Archives of Microbiology (2018) 200:1205–1216
https://doi.org/10.1007/s00203-018-1537-8

ORIGINAL PAPER

Lipopeptide biodiversity in antifungal *Bacillus* strains isolated from Algeria

Lamia Abdellaziz[1,2] · Marlène Chollet[2] · Ahmed Abderrahmani[1,2] · Max Béchet[2] · Lamia Yaici[1,2] · Gabrielle Chataigné[2] · Anthony Arguelles Arias[3] · Valérie Leclère[2] · Philippe Jacques[2,3]

Received: 22 November 2017 / Revised: 18 May 2018 / Accepted: 29 May 2018 / Published online: 9 June 2018

Abstract

Several *Bacillus* strains have been well studied for their ability to control soil-borne plant diseases. This property is linked to the production of several families of lipopeptides. Depending of their structure, these compounds show antifungal and/or plant systemic resistance inducing activities. In this work, the biodiversity of lipopeptides produced by different antifungal *Bacillus* strains isolated from seeds, rhizospheric, and non-rhizospheric soils in Algeria was analyzed. Sixteen active strains were characterized by PCR for their content in genes involved in lipopeptide biosynthesis and by MALDI-ToF for their lipopeptide production, revealing a high biodiversity of products. The difficulty to detect kurstakin genes led us to design two new sets of specific primers. An interesting potential of antifungal activity and the synthesis of two forms of fengycins differing in the eighth amino acid (Gln/Glu) were found from the strain 8. Investigation of its genome led to the finding of an adenylation domain of the fengycin synthetase predicted to activate the glutamate residue instead of the glutamine one. According to the comparison of both the results of MALDI-ToF-MS and genome analysis, it was concluded that this adenylation domain could activate both residues at the same time. This study highlighted that the richness of the Algerian ecosystems in *Bacillus* strains is able to produce: surfactin, pumilacidin, lichenysin, kurstakin, and different types of fengycins.

Keywords Lipopeptides · Kurstakin · Specific primers · Fengycin · Algerian *Bacillus* strains

Introduction

Lipopeptides are amphiphilic compounds produced by different micro-organisms and showing a high biodiversity of structure and functional properties (Coutte et al. 2017). Among these lipopeptide-producing micro-organisms,

Communicated by Pierre Béguin.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00203-018-1537-8) contains supplementary material, which is available to authorized users.

✉ Philippe Jacques
philippe.jacques@ulg.ac.be

Lamia Abdellaziz
lamiaabdellaziz@yahoo.fr

Marlène Chollet
marlene.chollet@univ-lille1.fr

Ahmed Abderrahmani
abderahmani_ahmed@yahoo.com

Max Béchet
max.bechet@univ-lille1.fr

Lamia Yaici
lamia.yaici@yahoo.fr

Gabrielle Chataigné
gabrielle.chataigne@polytech-lille.fr

Anthony Arguelles Arias
aarguelles@ulg.ac.be

Valérie Leclère
valerie.leclere@univ-lille1.fr

[1] Microbiological Team, Cellular and Molecular Biological Laboratory, Faculty of Biological Sciences, University of Sciences and Technology Houari Boumediene, Algiers, Algeria

[2] Univ. Lille, INRA, ISA, Univ. Artois, Univ. Littoral Côte d'Opale, EA 7394-ICV-Institut Charles Viollette, 59000 Lille, France

[3] Microbial Processes and Interactions, Gembloux Agro-Bio Tech, TERRA Teaching and Research Centre, University of Liege, Gembloux, Belgium

Bacillus spp. are widely studied for their production of five different families named fengycins/plipastatins, surfactins, iturins, kurstakins (Jacques 2011), and locillomycins (Luo et al. 2015a). Due to their antifungal activity and/or their ability to induce systemic resistance in plant, some of these biosurfactant molecules could be used in agriculture as biopesticides instead of chemicals (Deravel et al. 2014).

Kurstakins are lipoheptapeptides containing a cyclic or linear peptide sequence as (D-Thr)-Gly-Ala-Gln-His-(D-Gln)-Ser linked to a fatty acid chain of 11–14 carbon atoms, β-hydroxylated or not (Hathout et al. 2000; Béchet et al. 2012). Fengycins are lipodecapeptides with a peptide chain as Glu-(D-Orn)-Tyr-(D-a-Thr)-Glu-(D-Ala/Val)-Pro-Gln-(D-Tyr)-Ile partially cyclized and linked to a β-hydroxy fatty acid of 14–18 carbon atoms (Ongena and Jacques 2008). Iturins are lipoheptapeptides with different peptide chains linked to a β-amino fatty acid chain between 14 and 17 carbon atoms (Peypoux et al. 1978), and surfactins are lipoheptapeptides with different peptide chains linked with a β-hydroxy fatty acid of 13–18 carbon atoms (Jacques 2011). Locillomycins are partially cyclized nonapeptides with the peptide sequence Thr-Gln-Asp-Gly-Asn-Asp-Gly-Tyr-Val linked to a fatty acid chain of 13–15 carbon atoms (Luo et al. 2015a).

All these lipopeptides are synthesized by non-ribosomal peptide synthetases (NRPS), which are megaenzymes organized into modules. Each module incorporates an amino acid and is composed of at least three main domains involved in adenylation (500–600 AA in length), thiolation (80–100 AA), and condensation (450 AA), insuring, respectively, the recognition and activation, tethering of the amino acids on the NRPS, and the peptide bond formation. In addition, the domain of thioesterase (280 AA), present in the last module, releases the neoformed peptide and catalyzes in some cases its cyclization. Moreover, secondary domains such as those involved in epimerization and methylation modify the peptide structure (Marahiel et al. 1997).

Kurstakin operon is organized into three synthetases (KrsA, KrsB, and KrsC) followed by a gene coding phosphopantetheinyl transferase (Ppant), the latter being located upstream from an additional thioesterase domain named KrsD. These synthetases are responsible for the incorporation of D-Thr (KrsA), Gly-Ala (KrsB), and Ser-His-Gln-D-Gln (KrsC) (Abderrahmani et al. 2011). Fengycin operon is known to be composed of five synthetases PpsA/FenA, PpsB/FenB, PpsC/FenC, PpsD/ FenD, and PpsE/FenE, which are responsible for the incorporation of (Glu, D-Orn), (Tyr, D-a-Thr), (Glu, D-Ala/D-Val), (Pro, Gln, D-Tyr), and (Ile), respectively (Jacques 2011).

Different approaches have been recently developed for the screening of lipopeptidic compounds (Biniarz et al. 2017). MALDI-ToF-MS is usually used to check lipopeptide production while degenerate primers have been designed to detect by PCR the presence of genes involved in their biosynthesis (Tapi et al. 2010; Abderrahmani et al. 2011). In addition, considering the facility to get genome sequencing and the availability of softwares to decipher the synthetase organization in modules and domains and to predict the recruited monomers by each adenylation domain, it is now possible to identify the genomic potential of a strain regarding the production of lipopeptides (Weber et al. 2015; Flissi et al. 2016). In this work, these different approaches were combined to explore the biodiversity of lipopeptides produced in Algerian ecosystems.

