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Caracterización genética y molecular de una cepa entomopatógena activa frente a la mosca de la fruta del Mediterráneo (*Ceratitis capitata*) para la búsqueda de factores de virulencia

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Caracterización genética y molecular de una cepa entomopatógena activa frente a la mosca de la fruta del Mediterráneo (*Ceratitis capitata*) para la búsqueda de factores de virulencia

Memoria presentada

por

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A mis padres,

A Víctor,

A la memoria de mi papito Lalo.

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“No te des por vencido, ni aún vencido,
no te sientas esclavo, ni aún esclavo;
trémulo de pavor, piénsate bravo,
y acomete feroz, ya mal herido.

Ten el tesón del clavo enmohecido
que ya viejo y ruin, vuelve a ser clavo,
no la cobarde estupidez del pavo
que amaina su plumaje al primer ruido.

Procede como Dios que nunca llora;
o como Lucifer, que nunca reza;
o como el robledal, cuya grandeza
necesita del agua y no la implora...”

(Pedro Bonifacio Palacios)

"No existen suficientes experimentos que puedan demostrar que tengo razón,
pero basta uno sólo para mostrar que estoy equivocado"

(Albert Einstein)

"No temas a las dificultades: lo mejor surge de ellas"

(Rita Levi–Montalcini)

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µg	Microgramo (microgram)
µl	Microlitro (Microliter)
µm	Micrómetro (micrometer)
µM	Micromolar
16S ARNr	Subunidad 16S del ácido ribonucleico ribosomal
2D	Dos dimensiones (2-dimensional)
aa	Aminoácido (amino acid)
ADN	Ácido desoxirribonucleico, en inglés **DNA** (Deoxyribonucleic Acid)
ADP	Adenosín difosfato
Afp	Profago de anti-alimentación, del inglés "Antifeeding Prophage"
AMPs	Péptidos antimicrobianos
ARN	Ácido ribonucleico, en inglés **RNA** (Ribonucleic Acid)
ARNasa	Enzima ribonucleasa
ATP	Adenosín trifosfato
Bin	Toxinas binarias proteicas, del inglés "Binary toxin"
Bt	*Bacillus thuringiensis*
CCC	Círculo cerrado covalente, del inglés "Covalently Closed Circular"
CDS	Secuencia codificante, del inglés "Coding DNA Sequence"
CFU	Unidades formadoras de colonia, del inglés "Coloni-Forming Units"
CMV	Virus del mosaico del pepino, del inglés "Cucumber Mosaic Virus"
Cry	Proteína formadora de cristal, del inglés "Crystal"
C-terminal	Extremo carboxilo terminal
Cyt	Proteína citolítica, del inglés "Cytolitic"
Da	Dalton
EFSA	Autoridad Europea de seguridad alimentaria, del inglés "European Food Safety Authority"
EPS	Sustancia polimérica extracellular, del inglés "Extracellular Polymeric Substance"
g	Gramo (gram)
g	Gravedades (gravity)
GC o G+C	Guanina y Citosina (Guanine Cytosine)
h	Hora (hour)
IEF	Isoelectroenfoque, del inglés "Isoelectric Focusing"
J	Joule
kDa	Kilodaltons
l	Litro (liter)
LB	Luria-Bertani
LC-MS/MS	Cromatografía líquida acoplada a espectrometría de masas, del inglés "Liquid Chromatography – Mass Spectrometry"

M	Molar
m	Meter (meter)
MALDI-TOF	Desorción/ionización láser asistida por matriz acoplada a detector de iones, del inglés "Matrix-Assisted Laser Desorption/Ionization – Time Of Flight"
MIC	Concentración mínima inhibitoria, del inglés "Minimum Inhibitory Concentration"
min	Minuto (minute)
ml	Mililitro (Milliliter)
mm	Milímetro (millimeter)
mM	miliMolar (milliMolar)
MS	Espectrometría de masas, del inglés "Mass Spectrometry"
Mtx	Toxina mosquitocida, del inglés "Mosquitocidal toxins"
nc RNA	ARN no codificante (non-coding RNA)
NCBI	National Center for Biotechnology Information
ng	Nanogramo (nanogram)
nm	Nanómetro (nanometer)
N-terminal	Extremo amino-terminal
ºC	Grados centígrados (Celsius degrees)
OD	Densidad óptica, del inglés "Optical Density"
ORF	Marco abierto de lectura, del inglés "Open Reading Frame"
pADAP	Plásmido asociado a la enfermedad ambar
PAGE	Electroforesis en gel de poliacrilamida, del inglés "Polyacrylamide Gel Electrophoresis"
pb	Pares de bases**,** en inglés **bp** (base pairs)
PBS	Tampón fosfato salino, del inglés "Phosphate-Buffered Saline"
PCN	Número de copias del plásmido, del inglés "Plasmid Copy Number"
PCR	Reacción en cadena de la polimerasa, del inglés "Polymerase Chain Reaction"
pH	Potencial Hidrógeno
p*I*	Punto isoeléctrico (isoelectric point)
PVC	*Cassette* de virulencia de *Photorhabdus*
qRT-PCR	PCR cuantitativo en tiempo real, del inglés "Quantitative Real Time PCR"
RCR	Replicación de círculo rodante, del inglés "Rolling Circle Replication"
RIDL	Liberación de insectos con un dominante letal, del inglés "Release of Insects carrying a Dominant Lethal"
rpm	Revoluciones por minuto (Revolutions per minute)
rRNA	ARN ribosómico (ribosomal RNA)
RT	Temperatura ambiente, del inglés "Room Temperature"
s	Segundo (second)

SDS	Dodecil sulfato de sodio, el inglés “Sodium Dodecyl Sulfate”
SEM	Microscopía electrónica de barrido, del inglés “Scanning Electron Microscopy”
Sip	Proteína insecticida secretada, del inglés “Secreted insecticidal protein”
ssDNA	ADN de cadena simple (DNA single-strand)
Tc	Complejo tóxico
TEM	Microscopía electronic de transmission, del ingles “Transmission Electron Microscopy”
TIE	Técnica del insecto estéril
tRNA	ARN de transferencia (transfer RNA)
U	Unidad (unit)
UV	Ultra violeta (ultraviolet)
V	Volt
Vip	Proteínas insecticidas vegetativas, del inglés “Vegetative insecticidal proteins”
vol/vol	Volumen/volume (volume/volume)
W	Watt
wt/vol	Peso/volumen (weight/volume)

RESUMEN

En este trabajo se presenta la caracterización realizada sobre la cepa *Bacillus pumilus* 15.1, previamente aislada por nuestro grupo de investigación. Esta cepa presenta una importancia relevante en Biotecnología debido a su actividad tóxica frente a larvas de *Ceratitis capitata*, una plaga ampliamente distribuida a nivel mundial.

La caracterización realizada en este trabajo aporta información importante sobre el metabolismo (utilización de fuentes de carbón y nitrógeno), actividades enzimáticas, formación de biopelículas, motilidad, suceptibilidad a los antibióticos, y resistencia a agentes externos. Una de las características más relevantes descubiertas como consecuencia de esta caracterización fue la gran resistencia que presentaron las esporas y las células vegetativas de *B. pumilus* 15.1 al efecto de la radición UV-C; las células vegetativas alcanzaron valores de supervivencia mucho más altos que los de las cepas de *Bacillus thuringiensis* (*Bt*) y *Bacillus subtilis* ensayadas en este estudio.

La caracterización realizada permitió descubrir que *B. pumilus* 15.1 posee dos elementos extracromosómicos; uno de ellos, el plásmidos pBp15.1S fue subclonado, secuenciado, ensamblado y su secuencia analizada en busca de factores de virulencia.

En la caracterización morfológica de la cepa *B. pumilus* 15.1, durante el crecimiento del cultivo bacteriano, se observó la producción de cristales paraesporales semejantes a los producidos por la bacteria *Bt* y responsables principales de la toxicidad entomopatógena de la bacteria. En este trabajo se caracterizaron los cristales paraesporales de la cepa *B. pumilus* 15.1. Para determinar el origen de su codificación, se obtuvo una cepa curada de elementos extracromosómicos denominda *B. pumilus* 15.1C. Se relacionó la producción de los cristales con el perfil proteico de la cepa con lo cual se pudo deducir que el componente mayoritario de los cristales paraesporales observados fue una proteína de 45 kDa. La caracterización de esta proteína mostró que es resistente a la tripsina y que presenta un peculiar mecanismo de solubilización. Se realizaron análisis de

MALDI-TOF y secuenciación del extremo N-terminal de la proteína con objeto de proceder a su identificación.

Además, mediante la técnica *shotgun* se obtuvo la secuencia del genoma de *B. pumilus* 15.1, el cual fue ensamblado, anotado y puesto a disposición de la comunidad científica en la base de datos del NCBI. Para buscar posibles factores de virulencia en el genoma de la cepa, se creó una base de datos con todos los factores tóxicos frente a insectos de bacterias Gram-positivas y Gram-negativas. El resultado de esta búsqueda fue el hallazgo de que en el genoma de *B. pumilus* 15.1existen genes codificantes para proteínas homólogas a dos proteínas recientemente descritas como factores de virulencia en otras bacterias, además de quitinasas, metaloproteasas, citolisinas y antibióticos.

En este trabajo de investigación se describe por primera vez la caracterización de una cepa entomopatógena de la especie *B. pumilus*, además de proporcionar información sobre los posibles factores de virulencia de la cepa *B. pumilus* 15.1.

1. INTRODUCCIÓN GENERAL

1.1 Microorganismos entomopatógenos

Las millonarias pérdidas agrícolas debidas a plagas de insectos que atacan tanto los cultivos como los lugares de almacenamiento alcanzan un tercio de la producción mundial (Sree and Varma 2015). Los insectos son uno de los grupos más diversos sobre la tierra, con más de un millón de especies descritas, y cuya diversidad y capacidad de adaptación a diferentes ambientes los ha convertido en hospedadores o fuente de alimento de una gran variedad de parásitos (Bode 2009). A los parásitos capaces de causar la muerte al insecto huésped se los denomina entomopatógenos. Otros enemigos naturales de los insectos incluyen depredadores y patógenos de insectos. Los organismos entomopatógenos representan una alternativa sostenible de control a largo plazo comparado con los productos químicos mayoritariamente utilizados, cuyo espectro de especificidad es muy bajo y por lo general son de larga acción residual.

Desde hace mucho tiempo, estos enemigos naturales se han utilizado con mucho éxito en el manejo integrado de plagas, como por ejemplo en el caso de *Anagyrus lopezi* (Hymenoptera: Encyrtidae) una pequeña avispa que mata a la cochinilla de la yuca, *Phenacoccus manihoti* (Homoptera: Pseudococcidae). En 1973, la cochinilla de la yuca se convirtió en una plaga en África llegando a invadir 27 países. En 1981, la avispa parasitoide *A. lopezi* fue liberada como agente de control biológico. A finales de esa década se consiguió una reducción entre el 90% y 95% de la plaga (Neuenschwander 2004).

Entre los agentes de control biológico, los microorganismos entomopatógenos presentan sobre los demás varias ventajas, cómo su facilidad de producción y aplicación, especificidad, viabilidad económica y seguridad ambiental (Sree and Varma 2015). Para que un microorganismo entomopatógeno tenga éxito debe ser capaz de persistir en el ambiente, multiplicarse en el hospedador y dispersarse hacia otros hospedadores susceptibles. Los grupos más importantes de microorganismos entomopatógenos son: bacterias, virus, hongos, nematodos y protozoos; los cuales han desarrollado diferentes estrategias para atacar, penetrar en el organismo y matar

a su hospedador. Generalmente, los hongos patógenos penetran a través de la cutícula; mientras que las bacterias, los virus y los protozoos lo hacen por el intestino medio tras la ingestión. Los nematodos penetran principalmente por aperturas naturales en el cuerpo del hospedador (Ruiu *et al.* 2013).

Además hay que tener en cuenta que los insectos no sólo causan problemas en la agricultura, sino también son una fuente de diseminación de microorganismos y parásitos que causan serios problemas en la salud humana o animal. Por lo tanto, los entomopatógenos son también utilizados para disminuir poblaciones de insectos vectores de enfermedades.

1.2 Bacterias entomopatógenas

Los insectos poseen un gran número de bacterias asociadas a ellos. La mayoría son saprofitas y comensales, y algunas son simbióticas. Se han descrito pocas especies bacterianas patógenas de insectos, sin embargo en los últimos años ha aumentado el interés de la comunidad científica por su potencial para el control de plagas en la agricultura y el control de vectores de enfermedades (van Emden and Service 2004).

Las bacterias son el grupo de patógenos de insectos que ha tenido más éxito en su uso comercial. Sin embargo, el estudio y la caracterización de cepas bacterianas no son labores fáciles, ya que muchas de ellas no comparten el o los factores de virulencia ni tampoco la manera de actuar para provocar la muerte del insecto. No existe un patrón común que abarque a todas las cepas entomopatógenas descritas de cómo deben invadir, persistir, actuar y propagarse en el medio de manera exitosa. En muchos casos no se conoce ni el modo de acción ni el papel exacto de una toxina en el estilo de vida de muchas bacterias, siendo muchas veces difícil elucidarlo ya que hay genes que codifican toxinas similares en bacterias que no han sido previamente descritas como entomopatógenas (Vallet-Gely *et al.* 2008).

Se puede decir de manera general que las bacterias acceden al insecto por medio de la ingestión, aunque si existe alguna herida en la cutícula pueden ingresar por ahí hasta llegar al hemocele. Pero también hay bacterias que ingresan en el insecto por

medio de un transporte asistido (Vallet-Gely *et al.* 2008), como es el caso de *Photorhabdus luminescens* y *Xenorhabdus nematophila*, que se encuentran en asociación simbiótica con nematodos entomopatógenos los cuales las transportan hasta el interior del insecto donde las bacterias son liberadas y atacan al hospedador hasta causarle la muerte (Forst and Nealson 1996).

El modo de acción es complejo, de hecho esta es una de las ventajas por la cual han sido reconocidos como agentes de control biológico eficaces, ya que a diferencia de los compuestos químicos convencionales, las bacterias dirigen su acción a diferentes puntos haciendo menos probable el desarrollo de resistencia por parte del insecto (Ruiu 2015).

Muchas cepas bacterianas entomopatógenas pertenecen a géneros bacterianos que no se encuentran normalmente asociados a insectos. Vallet-Gely y colaboradores (2008) proponen que la capacidad para interactuar con un insecto es lo que hace que la bacteria pueda ser entomopatógena, es decir, que la patogenicidad va a estar mediada por factores que debe superar la bacteria. Para que una bacteria sea entomopatógena se debe cumplir que: (1) las bacterias ocupen el mismo nicho ecológico que el insecto, (2) posean la capacidad de persistir en los insectos, (3) evadan o toleren la respuesta del hospedador y (4) logren una infección persistente que sea capaz de colonizar los tejidos del insecto y/o producir toxinas que pongan en peligro la fisiología del hospedador.

Las bacterias entomopatógenas más conocidas y utilizadas son las formadoras de esporas de la familia Bacillaceae, especialmente algunas especies del género *Bacillus*. El interés por este género bacteriano se debe a la aptitud de sus cepas para ser tratadas industrialmente para la producción masiva y la aplicación en el campo como insecticida microbiológico (van Emden and Service 2004).

1.2.1 El género *Bacillus*

Son bacterias Gram-positivas, buenas secretoras de proteínas y metabolitos, fáciles de cultivar y altamente eficientes para el control de plagas y enfermedades (Berg and Hallmann 2006). Poseen una morfología alargada simulando un bastón, son aerobias estrictas o anaerobias facultativas, ubicuas en la naturaleza; incluyen

especies de vida libre, patógenas y varias especies que forman parte natural de la flora intestinal humana (Todar 2012).

Una característica particular de este género es su habilidad para formar endosporas bajo condiciones de estrés físico o químico, lo que le permite sobrevivir y permanecer metabólicamente activas por largos períodos de tiempo (Errington 2010), haciéndolos apropiados para la formulación de productos viables y estables para el control biológico.

Existen otras bacterias capaces de producir endosporas, cómo las pertenecientes a los géneros *Clostridium*, *Sporosarcina*, *Sporohalobacter*, *Sporolactobacillus*, *Anaerobacter*, *Desulfotomaculum*, *Helicobacterium* y *Heliophilum* (Nicholson *et al.* 2000); sin embargo los géneros más estudiados son *Bacillus* y *Clostridium* (Nicholson *et al.* 2000; Moller and Hederstedt 2006).

El género *Bacillus* ha cobrado importancia por su producción de toxinas, capaces de lisar eritrocitos de diferentes especies animales (hemolisinas) o de presentar propiedades patógenas frente a insectos (Hoult and Tuxford 1991). Los *Bacillus* con propiedades entomopatógenas utilizan una gran variedad de proteínas tóxicas para producir la invasión, infección y finalmente la muerte de su hospedador a través de su acción en el intestino medio del insecto. La mayoría de estas toxinas se producen en grandes cantidades durante la esporulación y tienen la característica de encontrarse localizadas en cristales paraesporales (de Maagd *et al.* 2003).

Las bacterias entomopatógenas pertenecientes a este género son agentes naturales de control biológico de plagas de insectos y han sido utilizadas como base de varios insecticidas biológicos comerciales. Las especies más relevantes son *Bacillus thuringiensis* (*Bt*) y *Lysinibacillus sphaericus* (anteriormente llamada *Bacillus sphaericus* y que ahora pertenece al género *Lysinibacillus*). Otras especies de la familia Paenibacillaceae, antes incluidas en el género *Bacillus*, son también importantes para el biocontrol, entre ellas *Brevibacillus laterosporus*, *Paenibacillus larvae*, *Paenibacillus lentimorbis* y *Paenibacillus popilliae* (Ruiu 2015).

L. sphaericus es conocida por la producción de toxinas insecticidas para el control de mosquitos vectores de enfermedades humanas (Jones *et al.* 2007). Esta especie forma esporas esféricas junto con cristales localizados dentro del exosporium que son altamente tóxicos para larvas de dípteros (Broadwell and

Baumann 1986). Estos cuerpos paraesporales contienen toxinas binarias proteicas (Bin) que actúan de forma similar a las proteínas Cry. Durante la fase vegetativa, *L. sphaericus* es capaz de producir toxinas contra mosquitos conocidas como proteínas Mtx, que juegan un papel menor debido a su baja producción y rápida degradación (Wirth *et al.* 2007). También se ha descrito la producción de dos toxinas Cry, Cry48Aa y Cry49Aa, que exhiben alta especificidad contra *Culex quinquefasciatus* (Jones *et al.* 2007; Jones *et al.* 2008).

El género *Paenibacillus* incluye diferentes especies que han mostrado toxicidad contra insectos, entre las cuales está *P. larvae* que es el agente causal de la enfermedad loque americana que afecta a las larvas de las abejas (*Apis mellifera*). Se han descrito algunos factores de virulencia para *P. larvae*, aunque no es totalmente conocido el mecanismo de acción de los mismos (Fünfhaus *et al.* 2013; Garcia-Gonzalez *et al.* 2014). Otros miembros de éste género son *P. lentimorbus* y *P. popilliae* que son los causantes de la enfermedad lechosa de las larvas del escarabajo (de Maagd *et al.* 2003). Estas especies poseen genes que codifican proteínas Cry (Zhang *et al.* 1997; Yokoyama *et al.* 2004), siendo estas proteínas el factor tóxico de *P. lentimorbus* (Yokoyama *et al.* 2004). Para *P. popilliae* los cristales paraesporales compuestos por proteínas Cry no son los responsables directos de la acción insecticida, posiblemente su contribución en la patogenicidad está en el daño que producen en la pared del intestino del insecto permitiendo la entrada de células vegetativas a la hemolinfa (Zhang *et al.* 1997).

En cuanto al género *Bacillus*, *Bt* es la especie con más éxito comercial y de la que se tiene más conocimiento científico; sus cepas son conocidas por la producción de toxinas Cry que constituyen el principal factor tóxico en bioinsecticidas y cultivos transgénicos. Los detalles de esta especie serán tratados más adelante.

Otra especie conocida es *B. laterosporus* cuya actividad bioinsecticida frente a un amplio rango de especies de los órdenes Coleoptera, Lepidoptera, Diptera y algunos nematodos y moluscos es muy conocida; además posee actividad antimicrobiana frente a bacterias fitopatógenas y hongos (Ruiu 2015). Esta especie produce dos tipos de cristales paraesporales (Figura 1); uno de ellos característico de esta especie, tiene forma de canoa y está unido a la espora (Smirnova *et al.* 1996). Son varios los

factores tóxicos de *B. laterosporus*, entre ellos los cristales paraesporales y las proteínas secretadas ISP que tienen homología con las proteínas Vip de *Bt.*

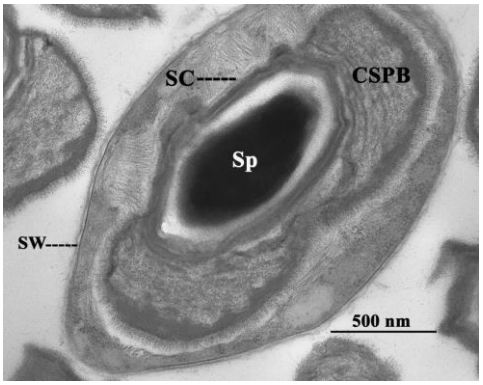

Figura 1. Cuerpos paraesporales producidos por *B. laterosporus*. Se muestra la pared del esporangio (SW), la espora (Sp), la capa de la espora (SC) y el cuerpo paraesporal en forma de canoa (CSPB). Tomado de (Ruiu 2013).

La ubicuidad en la naturaleza, la producción de antibióticos, la formación y resistencia de sus endosporas, la toxicidad de sus esporas, y la producción de cristales proteicos tóxicos para varios órdenes de insectos hacen del género *Bacillus* un género muy relevante en Medicina, Agricultura, Bioquímica y en la empresa farmacéutica (Todar 2012).

1.2.1.1 *Bacillus thuringiensis* (*Bt*)

Es una bacteria aerobia o anaerobia facultativa, flagelada y esporulante. Se considera un microorganismo del suelo ya que en los primeros estudios fue el medio más frecuente de donde se obtuvieron sus aislados (Martin and Travers 1989). Posteriormente han sido aisladas de diferentes hábitats, incluidos insectos, productos almacenados, partes de plantas (Schnepf *et al.* 1998), telarañas (Sauka *et al.* 2010), agua (Iriarte *et al.* 2000) y del intestino de pequeños roedores del orden Rodentia e Insectivora (Swiecicka *et al.* 2002).

Durante su ciclo de vida presenta dos fases principales, el crecimiento vegetativo y la esporulación (Itoua-Apoyolo *et al.* 1995). En el crecimiento vegetativo la bacteria está en un medio rico de nutrientes y se produce un crecimiento

exponencial; cuando los nutrientes escasean la bacteria entra en fase estacionaria y forma la endospora y las inclusiones cristalinas. La endospora se desarrolla en un esporangio formado por la célula madre y la espora. Los cristales se acumulan en la célula madre durante el proceso de esporulación. En algunas cepas de *Bt* el cristal se encuentra dentro del exosporium, aunque en la mayoría de los casos las dos estructuras no están físicamente conectadas (Crickmore 2006). La fase de esporulación consta de siete estadios (Bechtel and Bulla 1976), los cuales se inician cuando hay limitación de nutrientes y se cierra en la fase lítica, en donde la endospora se lisa y tanto la exospora como los cristales son liberados. Las esporas se mantienen viables bajo condiciones desfavorables por largos períodos de tiempo hasta que el medio vuelva a ser óptimo y regresan a un crecimiento vegetativo.

La producción de cristales paraesporales es lo que distingue filogenéticamente a *Bt* de *Bacillus anthracis* (patógeno obligado de mamíferos) y de *Bacillus cereus* (organismo del medio ambiente y patógeno humano oportunista) (Ruan *et al.* 2015). Estos cristales son los que contienen una o más proteínas insecticidas. Las cepas de *Bt* aisladas han mostrado alto grado de especificidad frente a diferentes órdenes de insectos como Lepidoptera, Diptera, Coleoptera, Himenoptera, Homoptera, Ortoptera, Phthiraptera o Mallophaga y Acari; y en otros invertebrados como Nemathelminthes, Platyhelminthes, y Sarcomastigorphora (Bravo 1997; de Maagd *et al.* 2003).

Las inclusiones cristalinas producidas por *Bt* están constituidas fundamentalmente de proteínas llamadas δ-endotoxinas, cuya producción está estrechamente ligada al proceso de esporulación, ya que la expresión de los genes está determinada a nivel transcripcional por factores sigma específicos de la célula madre; aunque hay excepciones, como la Cry3A, en la cual los genes son transcritos durante el crecimiento vegetativo (Baum and Malvar 1995). A pesar de que la transcripción se produzca en estadios tempranos, el cristal sólo es ensamblado durante la esporulación (Crickmore 2006). Varios factores condicionan la cristalización de las δ-endotoxinas, entre estos están la estructura secundaria, la formación de puentes disulfuro entre residuos cisteína y la presencia de componentes adicionales en el cristal; también los mecanismos de cristalización

varían entre las distintas δ-endotoxinas (Agaisse and Lereclus 1995; Baum and Malvar 1995).

La morfología que adquieren los cristales puede ser muy diversa y una célula de *Bt* puede producir más de un cristal paraesporal (Figura 2). Se han reportado cristales romboides, bipiramidales, heterogéneos (Kaelin *et al.* 1994), esféricos, irregulares/amorfo y cúbicos (Karamanlidou *et al.* 1991).

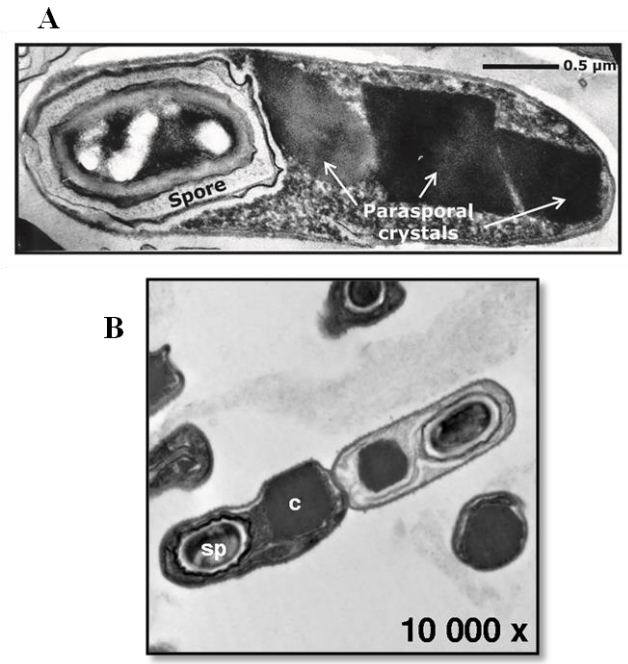

Figura 2. Microscopía electrónica de transmisión de células esporuladas de *Bt*. A) *B. thuringiensis* subsp. *morrisoni* cepa C18, contiene 15 genes *cry* y los cristales indicados con flechas varían en forma y tamaño. Tomado de (Ibrahim *et al.* 2010). B) *B. thuringiensis* subsp. *thuringiensis* cepa IS5056, en donde sp es la espora y c el cristal. Tomado de (Swiecicka *et al.* 2008).

No todas las cepas de *Bt* que producen cristales paraesporales han sido descritas como tóxicas para los insectos bioensayados (Bernhard *et al.* 1997; Benintende *et al.* 1999). Ohba y Aizawa (1986) sugieren que los cristales paraesporales no tóxicos predominan con respecto a los que han mostrados toxicidad en bacterias. Sin embargo los cristales paraesporales son sólo uno de los factores tóxicos producidos por *Bt*, ya que esta bacteria es capaz de expresar una gama amplia de factores

insecticidas como son las proteínas Vip, Sip, proteína de 41.9 kDa, sphaericolisina, alveolisina y β-exotoxinas, entre otras (Palma *et al.* 2014).

Bt está equipado genéticamente para tener un estilo de vida patógeno. Raymond y colaboradores (2010) describen todas las barreras y cómo *Bt* las asume para lograr ser infeccioso. En la Figura 3 se representa este proceso: una vez que las esporas y los cristales son ingeridos por el insecto, *Bt* tiene que combatir el ambiente hostil del intestino, para esto sintetiza una superficie celular modificada que le ayuda a protegerse de los péptidos antimicrobianos (AMPs) sintetizados por el hospedador y para competir con otras bacterias del intestino, *Bt* produce sus propios AMPs y bacteriocinas, y posiblemente expresa bombas de eflujo de drogas para combatir los antibióticos producidos por sus competidores. Cuando ha sobrevivido al ambiente del intestino, *Bt* debe atacar al hospedador; es aquí donde actúan los factores de virulencia de la cepa, como por ejemplo las toxinas Cry que destruyen las células epiteliales que recubren el intestino medio. La germinación de la espora permite la expresión y producción de otros factores de virulencia que están involucrados en la destrucción del tejido del intestino medio como las fosfolipasas, enterotoxinas y las proteasas. Para seguir la invasión del insecto, *Bt* debe cruzar la matriz peritrófica compuesta de quitina; aunque el mecanismo no está completamente claro, este proceso podría estar facilitado por la producción de quitinasas y enancinas. Una vez que han cruzado la barrera del intestino, las células vegetativas y/o las esporas pueden entrar al hemocele. También podrían ingresar al hemocele a través de espiráculos o brechas en la cutícula. Dentro del hemocele la bacteria debe evitar la destrucción que puede estar causada por el sistema inmune humoral innato y los hemocitos circulantes; para esto la bacteria produce metaloproteasas que podrían ayudar a protegerlo o a facilitar su escape. En la patogénesis de *Bt* se ha descrito que puede provocar septicemia, la cual podría estar producida por la expresión de enterotoxinas. Además, la producción de sistemas de adquisición de hierro de *Bt* puede contrarrestar el secuestro de hierro del hospedador y así facilitar la colonización. La capacidad para competir con otras bacterias en este proceso podría estar facilitada por la producción de desactivadores (*quenchers*) de quórum y antibióticos (Raymond *et al.* 2010).

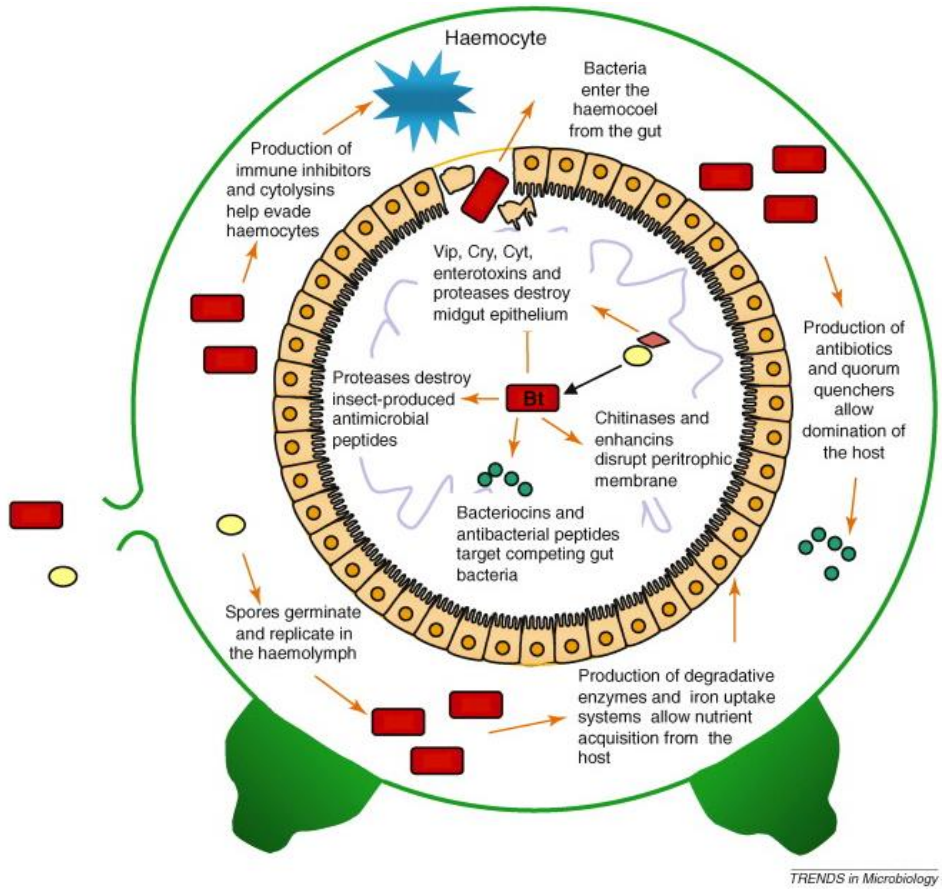

Figura 3. Interacción entre *Bt* y su insecto diana, después de la ingestión o introducción en el hemocele. El diagrama representa una sección transversal de una oruga en donde se muestra los diversos compartimentos, barreras y algunas defensas inmunitarias innatas encontradas por *Bt*. La sección se ve desde el lumen del intestino medio hacia la matriz peritrófica (línea azul), las células epiteliales del intestino medio, el hemocele y finalmente la cutícula (línea verde). *Bt* se muestra en su forma vegetativa (rectángulo rojo) y en forma de espora (óvalo amarillo) con los cristales (rombo rosa). Tomado de (Raymond *et al.* 2010).

El conocimiento de las bases genéticas de la toxicidad de *Bt*, así como la utilización de diferentes técnicas de transformación genética, han permitido desarrollar productos modificados que han puesto en el mercado nuevas toxinas. Además se han podido generar plantas transgénicas que solucionan el inconveniente de la baja persistencia en el medio ambiente de los productos *Bt* y han permitido dirigir la toxicidad a partes específicas de las plantas (Zaidi *et al.* 2005).

Todas estas características, hacen de los productos basados en las toxinas de *Bt* sean una herramienta útil, ventajosa y muy aplicable para el control de plagas y vectores de enfermedades.

1.2.2 Otras especies o cepas entomopatógenas

Como se ha indicado el ejemplo más conocido y estudiado de bacteria entomopatógena es *Bt*, sin embargo hay otros géneros bacterianos conocidos por tener cepas entomopatógenas con diferentes modos de acción. Miembros de la familia Enterobacteriaceae han sido reconocidos como especies patógenas para insectos, como es el caso de ***Serratia entomophila*** y ***Serratia proteamaculans*** que causan la muerte del escarabajo del pastizal de Nueva Zelanda, *Costelytra zealandica* (Coleoptera: Scarabaeidae), agente causal de lo que se conoce como enfermedad ambar. Después de dos o tres días de la ingestión de *S. entomophila*, las larvas de *C. zealandica* dejan de alimentarse, lo que produce un aclaramiento en el intestino, dando como resultado el característico color ambar de las larvas (Glare *et al.* 1993). Los factores de virulencia de esta cepa han sido localizados en el plásmido pADAP (del inglés "amber disease-associated plasmid") (Glare *et al.* 1993), en el cual se encuentran los genes que codifican para las proteínas SepA, SepB y SepC que son esenciales para la producción de los síntomas de la enfermedad ambar y que tienen similitud significativa en su secuencia con los factores insecticidas producidos por la bacteria *P. luminescens* (Hurst *et al.* 2000). En pADAP también se encuentran codificadas unas partículas parecidas a la cola de un fago, conocidas como Afp (del inglés "antifeeding prophage") las cuales son necesarias para el cese de la alimentación de las larvas (Hurst *et al.* 2004).

Serratia secreta grandes cantidades de enzimas extracelulares, por ejemplo *Serratia marcescens* produce proteasas, lipasas, nucleases, quitinasas (Hines *et al.* 1988), hemolisinas (Hertle 2002), serralisina metaloproteasa (Ishii *et al.* 2014a; Tambong *et al.* 2014) que están relacionadas con la virulencia de la bacteria.

Photorhabdus y ***Xenorhabdus*** son bacterias mótiles Gram-negativas que están simbióticamente asociadas con nematodos de las familias Steinernematidae y Heterorhabditidae, respectivamente. Estas bacterias comparten características en su ciclo de vida: viven en el intestino de nematodos entomopatógenos que penetran en

el hemocele del insecto a través de la cutícula o del espiráculo respiratorio; dentro del hemocele las bacterias son liberadas y juntos, nematodos y bacterias, matan rápidamente al insecto en estado larval; aunque en muchos casos la bacteria sola es altamente virulenta (Forst and Nealson 1996).

P. luminescens y ***X. nematophila*** sintetiza inclusiones cristalinas proteicas en la fase media y final del crecimiento exponencial del cultivo (Figura 4) que constituyen el 40% de la proteína total celular (Couche and Gregson 1987; Bowen and Ensign 2001). Sin embargo estos cristales proteicos no son los responsables de la toxicidad de las cepas y no tiene similitud significativa con otras secuencias de aminoácidos conocidas (Bintrim and Ensign 1998; Goetsch *et al.* 2006); estos cristales están implicados en el desarrollo del nematodo (You *et al.* 2006). En la acción patogénica de estas bacterias intervienen lipasas, fosfolipasas, proteasas, lipopolisacáridos, compuestos antifúngicos y antimicrobiano, y varias proteínas tóxicas (Forst and Nealson 1996). La secuenciación del genoma de diversas cepas entomopatógenas de estas especies han mostrado la codificación de una multitud de proteínas insecticidas, algunas altamente específicas al insecto diana y otras más generales (Hinchliffe *et al.* 2010). Entre las proteínas insectidas más estudiadas de *P. luminescens* están los complejos de toxina Tcs, las proteínas Pir, las toxinas Mcf y los casetes de virulencia PVC "*Photorhabdus* virulence cassetes" (Rodou *et al.* 2010); y de *X. nematophila* el complejo tóxico Xpt (Sheets *et al.* 2011), GroEL (Joshi *et al.* 2008) y la subunidad de pilina de 17 kDa (Khandelwal *et al.* 2004); aunque no son las únicas toxinas reportadas para estas especies.

P. luminescens y *X. nematophila* pueden multiplicarse y matar al insecto hospedador en 24 o 48 horas y actúan sobre un amplio rango de insectos de diferentes órdenes (Lepidoptera, Coleoptera y Dictyoptera) (Bowen *et al.* 1998) a diferencia de las δ-endotoxinas de *Bt* que usualmente exhiben especificidad para un grupo de insectos.

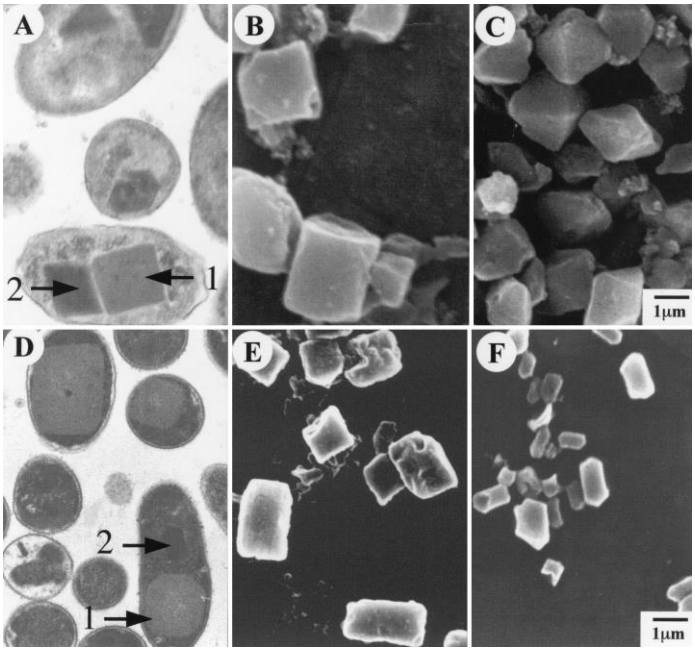

Figura 4. Microscopía de cepas de *P. luminescens.* Microscopía electrónica de transmisión de A) cepa NC1 y D) cepa Hm, donde se muestra que contienen dos inclusiones proteicas distintas. Microscopía electrónica de barrido de las inclusiones proteicas separadas, donde B y C) cepa NC1; E y F) cepa Hm. Tomado de (Bowen and Ensign 2001).

Otras enterobacterias del género ***Yersinia*** han mostrado ser tóxicas para insectos; la toxicidad está relacionada con la producción de toxinas Yen-Tc homólogas a las proteínas Tc de *Photorhabdus* spp., como en el caso de ***Yersinia entomophaga*** activa frente a Coleópteros y Lepidópteros (Hurst *et al.* 2011). También ***Pseudomona entomophila*** y ***Pseudomonas taiwanensis*** con un amplio espectro insecticida, codifican proteínas Tc asociadas a la toxicidad de las cepas (Vodovar *et al.* 2006; Chen *et al.* 2014).

Clostridium bifermentans serovar *malaysia* fue la primer bacteria anaerobia estricta altamente tóxica para mosquitos y mosca negra. Posee dos proteínas Cry, una de ellas, la Cry16Aa (llamada Cbm71) implicada en la toxicidad (Barloy *et al.* 1996).

Más recientemente han sido descritas especies de Beta-proteobacterias que muestran un amplio espectro insecticida. Este grupo incluye cepas de *Burkholderia* spp. y *Chromobacterium* spp. Ciertas especies de Actinobacteria han generado

interés científico y comercial por su producción de metabolitos que actúan como potentes insecticidas, como es el caso de las especies *Streptomyces* y *Saccharopolyspora* (Ruiu 2015).

