	1.78E+04	2.72E+00	4.35E-01
3-hydroxyisobutyrate dehydrogenase (EC 1.1.1.31)	2.06E-03	5.55E+04	5.98E+05	2.90E+04	7.08E+04	9.29E-02	-1.03E+00
Acyl carrier protein	2.10E-03	1.61E+04	5.17E+03	1.79E+03	1.05E+03	3.11E+00	4.93E-01

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
Acetylornithine deacetylase/Succinyl-diaminopimelate desuccinylase and related deacylases	2.41E-03	2.95E+04	2.03E+05	3.00E+04	3.20E+04	1.45E-01	-8.37E-01
SSU ribosomal protein S11p (S14e)	2.60E-03	2.04E+02	9.63E+02	1.37E+02	1.41E+02	2.12E-01	-6.74E-01
hypothetical protein	2.83E-03	1.86E+05	2.22E+06	2.25E+05	3.74E+05	8.37E-02	-1.08E+00
Phosphoenolpyruvate carboxykinase [ATP] (EC 4.1.1.49)	2.96E-03	3.86E+04	4.80E+05	2.47E+04	6.40E+04	8.03E-02	-1.10E+00
Phosphatidylinositol-specific phospholipase C (EC 4.6.1.13)	3.19E-03	1.13E+04	2.08E+05	1.03E+04	2.84E+04	5.41E-02	-1.27E+00
Ornithine aminotransferase (EC 2.6.1.13)	3.21E-03	4.33E+04	2.05E+05	3.16E+04	3.10E+04	2.12E-01	-6.75E-01
Streptomycin biosynthesis StrF domain protein	3.23E-03	1.07E+03	6.28E+03	1.00E+03	1.02E+03	1.70E-01	-7.70E-01
Urocanate hydratase (EC 4.2.1.49)	3.27E-03	9.98E+04	7.02E+05	1.04E+05	1.24E+05	1.42E-01	-8.47E-01
Ferredoxin--sulfite reductase, bacillial type (EC 1.8.7.1)	3.98E-03	6.23E+03	4.80E+04	9.00E+03	6.43E+03	1.30E-01	-8.86E-01
FIG01227664: hypothetical protein	4.17E-03	1.94E+04	1.49E+05	1.35E+04	2.54E+04	1.31E-01	-8.84E-01
Catalyzes the cleavage of p-aminobenzoyl-glutamate to p-aminobenzoate and glutamate, subunit A	4.22E-03	8.33E+03	3.55E+04	5.62E+03	5.71E+03	2.35E-01	-6.29E-01
Immune inhibitor A precursor	4.80E-03	4.37E+05	2.13E+05	3.79E+04	5.13E+04	2.05E+00	3.12E-01
Ribulose-5-phosphate 4-epimerase and related epimerases and aldolases	5.35E-03	2.65E+04	7.82E+04	1.21E+04	8.29E+03	3.39E-01	-4.70E-01
Pantothenate kinase type II, eukaryotic (EC 2.7.1.33)	5.42E-03	4.53E+03	3.71E+04	1.81E+03	5.55E+03	1.22E-01	-9.13E-01
Gamma-aminobutyrate:alpha-ketoglutarate aminotransferase (EC 2.6.1.19)	5.85E-03	1.14E+05	3.01E+05	3.80E+04	4.52E+04	3.79E-01	-4.21E-01

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase (EC 2.1.1.14)	6.42E-03	8.18E+04	1.45E+06	5.87E+04	2.30E+05	5.63E-02	-1.25E+00
Branched-chain amino acid aminotransferase (EC 2.6.1.42)	6.56E-03	9.40E+03	3.25E+05	3.38E+03	4.53E+04	2.90E-02	-1.54E+00
transcriptional regulator, XRE family	6.78E-03	1.07E+08	4.36E+07	1.40E+07	6.71E+06	2.46E+00	3.90E-01
hypothetical Membrane Spanning Protein	7.23E-03	1.02E+05	2.92E+05	3.71E+04	4.93E+04	3.48E-01	-4.58E-01
aminopeptidase	7.49E-03	5.19E+04	2.27E+05	3.14E+04	4.54E+04	2.29E-01	-6.40E-01
5'-methylthioadenosine nucleosidase (EC 3.2.2.16) / S-adenosylhomocysteine nucleosidase (EC 3.2.2.9)	7.52E-03	5.17E+03	3.39E+04	5.58E+03	7.51E+03	1.53E-01	-8.16E-01
Cytochrome c oxidase polypeptide III (EC 1.9.3.1)	7.66E-03	2.52E+03	4.09E+04	2.20E+03	7.33E+03	6.17E-02	-1.21E+00
hypothetical protein	7.75E-03	1.24E+04	8.61E+04	6.37E+03	1.59E+04	1.44E-01	-8.42E-01
NADP-dependent malic enzyme (EC 1.1.1.40)	7.88E-03	3.79E+03	1.96E+04	1.40E+03	3.45E+03	1.93E-01	-7.14E-01
Cystine-binding periplasmic protein precursor	7.98E-03	4.22E+04	5.34E+05	9.20E+03	7.96E+04	7.91E-02	-1.10E+00
transcriptional regulator, XRE family	8.24E-03	1.43E+06	4.70E+05	2.08E+05	7.91E+04	3.05E+00	4.84E-01
hypothetical Membrane Spanning Protein	8.27E-03	1.97E+03	1.86E+04	2.54E+03	4.29E+03	1.06E-01	-9.75E-01
hypothetical protein	8.34E-03	5.91E+02	6.78E+03	2.90E+02	1.16E+03	8.72E-02	-1.06E+00
Fe-bacillibactin uptake system FeuA, Fe-bacillibactin binding	8.54E-03	8.87E+03	4.35E+04	7.18E+03	9.42E+03	2.04E-01	-6.90E-01
DUF124 domain-containing protein	8.60E-03	5.89E+03	4.12E+04	7.94E+03	9.57E+03	1.43E-01	-8.46E-01
Cysteine synthase (EC 2.5.1.47)	8.94E-03	4.31E+04	5.19E+05	5.45E+04	1.16E+05	8.31E-02	-1.08E+00
Polyribonucleotide nucleotidyltransferase (EC 2.7.7.8)	9.04E-03	2.92E+04	6.44E+04	9.70E+03	7.78E+03	4.54E-01	-3.43E-01
Translation elongation factor Ts	9.07E-03	1.86E+03	7.93E+03	1.27E+03	1.68E+03	2.35E-01	-6.30E-01

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
Pyruvate carboxylase (EC 6.4.1.1)	9.56E-03	2.69E+04	5.32E+05	1.02E+04	8.94E+04	5.05E-02	-1.30E+00
Peptidase, M42 family	9.73E-03	3.85E+04	1.75E+05	3.67E+04	3.55E+04	2.20E-01	-6.58E-01
Methionine ABC transporter substrate-binding protein	9.79E-03	4.15E+04	1.40E+05	2.77E+04	2.28E+04	2.96E-01	-5.28E-01
hypothetical protein	1.00E-02	1.76E+04	1.32E+05	2.29E+04	3.25E+04	1.33E-01	-8.75E-01
Porphobilinogen deaminase (EC 2.5.1.61)	1.01E-02	1.27E+03	2.68E+04	6.98E+02	4.76E+03	4.75E-02	-1.32E+00
hypothetical protein	1.02E-02	7.44E+03	2.40E+04	4.71E+03	3.12E+03	3.09E-01	-5.10E-01
Pyrimidine-nucleoside phosphorylase (EC 2.4.2.2)	1.10E-02	1.51E+04	1.27E+05	1.08E+04	2.75E+04	1.19E-01	-9.24E-01
3-ketoacyl-CoA thiolase [isoleucine degradation] (EC 2.3.1.16)	1.21E-02	9.78E+04	9.55E+05	7.46E+04	2.11E+05	1.02E-01	-9.89E-01
SSU ribosomal protein S2p (SAe)	1.26E-02	2.80E+03	2.11E+04	3.56E+02	3.68E+03	1.33E-01	-8.77E-01
Aspartate-semialdehyde dehydrogenase (EC 1.2.1.11)	1.42E-02	3.14E+04	1.18E+05	2.04E+04	2.76E+04	2.65E-01	-5.76E-01
GMP synthase [glutamine-hydrolyzing], amidotransferase subunit (EC 6.3.5.2) / GMP synthase [glutamine-hydrolyzing], ATP pyrophosphatase subunit (EC 6.3.5.2)	1.43E-02	4.53E+03	2.78E+04	3.32E+03	6.82E+03	1.63E-01	-7.87E-01
Chaperone protein DnaK	1.46E-02	3.35E+04	1.19E+05	1.86E+04	2.72E+04	2.83E-01	-5.49E-01
hypothetical Membrane Spanning Protein	1.51E-02	1.12E+04	2.00E+04	2.58E+03	2.67E+03	5.62E-01	-2.51E-01
aldehyde dehydrogenase family protein	1.55E-02	5.87E+03	2.67E+04	2.33E+03	5.89E+03	2.20E-01	-6.58E-01
Methionine ABC transporter substrate-binding protein	1.55E-02	4.10E+04	2.26E+05	3.01E+04	5.73E+04	1.81E-01	-7.41E-01
Chitinase (EC 3.2.1.14)	1.57E-02	1.42E+05	5.42E+05	1.20E+05	1.23E+05	2.61E-01	-5.83E-01
hypothetical Membrane Spanning Protein	1.65E-02	2.45E+04	6.07E+04	1.03E+04	1.18E+04	4.03E-01	-3.94E-01
hypothetical protein	1.66E-02	8.06E+03	2.01E+04	3.50E+03	3.86E+03	4.02E-01	-3.96E-01

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
Methionine ABC transporter substrate-binding protein	1.66E-02	1.33E+04	2.87E+04	5.11E+03	3.30E+03	4.63E-01	-3.35E-01
Catalase (EC 1.11.1.6)	1.77E-02	2.01E+05	4.56E+06	1.18E+05	1.05E+06	4.41E-02	-1.36E+00
Proline dipeptidase (EC 3.4.13.9)	1.78E-02	1.31E+04	9.10E+04	7.11E+03	2.20E+04	1.44E-01	-8.40E-01
hypothetical protein	1.87E-02	9.38E+03	2.13E+05	6.77E+03	5.09E+04	4.41E-02	-1.36E+00
hypothetical Membrane Spanning Protein	1.92E-02	1.56E+05	5.35E+05	1.17E+05	1.26E+05	2.92E-01	-5.35E-01
Butyryl-CoA dehydrogenase (EC 1.3.99.2)	1.95E-02	1.33E+04	8.77E+04	1.05E+04	2.38E+04	1.51E-01	-8.20E-01
Alkyl hydroperoxide reductase protein F (EC 1.6.4.-)	1.96E-02	1.87E+04	5.85E+05	9.84E+03	1.41E+05	3.19E-02	-1.50E+00
Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein OppA (TC 3.A.1.5.1)	2.07E-02	2.35E+05	1.47E+06	1.13E+05	3.67E+05	1.60E-01	-7.97E-01
3-ketoacyl-CoA thiolase (EC 2.3.1.16) @ Acetyl-CoA acetyltransferase (EC 2.3.1.9)	2.11E-02	4.78E+04	8.81E+05	3.28E+04	2.23E+05	5.43E-02	-1.27E+00
Long-chain-fatty-acid--CoA ligase (EC 6.2.1.3)	2.19E-02	2.62E+04	2.20E+05	3.27E+04	6.70E+04	1.19E-01	-9.24E-01
Threonine synthase (EC 4.2.3.1)	2.30E-02	2.09E+04	1.38E+05	2.21E+04	4.20E+04	1.51E-01	-8.20E-01
NADH dehydrogenase (EC 1.6.99.3)	2.31E-02	3.64E+03	8.21E+04	2.59E+03	2.17E+04	4.44E-02	-1.35E+00
EPSX protein	2.33E-02	3.61E+05	5.75E+04	1.04E+05	4.55E+04	6.29E+00	7.99E-01
4-hydroxyphenylpyruvate dioxygenase (EC 1.13.11.27)	2.33E-02	1.42E+05	1.56E+06	1.48E+05	4.52E+05	9.10E-02	-1.04E+00
2-methylcitrate dehydratase (EC 4.2.1.79)	2.34E-02	7.67E+04	1.15E+06	1.13E+04	2.91E+05	6.66E-02	-1.18E+00
hypothetical protein	2.35E-02	2.19E+04	2.65E+05	1.30E+04	7.00E+04	8.27E-02	-1.08E+00
FIG01225390: hypothetical protein	2.37E-02	1.75E+03	8.74E+03	2.47E+03	1.19E+03	2.01E-01	-6.98E-01
hypothetical protein	2.39E-02	6.05E+04	4.25E+05	7.97E+04	1.35E+05	1.42E-01	-8.47E-01
Autoinducer 2 (AI-2) aldolase LsrF (EC 4.2.1.-)	2.42E-02	2.76E+04	1.50E+05	7.02E+03	3.61E+04	1.84E-01	-7.36E-01

