ANTIMICROBIAL ACTIVITY IN BACILLUS SPP. FROM PLANT ENVIRONMENTS AGAINST PLANT PATHOGENS. RELATIONSHIPS WITH CYCLIC LIPOPEPTIDE GENES AND PRODUCTS

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PhD Thesis

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Dirigida per:

Dr. Emilio Montesinos i Seguí

Dr. Jordi Cabrefiga i Olamendi

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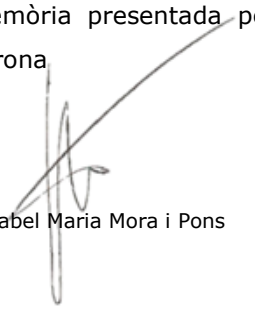

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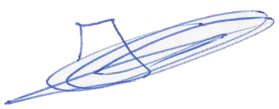

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DECLAREM:

Que el treball titulat "Antimicrobial activity in *Bacillus* spp. from plant environments against plant pathogens. Relationships with cyclic lipopeptide genes and products", que presenta Isabel Maria Mora i Pons per a l'obtenció del títol de doctora per la Universitat de Girona, ha estat realitzat sota la nostra direcció.

I, perquè així consti i tingui els efectes oportuns, signen aquest document a Girona, el 13 de Juny de 2013.

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Anna Bonaterra i Carreras, Professora Titular de l'Àrea de Producció Vegetal de la Universitat de Girona, i directora dels projectes de recerca "Control biotecnológico del fuego bacteriano. Utilización de cepas bacterianas Gram-positivas productoras de bacteriocinas y ciclolipopeptidos." (Ref. AGL2009-13255-C02-01/AGR) i "Nuevas estrategias de control del fuego bacteriano. Bacterias del ácido láctico productoras de bacteriocinas (BALFUEBA)" (Ref. AGL2012-39880-C02-01) de la Comisión Interministerial de Ciencia y Tecnologia, en els que es circumscriu la tesi doctoral titulada "Antimicrobial activity in *Bacillus* spp. from plant environments against plant pathogens. Relationships with cyclic lipopeptide genes and products" amb el número de registre 1235 de dia 15-07-2010 realitzada per **Isabel Maria Mora Pons**,

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Aquesta tesi està sotmesa a la propietat intel·lectual compartida amb els investigadors del grup de Patologia Vegetal i del Institut de Tecnologia Agroalimentària de la Universitat de Girona que participen en els esmentats projectes (Article 2. Apartat 2. RD 1326/2003 de 24-10-2003; Llei de la Propietat Intel·lectual, RD 1/1996 de 12-04-1996)

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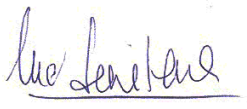

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Publications

Mora, I., Cabrefiga, J. and Montesinos, E. 2011. Antimicrobial peptide genes in *Bacillus* strains from plant environments. Int Microbiol. 14:213-223.

Abbreviations

ACP	Acyl-carrier-protein domain
AHL	N-acyl-L-homoserine
AMP	Antimicrobial peptide
AT	Acyltransferase
AUGC	Area under the growth curve
BCA	Biocontrol agent
bp	Base pair
cfu	Colony-foming units
CHCA	α-cyano-4-hydroxycinnamic acid
cLP	Cyclic lipopeptide
D	Decimal reduction time
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EFSA	European Food Safety Authority (EU)
ESI-MS	Electrospray ionization-mass spectrometry
f.w.	Fresh weight
FDA	Food and Drug Administration (USA)
GAI	Global activity index
GRAS	Generally recognized as safe
IR	Induced resistance
ISR	Induced systemic resistance
JA	Jasmonic acid
kb	Kilobase
Kd	Death constant
KS	Ketosynthase
LAR	Local acquired resistance
LB	Lysogeny broth
LP	Lipopeptide
MALDI-TOF	Matrix-assisted laser desorption/ionization time-of-flight
mAU	Mili-absorbance unit

MLTS	Multilocus sequence typing
m/z	Mass divided by charge
NA	Nutrient agar
NB	Nutrient broth
nm	Nanometer
NRP	Non-ribosomal peptide
NRPS	Non-ribosomal peptide synthetase
OD	Optical density
ORF	Open reading frame
QPS	Qualified presumption of safety
PCR	Polymerase chain reaction
PM	Production medium
PDA	Potato dextrose agar
PDB	Potato dextrose broth
PGPR	Plant growth promoting rhizobacteria
PK	Polyketides
PKS	Polyketide synthetase
PVP	Polyvinylpyrrolidone
RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
Rif^+	Rifampicin resistant
RNA	Ribonucleic acid
SA	Salicylic acid
SAR	Systemic acquired resistance
SDS	Sodium dodecyl sulfate
TFA	Trifluoroacetic acid
Tris	Tris(hydroxymethyl)aminomethane

List of Tables

List of Figures

Table of Contents

Summary

Bacillus is one of the most well-studied and characterized genus of Gram-positive bacteria. The interest in this genus is due to its ability to form endospores, and to produce metabolites of interest in the agronomic, pharmaceutical and industrial fields. *Bacillus* is a complex genus at the genotypic, phenotypic, metabolic, taxonomic, and ecologic level, allowing them to be very versatile in different environments, especially in soil. In agriculture, *Bacillus subtilis* and related species have strong interest because it has been recognized as 'Generally Recognized as Safe' (GRAS) by the Food and Drug Administration (FDA, USA) and 'Qualified Pressumption of Safety' (QPS) by the European Food Safety Authority EFSA, EU) and several strains are biocontrol agents or biofertilizers. Its ability to sporulate allows to prepare formulations to increase shelf-life of biopesticide products. The production of secondary metabolites with antimicrobial properties is a key mechanism in the control of fungal and bacterial plant diseases. This applied interest has result in the study of several complete genomes of *Bacillus subtilis* and related species which have identified genes involved in metabolic pathways of products related with the biocontrol of plant pathogens, as well as their involvement in the ecology of these bacteria.

The present PhD thesis deal with isolation of *Bacillus* from plant environments, and their characterization, with the main objective of determinating relationships between antimicrobial activity, genes and products. It is expected that this knowledge could contribute to improve the efficiency of screening procedures to find better and novel BCAs.

In this work, we have optimized, verified and validated PCR tools that allowed a specific detection of *Bacillus* in natural samples by the amplification of *16S rDNA* gene with high sensitivity (10^2 cfu/ml), and of six genes of antimicrobial peptides biosynthetic pathways, specifically of the surfactin (*srfAA*), bacilysin (*bacA*), bacillomycin (*bmyB*), fengycin (*FenD*),

subtilin (*spaS*) and iturin (*ituC*). The sensitivity of primers designed for the detection of the AMP genes depends on the strain, and PCR detection of *ituC* and *fenD* genes showed a high sensitivity, with values of $5x10^2$ cfu/ml, while the lowest sensitivity was observed for *spaS* gene , with values above $1x10^4$ cfu/ml. In addition, the prevalence of genes *srfAA*, *bacA*, *bmyB* and *fenD* has been determined in reference strains, and a multiplex PCR for these genes was designed, optimized and validated. However, the sensitivity of this tool was reduced in comparison to simple PCR, with sensitivity values of $1x10^5$ cfu/ml. In order to obtain optimum results using the PCR tools it was necessary the optimization of a selective enrichment procedure for *Bacillus* from natural samples. This enrichment procedure allowed us to increase the *Bacillus* populations in sample extracts up to detectable levels by PCR. The selective enrichment procedure consisted of a heat treatment at 80 °C for 10 min followed by a stage of enrichment at 40 °C for 24 h, with a previous dilution to 1:100 of the extract before to be enriched in order to remove potential PCR inhibitors from the natural sample. The application of the selective enrichment procedure followed by PCR allowed increase the detection and isolation of population levels of *Bacillus*, especially in samples of aerial plant parts with undetectable *Bacillus* with the standard procedure of isolation. The detection of AMP genes *srfAA*, *bacA*, *bmyB*, *fenD*, *spaS* and *ituC* was also increased two to three times compared to the standard procedure, without altering the AMP genes distribution in samples. Once the selective enrichment procedure and PCR targeted to AMP genes were validated, we have build-up a collection of 184 *Bacillus* isolates from 183 natural samples. Samples were collected from soil, rhizosphere and aerial plant parts of several species from various geographical locations of the Eastern Mediterranean. Each isolate was characterized by the presence of the six biosynthetic genes (*srfAA*, *bacA*, *bmyB*, *fenD*, *spaS* and *ituC*), the production of cyclic lipopeptides corresponding to the families of iturins, fengycins and surfactins, and the antimicrobial activity against eight bacterial and five fungal plant pathogens of agronomic interest. The frequency distribution of biosynthetic genes in isolates showed a normal distribution, with most isolates showing between 2 and 4 simultaneous AMP. genes.

The most detected genes were *srfAA* (69 %), *bacA* (61 %), *bmyB* (55 %) and *fenD* (40 %). The ability of the isolates to produce isoforms of iturins, fengycins and surfactins has been demonstrated in a selection of 64 out of 184 *Bacillus* isolates. Surfactins were produced in 98 % of the isolates, followed by iturins (91 %) and fengycins (80 %). However, we were not able to determine the production of bacilysin and subtilin because of the limitations of the methodology used. Interestingly, 99 % of the isolates showed antimicrobial activity towards at least one of the pathogens studied. Specifically, 72 % of the isolates were active against at least 6 out of 8 bacterial pathogens, and 37 % against 3 out of 5 fungal pathogens.

The characterization of *Bacillus* isolates at the level of AMP genes, cLPs production, and antimicrobial activity against plant pathogens, allowed establishing relationships between these parameters. There was a significant direct relationship between the presence of high number of simultaneous AMP genes and antibacterial activity in LB agar, as well as between the presence of *fenD* gene, production of fengycins and the antimicrobial activity. In spite of the heterogeneity in the detection of biosynthetic AMP genes, AMP products and antimicrobial activity within the *Bacillus* isolates collection, the correspondence analysis confirmed the presence of two consistent groups. Thus, the first one has been characterized by the simultaneous presence of antimicrobial peptide genes, production of related cyclic lipopeptides and high antimicrobial activity, while the second has been characterized by the absence of these three characters. Therefore, the prevalence of AMP genes in plant-associated populations of *Bacillus* has been demonstrated, and their relationship with the antagonistic capacity of the respective isolates and the synthesis of the expression products was determined.

Here, we have provide the procedures to build-up *Bacillus* isolates collections with a high yield ofstrains having multiple AMP genes simultaneously, and producing several cyclic lipopeptides at the same time, with a wide range of antimicrobial activity against bacterial and fungal plant pathogens. It is expected that such strains would be suitable candidates as biological control agents of plant diseases.

Resum

Bacillus és un dels gèneres de bacteris Gram-positius més ben estudiat i caracteritzat. L'interès per aquest gènere es deu a la seva capacitat per formar endòspores, i a la de producció de metabòlits d'interès en els camps agronòmic, farmacèutic i industrial. *Bacillus* és un gènere complex a nivell genotípic, fenotípic, metabòlic, taxonòmic i ecològic, fet que li permet ser àmpliament versàtil en diferents ambients, especialment en el sòl. En agricultura, *Bacillus subtilis* i les espècies relacionades són de gran interès a causa del seu reconeixement com 'Generally Recognized as Safe' (GRAS) per la Food and Drug Administration (FDA, USA) i 'Qualified presumption of Safety' (QPS) per la European Food Safety Authority (EFSA, EU), les quals han estat descrites com agents de biocontrol o biofertilitzants. La seva capacitat d'esporulació permet incrementar la vida útil dels biopesticides, i la producció de metabòlits secundaris amb propietats antimicrobianes esdevé el mecanisme clau en el control de malalties de plantes causades per fongs i bacteris. El gran interès per aquest grup ha propiciat l'estudi de diferents genomes complets de *Bacillus subtilis* i d'especies relacionades, a partir del qual s'han identificat els gens implicats en les rutes metabòliques dels productes relacionats amb el biocontrol de patògens de plantes, així com la seva implicació en l'ecologia d'aquests bacteris.

La present tesi de doctorat tracta l'aïllament i la caracterització de *Bacillus* procedents d'ambients relacionats amb les plantes, amb el principal objectiu de determinar les relacions entre l'activitat antimicrobiana, els gens i els respectius productes. S'espera que aquest coneixement pugui contribuir en la millora de l'eficàcia del procés de selecció per trobar nous i millors agents de biocontrol de malaltïes de les plantes.

En aquest treball s'han optimitzat, verificat i validat eines moleculars basades en la PCR que permeten la detecció específica de *Bacillus* en mostres naturals mitjançant l'amplificació del gen *16S rDNA* amb una elevada sensibilitat (10^2 cfu/ml), i de sis gens relacionats amb les rutes

biosintètiques de pèptids antimicrobians, específicament de la surfactina (*srfAA*), bacilisina (*bacA*), bacilomicina (*bmyB*), fengicina (*FenD*), subtilina (*spaS*) i iturina (*ituC*). La sensibilitat dels encebadors dissenyats per a la detecció dels gens biosintètics depèn de la soca, resultantser els més sensibles els gens *fenD* i *ituC*, presentant una sensibilitat de $5x10^2$ cfu/ml, mentre el gen *spaS* va ser el menys sensible, amb valors per sobre de $1x10^4$ cfu/ml. A més a més, es va determinar la prevalença dels gens *srfAA*, *bacA*, *bmyB* i *fenD* en soques de referència, fet pel qual es va dissenyar, optimitzar i validar una PCR multiplex. Tanmateix la sensibilitat d'aquesta eina es va veure reduïda en comparació a la PCR senzilla, amb nivells de sensibilitat de $1x10^5$ cfu/ml.

Amb la finalitat d'obtenir resultats òptims mitjançant la utilització de les eines molecular va ser necessària la optimització d'un procés d'enriquiment selectiu per *Bacillus* en mostres naturals. Aquest procés d'enriquiment va permetre un increment de les poblacions de *Bacillus* en extractes naturals fins a valors detectables per PCR. El procés d'enriquiment selectiu va consistir en un tractament tèrmic a 80 ºC durant 10 min seguit d'un estadi d'enriquiment a 40 ºC durant 24 h, amb la prèvia dilució 1:100 de l'extracte tractat tèrmicament per tal d'eliminar els inhibidors de la PCR procedents de la mostra natural. L'aplicació d'aquest procés d'enriquiment selectiu seguit de PCR va permetre un increment de la detecció i aïllats de *Bacillus*, especialment en mostres de part aèria de plantes, on prèviament no s'havien pogut detectar utilitzant el procés estàndard d'aïllament. La detecció dels AMP gens *srfAA*, *bacA*, *bmyB*, *fenD*, *spaS* i *ituC* també es va veure incrementada entre dues i tres vegades en comparació al procés estàndard, sense alterar la distribució dels gens biosintètics en mostres naturals.

Un cop validat el procés d'enriquiment selectiu i les eines moleculars dirigides als gens biosintètics, es vaconstituïruna col·lecció de 184 aïllats de *Bacillus* procedents de 183 mostres naturals. Les mostres van ser recol·lectades de sòl, rizosfera i part aèria de plantes de diferents espècies procedent de diverses zones geogràfiques de l'est del Mediterrani. Cada aïllat va ser caracteritzat segons la presència dels sis gens biosintètics (*srfAA*, *bacA*, *bmyB*, *fenD*, *spaS* i *ituC*), la producció de lipopèptids cíclics

correspondents a les families de les iturines, fengicines i surfactines, il'activitat antimicrobiana contra vuit bacteris i cinc fongs patògens de plantes d'interès agrícola. La freqüència dels gens biosintètics entre els aïllats presentava una distribució normal, on la majoria dels aïllats presentaven entre 2 i 4 gens biositètics de forma simultània. Els gens més detectats van ser *srfAA* (69 %), *bacA* (61 %), *bmyB* (55 %) i *fenD* (40 %). La capacitat dels aïllats de produir isoformes dels pèptids ciclics iturina, fengicina i surfactina es va demostrar en 64 dels 184 aïllats de *Bacillus*. La producció de surfactines es va detectar en un 98 % dels aïllats, seguit de les iturines (91 %) i de les fengicines (80 %). Tanmateix, no vam poder determinar la producció de bacilisina i de subtilina a causa de les limitacions en les metodologies utilitzades. De manera interessant, el 99 % dels aïllats presentaven activitat antimicrobiana al menys contra un dels patògens estudiats. Concretament, un 72 % dels aïllats eren actius almenys contra sis dels vuit bacteris patògens, i un 37 % contra tres dels cinc patògens fúngics.

La caracterització dels aïllats de *Bacillus* a nivell de detecció de gens biosintètics, producció de lipopèptids cíclics i activitat antimicrobiana contra patògens de plantes va permetre establir relacions entre aquests paràmetres. Es va observar una relació directe i significativa entre la presència simultània d'un elevat nombre de gens biosintètics i l'activitat antibacteriana en medi LB, així com entre la presència del gen *fenD*, la producció de fengicines i l'activitat antimicrobiana. Tot i l'heterogeneitat entre els aïllats de *Bacillus* quant a la detecció dels gens biosintètics, producció de pèptids antimicrobians i activitat antimicrobiana, l'anàlisi de correspondències va confirmar la presencia de dos grups consistents. El primer d'ells es caracteritzava per una elevada i simultània presencia de gens biosintètics, la producció dels lipopèptids cíclics relacionats amb els gens i una elevada activitat antimicrobiana. Per altra banda, el segon grup estava format per aïllats de *Bacillus* caracteritzats per la carència d'aquest tres caràcters. Llavors, s'ha demostrat la prevalença dels gens biosintètics en poblacions de *Bacillus* associats a plantes, així com la relació entre la capacitat antagonista dels aïllats i la síntesi dels productes d'expressió.

Mitjançant aquest treball hem proporcionat els procediments per a la construcció de col·leccions d'aïllats de *Bacillus* amb un elevat rendiment de soques que presenten multiples gens biosintètics de forma simultània, i que produeixen diversos lipopèptids cíclics al mateix temps, amb un ampli espectre d'activitat contra bacteris i fongs fitopatògens. D'aquesta manera, s'espera que aquestes soques siguin candidats adequats com a agents de control biològic de malalties de les plantes.

Resumen

Bacillus es uno de los géneros de bacterias Gram-positivas mejor estudiadas y caracterizadas. El interés por este género se debe a su capacidad para formar endosporas, y a la producción de metabolitos de interés en los campos agronómico, farmacéutico e industrial. *Bacillus* es un género complejo a nivel genotípico, fenotípico, metabólico, taxonómico y ecológico, hecho que le permite una gran versatilidad en diferentes ambientes, especialmente en el suelo. En agricultura, *Bacillus subtilis* y las especies relacionadas son de gran interés debido a su reconocimiento como 'Generally Recognized as Safe' (GRAS) por la Food and Drug Administartion (FDA, USA) y 'Qualified Presumtion of Safety' QPS por la European Food Safety Authority (EFSA, EU), las cuales han sido descritas como agentes de biocontrol o biofertilitzantes. Su capacidad de esporular permite incrementar la vida útil de biopesticidas, y la producción de metabolitos secundarios con propiedades antimicrobianas es el mecanismo clave en el control de enfermedades de plantes causadas por hongos y bacterias. El gran interés por este grupo ha propiciado el estudio de diferentes genomas completos de *Bacillus subtilis* y de especies relacionadas, a partir del cual se han identificado los genes implicados en las rutas metabólicas de los productos relacionados con el biocontrol de patógenos de plantas, así como su implicación en la ecología de estas bacterias.

La presente tesis doctoral trata el aislamiento y caracterización de *Bacillus* procedentes de ambientes relacionados con las plantas, con el principal objetivo de determinar las relaciones entre la actividad antimicrobiana, los genes y sus productos. Se espera que este conocimiento pueda contribuir en la mejora de la eficacia del proceso de cribaje para encontrar nuevos y mejores agentes de biocontrol.

En este trabajo, se han optimizado, verificado y validado herramientas moleculares basadas en la PCR que permiten la detección específica de *Bacillus* en muestras naturales mediante la amplificación del gen *16S rDNA*

con una elevada sensibilidad (10^2 cfu/ml), y de seis genes relacionados conlas rutas biosintéticas de péptidos antimicrobianos, concretamente los de la surfactina (*srfAA*), bacilisina (*bacA*), bacilomicina (*bmyB*), fengicina (*FenD*), subtilina (*spaS*) e iturina (*ituC*). La sensibilidad de los cebadores diseñados para la detección de genes biosintéticos depende de la cepa, en este caso los genes *fenD* e *ituC* fueron los más sensibles, presentando una sensibilidad de $5x10^2$ cfu/ml, mientras que el gen *spaS* fue el menos sensible, con valores por encima de $1x10^4$ cfu/ml. Además, se determinó la prevalencia delos genes *srfAA*, *bacA*, *bmyB* y *fenD* en cepas de referencia, por lo cual se diseñó, optimizó y validó una PCR multiplex. No obstante, la sensibilidad de esta herramienta se vio reducida en comparación a la PCR simple, con niveles de sensibilidad de $1x10^5$ cfu/ml.

Con la finalidad de obtener resultados óptimos mediante la utilización de las herramientas moleculares fue necesaria la optimización de un proceso de enriquecimiento selectivo para *Bacillus* en muestras naturales. Este proceso de enriquecimiento permitió un incremento de las poblaciones de *Bacillus* en extractos naturales hasta valores detectables mediante PCR. El proceso de enriquecimiento selectivo consistió en un tratamiento térmico a 80 ºC durante 10 min, seguido de un estadio de enriquecimiento a 40 ºC durante 24 h, con la previa dilución 1:100 del extracto tratado térmicamente con la intención de eliminar los inhibidores de la PCR procedentes de la muestra natural. La aplicación de este proceso de enriquecimiento selectivo seguido de PCR permitió el incremento de *Bacillus*, especialmente en muestras de parte aérea de plantas, donde previamente no se habían podido detectar utilizando el proceso estándar de aislamiento. La detección de los genes biosintéticos *srfAA*, *bacA*, *bmyB*, *fenD*, *spaS* e *ituC*, también se vio incrementada entre dos y tres veces en comparación con el proceso estándar, sin alterar la distribución de los genes biosintéticos en muestras naturales.

Una vez validado el proceso de enriquecimiento selectivo y las herramientas moleculares dirigidas a los genes biositnéticos, se constituyó una colección de 184 aislados de *Bacillus* procedentes de 183 muestras naturales. Las muestras fueron recolectadas del suelo, rizosfera y parte aérea de plantas de diferentes especies procedentes de diversas zonas

geográficas del Este del Mediterráneo. Cada aislado fue caracterizado según la presencia de los seis genes biosintéticos (*srfAA*, *bacA*, *bmyB*, *fenD*, *spaS* e *ituC*), la producción de lipopéptidos cíclicos correspondientes a las familias de las iturinas, fengicinas y surfactinas, y la actividad antimicrobiana contra ocho bacterias y cinco hongos fitopatógenos de interés agrícola. La frecuencia de los genes biosintéticos entre los aislados presentaba una distribución normal, donde la mayoría de los aislados presentaron entre 2 y 4 genes biosintéticos simultáneamente. Los genes más detectados fueron *srfAA* (69 %), *bacA* (61 %), *bmyB* (55 %) y *fenD* (40 %). La capacidad de los aislados de producir isoformas de los péptidos cíclicos iturina, fengicina y surfactina se demostró en 64 de los 184 aislados de *Bacillus*. La producción de surfactinas se detectó en un 98 % de los aislados, seguido de las iturinas (91 %) y de las fengicinas (80 %). No obstante, no se pudo determinar la producción de bacilisina y subtilina debido a las limitaciones en las metodologías utilizadas. De forma interesante, el 99 % de los aislados presentaban actividad antimicrobiana al menos contra uno de los patógenos estudiados. Concretamente, un 72 % de los aislados eran activos al menos contra seis de los ocho, y un 37 % contra tres de los cinco patógenos fúngicos.

La caracterización de los aislados de *Bacillus* a nivel de detección de genes biosintéticos, producción de lipopéptidos cíclicos y actividad antimicrobiana contra patógenos de plantas permitió establecer relaciones entre estos parámetros. Se observó una relación directa y significativa entre la presencia simultánea de un elevado número de genes biosintéticos y la actividad antibacteriana en medio de cultivo LB, así como entre la presencia del gen *fenD*, la producción de fengicinas y la actividad antimicrobiana. A pesar de la heterogeneidad entre los aislados de *Bacillus* en relación a la detección de los genes biosintéticos, producción de péptidos antimicrobianos y la actividad antimicrobiana, el análisis de correspondencias confirmó la presencia de dos grupos consistentes. El primero de ellos se caracterizó por una elevada y simultánea presencia de genes biosintéticos, la producción de lipopéptidos cíclicos relacionados con los genes y una elevada actividad antimicrobiana. Por otro lado, el segundo grupo estaba formado por aislados de *Bacillus* caracterizados por la ausencia de estos tres caracteres. De este modo, se ha demostrado la

prevalencia de los genes biosintéticos en poblaciones de *Bacillus* asociados a plantas, así como la relación entre la capacidad antagonista de los aislados y la síntesis de los productos de expresión.

A partir de este trabajo hemos proporcionado los procedimientos necesarios para la construcción de colecciones de aislados de *Bacillus* con un elevado rendimiento de cepas que presentan múltiples genes biosintèticos de forma simultánea, y que producen varios lipopéptidos cíclios al mismo tiempo, con un amplio espectro de actividad contra bacterias y hongos fitopatógenos. De este modo, se espera que estas cepas puedan ser candidatos adecuados como agentes de biocontrol de enfermedades de plantas.

CHAPTER 1

General Introduction

1. Biology, ecology and applications of *Bacillus*

The *Bacillus* genus was described for the first time in 1872 by F. Cohn, 37 years after the discovery of a strain initially included in *Vibrio* genus, by Ehrenberg (1835). Roughly, bacteria included in *Bacillus* genus are grouped in the family *Bacillaceae* which differed from other families of *Bacillales* order for the production of spores. Thus*, Bacillus* species are part of a group of Gram-positive endospore forming bacteria, a very diverse group including from pathogenic to beneficial species. According to Bergey's manual (Logan and de Vos 2009) in 2000 over 90 species have been described. Furthermore it is a wide distributed genus at ecological level, for its ubiquity in soil, air, and water, and arealso involved in a wide range of chemical process, becoming one of the most diverse and commercially useful bacteria groups (Harwood 1989).

Some members of the *Bacillus* genus are *B. amyloliquefaciens*, *B. anthracis*, *B. cereus* and *B. subtilis*. Concretely, *B. subtilis* is one of the best known and most extensively studied Gram-positive bacteria, and is an established model organism for research on Gram-positive bacteria. In addition, the genome of several *Bacillus* species is already sequenced, which has permitted a deeper understanding in the biology of this species. While is known about *B. subtilis* at the molecular level, relatively little is known about its ecology and evolution (Earl *et al*. 2007). Even so, research in *B. subtilis* is remarkably diverse, spanning genetics, biochemistry, cell biology, and ecology, and as a result, has had an enormous impact on both basic and applied biology (Driks 2004).

1.1 Genome

In 1997 it was published the first complete genome of a *Bacillus* strain. This corresponds to *B. subtilis* strain 168 (Kunst *et al*. 1997), a tryptophan auxotrophic mutant derived from the *B. subtilis* ATCC6051, isolated from Cohn in 1872 (Wipat and Harwood 1999). *Bacillus subtilis* is the type species for the genus, in which more than hundred species have been identified. Historically, *B. subtilis* is one of the model organisms for prokaryotic research. The research interest in *B. subtilis* was motivated by the opportunity to investigate the developmental system of sporulation, and by the ability of the bacterium to synthesize and secrete quantities of industrially important enzymes and antibiotics (Devine 1995). A second stimulus for research derives from the industrial importance of members of this bacterial genus. *Bacillus* species produce three types of industrially important products: enzymes, antibiotics and insecticides. Enzymes, such as proteases, amylases, glucanases and cellulases, are produced by various *Bacillus* species that, when grown on simple media under industrial fermentation conditions, can produce up to 20 g of enzyme per liter of supernatant (Ferrari *et al*. 1953). Thus, the reasons for sequencing the genome of *B. subtilis* derive from its importance as a model system for prokaryotic research, the industrial importance of the *Bacillus* genus and the small size of its genome (Kunst *et al*. 1997).

The genome of *B. subtilis* 168 is composed of 4,214,810 base pairs and comprises 4,100 protein-coding genes. Of these protein-coding genes, 53 % are represented once, while a quarter of the genome corresponds to several gene families that have been greatly expanded by gene duplication, the largest family containing 77 putative ATP-binding transport proteins. In addition, a large proportion of the genetic capacity is devoted to the utilization of a variety of carbon sources, including many plant-derived molecules. The identification of five signal peptidase genes, as well as several genes for components of the secretion apparatus, is important given the capacity of *Bacillus* strains to secrete large amounts of industrially important enzymes. Many of the genes are involved in the synthesis of secondary metabolites, including antibiotics that are more typically associated with *Streptomyces* species (Kunst *et al*. 1997). In *B. subtilis*, all genes specifying antibiotic biosyntheses combined are coded by 350 kb; however, an average of about 4–5 % of a *B. subtilis* genome is devoted to antibiotic production (Stein 2005). The potential of a given *B. subtilis* strain for antibiotic synthesis is comparable with *B. amyloliquefaciens* with around 8 % of the genome (Chen *et al*. 2009a, Koumoutsi *et al*. 2004) and with the potential of *Streptomyces* such as *Streptomyces avermitilis* with 6.4 % of the genome (Omura *et al*. 2001). The marked differences of *B. subtilis* strains with regards to their produced antibiotic spectra suggest that the antibiotic specifying loci must have been recent acquisitions. For this reason, horizontal exchange of genetic material enabled via uptake of phage, plasmid or naked DNA by genetically competent cells has been proposed as a feasible possibility to explain this divergence (Stein 2005).

In the last 10 years, several *Bacillus* strains of different species have been sequenced, such as *B. anthracis* (Read *et al*. 2003), *B. licheniformis* (Veith *et al*. 2004), *B. amyloliquefaciens* (Chen *et al*. 2007), *B. thuringiensis* (Challacombe *et al*. 2007), *B. cereus* (Klee *et al*. 2010), *B. megaterium* (Eppinger *et al*. 2011), *B. pumilus* (Gioia *et al*. 2007) among others. Besides, the genome of *Bacillus subtilis* strain 168 that was updated recently (Barbe *et al*. 2009, Srivatsan *et al*. 2008) (Fig. 1.1).

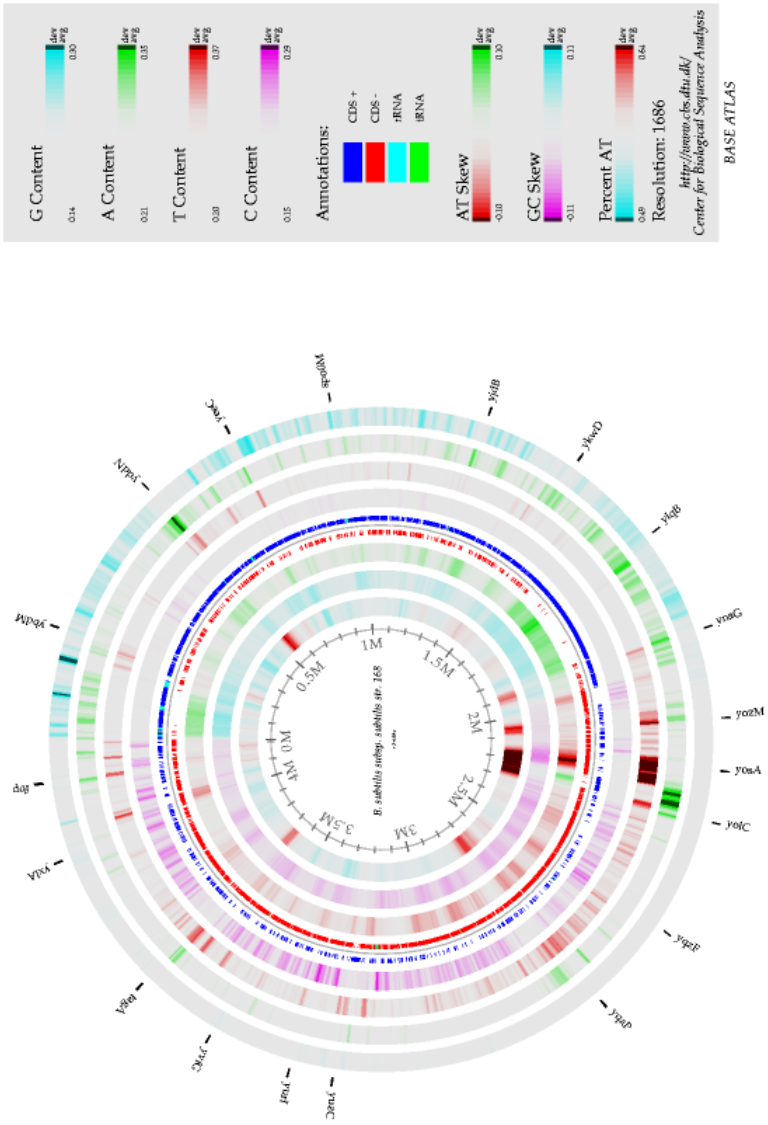

Figure 1.1. Base atlas of *Bacillus subtilis* 168 genome. http://www.cbs.dtu.dk/services/GenomeAtlas-2.0.

1.2 Endospores

Endospores were first described by Cohn (1872) in *B. subtilis* and later by Koch (1876) in the pathogen, *B. anthracis*. Endospore formation, universally found in *Bacillus* genus, is thought to be a strategy for survival in the soil environment, wherein these bacteria predominate. Aerial distribution of the dormant spores probably explains the occurrence of aerobic spore-formers in most habitats examined. *Bacillus* species generally exist as epiphytes and endophytes in the spermosphere and rhizosphere and are also found in many other environments (Zhang *et al*. 2008b). Cohn demonstrated the heat resistance of endospores in *B. subtilis*, and Koch described the developmental cycle of spore formation in *B. anthracis*. Endospores are so named because they are formed intracellularly, although they are eventually released from this mother cell as free spores. Endospores have proven to be the most durable type of cell found in nature, and in their cryptobiotic state of dormancy they can remain viable for extremely long periods of time, perhaps millions of years.

Endospores are not formed normally during active growth and cell division. Rather, their differentiation begins when a population of vegetative cells passes out of the exponential phase of growth, usually as a result of nutrient depletion or other stressing factors. Typically one endospore is formed per vegetative cell. The mature spore is liberated by lysis of the mother cell in which it was formed. Mature spores have no detectable metabolism, a state that is described as cryptobiotic. They are highly resistant to environmental stresses such as high temperature (some endospores can be boiled for several hours and retain their viability), irradiation, strong acids, disinfectants, etc. (Setlow 2006). Although cryptobiotic, they retain viability indefinitely such that under appropriate environmental conditions, they germinate into vegetative cells. Endospores are formed by vegetative cells in response to environmental signals that indicate a limiting factor for vegetative growth, such as exhaustion of an essential nutrient. They germinate and become vegetative cells when the environmental stress is relieved. Hence, endospore-formation is a mechanism of survival rather than a mechanism of reproduction.

Although *Bacillus* sporulation is induced by starvation, the sporulation developmental program is not initiated immediately when growth slows due to nutrient limitation. A variety of alternative responses can occur, including the activation of flagellar motility to seek new food sources by chemotaxis, the production of antibiotics to destroy competing soil microbes, or the secretion of hydrolytic enzymes to scavenge extracellular proteins and polysaccharides (Stephens 1998). Specifically, sporulation of *B. subtilis* is regulated through four key transcription factors, the RNA polymerase sigma factors σ^F, σ^E, σ^G and σ^K. The activation of these key transcription factors induce the activation of the response regulator Spo0A. More than 200 genes have been involved in sporulation induction and regulation, as well as spore formation, division, germination and outgrowth (Piggot and Losick 2002).

Their ability to sporulate increase cell survival, and permits a high prevalence under adverse conditions (Driks 2004). Moreover permits a good development of reliable formulations to increase the shelf-life of starters or inoculants, and spores can be easily formulated, and have high viability compared with vegetative cells (Wulff *et al*. 2002).

1.3 Taxonomy

Bacillus is a diverse bacterial genus characterized by Gram-positive, aerobic or facultative anaerobiotic bacteria forming dormant ellipsoidal endospores. *Bacillus* species were some of the first bacteria ever characterized in a systemic manner in the late 19th century (Maughan and Van der Auwera 2011). Its description was carried out by F. Cohn (1872) with the discovery of the sporulation in *Bacillus*. They are in general mesophilic with regard to temperature and neutrophilic with respect to pH for growth (Fritze 2004, Ki *et al*. 2009, Kloepper *et al*. 1991, Loganand Berkeley 1984, Shoda *et al*. 2000).

The genus *Bacillus* has undergone considerable taxonomic change, especially with the development of new methods for the characterization and identification of bacterial strains, in particular on the genetic level, of which the 16S rRNA/DNA sequence analysis has exerted major influence (Fritze 2004). Nowadays, the group of aerobic endospore-formig bacteria

embraces more than 25 genera and more than 250 species with different phenotypes and genotypes (www.bacterio.cict.fr). Nevertheless, the main studied *Bacillus* species were included in two specific groups. The first one refers to the *Bacillus subtilis* group, which are closely related and thus not easily distinguishable. It includes among others *B. subtilis*, *B. pumilus*, *B. licheniformis*, *B. trophaeus* and *B. amyloliquef*aciens. The second group refers to the *Bacillus cereus* group, which include *B. cereus, B. thuringiensis, B. mycoides* and *B. anthracis*.

The knowledge of *Bacillus* spp. and their identification have been available for a long time, and it was still considered to be complicated during several years (Logan and Berkeley 1984). Macroscopical identification by Bergey's Manual (Suihko and Stackerbrandt 2003) and biochemical tests were the first type of identification which was used. In this way, most of the tests conducted have been based on physiological and nutritional tests, in which different media were the first factors to study, but the different requirements for growth media containing unusual ingredients increases the standardization problems. Later, it was developed a biochemical method based on 50 tests, called API50CHB, showing greatest reproducibility and discriminatory value (Logan and Berkeley 1984, Suihko and Stackerbrandt 2003, Wulff *et al*. 2002). According to a study of aerobic endospore-forming bacteria performed by Priest *et al*. (1988), 368 strains were analyzed using more than 100 characters, and among 80 taxa of approximate species could be assigned to 6 Cluster-groups. Clearly, *B. circulans* taxa with a high heterogeneity among strains formed a single cluster (including *B. circulans*, *B. alvei*, *B. macerans* and *B. polymyxa*). The second cluster-group encompassed a variety of species described to date, such as *B. cereus* group (mainly composed by *B. cereus*, *B. mycoides* and *B. thuringiensis*) and *B. subtilis* group (composed by *B. amyloliquefaciens*, *B. licheniformis*, *B*. *pumilus*, *B. megaterium* and *B. subtilis*). From third to sixth cluster-groups increase their heterogeneity and reduce the number of strains. Thus, the third cluster included *B. carotarum* and *B. firmus* strains. The fourth cluster was mainly composed by *B. brevis* strains. The fifth cluster was formed by *B. firmus* and *B. lentus*, and at last the sixth cluster showed *B. coagulans* and *B. stearothermophilus* strains.

Bacillus genus, show small differences in *16S rRNA* gene sequenced (Wulff *et al*. 2002). Some studies revealed as the addition of phenotypic information to topology trees analyzed using *16S rDNA* locus dispersed the tree instead to refine it, confirming the heterogeneity of *Bacillus* genus. These results conclude than strains with similar phenotypes were not necessarily closely related at the *16S rDNA* level (Maughan and Van der Auwera 2011).

Several studies on *Bacillus* spp. combined molecular and biochemical methods such as DNA-DNA hybridation fingerprinting (Cho *et al*. 2004, Miranda *et al*. 2008), restriction enzyme DNA patterns (Suihko and Stackerbrandt 2003), mass spectrometry characterization of bacteria by matrix-assisted laser desorption-ionization (MALDI) time-of-flight mass spectrometry (TOF/MS) (Lasch *et al*. 2009), and combination of different genes sequencing, as *16S rRNA*, *rpoB* and *gyrP* (Ki *et al*. 2009, Ko *et al*. 2003, Miranda *et al*. 2008, Suihko and Stackerbrandt 2003). For example, the *Bacillus cereus* group has three high representative *Bacillus* species: *B. cereus, B. thuringiensis and B. anthracis*, which show great phenotypic and biochemical difficulties to differentiate the species. In *B. anthracis*, which is the responsible agent of anthrax, different identification tools have been developed by multilocus sequence typing (MLST) (Kim *et al*. 2005). Thus, combination of *rpoB* gene encoding RNA polymerase β-subunit with other genes, as *gyrB* gene, is used to specific identification of *B. anthracis* (Ko *et al.* 2003). Likewise, *B. anthracis* was identified and differentiated from other *Bacillus cereus* group species by mass spectrometry (Lasch *et al*. 2009), or by (RFLP) (Shangkuan *et al*. 2000) and (RAPD) (Daffonchio *et al*. 1999). Similar issues have been reported in the *B. subtilis* group where phenotypical studies are not enough to differentiate *B. subtilis* and *B. amyloliquefaciens*, and in most cases it is better to combine biochemical with molecular methods (Marten *et al*. 2000, Romero *et al*. 2004, Suihko and Stackerbrandt 2003).

1.4 Diversity and ecology

The genus *Bacillus* is both taxonomically and metabolically diverse. The primary habitat is the soil, though can be found associated with the surface

of the roots or the aerial part of the plant, which can help in obtaining nutrients, at the same time that prevents diseases to the plant and helps in the production of phytohormones (Kloepper *et al*. 1991, Shoda 2000). *Bacillus* spp. is ubiquitous in agricultural systems and the ability to sporulate and their metabolic diversity are significant factors that have led to their successful colonization of a wide variety of environments (Wipat and Harwood 1999). Moreover, their ability to form endospores that allow them to survive for extended periods under adverse environmental conditions. Common physiological traits important to their survival in several environments include production of a multilayered cell wall structure, formation of stress-resistant endospores, and secretion of antimicrobial peptides (AMPs), peptide signal molecules and extracellular enzymes, which permit them to survive in an elevate number of environments (Mahaffee and Kloepper 1997, McSpadden-Gardener 2004). Population levels of these bacteria generally range from 3 to 6 log cells per gram fresh weight (McSpadden-Gardener 2004, Mora *et al*. 2011). Molecular methods have permitted to determine the diversity and distribution of *Bacillus* spp. in different environments around the world (Ahaotu*et al*. 2013, Bento *et al*. 2005, Ki *et al*. 2009).

B. subtilis is often found in the plant rhizosphere (Bais *et al*. 2004, Earl *et al*. 2008, Morikawa 2006) and animal gastrointestinal tracts, where it has an active role and it has been considered as a probiotic (Earl *et al*. 2008). *B. subtilis* has also been isolated from aquatic environments, and at the same time, it has been found in the gastrointestinal tracts of marine organisms (Earl *et al*. 2008, Miranda *et al*. 2008). Recent studies have suggested that the ability of *B. subtilis* to efficiently colonize surfaces of plant roots is a prerequisite for phytoprotection. This process relies on surface motility and efficient biofilm formation (Arguelles-Arias *et al.* 2009, Morikawa 2006). This is supported by the fact that various strains of *B. subtilis* have been shown to be capable of forming biofilms (Branda *et al*. 2001, Bais *et al*. 2004, Hamon and Lazazzera 2001). Moreover, it has been reported that *B. subtilis* forms biofilms on inert surfaces under the control of a variety of transcription factors (Hamon and Lazazzera 2001, Stanley *et al*. 2003). Additionally, some studies reported that biofilm formation is dependent on the secretion of surfactin, a lipopeptide (LP) commonly

produced by *Bacillus* species (Branda *et al*. 2004, Kinsinger *et al*. 2003). Absence of essential genes involved in the synthesis of surfactin in *B. subtilis* 168 resulted in a low performance on biofilm development (McLoon *et al*. 2011). In addition, there is a growing recognition that biosurfactant production not only affects biofilm architecture but can influence the attachment of bacterial cells to surfaces (Davey *et al*. 2003) and also the surface movement by swarming (Connelly *et al*. 2004, Kearns and Losick 2003). Thus, quorum sensing system of *B. subtilis* plays a key role in the development of genetic competence and other physiological systems when cells enter the stationary growth phase. Concretely, the comQ/X/P/A quorum-sensing system controls various cell density-dependent phenotypes such as the production of degradative enzymes and antibiotics and the development of genetic competence (Schneider *et al*. 2002, Weinrauch *et al*. 1991).

Moreover, *B. subtilis* group has long been viewed as a strict aerobe, but it also grows anaerobically either by utilizing nitrate or nitrite as an electron acceptor or by fermentation in the absence of electron acceptors (Nakano and Zuber 1998). The switch between aerobic and anaerobic metabolism in *B. subtilis* is regulated mainly at the transcriptional level and, in some cases by modulation of enzyme activity. Nitrate respiration makes sense from an ecological standpoint because soil, contains numerous anaerobic microenvironments. Furthermore, colony and biofilm formation results in oxygen depletion for cells located within cell aggregates (Nakano and Zuber 2002). The genome sequence of *B. sutilis* 168 has helped to provide information on its lifestyle and the habitats where it resides (Kunst *et al*. 1997).

1.5 *Bacillus* in industry

Bacillus species are attractive industrial organisms for a variety of reasons, including their high growth rates leading to short fermentation cycles, or times, their capacity to secrete proteins into the extracellular medium. In addition, much is now known about the biochemistry, physiology, and genetics of *B.subtilis* and other species, which facilitates further development and greater exploitation of these organisms in industrial

processes (Schallmey *et al*. 2004). Another important aspect is that several *Bacillus* species, such as *B. subtilis*, *B. amyloliquefaciens* and *B. licheniformis*, are qualified with the GRAS (generally Recognized as Safe) status by the FDA (Food and Drug Administration) of USA, and as QPS (Qualified Presumption of Safety) by EFSA (European Food Safety Authority).

Strains of *Bacillus subtilis* and related species are used for the production of five main types of products: enzymes, antibiotics, fine biochemicals including flavor enhancers, food supplements and insecticides. Proteases and hydrolytic enzymes are the most commercialized and used enzymes, which cover a wide range of application from detergent or pharmaceutical to food additives. It is estimated that *Bacillus subtilis* related species produce about 50 % of the total enzyme market, that are used mostly for production and extraction of alkaline proteases, amylases, protease, inosine, ribosides, and amino acids (Schallmey *et al*. 2004). Specifically, the serine endopeptidase sutbilisin (Bott *et al*. 1988), produced by *B. licheniformis*, *B. pumilus* and *B. subtilis* (Schallmey *et al*. 2004), due to having a serine, histidine and aspartic acid residues gives them the ability to act in a wide pH range of 6-13 and high stability at high temperatures (Polgár 2005). The main application of this enzyme is in detergent industry due to its physical characteristics (Harwood 1989). However, other *Bacillus* industrial enzymes are used in other sectors (Table 1.1) such amylases, glucanases, or cellulases among others. For example, α-amylase synthesized by *B. licheniformis*, operates at 95 ºC and is used for liquefying starches that only gelatinizes at 100 ºC (Slott *et al*. 1974), or glucose isomerase from *B. coagulans*, used in the starch hydrolysis process, because it has the optimum pH and temperature process (Keating *et al*. 1998).

Antimicrobial compounds produced by *Bacillus* species during the early stages of sporulation are also interesting in feed and bioremediation industry. Bacitracin A is a cyclic oligopeptide synthesized by *B. licheniformis* and the principal commercial product (Schallmey *et al*. 2004), and is used as a dietary antibiotic mainly in poultry consumption, especially in chicken (Nurmi and Rantala 1974). *B. subtilis* and related species are the most

known producers of antimicrobial compounds, from which the most important and studied is surfactin (Table 1.1), a cyclic lipopeptide (cLP) with exceptional surfactant activity and emulsification properties. This cLP shows antimicrobial and antitumoral activity, as well as potential applications in bioremediation (Stein 2005). For example, the degradation of dibromodiphenyl ether in river sediments is stimulated by the addition of surfactin into the sediment (Huang *et al*. 2012), or their use for remove heavy metal and organic contaminants from soils (Mulligan 2005).

Other compounds produced by *Bacillus* and modified *Bacillus* strains are used in pharmaceutical and feed industries. Some of these products are vitamins (like riboflavin), purine nucleotides (Table 1.1), or D-Ribose among others (Schallmey *et al*. 2004).

Table 1.1. Examples of *Bacillus* spp. products used in industry.

Product	**Application**	**Related species**	**References**
Protease	Detergent	*B. licheniformis*	Gupta *et al*. 2002
	Feed additive	*B. thermoprotyolyticus*	Rao *et al*. 1998
Amylase	Starch hydrolysis	*B. subtilis*	Aiyer 2005
	Detergent	*B. subtilis*	Roy *et al*. 2012
Cellulase	Bioenergy	*B. subtilis*	Nakamura *et al*. 1991
Glucanase	Feed additive	*B. licheniformis*	Teng *et al*. 2007
Lipopeptides	Bioremediation	*B. subtilis*	Awashti *et al*. 1999
Lantibiotics	Food preservation	*B. subtilis*	Lee and Kim 2011
Endotoxins	Insecticide	*B. thuringiensis*	Bizzarri and Bishop 2007
Purine nucleotides	Biopolymers	*B. subtilis*	Fan *et al*. 2004
	Feed additives	*B. subtilis*	Li *et al*. 2011

Finally, the fact that *Bacillus* are spore forming bacteria, make them feasible to be used as probiotics, conferring advantages over non–spore formers such as *Lactobacillus* (De Vecchi and Drago 2006). Spore coat allows surviving extreme environmental conditions, such UV radiation, extreme temperatures, exposure to solvents and enzymes (Setlow 2006). Thus, when dehydrated spores are exposed to appropriate nutrients and

water, they will germinate (Driks 2004, Nicholson 2002). For this reason, *Bacillus* can be stored at room temperature in a desiccated form without affected their viability, as well as, they have the ability to survive the acid pH of the stomach (Kim *et al*. 2009). In addition, several studies have been focused on *Bacillus* safety (Endres *et al*. 2009, Hong *et al*. 2008, Lesellier *et al*. 2006, Williams *et al*. 2009). Since the late 50's, when the first *Bacillus* probiotic was registered as a medical supplement in Italy, the research on *Bacillus* as a probiotic became more widespread. The use of probiotics ranges from human consumption as dietary supplements to animals as growth promoters or enhancing the growth of cultured seafood. *B. subtilis* is the most studied species, their benefits have been associated with comsumtion of Natto, which include stimulation of the immune system and reduction blood clotting by fibrinolysis. Even some studies have shown the capacity of *B. subtilis* to reduce infection by *Salmonella enterica* serotype Enteritidis and *Clostridium perfringens* in young chickens (La Ragione and Woodward 2003, Thirabunyanon and Thongwittaya 2012) and also competitively exclude *Escherichia coli* O78:K80 in poultry (La Ragione *et al*. 2001). But *B. subtilis* is not the only *Bacillus* species which is used as a probiotic. The combination of *B. subtilis* and *B. licheniformis* species is used in animal feed, and *B. coagulans*, is used to acts against enteric microbes by the action of the coagulin bacteriocin (Drago and De Vecchi 2009). Even strains of *B. cereus* specie are used in animal feed (Vilà *et al*. 2009).

1.6 *Bacillus* in plant health

The autochthonous microbiota of plants represents a large source of microorganisms that can be used to obtain biocontrol agents (BCAs) of plant diseases, especially, bacteria that represent the predominant microbiota on these environments due to they have a high degree of fitness and are generally good colonists of plant surfaces, which is one of the most important factors in a successful biocontrol process. The microbiota of plants is mainly composed of Gram-negative bacteria of the species *P. fluorescens*, *P. agglomerans* and from genera such as *Aerobacter*, *Xanthomonas* and *Flavobacterium*. Gram-positive bacteria are less abundant though bacteria from genera *Clavibacter*, *Bacillus* and *Lactobacillus* can be found (Cook and Baker 1983). However, only strains

mainly of the species *P. fluorescens*, *P. agglomerans* and of *Bacillus* have been described as BCAs. Several BCAs have been capable to control plant diseases, such as *Pseudomonas fluorescens* EPS62e (Pujol *et al*. 2005), *P. fluorescens* A506 (Johnson *et al*. 2004), *Bacillus amyloliquefaciens* FZB42 (Chen *et al*. 2009c) and *B. subtilis* QST713 (Lahlali *et al*. 2013), which are used in control of fire blight in apple and pear, and *Pantoea agglomerans* EPS125, which has been effective against postharvest fungal pathogens in different fruits (Francés *et al*. 2006). Nevertheless, some of these species, basically Gram-negative bacteria, have experimented limitations in the commercial process including formulation problems or biosafety concerns. For this reason, there is increasing interest in other bacterial groups like the genus *Bacillus*, that involves major advantages over other BCAs described above. Thus, *Bacillus subtilis* and related species are of particular interest because of their safety, widespread distribution in very diverse habitats, remarkable ability to survive adverse conditions due to the development of endospores, and the production of compounds that are beneficial for agronomical purposes (Earl *et al*. 2008, Emmert and Handelsman 1999, Kloepper *et al*. 2004, Montesinos 2007, Ongena and Jacques 2008, Phister *et al*. 2004).

In the agronomy field, *Bacillus* is of special interest due to the ability of several species to develop antagonism against a wide range of bacterial and fungal pathogens. Species of interest able to protect plants are most commonly *B. subtilis* and *B. amyloliquefaciens*. There are several examples describing the efficacy of *Bacillus* strains against plant pathogens, indicating that biological control by using antagonistic *Bacillus* strains instead of chemical pesticides to suppress crop diseases, offers a powerful contribution to environmental conservation (Bais *et al*. 2004, Zhang*et al*. 2008a). In recent years, commercial strains of *B. subtilis* have been marketed as BCAs for fungal diseases of crops (Chung *et al*. 2008, Hu *et al*. 2008, Romero *et al*. 2007), like *Bacillus subtilis* GB03, which is highly effective for crop protection from the pathogens *Fusarium* and *Rhizoctonia* (Emmert and Handelsman 1999), and *B. subtilis* QST713, effective in reducing root-hair colonization by the pathogen *Plasmodiophora brassicae* (Lahlali *et al*. 2011).

Members of *Bacillus* genus are among the beneficial bacteria most exploited as biopesticides to control plant diseases, mainly due to insecticidal strains of *Bacillus thuringensis* that accounts for more than 70 % of total sales (Cawoy *et al*. 2011). This bacterium was first applied in 1938 (Sanahuja *et al*. 2011), and its activity is mediated by the production of the Cry toxins that are part of the spore components and are highly toxic to insects (Arguelles-Arias *et al*. 2009, McSpadden-Gardener 2004). As for the rest, *Bacillus*-based products represent about half of the commercially available bacterial BCAs (Fravel 2005). The most important are *B. subtilis* strain GBO3 (Gusfatson Company, USA*), B. subtilis* strain QST713 (Agraquest, USA), *B. subtilis* MBI600 (Becker Underwood, USA), *B. amyloliquefaciens* strain FZB42 (Abitep GmbH, Germany), *B. licheniformis* strain SB3086 (Novozymes Biologicals, USA) and *B. pumulis* strain GB34 (Gustafson, USA). Thus, *Bacillus* species are important candidates for microbial control agents for plant diseases (Table 1.2).

Table 1.2. A list of plant pathogens controlled by *Bacillus* spp.

Pathogen	**Crops**	**References**
Fungi		
Fusarium oxysporum f. sp. lycopersici	Tomato	Baysal *et al*. 2008
Aspergillus flavus	Corn and certain nuts	Moyne *et al*. 2001
Rhizoctonia solani	Tomato	Asaka and Shoda 1996
Gaeumannomyces graminis	Wheat	Sari *et al*. 2006
Phytophthora capsici	Red pepper	Lee *et al*. 2008
Podosphaera fusca	Cucurbit	Romero *et al*. 2007
Botrytis cinerea	Tomato	Lee *et al*. 2006
Rosellinia necatrix	Advocado	Cazorla *et al*. 2007
Monilinia fructicola	Peach	Gueldner *et al*. 1988
Bacteria		
Ralstonia solanacearum	Mulberry	Ji *et al*. 2008
Erwinia amylovora	Apple and pear	Chen *et al*. 2009c
Xanthomonas oryzae pv. oryzae	Rice	Lin *et al*. 2001
P. syringae	Arabidopsis	Bais *et al*. 2004

Bacillus species have special characteristics that make them good candidates as BCAs, and the most remarkable is the ability to produce a wide variety of bioactive compounds valuable for agricultural applications (Liu *et al*. 2010), including metabolites with antimicrobial activity (Loeffler *et al*. 1986, McSpadden-Gardener 2004, Ongena and Jacques 2008) and surface-active properties (Banat *et al*. 2000, Singh and Cameotra 2004) and compounds implicated in the induction of plant defense responses and growth promotion (Henry *et al*. 2011, Jordan *et al*. 2009, Kloepper *et al*. 2004, Ongena *et al*. 2007). Several studies have been performed in an attempt to elucidate the mechanisms involved in biological control by *Bacillus* species, and antibiosis appears the most plausible mechanism in most of cases (Cawoy *et al*. 2011, Ongena and Jacques 2008, Raaijmakers *et al*. 2010). Production of these compounds contributes to control plant pathogens (Asaka and Shoda 1996, Hou *et al*. 2006, Leclère *et al*. 2006, Romero *et al*. 2007, Silo-Suh *et al*. 1994, Touré *et al*. 2004, Wang *et al*. 2012, Yuan *et al*. 2012) but also appears to contribute to the establishment and persistence of the antagonist in the plant (Bais *et al*. 2004, Davey *et al*. 2003). Most of these metabolites have been characterized as PKs or LPs with low molecular weight like surfactin, iturin and fengycin families (Montesinos 2003, Ongena and Jacques 2008, Stein 2005). For example, *B. amyloliquefaciens* GA1 was considered as a source of potent antibiotics, which show antifungal and oomicetide activity (Arguelles-Arias *et al*. 2009).

1.6.1 Mechanisms of action

Because of the complex interactions take place between the BCAs, host, pathogen and other microorganisms present in the microbiota of plants, it is difficult to study and determine the exact mechanism of action of a specific BCAs. The knowledge of mechanisms involved in the biocontrol may be crucial for its successful development and to improve its efficacy. The mode of action will determine the strategy in disease control, the Implication of its application and the improvement of efficacy and consistency (Handelsman and Parke 1989).

The mechanisms of action include the production of hydrolytic enzymes, competition for nutrients and space, and the induction of host resistance mechanisms, the direct interaction between the antagonist and the pathogen and the antibiosis (Montesinos and Bonaterra 2009). On several occasions the BCAs act not only by a single mechanism of action, but with a combination of them, where the importance of eachone is relative and is conditioned by environmental factors. The production of hydrolytic enzymes (included in hyperparasitism) permits to synthesize compounds by inducible complex process, which principally alters the fungal cell wall by hydrolysis (Lam and Gaffney 1993). The hydrolytic enzymes involved in this process are kinases, cellulases or glucosidases, which hydrolyze chitin, glucan and cellulose, respectively, the major components of fungal cell wall (Handelsman and Parke 1989). The capacity to synthesize hydrolytic enzymes has described in *Bacillus* strains (Lee *et al*. 2012), specifically the synthesis of chitinases have been reported in *Bacillus* spp. (Yamabhai *et al*. 2008).

The competition for nutrients and space is a preventive mechanism of action which permits the inhibition of pathogens without the direct effect over themselves. Competition occurs when the BCA and the pathogen share the necessity of any nutrient or limiting space. Control takes place when BCA shows a greater efficiency to establish on the plant surface, or to acquire limited nutrients respect pathogens. In relation to the assimilation of nutrients, high efficiency and assimilation rate in obtaining limiting nutrients such as nitrogen compounds, iron, carbon or oxygen, confers the capacity to limiting the pathogen growth. Some examples of this mode of action are *Bacillus pumilus* 3PPE and *B. amyloliquefaciens* 2TOE strains against grey mould of pear (Mari *et al.* 1996). In relation to the competition for space, some microorganisms have the capacity to colonize an injured surface quicker than pathogens, inhibiting the development of disease. This fact occurs when the antagonist show a capacity to adhesion, distribution, multiplication and survive on the environment more efficiently than pathogens. Thus, to colonize the surface of the plant is an essential feature to the biocontrol development. A clear example is the phenomenon of the formation of biofilms by *B. subtilis* strain 6051 against *P. syringae* in *Arabidopsis* (Bais *et al*. 2004).

Some microorganisms have the capacity to trigger pathogen-induced-resistance (IR) on plants conferring a protection system in front of a wide range of pathogens. The IR can be established in the tissue surrounding the site of initial infection, ''localized acquired resistance'' (LAR), and also in the distant, uninfected parts of the plant, ''systemic acquired resistance'' (SAR) (Hammerschmidt 2009, Ross 1957). SAR confers long-lasting protection against a broad spectrum of microorganisms, which is commonly activated during pathogens infections, is mediated by salicylic acid (SA) and is characterized by the accumulation of the large number of PR proteins. Most of these PR proteins are lytic enzymes, spreading signals or enzymes involved in the cell-wall reinforcement to resist the infections. It has been also described the induced systemic resistance (ISR) which is mediated by non-pathogenic bacteria and is not associated with PR gene expression or SA accumulation (Pieterse *et al*. 1996). In this case, response is mediated by the jasmonic acid (JA) and ethylene, and is activated by the action of plant growth promoting rhizobacteria (PGPR) (Kloepper *et al*. 2004, Pal and McSpadden-Gardener 2006, Pieterse *et al*. 1998). Different pathways can be activated by various microorganisms, multiple stimuli and different processes of the plant, which implies that the magnitude and duration of the induction of host defense changes with time. In particular, there are different types of resistance described the host induced by strains of *Bacillus*, as is the case of *B. mycoides* and *B. pumilus* (Pal and McSpadden-Gardener 2006), and *B. amyloliquefaciens* (Desoignies *et al*. 2013), which induce ISR in suggarbeet, and *B. subtilis*, which induces ISR in *Arabidopsis* (Pal and McSpadden-Gardener 2006). The capacity to increase the ISR has been associated with different *Bacillus* species as *B. amlolyquefaciens*, *B. cereus*, *B. mycoides*, *B. pumilus*, *B. sphaericus* and *B. subtilis* (Kloepper *et al*. 2004, McSpadden-Gardener 2004) that induce resistance to viral, bacterial, oomycete, fungal and nematode pathogens. Specifically, the plant root-colonizing *B. amyloliquefaciens* FZB42 is a naturally occurring isolate and has the ability to stimulate plant growth and suppress plant pathogens (Idris *et al*. 2004). *B. pumilus* PGPR-elicited induced resistance in wheat seedling roots against the take-all fungus, *Gaeumannomyces graminis* var. tritici (Sari *et al.* 2007), and *B. mycoides* strain BacJ in sugar beet (Bargabus *et al*. 2002).

The direct interaction with the pathogen is another mechanism of action of BCAs. It is based on the ability of some BCAs to recognize and interact with the pathogen, interfere with its growth and consequently with the development of the disease. One of the most studied cases is the hiperparasitism, in which pathogen is attacked directly by a specific BCA. This mechanism is only well known against fungal pathogens (Handelsman and Parke 1989) and nematodes (Chen *et al*. 1996) but not against bacterial pathogens. However, some studies revealed that clearly visible interaction were done on *Bacillus* strains against bacterial pathogens, although the appreciation of this mechanism was less sensitive in bacteria due to most divergences between tested pathogens in optimal growth conditions (Földes *et al*. 2000).

Quorum sensing inhibition or quorum quenching is the most recently described strategy of action to control plant pathogens. It is based on the inactivation of quorum sensing molecules, attenuating bacterial virulence (Liu *et al*. 2013). Quorum sensing is the regulation of gene expression in response to fluctuations in cell-population density by small signaling molecules (Miller and Bassler 2001). Many pathogenic bacteria use quorum sensing systems to regulate the expression of virulence genes in a density-dependent manner (Riedel *et al*. 2001, Winson *et al*. 1995). The small molecules that mediate these intercellular communication pathways are numerous and diverse in structure. Among this variety of signaling molecules, N-acyl-L-homoserine lactones (AHLs), are the most common and best known among pathogenic bacteria. In this way, the most extensively strategy of quorum quenching is mediated by AHL hydrolases (Liu *et al*. 2013). Some species of the *Bacillus* genus are able to produce an AHL lactonase, which rapidly catalyze the hydrolysis of the AHL ring in a number of different substrates (Dong *et al*. 2002). For example, the capacity of *B. thuringiensis* to suppress the quorum-sensing-dependent virulence of plant pathogen *Erwinia carotovora* has been reported (Dong *et al.* 2004).

Finally, antibiosis or production of metabolites with antibiotic activity is the last mechanism. Most of active BCAs are bacteria with capacity to synthesis metabolites, which are secreted to the environment and present some harmful properties among other microorganisms or organisms. These are

secondary compounds or derivatives of metabolism, with low molecular weight, which act at low concentrations reducing the growth or metabolic activity of other microorganisms (Fravel 1988). The antimicrobial compounds produced by BCAs are very different, in structure and performance range. In some cases it could be demonstrated direct implication of antibiotic production in biocontrol, as in the case of *Pseudomonas fluorescens* CHA0. In addition, the same microorganism may be able to synthesize more than one antibiotic at a time (Keel *et al*. 1989). *Bacillus* species are the best known secondary metabolites producing bacteria with antibiotic activity (Stein 2005, Montesinos 2007, Ongena and Jacques 2008).

1.7 Safety of *Bacillus* as a biocontrol agent

There is a relatively low number of microbial pesticides registered in Europe (Table 1.3). The registration process is the main limitation due to European regulations, included in the Directive 2009/128/CE and the Regulation 1107/2009 CE, which are very restrictive. The main aspects in regulatory systems on the BCAs are the clear identification and description of microorganism strain, as well as the characterization of its potential effects of toxicity on human health and on non-target organisms. However, in Europe there are more aspects to consider that are not required in other countries, like the demonstration of the efficacy of the formulated product and another important aspect is that the authorization is given only for use in specific crops. Thus, each product should be evaluated in several experiments, in different geographic regions and in different years (Alabouvette *et al*. 2006). These aspects drastically increase the cost and time of products registration and besides biological control products commonly fail to show a consistent effect under variable environmental conditions and finally are discarded. As an example, the first application for register of a biopesticide, *Paecilomyces fumosoroseus*, was submitted to the European Union in 1994 and approved in 2001 (Montesinos 2003). Another example of the limitations of European regulations is the commercial product Serenade, which is a preparation based on a strain of *B. subtilis* used in the USA against several diseases in many crops and registered in Europe only for a limited number of uses.

In addition to efficacy, biosafety is another key issue for microbial pesticides authorization in the EU and other countries. Some of the species of *Bacillus* are known pathogens such as *B. anthracis* which is pathogenic to humans and other animals, and *B. cereus* which is a common cause of food poisoning (Claus and Berkeley 1986, Norris *et al*. 1981). *B. thuringiensis*, *B. larvae*, *B. lentimorbus*, *B. popilliae*, and some strains of *B. sphaericus*are pathogenic to certain insects. However, most of the species included in this genus, such as *B. amyloliquefaciens*, *B. licheniformis, B. pumilus* and *B. subtilis*, are considered non pathogenic, and have been extensively used in humans as dietary supplements (Sanders *et al*. 2003), in animals as growth promoters and competitive exclusion agents (Cutting 2011) or in agriculture as active ingredients of biopesticides or growth promoters (Pérez-Garcia *et al*. 2011). Concretely, *B. subtilis* is considered safe for human consumption and has been assigned as 'Generally Recognized As Safe' status (GRAS) by the FDA (FDA) on a case-by-case basis, and has been referred as 'Qualified Presumption of Safety' (QPS) by the EFSA (EFSA 2011), whose aims are to harmonize the safety assessment of micro-organisms throughout the food chain. Although the species traditionally included in the *B. subtilis* group could be considered as a group for QPS purposes, in the first instance it would seem prudent to deal with them on an individual species basis. Other indications of pathological conditions associated with the *B. subtilis* group are rare. However, in many clinical reports on opportunistic infections related with *Bacillus*, the causative agent has not been identified to species level (EFSA 2005).

2. *Bacillus* as a source of antimicrobial peptides

Members of *Bacillus* are often considered microbial factories for the production of a vast array of biological active molecules potentially inhibitory for plant pathogens (Stein 2005). Advances in genome sequencing have revealed that *Bacillus* possess an unexpectedly high number of genes implicated in the biosynthesis of antibiotic-like compounds. In the case of *B. subtilis*, more than 4 % of the genome has

Table 1.3. Strains of biocontrol agents in commercial microbial biofungicides and bactericides registered in the EU.

Active ingredient	Target pathogen/disease	Host
Ampelomyces quisqualis AQ10	Strictly specific to the fungi causing powdery mildew and belonging to the family *Erysphaceae*	Grape
*Aureobasidium pullulans**	Soil-borne fungal plant pathogens, and fire blight caused by *Erwinia amylovora*	Horticulture, forestry, viticulture
Bacillus amyloliquefaciens subsp. *plantarum* D747*	Seedling fungal pathogens including *Fusarium* spp., *Pythium* spp. and *Rhizoctonia* spp.	Wide range of crops including cereals, legumes, soft fruit and cotton
Bacillus subtilis QST713	Bacteria (fire blight) and fungal root diseases, *Alternaria*, *Aspergillus*, *Venturia* grey mould	Grape, pome and stone fruits, horticultural crops
Coniothyrium minitans CON/M/91-08 (DSM 9660)	*Sclerotinia C. minitans* is a highly specialized hyperparasite. Its host range is restricted to certain screrotia-forming species within the Ascomyctonia and Deuteromycotina	Horicultural crops (soil decontamination)
Gliocladium catenulatum J1446	*Pythium*, *Rhizoctonia*, *Phytophthora*, *Fusarium*, *Didymella*, *Botrytis*, *Vericillium*, *Alternaria*, *Cladosporium*, *Helminthosporium* and *Penicillium*	Vegetables, herbs, strawberries, ornamentals and stored products
Phlebiopsis gigantean 14	*Heterobasidion annosus*	Coniferous trees
Pseudomonas chlororaphis MA 342	Seedborne fungi	Fieldgrown monocotyledonous crops (oat, rye, durum, barley, wheat triticale)
Pythium oligandrum M1	Soilborne pathogenic fungi and *Alternaria* and *Sclerotium*	Oil-seed rape in the field
Streptomyces K61 (formely *S. griseoviridis*)	Root rot, grey mould and food decay	Ornamentals, vegetables and herbs, under protection and outdoors

Active ingredient	Target pathogen/disease	Host
Trichoderma asperellum (formerly *T. harzianum*) strains ICC012, T11 and TV1	Soil-borne fungal plant pathogens (e.g., *Pythium* spp., *Phytophthora* spp., *Sclerotinia* spp., *Sclerotium* spp., *Thielaviopsis basicola*, *Rhizoctonia* spp., *Verticillium* spp.)	Horticulture, forestry, viticulture, nursery, glasshouse, greenhouse, open fields, protected crops, home gardening, house plants, ornamentals
Trichoderma atroviridae (formerly *T. harzianum*) strains IMI 206040 and T11	Soil-borne fungal plant pathogens	Strawberries outdoors and in greenhouse. On ornamental trees in the field
Trichoderma gamsii (formerly *T. viride*) ICC080	Soil-borne fungal plant pathogens (e.g., *Pythium* spp., *Phytophthora* spp., *Sclerotinia* spp., *Sclerotium* spp., *Thielaviopsis basicola*, *Rhizoctonia* spp., *Verticillium* spp.)	Horticulture, forestry, viticulture, nursery, glasshouse, greenhouse, open fields, protected crops, home gardening, house plants, ornamentals
Trichoderma harzianum strains T-22 and ITEM 908	Soil-borne fungal plant pathogens	Horticulture, forestry, viticulture, nursey, glasshouse, greenhouse, open fields, protected crops, home gardening, house plants, ornamentals
Trichoderma polysporum IMI 206039	Soil-borne fungal plant pathogens	Horticulture, forestry, viticulture, nursey, glasshouse, greenhouse, open fields, protected crops, home gardening, house plants, ornamentals
Verticillium albo-atrum (formerly *V. dahliae*) WCS850	Dutch elm disease	Elm

This table contains only products currently registered (updated 17/04/2013).
*Products that are pending of decision and temporarily authorized in certain countries.

been found potentially devoted to synthesis of polyketides (PKs), nonribosomal peptides (NRPs), lantibiotics as well as other unusual antibiotics (Arguelles-Arias *et al*. 2009, Chen *et al*. 2009a, Koumoutsi *et al*. 2004, Kunst *et al*. 1997, Schneider *et al*. 2007). Moreover, studies on the genome of *B. amyloliquefaciens* FZB42 reveal the presence of nine giant gene clusters directing synthesis of bioactive peptides and PKs by modularly organized mega-enzymes named non-ribosomal peptides synthetases (NRPSs) and polyketide synthetases (PKSs) (Chen *et al*. 2009b).