Materials and methods

Isolation and characterization of strains

Samples were randomly collected in Algeria from different types of soils and seeds. The soil samples were from the rhizosphere of *Ficus macrophylla, Macadamia integrifolia, Washingtonia robusta, Phyllostachys sp*., *Yucca guatemalensis, Carya oliviformis, Mangifera indica, Prunus dulcis, Echium creticum, Ficus carica*, and *Pinus halepensis* or from non-rhizospheric soils, polluted or not. The seed samples were from *Triticum durum, Vicia faba, Hordeum vulgare, Pisum sativum*, and *Oryza sativa*. Samples constituted of 20 g of soil or cereals were placed in 200 mL of sterile saline water. 1 mL samples of the appropriate decimal dilution were plated onto Luria Bertani (LB) agar medium and incubated at 30 °C for 48 h. Pure cultures were obtained by repeated streaking of isolated colonies on LB agar. The colonies were then kept on agar slides at room temperature.

One hundred Gram-positive bacilli were selected as *Bacillus* strains according to the following usual microbiological methods: sporulation, motility, facultative anaerobes, catalase activity, positive VP (Voges–Proskauer) test, negative indole production, starch, and lecithin hydrolysis. The presence of the Cry protein crystal checked under photonic microscopic observation was used to identify *Bacillus thuringiensis* strains.

The spot method was used to evaluate the antifungal activities of the *Bacillus* strains. Two-microliter spore suspensions of *Fusarium oxysporum* f. *sp. conglutinans, Rhizoctonia solani* isolate S010-1-1 (Asselbergh et al. 2007), *Galactomyces geotrichum* MUCL 28,959 (BCCM/MUCL Collection, Louvain-la-Neuve, Belgium), and *Botrytis cinerea* R16 (Van Beneden et al. 2009), containing approximately 10^4 spores mL^{-1} were spotted onto LB agar at 1 cm from the spot (2 µL containing about 10^6 cells mL^{-1}) of each tested strain. Petri dishes were incubated at 30 °C for 72 h. The antifungal activities were monitored by the ratio of the fungal growth radius in the presence (a) and in the

absence (b) of the bacteria (ratio = *a*/*b*). A significant antifungal activity is observed when this ratio is lower than 0.9.

Sixteen strains showing the highest antifungal activities were identified by both 16S rRNA and in some cases by *gyrB* genes PCR analyses using, respectively, the universal primers S1-F/S2-R (Jacobs 2007) and gyrB-F/gyrB-R (Yamamoto and Harayama 1995); these latter discriminating the species of *Bacillus subtilis* group, in particular *Bacillus licheniformis* and *B. subtilis*. After electrophoresis, the amplicons were extracted from 0.7% agarose gels using GeneJet Gel Extraction Kit (Thermo Fisher Scientific Fermentas, Vilnius, Lituania), and the 16S rRNA and *gyrB* amplified fragments were sequenced by Eurofins Genomics (Anzinger, Germany). The obtained sequences were compared with the 16S ribosomal RNA sequences (Bacteria and Archaea) and Genebank databases, respectively, using the Nucleotide Basic Local Alignement Tool (BLASTn) software provided on line by the National Center of Biotechnology and Information (NCBI, Bethedsa, MD, U.S.A.).

Two reference strains, *B. thuringiensis* CIP 110,222 from Pasteur Institute Collection (Paris, France) that was named strain 1 in this study and *B. subtilis* serovar *spizizenii* ATCC 6633 from American Type Culture Collection (Manassas, VA, U.S.A.) were used as positive and negative controls for the presence of kurstakin operon, respectively. All the strains presented in this study are listed in Table 1.

Lipopeptide detection by whole bacterial cell matrix-assisted laser-desorption time of flight mass spectrometry and LC-MS

The bacteria were cultured on LB agar plates, which were incubated at 30 °C for 72 h. Individual bacteria colonies were carefully removed from the agar surface with a tungsten wire loop and immediately suspended into a 1.5 mL Eppendorf tube containing a matrix solution (10 mg mL^{-1} cyano-4-hydroxycinnamic acid in 70% water, 30% acetonitrile, and 0.1% trifluoroacetic acid). The samples were vortexed and centrifuged at 2000*g*. For classical analysis, 1 µL of sample solution was spotted onto a MALDI-ToF MTP 384 target plate (Bruker Daltonik GmbH, Leipzig, Germany) according to the procedure of the dried-droplet preparation.

Mass profiles experiments were analyzed with an Ultraflex MALDI-ToF/ToF mass spectrometer (Bruker, Bremen, Germany) equipped with a smartbeam laser. Samples were analyzed using an accelerating voltage of 25 kV and matrix suppression in reflectron mode at *m*/*z* 750. The laser power was set to just above the threshold of ionization (around 35%). Spectra were acquired in reflector positive mode in the range of 800 at 3000 Da. Each spectrum was the result of 1000 laser shots per m/z segment per sample delivered in 10 sets of 50 shots distributed in three different locations on the surface of the matrix spot. The instrument was externally calibrated in positive reflector mode using bradykinin (1–7) $[M+H]^+$ 757.3991, angiotensin II $[M+H]^+$ 1046.5418, angiotensin I $[M+H]^+$ 1296.6848, substance P $[M+H]^+$ 1347.7354, bombesin $[M+H]^+$ 1619.8223, and ACTH (1–17) $[M+H]^+$ 2,093.0862.

Culture supernatants of strain 8 were analyzed by reverse phase UPLC–MS (UPLC, Waters, Acquity class H) coupled with a single quadrupole MS (SQDetector, Waters, Acquity) on an Acquity UPLC BEH C18 (Waters) 2.1 × 50 mm, 1.7 µM column using. A method based on acetonitrile/water (acidified with 0.1% formic acid) gradients that allowed the simultaneous detection of all three lipopeptide families. Elution was started at 30% acetonitrile (flow rate of 0.60 ml min^{-1}). After 2.43 min, the percentage of acetonitrile was brought up to 95% and held until 5.12 min. Then, the column was stabilized at an acetonitrile percentage of 30% for 1.87 min. Compounds were identified on the basis of their retention times compared with authentic standards (98% purity, Lipofabrik, Villeneuve d'Ascq, France) and the

Table 1 Strains used in this study

Name and serotype	Genotype	Origin
B. thuringiensis CIP 110,222	Wild type	*Vicia faba* L. (Algiers)
B. subtilis ATCC 6633	Wild type	ATCC, Manassas, VA, U.S.A
Escherichia coli JM109	*recA1, endA1, gyrA96, thi, hsdR17*; (rK−, mK+), *relA1, supE44*; Δ(*lac-proAB*), [F−, *traD36*; *proAB, lacIqZ*ΔM15]	Promega Corp., Madison, WI, U.S.A
Rhizoctonia solani S010-1-1		M. Höfte, Ghent University, Belgium, Asselbegh et al. (2007)
Galactomyces geotrichum MUCL 28,959		M. Höfte, Ghent University, Belgium, Van Beneden et al. (2009)
Botrytis cinerea R16		M. Höfte, Ghent University, Belgium (Van Beneden et al. 2009)
Fusarium oxysporum f. sp. *conglutinans*		M. Höfte, Ghent University, Belgium, Asselbegh et al. (2007)

masses detected in the SQDetector. Ionization and source conditions were set as follows: source temperature, 130 °C; desolvation temperature, 400 °C; nitrogen flow, 1000 l h^{-1}; cone voltage, 120 V.