El descubrimiento de nuevas especies bacterianas entomopatógenas y sus metabolitos insecticidas son el resultado de las continuas búsquedas por parte de las empresas y grupos de investigación que han tenido auge con los nuevos marcos legislativos que fomentan el uso de bioinsecticidas en los programas de Manejo Integrado de Plagas (Ruiu 2015).

1.3 *Bacillus pumilus*

1.3.1 Biología

Bacillus pumilus fue descrita por primera vez por Meyer y Gottheil en 1901. Es conocida también por los nombres de *Bacillus aminoglucosidicus* o *Bacillus mesentericus.* Taxonómicamente pertenece al dominio Bacteria, filo Firmicutes, clase Bacilli, orden Bacillales, familia Bacillaceae, género *Bacillus* (Skerman *et al.* 1989).

Se la considera ubicua en la naturaleza, comúnmente aislada de una gran variedad de fuentes ambientales; incluso en sitios poco comunes para el aislamiento de microorganismos como el interior de basalto del desierto de Sonora (Benardini *et al.* 2003), la planta de montaje de naves espaciales y la superficie de la Estación Espacial Internacional (La Duc *et al.* 2003; Newcombe *et al.* 2005).

Es una bacteria Gram-positiva, esporulante, de forma bacilar, aerobia o anaerobia facultativa, con bajo contenido en Guaninas y Citocinas en su genoma (aproximadamente 41%). Es una especie mesófila (Coorevits *et al.* 2008), también hay cepas que pueden ser termófilas y alcalófilas (Moallic *et al.* 2006). Es una especie móvil (posee flagelo), β-hemolítica en agar sangre, catalasa positiva, tolerante a las sales y susceptible a la penicilina (Tena *et al.* 2007; Porwal *et al.* 2009). Crece como una colonia lisa que se torna amarilla cuando aumenta el tiempo de incubación.

B. pumilus tiene propiedades tóxicas, efectos citopáticos en células Vero, actividad hemolítica, producción de lectinasa y actividad proteolítica en caseína (Hoult and Tuxford 1991). Es considerada como no patógena para humanos, pero debido a su producción de hemolisinas se la considera una bacteria patógena oportunista. La infección humana por *B. pumilus* es excepcional (Porwal *et al.* 2009). From *et al.* (2007) la relacionan con incidentes de contaminación alimentaria debido a la producción de un complejo de lipoproteínas conocidas como pumilacidinas previamente encontradas en otros *Bacillus* spp. causantes de contaminación alimentaria.

Ecológicamente *B. pumilus*, al igual que otras bacterias de su género, mantiene relación endofítica con ciertas plantas, favoreciendo el crecimiento y desarrollo, y dando protección frente a otros organismos del suelo que causan enfermedades, como hongos del género *Fusarium* (Benhamou *et al.* 1996) y *Rhizoctonia* (Cottyn *et al.* 2009).

Al ser una especie esporulante, puede permanecer en estado de inactividad durante largos períodos de tiempo cuando las condiciones ambientales son desfavorables y luego volver a germinar y a formar una célula vegetativa (Nicholson *et al.* 2000).

Sus esporas son resistentes al calor, desecación, radiación UV, radiación γ, H_2O_2 y a la escasez de nutrientes. Son más resistentes que otras bacterias de su género, pudiendo sobrevivir incluso a las prácticas de esterilización estándar (Gioia *et al.* 2007).

1.3.2 Aplicaciones Biotecnológicas de *B. pumilus*

1.3.2.1 Producción de Enzimas

Los microorganismos son una excelente fuente de enzimas comerciales, ya que pueden cultivarse en grandes cantidades dentro de un tiempo relativamente corto utilizando métodos de fermentación establecidos, lo que da como resultado una producción abundante y regular de la enzima deseada. *B. pumilus* es utilizada a nivel industrial por la gran cantidad de enzimas que es capaz de producir. Las primeras enzimas descritas producidas extracelularmente por esta bacteria fueron: celulasas,

liquenasas, pectato liasas, serín proteasas, serín-metal proteasas y deoxiribonucleasa-ribonucleasa (Priest 1977).

Las proteasas microbianas constituyen aproximadamente el 40% de la producción mundial de todas las enzimas. El género *Bacillus* produce las proteasas comerciales más utilizadas en la actualidad, siendo *Bacillus licheniformis*, *Bacillus subtilis* y *B. pumilus* las especies más conocidas a nivel industrial por la producción de proteasas alcalinas, usadas ampliamente en la industria alimentaria, farmacéutica, producción de detergentes y tratamiento del cuero (Jaouadi *et al.* 2008; Ibrahim *et al.* 2011).

Las proteasas alcalinas de *B. pumilus* han sido descritas para las siguientes aplicaciones: inactivación de ARNasas durante la purificación de ARN de células homogenizadas, coagulación de la leche de soja, limpieza de membranas de ultrafiltración, depilación del cuero, producción de hidrolizados de zeína y formulación de detergentes (Jaouadi *et al.* 2008). Destacan por ser más eficientes que las de otras bacterias, por ejemplo, se han descrito proteasas alcalinas de *B. pumilus* con mejor eficacia catalítica que la subtilisina, mayor estabilidad al pH, temperatura y desnaturalizantes químicos (Jaouadi *et al.* 2008); mayor eficiencia en la depilación de cuero (Wang *et al.* 2007); queratinasa más estable para la degradación de pelo de bovinos (Kumar *et al.* 2008); y colagenasa con alta especificidad (Wu *et al.* 2010).

Otra enzima producida por esta bacteria es la xilanasa, ampliamente investigada por la variedad de aplicaciones biotecnológicas utilizadas en la industria, como el blanqueo de la pulpa, el aumento del brillo del papel (Nagar *et al.* 2013), la mejora de la digestibilidad de los piensos, el tratamiento de residuos, la generación de energía y la producción de xilo-olisacáridos que son utilizados como probióticos (Kapoor and Kuhad 2007). El uso de esta enzima ha ayudado a controlar el daño medioambiental causado por los productos químicos utilizados en el blanqueo convencional y la eliminación de la lignina.

Destacan las lacasas, por su versatilidad como biocatalizadores para aplicaciones industriales. Catalizan la oxidación de sustancias orgánicas, polimerización de monómeros, degradación de polímeros y la oxidación de compuestos fenólicos. Industrialmente ha sido utilizada para la deslignificación de pulpa, la oxidación de

los contaminantes orgánicos, el blanqueo de mezclilla y la decoloración y transformación de colorantes textiles. La importancia de la lacasa de *B. pumilus* se caracteriza por su tolerancia a altas temperaturas y actividad en condiciones neutras hasta alcalinas, a diferencia de las lacasas de hongos que son activas sólo a pH ácidos (Reiss *et al.* 2011).

B. pumilus produce lipasas que son utilizadas en la industria para el desarrollo de sabores en la maduración de quesos, en productos de panadería y bebidas, para eliminar la grasa en productos cárnicos y pescados y aplicados como biocatalizadores (Kumar *et al.* 2012). Las cepas de *B. pumilus* productoras de lipasas presentan algunas ventajas, como por ejemplo la actividad a bajas temperaturas, la tolerancia alcalina, la actividad hidrolítica de ácidos grasos de cadena corta y media y algunos triglicéridos naturales (Arifin *et al.* 2013), o la tolerancia a un amplio rango de solventes orgánicos lo que lo hace muy atractivo para las aplicaciones industriales (Kumar *et al.* 2012).

También se ha descrito la producción de catalasa-peroxidasa (Sangar *et al.* 2012); esterasas (Rasool *et al.* 2005); enzimas degradadoras de glúcidos como las endoglucanasas (Ariffin *et al.* 2008), quitinasas (Ahmadian *et al.* 2007; Dehestani *et al.* 2010), pectinasas (Sharma and Satyanarayana 2006) y pectato liasa (Ouattara *et al.* 2011).

Algunas cepas de *B. pumilus* han sido descritas como degradadoras de compuestos xenobióticos, siendo efectivas en el tratamiento de contaminantes de efluentes industriales (Meyers *et al.* 1991), la degradación de biopolímeros (Hayase *et al.* 2004) y la eliminación de contaminantes ambientales (Calvo *et al.* 2004; Buzanello *et al.* 2014).

Recientemente, una enzima producida por *B. pumilus* (antes descrita como *Bacillus intermedius*) ha causado gran interés en la comunidad científica y médica porque ha mostrado ser una herramienta prometedora en el desarrollo de agentes específicos que atacan a patógenos y células malignas (Ulyanova *et al.* 2011). Se trata de la Binasa, una ARNasa secretada por la bacteria con propiedades citotóxica capaz de inducir apoptosis selectiva en las células cancerosas (Mitkevich *et al.* 2010; Mitkevich *et al.* 2011; Cabrera-Fuentes *et al.* 2013). También ha demostrado tener

actividad contra el virus de la influenza A (virus H1N1) (Shah Mahmud and Ilinskaya 2013).

1.3.2.2 Producción de Antibióticos

En general, el género *Bacillus* es conocido por la producción de compuestos naturales con actividad antagonista frente a muchas bacterias, hongos y virus patógenos y son usualmente usados en el tratamiento y/o prevención de diferentes infecciones. *B. pumilus* es conocida por la producción de antibióticos como la pumilina activa frente a bacterias Gram-positivas (Bhate 1955), la pumilacidina que es un antibiótico antiviral activo frente al virus del herpes simple y que protege contra úlceras gástricas (Naruse *et al.* 1990), la pumilicina 4 que tiene acción antibacteriana contra *Staphylococcus aureus* resistente a meticilina y *Enterococcus faecalis* resistente a vancomicina (Aunpad and Na-Bangchang 2007) y la pumiviticina que inhibe el crecimiento de un amplio rango de bacterias del ácido láctico y también de bacterias Gram-negativas patógenas como *Salmonella typhimurium* y *Proteus vulgaris* (Rajesh *et al.* 2012).

Se han descrito cepas de *B. pumilus* que producen bacitracina (Awais *et al.* 2007), tetaína (Krynski *et al.* 1952) e iturina (Cho *et al.* 2009). Otro antibiótico producido por *B. pumilus* es la amicoumacina A que pertenece al grupo de las isocoumarinas, tiene efecto antiinflamatorio y actividad gastroprotectora (Itoh *et al.* 1981).

Hay varios estudios de cepas capaces de inhibir el crecimiento de otros microorganismos cuyos compuestos antibacteriales (Hasan *et al.* 2009; Hassi *et al.* 2012) y antifúngicos (Munimbazi and Bullerman 1998) no han sido estudiados con detalle.

1.3.2.3 *B. pumilus* en Control Biológico

1.3.2.3.1 Control de Hongos, Bacterias y Virus en plantas

Algunas cepas de *B. pumilus* tienen actividad fungicida y han sido utilizadas como agentes de control biológico de hongos fitopatógenos. La propiedad de estas cepas para producir metabolitos extracelulares antifúngicos es una de las más

explotadas, ya que inhiben el crecimiento del micelio y la producción de micotoxinas de muchas especies de hongos como *Aspergillus*, *Penicillium* y *Fusarium*, causantes de grandes pérdidas económicas en los cultivos a nivel mundial (Munimbazi and Bullerman 1998).

Se han descrito cepas activas contra Mucoraceae (*Mucor*, *Rhizopus* y *Cunninghamella*) y *Aspergillus* (*Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus tereus* y *Aspergillus glacus*) (Bottone and Peluso 2003); *Phytophthora capsici*, *Verticillium dahliae*, *Fusarium oxysporum* f. sp. *cucumerinum*, *F. oxysporum* f. sp. *vasinfectum*, *Rhizoctonia solani*, *Phtophthora parasitica* var. *nicotiana* y *Ralstonia solanacearum* (Mei *et al.* 2010); y *Fusarium solani* (Ajilogba *et al.* 2013).

Hay cepas capaces de actuar frente a hongos y/o bacterias en condiciones adecuadas. Es así que, *B. pumilus* SE34 está descrita como una rizobacteria que induce resistencia sistémica en *Arabidopsis* frente a *Pseudomonas syringae* (Ryu *et al.* 2003); en plantas de tabaco frente a *Peronospora tabacina* (Zhang *et al.* 2002a; Zhang *et al.* 2002b); en plantas de tomate frente a *Phytophthora infestans* (Yan *et al.* 2002), *Xanthomonas campestris* pv. *vesicatoria*, *Xanthomonas vesicatoria* (Ji *et al.* 2006), virus del mosaico del pepino (CMV) y virus del moteado del tomate (ToMoV) (Zehnder *et al.* 2001); y en arroz frente a *Xanthomonas oryzae* (Chithrashree *et al.* 2011). Esta cepa activa las defensa de la planta, vía inducción de la resistencia sistémica; este proceso depende de la activación física o química de las barreras de la planta pero no es el único proceso por el cual se cree que la bacteria protege a la planta. Otra cepa con características similares es *B. pumilus* T4, que induce resistencia en *Arabidopsis* frente a *P. syringae* (Ryu *et al.* 2003) y *P. tabacina* (Zhang *et al.* 2002b).

La inoculación con *B. pumilus* INR7 es efectiva frente a enfermedades causadas por CMV, *R. solanacearum*, *R. solani*, *Sclerotium rolfsii* y *Colletotrichum gloeosporioides* en pimienta y tomate; *Cronartium quercuum* f. sp. *fusiforme* en pino; *Xanthomonas axonopodis* pv. *vesicatoria* y *R. solanacearum* en pimiento (Yi *et al.* 2013). En pimiento se ha descrito que esta bacteria, en combinación con benzotiadiazol, activa la expresión de genes de defensa en la planta y es así como ayuda en el control de la plaga (Yi *et al.* 2013).

El mecanismo de acción de algunas cepas para inducir resistencia en plantas se conoce bastante bien. Por ejemplo, en remolacha azucarera, *B. pumilus* 203-6 y 203-7 induce la resistencia sistémica por el incremento de la producción de quitinasa y de β-1,3-glucanasa, y precedido por la producción de peróxido de hidrógeno, de esta manera ayuda a la planta a protegerse de la mancha foliar producida por el hongo *Cercospora beticola* (Bargabus *et al.* 2004); en tabaco, la resistencia mediada por *B. pumilus* SE34 es determinada por el aumento de los niveles de ácido salicílico (Zhang *et al.* 2002a), mientras que en guisantes, esta misma cepa induce la resistencia por el fortalecimiento de las paredes celulares epidérmica y cortical mediante la producción de calosa y compuestos fenólicos (Choudhary and Johri 2009).

Así, la inducción de resistencia sistémica en plantas por *B. pumilus*, al igual que otras de su especie, depende de la cepa bacteriana, la planta huésped y en algunos casos del patógeno (Choudhary and Johri 2009).

Muchas de las cepas de *B. pumilus* han sido patentadas, incluso más de una vez con diferentes aplicaciones, por ejemplo la cepa NRRL (número de acceso B-30087) productora de metabolitos y antibióticos ha sido patentada por sus propiedades fungicidas frente a ciertos patógenos específicos de plantas (Lehman *et al.* 2001) y posteriormente presentada para el control de nematodos parásitos de plantas con el nombre de cepa QST2808 (Reed and Varghese 2015). Existen patentes de esta cepa con efecto sinérgico en combinación con otras especies bacterias o compuestos químicos (Lehman *et al.* 2008; Hellwege *et al.* 2015).

Esta aplicación ha sido industrialmente explotada y existen varios productos a la venta, cuyo ingrediente activo es *B. pumilus*, entre ellos están los fungicidas Sonata (unión de Ballad Plus® y Sonata® de AgraQuest), Yield Shield® de la compañía Bayer CropScience; Integral® F-33 de la compañía Becker Underwood, Inc; y PRO-MIX® BIOFUNGICIDE de Premier Tech Horticulture, entre otros.

1.3.2.3.2 Control de Insectos

Tradicionalmente, *B. pumilus* no ha sido considerada una especie entomopatógena. A pesar de la gran diversidad de actividades biológicas útiles que se han demostrado, sus aplicaciones se han centrado en el uso de bioplagicidas para

la prevención y tratamiento de enfermedades causadas por hongos fitopatógenos, principalmente en las raíces de ciertos cultivos.

Sin embargo, algunos estudios demuestran la patogenicidad de cepas de *B. pumilus* frente a algunos insectos (Heins *et al.* 1999; Ertürk *et al.* 2008; Molina *et al.* 2010; Yaman *et al.* 2010b).

En 1999, Heins y colaboradores patentaron un método para proteger a las plantas contra el gusano de las raíces del maíz (*Diabrotica virgifera*, *Diabrotica longicornis* y *Diabrotica undecimpunctata*), la rosquilla verde o gusano de la remolacha (*Spodoptera exigua*) y algunas especies de nematodos, mediante la aplicación de una cantidad determinada del sobrenadante obtenido de un cultivo completo de *B. pumilus* AQ717 sobre cualquier parte de la planta, incluidas las raíces o aplicaciones al suelo que la rodean.

La patente describe la producción de un metabolito tóxico y soluble de bajo peso molecular (<10,000 daltons), que se encuentra en el sobrenadante de un cultivo completo tras un período de incubación y agitación (Heins *et al.* 1999).

En otro estudio, un *Bacillus* aislado del suelo e identificado como *B. pumilus* por métodos bioquímicos, presentó actividad tóxica frente al escarabajo de la patata o dorífora (*Leptinotarsa decemlineata*), considerado como la plaga más importante en patatas, berenjenas y tomates. En ensayos de laboratorio, esta cepa alcanzó niveles de mortalidad de 95.7 y 26.7% en larvas y adultos respectivamente (Ertürk *et al.* 2008).

Otra cepa aislada del escarabajo de la corteza del abeto (*Dendroctonus micans*), también identificada por métodos bioquímicos, ha mostrado actividad en bioensayos frente a insectos de la misma especie de la cual fue aislada. Estas pruebas revelaron una toxicidad del 69.4 y 40.9% de mortalidad en larvas y adultos, respectivamente de *D. micans* (Yaman *et al.* 2010a).

Nuestro grupo de investigación, publicó el aislamiento y caracterización mediante comparación de secuencias parciales del gen 16S ARNr, de una cepa de *B. pumilus* entomopatógena activa frente a la mosca de la fruta del Mediterráneo (*Ceratitis capitata*). La cepa denominada *B. pumilus* 15.1 presentó rangos de mortalidad del 68 al 94% en larvas de *C. capitata*, dependiendo de las condiciones

bajo las cuales se realizan los bioensayos y se preparaba el cultivo (Molina *et al.* 2010).

1.3.2.4 Otras Aplicaciones

B. pumilus ha sido descrita como rizobacteria promotora del crecimiento vegetal (Ryu *et al.* 2003; Choudhary and Johri 2009), como una herramienta potencial que proporciona importantes beneficios a la agricultura por su capacidad de potenciar el crecimiento de la planta y por tanto el rendimiento del cultivo.

Es considerado como un microorganismo que al ser administrado en dosis adecuadas confiere beneficios en su huésped. Es así, que ha sido utilizada comercialmente como probiótico para el consumo humano y puesto en el mercado con los nombres de Nature's First Food y Biosubtyl I y II (Cutting 2011). Ha sido descrito por la capacidad de mejorar la respuesta inmune de peces (Sun *et al.* 2010) y protegerlo contra infecciones (Song *et al.* 2014; Ramesh *et al.* 2015) lo cual lo hace un atractivo probiótico para la acuicultura. Song y colaboradores (2014) describen la acción protectora de *B. pumilus* debido a la inhibición de *quorum sensing*. Esta propiedad de inhibir el *quorum sensing* de otras bacterias también ha sido descrita en otra cepa capaz de reducir los factores de virulencia de algunas bacterias Gram-negativas como *Chromobacterium violaceum*, *Pseudomonas aeruginosa* y *S. marcescens*, mediante la acción de una acilasa (Nithya *et al.* 2010).

El control bacteriano por medio de cepas de *B. pumilus* tiene potenciales aplicaciones clínicas y ambientales, por ejemplo la inhibición de la formación de biopelícula que controla la contaminación biológica de membranas en sistemas de filtración (Zhang *et al.* 2014) o el control de patógenos como *Vibrio* spp. (Nithya and Pandian 2010). Se ha reportado el uso con éxito de esporas de *B. pumilus* junto con *B. subtilis* y *B. megaterium* (cepas con grado de calidad alimentaria) para descontaminar superficies hospitalarias de los microorganismos causantes de las infecciones asociadas al cuidado de la salud (Vandini *et al.* 2014).

Algunas cepas de *B. pumilus* producen surfactina (Morikawa *et al.* 1992), que es un lipopéptido estudiado por sus amplias aplicaciones biológicas entre las que destaca la actividad antiviral, antitumoral y antiinflamatoria (Slivinski *et al.* 2012).

También existe descrita una cepa de *B. pumilus* capaz de producir compuestos anti-*Trypanosoma cruzi*; estos compuestos son 3-hidroxiacetilindol, N-acetil-B-

oxotriptamina y 3-formilindol, activos específicamente frente a este parásito (Martinez-Luis *et al.* 2012).

1.4 *Bacillus pumilus* 15.1

La cepa *B. pumilus* 15.1 fue aislada de vegetación en descomposición de caña silvestre (*Phragmites australis*) en Almuñecar, una localidad agrícola de la Costa Tropical de Granada, al sur de España, en donde la mosca de la fruta del Mediterráneo es considerada la plaga económicamente más perjudicial de los cultivos frutales de la zona. La cepa se identificó mediante secuenciación del gen 16S ARNr, y su secuencia fue depositada en la base de datos GenBank bajo el número de acceso EU978469 (Molina *et al.* 2010).

Esta cepa ha cobrado importancia por presentar toxicidad frente a *C. capitata*. Su identificación y caracterización indican que es posible encontrar una cepa natural de *Bacillus* que sea tóxica para esta plaga y que cepas de *B. pumilus* son entomopatógenas.

B. pumilus 15.1 es altamente tóxica para larvas de *C. capitata*, reportando mortalidad máxima del 94% en bioensayos. La toxicidad está determinada por las condiciones a las cuales el cultivo debe ser sometido. Así, la toxicidad se manifiesta únicamente cuando el cultivo esporulado tiene un tiempo de incubación de 96 horas a bajas temperaturas (4ºC o -20ºC) (Molina *et al.* 2010).

El factor de virulencia de esta cepa no es conocido, pero evidencias obtenidas en nuestro grupo de investigación nos sugiere que puede ser de origen proteico. Es importante la caracterización de esta nueva cepa, no sólo por su actividad entomopatógena sino también para conocer el curioso mecanismo de activación de dicha toxicidad.

1.5 Factores de virulencia bacteriana frente a insectos

1.5.1 Toxinas Cry y Cyt

Las proteínas Cry (del inglés "*Crystal*") y las proteínas Cyt (del inglés "*Cytolitic*") son δ-endotoxinas que no presentan homología de secuencia ni de estructura terciaria entre sí. Hasta la fecha se han descrito 74 familias de toxinas Cry y 3 familias de toxinas Cyt (Crickmore *et al.* 2014). La nomenclatura actual de las toxinas está basada únicamente en la identidad de las secuencias de aminoácidos y no tiene en cuenta la toxicidad; así toxinas activas frente a un mismo organismo pueden estar clasificadas en diferentes familias (Crickmore *et al.* 1998).

En *Bt* se han identificado la gran mayoría de estas toxinas, sin embargo no es la única especie capaz de producirlas. Muchas de las proteínas Cry de *Bt* han sido investigadas por sus propiedades insecticidas, pero hay otras que presentan actividad diferente. Por ejemplo, hay proteínas Cry de *Bt* con actividad citocida específica frente a células cancerígenas humanas de diversos orígenes y se las conoce como Parasporinas (Ohba *et al.* 2009), otras muestran actividad frente a protozoos patógenos de humanos, o hacia moluscos, incluso otras a bacterias (Palma *et al.* 2014). Cada δ-endotoxina tiene un espectro de actividad característico, con un rango de acción limitado a pocas especies, generalmente de un mismo orden. Este espectro puede depender de la combinación de δ-endotoxinas que la cepa produzca. En la Figura 5 se muestra un resumen del espectro de actividad insecticida de estas toxinas.

Se han descrito una gran cantidad de combinaciones de genes siendo las más comunes de la familia *cry*1 y *cry*2 (Porcar and Juarez-Perez 2003), la combinación *cry4*, *cry10*, *cry11*, *cyt1* y *cyt2* (Berry *et al.* 2002) o la combinación Cry48Aa-Cry49Aa que es altamente restrictiva a un único insecto diana (*C. quinquefasciatus*) (Jones *et al.* 2008). Estas combinaciones se deben principalmente a que estos genes están situados en plásmidos transmisibles por conjugación entre cepas y en general estas secuencias se encuentran flanqueadas por elementos transponibles, posibilitando la transmisión horizontal (Schnepf *et al.* 1998; de Maagd *et al.* 2001).

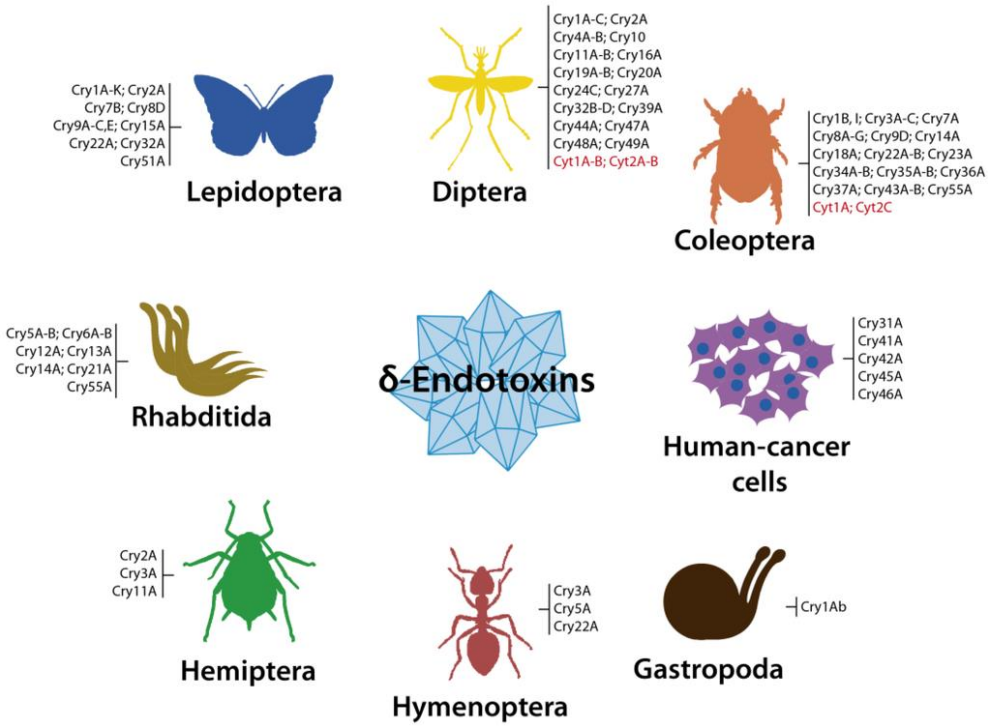

Figura 5. Gama de hospedadores conocidos sobre los que actúan las δ-endotoxinas de *Bt* (Cry y Cyt). Los grupos de toxinas separadas por guión indican que pertenecen a las familias abarcadas entre las letras, por ejemplo Cry1A-C indica familias de Cry1A, Cry1B y Cry1C. Los grupos de toxinas separadas por coma indican las familias a las cuales se refiere, por ejemplo Cry1B, I indica familias de Cry1B y Cry1I. El punto y coma separa los grupos o las toxinas individuales. Las toxinas Cry están en negro y las Cyt en rojo. Tomado de: (Palma *et al.* 2014).

Se ha visto un efecto sinérgico entre las esporas y las δ-endotoxinas de algunas cepas (Liu *et al.* 1998). No se conoce bien el mecanismo por el cual la espora tiene efecto potenciador, pero se sabe que no sólo depende de la germinación de la espora sino también de las proteínas presentes en su cubierta (Johnson *et al.* 1998).

Estas toxinas no pertenecen a una sola familia de proteínas. El grupo más grande son las conocidas como proteínas Cry de tres dominios; otros grupos son las Cyt, toxinas binarias, ETX_MTX2-like, Toxin_10 y aerolisinas; otras no pertenecen a ninguno de los grupos descritos como es el caso de la Cry22, Cry6 y la Cry55 (Palma *et al.* 2014).

Tres modelos diferentes han sido propuestos para explicar el modo de acción de las proteínas Cry de tres dominios: el modelo clásico (formación de poro), el modelo

de unión secuencial y el modelo vía de señalización (Vachon *et al.* 2012). Vachon y colaboradores (2012) plantean que el modelo clásico es el que está sustentado con más información experimental a pesar de que los procesos de formación de poro y de unión a un receptor sean poco conocidos.

Palma y colaboradores (2014) explican los tres modelos brevemente:

1) El modelo clásico propone: los cristales son ingeridos y solubilizados por el pH del lumen intestinal; la proteína nativa (protoxina) es activada proteolíticamente convirtiéndola en polipéptidos más pequeños resistentes a proteasa; parte de la toxina se une a receptores específicos de la superficie de las células epiteliales del intestino medio; formación de poros no selectivos, permeables a iones inorgánicos, aminoácidos y azúcares; estos poros producen la lisis de las células epiteliales lo que conduce a la muerte del insecto.
2) El modelo de unión secuencial sugiere que una vez que las toxinas son activas por las proteasas intestinales se unen a proteínas tipo cadherina (que serían las receptoras) y se produce un cambio conformacional y se forma una estructura pre-poro oligomérica; posteriormente la unión con un segundo receptor, como una aminopeptidasa, facilita la inserción del pre-poro dentro de la membrana lo que conduce la muerte del insecto.
3) El modelo vía señalización sugiere que la actividad tóxica es mediada por el receptor cadherina, iniciando una cascada de señalización dependiente de Mg^{2+}, adenilato ciclasa/proteína quinasa A y esto produce la muerte celular necrótica.

En cambio, las toxinas Cyt poseen un único dominio y no precisan receptores proteicos, interactúan directamente y de forma poco específica con la fracción lipídica de la membrana. Para esto se han propuesto dos modos de acción: 1) modelo de formación de poro, en el cual las toxinas Cyt se insertan en la membrana como monómeros y cuando alcanzan cierta densidad, éstos se oligomerizan conformando poros de membrana (Promdonkoy and Ellar 2003); y 2) modelo de acción tipo detergente, en el cual las toxinas Cyt se agregan de manera poco específica sobre la superficie de la membrana plasmática alterando su estructura o destruyéndola, de forma similar a la acción de un detergente (Butko 2003).

1.5.2 Toxinas Vip

Del inglés "vegetative insecticidal proteins", son proteínas sintetizadas y secretadas al medio en la fase de crecimiento vegetativo por *Bt* y *B. cereus* (de Maagd *et al.* 2003). Se han descrito 4 grupos: Vip1, Vip2, Vip3 y Vip4; su sistema de nomenclatura es el mismo que se aplica para las δ-endotoxina (Crickmore *et al.* 2014).

Vip1 y Vip2 constituyen una toxina binaria, es decir que se requiere ambas para que sea activa; tiene acción insecticida frente a varios coleópteros (de Maagd *et al.* 2003) y *Aphis gossypii* (Hemiptera) (Sattar and Maiti 2011). Vip3 tiene actividad frente a lepidópteros (Estruch *et al.* 1996), mientras que de Vip4 no se conoce la actividad ni su rango de hospedadores.

Los genes *vip1* y *vip2* se encuentran en un mismo operón, cada uno contienen un péptido señal N-terminal. El extremo N-terminal de Vip1 es procesado después de la secreción. Posiblemente su modo de acción es similar a las toxinas binarias A+B, en la que Vip2 es el dominio A citotóxico y Vip1 es el dominio de unión al receptor responsable de la translocación de Vip2 dentro de la célula huésped; una vez dentro Vip2 actúa como ADP-ribosiltransferasa en la actina lo que bloquea la polimerización y conduce a la muerte de la célula (Palma *et al.* 2014).

Vip3 tiene también péptido señal, pero no es procesado durante la secreción, y sigue presente en el péptido maduro secretado (Estruch *et al.* 1996; de Maagd *et al.* 2003). No se conoce el modo de acción de estas toxinas, posiblemente actúen por formación de poro; además se sabe que los fluidos del intestino del insecto o la tripsina digiere la proteína en 4 productos, el de mayor tamaño (66 kDa) es conocido como "trypsin resistant core" y es la fracción que tiene más actividad tóxica (Palma *et al.* 2014).

1.5.3 Toxina Sip

Del inglés "secreted insecticidal protein", hasta la fecha sólo se ha descrito una toxina de este tipo designada como Sip1A, obtenida del sobrenadante de un cultivo de *Bt* (Donovan *et al.* 2006). Tiene 46% de similitud y 21% de identidad con 141 aminoácidos de la proteína mosquitocida Mtx3 de *L. sphaericus*; no se conoce su

modo de acción, pero es letal para *Leptinotarsa decemlineata* y retrasa el crecimiento de *Diabrotica undecimpunctata howardi* y *Diabrotica virgifera virgifera* (Donovan *et al.* 2006).

1.5.4 β-exotoxinas

Conocida como thuringiensinas, son producidas por algunas cepas de *Bt* y son excretadas al medio en la fase vegetativa de la bacteria. Son toxinas insecticidas termoestables, solubles en agua y dializables. No se trata de ninguna toxina proteica sino de una pequeña molécula oligosacárida análoga al nucleótido adenina; se le atribuye acción insecticida en Lepidópteros, Dípteros, Coleópteros, Hemípteros, Isópteros, Orthópteros y Nematodos (Liu *et al.* 2014). La toxicidad de este compuesto se debe a que inhibe la ARN polimerasa dependiente de ADN, ya que la β-exotoxina puede competir con el ATP; probablemente esto hace que la toxina muestre propiedades tóxicas de amplio espectro, incluso en mamíferos (Liu *et al.* 2014). Es por esto que el uso de cepas productoras de β-exotoxina está prohibido en Europa, Estados Unidos y Canadá (Palma *et al.* 2014).

1.5.5 Toxinas Bin

Las toxinas Bin son producidas por *L. sphaericus* durante la esporulación, en la cual se forma un cristal paraesporal (Figura 6) que contiene una proteína de 42 kDa y otra de 51 kDa que actúan como una toxina binaria en la que ambas son requeridas para la toxicidad (Broadwell *et al.* 1990). Están codificadas en un mismo operón y se producen en cantidades equimolares; se las conoce como BinA (42 kDa) y BinB (51 kDa). Su modo de acción no es del todo conocido, se sabe que los cristales deben ser ingeridos, solubilizados y una vez liberada la protoxina, debe ser procesada para ser activa y para que se una a receptores específicos del epitelio; posteriormente ocurre el efecto citopatológico seguido de la muerte de la larva (Silva Filha *et al.* 2014). BinB se une al receptor, mientras BinA es el componente tóxico. BinB es miembro de las toxinas tipo aerolisina, probablemente con similitud en el mecanismo citolítico de formación de poro (Srisucharitpanit *et al.* 2014).

Son altamente activas frente a *Culex* y *Anopheles* spp. (Baumann *et al.* 1991), los cuales son vectores de enfermedades infecciosas en humanos. Algunas tienen un efecto sinérgico con toxinas Cry (Wirth *et al.* 2004). Recientemente se ha reportado actividad frente a células cancerígenas humanas (Luo *et al.* 2014).

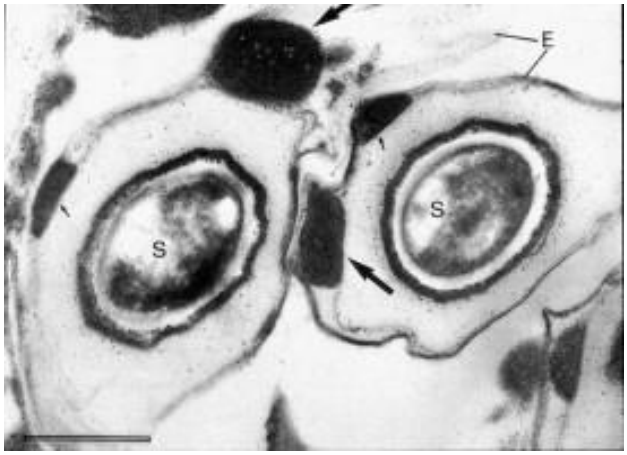

Figura 6. Microscopía electrónica de células esporuladas de *L. sphaericus*. Las pequeñas inclusiones oscuras (señaladas con una fecha) están formadas por BinA y BinB, las cuales están adheridas a la superficie interna de la membrana del exosporio (E). (S) indica la espora. Tomado de (Berry 2012).

1.5.6 Toxinas Mtx

Las toxinas Mtx (mosquitocidal toxins) son sintetizadas por *L. sphaericus* durante la fase vegetativa tanto en cepas con baja actividad como en cepas con alta actividad larvicida (Thanabalu *et al.* 1991). Su baja y/o inestable actividad puede deberse a bajos niveles de producción y a la degradación proteolítica durante la esporulación (Wirth *et al.* 2007).

Se han descrito 4 tipos de toxinas Mtx, agrupadas en dos familias; la familia Mtx1 y la familia Mtx2 compuesta por Mtx2, Mtx3 y Mtx4.

Mtx1 se produce como una proteína de 100 kDa cuyo extremo N-terminal tiene características de péptido señal aunque no hay evidencias de que esta proteína sea secretada al medio de cultivo (Thanabalu *et al.* 1991; Berry 2012). El procesamiento proteolítico de Mtx1 genera un producto de ~27 kDa que presenta homología con ADP-ribosiltransferasas y un producto de ~70 kDa con secuencias repetidas internas

(Berry 2012). La familia Mtx2 no tiene relación estructural con Mtx1. Mtx2 es miembro de la familia de toxinas formadora de poro; por lo que Mtx1 y Mtx2 tienen diferente mecanismo de acción (Wirth *et al.* 2014).

Ambas toxinas Mtx pueden actuar de forma sinérgica entre si, o con toxinas Bin o toxinas Cry de *Bt*; incluso se ha demostrado que pueden suprimir la resistencia a Cry en *C. quinquefasciatus* (Wirth *et al.* 2007; Wirth *et al.* 2014).

1.5.7 Sphaericolisina

Producida por *L. sphaericus*, es secretada en el medio de cultivo como una proteína de 53 kDa después de perder una parte de su secuencia N-terminal; al ser inyectada, ha mostrado toxicidad frente a la cucaracha *Blatella germanica* y en menor medida al lepidóptero *Spodoptera litura* (Nishiwaki *et al.* 2007). Es una toxina formadora de poros; pertenece a las citolisinas dependientes de colesterol entre las cuales está la perfringolisina O, alveolysina, cereolisina, thuringiolisina y la anthrolisina de *Bacillus*.

1.5.8 Capa S

En general, la capa S son glicoproteínas que tienen la capacidad intrínseca de auto-ensamblarse en matrices cristalinas bidimensionales tanto *in vivo* como *in vitro*, cubriendo completamente la superficie celular de las bacterias que la poseen durante todas las etapas del ciclo de crecimiento; pueden constituir hasta el 15% de las proteínas sintetizadas y juegan un papel fundamental para la bacteria en su entorno natural: le proporcionan una capa protectora contra agentes externos, sirve como mecanismo de adhesión y de reconocimiento de superficie (Poppinga *et al.* 2012) y absorbe metales (Allievi *et al.* 2011). Más recientemente han sido implicadas en la patogenicidad de varias bacterias, especialmente del género *Bacillus* (Lozano *et al.* 2011; Allievi *et al.* 2014).

Estas glicoproteínas están presentes tanto en células vegetativas como en esporas, pero a diferencia de las toxinas Mtx producidas durante el crecimiento exponencial, las proteínas de la capa S no son degradadas por la acción proteolítica durante el proceso de esporulación (Allievi *et al.* 2014). La actividad mosquitocida de estas

proteínas en cepas de *L. sphaericus* ha sido probada en cultivos vegetativos frente a larvas de *C. quinquefasciatus* (Lozano *et al.* 2011) y también en cultivos esporulados frente a larvas de *Aedes aegypti* y *Culex* sp. (Allievi *et al.* 2014). Allievi y colaboradores (2014) describen el aumento de la actividad tóxica cuando se ensaya con esporas y las proteínas de la capa S, y que la capa S está en asociación con los cristales de las toxinas Bin A-B.

No se conoce su mecanismo de acción, pero el análisis de su secuencia sugiere la presencia de un dominio de unión a quitina y un domino hemolítico, los cuales podrían estar implicados en la patogenicidad (Allievi *et al.* 2014). A pesar de que la secuencia de los genes *slpC* (capa S en *L. sphaericus*) de dos cepas distintas han mostrado ser idénticas, estas cepas presentan actividad diferente frente a los organismos bioensayados (Allievi *et al.* 2014).

En cepas de *Bt* se han reportado cristales paraesporales compuestos por proteína de la capa S de ~100 kDa (Peña *et al.* 2006; Guo *et al.* 2008). La capa S ha sido reportada como el factor de virulencia frente a *Epilachna varivestis* (Coleoptera) (Peña *et al.* 2006). Estas inclusiones son diferentes a las toxinas Cry en forma, densidad y estructura (Figura 7) (Guo *et al.* 2008). Los genes de la capa S en *Bt* son *slp1* y *slp2*, y sus productos se encuentran tanto en las células vegetativas como formando inclusiones cristalinas en cultivos esporulados (Zhou *et al.* 2011).

También en *P. larvae*, la proteína SplA (capa S en esta bacteria) ha sido reportado como el primer factor de virulencia funcional de la especie (Poppinga *et al.* 2012).