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
Histidine ammonia-lyase (EC 4.3.1.3)	2.62E-02	5.57E+04	4.53E+05	1.79E+04	1.18E+05	1.23E-01	-9.10E-01
Formate--tetrahydrofolate ligase (EC 6.3.4.3)	2.63E-02	3.13E+04	1.59E+05	3.34E+04	4.96E+04	1.97E-01	-7.06E-01
phosphotransbutyrylase (EC 2.3.1.19)	2.73E-02	4.69E+04	2.17E+05	7.91E+03	5.18E+04	2.16E-01	-6.65E-01
Ortholog yrbG, yetE, ykjA, ydfS, ydfR B.subtilis	2.75E-02	4.35E+06	1.56E+06	9.96E+05	1.02E+06	2.79E+00	4.46E-01
Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein OppA (TC 3.A.1.5.1)	2.78E-02	8.57E+06	2.08E+05	2.47E+06	7.66E+04	4.12E+01	1.62E+00
Biosynthetic Aromatic amino acid aminotransferase beta (EC 2.6.1.57) @ Histidinol-phosphate aminotransferase (EC 2.6.1.9)	2.83E-02	1.06E+04	8.33E+04	1.13E+04	2.68E+04	1.28E-01	-8.94E-01
Non-heme chloroperoxidase (EC 1.11.1.10)	2.94E-02	1.21E+04	6.37E+04	7.56E+03	1.90E+04	1.91E-01	-7.20E-01
Dihydrolipoamide dehydrogenase of pyruvate dehydrogenase complex (EC 1.8.1.4)	3.05E-02	4.39E+04	2.58E+05	4.81E+04	8.60E+04	1.70E-01	-7.69E-01
Pyruvate dehydrogenase E1 component beta subunit (EC 1.2.4.1)	3.05E-02	1.43E+04	4.99E+03	2.29E+03	3.77E+03	2.86E+00	4.56E-01
Acid phosphatase (EC 3.1.3.2)	3.09E-02	2.02E+04	3.00E+05	1.06E+04	8.94E+04	6.74E-02	-1.17E+00
N-acetylglucosamine-1-phosphate uridyltransferase (EC 2.7.7.23) / Glucosamine-1-phosphate N-acetyltransferase (EC 2.3.1.157)	3.11E-02	7.27E+03	3.85E+04	3.70E+03	1.13E+04	1.89E-01	-7.24E-01
alkaline serine protease, subtilase family	3.12E-02	1.06E+05	2.13E+06	1.43E+05	6.82E+05	4.97E-02	-1.30E+00
FIG002379: metal-dependent hydrolase	3.15E-02	4.24E+03	1.89E+04	5.11E+03	1.35E+03	2.25E-01	-6.48E-01
3-hydroxybutyryl-CoA dehydrogenase (EC 1.1.1.157)	3.17E-02	1.32E+04	3.77E+05	8.12E+03	1.16E+05	3.49E-02	-1.46E+00
Leucine dehydrogenase (EC 1.4.1.9)	3.17E-02	5.39E+05	3.96E+06	1.57E+05	1.12E+06	1.36E-01	-8.66E-01

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
S-layer protein, putative	3.22E-02	3.10E+03	1.09E+04	1.45E+02	2.49E+03	2.85E-01	-5.45E-01
3-oxoacyl-[acyl-carrier-protein] synthase, KASII (EC 2.3.1.41)	3.23E-02	4.06E+03	6.34E+04	1.72E+03	1.92E+04	6.41E-02	-1.19E+00
Nitrilotriacetate monooxygenase component B (EC 1.14.13.-)	3.25E-02	2.53E+04	5.05E+05	3.39E+04	1.64E+05	5.01E-02	-1.30E+00
hypothetical Membrane Spanning Protein	3.29E-02	8.62E+04	5.17E+05	9.87E+04	1.78E+05	1.67E-01	-7.78E-01
Dihydroorotase (EC 3.5.2.3)	3.41E-02	9.90E+03	5.54E+04	7.36E+03	1.80E+04	1.79E-01	-7.47E-01
Thioredoxin reductase (EC 1.8.1.9)	3.44E-02	2.45E+03	3.48E+04	2.63E+03	1.15E+04	7.05E-02	-1.15E+00
Intracellular serine protease	3.48E-02	2.93E+02	9.48E+02	2.55E+02	9.34E+01	3.09E-01	-5.10E-01
Acid phosphatase (EC 3.1.3.2)	3.52E-02	6.69E+04	2.76E+04	1.39E+04	2.59E+03	2.42E+00	3.84E-01
RND multidrug efflux transporter; Acriflavin resistance protein	3.55E-02	6.92E+05	5.47E+04	2.15E+05	1.33E+04	1.27E+01	1.10E+00
hypothetical protein	3.60E-02	1.88E+04	5.25E+04	1.24E+04	1.40E+04	3.59E-01	-4.45E-01
Cysteine desulfurase (EC 2.8.1.7)	3.62E-02	2.08E+04	5.66E+04	1.33E+04	3.47E+03	3.67E-01	-4.35E-01
Dihydroneopterin aldolase (EC 4.1.2.25)	3.67E-02	2.52E+03	6.22E+04	4.97E+02	2.04E+04	4.06E-02	-1.39E+00
Two-component response regulator SA14-24	3.82E-02	4.86E+04	2.19E+05	5.39E+04	7.51E+04	2.22E-01	-6.53E-01
Beta-lactamase (EC 3.5.2.6)	3.85E-02	1.13E+05	5.33E+06	3.92E+04	1.83E+06	2.11E-02	-1.68E+00
Phosphatidylinositol-specific phospholipase C (EC 4.6.1.13)	3.90E-02	1.30E+04	7.01E+04	6.36E+03	2.24E+04	1.85E-01	-7.32E-01
CBS domain protein AcuB	3.94E-02	3.85E+03	1.83E+04	1.51E+03	5.64E+03	2.10E-01	-6.77E-01
Aminopeptidase S (Leu, Val, Phe, Tyr preference) (EC 3.4.11.24)	3.96E-02	3.75E+04	2.87E+05	5.72E+04	1.10E+05	1.31E-01	-8.84E-01
hypothetical protein	3.99E-02	1.58E+03	4.25E+03	1.07E+03	3.23E+02	3.72E-01	-4.29E-01
Aminopeptidase YpdF (MP-, MA-, MS-, AP-, NP- specific)	3.99E-02	1.39E+04	7.63E+04	1.18E+04	2.68E+04	1.83E-01	-7.39E-01
Phosphopentomutase (EC 5.4.2.7)	4.00E-02	4.27E+04	1.95E+05	9.71E+03	5.68E+04	2.19E-01	-6.60E-01
acetyltransferase, GNAT family	4.01E-02	1.23E+03	7.23E+03	7.93E+02	2.43E+03	1.71E-01	-7.68E-01
RNA-binding protein Hfq	4.05E-02	3.87E+03	3.36E+05	5.46E+03	1.20E+05	1.15E-02	-1.94E+00

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
Electron transfer flavoprotein, beta subunit	4.08E-02	1.19E+05	2.92E+05	7.73E+04	5.99E+04	4.08E-01	-3.89E-01
N-acetyl-L,L-diaminopimelate deacetylase (EC 3.5.1.47)	4.21E-02	2.32E+03	1.82E+04	2.06E+03	6.56E+03	1.28E-01	-8.94E-01
Oligoendopeptidase F (EC 3.4.24.-)	4.26E-02	2.64E+05	5.87E+04	8.66E+04	2.99E+04	4.49E+00	6.52E-01
Vip4Aa-like_1	4.27E-02	1.34E+05	6.88E+04	2.68E+04	7.64E+03	1.95E+00	2.91E-01
Glucosamine--fructose-6-phosphate aminotransferase [isomerizing] (EC 2.6.1.16)	4.28E-02	3.80E+04	3.07E+05	1.16E+04	1.01E+05	1.24E-01	-9.07E-01
hypothetical Membrane Spanning Protein	4.30E-02	1.08E+05	3.67E+05	1.17E+05	5.68E+04	2.94E-01	-5.32E-01
oxidoreductase, aldo/keto reductase family	4.50E-02	1.64E+04	1.00E+05	6.73E+03	3.36E+04	1.64E-01	-7.85E-01
Acetyl-CoA:acetoacetyl-CoA transferase, alpha subunit (EC 2.8.3.8)	4.63E-02	7.08E+02	3.30E+04	2.65E+02	1.25E+04	2.14E-02	-1.67E+00
DNA-directed RNA polymerase beta subunit (EC 2.7.7.6)	4.66E-02	4.18E+04	1.51E+05	3.54E+04	5.19E+04	2.77E-01	-5.57E-01
2-hydroxy-3-oxopropionate reductase (EC 1.1.1.60)	4.68E-02	1.51E+04	1.00E+05	1.92E+04	3.97E+04	1.51E-01	-8.21E-01
hypothetical Membrane Spanning Protein	4.69E-02	7.10E+04	1.10E+06	5.11E+04	4.07E+05	6.48E-02	-1.19E+00
Imidazoleglycerol-phosphate dehydratase (EC 4.2.1.19)	4.74E-02	1.38E+04	5.27E+04	1.75E+04	6.68E+03	2.63E-01	-5.81E-01
Glutamyl-tRNA synthetase (EC 6.1.1.17) @ Glutamyl-tRNA(Gln) synthetase (EC 6.1.1.24)	4.81E-02	3.93E+04	9.29E+04	2.20E+04	3.86E+03	4.23E-01	-3.74E-01
2,3-bisphosphoglycerate-independent phosphoglycerate mutase (EC 5.4.2.1)	4.82E-02	3.97E+04	9.18E+04	1.69E+04	2.51E+04	4.33E-01	-3.64E-01
Glucose-6-phosphate isomerase (EC 5.3.1.9)	4.86E-02	1.71E+04	3.73E+05	4.53E+03	1.41E+05	4.59E-02	-1.34E+00

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
Phosphatidylinositol-specific phospholipase C (EC 4.6.1.13)	5.00E-02	1.56E+04	5.69E+04	1.93E+04	8.07E+03	2.74E-01	-5.62E-01
Peptide deformylase (EC 3.5.1.88)	5.03E-02	2.59E+03	3.14E+04	1.40E+03	1.19E+04	8.26E-02	-1.08E+00
Aconitate hydratase (EC 4.2.1.3) @ 2-methylisocitrate dehydratase (EC 4.2.1.99)	5.08E-02	2.25E+04	1.03E+05	3.27E+04	3.82E+04	2.18E-01	-6.62E-01
prophage LambdaBa02, site-specific recombinase, phage integrase family	5.21E-02	3.99E+04	5.49E+05	9.88E+03	2.10E+05	7.26E-02	-1.14E+00
Peptidyl-prolyl cis-trans isomerase (EC 5.2.1.8)	5.25E-02	8.49E+03	4.48E+04	3.06E+03	1.57E+04	1.89E-01	-7.23E-01
Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein OppA (TC 3.A.1.5.1)	5.32E-02	2.49E+06	3.13E+05	9.19E+05	9.07E+04	7.96E+00	9.01E-01
Isovaleryl-CoA dehydrogenase (EC 1.3.8.4)	5.33E-02	4.71E+04	6.41E+05	2.62E+04	2.51E+05	7.35E-02	-1.13E+00
2-oxoglutarate oxidoreductase, alpha subunit (EC 1.2.7.3)	5.36E-02	3.98E+04	2.51E+05	1.47E+04	9.14E+04	1.58E-01	-8.00E-01
Methylthioribose-1-phosphate isomerase (EC 5.3.1.23)	5.42E-02	1.20E+04	1.13E+05	8.84E+03	4.45E+04	1.06E-01	-9.74E-01
ATP synthase beta chain (EC 3.6.3.14)	5.54E-02	3.10E+04	1.04E+05	3.73E+04	2.30E+04	2.97E-01	-5.27E-01
1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate synthase (EC 1.17.7.1)	5.55E-02	1.28E+04	7.58E+04	1.44E+03	2.70E+04	1.68E-01	-7.74E-01
NADH-dependent butanol dehydrogenase A (EC 1.1.1.-)	5.70E-02	2.35E+03	9.11E+03	1.65E+03	3.42E+03	2.58E-01	-5.89E-01
Deblocking aminopeptidase (EC 3.4.11.-)	5.76E-02	3.29E+03	2.16E+04	1.28E+03	8.21E+03	1.52E-01	-8.18E-01
Acetylornithine aminotransferase (EC 2.6.1.11)	5.85E-02	1.24E+03	1.23E+04	1.04E+03	5.09E+03	1.00E-01	-9.98E-01
hypothetical Membrane Spanning Protein	5.88E-02	4.86E+05	1.30E+07	1.21E+05	5.53E+06	3.72E-02	-1.43E+00

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein OppA (TC 3.A.1.5.1)	5.99E-02	3.47E+04	4.97E+05	1.46E+04	2.06E+05	6.98E-02	-1.16E+00
hypothetical protein	6.04E-02	8.43E+03	2.79E+04	2.65E+03	9.38E+03	3.02E-01	-5.19E-01
Manganese superoxide dismutase (EC 1.15.1.1)	6.11E-02	3.63E+05	8.54E+06	8.41E+04	3.67E+06	4.25E-02	-1.37E+00
Ribosome-associated heat shock protein implicated in the recycling of the 50S subunit (S4 paralog)	6.11E-02	7.27E+03	4.62E+04	1.03E+04	2.04E+04	1.57E-01	-8.03E-01
hypothetical protein	6.22E-02	3.97E+03	2.18E+04	3.86E+03	9.23E+03	1.82E-01	-7.41E-01
Delta-1-pyrroline-5-carboxylate dehydrogenase (EC 1.5.1.12)	6.29E-02	3.07E+04	2.17E+05	3.01E+04	9.35E+04	1.41E-01	-8.50E-01
Cystathionine gamma-lyase (EC 4.4.1.1)	6.42E-02	1.50E+04	1.81E+05	1.60E+04	7.98E+04	8.29E-02	-1.08E+00
hypothetical protein	6.44E-02	4.46E+05	2.87E+05	8.43E+04	6.51E+04	1.56E+00	1.92E-01
hypothetical protein	6.56E-02	3.61E+04	3.25E+05	2.71E+04	1.40E+05	1.11E-01	-9.54E-01
Chitin binding protein	6.57E-02	1.21E+04	1.16E+05	7.34E+03	4.96E+04	1.05E-01	-9.81E-01
Ketol-acid reductoisomerase (EC 1.1.1.86)	6.62E-02	7.31E+02	6.38E+03	2.05E+02	2.67E+03	1.14E-01	-9.41E-01
Amidohydrolase AmhX	6.77E-02	1.19E+04	1.08E+05	3.77E+03	4.61E+04	1.10E-01	-9.60E-01
Ribonuclease PH (EC 2.7.7.56)	6.78E-02	3.59E+03	2.02E+04	2.50E+03	8.54E+03	1.77E-01	-7.51E-01
Pyruvate kinase (EC 2.7.1.40)	6.81E-02	3.02E+04	4.52E+05	4.80E+03	2.01E+05	6.69E-02	-1.17E+00
FIG01226061: hypothetical protein	6.89E-02	7.72E+03	1.61E+04	1.74E+03	4.47E+03	4.81E-01	-3.18E-01
Alanine dehydrogenase (EC 1.4.1.1)	6.89E-02	3.40E+05	8.44E+05	9.20E+04	2.66E+05	4.03E-01	-3.95E-01
CBS domain containing protein	7.00E-02	1.94E+04	1.45E+05	1.02E+04	6.23E+04	1.34E-01	-8.73E-01
ATP synthase alpha chain (EC 3.6.3.14)	7.01E-02	1.35E+04	5.58E+04	1.15E+04	2.35E+04	2.41E-01	-6.18E-01
MaoC family protein	7.02E-02	9.51E+03	6.41E+04	5.70E+03	2.76E+04	1.48E-01	-8.29E-01
Fumarate hydratase class I, aerobic (EC 4.2.1.2)	7.05E-02	3.56E+03	1.12E+04	4.15E+03	3.38E+03	3.17E-01	-4.99E-01
D-alanine aminotransferase (EC 2.6.1.21)	7.26E-02	8.94E+04	2.91E+05	1.15E+05	6.26E+04	3.07E-01	-5.13E-01
hypothetical protein	7.47E-02	1.72E+03	9.62E+03	1.64E+03	4.39E+03	1.78E-01	-7.49E-01