Antimicrobial peptides (AMPs) represent the predominant class, and most of them have been characterized as polypeptides of low molecular weight. Among these peptides, cyclic lipopeptides (cLPs) of the surfactin, iturin and fengycin families have well-recognized potential uses in biotechnology, biopharmaceutical and agricultural applications because of their bio-active properties (Ongena and Jacques 2008). Generally, these peptides exhibit highly rigid, hydrophobic and/or cyclic structures. Furthermore, cysteine residues are either oxidized to disulphides and/or are modified to characteristic intramolecular C–S (thioether) linkages,and consequently the AMPs are insensitive to oxidation (Stein 2005). Additionally, linear and cyclic peptides may have linked fatty acid chains or other chemical substitutions resulting in complex molecules as the LPs (Montesinos 2007, Stein 2005).

Antimicrobial compounds produced by *Bacillus* are classified according to their structure and biosynthetic mechanism. Two different biosynthetic pathways have been described the ribosomal synthesis of linear precursor peptides that are subjected to post-translational modification and proteolytic processing (Lee and Kim 2011, Stein 2005), and the non-ribosomal synthesis of peptides or PKs by large megaenzymes, the non-ribosomal peptide synthetases (NRPSs) or hybrid polyketide synthases (PKSs) (Arguelles-Arias *et al*. 2009, Chen *et al*. 2009a, Montesinos 2007, Ongena and Jacques 2008, Stein 2005).

2.1. Ribosomal peptide antibiotics

Bacillus spp. genus is able to produce ribosomally synthetized peptides, the so-called bacteriocins (Abriouel *et al*. 2011, Lee and Kim 2011). Based on their structure and biological activities, bacteriocins could be divided in three groups. Class I bacteriocins, also called lantibiotics, are characterized by their unusual amino acids such as lanthionine, methyllanthionine and dehydrated residues (Fickers 2012). Post-translational modification of ribosomally synthesized precursor allows the lanthionine formation, including dehydratation of serine and threonine residues and addition of neighboring cysteine thiol groups (Klein *et al*. 1993, Lee and Kim 2011, Stein 2005). Compared to lantibiotics, class II bacteriocins are non-modified peptides that are synthesized ribosomally and are characterized by a molecular weight below 5 kDa. Finally, class III bacteriocins are heat sensitive molecules with a molecular weight higher than 30 KDa, and megacin A-216 is the only representative among the *Bacillus* genus.

More specifically, lantibiotics are small molecules (3-10 kDa) and are inactivated by conventional proteolytic enzymes. In addition, most of them show a cationic and amphiphilic character (Lee and Kim 2011). Lantibiotics can be further divided by general structure, molecular weight and charge into groups A and B. Type A lantibiotics (21-38 amino acid residues) exhibited linear secondary structures and are positively charged. By contrast, type B lantibiotics, exhibit a globular structure and are non-charged. Type A lantibiotics group includes subtilin and ericins, and type B includes mersacidin, sublancin and subtilosin (Klein *et al.* 1993, Mannanov and Sattarova 2001, Stein 2005). Type A lantibiotics are active against Gram-positive target cells by forming voltage-dependent pores into the cytoplasmic membrane (Lee and Kim 2011, Parisot *et al*. 2008, Stein 2005, Stein and Entian 2002) or by inhibition of peptidoglycan synthesis (Bierbaum and Sahl 2009). The Gram-positive lantibiotic producers display different mechanisms to protect themselves of their own products. The ATP binding cassette transporter homologous proteins (LanFEG) permit to export the lantibiotic from the cytoplasmatic membrane into the extracellular space. Moreover, some lantibiotic producers possess membrane-bound lipoproteins LanI, which exhibit a sequestering-like

function that prevents high local concentrations of the lantibiotic close to the cytoplasmatic membrane and/or interferes with lantibiotic lipid II pore formation (Koponen *et al*. 2004, Stein 2005).

2.1.1 Subtilin

Subtilin is one of the most interesting and studied lantibiotics produced by *B. subtilis*. Its structure consists in 32 amino acid residues arranged in a cationic pentacyclic structure (Lee and Kim 2011, Stein 2005). As other lantibiotics, antibacterial activity rest on Gram-positive target cells by forming cationic peptide pores (Liu and Hansen 1992, Stein 2005). Subtilin antibacterial activity is easily lost during isolation and storage because is a relatively instable compound (Liu and Hansen 1992). The genetic studies reveal as subtilin gene cluster is composed for 10 genes (*spa BTCSIFEGRK*). The combination of these genes control the synthesis of different proteins evolved in the biosynthesis, transport and maturation of subtilin. Moreover, they are the responsible to control the immunity of producers (Lee and Kim 2011, Mannanov and Sattarova 2001). Subtilin biosynthesis is regulated by positive feedback, in which extracellular subtilin activates the two component regulatory system spaK (sensor histidine kinase) and SpaR (protein regulator) that binds to a DNA motif (spa-box) promoting the expression of genes for subtilin biosynthesis (*spaS* and *spaBTC*) and immunity (*spaIFEG*) (Kleerebezem 2004, Stein *et al*. 2003). On the other hand, *SpaRK* expression is controlled by the sporulation transcription Sigma H factor (Stein *et al*. 2004). Thus, subtilin production appears to be dual controlled, to culture density in quorum sensing mechanism in which subtilin plays a pheromone type role and in response to the growth phase (Bongers *et al.* 2005, Burkard *et al*. 2007, Lee and Kim 2011, Stein 2005).

2.1.2. Other bacteriocins

Ericin bacteriocins are type A lantibiotics. The ericin gene cluster contains two structural genes, *eriA* and *eriS*, although the open reading frames (ORFs) are closely related to corresponding genes of the subtilin cluster (Stein *et al.* 2002, Stein 2005). Ericin S structure is close related with subtilin and only differed in 4 amino acid residues. However, ericin A

structures varied in 16 amino acid residues and the ring conformation in comparison to ericin S. The lantibiotic mersacidin belongs to the type B lantibiotics which exhibit a more globular structure. It inhibits cell wall biosynthesis by complexing lipid II (Brötz *et al*. 1997). The mersacidin gene cluster consists of the structural gene *mrsA*, as well as genes involved in post-translational modification (*mrsM* and *mrsD*), transport (*mrsT*), immunity (*mrsFEG*) and regulation (*mrsR1*, *mrsR2*, *mrsK2*) (Guder *et al*. 2002). Mersacidin production occurs from the beginning of the stationary phase; however, the link between its mersacidin regulatory systems and the cellular regulation network of *B. subtilis* is yet unknown (Stein 2005). Some unusual lantibiotics as sublancin and subtilosin A were also described both active against Gram-positive bacteria. Sublancin shows two disulphide bridges in the structure, and subtilosin A is the unique macrocyclic bacteriocin, it has a macrocyclic structure with three inter-residual amino acid alpha-carbons (Huang *et al*. 2009, Stein 2005).

2.2. Non-ribosomal peptide antibiotics

The non-ribosomal synthesis of AMP is widespread among bacteria and fungi. Non-ribosomal peptides (NRPs) are synthesized by large multimodular NRPs synthetase (NRPSs) by elongation of activated monomers of amino and hydroxyl acid building blocks (Fickers 2012). NRPSs are organized in modules responsible for the incorporation of a specific amino acid. The modules consist of three main core domains that catalyze a specific reaction for the incorporation of a monomer (Ongena and Jacques 2008, Stein 2005). Firstly, the adenylation domain (A), with 550 aa, selects and activates the cognate amino acid as an amino acyladenylate. Then, thiolation domain (T), with 80 aa, covalently binds the activated monomer to the synthetase through a phosphopantetheinyl arm. Finally, the condensation domain (C), with 450 aa, catalyses the formation of the peptide linkage between the activated amino acids from two adjacent T modules (Fickers 2012, Ongena and Jacques 2008, Stein 2005). The linear assembly line-like arrangement of multiple of such core units (A–C) ensures the coordinated elongation of the peptide product. In most of cases, the non-ribosomal peptide biosynthesis is terminated by macrocyclization of the peptide product, whereby parts of the molecule

distant in the constructed linear peptide chain are covalently linked to one another (Kohli and Walsh 2003). These specialized domains enable NRPSs to synthesize an impressive number of diversified structures with broad range of biological activities that could not be obtained by the ribosomal machinery (Felnagle *et al.* 2008). Among these diversity of structures, cLPs including surfactin, iturin and fengycin families (Fig. 1.2) are widely spread in *B. subtilis* and related species (Stein 2005).

2.2.1. Surfactins

The surfactin family is considered the first family of cLP, and encompasses structural variants but all members are heptapeptides with an LLDLLDL chiral sequence linked by a β-hydroxy fatty acid consisting of 13–16 carbon atoms to form a cyclic lactone structure (Arguelles-Arias *et al*. 2009, Chen *et al*. 2009b, Leclère *et al*. 2006, Ongena and Jacques 2008, Peypoux *et al*. 1999, Stein 2005).

The compounds of its family are powerful biosurfactants with emulsifying and foaming properties (Carrillo *et al*. 2003, Chen *et al*. 2009b, Ongena and Jacques 2008, Peypoux *et al*. 1999, Stein 2005). Because of their amphyphilic nature, surfactins can also anchor into lipid layers and can interfere with biological membrane integrity in a dose-dependent manner. This could explain why surfactins display haemolytic, antiviral, and antibacterial activities (Ongena and Jacques 2008, Peypoux *et al*. 1999). Biosynthesis of surfactin is catalyzed by a synthetase complex that consists of four enzymatic subunits. Three are surfactin synthetases, SrfA, SrfB and SfrC, and the fourth is the acyltransferase SrfD, which plays an important role in the surfactin initiation reaction which consists in transfer the β-hytroxy fatty acid substrate from coenzyme A to the first module of SrfA (Steller *et al*. 2004). The surfactin synthetase complex is coded by the inducible operon named *srf* (25 kb), which is also responsible for sporulation and competence development (Hamoen *et al*. 2003). This operon is present in *B. amyloliquefaciens* FZB42 genome, and is organized in a similar manner as in *B. subtilis* 168 (Chen *et al*. 2009a , Koumoutsi *et al*. 2004, Stein 2005).

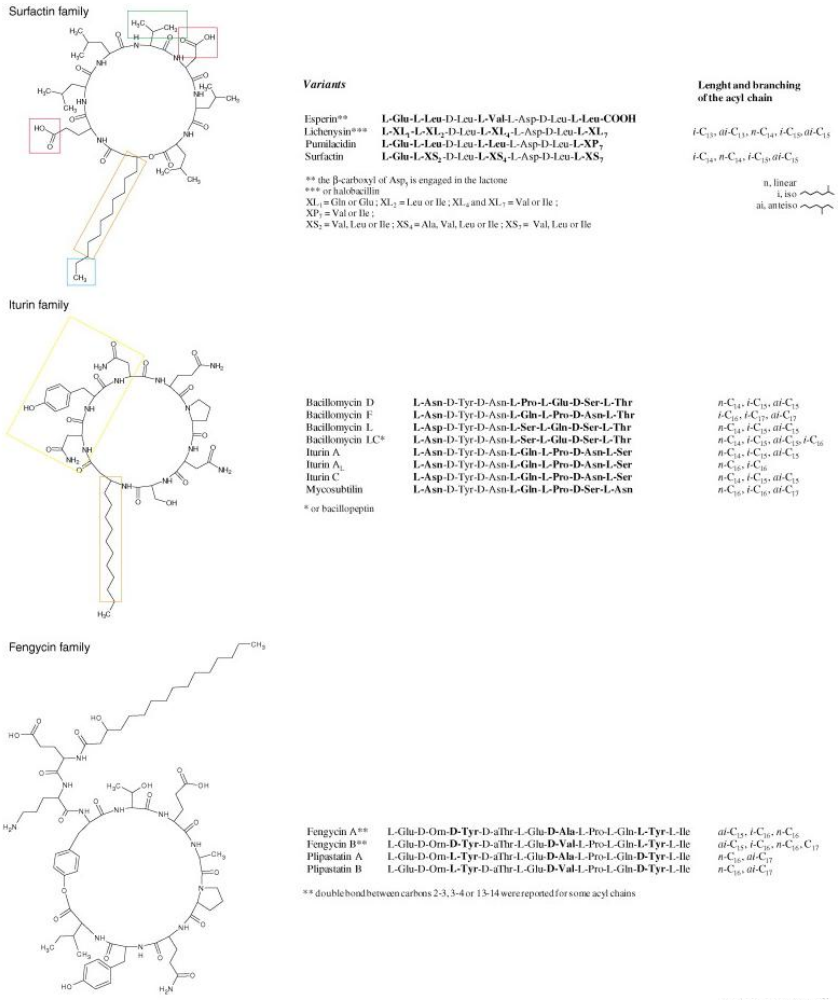

Figure 1.2.Structures of representative members and diversity within the three cyclic lipopeptide families synthesized by *Bacillus* species (taken from Ongena and Jacques 2008).

The synthesis of surfactin is growth-phase-dependent and is induced during transition to stationary phase (Chen *et al*. 2009b). The regulation is mediated via a complex network that governs cellular differentiation, including quorum sensing via extracellular ComX and the two-component regulatory system ComAP (Hamoen *et al*. 2003, Nakano *et al*. 1991). Moreover, several reports showed as surfactin biosynthesis is closely related to the early stages of sporulation (Nakano *et al*.1991, Yazgan *et al.* 2001a).

2.2.2. Iturins

Iturin A, C, D and E, bacillomycin D, F, and L, bacillopeptin and mycosubtilin were described as the main variants within the iturin family (Moyne *et al*. 2004, Ongena and Jacques 2008). Members of the iturin family are heptapeptides linked to a β-amino fatty acid with a length of 14 to 17 carbons (Arguelles-Arias *et al*. 2009, Leclère *et al*. 2005, Raaijmakers*et al*. 2010, Stein 2005). The members of the iturin family are strongly hemolytic and display a strong *in vitro* antifungal activity against a wide range of fungi and yeast, but a limited antibacterial and antiviral activity (Moyne *et al*. 2001, Romero *et al*. 2007, Thimon *et al*. 1995). The fungitoxicity of iturins relies on their membrane permeabilization properties (Bonmatin *et al*. 2003). However, the underlying mechanism is based on osmotic perturbation owing to the formation of ion-conducting pores and not membrane disruption or solubilization as caused by surfactins (Aranda *et al*. 2005, Patel *et al*. 2011). The NRPSs gene cluster of bacillomycin (*bam*/*bmy*), mycosubtilin (*myc*) and iturin A (*itu*) is composed of four large ORFs called *bmyD*, *bmyA*, *bmyB* and *bmyC* for bacillomycin, *fenF*, *mycA*, *mycB* and *mycC* for mycosubtilin and *ituD*, *ituA*, *ituB* and *ituC* for iturin (Duitman *et al*. 1999, Koumoutsi *et al*. 2004, Moyne *et al*. 2004, Tsuge *et al*. 2001). The first ORF, corresponding to *bmyD*, *ituD* and *fenF*, encodes malonyl-CoA transcylase. The last three genes code for the NRPSs responsible for the incorporation of the first residue for bmyA (or mycA or ituA), the following four residues for *bmyB* (or *mycB* or *ituB*) and the two last residues for *ituC* (or *mycC*) (Chen *et al*. 2009b, Ongena and Jacques 2008). Mycosubtilin and iturin A have almost the same structure expect that the last two amino acids in mycosubtilin are inverted in iturin A. In contrast,

bacillomycin variants are characterized for the presence of a terminal threonine (Thasana *et al*. 2010).

2.2.3. Fengycins

Fengycin and the closely related plipastatin, are cLPs containing an internal lactone ring in the peptidic moiety and with a β-hydroxy fatty acid with a side chain length of 14-18 carbon atoms (Arguelles-Arias *et al*. 2009, Wu *et al*. 2006, Chen *et al*. 2009a, Leclère *et al*. 2005, Ongena and Jacques 2008, Raaijmakers *et al*. 2010, Stein 2005). Fengycin family combines several exceptional structural properties, such cyclization and branching and unusual constituents, like the presence of ornithine and four D-amino acids (Chen *et al*. 2009a, Stein 2005, Vanittanakom *et al*. 1986). Compared with the previoulsy described cLPs (surfactins and iturins), fengycins are equally widespread in occurrence but relatively low in structure diversity. Fengycin A and fengycin B have been described as the only two types of structural variants which differ in the amino acid residue at position 6, namely fengycin A has an Ala, in contrast to fengycin B which has a Val at the same position. The mechanism of action of fengycins is less well known compared with other LPs. Fengycin family is specifically active against filamentous fungi and inhibits phospholipase A2, altering the structure and permeability of the cell membrane (Chen *et al*. 2009a, Koumoutsi *et al*. 2004, Ongena and Jacques 2008, Patel *et al*. 2011, Stein 2005). Fengycins are synthesized by NRPSs encoded by an operon with five ORFs *fenA-E* (Steller *et al*. 2004). The *fen* operon described in *B. amyloliquefaciens* FZB42 is about 25kb distant from operon *bmy* and is related to the *pps* operon in *B. subtilis* 168 that is situated at the same locus (Chen *et al*. 2009a).

2.2.4. Bacilysin

Bacilysin is one of the simplest peptide antibiotics known and has been described in certain *Bacillus* strains (Chen *et al*. 2009a, Chen *et al*. 2009c, Mannanov and sattarova 2001, Rajavel *et al*. 2009, Yazgan *et* al. 2001a). It is a dipeptide antibiotic which contains L-alanine residue at the N-terminus and a non-proteinogenic amino acid, L-anticapsin, at the C-terminus (L-alanyl-[2,3-epoxycyclohezanone-4]-L-alanine) (Chen *et al*. 2009b, Stein

2005). The peptide bound with L-alanine proceeds with a non-ribosomal mode catalyzed by an amino acid ligase (bacilysin synthetase) (Chen *et al.* 2009a). Bacilysin is active against a wide range of bacteria and yeast, and its action is performed by the anticapsin moiety, which becomes released after uptake into susceptible cells and blocks glucosamine synthetase, an essentials enzyme of cell wall biosynthesis (Chen *et al.* 2009a, Chen *et al*. 2009c). Bacilysin biosynthesis is regulated by the *bacABCDE* (from *ywfBCDEF*) gene cluster. The *bacABCDE* gene cluster in *B. amyloliquefaciens* FZB42 was collinear to *B. subtilis* 168 and its homology ranged between 84-93 % (Chen *et al*. 2009a, Yazgan *et al*. 2001a). Moreover, bacilysin production is regulated nutricional and feedback regulation, negatively by GTP via the transcriptional regulator *CodY* and *AbrB*, and positively by guanonsine tetraphosphate (ppGpp) and a quorum-sensing mechanism through the peptide pheromone PhrC (Stein 2005). Furthermore, unlike other LPs as surfactin, bacilysin biosynthesis is not close related on sporulation (Yazgan *et al.* 2001a).

2.3. Miscellaneous antibiotic compounds

In addition to antimicrobial peptides, PKs are the other dominant family of secondary metabolites having antimicrobial, immunosuppressive, antitumor, or other bioactivities. Although PKs are widespread secondary metabolites from other bacteria, only a few have been isolated and characterized from *Bacillus* (Schneider *et al*. 2007). Difficidin/oxydifficidin, bacillaene (Hofemeister *et al*. 2004) and macrolactin (Jaruchoktaweechai *et al*. 2000) are some examples of PKs produced by several wild type strains belonging to *B. subtilis* and *B. amyloliquefaciens*. Bacterial PKs are synthesized by type I polyketide synthases (PKSs), modularly organized assembly lines starting from acyl-CoA precursors by decarboxylative condensations (Khosla *et al*. 2007). In general, their biosynthetic pathway follows the same logic as in non-ribosomally synthesized peptides and requires at least three domains (Walsh 2004): an acyltransferase (AT) domain which selects the appropriate extender unit and transfers it to the acyl-carrier-protein (ACP) domain, where the thioester bond is formed fixing the growing polyketide to the synthase, and a ketosynthase (KS) domain which is responsible for the condensation between the extender unit

present in the ACP domain of the same module and the PK intermediate bound to the ACP domain of the preceding module. Additional secondary domains are the responsible to modify the growing PK molecule (Arguelles-Arias *et al*. 2009).

A series of new antibiotics have been recently isolated from well-known *B. subtilis* strains, including bacilysocin, an anti-microbial phospholipid, which can be isolated from *B. subtilis* 168 cells by extraction with butanol (Tamehiro *et al*. 2002). Its activity is more pronounced against eukaryotic organism *Saccharomyces cerevisiae*, in addition to fungi with nonfilamentous growth, like *Candida pseudotropicalis* and *Cryptococcus neorformans* (Hamdache *et al*. 2011). Amicoumacins are phenylpropanol derivate substances that are produced by several *B. subtilis* strains (Pinchuk *et al*. 2002). These compounds were isolated for first time in *B. pumilus* and exhibited antibacterial and suppressed inflammation and ulcer activity.

2.4. Involvement of antimicrobial peptides in biocontrol

Cell wall-degrading enzymes, AMP and other small molecules produced by *Bacillus* strains have been shown to contribute to plant pathogen suppression (Shoda 2000). However, LPs are the more often produced and studied antibiotic compounds among *Bacillus* species. The main functions of LPs described to date are their role in antagonism towards other microorganisms, motility and attachment to surfaces and more recently the induction of plant defense responses (McSpadden-Gardener 2004, Pérez-García *et al*. 2011, Raaijmakers *et al*. 2006).

The capacity to produce secondary metabolites, especially LPs, has been proposed to confer a competitive advantage in interactions with other microorganisms in natural habitats (Raaijmakers *et al*. 2010). Besides, several studies reveal as most *Bacillus* strains presented antibiosis activity against a wide range of plant pathogens, and sometimes against human pathogens too. Also, in most cases it had been possible to identify the active compounds responsible for this activity (Arguelles-Arias *et al*. 2009, Ongena and Jacques 2008, Raaijmakers *et al*. 2010, Stein 2005). For example, bacillomicyn D extracted from *B. subtilis* AU195 is active against

Aspergillus flavus (Moyne *et al*. 2001). Surfactin produced by *B. subtilis* 6051 is active against root infections by *P. syringae* in arabidopsis (Bais *et al*. 2004). Overexpression of mycosubtilin in *B. subtilis* ATCC6633 also led to a significant reduction of seedling infection by *Phytium aphanidermatum* in tomato (Leclere *et al*. 2005). Iturin A produced by *B. subtilis* RB14 was involved in the control of damping-off tomato caused by *Rhizoctonia solani* (Asaka and Shoda 1996). Although most of these studies focus on a single molecule as the active molecule, lypopeptides and other AMPs are known to act in a synergistic manner. Several studies described how LPs combine with each other to improve their activity, as bacillomycin, fengycin and iturin acts against powdery mildew on melon caused by *Podosphaera fusca* (Ongena and Jacques 2008, Romero *et al*. 2007). Also in the case of *B. amyloliquefaciens* GA1, the production of a wide variety of fengycins and other LPs, protected wounded apple fruits against gray mold disease caused by *Botrytis cinerea* (Arguelles-Arias *et al*. 2009). Bacillomycin D and fengycin are responsible for the main antifungal activity exerted by *B. amyloliquefaciens* FZB42 and was shown to suppress growth of *Fusarium oxysporum*, *Geaumannomycer grammis*, *Rhizoctonia solani*, *Alternaria alternata*, *Botrytis cinerea* and *Pythium aphanidermatum* (Chen *et al*. 2009a, Chen *et al*. 2009b, Koumoutsi *et al*. 2004). Moreover, it was described the antibacterial activity of bacilysin, jointly with difficidin poliketide, against *E. amylovora* in *B. amyloliquefaciens* FZB42 (Chen *et al*. 2009a, Chen *et al*. 2009c).

Besides antibiosis, many other properties have been attributed to the AMPs, as their implication in plant tissue colonization. LPs are generally involved in the colonization of surfaces, which act as chemical signals to move to the root surface by modifying cell surface properties (Ongena and Jacques 2008). The extracellular matrix of the biofilm protects bacteria against adverse environmental conditions, and also provides protection against protozoan predation and is a niche for horizontal gene transfer (Branda *et al*. 2001). Surfactins have an important role in the formation of stable biofilm, intervening in different process. Surfactins have been involved in pellicle formation at the air-water interface and permitted the modification of surface properties of *B. subtilis* jointly with iturin. Moreover, their secretion is related with the cell motility, facilitate cell spreading and

swarming (Arguelles-Arias *et al*. 2009, Ongena and Jacques 2008, Raaijmakers *et al*. 2010, Stein 2005). At the same way, fengycins can also play an important role in the colonization of surfaces. They can be applicable as anti-adhesion agents against biofilm and as biosurfactants for polycyclic aromatic hydrocarbons degradation (Romero *et al*. 2007). Biosurfactants can change the viscosity of surfaces, thereby influencing cell differentiation and motility. In plant-associated environments, biosurfactants may act as wet table agents of the hydrophobic cuticle of leaves, which may promote not only cell motility but even solubilization and diffusion of substrates for growth. The LP is thought to be involved in aggregation of the cells into dendrites and in the coordination of their movement throughout the swarm front. Enhancement of motility was also observed for mycosubtilin overproducers (Leclère *et al*. 2005).

Production of AMPs has also been related to the capacity in triggering induced systemic resistance (ISR) in plant systems (Choudhary and Johri 2009, Jourdan *et al*. 2009, Ongena *et al*. 2005). Surfactin and fengycin have been identified as bacterial determinants responsible for elicitation of ISR in bean and tomato leaves against *B. cinerea* (Ongena *et al.* 2007). A clear accumulation of nonpolar antifungal compounds also occurred in the treated plants with LPs, suggesting a pytoalexin-inducing activity of *Bacillus* LPs (Raaijmakers *et al*. 2010). In other systems, major changes in the defence response of plant cells, such as modifications in the pattern of phenolics, were observed upon treatment with *Bacillus* LPs (Jourdan *et al*. 2009, Ongena *et al*. 2005). Treatment of tobacco cell suspensions with low concentrations of surfactin induced several early plant defense-related events such as phosphorylation, Ca^{2+} dependent extracellular alkalinization and oxidative burst (Jourdan *et al*. 2009). Compared with defense-related events triggered by elicitors from plant pathogens, it is yet unclear whether the induction of the ISR response by LPs requires specific receptors in the plant membrane. It is postulated that some LPs may induce a disturbance or transient channeling in the plasma membrane, which in turn activates a cascade of molecular events leading to enchanced defence (Jourdan *et al*. 2009). However, to further exploit these potential beneficial effects, more knowledge is required about the key structural features and constituents of LPs involved in the induction of the defense responses in plants.

Context of this work

The current trend of crop protection has been reoriented to a rational use of pesticides and to a reduction of the number of registered active ingredients to those certainly unavoidable, more selective, less toxic and with a lower negative environmental impact (Montesinos 2003, Montesinos and Bonaterra 2009). In this context, the interest in new active ingredients based on microorganisms has increased. For this reason, the group of Plant Pathology of the University of Girona initiated its experience in biocontrol of plant diseases around 1993 with a study focused on the isolation of bacteria from plant environments to control *Stemphylium vesicarium* the causal agent of brown spot of pear (Montesinos *et al*. 1996), a disease of economic importance. Since then, the group has focused their studies in beneficial Gram-negative bacteria, on the isolation, characterization and improvement of BCAs, especially strains of *P. fluorescens* and *P. agglomerans* species, to control plant diseases of economical interest in agriculture, like fire blight and brown spot of pear, but also in postharvest rot losses. The studies have been focused in all the aspects of biocontrol, including determination of the mechanisms of action (Cabrefiga *et al*. 2007), dose-response relationships (Montesinos and Bonatera 1996, Francés *et al*. 2006), colonization and traceability (Pujol *et al*. 2005, Pujol *et al*. 2006), and physiological improvement of formulations (Bonaterra *et al*. 2005, Bonaterra *et al*. 2007, Cabrefiga *et al*. 2011). In the last decade, the group started a new research focused on Gram-positive bacterial strains as BCAs, including lactic acid bacteria (Trias *et al*. 2008) and *Bacillus* (Mora *et al*. 2011), with an interesting profile for biosafety, production and formulation. Accordingly, the present PhD thesis deal with isolation of *Bacillus* from plant environments, and their characterization, with the main objective of determinating relationships between antimicrobial activity, genes and products. It is expected that this knowledge could contribute to improve the efficiency of screening procedures to find better and novel BCAs.

General objectives

The main objectives of this thesis were:

1. To develop and evaluate a method to increase the efficiency of isolation of *Bacillus* strains with multiple AMP genes simultaneously.

2. To characterize a collection of *Bacillus* strains obtained from natural plant environments on basis of the presence of biosynthetic genes of AMPs, production of AMPs and antimicrobial activity against a broad range of bacterial and fungal plant pathogens.

3. To relate the presence of antimicrobial peptide biosynthetic genes with the production of surfactin, fengycin and iturin family cLPs, and the intensity of the antimicrobial activity of the *Bacillus* strains.

CHAPTER 2

A procedure for increasing the yield of isolation of *Bacillus* strains with multiple antimicrobial peptide genes from plant environments

Introduction

Plant disease control has been reoriented to a rational use of fungicides and bactericides and to non-chemical methods with decreased environmental impact. Microbial biopesticides offer an alternative or complement to chemical pesticides (Fravel 2005, Pal and McSpadden-Gardener 2006) and are composed of microbial strains, usually of microorganisms inhabitants of plant environments, including bacterial or fungal species or bacteriophages (Montesinos and Bonaterra 2009).

Commercial microbial biopesticides are under expansion in agriculture, and there is an increasing demand of biocontrol strains by the biopesticide industry. However, discovery of new biocontrol strains is a low efficiency process departing from large microbial collections which are submitted to a

reductional process of *in vitro*, *ex vivo* and *in planta* efficacy bioassays towards the target pathogen (Montesinos 2003, Montesinos and Bonaterra 2009, Pliego *et al*. 2011). Thus, appropriate screening procedures must be used to select the most suitable microorganisms for disease control in diverse plant environments. Selection of the adequate screening procedure will depend on the preferred mechanism of action but also on the biocontrol strategy (Pliego *et al*. 2011). In addition, efficiency in isolation of potential candidates can be a problem in some cases when species of interest are present in low amounts in natural samples or because they are overgrown by the accompanying microbiota with better performance under standard laboratory conditions. Thus, there is a need for enrichment and specific selection methods addressed to microbial groups with potential biocontrol properties. In this way, enrichment methods have been successfully used in the case of some *Bacillus* species to find bioinsecticides, due to its thermal tolerance compared to the more abundant non-sporulating bacteria found in plant environments (Johnson and Bishop 1996).

Species of *Bacillus*, like *B. subtilis* and *B. amyloliquefaciens,* have been the object of strong interest because they are safe, are present in most environments, and have remarkable abilities for synthesizing beneficial substances for agronomical purposes (Kloepper *et al*. 2004, Ongena and Jacques 2008). Also, the colonization aptitudes and outstanding ability to sporulate of *Bacillus* species, increase their survival and prevalence under adverse environmental conditions, facilitate the development of reliable formulations and increase the shelf-life of the formulated products (Chung *et al*. 2007, Collins and Jacobsen 2003).

Several strains of *Bacillus* have been reported to control plant diseases by different mechanisms of action including antibiosis, induction of defense responses in the host and competition for nutrient sources and space (Ongena and Jacques 2008, Raaijmakers *et al*. 2010). From these mechanisms, antibiosis by means of antimicrobial peptides (AMP) has been the focus of great interest. Cyclic lipopeptides (cLP) such as fengycin, iturin, bacillomycin and surfactin, that have a wide antimicrobial spectrum and intense surfactant activities (Stein 2005, Vater *et al*. 2002), which have been related with the biocontrol of several plant diseases (Bais *et al*. 2004,

Chen *et al*. 2009a, Chung *et al*. 2008, Ongena *et al*. 2005, Romero *et al*. 2007). In addition, multi-production of these cLPs has been described in *Bacillus* spp. strains with a broad range of activity against bacterial and fungal plant pathogens (Chen *et al*. 2009c, Joshi and McSpadden-Gardener 2006), and some studies have related production of multiple metabolites with the biological control activity (Chung *et al*. 2008, Romero *et al*. 2007).

The genus *Bacillus*, and specifically the species *B. subtilis* and *B. amyloliquefaciens*, are considered as microbial factories, due to their capacity to produce a broad spectrum of AMPs and other compounds, that has given up its high antimicrobial activity against variety of plant pathogens, as well as their implication in induction of systemic resistance in plants (Arguelles-Arias *et al*. 2009, Chen *et al*. 2009a, Ongena and Jacques 2008, Stein 2005). The production of different AMPs simultaneously is important for the efficient and broad range of antagonistic activity and biocontrol capacity in *Bacillus*. For example, the production of mixtures of bacillomycin, fengycin, and iturin A by *B. subtilis* has been related to the control of *Podosphaera fusca* in cucurbits (Romero *et al*. 2007), and the production of bacilysin, iturin, and mersacidin in *B. subtilis* ME488 was implicated in the suppression of *Fusarium* wilt of cucumber and *Phytophthora* blight of pepper (Chung *et al*. 2008). Accordingly, strains of *Bacillus* that scored positive for the AMP biosynthetic genes *bmyB*, *fenD*, *ituC*, *srfAA* and *srfAB* were more effective at inhibiting the growth of *R. solani* and *Pythium ultimum* than other *Bacillus* isolates that lacked one or more of those markers (Joshi and McSpadden-Gardener 2006). Also, the most effective *Bacillus* strains inhibiting *Sclerotinia sclerotiorum* harboured the surfactin and iturin A biosynthetic genes (Athukorala *et al*. 2009). In addition, the analysis of the genome in the commercial strain *B. amyloliquefaciens* FZB42 has revealed the presence of genes responsible for the synthesis of several antimicrobial compounds (Chen *et al*. 2009c), and similar genes have been reported in the commercialized *B. subtilis* strains GB03, QST713, and MBI600 (Joshi and McSpadden-Gardener 2006), and GA1 (Arguelles-Arias *et al*. 2009).

The use of molecular markers to prospect specifically biocontrol agents (BCAs) by means of the specific detection of genes involved in the synthesis

of secondary metabolites with antimicrobial properties seems to be a good strategy to increase the efficiency of screening procedures. The existing knowledge on mechanisms of action in several strains of *Bacillus* with proven biocontrol capacities have provide markers related to AMP biosynthetic genes like *bmyB*, *fenD*, *ituC*, *srfAA* and *srfAB* (González-Sánchez *et al*. 2010, Joshi and McSpadden-Gardener 2006). Therefore, the use of AMP genes as molecular markers to detect putative biocontrol strains combined with a selective enrichment procedure seems to be a good strategy to increase the yield of screening methods of *Bacillus* strains candidate to be developed as microbial biopesticides.

OBJECTIVES

The aim of the present work was to develop and evaluate a method to increase the efficiency of isolation of *Bacillus* strains with multiple AMP genes simultaneously. This method consists of a thermal treatment of the sample extract followed by growth at a restrictive temperature combined with molecular marker assisted screening targeted to several antimicrobial peptide genes.

Specific objectives were the:

- Design and evaluation of molecular tools based on antimicrobial peptide genes to screen field samples and pure cultures of *Bacillus* spp.
- Development and testing of a selective enrichment method for *Bacillus*.
- Evaluation of the efficacy of the screening procedure combining selective enrichment with molecular markers in comparison to the standard direct method of isolation.

MATERIALS AND METHODS

1. Development of PCR tools based on AMP genes to screen field samples and culture collections of *Bacillus* spp.

According to the objectives of this work we have develop tools for efficiently isolate potential BCAs pertaining to *Bacillus* spp. The marker-assisted screening method developed in this work was based on the detection of genus specific genes, and of genes involved in the biosynthetic pathways of the most important AMPs, which have been related with biocontrol. For this reason, molecular tools based on PCR have been designed, optimized and validated.

1.1 Bacterial strains

1.1.1 Strains and growth conditions

A collection of reference *Bacillus* and of non-*Bacillus* bacterial strains was built up to evaluate and validate the methodology of analysis (Table 2.1). *Bacillus* strains were selected on the basis of biocontrol capacities and the previously reported production of AMPs. These strains were obtained from commercial products, and from research groups.

Bacillus strains were cultured in Lysogeny broth (LB) agar at 28 ºC for 24 h, and non-*Bacillus* strains at 24 ºC for 24 h. For long-term preservation, bacteria were stored at -80 ºC in LB broth with 20 % glycerol. Bacterial suspensions were adjusted to a final concentration of 10^8 cfu/ml by standard curve that relates the cell concentration with the optical density. For non-*Bacillus* strains optical density was adjusted at 620 nm using a standard spectrophotometer (Aurius 2000 series, CE2021, CECIL Instruments Limited, Cambridge), while *Bacillus* strains concentrations were adjusted with NanoDrop 2000 Spectrophotometer (Thermoscientific, Barcelona, Spain) to get more accuracy in optical density determination. A standard curve was done for each genus among strains. The corresponding optical density values to fit the bacterial suspensions at 10^8 cfu/ml were the following: 0.25 for *P. fluorescens* strains, 0.15 for *P. agglomerans* strains and 0.04 for *Bacillus* strains. Bacterial concentrations were determined by plating 20 µl of the suspension onto LB agar, incubating at 28 °C for 24 h, and finally counting the grown colonies.

1.2 Selection of suitable molecular markers and design of primer sets for PCR

1.2.1 Selection of molecular markers

In order to determine the most interesting genes to be used as molecular markers of biocontrol, our interest was focused on reported specific genes of *Bacillus* as candidates to identify *Bacillus* at genus level, but also in genes

Table 2.1. Origin of *Bacillus* spp., *P. agglomerans* and *P. fluorescens* bacterial strains

Species	Strain	Source[x]	Reference
Bacillus amyloliquefaciens	FZB42	ABT	Koumoutsi, 2004
B. circulans	RGAF11	IAS	Landa *et al*. 2004
B. macerans	RGAF101	IAS	Landa *et al*. 2004
B. megaterium	RGAF46	IAS	Landa *et al*. 2004
B. megaterium	RGAF51	IAS	Landa *et al*. 2004
B. polymyxa	RGAF5	IAS	Landa *et al*. 2004
B. polymyxa	RGAF84	IAS	Landa *et al*. 2004
B. subtilis	QST713	NUF	Joshi and McSpadden, 2006
B. subtilis	EPS2004	This study	Mora *et al*. 2011
B. subtilis	RGAF9	IAS	Landa *et al*. 2004
B. subtilis	RGAF32	IAS	Landa *et al*. 2004
B. subtilis	UMAF6614	UMA	Romero *et al*. 2004
B. subtilis	UMAF6639	UMA	Romero *et al*. 2004
Bacillus sp.	RGAF66	IAS	Landa *et al*. 2004
Pantoea agglomerans	EPS10	UdG	Cabrefiga *et al*. 2007
	EPS13		
	EPS21		
	EPS125		
	EPS130		
	EPS132		
	EPS156		
	EPS203		
	EPS210		
	ESP230		
	ESP453		
Pseudomonas fluorescens	EPS 62e	UdG	Cabrefiga *et al*. 2007
	EPS82		
	EPS87		
	EPS89F		
	EPS95		
	EPS102		
	ESP173		
	EPS282		
	EPS353		
	EPS684		

[x]ABT, Abitep GmbH, Berlin (Germany); IAS-UCO, Instituto de Agricultura Sostenible-CSIC, Córdoba (Spain); NUF, Nufarm España, Barcelona (Spain); UMA, University of Málaga, Malaga (Spain), UdG, University of Girona, Girona (Spain).

involved in the synthesis of AMPs to be used as biocontrol markers. The search was focused on AMPs related with biocontrol (Chen *et al*. 2007, Joshi and McSpadden-Gardener 2006, Montesinos *et al*. 2007, Ongena and Jacques 2008, Stein 2005) though trying to include a wide range of compounds. In parallel, genomic sequences of genes of interest were obtained from the DNA sequence database at the National Center for Biotechnology Information (NCBI).

A selection of genes was done taking into account different structures, involvement in the biological control, and also the variability of sequences (Table 2.2). Finally, six sequences were selected as specific markers for AMP biosynthetic genes within the coding regions of *bmyB* (bacillomycin L synthetase B) (Hofemeister *et al*. 2004, Joshi and McSpadden-Gardener 2006, Koumoutsi *et al*. 2004, Koumoutsi *et al*. 2007), *fenD* (fengycin synthetase) (Joshi and McSpadden-Gardener 2006, Kunst *et al*. 1997), *ituC* (iturin A synthetase C) (Chung *et al*. 2008, Joshi and McSpadden-Gardener 2006, Moyne *et al*. 2004, Ongena and Jacques 2008), *srfAA* (surfactin synthetase subunit 1) (Chen *et al*. 2006, Chung *et al*. 2008, Hofemeister *et al*. 2004, Joshi and McSpadden-Gardener 2006, Koumoutsi *et al*. 2004, Kunst *et al*. 1997), *bacA* (bacilysin biosynthesis protein) (Chen *et al*. 2007, Chung *et al*. 2008), and *spaS* (Putative uncharacterized protein spaS) (Bongers *et al*. 2005, Burkard *et al*. 2007, Klein *et al*. 1992, Liu *et al*. 1992, Stein *et al.* 2002, Stein 2005). Two additional sequences were chosen as specific markers for *Bacillus* genus within the coding regions of the *16S rDNA* (16S ribosomal DNA) (Cazorla *et al*. 2007, Ronimus *et al*. 2003) and *spoVG* (putative septation protein spoVG) (Broggini *et al*. 2005, Kunst *et al*. 1997, Matsuno and Sonenshein 1999, Nakano *et al*. 1988, Schiött and Hederstedt 2000) due to these both genes showed a good specificity for *Bacillus* species.

1.2.2 Primers design

Before designing our own primers we have revised the possible existence of primers previously described in the literature for each of the biosynthetic genes of interest.

Table 2.2. Interesting AMPs described in *Bacillus* spp. according to their structure, related genes and strains in which have been reported.

Compound	Structure	Synthesis	Related Genes	Strains	References
Bacillomycin	Cyclc lipopeptide	Non-ribosomal	*bmy CBAD*	*B. amyloliquefaciens* FZB42	Chen *et al*. 2009b
Fengycin	Cyclc lipopeptide	Non-ribosomal	*fenABCDE* *ppsABCDE*	*B. amyloliquefaciens* FZB42 *B. subtilis* 168	Chen *et al*. 2009b Kunst *et al*. 1997
Iturin	Cyclc lipopeptide	Non-ribosomal	*ituABCD*	*B. subtilis* 168	Tsuge *et al*. 2005
Surfactin	Cyclc lipopeptide	Non-ribosomal	*srfABCD* *srfAA, AB, AC, AD*	*B. amyloliquefaciens* FZB42 *B. subtilis* 168	Chen *et al*. 2009b Kunst *et al*. 1997
Gramicidin	Cyclc lipopeptide	Non-ribosomal	*grsAB*	*B. brevis* ATCC9999	Krause and Marahiel 1988
Mycosubtilin	Cyclc lipopeptide	Non-ribosomal	*fenF, mycABC*	*B. subtilis* BBG100	Leclère *et al*. 2005
Bacilysin	Dipeptide	Non-ribosomal	*bacABCDE, ywfG* *ywfBCDEFG*	*B. amyloliquefaciens* FZB42 *B. subtilis* 168	Chen *et al*. 2009b Kunst *et al*. 1997
Chlorotetaine	Dipeptide	Non-ribosomal	*bacA and bacB*	*B. amyloliquefaciens* GA1	Arguelles-Arias *et al*. 2009
Anticapsin	Dipeptide	Non-ribosomal	*bacA and bacB*	*B. subtilis* 168	Steinborn *et al*. 2005
Bacillibactin	Siderophore	Non-ribosomal	*dhbABCDEF*	*B. amyloliquefaciens* FZB42 *B. subtilis* 168	Chen *et al*. 2009b Kunst *et al*. 1997
Macrolactin	Polyketide	Non-ribosomal	*mlnABCDEFGHI*	*B. amyloliquefaciens* FZB42	Chen *et al*. 2009b
Bacillaene	Polyketide	Non-ribosomal	*baeBCDE, acpk, baeGHIJLMNRS* *pksBCDE, acpk, pksGHIJLMNRS*	*B. amyloliquefaciens* FZB42 *B. subtilis* 168	Chen *et al*. 2009b Kunst *et al*. 1997
Difficidin	Polyketide	Non-ribosomal	*difAYXBCDEFGHIJKLM*	*B. amyloliquefaciens* FZB42	Chen *et al*. 2009b
Rhizocticin	Phosphono-oligopeptide	Non-ribosomal	*rhiA-rhiM*	*B. subtilis* ATCC6633	Borisova *et al*. 2010
Bacilysocin	Phospholipid	Non-ribosomal	*ytpA*	*B. subtilis* 168	Kunst *et al*. 1997
Subtilin	Lantibiotic	Ribosomal	*spaBTCSIFEGRK*	*B. subtilis* 168	Kunst *et al*. 1997
Subtilosin A	Lantibiotic	Ribosomal	*Sbo/albABCDEFG*	*B. subtilis* 168	Kunst *et al*. 1997
Sublancin	Lantibiotic	Ribosomal	*SunA, SunT*	*B. subtilis* 168	Kunst *et al*. 1997
Ericin	Lantibiotic	Ribosomal	*eriABCEFGIKRST*	*B. subtilis* A13	Stein *et al*. 2002
Mersacidin	Lantibiotic	Ribosomal	*mrsADEFGKMR1R2T*	*B. subtilis* 168	Altena *et al*. 2000

However, primers have not been described previously for some of the genes and most primers already described were designed using as a model partial gene sequences from few strains. For these reason, design of new primers for all the genes were considered necessary. Thus, specific primers targeting six AMP biosynthetic related genes (*srfAA*, *bacA*, *bmyB*, *fenD*, *ituC* and *spaS*) were designed from genomic sequences available in the Genbank (NCBI) (Table 2.3). Sequences for each gene were aligned by MULTALIN software (Corpet 1988) in order to obtain consensus sequences that were used to design the primers by means of Primer3 software (v.0.4.0) (Rozen and Skaletsky 2000). The gene sequences were introduced as well as the appropriate parameters according to the desired amplification. The most important parameters we considered were: amplicon length (bp), primer length (bp), annealing temperature (ºC) and guanine/cytosine percentage. Primers obtained were checked using nucleotide basic Local Alignment Search Tool v.2.2.6 (Blast-N; NCBI) to verify that not unspecific homologies exist with any other sequence available in the Genbank. Furthermore, two primers directed to molecular targets specific for *Bacillus* genus (*16S rDNA* and *spoVG* genes) were designed. In this case, the procedure to design the *spoVG* primers was the same as described above. In contrast, *16S rDNA* gene primers were obtained using a different design strategy. The sequences of *16S rDNA* obtained from the GenBank for different *Bacillus* species were aligned, with the aim of obtaining a consensus sequence for the *Bacillus* genus. The consensus sequence was then compared with other described sequences of *16S rDNA* for different species of non-*Bacillus*, especially *Pseudomonas* and *Pantoea* spp. and other common plant associated bacteria. The aim was to obtain homologous regions among *Bacillus* species but differential areas among species of other genus. Once these areas were determined, the primers were designed according to the procedure previously described.

1.3 Evaluation and optimization of PCR based methods for detection of molecular markers

Two strategies have been developed for the detection of molecular markers, one based on a single amplification of each gene individually and a second one based on multiplex PCR that allows the simultaneous amplification of

several genes in the same reaction. This option was included in order to reduce the cost and time consumption for massive strain/sample screening.

1.3.1 DNA extraction

DNA extractions were done using two different protocols depending on the necessities of each sample. The first protocol used was a commercial kit indicated for use in Gram-positive bacteria, the QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA) (Table 2.4). The second method was based on precipitation of DNA with isopropanol (Llop *et al*. 1999) (Table 2.5). Independently of the protocol used, DNA obtained was kept in water or in the corresponding buffer at -20 ºC for a long-term storage.

1.3.2. Single PCR

Single PCR was optimized for the detection of the eight selected genes, including primers described in the bibliography and also the primers designed in the present work (Table 2.3). Pure culture suspensions of *Bacillus* strains QST713, FZB42, UMAF6614, RGAF51 and EPS2004 (Table 2.1) adjusted to final concentrations of 10^7-10^8 cfu/ml were used to optimize the single PCR. DNA extraction was done by QIAamp DNA Mini Kit as previously described. Properly, controls based on water and known *Bacillus* strains were introduced in each PCR assay. Amplification reactions for single genes were performed in 50 µl consisting of 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs (Biotools, B & M Labs), 0.2 µM of each primer, 2.0 U of Taq DNA polymerase (Biotools) and 1-4 µl (aprox. 100 ng) of genomic DNA. $MgCl_2$ concentration was reduced to 0.75 mM for *16S rDNA* and *spoVG* gene primers to prevent as far as possible nonspecific amplifications. The following cycling conditions were used to amplify all targets: 4 min at 95 ºC, 40 cycles of 1 min at 94 ºC, 1 min at the corresponding annealing temperature, and 1 min at 72 ºC. A final extension step of 5 min at 72 ºC was followed by a 4 ºC soak. The annealing temperature was set to 58 ºC for primers directed to *16S rDNA*, *fenD*, *ituC*, *bacA*, *srfAA*, *spaS* genes, 55 ºC for *bmyB* gene and 52 ºC for *spoVG* gene.

Table 2.3. Oligonucleotide primers used to detect the selected genetic markers in *Bacillus* spp.

Primer	Expression Product	Sequence (→3′)	Gene	Melting T (°C)	Product size (bp)	References
16SBACF	16S rRNA	GCTTGCTCCCTGATGTTAGC	*16S rDNA*	59.9	163	Mora *et al*. 2011
16SBACR		CGGGTCCATCTGTAAGTGGT				Mora *et al*. 2011
SPOF	Spore protein	AATACCGATGGTCGCATGA	*spoVG*	59.5	226	Mora *et al*. 2011
SPOR		CAGAATCACCCAAACGATGA				Mora *et al*. 2011
FENDF	Fengycin	GGCCCGTTCTCTAAATCCAT	*fenD*	60.1	269	Mora *et al*. 2011
FENDR		GTCATGCTGACGAGAGCAAA				Mora *et al*. 2011
FNDF1	Fengycin	CCTGCAGAAGGAGAAGTGAAG	*fenD*	52.0	293	Joshi and McSpadden-Gardener 2006
FNDR1		TGCTCATCGTCTTCCGTTTC				Joshi and McSpadden-Gardener 2006
BMYBF	Bacillomycin	GAATCCCGTTGTTCTCCAAA	*bmyB*	59.9	370	Mora *et al*. 2011
BMYBR		GCGGGTATTGAATGCTTGTT				Mora *et al*. 2011
BMBF2	Bacillomycin	TGAAACAAAGGCATATGCTC	*bmyB*	52.0	395	Joshi and McSpadden-Gardener 2006
BMBR2		AAAAATGCATCTGCCGTTCC				Joshi and McSpadden-Gardener 2006
ITUCF	Iturin	GGCTGCTGCAGATGCTTTAT	*ituC*	60.1	423	Mora *et al*. 2011
ITUCR		TCGCAGATAATCGCAGTGAG				Mora *et al*. 2011
ITUCF1	Iturin	TTCACTTTTGATCTGGCGAT	*ituC*	52.0	575	Joshi and McSpadden-Gardener 2006
ITUCR3		CGTCCGGTACATTTTCAC				Joshi and McSpadden-Gardener 2006
SRFAF	Surfactin	TCGGGACAGGAAGACATCAT	*srfAA*	60.4	201	Mora *et al*. 2011
SRFAR		CCACTCAAACGGATAATCCTGA				Mora *et al*. 2011
SRFAF1	Surfactin	GAAAGAGCGGCTGCTGAAAC	*srfAA*	62.0	273	Joshi and McSpadden-Gardener 2006
SRFAR1		CCCAATATTGCCGCAATGAC				Joshi and McSpadden-Gardener 2006
BACF	Baclysin	CAGCTCATGGGAATGCTTTT	*bacA*	60.1	498	Mora *et al*. 2011
BACR		CTCGGTCCTGAAGGGACAAG				Mora *et al*. 2011
SPASF	Subtilin	GGTTTGTTGGATGGAGCTGT	*spaS*	59.6	375	Mora *et al*. 2011
SPASR		GCAAGGAGTCAGAGCAAGGT				Mora *et al*. 2011

Amplifications were carried out in a T3000 thermocycler (Biometra, Germany). Amplification products were separated by horizontal electrophoresis (Mini-Sub Cell GT, BioRad, Barcelona, Spain) on a 1.8 % agarose gel (Roche diagnostics, Mannheim, Germany) in 1X Tris-acetate EDTA (TAE) buffer, for 45 min at 90 V. Electrophoresis gel was stained with ethidium bromide, and gel images were captured with ChemiDoc XRS+ System (Bio-Rad, USA).

The specificity of primer pairs was tested in 14 strains of *Bacillus* spp., 10 strains of *P. agglomerans* and 10 strains of *P. fluorescens* (Table 2.1). Suspensions of each strain were prepared at 10^8 and 10^6 cfu/ml. DNA of *Bacillus* spp. was extracted by QIAamp DNA Mini Kit (Table 2.4), while DNA of non-*Bacillus* species was extracted with the isopropanol DNA extraction (Table 2.5). PCR assay was carried out using the procedure previously described.

The sensitivity of different primer pairs was determined in four *Bacillus* strains, specifically QST713, UMAF6614, RGAF51 and EPS2004 (Table 2.1) in order to determine the detection level for each primer. Suspensions of each strain were prepared at final concentrations of 10^8,10^7, 10^6, 10^5, 10^4, 10^3, 10^2 and 10^1 cfu/ml. DNA was extracted by QIAamp DNA Mini Kit as described in table 2.4. The PCR assay was carried out using the procedure previously described.

1.3.3. Multiplex PCR

Primer pairs were designed to provide amplification products of different length but similar annealing temperature. Multiplex PCR was designed only for genes *srfAA*, *bacA*, *bmyB* and *fenD* due to these genes showed a wide distribution among reference *Bacillus* strains. *ituC* and *spaS* genes were discarded due to their scarce distribution. DNA extraction was done by QIAamp DNA Mini Kit as previously described. Properly controls based on water and known *Bacillus* strains were introduced in each PCR assay.

Table 2.4. DNA extraction using QIAamp DNA Mini Kit (QIAGEN)

Step	Description
1	Place 1000 μl of sample into an microcentrifuge tube (1.5 ml) and collect the pellet bacteria by centrifugation for 10 min at 5000 g (7500 rpm)
2	Suspend bacterial pellet in 180 μl of the enzyme solution (20 mg/ml lysozyme; 20 mM Tris-HCl, pH8.2: 2 mM EDTA; 1.2 % Triton)
3	Incubate for at least 30 min at 37 ºC
4	Add 20 μl proteinase K and 200 μl Buffer AL. Mix by vortexing
5	Incubate at 56 ºC for 30 min and then for a further 15 min at 95 ºC
6	Centrifuge for a few seconds
7	Add 200 μl Buffer AL to the sample, mix by pulse-vortexing for 15 s, and incubate at 70 ºC for 10 min. Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the lid
8	Add 200 μl ethanol (96-100%) to the sample, and mix by pulse-vortexing for 15 s. After mixing, briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from inside the lid
9	Carefully apply the mixture (including the possible precipitate) to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 g (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube, and discard the tube containing the filtrate
10	Carefully open the QIAamp Mini spin column and add 500 μl Buffer AW1 without wetting the rim. Close the cap, and centrifuge at 6000 g (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube, and discard the collection tube containing the filtrate
11	Carefully open the QIAamp Mini spin column and add 500 μl Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed 20000 g (14000 rpm) for 3 min
12	Recommended: Place the QIAamp Mini spin column in a new 2 ml collection tube and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min
13	Place the QIAamp Mini spin column in a clean 1.5 ml microcentrifuge tube, and discard the collection tube containing the filtrate. Carefully open the QIAamp Mini spin column and add 200 μl Buffer AE or distilled water. Incubate oat room temperature for 1 min, and then centrifuge at 6000 g (8000 rpm) for 1 min
14	Repeat step 13

Table 2.5. Procedure for DNA extraction using the isopropanol method (Llop *et al*. 1999).

Step	Description
1	Place 500 µl of sample into an microcentrifuge tube (1.5 ml) and centrifuge at 10000 g for 10 min
2	Suspend bacterial pellet in 500 µl of extraction buffer (200 mM Tris HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5 % SDS, 2 % PVP), and mix by vortexing for 1 h at room temperature with continuous shaking
3	Centrifuge at 5000 g for 5 min
4	Recover 450 µl of the supernatant and add 450 µ l isopropanol, mix gently
5	Let stand 1 h at room temperature
6	Centrifuge at 13 000 g for 10 min
7	Discard the supernatant and dry the pellet under vacuum
8	Suspend pellet in 100 µl distilled water

The selected primer pairs designed in this work were SRFAF/SRFAR, BACAF/BACAR, FENDF/FENDR and BNYBF/BMYBR (Table 2.3). Amplification fragments length for these primer pairs ranged between 100 and 500 bp.

Amplification length was fixed in this range to get similar efficiency during PCR but sufficient differences in length, at least with a difference higher than 50 bp, in order to separate and distinguish them in agarose gels. The multiplex PCR optimization was carried out modifying final concentrations of PCR mixture components. Thus, a total of three PCR mixtures with different primers and $MgCl_2$ concentrations in a final volume of 25 µl were tested (Table 2.6). All tests were performed using the Biotools Taq DNA polymerase. The following cycling conditions were used to amplify all targets: 5 min at 95 ºC, 35 cycles of 1 min at 94 ºC, 1 min at 58 ºC (annealing temperature), and 1 min at 72 ºC. A final extension step of 5 min at 72 ºC was followed by a 4 ºC soak. Amplifications were carried out in a T3000 thermocycler, and amplification products were separated by horizontal electrophoresis on a 1.8 % agarose gel in 1X TAE buffer, for 45 min at 90 V as described in section 1.3.1. Electrophoresis gel was revealed

after staining with ethidium bromide, and gel images were captured using ChemiDoc XRS+ System (Bio-Rad, USA). Pure suspensions of *Bacillus* strains QST713, FZB42, UMAF6614, RGAF51 and EPS2004 adjusted at final concentrations of 10^8 cfu/ml were used to optimize the multiplex PCR.

A total of 14 *Bacillus* strains (Table 2.1), at a final concentration of 10^8 cfu/ml, were processed by multiplex PCR to evaluate its specificity using the PCR mixture 3, according to the results obtained by multiplex PCR optimization (Table 2.6). Amplification reactions in a total volume of 25 µl consisted of 1X PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs (Biotools, B & M Labs), 0.16 µM of SRFA primers, 0.4 µM of BACA primers, 0.24 µM of FEND primers, 0.24 µM of BMYB primers, 2.0 U of Taq DNA polymerase (Biotools) and 1-4 µl (aprox. 100 ng) of genomic DNA. The following cycling conditions were used to amplify all targets: 4 min at 95 ºC, 35 cycles of 1 min at 94 ºC, 1 min at the corresponding annealing temperature, and 1 min at 72 ºC. A final extension step of 5 min at 72 ºC was followed by a 4 ºC soak.A negative control with water was introduced in each PCR assay. Amplification reactions and PCR products analysis were performed as previously described for single PCR assays.

Table 2.6. Mixture components of multiplex PCR targeted to the biosynthetic genes *srfAA*, *bacA*, *fenD* and *bmyB*.

		Mixture (µl)		
Component	**Stock concentration**	**1**	**2**	**3**
Milli-Q water		12.05-16.05	8.95-12.95	10.15-14.15
Buffer $MgCl_2$ free	10X	2.5	2.5	2.5
$MgCl_2$	50mM	0.75	0.75	1.25
dNTPs	10mM	0.5	0.5	0.5
SRFAF	10µM	0.5	0.5	0.4
SRFAR	10µM	0.5	0.5	0.4
BACAR	10µM	0.5	1	1
BACAF	10µM	0.5	1	1
FENDF	10µM	0.5	1	0.6
FENDR	10µM	0.5	1	0.6
BMYBF	10µM	0.5	1	0.6
BMYBR	10µM	0.5	1	0.6
Taq polymerase	5U/µl	0.2	0.3	0.4
Sample	Aprox. 100 ng	1-5	1-5	1-5

The sensitivity of multiplex PCR was determined in five *Bacillus* strains (QST713, FZB42, UMAF6614, RGAF51 and EPS2004) at final concentrations of 10^7, 10^6, 10^5, 10^4, 10^3 and 10^2 cfu/ml. The PCR assay and amplifications were done in the same way as for multiplex PCR assays.

1.4 Validation of PCR tools in natural samples

DNA extracts obtained from direct plant or soil samples can contain organic components, such as humic substances or phenolics, which inhibit the Taq polymerase, leading to a failed amplification in some cases. The strategy used in the present study to reduce inhibitors interferences was to work with extracted DNA and with samples diluted during the enrichment process. Therefore, molecular tools developed in this work were evaluated using different dilution conditions in different type of samples, such as aerial plant parts, rhizosphere and soil. Plant extracts were obtained by homogenization of 1 g of sample in 10 ml of phosphate buffer (0.02 M Na_2HPO_4, 0.05 M KH_2PO_4) using a Stomacher (Masticator 400, IUL Instruments, United Kingdom) for 60 s. In addition, samples were serially diluted to obtain 1:10, 1:100 and 1:1000 dilutions, in order to determine the right dilution, which permits detection of the molecular targets by PCR and eliminate the effects of Taq polymerase inhibitors.

Plant extracts, both direct and diluted, were divided into aliquots of 1 ml, and then fortified with *Bacillus* QST713 to obtain a final concentration of 10^7 cfu/ml. DNA extraction of natural extracts amended with strain QST713 was done by the isopropanol DNA extraction procedure. Single PCR targeted to *16S rDNA*, *spoVG*, *srfAA*, *bmyB*, *fenD*, *ituC*, *bacA* and *spaS* genes, and multiplex PCR to *srfAA*, *bacA*, *fenD* and *bmyB* genes, were done following the optimized procedures previously described for single and multiplex PCR, respectively. Four external controls, corresponding to strain QST713 in aqueous solution at final concentration of 10^7 cfu/ml and also natural samples without amendment of known *Bacillus*, were included to verify the right performance of single and multiplex PCR and validate the detection level in optimum conditions.

The sensitivity of single and multiplex PCR for 4 strains of *Bacillus* (QST713, UMAF6614, RGAF51 and EPS2004) was also assessed in natural samples only using dilution 1:100 of the sample. *Bacillus* strains were amended at final concentrations of 10^7, 10^6, 10^5, 10^4, 10^3 and 10^2 cfu/ml. External controls consisting of tested *Bacillus* strains at final concentration of 10^7 cfu/ml, and a natural sample of three origins (aerial plant part, rhizosphere and soil) at 1:100 were included. The PCR assay and amplifications were performed in the same way than in the single and multiplex PCR specificity assays.

2. Development of a selective enrichment method

The main limitation in the application of molecular tools in natural samples is the detection level for targets. The detection level has two main components, the first provided by the molecular tool itself and the second is due to the amount of the target in the environment. In this stage of the work, the efforts were focused on increase the amount of *Bacillus* in samples in order to be detected by PCR.

2.1 Analysis of the presence and population level distribution of *Bacillus* spp. in natural environments

The presence of sufficient population levels of *Bacillus* in samples of natural environments is the critical point for a successful detection of molecular markers. However, to increase the performance of the methods to be developed it is necessary to know the abundance of plant associated *Bacillus* in field samples.

Forty-five samples collected from plant environments including aerial plant part, rhizosphere and soil, were used to detect and quantify *Bacillus* spp. (Table 2.7). The microbiota of samples was obtained after homogenization of 1 g of material in 10 ml of phosphate buffer using a Stomacher for 60 s. Each extract was serially decimal diluted in sterile distilled water,

Table. 2.7. Location and type of field samples used to obtain the strain collection of *Bacillus.*

Sample location	Zone	Latitude	Longitude	Sample type	Sample specie	Number of samples
Girona	Estartit (Baix Empordà)	42º 30' N	3º 11' E	Rhizosphere	*Artiplex sp.*	1
					Juncus maritimus	1
					Maresia nana	1
					Plantago sp.	1
				Soil	*nd*	4
	Torroella de Montgrí (Baix Empordà)	42º 02'	3º 07' E	Rhizosphere	*Brassica oleracea*	1
					nd	1
					Triticum aestivum	2
				Soil	*nd*	6
Lleida	Aigüestortes (Pallars Sobirà)	42º 34'	0º 55' E	Rhizosphere	*nd*	3
				Soil	*nd*	3
	Montenartró (Pallars Sobirà)	42º 27' N	1º 13' E	Soil	*nd*	2
Menorca	Binimel·là (Menorca)	40º 03' N	4º 03' E	Aerial plant part	*Hedysarum coronarium*	2
					nd	1
					Thymus vulgaris	1
	Cap de Cavalleria (Menorca)	40º 05' N	4º 08' E	Aerial plant part	*Desmazeria marina*	1
					Hyoseris radiata	1
					Limoniun sp.	1
					Myrtus communis	1
					nd	2
					Plantago crassifolia	1
					Phillyrea angustifolia	1
					Santolina chamaecuparissus	1
					Teucrium dunense	1
					Ulex parviflorus	1
				Rhizosphere	*Desmazeria marina*	1
					Limonium sp.	1
					Teucrium dunense	1
					Hyoseris radiata	1

nd, not determined

and 50 µl of each dilution was spread using an automatic spiral plater (Eddy Jet, IUL Instruments, United Kingdom) in LB agar supplemented with actidione (50 mg/l). Plates were incubated at 28 ºC and the colonies counted after 24 h using an automatic colony counter (Countermat, IUL Instruments, United Kingdom). *Bacillus* like colonies were identified on the basis of their morphology (Sneath 1986) and further confirmed by optical microscopy (Olympus BH2, New York Microscope Company, Inc., USA).

2.2 Development of a method for the traceability of *Bacillus* strains upon introduction in natural samples

In order to verify the methods to enrich selectively the population of *Bacillus* in natural plant samples, a procedure for monitoring introduced *Bacillus* was developed using spontaneous mutants resistant to an antibiotic.

2.2.1 Selection of antibiotic spontaneous mutants

The use of spontaneous mutants resistant to antibiotics is a simple and efficient methodology to monitor strains of interest in a complex bacterial community. Accordingly, in the specific case of our study it was essential to validate the survival of the artificial by inoculated strains during the selective enrichment procedure. Artificial introduction of *Bacillus* strains at known concentration allows determining the time course of this *Bacillus* population after the different enrichment procedure steps and validate the sensitivity threshold of molecular markers.

The selection of spontaneous mutants resistant to antibiotics was done with strains QST713, RGAF51, and UMAF6614 and consisted of plating 100 µl of a highly concentrated cell suspension in LB agar amended with rifampicin (100 mg/l). After incubation for 24-48 h at 28 ºC, grown colonies were selected as spontaneous mutants. Then, a validation of the growth properties of rifampicin spontaneous mutants was performed to ensure that growth potential was not modified respect to the wild type strain. The assay was based on the growth curves in LB broth by means of absorbance measurements over time and consisted of multiwell plates with 180 µl of LB

broth and 20 µl of strain suspension adjusted to 10^7 cfu/ml. Wild type strains QST713, RGAF51 and UMAF6639 and four rifampicin mutants of each strain were evaluated. Triplicates of each treatment were done.

The assay was carried out in an automatic optical density reader (Bioscreen C, Labsystems, Finland). Microplates were incubated for 72 h at 30 ºC with 20 s of shaking before hourly absorbance measurement at 600 nm. Comparison among strains was done using the area under the growth curve (AUGC), and was calculated as follows:

$$AUGC = \Sigma[(A_i+A_{i+1})/2]x(t_{i+1}-t_i)$$

Where A_i is initial optical density (OD), A_{i+1} is successive OD measurements at A_i, t_i is initial time of measurement of OD , and t_{i+1} is successive time of measurements at t_i. ANOVA was performed to test the differences in growth between wild type strains and the corresponding antibiotic resistant mutants. Means were separated according to the Tukey's test ($p \leq 0.05$). Statistical analysis was carried out by SPSS v15 (SPSS Inc. Chicago. USA).

2.2.2 Recovery of *Bacillus* spp. from natural samples fortified with antibiotic resistant mutants

The recovery of *Bacillus* spp. from natural samples was evaluated by the incorporation of rifampicin resistant strains as a tag. Sample extracts from soil and rhizosphere were obtained after homogenization of 1 g of material in 10 ml of phosphate buffer using a stomacher for 60 s. In parallel, *Bacillus* strain QST713 Rif^+ was adjusted at concentrations of 10^7, 10^6, 10^5 i 10^4 cfu/ml, and 100 µl of each suspension was independently added to 900 µl aliquots of natural extracts. A control consisting in mixture of 100 µl of distilled water in 900 µl of each extract was added. Population levels of total bacteria and of rifampicin resistant *Bacillus* strain were determined by means of plate counting using an automatic spiral plater (Eddy Jet, IUL Instruments, United Kingdom). Extracts were serially diluted and then 50 µl of the proper dilution were spread in LB agar plates and LB agar plates supplemented with rifampicin (100 mg/l) to quantify the total culturable population present in natural samples, and *Bacillus* Rif^+ artificially amended,

respectively. All plates were incubated at 28 ºC and the colonies counted after 24-48 h with an automatic counter (Countermat, IUL Instruments, United Kingdom).

2.3 Strategies for the development of a selective enrichment procedure for *Bacillus*

For the development of a reliable selective enrichment procedure two strategies were designed, one based on the counterselection with a differential antibiotic in the growth medium, and a second based in a thermal treatment of the sample before the enrichment stage.

2.3.1 Antibiotic resistance

Gram-positive bacteria are characterized by a differential cell envelope structure in comparison to Gram-negative bacteria. This particularity and other characteristics confer to *Bacillus* a differential resistance to some antibiotics respect to other common plant inhabitants, most Gram-negative bacteria. Antibiotic resistance to rifampicin (100 mg/l), nalidixic acid (20 mg/l), colistin (5 and 15 mg/l) and chloramphenicol (50 mg/l) was tested in 11 strains of *Bacillus* (QST713, FZB42, RGAF5, RGAF9, RGAF11, RGAF32, RGAF46, RGAF51, RGAF66, RGAF84 and RGAF101) (Table 2.1). Growth in LB broth supplemented with the above mentioned antibiotics was determined by optical density measurements. Thus, 20 µl of each strain suspension adjusted at 10^7 cfu/ml was mixed in a microtiter plate well with 180 µl of the LB amended with the corresponding antibiotic. Three replicates for each strain and antibiotic were done. Controls containing LB were included. Microbial growth was automatically determined by optical density measurement at 600 nm (Bioscreen C; Labsystems, Helsinki, Finland). Microplates were incubated at 28 °C with shaking cycles, and the measurements were done every 1 h for 2 days.

2.3.2 Thermal resistance

Sporulation capacity of *Bacillus* spp. compared to Gram-negative plant associated bacteria was the most important reason to include the step of

thermal treatment in the selective enrichment procedure. Thermal resistance was determined in five *Bacillus* strains (RGAF32, RGAF51, UMA6614, UMA6639 and EPS2004) and two strains of Gram-negative bacteria, concretely *P. fluorescens* EPS62e and *P. agglomerans* EPS125 (Table 2.1). Suspensions were adjusted to 10^6 cfu/ml and 1 ml of each suspension was distributed in microtubes of 1.5 ml. Then, suspensions were submitted to different thermal treatments consisting of exposure during 60 min to a range of temperatures from 60 to 120 ºC for *Bacillus* strains and from 40 to 100 ºC for non *Bacillus* strains (Universal oven Model UFB 400, Memmert Basics, Schwabach, Germany). Afterwards, surviving cells were determined every 15 min by plating 100 µl of the proper decimal dilutions onto LB agar. Plates were incubated at 28 ºC for *Bacillus* strains and at 24 ºC for non-*Bacillus* strains. Decimal reduction time (*D*), the time of exposure at a given temperature required to kill 90 % of the cells, was calculated for each strain and corresponding exposure temperature. Death constant (*Kd*) values were estimated using the thermal death kinetics curve at each temperature. Regression and parameter estimation were performed by a nonlinear least squares method using the NLIN method of the PC-Statistical Analysis System (SAS, version 8.2, SAS Inst. Cary, NY, USA).

2.4 Selective enrichment procedure

2.4.1 Optimization

Optimization of selective enrichment was guided by two parameters, duration of the thermal shock, to ensure decrease of non-*Bacillus* population but keeping high *Bacillus* population, and dilution during growth in rich medium, to ensure reduction of PCR inhibitors but allowing the recovery of *Bacillus* population (Figure 2.1).

A study of the behavior of an amended *Bacillus* strain into the natural extract was performed to optimize the selective enrichment procedure. A rhizosphere tomato extract was obtained by homogenization of 1 g of tomato roots in 10 ml of phosphate buffer for 60 s.

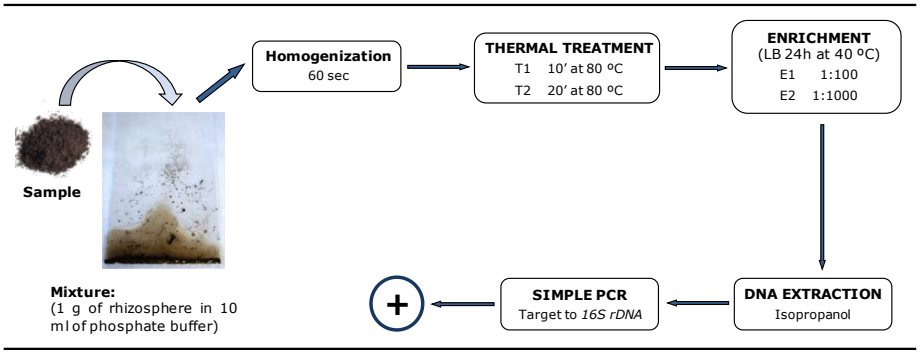

Figure 2.1. Scheme of the protocol used for optimization of the selective enrichment procedure.