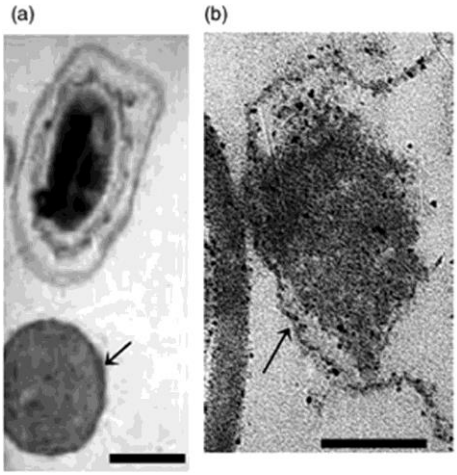

Figura 7. Microscopía electrónica de transmisión de las inclusiones paraesporales de la cepa CTC de *Bt.* a) Inclusiones tipo cristal y esporas libres de la célula madre en la etapa de esporulación. b) Las inclusiones tienden a descomponerse. La barra representa 1.0 µm. Tomado de (Guo *et al.* 2008).

1.5.9 Complejo de toxinas Tcs

Han sido identificadas como toxinas de alto peso molecular compuestas de múltiples subunidades. Fueron inicialmente descritas en *P. luminescens* mostrando toxicidad por vía oral en insectos (Bowen *et al.* 1998). Posteriormente se han encontrado en una variedad de bacterias tanto Gram-negativas como Gram-positivas. Este complejo se compone de tres tipos de subunidades de proteínas, A, B y C, y aunque su modo de acción no está completamente claro, se sabe que son biológicamente activas sólo cuando están presentes todas las subunidades (Waterfield *et al.* 2005). TcA forma una estructura pentamérica larga que se une a la membrana y como una jeringa forma un canal de translocación por el cual la citotoxina ingresa al citoplasma (Gatsogiannis *et al.* 2013), es decir, TcA forma un preporo y un poro que además tiene sitios de unión al receptor que son importantes para la especificidad del hospedador (Meusch *et al.* 2014). La región C-terminal de TcC es el principal componente citotóxico (Lang *et al.* 2010). TcC realiza una auto-proteólisis cuando se une con TcB formando un complejo en el cual está encapsulado el extremo C-terminal citotóxico (Busby *et al.* 2013). Meusch y

colaboradores (2014) explican el mecanismo molecular de acción de las toxinas Tc como un nuevo modelo de despliegue y translocación de proteínas.

El complejo de toxinas Tc ha sido descrito en especies asociadas a insectos como *Xenorhabdus*, *S. entomophila*, *Yersinia*, *Paenibacillus* (Ffrench-Constant and Waterfield 2005) y *Bt* (Blackburn *et al.* 2011). También se han encontrado genes con predicción de aminoácidos similares a los de los genes de Tc en bacterias no asociados a insectos como *P. syringae*, *Pseudomonas fluorescens* y *Fibrobacter succinogenes* (Ffrench-Constant and Waterfield 2005). En *Yersinia pseudotuberculosis* y *Yersinia pestis* los genes *tc* han demostrado toxicidad en cultivos de células de mamíferos, indicando que tienen un papel en la patogenicidad hacia mamíferos (Hares *et al.* 2008).

A pesar de que los genes *tc* tienen homología de secuencias entre las bacterias que los portan, han demostrado causar diferentes patologías en sus hospedadores. Mientras las cepas de *Yersenia* han mostrado ser altamente patógenas y capaces de causar septicemias rápidas (Hurst *et al.* 2011), aislados de *S. entomophila* causan enfermedades crónicas a través de la colonización del intestino durante un período largo antes de invadir el hemocele (Hurst *et al.* 2004).

Los genes *tc*-like están localizados en el cromosoma o en plásmidos (Dodd *et al.* 2006). En *Photorhabdus* los complejos tóxicos Tc están localizados en islas de patogenicidad (PAI), que son definidas como un largo segmento localizado en el cromosoma adquirido de manera horizontal y que codifican genes de virulencia (Waterfield *et al.* 2002). En *S. entomophila* están localizados en el plásmido pADAP (Hurst *et al.* 2000).

Otros complejos tóxicos Tc descritos son: XptA2, XptB1 y XptC1 de *Xenorhabdus* (Sheets *et al.* 2011); Sep compuestos por SepA, SepB y SepC de *S. entomophila* (Hurst *et al.* 2000) y que actúan junto a un profago (Hurst *et al.* 2004); en *Yersenia frederiksenii* los homólogos a SepA y SepB han sido designados como tcYF1 y tcYF2 (Dodd *et al.* 2006); en *Y. entomophaga* el complejo tóxico está formado por YenA,B, C y dos quitinasas (Hurst *et al.* 2011).

1.5.10 Otros factores de virulencia

Las bacterias entomopatógenas han desarrollado un arsenal de factores de virulencia; algunos como hemos mencionado anteriormente pueden actuar solos, otros han sido descritos como potenciadores del efecto patógeno de la cepa. A continuación se describen algunos de estos factores de virulencia.

Proteínas con dominios de **unión a quitina y actividad quitinasa** (o su acción conjunta) han sido descritos como factores de patogenicidad; si bien en bacterias no actúan solos, se ha demostrado que mejoran la actividad insecticida de toxinas Vip (Arora *et al.* 2003), Cry (Arora *et al.* 2013) y Bin (Cai *et al.* 2007); incluso en algunos casos, este sinergismo es altamente efectivo frente a insectos resistentes a las toxinas solas (Cai *et al.* 2007). La inhibición de la quitinasa en cepas de *Bt* ha mostrado reducción de la toxicidad (Sampson and Gooday 1998). La quitinasa, como un importante factor de virulencia, es ampliamente conocido en hongos entomopatógenos (Fan *et al.* 2007).

Las **proteasas** son conocidas como factores de virulencia en bacterias patógenas (Petersen and Tisa 2014); entre ellas las **metaloproteasas** presentes en la infección *in vivo* de *P. larvae* hacia larvas de abejas (Antunez *et al.* 2011b), y la **serralisina** segregada por *S. marcescens* que contribuye a la patogenicidad bacteriana. La serralisina actúa suprimiendo la inmunidad celular del insecto; específicamente, disminuye las propiedades adhesivas de las células de inmunovigilancia del hospedador (Ishii *et al.* 2014a) e inhibe la cicatrización de las heridas (Ishii *et al.* 2014b). También, las metaloproteasas han sido identificadas como vitales en la virulencia producida por algunas de las toxinas Cry de *Bt* (Fedhila *et al.* 2002).

Las **hemolisinas** han sido descritas como un factor de virulencia que causa la muerte del insecto en *Bt* (Tran *et al.* 2013). La acción de estas proteínas es formar poros en membranas (Hertle 2002).

Se sabe que los **antibióticos** juegan un papel fundamental para que la bacteria pueda competir y colonizar al insecto hospedador. Así, la **paenilamicina** producida por *P. larvae* es considerada un factor de virulencia, su citotoxicidad ha sido probada en una línea celular de *Trichoplusia ni* (Lepidoptera) que si bien no causa un efecto tóxico por sí solo, está involucrado en la determinación de la evolución de la enfermedad (Garcia-Gonzalez *et al.* 2014). También el antibiótico aminopoliol

zwittermicina A producido por *B. cereus* actúa sinérgicamente con *Bt* subsp. *kurstaki* e incrementa la mortalidad de *Lymantria dispar*, aunque si es administrado solo no causa efectos tóxicos (Broderick *et al.* 2000).

Otros factores de virulencia descritos son la **enolasa** (Antunez *et al.* 2011a) y la **sevadicina** (Garcia-Gonzalez *et al.* 2014).

Un particular factor de virulencia es el **profago** de la bacteria entomopatógena *S. entomophila*. El profago de anti-alimentación (anti-feeding prophage) llamado Afp es uno de los principales factores de virulencia determinantes para que se produzca la muerte del escarabajo del pastizal de Nueva Zelanda (Hurst *et al.* 2004). Afp actúa dentro de 1 a 3 días después de que la larva lo ingiera, causando el cese de la alimentación. Afp está codificado por 18 genes localizados en el plásmido pADAP; estas secuencias se encuentran en los genomas de otras bacterias insecticidas como por ejemplo *P. luminescens* TTO11 en donde se los conoce como PVCs (Rybakova *et al.* 2013). Las partículas de Afp se asemejan a las colas de los bacteriófagos (Figura 8) y comparten características con los fagos contráctiles y con los sistemas de secreción tipo VI (Rybakova *et al.* 2013). Pertenecen a las tailocinas que son fagos defectuosos que no tienen cabeza y no contienen ADN (Rybakova *et al.* 2013). Los primeros 16 genes codifican para las proteínas Afp1 a Afp16, que forman la columna vertebral de la tailocina Afp; mientras que los genes *afp*17 y *afp18* posiblemente son los que codifican el o los factores activos responsables de la actividad alimenticia (Hurst *et al.* 2004). Por su estructura se cree que la partícula Afp actúa como un dispositivo de entrega de toxina, una especie de jeringa que inyecta la toxina en las células (Heymann *et al.* 2013).

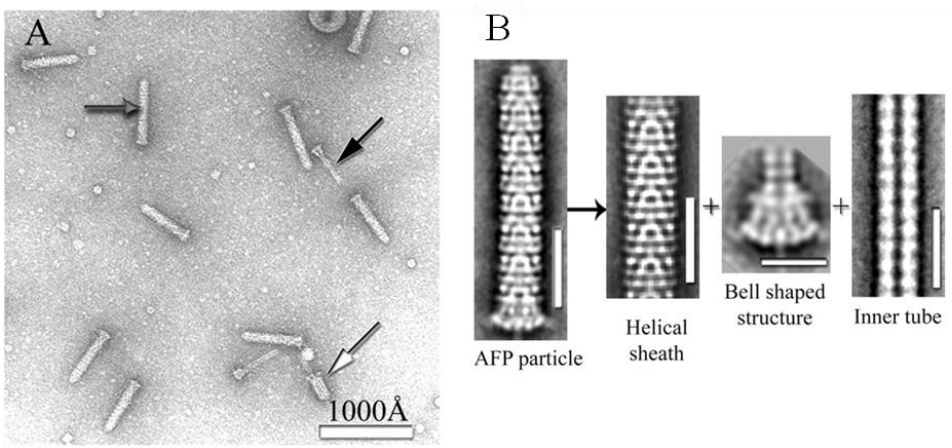

Figura 8. Microscopía electrónica de muestras con tinción negativa de partículas de Afp recombinantes parcialmente purificadas. A) Las flechas indican la configuración extendida y la configuración contráctil. B) Conformación de una partícula Afp en configuración extendida, donde se ve la vaina helicoidal + estructura en forma de campana + tubo interior. Tomado de (Sen *et al.* 2010).

1.6 *Ceratitis capitata*

La mosca de la fruta del Mediterráneo, *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae), es una de las plagas agrícolas más importantes a nivel mundial. Es una especie polífaga que infecta a más de 300 especies de frutas y verduras, siendo la plaga de mosca de la fruta con más hospedadores conocidos (Leftwich *et al.* 2014).

C. capitata es de origen africano, de la zona tropical del este sub-sahariano, específicamente de Kenia (Gasperi *et al.* 2002; Malacrida *et al.* 2007). A principios del siglo XIX fue identificada en las regiones del sur de Europa, donde subsecuentemente fue propagada hacia otras partes del globo terráqueo (Gasperi *et al.* 2002). Su presencia se ha reportado en la zona Mediterránea de Europa, en Oriente Medio, en muchas partes de África, Centro y Sur América, Hawaii, Australia, Florida y California (Figura 9) (Szyniszewska and Tatem 2014).

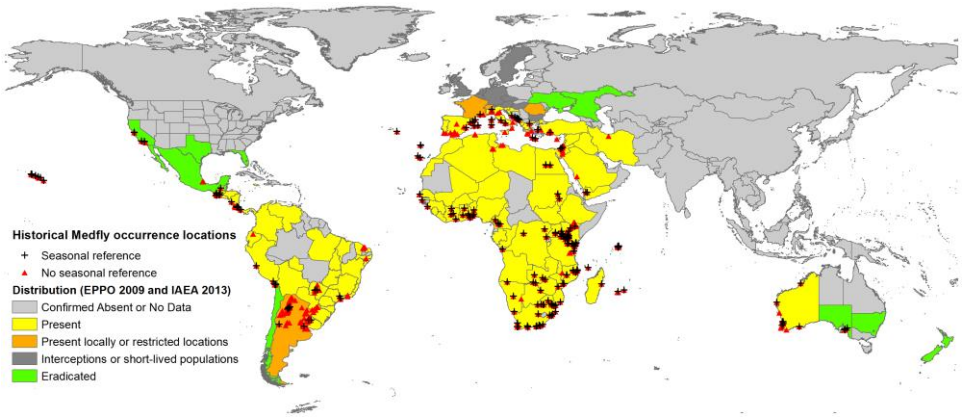

Figura 9. Mapa de distribución mundial de la ocurrencia de *Ceratitis capitata* entre los años 1980 y 2014. Los países o regiones donde *C. capitata* está presente están representados en color amarillo mientras que los lugares en donde la plaga ha sido erradicada están en verde. Tomado de (Szyniszewska and Tatem 2014).

El principal daño causado por *C. capitata* es la afección de frutos, provocada por las hembras que depositan sus huevos en el fruto y las larvas que se alimentan de él en su interior. Por otra parte, la picadura que efectúa la hembra en la ovoposición produce un pequeño orificio en la superficie del fruto, el cual es la entrada de microorganismos que provocan la pudrición del mismo convirtiéndolos en no aptos para la comercialización (Urbaneja *et al.* 2015). Las pérdidas económicas directas de esta plaga se suman a las cuantiosas pérdidas derivadas de la imposición de períodos de cuarentena y protocolos de exportación restrictivos, lo que convierte a *C. capitata* en una de las plagas con mayores repercusiones económicas y sociales para la agricultura.

Tradicionalmente, el control de esta plaga se ha realizado con el uso de insecticidas organofosforados y piretroides. En España, al igual que en otras regiones, el control de esta plaga se ha basado en tratamientos por pulverización-cebo que contienen malatión mezclado con proteína hidrolizada como atrayente alimenticio (Juan-Blasco *et al.* 2013). Sin embargo, el uso intensivo de malatión ha producido la resistencia de este insecticida en las poblaciones silvestre de *C. capitata* (Magaña *et al.* 2007). Los efectos nocivos del malatión, así como su falta de selectividad llevaron a su exclusión en la Unión Europea desde el 2009 (Urbaneja *et al.* 2009). Los productos fitosanitarios recomendados para el tratamiento de *C.*

capitata en España son: metil clorpirifos, lambda cihalotrin, etofenprox, spinosad, deltametrina y lufenuron; a pesar de que el control químico no es el sistema más recomendable ni deseable se usa cuando las poblaciones de mosca no han podido ser combatidas por otros métodos (Urbaneja *et al.* 2015). Recientemente se ha descrito resistencia a lamba cihalotrin en España (Arouri *et al.* 2014).

Una de las alternativas al control químico es el trampeo masivo. Consiste en trampas con compuestos atrayentes, sexuales o alimenticios, que pueden estar mezclados o no con insecticidas químicos. Este método es eficaz y no deja residuos tóxicos en los frutos, la desventaja es que consigue la protección adecuada cuando las poblaciones son bajas o moderadas (Martinez-Ferrer *et al.* 2012). Otra modalidad de las trampas es el uso de sustancias quimioesterilizantes, como el lufenuron que a bajas dosis produce esterilidad; este sistema está siendo comercializado por la empresa Syngenta con la unidad de control denominada Address®. El uso de trampas quimioesterilizantes ha empezado a demostrar su utilidad y efectividad tras varios años de aplicación (Urbaneja *et al.* 2015).

Junto con el trampeo masivo, la técnica del insecto estéril TIE (en inglés SIT) son los métodos más comunes utilizados para el control de *C. capitata* en la cuenca del Mediterráneo (Juan-Blasco *et al.* 2013). La TIE reduce el potencial reproductivo de las poblaciones a través de la liberación en masa de machos estériles que se aparean con las hembras silvestres. El mecanismo más común para conseguir la esterilidad es sometiendo a las pupas a radiación ionizada (Leftwich *et al.* 2014); los machos estériles compiten con los machos silvestres por el acceso a las hembras; los cruces entre machos estériles con hembras silvestres producen progenie no viable. A esta técnica también se la conoce como control autocida.

Otra técnica desarrollada para el control es RIDL (liberación de insectos que llevan un dominante letal). RIDL es una variación del método convencional TIE que se ha presentado como una alternativa a los inconvenientes de la radiación, en el cual se crea una cepa que lleva un gen condicional, dominante y letal sexo-específico (Thomas *et al.* 2000). En ésta técnica los insectos machos son homocigotos para un gen letal suprimible; el gen represor puede estar relacionado a alguna sustancia que no se encuentre en la naturaleza por ejemplo un aditivo en la dieta o un antibiótico. Los insectos RIDL liberados al cruzarse con hembras

silvestres producen una progenie heterocigota que al no encontrarse en presencia de la sustancia que reprime la expresión del gen letal produce la muerte de la progenie (Thomas *et al.* 2000). Esta técnica ha mostrado ser efectiva para el control de *C. capitata* (Leftwich *et al.* 2014).

1.6.1 Control Biológico frente a *C. capitata*

El principal método de control biológico contra esta plaga ha sido la liberación masiva de parasitoides y depredadores, con diferentes grados de éxito dependiendo de los países donde ha sido aplicado (Sivinski 1996).

Actualmente en España, la acción de los enemigos naturales frente a *C. capitata* no es suficiente para controlar por completo los daños producidos por esta plaga, sin embargo juega un papel importante para disminuir sus poblaciones (Urbaneja *et al.* 2015). Los depredadores utilizados son la araña *Pardosa cribata* Simon (Araneae: Lycosidae) que se alimenta de adultos de la mosca recién emergidos y el carábido *Pseudophonus rufipes* (De Geer) que se alimenta de las pupas (Urbaneja *et al.* 2015). Como especies parasitoides importadas se utilizan los bracónidos *Diachasmimorpha tryoni* (Cameron), *Diachasmimorpha longicaudata* (Ashmead) y *Fopius arisanus* (Sonan), los dos primeros parasitoides larvarios y el último parasitoide de huevos; mientras que los parasitoides autóctonos son los pteromálidos *Pachycrepoideus vindemmiae* (Rondani) y *Spalangia cameroni* Perkins, ambos parasitoides de pupas (Urbaneja *et al.* 2015).

Otro tipo de agentes de control biológico frente a *C. capitata* son los hongos entomopatógenos. Aislados de las especies *Metarhizium anisopliae* (Navarro-Llopis *et al.* 2015) y *Beauveria bassiana* (Imoulan and Elmeziane 2014) han mostrado buenos resultados de su actividad entomopatógena sobre diferentes estadios de *C. capitata*. Varias estrategias han sido ensayadas para la aplicación de estos agentes, siendo la conocida como "atrayente-contaminante" posiblemente la estrategia más exitosa (Navarro-Llopis *et al.* 2015). Consiste en atraer a las moscas hacia un dispositivo de inoculación donde éstas se contaminan con los conidios infectivos del hongo, lo cual favorece a la diseminación del patógeno. La eficacia de *M. anisopliae* con un dispositivo de atrayente contaminante ha sido evaluada en campo a media escala (40 hectáreas) durante 2 años con resultados satisfactorios, una reducción de

hasta un 86% de la población frente a parcelas de referencia (Navarro-Llopis *et al.* 2015).

También, los investigadores han realizado intensas búsquedas de bacterias entomopatógenas activas frente a la mosca de la fruta del Mediterráneo. Vidal-Quist y colaboradores (2009) y Aboussaid y colaboradores (2011) rastrearon 379 y 58 aislados de *Bt* respectivamente, buscando una cepa con actividad frente a esta plaga, sin embrago sus resultados mostraron mortalidades de sólo el 30% en larvas.

Como se describió en el apartado 1.3.2.3.2 de esta Introducción, nuestro grupo de investigación aisló una cepa de *B. pumilus* con actividad máxima de 94% frente a larvas de *C. capitata* (Molina *et al.* 2010).

Recientemente, Ruiu y colaboradores (2015) han caracterizado una cepa de *B. cereus sensu lato* con efectos letales y subletales contra larvas de *C. capitata*. Esta cepa ha mostrado una mortalidad de 82.64% en larvas de primer estadio. La toxicidad está asociada a la espora y a la fracción insoluble extra-espora, en la que se han detectado proteínas asociadas a la toxicidad hacia insectos como metaloproteasas, peptidasas, betaina aldehido deshidrogenasa y un homólogo a fosfolipasa patatina (Ruiu *et al.* 2015).

También, Elleuch y colaboradores (2015) bioensayaron 10 cepas de *Bt* que producen cristales paraesporales esféricos, 3 de estos aislados mostraron moderados niveles de toxicidad cuando una mezcla de esporas-cristales fueron ensayados contra *C. capitata*; sin embargo todas las cepas incluyendo la cepa comercial control *B. thuringiensis* serovar *israelensis* H14 mostraron rangos de mortalidad del 37 al 97% cuando se ensayaron únicamente los sobrenadantes de cultivos de 6 y 24 horas, lo cual indica que posiblemente la toxicidad se deba a una proteína excretada en la fase vegetativa. Como los propios autores lo dicen, habría que hacer más ensayos para identificar las moléculas responsables de la toxicidad exhibida por estas cepas hacia *C. capitata* (Elleuch *et al.* 2015) ya que no es muy frecuente que todas las cepas ensayadas den altos porcentajes de mortalidad, incluido el control comercial *Bt* serovar *israelensis* H14.

1.6.2 *B. pumilus* 15.1 y el control de la plaga *C. capitata*

C. capitata es altamente móvil y muestra resistencia a una amplia gama de condiciones ambientales, lo que facilita su prevalencia y la capacidad de reinvadir áreas de plagas con supresión (Juan-Blasco *et al.* 2013). El control de las poblaciones de *C. capitata* es un serio problema a nivel mundial. En España, la importancia económica de esta plaga reside principalmente en las pérdidas producidas en los cultivos de cítricos, producción destinada al consumo local y a la exportación.

Debido al impacto económico y social que causa esta plaga, el grupo de investigación CTS-183 de la Universidad de Granada, dentro de su línea de investigación Control Biológico de Plagas y Vectores, inició un proyecto cuyo objetivo fue la búsqueda de una nueva cepa perteneciente al género *Bacillus* con actividad insecticida frente a la mosca de la fruta del Mediterráneo. Como resultado se obtuvo un aislado perteneciente a la especie *B. pumilus* altamente tóxico para larvas de *C. capitata* (Molina *et al.* 2010). La toxicidad de esta cepa se expresó únicamente bajo condiciones muy determinadas: cuando el cultivo esporulado se incubó durante al menos 96 horas a 4ºC (Molina *et al.* 2010; Molina *et al.* 2012).

Según los resultados de nuestro laboratorio, el factor de virulencia responsable de la toxicidad de *B. pumilus* 15.1 es sintetizado durante la esporulación, ya que los cultivos vegetativos no presentaron toxicidad (Molina *et al.* 2010). Experimentos realizados con cultivos tratados con diferentes enzimas (proteasas) antes de ser bioensayados sugieren que el factor de virulencia de esta cepa puede ser de naturaleza proteica; también se bioensayaron cultivos autoclavados (121ºC durante 20 minutos) en donde la actividad tóxica fue completamente abolida, lo cual indica que la toxicidad no es causada por una exotoxina y que puede ser de origen proteico (Molina 2010).

B. pumilus no es una especie considerada entomopatógena, y como se explicó anteriormente en esta introducción, las tres cepas descritas con propiedades insecticidas no han sido caracterizadas, no se conoce el factor de virulencia ni su modo de acción. Por lo cual, nuestro grupo de investigación consideró necesaria la investigación a fondo de la cepa 15.1, centrándonos en la búsqueda del factor o de

los factores tóxicos y el análisis de propiedades que la hacen diferente al resto de las *B. pumilus* descritas.

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2. OBJETIVOS

En los últimos tiempos, debido a sus ventajas medioambientales, el control biológico de plagas ha cobrado una gran relevancia convirtiendo la búsqueda de nuevos agentes de control de plagas y vectores en una prioridad. Además en el caso concreto de los organismos entomopatógenos sigue existiendo un gran desconocimiento de los factores y mecanismos subyacentes que convierten a un organismo en tóxico para un insecto.

Con esta premisa, el objetivo general de este trabajo de investigación fue la caracterización de la cepa entomopatógena *Bacillus pumilus* 15.1. Para lograrlo se plantearon los siguientes objetivos específicos:

1. Caracterizar bioquímica, microbiológica y morfológicamente a la cepa *B. pumilus* 15.1.

2. Identificar y caracterizar elementos extracromosómicos en la cepa *B. pumilus* 15.1 con el fin de localizar e identificar potenciales factores de virulencia.

3. Caracterizar los cristales paraesporales sintetizados por la cepa *B. pumilus* 15.1.

4. Determinar la actividad de los cristales paraesporales de la cepa *B. pumilus* 15.1 mediante la realización de bioensayos frente a larvas de *Ceratitis capitata*.

5. Secuenciar y ensamblar el genoma de la cepa *B. pumilus* 15.1.

6. Anotar el genoma bacteriano de *B. pumilus* 15.1. Identificar secuencias homólogas a factores de virulencia conocidos por su actividad frente a insectos en otras cepas bacterianas.

3. RESULTADOS

Chapter I

An in-depth characterization of the entomopathogenic strain *Bacillus pumilus* 15.1 reveals that it produces inclusion bodies similar to the parasporal crystals of *Bacillus thuringiensis*

Garcia-Ramon, D., A. Molina, A. Osuna and S. Vílchez (2015) "An in-depth characterization of the entomopathogenic strain *Bacillus pumilus* 15.1 reveals that it produces inclusion bodies similar to the parasporal crystals of *Bacillus thuringiensis*". Under review at Applied Microbiology and Biotechnology.

Abstract

In the present work, the local isolate *Bacillus pumilus* 15.1 has been morphologically and biochemically characterized in order to gain a better understanding of this novel entomopathogenic strain active against *Ceratitis capitata*. This strain could represent an interesting biothechnological tool for the control of this pest. Here, we report on its nutrient preferences, extracellular enzyme production, motility mechanism, biofilm production, antibiotic suceptibility, natural resistance to chemical and physical insults and morphology of the vegetative cells and spores. The pathogen was found to be β-hemolytic and susceptible to penicillin, ampicillin, chloramphenicol, gentamicin, kanamycin, rifampicin, tetracycline and streptomycin. We also report a series of biocide, thermal and UV treatments that reduce the viability of *B. pumilus* 15.1 by several orders of magnitude. Heat and chemical treatments kill at least 99.9% of vegetative cells but spores were much more resistant. Bleach was the only chemical that was able to completely eliminate *B. pumilus* 15.1 spores. Compared to the *Bacillus subtilis* 168 spores, *B. pumilus* 15.1 spores were between 2.67-350 times more resistant to UV radiation while the vegetative cells of *B. pumilus* 15.1 were almost up to 3 orders of magnitude more resistant than the model strain. We performed electron microscopy for morphological characterization and we observed geometric structures resembling the parasporal crystal inclusions synthesized by *Bacillus thuringiensis.* Some of the results obtained here such as the parasporal inclusion bodies produced by *B. pumilus* 15.1 could potentially represent virulence factors of this novel and potentially interesting strain.

Introduction

Bacillus pumilus was described by Meyer and Gottheil in 1901 (Skerman *et al.* 1989). *B. pumilus* belongs to the *Bacillus* genus, which comprises of 299 recognized species to date (7/07/2015) (Euzeby 1997), several of them of high ecological, biotechnological and medical relevance. *B. pumilus* is ubiquitous in the environment

and has been isolated from soil (Garbeva *et al.* 2003; Padaria *et al.* 2014; Shagimardanova *et al.* 2014), sea and marine sediments (Nithya and Pandian 2010; Liu *et al.* 2013) as well as fermented foods (Yamanaka *et al.* 2007; Afifah *et al.* 2014). The bacterium is associated with animals (Parvathi *et al.* 2009; Zhang *et al.* 2009) and plants either as an endophyte (Asraful Islam *et al.* 2010; Ren *et al.* 2013) or an epiphyte (Cao *et al.* 2001). Furthermore, *B. pumilus* has been isolated from unusual places such as interior parts of Sonoran desert basalt (Benardini *et al.* 2003), from a spacecraft assembly facility and the surface of the International Space Station (La Duc *et al.* 2003; Newcombe *et al.* 2005).

B. pumilus has a high economic relevance owing to the wide range of applications that this microorganism and its products have in biotechnology, industry, biopharmaceutics and in environmental sectors. Many *B. pumilus* strains have been used for degrading xenobiotic compounds (Meyers *et al.* 1991; Hayase *et al.* 2004), as plant growth promoters (Thomas 2004; de-Bashan *et al.* 2010), as antimicrobial agents (Aunpad and Na-Bangchang 2007; Ouoba *et al.* 2007) or as animal and human probiotics (Duc le *et al.* 2004; Prieto *et al.* 2014).

Several *B. pumilus* strains are known to exhibit biological activity useful for biocontrol of many fungal species of *Aspergillus*, *Penicillium*, *Fusarium*, *Phytophthora*, *Rhizoctonia* and *Pythium* (Munimbazi and Bullerman 1998; Asraful Islam *et al.* 2010). Traditionally, *B. pumilus* has not been considered an entomopathogenic species. However, a few cases of *B. pumilus* strains with activity against insects have been reported (Heins *et al.* 1999; Ertürk *et al.* 2008; Molina *et al.* 2010; Yaman *et al.* 2010). The patented *B. pumilus* AQ717 strain, which is active against corn rootworm (Heins *et al.* 1999), was able to produce metabolites in the supernatant of the whole culture that were useful as biocontrol agents. Similarly, strains of *B. pumilus* were reported to be active against the Colorado potato beetle, *Leptinotarsa decemlineata* larvae (Ertürk *et al.* 2008) and the great spruce bark beetle, *Dendroctonus micans* larvae (Yaman *et al.* 2010). Although these strains are described to be active against insects, little is known about them and the toxicity causing agent.

We previously reported the isolation of a novel strain, *B. pumilus* 15.1, active against *Ceratitis capitata* (Diptera: Tephritidae) larvae, the Mediterranean fruit fly

(Molina *et al.* 2010), one of the worst world-wide distributed insect pests afecting more than 300 fruits and vegetables of agriculture importance (Szyniszewska and Tatem 2014). Furthermore, we also described a method for increasing the toxicity of this strain (Molina *et al.* 2009). *B. pumilus* could play an important role as a biological control agent in pest management in the future as many other new emerging entomopathogenic bacterial species (Ruiu 2015) so an in-depth characterization of it was needed in order to get practical knowledge of the strain and to further optimize its virulence. In this work, we centered our efforts on microbiological, morphological and biochemical characterization of *B. pumilus* 15.1 in order to acquire a better knowledge of this biotechnological-interesting strain. Our results provide some interesting insights into potential virulence factors of *B. pumilus* 15.1 strain that could be used for controling *C. capitata* population.

Materials and Methods

Bacterial strains and culture conditions

Ten bacterial strains were used in this study: the *B. pumilus* strain 15.1 (Spanish Type Culture Collection ref. CECT 7462) with entomopathogenic activity against *C. capitata* (Molina *et al.* 2010); the acrystalliferous strain of *B. thuringiensis* var. *israeliensis* IPS 78/11 (Ward and Ellar 1983); *B. thuringiensis* var. *kurstaki* HD1 (Dulmage 1970); two *B. pumilus* strains (*B. pumilus* M1 and M2) isolated from the sea and kindly provided by Dr. C. Calvo, (Uad *et al.* 2007); *B. subtilis* 168 (Burkholder and Giles 1947); *B. cereus* 569 (Benedict *et al.* 1945); *Pseudomonas putida* MAX10 (Manzanera *et al.* 2004); *Burkholderia cepacia* DSM 9241 (Palleroni and Holmes 1981; Yabuuchi *et al.* 1992); and *Escherichia coli* XL1Blue (Bullock *et al.* 1987). Luria-Bertani (LB) medium was routinely used for growing bacteria. When sporulation was required, liquid T3 medium (Travers *et al.* 1987) was used and cultures were kept at 30ºC and 240 rpm for 72 h. *E. coli* was grown at 37ºC while the rest of the strains were grown at 30ºC, unless otherwise stated.

Characterization of metabolic profile

Commercially available plates with 95 biochemical tests, frequently used to type bacterial strains, were used to determine the biochemical profile of several strains. Biolog GP2 MicroPlates™ (Biolog Catalog #1014) were used following manufacturer's instructions. The *Bacillus* strains were cultured overnight in LB medium at 30ºC and 240 rpm. The bacterial cultures were diluted in 18 ml of sterile gelling inoculating fluid (Biolog Catalog #72101) to a final suspension with 28% of transmittance, and 150 µl of the bacterial suspensions was transferred into each well. After 4 h and 24 h of incubation at 30ºC, wells were visually compared with the negative control well. All wells visually similar to the negative control were scored as negative and all wells with a noticeable purple color were scored as positive. Each strain was tested three times. Those compounds weakly used by the strain could show variation in color production in each repetition.

Protease, lipase, catalase and hemolytic activity

Extracellular protease activity was assayed by methods previously described with minor modifications. Very briefly, 10 µl of an overnight culture in LB of the strain were placed on LB plates supplemented with casein 2% (Montville 1983), or skimmed milk powder 3% (Rahman *et al.* 2007) and grown at optimal temperature. The ability to produce proteases was determined by the formation of clearing zones around the colonies on the solid turbid media after 24 and 48 h.

Extracellular lipase activity was assayed using LB plates containing olive oil 3% (vol/vol) and rhodamine B 0.1% (wt/vol) (Solaiman *et al.* 2001). Ten microliters of an overnight culture in LB were placed on testing plates and incubated at optimal temperature for 48 h. Extracellular lipase activity was revealed by the fluorescent halo appearing around the bacterial biomass when visualized under UV light (320 nm). Strains with no fluorescence halo were considered negative to extracellular lipase activity.

Catalase activity was detected by the production of O_2 when a colony was immersed with the use of a sterile loop in a 3% solution of hydrogen peroxide (Barbosa *et al.* 2005).

Hemolytic activity was determined on BD™ Columbia agar plates with 5% sheep blood (Catalog #254005). Ten microliters of an overnight culture in LB were placed on the testing plates and incubated at optimal temperature. Clear halos as a result of a hemolytic activity were measured after 48 h of incubation. All tests were performed in duplicates.

Motility assays

Motility assays were performed as previously described (Houry *et al.* 2010) with minor modifications. In brief, strains were grown in T3 medium at 240 rpm and 30ºC until cultures reached an optical density of 1 at 600 nm. Then, 5 µl of the cultures were placed on an LB soft-agar plate (0.3% agar) and incubated at 30ºC for 24 h.

For determining swarming motility, LB agar plates containing 0.5%, 1% and 1.5% agar were prepared 1 h before inoculation and dried with lids opened inside a laminar flow hood for 15 min (Hamze *et al.* 2011). Five microlitres of the bacterial culture, obtained as previously described, were placed on each plate and incubated at 30ºC for 24 h.

The extent of motility was determined as the diameter of the colony after 12 h and 24 h.

Biofilm formation

Biofilm formation was tested as previously described (O'Toole and Kolter 1998) with minor modification. Essentially, 5 ml of T3 medium were inoculated with the strain under study and incubated at 30ºC, 240 rpm for 4 h. Cultures were diluted (1:100) into 5 ml of fresh medium inside a 20 ml polystyrene tube and incubated at 30ºC for 24 h without agitation. After that, bacterial cultures were discarded and polystyrene tubes were rinsed with sterile distilled water to remove loosely associated bacteria. Biofilm formation was observed as a bacterial ring formed in the interface air and medium. Biofilms were stained by incubation with 6 ml of 1% crystal violet solution in water for 15 min at 30ºC. Then, tubes were washed with sterile distilled water repeatedly to remove the excess of the stain. Tubes were treated with 5 ml of 95% ethanol and vortexed until the blue ring on the walls of the

tube disappeared. Biofilm production was quantified by measuring the absorbance of the resulting solution at 600 nm. Four replicates for each strain were made.

Determination of minimum inhibitory concentration (MIC) for* B. pumilus *15.1

Minimum inhibitory concentration (MIC) is the lowest concentration of an antimicrobial agent, in this case antibiotic, that inhibits the growth of a microorganism after a certain incubation time. The MIC for eight antibiotics was determined for *B. pumilus* 15.1 using the broth dilution method (Ericsson and Sherris 1971). Briefly, a sporulated culture of *B. pumilus* 15.1 on T3 medium was used to make a bacterial suspension with approximately $1x10^5$ CFU/ml in fresh LB. The suspension was divided in 2 ml aliquot fractions (except for one with 4 ml) and placed in 30 ml plastic tubes. Antibiotics were diluted in the tubes containing 4 ml of medium, mixed thoroughly and used to make two-fold serial dilutions by transferring 2 ml into the next tube. Tubes were incubated at 26ºC without agitation for 48 h. A negative control consisting on LB medium without bacteria and incubated under the same conditions was included in the experiment. Antibiotic stock solutions were prepared according to the instructions of the manufacturer. The antibiotics and concentrations tested were streptomycin (from 10 to 0.625 µg/ml), ampicillin (from 10 to 0.0195 µg/ml), tetracycline (from 5 to 0.010 µg/ml), penicillin (from 6 to 0.047 µg/ml), rifampicin (from 5 to 0.010 µg/ml), chloramphenicol (from 30 to 0.234 µg/ml), gentamicin (from 20 to 0.039 µg/ml), and kanamycin (from 30 to 0.234 µg/ml). The lowest concentration of antibiotic preventing the appearance of turbidity was considered to be the minimal inhibitory concentration. All MIC experiments were performed in duplicates.

Heat, Chemical and UV resistance tests

Cultures were prepared by growing the bacterial strains for 72 h under standard growth conditions on T3 liquid medium and heated at 70ºC for 10 min in a water bath to obtain sporulation-synchronized cultures. This spore suspension was either used to obtain vegetative or spores cells of the strains under study. To obtain cells suspensions highly enriched in vegetative cells, 500 µl of the previously mentioned suspensions were used to inoculate 50 ml of T3 media. Cultures were incubated at

30ºC and 240 rpm until an optical density (at 600 nm) of 0.4-0.9 was reached. Then, vegetative cells were pelleted at 20,000 x *g* for 20 min and washed twice with phosphate-buffered saline (PBS) and finally resuspended in 50 ml of PBS. To obtain highly enriched spore suspensions, 500 µl of the synchronized culture were used to inoculate 50 ml of T3 media and incubated at 30ºC and 240 rpm for 72 h. Spores were harvested by centrifugation under the same conditions previously stated, washed twice with water and finally resuspended in 50 ml of sterile distilled water (Nicholson and Setlow 1990; Schichnes *et al.* 2006).

Heat resistance of vegetative cells and spores was examined by placing 1 ml of bacterial suspension (vegetative cell or spore suspension) in a 1.5 ml Eppendorf tube and heated in a water bath at 60ºC, 70ºC, 80ºC or 90ºC for 10 min. The viability of heat-treated cells was determined by plating ten-fold serial dilutions made in M8 modified medium ($Na_2HPO_4.7H_2O$, 7 g/l; KH_2PO_4, 3 g/l and NaCl, 0.5 g/l) on LB. Plates were incubated at 30ºC overnight, and colonies counted the following day. The number of surviving cells in each treatment was compared with the total number of viable cells before the heat treatment was applied.

Similarly, the effect of various biocides on *B. pumilus* viability was assayed on vegetative cells and spores. The biocides assayed were solutions of 70% ethanol; 4% formaldehyde; 1.6% $KMnO_4$; 1% bleach; 10 mM NaOH, (pH 12); 10 mM HCl, (pH 2), (Vilchez *et al.* 2008); 2% benzalkonium chloride; and 3% hydrogen peroxide. For the assay, 1 ml of cell suspension (either vegetative cells or spores) was spun down at 16,000 x *g* for 1 min. Pellets were resuspended in 200 µl of the biocide, and the mixtures were incubated for 30 min in agitation at room temperature. Cells were harvested by centrifugation as described above and pellets were washed twice in PBS and the final pellet resuspended in 1 ml of PBS. The number of surviving cells was determined by serial dilutions and plating as previously described.

For UV test, 10 ml of vegetative cell or spore suspensions with an absorbance of 0.125 at 600 nm were made. Within a laminar flow bench (Telstar® Bio II A/P) the bacterial suspensions were poured on lid-opened Petri dishes (60 mm diameter) and irradiated at different times (5, 10 and 30 min) under an UV *Lightech* G15T8 lamp producing 254 nm UV-C radiation. UV doses (in joules per square meter) received by bacterial suspensions during the irradiation time were calculated using the

following equation provided by Dr. Galvez Ruiz, from the Applied Physics Department of the University of Granada:

$$\left(\frac{W}{2\pi \times L \times D}\right) \times s = \frac{J}{m^2}$$

Where **D** is the distance between the lamp and the plates (0.7 m), ***W*** is the radiant power of the lamp (4.8 W), ***L*** is the length of the lamp (0.4 m) and ***s*** is the irradiation time in seconds (300, 600 and 1800 s). Using this equation, the UV doses that the cell suspensions received were calculated to be 818.51, 1637.02 and 4911.07 J/m^2 respectively.

Viable cells present in each treatment were determined by plating ten-fold serial dilutions on LB plates. All resistance experiments were performed in duplicates.

Statistical analysis

All experiments were performed at least twice. Means and standard deviation were provided and a one way analysis of variance (ANOVA) was calculated where required using StatPlus®.