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
Phosphonate ABC transporter phosphate-binding periplasmic component (TC 3.A.1.9.1)	7.59E-02	5.10E+02	8.09E+03	3.98E+02	3.88E+03	6.30E-02	-1.20E+00
D-Ribose 1,5-phosphomutase (EC 5.4.2.7)	7.80E-02	2.11E+04	1.86E+05	9.68E+03	8.59E+04	1.14E-01	-9.45E-01
5-Enolpyruvylshikimate-3-phosphate synthase (EC 2.5.1.19)	7.88E-02	1.09E+04	3.63E+04	1.07E+04	1.47E+04	3.01E-01	-5.21E-01
Deblocking aminopeptidase (EC 3.4.11.-)	7.92E-02	1.69E+04	6.81E+04	5.91E+03	2.77E+04	2.48E-01	-6.05E-01
FIG01226521: hypothetical protein	7.97E-02	1.79E+03	2.59E+04	1.04E+03	1.26E+04	6.91E-02	-1.16E+00
NAD synthetase (EC 6.3.1.5)	8.07E-02	3.18E+04	1.38E+05	3.33E+04	6.28E+04	2.31E-01	-6.36E-01
Xanthine permease	8.11E-02	1.33E+05	5.55E+05	4.10E+04	2.29E+05	2.39E-01	-6.21E-01
FIG001621: Zinc protease	8.13E-02	1.20E+04	5.76E+04	1.00E+04	2.64E+04	2.09E-01	-6.80E-01
Single-stranded DNA-binding protein	8.15E-02	1.31E+04	2.68E+05	2.52E+03	1.34E+05	4.87E-02	-1.31E+00
Phosphonate ABC transporter phosphate-binding periplasmic component (TC 3.A.1.9.1)	8.17E-02	1.47E+04	5.21E+04	1.77E+04	2.12E+04	2.83E-01	-5.49E-01
NLP/P60 family protein	8.31E-02	3.97E+04	1.09E+05	3.14E+04	4.02E+04	3.65E-01	-4.37E-01
ATP synthase delta chain (EC 3.6.3.14)	8.32E-02	2.97E+03	7.21E+03	1.61E+03	2.55E+03	4.12E-01	-3.85E-01
Glyoxalase family protein	8.36E-02	5.94E+03	2.57E+04	5.45E+03	1.18E+04	2.31E-01	-6.37E-01
hypothetical protein	8.40E-02	7.14E+03	1.83E+05	6.33E+03	9.47E+04	3.91E-02	-1.41E+00
FIG017263: hypothetical protein	8.50E-02	8.90E+04	3.60E+05	9.84E+04	1.64E+05	2.48E-01	-6.06E-01
Heat shock protein 60 family chaperone GroEL	8.64E-02	7.43E+05	2.43E+06	7.74E+05	1.01E+06	3.05E-01	-5.15E-01
Isocitrate lyase (EC 4.1.3.1)	8.92E-02	4.53E+04	6.60E+05	5.99E+04	3.50E+05	6.87E-02	-1.16E+00
hypothetical Membrane Spanning Protein	9.02E-02	5.74E+03	5.28E+04	4.51E+03	2.69E+04	1.09E-01	-9.64E-01
transcriptional regulator, XRE family	9.03E-02	2.80E+04	4.82E+05	1.32E+04	2.55E+05	5.80E-02	-1.24E+00
Tripeptide aminopeptidase (EC 3.4.11.4)	9.13E-02	1.00E+04	6.50E+04	3.11E+03	3.12E+04	1.54E-01	-8.13E-01
NADPH dehydrogenase (EC 1.6.99.1)	9.15E-02	1.24E+03	2.98E+04	5.81E+02	1.61E+04	4.18E-02	-1.38E+00

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
Nicotinamidase (EC 3.5.1.19)	9.22E-02	2.55E+03	7.46E+04	3.41E+03	4.10E+04	3.42E-02	-1.47E+00
Glycine dehydrogenase [decarboxylating] (glycine cleavage system P2 protein) (EC 1.4.4.2)	9.45E-02	2.39E+04	9.23E+04	9.71E+03	4.10E+04	2.59E-01	-5.87E-01
Lysine 2,3-aminomutase (EC 5.4.3.2)	9.50E-02	2.38E+04	1.35E+05	2.85E+04	6.99E+04	1.76E-01	-7.54E-01
Phosphate acetyltransferase (EC 2.3.1.8)	9.50E-02	8.72E+04	1.62E+05	2.82E+04	4.79E+04	5.37E-01	-2.70E-01
Methionyl-tRNA synthetase (EC 6.1.1.10)	9.81E-02	2.10E+04	1.06E+05	2.09E+03	4.97E+04	1.99E-01	-7.02E-01
hypothetical protein	9.82E-02	4.09E+04	1.06E+04	1.86E+04	4.75E+03	3.85E+00	5.85E-01
Chitin binding protein	1.00E-01	4.82E+05	1.07E+05	2.26E+05	3.24E+04	4.51E+00	6.54E-01
transcriptional regulator, XRE family	1.00E-01	1.41E+05	7.02E+04	4.65E+04	2.25E+04	2.01E+00	3.04E-01
Putative secretion accessory protein EsaA/YueB / Bacteriophage SPP1 receptor	1.01E-01	5.84E+03	1.46E+03	2.86E+03	1.30E+03	3.99E+00	6.01E-01
Homogentisate 1,2-dioxygenase (EC 1.13.11.5)	1.05E-01	2.69E+04	2.13E+05	2.53E+04	1.18E+05	1.26E-01	-8.99E-01
Exoenzymes regulatory protein AepA in lipid-linked oligosaccharide synthesis cluster	1.06E-01	2.78E+03	1.53E+04	1.34E+03	7.83E+03	1.82E-01	-7.40E-01
hypothetical protein	1.07E-01	2.66E+05	1.12E+06	4.85E+04	5.34E+05	2.37E-01	-6.26E-01
Aspartate aminotransferase (EC 2.6.1.1)	1.08E-01	5.54E+03	2.73E+04	3.93E+03	1.41E+04	2.03E-01	-6.93E-01
hypothetical Membrane Spanning Protein	1.10E-01	1.37E+05	5.11E+04	5.74E+04	2.09E+04	2.68E+00	4.28E-01
Purine nucleoside phosphorylase (EC 2.4.2.1)	1.10E-01	9.00E+03	1.91E+04	6.69E+03	5.09E+03	4.70E-01	-3.28E-01
hypothetical Membrane Spanning Protein	1.10E-01	2.37E+04	6.15E+04	2.35E+04	2.16E+04	3.85E-01	-4.14E-01
Membrane-associated zinc metalloprotease	1.11E-01	1.84E+04	5.09E+04	6.42E+03	2.15E+04	3.61E-01	-4.42E-01

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
Menaquinone-cytochrome C reductase iron-sulfur subunit	1.13E-01	9.17E+03	1.02E+05	4.26E+03	5.95E+04	8.98E-02	-1.05E+00
Cell division protein FtsZ (EC 3.4.24.-)	1.13E-01	5.44E+03	6.31E+04	2.12E+03	3.69E+04	8.62E-02	-1.06E+00
SSU ribosomal protein S19p (S15e)	1.14E-01	3.58E+03	2.60E+04	2.80E+03	1.47E+04	1.37E-01	-8.62E-01
Oligoendopeptidase F (EC 3.4.24.-)	1.14E-01	6.62E+04	8.23E+05	5.54E+04	4.91E+05	8.04E-02	-1.09E+00
Glutamate-1-semialdehyde aminotransferase (EC 5.4.3.8)	1.15E-01	1.09E+03	3.77E+03	4.96E+01	1.73E+03	2.88E-01	-5.41E-01
Fe-bacillibactin uptake system FeuA, Fe-bacillibactin binding	1.15E-01	4.29E+05	2.59E+05	1.14E+05	2.75E+04	1.66E+00	2.19E-01
Enoyl-CoA hydratase [isoleucine degradation] (EC 4.2.1.17) / 3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35)	1.17E-01	5.64E+02	8.40E+03	2.17E+02	5.11E+03	6.71E-02	-1.17E+00
O-acetylhomoserine sulfhydrylase (EC 2.5.1.49) / O-succinylhomoserine sulfhydrylase (EC 2.5.1.48)	1.18E-01	4.42E+04	1.37E+05	6.41E+04	2.29E+04	3.24E-01	-4.90E-01
Chitinase (EC 3.2.1.14)	1.19E-01	6.25E+04	2.23E+05	1.22E+04	1.07E+05	2.80E-01	-5.53E-01
4-hydroxy-tetrahydrodipicolinate synthase (EC 4.3.3.7)	1.20E-01	9.99E+04	2.92E+05	1.37E+05	6.36E+04	3.42E-01	-4.66E-01
Electron transfer flavoprotein, alpha subunit	1.20E-01	1.81E+05	2.65E+05	4.68E+04	5.62E+04	6.83E-01	-1.65E-01
Aminomethyltransferase (glycine cleavage system T protein) (EC 2.1.2.10)	1.20E-01	2.30E+03	1.31E+04	2.38E+03	7.55E+03	1.75E-01	-7.57E-01
6,7-dimethyl-8-ribityllumazine synthase (EC 2.5.1.78)	1.21E-01	3.38E+04	2.23E+05	3.28E+04	1.30E+05	1.52E-01	-8.18E-01
Azoreductase	1.24E-01	4.30E+04	2.96E+05	1.89E+04	1.72E+05	1.45E-01	-8.38E-01
FIG009210: peptidase, M16 family	1.24E-01	2.86E+04	9.72E+04	3.08E+04	4.92E+04	2.95E-01	-5.31E-01
Formamidase amiF (EC 3.5.1.49)	1.24E-01	7.29E+03	5.93E+04	1.98E+03	3.52E+04	1.23E-01	-9.10E-01
N-acetyl-gamma-glutamyl-phosphate reductase (EC 1.2.1.38)	1.27E-01	5.97E+03	1.88E+04	8.80E+03	3.63E+02	3.17E-01	-4.98E-01
SSU ribosomal protein S13p (S18e)	1.28E-01	1.54E+04	3.23E+05	1.23E+04	2.12E+05	4.77E-02	-1.32E+00

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
ClpB protein	1.28E-01	8.17E+03	2.98E+04	6.00E+03	1.58E+04	2.74E-01	-5.62E-01
Non-specific DNA-binding protein Dps / Iron-binding ferritin-like antioxidant protein / Ferroxidase (EC 1.16.3.1)	1.31E-01	1.94E+05	1.56E+06	8.67E+04	9.56E+05	1.25E-01	-9.04E-01
3'->5' exoribonuclease Bsu YhaM	1.31E-01	2.80E+02	5.73E+03	1.28E+02	3.81E+03	4.88E-02	-1.31E+00
Thioredoxin reductase (EC 1.8.1.9)	1.33E-01	1.54E+04	1.22E+05	2.07E+03	7.50E+04	1.26E-01	-8.98E-01
FIG007421: forespore shell protein	1.35E-01	3.32E+03	1.84E+04	2.40E+03	1.10E+04	1.80E-01	-7.45E-01
Membrane-attached cytochrome c550	1.35E-01	1.60E+03	1.79E+04	1.31E+03	1.16E+04	8.96E-02	-1.05E+00
transcriptional regulator, XRE family	1.35E-01	4.86E+04	1.05E+04	2.74E+04	4.17E+03	4.61E+00	6.64E-01
Purine nucleoside phosphorylase (EC 2.4.2.1); N-Ribosylnicotinamide phosphorylase (EC 2.4.2.1) ## possible	1.35E-01	3.05E+04	3.05E+05	8.52E+03	1.95E+05	1.00E-01	-1.00E+00
Carbamoyl-phosphate synthase large chain (EC 6.3.5.5)	1.36E-01	2.02E+03	2.18E+04	9.65E+02	1.42E+04	9.26E-02	-1.03E+00
Dihydroxyacetone kinase, ATP-dependent (EC 2.7.1.29)	1.36E-01	1.71E+03	3.92E+04	1.05E+03	2.68E+04	4.36E-02	-1.36E+00
Oligopeptide transport ATP-binding protein OppF (TC 3.A.1.5.1)	1.37E-01	7.00E+03	1.60E+04	3.80E+03	6.79E+03	4.39E-01	-3.57E-01
alkaline serine protease, subtilase family	1.38E-01	2.65E+04	1.96E+05	2.59E+04	1.25E+05	1.36E-01	-8.68E-01
carbonic anhydrase, family 3	1.40E-01	8.68E+03	2.48E+04	8.86E+03	1.20E+04	3.49E-01	-4.57E-01
Molybdenum ABC transporter, periplasmic molybdenum-binding protein ModA (TC 3.A.1.8.1)	1.40E-01	4.28E+05	3.78E+04	2.85E+05	2.09E+04	1.13E+01	1.05E+00
Glycine dehydrogenase [decarboxylating] (glycine cleavage system P1 protein) (EC 1.4.4.2)	1.42E-01	3.84E+04	1.47E+05	2.57E+04	8.25E+04	2.62E-01	-5.81E-01
DNA-directed RNA polymerase beta' subunit (EC 2.7.7.6)	1.42E-01	8.14E+03	1.76E+04	4.60E+03	7.27E+03	4.62E-01	-3.36E-01
hypothetical protein	1.43E-01	1.14E+04	9.64E+04	6.60E+03	6.29E+04	1.19E-01	-9.26E-01