Lipopeptide synthetase gene detection by PCR

DNA extraction

Total genomic DNAs were isolated from 1.5 mL of LB broth culture inoculated by one colony of *Bacillus* strain. DNA was extracted and purified using the Wizard DNA purification kit (Promega Corp., Madison, WI, U.S.A.) according to the supplier recommendations. DNA concentration was determined using the NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific, Villebon sur Yvette, France).

Polymerase chain reaction

The different primer pairs designed by Tapi et al. (2010) and Abderrahmani et al. (2011) were used to detect specifically lipopeptides synthetase genes coding for surfactin, mycosubtilin, bacillomycin, fengycin, and kurstakin synthetases. To design new sets of primers for the detection of kurstakin genes in all *Bacillus* strains producing kurstakin, 38 kurstakin operons (16 from *Bacillus cereus* and 22 from *B thuringiensis* strains) were identified from the NCBI genome databases using antiSMASH 3.0 (Weber et al. 2015). Primers were then determined from the consensus regions after alignment of the adenylation domains responsible for histidine incorporation (AH-F/AH-R). Primers designed in the thioesterase-Ppant-thioesterase cluster were determined from *B. cereus* BGSC 9842 genome using AmplifX 1.7.0 (Jullien 2012; CNRS, Aix Marseille Université, France). All the primers used in this study are listed in Table 2.

To optimize PCR conditions with the newly designed primers, positive control from the strain CIP 110,222 was used (Abderrahmani et al. 2011). The PCR thermal cycle program consisted of an initial denaturation step at 94 °C for 3 min followed by 29 cycles with a denaturation step at 94 °C for 1 min, an annealing step for 30 s at 42.4 °C for AH-F/AH-R and at 54 °C for P-Te1-F/P-Te2-R, followed by an extension step at 72 °C for 2 min. A final extension was performed at 72 °C for 10 min.

Table 2 Characteristics of the different primers used in this study

Primer name	Sequences (5′→3′)	Identified lipopeptides or non-ribosomal domains	Expected amplicon size (bp)	References
As1-F/Ts2-R	CGCGGMTACCGVATY GAGC[a] ATBCCTTTBTWDGAATGT CCGCC[a]	Surfactin (Srf)	419; 422; 425; 431	Tapi et al. (2010)
Am1-F/Tm1-R	CAKCARGTSAAAATY CGMGG[a] CCDASATCAAARAADT-TATC[a]	Mycosubtilin (Myc)	416; 419	Tapi et al. (2010)
Af2-F/Tf1-R	GAATAYMTCGGMCGTMT-KGA[a] GCTTTWADKGAATSBC-CGCC[a]	Fengycin (Fen)	443; 452; 455	Tapi et al. (2010)
Abl1-F/Tbl1-R	GATSAWCARGTGAAAA TYCG[a] ATCGAATSKCCGCCRARA TCRAA[a]	Bacillomycin (Bmy)	428; 431; 434	Abderrahmani et al. (2011)
Aks1-F/Tks1-R	TCHACWGGRAATCCA AAGGG[a] CCACCDKTCAAAKAARK-WATC[a]	Kurstakin (Krs)	1125; 1152; 1161; 1167; 1173	Abderrahmani et al. (2011)
AH-F/AH-R	AGGATCGTGCGACTCAGA TT CCATACTGATTAATTACC GCA	Histidine adenylation domain of kurstakin	307	This study
P-Te1-F/P-Te2-R	TCATTGATATGTAAATAT GC CCCATTGCAATAACTTCT CT	Thioesterase domains of kurstakin	1222	This study

[a]Using IUPAC DNA code [Y = C or T; M = A or C; K = G or T; W = A or T; D = A or G or T; S = C or G; B = C or G or T; R = A or G; H = A or C or T; V = A or C or G]

Cloning and sequencing conditions

25 µL of PCR mixture was analyzed by electrophoresis in 0.7% (w/v) agarose gel containing GelRed (0.01% v/v) staining. When a band of the appropriate size was obtained, a positive score was given. After extraction and purification from the gels using GeneJet Gel Extraction Kit (Thermo Fisher Scientific Fermentas), each amplicon was cloned into pGEM-T Easy vector (Promega Corp.). The ligation mixtures consisted of 3 µL of PCR product, 5 µL T4 DNA ligase buffer (2 X), 1 µL of pGEM-T Easy (50 ng μL^{-1}), and 1 µL of T4 DNA ligase. After overnight incubation at 16 °C, these mixtures were used to transform *Escherichia coli* JM109 competent cells, using the heat shock procedure as specified by the supplier. After about 90 min subculture in SOC medium, the bacteria were spread onto LB plates supplemented with ampicillin (Amp; Na salt; 100 µg mL^{-1}), isopropyl-β-D-galactopyranoside (IPTG; 200 µg mL^{-1}) and X-Gal (20 µg mL^{-1}), all provided by Sigma-Aldrich (St Louis, MO, U.S.A.). After 18 h of incubation at 37 °C, white colonies were cultured overnight in LB containing ampicillin. Plasmids were recovered using GeneJet Miniprep Plasmid Kit (Thermo Fisher Scientific Fermentas) and the presence of inserts was checked by restriction analysis using *Eco*RI (Thermo Fisher Scientific Fermentas). Cloned products were sequenced using the universal primers pUCM13R/F in an ABI PRISM dye terminator cycle sequencing kit (Eurofins Genomics). The sequences were analyzed by comparison with the GenBank database using BLAST software provided on line by the NCBI.

Genome sequencing, assembly, and annotation for the relevant strain

DNA was extracted using the Wizard DNA purification kit (Promega Corp.), to obtain a total genomic DNA concentration of 131.9 ng/µL diluted in 10 mM Tris–HCl pH 8.5 buffer. The sequencing, assembling, and the first annotation were provided by MicrobesNG (http://microbesng.uk/faq/) which is supported by the BBSRC (grant number BB/L024209/1), with the use of Miseq Illumina Platform. The assembled nodes provided were then annotated using RAST version 2.0 (Rapid Annotation using Subsystems Technology, a free annotation web server) (Aziz et al. 2008; Overbeek et al. 2014; Brettin et al. 2015). After that, the genome was annotated for secondary metabolites using antiSMASH 3.0 at the link http://antismash.secondarymetabolites.org (Weber et al. 2015).