Then, 100 µl of a suspension of *Bacillus* RGAF51 Rif$^+$ were amended in 900 µl of extract to attain final concentrations of 10^7, 10^6, 10^5 and 10^4 cfu/ml. Two thermal treatment conditions were tested. Thus, extracts consisting in 1 ml mixture were exposed to thermal shock (Cho *et al*. 2004, Rahman *et al*. 2006) at 80 ºC for 10 or 20 min, respectively. Population levels of total culturable bacteria and rifamicin resistant *Bacillus* after thermal shock were determined as previously described. Culturable population levels were determined by plate counting of the corresponding decimal dilution. Population levels of total culturable bacteria were determined in LB agar, while *Bacillus* RGAF51 Rif$^+$ population was determined in LB agar supplemented with rifampicin (100 mg/l).

All plates were incubated at 28 ºC and the colonies counted after 24-48 h. In addition, two enrichment conditions were tested after each thermal shock treatment consisting of dilution of extracts in LB broth at 1:100 and 1:1000. The enrichments were incubated at 40 ºC for 24 h. Population levels of total culturable bacteria and rifampicin resistant *Bacillus* after the enrichment process were determined as previously described. Three experimental repetitions were done for each condition. Apart from viable cell counting, the selective enrichment procedure was evaluated using the molecular tools previously developed. Thus, samples obtained in each of the three steps of selective enrichment (before heat treatment, after heat treatment and after enrichment) were taken to be processed by PCR for the detection of *Bacillus* targeting to the *16S rDNA* gene. DNA of all mixtures was extracted by the isopropanol DNA extraction procedure. RGAF51 Rif$^+$

was added as a positive control in the PCR assay at final concentrations of 10^6, 10^5, 10^4 and 10^3 cfu/ml.

2.4.2 Evaluation

Two assays to evaluate the selective enrichment procedure were performed. The first assay consisted of the amendment of rifampicin-resistant *Bacillus* strains to an artificial microcosms composed of strains of bacteria ubiquitous in plant environments, such as *P. agglomerans* EPS10, *P. agglomerans* EPS210, *P. fluorescens* EPS62e, or plant pathogenic *P. syringae* pv. syringae EPS94, *P. syringae* pv. tomato DC3000, *Xanthomonas axonopodis* pv. vesicatoria CFBP2135, *X. arboricola* pv. fragariae CFBP3549 and *Pectobacterium carotovorum sbsp. carotovorum* CECT225 (see Tables 2.1 and Table 3.1). Suspensions were adjusted to 10^8 cfu/ml individually and added in a mixture suspension to attain a final total concentration of 10^8 cfu/ml. The second assay consisted of adding rifampicin-resistant *Bacillus* strains to a natural plant microbiota extract obtained by homogenizing 1 g of grapevine leaves in 10 ml of phosphate buffer with a stomacher for 60 s. Both assays were repeated twice using different rifampicin-resistant *Bacillus* (*B. megaterium* RGAF51Rif$^+$ and *B. subtilis* UMAF6639Rif$^+$). *Bacillus* suspensions were added to 900 µl of the corresponding extract to obtain final concentrations of 10^6, 10^4 and 10^3 cfu/ml. In both assays, final mixtures were submitted to the selective enrichment procedure that consisted of a thermal shock at 80 ºC for 10 min followed by an enrichment, which consists of an inoculation (1:100) in LB broth at 40 ºC for 24h. Then, population levels were determined at tree stages of the procedure (before thermal treatment, after thermal treatment, and after complete enrichment) by plating properly decimal dilution in LB agar, for total culturable bacteria, and in LB agar supplemented with rifampicin (100mg/l) for the rifampicin-resistant *Bacillus* strains amended. All plates were incubated at 28 ºC for 24h. At the tree stages of the procedure the presence of *Bacillus* was confirmed by PCR targeted to *16S rDNA* gene as previously described. The experimental design consisted of three replicates of 3 tubes per treatment, and both assays were repeated three times.

2.5 Comparison of standard and selective enrichment methods

One of the objectives of the present work was to increase the yield of recovery of *Bacillus* from field samples, in order to increase the probability to find suitable BCAs. Accordingly, the standard method of isolation was compared with the selective enrichment procedure. In order to compare both methods, forty-five field samples from plant environments were collected from three locations of the North Eastern Mediterranean area, in Girona, Lleida and Menorca provinces of Spain, in late spring-early summer. Fifteen samples were from the aerial plant part, 15 from the rhizosphere, and 15 from the soil surrounding the plant root system. Plant sampled were of representative species of ten families of herbaceous plants typical from the Mediterranean area (Table 2.7). The standard method consisted of the recovery of *Bacillus* isolates directly from the sample extract and was based on the homogenization of 1 g of material in 10 ml of phosphate buffer using a stomacher for 60 s. Then, 100 µl of serial 10-fold dilutions of each extract were spread onto LB agar plates and incubated at 28 ºC for 24 h, from which total bacteria and *Bacillus* population levels were determined. *Bacillus* like colonies were identified on the basis of their colony morphology (Sneath 1986), and verified by optical microscopy and by PCR using the primers designed for the specific amplification of *16S rDNA* from *Bacillus*. The presence of *Bacillus* was also determined in the sample extract by PCR using the primers developed for the *16S rDNA*. Isolated strains and sample extracts were also analyzed by PCR using specific primers for the biosynthetic genes (*bmyB*, *fenD*, *ituC, srfAA*, *bacA* and *spaS*) designed in the present work (Table 2.3) according to the procedure previously described. The selective enrichment method was applied to the crude extract and consisted of a thermal treatment at 80 ºC for 10 min followed by enrichment of diluted extract (1:100) in LB broth for 24 h at 40 ºC. Only the extracts showing presence of *Bacillus* using *16S rDNA* specific primers were processed for later isolation of *Bacillus*. Colonies were confirmed as previously described for the standard procedure. Finally, the presence of biosynthetic genes (*bmyB*, *fenD*, *ituC, srfAA*, *bacA* and *spaS*) was determined in the extracts and in the *Bacillus* isolates obtained during the process.

Results

1. Development of PCR tools targeted to AMP genes for screening field samples and culture collections of *Bacillus* spp.

1.1 Selection of molecular markers and design of primer sets

1.1.1 Selection of molecular markers

Two generalist genes for detection of *Bacillus* in natural samples were selected, the *16S rRNA* gene, which encodes for the subunit of the ribosome in prokaryotes, and the *spoVG* gene, which encodes the G protein required in the stage V of the spore synthesis. *16S rRNA* gene was selected because it is widely used in phylogenetic studies and highly conserved between

species (Baysal *et al*. 2008, Cazorla *et al*. 2007, Ki *et al*. 2009, Kim *et al*. 2011). *spoVG* gene has not been described as a molecular marker, but its detection permits to identify *Bacillus* bacteria clearly, because it codes for the synthesis of the spore coat in this genus. Attention was also payed to the list of AMP genes described in *Bacillus* (Table 2.2). Most of these antimicrobial peptide genes have been described in the genomes already sequenced of *B. subtilis* 168 and *B. amyloliquefaciens* FZB42. The selection of antimicrobial peptides was performed using different criteria, including peptide structure and involvement in biocontrol. However, the main limitation was the availability of sequences for the genes involved in the biosynthesis of these peptides, because this information is essential for designing primers to be used for the detection of genes of interest. Thus, peptide genes without or with few sequences available in the database were discarded. Other peptide genes were discarded due to the lack of studies that demonstrate the antimicrobial activity of their gene products in *in vivo* assays. For this reasons, the selection was focused on those metabolites coded by genes with proven activity in biocontrol and with more information on the biosynthetic process. Finally, the genes for cLPs fengycin, iturin, bacillomycin and surfactin were selected, because they have been widely associated with biocontrol, and there are sufficient number of sequences available or previously reported primers (Baysal *et al*. 2008, Chung *et al*. 2008, Hsieh *et al*. 2008, Joshi and McSpadden-Gardener 2006, Moyne *et al*. 2004, Tsuge *et al*. 2005). In addition, the genes for the dipeptide bacilysin (Chen *et al*. 2009c, Hilton *et al*. 1988), and the lantibiotic subtilin (Bongers *et al*. 2005, Klein *et al*. 1992) were also chosen, both related to biocontrol, and with different structure than the cLPs, and because of the availability of enough sequences to permit a reliable design of primers.

1.1.2 Primer design

The strategy used for the design of primers directed to AMP genes was to select a consensus sequence from as many as possible sequences in order to increase the robustness of detection of the molecular tools. Thus, primers were designed in the most conserved regions of these consensus sequences. Design was also focused on obtaining similar melting temperatures among primers and PCR products with compatible length

(amplicon length differed 50 bp) in order to permit a further development of a multiplex PCR. Table 2.3 shows the final sequences and properties of primers included in this study. In addition, previously described primers by other authors directed to genes *fenD*, *ituC, bmyB* and *srfAA* were included (Joshi and McSpadden-Gardener 2006). The strategy of design used to detect *Bacillus* at genus level was similar to the strategy used for AMP genes in the case of *spoVG* gene, but slightly different for the *16S rDNA* gene, because this gene is present in all bacteria, in contrast to the other genes used in the present work, which are related to *Bacillus* genus. Thus, primers targeted to *16S rDNA* gene were designed by obtaining a consensus sequence for the *Bacillus* genus, which differed from sequences of other common plant associated bacteria.

1.2 Evaluation and optimization of methods for detection of molecular markers

1.2.1 Single PCR

The evaluation of the single PCR procedure was carried out using three *B. subtilis* strains (QST713, UMAF6639 and EPS2004), one *B. amyloliquefaciens* strain (FZB42) and one *B. megaterium* strain (RGAF51) included in the reference *Bacillus* strains collection. PCR products obtained for each primer pair showed the expected size (Fig. 2.2), suggesting a successful amplification of the genes of interest in at least one of the chosen strains. The strains showing a positive amplification were selected as positive controls for successive PCR assays. Interestingly, unspecific bands were not detected for any reaction. For the primer specificity analysis, *16S rDNA* and *spoVG* genes were amplified in the fourteen *Bacillus* strains, ten strains of *P. agglomerans* and ten strains of *P. fluorescens*, using the designed generalist primers for *Bacillus*. Primers set 16S rDNA amplified three *P. fluorescens* (EPS173, EPS353 and EPS684) at 10^8 cfu/ml, though only strain EPS317 was positive at the lowest concentration. However, the primers spoVG also amplified for three *P. agglomerans* (EPS10, EPS13 and EPS230) and four *P. fluorescens* (EPS173, EPS62e, EPS353 and EPS684) at both concentrations tested (10^6 and 10^8 cfu /ml).

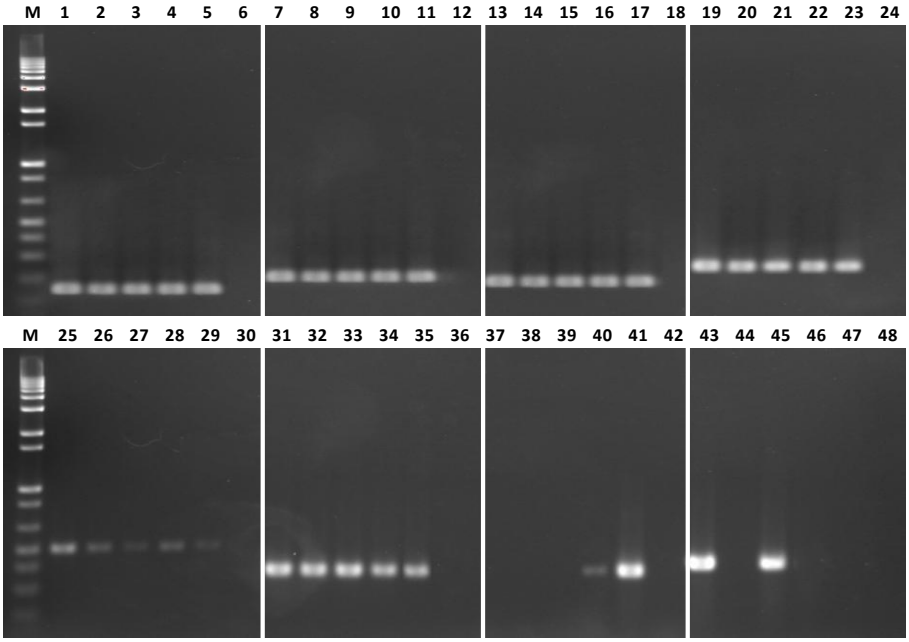

Figure 2.2. PCR analysis of general and biosynthetic gene sequences by means of primers designed in the present work, directed to *16S rDNA* (~163 bp) (1-5), *spoVG* (~226 bp) (7-11), *srfAA* (~201 bp) (13-17), *fenD* (~269 bp) (19-23), *bacA* (~498 bp) (25-29), *bmyB* (~370 bp) (31-35), *spaS* (~375 bp) (37-41) and *ituC* (~423 bp) (43-47). *Bacillus* strains QST713 (1, 7, 13, 19, 25, 31, 37, 43), FZB42 (2, 8, 14, 20, 26, 32, 38, 44), UMAF6614 (3, 9, 15, 21, 27, 33, 39, 45), RGAF51 (4, 10, 16, 22, 28, 34, 40, 46) and EPS2004 (5, 11, 17, 23, 29, 35, 41, 47). Negative control for each set of primers (6, 12, 18, 24, 30, 36, 42, 48). M, 1Kb Plus DNA Ladder (Invitrogen).

The study of the specificity of the PCR with genes related to biosynthesis of APMs revealed differences among the *Bacillus* strains used as reference (Fig. 2.3). *srfAA* gene was detected in all strains except in RGAF84, while other genes were very rare like *spaS,* only detected in EPS2004 and UMAF6614, or *ituC* only detected in three out of 14 *Bacillus* strains analyzed (Table 2.8). The pattern of distribution of the six AMP genes among the fourteen strains of *Bacillus* tested was dependent on the strain. None of the AMP genes was found in RGAF84. The remaining strains had *srfAA* (RGAF5); *srfAA* and *bacA* (RGAF9, RGAF11, RGAF101); *srfAA, bacA* and *bmyB* (RGAF66, RGAF32, RGAF46), *srfAA, bacA, bmyB* and *fenD* (FZB42); *srfAA, bacA, bmyB, fenD* and *ituC* (QST713, RGAF51, UMAF6639); *srfAA, bacA,bmyB, fenD* and *spaS* (UMAF6614, EPS2004). None of the strains had the six AMP genes simultaneously (Table 2.8).

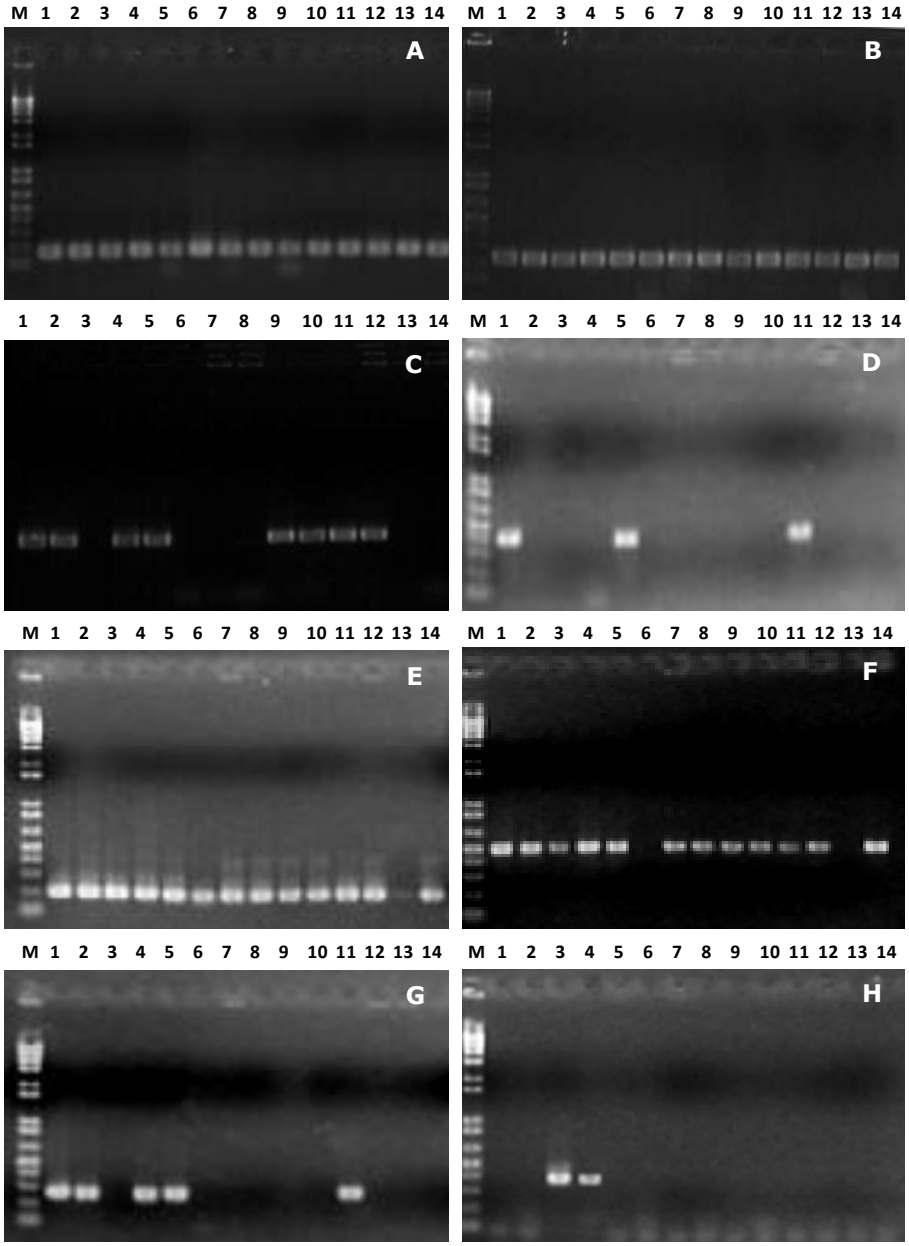

Figure 2.3.PCR analysis of *Bacillus* strains using primers directed to *16S rDNA* (~163 bp) (A), *rpoVG* (~226 bp) (B), *bmyB* (~370) (C), *ituC* (~423 bp) (D), *srfAA* (~201 bp) (E), *bacA* (~498 bp) (F), *fenD* (~269 bp) (G), *spaS* (~375 bp) (H). *Bacillus strains* QST713 (1), FZB42 (2), EPS2004 (3), UMAF6614 (4), UMAF6639 (5), RGAF5 (6), RGAF9 (7), RGAF11 (8), RGAF32 (9), RGAF46 (10), RGAF51 (11), RGAF66 (12), RGAF84 (13), RGAF101 (14). Within each panel: M, 1 Kb Plus Ladder (Invitrogen).

Table 2.8. Patterns of presence of antimicrobial peptide biosynthetic genes in 14 *Bacillus* spp. reference strains using the primers designed in the present work.

Strain	*srfAA*	*bacA*	*bmyB*	*fenD*	*ituC*	*spaS*
RGAF84	-	-	-	-	-	-
RGAF5	+	-	-	-	-	-
RGAF9	+	+	-	-	-	-
RGAF11	+	+	-	-	-	-
RGAF101	+	+	-	-	-	-
RGAF66	+	+	+	-	-	-
RGAF32	+	+	+	-	-	-
RGAF46	+	+	+	-	-	-
FZB42	+	+	+	+	-	-
QST713	+	+	+	+	+	-
RGAF51	+	+	+	+	+	-
UMAF6639	+	+	+	+	+	-
UMAF6614	+	+	+	+	-	+
EPS2004	+	+	+	+	-	+

Generally, unspecific amplifications for the AMPs genes were not observed in strains of the other bacterial species, except for *P. fluorescens* (EPS282 and EPS353) that amplified with *bmyB* primers at a concentration of 10^6 cfu/ml, but giving an amplification product of a different size from the expected. In relation to the sensitivity of the generalist primers, *16S rDNA* gene primers were more sensitive than SPOVG primers (Fig. 2.4). The threshold was established between $5x10^1$ and $5x10^4$ cfu/ml for the *16S rDNA*, and between $5x10^3$ and $5x10^6$ cfu/ml for *spoVG*, depending on the *Bacillus* strain (Table 2.9). On the basis of these results, primers for *16S rDNA* were used for confirmation of *Bacillus* but with diluted samples. The sensitivity of detection of the primers for the biosynthetic genes (*bmyB, fenD, ituC, srfAA*, *bacA* and *spaS*) showed a high variability among genes and strains (Table 2.9).

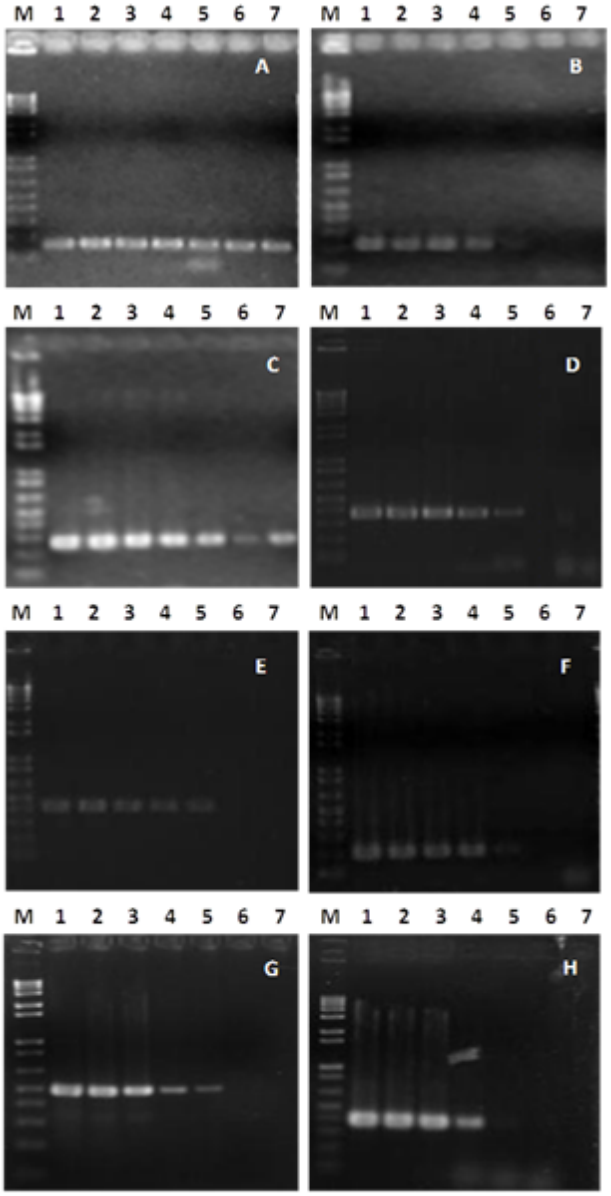

Figure 2.4.Sensitivity of the designed primers for PCR analysis. Product amplification: *16S rDNA* (~163 bp) (A),*spoVG* (~226 bp) (B), *fenD* (~269 bp) (C), *bmyB* (~370 bp) (D), *ituC* (~423 bp) (E), *srfAA* (~201 bp) (F), *bacA* (~498 bp) (G), *spaS* (~375 bp) (H). *Bacillus* strains RGAF51 (A and E) at concentrations of $5x10^7$, $5x10^6$, $5x10^5$, $5x10^4$, $5x10^3$, $5x10^2$, $5x10^1$ cfu/ml; UMA6614 (B and F) at concentrations of 10^8, 10^7, 10^6, 10^5, 10^4, 10^3, 10^2 cfu/ml; QST713 (C and D) at concentrations of $5x10^7$, $5x10^6$, $5x10^5$, $5x10^4$, $5x10^3$, $5x10^2$, $5x10^1$ cfu/ml; and EPS2004 (G and H) at concentrations of 10^8, 10^7, 10^6, 10^5, 10^4, 10^3, 10^2 cfu/ml. M 1Kb Plus Ladder (Invitrogen).

Table 2.9. Level of detection (cfu/ml) of six antimicrobial peptide biosynthetic genes in four reference strains of *Bacillus* using the previously reported primers (RP) and the primers designed in the present study (PS).

Gene	Strain							
	RGAF51		UMAF6614		QST713		EPS2004	
	RP [a]	PS	RP [a]	PS	RP [a]	PS	RP [a]	PS
16S rDNA	-- [b]	$5x10^1$	-- [b]	$5x10^3$	-- [b]	$5x10^3$	-- [b]	$5x10^2$
spoVG	-- [b]	$5x10^3$	-- [b]	$1x10^4$	-- [b]	$5x10^3$	-- [b]	$1x10^5$
srfAA	$1x10^6$	$5x10^2$	$1x10^6$	$1x10^4$	$1x10^4$	$5x10^3$	nt [c]	$1x10^3$
fenD	$1x10^5$	$5x10^2$	$1x10^5$	$1x10^2$	$1x10^3$	$5x10^1$	nd [d]	$1x10^2$
bmyB	nd [d]	$5x10^2$	$1x10^5$	$1x10^4$	$1x10^2$	$5x10^3$	nt [c]	$1x10^4$
ituC	$1x10^7$	$5x10^3$	nd [d]	nd [d]	$1x10^5$	$5x10^2$	nd [d]	nd [d]
bacA	-- [b]	$5x10^4$	-- [b]	$1x10^4$	-- [b]	$5x10^3$	-- [b]	$1x10^3$
spaS	-- [b]	nd [d]	-- [b]	$1x10^7$	-- [b]	nd [d]	-- [b]	$1x10^4$

a According Joshi and McSpadden-Gardener, 2006.
b Primers not designed for this gene in Joshi and McSpadden-Gardener, 2006.
c nt, not detected.
d nd, not determined.

Globally, the primers designed in the present work were more sensitive than the primers previously reported by Joshi and McSpadden-Gardener (2006). *fenD* gene primers pair was the most sensitive and more consistent among strains with a detection limit around 10^2 cfu/ml while *spaS* gene primers pair was the less sensitive and less consistent among strains with a detection limit between $1x10^4$ and $1x10^7$ cfu/ml (Fig. 2.4). Primers for genes *srfAA*, *bmyB*, *ituC* and *bacA* showed a wide threshold of detection, with values between $5x10^2$ and $1x10^4$ cfu/ml, depending on the strain.

1.2.2 Multiplex PCR

Five strains from the initial reference *Bacillus* strains collection were used to optimize the multiplex PCR. These strains were selected due to the previous knowledge about the detection of four interesting sequences thereon. PCR assays using the mixtures 1, 2 and 3 (Table 2.6) were carried out following the PCR conditions established for single PCR during 35 cycles. Results obtained using mixture 1 revealed that only the *srfAA* gene band was detected under these PCR conditions (Fig. 2.5-A). In order to allow an effective amplification of the *bacA*, *fenD* and *bmyB* genes, some modifications were introduced in the mixture 2, concretely the concentration of primers pairs was modified. Results using this mixture showed a good intensity for bands corresponding to *srfAA*, *fenD* and *bmyB* genes. However, the band corresponding to *bacA* gene was not detected yet (Fig. 2.5-B). Finally, primer and $MgCl_2$ concentrations were re-adjusted in the mixture 3 and all the expected bands were detected with equal intensity (Fig. 2.5-C).

Once optimized, the multiplex PCR was evaluated with the 14 reference *Bacillus* strains. Analysis of PCR products showed that Multiplex PCR faithfully reproduced the results obtained using the single PCR for each pair of primers individually showing combinations of 1, 2, 3 and 4 gene bands (Fig. 2.6). When sensitivity of the multiplex PCR was analyzed, it was observed an alteration on the sensitivity threshold compared to the single PCR.

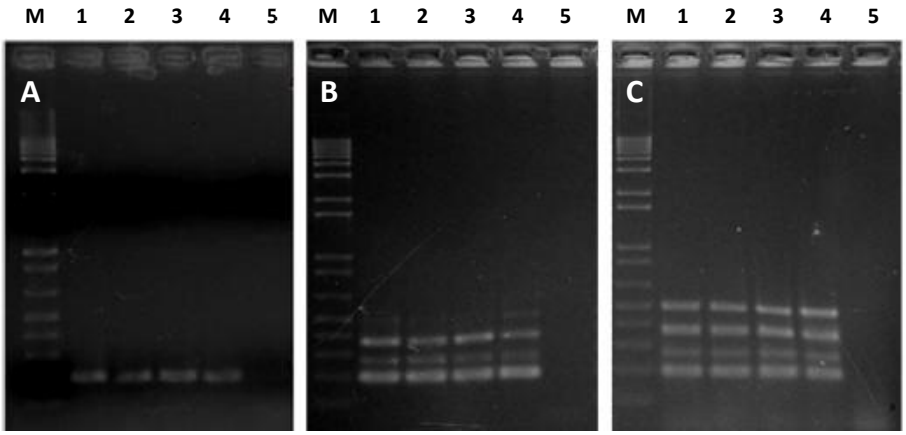

Figure 2.5. Multiplex analysis using different mixtures: Mixture 1 (A), Mixture 2 (B), Mixture 3 (C). *Bacillus* strains: QST713 (1), FZB42 (2), UMAF6614 (3), EPS2004 (4) and water control (5). M, 1Kb Plus Ladder (Invitrogen).

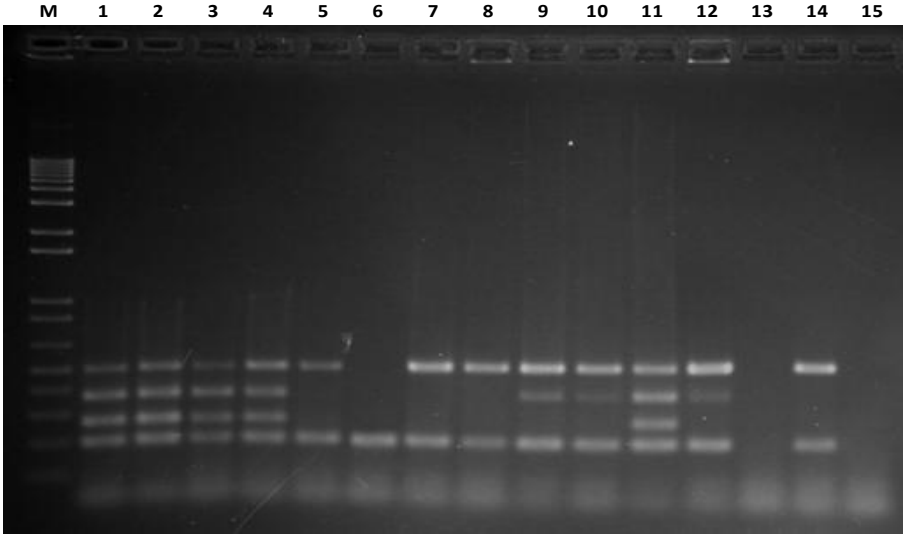

Figure 2.6. Analysis of *srfAA*, *fenD*, *bmyB* and *bacA* genes by multiplex PCR procedure using mixture 3 at 35 cycles of PCR. *Bacillus* strains QST713 (1), FZB42 (2), UMAF6614 (3), UMAF6639 (4), EPS2004 (5), RGAF5 (6), RGAF9 (7), RGAF11 (8), RGAF32 (9), RGAF46 (10), RGAF51 (11), RGAF66 (12), RGAF84 (13) RGAF101(14) and water control (15). M, 1Kb Plus Ladder (Invitrogen).

Results showed a reduction of the sensitivity using the multiplex PCR where the sensitivity values ranged from 10^5 to 10^6 cfu/ml (Fig. 2.7), a lower threshold than the sensitivity values detected by studying the sensitivity using the primers individually, where the least sensitive was observed for primer pairs directed to *bacA* gene, with a minimum detection level of $5x10^4$ cfu/ml.

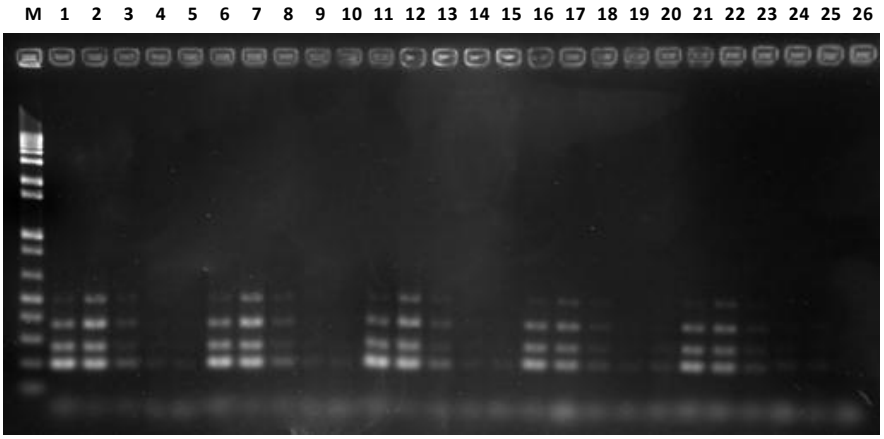

Figure 2.7. Analysis of the level of detection (cfu/ml) of primers directed to *srfAA*, *bacA*, *bmyB* and *fenD* genes by multiplex PCR in four reference strains of *Bacillus*, QST713 (1-5), FZB42 (6-10), UMAF6614 (11-15), RGAF51 (16-20) and EPS2004 (21-25) at concentrations from 10^7 to 10^2 cfu/ml and control (26). M, 1Kb Plus Ladder (Invitrogen).

1.3 Evaluation and validation of PCR tools in field samples

Results obtained after the use of single PCR to detect *16S rDNA*, *spoVG*, *srfAA*, *bacA*, *fenD*, *bmyB*, *ituC* and *spaS* sequences present in natural samples amended with strain QST713 showed differences according to the dilution of extract but not due to the type of sample. Thus, none of the genes were detected in undiluted samples or diluted 1:10 for all different types of natural samples. In contrast, amplification products for all gene sequences were detected in extracts amended with QST713 and diluted 1:100 and 1:1000 (Fig. 2.8). Identical results were obtained using the multiplex PCR, with no amplification in direct and diluted 1:10 samples, and with good amplification in samples diluted 1:100 and 1:1000 (Fig. 2.9). The sensitivity of single and multiplex PCR on natural samples diluted 1:100 was

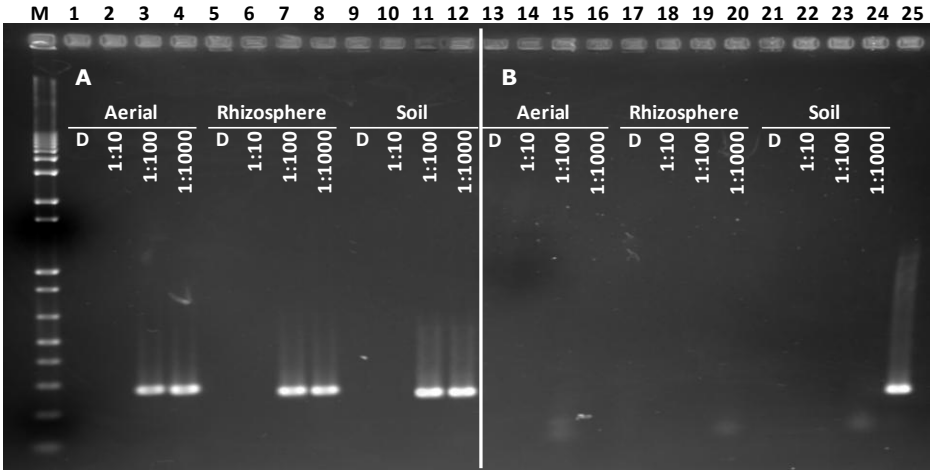

Figure 2.8. Single PCR analysis directed to *fenD* gene on natural extracts amended with *Bacillus* QST713 strain at final concentration of 10^7 cfu/ml (A) and natural extracts without addition of QST713 strain (B) obtained from aerial plant part, rhizosphere and soil, directly or previously diluted 1:10, 1:100, 1:1000. Positive control amplification products for QST713 at 10^7 cfu/ml (25). M, 1Kb Plus Ladder (Invitrogen).

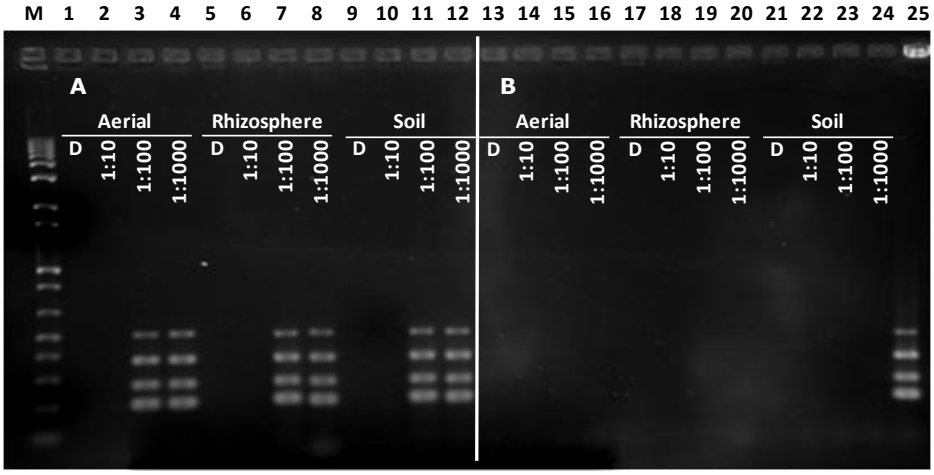

Figure 2.9. Multiplex PCR analysis of field extracts amended with QST713 strain at final concentration of 10^7 cfu/ml (A) and natural extracts (B) obtained from aerial plant part, rhizosphere and soil, directly (D) or previously diluted 1:10, 1:100, 1:1000. Positive control amplification products for QST713 at 10^7 cfu/ml (25). M, 1Kb Plus Ladder (Invitrogen).

evaluated, and showed a high variability among genes and strains as previously observed on the sensitivity assays in water during optimization. Particularly, sensitivity of single PCR also varied depending on the sample type. Thus, the sensitivity in aerial plant samples was only reduced 10 times in comparison to sensitivity previously observed in water solutions during evaluation and optimization assays.

In contrast, the sensitivity was extremely poor using rhizosphere or soil samples, reaching sensitivity with values of only 10^6 cfu/ml (Fig. 2.10), though there was a high variability among samples of the same type.On the other hand, the multiplex PCR sensitivity in strains QST713, UMAF6614, RGAF51 and EPS2004 was not affected when were applied to field samples in comparison with previous optimization assays done pure cultures in water, which was poor than in single PCR. Values of multiplex PCR sensitivity were around 10^5 cfu/ml in samples from aerial plant parts and rhizosphere, and around 10^6 cfu/ml in samples from soil (Fig. 2.11).

2. Development of a selective enrichment method

2.1 Distribution of *Bacillus* spp. population levels in natural environments

Assessment of population levels of *Bacillus* and its proportion compared to total bacterial population was done in 45 natural samples. Population levels of total bacteria were quite homogeneous with values between $1x10^5$ to $5x10^7$ cfu/g f.w., whereas the *Bacillus* population showed a high variability with values between not detected to 10^7 cfu/g f.w. (Table 2.10).

Bacillus colonies were not isolated in 31 out of 45 samples, especially in samples from the aerial plant part. Thus, *Bacillus* populations tend to be higher in roots and soil, with values ranging from 10^4 to 10^5 cfu/g f.w., than in leaves, fruits or flowers where *Bacillus* was not detected in most samples. Besides, the proportion of *Bacillus* in relation to the total microoganisms showed great variability, with values from >0.1 to 37.3 %.

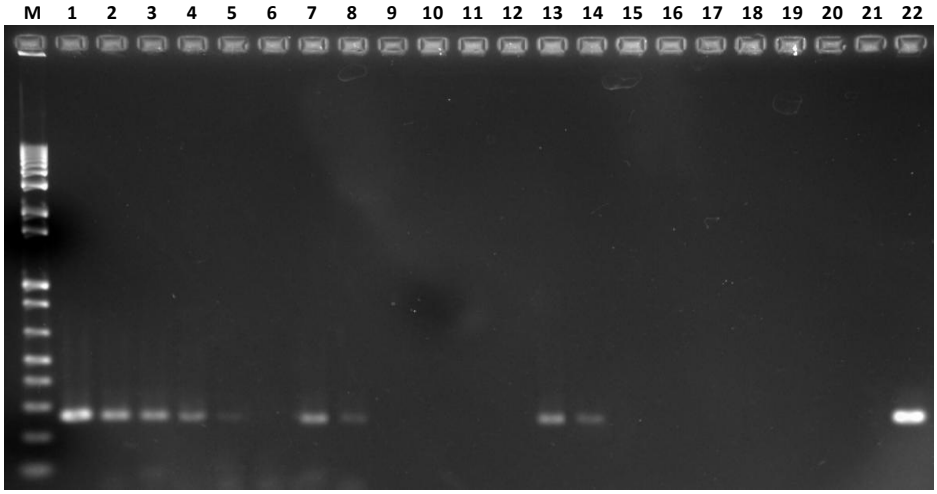

Figure 2.10. Single PCR analysis directed to *fenD* gene on field extracts amended with QST713 strain at final concentration form 10^7 to 10^2 cfu/ml, obtained from aerial plant part(1-6), rhizosphere (7-12) and soil (13-18), previously diluted 1:100. Negative control amplification products for field extracts aerial plant part, rhizosphere and soil previously diluted 1:100 (19, 20, 21), and positive control amplification products for QST713 at 10^7 cfu/ml (22). M, 1Kb Plus Ladder (Invitrogen).

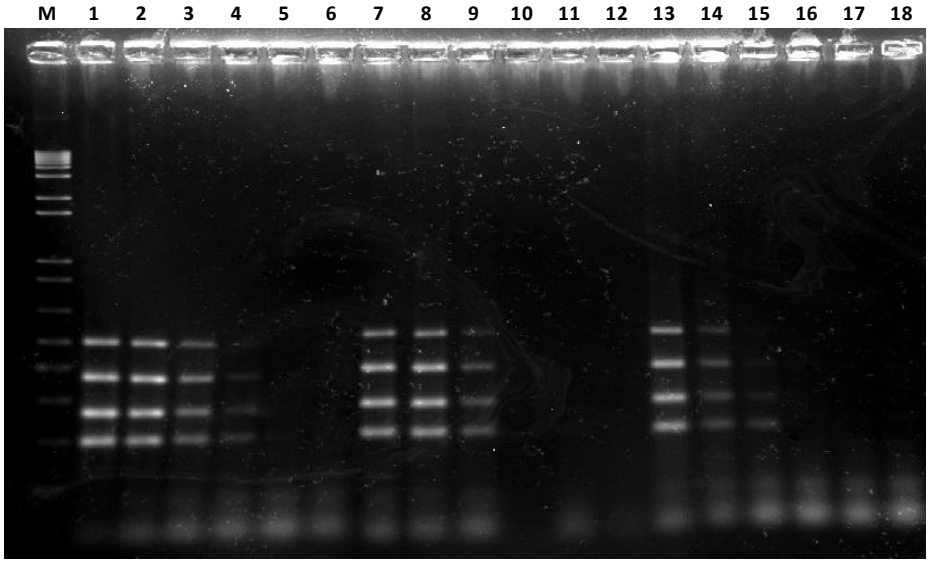

Figure 2.11. Multiplex PCR analysis to determine the sensitivity of detection of strain QST713 at final concentrations of 10^7-10^2 cfu/ml in natural samples of aerial plant part (1-6), rhizosphere (7-12) and soil (13-18) previously diluted 1:100. M, 1Kb Plus Ladder (Invitrogen).

Table 2.10. Population levels of total culturable bacteria and *Bacillus* spp. in 45 field samples from natural plant environments.

Sample Type	Sample	Total bacteria (log_{10} cfu/g f.w.)	*Bacillus* (log_{10} cfu/g f.w.)	Proportion of *Bacillus vs* Total bacteria (%)
Aerial plant	S1	6.60	nd	< 0.1
	S3	7.36	4.00	< 0.1
	S5	4.48	nd	< 0.1
	S6	4.30	nd	< 0.1
	S7	6.79	nd	< 0.1
	S8	5.68	nd	< 0.1
	S10	2.90	nd	< 0.1
	S11	5.00	nd	< 0.1
	S12	6.23	nd	< 0.1
	S13	6.85	nd	< 0.1
	S15	7.32	nd	< 0.1
	S16	7.60	nd	< 0.1
	S17	6.95	nd	< 0.1
	S18	2.60	nd	< 0.1
	S19	5.28	nd	< 0.1
Rhizosphere	S2	7.45	4.30	< 0.1
	S4	7.68	5.20	0.3
	S9	5.88	nd	< 0.1
	S14	7.36	nd	< 0.1
	S24	6.72	5.23	3.3
	S25	5.95	nd	< 0.1
	S26	7.59	nd	< 0.1
	S27	6.08	5.00	8.3
	S33	6.48	nd	< 0.1
	S34	7.62	7.20	37.4
	S35	5.51	nd	< 0.1
	S36	6.85	nd	< 0.1
	S43	6.30	4.78	3.0
	S44	6.60	4.30	0.5
	S45	6.08	nd	< 0.1
Soil	S20	6.26	nd	< 0.1
	S21	5.64	5.08	27.3
	S22	6.28	nd	< 0.1
	S23	4.95	nd	< 0.1
	S28	5.78	nd	< 0.1
	S29	7.20	6.15	8.7
	S30	6.32	nd	< 0.1
	S31	5.95	nd	< 0.1
	S32	5.70	nd	< 0.1
	S37	6.11	nd	< 0.1
	S38	6.00	5.11	13.0
	S39	6.66	4.90	1.7
	S40	5.49	4.48	9.7
	S41	5.34	nd	< 0.1
	S42	5.73	4.70	9.3

nd, not detected attending a detection limit of 10^1 cfu/g f.w.

It was also observed that the population of total bacteria and *Bacillus* spp. did not follow a normal distribution according to the analysis of frequencies and Shapiro-Wilk normality test (p value <0.05 in both cases) (Fig. 2.12). Although, total culturable bacterial levels in most of samples were between 10^6 and 10^7 cfu/g f.w. while *Bacillus* were less frequent, with population values between 10^4 and 10^5 cfu/g f.w. when were present.

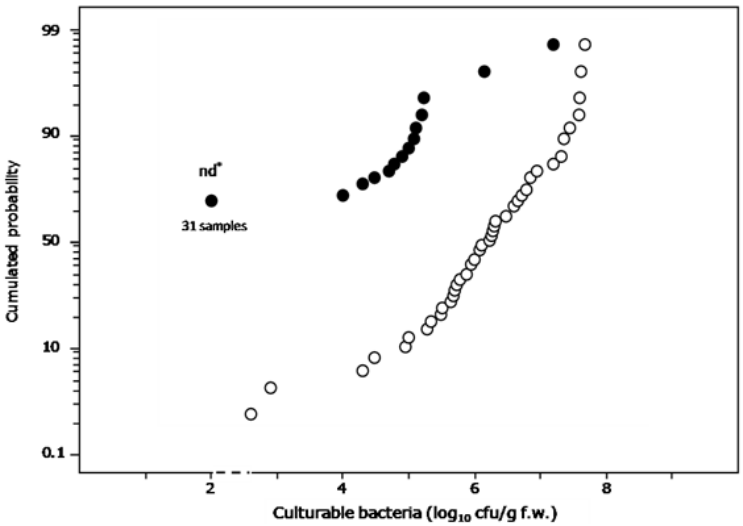

Figure 2.12. Cumulated probability of total aerobic bacteria (open circles) and *Bacillus* spp. population (black circles) of 45 natural samples. nd, *Bacillus* spp. not detected in 31 out of 45 samples processed.

2.2 Evaluation of the method for tracking *Bacillus* strains amended to field samples

2.2.1 Selection of spontaneous mutants

Four mutants resistant to rifampicin were obtained from each of the strains QST713, UMAF6639 and RGAF51. Growth of these spontaneous mutants was compared to the growth of wild type parental using the area under the growth curve (AUGC). Growth of 3 out of 4 RGAF51 and QST713 mutant strains, and 4 out of 4 UMAF6639 mutant strains was not significantly different of the respective wild type strain (Table 2.11). In contrast, the QST713 Rif^+1 and RGAF51 Rif^+4 mutant strains showed a lower growth rate.

2.2.2 Recovery of *Bacillus* spp. from fortified field samples

The recovery of mutant *Bacillus* strains was assessed in field sample extracts by the addition of strain QST713 Rif$^+$ at different concentrations in two independent assays (Fig. 2.13). Total culturable bacteria from rhizosphere extracts were $3.6x10^6$ to $1.4x10^6$ cfu/ml. Recovery of QST713 Rif$^+$ showed a 100 % efficiency in extracts amended with 10^3, 10^4 and 10^5 cfu/ml, independently of the assay. However, the yield of *Bacillus* recovered decreased in extracts fortified with 10^6 cfu/ml, though values of recovery were 87 and 74 % in assay 1 and 2, respectively.

Table *2.*11. Growth potential (AUGC) of wild type strains and the corresponding rifampicin resistant mutants in LB broth.

Strain	Area Under the Growth Curve [1]				
	Wilde type [2]	Rif1	Rif2	Rif3	Rif4
QST713	43,71	16,61*	40,67	41,40	43,58
RGAF51	39,07	39,10	38,42	37,56	25,62*
UMA6639	41,82	40,46	39,87	40,15	38,97

[1] AUGC values are the mean of 3 experimental repetitions
[2] Mean followed by (*) differ significantly ($p \leq 0,05$) according to Tukey's test.

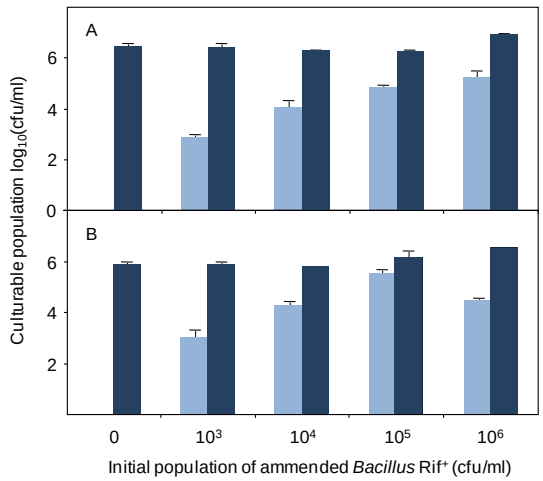

Figure 2.13. Total cuturable bacteria (■) and rifampicin resistant *Bacillus* strain (■) recovered from rhizosphere extracts initially amended at different concentrations of QST713 Rif$^+$ in two independent assays. Assay 1 (A), Assay 2 (B).

2.3 Evaluation of selection and enrichment strategies

2.3.1 Antibiotic resistance

Eleven *Bacillus* strains were studied to determine their resistance to four known antibiotics (rifampicin, nalidixic acid, colistin and chloramphenicol). Seven of the tested *Bacillus* strains (FZB42, RGAF5, RGAF9, RGAF11, RGAF32, RGAF51 and RGAF66) were sensitive to all antibiotics. Only strains QST713, RGAF46, RGAF84 and RGAF101 were naturally resistant to colistin, at both concentrations tested, but susceptible to the other antibiotics.

2.3.2 Thermal resistance

The four *Bacillus* strains tested (RGAF32, RGAF51, EPS2004 and UMAF6639) showed a higher tolerance to thermal shock than the non-*Bacillus* strains that were considerably more sensible (Fig. 2.14). The *Bacillus* strains showed a survival up to 40 % after 15 min at 80 ºC, while the strains of *P. fluorescens* and *P. agglomerans* survived less than 1 % (Fig. 2.15). These differences were particularly important at 60 ºC, where D_{60} values ranged from 343 to 1116 min in *Bacillus* strains, whereas in *P. fluorescens* and *P. agglomerans* strains ranged from 6 to 10 min. The maximum differences among *Bacillus* strains were observed at 120 ºC, where the most resistant strains showed D_{120} values of 20 min, compared to the rest of strains that showed D_{120} values around 4 min.

2.4 Selective enrichment procedure

2.4.1 Optimization

The selective enrichment procedure consisted in a heat treatment followed by an enrichment stage in LB broth at 40 ºC. The effect of the thermal treatment at 80 °C for 10 and 20 minutes, followed by enrichment of the extracts in LB broth diluted to 1:100 and 1:1000 was tested.

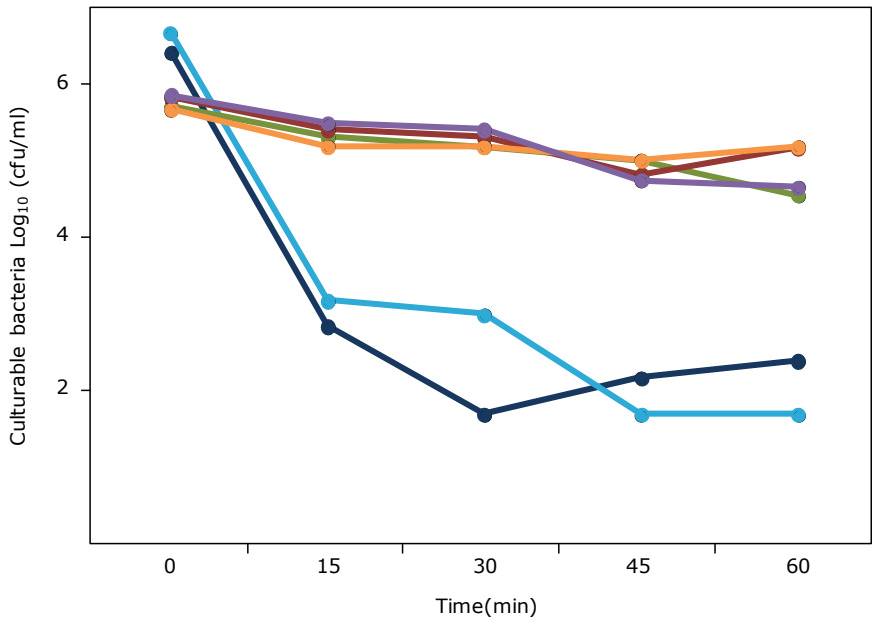

Figure 2.14. Kinetics of survival *B. subtilis* strains RGAF32 (—●—), EPS2004 (—●—), and UMAF6639 (—●—), *B. megaterium* RGAF51 (—●—), compared to the plant associated bacteria *Pseudomonas fluorescens* EPS62e (—●—) and *Pantoea agglomerans* EPS125 (—●—) after thermal treatment at 80 ºC at different exposure times.

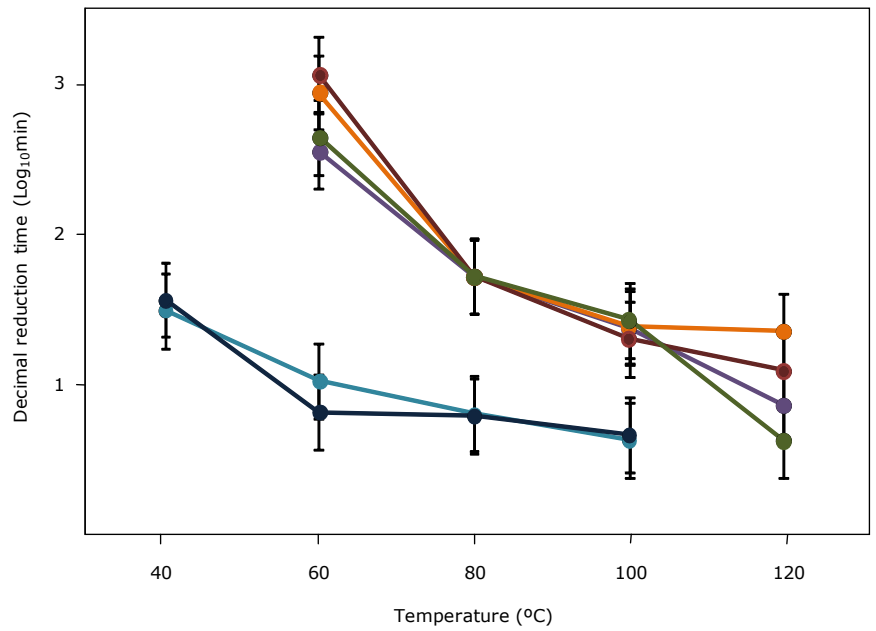

Figure 2.15. Thermal sensitivity of *B. subtilis* strains RGAF32 (—●—), EPS2004 (—●—), and UMAF6639 (—●—), *B. megaterium* RGAF51 (—●—), compared to the plant associated bacteria *Pseudomonas fluorescens* EPS62e (—●—) and *Pantoea agglomerans* EPS125 (—●—).

Total culturable bacteria populations and population of amended *Bacillus* RGAF51 Rif$^+$ at different initial concentrations (10^4, 10^5, 10^6 and 10^7 cfu/ml) were determined for each step of the procedure (untreated, after thermal treatment, at the end of the enrichment) (Fig. 2.16). In the extracts without addition of *Bacillus*, the population of total culturable bacteria decreased to undetectable levels after thermal treatment at both times of exposure tested with a reduction higher than 4 logs. Besides, these populations were not recovered after the enrichment stage in none of the dilutions assessed. In contrast, the population levels of *Bacillus* RGAF51 Rif$^+$, decreased to only around 2 logs after the thermal treatment and differences were not observed between the two thermal exposure times. However, significant differences in populations of *Bacillus* RGAF51 Rif$^+$ were observed depending on the dilution conditions during the enrichment step. Recoveries of *Bacillus* did not differ when sample extracts were amended with high or low initial concentrations (10^7 and 10^6 cfu/ml) achieving recovery values of $5x10^6$ cfu/ml in both cases. However, the recovery of *Bacillus* added at low initial concentrations (10^5 and 10^4 cfu/ml) was affected by the dilution during enrichment, with recovery values around 10^6 cfu/ml using dilution 1:100, but of only 10^3 cfu/ml with dilution 1:1000, independently of the thermal treatment applied. Moreover, *Bacillus* added at low initial concentrations were not recovered when the sample was submitted to a thermal treatment at 80 ºC for 20 min and to an enrichment in 1:1000 dilution.

2.4.2 Evaluation

The selective enrichment method was evaluated in two assays consisting of the addition of a *Bacillus* strain to mixture of Gram-negative strains (artificial microcosm) and field extracts. The addition of a *Bacillus* strain in a mixture of Gram-negative bacteria was performed in order to compare the survival of heat-sensitive bacteria, such as chosen Gram-negative strains with the thermoresistant *Bacillus* strain. Population levels of total culturable bacteria and of a *Bacillus* spp. were determined for each step of the procedure (initial untreated, after thermal treatment, at the end of the enrichment stage) (upper panel Fig. 2.17).

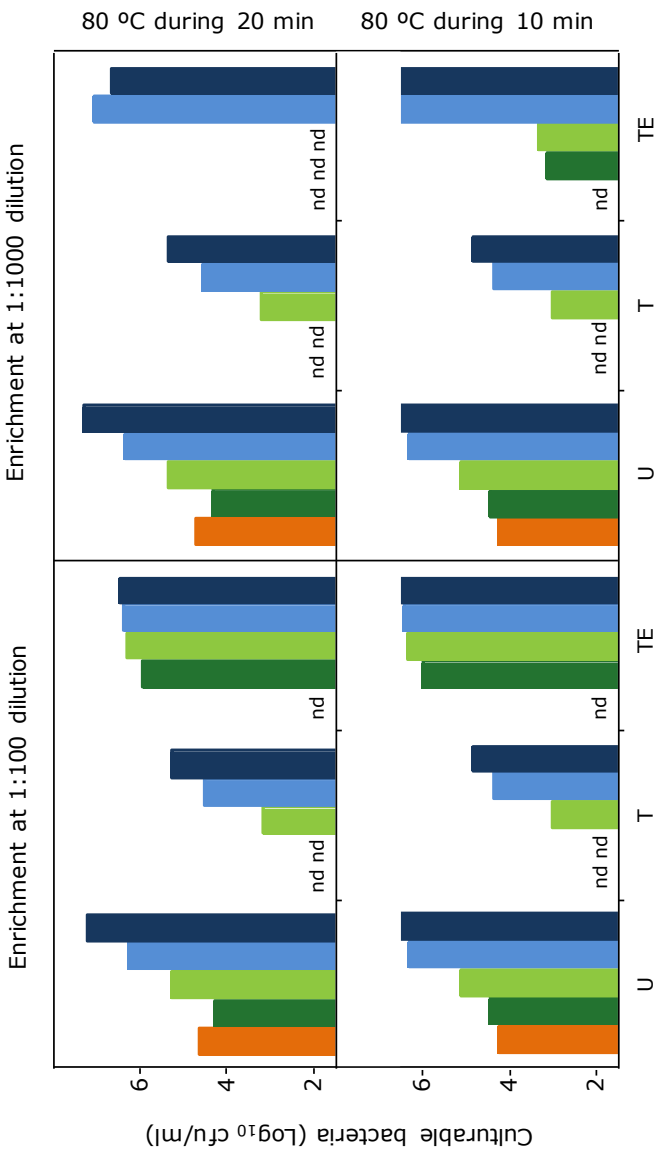

Figure 2.16. Population levels of total culturable bacteria and of *B. megaterium* RGAF51Rif^{+} from rhizosphere extract at different stages of the selective enrichment method. Untreated (U), after thermal treatment (T) and after thermal treatment and enrichment (TE). Different initial concentrations of RGAF51Rif^{+} were used (0 (■), 10^{4} (■), 10^{5} (■), 10^{6} (■) and 10^{7} cfu/ml (■)). Different extract dilution was used before the enrichment step (diluted 1:100 and diluted 1:1000). Different exposure time of thermal treatment were used 20 min and 10 min. Total culturable bacteria (■) were determined in LB agar whereas culturable rifampicin resistant bacteria (■, ■, ■) were determined on LB agar supplemented with rifampicin (100 mg/l). The minimum detection level was 1.7 $\log_{10}$ cfu/ml. nd, not detected.

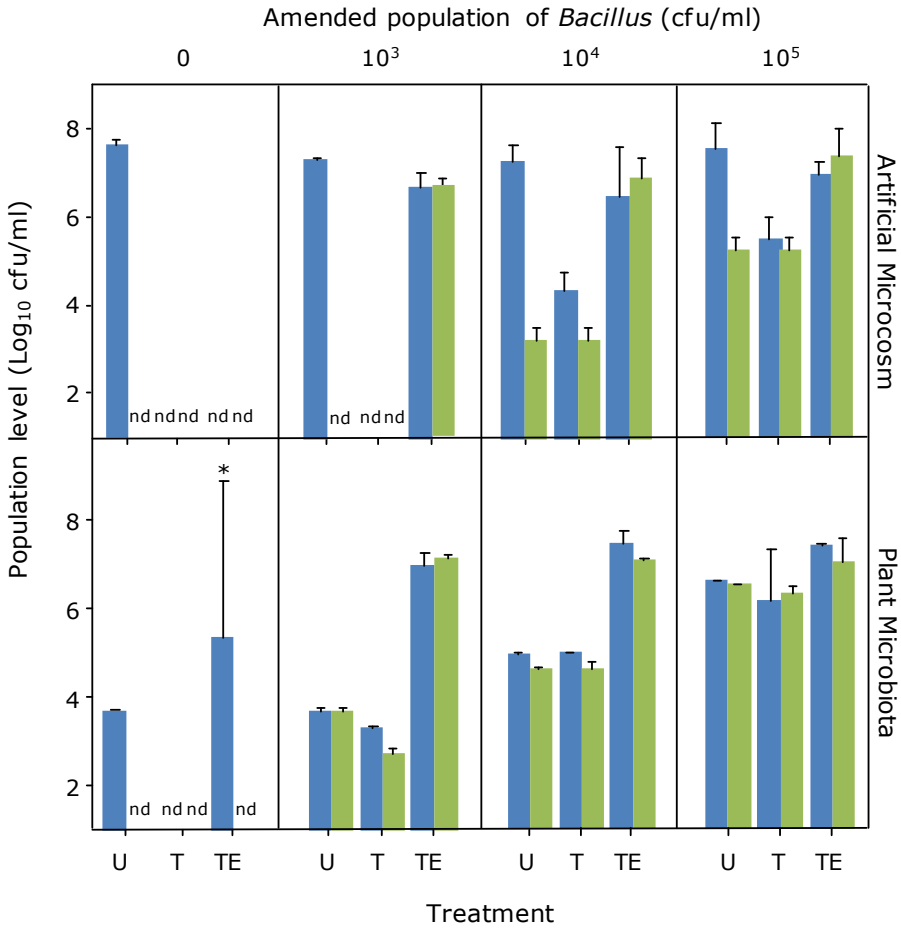

Figure 2.17. Population of total culturable bacteria (■) and *B. megaterium* RGAF51 Rif^+ (■) from artificial microcosm (upper panel) and natural plant microbiota (Lower pannel). Populations were determined in different stages of the selective enrichment method: Untreated (U), after thermal treatment (T) and after thermal treatment and enrichment (TE). Different initial concentrations of RGAF51 Rif^+ were used (0, 10^3, 10^4, and 10^6 cfu/ml). Total culturable bacteria were determined in LB agar whereas culturable rifampicin resistant bacteria were determined on LB agar supplemented with rifampicin (100 mg/l). The minimum detection level was 1.7 log_{10} cfu/ml. Confidence intervals over the bars correspond to three replicates. (*) Indicates that autochthonous *Bacillus* grew into one out of the three replicates. nd indicates non-detected colonies.

In the mixture of Gram-negative strains without addition of the *Bacillus* strain, the total bacterial culturable population decreased 6 logs, until undetectable values, after the thermal treatment, and bacteria were not recovered after the enrichment. When the rifampicin resistant *Bacillus* RGAF51 Rif^+ was added a reduction in the total culturable bacteria after the thermal treatment was also observed, although this reduction was related to the initial population of *Bacillus* added ($1x10^3$, $1x10^4$ and $1x10^6$ cfu/ml). The rifampicin resistant *Bacillus* population did not significantly changed after the thermal treatment and coincided with the total population of culturable bacteria. After the enrichment step *Bacillus* populations increased to values above 10^7 cfu/ml, independently of the initial populations inoculated.

Similar results were obtained in the assay performed with field sample extracts. Total culturable bacteria populations in the plant microbiota extract without the addition of *Bacillus* decreased to undetectable values after the thermal treatment (lower panel Fig. 2.17). Only in one of the three replicates a residual population was recovered after enrichment and this was due to presence of autochthonous *Bacillus*, which wasproved to be susceptible after isolation to the antibiotic. These results were confirmed by PCR with primers for *Bacillus* targeted to the *16S rDNA* gene (Fig. 2.18). Field extracts without addition of *Bacillus* did not gave amplification for the *16S rDNA* gene nor for the antimicrobial peptide genes *(bmyB, fenD, ituC, srfAA, bacA* and *spaS*), neither in the untreated samples nor after the thermal treatment, except for the above mentioned sample where autochthonous *Bacillus* were detected.

In contrast, all the samples amended with *Bacillus* RGAF 51 Rif^+ showed positive amplification for all primer pairs except in the samples amended to a final concentration of *Bacillus* of 10^4 and 10^3, just after the thermal treatment. Moreover, the intensity of bands was proportional to the initial concentration of *Bacillus* inoculated.

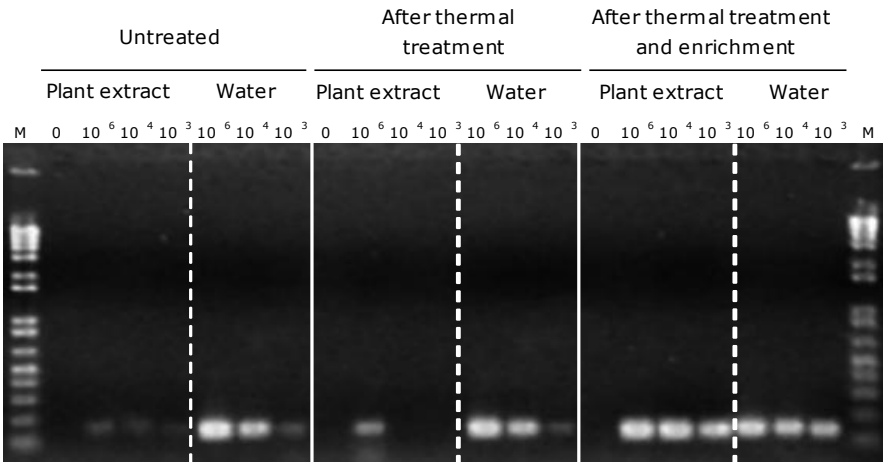

Figure 2.18. PCR detection of *Bacillus* by means of general primers (*16S rDNA*) in natural plant extract and water controls with or without amendment of *B. subtilis* RGAF51Rif^{+} at different concentrations and in different stages of the selective enrichment method. Initial concentrations of RGAF51Rif^{+} were 0, 10^3, 10^4, and 10^6 cfu/ml. M, 1Kb Plus DNA Ladder (Invitrogen).

2.5 Comparison of the standard and selective enrichment methods of isolation of *Bacillus* spp.

The population levels of total culturable bacteria and *Bacillus* in 45 field samples (15 from the aerial plant part, 15 from rhizosphere and 15 from soil), ranged from 6.01 to 7.08 $\log_{10}$ cfu/g f.w., and from 0.29 to 2.8 $\log_{10}$ cfu/g f.w., respectively (Table 2.12). Therefore, low presence of *Bacillus* was observed in field samples processed with the standard procedure, though the proportion of *Bacillus* spp. in the total population was higher in the rhizosphere and soil samples than in the aerial plant part. PCR using *16S rDNA* detected *Bacillus* spp. in 22 out of 45 samples and in 18 putative *Bacillus* colonies that were isolated (2 from aerial plant parts, 9 from rhizosphere, and 7 from soil) using the standard method (Fig. 2.19). After the application of the selective enrichment method, *Bacillus* population levels increased until high concentrations with a consistent presence among the different sample types. At the end of the whole process total culturable bacteria population levels were 7.29-8.10 $\log_{10}$ cfu/g f.w. and *Bacillus* spp. population levels were 6.41-7.55 $\log_{10}$ cfu/g f.w.

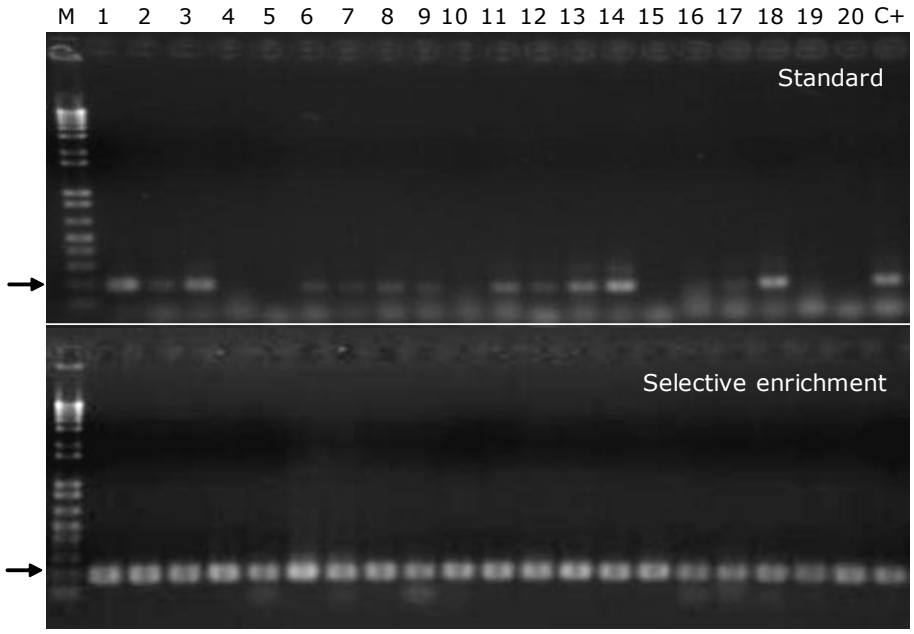

Figure 2.19. PCR analysis of *srfAA* gene in selected *Bacillus* strains and field samples. Strains FZB42, QST713 and RGAF51 (1-3), and 17 field samples (4-20) processed by the standard method or by the selective enrichment method. M, 1Kb Plus DNA Ladder (Invitrogen).

Also, all samples after the selective enrichment procedure showed amplification for the *16S rDNA* primers, confirming the presence of *Bacillus* spp. in all cases (Table 2.12). 55 putative *Bacillus* colonies were isolated, 14 from aerial plant samples, 19 from rizhosphere, and 22 from soil. Then, the yield of the selective enrichment, in terms of *Bacillus* isolation, was around three times higher than in the standard procedure.

Furthermore, 21.9 % of *Bacillus* spp. isolates originated from aerial plant part samples, while 38.3 % and 39.7 % of isolates were from rhizosphere and soil samples, respectively.

The presence of the six antimicrobial peptides genes (*bmyB, fenD, ituC, srfAA, bacA* and *spaS*) was determined in natural extracts and in the corresponding *Bacillus* isolates in both, the samples processed using the standard method and the samples processed with the selective enrichment procedure. The number of simultaneous AMP genes per sample and per

Table 2.12. Performance of the standard and selective enrichment methods for *Bacillus* spp. isolation from plant samples of different origin.