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
Putative Xaa-Pro dipeptidyl-peptidase (EC 3.4.14.11) (X-Pro dipeptidyl-peptidase) (X-prolyl-dipeptidyl aminopeptidase) (X-PDAP)	1.43E-01	1.72E+04	5.68E+04	2.35E+04	2.90E+04	3.03E-01	-5.18E-01
Cyclic beta-1,2-glucan modification transmembrane protein	1.44E-01	8.77E+03	1.67E+03	5.24E+03	2.12E+02	5.25E+00	7.20E-01
Choline binding protein A	1.44E-01	9.49E+04	1.97E+05	5.81E+04	7.60E+04	4.82E-01	-3.17E-01
Lysyl-tRNA synthetase (class II) (EC 6.1.1.6)	1.44E-01	6.12E+04	1.74E+05	7.66E+04	7.63E+04	3.51E-01	-4.55E-01
hypothetical protein	1.45E-01	3.90E+03	1.34E+05	8.28E+02	9.66E+04	2.91E-02	-1.54E+00
hypothetical protein	1.45E-01	6.20E+05	3.94E+05	5.04E+04	1.74E+05	1.57E+00	1.96E-01
Chitosanase	1.45E-01	3.10E+05	1.33E+06	1.61E+05	7.75E+05	2.33E-01	-6.32E-01
metallo-beta-lactamase family protein	1.48E-01	3.64E+03	4.73E+04	3.09E+03	3.30E+04	7.69E-02	-1.11E+00
6-phosphofructokinase (EC 2.7.1.11)	1.48E-01	8.61E+04	2.81E+05	3.63E+04	1.51E+05	3.06E-01	-5.14E-01
Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein OppA (TC 3.A.1.5.1)	1.52E-01	4.87E+05	1.20E+06	1.44E+05	5.60E+05	4.07E-01	-3.91E-01
Hypothetical radical SAM family enzyme in heat shock gene cluster, similarity with CPO of BS HemN-type	1.52E-01	5.58E+05	1.61E+06	1.34E+05	8.16E+05	3.47E-01	-4.60E-01
NAD-specific glutamate dehydrogenase (EC 1.4.1.2)	1.53E-01	6.45E+03	7.15E+04	6.10E+03	5.04E+04	9.03E-02	-1.04E+00
LSU ribosomal protein L21p	1.53E-01	2.70E+03	5.33E+03	8.09E+02	2.12E+03	5.06E-01	-2.96E-01
DNA polymerase X family	1.56E-01	1.50E+04	3.72E+04	1.09E+04	1.80E+04	4.02E-01	-3.96E-01
hypothetical protein	1.57E-01	4.91E+03	1.91E+04	7.81E+03	1.13E+04	2.57E-01	-5.90E-01
Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein OppA (TC 3.A.1.5.1)	1.57E-01	3.13E+03	9.99E+03	3.52E+03	5.54E+03	3.13E-01	-5.05E-01
response regulator, putative	1.58E-01	6.69E+02	7.74E+03	7.74E+02	5.59E+03	8.64E-02	-1.06E+00
NAD-dependent glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12)	1.58E-01	1.37E+04	8.64E+04	5.82E+03	5.73E+04	1.59E-01	-7.99E-01

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
hypothetical protein	1.60E-01	7.70E+03	7.81E+04	6.74E+03	5.60E+04	9.85E-02	-1.01E+00
UDP-glucose 4-epimerase (EC 5.1.3.2)	1.63E-01	2.28E+04	1.85E+05	1.38E+04	1.30E+05	1.23E-01	-9.08E-01
hypothetical protein	1.63E-01	3.48E+04	4.42E+05	8.02E+03	3.27E+05	7.88E-02	-1.10E+00
alternate gene name: yznA	1.65E-01	5.77E+04	3.40E+04	1.86E+04	1.52E+04	1.70E+00	2.29E-01
Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein OppA (TC 3.A.1.5.1)	1.68E-01	1.75E+05	1.36E+04	1.32E+05	1.82E+03	1.28E+01	1.11E+00
Heat shock protein 60 family co-chaperone GroES	1.68E-01	5.40E+04	1.00E+05	3.13E+04	3.54E+04	5.40E-01	-2.67E-01
PlcB, ORFX, ORFP, ORFB, ORFA, ldh gene	1.72E-01	2.79E+03	6.41E+04	2.46E+03	5.09E+04	4.35E-02	-1.36E+00
FIG01225420: hypothetical protein	1.74E-01	1.92E+04	5.45E+04	7.24E+03	3.01E+04	3.52E-01	-4.54E-01
hypothetical protein	1.74E-01	7.72E+04	2.21E+05	1.16E+05	9.21E+04	3.50E-01	-4.56E-01
ATP synthase gamma chain (EC 3.6.3.14)	1.74E-01	1.33E+04	1.09E+05	9.04E+03	8.01E+04	1.22E-01	-9.13E-01
DNA-directed RNA polymerase alpha subunit (EC 2.7.7.6)	1.77E-01	4.05E+03	4.36E+04	3.05E+03	3.36E+04	9.30E-02	-1.03E+00
hypothetical protein	1.78E-01	1.11E+03	2.07E+04	9.85E+02	1.67E+04	5.35E-02	-1.27E+00
hypothetical Membrane Spanning Protein	1.80E-01	5.01E+05	7.73E+05	1.29E+05	2.39E+05	6.49E-01	-1.88E-01
Petrobactin ABC transporter, periplasmic binding protein	1.80E-01	3.31E+04	6.39E+05	6.31E+03	5.19E+05	5.18E-02	-1.29E+00
hypothetical protein	1.82E-01	2.31E+03	7.42E+03	8.23E+02	4.46E+03	3.12E-01	-5.06E-01
Serine hydroxymethyltransferase (EC 2.1.2.1)	1.88E-01	2.80E+03	2.16E+04	3.75E+03	1.69E+04	1.29E-01	-8.88E-01
Dihydrolipoamide succinyltransferase component (E2) of 2-oxoglutarate dehydrogenase complex (EC 2.3.1.61)	1.91E-01	2.79E+03	1.87E+04	3.45E+03	1.44E+04	1.49E-01	-8.27E-01
Microbial collagenase (EC 3.4.24.3)	1.92E-01	6.82E+04	1.49E+05	1.83E+04	7.30E+04	4.59E-01	-3.38E-01
N-succinyl arginine/lysine racemase	1.92E-01	9.02E+02	7.77E+03	1.06E+03	6.19E+03	1.16E-01	-9.35E-01
Isoleucyl-tRNA synthetase (EC 6.1.1.5)	1.92E-01	9.82E+03	2.07E+04	9.87E+03	1.84E+03	4.74E-01	-3.25E-01

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
Hypothetical protein YfkI	1.94E-01	7.10E+03	1.84E+04	1.04E+04	4.42E+03	3.87E-01	-4.13E-01
wall-associated protein, putative	1.94E-01	7.73E+03	1.88E+04	1.02E+04	3.96E+03	4.12E-01	-3.85E-01
Triosephosphate isomerase (EC 5.3.1.1)	1.94E-01	1.70E+05	5.68E+05	1.32E+05	3.67E+05	3.00E-01	-5.23E-01
Acetolactate synthase small subunit (EC 2.2.1.6)	1.94E-01	5.40E+02	2.90E+03	4.85E+02	2.16E+03	1.86E-01	-7.30E-01
Arginine decarboxylase (EC 4.1.1.19)	1.94E-01	4.91E+02	4.56E+03	2.60E+02	3.68E+03	1.07E-01	-9.69E-01
transcriptional regulator, XRE family	1.95E-01	9.41E+02	9.25E+03	1.35E+03	7.59E+03	1.02E-01	-9.92E-01
Non-specific DNA-binding protein Dps / Iron-binding ferritin-like antioxidant protein / Ferroxidase (EC 1.16.3.1)	1.96E-01	2.11E+05	2.14E+06	1.32E+05	1.75E+06	9.86E-02	-1.01E+00
DUF1696 domain-containing protein	2.01E-01	1.27E+04	5.58E+04	2.47E+03	3.98E+04	2.27E-01	-6.43E-01
Cysteine desulfurase (EC 2.8.1.7), SufS subfamily	2.06E-01	7.20E+03	3.77E+04	7.46E+03	2.91E+04	1.91E-01	-7.19E-01
FIG01225418: hypothetical protein	2.09E-01	4.86E+04	8.57E+03	3.85E+04	9.77E+03	5.67E+00	7.53E-01
Thermostable carboxypeptidase 1 (EC 3.4.17.19)	2.10E-01	4.03E+04	1.45E+05	1.47E+04	1.00E+05	2.77E-01	-5.57E-01
transcriptional regulator, XRE family	2.13E-01	6.21E+03	5.03E+02	5.48E+03	1.19E+02	1.23E+01	1.09E+00
metallo-beta-lactamase family protein	2.13E-01	7.01E+02	2.06E+03	9.53E+02	1.25E+03	3.40E-01	-4.68E-01
Phosphonate ABC transporter phosphate-binding periplasmic component (TC 3.A.1.9.1)	2.14E-01	3.34E+03	1.29E+04	1.31E+03	9.26E+03	2.59E-01	-5.86E-01
Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein OppA (TC 3.A.1.5.1)	2.16E-01	1.96E+06	1.25E+06	6.99E+05	1.07E+05	1.57E+00	1.97E-01
transcriptional regulator, XRE family	2.17E-01	5.23E+05	2.86E+05	2.32E+05	1.33E+05	1.83E+00	2.62E-01
Pyruvate dehydrogenase E1 component alpha subunit (EC 1.2.4.1)	2.19E-01	8.97E+03	1.45E+04	3.88E+03	5.26E+03	6.16E-01	-2.10E-01
Oligoendopeptidase F (EC 3.4.24.-)	2.20E-01	1.02E+04	9.21E+04	2.13E+03	8.05E+04	1.11E-01	-9.54E-01
Polypeptide composition of the spore coat protein CotJC	2.21E-01	5.19E+05	2.08E+05	3.08E+05	3.65E+04	2.50E+00	3.98E-01
hypothetical protein	2.22E-01	9.44E+03	2.72E+04	1.42E+04	1.58E+04	3.47E-01	-4.59E-01

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
FIG01225999: hypothetical protein	2.22E-01	1.86E+05	3.63E+05	1.36E+05	1.61E+05	5.13E-01	-2.90E-01
FIG01239190: hypothetical protein	2.24E-01	2.71E+05	3.99E+06	2.51E+05	3.71E+06	6.79E-02	-1.17E+00
Two-component response regulator SA14-24	2.24E-01	1.52E+03	6.04E+03	1.46E+03	4.58E+03	2.52E-01	-5.99E-01
Hemolysin BL lytic component L2	2.25E-01	1.59E+06	2.28E+05	1.37E+06	1.33E+05	6.97E+00	8.43E-01
hypothetical protein	2.27E-01	6.10E+05	8.38E+04	5.30E+05	4.71E+04	7.27E+00	8.62E-01
Glycerate kinase (EC 2.7.1.31)	2.27E-01	1.04E+05	1.83E+05	7.26E+04	6.17E+04	5.70E-01	-2.44E-01
FIG01226746: hypothetical protein	2.28E-01	2.41E+03	5.91E+03	3.25E+03	2.72E+03	4.07E-01	-3.90E-01
Maltose/maltodextrin ABC transporter, permease protein MalG	2.28E-01	6.43E+04	1.56E+04	4.96E+04	1.01E+04	4.13E+00	6.16E-01
Hemoprotein HemQ, essential component of heme biosynthetic pathway in Gram-positive bacteria	2.29E-01	7.74E+04	1.70E+05	4.62E+04	9.45E+04	4.56E-01	-3.41E-01
Subtilase family domain protein	2.29E-01	1.10E+05	6.98E+04	4.14E+04	2.17E+04	1.58E+00	1.99E-01
Beta-lysine acetyltransferase (EC 2.3.1.-)	2.29E-01	3.41E+03	5.77E+04	2.50E+03	5.50E+04	5.91E-02	-1.23E+00
FIG007959: peptidase, M16 family	2.30E-01	6.03E+03	1.80E+04	6.74E+03	1.22E+04	3.35E-01	-4.75E-01
hypothetical Membrane Spanning Protein	2.30E-01	2.30E+06	4.39E+06	2.11E+06	1.26E+06	5.24E-01	-2.80E-01
Cytosol aminopeptidase PepA (EC 3.4.11.1)	2.32E-01	2.76E+05	6.70E+05	4.07E+05	1.01E+05	4.12E-01	-3.85E-01
Phosphatidylinositol-specific phospholipase C (EC 4.6.1.13)	2.34E-01	2.80E+05	7.08E+05	4.38E+05	2.55E+05	3.95E-01	-4.03E-01
LSU ribosomal protein L29p (L35e)	2.38E-01	1.07E+04	6.33E+03	4.66E+03	1.71E+03	1.70E+00	2.30E-01
Non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase (NADP) (EC 1.2.1.9)	2.38E-01	4.14E+03	1.57E+04	7.86E+02	1.20E+04	2.64E-01	-5.79E-01
hypothetical Membrane Spanning Protein	2.39E-01	1.91E+05	5.55E+05	1.47E+05	3.87E+05	3.44E-01	-4.63E-01
D-alanine aminotransferase (EC 2.6.1.21)	2.41E-01	1.29E+04	4.12E+04	3.92E+03	2.99E+04	3.13E-01	-5.04E-01