Results

Identification and characterization of the isolates

Ninety-two *Bacillus* strains were isolated from the different collected samples. All the strains were characterized by phenotypic and biochemical tests confirming the affiliate of the strains to *Bacillus* genus (see Materials and methods). Among these isolates, 89 strains were active against at least 1 fungus and 9 among these latter were active against the 4 fungi. The origins of these nine strains were rhizospheric soils for four of them (strains 8, 2, 16, and 3 from *Prunus dulcis, Yucca guatemalensis, Macadamia integrifolia*, and *Phyllostachys* sp., respectively) and seeds for the five other ones (strains 7, 10, and 12 from *Triticum durum*, strain 5 from *Vicia faba* and strain 6 from *Hordeum vulgare*). Looking the broader spectrum of the activity displayed by these strains, they were selected beside seven other ones active against at least two fungi for further analyzes. The last strains were 4, 9, 11, and 13 from rhizospheric soils of *Prunus dulcis, Echium creticum, Ficus carica*, and *Phyllostachys sp.*, respectively, strain 14 from *Triticum durum* seeds, and strains 16 and 17 from non-rhizospheric soils. Some examples of antifungal activities are shown in Fig. 1. The selected strains were first identified by 16S rRNA gene sequencing (Table 3). Ten strains belong to the *B. cereus* group (strains 4, 6, 7, 9, 10, 11, 12, 15, 16 and 17) and were identified as *B. thuringiensis* (based on 16S rRNA sequence analysis and endotoxin production checked under microscopic observation). Three strains were identified as *Bacillus pumilus* (strains 2, 3 and 5) and two strains belonged to *B. subtilis* (strain 8) and *B. licheniformis* (strain 14). This last result was confirmed by the sequence of the gyrB gene. The last one (strain 13) belonged to *Bacillus megaterium*.

Matrix-assisted laser-desorption/ionization time of flight mass spectrometry and LC-MS

MALDI-ToF analyses were performed on the colonies of the 16 selected strains and a control strain (*B. thuringiensis* CIP 110,222) (Table 4). Depending on the strains, these analyses showed surfactins (surfactin, pumilacidin, and lichenysin) production represented by *m/z* in the interval 994–1074 corresponding to the protonated, sodium or potassium forms of different homologous compounds (surfactins with a fatty acid chain of C13, C14, and C15). Kurstakins were mainly highlighted by the m/z 916, 928, 930, and 944, corresponding to C11 $[M+K]^+$, C13 $[M+Na]^+$, C12 $[M+K]^+$, and C13 $[M+K]^+$ homologous compounds, respectively. Finally, fengycin molecules

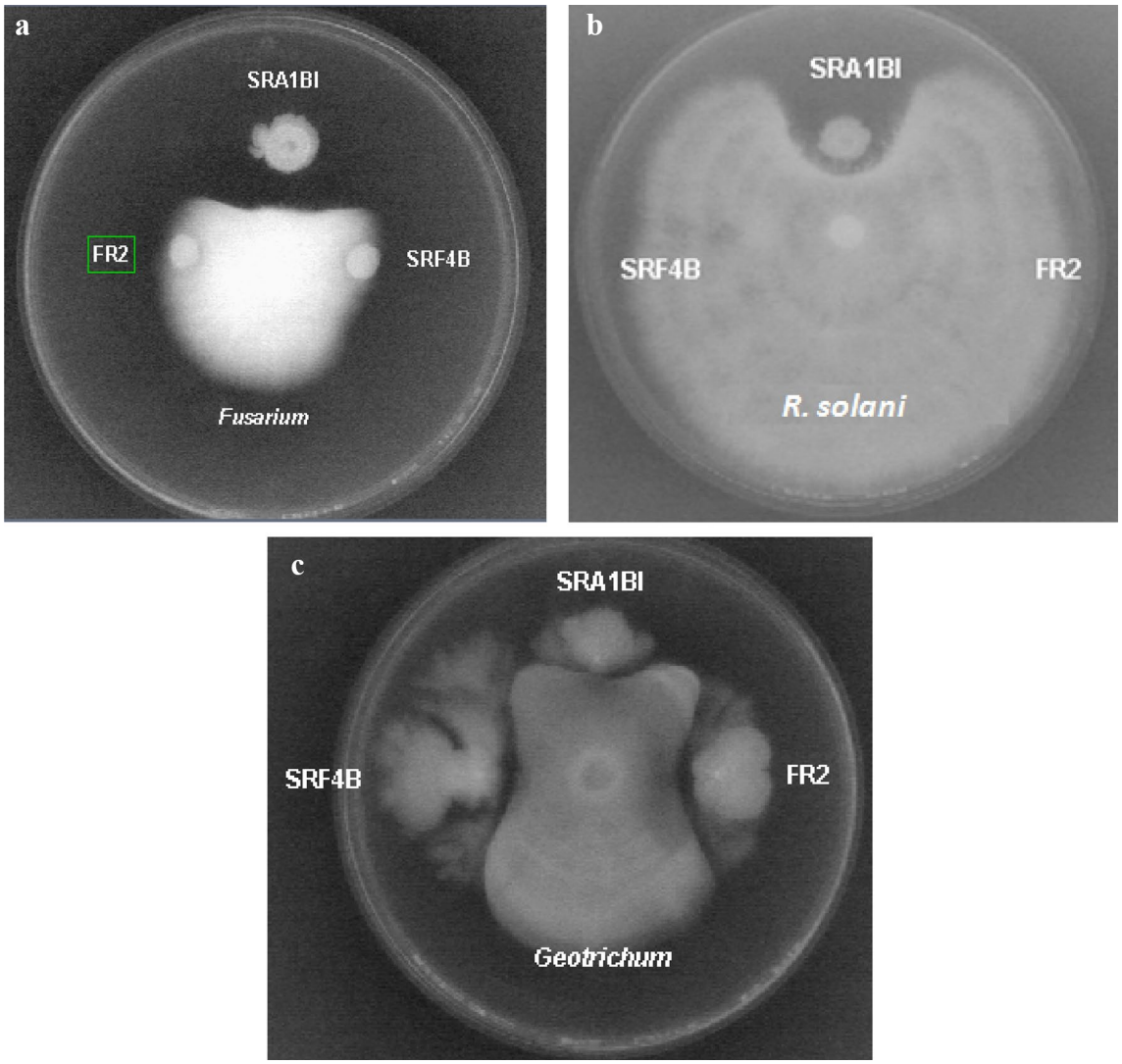

Fig. 1 Antifungal activity of strains 8 (SRA1Bl), 11 (FER2), and 12 (SRF4B) against **a** *F. oxysporum*, **b** *R. solani*, and **c** *G. geotrichum* (Lamia Abdellaziz, Marlène Chollet, Ahmed Abderrahmani, Max Béchet, Lamia Yaici, Gabrielle Chataigné, Anthony Arguelles Arias, Valérie Leclère, Philippe Jacques)

are detected with an m/z between 1477 and 1543.7 corresponding to the three homologous compounds with C15, C16, and C17. In strain 8, two isoforms of fengycin B C17 were observed in the same spectrum with *m*/z of 1499.6 and 1527.7 $[M+Na]^+$ and *m*/*z* of 1500.7 and 1528.7 which could be thus related to the production of fengycin with a different amino acid composition (Fig. 2). This result was confirmed by LC-MS analysis (data not shown).