Method	Origin	Number of samples	Total culturable bacteria (Log_{10} cfu/g f.w.)	*Bacillus* like colonies (Log_{10} cfu/g f.w.)	Positive samples for *Bacillus* 16S rDNA	Confirmed *Bacillus* isolates
Standard	Aerial plant part	15	6.01 ± 1.59	0.29 ± 1.11	14	2
	Rhizosphere	15	7.08 ± 0.65	2.54 ± 2.88	5	9
	Soil	15	6.62 ± 0.75	2.80 ± 2.75	3	7
	Total	145	6.57 ± 1.00	1.88 ± 2.60	22	18
Enrichement	Aerial plant part	15	8.10 ± 2.63	6.60 ± 3.32	15	14
	Rhizosphere	15	8.03 ± 1.49	7.55 ± 2.5	15	19
	Soil	15	7.29 ± 0.78	6.41 ± 2.31	15	22
	Total	45	7.81 ± 1.80	6.85 ± 2.73	45	55

Bacillus isolate is shown in Figure 2.20. The methods differed in the frequency of AMP genes per sample, thus none of the genes was detected in 20 out of 45 samples processed by the standard method, while only 5 out of 45 samples did not showed the presence of AMP genes by the selective enrichment procedure. In addition, only 11 out of 45 samples gave 3 or more AMP genes with the standard method, whereas in the selective enrichment method 37 out of 45 samples had 3 or more AMP genes.

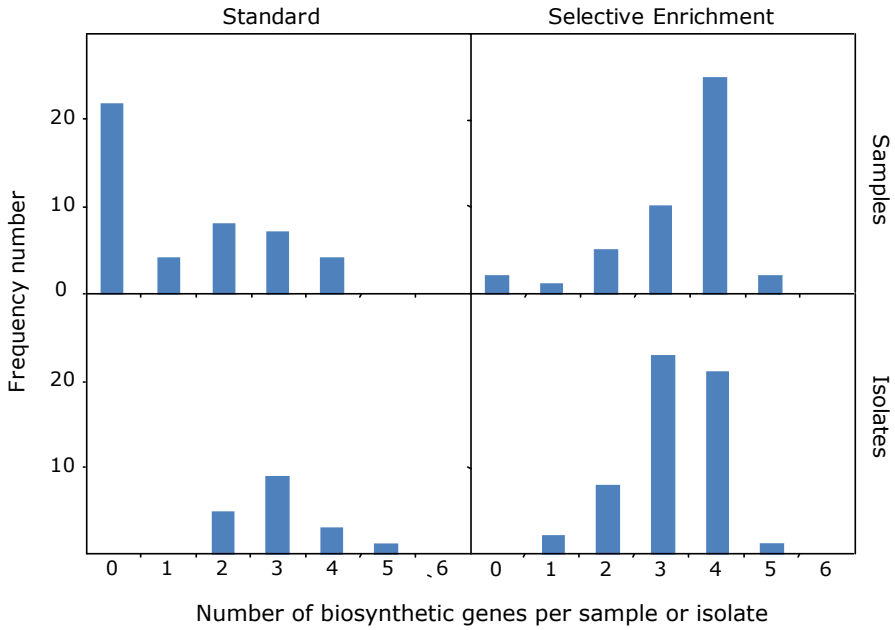

Figure 2.20. Number of simultaneous biosynthetic genes detected by PCR in field samples processed with the standard or with the selective enrichment method, and in the corresponding *Bacillus* isolates.

The distribution of frequencies of the six AMP genes for samples and for the *Bacillus* isolates depending on the method used, standard or selective enrichment method, is shown in Fig. 2.21. In field samples and isolates, the distribution of frequencies were similar (Chi-square 0.900), independently of the method used, being the most common biosynthetic genes *srfAA, bacA, fenD* and *bmyB* (20 to 40 % in each case). The other genes, *ituC* and *spaS*, were not detected at the standard treatment, but were detected in frequencies lower than 2 % in the selective enrichment procedure.

An increase of the frequency of each biosynthetic gene was observed by processing samples using the selective enrichment in comparison with the standard method, in the same way as for the isolates obtained by the selective enrichment method. Although, the frequency of each gene increased in samples processed by the selective enrichment method, the frequency distribution was not altered (Fig. 2.21).

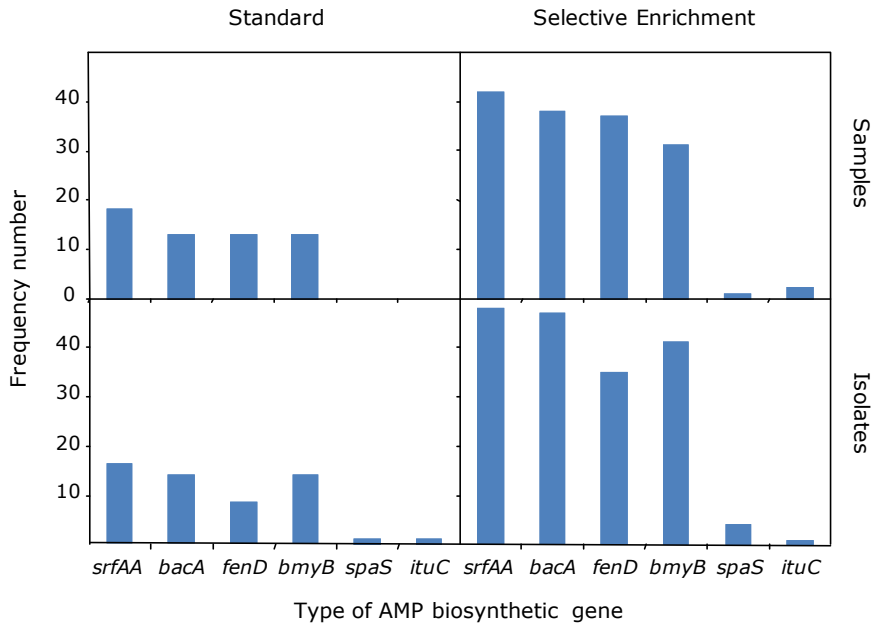

Figure 2.21. Frequency of field samples and the corresponding *Bacillus* isolates on basis of the presence of AMP biosynthetic genes processed directly by the standard or by the selective enrichment method.

Discussion

Studies of the presence and diversity of AMP biosynthetic genes in natural populations of *Bacillus* may be useful for discovering new strains with broad-range and better efficacy of pathogen control, as well as improved fitness in plant environments. Most of the studies focused in the development of BCAs are based on the selection of strains with antagonist activity *in vitro* towards interest pathogens and therefore biocontrol activity related with production of antimicrobial compounds. However, one of the limitations of this procedure is the low yield obtained in the selection of future BCAs (Adesina *et al*. 2007, Cazorla *et al*. 2007, Kim *et al*. 2008, Li *et al*. 2008, Wu *et al*. 2007). An alternative to the standard method would be the use of biocontrol markers in order to select quickly and easily the potential biocontrol candidates.

Concretely in *Bacillus*, the sequenced genomes of *B. subtilis* 168 and *B. amyloliquefaciens* FZB42, and of relevant genes in other strains related to the secondary metabolite production (e.g. antimicrobial peptides) have provide information to find functional molecular markers. This fact has facilitated the development of PCR tools to analyze the prevalence and distribution of this marker in commercial biocontrol *Bacillus* strains and in isolates from natural populations. Thus, Joshi and McSpadden-Gardener (2006) observed that strains of *Bacillus* with proven biocontrol capacities presented sequences with high similarity with genes involved in antibiotic synthesis (*bmyB*, *fenD*, *ituC*, *srfAA* and *srfAB*), and also observed that strains containing these genes exhibited a greater inhibition of the growth of *Rhizoctonia solani* and *Pythium ultimum* than other *Bacillus* isolates that lacked the markers. More recently, the presence of biosynthetic genes of fengycin and bacillomycin D in *Bacillus* spp. strains has been related with the antagonistic activity against fungal pathogens of canola and wheat and also with the production of the respective products (Ramarathnam *et al*. 2007). These results suggest that marker gene sequences can be used as a criterion for selecting novel strains that might be better suited for biocontrol applications. By using PCR, large collections of *Bacillus* spp. could be screened rapidly, prior to phenotypic tests, accelerating the rate of discovery of new and useful biocontrol strains and plant growth-promoting rhizobacteria (Joshi and McSpadden-Gardener 2006).

In the present study, we developed a procedure to detect specific markers associated to *Bacillus* and to antimicrobial peptide genes directly in field sample extracts. The methodology has been optimized for eight molecular markers, two that discriminate at genus level and six that discriminate the potential production of antimicrobial peptides. Generalist markers of *Bacillus* were based on *16S rRNA* and *spoVG* genes. These genes were selected because *16S rRNA* gene is used as a framework for the modern classification of bacteria, including those in the genus *Bacillus* (Cazorla *et al.* 2007, Fritze 2004, Ki *et al.* 2009, Miranda *et al.* 2008, Wulff *et al*. 2002) while *spoVG* gene encodes a protein that is involved in the fifth stage of spore formation on *Bacillus* spp. (Asai *et al.* 1998, Matsuno and Sonenshein 1999). These generalist markers worked relatively well showing a good spectrum of positive detection among all strains of *Bacillus* tested.

However, markers based on s*poVG* gene were discarded because the sensitivity threshold observed was poorer compared to the *16S rDNA* sequence. In addition, the results of specificity were also better for *16S rDNA* sequence that showed only one unspecific amplification of a *Pseudomonas* spp. strain at the highest concentration tested, indicating the capacity to discriminate *Bacillus* spp. respect to the most common inhabitants of natural environments, *Pantoea* spp. and *Pseudomonas* spp. In the case of *spoVG* sequence, a low discriminative capacity was observed because a positive amplification with several strains of *Pseudomonas* spp. and *Pantoea* spp. was observed at the lowest concentration.

Additionally, antimicrobial peptide biosynthetic genes *bmyB* (bacillomycin), *fenD* (fengycin), *ituC* (iturin), *srfAA* (surfactin), *bacA* (bacilysin) and *spaS* (subtilin) were selected as specific molecular markers due to the fact that their expression products have been reported to inhibit several plant pathogenic microorganisms and in several cases have been implicated in the mechanism of action of *Bacillus* BCAs against plant diseases (Arguelles-Arias *et al*. 2009, Chen *et al*. 2009b, Joshi and McSpadden-Gardener 2006). The specificity and sensitivity of the primers previously described (Joshi and McSpadden-Gardener 2006) was low, with detection threshold below 10^5 cfu/ml in most of strains and genes tested. This detection level was considered too poor to use these primers for the detection of AMP genes in field samples and consequently new primer pairs were designed in the present work in order to improve sensitivity. New primers were evaluated and validated with reference strains of *Bacillus* for which there is information available. Our results agreed with the presence of genes *srfAA*, *bacA*, *bmyB* and *fenD* in strain FZB42 and of *srfAA*, *bmyB*, *fenD* and *ituC* genes in strain QST713 (Joshi and McSpadden-Gardener 2006, Koumoutsi *et al*. 2004). Interestingly, a fifth AMP gene, *bacA*, was detected in strain QST713. Although these AMP gene markers have not been reported previously in other strains, our results are consistent with the production of bacillomycin, fengycin, and surfactin detected by other authors in strain UMAF6614, and of iturin, fengycin and surfactin in strain UMAF6639 (Romero *et al.* 2007). Note that we detected gene markers for bacilysin in both strains, for bacillomycin in UMAF6639, and for subtilin in UMAF6614.

The sensitivity of the specific primers was dependent on the *Bacillus* strain and target gene, and was high for *fenD* and *ituC* (<5x10^2 cfu/ml), moderate to low for *bmyB*, *srfAA* and *bacA* (between 10^2 and 10^4 cfu/ml) and very poor for *spaS* (>10^4 cfu/ml). The sensitivity was better than in primers previously reported for the genes *srfAA*, *bmyB*, *fenD* and *ituC* (Joshi and McSpadden-Gardener 2006, Koumoutsi *et al*. 2004). These sensitivity levels are in agreement with results obtained in studies of detection of plant pathogenic bacteria like *X. vesicatoria* (Moretti *et al*. 2009), *P. syringae* pv. tomato (Zaccardelli *et al*. 2005) or *E. amylovora* (Bereswill *et al*. 1992). A good sensitivity is important for screening directly in field sample extracts where often a combination of low levels of the target gene and presence of compounds affecting the polymerase chain reaction limit this type of analysis (Desai andMadamwar 2007). Results observed in the present work for simple PCR to detect *16S rDNA*, *spoVG*, *srfAA*, *bacA*, *bmyB*, *fenD*, *ituC* and *spaS* genes, in natural samples fortified with a known *Bacillus* strain, indicated that it was necessary to apply a previous step based on dilution of natural extracts to optimize detection conditions to dilute interfering compounds in the PCR reaction, as has been described in other reports (Maes *et al*. 1996, Pan *et al*. 1997). Amplification results on diluted natural extracts fortified with a *Bacillus* strain indicated that dilutions 1:100 and 1:1000 were enough to eliminate interference from natural inhibitors. However, sensitivity under these conditions was around 10 times lower than the observed under optimal conditions with no interfering substances.

In order to reduce the time consumed and cost of screening, a multiplex PCR directed to genes *srfAA*, *bacA*, *bmyB* and *fenD* was optimized. Results agreed with those obtained for simple PCR. In contrast, sensitivity levels using multiplex PCR were poorer than in the simple PCR. This was due to the fact that multiplex PCR was optimized to get similar amplification signal for the four genes included, so sensitivity was drastically reduced to values above 10^5 cfu/ml. When multiplex PCR was tested in natural extracts fortified with a known *Bacillus* strain, similar results than simple PCR were observed and amplification reactions did not work in the dilution 1:10 of the natural extract but gave good results in extracts diluted 1:100 and 1:1000.

Once determined the detection levels for the simple and multiplex PCR, the population levels of *Bacillus* spp. were assessed in natural plant samples in order to discriminate the potential of these tools. Population levels of *Bacillus* spp. in the field samples of the Mediterranean Eastern area reported here varied within six orders of magnitude with frequent values below the detection level of the simple and multiplex PCR developed here. In addition, *Bacillus* population levels were frequently very low with values below 2.8 log10 cfu/g f.w. Our results are in accordance with other reports in which they were either not detected or varied strongly with sample type and origin, with isolates being more frequently obtained from soil or rhizosphere than from aerial plant parts (Adesina *et al*. 2007, Han *et al*. 2009, Martínez-Alonso *et al*. 2010).

Due to these problems, a method to increase *Bacillus* population levels in natural samples previous to the application of the molecular tools was required in order to work with natural extracts. Consequently, a selective enrichment procedure was developed and validated. Two enrichment strategies were tested, one based on the counterselection with a differential antibiotic amended to the growth medium and a second based in a thermal treatment of the sample extract. The first method based on the resistance to antibiotics was discarded because all the strains evaluated were sensible to many antibiotics, except two strains that were sensible to colistin. The second method was based on the capacity of *Bacillus* to resist high temperatures in contrast to non-sporulating bacteria that are sensible to temperatures above 60-70 °C (Cortezzo and Setlow 2005, Okahisa *et al*. 2008). Thus, sensitivity was evaluated by exposure to different temperature treatments. Results indicated that optimal conditions for selectivity in the natural samples to reduce non-*Bacillus* population levels and keep high population levels of *Bacillus* were temperatures between 70 and 90 ºC during 15 min. Finally, thermal treatment at 80 ºC for 10 min was chosen because D value (Decimal reduction time) of *Bacillus* strains at 80 ºC was approximately 8 times higher than of non-sporulating strains, and exposure to this temperature for only 10 min drastically reduced the population of non-sporulating bacteria without affecting significantly *Bacillus* populations. Besides, these results agreed with other reports (Cho *et al*. 2004, Rahman *et al.* 2006).

Once the thermal conditions were selected, the enrichment procedure was designed as a thermal treatment at 80 ºC for 10 min followed by a cultivation stage at 40 ºC for 24 h with a previous dilution of 1:100 in LB broth to dilute PCR inhibitors. In general, these enrichment methods facilitate target detection by increasing their numbers and decreasing inhibitors and have proven successful in detecting and identifying bacteria in seeds, soil samples and symptomless plant tissues (Ito *et al*. 1998, Manulis *et al*. 1998, Penyalver *et al.* 2000, Sakthivel *et al*. 2001, Weller *et al*. 2000, Weller and Stead 2002). The selective enrichment method was validated for its capacity to recover populations of *Bacillus* in complex communities, concretely in an artificial microcosm and in microbiota extracts from field samples, showing the capacity to recover initial populations of *Bacillus* equal or above to 10^3 cfu/ml. Then, molecular tools gave good results in all the samples processed using the selective enrichment procedure, because population levels of *Bacillus* were above 10^6 cfu/ml at the end of the enrichment procedure. Therefore, using this method an increase of *Bacillus* isolation yield was expected in accordance with the procedure reported in *B. thuringiensis* to increase the yield of isolation of active strains for insect pest control (Bizarri and Bishop 2007).

The comparison of the standard versus the selective enrichment procedures in natural samples showed the increase in yield of isolation of *Bacillus* isolates when applying the selective enrichment procedure. Thus, from a total of 73 *Bacillus* isolates obtained from 45 samples processed in parallel with both methods, only 18 of them were obtained using the standard procedure, while 55 were obtained using the selective enrichment procedure.

However, there were significant differences between both procedures. Only 51 % of natural samples gave signal for one or more antimicrobial peptides markers when applying the standard method, while 95% of samples gave signal for one or more antimicrobial peptides markers when applying the selective enrichment procedure. The number of AMP genes detected per sample simultaneously, depended on the method used, being the selective enrichment procedure, the method which gave detection of genes in more samples. The most frequent genes detected in our study were *srfAA, bacA,*

fenD and *bmyB*, with a scarce representation of *ituC* and *spaS*. However, these results apparently contrasts with a study of six AMP biosynthetic genes (bacillomycin D, iturin A, surfactin, mycosubtilin, fengycin, and zwittermicin A), within a collection of strains active against *S. sclerotiorum.* In this study the majority of strains harboured sufactin and iturin genes (Athukorala *et al*. 2009). Our results are in agreement with the higher frequency of surfactin genes, but not in the case of iturin genes. This can be due to the fact that in the above report the strain collection included only strains active against the target pathogen, whereas in our case the strains were unselected for a specific pathogen. It may be also due to the fact that the authors used PCR primers to *ituA* instead of *ituC* used in our work.

Finally, the distribution of AMP genes in samples was not significantly different between both methods, providing evidence that the selective enrichment procedure did not distort the actual *Bacillus* population structure in the original sample.

In conclusion, the procedure developed and evaluated here increases the yield of isolation of *Bacillus* strains containing simultaneous AMP genes. It is expected that the resulting strain collections would have a high probability to harbour efficient *Bacillus* BCAs with mechanisms of action relying on antibiosis against the target pathogen, mediated by antimicrobial peptides.

The content of this chapter has been previously published as indicated below:

Mora, I., Cabrefiga, J. and Montesinos, E. 2011. Antimicrobial peptide genes in *Bacillus* strains from plant environments. Int Microbiol. 14:213-223.

Abstract

The presence of the antimicrobial peptide (AMP) biosynthetic genes *srfAA* (surfactin), *bacA* (bacilysin), *fenD* (fengycin), *bmyB* (bacillomycin), *spaS* (subtilin), and *ituC* (iturin) was examined in 184 isolates of *Bacillus* spp. obtained from plant environments (aerial, rhizosphere, soil) in the Mediterranean land area of Spain. Most strains had between two and four AMP genes whereas strains with five genes were seldom detected and none of the strains had six genes. The most frequent AMP gene markers were *srfAA*, *bacA*, *bmyB*, and *fenD*, and the most frequent genotypes *srfAA*-*bacA*-*bmyB* and *srfAA*-*bacA*-*bmyB*-*fenD*. The dominance of these particular genes in *Bacillus* strains associated with plants reinforces the competitive role of surfactin, bacillomycin, fengycin, and bacilysin in the fitness of strains in natural environments. The use of these AMP gene markers may assist in the selection of putative biological control agents of plant pathogens.

CHAPTER 3

Characterization of *Bacillus* from plant environments. Antimicrobial peptide genes, products and activity

Introduction

Collections of antagonistic strains to plant pathogens have been extensively used to determine biocontrol markers to be used to improve strategies to select biocontrol agents (BCAs) of plant diseases faster and cheaper. Improvement of the selection process is essential because the success on the development of biocontrol strategies depends on the ability of a screening procedure that permits to identify appropriate candidates. The use of new techniques to identify biocontrol markers can enhance the ability to screen determinants involved in antimicrobial activity but also in other processes like colonization, motility or induction of plant defense responses. Some examples of markers of biocontrol that have been reported are the production of 2,4-diacetylphloroglucinol in *P. fluorescens* that is closely related with a superior disease suppression ability in the *Pythium*-cucumber

and *Fusarium*-tomato pathosystems compared to non-producing strains (Rezzonico *et al*. 2007) or the degradation of nicotinic acid for the selection of antagonists of *E. amylovora* (Paternoster *et al*. 2010).

In the case of *Bacillus* genus, most programs for developing BCAs have been based on strains isolated from agricultural systems in environments where bacterial populations vary in species diversity (Chandna *et al*. 2012) and quantity (McSpadden-Gardener 2004, Mora *et al*. 2011), which in most cases are rather rare. However, not all isolates can be defined as functional providing benefits on plants, because only a selection of them has the right features to be effective in biocontrol under certain conditions (McSpadden-Gardener 2004). For this reason, the identification of main characteristics that give to these isolates the skills to be good BCAs is very important in order to improve screening procedures. Thus, the screening may be oriented to obtain isolates presenting the specific characteristics, either obtaining strains active against a single pathogen or against a broad range of plant pathogens.

The selection of BCAs in the genus *Bacillus* has been focused in the species *B. subtilis* and closely related species like *B. amyloliquefaciens*. The importance of these species is mainly associated with two main factors, the first is the distribution of these species in the plant environments where they are predominant among *Bacillus* species (Arias *et al*. 1999) and the second the capacity to produce a wide variety of antimicrobial metabolites, including antimicrobial peptides (AMPs) (Montesinos 2007, Stein 2005), which inhibit growth of bacterial, fungal and nematode plant pathogens. The importance of the production of these metabolites is associated with the suppression of plant diseases, by means of the inhibition of pathogen growth, the induction of plant host defenses or the promotion of plant growth. Some examples are the production of bacillomycin D by the strain *B. subtilis* AU195 in the control of *Aspergillus flavus* (Moyne *et al*. 2001), bacillomycin and fengycin by *B. amyloliquefaciens* FZB42 in the control of *Fusarium oxysporum* (Koumoutsi *et al*. 2004), iturin A by *B. subtilis* QST713 in the control of *B. cinerea* and *Ralstonia solani* (Kloepper *et al*. 2004, Paulitz and Bélanger 2001), iturin A, bacillomycin and fengycin by *B. subtilis* UMAF6614 and UMAF6639 in the control of *Podosphaera fusca* (Romero *et*

al. 2007), or mycosubtilin by *B. subtilis* BBG100 in the control of *Pythium aphanidermatum* (Leclère *et al*. 2005). However, most of the studies on these metabolites have been focused on the inhibition of fungal pathogens, while scarce studies have been focused on the antibacterial properties of these metabolites (Chen *et al*. 2009c). This relies on the secondary metabolites most widely studied to date, the cyclic lipopeptides (cLPs), with proven antifungal activity. No conclusive evidence has been found of the antibacterial activity of iturin and fengycin families (Maget-Dana and Peypoux 1994, Koumoutsi *et al*. 2004), although some studies support the activity of the surfactin in the reduction of infections of *P. syringae* in *Arabidopsis* plants *in vitro* (Bais *et al*. 2004) or of iturin in the inhibition of infections caused by *Xanthomonas campestris* pv. cucurbitae and *Pectobacterium carotovorum* subsp. *carotovorum* in detached melon leaves (Zeriouh *et al*. 2011). Other active antimicrobial compounds, such as bacilysin, subtilin or difficidin, have been related with antibacterial activity. As examples, the dipeptide bacilysin and the poliketide difficidin have been related with the inhibition of *Erwinia amylovora,* the causal agent of fire blight (Chen *et al*. 2009c); or the lantibiotic subtilin has been involved in voltage-dependent multi-state pore formation in *Staphylococcus simulans* (Schüller *et al*. 1989).

CLPs have been the most well studied secondary metabolites in *Bacillus* such as gene clusters involved in their biosynthesis (Arguelles-Arias *et al*. 2009, Chen *et al*. 2009a, Kunst *et al*. 1997); the capacity of cLPs to inducing a global defense response in the plant known as induced systemic resistance (ISR) (Bais *et al*. 2004, Ongena *et al*. 2007, Zeriouh *et al*. 2011); and their structure and its relationship with the antimicrobial activity (Ongena and Jacques 2007, Stein 2005). In addition, the recent characterization of genomes of several *Bacillus* strains (Chen *et al*. 2007, Eppinger *et al*. 2011, Kunst *et al*. 1997, Rückert *et al*. 2011) have helped to increase the knowledge of genes involved in the synthesis and regulation of these compounds, including antimicrobial peptides. Additionally, the implication of in the plant health of cLPs and other AMPs such as bacteriocins and dipeptides, poliketides and other antimicrobial compounds in the plant health have been conduced to identify the related genes involved in their biosynthesis (Kunst *et al.* 1997*,* Koumoutsi *et al*. 2004).

The use of PCR methods to detect genes involved in the biosynthesis of these antimicrobial metabolites offer the possibility to screen large collections of strains obtained from a wide range of natural environments. In this way, several primers for PCR analysis have been reported for some antimicrobial biosynthetic genes in *Bacillus*. For example, the detection of fengycin and bacillomycin D biosynthetic genes among *Bacillus spp.* strains antagonistic to fungal pathogens of canola and wheat (Ramarathnam *et al*. 2007); of AMP biosynthetic genes of bacillomycin D, iturin A, surfactin, mycosubtilin, fengycin, and zwittermicin A over a collection of strains active against *S. sclerotiorum* (Athukorala *et al*. 2009); of iturin operon or *sfp* gene in a collection of *Bacillus* spp. natural isolates where most of them were active against a broad range of bacterial pathogens and especially against *Xanthomonas oryzae* pv. oryzae (Berić *et al*. 2012); or of five genes involved in cLP synthesis (*bmyB*, *fenD*, *ituC*, *srfAA* and *srfAB*) in a collection of strains isolated for the biocontrol of *Rhizoctonia solani* and *Pythium ultimum* (Joshi and McSpadden-Garddener 2006). Additionally, in other studies, the presence of some biosynthetic genes has been related with the production of cLPs, such as surfactin, iturin A and fengycin, and iron-siderophore bacillibactin, antibacterial polyketide macrolactin, bacillaene and difficidin, the dipeptide antibiotic bacilysin and the chlorinated derivate chlorotetaine (Arguelles-Arias *et al*. 2009).

The occurrence of cLPs mediated antibiosis has led to the suggestion that non-ribosomal peptide synthetases may be used as markers for identifying and selecting novel biocontrol agents from environmental samples (Giacomodonato *et al.* 2001). However, the recent discovery that multiple mechanisms of biological control and plant growth promotion can occur in some strains of *B. subtilis* indicates that such an approach would be feasible (McSpadden-Gardener 2004). Thus, the potential utility of the defined genetic markers to further define the biocontrol activities of *B. subtilis* must be evaluated.

OBJECTIVES

The aim of this work was the characterization of a large collection of *Bacillus* strains obtained from natural plant environments on basis of the presence of AMP biosynthetic genes, production of AMPs and antimicrobial activity against a broad range of bacterial and fungal plant pathogens.

Specific objectives were to:

- Build-up of a collection of plant-associated *Bacillus* from wide origin.
- Characterize isolates based on the presence of six antimicrobial peptide genes, the capacity to synthesize antimicrobial peptides, and their antimicrobial activity against fungal and bacterial plant pathogens.

MATERIALS AND METHODS

1. Strains and growth conditions

Bacterial and fungal plant pathogens were used as target pathogens in antimicrobial activity assays with the *Bacillus* isolates (Table 3.1). Plant pathogens were selected according to their importance in agriculture and to belong to different groups of interest. These strains were kindly provided by public research groups or obtained from culture collections.

Bacterial plant pathogens were cultured in LB agar at 24 ºC for 24 h. For long-term preservation, strains were stored at -80 ºC in LB broth containing 20 % glycerol. Fungal plant pathogens were cultured in potato dextrose agar (PDA) at room temperature for 7 days, except *P. cinnamomi* and *P. cactorum* that were grown during 14 days. For long-term preservation, fungal pathogens were stored in PDA agar at 4 ºC, with periodical subculture.

Table 3.1. Bacterial and fungal plant pathogens used in the present study as targets for antimicrobial activity studies.

Species	**Strain**	**Source[x]**	**Reference**
Rhizobium radiobacter	CECT472	CECT	--
Clavibacter michiganensis sbsp. michiganensis	CECT790	CECT	--
Erwinia amylovora	PMV6076	INRA	Barny *et al*. 1990
Pectobacterium carotovorum sbsp. carotovorum	CECT225	CECT	--
Pseudomonas syringae pv. syringae	EPS94	UdG	Moragrega *et al*. 1998
Ralstonia solanacearum	CECT125	CECT	--
Xanthomonas arboricola pv. fragariae	CFBP3549	CFBP	--
Xanthomonas axonopodis pv. vesicatoria	CFBP3275	CFBP	Ferre *et al*. 2006
Phytophthora cactorum	F490	PHL	Agustí *et al*. 2011
Botrytis cinerea	CECT2996	CECT	--
Pythium ultimum	CECT2364	CECT	--
Fusarium oxysporum f. sp. lycopersici	ATCC201829	ATCC	Badosa *et al*. 2009
Phytophthora cinnamomi	CECT2965	CECT	--
Penicillium expansum	EPS26	UdG	Badosa *et al*. 2009

[x]ATCC, American Type Culture Collection; CECT, Spanish Collection of Type Cultures; CFBP, French Collection of Plant Pathogenic Bacteria; INRA, Institut National de la Recherche Agronomique (France); PHL, Plant health laboratory, Huelva (Spain); UdG, University of Girona (Spain).

2. Collection of *Bacillus* isolates from plant environments

Bacillus isolates were obtained from 183 field samples including different plant environments (143 samples of the aerial plant part, 25 samples of the rhizosphere, 15 samples of bare soil), and 35 plant species (cultivated, wild-type), taken from seven sampling sites (mainly in Catalunya and Balearic Islands) in Spain (Table 3.2). Most samples were obtained from aerial plant parts because of the interest to obtain biocontrol agents that could work against aerial plant diseases.The bacterial microbiota was obtained from each sample after homogenization of 1g of material in 10 ml of phosphate buffer (pH 7, 0.02M Na_2HPO_4, 0.05M KH_2PO_4) using a paddles homogenizer (Masticator, IUL Instruments, UK) for 60s.

Plant extracts were mixed with equal volume of 20 % glycerol in LB broth, and stored at -80 ºC. To enhance recovery of strains, samples were processed using the selective enrichment procedure described in the previous chapter. Briefly, plant extracts were exposed to 80 ºC for 10 min and then diluted 1:100 in LB and enriched during 24 h at 40 ºC. Only enriched extracts showing positive response in PCR directed to *16S rDNA* of *Bacillus* were processed for the isolation of *Bacillus*. Thus, aliquots of 100 µl of the enriched samples were seeded onto LB agar plates and incubated at 28 ºC for 24-48 h. A maximum of three different colonies of the typical *Bacillus* morphology (Sneath 1986) were purified per sample. Then, isolates were identified by Gram stain, motility and presence of endospores.

Moreover, *Bacillus* isolates were also confirmed by PCR using the primers developed for the specific detection of *16S rDNA* from *Bacillus.* Pure cultures were stored at –80 ºC in 20 % glycerol in LB broth.

3. Characterization of *Bacillus* isolates

Bacillus isolates were characterized by the presence of AMP biosynthetic genes and their antimicrobial activity against a broad range of fungal and bacterial pathogens. In addition, production of AMPs among a selected group of isolates was determined once growth conditions were optimized

Table 3.2. Distribution and number of samples obtained from different locations in the Eastern Mediterranean area of Spain, and used for isolation of *Bacillus*.

Province	Zone	Latitude	Longitude	Sample type	Number of samples
Barcelona	Pacs del Penedès (Baix Penedès)	41º 21' N	1º 40' E	Aerial plant part	7
Girona	Canet de la Tallada (Baix Empordà)	42º 03' N	3º 04' E	Aerial plant part	12
	Estartit (Baix Empordà)	42º 03' N	3º 11' E	Aerial plant part	23
				Rhizosphere	13
				Soil	4
	Mas Badia (Baix Empordà)	42º 14' N	2º 51' E	Aerial plant part	4
	Torroella de Montgrí (Baix Empordà)	42º 02' N	3º 07' E	Aerial plant part	3
				Rhizosphere	4
				Soil	6
Lleida	Aigüestortes (Pallars Sobirà)	42º 34' N	0º 55' E	Rhizosphere	3
				Soil	3
	Les Borges Blanques (Les Garrigues)	41º 31' N	0º 52' E	Aerial plant part	6
	Montardit de Baix (Pallars sobirà)	42º 22' N	1º 06' E	Aerial plant part	10
	Montenartró (Pallars Sobirà)	42º 27' N	1º 13' E	Aerial plant part	4
				Soil	2
	Vallferrera (Pallars Sobirà)	42º 35' N	1º 19' E	Aerial plant part	1
Màlaga	Velez-Màlaga	36º 46' N	4º 06' W	Aerial plant part	3
Menorca	Binimel·la (Menorca)	40º 03' N	4º 03' E	Aerial plant part	8
	Cap de Cavalleria (Menorca)	40º 05' N	4º 08' E	Aerial plant part	11
				Rhizosphere	4
	Es Mercadal (Menorca)	39º 59' N	4º 05' E	Aerial plant part	10
Navarra	Navarra	42º 41' N	1º 40' W	Aerial plant part	4
Saragossa	Aldeanuaela de Ebro	42º 13' N	1º 53' W	Aerial plant part	1
	Alfamen	41º 36' N	1º 14' W	Aerial plant part	1
	Almunia de Doña Godina	41º 30' N	1º 24OW	Aerial plant part	4
Sevilla	Bollullo de la Mitación	37º 20' N	6º 08' W	Aerial plant part	2
Tarragona	Les Borges del Camp (Baix Camp)	41º 10' N	1º 01' E	Aerial plant part	20
				Rhizosphere	1
València	Pobla del Duc	38º 54' N	0º 25' W	Aerial plant part	1
	Riola	39º 11' N	0º 20' W	Aerial plant part	8

and suitable extraction, purification and identification protocols were established.

3.1 Analysis of biosynthetic AMP genes

A total of 184 isolates of *Bacillus* were characterized for the presence of antimicrobial peptide biosynthetic genes by PCR using the specific primers for *srfAA*, *bacA, bmyB, fenD, spaS* and *ituC*, described in Chapter 2 section 1.3.2, in order to determine the patterns of presence of AMP biosynthetic genes for each strain.

3.2 Analysis of AMPs produced by *Bacillus* strains

The relationship between the detection of 6 biosynthetic genes and the production of the metabolites associated to these genes was studied by screening the *Bacillus* collection and followed of a confirmation of the products.

3.2.1 Verification of antimicrobial activity in culture supernatants

In order to verify the implication of AMPs in the inhibition of different pathogens, activity of culture supernatants was compared with *Bacillus* cells. Moreover, techniques for the quantification of the activity of culture supernatants were optimized.

Comparison between activity of cells and culture supernatants

The antimicrobial activity of cells and supernatants of cultures of *B. amyloliquefaciens* EPS2059 was tested *in vitro* using the agar incorporation assay against eight bacterial plant pathogens (*E. amylovora* PMV6076, *P. syringae* p*v.* syringae EPS94, *X. arboricola* pv. fragariae CFBP3549, *X. axonopodis* pv. vesicatoria CFBP3275, *R. radiobacter* CECT472*, R. solanacearum* CECT125, *C. michiganensis* sbsp. *michiganensis* CECT790 and *P. carotovorum* sbsp*. carotovorum* CECT225); and also against four fungal plant pathogens (*P. cactorum* F490, *P. cinnamomi* CECT2965, *F. oxysporum* f. sp. *lycopersici* ATTC201829, and *P. expansum* EPS26).

The activity assay against bacterial indicators was performed in LB agar. Bacterial suspensions were adjusted to a final concentration of 10^8 cfu/ml using a standard curve that relates the cell concentration of each bacterial genus with the optical density. Agar overlays were prepared by mixing 4.5 ml of melted agar (7 ‰ at 45 ºC) and 0.5 ml of each bacterial pathogen (10^8 cfu/ml) and then were spread covering the surface of the agar plate. The assay against fungal indicators was performed in PDA. Conidia of *F. oxysporum* and *P. expansum* were obtained by scraping the culture surface with a wet cotton swab and suspending the material in distilled water. Concentration was adjusted with a hemocytometer to 10^6 conidia/ml. Agar overlays were prepared by mixing 4.5 ml of melted agar and 0.5 ml of each fungal suspension. In contrast, *P. cinnamomi* and *P. cactorum* strains were not tested using the agar incorporation test. Instead, agar-plugs (5x5 mm) of agar grown cultures were placed in the middle of PDA plates.

Bacillus strains grown overnight in LB agar plates at 28 ºC were picked in the surface of the agar overlay with toothpicks. Plates were incubated at 25 ºC and growth inhibition was assessed at 3-to-5 days depending on the bacterial pathogen and at 5-9 days depending on the fungal pathogen. Two independent replicates of each *Bacillus* isolate were done.

Bacillus culture supernatants were obtained from culture suspensions grown in LB broth medium at 28 ºC and 180 rpm in an orbital shaker incubator (COMECTA WY-200, Selecta SA, Barcelona, Spain) for 16 h, in the first trial, and 36 h in the second trial. After the incubation, cells were removed by centrifugation at 12000 rpm for 15 min and filtered with a cellulose acetate filter in a syringe (0.2 µm pore size, Whatman, GE Healthcare Bio-Sciences Corp.,Piscataway, USA) in order to remove any cells and spores in the sample that could interfere in activity assays. Then, 20 µl of filtered supernatant were deposited onto the surface of the agar plates. Finally, plates were incubated at 25 ºC and growth inhibition was assessed at 1-3 days depending on the bacterial pathogen and at 3-5 days depending on the fungal pathogen. Two independent replicates of each pathogen were done.

The intensity of inhibition was determined using the following scale according to the diameter of the inhibition halus: 0, no inhibition; 1, low inhibition (< 10 mm); 2, moderate inhibition (from 10 to < 20 mm), and 3, high inhibition (≥ 20 mm). In the agar-plug spread test, the intensity of inhibition was quantified using the same scale.

Assessment of antimicrobial activity of culture supernatants in liquid assay

In order to quantify more accurately the antimicrobial activity of supernatant cultures, an *in vitro* assay in liquid broth based on monitoring the growth of bacterial and fungal pathogens was performed. Four *Bacillus* strains culture supernatants (EPS2018, EPS2030, EPS2059 and EPS2077) were assayed against six bacterial pathogens (*E. amylovora* PMV6076, *P. syringae* p*v.* syringae EPS94, *X. arboricola* pv. fragariae CFBP3549, *R. radiobacter* CECT472*, R. solanacearum* CECT125 and *P. carotovorum* sbsp*. carotovorum* CECT225). *Bacillus* strain culture supernatants from strains (EPS2030, EPS2059 and EPS2135) were used against two fungal pathogens (*F. oxysporum* f. sp. lycopersici ATTC201829, and *P. expansum* EPS26).

To prevent growth of *Bacillus* spores or resting cells after filtration, spontaneous mutants of target bacteria resistant to rifampicin were obtained, except in the case of *E. amylovora* PMV6076 which was previously resistant to chloramphenicol (Barny *et al*. 1990). The selection was carried out as previously described for the selection of spontaneous mutants of *Bacillus* spp. in Chapter 2 section 2.2.1.

For the assay, *Bacillus* culture supernatants were obtained from grown cultures at 28 ºC for 48 h and 180 rpm in LB broth medium. Then, cells were removed by centrifugation at 12000 rpm for 15 min and filtrated with a cellulose acetate filter in a syringe (0.2 µm pore size) in order to remove any cells and spores.

The assay consisted of a 96-microwell plate containing per well 20 µl of each *Bacillus* culture supernatant, 160 µl of the corresponding medium (LB amended with the antibiotic for bacteria and potato dextrose broth (PDB)

for fungi) and 20 µl of the corresponding bacterial (10^8 cfu/ml) and fungal (10^6 conidia/ml) suspension in a total volume of 200 µl. Positive controls containing water instead of culture supernatant and negative controls containing culture supernatant without bacterial suspension were included. The assay was carried out in a spectral scanning multimode reader (Varioskan Flash, Labsystems, Helsinky, Finland) for bacterial pathogens and in a multiwell reader system (Bioscreen C, Labsystems, Helsinky, Finland) for fungal pathogens. Microplates were incubated at 25 ºC with moderate shaking for 24 h and 72 h for antibacterial and antifungal activity assays, respectively. Absorbance at 600 nm was recorded every hour during the incubation period. Three replicates for each plant pathogen and *Bacillus* culture supernatant were done. The assay was repeated twice. Growth inhibition was determined as the area under the growth curve (AUGC) in relation to control, and was calculated as previously described in Chapter 2 section 2.2.1.

3.2.2 Optimization of AMPs production

Production of AMPs is influenced by nutrients, temperature and time of incubation. For this reason, the best growth conditions of *Bacillus* strains were determined.

Effect of growth conditions in antimicrobial activity

In order to optimize the best growth conditions to improve AMPs production, strain EPS2059 was studied. Specifically, pH, cell concentration and percentage of sporulation were determined in cultures grown at 24, 28 and 37 ºC for 72 h in LB and Production Medium (PM) (Walker and Abraham 1970).

The *Bacillus* suspension was adjusted to 10^8 cfu/ml from cultures grown at 28 ºC for 24 h. Erlenmeyer flasks of 500 ml containing 100 ml of LB or PM medium were inoculated with 2 ml of the bacterial suspension. Cultures were incubated during 72h at 24, 28 and 37 ºC and 180 rpm in an orbital shaker incubator (Comecta WY-200, Selecta Group, Barcelona, Spain). Aliquots containing 6 ml of each culture were collected after 0, 24, 48 and

72 h of incubation. These aliquots were used to determine the parameters under study (pH, cell concentration and percentage of sporulation). For the pH determination, 50 µl of culture were deposited above a piece of universal indicator paper (Riedel-deHaën). Cell concentration was determined by spreading decimal dilutions in LB agar plates using an automatic spiral plater (Eddy Jet, IUL Instruments, United Kingdom).

Plates were incubated at 28 ºC and colonies were counted after 24 h using an automatic counting system (Countermat, IUL Instruments, United Kingdom). Finally, spores were determined using the same procedure used to determine cell density but previously exposing the cultures at 80 ºC for 10 min.

Finally, culture supernatants at different incubation times (24, 48 and 72 h) were obtained from 5 ml aliquots of grown cultures in each incubation condition, by centrifugation at 12000 rpm for 15 min and filtration through cellulose acetate syringe filters of 0.2 mm pore size. Culture supernatants were stored at -80 ºC and used in the antimicrobial activity assay.

Activity of supernatants

Antimicrobial activity of culture supernatants was determined using a growth inhibition assay in liquid culture. The activity was tested against the two bacterial plant pathogens, *E. amylovora* PMV6076 and *P. syringae* pv. syringae EPS94. As described in the previously Chapter 2, section 2.2.1, spontaneous mutants resistant to chloramphenicol, for *E. amylovora*, and to rifampicin, *P. syringae* pv. syringae, were used.

The assay was performed in 96 multiwell plates containing 20 µl of corresponding bacterial suspensions with 20 µl of *Bacillus* culture supernatant (EPS2059 strain grown in LB broth and PM during 0, 24, 48 and 72 h at 24, 28 and 37 ºC), and 160 µl of LB broth amended with the corresponding antibiotic (chloramphenicol 20 mg/ml for *E. amylovora* PMV6076, and rifampicin 50 mg/ml for *P. syringae* pv. syringae EPS94) into a total volume of 200 µl. Positive controls containing water instead of *Bacillus* culture supernatant, and negative controls containing water instead

of plant pathogen were included. The assay was carried out in an automatic optical density reader (Varioskan Flash). Microplates were incubated for 20 h at 25 ºC with 20 s of shaking before hourly absorbance measurement at 600 nm. Three replicates for each plant pathogen strain and *Bacillus* culture supernatant were done. The assay was repeated twice. Growth inhibition was determined using the AUGC in comparison to the control, and was calculated as previously described in Chapter 2 section 2.2.1.

3.2.3 Production and extraction of AMPs from culture supernatants

In order to identify the AMPs contained in the culture supernatants, extraction of the AMPs from medium components is required. Several protocols have been described including solid phase extraction, acid precipitation, ammonium sulphate precipitation or extraction by means of organic solvents like methanol or n-butanol. All these protocols, introducing some variations, were preliminary tested during the present work, though the best results were obtained using an organic phase separation with isoamyl alcohol (data not shown).

Supernatants obtained from strain EPS2059 grown in LB and PM were used to verify the performance of extraction process. Culture supernatants were obtained as previously described in previous sections. Briefly, 2 ml of *Bacillus* suspension adjusted to 10^8 cfu/ml were inoculated in 100 ml of LB broth medium contained in 500 ml Erlenmeyer flasks. Cultures were incubated at 28 ºC for 48 h and 180 rpm in an orbital shaker incubator. After 48 h of incubation, cell-free supernatants were obtained by centrifugation at 12000 rpm for 15 min and filtered by cellulose acetate syringe filters of 0.2 µm pore size. Isoamyl alcohol was added 50 % (v/v) to the supernatant and the mixture was gentle shacked. The organic phase was separated by centrifugation at 12000 rpm for 5 min at room temperature. Organic and aqueous phases were collected independently, and then the organic phase was evaporated under vacuum (Scan Speed 40 Teflon, AAPPTEC, LCC, Louisville, USA), while aqueous phase was lyophilized (Sharp Freeze-110, AAPPTEC, LCC, Louisville, USA). Dried concentrate aliquots were stored at -80 ºC.

The antimicrobial activity of organic and aqueous phases obtained from culture supernatants was determined qualitatively using the agar incorporation test and quantitatively using growth inhibition assay in liquid media based on growth kinetics of target pathogens.

The qualitative analysis of the antimicrobial activity against 6 bacterial pathogens (*E. amylovora* PMV6076, *P. syringae* pv. syringae EPS94, *Rhizobium radiobacter* CECT472, *Pectobacterium carotovorum sbsp. carotovorum* CECT225, *Xanthomonas axonopodis* pv. vesicatoria CFBP3275, *Ralstonia solanacearum* CECT125) was performed in LB using the agar incorporation test as previously described. Culture supernatants and the corresponding organic and aqueous phases of *Bacillus* strain EPS2059 grown in LB medium, all of them grown and processed as described in previous Chapter 2 (section 3.2.1), were diluted in sterile distilled water until the original concentration and deposited on the agar surface by applying a drop of 20 µl. Plates were incubated at 25 ºC and growth inhibition was assessed at 1-to-3 days. Two independent replicates of each plant pathogen were done. The capacity of inhibition was determined according to the presence or absence of inhibition halus.

The quantitative analysis of antimicrobial activity of supernatant cultures and the corresponding phases was determined by an *in vitro* assay in liquid broth based on monitoring the growth of bacterial pathogens. Assay was performed against *X. axonopodis* pv. vesicatoria CFBP3275 and *E. amylovora* PMV6076. The protocol used was the same as in section 3.2.1. Again, to avoid the hypothetically growth of possible *Bacillus* spores, spontaneous mutant of the pathogens resistant to antibiotics were used, specifically *X. axonopodis* pv. vesicatoria CFBP3275 resistant to rifampicin and *E. amylovora* PMV6076 resistant to chloramphenicol. The assay was carried out following the same procedure described in section 3.2.2 to determine activity of supernatants.

3.2.4 AMPs purification and partial identification

Antimicrobial peptides can be purified and identified by biochemical techniques. In this work, a combination of two techniques was used in order

to purify and identify components contained in the culture supernatants. Thus, crude culture supernatants and the corresponding organic and aqueous phases were analyzed by high-performance liquid chromatography (HPLC) and characteristic elution peaks were separated and collected by preparative HPLC. Components were identified according to their mass using two techniques of mass spectrometry, specifically matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF), that allows the analysis of biomolecules which tend to fragment when ionized on a solid matrix, and electrospray ionization mass spectrometry (ESI-MS) to analyze with high accuracy the composition of different chemical elements and isotopes separating the atomic nuclei according to their mass-charge ratio (m/z). To optimize analytical techniques two commercial standards were used: iturin A (1 mg/ml) and surfactin (10 mg/ml) (Sigma-Aldrich, USA). Once optimized each analytical technique, *B. amyloliquefaciens* EPS2059 culture supernatants and the corresponding organic and aqueous phases were analyzed. For the analysis, samples were dissolved in distilled water supplemented with trifluoroacetic acid (TFA) 0.085 % instead of water, until a final concentration tenfold concentrated respect the original extract.

Analytic HPLC

The analytical HPLC was performed on Agilent 1200 HPLC system (Agilent Technologies, Palo Alto, CA) equipped with a Multi Wavelength Detector, using a reverse-phase Kinetex XB-C18 column (column dimensions, 75 x 4.5 mm; particle size, 2.6 µm; pore size 100 A; Phenomenex, Madrid, Spain). The elution was monitored using a UV detector at 220 nm and the purity of each one was estimated with the integrated area under peaks. Samples were analyzed using KINETEX4 procedure with a flow rate of 1.85 ml/min using 0.1 % aqueous TFA as solvent A, and 0.085 % TFA in acetonitrile as solvent B. The gradient of solvent B (%) used in relation to time was: 0 min – 2 %, 18 min – 50 %, 22 min – 100 %, 25.5 min – 2 %.

Preparative HPLC

The equipment used was an Agilent Technologies 1200 Series equipped with a Multi Wavelength Detector, an autosampler and an automatic collector.

Purification was carried out with a Spherisorb ODS2 C-18 column (10×250 mm, 5 µm particle size), with a flow rate of 6 ml/min, using again 0.1 % aqueous TFA as solvent A, and 0.085 % TFA in acetonitrile as solvent B. With a gradient of solvent B (%) used in relation to time of: 0 min – 2 %, 4 min – 40 %, 20 min – 50 %, 34 min – 100 %, 37 min – 2 %. Peptide detection was performed at 220 nm and the peak-based collection was of maximum of 0.5 min.

Electrospray ionization-mass spectrometry (ESI-MS)

ESI-MS analyses were done in an Esquire 6000 ESI ion Trap LC/MS (Bruker Daltonics, Bruker corporation, Billerica, USA) equipped with an electrospray ion source at the Technical Research Services of the University of Girona. The instrument was operated either in the positive ESI(+) or negative ESI(-) ion mode. Samples of 5 µl, consisting in purified elution peaks were introduced into the mass spectrometer ion source directly through a HPLC autosampler. The mobile phase flow (100 µl/min of 80:20 v/v Acetonitrile/H_2O) was delivered by a 1200 Series HPLC pump (Agilent). Nitrogen was employed as both drying and nebulizing gas. Association of the substrate were often observed, the latter appeared from reactions with traces of cations present, even in HPLC-grade solvent.

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF)

Mass analysis was performed in Bruker Daltonics Ultraflex (Bruker Daltonics, Bruker corporation, Billerica, USA) apparatus consisting of a linear ion analyzer, a nitrogen laser and an electrostatic ion reflector at the Technical Research Services of the University of Girona. For this analysis, dried samples, consisting in purified elution peaks, were suspended in 70 % acetonitrile in water containing 0.1 % of trifluoroacetic acid (v/v). Then, 1 µl of each sample was mixed with 1 µl of matrix solution, which consisted of a saturated solution of a-cyano-4-hydroxycinnamic acid in 30 % aqueous acetonitrile containing 0.1 % TFA (v/v). 1 µl of the mixture was deposited in spots on a MALDI plate and dried with the help of air flow cabin. The acceleration and reflector voltages were 20-28.5 and 23-30 kV, respectively. Mass spectra were taken and recorded by using a nitrogen

laser (337 nm, repetition rate 20 Hz) for desorption and ionization (acquisition mass range: 500-3000 m/z, low mass gate: 200 m/z). All experiments were carried out with the reflector positive ion mode. Between 100 and 500 laser shots were accumulated for each mass spectrum. Data was analyzed with the software flexAnalysis v 2.0 (Bruker Daltonics).

3.2.5 Relative quantification of AMPs

In order to quantify the identified antimicrobial peptides produced by *Bacillus* strains, two commercial standards were used, iturin A and surfactin (Sigma-Aldrich, USA). Both lyophilized standards were suspended in 1 ml of methanol, in order to obtain a solution with a final concentration of 1 mM and 10 mM, respectively. From both initial solutions, serial dilution aliquots were performed to obtain different concentrations, ranging from 200 µM to 1 mM. All aliquots were independently analyzed three times by analytical HPLC, using the same procedure described in section 3.2.4, always using an injection volume of 20 µl. The relative area [mAU*s] and height [mAU] resulting from each peak was analyzed in order to obtain a standard curve to relate absorbance with the concentration of the corresponding standard.

3.2.6 Determination of AMPs production among *Bacillus* isolates

Antimicrobial peptide production in 64 representative strains out of 184 *Bacillus* isolated from field samples was determined using HPLC. HPLC analytic profiles were obtained according to the procedure described in section 3.2.4. Resulting chromatographic profiles were related with iturin, fengycin and surfactin peaks identified in strain EPS2059. In addition, relative production of the three families of cLPs was determined with the standard curves of the commercial standards of iturin A and surfactin.

3.3 Antimicrobial activity assays

The antimicrobial activity ofthe 184 *Bacillus* isolates was assayed *in vitro* using the agar incorporation test against eight bacterial and six fungal plant pathogens (Table 3.1).

The activity assay against the eight bacterial indicators was performed in LB and nutrient agar (NA) as previously described in the section 3.2.1. In both cases, for the bacterial and fungal assays, plates were incubated at 25 ºC and growth inhibition was assessed at 3-to-5 days depending on the bacterial pathogen and at 5-9 days depending on the fungal pathogen. Three independent replicates of each *Bacillus* isolate were done.

The intensity of inhibition was determined using the following scale according to the diameter of the inhibition halus: 0, no inhibition; 1, low inhibition (< 10 mm); 2, moderate inhibition (from 10 to < 20 mm), and 3, high inhibition (≥ 20 mm). In the agar-plug spread test (as described in the section 3.2.1), the intensity of inhibition was quantified using the same scale determined by halus diameter surrounded the *Bacillus* colony. A global activity index (GAI) for each *Bacillus* strain was calculated by computing the sum of the scores for the eight plant pathogenic bacteria or for the six plant pathogenic fungi. The maximum value of GAI was 24 for the plant pathogenic bacteria and 15 for the plant pathogenic fungi.

Results

1. Build-up of a collection of *Bacillus* isolates from plant environments

A total of 183 samples from the Mediterranean eastern land area, especially from Catalonia and Balearic Islands, were processed using the selective enrichment process assisted by PCR. A total of 33 samples were discarded due to be negative for *16S rDNA* gene of *Bacillus* spp. (Fig. 3.1). Most of these negative samples were obtained from aerial plant parts, only one sample was obtained from rhizosphere and none from soil. Thus, *Bacillus* was not detected in 22.3 % of the aerial plant part, while it was detected in most of the samples from rhizosphere (96 %) and soil (100 %). Additionally, *Bacillus* isolates were not obtained from 16 positive samples for *16S rDNA*, which were obtained from aerial plant parts, except oneobtained from the rhizosphere.

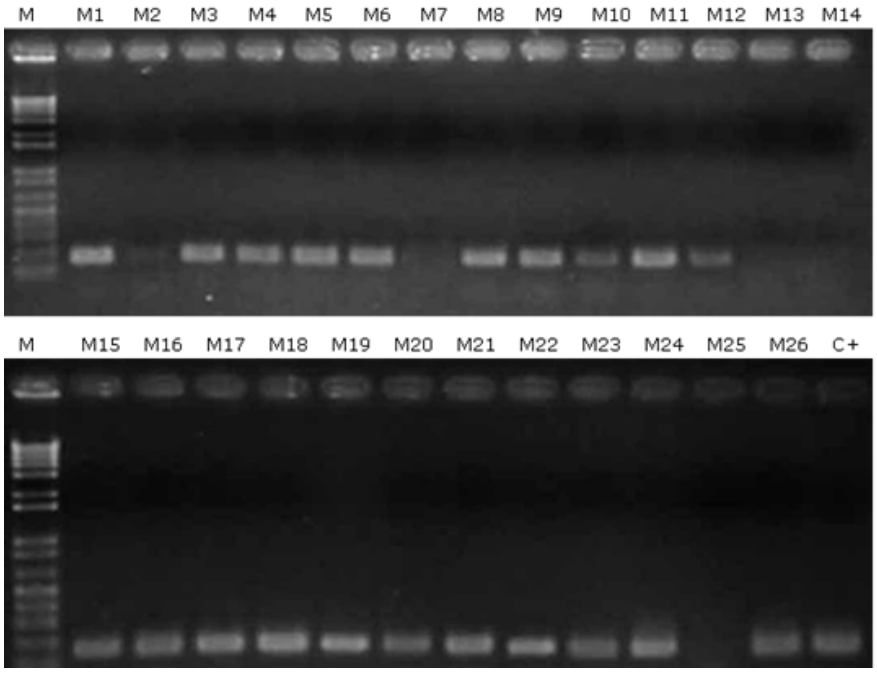

Figure 3.1. PCR analysis of *16S rDNA* gene *(*~163 bp) in field sample extracts. Internal control with *Bacillus* strain QST713 (C+). M, 1Kb Plus Ladder (Invitrogen).

At the end of the screening process, 184 putative *Bacillus* isolates (based on colony morphology) were obtained from 132 samples that gave a positive signal for *Bacillus 16S rDNA* gene (Fig. 3.2-A). For each sample only colonies with different morphology were selected to avoid siblings. Then, isolates in pure culture were characterized using Gram stain, endospore production and chain formation by examining wet mounts with phase-contrast microscopy (Fig. 3.2-B).

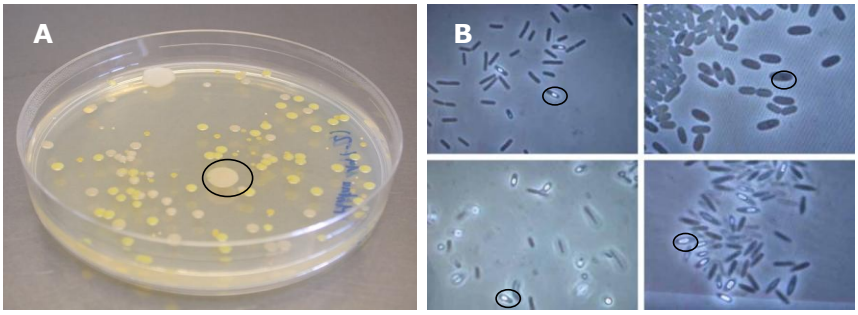

Figure 3.2. Typical *Bacillus* spp. colony morphology in LB agar (A). Detail of endospores (B).

Finally, the 184 isolates were reconfirmed as *Bacillus* using the primers for *16S rDNA* genes designed for specific detection of *Bacillus* (Fig.3.3). It was observed that they were more frequently isolated from rhizosphere (average of 1.72 isolates per sample) and soil (average of 1.93 isolates per sample) than from aerial plant parts (average of 0.78 isolates per sample) (Fig. 3.4).

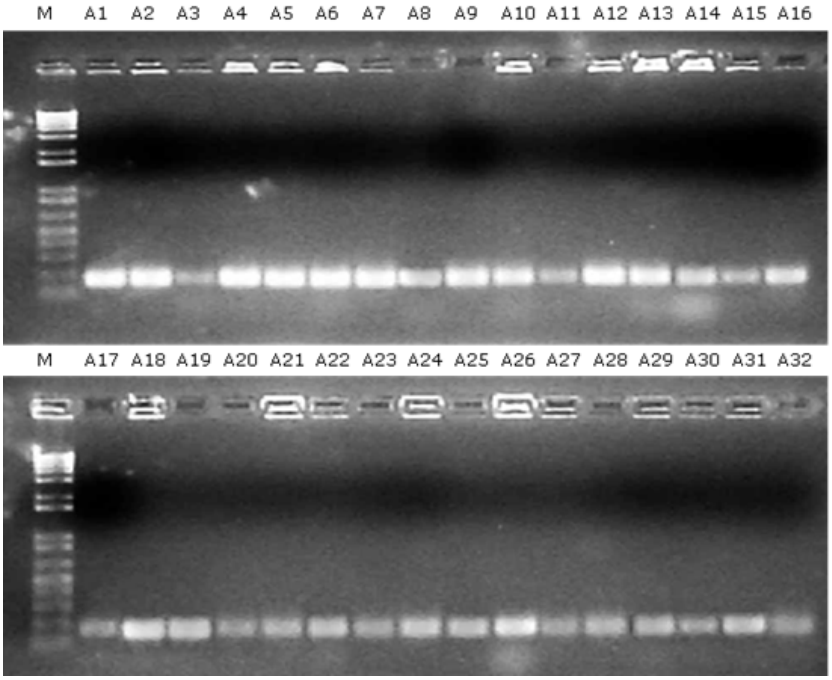

Figure 3.3. PCR analysis of *16S rDNA* gene *(*~163 bp*)* of *Bacillus* isolates A1 to A32. M, 1Kb Plus Ladder (Invitrogen).

2. Characterization of *Bacillus* isolates

2.1 Analysis of AMPs biosynthetic gene patterns

The analysis of the presence of AMP biosynthetic genes within the collection of 184 *Bacillus* isolates indicated a prevalence of AMP gene markers within the *Bacillus* population (Fig. 3.5). The distribution of the number of genes per strain and the frequency of each gene within the population are shown in Figure 3.6.

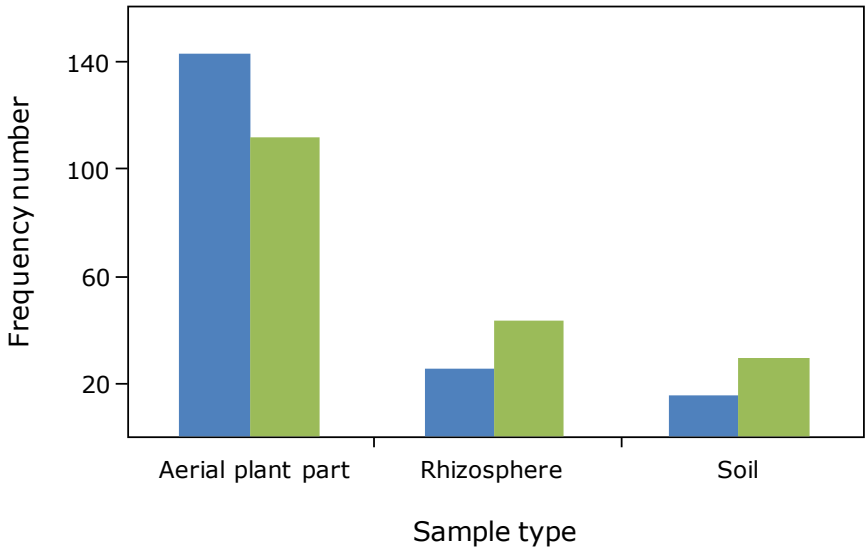

Figure 3.4. Number of field samples (■), and total *Bacillus* isolates (■) obtained in the survey depending on the type of field sample.

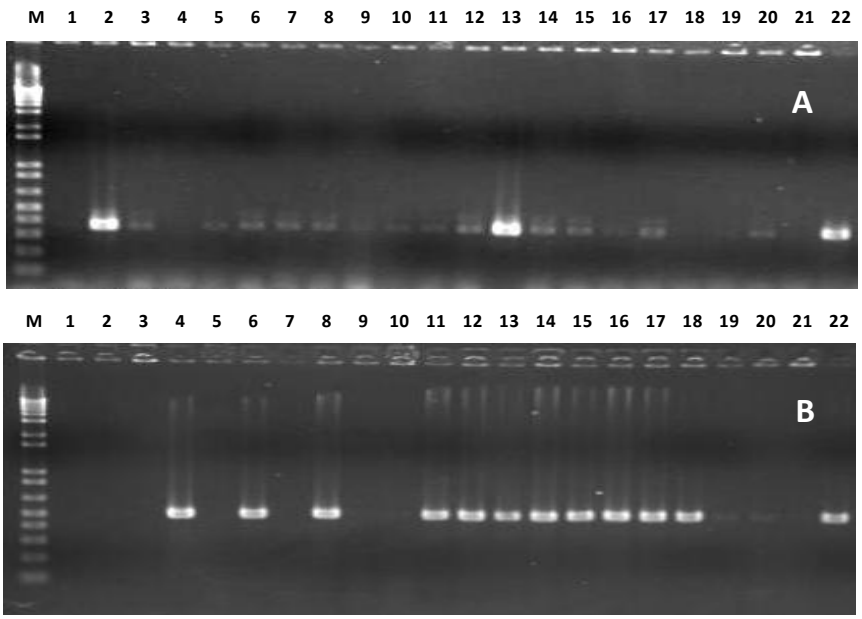

Figure 3.5. PCR analysis of *bmyB* (~370 bp) *(A) and bacA* gene (~498 bp) *(B)* of *Bacillus* isolates from field extracts, from A101 to A121 (1-21). Internal control with *Bacillus* strain QST713 (22). M, 1Kb Plus Ladder (Invitrogen).

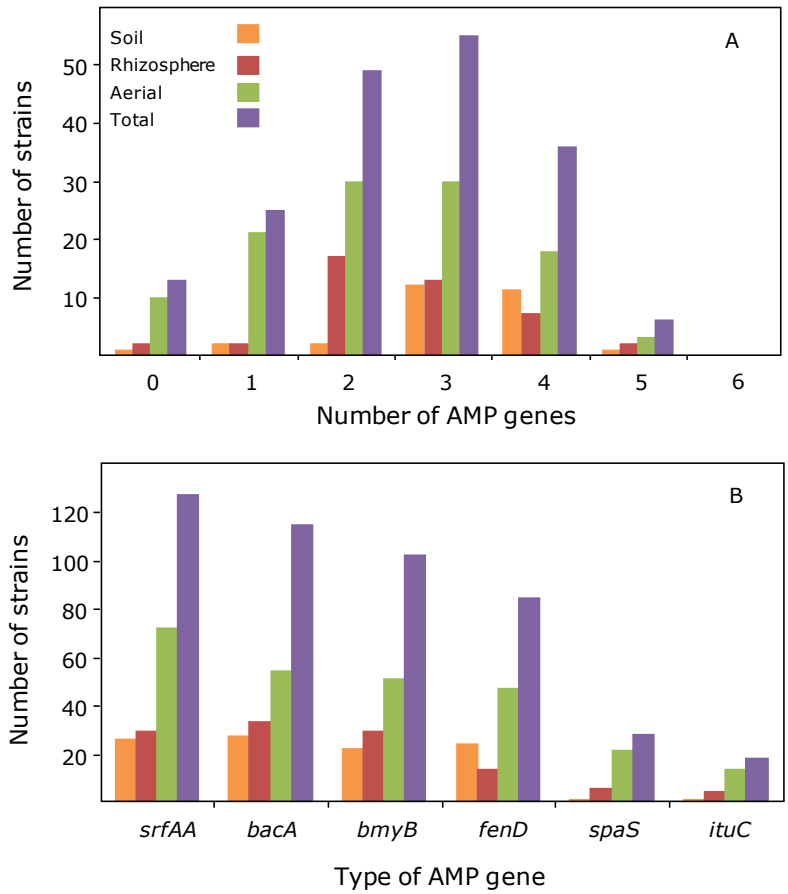

Figure 3.6. Frequency distribution of the number (A) and type (B) of antimicrobial peptide biosynthetic genes in 184 of *Bacillus* isolates from field samples. Data are presented in separate by sample origin (soil, rhizosphere, aerial plant part or combined).

The number of simultaneous genes per isolate followed a normal distribution with a tendency to have 2-3 genes simultaneously (Fig. 3.6-A). Most isolates had at least one of the biosynthetic genes (171 isolates, 92.9 %) and most of the isolates had 2-to-4 simultaneous genes (76.1 %). The presence of five genes simultaneously was rare (only six strains) and none of the isolates had six genes. Moreover, only 12 isolates had none of these genes. The distribution of the number of genes was similar between aerial and rhizosphere samples, but differed with soil isolates where the numbers of genes tended to be higher (3-to-4).

The most frequent genes observed were *srfAA* (69.0 %), *bacA* (61.4 %) and *bmyB* (55.4 %), followed by *fenD* (40.2 %). The genes *spaS* (15.2 %) and *ituC* (9.8 %) were the least frequent (Fig. 3.6-B). In addition, the frequency of the different gene patterns of AMP gene markers among isolates was studied (Fig. 3.7). The most frequent genotypes were *srfAA-bacA-bmyB* (15.2 %) and *srfAA-bacA-bmyB-fenD* (14.1 %). Isolates containing at least *srfAA*, *srfAA-bacA* or *srfAA-bacA-bmyB* accounted for 69, 46.7 and 34.8 %, respectively.

Finally, the frequency of detection of each AMP biosynthetic gene was studied depending on the type of sample (Fig. 3.8). The frequency of detection of *srfAA*, *bacA* and *bmyB* genes in soil samples was higher than in rhizosphere and aerial plant part samples. On the contrary, genes *spaS* and *ituC* were mainly detected in aerial plant parts. *fenD* gene was detected in a similar proportion among the three types of natural samples.

2.2 Analysis of AMPs produced by *Bacillus* strains

2.2.1 Verification of antimicrobial activity in culture supernatants

Comparative activity of cells against culture supernatants

B. amyloliquefaciens EPS2059 was used as a model for antimicrobial activity against eight bacterial plant pathogens (*C. michiganensis* sbsp. *michiganensis* CECT790, *E. amylovora* PMV6076, *P. cartovorum sbsp*. *carotovorum* CECT225, *P. syringae* pv. syringae EPS94, *R. solanacearum* CECT125, *R. radiobacter* CECT472, *X. arboricola* pv. fragariae CFBP3549, and *X. axonopodis* pv. vesicatoria CFBP3275) and four fungal plant pathogens (*P. cactorum* F490, *P. cinnamomi* CECT2965, *P. expansum* EPS26, and *F. oxysporum* f. sp. *lycopersici* ATTC201829). Antimicrobial activity against bacterial plant pathogens was analyzed in LB agar after 24, 48 and 72 h of incubation at 25 ºC (Fig. 3.9). Bacterial and culture supernatants of strain EPS2059 showed antagonism after 24 h of incubation (Table. 3.3).

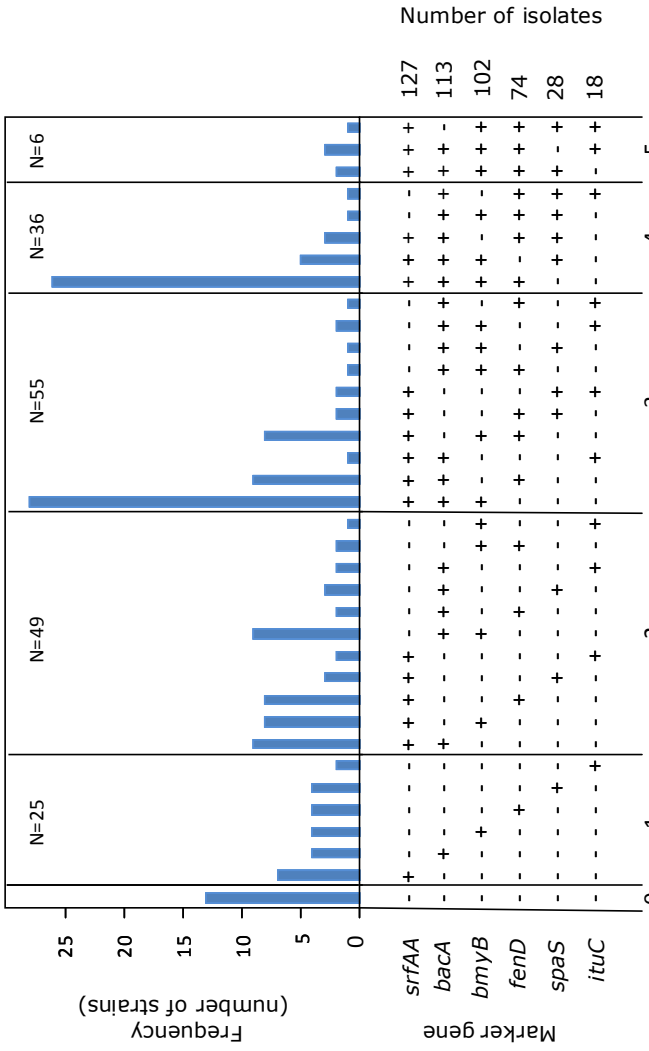

Figure 3.7. Frequency distribution of patterns of antimicrobial peptide biosynthetic gene markers in 184 strains of *Bacillus* spp. isolated from field samples. The number of strains (N) within each group of simultaneous number of gene markers is indicated in the upper part of the panels. Number of total isolates showing each gene was represented in the right part of the panel. The presence of antimicrobial peptide biosynthetic gene markers is also indicated: +, presence; -, absence.