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
GTP cyclohydrolase I (EC 3.5.4.16) type 1	2.43E-01	3.04E+04	5.80E+04	2.41E+04	2.53E+04	5.24E-01	-2.81E-01
Enolase (EC 4.2.1.11)	2.44E-01	4.53E+05	8.06E+05	6.53E+04	3.77E+05	5.62E-01	-2.51E-01
Butyryl-CoA dehydrogenase (EC 1.3.99.2)	2.45E-01	2.06E+04	4.85E+04	2.16E+04	2.77E+04	4.24E-01	-3.73E-01
Nucleoside diphosphate kinase (EC 2.7.4.6)	2.45E-01	3.77E+05	1.13E+06	1.35E+05	8.08E+05	3.33E-01	-4.77E-01
comA operon protein, putative	2.45E-01	1.06E+03	5.10E+03	1.36E+03	4.35E+03	2.09E-01	-6.80E-01
Tellurium resistance protein TerD	2.50E-01	4.78E+04	1.42E+05	1.15E+04	1.03E+05	3.36E-01	-4.74E-01
hypothetical protein	2.51E-01	1.74E+03	3.27E+04	9.49E+02	3.36E+04	5.32E-02	-1.27E+00
ATP synthase F0 sector subunit b	2.52E-01	4.35E+04	1.71E+04	2.90E+04	1.13E+04	2.54E+00	4.05E-01
hypothetical Membrane Spanning Protein	2.52E-01	3.58E+03	9.21E+02	2.93E+03	1.10E+03	3.89E+00	5.90E-01
Protein of unknown function identified by role in sporulation (SpoVG)	2.54E-01	1.05E+04	2.43E+04	1.33E+04	1.21E+04	4.30E-01	-3.66E-01
4-hydroxy-tetrahydrodipicolinate reductase (EC 1.17.1.8)	2.57E-01	2.03E+04	5.92E+04	1.60E+04	4.34E+04	3.43E-01	-4.64E-01
Phage major capsid protein	2.57E-01	2.39E+04	4.19E+03	2.18E+04	3.18E+03	5.71E+00	7.56E-01
alternate gene name: ipa-62r	2.60E-01	3.09E+04	2.88E+05	2.11E+04	2.86E+05	1.07E-01	-9.70E-01
Tellurium resistance protein TerD	2.60E-01	1.12E+03	3.87E+03	2.32E+02	3.06E+03	2.90E-01	-5.37E-01
HPr kinase/phosphorylase (EC 2.7.1.-) (EC 2.7.4.-)	2.62E-01	7.92E+03	2.73E+04	7.46E+03	2.19E+04	2.90E-01	-5.38E-01
Fructose-bisphosphate aldolase class II (EC 4.1.2.13)	2.67E-01	3.62E+03	8.52E+03	2.09E+03	5.61E+03	4.25E-01	-3.71E-01
Secreted and spore coat-associated protein 1, similar to biofilm matrix component TasA and to camelysin	2.70E-01	1.73E+04	1.06E+04	2.80E+03	7.76E+03	1.63E+00	2.13E-01
collagen triple helix repeat domain protein	2.71E-01	1.58E+03	3.40E+04	1.73E+03	3.73E+04	4.64E-02	-1.33E+00

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
S-ribosylhomocysteine lyase (EC 4.4.1.21) / Autoinducer-2 production protein LuxS	2.74E-01	9.86E+03	4.09E+04	6.73E+03	3.62E+04	2.41E-01	-6.18E-01
FIG01228215: hypothetical protein	2.77E-01	3.23E+04	2.39E+03	3.51E+04	2.21E+03	1.35E+01	1.13E+00
Penicillin-binding protein	2.78E-01	3.62E+03	5.74E+03	1.30E+03	2.46E+03	6.31E-01	-2.00E-01
Isocitrate dehydrogenase [NADP] (EC 1.1.1.42)	2.80E-01	2.22E+04	4.86E+04	3.12E+04	1.11E+03	4.56E-01	-3.41E-01
FIG146262: hypothetical protein	2.80E-01	4.00E+02	1.92E+03	3.87E+02	1.80E+03	2.09E-01	-6.80E-01
hypothetical Membrane Spanning Protein	2.81E-01	1.20E+03	3.80E+03	1.58E+03	3.06E+03	3.14E-01	-5.02E-01
Chorismate mutase I (EC 5.4.99.5) / 2-keto-3-deoxy-D-arabino-heptulosonate-7-phosphate synthase I beta (EC 2.5.1.54)	2.82E-01	6.05E+03	3.25E+04	1.28E+03	3.14E+04	1.86E-01	-7.30E-01
Imidazolonepropionase (EC 3.5.2.7)	2.84E-01	5.76E+03	4.43E+04	4.89E+03	4.60E+04	1.30E-01	-8.86E-01
Ferrichrome-binding periplasmic protein precursor (TC 3.A.1.14.3)	2.86E-01	2.36E+03	7.95E+03	1.47E+03	6.74E+03	2.97E-01	-5.28E-01
FIG01227877: hypothetical protein	2.87E-01	7.91E+02	3.33E+03	8.52E+02	3.08E+03	2.37E-01	-6.24E-01
UTP--glucose-1-phosphate uridylyltransferase (EC 2.7.7.9)	2.98E-01	6.35E+05	4.38E+04	7.35E+05	3.53E+03	1.45E+01	1.16E+00
hypothetical protein	3.03E-01	4.96E+03	7.46E+03	2.72E+03	2.43E+03	6.66E-01	-1.77E-01
Sip1Aa-like_2	3.04E-01	4.70E+04	2.24E+03	5.66E+04	1.18E+03	2.10E+01	1.32E+00
hypothetical protein	3.11E-01	5.32E+06	2.72E+06	3.35E+06	4.74E+05	1.95E+00	2.91E-01
Alkyl hydroperoxide reductase protein C (EC 1.6.4.-)	3.11E-01	1.09E+05	2.88E+05	3.68E+04	2.30E+05	3.80E-01	-4.20E-01
hypothetical protein	3.11E-01	5.07E+02	2.84E+03	5.62E+02	3.02E+03	1.79E-01	-7.48E-01
Methionine aminopeptidase (EC 3.4.11.18)	3.12E-01	5.33E+03	1.01E+05	3.24E+03	1.24E+05	5.28E-02	-1.28E+00
hypothetical Membrane Spanning Protein	3.13E-01	2.05E+04	4.20E+04	9.81E+03	2.79E+04	4.88E-01	-3.12E-01

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
hypothetical Membrane Spanning Protein	3.16E-01	2.44E+05	3.40E+05	1.17E+05	8.08E+04	7.18E-01	-1.44E-01
response regulator, putative	3.18E-01	4.03E+03	1.97E+04	3.20E+03	2.06E+04	2.05E-01	-6.88E-01
hypothetical protein	3.20E-01	7.11E+03	2.72E+04	4.62E+03	2.65E+04	2.62E-01	-5.82E-01
DNA polymerase IV (EC 2.7.7.7)	3.21E-01	1.17E+03	3.72E+03	1.44E+03	3.36E+03	3.15E-01	-5.02E-01
Vip4Aa1-like_2	3.23E-01	3.08E+04	1.47E+05	7.31E+03	1.55E+05	2.09E-01	-6.80E-01
2-oxoglutarate oxidoreductase, beta subunit (EC 1.2.7.3)	3.24E-01	7.16E+03	3.02E+05	5.30E+03	3.94E+05	2.37E-02	-1.63E+00
Outer spore coat protein E	3.24E-01	8.48E+04	2.51E+05	1.31E+05	2.11E+05	3.38E-01	-4.71E-01
Thioredoxin	3.34E-01	2.40E+04	3.06E+04	5.36E+03	8.62E+03	7.84E-01	-1.06E-01
Histidinol dehydrogenase (EC 1.1.1.23)	3.34E-01	9.07E+03	2.13E+04	1.26E+04	1.45E+04	4.27E-01	-3.70E-01
Protein GerPF, required for proper assembly of spore coat, mutations lead to super-dormant spore	3.55E-01	1.76E+03	3.25E+03	2.07E+03	1.22E+03	5.40E-01	-2.67E-01
Acetate kinase (EC 2.7.2.1)	3.60E-01	2.29E+03	2.09E+04	1.56E+03	2.74E+04	1.10E-01	-9.60E-01
Spore coat protein Z	3.61E-01	4.44E+02	6.32E+03	2.26E+02	8.67E+03	7.02E-02	-1.15E+00
MazG nucleotide pyrophosphohydrolase	3.64E-01	3.05E+03	8.76E+03	4.77E+03	8.09E+03	3.48E-01	-4.58E-01
Thiol peroxidase, Tpx-type (EC 1.11.1.15)	3.75E-01	2.00E+04	3.36E+04	1.42E+04	1.86E+04	5.95E-01	-2.25E-01
3-dehydroquinate synthase (EC 4.2.3.4)	3.88E-01	7.68E+04	3.17E+04	7.12E+04	7.13E+03	2.42E+00	3.84E-01
substrate-binding family protein, putative	3.92E-01	6.84E+03	4.09E+03	1.26E+03	4.39E+03	1.67E+00	2.24E-01
NAD kinase (EC 2.7.1.23)	3.93E-01	3.81E+02	2.31E+03	1.96E+02	3.09E+03	1.65E-01	-7.83E-01
hypothetical protein	3.96E-01	2.29E+04	1.44E+05	9.44E+03	1.96E+05	1.59E-01	-7.98E-01
Glycine cleavage system H protein	3.98E-01	2.03E+04	5.08E+04	3.32E+04	4.43E+04	3.99E-01	-3.99E-01
Spore cortex-lytic enzyme CwlJ	3.98E-01	4.05E+02	2.88E+03	2.78E+02	4.03E+03	1.41E-01	-8.52E-01
Putative deoxyribonuclease YcfH	3.99E-01	1.54E+03	2.91E+04	1.54E+03	4.50E+04	5.28E-02	-1.28E+00
Hypothetical protein ywlG	4.02E-01	4.85E+02	9.93E+02	2.69E+02	8.27E+02	4.88E-01	-3.11E-01

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
Sulfate adenylyltransferase, dissimilatory-type (EC 2.7.7.4)	4.04E-01	3.05E+04	4.32E+04	2.03E+04	9.64E+03	7.07E-01	-1.51E-01
transcriptional regulator, XRE family	4.05E-01	1.28E+03	1.63E+02	1.84E+03	7.46E+01	7.82E+00	8.93E-01
Lipase (EC 3.1.1.3)	4.11E-01	1.56E+05	2.71E+04	2.16E+05	8.48E+03	5.75E+00	7.60E-01
Acetolactate synthase large subunit (EC 2.2.1.6)	4.14E-01	3.84E+04	5.73E+04	3.17E+04	1.16E+04	6.69E-01	-1.74E-01
Chitin binding protein	4.15E-01	3.66E+05	2.52E+05	1.92E+05	6.71E+04	1.45E+00	1.62E-01
Acetyltransferase AcuA, acetyl-CoA synthetase inhibitor	4.23E-01	2.41E+04	1.57E+05	4.61E+03	2.31E+05	1.53E-01	-8.16E-01
FIG01227780: hypothetical protein	4.27E-01	3.67E+04	2.34E+04	2.32E+04	6.28E+03	1.57E+00	1.96E-01
Phosphatidylinositol-specific phospholipase C (EC 4.6.1.13)	4.42E-01	4.65E+03	2.60E+03	3.68E+03	1.26E+03	1.79E+00	2.52E-01
LSU ribosomal protein L2p (L8e)	4.44E-01	4.03E+04	5.81E+04	3.26E+04	7.11E+03	6.93E-01	-1.59E-01
Ser/Thr protein phosphatase family protein	4.49E-01	3.02E+04	4.76E+04	1.67E+04	3.05E+04	6.35E-01	-1.97E-01
Phenylalanine-4-hydroxylase (EC 1.14.16.1)	4.49E-01	4.04E+04	7.97E+04	1.77E+04	7.25E+04	5.07E-01	-2.95E-01
Phenylalanyl-tRNA synthetase domain protein (Bsu YtpR)	4.54E-01	1.08E+03	2.39E+03	7.92E+02	2.44E+03	4.50E-01	-3.47E-01
NADH-ubiquinone oxidoreductase chain D (EC 1.6.5.3)	4.59E-01	1.67E+04	2.00E+03	2.81E+04	2.17E+03	8.36E+00	9.22E-01
Glycine betaine ABC transport system, glycine betaine-binding protein OpuAC	4.61E-01	3.23E+05	5.14E+05	1.13E+05	3.61E+05	6.28E-01	-2.02E-01
Pyridoxamine 5'-phosphate oxidase (EC 1.4.3.5)	4.64E-01	1.26E+05	1.71E+04	2.11E+05	4.71E+03	7.40E+00	8.69E-01
5'-nucleotidase (EC 3.1.3.5)	4.67E-01	3.76E+04	1.55E+05	7.80E+03	2.28E+05	2.43E-01	-6.15E-01
Ribosomal subunit interface protein	4.72E-01	4.49E+04	8.46E+03	7.17E+04	3.48E+03	5.31E+00	7.25E-01
Arginine/ornithine antiporter ArcD	4.74E-01	2.76E+04	1.21E+04	3.06E+04	7.59E+02	2.27E+00	3.57E-01
sporulation kinase A	4.81E-01	7.95E+03	1.56E+04	2.05E+03	1.54E+04	5.10E-01	-2.92E-01

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
Predicted ATPase related to phosphate starvation-inducible protein PhoH	4.91E-01	1.66E+03	4.39E+02	2.51E+03	4.29E+02	3.78E+00	5.78E-01
Amino acid ABC transporter, amino acid-binding protein	5.00E-01	3.52E+04	4.66E+04	2.20E+04	1.42E+04	7.55E-01	-1.22E-01
SSU ribosomal protein S1p	5.06E-01	5.87E+03	2.00E+04	1.52E+03	3.04E+04	2.94E-01	-5.32E-01
FIG012187: hypothetical protein	5.07E-01	2.08E+03	1.49E+03	1.25E+03	4.14E+02	1.40E+00	1.45E-01
Branched-chain alpha-keto acid dehydrogenase, E1 component, alpha subunit (EC 1.2.4.4)	5.19E-01	7.10E+04	3.95E+04	6.81E+04	2.94E+04	1.80E+00	2.55E-01
hypothetical protein	5.20E-01	5.59E+05	3.82E+05	3.92E+05	1.01E+05	1.46E+00	1.65E-01
Manganese-dependent inorganic pyrophosphatase (EC 3.6.1.1)	5.21E-01	8.67E+04	1.16E+05	6.42E+04	2.22E+04	7.48E-01	-1.26E-01
FIG01235195: hypothetical protein	5.30E-01	1.09E+04	1.64E+04	8.15E+03	1.11E+04	6.64E-01	-1.78E-01
hypothetical protein	5.34E-01	3.31E+03	1.51E+03	4.12E+03	1.32E+03	2.19E+00	3.41E-01
hypothetical protein	5.36E-01	3.52E+03	5.09E+03	3.51E+03	1.60E+03	6.92E-01	-1.60E-01
Aliphatic amidase AmiE (EC 3.5.1.4)	5.46E-01	2.15E+04	6.64E+03	3.57E+04	6.02E+03	3.24E+00	5.11E-01
Phosphatidylinositol-specific phospholipase C (EC 4.6.1.13)	5.50E-01	3.22E+04	1.10E+04	5.10E+04	1.35E+04	2.93E+00	4.68E-01
Collagen adhesion protein	5.52E-01	5.65E+05	3.97E+05	4.08E+05	7.35E+04	1.42E+00	1.53E-01
acetyltransferase, GNAT family	5.53E-01	1.65E+04	5.06E+03	2.80E+04	1.77E+03	3.27E+00	5.14E-01
Branched-chain amino acid ABC transporter, amino acid-binding protein (TC 3.A.1.4.1)	5.53E-01	4.17E+03	6.37E+03	4.56E+03	3.73E+03	6.54E-01	-1.84E-01
FIG01233253: hypothetical protein	5.55E-01	2.75E+04	1.12E+04	4.01E+04	5.92E+03	2.45E+00	3.90E-01
Transcription termination protein NusA	5.58E-01	1.06E+04	4.02E+03	1.63E+04	2.55E+03	2.64E+00	4.21E-01
ATP-dependent hsl protease ATP-binding subunit HslU	5.59E-01	4.09E+03	6.46E+03	1.57E+03	5.82E+03	6.34E-01	-1.98E-01
Homoserine O-acetyltransferase (EC 2.3.1.31)	5.62E-01	6.86E+03	1.02E+04	3.97E+03	7.84E+03	6.75E-01	-1.71E-01