The results showed that the three strains of *B. pumilus* (2, 3, and 5) produced pumilacidin lipopeptide, whereas *B. megaterium* (13) and *B. licheniformis* (14) synthesized surfactin and lichenysin, respectively. No lipopeptide production was observed for the *B. thuringiensis* strains 9, 11, and 16, whereas the other strains from the same species (4, 6, 7, 12, 15, and 17) produced kurstakin. Fengycin was only produced by *B. subtilis* strain 8.

Detection by PCR of lipopeptide synthetase genes

PCR detection of operon encoding lipopeptide synthetase was thus performed to confirm the presence of the genes involved in the biosynthesis of these lipopeptides. The results obtained with the five usual primer pairs detecting lipopeptide genes in *Bacillus* strains (Aks1-F/Tks1-R, Abl1-F/Tbl1-R, As1-F/Ts2-R, Am1-F/Tm1-R, and Af2-F/Tf1-R) are presented in Table 4 and supplementary data. Except for kurstakin, a perfect correlation between MALDI-ToF lipopeptide detection and PCR lipopeptide gene amplification

Table 3 Results of the antifungal activity and the identification based on sequences of 16S rRNA for the 16 selected strains

Strains	Origins of strains	*R. solani*	*G. geotrichum*	*B. cinerea*	*F. oxysporum*	GenBank accession number	16S rRNA sequence of the closest strain + *gyrB* sequence of the closest strain if necessary	Identity by Blastn (%)
1: CIP 110222	Ref	0.89	0.50	0.75	1	MF818011	*B. thuringiensis* DH-3183	99
2: JeYg5	Ygr	0.44	0.80	0.80	0.96	KT965085	*B. pumilus* DT83	99
3: JeB10	Br	0.67	0.67	0.43	0.67	KT965081	*B. pumilus* B7	100
4: SRA1J	Pdr	1	0.83	1	0.88	KT965090	*B. thuringiensis* NBIN-863	100
5: FvBj3	Vfs	0.35	0.40	0.60	0.78	KT965082	*B. pumilus* B7	100
6: Org(1)5	Hvs	0.67	0.69	0.80	0.67	KT965086	*B. thuringiensis* DH-31	100
7: BL2-4	Tds	0.50	0.80	0.75	0.69	KT965077	*B. thuringiensis* 64ZG7	99
8: SRA1Bl	Pdr	0.42	0.75	0.40	0.59	KT965089	*B. subtilis* BI1 *B. subtilis* str. E3	99 99
9: Btrp	Ecr	0.90	0.80	1	0.69	KT965079	*B. thuringiensis* DH-31	99
10: Blt1	Tds	0.54	0.71	0.60	0.67	KT965078	*B. thuringiensis* DH-31	100
11: SRF4B	Fcr	1	0.60	1	0.86	KT965091	*B. thuringiensis* 263XG9	99
12: FR2	Tds	0.94	0.71	0.75	0.75	KT965080	*B. thuringiensis* 263 XG9	99
13 : Jeb1	Br	1	0.85	1	0.72	KT965083	*B. megaterium* NBRC 15308	99
14 : BL (5) 8	Tds	0.36	1	1	0.87	KT965076	*B. licheniformis* DSM 13 *B. licheniformis* ATCC 14580	100 99
15 : JeMi2	Mir	0.37	0.63	0.57	0.80	KT965084	*B. thuringiensis* Bt407	99
16 : SNRR4B	NRS	1	0.40	1	0.67	KT965088	*B. thuringiensis* DH-31	100
17: OS2	NRS	0.73	1	1	0.77	KT965087	*B. thuringiensis* ATCC 10792	99

In some cases, the identification was complemented with *gyrB* sequences

Antifungal activity = Ratio of fungal colony radius facing the bacteria/Fungal colony radius in the absence of the bacteria. A ratio of 1 meant absence of activity

Ref (Reference strain) CIP110222. Ygr: *Yucca guatemalensis*; rhizosphere. Br: *Phyllostachys* sp.; rhizosphere. Pdr: *Prunus dulcis;* rhizosphere. Vfs: *Vicia faba*; seeds. Hvs: *Hordeum vulgare*; seeds. Tds: *Triticum durum;* seeds. Ecr: *Echium creticum*; rhizosphere. Fcr: *Ficus carica*; rhizosphere. Mir: *Macadamia integrifolia*; rhizosphere. NRS: non-rhizospheric soil

was observed. A new set of primers was thus designed for a better detection of kurstakin genes.

Detection of kurstakin genes using new specific primer pairs

The alignment of the nucleic sequence of the histidine adenylation domain and the thioesterase1-phosphopantetheinyl transferase-thioesterase2 region of *B. cereus* G9842 with those of four strains of *B. thuringiensis* (*Bt* var *pondicheriensis* BGSC 4BA1, *Bt* var *kurstaki* str. T03a001, *Bt* BMB171, and *Bt* var *pulsiensis* BGSC 4CC1) showed that these regions are similar for all the strains. Therefore, these sequences were used to define two primer pairs.

These primers (Table 2) were tested *in silico* with AmplifX software (Jullien 2012) on 16 *B. cereus* and 22 *B. thuringiensis* genomes of kurstakin producing strains found in NCBI Genome databases. Using AH primers, an amplicon of the expected size (307 bp) was obtained. At the opposite, primers P-Te1-F/P-Te2-R led to amplicons with different sizes (1218; 1222; 1238 bp) suggesting a variable length of the region comprised between the two thioesterase domains.

These primer pairs were then tested *in vivo* on the DNAs from *B. thuringiensis* strains 7, 11, 12, and *B. subtilis* strain 8. The *B. thuringiensis* CIP 110,222 and *B. subtilis* 6633 DNA were used as positive and negative controls, respectively (Table 5).

As expected, AH-F/AH-R gave an amplicon of 307 bp for all the tested *B. thuringiensis* strains (1, 7, 11, and 12) and no amplification for the strain 8 and the negative control. Using P-Te1-F/P-Te2-R, an amplification was obtained with the positive (1222 bp) and the negative (604 bp) controls, beside strains 7 (1,222 bp), 11 (1,240 bp), and 12 (1,202 bp). For the negative control that harbors surfactin and mycosubtilin genes, the small size of the amplicon corresponding to a Ppant gene emphasizes different arrangement of the region Ppant-Te as it can be observed for the kurstakin gene. The results obtained with the two sets of primers confirmed the presence of kurstakin operon in strain 7 that was not detected using the Aks1-F/Tks1-R.