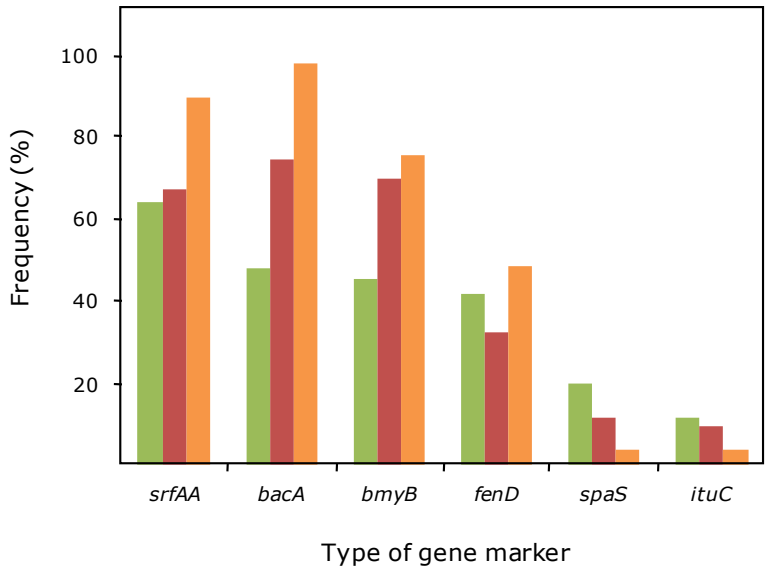

Figure 3.8. Frequencies of each biosynthetic gene for six AMP genes (*srfAA*, *bacA*, *bmyB*, *fenD*, *spaS* and *ituC*) among strains in function of the type of sample (aerial plant part (■), rhizosphere(■), or soil (■)).

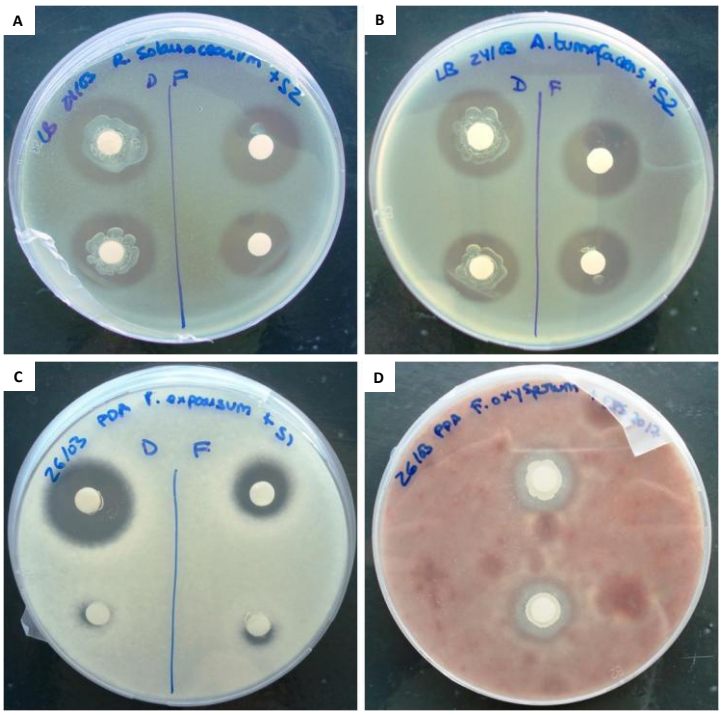

Figure 3.9. Antimicrobial activity of *B. amyloliquefaciens* EPS2059 in LB agar against *R. solanacearum* CECT125 (A), *R. radiobacter* CECT472 (B); and in PDA against *P. expansum* EPS26 (C), and *F. oxysporum* f. *sbsp. lycopersici* ATTC201829 (D).

Table 3.3. Antimicrobial activity of *B. amyloliquefaciens* EPS2059 cells and culture supernatants against 8 bacterial and 4 fungal plant pathogens.

	Phytopathogens	EPS2059[a]		
		Crude culture activity	Supernatant activity	
			First trial[b]	Second trial[c]
Bacterial	*E. amylovora* PMV6076	3	1	2
	P. carotovorum sbsp. carotovorum CECT225	2	nt[d]	2
	P. syringae pv. syringae EPS94	3	1	2
	X. axonopodis pv. vesicatoria CFBP3275	3	nt[d]	3
	X. arboricola pv. fragariae CFBP3549	2	1	3
	R. solanacearum CECT125	2	0	2
	C. michiganensis sbsp. michiganensis CECT790	2	2	3
	R. radiobater CECT472	2	1	2
Fungal	*P. cinnamomi* CECT2965	1	nt[d]	2
	P. cactorum F490	2	nt[d]	3
	F. oxysporum f. sp. lycopersici ATCC201829	0	nt[d]	2
	P. expansum EPS26	3	nt[d]	2

[a]Activity index according to the diameter of the inhibition halus: 0, no inhibition; 1, low inhibition (< 10 mm); 2, moderate inhibition (from 10 to < 20 mm), and 3, high inhibition (≥ 20 mm)

[b]Trial performed with *Bacillus* supernatant from grown cultures for 16h.

[c]Trial performed with *Bacillus* supernatant from grown cultures for 36h.

[d]nt, Not tested.

In the case of bacterial plant pathogens *X. arboricola* pv. fragariae CFBP3549, *X. axonopodis* pv. vesicatoria CFBP3275 and *C. michiganensis* sbsp. *michiganensis* CECT790, their susceptibility was analyzed after 48 h of incubation due to the slower growth.

Antimicrobial activity of *B. amyloliquefaciens* EPS2059 showed a mean activity index value around 2 (inhibition halus from 10 to <20mm). In contrast, to crude cultures values obtained for supernatants tend to be lower in the first trial but equal in the second trial. Supernatants obtained after 16 h of incubation resulted less active than supernatants obtained after 36 h of incubation in the same medium and temperature (Table 3.3).

Several culture supernatants were tested for their antimicrobial activity with the broth assay against six bacterial and two fungal plant pathogens, using growth kinetics of plant pathogens (Fig. 3.10). AUGC was obtained from cultures grown in LB broth for 20 h of incubation at 25 ºC (Fig 3.11), showing differences between *Bacillus* strains. Thus, most active supernatants showed strong growth inhibition values respect to the non treated control, while in other cases, like for supernatant from strain EPS2030, the inhibition was very low.

Results obtained in both trials were similar except in some specific treatments, especially in treatments showing low or moderate activity. Supernatants from strain EPS2059 were the most active against the six bacterial pathogens tested, whereas supernatants from strain EPS2030 were the less active. In all cases, the less sensitive bacterial plant pathogen was *P. syringae* pv. syringae EPS94, while the most sensitive bacteria were *R. radiobacter*, *R. solanacearum* and *X. arboricola* pv. fragariae.

In relation with antifungal activity culture supernatants showed antagonism after 72 h of incubation against both fungal plant pathogens, *F. oxysporum* f. *sbsp. lycopersici* ATTC201829 and *P. expansum* EPS26 (Fig. 3.12). While supernatants of strains EPS2059 were the most effective, EPS2030 and EPS2135 culture supernatants showed a very low effect above 8 and 2 % (Fig. 3.13).

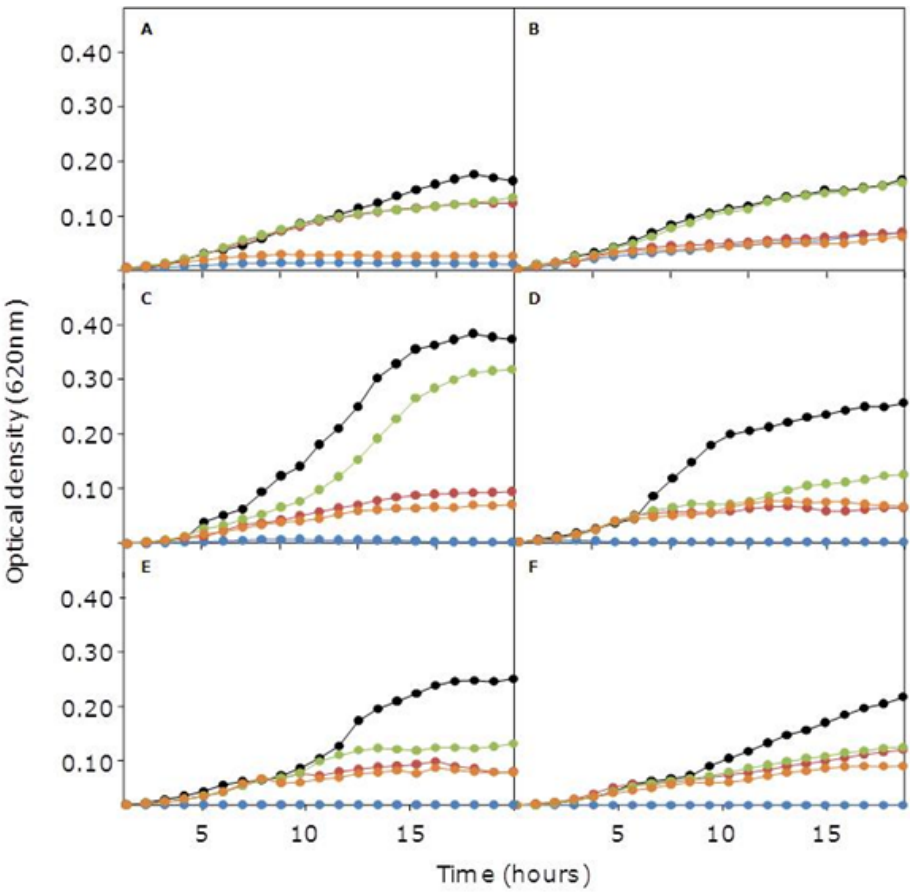

Figure 3.10. Growth kinetics of six bacterial plant pathogens after 20 h of culture in LB at 25 ºC, with the addition of different *Bacillus* culture supernatants. Untreated (●),EPS2018 (●), EPS2030 (●), EPS2059 (●) and EPS2077 (●). Bacterial plant pathogens tested were: *E. amylovora* PMV6076 (A), *P. syringae* pv. syringae EPS94 (B), *P. carotovorum sbsp*. *carotovorum* CECT225 (C), *R. radiobacter* CECT472 (D), *R. solanacearum* CECT125 (E), and *X. arboricola* pv. fragariae CFBP3549 (F).

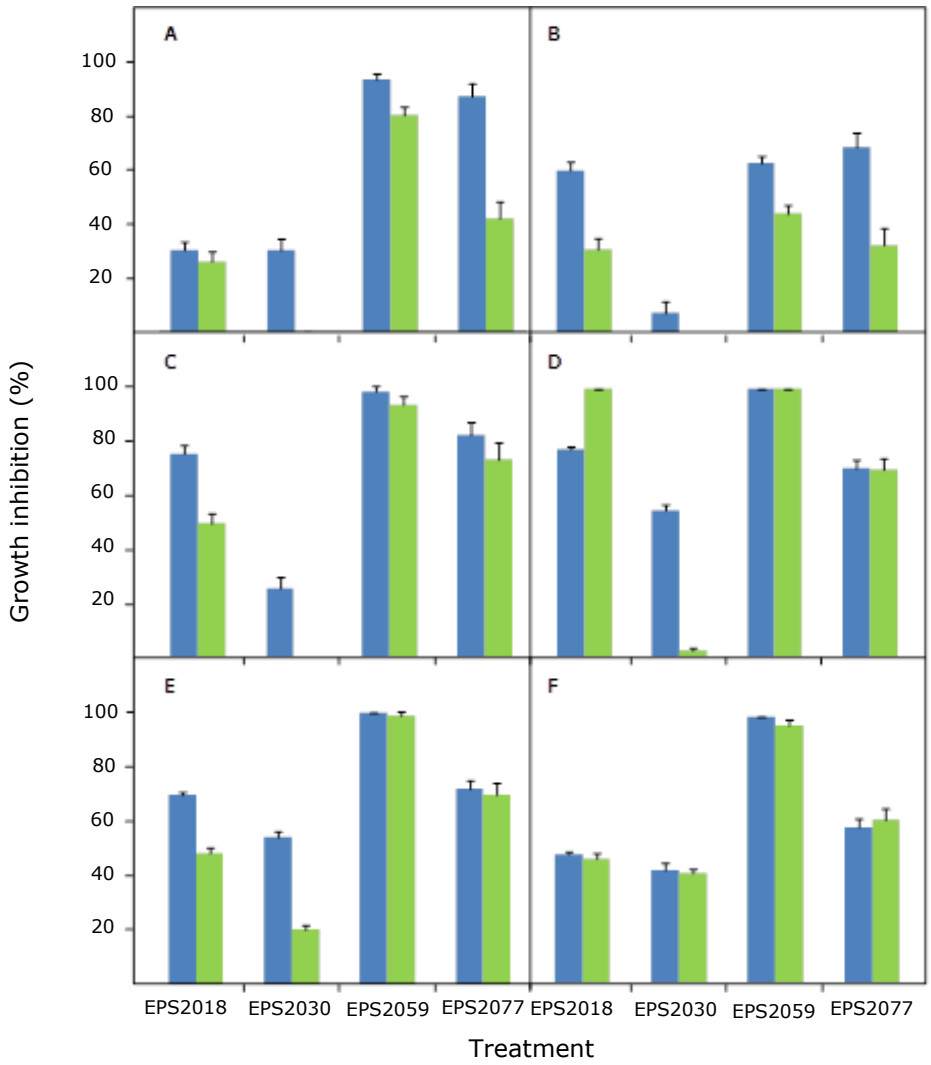

Figure 3.11. Growth inhibition of different bacterial plant pathogens, after 20 h of culture in LB at 25 ºC, with the addition of different *Bacillus* culture supernatants (EPS2018, EPS2030, EPS2059 and EPS2077), in two different trials: First trial (■), second trial (■). Bacterial plant pathogens tested were: *E. amylovora* PMV6076 (A), *P. syringae* pv. syringae EPS94 (B), *P. carotovorum sbsp*. *carotovorum* CECT225 (C), *R. radiobacter* CECT472 (D), *R. solanacearum* CECT125 (E), and *X. arboricola* pv. fragariae CFBP3549 (F). Growth inhibition was calculated on basis of the AUGC, respect the non-treated control.

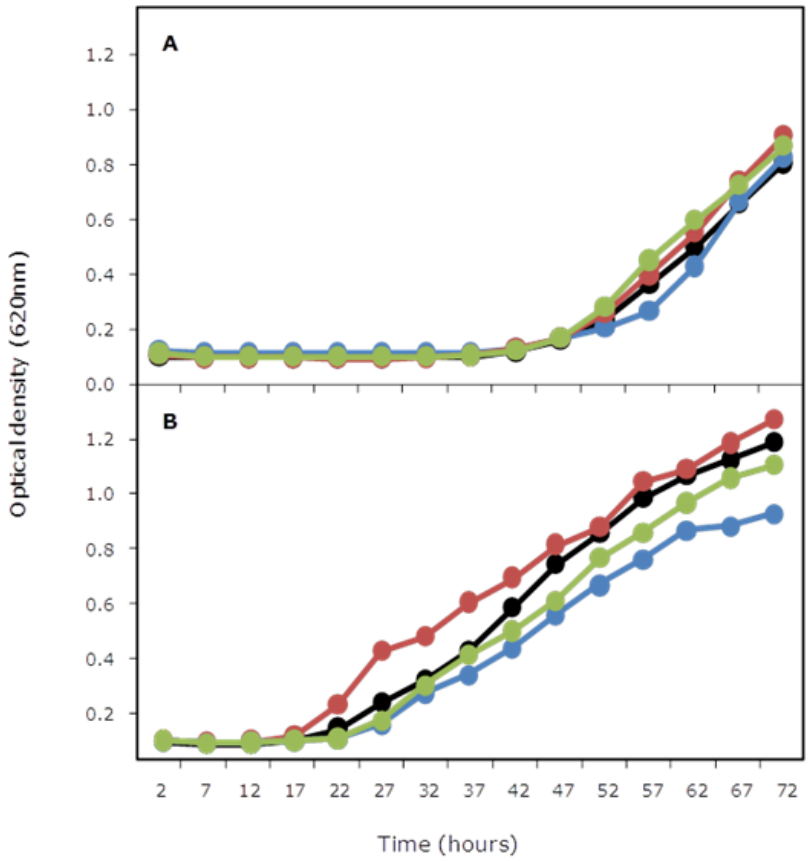

Figure 3.12. Growth kinetics of two fungal plant pathogens after 72 h of culture in PDB at 25 ºC, with the addition of different *Bacillus* supernatant cultures untreated (●), EPS2030 (●), EPS2059 (●) and EPS2135 (●). Fungal plant pathogens tested were: *F. oxysporum f. sbsp. lycopersici ATCC201829* (A) and *P. expansum* EPS26 (B).

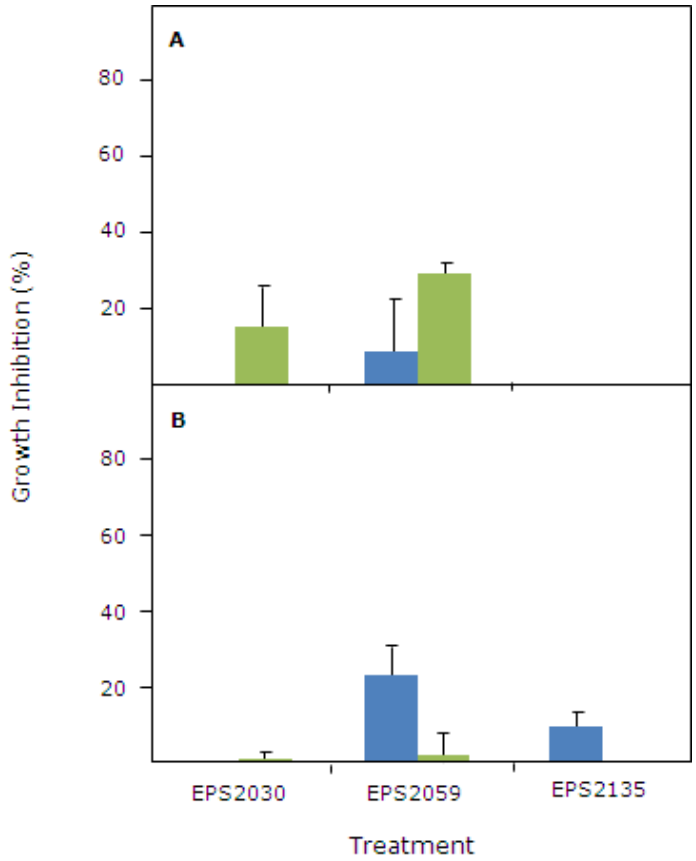

Figure 3.13. Growth inhibition of two fungal plant pathogens, after 72 h of culture in PDB at 25 ºC, with the addition of different *Bacillus* supernatant cultures (EPS2030, EPS2059 and EPS2135), in two different trials: First trial (■), second trial (■). Fungal plant pathogens tested were: *F. oxysporum f. sbsp. lycopersici ATCC201829* (A) and *P. expansum* EPS26 (B). Growth inhibition was calculated repect the non-treated control in AUGC.

2.2.2. Optimization of AMPs production

Cell concentration, pH and percentage of sporulation were analyzed in *B. amyloliquefaciens* EPS2059 cultures, after 0, 24, 48 and 72 h of growth at different temperatures (24, 28 and 37 ºC) using two kind of broth medium (LB and PM) (Fig. 3.14). According to the results, cell concentration, pH and percentage of sporulation were higher using LB medium and achieve better results earlier at high temperatures.

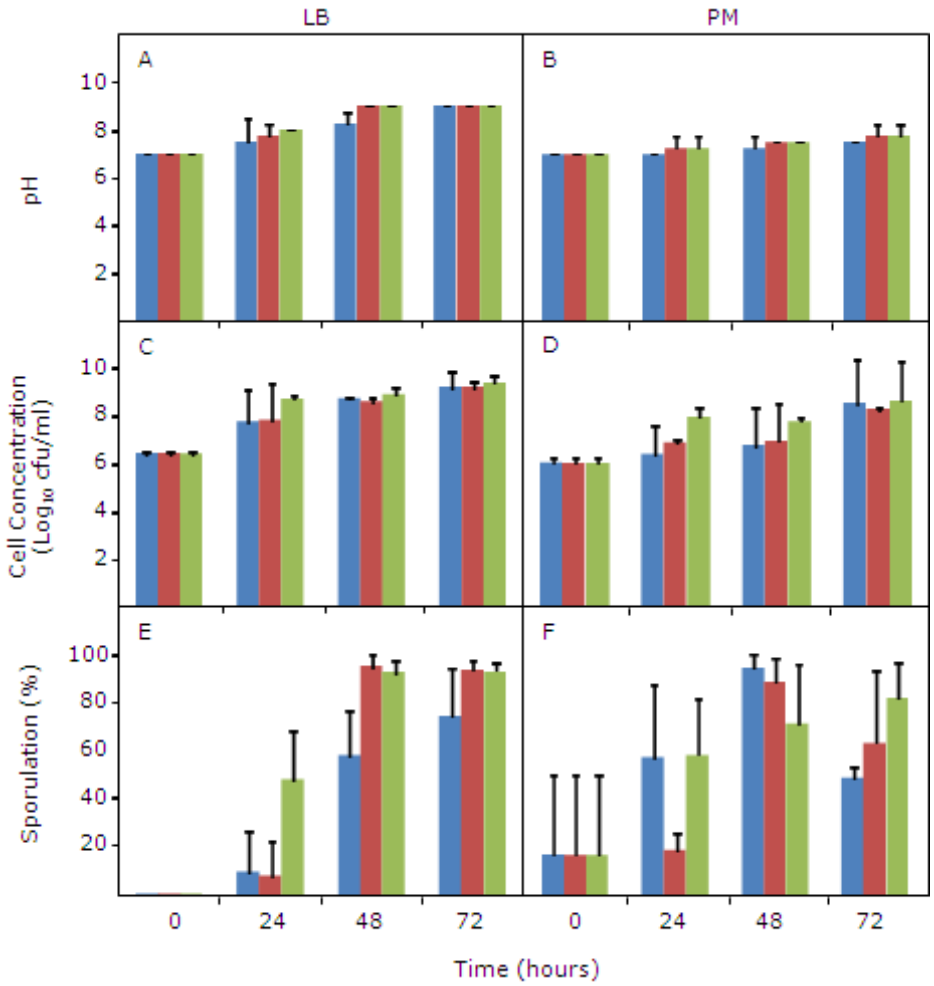

Figure 3.14. Evolution of pH, cell concentration and sporulation in EPS2059 cultures in LB broth and PM media after 0, 24, 48 and 72 h of incubation at 24 (■), 28 (■) and 37 ºC (■).

Specifically, initial pH was 7 independently of the medium used, and it increased over time in all cases. A higher alkalinization of grown culture using LB broth was observed, increasing at higher temperatures during the first 24 h. Maximum pH value using LB broth medium was 9, while pH values were above 8 using PM medium. Cell concentration also increased, from 10^5 cfu/ml to values above 10^9 cfu/ml after 72 h of incubation. Using LB broth medium, a higher increase of cell concentration levels were obtained at high temperatures of incubation. Finally, percentage of sporulation increased up to 100 % of sporulation in 48 and 72 h for cultures in LB broth and high temperatures allowed achieving the highest

percentage of sporulation earlier. On the contrary, values around 100 % of percentage of sporulation using PM culture medium in most cases were not observed.

Antimicrobial activity of culture supernatants to *E. amylovora* PMV6076 and *P. syringae* pv. syringae EPS94 was dependent on the culture medium, temperature and time of incubation. A different behavior of the activity of culture supernatants were observed between the two plant pathogen bacteria tested. *P. syringae* pv. syringae EPS94 was less sensitive to antimicrobial metabolites produced by *Bacillus* strains than *E. amylovora* PMV6076 (Fig. 3.15).

Culture supernatants obtained using PM medium were more active against *E. amylovora* PMV6076 than culture supernatants obtained using LB broth medium. Thus, growth inhibition produced by supernatants obtained from cultures grown in PM was around 100 % independently of time and temperature of incubation, except in the case of supernatants from cultures grown at 37 ºC that showed lower antibacterial activity than supernatants grown at 24 and 28 ºC. Antimicrobial components produced during culture of *Bacillus* in PM remained active up to 72 h of incubation at 24 and 28 ºC, and decreased slightly at 37 ºC over time.

In contrast, activity of supernatants obtained in LB showed differences in time and temperature of incubation, with the best activity in cultures grown for 48 h at 28 ºC. In the case of *P. syringae* pv. syringae EPS94, activity values of supernatant cultures obtained using PM and LB broth showed slightly differences (Fig. 3.15), although the activity values in PM medium were higher. Unlike the previous test, antimicrobial components produced during culture in both culture media remained active up to 72 h of incubation at 24, 28 and 37 ºC, and globally, antibacterial activity was improved by increasing the incubation temperature. The best activity levels were observed in cultures grown for 48 h at 28 ºC, and levels of growth inhibition were slightly lower against *P. syringae* pv. syringae than *E. amylovora*.

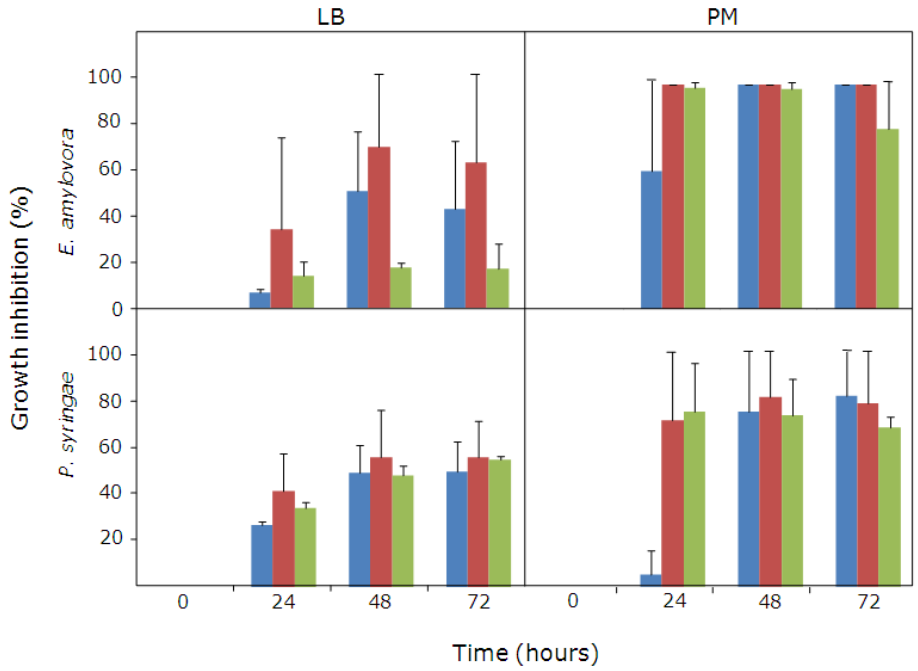

Figure 3.15. Growth inhibition of *E. amylovora* PMV6076 and *P. syringae* pv. syringae EPS94 after 20 h growth in LB broth supplemented with supernatants of EPS2059 obtained after 0, 24, 48 and 72 hours of incubation at 24 (■), 28 (■) and 37ºC (■) in LB and PM broth. Growth inhibition was calculated on basis of the AUGC. Values are the mean of three independent assays.

2.2.3 Production, extraction and partial identification of AMPs from culture supernatants

Organic fractions of *Bacillus* culture supernatants exhibited a higher inhibitory activity in comparison to aqueous fractions. Even, no activity was observed in the aqueous fraction against any of the pathogens tested (Fig. 3.16). The degree of activity of *Bacillus* extracts was dependent on target pathogen species. Thus, *E. amylovora* PMV6076 and *P. syringae* pv. syringae EPS94 were the less sensitive strains, while *X. axonopodis* pv. vesicatoria CFBP3275 and *R. solanacearum* CECT125 were the most sensitive strains. No activity was detected in controls of dried organic and aqueous phases obtained from non-inoculated fresh sterile LB broth.

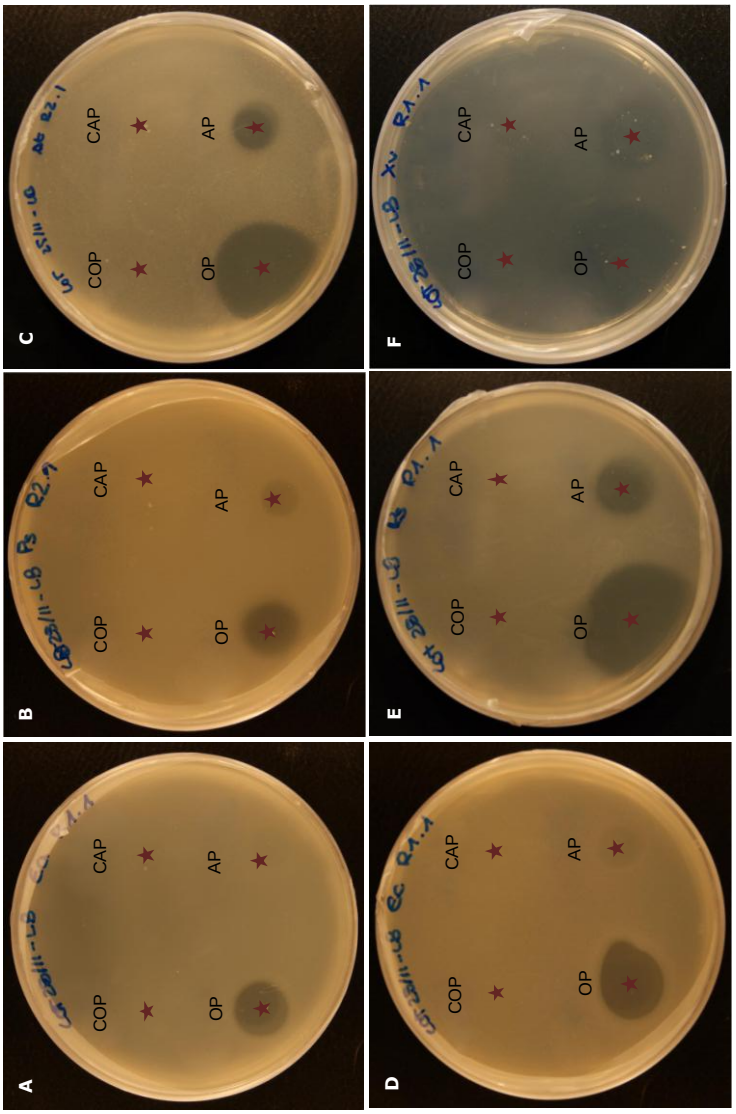

Figure 3.16. Antimicrobial activity of organic (OP) and aqueous (AP) phases obtained from *B. amyloliquefaciens* EPS2059 culture supernatants against *E. amylovora* PMV6076 (A), *P. syringae* pv. syringae EPS94(B), *R. radiobacter* CECT472 (C), *P. carotovorum sbsp. carotovorum* CECT225(D), *R. solanacearum* CECT125 (E) and *X. axonopodis* pv. vesicatoria CFBP3275 (F). Controls of phase separation procedure using organic (COP) and aqueous (CAP) phases from LB broth.

Quantitative analysis of the antimicrobial activity of supernatant cultures and the corresponding organic and aqueous phases against *E. amylovora* PMV6076 and *X. axonopodis* pv. vesicatoria CFBP3275 was also assayed with kinetic growth curves in liquid medium (Fig. 3.17). Growth inhibition of organic phase extracts maintained the same activity than crude culture supernatants, while the aqueous phase lost most of the activity. Again, growth inhibition values were dependent on the plant pathogen. Growth inhibition of *E. amylovora* PMV6076 was slightly higher than of *X. axonopodis* pv. vesicatoria CFBP3275 using culture supernatants and organic phase extract. While growth inhibition of *E. amylovora* PMV6076 was lower than of *X. axonopodis* pv. vesicatoria CFBP3275 using aqueous phase.

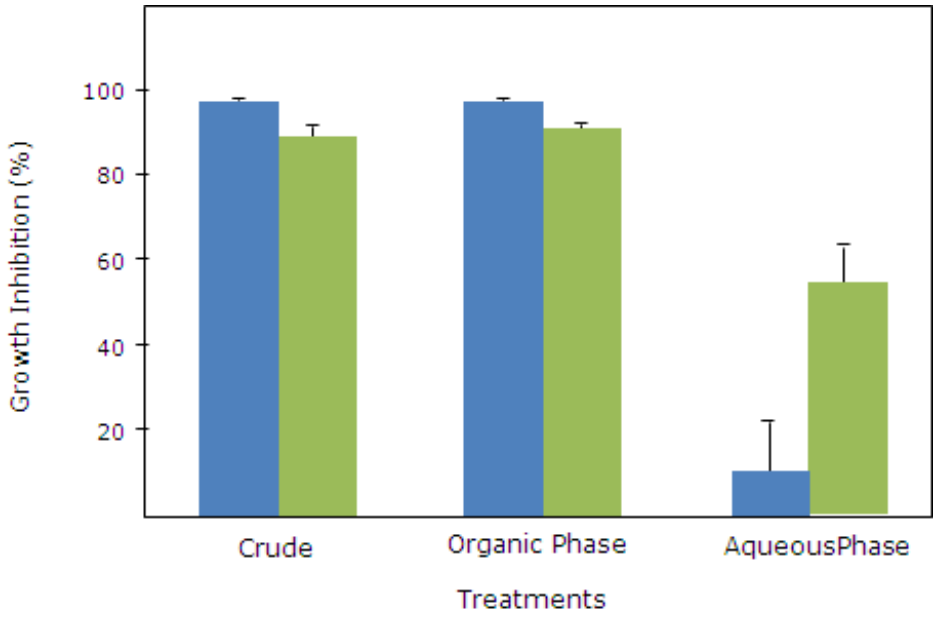

Figure 3.17. Growth inhibition of *E. amylovora* PMV6076 (■) and *X. axonopodis* pv. vesicatoria CFBP3275 (■) cultures after 20 h of growing in LB broth supplemented with crude culture supernatant or the corresponding organic and aqueous phases of *B. amyloliquefaciens* EPS2059 grown in optimal conditions. Results correspond to reduction of AUGC respect the non-treated control.

Peptide purification and identification

Peptide purification and identification was carried out using protocols optimized for the identification of known cyclic lipopeptides by chromatographic techniques, specifically High Performance Liquid Cromatography (HPLC) and mass spectrometry, by means of Electrospray Ionization-Mass Spectrometry (ESI-MS) and Matrix-Assisted Laser desorption/Ionization Time-Of-Flight (MALDI-TOF).

These procedures were optimized in the present work to detect the two commercial standard cLPs, iturin A and surfactin. Retention time values for purified iturin A and surfactin were determined using analytic HPLC. Iturin A retention time was between 3 and 5 min, and surfactin retention time was between 11 and 16 min (Fig. 3.18).

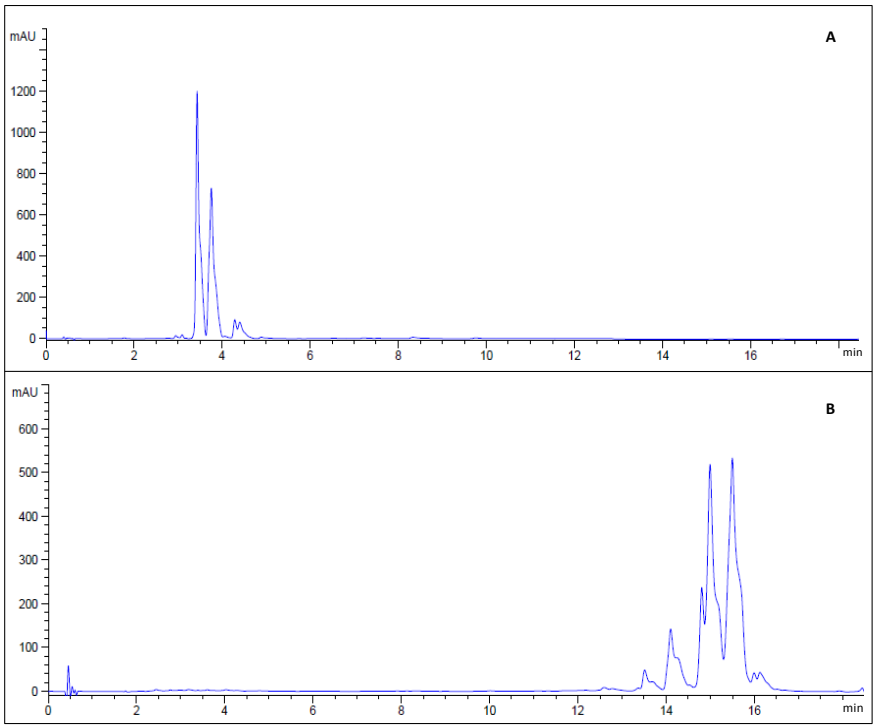

Figure 3.18. HPLC chromatographic profile of standards iturin A (1mg/ml) (A) and surfactin (10mg/ml) (B), using a reverse-phase Kinetex XB-C18 column.

Furthermore, the molecular weight of iturin A and surfactin was determined by mass spectrometry techniques. The molecular weight of iturin A and surfactin was very similar, and mass spectrum ranged between 1028 and 1109 m/z for iturin A and between 1016 and 1095 m/z for surfactin (Fig. 3.19). The range of mass spectrum values was related with the presence of different isoforms with different numbers of carbon and positive ions in each cLP.

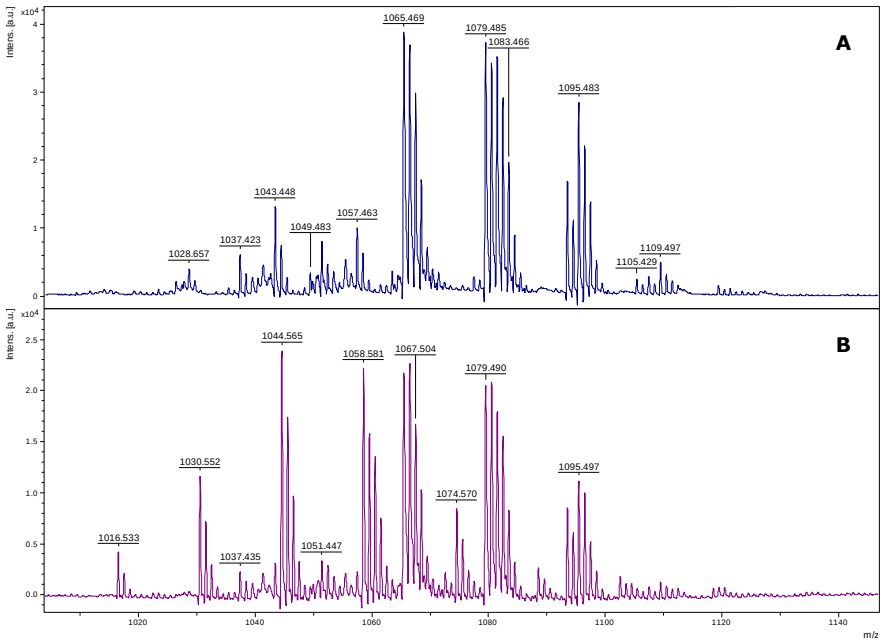

Figure 3.19. MALDI-TOF mass spectra of standards iturin A (A) and surfactin (B).

Once chromatographic and mass spectrometry methods were optimized, organic and aqueous phases obtained from culture supernatants of *B. amyloliquefaciens* EPS2059 were analyzed. Differential peaks were observed in both phases, and two main regions could be defined. A first region, between 0 and 3 min retention time was characterized by the presence of peaks in both phases, but also included peaks associated with products mainly from the culture medium. A second region, from 3 to 18 min was characterized by peaks clustered in groups only present in the organic phase and not in the medium or in the aqueous phase (Fig. 3.20).

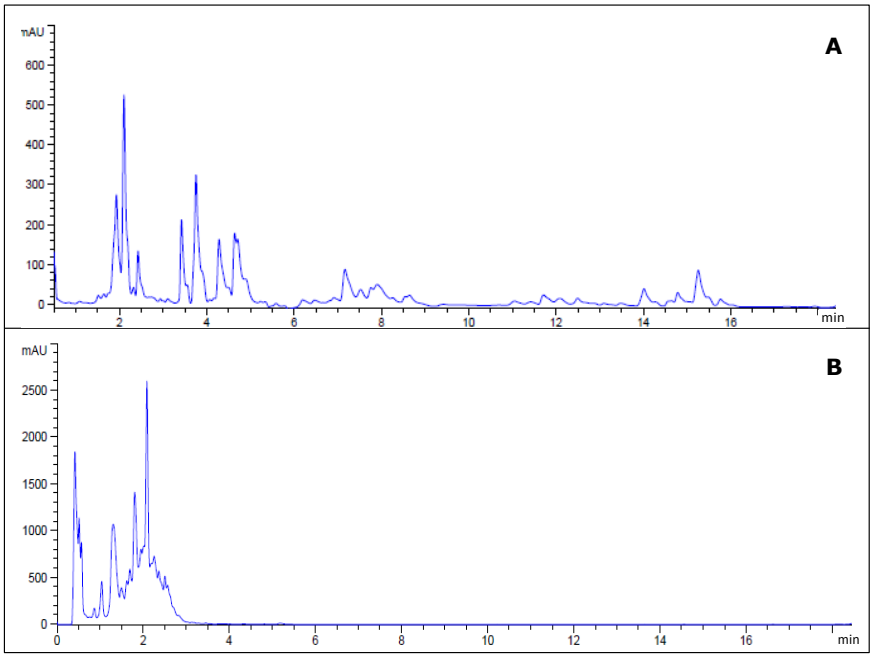

Figure 3.20. HPLC chromatographic profile of organic (A) and aqueous (B) phases of *B. amyloliquefaciens* EPS2059 culture supernatant, using a reverse-phase Kinetex XB-C18 column.

The organic phases of *B. amyloliquefaciens* EPS2059 obtained from LB or PM broth culture were analyzed by HPLC (Fig. 3.21). Chromatographic profiles obtained after growth in LB broth showed peaks from 0 to 3 minutes (Fig. 3.21-A), which were not detected in PM medium (Fig. 3.21-B). These peaks were associated with culture medium components because were coincident with the chromatographic profile of LB medium as reference control.

Using PM medium as culture broth, a reduction of background was observed, although intensity of peaks decreased considerably. Globally, peak profiles were maintained independently of the medium used. Retention peaks obtained from organic phases were purified by preparative HPLC.

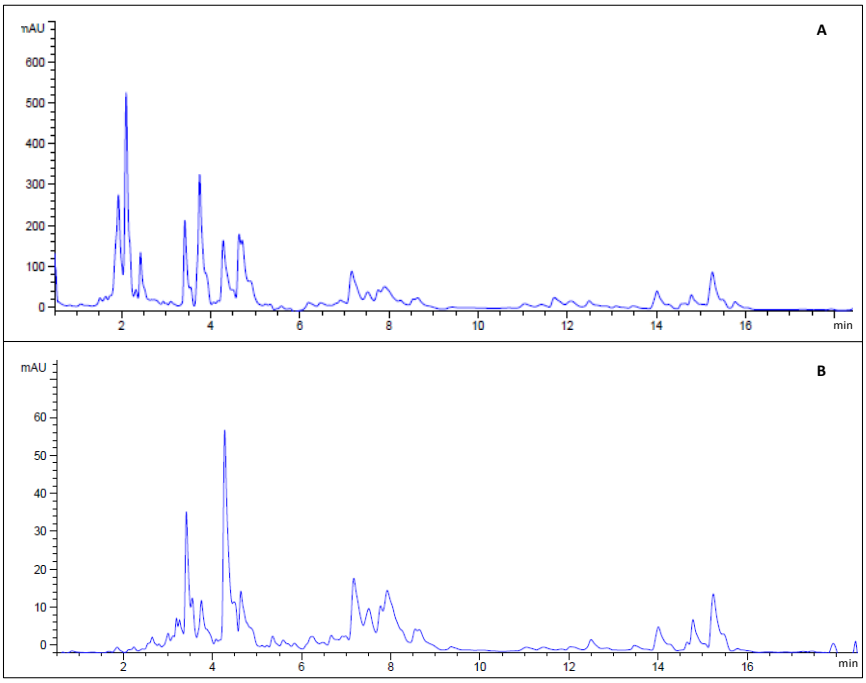

Figure 3.21. Analytical HPLC chromatographic profile of organic phase of strain EPS2059 grown in LB broth and PM medium, using a reverse-phase Kinetex XB-C18 column.

The aliquots obtained from the *Bacillus* extract, 26 in total, were evaporated under vacuum and rehydrated in distilled water with 0.1 % of TFA to be analyzed by MALDI-TOF. Different molecular masses were determined from each aliquot, which were associated with peptides described in the literature. According to the literature database, 13 molecular mass spectra agreed with: macrolactin (403 m/z), oxydifficidin (579 m/z), possible siderophore (597 m/z), kurstakin (907 m/z), lichenysin (991-1065 m/z), polymyxin (1130-1189 m/z), plantazolin (1336 m/z), bacitracin (1394-1422 m/z), surfactin (994-1096 m/z), iturin (1017-1082 m/z), bacillomycin (1031-1097 m/z), mycosubtilin (1071-1151 m/z), and fengycin (1435-1529 m/z). The identity of these molecular mass spectra was confirmed by ESI-MS, but only the cLPs surfactin, iturin, bacillomycin, mycosubtilin and fengycin were identified by fragmentation of peaks of interest (Fig. 3.22).

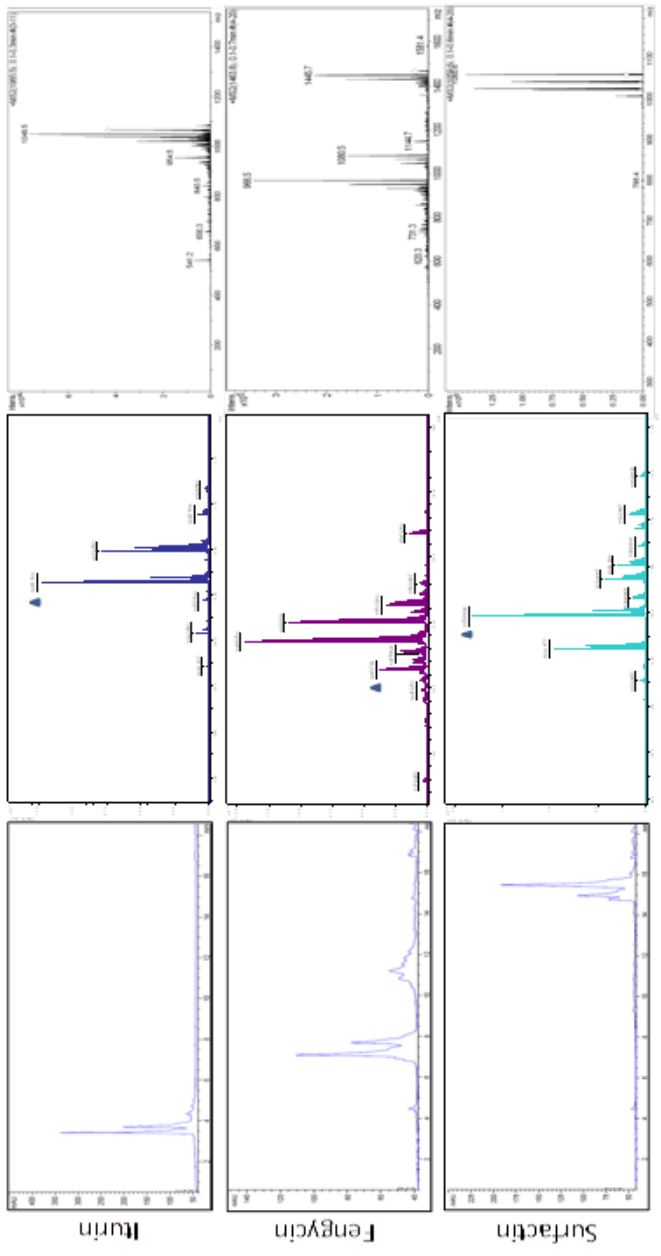

Figure 3.22. Analytical HPLC chromatographic profiles and mass spectra of collected peaks by means of preparative HPLC corresponding to cyclolipopeptide families present in organic phase of culture supernatant of strains EPS2059 grown in LB broth

Figure 3.22. Analytical HPLC chromatographic profiles and mass spectra of collected peaks by means of preparative HPLC corresponding to cyclic lipopeptide families present in organic phase of culture supernatants of strain EPS2059 grown in LB broth.

The mass spectra of the cLPs were mainly associated with 4 areas of the analytical HPLC chromatograpy profile (Fig. 3.23). The first area, between 3 and 5 min was related with the presence of iturin family compounds, including iturin, bacillomycin and mycosubtilin (I1, I2, I3 and I4). The second and third area, which includes peaks observed from 6 to 9 minutes and from 11 to 14 minutes, were related with presence of compounds of fengycin family (F1, F2, F3, F4, F5, F6 and F7). Finally, the fourth area was related with surfactin family compounds, which ranged from 14 to 16 min (S1, S2, S3 and S4). Each one of these elution peaks was typified as an isoform according to the majority cyclic lipopeptide isoform detected on basis of m/z analysis.

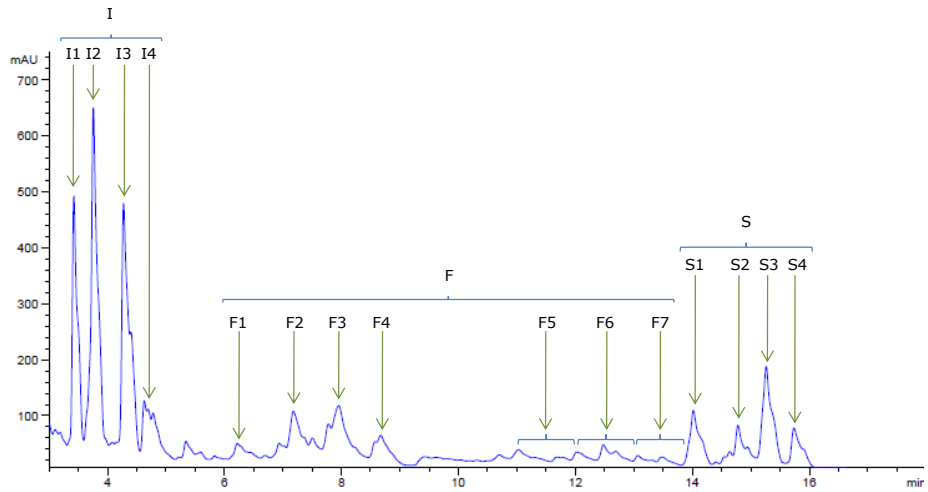

Figure 3.23. HPLC chromatographic profile of *Bacillus* extract illustrating 15 peaks related with the presence of iturin (I), fengycin (F) and surfactin (S) cyclic lipopeptides, grouped in three clusters according to the related family.

2.2.4 Quantification of the identified AMPs

Elution profiles at 220 nm obtained by analitycal HPLC from commercial iturin and surfactin standards showed the presence of different peaks, concretely 4 peaks for the iturin, between elution time 3.3 to 4.6 min, and 6 for the surfactin, between elution time 13.4 to 16.4 min. The presence of

multiple peaks due to isoforms complicates the quantitative analysis. Then, standard curves which correlates the absorption signal with the product concentration were optimized for iturin A and surfactin standards, using the area of all peaks observed for each product [mAU*s] (Fig. 3.24).

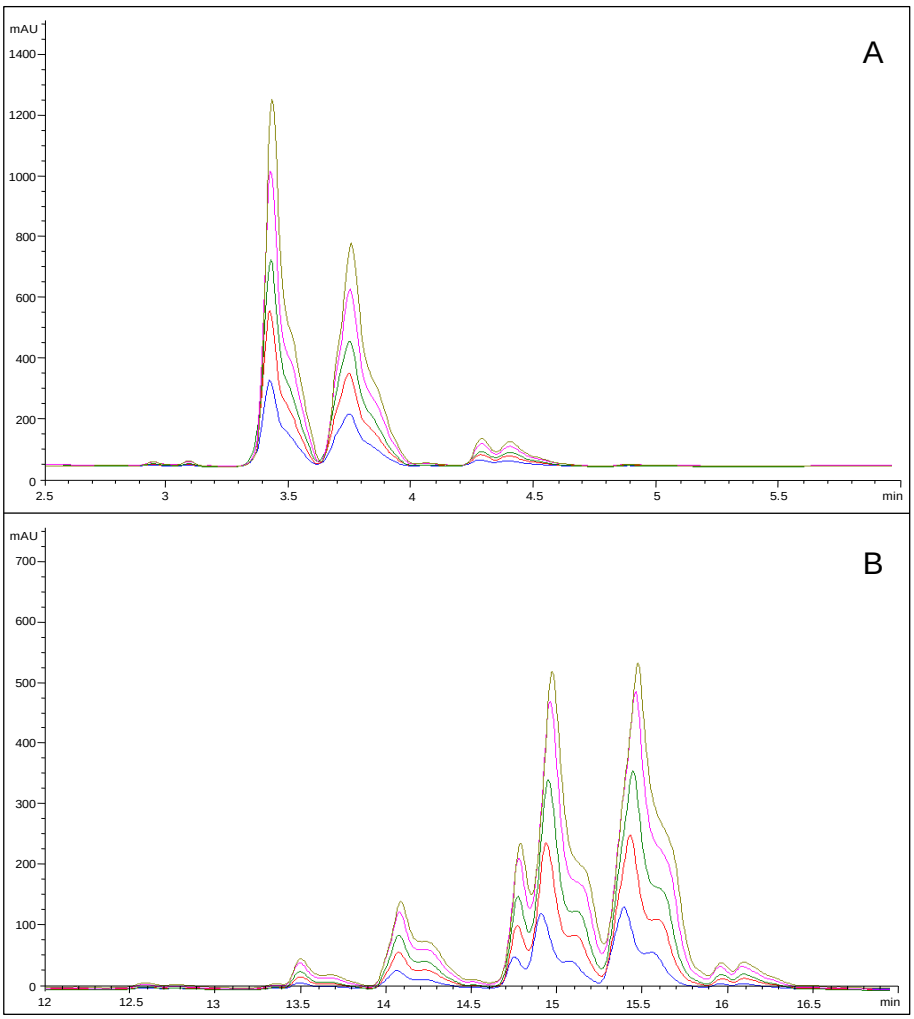

Figure 3.24. Analytical HPLC chromatographic profiles of different aliquots of comertial standards iturin A (A) and surfactin (B). Iturin A tested concentrations were: 200 µM (—), 400 µM (—), 600 µM (—), 800 µM (—) and 1 mM (—). Surfactin tested concentrations were:, 2 mM (—), 4 mM (—), 6 mM (—), 8 mM (—) and 10 mM (—).

Cyclic lipopeptides identified in EPS2059 were quantified as relative amounts to the standards iturin and surfactin. Taking into account cell concentration of cultures grown in LB broth medium of $1x10^9$ cfu/ml and the relative quantity of iturin family compounds, and surfactin families were estimated as 12.5 and 3.9 µg/ml, or 2.2 and 0.7 µg per mg of dried weight, respectively. Despite having no commercial standard for fengycin, this was related to standard curves, both iturin A and surfactin, being relative quantifications 11.3 µg/ml (19.5 µg/mg d.w.) or 8.4 µg/ml (1.4 µg/mg d.w.), respectively. Cultures grown in PM medium showing a cell concentration of $2x10^8$ cfu/ml, the relative quantity of iturin and surfactin family compounds were 4.5 and 1.0 µg/ml or 7.7 and 1.7 µg/mg d.w., respectively. And fengycin related products related to iturin A and surfactin standard curves were 6.9 µg/ml (11.9 µg/mg d.w.) or 5.2 (8.9 µg/mg d.w.), respectively.

2.2.5 AMPs production in *Bacillus* isolates from plant environments

The chromatographic profiles of 64 out of 184 *Bacillus* isolates were determined using analytic HPLC in order to identify the components of each peak, using techniques of mass spectrometry as for strain EPS2059. The 64 chromatographic profiles were very different (Fig. 3.25), providing information of each isolate in relation to the production of antimicrobial compounds, as well as on the major components produced. The frequency of the presence of different cyclic lipopeptides among isolates was studied. The simultaneous production of iturins, fengycins and surfactins was determined in 75 % of isolates, and the production of two out of the three families of cyclic lipopeptides was observed in 18.75 %. In addition, the frequency of patterns of cyclic lipopeptides isoforms produced was determined, showing a strong heterogeneity of products depending on the isolate (Fig. 3.26). Besides, when frequency of isolates was analyzed according to the production of multiple cyclic lipopeptide isoforms (produce more than 50 % of detectable isoforms), it was observed that most of isolates produced multiple isoforms of fengycin and surfactin (53 %). Moreover, only a low percentage of the isolates produced multiple isoforms of fengycin and sufactin individually. In contrast, frequency of isolates

producing multiple isoforms of iturin was similar between isolates that only produce iturin and isolates producing multiple cyclic lipopeptides.

In addition, the quantification of patterns profile of each family of cyclic lipopeptides for each isolate was done, being maximum values of the iturin family compounds of 108 µg/ml and for surfactin family compounds of 22.5 µg/ml. Based on standard curve of iturin A, maximum values of fengycin family products were around 73.7 µg/ml.

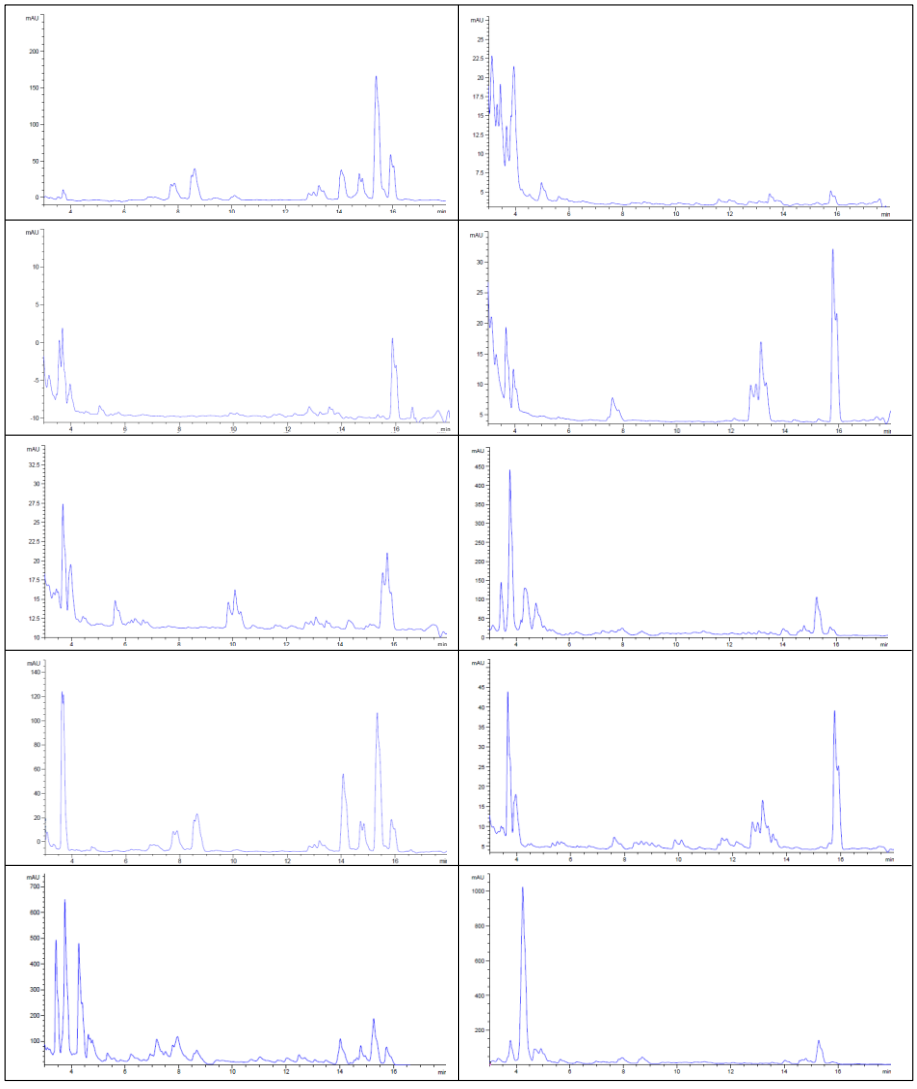

Figure 3.25. HPLC chromatographic profiles of 10 out of 64 *Bacillus* isolates.

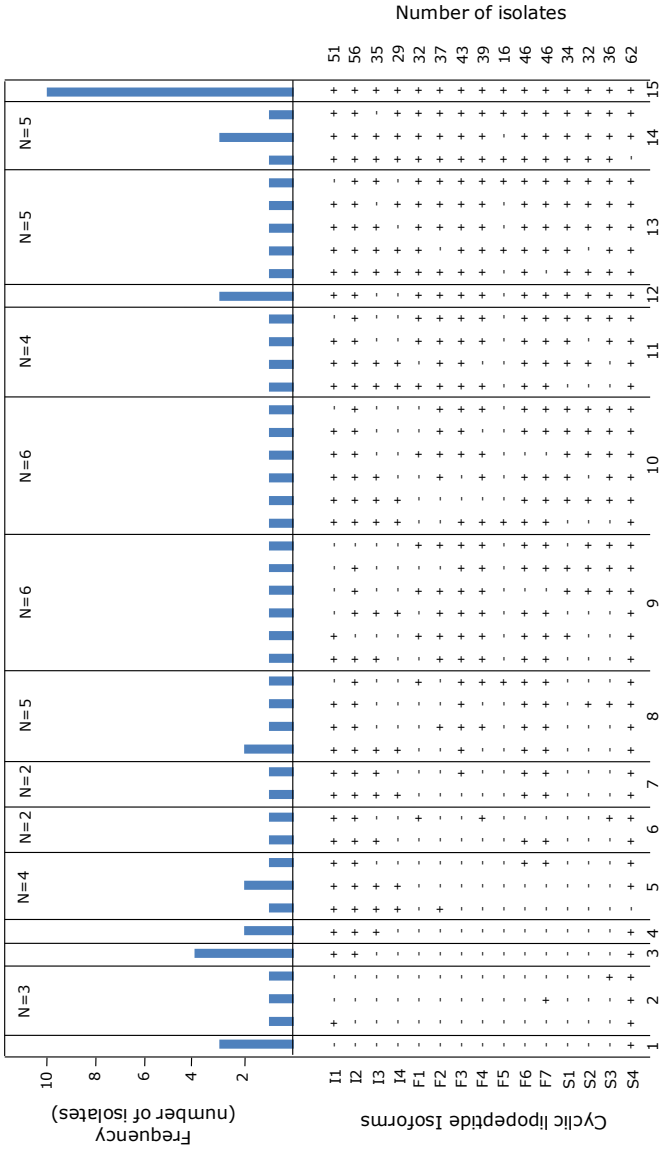

Figure 3.26. Frequency distribution of patterns of cyclic lipopeptide isoforms in 64 *Bacillus* isolates from field samples. The number of isolates (N) within each group of simultaneous cyclic lipopeptide is indicated in the upper part of the panels. Total number of isolates showing each cyclic lipopeptide isoform was showed in the right part of the panel. The presence of cyclic lipopeptides is also indicated: +, presence; -, absence.

2.3 Antimicrobial activity

The collection of 184 *Bacillus* isolates from field samples was characterized according to their antimicrobial activity on agar media against eight bacterial plant pathogens and five fungal plant pathogens (Fig. 3.27).

Antimicrobial activity against bacterial pathogens was analyzed in LB and NA medium with the agar overlay method after 24, 48 and 72 h of incubation at 25 ºC. Most of the isolates showed antagonism after 24 h of incubation and were active against at least one bacterial pathogen. Concretely, in LB medium a 91.3 % of strains showed inhibitory effect against at least one of plant pathogenic bacteria, and a 26.6 % were highly inhibitory (global antibacterial activity >8 over a maximum of 24) (Fig. 3.28-A). While in NA medium 99.4 % of strains showed inhibitory effect and a 36.4 % were highly inhibitory (Fig. 3.28-B). There was a differential activity of the isolates against the plant pathogenic bacteria depending on the medium used for the assay. An increase of the number of active strains against *E. amylovora*, *P. syringae* and *R. solanacearum* and a decrease of the number of active strains against *X. arboricola* was observed in NA medium in relation to LB medium. In contrast, the number of active strains against *P. carotovorum*, *X. axonopodis*, *R. radiobacter* and *C. michiganensis* was similar in both media. The frequency of active *Bacillus* isolates against bacterial plant pathogens was studied. Differences in antibacterial activity were observed according to the growth media used. In this context, increased the number of *Bacillus* isolates in NA medium (Fig. 3.29) was observed in comparison to active isolates detected in LB agar (Fig. 3.30). *In vitro* activity assays in NA medium showed a 63 % of isolates capable of inhibiting at least 5 bacterial plant pathogens. In contrast, only a 31 % of isolates were able to inhibit the same number of bacterial pathogens when assays were performed in LB agar. Globally, 72.3 % of isolates showed a strong antibacterial activity, which inhibited the growth in the range of 6 to 8 bacterial plant pathogens (Fig. 3.31). Antimicrobial activity against fungal plant pathogens was analyzed in PDA medium after 24, 48 and 72 h of incubation at 25 ºC, except for *P. cactorum* F490 and *P. cinnamomi* CECT2965 that were analyzed after 96, 120 and 144 h, due to both had a slower growth.

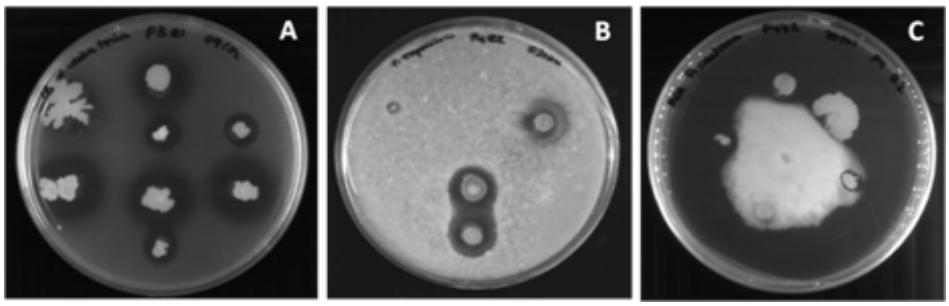

Figure 3.27. Antimicrobial activity *in vitro* of some of the *Bacillus* isolates against *X. axonopodis* pv. vesicatoria (A), *P. expansum* (B), and *P. cactorum* (C).

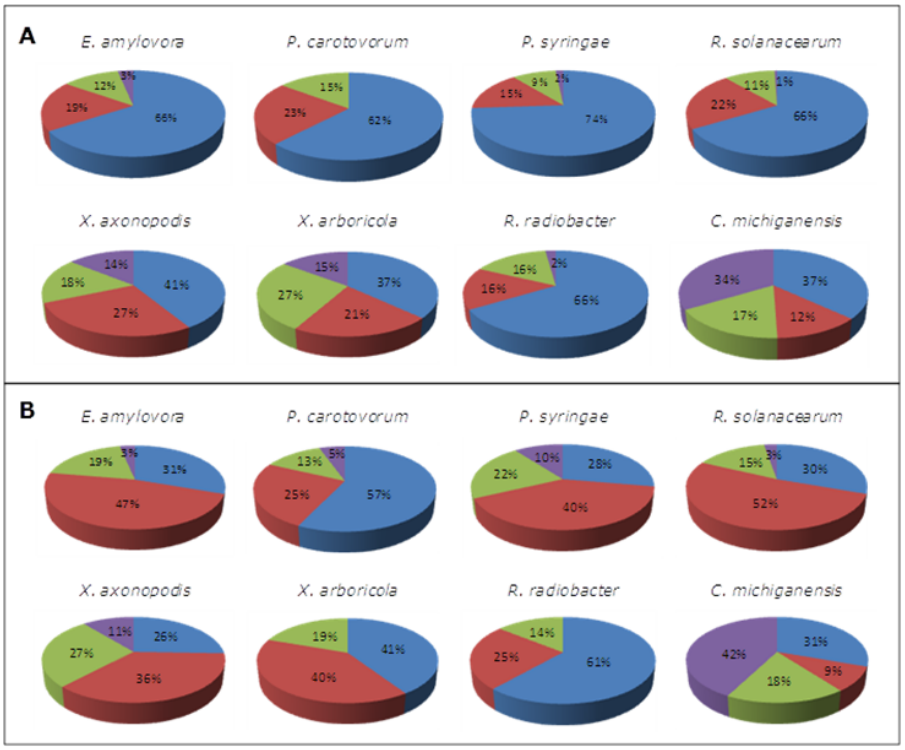

Figure 3.28. Frequency distribution of the antibacterial activity of 184 *Bacillus* isolates against 8 bacterial plant pathogens tested in LB (A) and NA (B) medium. Activity index: 0, no inhibition (■); 1, low inhibition (< 10 mm halus) (■); 2, moderate inhibition (from 10 to <20 mm halus) (■); 3, high inhibition (≥ 20 mm halus) (■).

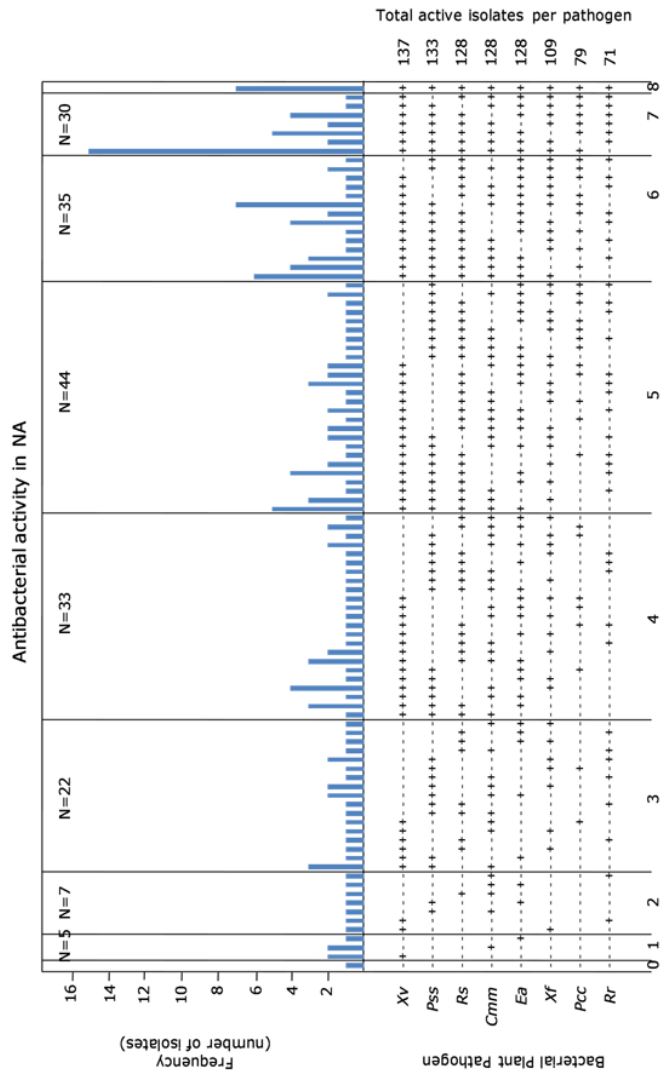

Figure 3.29. Frequency distribution of patterns of antibacterial activity tested in NA of 184 *Bacillus* isolates from field samples. The number of isolates (N) within each group with antibacterial activity against the same number of bacterial plant pathogens is indicated in the upper part of the panels. Total number of active isolates per pathogen was showed in the right part of the panel. The presence of active isolates is also indicated: +, presence; -, absence.

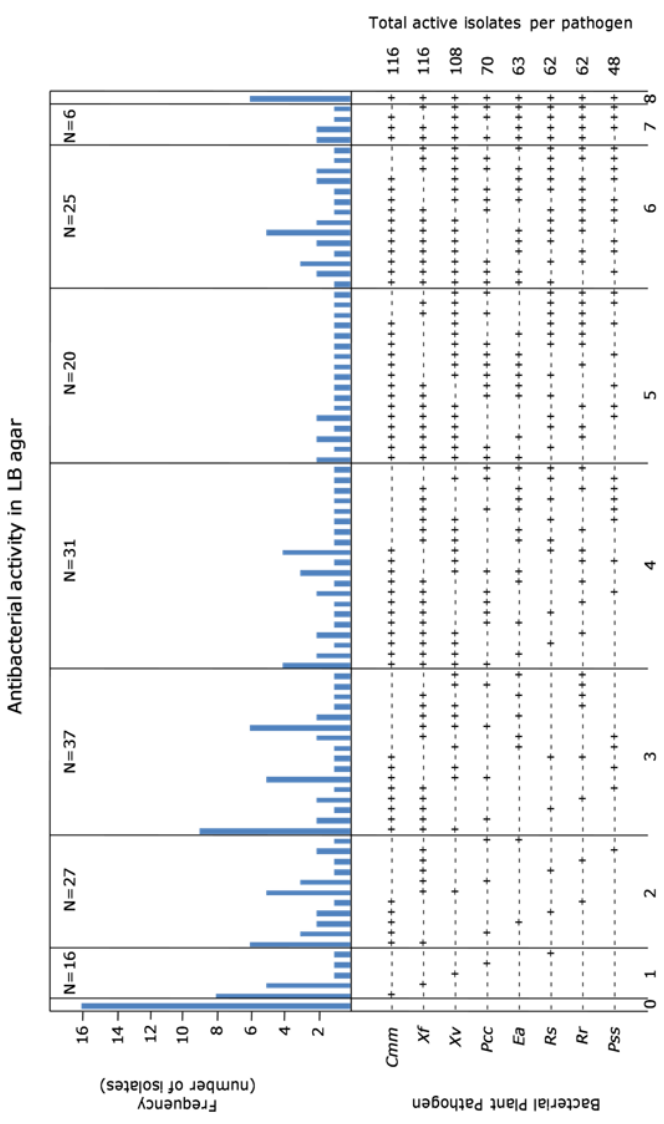

Figure 3.30. Frequency distribution of patterns of antibacterial activity tested in LB agar of 184 *Bacillus* isolates from field samples. The number of isolates (N) within each group with antibacterial activity against the same number of bacterial plant pathogens is indicated in the upper part of the panels. Total number of active isolates per pathogen was showed in the right part of the panel. The presence of active isolates is also indicated: +, presence; -, absence.