Transmission and scanning electron microscopy

Bacterial cells were taken from synchronized cultures of *B. pumilus* 15.1 in T3 grown at 30ºC and 240 rpm along time. Samples (1 - 4.5 ml) were pelleted at 12,700 x *g* for 10 min, washed with 1 ml of PBS and finally fixed in 1 ml of 2.5% glutaraldehyde, and 4% paraformaldehyde in cacodylate buffer (0.05 M, pH 7.2). Samples were kept at 4ºC for a maximum period of 24 h, then washed three times following this procedure: centrifugation at 12,700 x *g* for 5 min, rinse with washing buffer (cacodylate buffer 0.1M, pH 7.2) and incubation at 4ºC for 20 min. Finally, samples were resuspended in washing buffer and sent to the Biological Sample Preparation Laboratory at the Scientific Instrumentation Center of the University of Granada (CIC-UGR) for processing. Sample preparation for SEM was performed as previously described (Anderson 1951; Mazia *et al.* 1975; Sanders *et al.* 1975). Briefly, cell suspensions were placed on a surface with 0.1% poly-*l*-lysine and allowed to fix for 14-20 h at 4ºC in saturate atmosphere of glutaraldehyde. Post-fix

was made with 1% osmium tetroxide for 1 h. Finally, samples were critical-point dehydrated (Anderson 1951) using carbon dioxide and coated with carbon (EMITECH K975X). For TEM, samples were treated following the method described by Renau and Megias (Renau and Megias 1998). Briefly, samples were fixed with 1% osmium tetroxide and 1% potassium ferricyanide for 1 h at 4ºC. Then samples were embedded in EMbed 812 resin. Ultrathin sections (50 - 70 nm) were obtained (Ultracut R, LEICA) with DIATOME diamond knife from the polymerized resin blocks and mounted on 200 mesh-copper grids. Grids were post-stained with 1% uranyl acetate and subsequently with lead citrate following the methodology previously described (Reynolds 1963).

Samples were observed under a Transmission Electron Microscopy (ZEISS EM 902) and a Scanning Electron Microscopy (HRSEM Zeiss DSM 950) at the Microscopy Service of the CIC-UGR.

Results

Metabolic profile of B. pumilus *15.1 strain*

The metabolic profile of *B. pumilus* 15.1, M1 and M2 and *B. thuringiensis* 78/11 strains was determined by the GP2 MicroPlate™ test, specific for Gram-positive bacteria from Biolog®. The system uses 95 organic compounds to determine the carbon metabolic fingerprint of microorganisms for identification purposes, therefore this test is useful to make a metabolic characterization of the bacteria of interest (Garland and Mills 1991). In the test, a change in the color of the culture is observed (it turns purplish) when a strain is able to use the compound in the well of the microplate test. The appearance of purple color in all repetitions is shown in Table 1 as (+), in at least one repetition as (±) (faint color) and in none of the repetitions as (-). Table 1 only lists the substrates utilized by at least one of the strains tested. The test revealed that the strain with greater metabolic diversity was *B. pumilus* 15.1 (metabolized 24 different carbon sources in at least one repetition). *B. pumilus* M1, M2 and *B. thuringiensis* 78/11 metabolized 19, 16 and 18 compounds, respectively. *B. pumilus* 15.1 strain was able to metabolize 8 monosaccharides, 3 disaccharides, 1 trisaccharide, 4 glycosides, 2 polyols; 3 organic

acids and their derivatives, 1 amino acid and 2 alcohols from the pool of carbon sources tested.

Sixty carbon sources were not utilized by any of the *Bacillus* strains tested. Slight differences in the carbon source profile were observed in the *B. pumilus* strains tested. The strain 15.1 shared 86 identical results with M1 strain and 83 with M2 strain while only 71 with *B. thuringiensis* 78/11.

Table 1. Biolog® GP2 microplate assay results with *B. pumilus* 15.1, M1 and M2, and *B. thuringiensis* IPS 78/11.

Compound[a]	Strain			
	Bp15.1	M1	M2	Bt78/11
Carbohydrates				
N-Acetyl-β-D-Mannosamine	±	-	-	-
L-Arabinose	+	-	+	+
Arbutin	+	+	+	-
D-Cellobiose	-	+	+	-
D-Fructose	+	+	+	-
Gentiobiose	±	-	+	-
α-D-Glucose	+	+	-	+
Maltotriose	±	+	+	±
D-Mannitol	±	+	-	-
D-Mannose	+	+	+	-
3-Methyl Glucose	+	+	+	±
β-Methyl-D-Glucoside	+	+	+	-
D-Psicose	±	+	-	-
D-Ribose	+	+	+	±
Salicin	+	+	-	-
D-Sorbitol	±	+	+	-
Sucrose	±	+	+	-
D-Tagatose	-	+	-	-
D-Trehalose	+	+	+	+
D-Xylose	+	+	+	+
Surfactants				
Tween 40	-	-	-	±
Tween 80	-	-	-	±
Carboxylic acids				
α-Ketovaleric Acid	-	-	-	±
L-Malic Acid	+	+	+	-
Pyruvatic Acid Methyl Ester	+	-	-	-
Pyruvic Acid	+	-	-	±
Amino acids				
L-Alanine	-	-	-	±
L-Alanyl-Glycine	-	-	-	±
L-Asparagine	-	-	±	±
L-Glutamic Acid	-	-	-	±
L-Serine	±	-	-	+
Alcohols				
2,3-Butanediol	±	-	-	-
Glycerol	±	±	-	-
Other compounds				
Uridine	-	-	-	±
Adenosine-5'-Monophosphate	-	-	-	±

[a] From the 95 compounds tested, only are shown those that were utilized by at least one of the strains assayed.

(+) strong color in all repetitions; (-) no color; (±) weak color in at least in one repetition.

Enzyme production and hemolytic activity of* B. pumilus *15.1

The ability of *B. pumilus* 15.1 to secrete different extracellular enzymes was tested in addition to its hemolytic activity. The results obtained for *B. pumilus* 15.1 and other bacterial strains are summarized in Table 2.

Five out of nine strains tested (*B. pumilus* 15.1, M1 and M2; *B. subtilis* 168 and *B. cereus* 569) were found to produce extracellular proteases as indicated by the presence of clear zones of hydrolysis around the bacterial colonies. The strain *B. pumilus* 15.1 formed the biggest halo of all strains tested on both substrates.

Lipase activity was performed in agar plates containing trioleoylglycerol (olive oil) and rhodamine and monitored by observation under an UV light. Since only the *B. cepacia* DSM 9241 strain produced a fluorescent halo the strain *B. pumilus* 15.1 was considered as negative in the production of extracellular lipase.

Oxygen bubbles generated as a result of the decomposition of the hydrogen peroxide was used as an indicator for catalase activity. Hemolytic activity was detected on 5% sheep blood agar plates. Four out of the nine strains tested exhibited hemolytic activity (*B. pumilus* 15.1 and M2, *B. thuringiensis* IPS 78/11 and *B. cereus* 569). The halo from *B. pumilus* 15.1 was clear; the agar around the colony appeared lightened and transparent, suggesting β-hemolysis activity.

Table 2. Protease, lipase, catalase and hemolysin production of nine bacterial strains used in the study.

Bacterial strain	Activity				
	Protease		Lipase	Catalase	Hemolysis
	Casein (2%)[a]	Milk powder (3%)[a]			
B. pumilus 15.1	10 ± 1	7 ± 0	-	+	+
B. pumilus M1	7 ± 1	2.5 ± 0	-	+	-
B. pumilus M2	6 ± 1	4 ± 1	-	+	+
B. thuringiensis IPS 78/11	0	0	-	+	+
B. subtilis 168	1.5 ± 0	3 ± 0	-	+	-
B. cereus 569	0	3 ± 0	-	+	+
E. coli XL1 Blue	0	0	-	+	-
P. putida MAX10	0	0	-	+	-
B. cepacia DSM 9241	0	0	+	+	-

[a] Values represent means ± standard deviations of the distance between the edge of the bacterial colony and the edge of the clearing halo in mm.
(+) Activity detected; (-) Activity not detected.

Motility assays

We assayed the two most common motility mechanisms on bacteria on 0.3% agar plates (for swimming) and 0.5%, 1% and 1.5% agar plates (for swarming) (Harshey 2003) in nine bacterial strains (Table 3). Motility was measured as the ability of the strain to expand further than the diameter of the drop placed on the plate (around 7 mm). *B. pumilus* 15.1 was a motile strain showing both swimming and swarming motility. Its colony diameter on the 0.3%, 0.5% and 1% agar plates was one of the largest compared to other strains. Strain *B. pumilus* 15.1 was able to completely colonize each of the 0.3% and 0.5% agar plates, a striking observation seen in *B. pumilus* M2 strain as well. Only on the 1.5% agar plate, *B. pumilus* 15.1 did not show any kind of motility. Previously, swarming studies in *B. subtilis* showed the motility was limited at 1% agar and was abolished at 1.5% (Kearns and Losick 2003; Hamze *et al.* 2009), therefore this could be the case for *B. pumilus* 15.1.

Table 3. Swimming and swarming motility (after 12 h and 24 h) of nine bacterial strains measured as the colony diameter when grown on different agar concentrations.

Strains	Agar concentration											
	0.3%			0.5%			1%			1.5%		
	12 h	24 h		12 h	24h		12 h	24 h		10 h	24 h	
***B. pumilus* 15.1**	42±3	80[a]	+	27±3	80[a]	+	11±2	53±2	+	6±1	7±1	-
***B. pumilus* M1**	12±2	55±4	+	6±0	38±3	+	6±1	7±1	-	6±1	9±0	-
***B. pumilus* M2**	50±3	80[a]	+	35±5	80[a]	+	14±1	15±1	+	6±1	8±0	-
***B. subtilis* 168**	13±2	18±3	+	12±2	20±3	+	12±1	18±1	+	7±0	12±2	+
***B. cereus* 569**	10±1	80[a]	+	10±2	18±2	+	8±2	13±2	+	6±0	9±0	-
***B. thuringiensis* 78/11**	6±0	10±0	-	7±0	8±0	-	7±1	8±1	-	6±1	7±1	-
B. thuringiensis* var. *kurstaki	8±2	28±3	+	9±2	25±3	+	7±1	11±2	+	7±0	11±1	+
***E. coli* XL1 Blue**	7±0	8±0	-	6±1	8±0	-	6±1	7±1	-	7±0	7±1	-
***B. cepacia* DSM 9241**	10±1	11±0	-	8±1	9±0	-	8±1	9±0	-	8±0	9±0	-

(+) the strain shows motility on the medium assayed.

(-) the strain shows no motility on medium assayed [colony showed a size similar of the drop placed onto the agar plate (aprox. 7 mm)].

[a] The diameter of the colony is equal as the Petri dish diameter.

Biofilm formation

The ability of *B. pumilus* 15.1 to form biofilms was assayed by staining the cells that are able to adhere to an abiotic surface (polystyrene). As the strain was routinely grown in T3 medium (Molina *et al.* 2010), where it reveals its toxicity against *C. capitata*, all the biofilm assays were performed in this medium. Biofilm formation was quantified by staining and disrupting the ring formed (at the liquid-air interface of a static culture) followed by measuring the A_{600nm} of the resulting ethanol suspension. We found that the ability to form biofilms of *B. pumilus* 15.1 (2.3±0.35) and M2 (3.38±0.65) was significantly higher than that of the other strains assayed (Figure 1). In T3 medium, the strains *B. pumilus* M1 (0.115±0.05), *B. subtilis* 168 (0.0398±0.001), *B. cereus* 569 (0.0398±0.001), *B. thuringiensis* IPS 78/11 (0.0375±0.001), *B. thuringiensis* var. *kurstaki* (0.0357±0.001) and *B. cepacia* DSM 9241 (0.133±0.04) exhibited a poor ability to form biofilms on polystyrene. Statistical analysis (ANOVA) showed significant differences between strains ($F_{8,35}$=58.130, P= 0.000).

Given the ability of *B. pumilus* 15.1 to form biofilms, we next evaluated the variation of this capability over time. Biofilm quantification was then performed after 24 h, 48 h and 72 h of culture incubation following the same procedure as before. The average and standard error of the A_{600nm} values obtained were 2.3±0.2, 2.55±0.35 and 2.8±0.25 for 24, 48 and 72 h respectively. These results showed a slight increase in the biofilm formation over time.

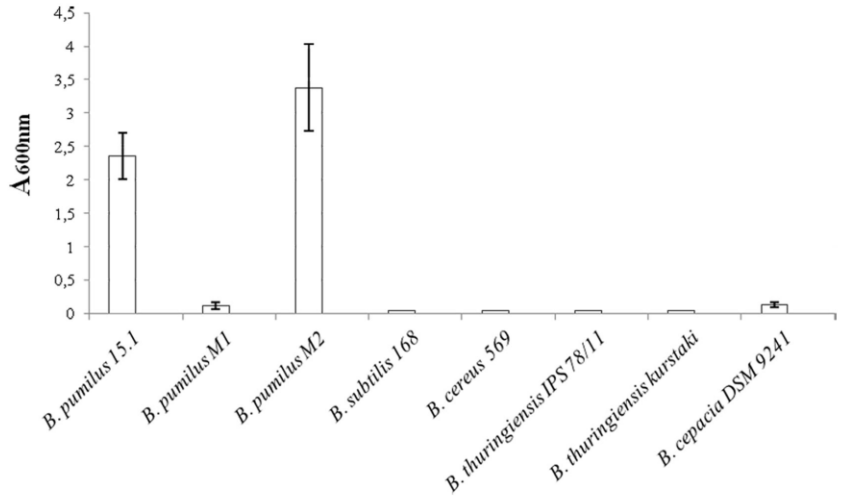

Figure 1. Biofilm formation measured by crystal violet staining of the rings formed in the interface air-liquid on *B. pumilus* 15.1, *B. pumilus* M1, *B. pumilus* M2, *B. subtilis* 168, *B. cereus* 569, *B. thuringiensis* IPS 78/11, *B. thuringiensis* var. *kurstaki* and *B. cepacia* DSM 9241 static cultures. Bars represent average A_{600nm} values and standard errors.

Antibiotic susceptibility analysis of B. pumilus 15.1 *strain*

The susceptibility of the strain *B. pumilus* 15.1 to the most commonly used antibiotics in a laboratory was determined in order to further characterize the strain. Using the broth dilution method, we determined the MIC for eight antibiotics and compared them with their interpretative breaking point for *Bacillus spp.* according to the European Food Safety Authority (EFSA) (EFSA 2008) (Table 4).

In general, all the estimated MICs for *B. pumilus* 15.1 were very low and in all cases lower than the interpretative breaking point considered by EFSA, so we conclude that *B. pumilus* 15.1 does not show inherent or acquired resistance to any of the antibiotics tested. *B. pumilus* 15.1 showed a MIC value close to the interpretative breakpoint only for Chloramphenicol.

Table 4. MIC values of eight different antibiotics for *B. pumilus* 15.1 determined after 48 h.

Antibiotic	MIC (µg/ml)	
	B. pumilus 15.1[a]	*Bacillus spp.*[b]
Ampicillin	0.16 ± 0.00	Nd
Chloramphenicol	7.50 ± 0.00	8
Gentamicin	2.50 ± 0.00	4
Kanamycin	3.75 ± 0.00	8
Penicillin	<0.05 ± 0.00	Nd
Rifampicin	0.16 ± 0.00	Nd
Tetracycline	0.62 ± 0.08	8
Streptomycin	5.00 ± 0.00	8

[a] MIC average values and standard deviation.

[b] Interpretative breakpoint for *Bacillus* spp. according to EFSA (EFSA 2008). Strains with MICs higher than the breakpoints are considered as resistant. Nd not determined by EFSA.

Natural resistance of B. pumilus *15.1 cells to physical and chemical insults*

As part of the characterization of *B. pumilus* 15.1, we determined the resistance of cell suspensions highly enriched with spores or vegetative cells to various physical and chemical stresses. In this series of experiments, we included the *B. subtilis* strain 168 as a control, it being a model microorganism for Gram-positive bacteria. Table 5 shows the survival of vegetative cells and spores of both strains after each treatment expressed as the logarithmic (log_{10}) of the total number of viable cells and the survival percentage compared to the control (bacterial suspension before treatment).

As expected, spores were much more resistant than vegetative cells to any of the insults tested. Regarding heat resistance, the surviving vegetative cells were, on an average, 1 to 3 orders of magnitude lower than the surviving spores except for the treatment at 90ºC.

In each temperature tested, spores of *B. subtilis* were more resistant to heat treatments than spores of *B. pumilus* 15.1, therefore we could conclude that the strain under study is more heat-sensitive than the model strain. In addition, at high temperatures (90ºC), *B. pumilus* was especially sensitive showing a decrease of spore viability of 5 orders of magnitude (0.004% survival).

To test the susceptibility of vegetative cells and spores to several biocides, a pellet with a known number of cells was resuspended in a biocide solution for 30 min following which the viability was estimated by plating. In general and as expected, vegetative cells were more susceptible to the biocide tested than spores in both strains. Only bleach was able to kill completely spores of the two strains tested. The rest of the biocides only had a slight effect on spore viability. The effect of formaldehyde, $KMnO_4$, bleach, benzalkonium chloride and hydrogen peroxide on vegetative cells was very dramatic but not the rest of biocides. *B. pumilus* 15.1 vegetative cells were especially sensitive to formaldehyde while *B. subtilis* vegetative cells were slightly resistant. On the contrary, *B. pumilus* 15.1 vegetative cells were more resistant to alkaline media than *B. subtilis*. Both spores were particularly resistant to ethanol, $KMnO_4$, NaOH (pH 12), HCl (pH 2) and hydrogen peroxide.

The resistance of spores and vegetative cells of *B. pumilus* 15.1 to UV light was assayed and compared with the resistance of *B. subtilis* 168 due to its reported UV resistance (Nicholson *et al.* 2000; Newcombe *et al.* 2005). The bacterial suspensions were UV irradiated for 5, 10 and 30 min with 815.5, 1,637.0 and 4,911.1 J/m^2 radiation energy, respectively. The results showed that it was necessary to supply 4,911.1 J/m^2 of radiation energy to completely reduce the viability of the *B. subtilis* vegetative cells under the detection limit. Nevertheless, surviving *B. pumilus* 15.1 vegetative cells were detected at this radiation energy. Both spores and vegetative cells of *B. pumilus* 15.1 were, in general, more resistant to UV than *B. subtilis* 168 cells. In particular, *B. pumilus* 15.1 spores were between 2.7 and 350 times more resistant than the *B. subtilis* spores while *B. pumilus* 15.1 vegetative cells were between 1.71-2.59 orders of magnitude more resistant than *B. subtilis* vegetative cells.

Table 5. Number of viable vegetative cells and spores (in logarithms) of *B. pumilus* 15.1 and *B. subtilis* 168 and viable cell percentage after treatment with several insults.

Treatment	*B. pumilus* 15.1		*B. subtilis* 168	
	Vegetative cells	Spores	Vegetative cells	Spores
Heat				
Control	8.45 ± 0.13 (100%)	7.99 ± 0.13 (100%)	8.31 ± 0.25 (100%)	7.93 ± 0.19 (100%)
60°C	ND	6.95 ± 0.17 (9.22%)	ND	7.03 ± 0.29 (14.4%)
70°C	4.46 ± 0.42 (0.015%)	6.32 ± 0.21 (2.27%)	4.38 ± 0.34 (0.013%)	6.92 ± 0.19 (9.95%)
80°C	3.72 ± 0.29 (0.002%)	4.37 ± 0.40 (0.03%)	4.04 ± 0.17 (0.0049%)	5.81 ± 0.38 (0.99%)
90°C	3.47 ± 0.25 (0.0012%)	2.97 ± 0.81 (0.004%)	2.35 ± 0.49 (0.0002%)	4.29 ± 0.31 (0.03%)
Chemical				
Control	7.18 ± 0.07 (100%)	8.23 ± 0.40 (100%)	7.36 ± 0.32 (100%)	8.13 ± 0.18 (100%)
70% ethanol	4.81 ± 0.73 (1.17%)	7.30 ± 0.65 (17.36%)	3.51 ± 0.39 (0.015%)	7.93 ± 0.08 (58.23%)
4% formaldehyde	0.00 ± 0.00 (0%)	6.32 ± 0.20 (0.85%)	2.19 ± 0.41 (0.0008%)	6.74 ± 0.42 (5.65%)
1.6% $KMnO_4$	0.00 ± 0.00 (0%)	7.40 ± 1.03 (50.53%)	0.00 ± 0.00 (0%)	7.70 ± 0.52 (61.22%)
1% bleach	0.00 ± 0.00 (0%)	0.00 ± 0.00 (0%)	0.00 ± 0.00 (0%)	0.00 ± 0.00 (0%)
10 mM NaOH (pH 12)	2.29 ± 0.11 (0.001%)	7.43 ± 0.65 (23.39%)	0.85 ± 0.85 (0.00008%)	7.27 ± 0.64 (29%)
10 mM HCl (pH 2)	2.50 ± 0.22 (0.002%)	7.49 ± 0.53 (21.20%)	2.93 ± 0.45 (0.004%)	6.71 ± 0.33 (4.56%)
2% benzalkonium chloride	0.00 ± 0.00 (0%)	6.00 ± 0.23 (0.42%)	0.00 ± 0.00 (0%)	5.62 ± 0.88 (1.07%)
3% hydrogen peroxide	0.35 ± 0.35 (0.00002%)	7.43 ± 0.50 (16.92%)	0.00 ± 0.00 (0%)	7.57 ± 0.35 (32.40%)
UV-C				
Control	7.75 ± 0.15 (100%)	7.79 ± 0.17 (100%)	7.18 ± 0.14 (100%)	7.17 ± 0.02 (100%)
815.5 J/m^2	4.50 ± 0.05 (0.053%)	7.23 ± 0.07 (26.06%)	2.79 ± 0.01 (0.004%)	5.69 ± 0.09 (3.39%)

1,637.0 J/m^2	4.25 ± 0.05 (0.03%)	6.63 ± 0.03 (6.52%)	2.33 ± 0.03 (0.001%)	5.33 ± 0.48 (2.44%)
4,911.1 J/m^2	2.59 ± 0.02 (0.00065%)	4.66 ± 0.01 (0.07%)	0.00 ± 0.00 (0%)	0.86 ± 0.86 (0.0002%)

In order to further characterize this feature, we performed more UV tests with other sporulating bacteria. We compared *B. pumilus* 15.1 vegetative cell and spore resistance to *B. pumilus* M1 and M2, *B. thuringiensis* var. *israeliensis* IPS 78/11 and var. *kurstaki* HD1, *B. subtilis* 168 and *B. cereus* (Figure 2). Survival studies after exposure of vegetative cell and spores to UV-C (254 nm) radiation demonstrated that *B. pumilus* 15.1 (vegetative cell and spores) were much more resistant to UV-C than any of the *Bacillus sp.* tested in this study, except for *B. pumilus* M2 that proved to be equally resistant. Only *B. pumilus* 15.1 and M2 vegetative cells were able to survive the maximal radiation applied.

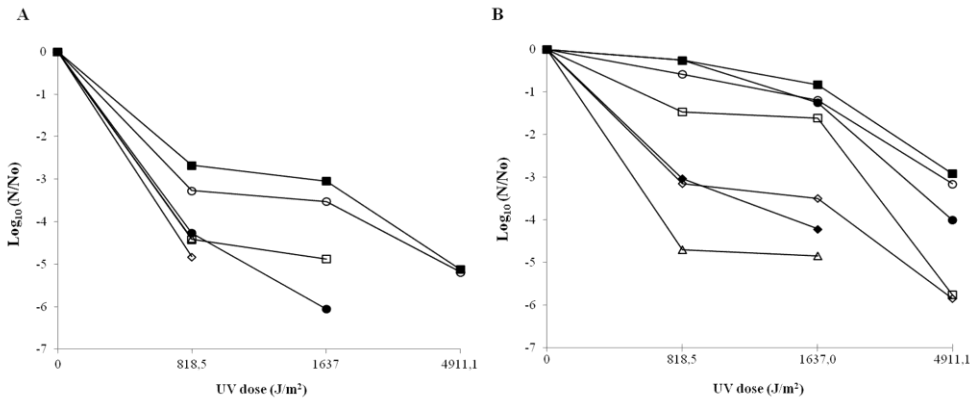

Figure 2. UV-C (254 nm) inactivation curves of vegetative cells (A) and spores (B) of *B. pumilus* 15.1 (open circles), *B. pumilus* M1 (closed circles), *B. subtilis* 168 (open squares), *B. pumilus* M2 (closed squares), *B. thuringiensis* var. *kurstaki* HD1 (open rhomb), *B. thuringiensis* 78/11 (closed rhomb) and *B. cereus* (open triangle). N/No was calculated by dividing the viable cells titer at any given UV dose (N) by the cells titer obtained from the non-irradiated suspension (No).

Characterization of* B. pumilus *15.1 strain by electron microscopy

In order to morphologically characterize the vegetative cells and spores of the *B. pumilus* 15.1 strain, cultures of this bacterium were analyzed under transmission and scanning electron microscopy along time.

Scanning electron microscopy

The surface structure and morphology of *B. pumilus* 15.1 was visualized over time by scanning electron microscopy. At the beginning of a *B. pumilus* 15.1 culture (6 h) (Figure 3 A and B), all cells were in vegetative phase, possessed a bacillar shape approximately 1.4 - 1.9 µm long and 0.49 - 0.50 µm wide, displayed an intact smooth surface and some of them were even observed at binary fission (white arrow). As time progressed, cells became shorter and showed an irregular surface (Figure 3C). Upon visualizing multiple fields of the sample, we observed the presence of several clusters of cells (vegetative and spores) joined together by an extracellular material. The extracellular material appeared to be composed of tangled thread-like strands forming a complex network. These filaments are very thin in early cultures (Figure 3B) but as time progressed, they seem to thicken and serve as deposit for small spheres of other (or the same) material that completely surrounded the cells (Figure 3D). A close observation of these cell clusters showed some appendages connecting the cells (Figure 3D, black circle) that were different from the surrounding material. These appendages seemed to be tubular protrusions formed from the cell wall.

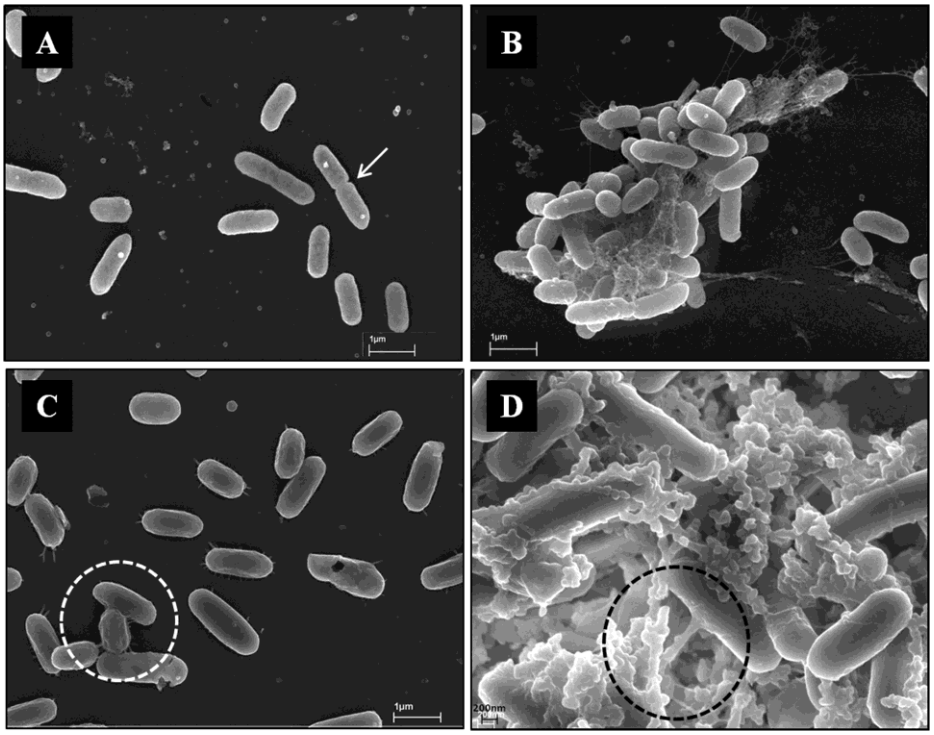

Figure 3. Scanning electron microscopy of a culture of *B. pumilus* 15.1. Magnification is shown at the bottom of each panel. Panel A: a 6 h culture sample, white arrow showing a cell in binary fission. Panel B: a 6 h culture sample, vegetative cells forming a cluster with very thin filaments. Panel C: a 50 h culture sample, spores with small filaments connecting cells with others (white circle). Panel D: a 72 h culture sample, cells forming cluster joined together by an extracellular material, and filaments that seem to be composed by the cell wall (black circle).

Transmission electron microscopy

A culture of *B. pumilus* 15.1 in T3 medium was monitored by transmission electron microscopy over time as well. Samples that were taken after 6 and 11 h of incubation (exponential growth phase of the culture) showed vegetative cells with a typical bacillar shape. The vegetative cells were 1.39 - 1.8 µm long and 0.44 - 0.49 µm wide (Figure 4A). When the culture progressed, a transformation of the morphology of the cells was observed. Twenty-nine hours after the start of the culture, 90% of cells were mother cells with a prespore inside (Figure 4B). Free spores were observed in samples taken from 33 h. *B. pumilus* 15.1 spores size ranged between 1.05 - 1.25 µm in length and 0.45 - 0.70 µm in width.

Upon a detailed analysis of the spores (Figure 4C), we observed that (from inside to outside) the spore core was surrounded by an inner membrane, a germ cell wall and a protective cortex, followed by a thick spore coat organized as lamella-like inner coat layer and an electro-dense outer coat layer. When the spore had not yet been released, a layer corresponding to the cell wall of the mother cell surrounding the spore was frequently observed.

In samples taken from the culture 44 h after the start of incubation, we observed electron-dense polygonal shaped structures outside of the spores. The number of these structures increased with the incubation time and with the number of total spores of the culture (Figure 4D). A close inspection of the samples revealed geometric structures resembling the typical parasporal crystals observed in *B. thuringiensis* culture and composed of crystalline inclusions (δ-endotoxin) responsible for insecticidal activity (Ward and Ellar 1983). The size and shape of the inclusions in *B. pumilus* 15.1 was very variable as shown in Figure 4E, 4F and 4G. Most of them showed square and bipyramidal shape, others appeared as triangles, trapezoids and even an amorphous shape. The crystals showed striking uniformly spaced grids observable by electron microscopy (Figure 4H).

Such structures were observed not only liberated into the medium together with the spore but also inside the vegetative cells (Figure 4I and J) demonstrating the intra-cellular origin of these inclusions.

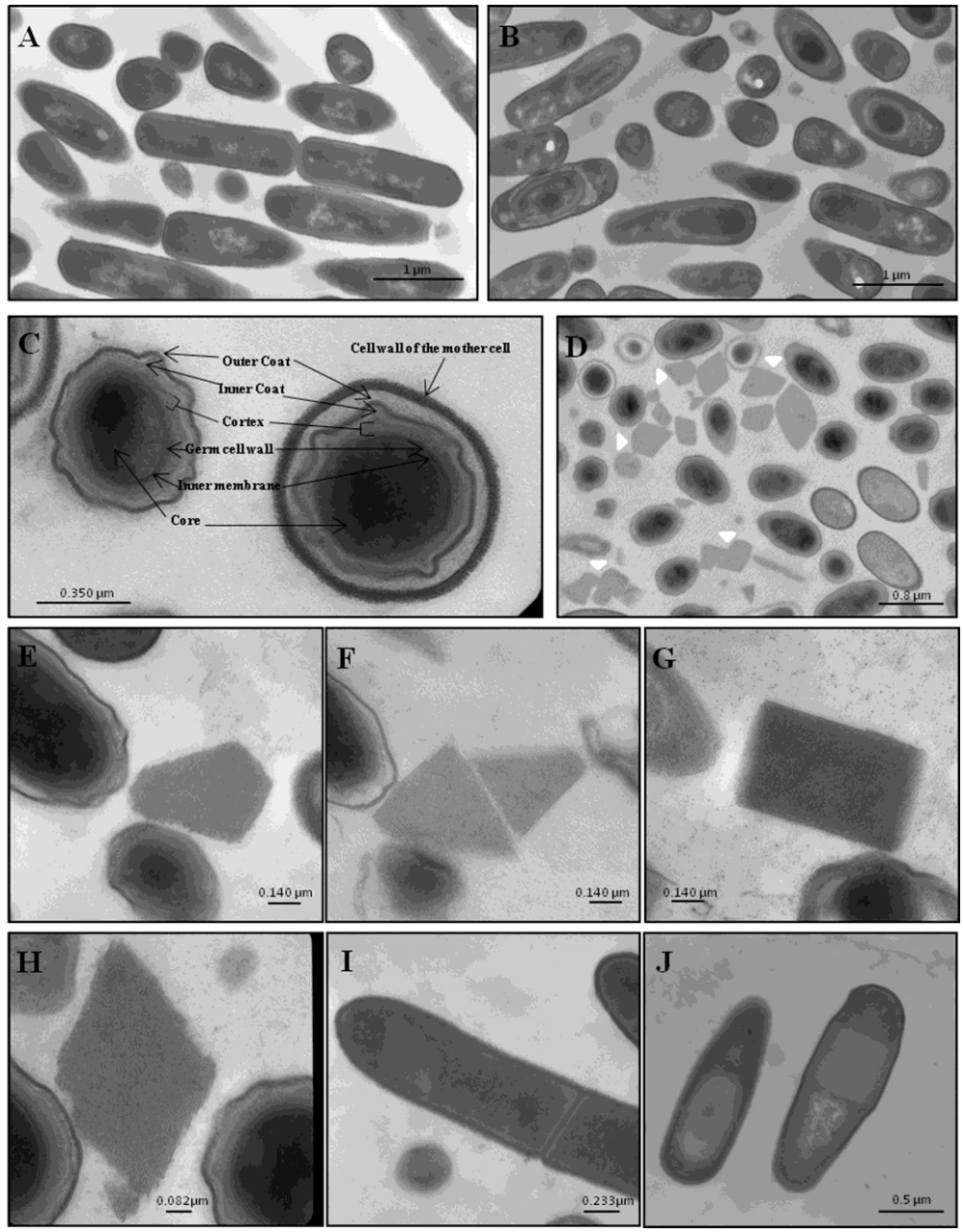

Figure 4. Transmission electron microscopy of a *B. pumilus* 15.1 culture along time. Panel A: *B. pumilus* 15.1 vegetative cells in a 6 h culture. Panel B: mother cells in a 29 h culture with a prespore inside. Panel C: Details of the spore. Panel D: spores and parasporal structures (indicated with white triangles) in a 44 h culture. Panel E, F and G: Crystals observed with different sizes and shapes. Panel H: Details of a crystal showing a highly organized grid pattern. Panel I and J: Details of crystals forming inside of vegetative cells.

Discussion

In this work we report some of the main observations made whilst characterizing *B. pumilus* 15.1, a novel entomopathogenic strain active against *C. capitata* larvae, previously isolated by our group (Molina *et al.* 2010) that could represent an important biotechnological tool for the control of the Mediterranean fruit fly. As *B. pumilus* 15.1 is a natural isolate, little is known about it, so basic research and characterization was needed in order to identify some of the most relevant characteristics of the strain. We isolated the strain *B. pumilus* 15.1 from a partially decomposed common reed plant and demonstrated its insecticidal potency towards *C. capitata* larvae in very particular conditions (Molina *et al.* 2010). Given the strain was taxonomically classified by 16S rRNA sequencing as a *B. pumilus* strain, not a very frequent enthomopathogenic bacterium, we found *B. pumilus* 15.1 extremely interesting from a scientific point of view. Hence, we set about to gain a deeper knowledge of this completely uncharacterized strain.

Our first approach in the characterization of this strain was to determine potential compounds that could be used by *B. pumilus* 15.1 as carbon or nitrogen sources. To study this, we used the BIOLOG® GP2 microplate system and conclude that *B. pumilus* 15.1 was able to metabolize 24 compounds, most of them carbohydrates and possessed the greatest metabolic ability amongst the tested strains. The differences observed in the metabolic profiles of the three *B. pumilus* strains compared could be explained by the different habitats they were isolated from (15.1 strain from a partially decomposed common reed plant, and M1 and M2 from the sea).

The results of the metabolic profile characterization of *B. pumilus* 15.1 will be of great interest in culture media optimization or in future studies of virulence characterization given that many of the virulence factor expressing genes (and antibiotic resistance) are greatly affected by the presence of other compounds in the culture media (Gorke and Stulke 2008; Somerville and Proctor 2009).

The catalase positive *B. pumilus* 15.1 strain was sensitive to most of the antibiotic commonly used in a laboratory. This susceptibility makes the *B. pumilus* 15.1 strain useful as a host for heterologous expression using common plasmids available for *Bacillus* with different antibiotic resistance genes.

We have shown that *B. pumilus* 15.1 was one of the most motile strains tested in our study and that the bacterium probably moves by swimming and swarming mechanisms. Swarming is a strategy developed by microorganisms to ensure their rapid expansion in the natural environment and plays an important role in the colonization of the host in some pathogens. This is the case in model organisms for swarming mechanism such as *Proteus mirabilis*, that invades human urothelial cells (Allison *et al.* 1992) and *Pseudomonas aeruginosa*, an opportunistic pathogen of acute infections (Montie *et al.* 1982). *B. pumilus* 15.1 showed a typical colony morphology and terraced structure or concentric rings (data not shown) described in *P. mirabilis* (Matsuyama and Matsuyama 2001) and other *B. pumilus* strains (Bottone and Peluso 2003).

Bacillus genus is characterized by the production of spores, highly resistant cell forms differentiated when the conditions are adverse for life. Spores are much more resistant to heat, radiation and chemicals than vegetative cells (Nicholson *et al.* 2000; Melly *et al.* 2002). Given this resistance, lots of treatments have been developed for efficient and cost-effective disinfecting and control methods of *Bacillus*. In this sense, we identified a series of biocides, thermal and UV treatments to reduce the viability of *B. pumilus* 15.1 by several orders of magnitude not only of vegetative cells but also of spores (Table 5) but the only chemical insult that was completely able to reduce the number of viable spores under the detection limit (in the time assayed, 30 min) was a solution of 1% bleach.

The most remarkable feature of the *B. pumilus* 15.1 strain from all of the resistance tests performed was its resistance to UV radiation. Resistance to UV of *B. pumilus* spores has been previously reported, but as far we know, this has not been the case with the vegetative cells. The *B. pumilus* SAFR-032 strain, isolated from a spacecraft assembly facility is reported to be one of the most UV resistant spores on earth (Link *et al.* 2004; Newcombe *et al.* 2005). Surprisingly, *B. pumilus* 15.1 vegetative cells remained viable after 10 min of exposure at UV-C (254 nm), a dose much higher than that reported by Newcombe, *et al.*, (Newcombe *et al.* 2005). Whilst *B. pumilus* SAFR-032 showed a 3-log reduction when radiated with 200 J/m^2, *B. pumilus* 15.1 reduced 3.25-log when a 815.5 J/m^2 dose and 5.16-log when a 4,911.1 J/m^2 dose were applied. Furthermore, *B. pumilus* 15.1 spores required a

1,637 J/m^2 dose to kill 93.48% of the population of the spores similar to the reported by Gioia *et al*, (2007) with *B. pumilus* SAR-0.32 spores (1,500 J/m^2 killed 90% of the population). The UV resistance showed by *B. pumilus* 15.1 could represent a huge advantage in biological control programs as one of the main drawbacks of microbial control with living agents is that the decrease of their viability under field conditions due to UV damage (Pusztai *et al.* 1991).

B. pumilus 15.1 formed cell clusters in both vegetative cells and spore stages (Figure 4 C and D). Some of *B. pumilus* 15.1 cells in liquid cultures seem to be encapsulated within a matrix (Figure 4 D) that resembled the extracellular polymeric substance (EPS), typical of some bacterial strains and primarily composed by polysaccharides (Cescutti *et al.* 1999; Dogsa *et al.* 2005). The production of this EPS is essential for biofilm formation and it is normally observed in bacteria grown in static cultures not in liquid cultures as in our case.

Apart from the phenotypic and general characterization of *B. pumilus* 15.1 strain we obtained some insights on possible virulence factors of the 15.1 strain. In general, virulence of an entomopatogenic strain is a complex mechanism not driven by one unique virulence factor but by several that act sequentially or synergically to achieve the objective: to conquer and to colonize the host. A lot of work needs to be done in order to elucidate the complex mechanism of action of *B. pumilus* 15.1, and although this was not the scope of this work, some of the results obtained here allow us to speculate on possible virulence factors.

Several proteases synthesized by entomopathogenic bacteria have been shown to be virulence factors. It is thought they play a significant role in invasion by digesting the host tissue and allowing other virulence factors reach their target. This is the case for serralysin in *Xenorhabdus* (Massaoud *et al.* 2010), the alkaline zinc metalloprotease PrtA in *Photorhabdus* (Bowen *et al.* 2003; Bishop 2014), the zinc metalloprotease InhA in *B. thuringiensis* (Fedhila *et al.* 2002) or the recently described extracellular metalloproteases from *Bacillus cereus* active against *C. capitata* larvae (Ruiu *et al.* 2015). *B. pumilus* 15.1 strain proved to be a good extracellular protease producer, rendering the biggest halo in casein and in skimmed milk test compared to the other bacterial strains in this study as other studies demonstrated before (Yu *et al.* 2014).