Table 4 Analyses of the sequences obtained with the Aks1-F/Tks1-R primers pair using Blastx and MALDI-ToF-MS

Strain	Amplicon size (bp)	Genebank accession number	Blastx	Identity (%) and (*E* value of gene identity)	Peak list (*m/z*)	Lipopeptide production
1	1152	WP_016124572.1	Amino acid adenylation domain-containing protein [*B. cereus*]	99 (0.00)	916.4; 928.4; 930.4; 944.4; 958.4	K
2	–				1058.7; 1072.7; 1088.7; 1100.7; 1116.7	P
3	–				1058.7; 1072.7; 1088.7; 1100.7; 1116.7	P
4	1137	WP_016108417.1	Amino acid adenylation domain-containing protein [*B. cereus*]	96 (0.00)	930.4; 944.4	K
5	–				1072.7; 1088.7; 1116.7	P
6	1138	WP_000061913.1	Hypothetical protein [*B. thuringiensis*]	98 (0.00)	916.4; 930.4; 944.4	K
7	1143	WP_001133922.1	Diguanylate cyclase [*B. cereus*]	99 (0.00)	916.4; 944.4	K
8	1144	AAB80955.2	Fengycin synthetase FenA [*B. subtilis*]	97 (0.00)	1030.7; 1058.7; 1074.7 1477.7; 1499.6; 1500.7; 1515.7; 1517.8; 1527.7; 1528.7; 1543.7	S F
9	–				No peaks corresponding to lipopeptides	–
10	–					
11	1160	WP_000503049.1	Peptide synthetase [*B. cereus*]	98 (0.00)	No peaks corresponding to lipopeptides	–
12	1152	EEM37687.1	Hypothetical protein bthur0004_65120 [*B. thuringiensis* serovar *sotto* T04001]	99 (0.00)	944.4	K
13	–				1022.7; 1058.7; 1072.7; 1088.7	S
14	–				1007.7; 1043.7; 1057.7; 1073.7	L
15	1161	WP_003192746.1	Peptide synthetase [*B. mycoides*]	96 (0.00)	930.4; 944.4	K
16	1125	WP_016109989.1	Amino acid adenylation domain-containing protein [*B. cereus*]	97 (0.00)	No peaks corresponding to lipopeptides	–
17	1167	CDN35947.1	Unnamed protein product [*B. thuringiensis* DB27]	97 (0.00)	944.4	K

Strain 1: kurstakin and fengycin (positive) control

⁻Absence or weak amplification; *K* kurstakin, *F* fengycin, *P* pumilacidin, *S* surfactin, *L* lichenysin

Genome analysis of NRPS operons in the strain 8

As previously mentioned, strain 8 showed a strong antifungal activity and produced novel forms of fengycins. The genome of this strain was thus analyzed to check the potentially produced antifungal secondary metabolites and the specificity of the adenylation domain of the fengycin synthetases. The antiSMASH 3.0 analysis of strain 8 genome led to find five secondary metabolite gene clusters which are related to bacillibactin (siderophore produced by *Bacillus* species), bacillaene (an antibacterial peptide), surfactin, bacilysin, and fengycin. Only the two last ones are known to be responsible for the synthesis of antifungal molecules, so we focused on these two operons. The bacilysin operon was complete with *bacA bacB bacC bacD bacE* and *ywfH*. The fengycin operon was partially sequenced, with incomplete *fenA fenB fenD* and *fenE. However*, the analysis of the specificity of the adenylation domains was possible and showed usual results for the different domains instead for FenD (Gene bank accession number MF818010) for which glutamate prediction instead

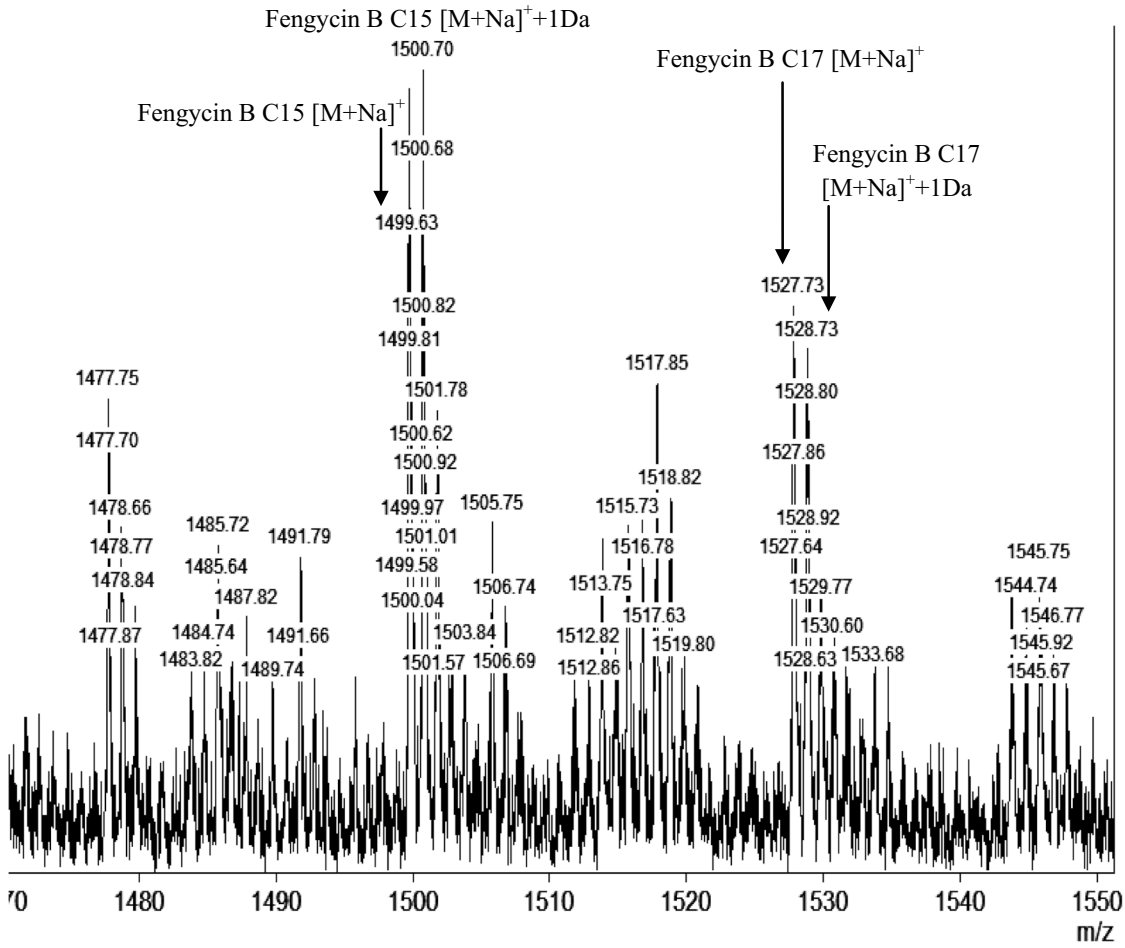

Fig. 2 MALDI-ToF-MS spectrum of strain 8 showing fengycins peaks (1,499.6; 1,527.7, and 1,500.7; 1,528.7 *m/z*) (Lamia Abdellaziz, Marlène Chollet, Ahmed Abderrahmani, Max Béchet, Lamia Yaici, Gabrielle Chataigné, Anthony Arguelles Arias, Valérie Leclère, Philippe Jacques)