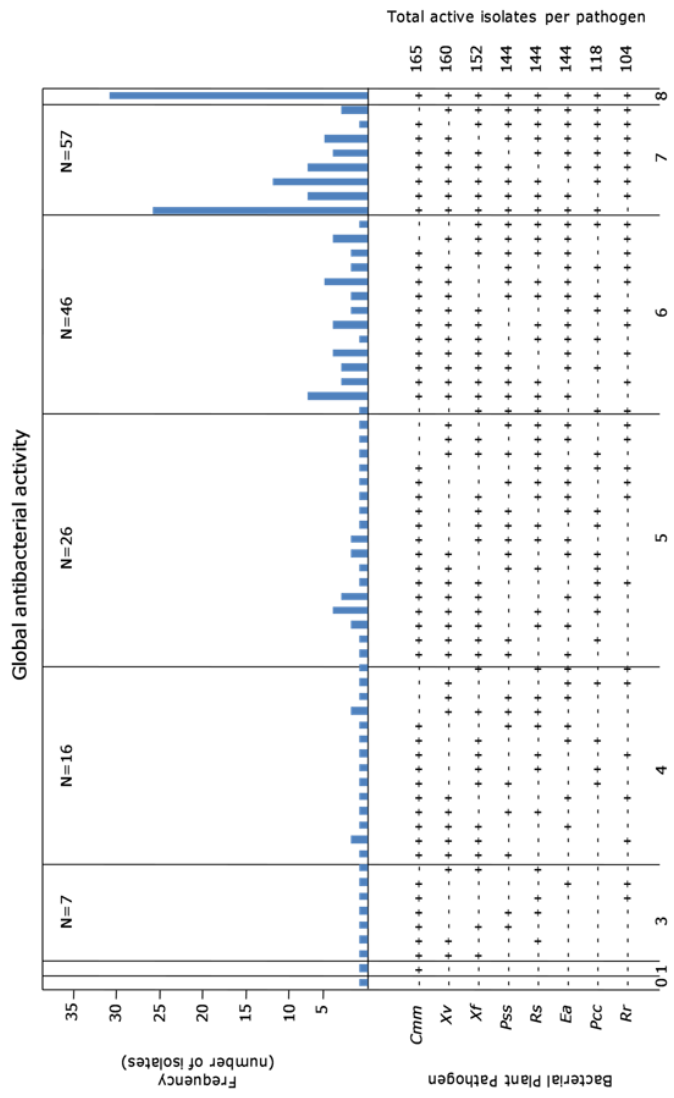

Figure 3.31. Frequency distribution of patterns of global antibacterial activity tested in LB agar and NA of 184 *Bacillus* isolates from field samples. The number of isolates (N) within each group with antibacterial activity against the same number of bacterial plant pathogens is indicated in the upper part of the panels. Total number of active isolates per pathogen was showed in the right part of the panel. The presence of active isolates is also indicated: +, presence; -, absence.

An 84.8 % of isolates showed inhibitory effect against at least one plant pathogenic fungus, and a 26.1 % were highly inhibitory (global antifungal activity >5 over a maximum of 15). The most susceptible plant pathogenic fungi were *P. cactorum, P. ultimum* and *P. cinnamomi*, being the least susceptible *F. oxysporum* and *P. expansum* (Fig. 3.32). The frequency of active *Bacillus* isolates against fungal plant pathogens was also studied. In this case, 37 % of isolates showed a strong antifungal activity, which inhibited the growth of 3 to 5 fungal plant pathogens (Fig. 3.33).

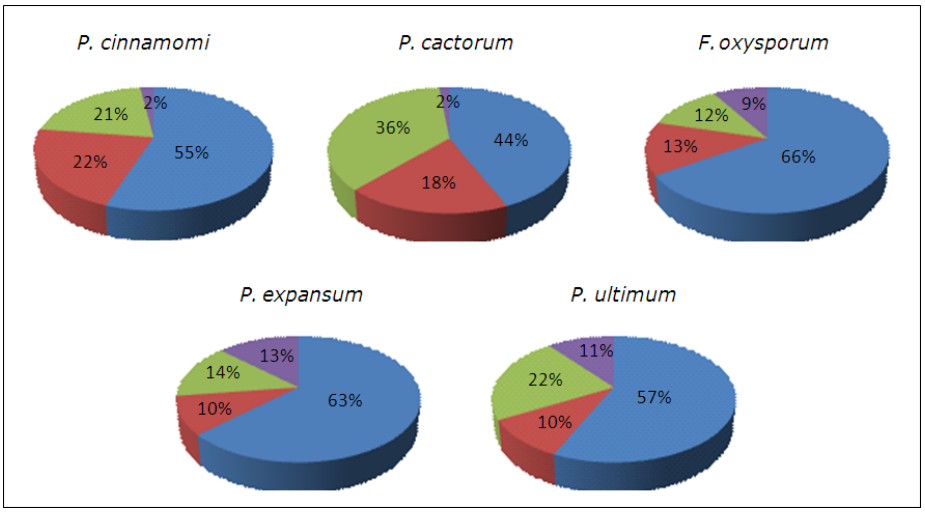

Figure 3.32. Frequency distribution of antifungal activity of 184 *Bacillus* isolates against 5 fungal plant pathogens tested in PDA medium. Activity index: (■) 0, no inhibition; (■) 1, low inhibition (< 10 mm halus); (■) 2, moderate inhibition (from 10 to <20 mm halus); (■) 3, high inhibition (≥ 20 mm halus).

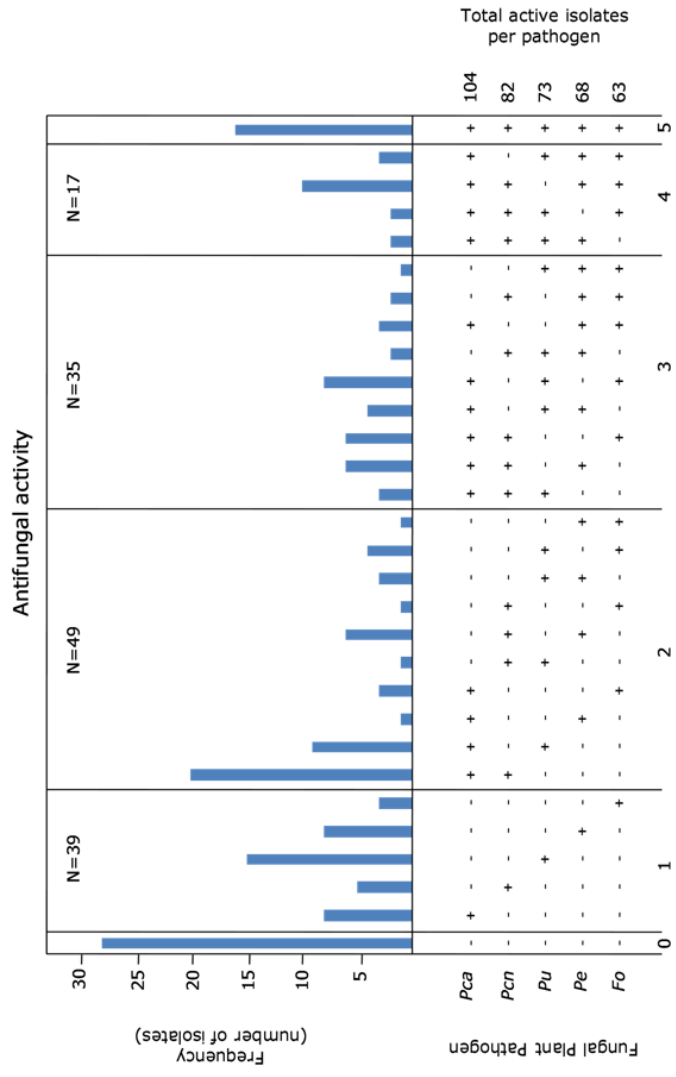

Figure 3.33. Frequency distribution of patterns of antifungal activity of 184 *Bacillus* isolates from field samples. The number of isolates (N) within each group with antifungal activity against the same number of fungal plant pathogens is indicated in the upper part of the panels. Total number of active isolates per pathogen was showed in the right part of the panel. The presence of cyclolipopeptides is also indicated: +, presence; -, absence.

Discussion

In the present study we have constituted a *Bacillus* collection of 184 isolates, obtained from 183 field samples covering a wide range of plant environments (soil, rhizosphere and aerial plant parts) within a Mediterranean Eastern land area in Spain. Most of them were isolated from soil and rhizosphere (Earl *et al*. 2008, McSpadden-Gardener 2004).

The screening of the collection indicated that there was a prevalence of AMP gene markers within the population of *Bacillus* since most isolates had at least one marker. Interestingly, the number of gene markers per isolate followed a normal distribution with most frequent values from 2 to 4, and *srfAA*, *bmyB*, *bacA*, *fenD* as dominant, with a scarce representation of *ituC* and *spaS*. This high presence of certain AMP gene markers among the population is in agreement with the potential of the sequenced strains *B.*

subtilis 168 and *Bacillus amyloliquefaciens* FZB42 for antibiotic synthesis which have six AMP operons that account for a 7.5 % of the genome (Koumoutsi *et al*. 2004). Similar results were observed in a study of the gene markers of bacillomycin D, iturin A, surfactin, mycosubtilin, fengycin and zwittermicin A within a collection of strains antagonistic to *S. sclerotiorum*, where the majority of strains were found to harbor surfactin and iturin related genes (Athukorala *et al*. 2009). Thus, our results are in agreement with the prevalence of the surfactin gene, but not in the case of iturin gene. This may be due to the fact that in the above report the strain collection included only strains active against the target pathogen, whereas in our study the isolates were not selected on basis of the antimicrobial activity against a specific pathogen. In addition, those authors analyzed *ituA* rather than *ituC* gene. The dominance of these particular genes in plant environments reinforces the competitive role of surfactin, bacillomycin, fengycin and bacilysin in the fitness of strains in natural environments. Surfactin production is widely spread among *B. subtilis* (Peypoux *et al*. 1999), and *B. amyloliquefaciens* (Koumoutsi *et al*. 2004) and it has been implicated in cell attachment and detachment to surfaces during biofilm formation and in swarming motility (Kinsinger *et al*. 2005, Ongena and Jacques 2008, Raaijmakers *et al*. 2010). It has been recently documented that the colonization of plant roots by *B. subtilis* is associated to surfactin production and biofilm formation and was implicated in the protection of *Arabidopsis thaliana* against the infection by the pathogen *P. syringae* pv. tomato (Bais *et al*. 2004). Bacillomycin, a member of the iturin family, has been reported together with fengycin to exhibit strong antifungal activity (Thimon *et al*. 1995), being in *B. amyloliquefaciens* FZB42 responsible of the main antagonistic activity against *F. oxysporum* (Koumoutsi *et al*. 2007). It has been also reported that bacilysin is active against a wide range of bacteria (Chen *et al*. 2009c, Loeffler *et al*. 1986).

The fact that the most abundant pattern observed in our isolates was *srfAA-bmyB-bacA* alone or with *fenD*, could be due to the existence of a certain degree of linkage between these genes, or to horizontal genetic exchange followed by a positive selection pressure within the population. In relation to the possibility of linkage, the probability of paired associations of these gene markers observed in our isolates is not related to the relative distance

of the genes, on the basis of genome sequences and relative distances in known strains of *Bacillus* (Chen *et al*. 2009b, Koumoutsi *et al*. 2004, Kunst *et al*. 1997). For example, *srfAA-bacA* appeared in 46.7 % isolates and the average distance reported is c.a. 700 kb, *bmyB-fenD* appeared in 23.9 % isolates and the average distance reported is c.a. 30 kb, *bacA-bmyB* appeared in 42.4 % isolates and the average distance reported is c.a. 2100 kb, *bacA-fenD* appeared in 26.6 % strains and the average distance reported is c.a. 2300 kb, *srfAA-fenD* appeared in 33.7 % isolates and the average distance reported is c.a. 1500 kb, and s*rfAA-bmyB* appeared in 44.0 % isolates and the average distance reported is c.a.1500 kb. More feasible is the possibility of a horizontal exchange of genetic material by means of the uptake of phage, plasmid or naked DNA by genetically competent cells, as has been suggested to explain the clustered of non-ribosomal protein synthetases (NRPSs) genes among *Bacillus* strains 168, FZB42 and GA1 supported by the presence of insertion sequence elements (Arguelles-Arias *et al*. 2009, Earl *et al*. 2007). Then, the predominance in the population of the above mentioned four genes could be due to the benefit provided by the complementary mechanism of action among the gene products like the biosurfactant and biofilm activating properties of surfactin, the antifungal activity of bacillomycin and fengycin, and the antibacterial activity of bacilysin.

The screening of the collection of *Bacillus* isolates for the antimicrobial activity showed that most of the *Bacillus* isolates inhibited at least one plant pathogenic bacteria in the two growth media used (LB and NA), and also a great percentage of these isolates inhibited two or more pathogenic bacteria. The percentage was greater in NA medium where a 99 % of isolates inhibited at least one pathogenic bacterium. These results are in agreement with the report of antagonistic activity of several *Bacillus* species against *X. campestris* pv. campestris (Wulff *et al*. 2002) and *E. amylovora* (Chen *et al*. 2009c, Jock *et al*. 2002). Similarly, *Bacillus* isolates inhibited most of plant pathogenic fungi in PDA medium. However, the level of inhibition depended on the antagonistic isolate, but also on the plant pathogen and the growth medium. Differences in sensitivity of different bacterial species to *Bacillus* isolates could be due to the several compounds produced by *Bacillus*, like iturins and fengycins, that are membrane pore

formers and its specificity depends on cell envelope properties of the target pathogens that differ between Gram-positive and Gram-negative bacteria, and between the species of the plant pathogenic bacteria. Accordingly, differences in susceptibility to synthetic AMPs that are membrane disruptors, have been reported in plant pathogenic bacteria, being *X. axonopodis* pv. vesicatoria highly susceptible, compared to the less sensible *P. syringae* and *E. amylovora* (Badosa *et al*. 2007, Monroc *et al*. 2006). It is also possible that this differential effect is due to pathogen self-defense mechanisms to counteract microbial antagonism (Duffy *et al*. 2003). As the same way, iturins and fengycins are responsible for the main antifungal activity of *Bacillus*, and their activity relies on their membrane permeabilization properties (Aranda *et al*. 2005, Ongena and Jacques 2008, Romero *et al*. 2007).

Differences in the activity of *Bacillus* isolates depending on the type of culture medium used could be due to the known effect of the composition in the differential production of secondary metabolites, especially in *Bacillus* (Gu *et al*. 2005, Sharp *et al*. 1989). This could explain the high number of active *Bacillus* isolates in NA medium especially against *E. amylovora*, *P. syringae*, *R. solanacearum* and *X. axonopodis*. Results of antagonism *in vitro* indicated that *Bacillus* isolates have a great potential in the inhibition of bacterial and fungal pathogens by means of the production of antimicrobial peptides. Besides, most of strains showed a broad range of activity against several bacterial and plant pathogens, suggesting the production of several metabolites with different activity against bacteria as against fungi.

Characterization of isolates was beyond the determination of antimicrobial activity, and demonstrated their ability of producing AMPs. Production of AMPs was determined focusing on different aspects, such as the influence of growth medium, temperature and time of incubation and fractionation of cell culture. In recent years it has been emphasized on the optimization of the production of AMPs and related products, as well as detection and purification. Although there are many studies focused on the detection and purification of different metabolites with antimicrobial activity (Arguelles-Arias *et al*. 2009, Chen *et al*. 2009c, Patel *et al*. 1995, Schneider *et al*.

2007, Stein *et al.*2004, Steinborn *et al*. 2005, Yazgan *et al*. 2001a), most of them focus on cLPs (Arguelles-Arias *et al*. 2009, Bais *et al*. 2004, Chen *et al*. 2010, Leclère *et al*. 2005, Malfanova *et al*. 2012, Moyne *et al*. 2001, Romero *et al*. 2007, Steller *et al*. 2004, Vater *et al*. 2002, Zeriouh *et al*. 2011). Growth medium used differed, but generally a minimum medium amended with salts and sources of nitrogen is used. Incubation characteristics as temperature, time and shaking, are chosen depending on the type of secondary metabolite to be produced. In most cases, the optimum temperature ranges between 25 and 37 °C, while the incubation time and shaking factors were more variable, ranging from 10 to 240 h and 0 to 250 rpm, respectively.

In the present work, the production, extraction and identification of cLPs were optimized monitored using the antimicrobial activity. In this case it was determined that the cell-free supernatant was active. Besides, it was observed that an increase of the incubation time increased growth inhibition of the bacterial plant pathogens evaluated. These results were in agreement with other studies, such as antifungal assays performed by Moyne *et al.* (2001) in *Aspergillus flavus*, in which an increase of growth inhibition was observed when was treated with filtered culture supernatants of *B. subtilis* AU195 collected each 24 h for 7 days. From these evidences, a study with strain EPS2059 grown in two growth media (LB and PM) was carried out in order to determine the evolution of the antimicrobial activity during 72 h of incubation at different temperatures (24, 28 and 37 ºC), and its relation with growth parameters such as pH, percentage of sporulation and cell concentration. For both growth media, pH and cell concentration increased regularly over time, though the increase was higher in rich medium (LB) than in poor medium (PM). For these two parameters, a slight effect of temperature was observed, and the highest values of pH and cell concentration were observed when cultures were incubated at 37 ºC. In contrast, a higher effect of temperature was observed in percentage of sporulation, especially in rich medium. According to some authors, medium composition had significant effects in the percentage of sporulation, being a poor medium and the increase of temperature favorable for sporulation (Jabbari *et al*. 2011, Ramírez-Peralta *et al*. 2012). In the same way, surfactin biosynthesis was associated to the sporulation process because

induction of different genes, such as *spo* or *srfAA*, were related with both processes (Jabbari *et al.*2011, Morikawa 2006, Nakano *et al.* 1988). Then, the aggregates of spores and cells prematurely observed in poor medium could be due to a rapid sporulation and production of surfactins and could explain the variability in percentage of sporulation observed in poor medium due to the difficulties for cell and spore counting, thus allowing to underestimate viable counts.

LB medium increase cell culture and sporulation rates compared to PM medium suggesting a higher production of secondary metabolites. Supernatants obtained from cultures grown in PM medium were more active against *E. amylovora* and *P. syringae* pv. syringae than supernatants from LB cultures. However, the analysis of the production of cLPs indicated that the increase of antimicrobial activity was not related with an increase in the production of detectable antimicrobial compounds, because the production of detectable cLPs in PM growth medium decreases ten times in relation to LB medium. Therefore, the more feasible hypothesis would be the inactivation of antimicrobial metabolites due to binding to media components as has been suggested by some authors (Schwab *et al*. 1999). Other studies on cationic antimicrobial peptides describe that NaCl reduce the attraction between the peptide and the cell membrane in the target pathogen, so that increasing the concentration of NaCl reduces the antimicrobial activity of the peptides (Ganz and Lehrer 1999). Also, the presence of cations reduces the activity of MSI-78, an analog of the frog skin magainin (MacDonald *et al.* 1997).

Fractionation of supernatant cultures was required to purify the cLPs. Different fractionation techniques have been used for in culture supernatants of *Bacillus* strains, such as acid precipitation with HCl (Chen *et al.* 2010, Troyano *et al.* 2009, Vater *et al.* 2002); organic fractionation with n-butanol (Arrebola *et al.* 2010, Hofemeister *et al.* 2004, Leenders *et al.* 1999, Stein *et al.* 2008, Tamehiro *et al.* 2002); or ammonium sulphate precipitation (Liu *et al.* 2010, Moyne *et al.* 2001, Yazgan *et al.* 2001b). In this work, peptide fractionation was carried out using isoamyl alcohol, instead of n-butanol. Antimicrobial activity assays performed using organic and aqueous phases obtained from filtered culture supernatants confirmed

that most of the antimicrobial compounds remained in the organic phases, showing similar growth inhibition values than direct filtered culture supernatants. Nevertheless, antimicrobial activity depends on pathogen species. Thus, aqueous fraction also showed highly inhibitory effect in growth of *X. axonopodis,* the most sensitive species.

Presence of antimicrobial metabolites in both fractions was determined with analytic HPLC profiles, and confirmed the presence of differential and characteristic clusters of peaks in the organic phase in comparison to the aqueous phase. These peaks were analyzed by means of mass spectrometry in order to determine the predominant molecular masses and associate with previous described peptides.

MALDI-TOF and ESI-MS analysis were used to characterize products from each preparative HPLC peak. These methods improve identification of peptides, but the absence of interfering signals must be confirmed, because cluster formation with α-cyano-4-hydroxycinnamic acid (CHCA) matrix may occur. Previous reports concluding that using CHCA matrix some signals dominating in the range below 1200 m/z in MALDI spectra are associated to matrix and might be removed especially when the concentration of analyzed peptides is lower than 10 fmol/µl (Smirnov *et al.* 2004). From mass spectra analysis of *Bacillus* strain EPS2059 and the corresponding relationship with molecular mass of antimicrobial peptide previously described, three families of cyclic lipopeptides were identified, concretely surfatin, fengycin and iturin families. Masses contained in the peak cluster between 994 and 1096 *m/z* were attributed to surfactin isoforms; masses between 1017 and 1151 *m/z* were attributed to iturin isoforms, including variants of iturin (1017-1082 m/z), bacillomycin (1031-1097 m/z) and mycosubtilin (1071-1151 m/z); while the masses between 1435 and 1529 m/z were attributed to variants of fengycin (Arguelles-Arias *et al.* 2009, Chen *et al.* 2008, Chitarra *et al.* 2003, Hofemeister *et al.* 2004, Koumoutsi *et al.* 2004, Price *et al.* 2007, Romero *et al.* 2007, Stein 2008).

As a result of analytical HPLC combined with mass spectrometry, three clusters of peaks were characterized in our *Bacillus* isolates, corresponding to cLPs from surfactin, iturin and fengycin families. The iturin family was

composed of the less polar cLPs, being the first to be detected, followed by fengycins and surfactins. The same clusters of peaks have been previously described in other studies, in which similar analytical HPLC conditions have been used (Arrebola *et al.* 2010, Malfanova *et al.* 2012). In agreement, iturins and fengycins were less separated due to the similar polarity between iturins with a high molecular mass and fengycins with lower molecular mass. However, a second cluster of fengycins was indentified in the present work that was not identified by other reports. This fact could be due to the differences in HPLC conditions that allow separating fengycins with low molecular mass from those of high molecular mass. Based on calibration curves for standard iturin A and surfactin, the concentration of iturins, fengycins and surfactins in the cultures was determined. A maximum concentration of cLPs was obtained using LB broth instead of PM medium. However, iturins, fengycins or surfactins production depends on strain, where wide range of differences was observed in the detection and concentration of various synthesized cLPs.

In conclusion, the characterization of isolates based in AMP genes, antimicrobial activity and AMP production revealed a strong diversity for AMP gene patterns, as well as for production of AMPs and antimicrobial activity.

CHAPTER 4

Relationships between AMP genes, products and antimicrobial activity in *Bacillus*

Introduction

Selection and evaluation of microbial strains for their ability to control plant diseases is a time and energy consuming process. Molecular approaches have the potential to simplify screening processes and increase yield in obtaining promising biocontrol strains. Some studies have applied molecular approaches in the development of biocontrol strategies. For example the adaptation of a PCR-based method to search for peptide-producing microorganisms using degenerate primers from conserved regions of peptide synthetase genes, resulted in the selection of *Bacillus* strains with antifungal activity against *Sclerotinia sclerotiorum* (Giacomodonato *et al*. 2001). A similar approach has been used to detect strains of antibiotic-producing fluorescent *Pseudomonas* in soils, using colony hybridization

(Raaijmakers *et al*. 1997). However, other approaches can be used in the absence of knowledge on the mechanisms involved in biocontrol, such as PCR-based suppressive-substractive hybridization to identify new markers (Benitez and McSpadden-Gardener 2009, Leveau *et al*. 2006). Recently, the evolution of molecular tools has permitted the development of new screening strategies, such as the sequence-based T-RFLP-derived molecular markers to direct the identification and isolation of novel bacteria (Benitez and McSpadden-Gardener 2009).

Because one of the main mechanisms of biocontrol of plant pathogens by *Bacillus* strains relies in the production of antimicrobial compounds, most studies have been focused in selecting strains that produce these antimicrobial compounds. Several of these studies have tried to elucidate the relevance of key biosynthetic genes in biocontrol. For example the study of distribution of four AMP biosynthetic genes over a collection of strains active against *S. sclerotiorum* (Athukorala *et al*. 2009) or the report of the intensity of the antagonistic activity against several fungal pathogens in relation to the presence-absence of eight antimicrobial genetic markers (Joshi and McSpadden-Gardener 2006). However, in most cases the relationship between the presence of biosynthetic genes and the production of the corresponding products has not been studied in a population of *Bacillus* isolates. This has been only partially confirmed in the strain *B. amyloliquefaciens* GA1 where the presence of eight giant gene clusters directing the synthesis of bioactive peptides and polyketides has been related with the production of cLPs, such as surfactin, iturin A and fengycin, iron-siderophore bacillibactin, antibacterial polyketides macrolactin, bacillaene and difficidin, the dipeptide antibiotic bacilysin and the chlorinated derivate chlorotetaine (Arguelles-Arias *et al*. 2009).

The availability of complete genome sequences of several *Bacillus* strains and the knowledge of the molecular determinants of related metabolites permits the reliable development of molecular markers with a great potential in the identification of isolates with biocontrol properties. However, as far as we know, neither the distribution of the number and patterns of AMP genes nor its relationships with the antagonistic activity against plant pathogenic bacteria and the production of AMPs have been reported in

detail in strain collections of natural populations of plant-associated *Bacillus*. Therefore, we consider that it is important to know the relationships between the distribution of AMP genes and antagonistic activity in natural populations in order to select the best molecular markers to be used in the isolation process mediated with molecular tools.

OBJECTIVES

The aim of the present study was to analyze a large collection of plant-associated *Bacillus* from wide origin to study the relationships of AMP biosynthetic genes with the production of cLPs, particullarly surfactins, fengycins and iturins, and their antimicrobial activity.

Specific objectives were to determine relationships between:

- Antimicrobial peptide genes and antimicrobial activity.
- Antimicrobial peptide genes and production of cyclic lipopeptides.
- Production of cyclic lipopeptides and antimicrobial activity.

MATERIALS AND METHODS

The relationships between the presence of AMPs biosynthetic genes, production of AMPs and antimicrobial activity in isolates of *Bacillus* from plant environments was determined with Chi-squared distribution with Pearson's statistic and correspondence multivariate analyses using the IBM SPSS Statistics 19.0 package (IBM corporation, USA).

1. AMP genes and antimicrobial activity

A collection of 184 *Bacillus* isolates were analyzed according to the patterns of AMP biosynthetic genes and antimicrobial activity against five fungal and eight bacterial plant pathogens, as determined in the previous chapter. The frequency of isolates according to the degree of antimicrobial activity

against the pathogenic bacteria in LB and NA agar (classified as Global Activity Index, GAI low ≤8; GAI high >8 over a maximum of 24), and pathogenic fungi tested in PDA (GAI low ≤5; GAI high >5 over a maximum of 15) was determined. Then, it was related to the number of simultaneous biosynthetic genes (low <3, high ≥3) and the type of AMP genes present (*srfAA*, *bmyB*, *fenD, bacA*, *spaS* and *ituC*).

2. AMP genes and AMP products

AMPs production and the presence of AMP genes were related in a selection of 64 out of 184 *Bacillus* isolates. The frequency of isolates according to the type (*srfAA*, *bacA*, *bmyB*, *fenD*, *spaS* and *ituC*) and presence of simultaneous AMP biosynthetic genes (high or low) was related with the production of the three families of cLPs (iturins, fengycins and surfactins), and to the corresponding isoforms (I1, I2, I3, I4, F1, F2, F3, F4, F5, F6, F7, S1, S2, S3 and S4) according to the elution HPLC peak profiles (Fig. 3.23).

3. AMP products and antimicrobial activity

The 64 selected *Bacillus* isolates were analyzed according to AMPs production and antimicrobial activity against several fungal and bacterial plant pathogens. The frequency of production of the three families of cLPs (iturins, fengycins and surfactins), and the corresponding isoforms (I1, I2, I3, I4, F1, F2, F3, F4, F5, F6, F7, S1, S2, S3 and S4) according to the elution HPLC peak profileswere related to the antimicrobial activity (high or low GAI) obtained in NA and LB agar against bacteria and in PDA against fungi.

4. Global analysis of genes, AMPs and antimicrobial activity

A global analysis was performed on 64 *Bacillus* isolates to obtain relationships between the type of AMP gene (*srfAA*, *bacA*, *bmyB*, *fenD*, *spaS* and *ituC*), presence of simultaneous AMP biosynthetic genes, antimicrobial activity against eight pathogenic bacteria in NA and LB agar, and five pathogenic fungi in PDA, and the production of cLPs (iturins, fengycins and surfactins) and their isoforms (I1, I2, I3, I4, F1, F2, F3, F4, F5, F6, F7, S1,

S2, S3 and S4). Sample origin (aerial plant part, rhizosphere, soil) from each isolate was also introduced in the analysis as an informative variable. In addition to previous statistical tests, and to find groups of isolates, a herarchical clustering following Ward's minimum variance criterion was performed, which minimizes the total within-cluster variance according to squared Euclidean distance.

Results

The relationship between AMP genes, AMPs production and antimicrobial activity was studied in an extensive collection of isolates. This collection was formed by 184 *Bacillus* isolates, obtained from 183 field samples, which were randomly collected from different plant environments (143 samples of the aerial plant part, 25 samples of the rhizosphere, 15 samples of bare soil), and 35 plant species (cultivated, wild-type), taken from seven sampling sites (mainly in Catalunya and Balearic Islands) in Spain as previously described (Table 3.2). The isolates obtained by selective enrichment procedure and PCR screening were characterized by the pattern of six AMP biosynthetic genes (*srfAA*, *bacA*, *bmyB*, *fenD*, *spaS* and *ituC*), the activity against eight plant pathogens using two growth media, and against five fungal plant pathogens. In addition, production of AMPs from 64 out of 184 *Bacillus* isolates was determined using HPLC analitycal technique in order to determine the capacity of production of three families of cLPs.

1. AMP genes and antimicrobial activity

1.1. Number of simultaneous AMP genes per isolate and antimicrobial activity

The simultaneous presence of AMP genes was related with the degree of antibacterial and antifungal activity in 184 *Bacillus* isolates. In relation to the activity against bacterial pathogens, it was observed that most of *Bacillus* isolates with high antibacterial activity in LB medium had 2 to 5 AMP genes, with a clear tendency to increase the proportion of highly active antimicrobial isolates (intensity of inhibition of 2 and 3) when increased the number of simultaneous AMP genes per isolate (Fig. 4.1). This effect was especially important in the most susceptible bacterial pathogens, *C. michiganensis* and *X. axonopodis* pv. vesicatoria, for *Bacillus* isolates containing at least three simultaneous AMP genes (Fig. 4.2). In contrast, the antibacterial activity in NA medium was not influenced by the increase of the number of simultaneous AMP genes per isolate (Fig. 4.2). Similar behavior was observed when the activity against fungal pathogens was analyzed. In this case, the proportion of highly active isolate was similar in isolates with 1, 2, 3 or 4 simultaneous genes, and only a slight increase was observed in isolates containing 5 simultaneous genes (Fig. 4.3). In the case of *P. cinnamomi* and *P. cactorum*, it was observed a certain tendency of increase of the number of highly active isolates related to the increase in the number of simultaneous genes (Fig. 4.3).

In order to further analyze these results, the distribution of isolates according to the global activity index (GAI) was related with the number of simultaneous AMP genes (Fig. 4.4). The percentage of highly active isolates (GAI >8) in LB increased with the number of simultaneous genes per isolate, being 7.6 % for 0 genes, 12.0 % for one gene, 14.3 % for two genes, 30.9 % for three genes, 47.2 % for four genes and 66.7 % for five genes. In NA medium the percentage of highly active isolates also increased with the number of simultaneous genes per isolate, being 15.4 % for 0 genes, 24.0 % for one gene, 40.8 % for two genes, 30.9 % for three genes, 47.2 % for four genes and 66.7 % for five genes.

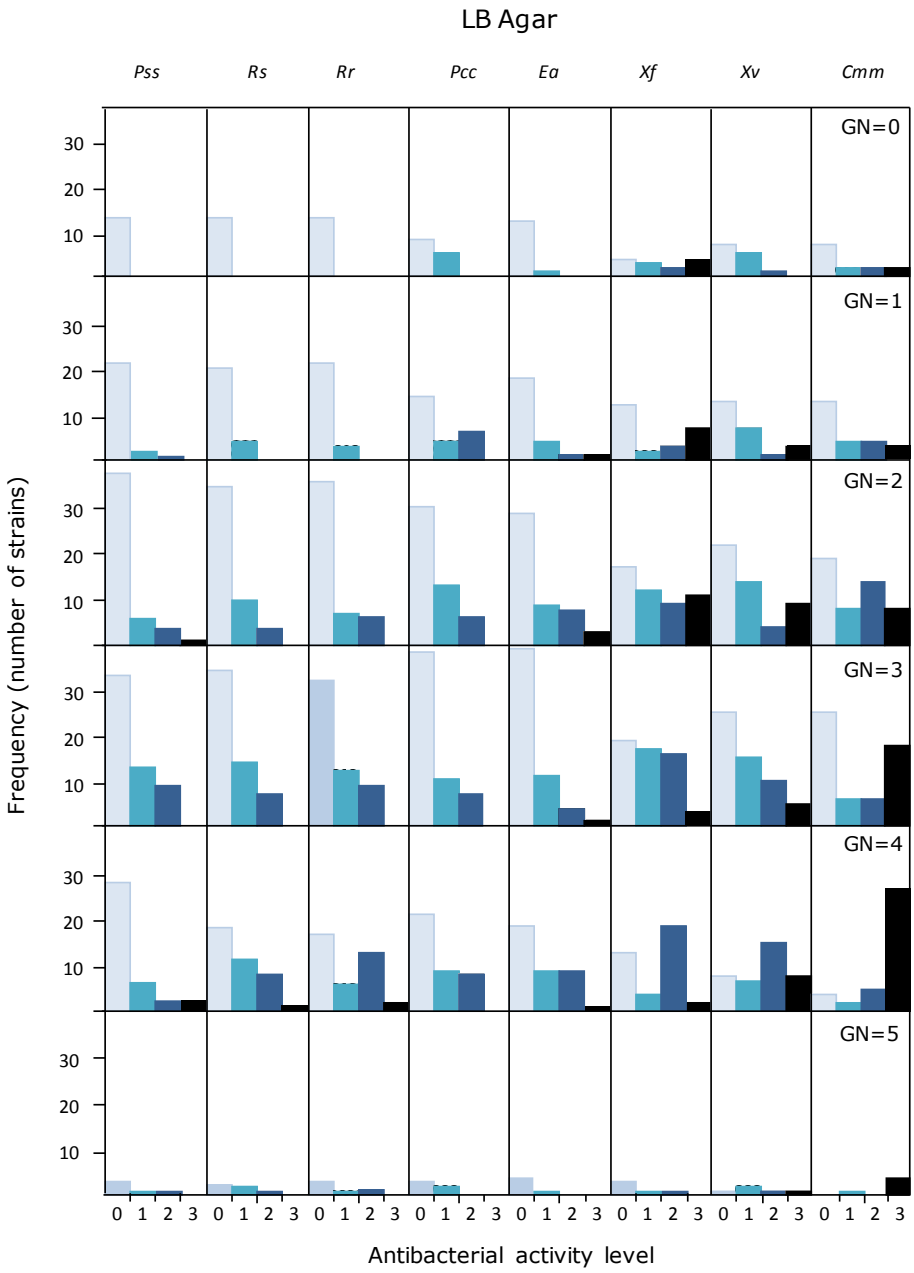

Figure 4.1. Frequency distribution of *Bacillus* isolates according to the antibacterial activity in LB agar against eight plant pathogenic bacteria in relation to the number of simultaneous AMP genes per isolate. Pss, *Pseudomonas syringae* pv. syringae EPS94; Rs, *Ralstonia solanacearum* CECT125; Rr, *Rhizobium radiobacter* CECT472; Pcc, *Pectobacterium carotovorum sbsp. carotovorum* CECT225; Ea, *Erwinia amylovora* PMV6076; Xf, *Xanthomonas arboricola* pv. fragariae CFBP3549; Xv, X. *axonopodis* pv. vesicatoria CFBP3275; Cmm, *Clavibacter michiganensis subsp. michiganensis* CECT790. The distributions are separated by the number of simultaneous AMP gene marker per isolate (GN) that are indicated in the upper right corner of each panel.

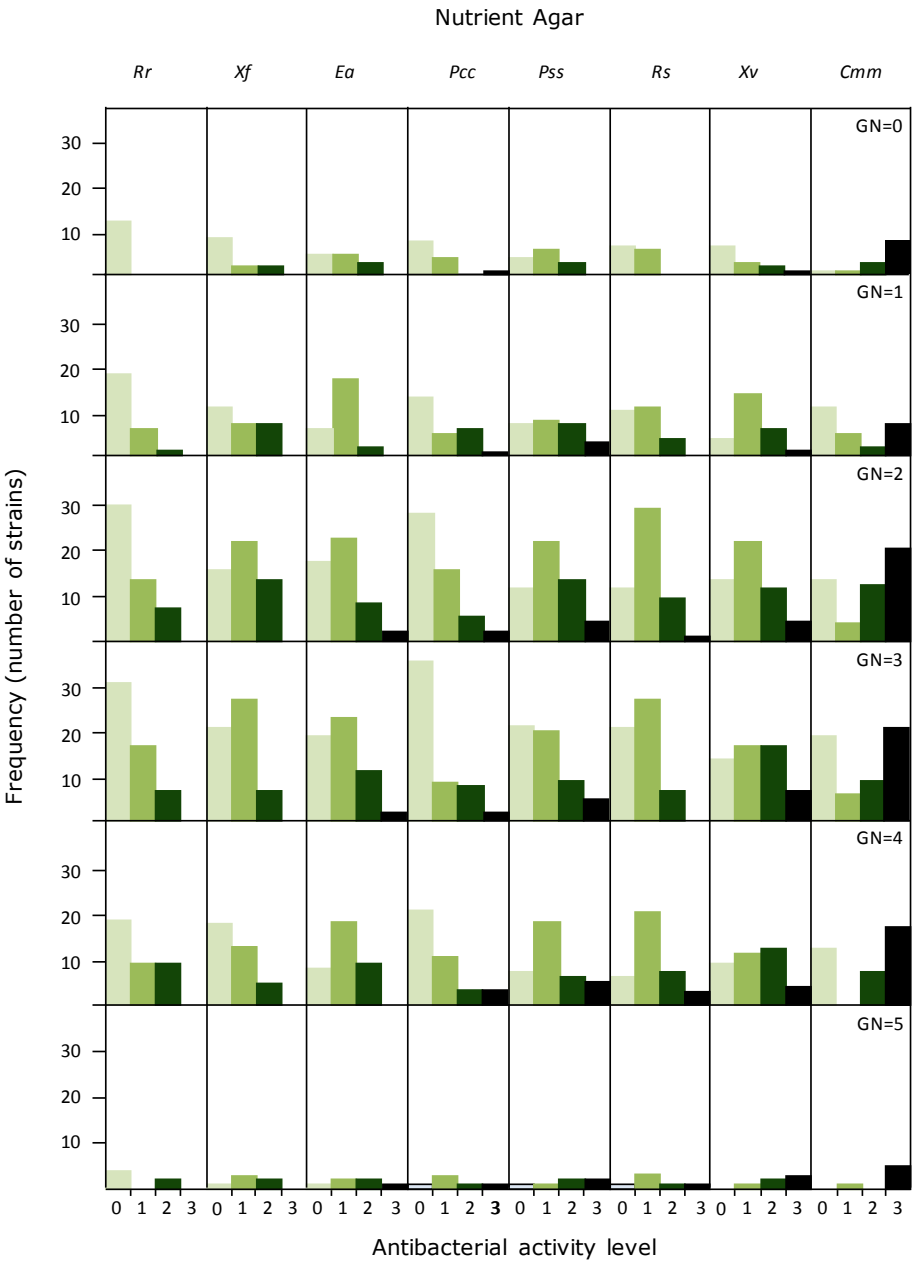

Figure 4.2. Frequency distribution of *Bacillus* isolates according to the antibacterial activity in Nutrient agar against eight plant pathogenic bacteria in relation to the number of simultaneous AMP genes per isolate. Pss, *Pseudomonas syringae* pv. syringae EPS94; Rs, *Ralstonia solanacearum* CECT125; Rr, *Rhizobium radiobacter* CECT472; Pcc, *Pectobacterium carotovorum sbsp. carotovorum* CECT225; Ea, *Erwinia amylovora* PMV6076; Xf, *Xanthomonas arboricola* pv. fragariae CFBP3549; Xv, X. *axonopodis* pv. vesicatoria CFBP3275; Cmm, *Clavibacter michiganensis subsp. michiganensis* CECT790. The distributions are separated by the number of simultaneous AMP gene marker per isolate (GN) that is indicated in the upper right corner of each panel.

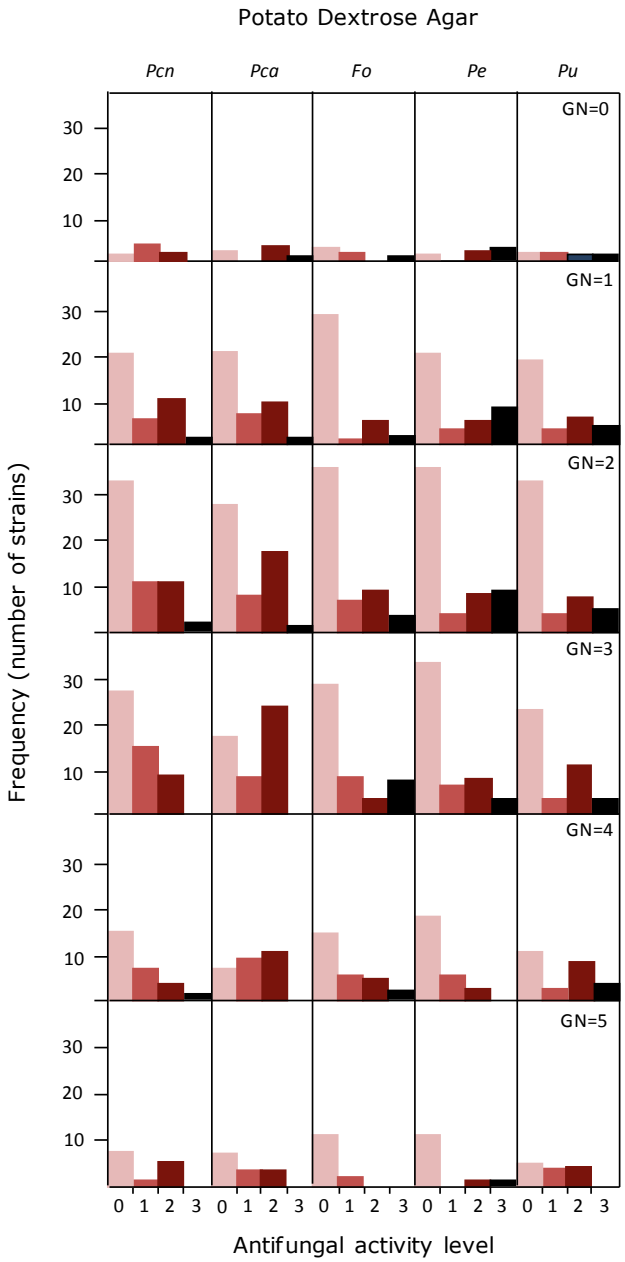

Figure 4.3. Frequency distribution of *Bacillus* isolates according to the antifungal activity in PDA against five plant pathogenic fungi in relation to the number of simultaneous AMP genes per isolate. Pcn, *Phytophthora cinnamomi* CECT2965; Pca, *P. cactorum* F490; *Fo*, *Fusarium oxysporum* sp *lycopersici* ATTC201829 (FOL3 race 2); *Pe*, *Penicillium expansum* EPS26; *Pu*, *Pythium ultimum* CECT2364. The distributions are separated by the number of simultaneous AMP gene marker per isolate (GN) that are indicated in the upper right corner of each panel.

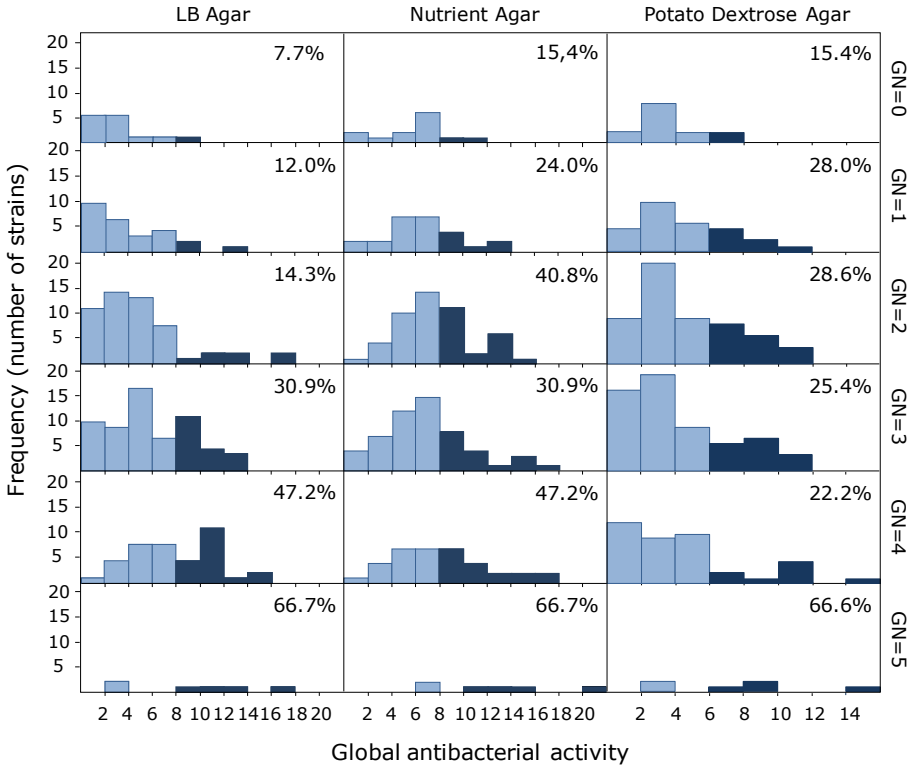

Figure 4.4. Frequency distribution of *Bacillus* isolates based on the global antibacterial activity index(GAI) against bacterial plant pathogens in NA and LB agar, and against fungal plant pathogens in PDA agar, in relation to the number of simultaneous AMP genes per isolate. Each panel corresponds to the distribution of isolates grouped by the number of antimicrobial peptide gene markers. Upper right corner in each panel indicate the number of simultaneous genes (GN) and the percentage of isolates with a high activity, a high GAI for antibacterial assays performed in LB agar and NA medium corresponds to total inhibitory activity higher than 8 (also as highlighted black bars), and a high GAI for antifungal assays performed in PDA medium corresponds to activity higher than 5.

A similar behavior was observed against fungal pathogens with an increase of highly active isolates when increasing the number of genes. However, the percentage of highly active isolates remains stable with one, two, three and four biosynthetic genes, with percentages of 28.6 %, 25.4 % and 22.2 %, respectively, being the maximum percentage of highly active isolates (66.6 %) for five genes.

Globally, the frequency distribution of isolates with high or moderate antimicrobial activity evaluated against eight species of plant pathogenic bacteria in LB medium showed significant differences ($p<0.05$) according to the number of simultaneous AMP biosynthetic genes detected.

The frequency of highly active isolates with simultaneous AMP genes was three times greater than the frequency of isolates with a low number of AMP genes. In contrast, no differences were observed in frequencies of isolates when antibacterial activity was tested in NA and antifungal activity in PDA medium (Fig. 4.5).

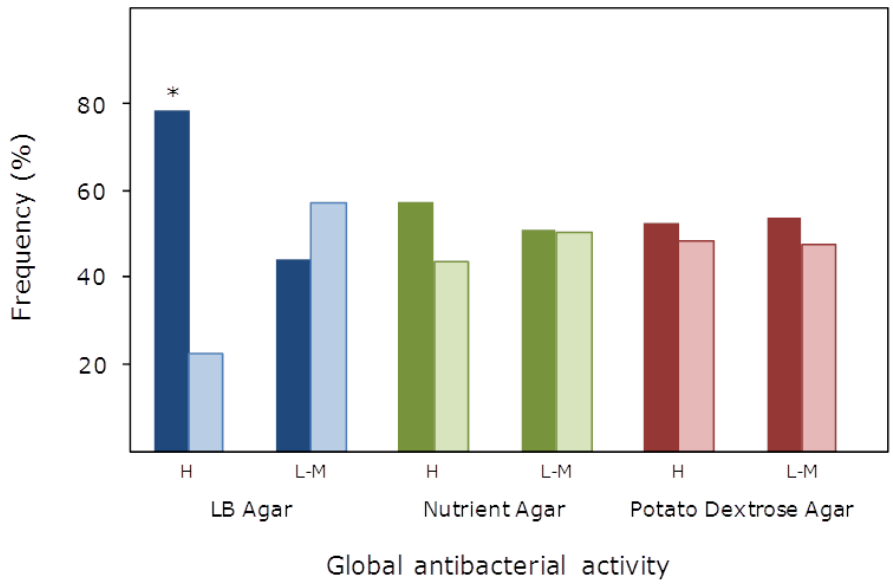

Figure 4.5. Frequency of isolates with high presence of simultaneous AMP genes (dark bars) or low presence of AMP genes (light bars) according to the high (H) or low-moderate (L-M) global antimicrobial activity index (GAI) in LB, NA and PDA media. Asterisk indicates that the proportion of isolates for simultaneous AMP genes is significantly different between high and low antimicrobial isolates, according to the Chi-squared tests ($P<0.05$).

1.2. Type of AMP genes per isolate and antimicrobial activity

Figure 4.6 shows the proportion of isolates with a given specific antimicrobial peptide biosynthetic gene comparing isolates with high and isolates with low antibacterial activity. In LB medium the proportion of isolates with *srfAA*, *bmyB* and *fenD* genes was significantly higher in the population of highly active isolates (GAI >8) than in the low or non-active. In contrast, no significant differences in antimicrobial activity were observed in isolates with *bacA*, *spaS* and *ituC* genes.

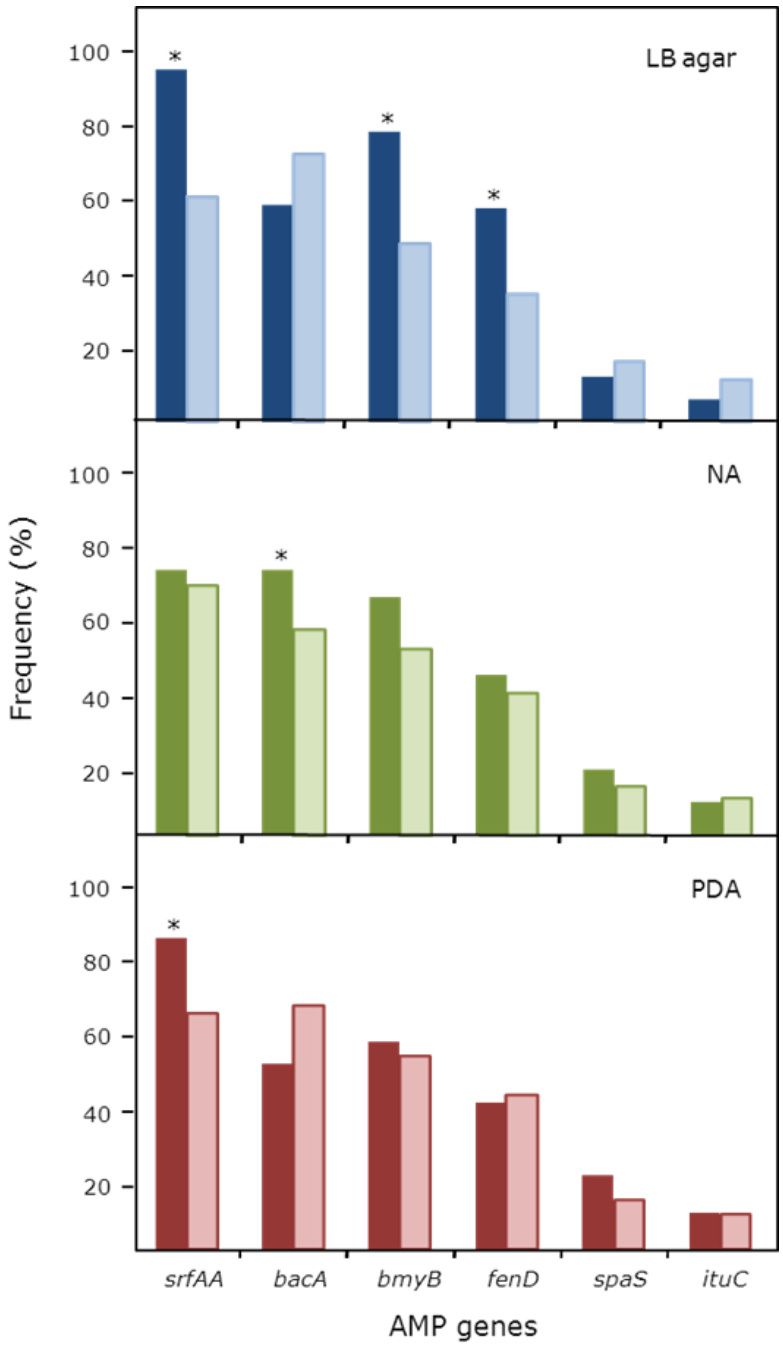

Figure 4.6. Proportion of isolates with a given antimicrobial peptide biosynthetic gene within isolates with high (dark bars) or low-moderate (light bars) global antimicrobial activity index (GAI) in LB agar, NA and PDA medium. Asterisk indicates that the proportion of isolates with presence of specific AMP gene is significantly different between isolates with high and low antimicrobial activity, according to Chi-squared test ($P<0.05$).

In NA medium, the proportion of isolates with *bacA* was significantly higher in the population of active isolates, and the frequency of isolates with *bmyB* gene was also higher in the population of highly active isolates than in the low or non-active though the differences were not significant. Finally, the proportion of isolates with *srfAA* gene differed significantly between isolates with high and low antifungal activity, higher proportion in highly active isolates (GAI >5) than in the low or non-active.

When the pattern of AMP genes was compared to the antimicrobial activity, it was observed that among isolates with high antibacterial activity in LB agar the pattern *srfAA-bacA-bmyB* was present in a 63.3 % of isolates, whereas only in 24.3 % in isolates with low antibacterial activity. Also the pattern *srfAA-bacA-bmyB-fenD* was present in 38.8 % of isolates with high antibacterial activity in LB medium, whereas only in 8.8 % of isolates with low antibacterial activity. In contrast, low differences were observed in the frequency of patterns *srfAA-bacA-bmyB* and *srfAA-bacA-bmyB-fenD* among isolates with high and low antimicrobial activity in NA and PDA medium. Although the pattern *srfAA-bacA-bmyB* was present in 40.3 % of isolates with high antibacterial activity in NA medium and in 32.0 % of isolates with high antifungal activity, the same pattern was present in in 31.6 % of isolates with low antibacterial activity in NA medium and 35.8 % of isolates with low antifungal activity. Similar results were observed with the pattern *srfAA-bacA-bmyB-fenD* that was present in 22.4 % and 20.0 % of isolates with high activity and in 13.7 % and 15.7 % of isolates with low activity against bacteria in NA medium and against fungi, respectively.

1.3. Correspondence analysis of the number and type of AMP genes and antimicrobial activity

The correspondence analysis performed between antimicrobial activity (low GAI/high GAI), number of simultaneous genes (low <3, high ≥3) and type of antimicrobial peptide biosynthetic genes (presence, absence) is shown in Figure 4.7. For the antibacterial activity, the first dimension was described by the number of AMP genes per isolate (0.745), *srfAA* (0.440), *bmyB* (0.438), *bacA* (0.398) and antibacterial activity (0.264) in LB medium.

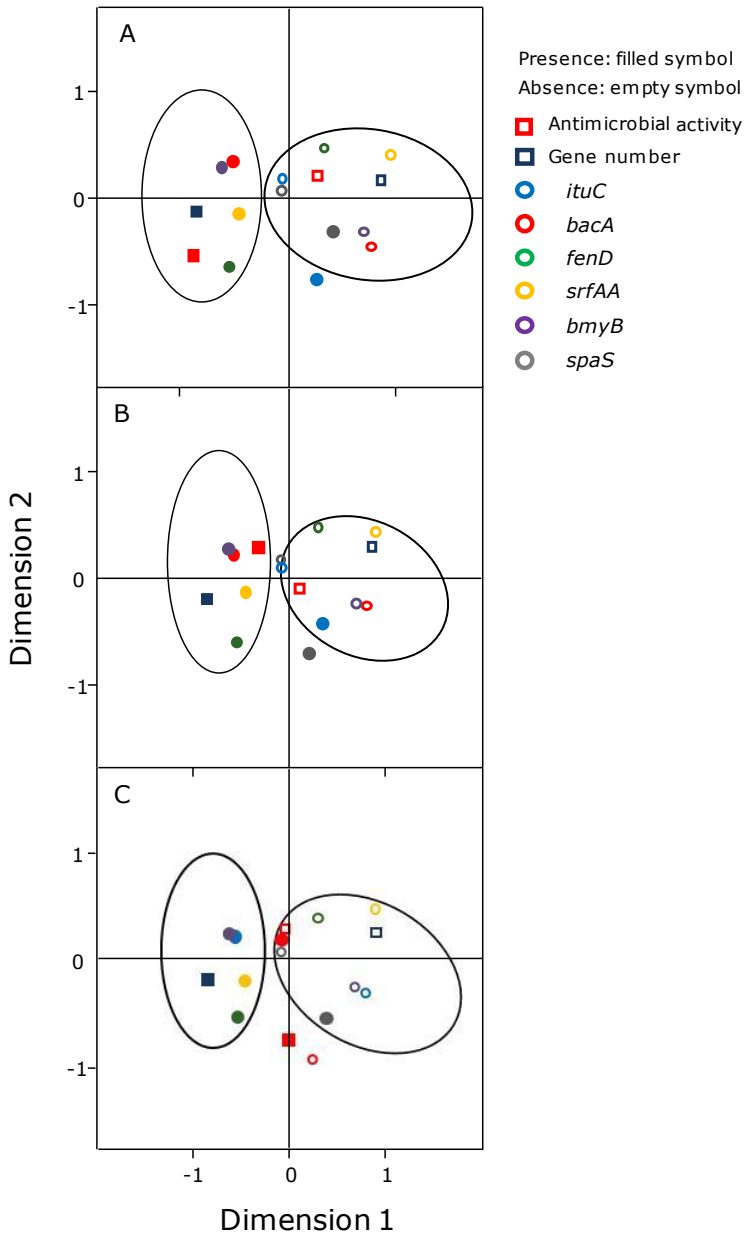

Figure 4.7. Map of the correspondence analysis of the antibacterial activity (GAI low or high), AMP biosynthetic gene number (lower or equal/higher than three simultaneous genes) and presence of *srfAA*, *bacA*, *fenD*, *bmyB*, *spaS* or *ituC* gene markers, for the 184 *Bacillus* isolates from field samples. Colored and white symbols correspond to a positive or negative score, respectively. The two main groups of variable scores defined by the dimensions are highlighted. *In vitro* testing was performed against bacteria in LB (A), NA (B) and against fungi in PDA (C).

However, in NA medium it was represented by the number of AMP genes per isolate (0.756), *bacA* (0.466), *bmyB* (0.451), and *srfAA* (0.406), but not by the antibacterial activity. The second dimension was mainly composed of *fenD* (0.287 in LB and 0.347 in NA) (Fig. 4.7, A and B). Also, the analysis for antifungal activity showed that the first dimension was represented by the same variables, *bacA* (0.459), *bmyB* (0.448), *srfAA* (0.414) and the number of AMP genes per isolate (0.768) though not represented by the antifungal activity. However, the second dimension was mainly composed of *fenD* (0.260) and the antifungal activity (0.248) (Fig. 4.7-C).

Hence, the analysis confirmed two main groups of variable scores defined by the two dimensions, explaining 45.1 % of the variability in LB, 43.1 % of the variability in NA for antibacterial assays, and 43.4 % of the variability in PDA medium for antifungal activity. In the case of antibacterial activity, independently of the medium used, the first group was composed of isolates with low antibacterial activity associated to a low number of AMP genes and absence of *srfAA*, *bacA*, *bmyB*, and *fenD* genes. The second group was composed of isolates with high antibacterial activity associated to multiple presence of AMP genes (*srfAA*, *bacA*, *bmyB*, and *fenD*). Besides, antibacterial activity was defined more clearly in LB medium than in NA medium. Finally, in the case of antifungal activity, the first group was composed of isolates with high antifungal activity associated to low number of AMP genes and the presence of *ituC* gene. The second group was composed of isolates with high number of simultaneous AMP genes associated to presence of *srfAA*, *bacA*, *bmyB* and *fenD* genes.

2. AMP genes and AMP products

In order to determine the relationship of the production of AMPs with the presence of AMP genes, a selection of 64 *Bacillus* isolates from natural environments was used. In particular, AMP products were analyzed by a binary array according to the individual peak detection and grouping peaks in families of cLPs, which were related to simultaneous detection of biosynthetic genes and also with the presence of specific AMP genes

associated to the identified products. Thus, *ituC* and *bmyB* genes were related with the production of iturins, *fenD* gene was related with the production of fengycins and *srfAA* gene was related with the production of surfactins.

According to the results, the production of iturins, fengycins and surfactins was not related to the simultaneous detection of biosynthetic genes within the isolates, being the production of these compounds similar in isolates with high and low number of AMP genes (Fig. 4.8-A). However, a tendency to increase the number of products produced by isolates with high simultaneous biosynthetic genes was observed, and significant differences were observed in the production of certain compounds included in each family of cLPs. Particularly, six groups of compounds corresponding to fractions I3, F1, F2, F5, S1 and S2 (Fig. 4.9-B, C and D) were detected more frequently among isolates containing high number of AMP genes simultaneously.

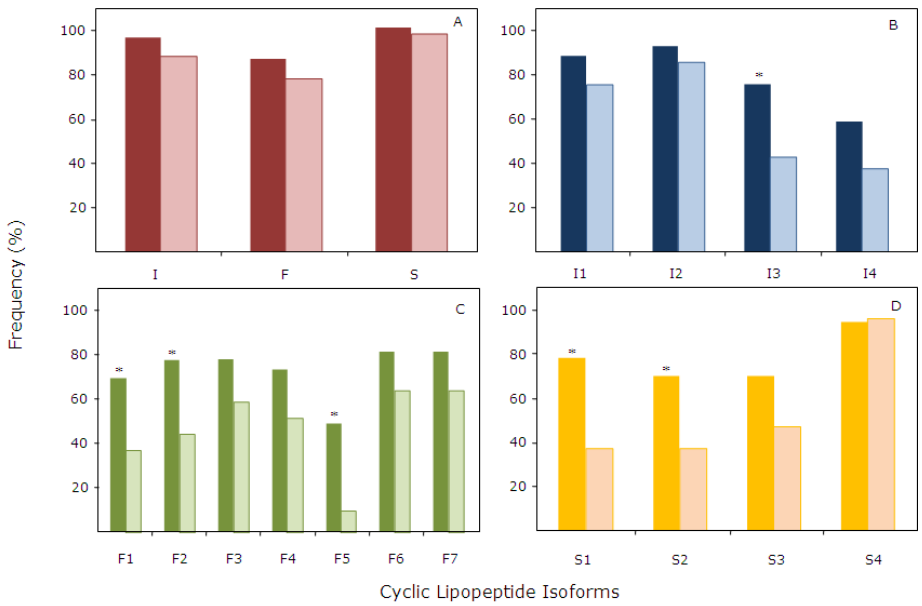

Figure 4.8. Proportion of AMP producing isolates in a population with high (dark bars) or low (light bars) presence of simultaneous AMP genes. Global production of cyclic lipopeptides itruin (I), fengycin (F) and surfactin (S) families (A), production of iturin isoforms (I1, I2, I3 and I4)(B), specific fengycin isoforms (F1, F2, F3, F4, F5, F6 and F7) (C) and surfacin isoforms (S1, S2, S3 and S4)(D). Asterisk indicates that the proportion of producing isolates is significantly different between the presence or absence of simultaneous AMP genes detected, according to Chi-squared test ($P<0.05$).

Additionally, the proportion of isolates with the individual genes *bmyB* (50 %), *ituC* (21.8 %), *fenD* (34 %) and *srfAA* (48 %) in the *Bacillus* isolates was lower than the proportion of isolates producing iturins (90.6 %), fengycins (81.2 %) and surfactins (98.4 %), respectively. Only the presence of *fenD* gene was significantly related with the production of fengycins within the isolates under study (Fig. 4.10).

While the presence of biosynthetic genes *bmyB*, *ituC* and *srfAA* was not significantly correlated with the production of iturins and surfactins, respectively, except in the case of I1. In the 100 % of isolates containing the biosynthetic genes *ituC*, *fenD* and *srfAA* it was detected the production of iturins, fengycins and surfactins, respectively. In contrast, only 87 % of isolates with *bmyB* gene produced iturins.

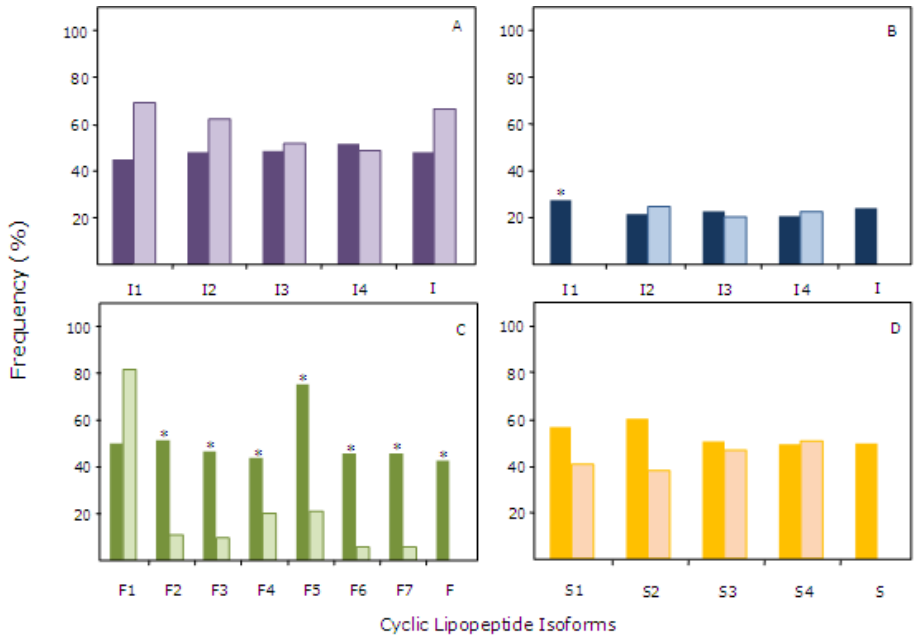

Figure 4.9. Proportion of isolates with *bmyB* (A), *ituC* (B), *fenD* (C) and *srfAA* (D) genes that produce (dark bars) or not produce (light bars) the corresponding cyclic lipopeptides. HPLC elution peaks associated to iturins (I1, I2, I3 and I4), fengycins (F1, F2, F3, F4, F5, F6 and F7), and surfactins (S1, S2, S3, and S4), and group of peaks according to cyclic lipopeptide family iturins (I), fengycins (F) and surfactins (S). Asterisk indicates that the proportion of isolates within individual AMP gene was detected is significantly different between isolates that produce or not produce the corresponding AMP, according to Chi-squared test ($P<0.05$).

Finally, the correspondence analysis that related the type of AMPs, according to the 15 chromatography peaks, and the type of AMP genes was performed. The analysis confirmed two main groups of variable scores defined by the two dimensions, explaining a 49 % of the variability.

The first dimension was described by the presence of fengycins, surfactins and the gene *fenD*: S1 (0.703), F3 (0.662), F4 (0.649), F2 (0.648), S2 (0.636), F1 (0.626), F6 (0.584), S3 (0.574), F7 (0.482) and *fenD* (0.340). The second dimension was described by the presence of iturins: I3 (0.590), I4 (0.390), I1 (0.327), I2 (0.201). There were two groups of variables. The first group was mainly composed of isolates with low presence of *srfAA*, *bacA*, *fenD* and *bmyB*, as well as, low production of iturins, fengycins and surfactins. The second group was composed of isolates with antimicrobial peptide genes (*srfAA*, *bacA*, *bmyB* and *fenD*) and high production of iturins, fengycins and surfactins (Fig. 4.10).

3. AMP products and antimicrobial activity

The production of AMP products was related with the antimicrobial activity in *Bacillus* isolates from natural environments. As in the previous section, AMP products were analyzed according to the individual peak detection and grouping peaks (isoforms) in the cLPs families, and were related to the antimicrobial activity against eight bacterial plant pathogens and five fungal plant pathogens.

The proportion of active isolates in LB agar (28 %), NA agar (56 %) and PDA agar (42 %) was lower than the proportion of isolates producing iturins (90.6 %), fengycins (81.2 %) and surfactins (98.4 %). However, 100 % of the active isolates against bacterial plant pathogens in LB agar medium produced at least one type of cLPs. In addition, more than 85 % of the active isolates in NA medium and more than 90 % of the active isolates in PDA medium also produced at least one type of cLPs. Figure 4.11 illustrates the frequency of isolates that produce the three families of cLPs (iturins, fengycins and surfactins), as well as the individual products isoforms in a population with high or low-moderate antimicrobial activity.