B. pumilus 15.1 showed a quite significant hemolysis activity. This could represent another virulence factor of this strain as other erythrocyte-active proteins such us sphaericolysin (Nishiwaki *et al.* 2007) from *Lysinibacillus sphaericus* (formerly known as *Bacillus sphaericus*) or alveolysin from *Paenibacillus alvei* (formerly known as *Bacillus alvei*), proteins similar to well known non-entomopatogenic toxins such as listeriolysin O (Rouquette and Berche 1996), perfringolysin O, pneumolysin, and streptolysin O [see (Heuck *et al.* 2010) for an extensive review of their mode of action].

The parasporal crystals observed outside of the cells (Figure 4D, E, F, G, and H), inside of mother cells (data not shown) and inside of some vegetative cells (Figure 4I and J) resembled to those produced by the classical entomopathogen *B. thuringiensis* during sporulation (Bechtel and Bulla 1976). The production of inclusion bodies of crystalliferous nature is a common feature in entomopathogenic *Bacillus* species such as *Lysinibacillus sphaericus* (Kalfon *et al.* 1984), *Brevibacillus laterosporus* (Zubasheva *et al.* 2010; Ruiu 2013), *Paenibacillus popilliae* (Weiner 1978) and more than often, they are the main virulence factor responsible for the mortality of the insect. This type of crystal inclusion has even been described in the non-entomopatogenic strain *B. licheniformis* (Yan *et al.* 2007). To our knowledge, this is the first time that a parasporal body of this nature has been reported in a *B. pumilus* strain.

As the parasporal crystals produced by *B. thuringiensis* are the main virulence factor responsible for toxicity against insects, we could also speculate that these inclusions produced by *B. pumilus* 15.1 might represent a virulence factor as well. Crystals in *B. thuringiensis* are mainly composed of Cry and Cyt proteins and in many cases *cry* and *cyt* genes are codified in plasmids. The possibility that a *B. pumilus* strain acquired a plasmid from *B. thuringiensis* could explain why a non-classical entomopathogenic species such as *B. pumilus* showed insecticidal potency against insects. Studies on the nature of the inclusions, their involvement in toxicity and genome characterization of *B. pumilus* 15.1 strain are currently underway in order to prove our hypothesis.

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Chapter II

Identification, sequencing and comparative analysis of pBp15.1S plasmid from the newly described entomopathogen *Bacillus pumilus* 15.1

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Abstract

The *Bacillus pumilus* 15.1 strain, a recently described entomopathogenic strain active against *Ceratitis capitata*, contains at least two extrachromosomal elements, pBp15.1S and pBp15.1B. Given that *B. pumilus* is not a typical entomopathogenic bacterium, the acquisition of this extrachromosomal DNA may explain why *B. pumilus* 15.1 is toxic to an insect. One of the plasmids present in the strain, the pBp15.1S plasmid, was subcloned, sequenced and analyzed using bioinformatics to identify any potential virulence factor. The pBp15.1S plasmid was found to be 7,785 bp in size with a GC content of 35.7% and 11 putative ORFs. A replication module typical of small rolling circle plasmid and a sensing and regulatory system specific for plasmids was found in pBp15.1S. Additionally, we demonstrated the existence of ssDNA in plasmid preparations suggesting that pBp15.1S replicates by the small rolling circle mechanism. A gene cluster present in plasmid pPZZ84 from a distantly isolated *B. pumilus* strain was also present in pBp15.1S. The plasmid copy number of pBp15.1S in exponentially growing *B. pumilus* cells was determined to be 33 copies per chromosome. After an extensive plasmid characterization, no known virulence factor was found so a search in the other extrachromosomal elements of the bacteria is needed.

Introduction

Extrachromosomal DNA is common in the bacteria kingdom. Usually found as covalently closed circular DNA (CCC DNA) or plasmids, it endows the bacteria with additional biochemical capabilities different from those encoded by the chromosome, and sometimes with very special features. Bacterial plasmids, together with bacteriophages and transposons, are considered as the main tools for prokaryotes to evolve (Norman *et al.* 2009) as these elements carry a pool of genes available for different bacterial species.

The occurrence of CCC DNA or plasmids in *B. pumilus* species is not very high. Some reports revealed that only 15% of the studied strains showed some kind of CCC DNA (Lovett and Bramucci 1975b), but others showed that no plasmids were

identified at all (Yoshimura *et al.* 1983). Most plasmids found in *B. pumilus* strains (Table 1) are cryptic and do not determine for any antibiotic marker. There are only a few plasmids that have been extensively studied, but interestingly their presence in the *B. pumilus* strains has been associated with very surprising phenotypes.

Many bacterial virulence factors active against insects are encoded by plasmids. This is the case for the Cry toxins, proteins with insecticidal activity against a wide range of insect orders and synthesized mainly by *Bacillus thuringiensis.* Most of the known Cry toxins show plasmid codification, mainly in megaplamids, such as pBtoxis in *B. thuringiensis* var. *israeliensis* (Berry *et al.* 2002), or pIS56-285, pIS56-107 and pIS56-63 in *B. thuringiensis* var. *thuringiensis* (Murawska *et al.* 2013). In addition, *cry* genes can reside in small plasmids as well, such as pBMBt1 from *B. thuringiensis* subsp. *darmstadiensis* (6,700 bp) (Loeza-Lara *et al.* 2005) or pBMB0228 from *B. thuringiensis* strain YBT-1518 (17,706 pb) (Guo *et al.* 2008).

In the case of the facultative entomopathogenic bacteria *Serratia enthomophila*, responsible for amber disease of the New Zealand grass grub *Costelytra zealandica*, the genes responsible for its virulence, *sep*A, *sep*B and *sep*C (Hurst *et al.* 2000) are also located in the large plasmid pADAP (Glare *et al.* 1993).

Lysinibacillus sphaericus, another entomopathogenic species has been successfully used in biological control programs to reduce populations of mosquito larvae transmitting-diseases such as malaria, filariasis, and arboviral diseases. This bacterium harbours the plasmids (pBsph) that contain the virulence factors, BinA and BinB (apart from the duplicate copy on the chromosome) mainly responsible for the death of the larvae (Hu *et al.* 2008).

A preliminary molecular analysis of the recently described *B. pumilus* 15.1 strain (Molina *et al.* 2010), an entomopathogenic strain active against *C. capitata*, the Mediterranean fruit fly (Medfly), showed the presence of at least two extrachromosomal elements when total DNA is extracted from the strain (Molina 2010). *B. pumilus* is not a classic entomopathogen, therefore we hypothesized that the strain 15.1 may have acquired the capability of being toxic to *C. capitata* larvae due to the uptake of extrachromosomal material from another entomopathogenic strain.

Table 1. Plasmids described in *B. pumilus* strains.

Plasmid name	Size	Sequence access No.	Copies per chromosome	Strain	Reference	Phenotype
p576	$30.4x10^6$ Da / 43.434 kb		2	*B. pumilus* NRS576	(Lovett 1973), (Lovett and Bramucci 1975a), (Singh *et al.* 2010).	Lack of the plasmid increases sporulation frequency. Colony appearance changed[a]
pPL7065	$6.4x10^6$ Da - $4.7x10^6$ Da / 7.607 kb	AY230134.1	10-20	*B. pumilus* ATCC 7065	(Lovett and Burdick 1973) (Lovett *et al.* 1977)	Cryptic killing activity
pMB1	$6.8x10^6$ Da		10 or more	*B. pumilus* ATCC 12140	(Lovett and Bramucci 1975b)	Cryptic
pMB2	$5.3x10^6$ Da		10 or more	*B. pumilus* ATCC 12140	(Lovett and Bramucci 1975b)	Cryptic
pPL10	$4.4x10^6$ Da /7.028 kb	NC_001858	20	*B. pumilus* ATCC 12140 L10 mutant	(Lovett *et al.* 1976)	Killing activity (bacteriocin-like activity)
pMGD302	60 kb		ND	*B. pumilus* 302.41	(Hendrick *et al.* 1991a)	Cryptic
pMGD296	42 kb		ND	*B. pumilus* 296.51	(Hendrick *et al.* 1991a; Hendrick *et al.* 1991b)	Cryptic
pMGD150	40 kb		ND	*B. pumilus* 15006E	(Hendrick *et al.* 1991b)	Cryptic
pSH1452	6.081 kb	U53767.1	ND	*B. pumilus* SH1451	(Hasnain and Thomas 1996)	Salt tolerant phenotype
pPZZ84	6.817 kb	GU144016	46	*B. pumilus* PZZ84	(Zhang *et al.* 2010)	Cryptic

[a] Wild type colonies ($plasmid^+$) were brown-translucent in tryptose-blood agar base or AK sporulation agar media, while spontaneous mutant colonies ($plasmid^-$) were opaque white after 3 days incubation (Lovett and Bramucci 1974).

The fact that there are not many *B. pumilus* strains bearing extrachromosomal DNA and that very few are well characterized prompted us to extensively characterize one of the plasmids found in *B. pumilus* 15.1. Our aim was to reveal if it contains potential genes responsible for its virulence against the Medfly.

Here we report the isolation, cloning, sequencing, comparative analysis and characterization of the small plasmids found in the *B. pumilus* 15.1 strain.

Materials and Methods

Bacterial strains and growth conditions

The bacterial strains used in this study were *B. pumilus* 15.1, *B. pumilus* M1 (Uad *et al.* 2007), and *Escherichia coli* XL1-Blue bearing pUC19 plasmid and *E. coli* DH5α. All the bacterial strains were grown in Luria-Bertani (LB) medium (Scharlau S.L.). *B. pumilus* 15.1 and M1 were grown at 30ºC in a rotary shaker at 240 rpm, whilst *E. coli* XL-1 Blue and DH5α strains were grown at 37ºC and 240 rpm. *E. coli* XL-1 Blue bearing pUC19 required 100 µg/ml of ampicillin when cultured.

DNA manipulation and analysis

Total DNA from *B. pumilus* strains was obtained from a mid-log culture following the protocol described by Reyes-Ramirez and Ibarra (2008). Plasmids pBp15.1S and pUC19 were extracted from *B. pumilus* 15.1 and *E. coli* XL-1 Blue, respectively, using QIAprep® Spin Miniprep Kit (Qiagen) according to the manufacturer's instructions with some modifications. When plasmid was obtained from *B. pumilus* 15.1, cells were re-suspended in TES buffer (30 mM Tris base, 5 mM EDTA, 50 mM NaCl; pH 8.0), supplemented with 20% sucrose, 2 mg/ml lysozyme and 1 µl/ml RNAse right before use instead of using P1 buffer provided by the kit. Cell suspension was incubated for 30 min at 37ºC in order to allow an extensive cell wall digestion before before adding P2 buffer (provided by the kit). Total DNA and plasmid concentration was determined spectrophotometrically in a NanoDrop® Spectrophotometer ND-1000.

One microgram of pBp15.1S was individually digested with *Sma*I, *Hin*dIII, *Xba*I, *Eco*RI, *Bam*HI, *Bgl*II and *Sal*I the enzymes from New England Biolabs according to the manufacturer's instructions. DNA samples were analyzed on 0.8% (wt/vol) agarose gels made in TBE buffer (for total DNA extractions) or TAE buffer (for DNA digestions), electrophoresed and visualized on a UVP BioDoc-It™ Imaging System.

DNA cloning and sequencing

*Hin*dIII or *Eco*RI-digested pBp15.1S DNA was ligated to *Hin*dIII or *Eco*RI-digested pUC19 after enzyme inactivation using T4 DNA Ligase (Invitrogen) according to the manufacturer's instructions. Chemically *E. coli* DH5α competent cells were transformed with ligation reactions by heat shock and white colonies were screened by the Toothpick Plasmid Assay (Sambrook *et al.* 1989) in a 1% agarose gel (in TAE buffer) to identify bacterial colonies containing plasmids larger than pUC19. Selected clones were kept for subsequent plasmid extraction and digestion with *Hin*dIII or *Eco*RI to verify the size of the inserted fragment. Both strands of each selected plasmid were sequenced using forward and reverse M13 universal primers (M13F: 5'TGTAAAACGACGGCCAGT3' and M13R: 5'CAGGAAACAGCTATGACC3') in a four capillary automatic sequencer (Applied Biosystems/Hitachi, model 3130). When forward and reverse sequences did not overlap, primer-walking technique was carried out. DNA sequences were assembled using the Lasergene software package from DNASTAR, Inc. and complete sequence of the pBp15.1S plasmid was determined.

Bioinformatic analyses

The pBp15.1S DNA sequence was analyzed with different bioinformatic tools. Open reading frames (ORFs) were deduced and annotated by using NCBI Glimmer v3.02 (Salzberg *et al.* 1998; Delcher *et al.* 1999), GeneMark.hmm PROKARYOTIC v2.8 (Besemer *et al.* 2001) and ORF finder at NCBI (Wheeler *et al.* 2003). The ORF sequences predicted by these tools were BLASTed using the blastn and blastp algorithms with default parameters against the GenBank database (Date: 10/07/2015). In order to obtain information about their functionality the ORF sequences were used as queries against the protein family databases STRING v9.1 (Franceschini *et al.* 2013) and CATH v4.0 (Sillitoe *et al.* 2013), the protein domain databases ScanProsite (de Castro *et al.* 2006), Pfam v27.0 (Finn *et al.* 2014), InterPro (Hunter *et al.* 2012; Jones *et al.* 2014) and CDD (Marchler-Bauer *et al.* 2013). They were also analyzed with Blast2GO (Conesa *et al.* 2005; Conesa and Gotz 2008; Gotz *et al.* 2008; Gotz *et al.* 2011) in order to obtain possible functional or location information. The results were later manually inspected to annotate and

predict the biological function of these ORFs. The selected ORFs were also analyzed for signal peptide sequences with SignalP v4.0 (Petersen *et al.* 2011). Moreover, the plasmid was screened for the presence of direct and inverted repeat sequences using GeneQuest from DNASTAR® Lasergene v11. The remaining non-annotated plasmid sequences were compared against the Rfam database v11.0 (Burge *et al.* 2013) to determine the presence of non-coding RNAs and ribo-switches. The presence of non-coding RNAs and the secondary structure of the plasmid was also checked looking for Rho-independent terminators with TransTermHP v2.08 (Kingsford *et al.* 2007). SnapGene® Viewer 2.4.3 software was used for drawing purposes. The sequence alignments created in the annotation process were made with the MegAlign of DNASTAR® and Clustal W v2.0 programmes (Larkin *et al.* 2007).

Plasmid copy number determination

Plasmid copy number was determined by quantitative real time PCR as previously described (Lee *et al.* 2006; Skulj *et al.* 2008; Zhong *et al.* 2011). Based on the genome sequence of *B. pumilus* SAFR-032, obtained from the public database of the NCBI (GenBank: CP000813.1), the *smc* gene was selected for being a single copy on the chromosome (Hirano 1998; Bartosik and Jagura-Burdzy 2005) and chosen as a reference in the q-PCR. Primers sequences used are shown in Table 2. Primer design was performed using Primer3web version 4.0.0 following BIO-RAD's instructions for the SsoFast™ EvaGreen® Supermixes.

Table 2. Primers used for plasmid copy number (PCN) determination in real-time q-PCR assays.

Primer name	Sequence 5'- 3'	Product size (bp)	Target
smc_F	GCTGAAAATCTCGCTTGCCA	177	Chromosome
smc_R	TCTTCCAGTTGTTCGGCTCC		Chromosome
orf7_F	GCCGGTCAGAAATTCATAGCTG	199	pBp15.1S
orf7_R	TAGCAACCACTCGAGTTCCAC		pBp15.1S

Chromosomal and plasmid amplicons were individually prepared by PCR using 300 ng of total bacterial DNA, 10 µl of 10X PCR buffer, 8 µl of $MgCl_2$ (25 mM), 2

µl of deoxynucleoside triphosphates (dNTPs) (10 mM each), 3.3 µl of F_primer and R_primer (10 µM each), 0.5 µl of *Taq* polymerase (5 U/µl), and enough MiliQ water so that the final volume of the mixture was 100 µl. PCR mixtures were denatured at 95ºC for 5 min, followed by 30 cycles at 95ºC for 30 s, 55ºC for 30 s, and 72ºC for 30 s and then a final extension at 72ºC for 5 min. Amplification was checked by electrophoresis on a 0.8% (wt/vol) agarose gel in TAE buffer. PCR products were cleaned using a QIAquick® PCR Purification kit (Qiagen) and concentration was determined spectrophotometrically. These PCR products were used to construct the standard curves by making five serial dilutions of both plasmid and genomic amplicons. For determining the plasmid copy number, 50 ng of total DNA of *B. pumilus* 15.1 from different extractions were placed as unknown samples in the reaction. All reactions were done in triplicate. Real-Time q-PCR reactions were performed in 10 µl mixture containing 1X SsoFast™ EvaGreen® Supermix (Bio-Rad), 300 nM of each primer, and DNA template. Real-Time q-PCR was performed in a Bio-Rad CFX96™ Real-Time System with the following cycling conditions: 98ºC for 2 min, followed by 39 cycles at 98ºC for 5 s and 55ºC for 10 s. SYBR Green fluorescent emission was measured at the end of the elongation step. Subsequently, a melting curve programme was applied with a continuous fluorescent measurement starting at 65ºC and ending at 95ºC (ramping rate of 0.5ºC/s). Cycle threshold (Ct) and concentration (Co) were determined automatically and the standard curve was plotted using Bio-Rad CFX Manager 3.0 software. The plasmid and genomic quantity was obtained interpolating data of unknown DNA samples into the standard curves. Plasmid copy number was calculated using the equation described by Lee *et al.* (2006).

Detection of ssDNA by PCR

To detect ssDNA we followed the method described by (Booth *et al.* 2001) with slight modifications. Twenty nanograms of pBp15.1 were used as a template in an extension reaction from primer ssDNA2 (5' TTTTATGGTATCTATCATTGTATCCACCGCC 3'), designed on the plasmid sequence (underlined). The same amount of pBp15.1 DNA was treated with S1 nuclease (Thermo Fisher Scientific) following the manufacturer's instructions. To

create an ssDNA control, 20 ng of pBp15.1 DNA were boiled at 95ºC for 5 min and cooled rapidly on ice. The three samples were added to 20 µl of an extension mix containing dNTPs (250 µM), primer ssDNA2 (330 nM), PCR buffer (1X), $MgCl_2$ (2 mM), and *Taq* Polymerase from EMBL (0.5 U/µl) and incubated at 40ºC for 5 min, and then up to 72ºC with a temperature ramp of 0.1ºC/s in a Bio-Rad CFX96™ thermocycler. Two microlitres of the extension reaction were mixed with 18 µl of a PCR master mix containing dNTPs (250 µM), primer ssDNA1 (330 nM) (5' TTTTATGGTATCTAT 3'), primer orf7_F (330 nM) (Table 2), PCR buffer (1X), $MgCl_2$ (2 mM), and *Taq* Polymerase from EMBL (0.5 U/µl). PCR was performed using a denaturing cycle at 95ºC for 5 min, 30 cycles of amplification (95ºC for 30 s, 40ºC for 30 s, 72ºC for 1 min) and a final cycle for extension at 72ºC for 5 min. After that, 5 µl of each sample were analyzed on a 0.8% agarose gel in TAE 1X containing SYBR Safe.

Nucleotide sequence accession number

The sequence of pBp15.1S from *B. pumilus* 15.1 determined in this study was deposited at the GenBank database under the accession No. KM348008.

Results and Discussion

Plasmid detection, pBp15.1S sub-cloning and sequencing

When a total DNA extraction from *B. pumilus* 15.1 was analyzed by agarose electrophoresis at least two extrachromosomal DNA elements were detected (Figure 1A lane 1), one of an apparent size of approximately 7 kb and other with an apparent size bigger than the band corresponding to the bacterial chromosome. The *B. pumilus* M1 strain was used as a control to identify the chromosomal DNA size (Figure 1A, lane 2). We concluded that the strain *B. pumilus* 15.1 contained at least one plasmid (named pBp15.1S) and one megaplasmid (named pBp15.1B) in its cytoplasm. Here we are focusing on the characterization of the pBp15.1S plasmid.

When pBp15.1S was extracted and separated from the megaplasmid pBp15.1B (by using a plasmid extraction kit with size limitation) and digested with 7 restriction enzymes (*Sma*I, *Hin*dIII, *Xba*I, *Eco*RI, *Bam*HI, *Bgl*II and *Sal*I) (data not

shown) only *Hin*dIII and *Eco*RI rendered fragments smaller than 7 kb. *Hin*dIII digestion rendered 2 fragments of approximately 2.5 and 1.5 kb respectively (Figure 1B) whilst *Eco*RI digestion rendered 3 fragments of 4, 2.5 and 1.1 kb respectively (Figure 1C). Given that the size of the plasmid was around 7 kb, it was postulated the existence of either a large number of small *Hin*dIII fragments or that there were two DNA fragments (possibly the 2.5 fragment) showing a similar size that was not resolved in a 0.8% agarose gel.

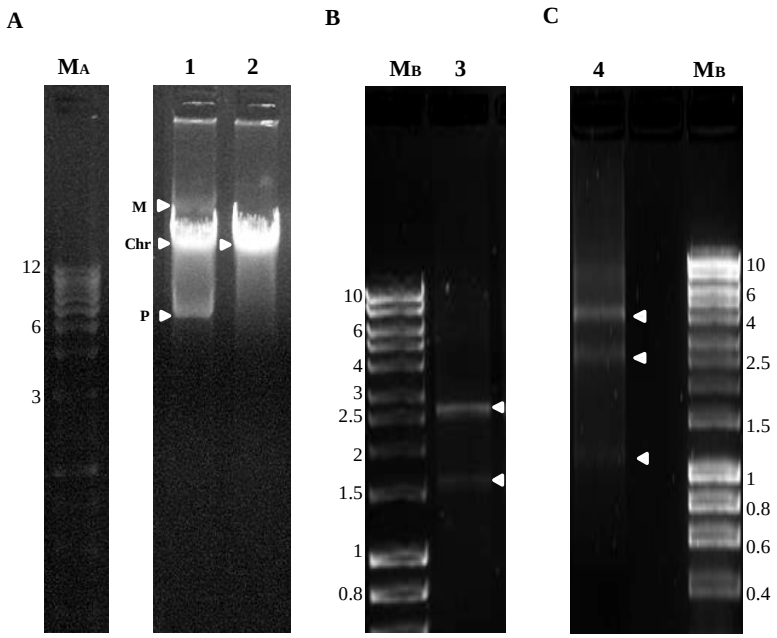

Figure 1. DNA electrophoresis in 0.8% agarose gel. (A) Total DNA extraction from *B. pumilus* 15.1 (lane 1) and *B. pumilus* M1 (lane 2); M_A shows a molecular weight marker (250 bp-12 kb from Stratagene). *Hin*dIII (B) and *Eco*RI (C) digestion of the pBp15.1S plasmid. M_B shows a molecular weight marker (HyperLadder I from Bioline) in kb.

*Eco*RI and *Hin*dIII fragments were cloned into pUC19 plasmid and positive transformants were screened, isolated and sequenced. The name of the plasmids obtained, the exact size of the fragments inserted and the position of the restriction sites at the complete sequence of the pBp15.1S plasmid are detailed in Table 3.

Table 3. Recombinant plasmids obtained from pBp15.1S *Eco*RI and *Hin*dIII digestion, insert size and position of restriction sites at the complete sequence.

Digestion	Recombinant plasmid names	Insert size (bp)	Position at the complete sequence
*Eco*RI	pDG_7	4084	6165-2463
	pDG_4	2588	2464-5051
	pDG_3	1113	5052-6164
*Hin*dIII	pDG_5	2615	7058-1887
	pDG_2	2564	4494-7057
	pDG_6	1583	1888-3470
	pDG_1	379	4115-4493

Sequence assembling and comparative analysis of the pBp15.1S plasmid

The sequence of each clone was assembled with the help of Lasergene software from DNASTAR and ClustalW algorithm and the obtained complete sequence of pBp15.1S was submitted to the GenBank. Sequence analysis showed that the plasmid pBp15.1S is a circular molecule with 7,785 bp in length and with a G+C content of 35.7%. A restriction enzyme analysis using SeqBuilder of DNASTAR showed that *Xba*I and *Bgl*II enzymes have a unique cleavage site on pBp15.1S, *Eco*RI have 3 sites, *Hin*dIII have 6 sites and that *Sma*I, *Bam*HI, and *Sal*I do not have the specific cleavage site (Figure 2), confirming our previous experimental results on DNA digestion.

ORF analysis

Analysis using several algorithms and approaches showed that pBp15.1S plasmid had 30 potential ORFs, but only 11 showed similarities to known proteins from the NCBI non-redundant microbial protein database (Table 4, Figure 2).

ORF1 showed 94% identity with the Rep protein from *Marinomonas* sp. D104, involved in the initiation of replication. The search against Pfam revealed a strong match between ORF1 and the Rep_1 family (PF01446) and annotation predicted with Bast2GO for this ORF was DNA replication as a biological process, DNA binding as a molecular function and as a cellular component extrachromosomal circular DNA. The putative protein encoded by ORF1 showed the three conserved

motifs characteristic of RCR (rolling circle replication) Rep proteins described by Ilyina and Koonin (1992) and one motif described by Yang and McFadden (1993). Upstream of the *rep* gene, a non-coding region (spanning from 298-354 bp) known as double strand origin (*dso*) or plus-origin was identified. This region was similar to the approximately 58 bp *dso* region of the *B. subtilis* plasmid pC194 described by Gros *et al.* (1987) and similar to the *dso* region from other *B. subtilis* (and related species) plasmids with small rolling circle replication mechanisms (Guglielmetti *et al.* 2007). In this region the conserved nick-site sequence 5'TCTTG*ATAC3' was identified between position 308 and 316. The nick-site (*) was 160 bp upstream of the start codon of the Rep protein. The *dso* region and the *rep* gene observed are predicted to constitute the replication module of the pBp15.1S plasmid.

Table 4. Comparative analysis of ORFs found in plasmid pBp15.1S from *B. pumilus* 15.1.

ORF name	Strand	Size (bp)	(Start - stop)	Size (aa)	Best BLAST match [Comments]	Accession No.	Identity[a] (%)	No. positive/No. examined[b] (%)	E-value[c]
ORF1	+	1023	473 - 1495	340	Rep protein, *Marinomonas* sp. D104 [pfam01446; gene = "rep40" /note="involved in initiation of replication"]	ETI59042.1	320/340 (94%)	334/340 (98%)	0
					Rep protein, *Bacillus subtilis* IAM1232 plasmid pTA1040	AAC44406.1	317/338 (94%)	328/338 (97%)	0
					Rep, *Bacillus pumilus* SH1451 plasmid pSH1452	AAB71488.1	311/341 (91%)	329/341 (96%)	0
ORF2	-	606	1533-2138	201	Product unknown, *Bacillus pumilus* ATCC 12140 plasmid pPL10 [name: Orf5, putative exported protein]	AAB91479.1	185/201 (92%)	189/201 (94%)	8E-122
ORF3	+	180	2226-2405	59	Hypothetical protein, *Bacillus* sp. SB47.	WP_026579015	52/59 (88%)	55/59 (93%)	2E-26
					Hypothetical protein, *Bacillus sonorensis*.	WP_029419235	33/59 (56%)	44/59 (74%)	1E-14
ORF4	-	372	2644-3015	123	Hypothetical protein IAU_04559, *Bacillus cereus* IS075	EJP86132.1	36/93 (39%)	50/93 (54%)	2E-09
					Hypothetical protein Bsph_1099, *Lysinibacillus sphaericus* C3-41.	YP_001696839	35/89 (39%)	47/89 (52%)	1E-08
ORF5	-	183	3095-3277	60	Hypothetical protein BAT_3812, *Bacillus pumilus* ATCC 7061	EDW20251.1	20/28 (71%)	22/28 (79%)	2E-04

ORF name	Strand	Size (bp)	(Start - stop)	Size (aa)	Best BLAST match [Comments]	Accession No.	Identity[a] (%)	No. positive/No. examined[b] (%)	E-value[c]
ORF6	-	225	3338-3562	74	Hypothetical protein, *Bacillus pumilus*	WP_017358598.1	41/66 (62%)	54/66 (82%)	1E-19
ORF7	+	624	3642-4265	207	Hypothetical protein, *Bacillus pumilus* ZZ84 plasmid pPZZ84 [note="ZZ1"]	ACZ28698.1	199/207 (96%)	204/207 (99%)	2E-142
ORF8	-	513	4388-4900	170	ZZ2, *Bacillus pumilus* ZZ84 plasmid pPZZ84	ACZ28699.1	155/170 (91%)	162/170 (95%)	4E-106
					ORF4, *Bacillus subtilis* plasmid pPOD2000	AAA99154.1	100/104 (96%)	102/104 (98%)	2E-65
ORF9	+	225	5686-5910	74	ZZ4, *Bacillus pumilus* ZZ84 plasmid pPZZ84	ACZ28701.1	62/74 (84%)	63/74 (85%)	1E-28
ORF10	+	1119	6291-7409	372	RapA, *Bacillus subtilis*, plasmid pPOD2000	AAA99153.1	372/372 (100%)	372/372 (100%)	0
					RapA, *Bacillus pumilus*, ATCC 7065 plasmid pPL7065 [response regulator aspartate phosphatase]	AAO72995.1	364/372 (98%)	368/372 (99%)	0
ORF11	+	117	7399-7515	38	RapAB, *Bacillus subtilis*, plasmid pPOD2000	AAA99152.1	38/38 (100%)	38/38 (100%)	7E-17

[a] Identity is the degree of correspondence between two sequences. Number of amino acid identities/Number of amino acids compared.

[b] Positive scores indicate substitutions that occur frequently/Number of amino acids examined.

[c] E-value represents the number of different alignments with scores equivalent to or better than score, that are expected to occur in a database search by chance.

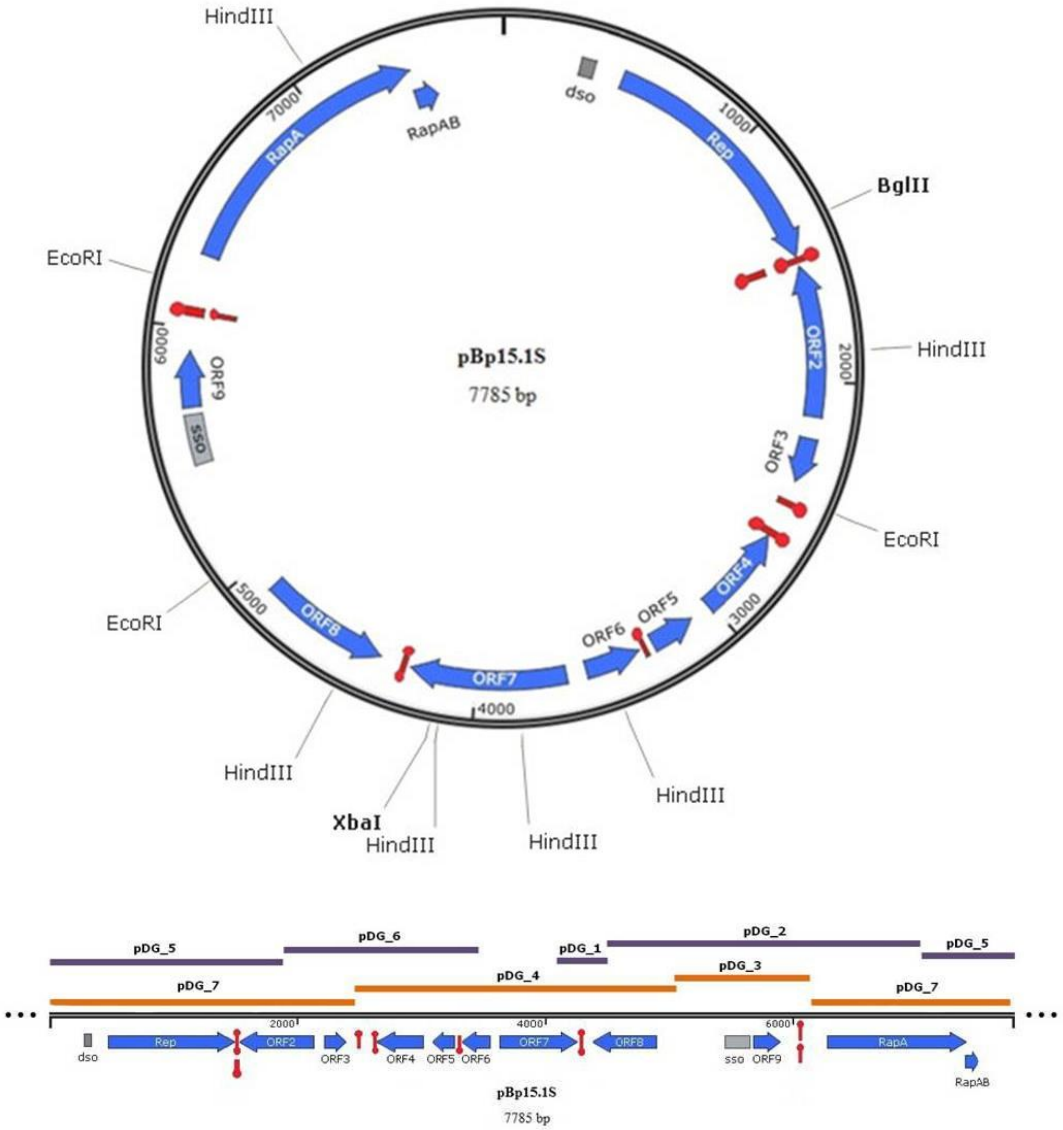

Figure 2. Circular map of pBp15.1S from *B. pumilus* strain 15.1. The positions of the eleven ORFs are shown as arrows including Rep, RapA and RapAB ORFs. The positions of the putative *dso* and *sso* regions are indicated by boxes and ITs are indicated by red hairpins. Inserts present in pDGi plasmids are detailed as well in a linear map.

ORF2 showed identity with a hypothetical protein with an unknown function that it is highly conserved in plasmids from *B. subtilis* and *B. pumilus* strains. Using the SignalP algorithm it was shown that ORF2 contained a predicted signal peptide at the N terminal end and a cleavage site between position 34 and 35.

The ORF3, ORF4, ORF5 and ORF6 showed similarity with different hypothetical proteins from the *Bacillus* genus. The SignalP analysis showed that only ORF4 had a predicted signal peptide and a cleavage site between the amino acid 26 and 27.

ORF7, ORF8 and ORF9 showed a high identity with the hypothetical proteins ZZ1 (96%), ZZ2 (91%) and ZZ4 (84%) from the plasmid pPZZ84 of *B. pumilus* ZZ84 strain. Apart from the identity, ORF7, ORF8 and ORF9 showed the same gene microsynteny and orientation as ZZ1, ZZ2, and ZZ4. ORF7 contained a predicted signal peptide and a cleavage site between the position 30 and 31 of the hypothetical protein. ORF8 showed two relevant protein domains, the MerR_1 domain (position 9-76) and the DUF3967 domain (position 135-169). MerR_1 or MerR-type HTH family (PF13411) is a transcriptional regulator with helix-turn-helix DNA binding regions at the N-terminal end. Most MerR-type transcriptional regulators respond to environmental stimuli, such as heavy metals, oxidative stress or antibiotics (Brown *et al.* 2003). DUF3967 (PF13152) is a protein family of unknown function frequently found in the Firmicutes phylum.

Even in the inter-genetic regions between ORF7, ORF8 and ORF9 were similar to those present in plasmid pPZZ84. The gene cluster in pPZZ84 comprises four ORFs (ZZ1-ZZ4) whilst in plasmid pBp15.1S only comprises three (ORF7-ORF9). A detailed analysis and comparison of the sequences of both plasmids showed that ZZ3 ORF was almost completely deleted from the cluster present in plasmid pBp15.1S (and hence not detected as a putative ORF).

The last two ORFs found in the plasmid, ORF10 and ORF11 showed an overlapping distribution. Thus, the putative N-terminal end of the ORF11 begins at the last codons of ORF10. An analysis of ORF10 and ORF11 predicted the presence of a signal peptide and a cleavage site between position 23 and 24 in ORF11. ORF10 and ORF11 showed high identity with RapA and RapAB proteins from the *B. subtilis* pPOD2000 plasmid (100% identity) and *B. pumilus* pPL7065 plasmid (98% identity). Although the RapAB is not annotated as such in plasmid pPL7065, it contains a region 97% identical to RapAB from pPOD2000.

A Pfam analysis of ORF10 showed significant alignments at positions 178-247 and 256-280 with TPR_12 (PF13424) and TPR_1 (PF00515) family. The TPR

family is characterized by the presence of tetratricopeptide repeats, a 34 aa structural motifs present in proteins from diverse organisms and in a variety of subcellular locations. Proteins with this motif are involved in functions such as cell cycle control, transcriptional control, mitochondrial and peroxisomal protein transport, neurogenesis and protein folding (Lamb *et al.* 1995; D'Andrea and Regan 2003). The gene ontology analysis assigned a hydrolase activity in ORF10 as a molecular function.

RapA belongs to the family of Rap proteins, response regulator aspartyl-phosphate phosphatases able to directly (Core and Perego 2003; Ogura *et al.* 2003) or indirectly (Perego *et al.* 1994; Jiang *et al.* 2000) inhibit the transcriptional factors involved in important cellular process such as sporulation, bacterial competence, or the production of enzymes or antibiotics. For example, RapA phosphatase prevents sporulation in *B. subtilis* by dephosphorilation of the SpoOF-P factor, and Rap I controls the excision of integrative and conjugative elements (ICEs) from the chromosome (Auchtung *et al.* 2005). The phosphatase activity of RapA is regulated by a small protein, PhrA, encoded by a downstream overlapping ORF (Perego and Hoch 1996). This overlap is a very frequent organization among the Rap proteins. More specifically, PhrA is synthetized as a 44 aa precursor that is processed by a series of proteolytic events resulting in the active pentapeptide ARNQT (Perego 1997). This pentapeptide specifically inhibits the phosphatase activity of RapA.

RapA proteins have been previously described to be present in the genome of *B. pumilus* SAFR-032 (No. access CP000813.1). This strain contains four RapA proteins, and a RapH, RapD1, RapD2, RapE and RapK belonging to the same family. From these 9 proteins only RapA1, RapA2 and RapH showed the two-protein system Rap-Phr. These three chromosomal Rap-Phr members showed some identity with the plasmidic ORF10 [40% with RapA1 (ABV61825.1), 40% with RapA2 (ABV61893.1), and 37.9% with RapH (ABV61816.1)] but very low identity with ORF11 [23.68% with PhrA1 (ABV61826.1), 10.53% with PhrA2 (ABV61894.1) and 10.53% PhrH (ABV61817.1)].

Taking into account all of the above-mentioned facts, we postulate that ORF10 and ORF11 from pBp15.1S may represent a sensing and regulatory system specific for the plasmid, different from those chromosomally encoded.

Transcription terminator, secondary structure and non-coding RNA analysis

TransTermHP v2.08 was used for predicting intrinsic terminators (ITs) in pBp15.1S sequence. Eleven putative ITs with high confidence value (80-100) were found in five out of the eleven ORFs predicted in the plasmid (Figure 2). All, except the one between ORF5 and ORF6, were predicted to be at the 3' end of a putative ORF. This terminator was located 28 nt upstream of the ORF5 start codon, probably in the promoter region.

An analysis of the pBp15.1S sequence with DNASTAR software searching for direct and inverted repeats was also performed (data not shown). A region rich in inverted repeats was localized between position 5459 and 5657, showing 5 inverted repeats that may generate stem-loop structures. This region was almost identical (93% identity) to the annotated *sso* region (single strand origin) of the *B. subtilis* plasmid pTA1040 (ID No. U32378.1). Conserved motifs typical of *ssoT* type were identified (Figure 3) (Seery and Devine 1993), so it is highly probable that this region may represent the *sso* region, where host polymerases start the conversion of the ssDNA into dsDNA (synthesis of the lagging strand). Accordingly to their sequence, *sso* regions can be classified into groups and plasmids pPL7065, pPOD2000, pPL10, pTA1040, and pSH1452 belonging to the *ssoT2* subgroup (Guglielmetti *et al.* 2007), so given its high identity pBp15.1S could also be classified as a member of this subgroup.

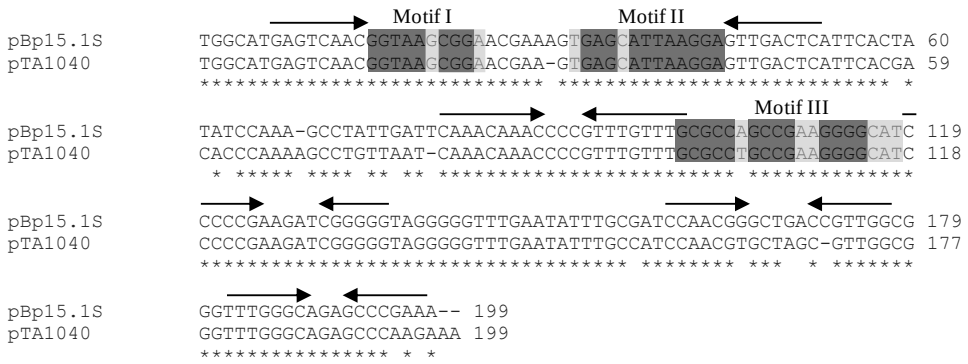

Figure 3. Clustal alignment of the 5459-5657 region of pBp15.1S and the annotated *sso* region of the *B. subtilis* pTA1040 plasmid. The figure shows the inverted repeats detected in pBp15.1S sequence as arrows. Conserved motifs in *ssoT* are shaded, and *ssoT2* subclass is light shaded.