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
endonuclease/exonuclease/phosphatase family	5.63E-01	3.01E+05	3.80E+05	1.12E+05	1.83E+05	7.91E-01	-1.02E-01
Ferredoxin	5.64E-01	1.36E+03	2.08E+03	8.87E+02	1.72E+03	6.53E-01	-1.85E-01
Branched-chain alpha-keto acid dehydrogenase, E1 component, beta subunit (EC 1.2.4.4)	5.70E-01	2.98E+04	3.87E+04	1.72E+04	1.81E+04	7.70E-01	-1.14E-01
putative cytochrome P450 hydroxylase	5.71E-01	1.30E+04	2.08E+04	1.69E+04	1.39E+04	6.24E-01	-2.05E-01
neutral metalloprotease, putative	5.76E-01	5.51E+05	4.23E+05	3.26E+05	1.22E+05	1.30E+00	1.15E-01
FIG01226324: hypothetical protein	5.86E-01	5.77E+04	1.01E+05	9.74E+04	7.95E+04	5.72E-01	-2.42E-01
Chitin binding protein	5.86E-01	1.13E+06	1.32E+06	3.56E+05	4.34E+05	8.55E-01	-6.82E-02
hypothetical protein	5.87E-01	2.84E+03	4.44E+03	4.19E+03	1.58E+03	6.40E-01	-1.94E-01
FIG01225999: hypothetical protein	5.97E-01	1.46E+05	9.24E+04	1.46E+05	5.55E+04	1.58E+00	2.00E-01
FIG01233823: hypothetical protein	5.99E-01	1.35E+05	8.01E+04	1.48E+05	6.22E+04	1.69E+00	2.27E-01
Succinate dehydrogenase iron-sulfur protein (EC 1.3.99.1)	6.09E-01	1.01E+03	1.55E+03	8.21E+02	1.41E+03	6.56E-01	-1.83E-01
Aminopeptidase Y (Arg, Lys, Leu preference) (EC 3.4.11.15)	6.10E-01	1.10E+05	1.27E+05	3.43E+04	4.21E+04	8.64E-01	-6.37E-02
Phosphoglycerate kinase (EC 2.7.2.3)	6.12E-01	8.47E+04	6.04E+04	6.72E+04	3.04E+04	1.40E+00	1.46E-01
Vip2Ac-like_1	6.13E-01	3.25E+04	2.76E+04	1.34E+04	7.51E+03	1.18E+00	7.20E-02
SSU ribosomal protein S4p (S9e)	6.28E-01	2.30E+04	3.20E+04	2.59E+04	1.21E+04	7.20E-01	-1.43E-01
hypothetical protein	6.30E-01	1.56E+03	3.17E+03	2.44E+03	4.63E+03	4.91E-01	-3.09E-01
Butyryl-CoA dehydrogenase (EC 1.3.99.2)	6.45E-01	6.46E+06	4.47E+06	5.87E+06	3.46E+06	1.45E+00	1.60E-01
Phosphonate ABC transporter phosphate-binding periplasmic component (TC 3.A.1.9.1)	6.60E-01	3.21E+03	1.64E+03	5.28E+03	7.06E+02	1.96E+00	2.91E-01
hypothetical Membrane Spanning Protein	6.66E-01	3.41E+06	2.69E+06	2.15E+06	1.57E+06	1.27E+00	1.03E-01
Ribosome recycling factor	6.76E-01	3.45E+04	2.35E+04	3.89E+04	6.30E+03	1.47E+00	1.66E-01
Respiratory nitrate reductase alpha chain (EC 1.7.99.4)	6.76E-01	4.91E+04	6.78E+04	6.67E+04	5.04E+03	7.25E-01	-1.40E-01

p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)	Description
Phage shock protein A	6.77E-01	2.38E+03	3.49E+03	1.23E+03	3.90E+03	6.81E-01	-1.67E-01
hypothetical Membrane Spanning Protein	6.82E-01	1.09E+04	8.17E+03	7.99E+03	6.78E+03	1.33E+00	1.23E-01
FIG01226054: hypothetical protein	6.94E-01	3.34E+04	4.68E+04	5.05E+04	1.16E+04	7.13E-01	-1.47E-01
O-succinylbenzoic acid--CoA ligase (EC 6.2.1.26)	6.99E-01	7.39E+03	4.39E+03	1.16E+04	2.14E+03	1.68E+00	2.26E-01
Cell division trigger factor (EC 5.2.1.8)	7.02E-01	9.61E+02	6.58E+02	1.15E+03	4.42E+02	1.46E+00	1.65E-01
hypothetical Membrane Spanning Protein	7.03E-01	3.14E+05	4.14E+05	3.77E+05	1.67E+05	7.58E-01	-1.21E-01
Ribonuclease J2 (endoribonuclease in RNA processing)	7.11E-01	2.14E+04	2.71E+04	1.96E+04	1.50E+04	7.89E-01	-1.03E-01
L-serine dehydratase, alpha subunit (EC 4.3.1.17)	7.13E-01	1.57E+05	1.84E+05	1.04E+05	4.30E+04	8.55E-01	-6.80E-02
Lipase	7.23E-01	3.65E+03	2.32E+03	5.63E+03	6.54E+02	1.57E+00	1.97E-01
Cell surface protein IsdA1	7.38E-01	2.50E+03	1.71E+03	3.35E+03	1.63E+03	1.46E+00	1.65E-01
Prolyl-tRNA synthetase (EC 6.1.1.15), archaeal/eukaryal type	7.51E-01	1.01E+04	1.41E+04	9.46E+03	1.73E+04	7.19E-01	-1.44E-01
Transcriptional regulator, MerR family	7.58E-01	6.04E+03	8.04E+03	3.35E+03	9.49E+03	7.51E-01	-1.24E-01
Unspecified monosaccharide ABC transport system, substrate-binding component	7.58E-01	9.71E+03	1.22E+04	1.01E+04	8.14E+03	7.96E-01	-9.89E-02
2',3'-cyclic-nucleotide 2'-phosphodiesterase (EC 3.1.4.16)	7.61E-01	2.76E+05	2.52E+05	2.47E+04	1.21E+05	1.10E+00	4.04E-02
Oligopeptide transport ATP-binding protein OppD (TC 3.A.1.5.1)	7.64E-01	3.13E+04	3.98E+04	4.32E+04	2.56E+03	7.84E-01	-1.05E-01
transcriptional regulator, XRE family	7.83E-01	8.54E+05	8.13E+05	9.48E+04	2.19E+05	1.05E+00	2.17E-02
ATP synthase epsilon chain (EC 3.6.3.14)	7.85E-01	4.65E+03	3.26E+03	7.25E+03	3.43E+03	1.42E+00	1.54E-01
Cell wall surface anchor family protein	7.86E-01	2.57E+04	2.07E+04	2.55E+04	1.46E+04	1.24E+00	9.42E-02
N-formylglutamate deformylase (EC 3.5.1.68)	7.86E-01	6.63E+04	7.05E+04	1.39E+04	2.05E+04	9.41E-01	-2.65E-02

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
Alcohol dehydrogenase (EC 1.1.1.1); Acetaldehyde dehydrogenase (EC 1.2.1.10)	7.88E-01	1.72E+04	1.54E+04	7.79E+03	7.07E+03	1.11E+00	4.66E-02
hypothetical protein	7.99E-01	5.49E+04	4.92E+04	8.01E+03	3.36E+04	1.12E+00	4.78E-02
LSU ribosomal protein L36p	8.41E-01	5.83E+03	4.78E+03	7.94E+03	8.56E+02	1.22E+00	8.60E-02
FIG01229284: hypothetical protein	8.43E-01	5.73E+03	6.81E+03	8.25E+03	1.88E+03	8.41E-01	-7.54E-02
Cell surface protein IsdA, transfers heme from hemoglobin to apo-IsdC	8.47E-01	2.03E+03	2.39E+03	2.58E+03	1.64E+03	8.47E-01	-7.20E-02
hypothetical Membrane Spanning Protein	8.49E-01	1.74E+05	1.94E+05	1.54E+05	4.29E+04	8.98E-01	-4.66E-02
Glutamyl aminopeptidase (EC 3.4.11.7); Deblocking aminopeptidase	8.54E-01	1.68E+05	1.36E+05	2.66E+05	4.69E+04	1.24E+00	9.25E-02
transcriptional regulator, XRE family	8.68E-01	6.34E+05	5.65E+05	3.59E+05	5.61E+05	1.12E+00	5.01E-02
Ornithine carbamoyltransferase (EC 2.1.3.3)	8.69E-01	7.55E+03	8.69E+03	6.40E+03	9.13E+03	8.69E-01	-6.12E-02
Ribonuclease P protein component (EC 3.1.26.5)	8.70E-01	9.44E+05	8.90E+05	2.97E+05	4.38E+05	1.06E+00	2.54E-02
Zinc metalloproteinase precursor (EC 3.4.24.29) / aureolysin	8.71E-01	2.13E+05	2.08E+05	3.66E+04	3.57E+04	1.02E+00	1.06E-02
Tellurium resistance protein TerD	8.80E-01	7.70E+04	7.10E+04	3.72E+04	5.28E+04	1.09E+00	3.55E-02
Methylmalonate-semialdehyde dehydrogenase (EC 1.2.1.27)	8.87E-01	1.35E+05	1.18E+05	1.85E+05	2.15E+04	1.15E+00	5.90E-02
LSU ribosomal protein L11p (L12e)	8.93E-01	3.55E+04	3.07E+04	5.35E+04	1.96E+04	1.16E+00	6.40E-02
Fumarylacetoacetate hydrolase family protein	9.02E-01	2.01E+04	1.82E+04	2.20E+04	1.29E+04	1.11E+00	4.44E-02
Stage IV sporulation protein A	9.07E-01	1.36E+03	1.53E+03	1.94E+03	1.15E+03	8.92E-01	-4.97E-02
Heat shock protein GrpE	9.17E-01	8.40E+03	7.63E+03	1.11E+04	2.70E+03	1.10E+00	4.15E-02
Stage V sporulation protein required for dehydratation of the spore core and assembly of the coat (SpoVS)	9.43E-01	1.30E+03	1.36E+03	1.27E+03	4.88E+02	9.55E-01	-2.02E-02

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
Tryptophan 2,3-dioxygenase (EC 1.13.11.11)	9.44E-01	4.07E+03	4.33E+03	2.37E+03	5.47E+03	9.39E-01	-2.72E-02
Hypothetical protein SAV1845	9.51E-01	1.97E+04	1.91E+04	7.77E+03	1.10E+04	1.03E+00	1.15E-02
LSU ribosomal protein L7/L12 (P1/P2)	9.67E-01	4.44E+04	4.54E+04	3.54E+04	3.77E+03	9.79E-01	-9.16E-03
IMP cyclohydrolase (EC 3.5.4.10) / Phosphoribosylaminoimidazolecarboxamide formyltransferase (EC 2.1.2.3)	9.83E-01	1.87E+04	1.91E+04	2.74E+04	5.39E+03	9.80E-01	-8.80E-03
Signal transduction histidine kinase	9.87E-01	2.34E+03	2.30E+03	3.77E+03	4.57E+02	1.02E+00	7.36E-03
ATP-dependent Clp protease, ATP-binding subunit ClpC / Negative regulator of genetic competence clcC/mecB	9.99E-01	8.32E+03	8.32E+03	1.13E+04	1.88E+03	1.00E+00	3.54E-04