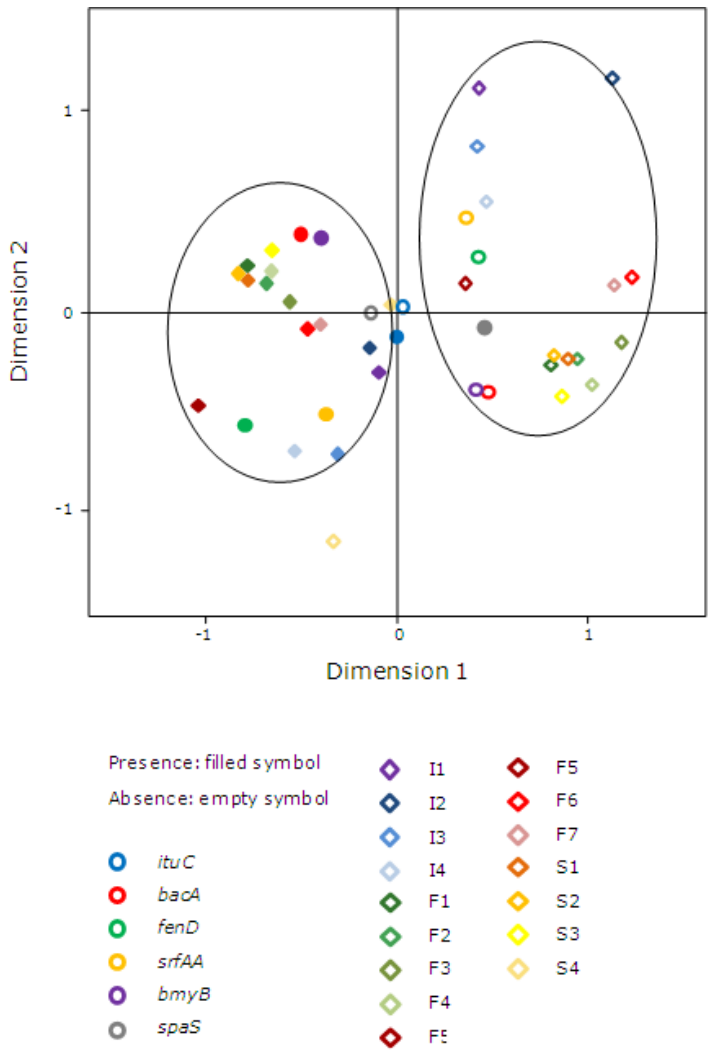

Figure 4.10. Map of the correspondence analysis of the type of AMPs following the 15 chromatography isoform peaks for iturins (I1, I2, I3 and I4), fengycins (F1, F2, F3,F 4, F5, F6 and F7) and surfactins (S1, S2, S3 and S4, and presence of *srfAA*, *bacA*, *fenD*, *bmyB*, *spaS* or *ituC* gene markers, for the 64 *Bacillus* isolates from field samples. Colored and white symbols correspond to a positve or negative score, respectively. The two main groups of variable scores defined by the dimensions are highlighted

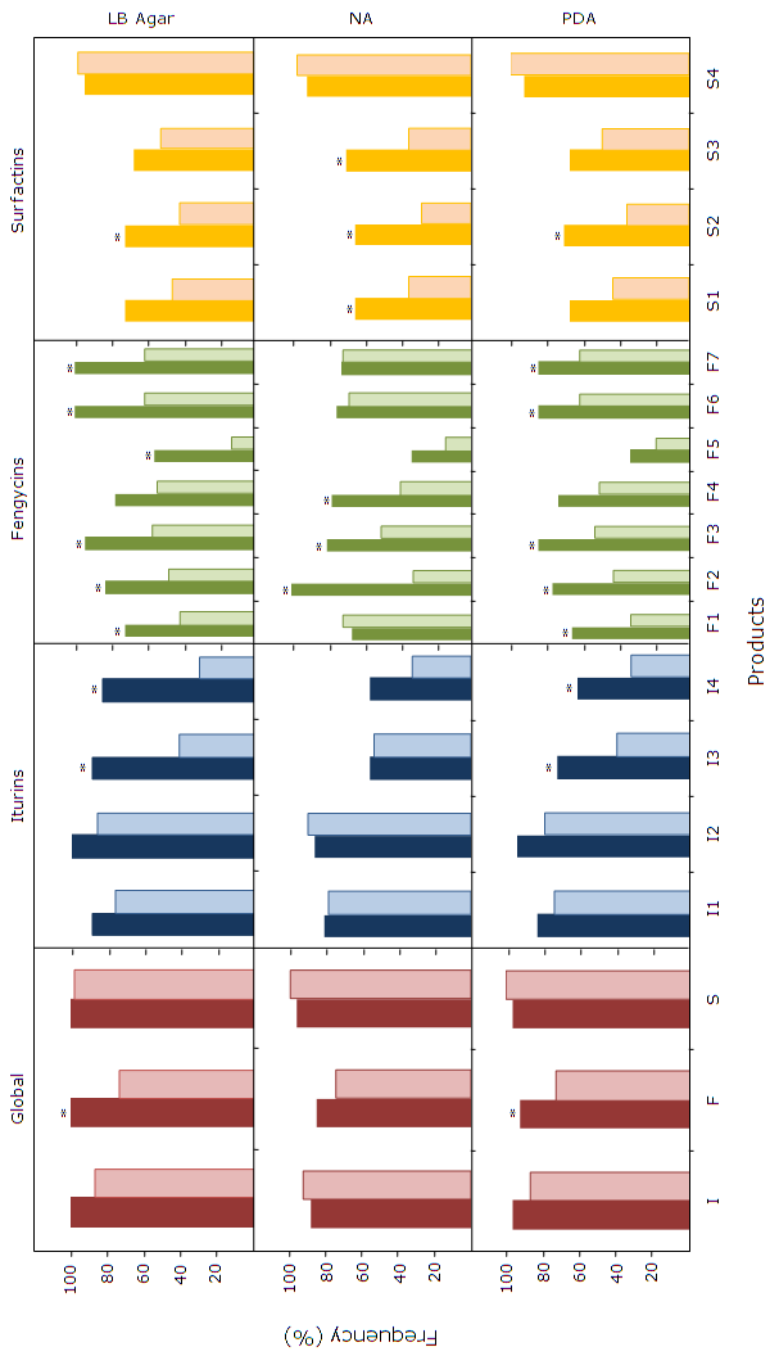

Figure 4.11. Proportion of isolates that produce iturins (I, I1, I2, I3 and I4), fengycins (F, F1, F2, F3, F4, F5, F6 and F7) and surfactins (S, S1, S2, S3 and S4) in a population with high (dark bars) or low (light bars) antimicrobial activity against 8 bacterial plant pathogens in LB and NA 5 fungal pathogens in PDA. Asterisk indicates that the proportion of isolates that produce a family or a specific type of AMP is significantly different between active on not active isolates, according to Chi-squared test (P<0.05).

According to the results, only the production of fengycins was significantly higher in active isolates in LB and PDA.While no significant differences were observed in other cases. When products were analyzed individually, it was observed that several products were more frequently detected among highly active isolates rather than in the low active. Concretely, fenycins F1, F2, F3, F6 and F7, iturins I3 and I4, and surfactin S2 were detected more frequently in isolates highly active against bacterial pathogens in LB, and against fungal pathogens in PDA. On the contrary, only fengycins F2, F3 and F4, and surfactins S1, S2 and S3 were detected more frequently in active isolates against bacterial pathogens in NA. In addition, the production of F5 was significantly higher in active isolates against bacterial pathogens in LB.

The correspondence analysis that related the type of AMPs, following the 15 HPLC chromatography peaks, and the antimicrobial activity against bacterial and fungal plant pathogens is shown in Figure 4.12. The analysis confirmed two main groups of variable scores defined by the two dimensions, explaining a 50.7 % of the variability. The first dimension was described by the presence of fengycins and surfactins: F3 (0.697), S1 (0.690), F4 (0.662), F2 (0.659), S2 (0.642), F1 (0.630), S3 (0.564), and F6 (0.630). The second dimension was described by the presence of iturins: I3 (0.577), I4 (0.381), I1 (0.321), I2 (0.201). There were two groups of variables, the first group was mainly composed of isolates with low production of iturins, fengycins and surfactins, as well as, low-moderate antibacterial activity in LB and NA media and low-moderate antifungal activity in PDA. The second group was composed of isolates with high production of iturins, fengycins and surfactins, and high antibacterial activity in both medium, LB and NA, and high antifungal activity in PDA.

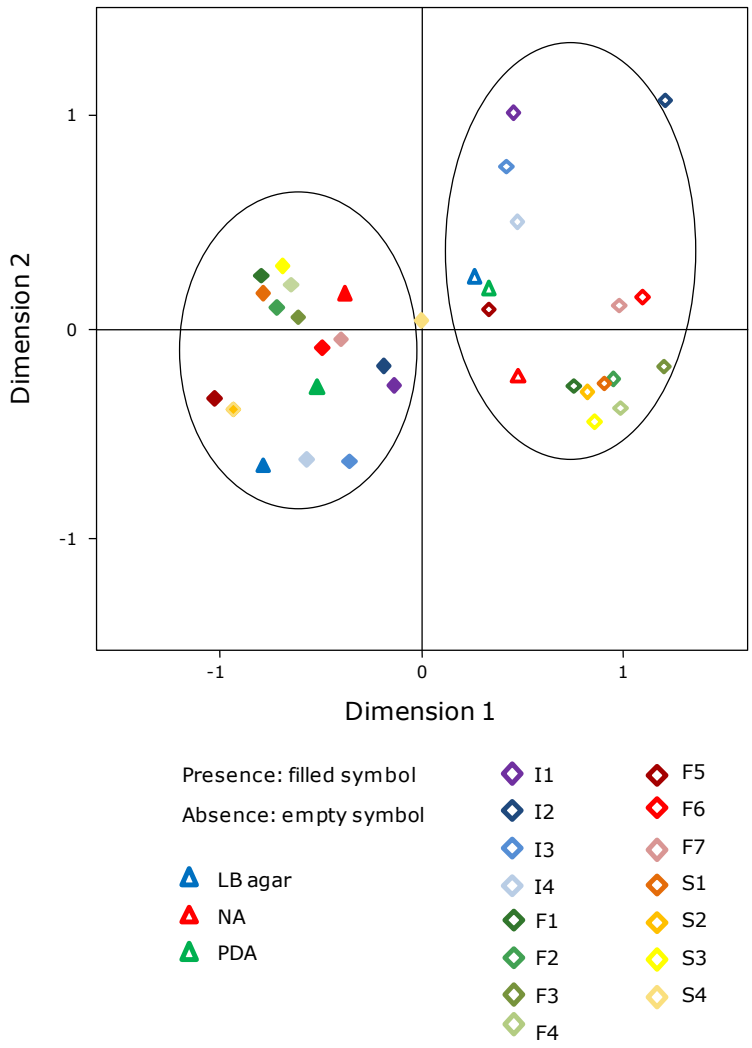

Figure 4.12.Map of the correspondence analysis of the type of AMPs following the 15 HPLC chromatography peaks (isoforms) for iturins (I1, I2, I3 and I4), fengycins (F1, F2, F3,F4, F5, F6 and F7) and surfactins (S1, S2, S3 and S4) and antimicrobial activity. Antibacterial activity was tested in LB agar and NA medium and antifungal activity was tested in PDA medium, for the 64 *Bacillus* isolates from field samples. Colored and white symbols correspond to a positive or negative score, respectively. The two main groups of variable scores defined by the dimensions are highlighted.

4. Global analysis of genes, AMPs and antimicrobial activity

To study the relationships between AMP genes, AMPs production and antimicrobial activity, the 64 *Bacillus* isolates in which all these parameters were measured were used. In addition the reference isolate QST713 was included, and also the origin of each sample type was added as an informative variable.

A binary array was performed including 24 variables. These variables were the presence of AMP products according to the HPLC elution peaks (I1, I2, I3, I4, F1, F2, F3, F4, F5, F6, F7, S1, S2, S3 and S4), presence of AMP genes (*srfAA*, *bacA*, *bmyB*, *fenD*, *spaS* and *ituC*) and activity against bacterial (in LB and NA media) and fungal (in PDA medium) plant pathogens. The isolates were classified by a hierarchical cluster analysis using the Ward's minimum variance criterion calculated by total within-cluster variance according to squared Euclidean distance. Figure 4.13 shows the resulting dendogram where four groups at rescaled distance of 8 were obtained, each one with 16, 14, 21 and 14 isolates. Each group of isolates was made up according to significant variables (Table 4.1). Some variables, such as detection of I1 and S2 products, and the presence of *bmyB*, *ituC* and *spaS* genes were not significant to determine the association of variables.

The greatest differences were found between groups A and D, so that the first group included antimicrobial active isolates that produced most products identified by chromatographic and mass spectra techniques, and from which was detected a high number of simultaneous antimicrobial genes. The fourth group D consisted of low active isolates, concretely in PDA and LB agar medium, from which few cyclic lipopeptides were detected. Group B was characterized by less active isolates than in the first group, non active in LB, but a large percentage of active isolates in NA medium and a relative low percentage of isolates showed antifungal activity. On the other hand, detection of simultaneous biosynthetic genes was still observed, but much less frequently than in the group A.

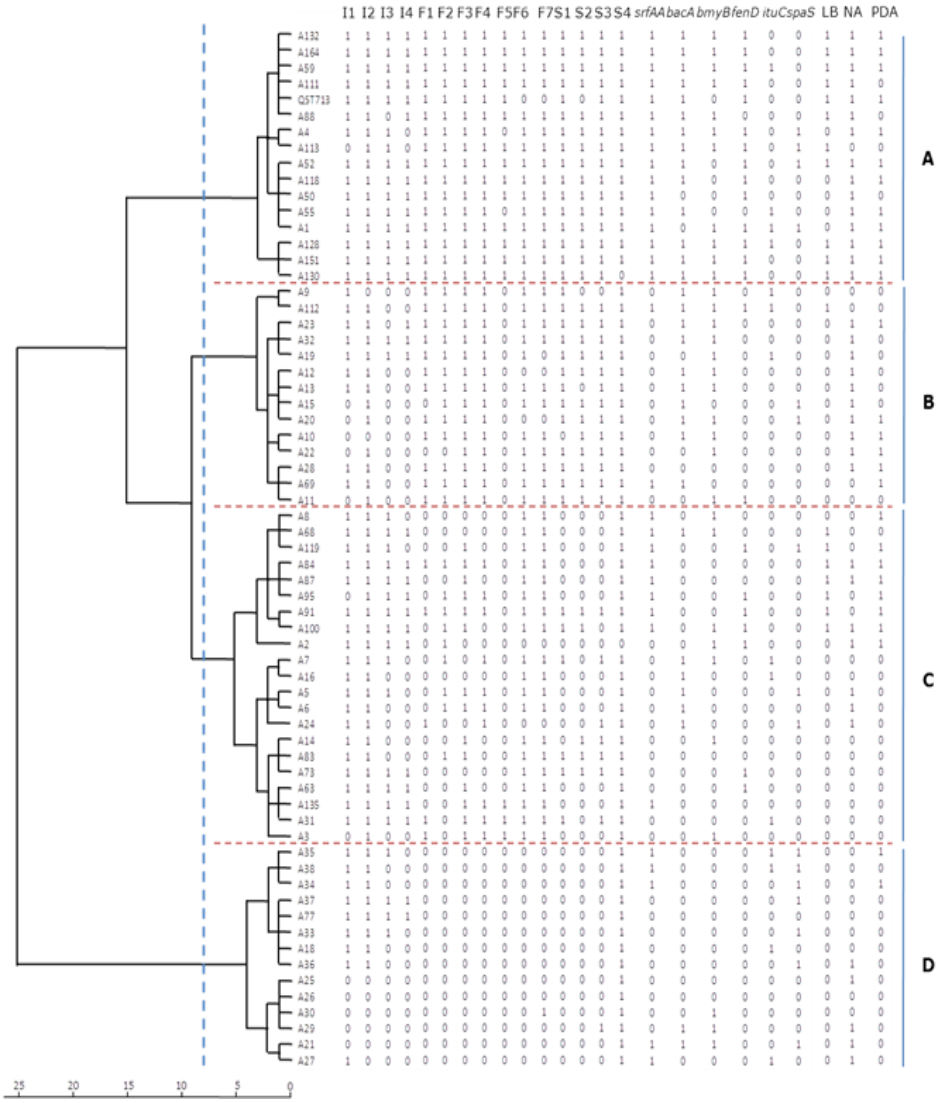

Figure 4.13. Dendrogram of the relatedness between 64 isolates of *Bacillus,* and strain QST713 as a reference, according to the detection of 6 AMP biosynthetic genes (*srfAA, fenD, bmyB, bacA, spaS* and *ituC*), production of components related to the three families of antimicrobial cyclic lipopeptides (iturins (I), fengycins (F) and surfactins (S)), and antimicrobial activity, depending on the growth medium used during the assay (LB and NA for bacteria and PDA for fungi). The similarity matrix between bacterial isolates was calculated using the squared euclidean distance at the discretion of Ward's. Cluster analysis was performed by applying the Agglomerative Hierarchical group method through cluster analysis procedure of IBMSPSS package.

Table 4.1. Significant variables that influence the classification of groups obtained by cluster analysis according to Ward's method in 64 isolates of *Bacillus.*

	Group A		**Group B**		**Group C**		**Group D**	
	presence	% of isolates	presence	% of isolates	presence	% of isolates	Presence	% of iisolates
I2							-	< 70
I3	+	≥ 80						
I4	+	≥ 80	-	≥ 80				
F1	+	≥ 90			-	≥ 80	-	≥ 90
F2	+	100	+	≥ 90	-	≥ 70	-	≥ 90
F3	+	100					-	100
F4	+	≥ 90	+	≥ 90			-	≥ 90
F5	+	≥ 70	-	100			-	100
F6	+	100			+	≥ 90	-	100
F7	+	100			+	≥ 90	-	≥ 90
S1	+	100	+	≥ 90	-	≥ 80	-	≥ 80
S2	+	100	+	≥ 80	-	≥ 80	-	100
S3	+	≥ 90	+	≥ 90	-	≥ 80	-	≥ 80
srfAA	+	100	-	≥ 90				
bacA	+	≥ 70					-	≥ 80
fenD	+	≥ 80	-	≥ 90			-	≥ 90
LB	+	≥ 70	-	≥ 90			-	100
NA	+	≥ 80			-	≥ 70		
PDA	+	≥ 70						

Gaps indicate that the variable was not particularly influential for the classification of the specific group.

However, the production of cyclic lipopeptides decreased slightly, especially of iturins. Group C consisted of low active isolates against bacteria and fungi. Simultaneous biosynthetic genes was slightly reduced in comparison to group B, but drastically reduced in comparison to group A. Although it was detected multiproduction of antimicrobial components related with iturin, fengycin and surfactin families, in this group there was a considerable reduction of the production of surfactins and fengycins respect to isolates from groups A and B. In detail, group A consists of isolates producing I3, I4, F1, F2, F3, F4, F5, F6, F7, S1, S2 and S3, containing *srfAA*, *bacA* and *fenD* genes, and high active in LB agar, NA and PDA medium. Group B was mainly composed of isolates that produce I4, F2, F4, F5, S1, S2 and S3, presence of *srfAA* and *fenD* genes, and high antibacterial activity in LB agar. Isolates in the group C produce F6 and F7, but not produce or produce in very low concentrations F1, F2, S1, S2 and S3, and were not active against bacterial plant pathogens in NA. Finally, group D

consist of isolates with low or absence of I2, F1, F2, F3, F4, F5, F7, S1, S2 and S3, low presence of *bacA* and *fenD* genes, and low antibacterial activity in LB agar.

The correspondence analysis performed using these variables is shown in Figure 4.14. The analysis confirmed two main groups of variable scores defined by the two dimensions, explaining a 43.72 % of the variability. The first dimension was represented by the AMP products, specifically with surfactin S1 (0.638) and S2 (0.585) and fengycins F2 (0.592), F3 (0.630) and F4 (0.592). The second dimension was mainly described by the AMP products related with iturin family I2 (0.310), I3 (0.493) and I4 (0.274), and biosynthetic gene *srfAA* (0.245). The variables were divided in two groups. The first group, situated on the left part of the panel was composed of high number of simultaneous AMP genes, presence of *srfAA*, *bacA*, *bmyB* and *fenD* genes and production of AMPs related with iturin fengycin and surfactin families (I1, I2, I3, I4, F1, F2, F3, F4, F5, F6, F7, S1, S2, S3 and S4) and associated to a high antibacterial and antifungal activity. The second group showed on the right part of panel, was composed of low number of simultaneous AMP genes, absence of *srfAA*, *bacA*, *bmyB* and *fenD* genes, and low production of AMPs related with iturin, fengycin and surfactin families.

The four groups of isolates obtained by means of hierarchical cluster analysis were associated to different areas of the map of correspondence analysis (Fig. 4.14). Thus, group A was located in the lower left panel, characterized by isolates with *fenD* and *srfAA* genes, active against bacterial plant pathogens in both tested growth medium, mainly iturin producers and isolated from soil samples. Group B was located in the upper left panel, characterized by isolates with *bacA* and *bmyB* genes, active against fungal plant pathogens, fengyicin and surfactin producers and isolated from rhizosphere samples. Groups C and D were located in lower right panel, mainly composed of isolates without *bacA* and *bmyB* genes, not active against fungal plant pathogens, not producers of fengycins and surfactins and isolated from aerial plant parts.

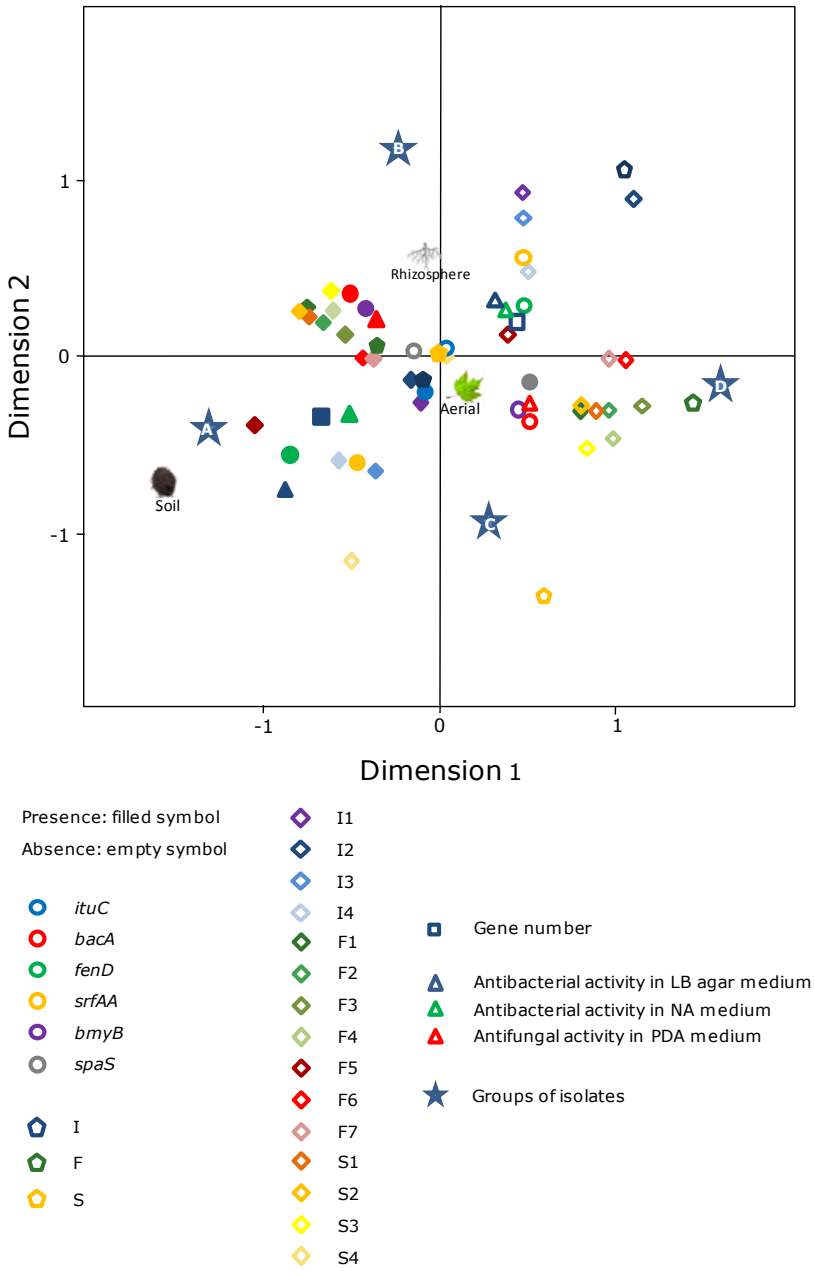

Figure 4.14. Map of the correspondence analysis of 64 *Bacillus* isolates according to AMP biosynthetic gene number (lower or equal/higher than three simultaneous genes), presence of *srfAA*, *bacA*, *fenD*, *bmyB*, *spaS* or *ituC* gene markers, production of AMPs related to iturin, fengycin or surfactin families I1, I2, I3, I4, F1, F2, F3, F4, F5, F6, F7, S1, S2, S3 or S4 (presence/absence) and adding the supplementary information of the antibacterial activity (GAI low or high). Colored and white symbols correspond to a positive or negative score, respectively. The two main groups of variable scores defined by the dimensions are highlighted. Distribution of four groups obtained after *Bacillus* classification according to their characteristics is indicated with a star.

Discussion

The characterization of a collection of *Bacillus* isolates from natural environments according to the presence of six AMP gene markers, production of cLPs and capacity to inhibit bacterial and fungal plant pathogens allowed to determine the relationships between these characteristics in a *Bacillus* population.

In this study, the clustering analysis of 64 isolates from natural environments revealed four groups, two of them well defined and consistent. The first of these groups was mainly composed of the most antimicrobial and AMP products with a higher number of simultaneous AMP genes. The second and third groups were very heterogeneous according to the presence of AMP genes and antimicrobial activity, but differed in terms of surfactin production. Finally, the fourth group was mainly composed of the less active isolates, fewer producers with none or low number of AMP

genes. The homogeneity of the first group allowed us to speculate that selecting an isolate increase the chances to obtain a potential biocontrol agent with consistent production of CLPs and with wide range of antimicrobial activity. Therefore, this opens the possibility to determine a characteristic pattern or markers from isolates of this group in order to define a profile related with the potential biocontrol capabilities.

These relationships were analyzed in a different maner by a correspondence analysis. Only around 43 % of the variability was explained on the basis of these three parameters. The unexplained variability is probably due to the methodological limitations, such as the limited sensitivity and specificity of primers used, the limited production of AMPs in the media used or the limited number of genes and products analyzed. Thus, it is possible that some strains can produce other AMPs, different from the six studied by PCR and the four detected by HPLC in the present work, that might be implicated in the synthesis of antimicrobial compounds reported in *Bacillus*, such as the poliketides difficidin (Chen *et al*. 2009c), bacillaene (Patel *et al*. 1995), the dipeptide bacilysin (Chen *et al*. 2009c) or lantibiotics subtilin and ericin (Stein 2008).

Nevertheless, the correspondence analysis confirmed the results obtained with cluster analysis. Two main groups were clearly differentiated, a first including isolates with high presence of biosynthetic genes, high antimicrobial activity and high production of AMPs. Contrarily, the second group consisted of low active isolates, with low presence of biosynthetic genes and low production of AMPs. This was very interesting, because there was a close relationship between the high presence of molecular markers and the production of their associate expression products, as well as higher antimicrobial activity.

When the first group was analyzed more in detail, it was observed a close cluster of isolates with high antibacterial activity, especially in LB medium, obtained from soil samples associated with the presence of *fenD* and *srfAA* genes, and with the production of compounds included in the fractions I3, I4 and above all F5. In addition, antifungal activity was associated to isolates from rhizosphere samples, presence of genes *bacA* and *bmyB*, and

the production of compounds related to fractions F1, F2, F3, F4, F6, F7, S1, S2 and S3. However, the division of antibacterial and antifungal isolates using the correspondence map was not clear and even less when the four groups of isolates from Ward's classification were located on the correspondences map.

The characterization of *Bacillus* isolates allowed also to determine the pairwise relationships between antimicrobial activity, the presence of type and number of simultaneous AMP marker genes and multiple AMP production.

The presence of AMP genes was related to the antagonism against plant pathogens. Thus, an increase of activity was found when the number of simultaneous genes increased, especially in isolates containing 5 simultaneous genes. This is in agreement with the observation that antimicrobial activity, especially against fungal pathogens, seems to be related with the multi-production of AMPs (Romero *et al*. 2007) and also with the presence of multiple AMP biosynthetic genes (Joshi and McSpadden-Gardener 2006). Although a general effect of the number of simultaneous genes on the antimicrobial activity was observed, a different behavior was observed in function of medium used to test the antimicrobial activity. However, a significant effect in the percentage of high active isolates according to the number of simultaneous genes was only observed when the activity was tested in LB. In contrast, when the activity was tested in NA and PDA, there was not significant relative presence of simultaneous biosynthetic genes.

The correspondence analysis relating antibacterial activity, AMP gene number and presence of *srfAA*, *bacA*, *fenD*, *bmyB*, *spaS* and *ituC* genes for the 184 *Bacillus* isolates confirmed the relationships between antibacterial activity in LB medium, the number and type of simultaneous AMP genes within isolates and also with the presence of some specific genes, concretely *bmyB*, *fenD* and *srfAA* genes. This is in agreement with the presence of several simultaneous markers of antimicrobial peptide genes in commercial biocontrol strains of *Bacillus* like QST713 and FZB42 previously reported (Joshi and McSpadden-Gardener 2006, Koumoutsi *et al*. 2004).

Also the fact that *Bacillus* strains producing multiple AMPs are efficient in the control of *Fusarium* wilt of chickpea (Landa *et al.* 2004) and of powdery mildew of cucurbits (Cazorla *et al*. 2007, Romero *et al*. 2004), contain 3-to-5 AMP gene markers simultaneously. In our population analysis only around 49 % of the variability was explained by the frequency of the six AMP gene markers studied. The unexplained variability can be attributed to different factors, either related to undetectable genes due to low specificity of primers used, or related with the differential AMP gene expression due to the fact that in some isolates the genes are not expressed under the laboratory conditions used in the present work or to different expression levels of some genes among isolate. These results also indicated that probably other factors not included in the analysis take part in the antimicrobial activity.

Correspondence analysis did not confirm the relationship between antifungal activity and the number and type of AMP genes, suggesting that the markers used in this study were not related with antifungal activity against the fungi tested in the present work or due to assay conditions did not favor the expression of codified products. However, it is also possible that *Bacillus* isolates can express other AMP genes, different from the six studied, that are implicated in the synthesis of antimicrobial compounds reported in *Bacillus*, like iturin A or polyketides, with high antifungal activity (Mannanov and Sattarovam 2001, Stein 2005).

The relationship between the presence of high number of simultaneous AMP biosynthetic genes and production of cLPs including all isoforms corresponding to iturin, fengycin and surfactin families was not confirmed. However, detection of some specific iturins, fengycins and surfactins (certain isoforms) was significantly related with the presence of simultaneous AMP genes. This lack of global relationship between the number of AMP genes and the production of AMPs is probably due to the high efficiency in the detection of AMPs by HPLC in comparison to the limitations of detection and specificity levels by PCR. This effect was especially marked in iturins, some fengycins (F6, F7) and surfactins (S4). When the presence of *ituC*, *bmyB*, *fenD* and *srfAA* genes was related with AMP products iturins, fengycins and surfactins, it was observed that only

the production of fengycins, both individually or as family products, was significantly related with the presence of the *fenD* gene. Moreover, all the isolates that showed *ituC*, *fenD* and *srfAA* genes were producers of iturin, fengycin and surfactin related products. Even, most isolates that were fengycin and surfactin producers did not show *fenD* and *srfAA* genes using the primers designed in the present work. This can be due to limitations in terms of sensitivity of both molecular and chromatographic techniques, but also associated with the intrinsic diversity at genetic level among *Bacillus* isolates that limit the capacity of a primer set to detect all the variations of a specific gene.

In contrast, not all the isolates containing the genes *bmyB* produced bacillomycin or any other iturin, suggesting that detection of these genes not always is corresponded with the production of the codified product. This is in agreement with some reports describing that the presence of antibiotic biosynthetic operons in bacterial strains was not always associated with the production of the gene product. This fact could be due mainly to gene mutation as it has been described in strain 168 that fails to produce the antibacterial peptides surfactin and fengycin due to a frameshift mutation in *sfp* encoding a 4' phosphopantetheinyl transferase which is required to activate (via posttranslational modification) the surfactin and fengycin synthases (Mootz *et al*. 2001, Nakano *et al*. 1991). Also, it is possible a differential production of cLPs in function of growth conditions during the production process (Fahim *et al.* 2012).

Finally, the correspondence analysis confirmed that most isolates with the capability to produce cLPs were multiproducers, and have several simultaneous biosynthetic genes. However, isolates that do not produce or produce few cLPs showed a low presence of biosynthetic genes simultaneously.

The relationship between antimicrobial activity and AMP production was also analyzed. It's important to emphasize that AMP production was performed only in LB broth, while antibacterial activity was carried out in LB and NA, and antifungal activity was determined in PDA. Globally, only the production of fengycin family products was significantly related with the antibacterial

and antifungal activity. Production of surfactin and iturin family products was not related with antimicrobial activity due to their production was detected over 90 % of isolates, and therefore equally distributed among active and not active isolates. According to other studies, surfactins and iturins were the most common lipopeptides synthesized by *Bacillus*, especially surfactins, which perform other functions in addition to antimicrobial activity, such as the ability to form biofilms (Bais *et al*. 2004, Hofemeister *et al*. 2004).

When products from individual elution fractions were analyzed (cLPs isoforms), it was observed that products related to iturin family with higher fatty acid chain length showed a significant relation with antimicrobial activity tested in LB agar and PDA medium. These results were supported by the fact that the length of fatty acid chain of iturins is known to play an important role in their antifungal activity, which increases with increasing the number of carbon atoms (Malfanova *et al*. 2012). Most products related with fengycin family corresponding to the first cluster of peaks detected by analytical HPLC were significantly related with a wide antimicrobial activity, independently of the type of pathogen and growth medium, while fengycin products corresponding to the second cluster of peaks were related only with antibacterial and antifungal activity. Although antibacterial activity of fengycin type cLPs has not been reported in the bibliography, fengycin has shown synergistic effect on antifungal activity in combination with other cLPs such as surfactin and iturin A (Arrebola *et al*. 2010, Athukorala *et al*. 2009, Chen *et al*. 2008) and probably this synergistic activity could take part also against bacterial pathogens. Besides, fengycin production might be a good indicator of potential biocontrol agents against bacterial pathogens due to their ability to induce defenses in plants (Ongena and Jacques 2008). Surfactin isoforms were very common in the *Bacillus* population studied, being C15 the most abundant homologue in agreement with Malfanova *et al.* (2012). The presence of this homologue did not indicate whether the studied population was high or low-moderate active, neither antibacterial nor antifungal. However, the presence of surfactin isoforms with fewer carbon atoms allowed differentiating active from non or low active isolates. Globally, the antibacterial activity in NA could be attributed to the presence of surfactins, and the antibacterial activity in LB

and the antifungal activity could be attributed to the presence of iturins and fengycins.

In conclusion, prevalence of AMP genes in plant-associated populations of *Bacillus* has been demonstrated, and the relationship of the presence of these genes with the antagonistic capacity of the respective isolates and with the synthesis of the expression products was determined.

CHAPTER 5

General Discussion

The interest in bacterial species and strains of the genus *Bacillus* has increased over the last decades in agriculture, animal and food science, and in industry. The importance of these bacteria is due to their high growth rates leading to short fermentation cycles and the capacity to secrete proteins into the extracellular medium, as well as their ability to produce a wide spectrum of products of biotechnological interest, such as enzymes, antibiotics or fine biochemicals, which are used as detergents, flavor enhancers, food supplements and insecticides (Schallmey *et al*. 2004). Particullarly, in the agronomy field, *Bacillus subtilis* and related species are of interest due to their aptitudes in biocontrol of a wide range of bacterial and fungal plant pathogens (Bais *et al*. 2004, Baysal *et al*. 2008, Landa *et al*. 1997, Romero *et al*. 2007). In addition, these species are qualified as safe by public organisms such as the Food and Drug Administration (FDA), in USA, and the European Food Safety Authority (EFSA), in Europe. This aspect is very important in order to get the permit from the authorities at the time of registration and commercialization of a microbial pesticide (Montesinos and Bonaterra 2009).

Owing to the great interest for the *Bacillus* genus, several studies at genotypic, phenotypic, taxonomic and ecological level have been done, showing the great taxonomical and metabolical diversity of this genus, that allow them to live in many environments. Accordingly, the capacity of *Bacillus* to produce highly resistant endospores confers the cells the ability to disperse to new habitats and survive as resting spores until they encounter suitable conditions for germination. *B. subilis* and related species are generally associated with plant root surfaces or bare soils (Earl *et al*. 2008). Accordingly, most *Bacillus* strains capable of controlling plant diseases have been isolated from soil, such as strains QST713 (Borris *et al*. 2011), registered as active substance of a commercial pesticide, or strain FZB42 (Idris *et al*. 2004), the main component of a biofertilizer for use in organic farming.

Although *Bacillus* is widespread, one of the main limitations to study its populations is the difficulty of detection and isolation from their habitats by standard microbiological methods. This limitation has been demonstrated with molecular techniques such as PCR-DGGE, by means of which large populations of unculturable *Bacillus* in soil and associated to plants have been identified (Garbeva *et al*. 2003). Culturable populations of *Bacillus* achieved in the present work ranged from 4 to 7 log cfu/g f.w., but frequently were undetected. This finding was in agreement with the report of McSpadden-Gardener (2004) who described that *Bacillus*culturable populations ranged from 3 to 6 log cell/g f.w. However, during our work, *Bacillus* could not be isolated in most of the samples using standard isolation procedures that are suitable for other bacterial plant inhabitants. Concretely, *Bacillus* isolates were obtained from a 46.6 % of rhizosphere samples, 40 % of soil samples, and only in a 6.6 % of the aerial plant part samples. Due to the wide range of population levels find in the different plant environments studied, we have processed the samples by molecular techniques in order to detect unculturable *Bacillus*. The analysis of the natural samples by PCR directed to *16S rDNA* gene allowed us to confirm that there was a high percentage of *Bacillus* (86.6 %) in aerial plant part samples, which were detected by PCR but were not isolated using standard microbiological procedures. However, a reduced number of *Bacillus* were detected by PCR in soil and rhizosphere samples, while were recovered with

higher frequency by culturable methods. Thus, PCR targetted to AMPs genes permitted us to detect in a more sensitivity manner the *Bacillus* population associated to plants. Unfortunately, we have detected the presence of interfering compounds mainly in soil, such as humic substances or phenolics, which were PCR inhibitors, and make more difficult the detection of *Bacillus*. This is in agreement with previous reports in rhizosphere and soil samples (Braid *et al*. 2003, Matheson *et al*. 2010).

The aim of the present work was to study the distribution of antimicrobial peptide genes and their related products, as well as the antimicrobial activity of *Bacillus* populations from plant environments. Due to the low yield in obtaining culturable *Bacillus* directly from natural samples, a procedure to enrich populations in the sample extracts (biological amplification) was necessary. For this reason, a selective enrichment procedure followed by PCR to detect specific molecular markers associated to antimicrobial peptide genes was designed, optimized, verified and validated, in order to screen large collections of field samples. The PCR tools permitted also to screen large strain collections.

In a first stage, six pairs of primers targeted to the genes *srfAA* (surfactin), *bmyB* (bacillomycin), *ituC* (iturin), *fenD* (fengycin), *bacA* (bacilysin) and *spaS* (subtilin) were designed, all of them are involved in biosynthetic pathways of certain antimicrobial peptides described in *Bacillus*. These genes were chosen according to the structural and functional characteristics of their products and also on the basis of its involvement in biocontrol of plant pathogens (Arguelles-Arias *et al*. 2009, Chen *et al*. 2009, Joshi and McSpadden-Garddener 2006, Montesinos 2007, Stein 2005). The specificity of the designed primers to *Bacillus* strains was confirmed, in such a way that no amplification products were observed by testing other bacteria commonly present in the same environments. In addition, the sensitivity of the primers was dependent on the *Bacillus* strain and target gene. Thus, primers for *fenD* and *ituC* genes showed a high sensitivity ($<5x10^2$ cfu/ml), whereas for*bmyB*, *srfAA* and *bacA* genes showed moderate to low sensitivity (between 10^2 and 10^4 cfu/ml), while for the *spaS* gene showed a very poor sensitivity. Globally, sensitivity was slightly better for our primers than in primers previously reported for the same genes by other authors

(Joshi and McSpadden-Gardener 2006, Koumoutsi *et al*. 2004). In addition, to reduce the time and costs of screening, a multiplex PCR directed to the most sensitive and frequent genes (*srfAA*, *bacA*, *bmyB* and *fenD*) was developed and optimized, but a poorer sensitivity level (10^5 cfu/ml) than in the single PCR was obtained. Unfortunately, the threshold detection level of single and multiplex PCR was higher than the actual population levels of *Bacillus* observed in most natural samples. Therefore the risk of achieving false negative results in samples was high when using PCR alone. Moreover, this problem was partially solved upon optimization of the detection tools because dilutions of 1:100 or 1:1000 of the natural extracts improved detection of specific *Bacillus* genes, especially in soil and rhizosphere samples, were inhibitory compounds to PCR are generally present in high amounts.

Thus, in order to achieve populations of *Bacillus* in the sample extracts at levels detectable by PCR, an enrichment of the samples was necessary. In the present work, a selective enrichment procedure based on a thermal treatment at 80 ºC for 10 min followed by a cultivation stage at 40 ºC for 24 h with previous dilution 1:100 in LB broth, was developed and validated. With the application of this procedure, the population of accompanying non-sporulating bacteria was drastically reduced, while *Bacillus* populations were enriched up to 10^6 cfu/ml independently of the sample origin. Resulting population levels were optimal to apply the molecular techniques according to the sensitivity levels obtained. The combination of these two strategies allowed us to increase the yield of *Bacillus* isolation, while the detection of *srfAA*, *bacA*, *bmyB*, *fenD*, *spaS* and *ituC* genes increased between two to three times above the detection obtained in non-enriched natural samples. Interestingly, the distribution of genes within the population of isolates obtained compared to untreated samples (not enriched) was not altered, leading to build-up a large collection of *Bacillus* isolates representative of the plant environment.

Once the isolation and detection tools were optimized and validated, a collection of 184 *Bacillus* isolates from a wide range of plant environments within a Mediterranean Eastern land area in Spain was constituted. These isolates were obtained from 132 out of 183 natural samples mainly from

aerial plant parts. *Bacillus* was isolated from all samples where its presence has been previously detected by molecular methods (100 % of soil, 93 % of rhizosphere and 65 % of aerial plant part samples). In some cases, more than one *Bacillus* isolate was obtained from each rhizosphere or soil sample.

This collection was firstly characterized according to the presence of *srfAA*, *bmyB*, *ituC*, *fenD*, *bacA* and *spaS* genes in each isolate. Most isolates had at least one of the six AMP genes, indicating a prevalence of AMP gene markers within the population of *Bacillus*. Besides, the number of simultaneous AMP genes per isolate followed a normal distribution, where a 76 % of *Bacillus* isolates presented between 2 and 4 AMP genes. The most prevalent genes were *srfAA*, *bacA*, *bmyB* and *fenD*, with a frequency of detection of 69, 61, 55 and 40 %, respectively. Among the studied genes, *srfAA* was the most frequently detected, in agreement with Athukorala *et al*. (2009), who described the high prevalence of the *srf* gene cluster in *Bacillus* isolates from their collection. Surfactin is considered a powerful biosurfactant and its antimicrobial activity has been related with the capacity to alter cell membrane integrity (Carrillo *et al*. 2003). In addition, the synthesis of surfactin is closely related with the sporulation process (Nakano *et al*. 1998), in the biofilm formation and in swarming mobility (Branda *et al*. 2001, Kinsinger *et al*. 2005). Interestingly, *srf* gene cluster regulation is linked to the regulation of *spo* genes that are involved in early stages of sporulation, confirming the importance of the presence of the *srfAA* gene in most isolates of our *Bacillus* collection. *bacA*, *bmyB* and *fenD* genes were also frequently detected in *Bacillus* isolates of our collection, in agreement with the widespread presence of these genes among genomes of known strains of *B. subtilis* and related species described as BCAs (Arguelles-Arias *et al*. 2009, Athukorala *et al*. 2009, Chen *et al*. 2007, Joshi and McSpadden-Gardener 2006). This finding emphasizes the role of bacillomycin, iturin, fengycin and bacilysin in the fitness of *Bacillus* in natural habitats and their involvement in the control of plant diseases (Branda *et al*. 2001, Chen *et al*. 2013, Ongena *et al*. 2007).

In addition to the AMP gene distribution, a second characterization of the *Bacillus* isolates was performed to verify the production of antimicrobial peptides related to the six biosynthetic genes detected by PCR. Production,

extraction and identification protocols were optimized and verified in our laboratory. This characterization was carried out in 64 *Bacillus* isolates, and permitted to indentify the production of three families of cyclic lipopeptides: surfactins, fengycins and iturins. Unfortunately, determination of production of bacilysin and subtilin was not possible in the present work due to technical reasons. Surfactins were the most abundantly produced cLPs (98.4 %), followed by iturins (90.6 %), which include bacillomycins, and lastly fengycins (79.7 %). Globally, a 75 % of isolates produced at least one of the three isoforms of cLPs families simultaneously, in agreement with the results obtained with the presence of AMP genes. It is also in agreement with the fact that these genes are simultaneously present in the genome of the *Bacillus* strains sequenced to date (Kunst *et al*. 1997, Chen *et al*. 2007).

The isolates of *Bacillus* were finally characterized using antimicrobial activity against a broad range of fungal and bacterial plant pathogens. Globally, antibacterial activity was observed in a 99 % of *Bacillus* isolates, inhibiting at least one plant pathogenic bacteria, and in 72 % that inhibited at least 6 out of 8 bacterial pathogens tested. Although the antibacterial activity was very widespread in *Bacillus* isolates, it was dependent on the composition of the growth medium used in the assay. The effect of culture medium composition could be due to differential growth of the indicator plant pathogenic bacteria, the different production of antimicrobial peptides by the isolate, or to the loss of activity of given synthesized AMPs due to interacion with growth media components. Some authors refer the differential inhibition of bacteria on basis of the growth medium, as for example, a poor growth of *Erwinia* and related species in NA growth medium was observed (Kado 2006). In contrast, *Clavibacter* spp. has better growth in peptone based growth media, like NA (Evtushenko and Takcuchi 2006), whereas *Agrobacterium* spp. sensitivity to NaCl causes a decrease in growth in rich salt media, such as LB (Matthyse 2006). Our results agree with these studies because we find differences of antibacterial activity between LB and NA, where *Clavibacter michiganensis sbsp. michiganensis* and *Xanthomonas arboricola* pv. fragariae showed a poor growth in LB than NA, while *Erwinia amylovora* showed a better growth in LB medium. These differences in bacterial indicator growth could result in a greater inhibition

by *Bacillus* isolates, provided that the AMP production in both media was the same, and its activity was not altered by growth media components.

Moreover, it should be considered the presence of AMPs inhibitors within the components of growth media, preventing their action against the plant pathogenic bacteria. In this way, a drastic reduction of the activity of *Bacillus* supernatants against *E. amylovora* has been observed in LB medium, especially due to tryptone and yeast extract components (data not shown). These results are in agreement with the fact that some AMPs lose their antimicrobial activity due to the high level of protein binding (Tawora *et al*. 2000) or due to peptide binding with complex carbohydrates (Schwab *et al*. 1999). Antifungal activity was only tested in PDA agar, so that we could not compare the antifungal activity in different growth media. However, we would expect to find differences in fungal growth depending on the growth medium components, and also presence of inhibitors of AMPs within the components of the medium, causing variations in the activity.

Once the collection of *Bacillus* isolates has been characterized according to the simultaneous presence of AMP genes, antimicrobial peptides and antimicrobial activity, we searched for possible relationships. It has to be taken into account that the collection of isolates is representative of the naturally occuring *Bacillus* (no significant differences between AMP genes distributions between direct or enriched samples, or between isolates by the two procedures). Therefore this study would reflect relationships at the level of natural culturable populations of *Bacillus*.

The relationships between AMP genes, the corresponding AMP production and the antimicrobial activity are not strictly direct, because there are other factors involved (Figure 5.1). These factors can be extrinsic, such as culture conditions, or intrinsic, such as the genetic diversity among *Bacillus* strains. Thus, expression of a specific gene is dependent on culture conditions like nutrients, temperature and pH that affect the proper production of secondary metabolites (Zhao *et al*. 2013). In the same way, antagonistic activity is also dependent on the conditions of assay, pathogen target and culture medium, as it has been previously discussed in relation to the differential sensitivity of target pathogen, the inactivation of certain AMPs

by growth medium components or the differential productionof AMPs. In contrast to extrinsic factors that can be modulated in the laboratory, the intrinsic factors are inherent to each *Bacillus* isolate and are related to the genetic background and the differential product expression, but also to the concentration and the stability of the products synthesized. In relation to the genetic diversity, it is well known the variability of the sequences involved in the synthesis for a given AMP in *Bacillus*. For example, phylogenetic classification of *Bacillus* strains has been performed using non-ribosomal peptide synthetases genes NRPS of different cLPs such as bacillomycin, fengycin, iturin, mycosubtilin and surfactin (Roongsawang *et al*. 2005).

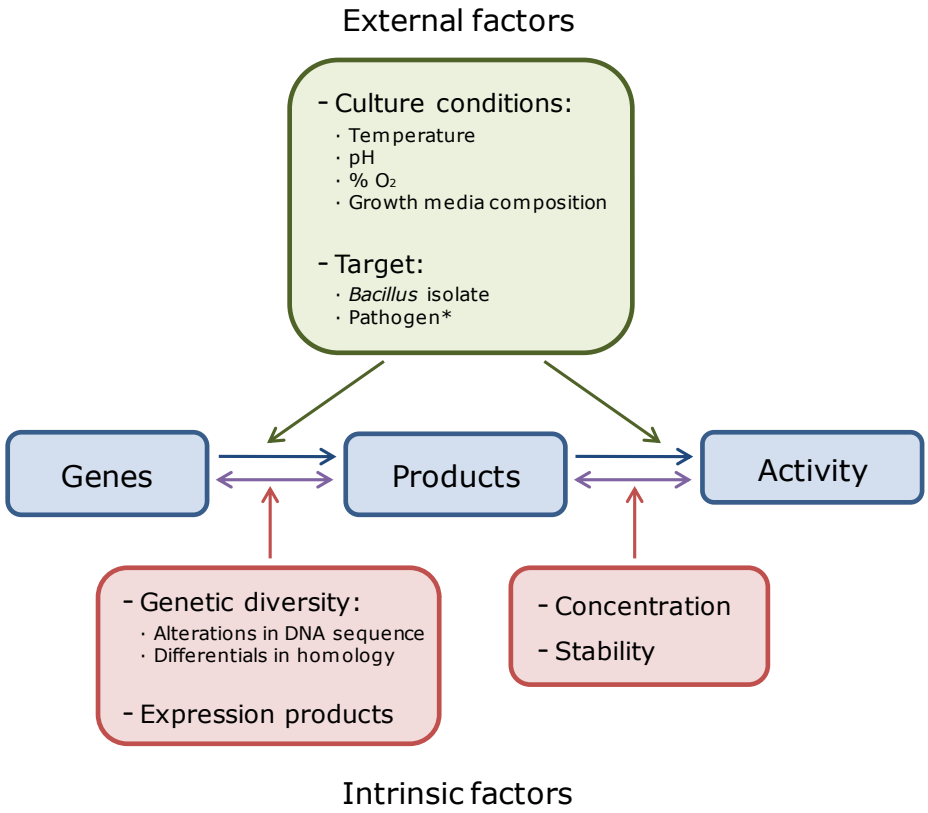

Figure 5.1. Diagram of factors affecting the production of antimicrobial peptides and antimicrobial activity in relation to the corresponding gene background.

In other cases, genetic variations of some regions are responsible of an abnormal synthesis of a specific product, as it has been described in the *Bacillus* subtilis 168. This strain contains three gene clusters controlling non-ribosomal synthesis of surfactin, fengycin and the siderophore bacillibactin, but it is deficient in their syntheses due to a frame shift mutation in the *sfp* gene which converts peptidyl carrier proteins domains to their active form (Mootz *et al*. 2001). Finally, the concentration and stability of AMPs are closely linked to the antimicrobial activity, therefore, the production of a specific AMP do not ensure the activity if not enough amounts are produced.

Methodological limitations can also interfere with the finding of relationships between genes, products and antimicrobial activity of the strains in the collection of *Bacillus*. One limitation is at the level of the PCR molecular tools due to the primers design that may be affected by the lack or scarce information information related to the gene of interest, which limits the reliability of the designed primers. Thus, a low sensitivity can be due to annealing problems of primers to target DNA, resulting in false negative detection. In addition, limitations related with exceptions in gene conservation at inter and intra species level, may result in a lack of detection of the gene with the designed primers, but the strain has obviously the ability to produce the expected product (Alvarez *et al*. 1996).

According to our results, only the cLPs corresponding to iturin, fengycin and surfactin families have been adequately identified. Therefore, only the presence of the biosynthetic genes *bmyB*, *ituC*, *fenD* and *srfAA* was related with the production of the corresponding products. Among the four genes analyzed, only *fenD* was significantly correlated with the production of fengycin. This limited number of significant correlations highlights the limitations provided by the PCR to detect the molecular markers, in comparison to the high sensitivity and fidelity of HPLC chromatographic techniques to detect the corresponding expression products. Concretely, the sensitivity of primers directed to the *fenD* gene was high with values around $1x10^2$ cfu/ml, which remained rather stable between different strains. In addition, the study of *fenD* sequences of different *Bacillus* strains obtained from GenBank showed a high homology at inter and intra species level,

which lead to a significant correlation between the detection of *fenD* gene with the production of fengycins. In contrast, a high sensitivity of *ituC* primers observed with reference strains was not related to a frequent detection of the *ituC* gene among *Bacillus* isolates of our collection, probably due to the heterogeneity of the *ituC* gene sequences. However, *srfAA* and *bmyB* gene primers presented slight sensitivity limitations that difficult the detection of both genes within the isolates collection. In addition, in the iturin family we have also to consider that there are several genes involved in the synthesis of various isoforms, such as iturins, bacillomycins and mycosubilins. However, in this work the presence of isoforms of the iturin family without making distinctions between them may be responsible of the inability to find relationships between *bmyB* and *ituC* genes and their corresponding products.

Antibacterial activity was also related with the presence of AMP genes. It was observed that the increase of the number of simultaneous AMP genes per strain was related with the increase of antimicrobial activity, particularly, for the genes *srfAA*, *bacA*, *bmyB* and *fenD*. However, the antibacterial activity was conditioned by the growth medium used, as previously discussed, and only the antibacterial activity tested in LB agar was correlated with the detection of simultaneous AMP genes, which was closely related with the presence of *srfAA*, *bmyB* and *fenD* biosynthetic genes. Contrarily, the antibacterial activity tested in NA was correlated with the presence of the *bacA* gene, and the antifungal activity with the detection of *srfAA*.

A similar behavior was observed when comparing the global relationship of cLPs production with the antimicrobial activity. A higher antimicrobial activity was observed related to an increase of the number of isoforms of the three families of cLPs. However, not all isoforms were related to the antimicrobial activity. The presence of fengycin isoforms was correlated with the antimicrobial activity regardless of the target pathogen and the growth medium used. Contrarily, iturin isoforms were mainly related with the antifungal and antibacterial activities in LB agar, while surfactin isoforms were related with the antibacterial activity in NA agar. However, it was difficult to relate the production of cLPs with the activity on NA and PDA

taking into account that were only produced in LB medium, and the production and action may be different in other culture media, such as NA and PDA.

This work was focused on global relationships between the presence of AMP genes and production of the corresponding AMPs and the antimicrobial activity in a collection of *Bacillus* isolates obtained from habitats strongly related with plant environment. However, our approach was designed as a population study because it included isolates from field origin and a wide variety of environments, and therefore the high variability in most parameters may affect robustness of the correlations obtained. Other approaches reported to establish relationships deal with defective mutants for a target AMP gene (Chen *et al*. 2009c, Romero *et al*. 2007b, Yánez-Mendizábal *et al*. 2012), but lack of ecological significance at population level. Also, in most cases the study is focused on a single antagonistic strain against a single target pathogen and only taking into account one specific AMP gene (Arguelles-Arias *et al*. 2009, Athukorala *et al*. 2009).

Another relevant aspect is that production of cLPs is dependent on environmental conditions, and therefore *in vitro* studies will give different results than *in planta* experiments. However, most studies focused on *Bacillus* as BCA only determine the production of AMPs under *in vitro* conditions (Arguelles-Arias *et al*. 2009, Malfanova *et al*. 2011, Romero *et al*. 2007) However, recent reports have demonstrated that the production of cLPs is substantially modulated in the rhizosphere compared to laboratory conditions, even varying the relative proportions between the homologues (Nihorimbere *et al*. 2012). Therefore, it seems difficult to extrapolate *in vivo* AMPs production, taking into account the production obtained under laboratory conditions. In any case, the aim of the present work was to know the potential of our isolates for producing AMPs and develop antimicrobial activity, and to relate this potential to the corresponding AMP genes.

In spite of the great heterogeneity observed in relation of the presence of AMP biosynthetic genes, production of cLPs and antimicrobial activity, two consistent groups of *Bacillus* were established within our collection. The first group includes *Bacillus* isolates with a high presence of simultaneous AMP

genes, specifically *srfAA*, *bacA*, *bmyB* and *fenD*, strong capacity to produce a wide range of iturins, fengycins and surfactins, and intense antimicrobial activity. The reference strain *Bacillus* QST713 in this group. In addition, a second group was composed for *Bacillus* isolates with a low number of simultaneous AMP genes, with poor production of cLPs, and low antimicrobial activity. Thus, the most relevant features for the discrimination of both groups were the presence of *bacA* and *fenD* genes, the production of fengycins and surfactins, and the antibacterial activity tested in LB agar medium. Interestingly, the first group theoretically includes isolates with a great potential in biocontrol and was mainly composed of rhizosphere and soil isolates.

Conclusions

1. The distribution of the population levels of *Bacillus* spp. in plant-associated environments from the Mediterranean Eastern area is highly variable and ranges from 4 to 7 $\log_{10}$ cfu/g f.w. when detected, but it is undetectable in most samples. *Bacillus* was more abundant in the rhizosphere and soil than in aerial plant parts.

2. A PCR procedure to detect specific molecular markers associated to *Bacillus* (*16S rDNA* gene) and to the presence of antimicrobial peptide genes (*srfAA*, *bacA*, *bmyB*, *fenD*, *spaS* and *ituC* genes) was developed, optimized, and validated in pure culture strains and field sample extracts.

3. A selective enrichment procedure has been developed, optimized, and validated to increase the yield of *Bacillus* population in natural sample extracts, thus facilitating strain isolation procedures and PCR detection of specific gene markers.

4. An increase of the yield of isolation of *Bacillus* strains containing simultaneous AMP genes was obtained with the selective enrichment procedure combined with PCR targeted to antimicrobial peptides genes (AMP) in comparison to the standard isolation method. And the resulting distribution of the AMP genes in enriched samples was not modified in relation to the unenriched natural samples.

5. A strain collection of 184 plant-associated *Bacillus* isolates has been built-up with the selective enrichment procedure followed by molecular marker detection, from 183 samples, mostly from aerial plant parts, including different plant environments.

6. The *Bacillus* collection showed a strong variability among isolates in relation to the presence of antimicrobial peptide genes (*srfAA*, *bacA*, *bmyB*, *fenD*, *spaS* and *ituC*), the production of cyclic lipopeptides (surfactins, fengycins and iturins) and the antimicrobial activity against bacterial and fungal plant pathogens (*Rhizobium radiobacter*, *Clavibacter michiganensis sbsp. michiganensis*, *Erwinia amylovora*, *Pectobacterium carotovorum sbsp. carotovorum*, *Pseudomonas syringae* pv. syringae, *Ralstonia solanacearum*, *Xanthomonas arboricola* pv. fragariae, *Xanthomonas axonopodis* pv. vesicatoria, *Phytophthora cactorum*, *Botrytis cinerea*, *Pythium ultimum*, *Fusarium oxysporum f. sp. lycopersici*, *Phytophthora cinnamomi*, *Penicillium expansum*).

7. The frequency distribution of the number of gene markers per isolate followed a normal distribution, with most frequently observed 2 to 4 genes per isolate, and a prevalence of *srfAA*, *bmyB*, *bacA* and *fenD* gene markers (69, 61, 55 and 40 % of isolates, respectively).

8. The presence of iturins, fengycins, and surfactins among the isolates showed a high variability in the type and isoforms, and production levels among isolates. A prevalence of the production of iturins, fengycins, and surfactins was observed within the population (98, 91 and 80 %, respectively).

9. *A*ntimicrobial activity was very variable and depends on the pathogen target and growth medium. Over 72 % of isolates inhibited at least 6 out of 8 bacterial plant pathogens and a 37 % inhibited at least 3 out of 5 fungal plant pathogens. *E. amylovora* (in LB), *R. radiobacter* (in LB and NA) and *F. oxysporum* has been the less sensible target pathogens to *Bacillus* isolates activity, being *C. michiganensis* and *P. cactorum* the most sensitive plant pathogens. In addition a 26.6 % of *Bacillus* isolates have been highly active against bacterial and 26.1 % against fungal phytopathogens according to the Global Activity Index (GAI).

10. There was a significant direct relationship between the presence of high number of simultaneous AMP genes and antibacterial activity in LB agar, as well as between the presence of *fenD* gene, production of fengycins and the antimicrobial activity.

11. The global correspondence analysis confirmed the presence of two consistent groups in the collection of *Bacillus* isolates; the first characterized by the simultaneous presence of antimicrobial peptide genes, production of related cyclic lipopeptides and high antimicrobial activity; and the second characterized by the absence of these three characters.

12. The prevalence of AMP genes in plant-associated populations of *Bacillus* has been demonstrated, and their relationship with the antagonistic capacity of the respective isolates and the synthesis of the expression products was determined.

References

A

Abriouel, H., Franz, C.M.A.P., Omar N.B. and Gálvez, A. 2011. Diversity and applications of *Bacillus* bacteriocins. FEMS Microbiol Rev. 35:201-232.

Adesina, M.F., Lembke, A., Costa, R., Speksnijder, A. and Smalla, K. 2007. Screening of bacterial isolates from various European soils for *in vitro* antagonistic activity towards *Rhizoctonia solani* and *Fusarium oxysporum*: Site-dependent composition and diversity revealed. Soil Biol Biochem. 39:2818-2828.

Agusti, L., Bonaterra, A., Moragrega, C., Camps, J. and Montesinos, E. 2011. Biocontrol of root rot of strawberry caused by *Phytophthora cactorum* with a combination of two *Pseudomonas fluorescens* strains. J Plant Pathol. 93:363-372.

Ahaotu, I., Anyogu, A., Njoku, O.H., Odu, N.N., Sutherland, J.P. and Ouoba, L.I.I. 2013. Molecular identification and safety of *Bacillus* species involved in the fermentation of African oil beans (*Pentaclethra macrophylla* Benth) for production of Ugba. Int J Food Microbiol. 162:95-104.

Aiyer, P.V. 2005. Amylases and their applications. Afr J Biotechnol. 4:1525-1529.

Alabouvette, C., Olivain, C. and Steinberg, C. 2006. Biological control of plant diseases: the European situation. Eur J Plant Pathol. 114:329-341.

Altena, K., Guder, A., Cramer, C. and Bierbaum, G. 2000. Biosynthesis of the Lantibiotic Mersacidin: Organization of a Type B lantibiotic gene cluster. Appl Environ Microb. 66:2565-2571.

Alvarez, A.J., Buttner, M.P., and Stetzenbach, L.D. 1995. PCR for bioaerosol monitoring: Sensitivity and environmental interference. Appl Environ Microb. 61:3639-3644.

Aranda, F.J., Teruel, J.A. and Ortiz, A. 2005. Further aspects on the hemolytic activity of the antibiotic lipopeptide iturin A. Biochim Biophys Acta. 1713:51-56.

Arguelles-Arias, A., Ongena, M., Halimi, B., Lara, Y., Brans, A., Joris, B. and Fickers, P. 2009. *Bacillus amyloliquefaciens* GA1 as a source of potent antibiotics and other secondary metabolites for biocontrol of plant pathogens. Microb Cell Fact. 8:63-74.

Arias, R.S., Sagardoy, M.A. and Van Vuurde, J.W.L. 1999. Spatio-temporal distribution of naturally occurring *Bacillus* spp. and other bacteria on the phylloplane of soybean under field conditions. J Basic Microb. 39:283-292.

Arrebola, E., Jacobs, R. and Korsten, L. 2010. Iturin A is the principal inhibitor in the biocontrol activity of *Bacillus amyloliquefaciens* PPCB004 against postharvest fungal pathogens. J Appl Microbiol. 108:386-395.

Asai, K., Fujita, M., Kawamura, F., Takahashi, H., Kobayashi, Y. and Sadaie, Y. 1998. Restricted transcription from sigma H or phosphorylated Spo0A dependent promoters in the temperature-sensitive *sec A341* mutant of *Bacillus subtilis*. Biosci Biotech Biochem. 62:707-1713.

Asaka, O. and Shoda, M. 1996. Biocontrol of *Rhizoctonia solani* damping-off of tomato with *Bacillus subtilis* RB14. Appl Environ Microb. 62:4081-4085.

Athukorala, S.N.P., Fernando, W.G.D. and Rashid, K.Y. 2009. Identification of antifungal antibiotics of *Bacillus* species isolated from different microhabitats using polymerase chain reaction and MALDI-TOF mass spectrometry. Can J Microbiol. 55:1021-1032.

Awashti, N., Kumar, A., Makkar, R. and Cameotra, S. 1999. Enhanced biodegradation of endosulfan, a chlorinated pesticide in presence of a biosurfactant. J Environ SCI Heal B. 34:793-803.

B

Badosa, E., Ferre, R., Planas, M., Feliu, L., Besalú, E., Cabrefiga, J., Bardají, E. and Montesinos, E. 2007. A library of linear undecapeptides with bactericidal activity against phytopathogenic bacteria. Peptides. 28:2276-2285.

Badosa, E. Ferré, R., Francés, J., Bardají, E., Feliu, L., Planas, M. and Montesinos, E. 2009. Sporicidal activity of synthetic antifungal undecapeptides and control of *Penicillium* rot of apples. Appl Environ Microb. 75:5563-5569.

Bais, H.P., Fall, R. and Vivanco, J.M. 2004. Biocontrol of *Bacillus subtilis* against infection of *Arabidopsis* roots by *Pseudomonas syringae* is facilitated by biofilm formation and surfactin production. Plant Physiol. 134:307-320.

Banat, I.M., Makkar, R.S. and Cameotra, S.S. 2000. Potential commercial applications of microbial surfactants. Appl Microbiol Biot. 53:495-508.

Barbe, V., Cruveiller, S., Kunst, F., Lenoble, P., Meurice, G., Sekowska, A., Vallenet, D., Wang, T., Moszer, I., Medigue, C. and Danchin, A. 2009. From a consortium sequence to a unified sequence: the *Bacillus subtilis* 168 reference genome a decade later. Microbiol-SGM. 155:1758-1775.

Bargabus, R.L., Zidack, N.K., Sherwood, J.E. and Jacobsen, B.J. 2002. Characterisation of systemic resistance in sugar beet elicited by a non-pathogenic, phyllosphere-colonizing *Bacillus mycoides*, biological control agent. Physiol Mol Plant P. 61:289-298.

Barny, M. A., Guinebretière, M.H., Marçais, B., Coissac, E., Paulin, J.P. and Laurent, J. 1990. Cloning of a large gene cluster involved in *Erwinia amylovora* CFBP1430 virulence. Mol Microbiol. 4:777-786.

Baysal, O., Çalişkan, M. and Yeşilova, O. 2008. An inhibitory effect of a new *Bacillus subtilis* strain (EU07) against *Fusarium oxysporum f. sp. radicis-lycopersici*. Physiol Mol Plant P. 73:25-32.

Benitez, M.S. and McSpadden-Gardener, B.B. 2009. Linking sequence to function in soil bacteria: Sequence-directed isolation of novel bacteria contributing to soilborne plant disease suppression. Appl Environ Microb. 75:915-924.

Bento, F.M., De Oliveira Camargo, F.A., Okeke, B.C. and Frankenberger Jr., W.T. 2005. Diversity of biosurfactant producing microorganisms isolated from soils contaminated with diesel oil. Microbiol Res. 160:249-255.

Bereswill, S., Pahl, A., Bellemann, P., Zeller, W., Geider, K. 1992. Sensitive and species-specific detection of *Erwinia amylovora* by polymerase chain reaction analysis. Appl Environ Microb. 58:3522-3526.

Berić, T., Kojić, M., Stanković, S., Topisirović, L., Degrassi, G., Myers, M., Venturi, V. and Fira, D. 2012. Antimicrobial activity of *Bacillus* sp. natural isolates and their potential use in the biocontrol of phytopathogenic bacteria. Food Technol Biotech. 50:25-31.

Bierbaum, G. and Sahl, H.G. 2009. Lantibiotics: Mode of action, biosynthesis and bioengineering. Curr Pharm Biotechno. 10:2-18.

Bizzarri, M.F., Bishop, A.H. 2007. Recovery of *Bacillus thuringiensis* in vegetative form from the phylloplane of clover (*Trifolium hybridum*) during a growing season. J Invertebr Pathol. 94:38-47.

Bonaterra, A., Camps, J. and Montesinos, E. 2005. Osmotically induced trehalose and glycine betaine accumulation improves tolerance to desiccation, survival and efficacy of the postharvest biocontrol agent *Pantoea agglomerans* EPS125. FEMS Microbiol Lett. 250:1-8.

Bonaterra, A., Cabrefiga, J., Camps, J. and Montesinos, E. 2007. Increasing survival and efficacy of a bacterial biocontrol agent of fire blight of rosaceous plants by means of osmoadaptation. FEMS Microbiol Ecol. 61:185-195.

Bongers, R.S., Veening, J.W., Van Wieringen, M., Kuipers, O.P., Kleerebezem, M. 2005. Development and characterization of a subtilin-regulated expresión system in B*acillus subtilis*: Strict control of gene expression by addition of subtilin. Appl Environ Microb. 71:8818-8824.

Bonmatin, J.M., Laprevote, O. and Peypoux, F. 2003. Diversity among microbial cyclic lipopeptides: Iturins and surfactins. Activity-structure relationships to design new bioactive agents. Comb Chem High T Scr. 6:541-556.

Borisova, S.A., Circello, B.T., Zhang, J.K., van der Donk, W.A. and Metcalf, W.W. 2010. Biosynthesis of rhizocticins, antifungal phosphonate oligopeptides produced by *Bacillus subtilis* ATCC6633. Chem Biol. 17:28-37.

Borriss, R., Chen, X.H., Rueckert, C., Blom, *et al*. 2011. Relationship of *Bacillus amyloliquefaciens* clades associated with strains DSM7^T and FZB42^T: A proposal for *Bacillus amyloliquefaciens subsp*. *amyloliquefaciens* subsp. nov. and *Bacillus amyloliquefaciens subsp*. *plantarum* subsp. nov. based on complete genome sequence comparisons. Int J Syst Evol Micr. 61:1786-1801.

Bott, R., Ultsch, M., Kossiakoff, A., Graycar, T., Katz, B. and Power, S. 1988. The three-dimensional structure of *Bacillus amyloliquefaciens* subtilisin at 1.8 A and an analysis of thestructural consequences of peroxide inactivation. J Biol Chem. 263:7895-7906.

Braid, M.D., Daniels, L.M., and Kitts, C.L. 2003. Removal of PCR inhibitors from soil DNA by chemical flocculation. J Microbiol Meth. 52:389-393.

Branda, S.S., González-Pastor, J.E., Ben-Yehuda, S., Losick, R. and Kolter, R. 2001. Fruiting body formation by *Bacillus subtilis.*P Natl Acad Sci USA. 98:11621–11626.

Branda, S.S., González-Pastor, J.E., Dervyn, E., Ehrlich, S.D., Losick, R. and Kolter, R. 2004. Genes involved in formation of structured multicellular communities by *Bacillus subtilis.* J Bacteriol. 186:3970-3979.

Broggini, G.A.L., Duffy, B., Holliger, E., Scharer, H.J., Gessler, C. and Patocchi, A. 2005. Detection of the fire blight biocontrol agent *Bacillus subtilis* BD170 (Biopro®) in a Swiss apple orchard. Eur J Plant Pathol. 111: 93-100.

Brötz, H., Bierbaum, G., Reynolds, P.E. and Sahl, H.G. 1997. The lantibiotic mersacidin inhibits peptidoglycan biosynthesis at the level of transglycosylation. Eur J Biochem. 246:193-199.

Burkard, M., Entian, K.D. and Stein, T. 2007. Development and application of a microtiter plate-based autoinduction bioassay for detection of the lantibiotic subtilin. J Microbiol Meth. 70:179-185.

C

Cabrefiga, J., Bonaterra, A. and Montesinos, M. 2007. Mechanisms of antagonism of *Pseudomonas fluorescens* EPS62e against *Erwinia amylovora*, the causal agent of fire blight. Int Microbiol. 10:123-32.

Cabrefiga, J., Francés, J., Montesinos, E. and Bonaterra, A. 2011. Improvement of fitness and efficacy of a fire blight biocontrol agent via nutritional enhancement combined with osmoadaptation. Appl Environ Microb. 77:3174-3181.

Carrillo, C., Teruel, J.A., Aranda, F.J. and Ortiz, A. 2003. Molecular mechanism of membrane permeabilization by the peptide antibiotic surfactin. Biochim Biophys Acta. 1611:91-97.

Cawoy, H., Bettiol, W., Fickers, P., and Ongena, M. 2011. *Bacillus*-based biological control of plant diseases. Pesticides in the modern world-pesticides and management. *1*:273-302.

Cazorla, F.M., Romero, D., Pérez-García, A., Lugtenberg, B.J.J., de Vicente, A. and Bloemberg, G. 2007. Isolation and characterization of antagonistic *Bacillus subtilis* strains from the avocado rhizoplane displaying biocontrol activity. J Appl Microbiol. 103:1950-1959.

Challacombe, J.F., Altherr, M.R., Xie, G., Bhotika, *et al*. 2007. The complete genome sequence of *Bacillus thuringiensis* Al Hakam. J Bacteriol. 189:3680-3681.

Chandna, P., Mallik, S. and Kuhad, R.C. 2012. Assessment of bacterial diversity in agricultural by-product compost by sequencing of cultivated isolates and amplified rDNA restriction analysis. Appl Microbiol Biot. 1-13. (*Article in press*).

Chen, Z.X., Dickson, D.W., McSorley, R., Mitchell, D.J., Hewlett, T.E. 1996. Suppression of *Meloidogyne arenaria* race 1 by soil application of endospores of *Pasteuria penetrans*. J Nematol. 28: 159-168.

Chen, X.H., Vater, J., Piel, J., Franke, P., *et al.* 2006. Structural and functional characterization of three polyketide synthase gene clusters in *Bacillus amyloliquefaciens* FZB 42. J Bacteriol. 188:4024-4036.