The sequence of pBp15.1S was analyzed using Rfam for the search of relevant non-coding RNAs. The *in silico* analysis showed a region of the plasmid (between 5464–5520 positions) with a high score (E-value 1.9e-10) with the *Bacillus*-plasmid RNA family (RF01691). pBp15.1S has this RNA-motif inside the predicted *sso* region, as well as other *B. pumilus* plasmids such as pPL10, pPZZ84, pL7065, pSH1452 and pMMH1 and other plasmids from *B. cereus*, *B. thuringiensis* or *B. subtilis*. Although the function of the RNA *Bacillus*-plasmid motif is unknown, its secondary structure suggests a cis-antisense RNA that might regulate plasmid copy number (Weinberg *et al.* 2010) or a RNA that acts as a primer to promote the lagging strand replication of the plasmid (Guglielmetti *et al.* 2007).

Determination of the copy number of pBp15.1S

In order to determine the copy number of plasmid pBp15.1S present in *B. pumilus* 15.1 cells at the exponential growth phase, a quantitative real time PCR method was used (Lee *et al.* 2006). For quantification purposes, the chromosomal *smc* gene was used as a reference of a single copy gene and a region of 199 bp was used for plasmid detection. Five-fold serial dilutions of PCR-amplified DNA (0.05-0.000005 ng/µl) were used to construct linear standard curves (r^2>0.99). Based on the data obtained, the copy number of pBp15.1S was calculated to be approximately 33±2 copies per chromosome.

Detection of ssDNA in pBp15.1 S

Data derived from the *in silico* analysis of the pBp15.1S sequence showed a predicted *dso* region and a *rep* gene, essential elements for the replication of plasmids with a Rolling Circle Replication mechanism. In order to demonstrate that pBp15.1S follows a RCR mechanism we tried to demonstrate the presence of ssDNA intermediates. For that, an extension from primer ssDNA2, followed by a PCR with primers orf7 and ssDNA1 was made using a preparation of pBp15.1S and a S1 treated pBp15.1S as templates. As a positive control, a boiled and cooled on ice preparation of pBp15.1S was performed in parallel. The results show (Figure 4) that ssDNA was present in the pBp15.1S preparation (lane 3) while it was not detected

when pBp15.1S was treated with S1 enzyme (lane 4), suggesting that the replication mechanism for pBp15.1S was a RCR mechanism.

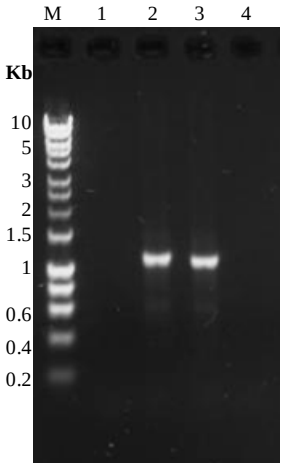

Figure 4. PCR products obtained with primers orf7 and ssDNA1 using molecules created by primer extensions from ssDNA2 as templates. Lane 1 contained no template (PCR negative control). In lane 2 template was obtained by extension from a boiled and cooled on ice pBp15.1S preparation (positive control). In lane 3 template was obtained by extension from a pBp15.1S preparation and in lane 4 from a S1 treated pBp15.1S preparation. M shows Molecular weight marker in kb (HyperLadder I from Bioline).

Conclusions

The plasmid pBp15.1S from *B. pumilus* 15.1 is a 7,785 bp circular covalently closed molecule, with approximately 33 copies per chromosome that codified for 11 relevant ORFs, with only 3 of them being similar to known proteins. ORF1 was homologous to the Rep protein, described as involved in plasmid replication and ORF 10 and 11 were homologous to the RapA and RapAB proteins, belonging to the Rap protein family and involved in the regulation of many important cells processes. The remaining ORFs were highly similar to other plasmid proteins but no known function could be identified for them.

A conserved region for the initiation of replication, the double strand origin (or *dso* region), and for the initiation of the conversion of the ssDNA to dsDNA, the single strand region (or *sso*), was identified by sequence comparison and by

secondary structure analysis. In addition, the existence of ssDNA was demonstrated experimentally, suggesting that pBp15.1S plasmid has a small rolling cycle (SRC) replication mechanisms, as many other plasmids from Gram-positive bacteria (Khan 2005).

Apart from ORF1, ORF10 and ORF11, the rest of the ORFs present in pBp15.1S have a so far unidentified function. However, a group of three ORFs (ORF7-ORF9) showed similarity with plasmid pPZZ84 from the *B. pumilus* strain ZZ84 (Zhang *et al.* 2010), The fact that this gene cluster has been found in two plasmids isolated from very distantly isolated *B. pumilus* strains may indicate that the genes from the cluster are related in their function.

The analysis of the pBp15.1S sequence did not show similarity with any virulence factors known either in bacteria or any other organisms, so for the moment it is difficult to relate the presence of the plasmid in the strain *B. pumilus* 15.1 with its toxicity against *C. capitata*, unless any of the putative ORFs with unknown functions codify for any virulence factor not yet described. However, four out of the 11 ORFs found in pBp15.1S (ORF2, ORF4, ORF7, and ORF11) were predicted to show a signal peptide and a processing site, so they could be considered as secreted proteins. As many virulence factors are secreted outside the cell, we still cannot rule out the possibility that these proteins are related with toxicity. This hypothesis is currently being experimentally ratified.

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Chapter III

Characterization of the parasporal crystals produced by *Bacillus pumilus* 15.1

Garcia-Ramon, D.C., Berry, C. and Vilchez, S., "Characterization of the parasporal crystals produced by *Bacillus pumilus* 15.1" (Paper under preparation).

Abstract

The insecticidal crystal proteins (Cry proteins) are the most studied virulence factors produced by entomopathogenic bacteria from *Bacillus* genus. In this work, the crystals produced by *Bacillus pumilus* 15.1, similar to the one produced by *B. thuringiensis* were characterized at molecular level. To investigate the codification origin of the crystals, a cured strain (*B. pumilus* 15.1C) was obtained by a novel curing strategy. The parasporal crystals were also observed in the cured strain *B. pumilus* 15.1C, indicating that the crystals have a chromosomal codification. Parasporal crystals were successfully purified from the spores as those isolated from *B. thuringiensis* by a discontinuous sucrose gradient. The crystals were detected by transmission electronic microscopy in the same fractions where an over-expressed 45 kDa protein was present. The characterization of the 45 kDa protein showed that the protein spontaneously solubilizes when crystals are kept at low temperatures and that the protein is resistant to trypsin. The crystals produced by *B. pumilus* 15.1 did not show significant toxicity when bioassayed against *Ceratitis capitata* larvae.

Introduction

Many bacterial toxins are proteins encoded by bacterial chromosome genes, plasmids or phages (Lubran 1988). During the interaction between pathogens and their host, secreted protein toxins play an important role in the specificity or the outcome of the infection (Lemichez and Barbieri 2013). These toxins have different structures and modes of action. Many species of bacteria belonging to the genus *Bacillus* produce entomopathogenic toxins of protein nature. The most studied protein toxins are the Cry and Cyt toxins, produced mainly by *Bacillus thuringiensis* (*Bt*). Cry and Cyt toxins are a group of proteins frequently produced during the sporulation phase that usually form a parasporal crystal, which is responsible for the toxicity on a wide range of insects (Bechtel and Bulla 1976; Bravo *et al.* 2007). The insecticidal activity of parasporal crystal proteins produced by *Bt* has been extensively used as the base of many commercial products. The ability of producing

parasporal crystals is not restricted to *Bt* as some strains of *Lysinibacillus sphaericus* (Jones *et al.* 2007), *Clostridium bifermentans* (Barloy *et al.* 1996), *Paenibacillus popilliae* (Zhang *et al.* 1997), *P. lentimorbus* (Yokoyama *et al.* 2004), also produce parasporal inclusion active against insects.

The mechanisms of action proposed for Cry toxins comprise three major steps: (*i*) solubilization and proteolytic activation of the protoxin in the insect midgut, (*ii*) binding of the activated fragment to midgut receptor/s, and (*iii*) insertion of the toxin into the midgut apical membrane to cause destruction of membrane (Palma *et al.* 2014). Although some aspects of the mechanism of action remains unclear, it is commonly accepted that the crystal proteins need to be solubilized to be processed for proteases present in the midguts of the target insect (Haider *et al.* 1986). Serine proteases are important in both solubilization and activation of *Bt* protoxins. Proteases remove amino acid residues from N-terminal and C-terminal ends, producing a protease-resistant toxin core that has biological activity (Lightwood *et al.* 2000; de Maagd *et al.* 2001). In some insects, changes in their proteases profile of their guts have been associated with resistance to *Bt* toxin (Li *et al.* 2004; Karumbaiah *et al.* 2007).

Recently, a new parasporal crystal toxic toward insects has been described (Huang *et al.* 2009; Lin *et al.* 2015) in *Bacillus bombysepticus*. Similarly to *Bt*, *B. bombysepticus* produces parasporal crystals during the sporulation (Huang *et al.* 2009). *B. bombysepticus* is able to infect *Bombix mori* and cause septicaemia (Huang *et al.* 2009; Lin *et al.* 2015). Lin *et al.* (2015) demonstrated that the parasporal crystal, called PC, is capable of causing silkworm death via receptor interactions in a way similar to *Bt* crystal toxins. The PC needs to be solubilized and activated with trypsin (protoxin has an approximate molecular weight of 42 kDa while the toxin is 22 kDa) to be biologically active. Proteins contained in the PC do not exhibit any homology with other bacterial toxins; however they showed conserved residues similar to some found in *Bt* Cry toxins (Lin *et al.* 2015).

Our research group reported a *Bacillus pumilus* strain toxic toward the Mediterranean fruit fly, *Ceratitis capitata* (Molina *et al.* 2010). Previous assays showed that the toxicity of *B. pumilus* 15.1 can be inactivated either by heat or by proteases, suggesting that the virulence factor produced by this strain could be of

proteinaceous nature (Molina 2010). In addition, previous reports have demonstrated that *B. pumilus* 15.1 produces parasporal crystals similar to Cry toxins (Garcia-Ramon *et al.* 2015a) that could be related to its toxicity against *C. capitata*. In this work we centred our efforts in characterizing the crystal inclusions produced by *B. pumilus* 15.1 during the sporulation phase in order to deep into the knowledge of this strain and to test if the production of parasporal crystals is related to the toxicity shown toward *C. capitata*.

Materials and Methods

Bacterial strain and growth conditions

The bacterial strain used in this study was *Bacillus pumilus* 15.1 (Molina *et al.* 2010). Luria-Bertani (LB) medium was routinely used for growing bacteria. When sporulation was required, T3 medium (Travers *et al.* 1987) was used for incubation at 30ºC for 72 h at 240 rpm. When indicated, a cold treatment of the cultures was made, as recommended by Molina *et al.* (2010).

Protein expression profile determination at different conditions

B. pumilus 15.1 was grown in 3 ml of LB at 30ºC and 240 rpm overnight and used as pre-inoculum. First, we analyzed the protein profile of a culture of *B. pumilus* 15.1 over the time. For this, five hundred microlitres of pre-inoculum were used to inoculate 50 ml of T3 medium. Culture was kept at 30ºC and 240 rpm for 72 h. Five mililitres samples were taken 12, 24, 48 and 72 h after the beginning of the culture.

Second, the protein profile of *B. pumilus* 15.1 was also determined using different $MnCl_2$ concentration in the T3 medium (0, 0.0005, 0.005, 0.05 and 0.5 g/L of $MnCl_2$). Cultures were obtained following the same methodology described above. Five mililitres samples were taken 72 h after the beginning of the culture.

All samples were centrifuged for 1 min at 16,000 x *g*. Pellets were resuspended in 50 µl of PBS and analyzed by SDS-PAGE, stained with Coomassie brilliant blue, according to the standard procedures. Precision Plus Protein™ Standards (Bio-rad) molecular weight marker was used for all SDS-PAGE gels.

Discontinuous sucrose gradient

Sporulated cultures (72 h of incubation), grown in T3 medium as describe above, were harvested by centrifugation (20,000 x *g*, 4ºC for 20 min). The pellets were washed with ice-cold PBS tree times and resuspended in 1/100 of the original culture volume of ice-cold sterile water. The spore-crystal mixture was layered on top of a 28 ml discontinuous sucrose gradient, comprising 7 ml solutions with 67%, 72%, 79% and 84% (wt/vol) sucrose. Centrifugation was carried out in a Beckman J-30 I ultracentrifuge, in a JS 24.38 rotor at 53,000 x *g* for 16 h at 10ºC. Eight culture fractions were collected and washed tree times in ice-cold PBS by centrifugation at 23,730 x *g* for 15 min at 4ºC, to remove the sucrose. The final pellets were resuspended in ice-cold deionized water at a final concentration of 500X. Samples were taken for microscopy visualization or analyzed by SDS-PAGE or stored at -20ºC for subsequent analysis.

Protein analysis by 2D gel electrophoresis

Analyses by 2-dimensional (2D) gel electrophoresis were carried out according to the manufacturer's recommendations (Biorad). Briefly, 15 µl of each protein sample were mixed with 115 µl of re-hydratation buffer (7 M urea, 2 M thiourea, 4% CHAPS, 10 mM DTT and 0.2% ampholytes) and loaded onto IPG strips (Ready Strip™ IPG Strips 11 cm, pH 3-10, Bio-Rad). The strips were re-hydrated at 20ºC for 16 h (passive rehydration) in a Protean® IEF Cell (Bio-Rad). Isoelectric focusing (IEF) was carried out using the following four-step program: (*i*) 250 V for 1 h in a linear mode; (*ii*) 4,000 V for 2 h in a linear mode; (*iii*) 4,000 V until 18,000 Vh in a rapid mode; (*iv*) 500 V until 50 µA per strip in a rapid mode. After IEF, strips were equilibrated for 10 min in equilibration buffer I [6 M urea, 0.375 M Tris pH 8.8, 2% SDS (wt/vol), 20% glycerol (vol/vol)] containing 130 mM DTT, followed by an incubation in equilibration buffer II, containing 135 mM iodoacetamide instead of DTT, for 10 min. Proteins were then separated by their molecular weight by placing the strip on the top of a 12% SDS-PAGE in a vertical electrophoretic unit (Bio-Rad). Electrophoresis was performed at 120 V for 60 min. Two dimension gels were stained with silver nitrate or Coomassie blue.

Protein digestion with proteases

The fraction 6 obtained from a sucrose gradient, frozen at -20ºC, was thawed on ice and centrifuged at 13,000 rpm for 3 min and the supernatant was collected in a fresh tube. Protein concentration was determined in the supernatants by Bradford (Sigma), following the manufacturer's recommendations and using bovine serum albumin BSA (Sigma) as a standard. Supernatant fractions were incubated with four different proteolytic enzymes: trypsin, chymotrypsin and papain from Sigma, and proteinase from *Bacillus subtilis* from Fluka. Buffers and incubation temperatures of each enzyme were carried out according the instructions provided by the suppliers. The standard ratio used for protease treatment was 10:1 (w/w) (protein:protease). Samples were incubated for 1 h and a BSA control was carried out in parallel to verify protease activity. A sample without proteases was also incubated under the same conditions as a control. The digested proteins were analyzed by SDS-PAGE.

For comparative purposes, the solubilized Cry1Aa13 (expressed in *Escherichia coli*) was also digested at the same protein:trypsin ratios (between 1:1 to 1:500, protein:trypsin). All The digested proteins were analyzed by SDS-PAGE.

Transmission electron microscopy

Fresh aliquots from the sucrose gradient fractions were pelleted and washed following the methodology previously described (Garcia-Ramon *et al.* 2015a) to be sent to the "Biological Sample Preparation Laboratory" at the Scientific Instrumentation Center of the University of Granada (CIC-UGR) for processing. Samples were observed under a Transmission Electronic Microscopy (LIBRA 120 PLUS de Carl Zeiss SMT to 120 KV) in the Microscopy Service of the CIC-UGR. Ten images of 12.6 µm in size were used for determine the crystals:spore radio.

Plasmid curing procedures

Three procedures reported in the bibliography were tested for the curing of the extrachromosomal elements present in the strain *B. pumilus* 15.1 without any success. In the first place, the methods described by Ward and Ellar (1983) and Mahillon *et al.* (1988), based on culturing the strain at high temperature were used with slight modifications. *B. pumilus* 15.1 strain was grown in 3 ml LB for 24 h at

42ºC and 240 rpm. Successive dilutions of the culture (1:100) into fresh media were made after 12 h of incubation during a total period of 72 h. The second method tested was performed as described above, with the difference that LB medium was supplemented with 0.002% SDS (Sivropoulou *et al.* 2000). In the third procedure, the *B. pumilus* 15.1 strain was grown in LB supplemented with 0.03% acridine orange or 0.12% promethazine for 24 h, either at 30ºC or at 42ºC. Bacterial cultures were transferred (1:100 dilution) into a fresh LB medium supplemented with the interfering compounds every 12 h for 5 days.

Cells derived from these procedures were plated on LB medium and incubated for 12-24 h at 30ºC. Randomly selected colonies were used for total DNA extraction using the methodology described by Reyes-Ramirez and Ibarra (2008). Total DNA was analyzed by electrophoresis in a 0.8% (wt/vol) agarose gel with SYBR Green from Invitrogen.

Given the lack of success in curing the extrachromosomal element in *B. pumilus* 15.1 a new method was developed. For that, *B. pumilus* 15.1 was cultured in 5 ml of LB medium up to an optical density at 600 nm of 0.9 to 1.1. One millilitre of the culture was pelleted at 16,000 x *g* for 1 min. The pellet was resuspended in 1 ml PBS containing 2% (wt/vol) lysozyme and 20% (wt/vol) sucrose, and was incubated at 37ºC for 90 min. In this period of time, more than 90% spheroplast formation was achieved and monitored under the microscope. The spheroplast suspension was diluted 1:100 in LB medium supplemented with 0.03% acridine orange or 0.12% promethazine and cultured at 30ºC and 240 rpm for 48 h until growth was observed. Serial dilutions were plated on LB plates and incubated at 30ºC overnight. Colonies with unusual morphology compared to the parental strain were selected. The verification of the lack of the plasmid was done by total DNA extraction and electrophoresis as described above.

Plasmid copy number determination

Plasmid copy number was determined by quantitative real time PCR as previously described (Garcia-Ramon *et al.* 2015c). Briefly, total DNA was used to amplify *smc* gene that is present in a single copy on the chromosome with smc_F and smc_R primers, and orf7_F and orf7_R primers were used to amplify a unique

region in the pBp15.1S. Plasmid copy number was calculated using the equation described by (Lee *et al.* 2006).

Southern blot analysis

Total DNA was electroforesed on a 0.8% (wt/vol) agarose gel and stained with ethidium bromide and transferred to a nylon membrane. Eight hundred fifty five nanogram of the PCR product amplified with orf7F and orf7R primers (Garcia-Ramon *et al.* 2015c) and cleaned with QIAquick® PCR Purification kit (Qiagen) were used as a probe.

DNA labelling, transfer and fixation to the membrane, hybridization and immunological detection were performed with a DIG DNA Labeling and Detection Kit (Roche No. 11093657910) following the instructions provided by the suppliers.

Mass spectrometric analysis of protein samples

Bands or spots identified for analysis from the 1D or 2D SDS-PAGE gels were individually excised and sent to "Centro de Investigación Principe Felipe", Valencia-Spain, for the peptide identification by Matrix-Assisted Laser Desorption Ionization-Time Of Flight Mass Spectrometry (MALDI TOF-MS). Protein samples were digested with trypsin before being analyzed by MALDI MS (4700 Proteomics analyser of the Applied Biosystems). Searches on public databases were performed using MASCOT search engine (Matrix-Science, London, UK).

The services from "SCSIE University of Valencia Proteomics Unit" and "CBMSO Protein Chemistry Facility" that belong to ProteoRed Proteomics Platform were also used. At the SCSIE University of Valencia Proteomics Unit a MALDI-TOF MS/MS analysis (5800 MALDI TOFTOF ABSciex) was performed. The MS and MS/MS information was analyzed by MASCOT via the Protein Pilot (ABSciex). Database search was performed on NCBInr.

At the CBMSO Protein Chemistry Facility (Madrid) a Liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis (Orbitrap-LTQ-Velos-Pro) was performed and the search was made on UniProt-Bacillus y UniProt-Bacillus pumilus databases, using Proteome Discoverer 1.4 software.

N-terminal amino acid sequencing

The solubilized and trypsinized protein of 45 kDa was separated in a 12% acrylamide SDS PAGE gel with Tris Tricine running buffer. Separated proteins were blotted onto PVDF membrane using a semi-dry transfer blotter. N-terminal sequencing was performed by Abingdon Health Laboratory services, Birmingham, UK.

The sequence obtained was compared with other protein sequences at the database using the BLAST algorithm. The amino acid sequence was searched on the genome of *B. pumilus* 15.1 (GenBank LBDK00000000.1).

Primer design and PCR amplification of the hypothetical protein YuaB

To amplify the *yuaB* gene by PCR, the primers YuabF (5' AAAAAGATCTAACCAAATGCGCTATTCCCC 3') and YuabR (5' AAGAATTCCTTTGTCAACAATCTGAAGCGC 3') were designed based on the sequence of the *yuab* gene from the genome of *B. pumilus* 15.1 (Garcia-Ramon *et al.* 2015b). Total DNA from the wild type and the cured strains were used. PCR was carried out using approximately 500 ng of total DNA, 10 µl of 10X PCR buffer, 8 µl of $MgCl_2$ (25 mM), 2 µl of deoxynucleoside triphosphates (dNTPs) (10 mM each), 3.3 µl of each primer (20 µM), 1 µl of Taq polymerase (5 U/µl), and enough MiliQ water so that the final volume of the mixture was 100 µl. The PCR mixture was denatured at 95ºC for 5 min, followed by 30 cycles of 95ºC for 1 min, 55ºC for 1 min, and 72ºC for 1 min and then a final extension at 72ºC for 5 min. Amplification was checked by electrophoresis on a 1% (wt/vol) agarose gel with SYBR Green Invitrogen.

C. capitata *larva bioassays*

The insecticidal activity of parasporal inclusion suspensions obtained from *B. pumilus* 15.1 strains (wild type and cured strains) was determined in bioassays with first-instar larvae of *C. capitata*. The fractions tested were resuspended in deionized water and were 41 times more concentrated than the original culture. The bioassays were performed as described by Molina *et al.* (2010) with some modifications. Briefly, 100 µl of a concentrated fraction were dispensed into each well and mixed

with 500 µl of artificial diet. One larva of *C. capitata* was placed in each well. The bioassays were performed in 48-well sterile Cellstar microplates (Greiner Bio-one) at 25ºC. Deionized water was used as negative control. Mortality was recorded 10 days after the beginning of the bioassay. The assay was done twice using samples obtained in different dates.

Results

B. pumilus *15.1 overexpresses a 45 kDa protein during sporulation*

In order to carry out a molecular study of *B. pumilus* 15.1 we decided to analyze the protein profile of the pellet fraction of a *B. pumilus* 15.1 culture along the sporulation process. Samples at 12, 24, 48 and 72 h were taken and processed, and the pellet fractions were analyzed in a 12% polyacrylamide gel. The protein profile of the vegetative cells (taken after 12 h of incubation) was completely different compared to the protein profiles found in sporulating or sporulated cells (samples taken at 24, 48 and 72 h) (Figure 1). Cells immersed in the sporulation process (24, 48 and 72 h of incubation) showed the overexpression of a 45 kDa protein. The amount of the 45 kDa protein increased along the incubation time and it was maximal after 72 h of incubation. Accordingly to these results, the expression of this 45 kDa protein seemed to start when the cell entered in the sporulation phase and it seems to be accumulated in the pellet fraction of the culture, either associated with the spore or as insoluble protein.

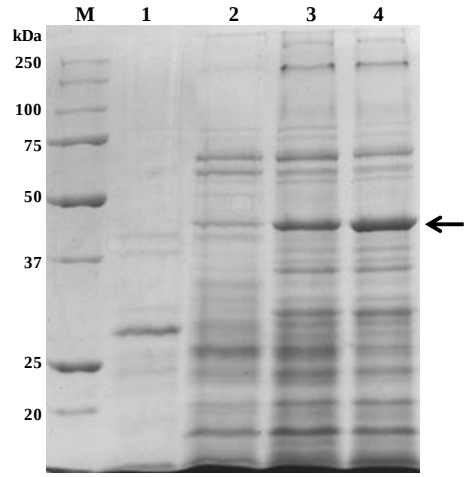

Figure 1. Protein profile of a culture of *B. pumilus* 15.1 analyzed by SDS-PAGE on a 12% polyacrylamide gel. Pellet fractions from a *B. pumilus* 15.1 culture taken after 12 h (lane 1), 24 h (lane 2), 48 h (lane 3) and 72 h (lane 4) were analyzed together with a molecular weight marker (Precision Plus Bio-rad) (lane M). The arrow shows the over-expressed 45 kDa protein.

The expression of the 45 kDa protein from B. pumilus *15.1 is dependant on the presence of Mn^{2+} ions in the growing medium*

Accordingly the composition of T3 medium, Mn^{2+} ions are the only oligoelements present in it. We evaluated the influence of the $MnCl_2$ concentration in the culture medium on the expression of the 45 kDa protein. T3 medium contains 0.005g/L of $MnCl_2$, so we assayed concentrations from 0 to 0.5 g/L in order to test the effect of Mn^{2+} ions in the protein expression profile. The results showed that the 45 kDa protein was present at all Mn^{2+} concentrations tested (Figure 2), showing maximal expression at 0.0005 g/L and 0.005 g/L of $MnCl_2$ (Figure 2, lanes 1 and 2).

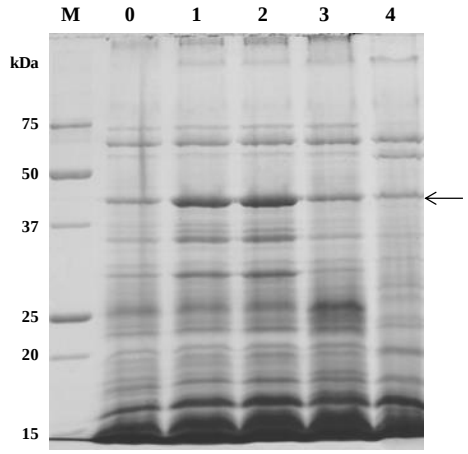

Figure 2. Protein profile of the pellet fractions of *B. pumilus* 15.1 cultures grown on T3 medium in the presence of different concentrations of $MnCl_2$. The standard conditions for $MnCl_2$ were 0.005 g/L (lane 2). Lane 0 shows a pellet fraction of a culture without $MnCl_2$, lane 1 with $MnCl_2$ 0.0005 g/L, lane 3 with $MnCl_2$ 0.05 g/L, and lane 4 with $MnCl_2$ 0.5 g/L. Lane M shows a molecular weight marker (Precision Plus Bio-rad) in kDa. The arrow shows the 45 kDa protein.

The overexpressed 45 kDa protein does not form part of the spore

The overexpressed 45 kDa protein was found in the pellet fraction of a sporulated *B. pumilus* 15.1 culture. We were interested in knowing if this over-produced protein was a constituting part of the spore or it was produced as an insoluble protein that could be related to the crystals produced by *B. pumilus* 15.1 and previously described by our group (Garcia-Ramon *et al.* 2015a). In order to do that, the pellet fraction of a 72 h culture of *B. pumilus* 15.1 was obtained in T3 medium and its components separated in a discontinuous sucrose gradient. After centrifugation, 8 fractions of the culture were obtained (Figure 3A) and analyzed by SDS-PAGE (Figure 3B).

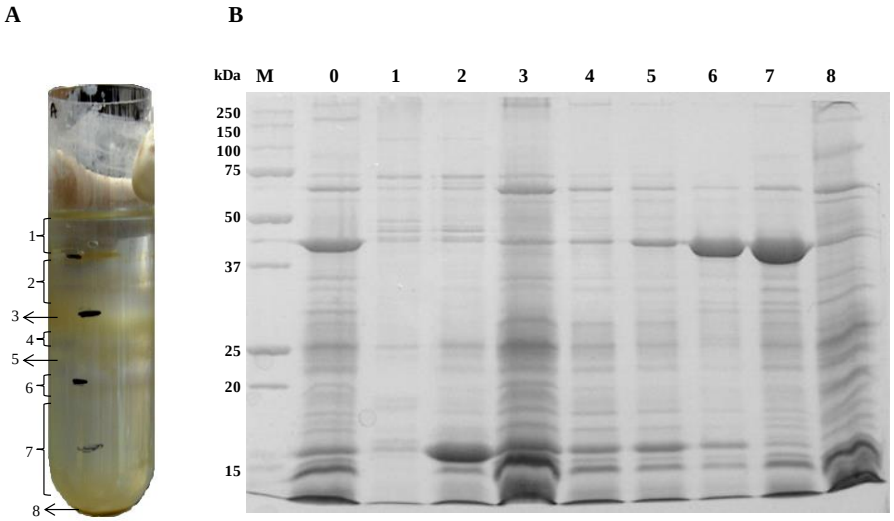

Figure 3. Panel A: Fractions obtained from the pellet of a 72 h *B. pumilus* 15.1 culture in T3 after separation in a discontinuous sucrose gradient. Panel B: SDS-PAGE analysis of the 8 fractions extracted from the pellet of a *B. pumilus* 15.1 culture. Lane 0 contains a 250X concentrated pellet before the sucrose gradient; lanes 1-8 contain the 8 sucrose fractions obtained after the gradient (concentrated 500X); lane M shows a molecular weight marker (Precision Plus Bio-rad) in kDa.

The overexpressed 45 kDa protein was observed mainly in the fractions 6 and 7 of the sucrose gradient well separated from the spores (fraction 8). Another intense band with 17 kDa in size was also observed in fraction 2.

These results seems to indicate that the over-expressed 45 kDa protein is not a constituting part of the spore and can be separated from them, meaning that is a secreted and insoluble protein.

Crystals produced by B. pumilus *15.1 are made of the overexpressed 45 kDa protein*

The 8 fractions obtained after the sucrose gradient were observed under transmission electron microscopy with the aim to know in which fraction of the gradient the parasporal crystals were present. An inspection of the images taken showed that the pellet of the culture, right before the sucrose gradient (fraction 0), was composed by a mix of crystals and spores as we previously described (Garcia-Ramon *et al.* 2015a). The crystals:spore ratio observed in fraction 0 was

approximately 0.09:1 (data not shown). Fractions 1 to 5 showed only bacterial debris and very few vegetative cells and spores, being fraction 2 particularly enriched with empty cell membranes, possibly generated from the mother cell or sporangium after the release of the spore (Figure 4A). Fraction 6 was obtained from the interface formed between the 72% and the 79% sucrose solutions, while fraction 7 was obtained taking together the 79% and 84% sucrose layers as no interface was observed. Fractions 6 (Figure 4B) and 7 (Figure 4C) were enriched in parasporal crystals with very variable size and shape. The crystals:spore ratio in these fractions was 1.6:1 and 1.25:1 respectively, meaning that a high degree of purification from the spores was achieved compared to the original mixture. In fraction 8, corresponding to the pellet of the sucrose gradient, only spores (and no crystals) were observed (Figure 4D).

The results presented here demonstrate that *i)* crystals produced in *B. pumilus* 15.1 can be partially purified from the spores using a sucrose gradient, and *ii)* the crystals are enriched in fractions 6 and 7, the same fractions were the 45 kDa protein was detected in the SDS-PAGE analysis. These results may suggest that crystals are composed by the 45 kDa protein observed in SDS-PAGE gels.

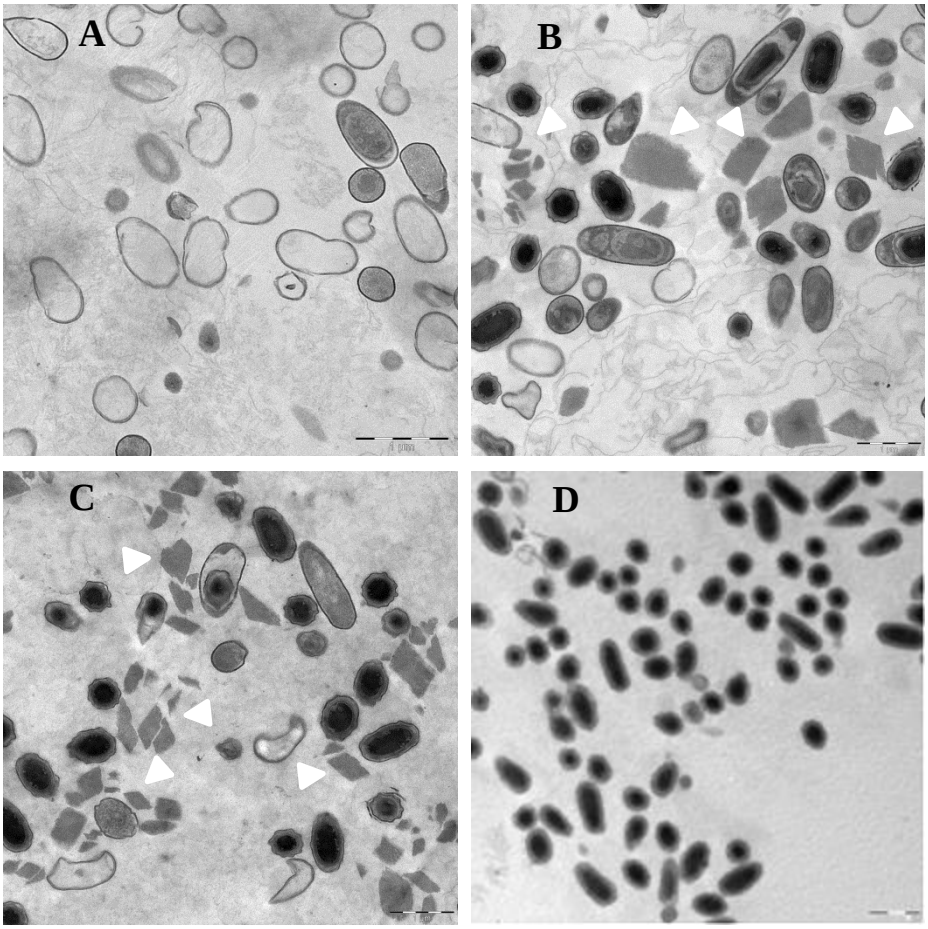

Figure 4. Transmission electron microscopy of the most relevant fractions obtained from a pellet of *B. pumilus* 15.1 after separation in a sucrose gradient. Panel A shows fraction 2, mainly composed by bacterial debris from cell walls from the mother cells. Panel B and C show fraction 6 and 7 respectively, where the parasporal inclusions were localized (white arrows). Panel D shows fraction 8, composed mainly by spores.

Investigating the codification source of the crystals produced by B. pumilus *15.1*

We have recently described that the *B. pumilus* 15.1 strain bears one plasmid (pBp15.1S) and one megaplasmid (pBp15.1B) in its cytoplasm (Garcia-Ramon *et al.* 2015c). Many crystals-forming proteins, such as Cry and Cyt toxins produced by *B. thuringiensis*, are encoded by extrachromosomal elements (plasmids and megaplasmids) (de Maagd *et al.* 2003). In order to determine if the crystals produced by *B. pumilus* 15.1 strain had chromosomal or extrachromosomal codification we decided to cure the strain from its extrachromosomal elements.

Obtaining* B. pumilus *15.1 variants without extrachormosomal elements

For that, different methodologies described in the bibliography such as heat and SDS treatment, acridine orange and promethazine treatment (detailed in Materials and Methods section) were used without any success (data not shown).

In a previous characterization of the *B. pumilus* 15.1 strain under electron microscopy (Garcia-Ramon *et al.* 2015a) we observed that the strain showed a particulary thick cell wall. We hypothesized that the lack of effect of the compounds tested for plasmid curing was caused by the difficulty of these compounds to penetrate in the cell and specifically interfere with plasmid replication. For that reason, we designed a strategy in order to improve the success of compound internalization and hence the success of plasmid curing. The strategy consisted in obtaining spheroplasts from *B. pumilus* 15.1 with the use of lysozyme previous to the treatment with the replication-interfering compounds. We tested our hypothesis with acridine orange and promethazine, two very well known curing compounds. *B. pumilus* 15.1 spheroplasts were obtained from vegetative cells as detailed in the Materials and Methods section and then they were diluted in LB medium containing acridine orange (0.03%) or promethazine (0.12%). As controls, the same number of vegetative cells, without the lysozyme treatment, was cultured under the same conditions in the presence of the replication-interfeering compounds. When total DNA was extracted from one colony obtained from each treatment (Figure 5) no extrachromosomal elements were observed in those cells previously treated with lysozyme (Figure 5, lanes 3 and 4). On the contrary, those cells not treated with lysozyme (Figure 5, lanes 5 and 6) showed the extrachromosomal elements in their cytoplasm. The use of the spheroplasts instead of the vegetative cells seams to improve the efficiency of acrydine and promethazine in curing the strain *B. pumilus* 15.1. The strain selected for further studies was the one obtained from the acridine orange treatment and it was named *B. pumilus* 15.1C [cured from the plasmid (pBp15.1S) and megaplasmid (pBp15.1B)].

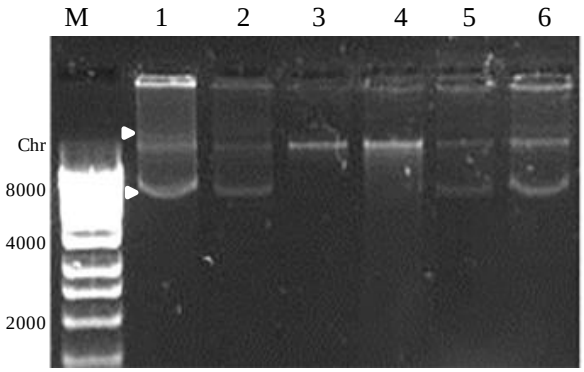

Figure 5. DNA electrophoresis in 0.8% agarose gel of total DNA extracted from several *B. pumilus* 15.1 variants. Wild-type strain is shown in lanes 1 and 2. Variants obtained with the previous formation of spheroplasts are shown in lanes 3 (treated with acridine orange) and 4 (treated with promethazine). Lanes 5 and 6 show two variants treated with acridine orange and promethazine respectively without lysozyme treatment. M: Molecular weight marker (HyperLadder I from Bioline) in base pairs. White arrows indicate the megaplasmid (pBp15.1B) and the plasmid (pBp15.1S).

Checking that* B. pumilus *15.1C lacks of pBp15.1S

As the sequence of pBp15.1S (accession number KM348008) was previously described by our research group (Garcia-Ramon *et al.* 2015c), we checked the absence of the plasmid in the strain *B. pumilus* 15.1C by qRT-PCR and southern blot.

Following the methodology previously described for the quantification of pBp15.1S copy number by qRT-PCR (Garcia-Ramon *et al.* 2015c) we determined the copy number of the plasmid in the strain *B. pumilus* 15.1C. No significant amplification of the probe was obtained (data not shown), so it was estimated that the copy number of pBp15.1S in *B. pumilus* 15.1C was 0 [opposed to the 33 copies of plasmid per chromosome in *B. pumilus* 15.1 (Garcia-Ramon *et al.* 2015c)].

Southern blot analysis was also performed in order to support this result. For that, a Dig-labeled probe designed in the *orf7* of the plasmid pBp15.1S was used for hybridization. No signal was observed in the lane corresponding to total DNA from *B. pumilus* 15.1C (Figure 6 Panel B, lane 2). This result agrees with the previously obtained so we can safely state that *B. pumilus* 15.1C lacks of the plasmid pBp15.1S. In any of the total DNA extractions made from *B. pumilus* 15.1C the megaplasmid was not observed either.

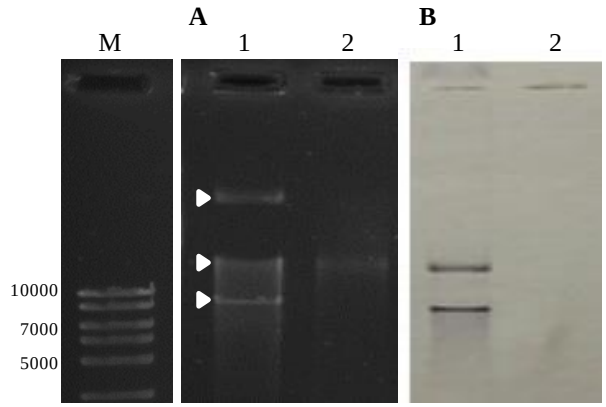

Figure 6. DNA electrophoresis (Panel A) and Southern blot (Panel B) of total DNA from *B. pumilus* 15.1 wild type (lanes 1) and *B. pumilus* 15.1C (lanes 2). Electrophoresis was performed in a 1% agarose gel and stained with ethidium bromide. Sothern blot was performed with a DIG labeled probe designed in the *orf*7 of the plasmid pBp15.1S (Garcia-Ramon *et al.* 2015c). M: Molecular weight marker (HyperLadder I from Bioline) in base pairs. The arrows indicate the megaplasmid, the chromosome and the plasmid.

The gene codifying for the 45 kDa protein in B. pumilus *15.1 has chromosomal location*

The protein profile of the pellet fraction of a culture of *B. pumilus* 15.1C in T3 medium was obtained, fractioned in a discontinuous sucrose gradient, analyzed by SDS-PAGE (Figure 7) and compared to the *B. pumilus* 15.1 protein profile. Although the general pattern of proteins was conserved, two main differences were observed. The first one was that the expression of the 45 kDa protein seems to be higher in the cured strain compared to the wild type (Figure 7A and B). The 45 kDa protein was observed mainly in fractions 6 and 7 of the gradient as in the wild type strain, being most abundant in fraction 6. The second difference was that the 17 kDa protein present in fraction 2 of a pellet of the wild type strain was completely missing in the cured strain (Figure 7, Panel A, lane 2 or Panel B, lane 2).