Table 5 Characterization of the sequenced PCR products using Blastn and PKS/NRPS analyses

Strain	Primers	Amplicon size (bp)	Match with probable regions	Query cover (%)	Identity (%)
6633 (-)*	AH-F/AH-R	–			
	P-Te1-F/P-Te2-R	604	*B. cereus* ATCC 14,579 (Ppant-Te2) AE016877.1	47	99
1 (+)**	AH-F/AH-R	307	*B. bombysepticus* str. Wang (graphics *krsC*) Query_131397	100	100
	P-Te1-F/P-Te2-R	1222	*B. cereus* ATCC 14,579 (after Te1, Ppant and some Te2) AE016877.1	100	99
7	AH-F/AH-R	307	*B. bombysepticus* str. Wang (graphics *krsC*) CP007512.1	100	100
	P-Te1-F/P-Te2-R	1222	*B. thuringiensis* BMB171 (4′-phosphopantetheinyl transferase thioesterase) (between Te1 and Ppant-Ppant-Te2) CP001903.1	100	99
8	AH-F/AH-R	–			
	P-Te1-F/P-Te2-R	–			
11	AH-F/AH-R	307	*B. thuringiensis* HD-1002 (*krsC*) CP009351.1	100	97
	P-Te1-F/P-Te2-R	1,240	*B. cereus* FM1 plasmid (between Te1 and Ppant-Ppant-Te2) CP009368.1	98	98
12	AH-F/AH-R	307	*B. thuringiensis* HD-1002 (*krsC*) CP009351.1	100	100
	P-Te1-F/P-Te2-R	1,202	*B. thuringiensis* HD-771 (betweenTe1 and Ppant-Ppant-Te2) CP003752.1	99	99

(…): Result of the depth analysis (it was done by analyzing the nearest operon found by Blastn from its graphics using the different prediction softwares)

*6633 (-) Negative control *B. subtilis* ATCC 6633

**1(+) Positive control *B. thuringiensis* CIP 110222

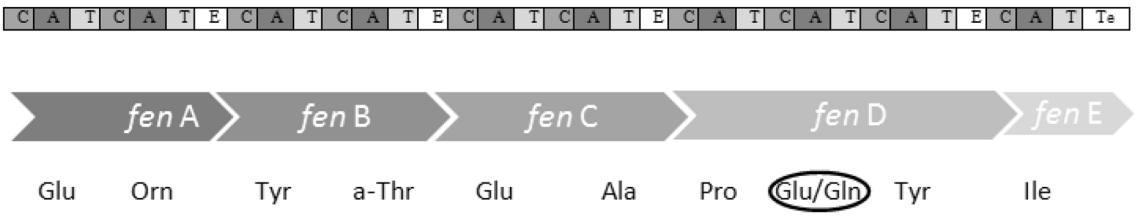

Fig. 3 The two forms of fengycin operon from the strain 8 with the eighth adenylation domain that recognizes and activates either glutamate or glutamine. (Lamia Abdellaziz, Marlène Chollet, Ahmed Abderrahmani, Max Béchet, Lamia Yaici, Gabrielle Chataigné, Anthony Arguelles Arias, Valérie Leclère, Philippe Jacques)

of glutamine was found (Fig. 3). The Stachelhaus code of this domain was DAWHFGSVDK. This signature differs from this (DAQDLGVVDK) determined for glutamine activating domain and from those determined for glutamic acid activating domain (DAKDLGVVDK or DAWHFGGVDK (Stachelhaus et al. 1999).

Discussion

An interesting diversity of *Bacillus* species was highlighted in the herein studied ecosystems, displaying both antifungal activity and lipopeptide production. Among these strains, 16 displayed high levels of antifungal activity with a large spectrum for some of them (2, 3, 5, 6, 7, 8, 10, 12, and 15), active against *F. oxysporum* f. sp. *conglutinans, R. solani* isolate S010-1-1, *G. geotrichum* MUCL 28,959 and *B. cinerea* R16. These strains were identified as follows: three *B. pumilus*, ten *B. thuringiensis*, one *B. megaterium*, one *B. subtilis*, and one *B. licheniformis*. The main source of the broader activity spectrum strains was seeds (five strains) with those from *Triticum durum* (strains 7, 10 and 12) in the first position. After seeds, came soils with four strains (2, 3, 8, and 16). By comparison, rhizospheric soils gave more broader activity spectrum than non-rhizospheric ones and this is in correlation with the report of Raaijmakers et al. (2009), who explained it by the higher competition (free water, nutrient and place) occurring in the rhizospheric soil than in the others. These results gave an overview of the richness of Algerian ecosystems that were the source of an interesting antifungal strains that could be exploited in the control of fungal phytopathogens.

The investigation of lipopeptide production by MALDI-ToF-MS of the 16 strains showed that the three *B. pumilus* strains produced pumilacidin, *B. megaterium* and *B. subtilis* synthesized surfactin, *B. licheniformis* formed lichenysin and most of *B. thuringiensis* produced kurstakin. These results are in agreement with the literature. Indeed, pumilacidin, surfactin, lichenysin and kurstakin were discovered from *B. pumilus, B. subtilis B. licheniformis, B. thuringiensis* HD1, respectively (Hathout et al. 2000; Jacques 2011). The correlation between the production of lipopeptides and the antifungal activity was observed in the majority of strains. In fact, the strains which are active against phytopathogenic fungi produce one or two lipopeptides, particularly those known for their antifungal action, namely fengycin and kurstakin. Thus, like the antifungal activity, the lipopeptide synthesis was linked to the strain environment. Indeed, the majority of the lipopeptide synthesis was noted from strains isolated from seeds of mainly *Triticum durum*, particularly in rhizospheric soils. This latter result could be due to a link between the broad spectrum of antifungal activity and the synthesis of lipopeptides. Interestingly, production of iturinic compounds was not detected in the selected strains.

The use of PCR primers described by Tapi et al. (2010) to detect lipopeptide synthetase genes led to expected results for the As1/Ts1, Am1/Tm2, Abl1/Tbl2, and Af1/Tf2 which amplify genes from surfactin, iturin and fengycin operons respectively. In the absence of iturin operon, Abl1/Tbl2 primers amplified peptides synthetases or diguanylate cyclase as observed by Abderrahmani et al. (2011). Nevertheless, the use of primers Aks1/Tks1 designed by these authors to specifically detect kurstakin synthetase genes did not allow their detection in strain 7, even if it produced kurstakin as determined by MALDI-ToF-MS.

New specific primers were thus designed to detect especially kurstakin genes. Two regions were used: the adenylation domain of histidine that is rarely found in other peptides available in Norine databases and the region comprised between the two thioesterase domains. The primers designed in the first specific region allowed the detection by in silico and in vivo PCR of the gene *krs*C in all the tested strains possessing the kurstakin operon. This indicates that these primers would be efficient to detect kurstakin genes. On the contrary, the primers designed in the thioesterase-Ppant-thioesterase region did not always give a positive result with the tested strains possessing the kurstakin operon.