Shared proteins of the Bt isolate E-SE10.2 with the Bt isolate O-V84.2

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
Aldehyde dehydrogenase (EC 1.2.1.3)	1.10E-04	4.51E+03	7.69E+04	5.70E+03	4.15E+03	5.87E-02	-1.23E+00
Malate dehydrogenase (EC 1.1.1.37)	2.11E-03	1.15E+04	6.81E+04	1.03E+04	8.23E+03	1.68E-01	-7.74E-01
Peptidase T (EC 3.4.11.4)	5.29E-03	8.54E+04	6.12E+05	5.56E+04	1.10E+05	1.39E-01	-8.55E-01
SSU ribosomal protein S7p (S5e)	1.34E-02	1.69E+04	6.59E+04	1.49E+04	1.32E+04	2.56E-01	-5.91E-01
Flagellin protein FlaA	2.37E-02	9.47E+06	9.96E+05	2.52E+06	5.70E+05	9.50E+00	9.78E-01
Molybdenum cofactor biosynthesis protein MoaB	3.26E-02	2.74E+03	1.46E+04	2.68E+03	4.88E+03	1.88E-01	-7.27E-01
Chaperone protein DnaK	4.93E-02	3.40E+04	9.35E+04	2.66E+04	2.56E+04	3.64E-01	-4.39E-01
ABC transporter, ATP-binding protein	6.88E-02	8.92E+04	2.70E+05	9.72E+04	3.92E+04	3.31E-01	-4.81E-01
Glycerophosphoryl diester phosphodiesterase (EC 3.1.4.46)	9.23E-02	1.38E+03	3.32E+04	9.67E+02	1.81E+04	4.15E-02	-1.38E+00
Heat shock protein 60 family chaperone GroEL	9.73E-02	7.45E+05	2.34E+06	7.79E+05	9.95E+05	3.18E-01	-4.98E-01
Porphobilinogen synthase (EC 4.2.1.24)	1.44E-01	3.39E+03	3.48E+04	2.24E+03	2.34E+04	9.73E-02	-1.01E+00
methyl-accepting/DNA response regulator, putative	1.58E-01	4.28E+04	7.92E+04	1.87E+04	2.95E+04	5.40E-01	-2.68E-01
hypothetical protein	1.74E-01	7.61E+04	4.72E+03	5.98E+04	3.20E+03	1.61E+01	1.21E+00
DNA gyrase subunit A (EC 5.99.1.3)	1.84E-01	6.58E+03	5.43E+04	5.79E+03	4.18E+04	1.21E-01	-9.17E-01
hypothetical protein	3.15E-01	5.46E+03	1.15E+04	7.15E+03	5.65E+03	4.73E-01	-3.25E-01
NLP/P60 family protein	3.19E-01	1.28E+04	2.24E+04	1.22E+04	7.27E+03	5.71E-01	-2.44E-01
Nicotinate-nucleotide adenylyltransferase (EC 2.7.7.18)	3.29E-01	3.36E+03	1.18E+02	4.39E+03	3.80E+01	2.85E+01	1.45E+00
hypothetical protein	3.36E-01	4.00E+04	8.36E+03	4.36E+04	4.59E+03	4.78E+00	6.80E-01
Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein OppA (TC 3.A.1.5.1)	3.67E-01	2.56E+04	4.90E+03	3.10E+04	8.92E+02	5.23E+00	7.18E-01
Phage lysin; N-acetylmuramoyl-L-alanine amidase, family 3 (EC:3.5.1.28)	3.96E-01	2.15E+03	8.74E+01	3.33E+03	7.88E+01	2.46E+01	1.39E+00
N-acetylmuramoyl-L-alanine amidase (EC 3.5.1.28)	4.22E-01	1.51E+04	8.99E+02	2.45E+04	1.43E+02	1.68E+01	1.22E+00

Description	p-value	Mean 24	Mean 48	Sigma 24	Sigma 48	Fold Change 24/48	Log (Fold Change)
hypothetical protein	4.26E-01	5.27E+03	2.07E+02	8.85E+03	6.57E+01	2.55E+01	1.41E+00
hypothetical protein	4.59E-01	4.36E+04	6.65E+03	7.04E+04	3.81E+03	6.56E+00	8.17E-01
FIG01226217: hypothetical protein	4.69E-01	7.97E+03	1.50E+03	1.26E+04	8.99E+02	5.33E+00	7.26E-01
#¡REF!	4.89E-01	4.62E+03	8.45E+03	2.16E+03	7.80E+03	5.47E-01	-2.62E-01
hypothetical protein	5.12E-01	1.09E+05	2.75E+05	1.31E+05	3.55E+05	3.96E-01	-4.03E-01
NAD-dependent malic enzyme (EC 1.1.1.38)	5.26E-01	4.11E+03	1.07E+04	1.28E+03	1.50E+04	3.84E-01	-4.16E-01
enterotoxin / cell-wall binding protein	5.33E-01	2.57E+04	7.88E+03	4.12E+04	8.23E+03	3.27E+00	5.14E-01
Fe-S oxidoreductase	5.50E-01	2.28E+03	3.33E+03	7.11E+02	2.54E+03	6.83E-01	-1.66E-01
FIG01231653: hypothetical protein	6.49E-01	1.61E+04	2.51E+04	8.72E+03	2.89E+04	6.42E-01	-1.93E-01
Spore cortex-lytic enzyme, N-acetylglucosaminidase SleL (EC 3.2.1.-)	7.05E-01	1.29E+03	2.03E+03	1.92E+03	2.48E+03	6.34E-01	-1.98E-01
Electron transfer flavoprotein, alpha subunit	7.49E-01	1.64E+05	1.42E+05	5.55E+04	9.37E+04	1.15E+00	6.22E-02
Glutamine ABC transporter, periplasmic glutamine-binding protein (TC 3.A.1.3.2)	7.81E-01	3.03E+03	3.96E+03	4.38E+03	3.19E+03	7.64E-01	-1.17E-01
Translation elongation factor Tu	8.31E-01	5.52E+04	4.50E+04	7.08E+04	2.35E+04	1.23E+00	8.90E-02
2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N-acetyltransferase (EC 2.3.1.89)	8.64E-01	1.28E+04	1.10E+04	6.67E+03	1.52E+04	1.16E+00	6.58E-02
Succinate dehydrogenase flavoprotein subunit (EC 1.3.99.1)	9.34E-01	2.37E+04	2.19E+04	3.29E+04	2.78E+03	1.08E+00	3.41E-02
Beta-lysine acetyltransferase (EC 2.3.1.-)	9.90E-01	1.18E+04	1.20E+04	2.01E+04	6.52E+03	9.86E-01	-5.92E-03

Chapter 2

SUPPLEMENTARY MATERIAL:

Analysis of cross-resistance to Vip3 proteins in eight insect colonies, from four insect species, selected for resistance to *Bacillus thuringiensis* insecticidal proteins

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Table S1. Confirmation of resistance in the susceptible and Cry1Ac, Cry2Ab, Vip3Aa, and Vip3Aa/Cry2Ab-resistant *H. armigera* insect colonies.

Toxins	Dose (μg/cm^2)	Mortality[1] ± SE[2]				
		Susceptible	Cry1Ac-resistant	Cry2Ab-resistant	Vip3A-resistant	Vip3Aa/Cry2Ab-resistant
Buffer[3]	-	0 ± 0	6.3 ± 4.6	1.0 ± 1.5	2.1 ± 2.1	0 ± 0
Cry1Ac	0.25	100 ± 0	11.7 ± 7.9	94.8 ± 7.4	100 ± 0	95.8 ± 4.2
Cry2Ab	0.25	100 ± 0	72.4 ± 8.0	1.4 ± 2.0	100 ± 0	4.2 ± 4.2
Vip3Aa	20	100 ± 0	96.9 ± 5.4	86.2 ± 6.8	6.3 ± 6.3	10.6 ± 6.1

[1] Mortality: Number of death larvae in each treatment, expressed as percentage
[2] SE: Standard error of the mean
[3] Buffer: 20 mM Tris, 150 mM NaCl, pH 9

Table S2. Comparison of LC_{50} values of Cry and VIP3Aa protoxins for Dipel-susceptible and -resistant *Plodia interpunctella* strains.

Toxin	Susceptible[1]	Dipel-Resistant[2]
Cry1Ab	0.02	2.37
Cry1Ac	0.08	>250
Cry1F	15.3	280
Dipel	4.80	UD
ViP3Aa	15.9	78.7
Vip3Ca	67.5	13.7

[1]Data in μg applied to a 15 mg diet disk; from Oppert et al., 2010 and this study

[2]UD – unable to determine

Table S3. Confirmation of resistance in the susceptible and Cry1Ab-resistant *O. furnacalis* insect colonies

O. furnacalis strains	LC_{50} (FL_{95})	Resistance Ratio[1]	Slope ± SE[2]
	μg/g diet		
Susceptible	0.23 (0.17 - 0.30)	-	1.16 ± 0.11
Cry1Ab-resistant	183 (155 - 213)	794	2.41 ± 0.26

[1] Resistance Ratio was calculated divided the LC_{50} value of the resistant strain by the LC_{50} value of the susceptible strain respectively.
[2] SE: Standard error of the slope.

Chapter 3

Supplementary material

Changes in gene expression and apoptotic response in *Spodoptera exigua* larvae exposed to sublethal concentrations of Vip3 insecticidal proteins

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Supplementary Information:

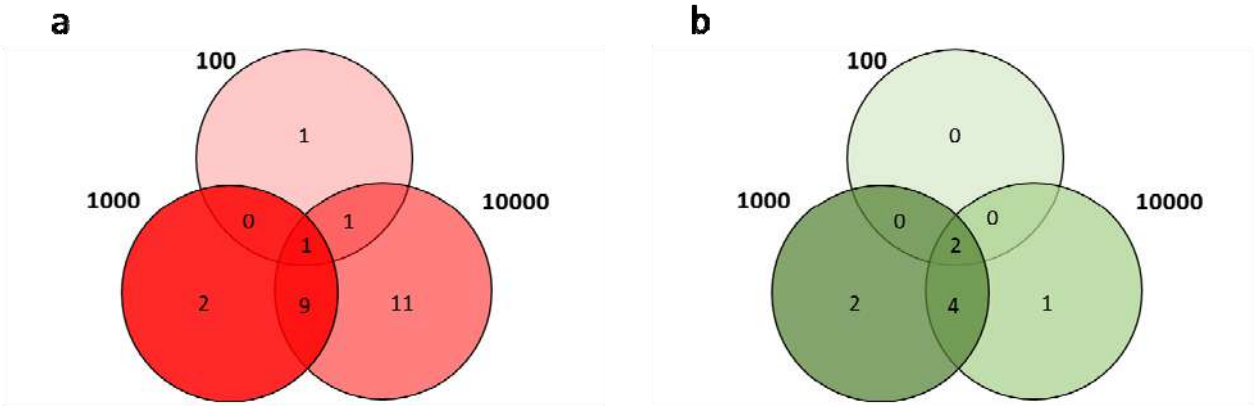
Supplementary Figure 1. Venn diagram showing up-regulated genes (panel a) and down-regulated genes (panel b) after 24 h challenged at 100, 1000, and 10000 ng/cm^2 of Vip3Ca.

Supplementary Figure 2. Correlation analysis between larval growth inhibition and the APN activity in the luminal fluid after exposure to four different concentrations of Vip3Ca protein for 24 h. Pearson r and p-value are shown in the graph.

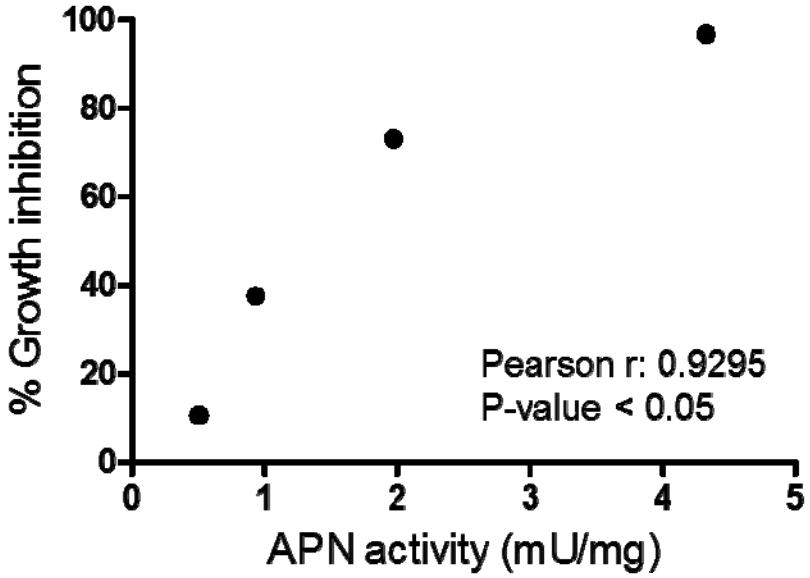
Supplementary Figure 3. Midgut tissue sections of *S. exigua* exposed for 24 h to Vip3Aa and Vip3Ca proteins were stained with hematoxylin and eosin. As controls, larvae fed with the empty vector (WK6) were used. Magnification was 100×. BM, basal membrane; AM, apical membrane and L, lumen.

Supplementary Table S1. List of primers for RT-qPCR used in this study and gene expression of each transcript after Vip3Ca challenged at 100, 1000, and 10000 ng/cm^2, respectively.

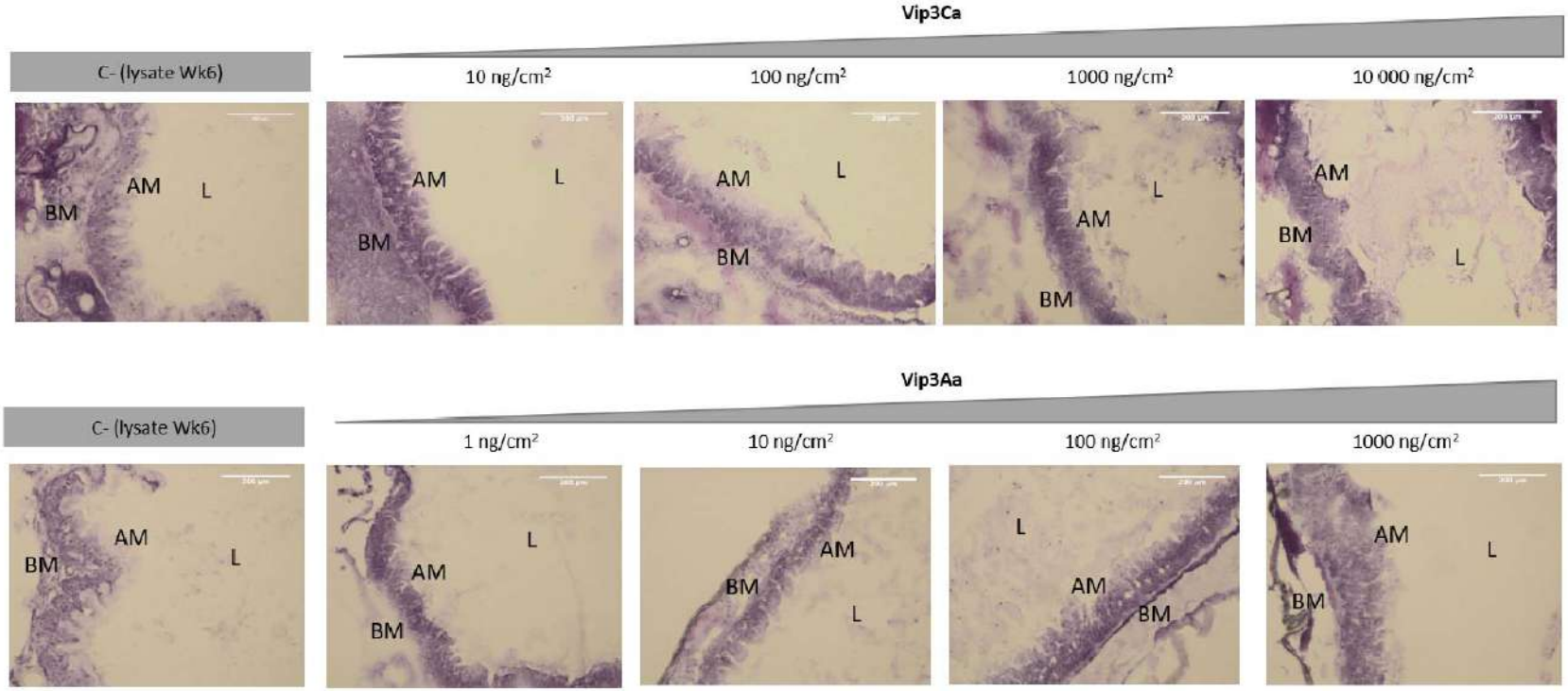
Supplementary Table S2. List of primers designed for the analysis of the expression levels of five apoptosis-related genes.