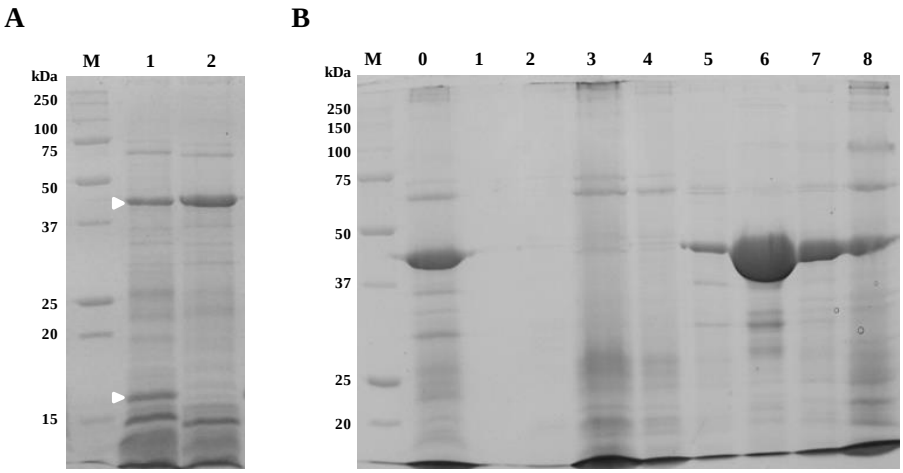

Figure 7. SDS-PAGE analysis of the pellets from *B. pumilus* 15.1 and *B. pumilus* 15.1C cultures (panel A, lanes 1 and 2 respectively) and the fractions obtained in a discontinuous sucrose gradient from the *B. pumilus* 15.1C pellet (panel B). White arrows show the protein of 45 kDa more intense in the cured strain and the 17 kDa protein present only in wild type strain. Lane M shows the molecular weight marker (Precision Plus Bio-rad) in kDa.

These results demonstrate that the overexpressed 45 kDa protein from *B. pumilus* 15.1 is not codified by any of the extrachromosomal elements present in the strain, as it is expressed regarding the presence of the plasmid and megaplasmid. For this reason, we can conclude that the gene codifying for the 45 kDa is localized in the chromosome of the strain. On the contrary, as the 17 kDa protein is not expressed in the cured strain, so it is highly probable that the gen is localized in one of the extrachromosomal elements.

When *B. pumilus* 15.1C was analyzed under transmission electron microscopy no morphological differences were observed compared to *B. pumilus* 15.1 strain (data not shown). The only remarkable difference was that the number of crystals in *B. pumilus* 15.1C cultures was higher than in *B. pumilus* 15.1. A quantification of the number of crystals and spores from different fields of the micrographs obtained showed that that ratio crystals:spore observed in a culture of *B. pumilus* 15.1C was 0.17:1 compared to the ratio 0.09:1 previously determined for *B. pumilus* 15.1. This result seems to indicate that the production of the crystals in the cured strain was higher (almost double) that in the wild type strain. The fact that the cured strain

produced more crystals and produced the 45 kDa protein in a higher level, it is consistent with the hypothesis that the crystals are composed by the 45 kDa protein.

As the 45 kDa and 17 kDa protein were the most interesting proteins in the sucrose gradients we decided to analyze both proteins by other means.

Analysis of the 45 kDa protein by MALDI-TOF

The 45 kDa proteins found in fractions 6 and 7 that were associated with the crystals synthesized by *B. pumilus* 15.1 and *B. pumilus* 15.1C were sent at Príncipe Felipe Research Center for its identification by fingerprinting using MALDI-TOF MS. The results obtained after the analysis are shown in Table 1. The fragmentation of the 45 kDa protein from *B. pumilus* 15.1 rendered 14 peptides that matched (40.8% of sequence coverage) with OxdD (Oxalato decarboxylase) from *B. pumilus* ATCC 7061.

The 45 kDa protein obtained from *B. pumilus* 15.1C strain was also identified as Oxalate decarboxylase (15 peptides with 41% sequence coverage with Oxalate decarboxylase of *B. pumilus* SAFR-032). We can conclude that the 45 kDa proteins observed in the wild type and the cured strains are the same protein and seem to be an Oxalate decarboxylase (Table 1).

Table 1. Results from the fingerprinting analysis of the 45 kDa proteins from *B. pumilus* 15.1 and *B. pumilus* 15.1C strains.

Protein	Protein identification	Accession number	MASCOT score	Sequence coverage (%)	Predicted mol. mass (Da)	Predicted p*I*
45 kDa protein from *B. pumilus* 15.1	Oxalate decarboxylase OxdD [*Bacillus pumilus* ATCC 7061]	EDW19970	148	40.8	43,795	5.2
45 kDa protein from *B. pumilus* 15.1C	Oxalate decarboxylase [*Bacillus pumilus* SAFR-032]	ABV61489	157	41	43,840	5.22

In order to confirm the results obtained and to rule out any possible contamination of the sample we decided to analyze the protein samples in 2D gels. Experimental conditions for 2D-PAGE using 11 cm ReadyStrip™ IPG strips (pH 3-10) for IEF and SDS 12% gels were determined to investigate the fractions of the sucrose gradient in the *B. pumilus* 15.1 and *B. pumilus* 15.1C strains. In fractions 5, 6 and 7, the most intense spots were at 45 kDa (data not shown). In these fractions, most of the 45 kDa proteins migrated at p*I* 5.5 (spot A), but at the end of the strip (p*I* around 10) a spot (spot B) was observed (Figure 8). The spot B was cut out from the Coomassie blue-stained 2D-gel and analyzed by MALDI-TOF for protein identification.

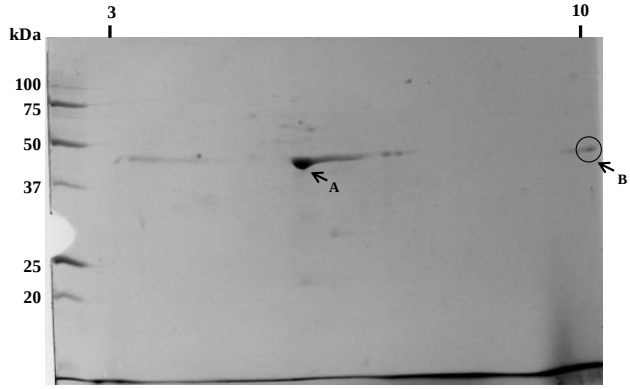

Figure 8. Two dimensional electrophoresis of fraction 5 obtained from the sucrose gradient of a *B. pumilus* 15.1C culture. pH (p*I*) range is shown in horizontal and molecular weight (kDa) is shown in vertical. p*I* ranged from 3 to 10. Arrow A shows p*I* 5.5. Arrow B shows p*I* ≥ 10, this spot was identified by MALDI-TOF analysis.

Oxalate decarboxylase has a theoretical p*I* of 5.22, the approximate pH shown for one of the spots in the 2D gel (Figure 8, arrow A). Hence, spot A could correspond to oxalate decarboxylase. The spot B was also identified by MALDI-TOF MS as Oxalate decarboxylase, showing the same peptide sequences and sequence coverage as spot A (Table 1). As far as we know there is no reported in the bibliography any Oxalate decarboxylase with a p*I* ≥10.

Identification of the 17 kDa protein by fingerprinting

The 17 kDa protein detected in fraction 2 of the gradient was present only in *B. pumilus* 15.1 wild-type but not in the cured strain. The protein band of 17 kDa was sent at Príncipe Felipe Research Center for its identification by MALDI-TOF MS as well.

Analysis of 17 kDa protein suggested that it contained an 11-amino-acid sequence (VLPAAGTYTFR) and a 24-amino-acid sequence (FYAEDTLDIQTRPVVVTPPDPCGC) with identity with the hypothetical protein BPUM_1610 of *B. pumilus* SAFR-032, accession number ABV62292.1 identified as being codified by *yuaB* gene. The coverage of the sequence was 19%. The predicted molecular weight was 19,297 Da and the predicted p*I* was 9.10.

The 17 kDa protein observed in* B. pumilus *15.1 is codified in the megaplasmid

The MALDI-TOF MS results suggested that the 17 kDa protein was the *yuaB* gene product. The gen *yuaB* was localized on the genome of *B. pumilus* 15.1 (Garcia-Ramon *et al.* 2015b) so primers based on this sequence were designed for its amplification by PCR. A product of 727 bp was detected only when DNA from the wild type strain was used as template in the PCR, but not when total DNA from *B. pumilus* 15.1C was used (Figure 9). PCR was repeated using as template different total DNA extractions from the wild type and cured strains, and the result was the same. These results confirmed that *yuaB* gene that codified for the 17 kDa hypothetical protein, was only present in *B. pumilus* 15.1 wild type and no in the cured strain. As the *yuaB* gene is not present in the sequence of pBp15.1S (Garcia-Ramon *et al.* 2015c), *yuaB* gene should be present in the megaplasmid pBp15.1B.

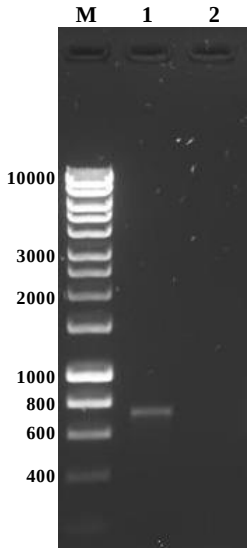

Figure 9. Agarose gel electrophoresis of PCR products after amplification with YuabF and YuabR primers. A PCR product of 727 bp was amplified with DNA from the wild type strain (lane 1) but not from the cured strain (lane 2). Lane M: molecular weight marker (HyperLadder I from Bioline) in base pairs.

Solubilization of the 45 kDa protein

In order to continue with the characterization of the 45 kDa protein, we studied the best conditions for the solubilization of the crystals. A serial of solubilization experiments at different buffer compositions and pHs were performed according to the bibliography (Koller *et al.* 1992; Naimov *et al.* 2008; Wang *et al.* 2012) with the pellet fraction of a frozen crystal preparation. For that, a frozen preparation of crystals was taken out from the freezer, centrifuged, supernatant discarded and aliquots of the pellet fraction resuspended in different buffers and incubated at 37ºC to facilitate solubilization. When we analyzed by SDS-PAGE both supernatant and pellet fractions after incubation we noticed that the amount of protein present in all preparations was very diminished compared to the one obtained in the sucrose gradient. It was then when we realized that most of the 45 kDa protein was not in the pellet fraction of the frozen pellet but in the supernatant fraction. Apparently, the insoluble crystals were solubilized when they were frozen at -20ºC. To verify this hypothesis, a new sucrose gradient was made and fresh fractions were used to check the solubilization process. The fraction 6 from the sucrose gradient was split into

two aliquot fractions. One of them was kept at -20ºC and the other at room temperature (RT). Ten microliters samples were taken over time from each aliquot fraction, centrifuged, pellet and supernatant separated, and analyzed by SDS-PAGE gels. The results are shown in Figure 10. When the crystal preparation was kept at RT the 45 kDa protein was observed only in the pellet fractions of the samples analyzed (Figure 10A). On the contrary, when the sample was kept at -20ºC, the concentration of the 45 kDa protein in the supernatant fraction increased as the incubation time at -20ºC progressed (Figure 10B). These results were interpreted as the 45 kDa protein was solubilized when incubated at low temperature.

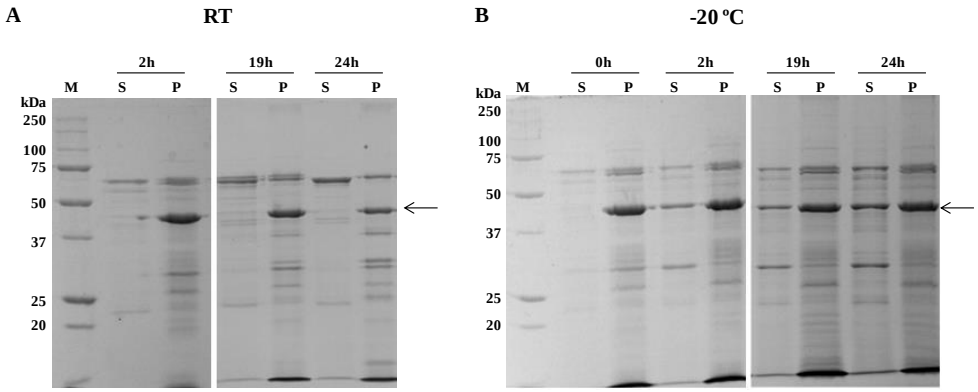

Figure 10. SDS-PAGE analysis of the fraction 6 obtained from a fresh sucrose gradient kept at room temperature RT (panel A) and low temperature (Panel B). The incubation at -20ºC solubilized the 45 kDa protein along time while incubation at RT made the protein to stay in the insoluble fraction. Lanes S represent the supernatant fractions and lanes P represent the pellet fractions of the samples. The arrows in panels point out the 45 kDa protein. Lanes M are a molecular weight marker (Precision Plus Bio-rad) in kDa.

In order to confirm our hypothesis that the low temperatures made the 45 kDa protein to solubilise, we analyzed the fractions by transmission electron microscopy. The sample incubated at RT contained parasporal crystals, while the sample incubated at -20ºC (for longer than 24 h) showed almost not crystal at all. This result was consistent with our previous observation that the crystals are made of the 45 kDa protein.

Proteins from the pellet and supernatant fractions obtained after incubation at -20ºC were sent for identification by MALDI-TOF MS. Once again, the results identified

the 45 kDa protein as Oxalate decarboxylase, not only in the pellet fraction (36% coverage) but also in the supernatant (34% coverage).

In order to discard the possibility that the 45 kDa protein observed was composed by more than one protein, 2.3 µg of soluble protein were sent to CBMSO Protein Chemistry Facility Service and a LC-MS/MS analysis was performed on the sample digested with trypsin and desalinated. The analysis of the sample showed relatively low number of fragmentation spectra of very good quality. A total of 27 groups of proteins were identified (Table 2). Oxalate decarboxylase presented the highest score (123.7), while other proteins rendered not significant scores. In addition, Oxalate decarboxylase was the only protein with the exact molecular weight as the estimated by gel electrophoresis.

Table 2. Protein hits from the results obtained by LC-MS/MS analysis of the soluble 45 kDa protein.

Accession	Description[a]	Score	Coverage	No peptide[b]	MW (kDa)[c]	calc. pI[d]
A8FB72	Oxalate decarboxylase	123.7	22.7	11	43.8	5.36
A8F9T1	1-pyrroline-5-carboxylate dehydrogenase	23.6	9.1	5	56.3	5.38
A8FDH2	Outer spore coat protein E	21	19.3	5	20.8	4.73
A8FEL3	Non-specific DNA-binding protein HBsu	20.11	44.57	5	9.8	9.31
A8FG02	Thioredoxin	17.06	27.88	3	11.3	4.60
A8FCS4	Dihydrolipoyl dehydrogenase	16.99	6.17	2	49.5	5.03
A8FDB6	Ribosome-recycling factor	15.01	18.92	3	20.8	5.43
A8FDJ7	N-acetylmuramoyl-L-alanine amidase	13.61	15.87	4	27.3	9.20
A8FA13	FMN-dependent NADH-azoreductase	12.78	13.27	2	23.0	5.58
A8FGW1	ABC superfamily ATP binding cassette transporter, binding protein	12.19	7.22	2	38.7	8.82
A8FBD7	Possible endonuclease	12.09	2.08	2	105.9	6.62
A8FHE1	Aldo/keto reductase	11.09	14.23	3	31.7	5.36
A8FFD2	Chaperone protein DNAK	8.52	3.92	2	66.1	4.83
A8F973	50S ribosomal protein L7/L12	8.25	21.49	2	12.6	4.59

A8FC23	Spore coat protein X	8.11	14.47	2	17.4	4.60
A8F9Z5	Uncharacterized protein	7.49	12.25	2	22.1	7.47
A8FB44	Possible rhamnogalacturan acetylesterase	7.33	11.96	2	23.3	5.02
A8FFN2	Glutamine ABC superfamily ATP binding cassette transporter, binding protein	7.01	4.83	2	29.6	7.78
A8FBX9	Oligopeptide ABC superfamily ATP Binding cassette transporter, binding protein	6.76	3.68	2	61.7	5.15
A8F9P2	TerD family tellurium resistance protein	6.61	13.99	2	20.5	4.65
A8F976	DNA-directed RNA polymerase subunit beta	5.77	3.09	3	133.9	5.06
A8FEL4	Stage IV sporulation protein A	5.75	4.88	2	55.3	4.86
A8FHJ4	Glyceraldehyde-3-phosphate dehydrogenase	5.55	7.46	2	35.7	5.17
A8FCU7	Cytochrome c oxidase subunit 2	3.89	5.63	2	40.2	8.19
A8FFS1	SpoVID-associated morphogenetic protein	3.74	5.80	2	48.0	7.02
A8FG47	Malate dehydrogenase	2.37	6.73	2	33.6	5.03
A8FBD3	Uncharacterized protein	1.98	9.47	2	19.0	8.03

[a] Protein identified belong to *B. pumilus* SAFR-032
[b] Peptides numbers found for reference proteins
[c] Predicted molecular weight in kDa
[d] Calculated p*I*

Protease stability of the 45 kDa protein

The soluble protein (obtained by incubation of the crystals at -20ºC) was digested with a range of proteases to determine if the protein was susceptible to their action or it was resistant to them as Cry toxins are. Trypsin, chymotrypsin, papain and proteinase from *B. subtilis* were tested at a 10:1 ratio (w/w) protein:enzyme. SDS-PAGE analysis revealed that the 45 kDa protein was not digested by neither trypsin nor chymotrypsin (Figure 11, lanes 2 and 3); while, papain and proteinase from *B.*

subtilis produced a complete digestion of the 45 kDa protein (Figure 11, lanes 4 and 5).

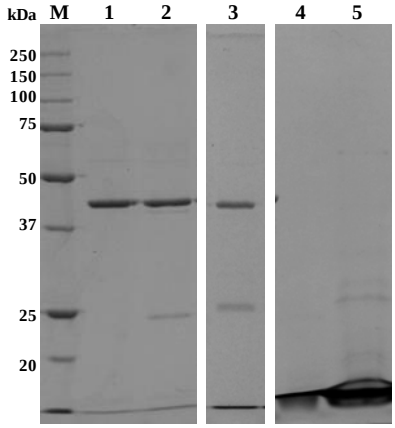

Figure 11. SDS-PAGE analysis of the 45 kDa protein digested with trypsin (lane 2), chymotrypsin (lane 3), papain (lane 4) and proteinase from *B. subtilis* (lane 5). Lane 1 shows the soluble 45 kDa protein with no protease treatment. Lane M, molecular mass marker (Precision Plus Bio-rad) in kDa.

An analysis of the resistance of the 45 kDa protein to trypsin digestion was performed with increasing concentrations of the protease (protein:trypsin ratios between 1:1 to 1:500). The solubilized Cry1Aa13 was used as control of digestion given its resistance of the core of the toxin to proteolysis with trypsin. The 66 kDa product resulting from trypsin digestion of the Cry1Aa13 was observed at all the protein:trypsin ratios (even 1:500 ratio) (Figure 12A). Similarly, the 45 kDa protein was resistant to proteolysis with trypsin (Figure 12B). Accordingly to these results we can consider that the 45 kDa protein is trypsin resistant as the protein can be still observed even in the presence of 500 times more trypsin than protein.

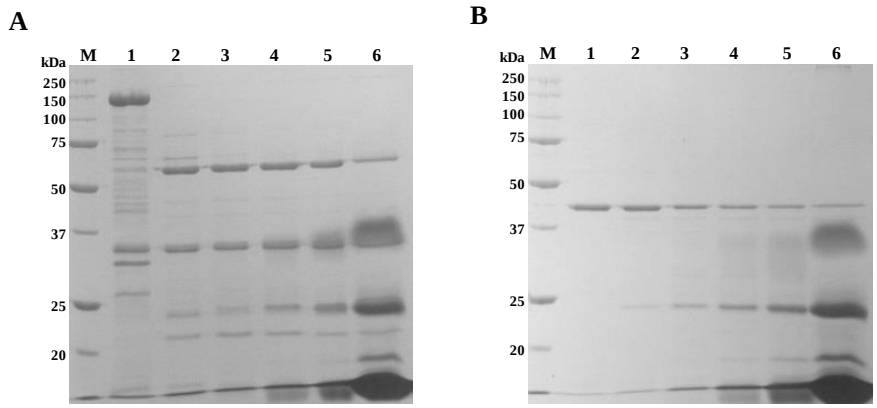

Figure 12. SDS-PAGE analysis of the Cry1Aa13 (Panel A) and 45 kDa protein (Panel B) digestion products obtained after trypsin treatment at protein:trypsin ratios 1:1 (lanes 2), 1:10 (lanes 3), 1:50 (lanes 4), 1:100 (lanes 5) and 1:500 (lanes 6). Lanes 1 show the proteins without trypsin treatment. Lane M, molecular mass marker (Precision Plus Bio-rad) in kDa.

In a similar way, we assayed the chymotrypsin resistance by digesting the 45 kDa protein with ratios 1:1 and 1:10 (protein:enzyme). As shown in Figure 13, when the amount of chymotrypsin incresased, the 45 kDa protein was partially digested (Figure 13, lanes 2 and 3).

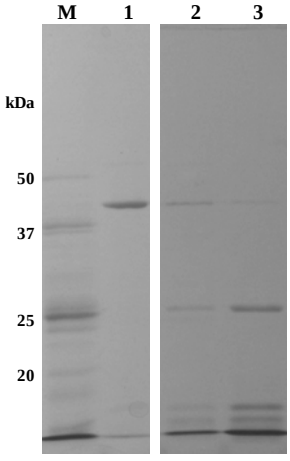

Figure 13. SDS-PAGE analysis of the 45 kDa protein digestion with chymotrypsin. Lane 1 shows the protein without chymotrypsin treatment. Protein:chymotrypsin ratio of 1:1 (lane 2) and 1:10 (lane 3) are shown. Lane M, molecular mass marker (Precision Plus Bio-rad) in kDa. The band of approximately 25 kDa corresponds to weight of chymotrypsin.

N-terminal sequence of 45 kDa protein

Finally, the N-terminal sequence of the 45 kDa protein was determined. The soluble 45 kDa protein was digested with trypsin, ran in a SDS-PAGE gel, transferred to membrane and sent to analysis. Amino terminal analysis yielded the sequence S-E-K-P-D/N-G-I-P. The SEKPNGIP sequence showed 100% identity and 100% sequence coverage with Oxalate decarboxylase from three strains of *B. pumilus* (accession numbers KIL13977; CUB14667; and KLL01117); the last one belongs to *B. pumilus* 15.1 strain. These accession numbers correspond to hypothetical proteins whose functions as oxalate decarboxylase have not been assayed yet. The N-terminal sequence obtained matched from the second amino acid of the predicted protein present in the genome of *B. pumilus* 15.1 (protein_id KLL01117.1). The search for the SEKPDGIP sequence in the databases did not render any positive result (with 100% of identities and 100% sequence coverage) to any protein.

Crystals produced by* B. pumilus *15.1 are not enough for toxicity

Fractions 6 and 7 from a sucrose gradient obtained from the wild type and cured strains containing the majority of the 45 kDa protein were tested in bioassays against first-instar larvae of *C. capitata* using deionized water as negative control for toxicity. In the first place, we tested activity of fresh fractions. Crystal obtained from *B. pumilus* 15.1 showed a mortality of the 4.2%, while crystals obtained from *B. pumilus* 15.1C showed 10.4% mortality. These mortality results were not significant compared to the negative control (6.25% mortality).

In the second place, we tested the activity of the crystal fractions after being frozen at -20ºC for 4 hours. Then, pellet and supernatant were separated and bioassayed separately. The pellet fraction of *B. pumilus* 15.1 caused 6.79% of mortality, while supernatant caused 18.8%. The pellet fraction of *B. pumilus* 15.1C caused 12% of mortality, while supernatant caused 14.6%. In the negative controls, where just water was bioassayed, a mortality of 2.08% was observed. A statistical analysis of these results showed that mortality differences between the fractions and the controls were not significant, so we cannot related the presence of the crystals with toxicity. Although it is likely that the 45 kDa protein is not able to induce the

mortality of *C. capitata* larvae by itself we cannot rule out the possibility that this protein may play some role in this process, together with other virulence factors.

Discussion

In the *Bacillus* genus, many virulence factors against insect are composed of crystals-forming proteins such as Cry, Cyt and Bin (Palma *et al.* 2014). The entomopathogenic *B. pumilus* 15.1 strain produces parasporal crystals during the sporulation phase that resemble the crystal toxins of *Bt* (Garcia-Ramon *et al.* 2015a). Here, we focused in the characterization of these crystal inclusions produced by *B. pumilus* 15.1. In order to establish if the parasporal crystals of *B. pumilus* 15.1 had an extrachromosomal codification or not, both plasmids of the strain were removed. The conventional plasmid curing methods, that involve culturing the plasmid bearing bacteria at high temperature and/or in the presence of replication-interfering chemical compounds, have been applied to many bacteria (Hara *et al.* 1982; Ward and Ellar 1983; Mahillon *et al.* 1988; Sivropoulou *et al.* 2000). Unfortunately, these techniques are not applicable for all strains (Rajini Rani and Mahadevan 1992; Feng *et al.* 2013). In fact, using the most conventional treatments (Ward and Ellar 1983; Mahillon *et al.* 1988; Ghosh *et al.* 2000; Molnar *et al.* 2003) we were not able to isolate a plasmid-free strain of *B. pumilus* 15.1. We assayed sub-inhibitory concentrations of SDS, acridine orange and promethazine combined with high temperature (42ºC), but the plasmids were no eliminated. Based on previous studies, it was proposed that cell wall/cell membrane could served as a barrier resulting in an inefficient plasmid elimination (Spengler *et al.* 2003). Hence, our curing strategy was based on obtaining spheroplast before the treatment with the replication-interfering compounds. The strategy resulted more efficient than the conventional methods used for spore-forming bacteria and faster, as no sucesive culturing was needed.

B. pumilus 15.1C, the cured strain obtained, showed a parasporal crystals production even higher than the wild type strain. This result proved that the crystals observed in *B. pumilus* 15.1 are chromosome-encoded. Although many Cry toxins are encoded by plasmids, there are some encoded in the chromosome such as

Cry18Aa1 (Zhang *et al.* 1997) or Cry16Aa1 (Barloy *et al.* 1996) while others are located on both chromosome and plasmids (Klier *et al.* 1982).

We were able to relate the production of parasporal crystals with the protein profile of *B. pumilus* 15.1 and *B. pumilus* 15.1C strains. As crystals were massively produced by *B. pumilus* 15.1 as cells transformed into spores (Garcia-Ramon *et al.* 2015a) and the overexpression of a 45 kDa protein was concomitant with the spore formation (Figure 1), we were tempted to postulate that the crystals could be made of the 45 kDa protein observed in the pellet fraction of the culture. The protein profile of the *B. pumilus* 15.1C was similar to the wild type strain, but the 45 kDa protein was more intense (Figure 7A); this result was consistent with the quantification of parasporal crystals from cured strain (almost double) and the wild type strain.

When sporulated cultures were subjected to a discontinuous sucrose density gradient using the technique described by Thomas and Ellar (1983), the most commonly adopted strategy for separating spores and crystals in *Bt*, the crystals of *B. pumilus* 15.1 and *B. pumilus* 15.1C were successfully separated from the spores. Crystals from *B. pumilus* 15.1 were retained on the interface formed between the solutions with 72% and 79% of sucrose like many Cry toxins (Thomas and Ellar 1983; Koller *et al.* 1992; Jones *et al.* 2007; Swiecicka *et al.* 2008). They were also found in the fraction composed by the 79% and 84% sucrose solutions.

To find out if the parasporal crystals were related to the toxicity shown by *B. pumilus* 15.1 strain against *C. capitata*, bioassays with fractions 6 of the sucrose gradient were done. Parasporal crystals of *B. pumilus* 15.1 and *B. pumilus* 15.1C showed no toxicity toward first-instar larvae of *C. capitata*. Our bioassays demonstrated that the toxic effect of *B. pumilus* 15.1 reported by Molina *et al*. (2010) cannot be exclusively associated with the parasporal crystals produced by this strain. Although the *B. pumilus* 15.1 crystals are not enough to cause mortality, we cannot rule out the possibility that crystals work in combination with other toxins and/or proteins in the virulence mechanism. Besides, it should be taken into consideration that not always the presence of parasporal inclusions ensures the toxicity of the bacterial strain. Many researchers have isolated non-toxic strains of *Bt*

(Bernhard *et al.* 1997; Benintende *et al.* 1999) and actually Ohba and Aizawa (1986) suggested that non-toxic parasporal inclusions bacterial predominate compared to the toxic ones. At least for now, the role of the parasporal crystal in the *B. pumilus* 15.1 virulence remains unclear.

Apart from the information of the crystal codification, the *B. pumilus* 15.1C strain showed us another useful information: the YuaB protein is codified by a gene localized in the megaplasmid, so it could be used as a marker for this extrachromosomic element. This protein showed homology with the hypothetical protein BPUM_1610 of *B. pumilus* SAFR-032; YuaB has been studied in *B. subtilus* where plays a role during biofilm formation (Ostrowski *et al.* 2011) being responsible to form a layer on the surface of the biofilm making the surface to be hydrophobic (Kobayashi and Iwano 2012). YuaB is a small secreted protein that is localized at the cell wall (Ostrowski *et al.* 2011) and in all literature reviewed (including UniProt database) *yuaB* has chromosome codification.

Many Cry toxins need to be solubilized and activated by proteases to act against insects (Bravo *et al.* 2007). The main digestive proteases of Diptera are serine proteases (de Maagd *et al.* 2001). Trypsin, a type of serine protease, can selectively hydrolyze proteins. It is considered to be the main hydrolase responsible for *Bt* toxin activation in the insect midgut (Li *et al.* 2004). When the proteolytical activation of Cry toxin has been carried out, the result is a protease resistant core that is biological active (Lightwood *et al.* 2000; de Maagd *et al.* 2001). Here, we demonstrated that the solubilized 45 kDa protein is resistant to trypsin at least as resistant as the core of the Cry1Aa13 toxin. But probably, the most interesting feature is the fact that the 45 kDa protein was solubilized at low temperature (-20ºC). To our knowledge, this is the first time a protein has such behavior.

All analysis made to identify the 45 kDa protein (by MALDI-TOF and N-terminal sequencing) showed the same result: the 45 kDa protein was identified as an oxalate decarboxylase. Nevertheless, the characteristics reported for oxalate decarboxylase enzyme are not similar to the characteristic of the 45 kDa protein of *B. pumilus* 15.1 presented in this study.

Oxalate decarboxylase (EC 4.1.1.2) catalyzes the conversion of oxalate to carbon dioxide and formate. The first bacterial oxalate decarboxylase was identified in *B. subtilis* (OxdC, formerly known as YvrK) as a cytosolic enzyme (Tanner and Bornemann 2000). Soon after a second hypothetical protein (YoaN) from *B. subtilis* exhibited a oxalate decarboxylase activity and was named OxdD (Tanner *et al.* 2001), which was found to be present in the interior layer of the spore coat (Costa *et al.* 2004). Oxalate decarboxylase belongs to the cupin superfamily and bicupin subclass, it has two β sandwich cupin domains (Tanner *et al.* 2001) each one containing one manganese binding site (Anand *et al.* 2002). In *B. subtilis*, OxdC and OxdD were spore-associated proteins (Kuwana *et al.* 2002) and the recombinant proteins overexpressed in *E. coli* were soluble showing oxalate decarboxylase activity only when expressed in the presence of manganese salts (Tanner *et al.* 2001).

In the bibliography, we did not find any data that relates oxalate decarboxylase with toxicity against insect. However, oxalate decarboxylase has been used in biological control of fungal plant diseases (Kesarwani *et al.* 2000; Dias *et al.* 2006). Phytopathogenic fungi, such as *Sclerotinia*, produce oxalic acid that has been associated with its pathogenesis; thus, enzymes able to degrade oxalic acid, as oxalate decarboxylase, have been used for making transgenic plants resistant to fungal pathogens (Kesarwani *et al.* 2000). This strategy has been successful in lettuce (Dias *et al.* 2006), and tobacco and tomato (Kesarwani *et al.* 2000) plants.

Although further studies are needed to learn more about the 45 kDa protein, such as its function and the role that plays in the virulence of *B. pumilus* 15.1 a great load of information has been obtained in this work.

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Chapter IV

Draft genome sequence of the entomopathogenic bacterium *Bacillus pumilus* 15.1, a strain highly toxic toward the Mediterranean Fruit Fly *Ceratitis capitata*

In silico analysis of the genome of *B. pumilus* 15.1 for the detection of potential virulence factors

Part of this chapter has been published as:

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Garcia-Ramon, D., L. Palma, C. Berry, A. Osuna and S. Vilchez. "*In silico* analysis of the genome of *B. pumilus* 15.1 for the detection of potencial virulence factors" (under preparation).

Abstract

Bacillus pumilus strain 15.1 shows high toxicity against *Ceratitis capitata* first instar larvae when cultured and processed under very particular conditions. The study of this strain is of great interest not only from a scientific and practical point of view as it could represent a novel biotechnological approach for the biological control of this pest. In this chapter we present the complete draft genome of *B. pumilus* 15.1 that consists of 3,795,691 bp with a G+C content of 41.3%, and 3,776 predicted protein-coding genes. Despite the fact that this strain forms parasporal crystals during sporulation, its genome did not exhibit any homologous sequences with previously known crystal-forming genes such as *cry*, *cyt* or *bin* genes; therefore, its toxic activity may be potentially attributed to unknown toxins or other virulence factors. In this chapter potential virulence-associated determinants were identified *in silico* based on sequence similarity to known microbial virulence factors. Moreover, while analysing the *B. pumilus* 15.1 genome, two lysogenic phages were identified and their existence was demonstrated experimentally by the induction of the lytic cycle by an environmental stress.

Introduction

The pathogenicity of a strain is a pretty complex issue. Not only depends on the activity of the bacterial culture itself, but also the medium composition and the growing conditions (Ben Khedher *et al.* 2011; Devidas *et al.* 2014).

Entomopathogenic bacteria belonging to the *Bacillus* genus, particularly the well-known *Bacillus thuringiensis* (*Bt*), produce crystalline inclusions during sporulation, composed by δ-endotoxins, also called Cry and Cyt proteins. Although Cry and Cyt proteins are the main virulence factor (Schnepf *et al.* 1998), full virulence is not possible without the intervention of many other virulence factors synthesized by the bacteria that help to overcome barriers and host defenses. Raymond *et al.* (2010) described several of the processes that *Bt* must undertakes to infect the target insect, such as protecting themselves from the hostile environment, competing with other gut bacteria for food resources, finding the right conditions for spore germination and finally invading the target insect. To carry out all these processes it is necessary

the synthesis of virulence factors such as antimicrobial peptides, bacteriocins, phospholipases, enterotoxins, proteases (including metalloproteases), chitinases, enhancins and iron acquisition systems. Although this repertoire of genes is present in the genome of *Bt*, the exact contribution of each factor is often unknown. Some of the virulence factors expressed by *Bt* are Vip (vegetative insecticidal protein), Sip (secreted insecticidal protein), Bin-like (binary toxin), Mtx-like (mosquitocidal toxin), β exotoxin, a 41.9 kDa protein, sphaericolysin and alveolysin (Palma *et al.* 2014a). This is just a representative example of the amount of virulence factors that bacteria require to be entomopathogenic. Other bacteria species show different virulence factors with a completely different mode of action, as mentioned above in the introduction of this thesis.

Since the advent of the genomic era, one of the most powerful techniques for revealing the molecular tools that a microorganism has for invading its host is the sequencing and comparative analysis of its genome. Only few *B. pumilus* genomes have been previously published. In this work, we report the first genome sequence of an entomopathogenic *B. pumilus* strain. Here we present *B. pumilus* 15.1 genome annotation by comparative sequence analysis focusing on searching for potential virulence factors.

Materials and Methods

Genome sequencing and computational analysis

Total DNA from *B. pumilus* 15.1 was isolated using the Wizard® Genomic DNA Purification Kit (Promega) following the manufacturer's instructions for DNA isolation from Gram-positive bacteria. The purified DNA was then used to construct a pooled Illumina library and sequenced using a HiSeq 2000 Sequencing System (Illumina Sequencing) in a single read mode with a read length of 50 bases (GATC Biotech, Konstanz, Germany). The reads produced by sequencing were sent to "Era7 Bioinformatics" (Granada, Spain) for *de novo* assembly by Velvet software (Zerbino and Birney 2008). Then we reduced the contig numbers by iterative mapping using Geneious Pro R8 software (Drummond *et al.* 2014).

BankIt tool was used to submit the contigs to GenBank database (NCBI); the annotation was done using NCBI Prokaryotic Genome Annotation Pipeline (PGAP).

Once the sequence was accepted in the GenBank, the genome of the *B. pumilus* 15.1 was automatically included in the Genome-NCBI for *Bacillus pumilus* (http://www.ncbi.nlm.nih.gov/genome/?term=Bacillus%20pumilus%2015.1) and a comparative dendrogram (based on genomic BLAST) was constructed.

Nucleotide Sequences Accession Numbers

Draft genome sequences were deposited at DDBJ/EMBL/GenBank under the accession **LBDK00000000** (version LBDK00000000.1.) following the database standards for sequence submission.

Comparative analysis for searching virulence factors

The genome of *B. pumilus* strain 15.1 was analyzed in order to search for insecticidal toxins and virulence factors commonly found in entomopathogenic bacteria. The 63 assembled contigs were analyzed with BLASTX (Altschul *et al.* 1990) by using a customized database (Palma *et al.* 2014b) updated by us adding amino acid sequences of known toxins and virulence factors of the following entomopathogenic bacteria: *B. thuringiensis*, *Bacillus cereus*, *Lysinibacillus sphaericus*, *Paenibacillus larvae*, *Photorhabdus luminescens*, *Photorhabdus temperate*, *Xenorhabdus japonica*, *Xenorhabdus nematophila*, *Pseudomonas spp*., *Yersinia spp*. and *Serratia entomophila*; we also included sequences of antibacterial and antifungal antibiotics from *Bacillus subtilis* and *Bacillus amyloliquefaciens*. The best BLASTX hits from the contigs against the updated database were analyzed. Protein sequence alignments were done using ClustalW2 (Larkin *et al.* 2007).

Bacteriophage lysis induction and visualization

A pre-inoculum of *B. pumilus* 15.1 was prepared culturing the bacteria in LB medium at 30ºC and 240 rpm overnight. Pre-inoculum was diluted 1/100 in 25 ml polystyrene sterile tubes containing 5 ml, 7 ml and 10 ml of T3 or LB medium respectively. The cultures were incubated at 30ºC and 240 rpm for 72 h. Growth was

recorded every 24 h by determining optical density of the culture. The experiment was carried out in triplicate.

The lysed cultures were sent to the Biological Sample Preparation Laboratory at the Scientific Instrumentation Center of the University of Granada (CIC-UGR) for processing and negative staining. Briedly, 40 µl of lysated culture were placed on carbon-coated copper grid and incubed at room temperature for 5 min. The grid was washed in two drops of MiliQ water and subsequently incubed for 30 s on 1% uranyl acetate. Samples were observed under a Transmission Electron Microscopy (LIBRA 120 PLUS de Carl Zeiss SMT to 120 KV) in the Microscopy Service of the CIC-UGR.

Results and Discussion

Draft genome sequence of B. pumilus *15.1 strain*

The whole-genome sequencing yielded 26,322,535 reads. The reads were assembled in contigs by using the Velvet software (Zerbino and Birney 2008) with the *de novo* assembly tool and default parameters and produced 134 contigs. The first draft assembly version was improved, in order reduce the contig number, by iterative mapping using Geneious Pro R8 software (Drummond *et al.* 2014). The initial 26,322,535 reads were sequentially mapped again over the 134 contig sequences and a second assembly was performed. This process was repeated three times by using "map to reference" and "*de novo* assemble" tools included in Geneious Pro R8. Contig number was effectively reduced to 63 contigs (Table 1) with no significant changes in the total size of the genome. The NCBI Prokaryotic Genome Annotation Pipeline (PGAP) added the gene annotation. The details of the assembled and annotated genome sequence of *B. pumilus* strain 15.1 are described in Table 1.

Table 1. Genome features of *Bacillus pumilus* 15.1.

Attribute (or Feature)	Value
Genome size (bp)	3,795,691
Contig sequences	63
GC content (%)	41.3
Extrachromosomal elements	1*
Total genes	3,840
rRNA genes	2 (16S, 23S)
tRNA genes	16
Protein coding genes	3,776
nc RNAs	1
Pseudogenes	45

*Note: only one of the two extrachromosomal elements present in the bacterium has been detailed in the Table 1 since this small plasmid is the only element that has been entirely assembled.