Chen, X.H., Koumoutsi, A., Scholz, R., Eisenreich, A., *et al.* 2007. Comparative analysis of the complete genome sequence of the plant growth–promoting bacterium *Bacillus amyloliquefaciens* FZB42. Nat Biotechnol. 25:1007-1014.

Chen, H., Wang, L., Su, C.X., Gong, G.H., Wang, P. and Yu, Z.L. 2008. Isolation and characterization of lipopeptide antibiotics produced by *Bacillus subtilis*. Lett Appl Microbiol. 47:180-186.

Chen, X.H., Koumoutsi, A., Scholz, R., Borriss, R. 2009a. More than anticipated-production of antibiotics and other secondary metabolites by *Bacillus amyloliquefaciens* FZB42. J Mol Microb Biotech. 16:14-24.

Chen, X.H., Koumoutsi, A., Scholz, R., Schneider, K., Vater, J., Süssmuth, R., Piel, J. and Borriss, R. 2009b. Genome analysis of *Bacillus amyloliquefaciens* FZB42 reveals its potential for biocontrol of plant pathogens. J Biotechnol. 140:27-37.

Chen, X.H., Scholz, R., Borriss, M., Junge, H., Mögel, G., Kunz, S. and Borris, R. 2009c. Difficidin and bacilysin produced by plant-associated *Bacillus amyloliquefaciens* are efficient in controlling fire blight disease. J Biotechnol. 140:38-44.

Chen Z.M., Li, Q., Liu, H.M., Yu, N., Xie, T.J., Yuan, M., Shen, P. and Chen, X.P. 2010. Greater enhancement of *Bacillus subtilis* spore yields in submerged cultures by optimization of medium composition through statistical experimental designs. Appl Microbiol Biot. 85:1353-1360.

Chitarra, G.S., Breeuwer, P., Nout, M.J.R., van Aelst, A.C., Rombouts, F.M. and Abee, T. 2003. An antifungal compound produced by *Bacillus subtilis* YM 10–20 inhibits germination of *Penicillium roqueforti* conidiospores. J Appl Microbiol. 94:159-166.

Cho, M.J., Kim, Y.K. and Ka, J.O. 2004. Molecular differentiation of *Bacillus* spp. antagonistic against phytopathogenic fungi causing damping-off disease. J Microbial Biotech. 14:599-606.

Choudhary, D.K. and Johri, B.N. 2009. Interactions of *Bacillus* spp. and plants--with special reference to induced systemic resistance (ISR). Microbiol Res. 164:493-513.

Chung, S., Lim, H.M. and Kim, S.D. 2007. Formulation of stable *Bacillus subtilis* AH18 against temperature fluctuation with highly heat-resistant endospores and micropore inorganic carriers. Appl Microbiol Biot. 76:217-224.

Chung, S., Kong, H., Buyer, J.S., Lakshman, D.K., Lydon, J., Kim, S.D. and Roberts, D.P. 2008. Isolation and partial characterization of *Bacillus subtilis* ME488 for suppression of soilborne pathogens of cucumber and pepper. Appl Microbiol Biot. 80:115-123.

Claus, D. and Berkeley, R.C.W. 1986. The genus *Bacillus*. In: Bergey's manual of systematic bacteriology, Vol 2. pp.1105-1139. Sneath PHA (Ed), The Williams and Wilkins Co, Baltimore.

Collins, D.P. and Jacobsen, B.J. 2003. Optimizing a *Bacillus subtilis* isolate for biological control of sugar beet cercospora leaf spot. Biol Control. 26:153-161.

Connelly, M.B., Young, G.M. and Sloma, A. 2004. Extracellular proteolytic activity plays a central role in swarming motility in *Bacillus subtilis*. J Bacteriol. 186:4159-4167.

Cook, R.J., and Baker, K.F. 1983. The nature and practice of biological control of plant pathogens, pp. 539. Am Phytopathol Soc Press, St. Paul, Minnesota.

Corpet, F. 1988. Multiple sequence alignment with hierarchical clustering. Nucleic Acids Res. 16:10881-10890.

Cortezzo, D.E. and Setlow, P. 2005. Analysis of factors influencing the sensitivity of spores of *Bacillus subtilis* to DNA damaging chemicals. J Appl Microbiol. 98:606–617.

Cutting, S.M. 2011. *Bacillus* probiotics. Food Microbiol. 28:214-220.

D

Daffonchio, D., Borin, S., Frova, G., Manachini, P.L. and Sorlini, C. 1999. PCR fingerprinting of whole genomes: the spacers between the *16S* and *23S rRNA* genes and of intergenic tRNA gene regions reveal a different intraspecific genomic variability of *Bacillus cereus* and *Bacillus licheniforrnis*. Int J Syst Bacteriol. 48:107-116.

Davey, M.E., Caiazza, N.C. and O'Toole, G.A. 2003. Rhamnolipid surfactant production affects biofilm architecture in *Pseudomonas aeruginosa* PAO1. J Bacteriol. 185:1027-1036.

Desai, C. and Madamwar, D. 2007. Extraction of inhibitor-free metagenomic DNA from polluted sediments, compatible with molecular diversity analysis using adsorption and ion-exchange treatments. Bioresource Technol. 98:761-768.

Desoignies, N., Schramme, F., Ongena, M. and Legreve, A. 2013. Systemic resistance induced by *Bacillus* lipopeptides in *Beta vulgaris* reduces infection by the rhizomania disease vector *Polymyxa betae*. Mol Plant Pathol. 14:416-421.

De Vecchi, E. and Drago, L. 2006. *Lactobacillus sporogenes* or *Bacillus coagulans*: Misidentification or mislabelling? Int J Probiot Prebiot. 1:3-10.

Devine, K.M. 1995. The *Bacillus subtilis* genome project: Aims and progress. Trends Biotechnol. 13:210-216.

Dong, Y.H., Gusti, A.R., Zhang, Q., Xu, J.L., and Zhang, L.H. 2002. Identification of quorum-quenching N-acyl homoserine lactonases from *Bacillus* species. Appl Envrion Microb. 68:1754-1759.

Dong, Y.H., Zhang, X.F., Xu, J.L. and Zhang, L.H. 2004. Insecticidal *Bacillus thuringiensis* silences *Erwinia carotovora* virulence by a new form of microbial antagonism, signal interference. Appl Environ Microb. 70:954-960.

Drago, L. and De Vecchi, E. 2009. Should *Lactobacillus sporogenes* and *Bacillus coagulans* have a future? J Chemotherapy. 21:371-377.

Driks, A. 2004. The *Bacillus* spore coat. Phytopathology. 94:1249-1251.

Duffy, B., Schouten, A. and Raaijmakers, J.M. 2003. Pathogen self-defense: Mechanisms to counteract microbial antagonism. Annu Rev Phytopathol. 41:501-538.

Duitman, E.H., Hamoen, L.W., Rembold, M., Venema, G., *et al*. 1999. The mycosubtilin synthetase of *Bacillus subtilis* ATCC6633: A multifunctional hybrid between a peptide synthetase, an amino transferase, and a fatty acid synthase. P Natl Acad Sci USA. 96:13294-13299.

E

Earl, A.M., Losick, R. and Kolter, R. 2007. *Bacillus subtilis* genome diversity. J Bacteriol. 189:1163-1170.

Earl, A.M., Losick, R. and Kolter, R. 2008. Ecology and genomics of *Bacillus subtilis*. Trends Microbiol. 16:269-275.

EFSA 2004. Opinion of the scientific panel on biological hazards on *Bacillus cereus* and other *Bacillus* spp. in food stuffs. EFSA J. 175:1-48. Available online: www.efsa.europa.eu/efsajournal.htm

EFSA 2011. Panel on biological hazards (BIOHAZ); Scientific opinion on the maintenance of the list of QPS biological agents intentionally added to food and feed (2011 update) EFSA J. 9(12):2497. Available online: www.efsa.europa.eu/efsajournal.htm

Emmert, E.A.B. and Handelsman, J. 1999. Biocontrol of plant disease: a (Gram-) positive perspective. FEMS Microbiol Lett. 171:1-9.

Endres, J.R., Clewell, A., Jade, K.A., Farber, T., Hauswirth, J. and Schauss, A.G. 2009. Safety assessment of a proprietary preparation of a novel Probiotic, *Bacillus coagulans*, as a food ingredient. Food Chem Toxicol. 47(6):1231-1238.

Eppinger, M., Bunk, B., Johns, M., Edirisinghe, J.N., *et al*. 2011. Genome sequences of the biotechnologically important *Bacillus megaterium* strain QM B1551 and DSM319. J Bacteriol. 193:4199-4213.

Evtushenko, L.I., and Takcuchi, M. 2006. The family Microbacteriaceae. In: Prokaryotes, Vol. 3, pp. 1020-1098. Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.H. and Stackebrandt, E. (Ed). Springer, New York.

F

Fahim, S., Dimitrov, K., Gancel, F., Vauchel, P., Jacques, P. and Nikov, I. 2012. Impact of energy supply and oxygen transfer on selective lipopeptide production by *Bacillus subtilis* BBG21. Bioresource Technol. 126:1-6.

Fan, H., Kitagawa, M., Raku, T. and Tokiwa, Y. 2004. Enzymatic synthesis of vinyl uridine esters catalyzed by Bioprase, alkaline protease from *Bacillus subtilis*. Biochem Eng J. 21:279-283.

Felnagle, E.A., Jackson, E.E., Chan, Y.A., Podevels, A.M., Berti, A.D., McMahon, M.D. and Thomas, M.G. 2008. Nonribosomal peptide synthetases involved in the production of medically relevant natural products. Mol Pharm. 5:191–211.

Ferrari, E., Jarnagin, A.S. and Schmidt, B.F. 1993. Commercial production of extracellular enzymes. In: *Bacillus subtilis* and other Gram-positive bacteria: Biochemistry, physiology, and molecular genetics , pp. 917-937. Sonenshein, A.L., Hoch, J.A. and Losick, R (Ed). American Society for Microbiology, New York.

Ferre, R., Badosa, E., Feliu, L., Planas, M., Montesinos, E. and Bardají, E. 2006. Inhibition of plant-pathogenic bacteria by short synthetic cecropin A-melittin hybrid peptides. Appl Environ Microb. 72:3302-3308.

Fickers, P. 2012. Antibiotic compounds from *Bacillus*: Why are they so amazing? Am J Biochem Biotechnol. 8:40-46.

Földes, T., Banhegyi, I., Herpai, Z., Varga, L. and Szigeti, J. 2000. Isolation of *Bacillus* strains from the rhizosphere of cereals and in vitro screening for antagonism against phytopathogenic, food-borne pathogenic and spoilage micro-organisms. J. Appl Microbiol. 89:840-846.

Francés, J., Bonaterra, A., Moreno, M.C., Badosa, E. and Montesinos, E. 2006. Pathogen aggressiveness and postharvest biocontrol efficiency in *Pantoea agglomerans*. Postharvest Biol Tec. 39:299-307.

Fravel, D.R. 1988. Role of antibiosis in the biocontrol plant-diseases. Annu Rev Phytopathol. 26:75-91.

Fravel, D.R. 2005. Commercialization and implementation of biocontrol. Annu Rev Phytopathol. 43:337-359.

Fritze, D. 2004. Taxonomy of the genus *Bacillus* and related genera: The aerobic endospore-forming bacteria. Phytopathology 94:1245-1248.

G

Ganz, T. and Lehrer, R.I. 1999. Antibiotic peptides from higher eukaryotes: Biology and applications. Mol Med Today. 5:292-297.

Garbeva, P., van Veen, J.A., and van Elsas, J.D. 2003. Predominant *Bacillus* spp. in agricultural soil under different management regimes detected via PCR-DGGE. Microb Ecol. 45:302-316.

Giacomodonato, M.N., Pettinari, M.J., Souto, G.I., Méndez, B.S. and López, N.I. 2001. A PCR-based method for the screening of bacterial strains with antifungal activity in suppressive soybean rhizosphere. World J Microb Biot. 17:51-55.

Gioia, J., Yerrapragada, S., Qin, X., Jiang, H., *et al*. 2007. Paradoxical DNA repair and peroxidase resistance gene conservation in *Bacillus pumilus* SAFR-032. PLoS ONE. 2,e928.

González-Sánchez, M.A., Perez-Jimenez, R.M., Pliego, C., Ramos, C., de Vicente, A. and Cazorla, F.M. 2010. Biocontrol bacteria selected by a direct plant protection strategy against avocado white root rot show antagonism as a prevalent trait. J Appl Microbiol. 109:65-78.

Gu, X.B., Zheng, Z.M., Yu, H.Q., Wang, J., Liang, F.L. and Liu, R.L. 2005. Optimization of medium constituents for a novel lipopeptide production by *Bacillus subtilis* MO-01 by a response surface method. Process Biochem. 40:3196-3201.

Guder, A., Schmitter, T., Wiedemann, I., Sahl, H.G. and Bierbaum, G. 2002. Role of the single regulator MrsR1 and the two-component system MrsR2/K2 in the regulation of mersacidin production and immunity. Appl Environ Microb. 68:106-13.

Gueldner, R.C., Reilly, C.C., Pusey, P.L., Costello, C.E., Arrendale, R.F., Cox, R.H., Himmelsbach, D.S., Crumley, F.G. and Cutler, H.G. 1988. Isolation and identification of Iturins as antifungal peptides in biological-contol of peach brown rot with *Bacillus subtilis*. J Agr Food Chem. 36:366-370.

Gupta, R., Beg, Q.K. and Lorenz, P. 2002. Bacterial alkaline proteases: molecular approaches and industrial applications. Appl Microbiol Biot. 59:15-32.

H

Hamdache, A., Lamarti, A., Aleu, J. and Collado, I.G. 2011. Non-peptide metabolites from the genus *Bacillus*. J Nat Prod. 74:893-899.

Hammerschmidt, R. 2009. Systemic Acquired Resistance. Adv Bot Res. 51:173-222.

Hamoen, L.W., Venema, G. and Kuipers, O.P. 2003. Controlling copentence in *Bacillus subtilis*: Shared use of regulators. Microbiology. 14:9-17.

Hamon, M.A. and Lazazzera, B.A. 2001. The sporulation transcription factor Spo0A is required for biofilm development in *Bacillus subtilis*. Mol Microbiol. 42:1199-1209.

Han, J.G., Xia, D.L., Li, L.B., Sun, L., Yang, K. and Zhang, L.P. 2009. Diversity of culturable bacteria isolated from root domains of *Moso Bamboo* (*Phyllostachys edulis*). Microb Ecol. 58:363-373.

Handelsman, J. and Parke, J.L. 1989. Mechanisms of biocontrol of soilborne plant pathogens. In: *Plant-Microbe Interactions*, Vol. 3, pp. 27-61. T. Kosuge and E.W. Nester (Ed). McGraw Hill, New York.

Harwood, C.R. 1989. *Bacillus*. In Biotechnology handbooks, Vol.2, pp. 1-4. Harwood, C.R. (Ed). Plenum Press, New York and London.

Henry, G., Deleu, M., Jourdan, E., Thonart, P. and Ongena, M. 2011. The bacterial lipopeptide surfactin targets the lipid fraction of the plant plasma membrane to trigger immune-related defense responses. Cell Microbiol. 13:1824-1837.

Hilton, M.D., Alaeddinglu, N.G. and Demain, A.L. 1988. Bacilysin by *Bacillus subtilis* B3 ranches from prephenate of the aromatic amino acid pathway. J Bacteriol. 170:482-484.

Hofemeister, J., Conrad, B., Adler, B., Hofemeister, *et al*. 2004. Genetic analysis of the biosynthesis of non-ribosomal peptideand polyketide-like antibiotics, iron uptake and biofilm formation by *Bacillus subtilis* A1/3. Mol Genet Genomics. 272:363–378.

Hong, H.A., Huang, J.M., Khaneja, R., Hiep, L.V., Urdaci M.C. and Cutting S.M. 2008. The safety of *Bacillus subtilis* and *Bacillus indicus* as food probiotics. J Appl Microbiol. 105:510-520.

Hou, X., Boyetchko, S.M., Brikc, M., Olson, D., Ross, A. and Hegedus, D. 2006. Characterization of the anti-fungal activity of a *Bacillus* spp. associated with sclerotia from *Sclerotinia sclerotiorum*. Appl Microbiol Biot. 72:644-653.

Hsieh, F.C., Lin, T.C., Meng, M. and Kao, S.S. 2008. Comparing methods for identifying *Bacillus* strains capable of producing the antifungal lipopeptide Iturin A. Curr Microbiol. 56:1-5.

Hu, Q.P., Xu, J.G., Song, P., Song, J.N., and Chen, W.L. 2008. Isolation and identification of a potential biocontrol agent *Bacillus subtilis* QM3 from Qinghai yak dung in China. World J Microb Biot. 24:2451–2458

Huang, T., Geng, H., Miyyapuram, V.R., Sit, C.S., Vederas, J.C. and Nakano, M.M. 2009. Isolation of a variant of subtilosin A with hemolytic activity. J Bacteriol. 191:5690-5696.

Huang, H.W., Chang, B.V. and Cheng, C.H. 2012. Biodegradation of dibromodiphenyl ether in river sediment. Int Biodeter Biodegr. 68:1-6.

I

Idris, E.E., Bochow, H. Ross, H. and Borriss, R. 2004. Use of *Bacillus subtilis* as biocontrol agent. VI. Phytohormonelike action of culture filtrates prepared from plant growth promoting *Bacillus amyloliquefaciens* FZB24, FZB42, FZB45 and *Bacillus subtilis* FZB37. J Plant Dis Protect. 111:583-597.

Ito, S., Ushijima, Y., Fujii, T., Tanaka, S., Kameya-Iwaki, M., Yoshiwara, S. and Kishi, F. 1998. Detection of viable cells of *Ralstonia solanacearum* in soil using a semiselective medium and a PCR technique. J Phytopathol. 146:379-384.

J

Jabbari, S., Heap, J.T. and King, J.R. 2011. Mathematical modelling of the sporulation-initiation network in *Bacillus subtilis* revealing the dual role of the putative quorum-sensing signal molecule PhrA. B Math Biol. 73:181-211.

Jaruchoktaweechai, C., Suwanborirux, K., Tanasupawatt, S., Kittakoop, P. and Menasveta, P. 2000. New macrolactins from a marine *Bacillus* Sc026. J Nat Prod. 63: 984-986.

Ji, X., Lu, G., Gai, Y., Zheng, C. and Mu, Z. 2008. Biological control against bacterial wilt and colonization of mulberry byan endophytic *Bacillus subtilis* strain. FEMS Microbiol Ecol. 65:565-573.

Jock, S., Völksch, B., Mansvelt, L. and Geider, K. 2002. Characterization of *Bacillus* strains from apple and pear trees in South Africa antagonistic to *Erwinia amylovora.* FEMS Microbiol Lett. 211:247-252.

Johnson, C. and Bishop, A.H. 1996. A technique for the effective enrichment and isolation of *Bacillus thuringiensis.* FEMS Microbiol Lett. 142:173-177.

Johnson, K.B., Stockwell, V.O. and Sawyer, T.L. 2004. Adaptation of fire blight forecasting to optimize the use of biological controls. Plant Dis. 88:41-48.

Jordan, D., Chin, J.J., Fahy, V.A., Barton, M.D., Smith, M.G. and Trott, D.J. 2009. Antimicrobial use in the Australian pig industry: Results of a national survey. Aust Vet J. 87:222-229.

Joshi, R. and McSpadden-Gardener, B.B. 2006. Identification and characterization of novel genetic markers associated with biological control activities in *Bacillus subtilis*. Phytopathology. 96:145-154.

Jourdan, E., Henry, G., Duby, F., Dommes, J., Barthelemy, J.P., Thonart, P. and Ongena, M. 2009. Insights into the defense-related events occurring in plant cells following perception of surfactin-type lipopeptide from *Bacillus subtilis*. Mol Plant Microbe Int. 22:456-468.

K

Kado, C.L. 2006. *Erwinia* and related genera. In: Prokaryotes, Vol. 6, pp. 443-450. Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.H. and Stackebrandt, E. (Ed). Springer, New York.

Katz, E. and Demain, A.L. 1977. The peptide antibiotics of *Bacillus*: Chemistry, Biogenesis, and possible functions. Bacteriol Rev. 41:449-474.

Kearns, D.B. and Losick, R. 2003. Swarming motility in undomesticated *Bacillus subtilis*. Mol. Microbiol. 49:581-590.

Keating, L., Kelly, C. and Fogarty, W. 1998. Mechanism of action and the substrate-dependent pH maximum shift of the α-amylase of *Bacillus coagulans*. Carbohyd Res. 309:311-318.

Keel, C., Voisard, C., Berling, C.H., Kahr, G. and Défago, G. 1989. Iron sufficiency, a prerequisite for suppression of tobacco black root rot by *Pseudomonas fluorescens* strain CHA0 under gnotobiotic conditions. Phytopathology. 75:584-589.

Khosla, C., Tang, Y., Chen, A.Y., Schnarr, N.A. and Cane, D.E. 2007. Structure and mechanism of the 6-Deoxyerythronolide b synthase. Annu Rev Biochem. 76:195-221.

Ki, J.S., Zhang, W. and Qian, P.Y. 2009. Discovery of marine *Bacillus* species by *16SrRNA* and *rpoB* comparisons and their usefulness for species identification. J Microbiol Meth. 77:48–57.

Kim, H.S., Yoon, B.D., Lee, C.H., Suh, H.H., Oh, H.M., Katsuragi, T., Tani, Y. 1997. Production and properties of a lipopeptide biosurfactant from *Bacillus subtilis* C9. J Ferment Bioeng. 84:41-46.

Kim, K., Cheon, E., Wheeler, K.E., Youn, Y., Leighton, T.J., Park, C., Kim, W. and Chunga, S.I. 2005. Determination of the most closely related *Bacillus* isolates to *Bacillus anthracis* by multilocus sequence typing. Yale J Biol Med. 78:1-14.

Kim, H.S., Sang, M.K., Jeun, Y.C., Hwang, B.K. and Kim, K.D. 2008. Sequential selection and efficacy of antagonistic rhizobacteria for controlling *Phytophthora* blight of pepper. Crop Prot. 27:436-443.

Kim, K.M., Kim, M.J., Kim, D.H., Park, Y.S. and Kang, J.S. 2009. Characterization of *Bacillus polyfermenticus* KJS-2 as a probiotic. J Microbiol Biotechn. 19:1013-8.

Kim, P.I., Ryu, J., Kim, Y.H. and Chi, Y.T. 2010. Production of biosurfactant lipopeptides iturinA, fengycin and surfactinA from *Bacillus subtilis* CMB32 for control of *Colletotrichum gloeosporioides*. J Microbiol Biotechn. 20:138-145.

Kim, M., Morrison, M. And Yu, Zhongtang. 2011. Status of the phylogenetic diversity census of ruminal microbiomes. FEMS Microbiol Ecol. 76:49-63.

Kinsinger, R., Shirk, M.C. and Fall, R. 2003. Rapid surface motility in *Bacillus subtilis* is dependent on extracellular surfactin and potassium ion. J Bacteriol. 185:5627-5631.

Kinsinger, R., Kearns, D., Halle, A., and Fall, R. 2005. Genetic requirements for potassium ion-dependent colony spreading in *Bacillus subtilis*. J Bacteriol. 187:8462-8469.

Klee, S.R., Brzuszkiewicz, E.B., Nattermann, H., Brüggemann, H., *et al*. 2010. The genome of a *Bacillus* isolate causing anthrax in chimpanzees combines chromosomal properties of *B. cereus* with *B. anthracis* virulence plasmids. PLoS ONE. 5, e10986.

Kleerebezem, M. 2004. Quorum sensing control of lantibiotic production; nisin and subtilin autoregulate their own biosynthesis. Peptides. 25:1405-1414.

Klein, C., Kaletta, C., Schnell, N. and Entian, K.D. 1992. Analysis of genes involved in biosynthesis of the lantibiotic subtilin. Appl Environ Microb. 58:132-142.

Klein, C., Kaletta, C. and Entian, K.D. 1993. Biosynthesis of the lantibiotic subtilin is regulated by a histidine kinase/response regulator system. Appl Environ Microb. 59:296-303.

Kloepper, J.W., Rodríguez-Kábana, R., McInroy, J.A. and Collins, D.J. 1991. Analysis of populations and physiological characterization of microorganisms in rhizospheres of plants with antagonistic properties to phytopathogenic nematodes. Plant Soil. 136:95-102.

Kloepper, J.W., Ryu, C.M. and Zhang, S. 2004. Induced systemic resistance and promotion of plant growth by *Bacillus* spp. Phytopathology. 94:1259-1266.

Ko, K.S., Kim, J.M., Kim, J.W., Jung, B.Y., Kim, W., Kim, I.J. and Kook, Y.H. 2003. Identification of *Bacillus anthracis* by *rpoB* sequence analysis andmultiplex PCR. J Clin Microbiol. 41:2908–2914.

Kohli, R.M. and Walsh, C.T. 2003. Enzymology of acyl chain macrocyclization in natural product biosynthesis. Chem Commun. 3: 297-307.

Koponen, O., Takala, T.M., Saarela, U., Qiao, M. and Saris, P.E.J. 2004. Distribution of the NisI immunity protein and enhancement of nisin activity by the lipid-free NisI. FEMS Microbiol Lett. 231:85–90.

Koumoutsi, A., Chen, X.H., Henne, A., Liesegang, H., Hitzeroth, G., Franke, P., Vater, J. and Borriss, R. 2004. Structural and functional characterization of gene clusters directing nonribosomal synthesis of bioactive cyclic lipopeptides in *Bacillus amyloliquefaciens* strain FZB42. J Bacteriol. 186:1084-1096.

Koumoutsi, A., Chen, X.H., Vater, J. and Borriss, R. 2007. DegU and YczE positively regulate the synthesis of bacillomycin by *Bacillus amyloliquefaciens* strain FZB42. Appl Environ Microb. 73:6953-6964.

Krause, M. and Marahiel, M. A. 1988. Organization of the biosynthesis genes for the peptide antibiotic gramicidin S. J Bacteriol. 170:4669-4674.

Kunst, F., Ogasawara, N., Moszer, I., Albertini, *et al*. 1997. The complete genome sequence of the gram-positive bacterium *Bacillus subtilis*. Nature. 390:249-256.

L

Lahlali, R., Peng, G., McGregor, L., Gossen, B.D., Hwang, S.F. and McDonald, M.R. 2011. Mechanisms of the biofungicide Serenade (*Bacillus subtilis* QST713) in suppressing clubroot. Biocontrol Sci Techn. 21:1351-1362.

Lahlali, R., Peng, G., Gossen, B.D., McGregor, L., Yu, F.Q., Hynes, R.K., Hwang, S.F., McDonald, M.R. and Boyetchko S.M. 2013. Evidence that the biofungicide Serenade (*Bacillus subtilis*) suppresses clubroot on canola via antibiosis and Induced Host Resistance. Phytopathology. 103:245-254.

Lam, S.T., and Gaffney, T.D. 1993. Biological activities of bacteria used in plant pathogen control, p. 291–320. In: Biotechnology in plant disease control.Chet, I. (Ed), Wiley-Liss, Inc., New York.

La Ragione, R.M., Casula, G., Cutting, S.M. and Woodward, M.J. 2001. *Bacillus subtilis* spores competitively exclude *Escherichia coli* O78:K80 in poultry. Vet Microbiol. 79:133-142.

La Ragione, R.M. and Woodward, M.J. 2003. Competitive exclusion by *Bacillus subtilis* spores of *Salmonella enterica* serotype Enteritidis and *Clostridium perfringens* in young chickens. Vet Microbiol. 94:245-256.

Landa, B.B., Hervás, A., Bettiol, W. and Jiménez-Díaz, R.M. 1997. Antagonistic activity of bacteria from the chickpea rhizosphere against *Fusarium oxysporum* f. sp. ciceris. Phytoparasitica. 25:305-318.

Landa, B.B., Navas-Cortes, J.A. and Jiménez-Diaz, R.M. 2004. Influence of temperature on plant-rhizobacteria interactions related to biocontrol potencial for supresion of *Fusarium* wilt of chickpea. Plant Pathol. 53:341-352.

Lasch, P., Beyer, W., Nattermann, H., Stämmler, A., Siegbrecht, E., Grunow, R. and Naumann, D. 2009. Identification of *Bacillus anthracis* by using matrix-assisted laser desorption ionization–time of flight mass spectrometry and artificial neural networks. Appl Environ Microb. 75:7229–7242.

Leclère, V., Béchet, M., Adam, A., Guez, J.S., *et al*. 2005. Mycosubtilin overproduction by *Bacillus subtilis* BBG100 enhances the organism's antagonistic and biocontrol activities. Appl Environ Microb. 71:4577-4584.

Leclère, V., Marti, R., Béchet, M., Fickers, P. and Jacques, P. 2006. The lipopeptides mycosubtilin and surfactin enhance spreading of *Bacillus subtilis* strains by their surface-active properties. Arch Microbiol. 186:475-483.

Lee, J.P., Lee, S.W., Kim, C.S., Son, J.H., Song, J.H., Lee, K.Y., Kim, H.J., Jung, S.J. and Moon, B.J. 2006. Evaluation of formulations of *Bacillus licheniformis* for the biological control of tomato gray mold caused by *Botrytis cinerea.* Biol Control. 37:329–337.

Lee, K.J., Kamala-Kannan, S., Sub, H.S., Seong, C.K. and Lee G.W. 2008. Biological control of *Phytophthora* blight in red pepper (*Capsicum annuum* L.) using *Bacillus subtilis.* World J Microb Biot. 24:1139-1145.

Lee, G.W., Ko, J.A., Oh, B.T., Choi, J.R., Lee, K.J., Chae, J.C. and Kamala-Kannan, S. 2012. Biological control of postharvest diseases of apples, peaches and nectarines by *Bacillus subtilis* S16 isolated from halophytes rhizosphere. Biocontrol Sci Techn. 22:351-361.

Lee, H. and Kim, H.Y. 2011. Lantibiotics, Class I bacteriocins from the genus *Bacillus*. J Microbiol Biotechn. 21:229-235.

Leenders, F., Stein, T., Kablitz, B., Franke, P. and Vater, J. 1999. Rapid typing of *Bacillus subtilis* strains by their secondary metabolites using matrix-assisted laser desorption/ionization mass spectrometry of intact cells. Rapid Commun Mass Sp. 13:943–949.

Lesellier, S., Palmer, S., Dalley, D.J., Davé, D., Johnson, L., Hewinson, R.G. and Chambers, M.A. 2006. The safety and immunogenicity of *Bacillus* Calmette-Guérin (BCG) vaccine in European badgers (*Meles meles*). Vet Immunol Immunop. 112:24–37.

Leveau, J.H.J., Gerards, S., Fritsche, K., Zondag, G., and van Veen, J.A. 2006. Genomic flank-sequencing of plasposon insertion sites for rapid identification of functional genes. J Microbiol Meth. 66:276-285.

Li, B., Xu, L.H., Lou, M.M., Li, F., Zhang, Y.D. and Xie, G.L. 2008. Isolation and characterization of antagonistic bacteria against bacterial leaf spot of *Euphorbia pulcherrima*. Lett Appl Microbiol. 46:450-455.

Li, H., Zhang, G., Deng, A., Chen, N. and Wen, T. 2011. De novo engineering and metabolic flux analysis of inosine biosynthesis in *Bacillus subtilis*. Biotechnol Lett. 33:1575-1580.

Lin, D., Qu, L.J., Gu, H. and Chen, Z. 2001. A 3 center dot 1-kb genomic fragment of *Bacillus subtilis* encodes the protein inhibiting growth of *Xanthomonas oryzae* pv. oryzae. J Appl Microbiol. 91:1044-1050.

Liu, B., Huang, L., Buchenauer, H. and Kang, Z. 2010. Isolation and partial characterization of an antifungal protein from the endophytic *Bacillus subtilis* strain EDR4. Pestic Biochem Phys. 98:305-311.

Liu, W. and Hanseng, J.N. 1992. Enhancement of the chemical and antimicrobial properties of subtilin by site-directed mutagenesis. J Biol Chem. 267:25078-25085.

Liu, C.F., Liu, D., Momb, J., Thomas, P.W, Lajoie, A., Petsko, G.A., Fast, W. and Ringe, D. 2013. A phenylalanine clamp controls substrate specificity in the quorum-quenching metallo-γ-lactonase from *Bacillus thuringiensis*. Biochemistry. 52:1603-1610.

Llop, P., Caruso, P., Cubero, J., Morente, C., López, M.M. 1999. A simple extraction method for efficient routine detection of pathogenic bacteria in plant material by polymerase chain reaction. J Microbiol Meth. 37:23-31.

Loeffler, W., Tschen, J.S.M., Vanittanakom, N., Kugler, M., Knorpp, E., Hsieh, T.F., Wu, T.G. 1986. Antifungal effect of bacilysin and fengymycin from *Bacillus subtilis* F-29-3 a comparison with activities of other *Bacillus* antibiotics. J Phytopathol. 115:204-213.

Logan, N.A. and Berkeley, R.C.W. 1984. Identification of *Bacillus* strains using the API system. J Gen Microbiol. 130:1871-1882.

Logan, N.A. and de Vos, P. 2009. Genus I. *Bacillus*. In. The Firmicutes. Bergey's manual of systematic bacterioloby, Vol. 3, pp. 21-128 (2 edition). Garrity, G.M. (Ed). Springer, New York.

M

MacDonald, D.L., York, D.W., Jones, L.M. and Zasloff, M.A. 1997. In vitro activity of MSI-78, an analog of the frog skin antibiotic magainin vs. ofloxacin (OFX) against aerobic and anaerobic clinical isolates from hospitals throughout the United States. In: Program and abstracts of the 37th Interscience Conference on Antimicrobial Agents and Chemotherapy, F-45, pp. 153. American Society for Microbiology, Washington, D.C.

Maes, M., Garbeva, P. and Crepel, C. 1996. Identification and sensitive endophytic detection of the fire blight pathogen *Erwinia amylovora* with 23S ribosomal DNA sequences and the polymerase chain reaction. Plant Pathol. 45:1139-1149.

Maget-Dana, R. and Peypoux, F. 1994. Iturins, a special class of pore-forming lipopeptides: biological and physicochemical properties. Toxicology. 87:151-74.

Mahaffee, W.F. and Kloepper, J.W. 1997. Temporal changes in the bacterial communities of soil, rhizosphere, and endorhiza associated with field-grown cucumber (*Cucumis sativus* L.). Microb Ecol. 34:210-223.

Malfanova, N., Franzil, L., Lugtenberg, B., Chebotar, V. and Ongena, M. 2012. Cyclic lipopeptide profile of the plant-beneficial endophytic bacterium *Bacillus subtilis* HC8. Arch Microbiol.194:893–899.

Mannanov R.N. and Sattarova, R.K. 2001. Antibiotics Produced by *Bacillus* Bacteria. Chem Nat Compd. 37:117-123.

Manulis, S., Haviv-Chesner, A., Brandl, M.T., Lindow, S.E. and Barash, I. 1998. Differential involvement of indole-3-acetic acid biosynthetic pathways in pathogenicity and epiphytic fitness of *Erwinia herbicola* pv. gypsophilae. Mol Plant Microbe Int. 11:634-642.

Mari, M., Guizzardi, M. and Pratella, G. C. 1996. Biological control of gray mold in pears by antagonistic bacteria. Biol Control. 7:30-37.

Marten, P., Smalla, K. and Berg, G. 2000. Genotypic and phenotypic differentiation of an antifungal biocontrol strain belonging to *Bacillus subtilis*. J Appl Microbiol. 89:463-471.

Martínez-Alonso, M., Escolano, J., Montesinos, E. and Gaju, N. 2010. Diversity of the bacterial community in the surface soil of a pear orchard based on *16S rRNA* gene analysis. Int Microbiol. 13:123-134.

Matheson, C.D., Gurney, C., Esau, N. and Leht, R. 2010. Assessing PCR inhibition from humic substances. Open Enzym Inhib J. 3:38-45.

Matthysse, A.G. 2006. The genus *Agrobacterium*. In: Prokaryotes, Vol. 5, pp. 91-114. Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.H. and Stackebrandt, E. (Ed). Springer, New York.

Matsuno, K., and Sonenshein, A.L. 1999. Role of *spoVG* in symmetric septation in *Bacillus subtilis.* J. Bacteriol. 181:3392–3401.

Maughan, H. and Van der Auwera, G. 2011. *Bacillus* taxonomy in the genomic era finds phenotypes to be essential though often misleading. Infect Genet Evol. 11:789-797.

McLoon, A.L., Guttenplan, S.B., Kearns, D.B., Kolter, R. and Losick, R. 2011. Tracing the domestication of a biofilm-forming bacterium. J Bacteriol. 193:2027-2034.

McSpadden-Gardener, B.B. 2004. Ecology of *Bacillus* and *Paenibacillus* spp. in agricultural systems. Phytopathology. 94:1252-1258.

Miller, M.B. and Bassler, B.L. 2001. Quorum sensing in bacteria. Annu Rev Microbiol. 55:165-199.

Miranda, C.A.C., Martins, O.B. and Clementino, M.M. 2008. Species-level identification of *Bacillus* strains isolates from marine sediments by conventional biochemical, *16S rRNA* gene sequencing and inter-tRNA gene sequence lengths analysis. Antonie van Leeuwenhoek. 93:297-304.

Monroc, S., Badosa, E., Feliu, L., Planas, M., Montesinos, E. and Bardají, E. 2006. De novo designed cyclic cationic peptides as inhibitors of plant pathogenic bacteria. Peptides. 27:2567-2574.

Montesinos, E. and Bonaterra, A. 1996. Dose-response models in biological control of plant pathogens: An empirical verification. Phytopathology. 86:464-472.

Montesinos, E., Bonaterra, A., Ophir, Y. and Beer, S.V. 1996. Antagonism of selected bacterial strains to *Stemphylium vesicarium* and biological control of brown spot of pear under controlled environment conditions. Phytopathology. 86:856-863.

Montesinos, E. 2003. Development, registration and commercialization of microbial pesticides for plant protection. Int Microbiol. 6:245-252.

Montesinos, E. 2007. Antimicrobial peptides and plant disease control. FEMS Microbiol Lett. 270:1-11.

Montesinos, E. and Bonaterra, A. 2009. Microbial pesticides. In: Encyclopedia of Microbiology (3rd edition), pp. 110-120. M. Schaechter (Ed). Elsevier Inc.

Mootz, H.D., Finking, R. and Marahiel, M.A. 2001. 4'-Phosphopantetheine transfer in primary and secondary metabolism of *Bacillus subtilis*. J Biol Chem. 276:37289-37298.

Mora, I., Cabrefiga, J. and Montesinos, E. 2011. Antimicrobial peptide genes in *Bacillus* strains from plant environments. Int Microbiol. 14:213-223.

Moragrega, C., Manceau, C. and Montesinos, E. 1998. Evaluation of drench treatments with phosphonate derivatives against *Pseudomonas syringae* pv. syringae on pear under controlled environment conditions. Eur J Plant Pathol. 104:171-180.

Moretti, C., Amatulli, M.T. and Buonaurio, R. 2009. PCR-based assay for the detection of *Xanthomonas euvesicatoria* causing pepper and tomato bacterial spot. Letters Appl Microbiol. 49:466-471.

Morikawa, M. 2006. Beneficial biofilm formation by industrial bacteria *Bacillus subtilis* and related species. J Biosci Bioeng. 101:1-8.

Moyne, A.L., Shelby, R., Cleveland, T.E. and Tuzun, S. 2001. Bacillomycin D: an iturin with antifungal activity against *Aspergillus flavus*. J Appl Microbiol. 90:622-629.

Moyne, A.L., Cleveland, T.E. and Tuzun, S. 2004. Molecular characterization and analysis of the operon encoding the antifungal lipopeptide bacillomycin D. FEMS Microbiol Lett. 234:43-49.

Mulligan, C.N. 2005. Environmental applications for biosurfactants. Environ Pollut. 133:183-198.

N

Nakamura, A., Fukumori, F., Horinouchi, S., Masaki, H., Kudo, T., Uozumi, T., Horikoshi, K. and Beppu, T. 1991. Construction and characterization of the chimeric enzymes between the *Bacillus subtilis* cellulase and an alkalophilic *Bacillus cellulose*. J Biol Chem. 266:1579-1583.

Nakano, M.M., Marahiel, M.A. and Zuber, P. 1988. Identification of a genetic locus required for biosynthesis of the lipopeptide antibiotic surfactin in *Bacillus subilis*. J Bacteriol. 170:5662-5668.

Nakano, M.M., Xia, L. and Zuber, P. 1991. Transcription initiation region of the *srfA* operon, which is controlled by the comp-comA signal transduction system in *Bacillus subtilis.* J Bacteriol. 173:5487-5493.

Nakano, M.M. and Zuber, P. 1998. Anaerobic growth of a 'strict aerobe' (*Bacillus subtilis*). Annu Rev Microbiol. 52:165-190.

Nakano, M.M. and Zuber, P. 2002. Anaerobiosis. In: *Bacillus subtilis* and its closests relatives, pp. 393-404. Soneshein, A.L, Hoch, J.A. and Losick, R. (Ed). American Society for Microbiology, Washington, D.C.

Nicholson, W.L. 2002. Roles of *Bacillus* endospores in the environment. Cell Mol Life Sci. 59:410-416.

Nihorimbere, B., Cawoy, H., Seyer, A., Brunelle, A. and Thonart, P. 2012. Impact of rhizosphere factors on cyclic lipopeptide signature from the plant beneficial strain *Bacillus amyloliquefaciens* S449. FEMS Microbiol Ecol. 79:176-191.

Norris, J.R., Berkeley, R.C.W., Logan, N.A. and O'Donnell, A.G. 1981. The genera *Bacillus* and *Sporolactobacillus*. In: The prokaryotes: a handbook on habitats, isolation and identification of bacteria, Vol. 2, pp. 1711-1742. Starr M., Stolp, H., Truper, H.G., Balows, A. and Schlegel, H. (Ed). Springer-Verlag, Berlin and Heidelberg.

Nurmi, E. and rantala, M. 1974. The influence of zinc bacitracin on the colonization of *Salmonella infantis* in the intestine of broiler chickens. Res Vet Sci. 17:24-27.

O

Okahisa, N., Inatsu, Y., Juneja, V.K. and Kawamoto, S. 2008. Evaluation and control of the risk of foodborne pathogens and spoilage bacteria present in Awa-Uirou, a sticky rice cake containing sweet red bean paste. Foodborne Pathog Dis. 5:351-359.

Omura, S., Ikeda, H., Ishikawa, J., Hanamoto, A., *et al*. 2001. Genome sequence of an industrial microorganism *Streptomyces avermitilis*: deducing the ability of producing secondary metabolites. P Natl Acad Sci USA. 98:12215–12220.

Ongena, M., Jacques, P., Toure, Y., Destain, J., Jabrane, A. and Thonart, P. 2005. Involvement of fengycin-type lipopeptides in the multifaceted biocontrol potential of *Bacillus subtilis*. Appl Microbiol Biot. 69:29-38.

Ongena, M., Jourdan, E., Adam, A., Paquot, M., Brans, A., Joris, B., Arpigny, J.L. and Thonart, P. 2007. Surfactin and fengycin lipopeptides of *Bacillus subtilis* as elicitors of induced systemic resistance in plants. Environ Microbiol. 9:1084-1090.

Ongena, M. and Jacques, P. 2008. *Bacillus* lipopeptides: versatile weapons for plant disease biocontrol. Trends Microbiol. 16:115-125.

P

Pal, K.K. and McSpadden-Gardener, B.B. 2006. Biological control of plant pathogens. Consulted 28/09/2011. Avaliable online: http://www.apsnet.org/edcenter/advanced/topics/Documents/PHI-BiologicalControl.pdf

Pan, Y.B., Grisham, M.P. and Burner, D.M. 1997. A polymerase chain reaction protocol for the detection of *Xanthomonas albilineans*, the causal agent of sugarcane leaf scald disease. Plant Dis. 81:189-194.

Parisot, J., Carey, S., Breukink, E., Chan, W.C., Narbad, A. and Bonev, B. 2008. Molecular mechanism of target recognition by subtilin, a Class I lanthionine antibiotic. Antimicrobl Agents Ch. 52:612-618.

Patel, P.S., Huang., S., Fisher, S., Pirnik, D., Aklonis, C., Dean, L., Meyers, E., Fernandes, P. and Meyers, E. 1995. Bacillaene, a novel inhibitor of procaryotic protein synthesis produced by *Bacillus subtilis*: Production, taxonomy, isolation, physico-chemical characterization and biological activity. J Antibiot. 48:997-1003.

Patel, H., Tscheka, C., Edwards, K., Karlsson, G. and Heerklotz, H. 2011. All-or-none membrane permeabilization by fengycin-type lipopeptides from *Bacillus subtilis* QST713. Biochim Biophys Acta. 1808:2000-2008.

Paternoster, T., Défago, G., Duffy, B., Gessler, C. and Pertot, I. 2010. Selection of a biocontrol agent based on a potential mechanism of action: Degradation of nicotinic acid, a growth factor essential for *Erwinia amylovora.* Int Microbiol. 13:195-206.

Paulitz, T.C. and Bélanger, R.R. 2001. Biological control in greenhouse systems. Annu Rev Phytopathol. 39:103-133

Penyalver, R., Garcia, A., Ferrer, A., Bertolini, E. and Lopez, M.M. 2000. Detection of *Pseudomonas savastanoi* pv. savastanoi in olive plants by enrichment and PCR. Appl Environ Microb. 66:2673-2677.

Pérez-García, A., Romero, D. and de Vicente, A. 2011. Plant protection and growth stimulation by microorganisms: biotechnological applications of Bacilli in agriculture. Curr Opin Biotech. 22:187-193.

Peypoux, F., Bonmatin, J. M. and Wallach, J. 1999. Recent trends in the biochemistry of surfactin. Appl Microbiol Biot. 51:553-563.

Phister, T.G., O'Sullivan, D.J. and McKay, L.L. 2004. Identification of bacilysin, chlorotetaine, and iturin A produced by *Bacillus* sp. strain CS93 isolated from pozol, a Mexican fermented maize dough. Appl Environ Microb. 70:631-634.

Pieterse, C.M.J., van Wees, S.C.M., Hoffland, E., van Pelt, J.A. and van Loon, L.C. 1996. Systemic resistance in *Arabidopsis* induced by biocontrol bacteria is independent of salicylic acid accumulation and pathogenesis-related gene expression. Plant Cell. 8:1225-1237.

Pieterse, C.M.J., van Wees, S.C.M., van Pelt, J.A., Knoester, M., Laan, R., Gerrits, N., Weisbeek, P.J. and van Loon, L.C. 1998. A novel signaling pathway controlling induced systemic resistance in *Arabidopsis*. Plant Cell. 10:1571-1580.

Piggot, P.J. and Losick, R. 2002. Sporulation genes and intercompartmental regulation. In: *Bacillus subtilis* and its closest relatives: From genes to cells, pp. 483–518. Sonenshein, A.L., Hoch, J.A. and Losick, R. (Ed). American Society for Microbiology, Washington, D.C.

Pinchuk, I.V., Bressollier, P., Sorokulova, I.B., Verneuil, B. and Urdaci, M.C. 2002. Amicoumacin antibiotic production and genetic diversity of *Bacillus subtilis* strains isolated from different habitats. Res Microbiol. 153:269-276.

Pliego, C., Ramos, C., de Vicente, A. and Cazorla, F.M. 2011. Screening for candidate bacterial biocontrol agents against soilborne fungal plant pathogens. Plant Soil. 340:505-520.

Polgár, L. 2005. Review: The catalytic triad of serine peptidases. Cell Mol Life Sci. 62:2161-2172.

Price, N.P.J., Rooney, A.P., Swezey, J.L., Perry, E. and Cohan, F.M. 2007. Mass spectrometric analysis of lipopeptides from *Bacillus strains* isolated from diverse geographical locations. FEMS Microbiol Lett. 271:83–89.

Priest, F.G., Goodfellow, M. and Todd, C. 1988. A numerical classification of the genus *Bacillus*. J Gen Microbiol. 134:1847-1882.

Pujol, M., Badosa, E., Cabrefiga, J. and Montesinos, E. 2005. Development of a strain-specific quantitative method for monitoring *Pseudomonas fluorescens* EPS62e, a novel biocontrol agent of fire blight. FEMS Microbiol Lett. 249:343-352.

Pujol, M., Badosa, E., Manceau, C. and Montesinos, E.2006. Assessment of the environmental fate of the biological control agent of fire blight, *Pseudomonas fluorescens* EPS62e, on apple by culture and real-time PCR methods. Appl Environ Microb. 72:2421-2427.

R

Raaijmakers, J.M., Weller, D.M. and Thomashow, L.S. 1997. Frequency of antibiotic-producing *Pseudomonas* spp. in natural environments. Appl Environ Microb. 63:881–887.

Raaijmakers, J.M., De Bruijn, I. and De Kock, M.J.D. 2006. Cyclic lipopeptide production by plant-associated *Pseudomonas* spp.: Diversity, activity, biosynthesis, and regulation. Mol Plant Microbe Int. 19:699-710.

Raaijmakers, J.M., de Bruijn, I., Nybroe, O. and Ongena, M. 2010. Natural functions of lipopeptides from *Bacillus* and *Pseudomonas*: more than surfactants and antibiotics. FEMS Microbiol Rev. 34:1037–1062.

Rahman, M.S., Ano, T. and Shoda, M. 2006. Second stage production of iturin A by induced germination of *Bacillus subtilis* RB14. J Biotechnol. 125:513-515.

Rajavel, M., Mitra, A. and Gopal, B. 2009. Role of *Bacillus subtilis BacB* in the synthesis of bacilysin. J Biol Chem. 46:31882-31892.

Ramarathnam, R., Bo, S., Chen, Y., Fernando, W.G.D., Gao, X.W. and de Kievit, T. 2007. Molecular and biochemical detection of fengycin and bacillomycin D producing *Bacillus* spp. antagonistic to fungal pathogens of canola and wheat. Can J Microbiol. 53:901-911.

Ramírez-Peralta, A., Zhang, P., Li, Y.-Q. and Setlow, P. 2012. Effects of sporulation conditions on the germination and germination protein levels of *Bacillus subtilis* spores. Appl Environ Microb. 78:2689-2697.

Rao, M. B., Tanksale, A. M., Ghate, M. S and Deshpande, V. V. 1998. Molecular and biotechnological aspects of microbial proteases. Microbiol Mol Biol R. 62:597–635

Read, T.D., Peterson, S.N., Tourasse, N., Baillie, L.W., *et al*. 2003. The genome sequence of *Bacillus anthracis* Ames and comparison to closely related bacteria. Nature. 423:81-86.

Rezzonico, F., Zala, M., Keel, C., Duffy, B., Moënne-Loccoz, Y. and Défago, G. 2007. Is the ability of biocontrol fluorescent pseudomonads to produce the antifungal metabolite 2,4-diacetylphloroglucinol really synonymous with higher plant protection? New Phytol. 173:861-872.

Riedel, K., Ohnesorg, T., Krogfelt, K.A., Hansen, T.S., Omori, K., Givskov, M. and Eberl, L. 2001. N-acyl-L-homoserine lactone-mediated regulation of the lip secretion system in *Serratia liquefaciens* MG1. J Bacteriol. 183:1805-1809.

Romero, D., Pérez-García, A., Rivera, M.E., Cazorla, F.M. and de Vicente, A. 2004. Isolation and evaluation of antagonistic bacteria towards the cucurbit powdery mildew fungus *Podosphaera fusca*. Appl Microbiol Biotl. 64:263-269.

Romero, D., de Vicente, A., Rakotoaly, R.H., Dufour, S.E., Veening, J.W., Arrebola, E., Cazorla, F.M., Kuipers, O.P., Paquot, M. and Pérez-García, A. 2007. The iturin and fengycin families of lipopeptides are key factors in antagonism of *Bacillus subtilis* toward *Podosphaera fusca*. Mol Plant Microbe Int. 20:430-440.

Ronimus, R.S., Parker, L.E., Turner, N., Poudel, S., Rückert, A. and Morgan H.W. 2003. A RAPD-based comparison of thermophilic bacilli from milk powders. Int J Food Microbiol. 85: 45-61.

Roongsawang, N., Siew, P.L., Washio, K., Takano, K., Kanaya, S. and Morikawa, M. 2005. Phylogenetic analysis of condensation domains in the nonribosomal peptide synthetases. FEMS Microbiology Lett. 252:143-151.

Ross, J.M. 1957. The pathogenesis of anthrax following the administration of spores by the respiratory route. J Pathol Bacteriol. 73:485-494.

Roy, J.K., Rai, S.K. and Mukherjee, A.K. 2012. Characterization and application of a detergent-stable alkaline α-amylase from *Bacillus subtilis* strain AS-S01. Int J Biol Macromol. 50:219–229.

Rozen, S. and Skaletsky, H.J. 2000. Primer3 on the WWW for general users and for biologist programmers. In: Bioinformatics methods and protocols: Methods in molecular biology, pp. 365-386. Krawetz, S. and Misener, S. (Ed). Humana Press, Totowa, N.J.

Rückert, C., Blom, J., Chen, X.H., Reva, O. and Borriss, R. 2011. Genome sequence of *Bacillys amyloliquefaciens* type strain DSM7T reveals differences to plant-associated *Bacillus amyloliquefaciens* FZB42. J Biotechnol. 155: 78-85.

S

Sakthivel, N., Mortensen, C.N. and Mathur, S.B. 2001. Detection of *Xanthomonas oryzae* pv. oryzae in artificially inoculated and naturally infected rice seeds and plants by molecular techniques. Appl Microbiol Biot. 56:435-441.

Sanahuja, G., Banakar, R., Twyman, R.M., Capell, T. and Christou, P. 2011. *Bacillus thuringiensis*: A century of research, development and commercial applications. Plant Biotechnol J. 9:283-300.

Sanders, M.E., Morelli, L., and Tompkins, T.A., 2003. Sporeformers as human probiotics: *Bacillus*, *Sporolactobacillus*, and *Brevibacillus*. Compr Rev Food Sci F. 2:101-110.

Sari, E., Etebarian, H.R., Roustaei, A. and Aminian, H. 2006. Biological control of *Gaeumannomycer graminis* on wheat with *Bacillus* spp. Plant Pathology J. 5:307-314.

Sari, E., Etebarian, H.R. and Aminian, H. 2007. The effects of *Bacillus pumilus*, isolated from wheat rhizosphere, on resistance in wheat seedling roots against the take-all fungus, *Gaeumannomyces graminis* var. *tritici.* J Phytopathol. 155:720-727.

Schallmey, M., Singh, A. and Ward, O.P. 2004. Developments in the use of *Bacillus* species for industrial production. Can J Microbiol. 50:1-17.

Schiött, T. and Hederstedt, L. 2000. Efficient spore synthesis in *Bacillus subtilis* depends on the CcdA protein. J Bacteriol. 182:2845-2854.

Schneider, K.B., Palmer, T.M. and Grossman, A.D. 2002. Characterization of *comQ* and *comX*, two genes required for production of comX pheromone in *Bacillus subtilis.* J Bacteriol. 184:410-419.

Schneider, K., Chen, X.H., Vater, J., Franke, P., Nicholson, G., Borriss, R. and Süssmuth, R.D. 2007. Macrolactin is the polyketide biosynthesis product of the pks2 cluster of *Bacillus amyloliquefaciens* FZB42. J. Nat Prod. 70:1417-1423.

Schüller, F., Benz, R. and Sahl, H.G. 1989. The peptide antibiotic subtilin acts by formation of voltage-dependent multi-state pores in bacterial and artificial membranes. Eur J Biochem. 182:181–186.

Schwab, U., Gilligan, P., Jaynes, J. and Henke, D. 1999. *In vitro* activities of designed antimicrobial peptides against multidrug-resistant cystic fibrosis pathogens. Antimicrob Agents Ch. 43:1435-1440.

Setlow, P. 2006. Spores of *Bacillus subtilis*: their resistance to and killing by radiation, heat and chemicals. J Appl Microbiol. 101:514-525.

Shangkuan, Y.H., Yang, J.F., Lin, H.C. adn Shaio, M.F. 2000. Comparison of PCR-RFLP, ribotyping and ERIC-PCR for typing *Bacillus anthracis* and *Bacillus cereus* strains. J Appl Microbiol. 89:452-462.

Sharp, J., Scawen, M.D. and Atikinson, T. 1989. Fermentation and downstream processing of *Bacillus*. In: Biotechnology handbooks, *Bacillus,* Vol. 2, pp. 255-292. Harwood, C.R. (Ed.). Plenum Press, London.

Shoda, M. 2000. Bacterial control of plant diseases. J Biosc Bioeng. 89:515-521.

Singh, P. and Cameotra, S.S. 2004. Potential applications of microbial surfactants in biomedical sciences. Trends Biotechnol. 22:142-146.

Smirnov, I.P, Zhu, S., Taylor, T., Huang, Y., Ross, P., Papayanopoulos, I.A., Martin, S.A and Pappin, D.J. 2004. Suppression of α-Cyano-4-hydroxycinnamic acid matrix clusters and reduction of chemical noise in MALDI-TOF mass spectrometry. Anal Chem. 76:2958-2965.

Slott, S., Madsen, G. and Norman, B.E. 1974. Application of a heat stable bacterial amylase in the starch industry. In: Enzyme Engineerin, Vol. 2, pp. 343-350. Pye, E.K. and Wingard Jr., L.B. (Ed). Plenum Press, New York and London.

Sneath, P.H.A. 1986. Nomenclature of bacteria. In: Biological Nomenclature Today, pp. 36-48. Ride, W.D.L. and Younès, T. (Ed). IRL Press, Oxford.

Srivatsan, A., Han, Y., Peng, J., Tehranchi, A.K., Gibbs, R., Wang, J.D. and Chen, R. 2008. High-precision, whole-genome sequencing of laboratory strains facilitates genetic studies. PLoS Genet. 4, e1000139.Stanley, N.R., Britton, R.A., Grossman, A.D. and Lazazzera, B.A. 2003. Identification of catabolite repression as a physiological regulator of biofilm formation by *Bacillus subtilis* by use of DNA microarrays. J Bacteriol. 185:1951-1957.

Stein, T. 2005. *Bacillus subtilis* antibiotics: strutures, syntheses and specific functions. Mol Microbiol. 56:845-857.

Stein, T. and Entian, K.D. 2002. Maturation of the lantibiotic subtilin: matrix-assisted laser desorption/ionization time-of-flight mass spectrometry to monitor precursors and their proteolytic processing in crude bacterial cultures. Rapid Commun Mass Sp. 16:103-110.

Stein, T., Heinzmann, S., Kiesau, P., Himmel, B. and Entian, KD. 2003. The *spa*-box for transcriptional activation of subtilin biosynthesis and immunity in *Bacillus subtilis*. Mol Microbiol. 47:1627-1636.

Stein, T., Düsterhus, S., Stroh, A. and Entian, K.D. 2004. Subtilosin production by two *Bacillus subtilis* subspecies and variance of the *sbo-alb* cluster. Appl Environ Microb. 70:2349-2353.

Stein, T., Borchert, S., Conrad, B., Feesche, J., Hofemeister, B., Hofemeister, J. and Entian, K.D. 2002. Two different lantibiotic-like peptides originate from the ericin gene cluster of *Bacillus subtilis* A1/3. J Bacteriol. 184:1703-1711.

Stein, T. 2008. Whole-cell matrix-assisted laser desorption/ionization mass spectrometry for rapid identification of bacteriocin/lantibiotic-producing bacteria. Rapid Commun Mass Sp. 22:1146-1152.

Steinborn, G., Hajirezaei, M.R. and Hofemeister, J. 2005. *bac* genes for recombinant bacilysin and anticapsin production in *Bacillus* host strains. Arch Microbiol. 183:71-79.

Steller, S., Sokoll, A., Wilde, C., Bernhard, F., Franke, P. and Vater, J. 2004. Initiation of surfactin biosynthesis and the role of the srfD-Thioesterase protein. Biochemistry-US. 43:11331-11343.

Stephens, C. 1998. Bacterial sporulation: A question of commitment? Curr Biol. 8:R45-R48.

Suihko, M.L. and Staackerbrandt, E. 2003. Identification of aerobic mesophilic bacilli isolated from board and paper products containing recycled fibres. J Appl Microbiol. 94:25-34.

T

Tamehiro, N., Okamoto-Hosoya, Y., Okamoto, S., Ubukata, M., Hamada, M., Naganawa, H. and Ochi, K. 2002. Bacilysocin, a novel phospholipid antibiotic produced by *Bacillus subtilis* 168. Antimicrob Agents Ch. 46:315-320.

Tawara, S., Ikeda, F., Maki, K., Morishita, Y., Otomo, K., Teratani, N., Goto, T., Tomishima, M., Ohki, H., Yamada, A., Kawabata, K., Takasugi, H., Sakane, K., Tanaka, H., Matsumoto, F. and Kuwahara, S. 2000. In vitro activities of a new lipopeptide antifungal agent, FK463, against a variety of clinically important fungi. Antimicrob Agents Ch. 44:57-62.

Teng, D., Fan, Y., Yang, Y-L., Tian, Z-G., Luo, J. and Wang J-H. 2007. Codon optimization of *Bacillus licheniformis* β-1,3-1,4-glucanase gene and its expression in *Pichia pastoris. Appl Microbiol Biot.* 74:1074-1083.

Thasana, N., Prapagdee, B., Rangkadilok, N., Sallabhan, R., Aye, S.L., Ruchirawat, S. and Loprasert, S. 2010. *Bacillus subtilis* SSE4 produces subtulene A, a new lipopeptide antibiotic possessing an unusual C15 unsaturated beta-amino acid. FEBS Lett. 584:3209-3214.

Thimon, L., Peypoux, F., Wallach, J. and Michel, G. 1995. Effect of the lipopeptide antibiotic, iturin A, on morphology and membrane ultrastructure of yeast cells. FEMS Microbiol Lett.128:101-106.

Thirabunyanon, M. and Thongwittaya, N. 2012. Protection activity of a novel probiotic strain of *Bacillus subtilis* against *Salmonella enteritidis* infection. Res Vet Sci.93:74-81.

Touré, Y., Ongena, M., Jacques, P., Guiro, A. and Thonart, P. 2004. Role of lipopeptides produced by *Bacillus subtilis* GA1 in the reduction of grey mould disease caused by *Botrytis cinerea* on apple. J Appl Microbiol. 96:1151-1160.

Trias, R., Bañeras, L., Montesinos, E. and Badosa, E. 2008. Lactic acid bacteria from fresh fruit and vegetables as biocontrol agents of phytopathogenic bacteria and fungi. Int Microbiol. 11:231-236.

Troyano, M., Bloch Jr., C., Carmona-Ribeiro, A.M. and di Mascio, P. 2009. Lipopeptides produced by a soil *Bacillus megaterium* strain. Microb Ecol. 57:367–378

Tsuge, K., Akiyama, T. and Shoda, M. 2001. Cloning, sequencing, and characterization of the iturin A operon. J Bacteriol. 183:6265-6273.

V

Vanittanakom, N., Loeffler, W., Koch, U. and Jung, G. 1986. Fengycin a novel antifungal lipopeptide antibiotic produced by *Bacillus subtilis* F-29-3. J antibiot. 39:888-901.

Vater, J., Kablitz, B., Wilde, C., Franke, P., Mehta, N. and Cameotra, S.S. 2002. Matrix-assisted laser desorption ionization–time of flight mass spectrometry of lipopeptide biosurfactants in whole cells and culture filtrates of *Bacillus subtilis* C-1 isolated from petroleum sludge. Appl Environ Microb. 68:6210-6219.

Veith, B., Herzberg, C., Steckel, S., Feesche, J., *et al.* 2004. The complete genome sequence of *Bacillus licheniformis* DSM13, an organism with great industrial potential. J Mol Microbiol Biotechnol. 7:204-211.

Vilà, B., Fontgibell, A., Badiola, I., Esteve-Garcia, E., Jiménez, G., Castillo, M. and Brufau, J. 2009. Reduction of *Salmonella enterica* var. Enteriditis conolization and invasion by *Bacillus cereus* var. Toyoi inclusion in poultry feeds. Poultry Sci. 88:975-979.

W

Walker, J.E., and Abraham, E.P. 1970. Isolation of bacilysin and a new amino acid from culture filtrates of *Bacillus subtilis*. Biochem J. 118:557-561.

Walsh, C.T. 2004. Polyketide and nonribosomal peptide antibiotics: modularity and versatility. Science. 303:1805-1810.

Wang, X., Luo, C. and Chen, Z. 2012. Genome sequence of the plant growth-promoting rhizobacterium *Bacillus* sp. strain 916. J Bacteriol. 194:5467-5468.

Weinrauch, Y., Msadek, T., Kunst, F. and Dubnau, D. 1991. Sequence and properties of *comQ*, a new competence regulatory gene of *Bacillus subtilis.* J Bacteriol. 173:5685-5693.

Weller, S.A., Elphinstone, J.G., Smith, N.C., Boonham, N. and Stead, D.E. 2000. Detection of *Ralstonia solanacearum* strains with a quantitative, multiplex, Real-Time, fluorogenic PCR (TaqMan) assay. Appl Environ Microb. 66:2853-2858.

Weller, S.A. and Stead, D.E. 2002. Detection of root mat associated *Agrobacterium* strains from plant material and other sample types by post-enrichment TaqMan PCR. J Appl Microbiol. 92:118-126.

Williams, L.D., Burdock, G.A., Jiménez, G. and Castillo, M. 2009. Literature review on the safety of Toyocerin®, a non–toxigenic and non-pathogenic *Bacillus cereus* var. toyoi preparation. Regul Toxicol and Pharm. 55:236-246.

Winson, M.K., Camara, M., Latifi, A., Foglino, M., Chhabra, S.R., Daykin, M., Bally, M., Chapon, V., Salmond, G.P. and Bycroft B.W. 1995. Multiple N-acyl-L-homoserine lactone signal molecules regulate production of virulence determinants and secondary metabolites in *Pseudomonas aeruginosa*. P Natl Acad Sci USA. 92:9427-9431.

Wipat, A. and Harwood, C.R. 1999. The *Bacillus subtilis* genome sequence: the molecular blueprint of a soil bacterium. FEMS Microbiol Ecol. 28:1-9.

Wulff, E.G., Mguni, C.M., Mansfeld-Giese, K., Fels, J., Lübeck, M. and Hockenhull, J. 2002. Biochemical and molecular characterization of *Bacillus amyloliquefaciens*, *B. subtilis* and *B. pumilus* isolates with distinct antagonistic potential against *Xanthomonas campestris* pv. campestris. Plant Pathol. 51:574-584.

Wu, C.Y., Chen, C.L., Lee, Y.H., Cheng, Y.C, Wu, Y.C., Shu, H.Y., Götz, F. and Liu, S.T. 2006. Nonribosomal synthesis of fengycin on an enzyme complex formed by fengycin synthetases. J Biol Chem. 282:5608-5616.

Wu, W.S., Wu, H.C. and Li, Y.L. 2007. Potential of *Bacillus amyloliquefaciens* for control of *Alternaria cosmosa* and *A. patula* of cosmos sulfurous (Yellow Cosmos) and *Tagetes patula* (French Marigold). J Phytopathol. 155:670–675.

Wulff, E.G., Mguni, C.M., Mansfeld-Giese, K., Fels, J., Lübeck, M. and Hockenhull, J. 2002. Biochemical and molecular characterization of *Bacillus amyloliquefaciens, B. subtilis* and *B. pumilus* isolates with distinct antagonistic potential against *Xanthomonas campestris* pv. campestris. Plant Pathol. 51:574-58.

Y

Yamabhai, M., Emrat, S., Sukasem, S., Pesatcha, P., Jaruseranee, N. and Buranabanyat, B. 2008. Secretion of recombinant *Bacillus* hydrolytic enzymes using *Escherichia coli* expression systems. J Biotechnol. 133:50-57.

Yánez-Mendizábal, V., Zeriouh, H., Viñas, I., Torres, R., Usall, J., de Vicente, A., Pérez-García, A. and Teixidó, N. 2012. Biological control of peach brown rot (*Monilinia* spp.) by *Bacillus subtilis* CPA-8 is based on production of fengycin-like lipopeptides. Eur J Plant Pathol. 132:609-619.

Yazgan, A., Özcengiz, G. and Mohamed A. 2001a. Tn10 insertional mutations of *Bacillus subtilis* that block the biosynthesis of bacilysin. Biochim Biophys Acta. 1518:87-94.

Yazgan, A., Özcengiz, G., Özcengiz, E., Kılınç, K., Marahiel, M.A. and Gürdal Alaeddinoglu, N. 2001b. Bacilysin biosynthesis by a partially-purified enzyme fraction from *Bacillus subtilis.* Enzyme Microb Tech. 29:400-406.

Yuan, J., Raza, W., Shen, Q. and Huang, Q. 2012. Antifungal activity of *Bacillus amyloliquefaciens* NJN-6 volatile compounds against *Fusarium oxysporum* f. sp. Cubense. Appl Environ Microb. 78:5942-5944.

Z

Zaccardelli, M., Spasiano, A., Bazzi, C., Merighi, M. 2005. Identification and in planta detection of *Pseudomonas syringae* pv. tomato using PCR amplification of hrpZPst. Eur J Plant Pathol. 111:85-90.

Zeriouh, H., Romero, D., García-Gutiérrez, L., Cazorla, F.M., De Vicente, A. and Pérez-García, A. 2011. The iturin-like lipopeptides are essential components in the biological control arsenal of *Bacillus subtilis* against bacterial diseases of cucurbits. Mol Plant Microbe Int. 24:1540-1552.

Zhang, C.X., Zhao, X., Jing, Y.X., Chida, T, Chen, H. and Shen, S.H. 2008a. Phenotypic and biological properties of two antagonist *Bacillus subtilis* strains. World J Microb Biot. 24:2179–2181.

Zhang B., Xie, C. and Yang, X. 2008b. A novel small antifungal peptide from *Bacillus* strain B-TL2 isolated from tobacco stems. Peptides. 29:350-355.

Zhao, P., Quan, C., Jin, L., Wang, L., Wang, J. and Fan, S. 2013. Effects of critical medium components on the production of antifungal lipopeptides from *Bacillus amyloliquefaciens* Q-426 exhibiting excellent biosurfactant properties. World J Microb Biot. 29:401-409.

Annex

BUFFERS and SOLVENTS

DNA extraction buffer [pH 7.5] (Llop *et al*. 1999)

Tris base [pH 7.5]	24.2 g
NaCl	14.6 g
EDTA	9.3 g
Sodium Dodecyl Sulfate (SDS)	5 g
Polyvinilpyrrolidone (PVP)	20 g
Distilled water	1 l

Sterilized by filtration

HPLC Solvent A

Trifluoroacetic acid (TFA)	1 ml
Distilled water	1 l

Filtered by cellulose nitrate filter (pore 0.45µm)

30 min homogenization and degasification

HPLC Solvent B

Trifluoroacetic acid (TFA)	0.85 ml
HPLC acetonitrile	1 l

Filtered by cellulose nitrate filter (pore 0.45µm)

30 min homogenization and degasification

MALDI-TOF solvent

Trifluoroacetic acid (TFA)	1 ml
HPLC acetonitrile	700ml
Distilled water	300ml

Filtered by cellulose nitrate filter (0.45µm pore size)

30 min homogenization and degasification

Phosphate buffer [pH 7.0]

Na_2HPO_4	7.1 g
KH_2PO_4	2.72 g
Distilled water	1 l

20 min sterilization by autoclaving (121 ºC)

Tris-Acetate-EDTA (TAE) buffer 50X [pH 8.0]

Tris base	242 g
Glacial acetic acid	57.1 ml
EDTA (0.5M) [pH 8.0]	100 ml
Distilled water	Adjust volume to 1 l

CULTURE MEDIA

Lysogeny Broth/Agar medium (LB) [pH 7.0]

Tryptone	10 g
Yeast extract	5 g
NaCl	10 g
Agar	15 g
Distilled water	1 l

20 min sterilization by autoclaving (121 ºC)

Nutrient Broth/Agar medium (NB/NA) [pH 7.2]

Glucose	2.5 g
Peptone	5 g
Yeast extract	3 g
NaCl	5 g
Distilled water	1 l

20 min sterilization by autoclaving (121 ºC)

Potato Dextrose Broth/Agar medium (PDB/PDA) [pH 5.6]

Potato-dextrose broth (DIFCO254920)	24 g
Potato-dextrose agar (DIFCO 0013-17-6)	39 g
Distilled water	1 l

20 min sterilization by autoclaving (121 ºC)

Production Medium (PM) [pH 7.0](Walker and Abraham, 1970)

Basal medium		954 ml
	KH_2PO_4	7 g/l
	$MgSO_4 \cdot 7H_2O$	0.5 g/l
	KCl	0.5 g/l
	$C_5H_{10}NNaO_5$	1 g/l
Ferric citrate solution		5 ml
	$FeCl_3 \cdot 6H_2O$	20 mg/ml
	$C_6H_9N_3O_9$	20 mg/ml
Oligodynamic solution		1 ml
	$ZnSO_4 \cdot 7H_2O$	0.1 mg/l
	$CoCl_2 \cdot 6H_2O$	0.1 mg/l
	$(NH_4)_2MoO_4$	0.1 mg/l
	$MnCl_2 \cdot 4H_2O$	1mg/l
	$CuSo_4$	0.01 mg/l

20 min sterilization by autoclaving (121 ºC)

Sucrose [342 g/l]	40 ml

Sterilized by filtration and added to sterilized medium