Supplementary Figure 1.



Supplementary Figure 2.



Supplementary Figure 3.

Supplementary Table S1: List of primers for RT-qPCR used in this study and gene expression of each transcript after Vip3Ca challenged at 100, 1000, and 10000 ng/cm ^{2}, respectively.

Number of pair of primer	Target gene	Primers		Source	Fold change ± SD		
		Forward (5' → 3')	Reverse (5' → 3')		expose 100 ng/cm^2	expose 1000 ng/cm^2	expose 10 000 ng/cm^2
1	*ATP synthase*	GTTGCTGGTCTGGTGGGATT	AGGCCTCAGACACCATTGAAA	Herrero et al., 2007	-	-	-
2	*Beta-glucan recognition protein*	AATTGGAAGCCATCTATCCTAAAGG	TGAGGTTTCCGTGGAATGC	Jakubowska et al., 2013	0.8 ± 0.6	5.0 ± 3.9*	2.7 ± 2.8
3	*Peptidoglycan recogniztion protein*	GTAGTACCGGAGTGTGTTAGTGATGAG	TTGTCCTATATCAGTGAATCCACGTT	Jakubowska et al., 2013	1.1 ± 0.8	1.7 ± 1.7	3.5 ± 4.4
4	*Prophenoloxidase activating enzyme*	AGCTGTGCGGCCCAGAT	TCGACACCGCAACATTCACT	Jakubowska et al., 2013	1.1 ± 0.7	3.0 ± 2.6	3.5 ± 3.6
5	*G-protein receptor*	GGCCGTCAGTGTGAAGAATATTAAGT	ACGGGAACAGCAAATTGTTGT	Jakubowska et al., 2013	1.3 ± 0.6	0.8 ± 0.9	1.0 ± 0.8
6	*TIN-ag-RP*	CGATGACTGTTGCCCAGACTAC	TGCAGCCCATGGTGTTATATTC	Jakubowska et al., 2013	0.8 ± 0.4	1.9 ± 1.7	2.6 ± 1.7
7	*Toll receptor*	TTCTTTAGTCTTTTCCAGAACATTGG	ACCTGATGCTGACAAAGACCTACA	Jakubowska et al., 2013	1.3 ± 0.7	0.3 ± 0.4	0.8 ± 0.5
8	*Imd*	GCTCCAAGGCCATCTACAGAGA	TCCTGATCTTCATTTTGATCTTGATT	Jakubowska et al., 2013	1.1 ± 1.1	0.06 ± 0.2	0.5 ± 0.3
9	*JAK-STAT*	CGCCCTTACAGGATCATCTCA	AGGCCGGATTCTAGGAGCTT	Jakubowska et al., 2013	0.7 ± 0.4	0.4 ± 0.2 *	0.5 ± 0.3
10	*SE_U12696 (REVIP)*	GGTCCAATTCCAACATGCACT	TGTAGGTCTTGTGAACGTGGTGT	Bel et al., 2013	0.1 ± 0.1 *	0.0015 ± 0.0014 *	0.006 ± 0.0033 *
11	*SE_U12832*	ACTGGTGCAGTTCCGAGCAT	AGCCCCAATACTGTGTCCCA	Bel et al., 2013	0.4 ± 0.3 *	0.07 ± 0.04 *	0.09 ± 0.06 *
12	*SE_U59986*	GCCATTGCCTTACCTTCTGG	GCTTCCAACAAAGTTCTCGTTGA	Bel et al., 2013	0.7 ± 0.4	0.07 ± 0.05 *	0.06 ± 0.03 *
13	*SE_U10224*	CGAAGGGAATGTTTGCGAAG	AGTTCGCTGACCAGAGAGTGC	Bel et al., 2013	0.6 ± 0.4	0.08 ± 0.04 *	0.2 ± 0.1 *
14	*SE_U08180*	ATTCGCCCGACCTCTTCAAT	TGTTTAGGATGAACTGGAACCATAAC	Bel et al., 2013	0.6 ± 0.4	0.06 ± 0.04 *	0.2 ± 0.1 *
15	*SE_U08346*	AGGTCATCTCCAGCTACGACG	CGTTGCACGATTCAAATTCG	Bel et al., 2013	0.6 ± 0.4	0.06 ± 0.04 *	0.1 ± 0.07 *
16	*SE_U56776*			Bel et al., 2013	5.9 ± 3.6 *	11.0 ± 36.0	29.3 ± 34.6 *
17	*SE_U20473*	CGGCCAAGAATTAGTTTCCAAA	AGACCGGGTACTCTGGCGTA	Bel et al., 2013	9.3 ± 6.9	6.4 ± 3.5 *	6.9 ± 6.1 *
18	*SE_U33476*	CAGTACAATGGCCGCTCTCAA	AAGGCAATGAGGAGCAGCAC	Bel et al., 2013	0.8 ± 0.9	9.8 ± 12.9	19.3 ± 11.6 *
19	*SE_17986*	CGAGTGCACCATGAACACCT	ATGACGGCGAGGAAAGAGAG	Bel et al., 2013	1.8 ± 1.3	19.2 ± 11.6 *	10.4 ± 8.9 *
20	*SE_U08322*	GCCGCTAAGAATGCAGCTAAA	TGATGCCCGTGGAAGCTT	Bel et al., 2013	8.8 ± 5.9 *	11.3 ± 5.6 *	6.7 ± 5.7 *
21	*SE_U08997*	CTCCCCGAAGCTGAGACCT	TGGTCTCCGGCTTTATTGGA	Bel et al., 2013	1.4 ± 0.4	1.4 ± 0.6	2.3 ± 1.2 *
22	*SE_13239*	TTGGGCATCAAGTCGCTAGA	GTCCCCCTTGATCTCGTCAA	Bel et al., 2013	2.9 ± 2.3	20.9 ± 23.0 *	7.8 ± 5.6 *
23	*SE_U09334*	CAGTCGCCGGCCAAATAC	CGGGCTCGGCTTTATAGACC	Bel et al., 2013	5.2 ± 3.6 *	3.2 ± 3.6	1.8 ± 1.6
24	*SE_U06544*	TCAATTTCCAATAAAGCCGGA	TCTCGTGCTCAGCAATGTGC	Bel et al., 2013	1.1 ± 1.0	1.2 ± 1.1	1.1 ± 1.0
25	*Gloverin*	CGAGGTGGCTACAAACAAGAC	CATATGCCTGGCCTTGAAG	Crava et al., 2015	1.1 ± 1.0	4.2 ± 3.6	31.7 ± 5.0 *
26	*Attacin 1*	CGTTCTTAGACCGCAAGGAC	CACGGAAGTGGTCGGGCT	Crava et al., 2015	1.5 ± 2.3	1.6 ± 2.6	2.2 ± 2.0
27	*Attacin 3*	CGGTTTATCAGCACCATTCGGT	CGCCTGGCAGCATCAAAG	Crava et al., 2015	1.3 ± 1.6	0.5 ± 0.7	2.9 ± 2.6
28	*Lebocin 1*	CACTACACCTGCCTGACTACA	GGCGAGGGTTGAAGGGA	Crava et al., 2015	1.2 ± 1.6	35.6 ± 19.5 *	14.7 ± 10.5 *
29	*Cecropin A1*	GTCATCGTAATCATCACATCAACTAC	ACGGCAGGCAGTTGCTCAG	Crava et al., 2015	0.2 ± 0.4	1.9 ± 2.1	1.2 ± 1.1
30	*Cecropin B*	GGATAAGCTGGTCTCCAAACAC	GTGTGCCAACTTATTCGAGAAC	Crava et al., 2015	1.3 ± 1.1	5.2 ± 8.9	2.5 ± 2.3 *
31	*Cecropin C*	CAGTGAGGAAGACTAGACGGC	ATGGAGCGTATACAAATGAACG	Crava et al., 2015	1.1 ± 0.9	4.5 ± 7.0	3,05 ± 2.8 *
32	*Cecropin D*	GCCAAAGCGCTAGGAAAGTAG	TCTGTTGCTGACTATTGAAGTAGG	Crava et al., 2015	0.6 ± 0.5	3.2 ± 5.3	2.7 ± 2.3
33	*Cecropin E*	TGGCCGTTGTGGGATCAG	GTATGTGTCAGGTCATAGGGACT	Crava et al., 2015	1.3 ± 1.1	1.2 ± 1.9	8,1 ± 8.3 *
34	*Cecropin F*	CCAAGGCGCTAGGATAAAC	GGCGGAATGAGTATTATGAGGT	Crava et al., 2015	1.1 ± 0.8	0.9 ± 3.2	-2.2 ± 0.2
35	*Spodoptericn*	TCGTGCGATTTCGAAGAAGC	GCAGATGCCGTAAGTGTAACCT	Crava et al., 2015	3.4 ± 3.7	10.2 ± 4.6 *	21,9 ± 28.4 *
36	*LYZ 1*	GAATCATGCAAAAGCTAACGGT	TGCCTCGCAATGCAAGCA	Crava et al., 2015	0.8 ± 0.7	3.0 ± 4.9	2.2 ± 1.9 *
37	*LYZ 2*	GACGAATTGCGATTTAGTTCAC	GAGCACTCTCACTGTTTACCAGAC	Crava et al., 2015	1.2 ± 0.9	3.7 ± 6.5	2.8 ± 2.1 *
38	*LYZ 3*	CCTAATTGAAGCGGAGGGTT	GTGGGAACCGTCTGAATTCG	Crava et al., 2015	1.4 ± 1.2	2.4 ± 4.7	11.1 ± 7.3 *
39	*LLP 1*	GCCTGATATTGAGAAATGTCCA	CTTGCCGTTCTCTTGTCTCTAGG	Crava et al., 2015	0.5 ± 0.4	0.5 ± 0.4	0.5 ± 0.3 *
40	*LLP 2*	CGCGGTCCAGCACTAAGAC	CGCCTAGATCTTCTCAACCTGG	Crava et al., 2015	1.1 ± 0.5	0.8 ± 0.7	-2.0 ± 0.5
41	*Diapausin A1*	GCGGTAGAATGGACTGTTACTGATG	CAAGAAGGTTATCACGAATACG	Crava et al., 2015	1.8 ± 1.4	2.8 ± 2.8*	21.6 ± 15.3 *
42	*Diapausin A2*	GCGGTAGAATGGACTGTTACTGATG	GCAAGTCAGGAATACTAAAGGGC	Crava et al., 2015	1.1 ± 0.6	0.6 ± 0.6	4.6 ± 4.0 *
43	*Diapausin A3*	GCGGTAGAATGGACTGTTACTGATG	CTAGAGAGCTGCGTTGTTCAC	Crava et al., 2015	2.2 ± 1.3	0.8 ± 0.7	29.0 ± 16.3 *
44	*Diapausin A6*	GCGGTAGAATGGACTGTTACTGATG	ATTAGGTTTCTAGGCTTGTGTCAC	Crava et al., 2015	3.1 ± 3.2	39.5 ± 49.0*	46.4 ± 38.8*
45	*Diapausin A7*	GCGGTAGAATGGACTGTTACTGATG	TAAGAAGATCCTCCACTACAAGG	Crava et al., 2015	3.9 ± 3.4	21.8 ± 16.4*	46.5 ± 40.8*
46	*Cobatoxin 1*	TCGAGGAGGTGGGAGATGTC	CGAACGGCTGGAGACTCTTC	Crava et al., 2015	0.8 ± 0.6	5.3 ± 3.4 *	4.7 ± 6.9
47	*Cobatoxin 2*	GAAGCTCGTATTGTTTGTCGT	CCTCAGCAAGTCGTCAATG	Crava et al., 2015	0.4 ± 0.2	0.4 ± 0.2*	0.8 ± 0.8
48	*Moricin*	AAGCGGCTCCAGGAAAGATACC	ATTGCCCGGAGACCTTTACCAA	Crava et al., 2015	1.6 ± 1.6	4.3 ± 2.8*	6.2 ± 6.6 *

* Genes whose expression was significantly up-(box in red) or down-regulated (box in green) after Vip3Ca challenge

Supplementary Table S2: List of primers design for the analysis of the expression levels of five apoptosis-related genes.

Target gene	Name	Length	Tm	%GC	5'-3' sequence	Amp Length
Se-Caspase-1	Se-Caspase-1 F	20	62	60	GCTTGAAGTCGCGTACTGGC	
	Se-Caspase-1 R	19	61	63	GTCTGCAGTCTGCTGGACG	137
Se-Caspase-2	Se-Caspase-2 F	20	59	55	ATCGATATCCCACCACGAGC	
	Se-Caspase-2 R	21	58	48	CAACACTAAATCCCAACGCTG	82
Se-Caspase-3	Se-Caspase-3 F	23	64	57	CACATGCTGACTTCCTCGTGCTG	
	Se-Caspase-3 R	25	63	52	CAGGTCCTCATGATGTTCCTCCA	122
Se-Caspase-4	Se-Caspase-4 F	22	60	55	CGAGGTACGAAGATCACCCAAG	
	Se-Caspase-4 R	21	60	57	GAGATCAGACTCCACTGGCAG	111
Se-Caspase-6	Se-Caspase-6 F	20	59	55	GAAGCTCTCCTTACCTGCCA	
	Se-Caspase-6 R	20	59	50	AGTACTTGCGTGCTGCATTG	76

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