B. pumilus 15.1 bears at least two extrachromosomal elements, one plasmid and one megaplasmid (Garcia-Ramon *et al.* 2015b). The plasmid sequence corresponds to contig 38 in the improved assembly. The plasmid was 7,785 bp in size with a GC content of 35.7%. The megaplasmid has not yet been assigned to any contig or contig sequences (data not shown), but it has been detected in total DNA extractions on agarose gels (Garcia-Ramon *et al.* 2015b). The *B. pumilus* 15.1 genome also contains prophage elements, even though a genome analysis revealed the presence of a CRISPR/Cas system (bacterial innate immune mechanism for protection from foreign DNA, including plasmids and phages) (Barrangou *et al.* 2007; Marraffini and Sontheimer 2008). Interestingly, during sporulation, *B. pumilus* 15.1 strain forms parasporal crystals that morphologically resemble to those produced by *B. thuringiensis* which are composed by Cry proteins (Garcia-Ramon *et al.* 2015a). The role of such crystalline inclusions in *B. pumilus* 15.1 has not yet been elucidated. No *cry*-like gene coding sequences were detected in the *B. pumilus* 15.1 genome but other genes encoding well-known entomopathogenic factors, such as chitinases (Cai *et al.* 2007), metalloproteases (Fedhila *et al.* 2002) and cytolysins (Nishiwaki *et al.* 2007) were detected.

The 21.8% of the coding sequences (825 proteins) were identified as hypothetical proteins; many of these proteins have no sequence similarity to any known proteins in the databases. Genomic comparisons of *B. pumilus* genomes available until date (30-08-2015) indicate that the strain 15.1 is closely related to *B. pumilus* SCAL1 strain (Figure 1). SCAL1 has been reported in the NCBI as a strain resistant to heat stress with plant growth promotion abilities. It is a root endophyte in tomato (*Lycopersicon esculentum*) that was isolated in Pakistan (BioProject accession PRJNA286914). Only 2 out of the 23 *B. pumilus* strains which their genomes are available in NCBI database, have been used as biologic control agents, the strains *B. pumilus* INR7 and *B. pumilus* 15.1. INR7 is a strain commercialized by Bayer Crop Science as a biocontrol product with capacity to elicit both induction systemic resistance, and plant growth promotion (Jeong *et al.* 2014). INR7 induce resistance against several plant pathogens, such as *Aspergillus*, *Penicillium*, *Fusarium* (Munimbazi and Bullerman 1998). *B. pumilus* 15.1 is the only strain with entomopathogenic properties.

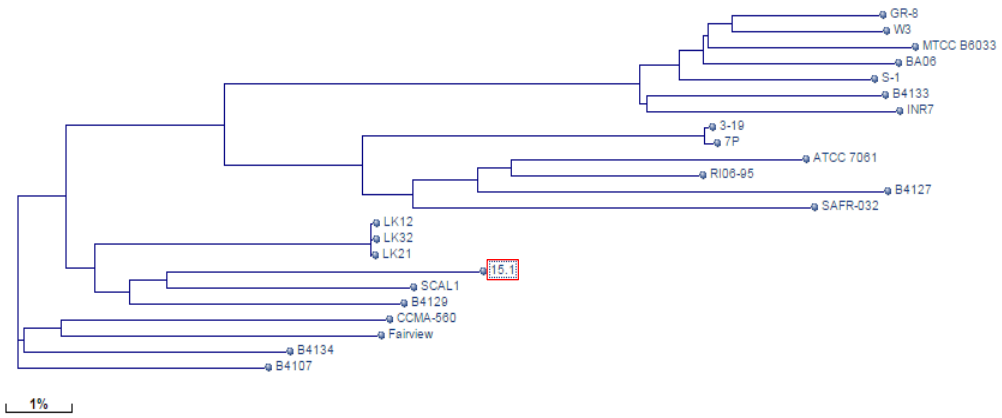

Figure 1. Dendrogram based on genomic BLAST of all *B. pumilus* genomes available in the NCBI genome database (August 2015). *B. pumilus* 15.1 is marked with a red rectangle.

Searching B. pumilus *15.1 genome for virulence factors*

Even though the genome annotation was carried out by the NCBI Prokaryotic Genome Annotation Pipeline (PGAP), we also analyzed the genome with BLAST (Altschul *et al.* 1990) using a customized insecticidal toxin database. To customize the search for virulence factors, we used a database constructed only with insecticidal proteins or virulence factors from bacteria available in public databases. The 63 contigs from *B. pumilus* 15.1 genome were Blasted against this database. A manual revision of the alignments was done.

Previously, we reported that the *B. pumilus* 15.1 produces parasporal crystals during sporulation (Garcia-Ramon *et al.* 2015a), that morphologically resemble to those produced by *Bacillus thuringiensis* and composed by Cry and Cyt proteins. However, in the genome of *B. pumilus* 15.1 there was not found any *cry*-like, *cyt*-like or *bin*-like gene. Common toxic proteins against insect from *Bacillus* genera, such as the mosquitocidal proteins Mtx1 and Mtx2, the secreted insecticidal protein Sip, and vegetative insecticidal proteins Vip1, Vip2 and Vip3, were also searched in the *B. pumilus* 15.1 genome. No significant homology with any of these virulence factors was detected. Nonetheless, homologies with other virulence factors were found. Taking into account the length of the sequence, the coverage of the alignment, the scores, the e-value and the identity percentage obtained, we selected the best alignments for further analysis. Table 2 shows the best hits obtained from the analysis. All of them showed an identity percent $\geq$ 50% and an alignment coverage ranging from 71.4% to 100%.

Table 2. List of proteins present in the genome of *B. pumilus* 15.1 similar to previously described virulence factors.

***B. pumilus* 15.1 Contig**	**Description protein reference**	**Genbank accession number**	**Length prot. ref.**	**Ident***	**Cover age**	**E-value**
18	Enolase [*Paenibacillus larvae*]	ETK29332.1	430	338/430 (78%)	100%	0.0
15	GroEL [*Xenorhabdus nematophila*]	AFW05298.1	548	303/525 (57%)	95.8%	0.0
11	Lipopeptide antibiotics Iturin [*Bacillus subtilis*]	BAM49288.1	165	84/158 (53%)	95.8%	2e-47
11	Surfactin synthase subunit 3 [*Bacillus amyloliquefaciens*]	CCP20389.1	1278	655/1275 (51%)	99.8%	0.0
53	Surfactin synthase subunit 1 [*Bacillus amyloliquefaciens*]	CCP20387.1	3584	1318/2567 (51%)	71.6%	0.0
41	Surfactin synthetase [*Bacillus amyloliquefaciens*]	CDG24630.1	3586	1291/2560 (50%)	71.4%	0.0

* Alignments with an identity percent ≥ 50%.

The two best hits obtained in the manual comparative analysis were Enolase and GroEL, both of them recently reported as virulence factors in insects (Joshi *et al.* 2008; Antunez *et al.* 2011; Kumari *et al.* 2014). In the search, we also include antibiotics as possible factors involved in the process of pathogenicity. As shown in Table 2, iturin and surfactins were found. Iturin and surfactin are lipopeptide antibiotics produced by bacteria that have a major role in the antagonism toward pathogenic fungi and others bacteria, so they are considered as biocontrol agents (Romero *et al.* 2007; Ji *et al.* 2013).

Enolase (phosphopyruvate hydratase, EC 4.2.1.11) is a enzyme catalyzing the conversion of 2-phospho-D-glycerate to phosphoenolpyruvate in the glycolytic pathway, and the reverse reaction in gluconeogenesis (Zhang *et al.* 1997). Traditionally, enolases are considered as cytoplasmic enzymes. However, several authors reported that enolases can be secreted or located on the cell surface so a possible function as virulence factor has been suggested (Bergmann *et al.* 2001; Lamonica *et al.* 2005; Antunez *et al.* 2010). In *Paenibacillus larvae*, the enolase production has been detected in vegetative cells, on the spores surface, secreted to the external growth medium and during the infection of honeybee larvae (Antunez *et al.* 2011). The spore-forming bacterium *P. larvae* is the causal agent of the American foulbrood, a widespread larval disease in honeybees, *Apis mellifera*. Antunez *et al.* (2011) evaluated the role of enolase during the infection of honeybee larvae and proved that enolase was highly toxic to honeybee larvae and immunogenic. Besides, they demonstrated that enolase from *P. larvae* was able to hydrolyze milk proteins, suggesting that it could be involved in larval tissue degradation, probably trough activating the plasmin(ogen) system.

In *B. pumilus* 15.1 genome, the enolase protein was located in contig 18 (GenBank KLK99626) and showed 78.6% amino acid identity with enolase (Eno) from *P. larvae* (GenBank ETK29332.1) (Figure 2).

Figure 2. Alignment of Enolase from *B. pumilus* 15.1 (GenBank KLK99626) and *P. larvae* (GenBank ETK29332.1).

GroEL is a member of the Hsp60 protein family. The heat-shock proteins (Hsp), also called stress proteins or molecular chaperones, are synthesized in response to environmental stress (Ranford *et al.* 2000). The 60 kDa chaperonins (Hsp60) are classified into two groups; Group I chaperonins are present in bacteria and in the endosymbiotic organelles of eukaryotes while Group II chaperonins are present in archaea and in the eukaryotic cytosols (Goyal *et al.* 2006). GroEL, that belongs to group I chaperonin, plays an important role in the folding of many proteins in the cell, under both normal and stress conditions (Ranford *et al.* 2000). It requires a 10 kDa co-chaperone protein called GroES, and ATP to carry out its function (Yoshida *et al.* 2001).

A homolog of GroEL has been reported in the symbiotic bacterium *Enterobacter aerogenes* (Yoshida *et al.* 2001), a bacterium that is present in the saliva of the parasitic antlion. When a culture of *E. aerogenes* is injected into cockroaches it

causes paralysis (Yoshida *et al.* 2001). Later, another report of the toxicity of GroEL was published (Joshi *et al.* (2008) demonstrating that GroEL is a virulence factor in *Xenorhabdus nematophila.* This protein is secreted in to the culture medium through membrane vesicles. XnGroEL showed oral insecticidal activity against *Helicoverpa armigera*. The insecticidal activity of GroEL was not related to its chaperoning activity, because no ATP or GroES was required. It showed a chitin binding ability, however, the exact mechanism of action has not been established yet (Joshi *et al.* 2008).

Recently, XnGroEL has been reported to confer resistance against *H. armigera* in transgenic tobacco (Kumari *et al.* 2014) and in tomatoes (Kumari *et al.* 2015). Also, it provided thermotolerance and protection against high salt concentration to the tomato plants (Kumari *et al.* 2015).

In our analysis, GroEL from *B. pumilus* 15.1 (GenBank KLK99918.1) share 60.48% sequence identity with GroEL from *X. nematophila* (GenBank AAN84781) (Figure 3). GroES was also found in the genome annotation of *B. pumilus* 15.1. It has been described as hypothetical protein (GenBank KLK99919.1) in contig 15. It has 47% identity with GroES from *X. nematophila* (Gen Bank ACA50470.1). There are no reports of GroES being involved in the toxicity. Interestingly, *X. nematophila* produces two no-toxic inclusions bodies, IP1 and IP2 (Couche and Gregson 1987). Only the gene encoding IP1, called *pixA*, has been identified (Goetsch *et al.* 2006); however, the PixA protein is not homologous with any sequence found in of *B. pumilus* 15.1. Both enolase and GroEL can be considered good candidates for the virulence of *B. pumilus* 15.1.

Figure 3. Alignment of GroEL from *B. pumilus* 15.1 (GenBank KLK99918.1) and *X. nematophila* (GenBank AAN84781).

Evidence of the presence of lysogenic phages in the genome of B. pumilus *15.1*

In the process of the comparative analysis of the *B. pumilus* 15.1 genome 27 CDS identified as phage proteins (phage transcriptional family protein, phage tail protein, phage portal protein, phage hydrolase, phage like protein, phage related protein, conserved phage protein and holing) were found. Phage proteins responsible for bacterial lysis include a holin, which function is associated with the collapse and permeabilization of the membrane of the host cell and an endolysin, that uses such permeabilization to reach and degrade the cell wall (Wang *et al.* 2000).

Experimental observation made in our group during the characterization of the strain *B. pumilus* 15.1 showed that cultures of the strain suddenly lyses when cells are grown under temperature or oxygen stress (Alarcón Morcillo 2011). This fact led us to consider the possibility that lysis could be produced by the induction of the lytic cycle of a lysogenic phage present in the genome of *B. pumilus* 15.1. To prove our hypothesis we induced the lysis of *B. pumilus* 15.1 by reducing the oxygen content in the culture medium. This was achieved by increasing the amount of medium in the growing tube. When the ratio medium:total volume of the tube was 1:5 the bacteria grew normally reaching an OD_{600nm} around 1.5. However, when the ratios were 1.4:5 and 2:5 the cultures grew normally reaching an OD_{600nm} of 1.5 but lysis was observed after 72 h and 48 respectively.

Samples from the lysate cultures (2:5 ratio grown for 72 h) were sent to Scientific Instrumentation Center of the University of Granada (CIC-UGR) to be negatively stained and observed by transmission electron microscopy. Bacteriophage particles were observed in all samples. Two kinds of bacteriophage particles were observed (Figure 4). The first type (Type 1 bacteriophage, Figure 4A and B), showed a small isomeric head with 31-35 nm in diameter, a neck with 36 nm in length, a contractile tail with 108-130 nm in length, and a tail needle with 63 nm in length. This type of phage was found more frequently in the samples. The second type (Type 2 bacteriophage, Figure 4C and D), showed a roundish icosahedral head with 51-56 nm in diameter and a tail with 90 -100 nm in length.

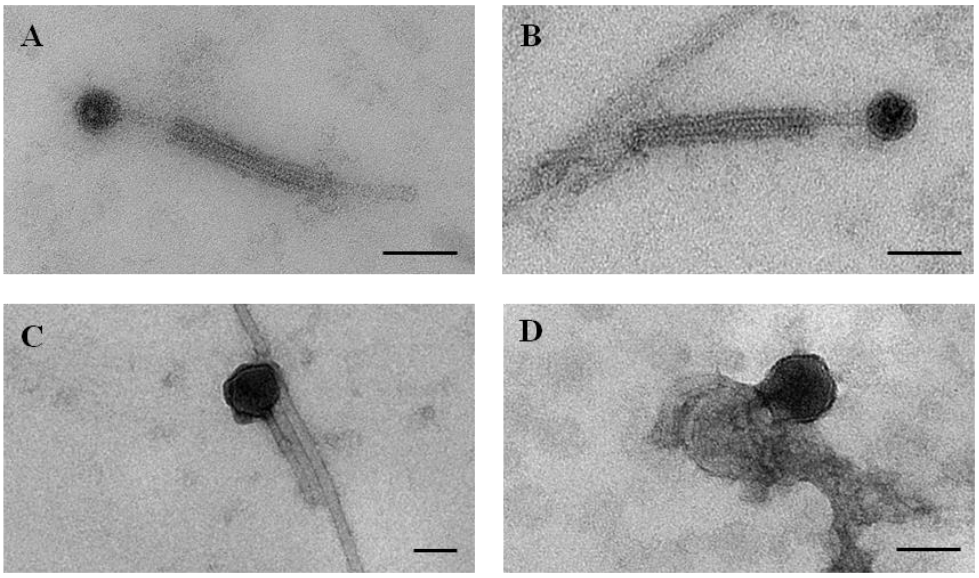

Figure 4. Transmission electron microscopy of type 1 (panels A and B) and type 2 (panels C and D) bacteriophage present in the lysates of a *B. pumilus* 15.1 culture obtained under oxygen restriction. Bars represent 50 nm.

The morphology of the type 1 bacteriophage from *B. pumilus* 15.1 resembles a phage particle obtained from *B. pumilus* GR8 recently reported by Yuan and Gao (2015). The phage in *B. pumilus* GR8 strain was inducible by mitomycin C and showed similar size of the phage components than *B. pumilus* 15.1 phage (Yuan and Gao 2015).

The study of phages in pathogenic bacteria is of great importance as the acquisition of virulence factors can be mediated by phage, converting a non-pathogenic bacterium to a pathogen (Gillis and Mahillon 2014). Moreover, several bacteriophages have been reported to increase or reduce bacterial virulence (Leon and Bastias 2015). Interestingly enough, a defective prophage from *Serratia entomophila* is considered one of the virulence factors in this bacterium. The antifeeding prophage, called Afp, morphologically resembles the sheathed tail of a bacteriophage (Heymann *et al.* 2013). Afp causes cessation of feeding; possibly this phage represent a novel toxin delivery system that utilizes a phage-type structure (Hurst *et al.* 2004). In the genome annotation of *B. pumilus* 15.1 strain, no homology with sequences of Afp was detected.

For now, the evidence showed that *B. pumilus* 15.1 strain has two lysogenic phages; however more experiments are needed in order to characterize the phages and their biological activities. These studies are being currently conducted.

Conclusions

In this work, *de novo* sequencing, a genome annotation pipeline, and the construction and analysis of customized database sequences, allowed us the rapid identification of possible virulence factors present in the *B. pumilus* 15.1 genome. The search revealed the presence of Enolase and GroEL proteins, which have been shown toxicity toward insects in *P. larvae* and *X. nematophila* respectively. On the other hand, the *B. pumilus* 15.1 annotated genome showed phage related proteins. Here, we demonstrated experimentally the presence of at least two types of bacteriophages in the genome of *B. pumilus* 15.1 which lytic cycles can be induced by reduction of oxygen in the culture medium.

These results represent an important advance in understanding the potential mechanism behind the toxicity and virulence of *B. pumilus* 15.1 against the Mediterranean fruit fly.

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4. DISCUSIÓN GENERAL

En el área de control biológico, una meta frecuente de los investigadores es la búsqueda de nuevas cepas bacterianas capaces de controlar a los insectos plaga de forma específica y eficaz. Esto incluye el descubrimiento de los factores tóxicos de la cepa y el modo de acción de los mismos. Bajo esta premisa, nuestro grupo de investigación describió el aislamiento de una nueva cepa entomopatógena activa frente a la mosca de la fruta del Mediterráneo *Ceratitis capitata* (Molina *et al.* 2010); tras este descubrimiento el nuevo objetivo que se planteó fue la caracterización de la cepa *B. pumilus* 15.1 en busca de potenciales factores de virulencia que pudieran explicar su actividad.

A pesar de que *B. pumilus* es una bacteria ubicua y que ha mostrado tener características relevantes como la resistencia a ambientes extremos (Benardini *et al.* 2003; Gioia *et al.* 2007) y la producción de enzimas de importancia industrial (Jaouadi *et al.* 2008; Reiss *et al.* 2011; Nagar *et al.* 2013; Kumar *et al.* 2014), existe relativamente poca información sobre esta especie. Por lo que, como primer objetivo de este trabajo, nos planteamos la caracterización a nivel bioquímico, microbiológico y morfológico de la cepa *B. pumilus* 15.1. De los resultados obtenidos en esta caracterización básica caben destacar dos características relevantes de la cepa *B. pumilus* 15.1 *i*) su elevada resistencia a la radiación UV-C (254 nm), en especial la sorprendente resistencia que mostraron las células vegetativas; y *ii*) la formación de inclusiones cristalinas sintetizadas en el proceso de esporulación.

Posiblemente la cepa más estudiada de *B. pumilus* sea la cepa SAFR-032, que destaca entre otras cosas por la gran resistencia que presentan sus células a diferentes tipos de estrés, en especial a la radiación UV (Link *et al.* 2004; Newcombe *et al.* 2005). La resistencia de *B. pumilus* 15.1 a la luz UV-C fue muy superior que la mostrada por *B. subtilis* 168 y las cepas de *Bt* ensayadas. Dicha resistencia podría suponer una ventaja desde el punto de vista práctico en la aplicación en campo, ya que resolvería uno de los problemas que presenta *Bt* y su inactivación con la luz UV. Comparando con los datos reportados por Gioia *et al.* (2007), las esporas de *B. pumilus* 15.1 son resistentes a la radiación UV prácticamente a los mismos niveles que *B. pumilus* SAFR-032; mientras que, las células vegetativas de *B. pumilus* 15.1 fueron más resistentes que las células vegetativas de SAFR-032 según los datos reportados por Newcombe *et al.* (2005).

Cuando se aplicó la mayor dosis de UV (4,911.1 J/m^2) en un cultivo con células vegetativas se obtuvo una reducción de 5 órdenes de magnitud, sin embargo aún quedaron células viables (un 0.00065%); por el contrario las células vegetativas de *B. subtilis* 168 no sobrevivieron a la más alta radiación ensayada. Nuestros datos de supervivencia para las células vegetativas de *B. subtilis* 168 fueron mucho más altos que los reportados por Newcombe *et al*. (2005) cuyos resultados mostraron que las células vegetativas de esta cepa no fueron capaces de sobrevivir a dosis superiores a 50 J/m^2, mientras que en nuestros ensayos las células vegetativas de *B. subtilis* 168 resistieron hasta 1,637 J/m^2 con un 0.001% de supervivencia. A diferencia de lo reportado por Newcombe *et al*. (2005), otros estudios sobre la resistencia de *B. subtilis* 168 han mostrado una supervivencia del 10% de las células vegetativas expuesta a radiaciones de 60 J/m^2 (Setlow 2001) y 80 J/m^2 (Nicholson *et al.* 2000).

Aunque desconocemos el proceso implicado en la resistencia de *B. pumilus* 15.1, posiblemente el hecho de que las células vegetativas permanezcan viables pueda explicarse por la formación de agrupaciones entre las células como los que se mostraron en estudios de microscopía (Figura 3 del Capítulo I), donde las bacterias que están dentro del enjambre (posiblemente formado por exopolisacáridos) podrían estar protegidas del efecto de la radiación. De hecho, Mir *et al*. (1997) haciendo ensayos de desinfección del agua utilizando cloro observaron que las células de *Bacillus mycoides* eran resistentes al tratamiento y que la bacteria formaba agregaciones más grandes cuando se la ponía en contacto con el cloro, por lo que plantearon que la agregación bacteriana podría ser una estrategia para sobrevivir en condiciones adversas.

Hasta donde llega nuestro conocimiento, esta es la primera vez que se describe una cepa de *B. pumilus* capaz de producir cristales paraesporales. Morfológicamente, estos cristales nos recuerdan a los formados por las proteínas Cry y Cyt producidas por especies entomopatógenas del género *Bacillus*, entre estas *Bt* (Bechtel and Bulla 1976), *L. sphaericus* (Kalfon *et al.* 1984), *B. laterosporus* (Hannay 1957; Montaldi and Roth 1990) y *P. popilliae* (Weiner 1978); los cuales son considerados como los factores de virulencia responsables de la muerte de los insectos. Mediante microscopía electrónica de transmisión se siguió el proceso de esporulación de *B. pumilus* 15.1, en donde se pudo observar que las inclusiones cristalinas se formaron

dentro de la célula junto a la espora y que fueron liberadas con la ruptura del esporangio, al igual que está descrito en *Bt* (Bechtel and Bulla 1976). Debido a la relación filogenética existente entre las dos especies, hipotetizamos que los cristales producidos por *B. pumilus* 15.1 podrían estar relacionados con la patogenicidad de la cepa frente a las larvas de *C. capitata*.

En *Bt*, la mayoría de cristales paraesporales están codificados en plásmidos. Con mayor frecuencia los genes *cry* están localizados en megaplásmidos (Berry *et al.* 2002; Murawska *et al.* 2013); sin embargo hay genes *cry* que pueden residir en plásmidos pequeños (Loeza-Lara *et al.* 2005; Guo *et al.* 2008). *B. pumilus* 15.1 presentó al menos dos elementos extracromosómicos: un plásmido pequeño, pBp15.1S, y un megaplásmido, pBp15.1B. Tras subclonar, secuenciar y realizar un análisis comparativo del plásmido pBp15.1S, se encontró que ninguno de los 11 ORFs putativos presentes codificaba para alguna proteína conocida relacionada con la toxicidad. En la secuencia se encontró homología con el módulo típico de la replicación por círculo rodante (RCR), el resto de ORF fueron asignados como proteínas hipotéticas, por lo que podemos decir que se trata de un plásmido críptico, hecho muy frecuente en la mayoría de los plásmidos encontrados en cepas de *B. pumilus* (Zhang *et al.* 2010; Garcia-Ramon *et al.* 2015b).

Sabiendo que pBp15.1S no tiene secuencias similares a las que codifican los cristales paraesporales reportados como tóxicos, ensayamos diferentes métodos de eliminación (también llamado curación) de plásmidos para ver si existía relación entre los plásmidos y la producción de inclusiones proteicas. A partir de que Gonzalez *et al.* (1981) obtuvieran mutantes acristalíferas de *Bt* eliminando los elementos extracromosómicos y comprobando que estas cepas carecían de toxicidad, la técnica de curado de plásmido es una estrategia común para iniciar el estudio de la genética de los cristales paraesporales. Al no conseguir resultados con las técnicas reportadas en la bibliografía (Ward and Ellar 1983; Mahillon *et al.* 1988; Ghosh *et al.* 2000; Molnar *et al.* 2003), aplicamos una nueva estrategia basada en la formación de esferoplastos en un paso previo a la aplicación de los compuestos naranja de acridina y promethazine, consiguiendo eliminar los plásmidos de *B. pumilus* 15.1 de forma exitosa. La cepa libre de elementos extracromosómicos, denominada *B. pumilus* 15.1C, también produjo cristales paraesporales

morfológicamente iguales a los producidos por la cepa nativa, lo cual implicó que los cristales paraesporales producidos por la cepa *B. pumilus* 15.1 son de codificación cromosómica. En el caso de los genes *cry*, la literatura describe que la mayoría de estos genes están localizados en plásmidos, aunque con menos frecuencia, insertados en el propio cromosoma bacteriano (Klier *et al.* 1982; Barloy *et al.* 1996; Zhang *et al.* 1997), por lo que el hecho de que la cepa *B. pumilus* 15.1C produjera cristales paraesporales no nos alejaba de la idea de poder relacionarlos con los genes *cry*.

En el análisis de los perfiles proteicos de las cepas *B. pumilus* 15.1 y *B. pumilus* 15.1C destacó la expresión una proteína de 45 kDa en el pellet del cultivo esporulado. Esta proteína fue parcialmente separada en un gradiente de sacarosa. Mediante microscopía se comprobó que en las fracciones que contenían la proteína de 45 kDa estaban retenidos los cristales paraesporales de la cepa. Utilizando la misma técnica de separación, muchas proteínas Cry han sido parcialmente purificadas; incluso muchas de ellas han sido retenidas en la interfase 72%/79% del gradiente de sacarosa (Thomas and Ellar 1983; Koller *et al.* 1992; Jones *et al.* 2007; Swiecicka *et al.* 2008) misma fracción en la cual la mayoría de cristales de *B. pumilus* 15.1 y *B. pumilus* 15.1C estuvieron presentes. Este resultado nos sugirió que los cristales paraesporales podrían estar compuestos por la proteína de 45 kDa.

La caracterización de la proteína de 45 kDa, que en principio fue obtenida como una proteína insoluble, mostró que se solubilizaba parcialmente al incubarla a -20ºC y quedaba en el sobrenadante de la suspensión de cristales tras la congelación. Este sorprendente fenómeno de solubilización en frío fue demostrado experimentalmente mediante la obtención de un nuevo gradiente y el seguimiento de su solubilización en condiciones de frío o a temperatura ambiente.

La solubilización de proteínas cuando son congeladas a -20ºC es un fenómeno que hasta donde llega nuestro conocimiento no ha sido descrito anteriormente. Normalmente cuando se requiere solubilizar una proteína, precisamente lo que se hace es aumentar su temperatura con el objetivo de aumentar su solubilidad. Pese a ser un fenómeno extraño, que por supuesto será caracterizado en profundidad en un futuro, no es un dato demasiado incongruente en el contexto de *B. pumilus* 15.1. Estudios anteriores de esta cepa (Molina *et al.* 2010) realizados por nuestro grupo de

investigación mostraron que la toxicidad de *B. pumilus* 15.1 únicamente se ponía de manifiesto cuando se bioensayaban cultivos que previamente debían ser incubados a bajas temperaturas (4 o -20ºC) durante al menos 4 días. Este hecho, para el cual no pudimos dar una explicación empírica en su momento, podría ahora estar soportado con esta nueva evidencia. Podemos hipotetizar que si los cristales observados en *B. pumilus* 15.1 son los responsables de la toxicidad en *C. capitata*, quizás sea necesaria la solubilización de los mismos para poder ejercer su mecanismo de acción, tal y como ocurre con los cristales proteicos producidos por *Bt*.

Otro hecho que recuerda al mecanismo de acción de las toxinas Cry es que la proteína de 45 kDa, una vez solubilizada fue resistente a la acción de las proteasas tripsina y quimotripsina. Existen varios ejemplos de proteínas resistentes a tripsina, incluso proteínas que tienen en sus secuencias los aminoácidos lisina y arginina (sitios diana de la tripsina) pueden ser resistentes a la proteólisis si la proteína está fuertemente plegada (Saveliev *et al.* 2013). Algunas proteínas Cry, Cyt, Vip o Sip al necesitar ser activadas por proteasas tienen un núcleo resistente a tripsina que es la parte tóxica de la proteína (Palma *et al.* 2014), la cual no puede ser digerida por las proteasas en el intestino de los insectos. La proteína de 45 kDa permaneció intacta al tratamiento con tripsina e incluso fue observada en tratamientos con 500 veces más proteasa que proteína, tal y como ocurre con el "*core*" de las toxinas Cry. Este hecho parece indicar que la proteína de 45 kDa podría resistir a la proteólisis en el intestino de dípteros cuyas proteasa digestivas son de tipo serin proteasas (de Maagd *et al.* 2001).

Sin embargo, pese a todas estas similitudes con las toxinas Cry, ningún gen con homología a los genes *cry* fue encontrado en el genoma de *B. pumilus* 15.1. Además, la identificación mediante fingerprinting de la proteína de 45 kDa obtenida en distintas condiciones siempre rindió el mismo resultado: los cristales parecieron estar formados por la enzima Oxalato descarboxilasa.

La secuenciación del extremo N-terminal de la proteína de 45 kDa solubilizada y tripsinizada también rindió como resultado una secuencia (SEKPNGIP) idéntica a la secuencia de la proteína predicha como oxalato descarboxilasa en el genoma de *B. pumilus* 15.1 (protein_id KLL01117.1) sin el iniciador metionina. Mientras que la secuencia (SEKPDGIP) no se encontró en las bases de datos. Pese a todas estas

evidencias, hasta donde llega nuestro conocimiento no existe descrita ninguna oxalato descarboxilasa que sea resistente a la tripsina, aunque esta proteína pertenece a la superfamilia de las cupinas, la cual tiene miembros no enzimáticos, dentro de los que se encuentran proteínas de reserva de las semillas que son resistentes a la proteólisis (Dunwell *et al.* 2000).

Se desconoce por qué la proteína de 45 kDa no presenta una metionina como aminoácido en primera posición. Quizás la proteína de 45 kDa haya sufrido un procesamiento post-traduccional por alguna proteasa intracelular endógena de *B. pumilus* 15.1. En *Bt* se ha descrito la producción de proteasas intracelulares especialmente al inicio de la esporulación y formación del cristal (Oppert 1999), que son capaces de hidrolizar las protoxinas recién sintetizadas o procesarlas en el cristal paraesporal y que además la acción de estas proteasas endógenas puede estar implicada en la especificidad de la toxina frente a un insecto determinado (Suresh Kumar and Venkateswerlu 1997).

Pese a los avances realizados en la caracterización de la proteína de 45 kDa se necesitan nuevos análisis que nos ayuden a identificarla y caracterizarla mejor. A pesar de que en este trabajo no se la pudo relacionar con la toxicidad de la cepa, ya que los cristales por sí solos no mostraron toxicidad en los bioensayos realizados, seguimos pensando que puede representar uno de los factores de virulencia más relevantes de la cepa *B. pumilus* 15.1, debido a hechos como que se produce en muy alta cantidad, forma cristales paraesporales, es resistente a la acción de proteasas, y se solubiliza cuando es sometida al frío, justo cuando la cepa muestra su toxicidad. Además, la proteína presenta características poco comunes que merecen la pena ser investigadas.

Dado que la proteína de 45 kDa, que forma parte de los cristales paraesporales, no presentó homología con ningún factor de virulencia conocido se emprendió la búsqueda de potenciales factores de virulencia en el genoma de *B. pumilus* 15.1. Tras obtener la secuencia genómica de la cepa mediante la técnica de *shotgun* y su ensamblaje en 63 contigs, fue anotada y depositada en GenBank (Garcia-Ramon *et al.* 2015a). Para alcanzar uno de los objetivos de esta tesis, se creó una base de datos personalizada con los factores de virulencia de cepas entomopatógenas y se realizó una búsqueda en el genoma de *B. pumilus* 15.1. No se encontró homología con

ninguna de las proteínas que conforman los cristales paraesporales de las cepas entomopatógenas, ya sean toxinas Cry, Cyt o Bin; por lo que no se pudo inferir la composición de los cristales ni su posible función en la cepa mediante estos estudios comparativos. Recientemente, Lin *et al.* (2015) describieron la producción de un cristal paraesporal (proteína PC) en *B. bombysepticus* que es letal para el gusano de la seda (*Bombix mori*); no hemos encontrado la secuencia de aminoácidos de la proteína PC por lo que no hemos podido buscar similitud con las secuencias en el genoma de *B. pumilus* 15.1. Sin embargo, los autores de ese trabajo aseguran que la secuencia de PC muestra menos del 11.4% de similitud con toxinas formadoras de poro de bacterias, por lo que la consideran una toxina diferente a las descritas hasta el momento, aunque comparte características del modo de acción de las toxinas Cry (Lin *et al.* 2015). Es posible que la proteína que conforma los cristales paraesporales de *B. pumilus* 15.1 sea nueva, y al igual que la proteína PC no tenga similitud de secuencia con proteínas descritas y por eso no haya sido identificada mediante huella peptídica ni en la anotación del genoma.

Las bacterias patógenas de insectos de diversos grupos taxonómicos y orígenes filogenéticos han mostrado tener notable similitud en los factores de virulencia que producen; esto se puede deber a que estos factores de virulencia generalmente están codificados en elementos genéticos móviles como plásmidos y bacteriófagos, por lo que puede existir transferencia horizontal (Castagnola and Stock 2014). Por esta razón, la base de datos creada contuvo factores de virulencia de bacterias Gram-positivas y Gram-negativas. En la búsqueda en esta base de datos encontramos que *B. pumilus* 15.1 presentó alta similitud de secuencia con una Enolasa de *P. larvae* y con GroEL de *X. nematophila*, dos proteínas recientemente descritas como altamente tóxicas para sus insectos diana (Joshi *et al.* 2008; Antunez *et al.* 2011). También se encontraron factores de virulencia que han sido descritos como una parte importante en el proceso global de virulencia, bien por favorecer la invasión del hospedador o bien por debilitar las defensas del mismo; tales como la quitinasa (Cai *et al.* 2007), metaloproteasa (Fedhila *et al.* 2002; Ishii *et al.* 2014), citolisina (Nishiwaki *et al.* 2007) y antibióticos (Nagorska *et al.* 2007).

En este trabajo se describió también la existencia de al menos dos fagos lisogénicos en la cepa *B. pumilus* 15.1, sin embargo la caracterización de los mismos

no formó parte de los objetivos de esta tesis. Consideramos que el estudio de los fagos de la cepa entomopatógena *B. pumilus* 15.1 es importante debido a la relación de los bacteriófagos con los factores de virulencia. Numerosos factores de virulencia codificados en profagos son los causantes directos de enfermedades como el botulismo, la dipteria o el cólera en humanos (Abedon and Lejeune 2005). A pesar de que los factores de virulencia codificados en profagos que infectan a organismos eucariotas superiores sean los más conocidos y estudiados, investigaciones recientes han mostrado que los profagos pueden estar involucrados también en la toxicidad de las cepas bacterianas frente a insectos; ya sea porque expresan factores de virulencia como es el caso del profago Afp de *S. entomophila* (Hurst *et al.* 2004), o porque causan reducción de la virulencia como es el caso del bacteriófago PPV que reduce la toxicidad de *S. marcescens* frente a larvas del lepidóptero *Parasemia plantaginis* (Friman *et al.* 2011).

De manera general, la virulencia es un mecanismo complejo dirigido por múltiples factores que actúan secuencial o sinérgicamente para conseguir su objetivo. Raymond *et al.* (2010) publicaron un artículo en el que se describe todo el proceso que debe llevar a cabo *Bt* en la infección y colonización de su insecto diana así como el arsenal de factores que necesita expresar para conseguir su objetivo. A pesar de que *Bt* es la especie en la que se han centrado la mayoría de los esfuerzos de la comunidad científica e industrial (Ruiu *et al.* 2013) aún no se conoce completamente el mecanismo de su virulencia.

Pocas veces *B. pumilus* ha sido descrita por su actividad entomopatógena (Heins *et al.* 1999; Ertürk *et al.* 2008; Molina *et al.* 2010; Yaman *et al.* 2010). La información que se tiene sobre los factores tóxicos de esta bacteria frente a insectos es escasa, no se sabe cuál es el factor de virulencia ni el modo de acción; y tampoco ha habido continuidad en la investigación de estos informes. Hasta donde llega nuestro conocimiento, esta es la primera vez que una cepa de *B. pumilus* con actividad entomopatógena es caracterizada a nivel bioquímico, microbiológico, morfológico, molecular y genético y aunque mucho se avanzado, muchas preguntas quedan aún por contestar. En esta tesis doctoral se describe lo que hasta hoy conocemos sobre la novedosa cepa *B. pumilus*15.1.

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5. CONCLUSIONES/CONCLUSIONS

De los resultados obtenidos en el presente trabajo se pueden extraer las siguientes conclusiones:

1. Las células vegetativas y las esporas de la cepa *B. pumilus* 15.1 son altamente resistentes a la radiación UV-C.

2. *B. pumilus* 15.1 posee al menos dos elementos extracromosómicos el plásmido pBp15.1S y el megaplásmido pBp15.1B. El plásmido pBp15.1S, un plásmido críptico de 7,785 pb que se encuentra en 33 copias de plásmido por célula, presenta un mecanismo de replicación en círculo rodante y ningún factor de virulencia conocido hasta el momento. La secuencia del megaplásmido está aún por identificar.

3. La cepa *B. pumilus* 15.1 produce cristales paraesporales que son de codificación cromosómica. Estas inclusiones cristalinas se forman dentro de la célula junto a la espora y son liberadas con la ruptura del esporangio. Dichos cristales paraesporales no son tóxicos por sí solos para larvas de primer estadio de *C. capitata* al menos bajo las condiciones ensayadas.

4. La cepa *B. pumilus* 15.1 sobreproduce una proteína de 45 kDa durante el proceso de esporulación, proteína que ha sido relacionada con los cristales paraesporales producidos por la misma. Esta proteína se solubiliza a bajas temperaturas y es resistente a la digestión por tripsina tal y como ocurre con las toxinas Cry de *B. thuringiensis*. La proteína ha sido identificada mediante huella peptídica y secuenciación del extremo N-terminal como una Oxalato descarboxilasa.

5. El genoma de *B. pumilus* 15.1 fue ensamblado en 63 contigs y presentó un total de 3,795,691 pb y un 41.3% de contenido en GC. En la anotación del genoma se generó un total de 3,776 proteínas predichas.

6. Los análisis comparativos de las secuencias obtenidas indicaron que *B. pumilus* 15.1 posee en su genoma secuencias similares a la Enolasa de *P. larvae* y a GroEL de *X. nematophila*, factores de virulencia para estas bacterias frente a sus insectos diana.

7. La actividad hemolítica, la producción de proteasas, la formación de biopelículas, la motilidad y la presencia de quitinasas, citolisinas, metaloproteasas y antibióticos en el genoma de *B. pumilus* 15.1 son factores a tener en cuenta en la virulencia de la cepa, ya que representan importantes factores de virulencia en otras cepas bacterianas.

The results obtained in this thesis led us to make the following conclusions:

1. The vegetative cells and the spores of *B. pumilus* 15.1 strain are highly resistant to UV-C radiation.

2. *B. pumilus* 15.1 bears at least two extrachromosomal elements, the plasmid pBp15.1S and the megaplasmid pBp15.1B. The pBp15.1S is a cryptic plasmid of 7,785 bp in size and 33 copies per chromosome. The pBp15.1S plasmid is replicated by the small rolling circle mechanism and does not codify for any known virulence factor. The sequence of the megaplasmid is still undetermined.

3. *B. pumilus* 15.1 produces parasporal crystals that show chromosomic codification. These crystalline inclusions are formed inside of the cell, together with the spore, and are released after the rupture of the sporangium. The parasporal crystals produced by *B. pumilus* 15.1 are not toxic for the first-instar larvae of *C. capitata* on their own, at least under the assayed conditions.

4. The *B. pumilus* 15.1 strain overexpresses a protein of 45 kDa during sporulation. This protein has been related with the parasporal crystals produced by the strain. The 45 kDa protein is solubilized at low temperature and is resistant to trypsin digestion as Cry toxins from *B. thuringiensis*. The protein has been identified by fingerprinting and the N-terminal sequencing as an Oxalate decarboxylase.

5. The *B. pumilus* 15.1 genome was assembled in 63 contigs showing 3,795,691 bp in total with a GC content of 41.3%. The genome annotation contains 3,776 predicted proteins.

6. The comparative analyses of the genome sequences showed that *B. pumilus* 15.1 have homologues to Enolase from *P. larvae* and GroEL from *X. nematophila*, which have shown toxicity toward target insects.

7. The hemolytic activity, the proteases production, the biofilm formation, the motility, and the presence of chitinases, cytolysins, metalloproteases and antibiotics in the *B. pumilus* 15.1 genome are factors worth taking into account as virulence factors, due to they represent important virulence factors in others bacteria strains.