Strain 8 belonging to the *B. subtilis* species showed the highest antifungal activity. Its genome sequence revealed a potential production of two antifungal compounds: bacilysin and fengycin. Bacilysin, whose mass is 270 Da (Walker and Abraham 1970), was not detected by

MALDI-ToF and LC-MS analyses. The MALDI-ToF-MS results showed peaks with usual fengycin *m*/*z* and with other unusual peaks that are upper than the first one by one Da. These findings agree with those of Li et al. (2012) that found 1505 Da (Fen B C17 $[M+H]^+$); 1477 Da (Fen B C15 $[M+H]^+$); 1491 Da ($[M+H]^+$); and 1464 Da, which corresponds to (1463 Da (Fen A C16 $[M+H]^+$) + 1) and Pathak et al. (2012) who analyzed the microheterogeneity of fengycin production by a banyan endophyte. Interestingly, these last authors confirmed that this mass difference is explained by the incorporation of a glutamate instead of a glutamine in the position eight of the peptide moiety.

The analysis of the signature of the different adenylation domains of the fengycin synthetase from strain 8 led to a good prediction of the usual amino acids encountered in fengycin except in position 8. In this case, the signature did not fit with that of an adenylation domain activating a glutamate or a glutamine. It differs from the glutamate incorporating signature by only one serine instead of one glycine. Maybe, this difference could explain the ability of this domain to incorporate both amino acid residues. The activation of more than one monomer by the same adenylation domain was reported by many authors. Indeed, Crawford et al. (2011) noted that modules 2 and 3 of xenematide synthetase would variably condense L-Trp and L-Phe with a preference for L-Trp. Similarly, select modules in the tyrocidine biosynthetic pathway that activate Phe or Tyr in tyrocidine A can also activate Trp to produce tyrocidines B-D, and the relative amount of each tyrocidine was dependent upon amino acid concentration in the culture medium (Mootz and Marahiel 1997). Likewise, in the iterative mechanism of locyllomycin synthesis, the second domain A of its operon activates glutamine in first then asparagine in iteration (Luo et al. 2015b). Esmaeel et al. (2016) have also revealed the presence of an adenylation domain with specificity for Ser and Thr in the second NRPS cluster found by the analysis of *Aeromonas salmonicida* genome. In the same way, Christiansen et al. (2011) found the coproduction of anabeopeptins bearing either of the chemically divergent amino acids Arg and Tyr in position 1 and the analysis of the first adenylation domain showed that it activates these two monomers in vitro. Phylogenetic analysis of different sequences of this domain revealed that strains with a promiscuous first adenylation domain derived from an ancestor that activates only Arg. Surprisingly, positive selection appears to affect only three codons within the encoding gene, suggesting that this remarkable promiscuity has evolved from point mutations only (Christiansen et al. 2011). Their last finding could concord with the occurring of serine instead of glycine in the signature found in our study. The replacement of a glutamine by a glutamate in the peptide moiety of fengycin will increase the negative charge of the lipopeptide and probably modify its biological activity, maybe explaining the high antifungal activity of strain 8.

Acknowledgements This work has been carried out in the framework of Alibiotech project which is financed by European Union, French State, and the French Region of Hauts-de-France and of INTERREG Va SMARTBIOCONTROL portfolio (Bioscreen Project) which is financed by European Union and Walloon Region.

References

Abderrahmani A, Tapi A, Nateche F, Chollet M, Leclère V, Wathelet B, Hacene H, Jacques P (2011) Bioinformatics and molecular approaches to detect NRPS genes involved in the biosynthesis of kurstakin from *Bacillus thuringiensis*. Appl Microbiol Biotechnol 92:571–581

Asselbergh B, Curvers K, Franca S-C, Audenaert K, Vuylsteke M, Van Breusegem F, Höfte M (2007) Resistance to *Botrytis cinerea* in *sitiens*, an abscisic acid-deficient tomato mutant, involves timely production of hydrogen peroxide and cell wall modifications in the epidermis. Plant Physiol 144:1863–1877

Aziz R-K, Bartels D, Best A-A, DeJongh M, Disz T, Edwards R-A, Formsma K, Gerdes S, Glass E-M, Kubal M, Meyer F, Olsen G-J, Olson R, Osterman A-L, Overbeek R-A, McNeil L-K, Paarmann D, Paczian T, Parrello B, Pusch G-D, Reich C, Stevens R, Vassieva O, Vonstein V, Wilke A, Zagnitko O (2008) The RAST server: rapid annotations using subsystems technology. BMC Genomics 9

Béchet M, Caradec T, Hussein W, Abderrahmani A, Chollet M, Leclère V, Dubois T, Lereclus D, Pupin M, Jacques P (2012) Structure, biosynthesis and properties of kurstakins, nonribosomal lipopeptides from *Bacillus* spp. Appl Microbiol Biotechnol 95:593–600

Biniarz P, Lucaszewicz M, Janek T (2017) Screening concepts, characterization and structural analysis of microbial-derived bioactive lipopeptides: a review. Crit Rev Biotechnol 37:393–410

Brettin T, Davis J-J, Disz T, Edwards R-A, Gerdes S, Olsen G-J, Olson R, Overbeek R, Parrello B, Pusch G-D, Shukla M, Thomason J-A, Stevens R, Vonstein V, Wattam A-R, Xia F (2015) RASTtk: a modular and extensible implementation of the RAST algorithm for building custom annotation pipelines and annotating batches of genomes. Sci Rep 5:8365

Christiansen G, Philmus B, Hemscheidt T, Kurmayer R (2011) Genetic variation of adenylation domains of the anabaenopeptin synthesis operon and evolution of substrate promiscuity. J Bacteriol 193:3822–3831

Coutte F, Lecouturier D, Dimitrov K, Guez J-S, Delvigne F, Dhulster P, Jacques P (2017) Microbial lipopeptide production and purification bioprocesses, current progress and future challenges. Biotechnol J DOI. https://doi.org/10.1002/biot.201600566

Crawford J-M, Portmann C, Kontnik R, Walsh C-T, Clardy J (2011) NRPS substrate promiscuity diversifies the xenematides. Org Lett 13:5144–5147

Deravel J, Lemière S, Coutte F, Krier F, Van Hese N, Béchet M, Sourdeau N, Höfte M, Leprêtre A, Jacques P (2014) Mycosubtilin and surfactin are efficient, low ecotoxicity molecules for the biocontrol of lettuce downy mildew. Appl Microbiol Biotechnol 98:6255–6264

Esmaeel Q, Chevalier M, Chataigné G, Subashkumar R, Jacques P, Leclère V (2016) Nonribosomal peptide synthetase with a unique iterative-alternative-optional mechanism catalyzes amonabactin synthesis in *Aeromonas*. Appl Microbiol Biotechnol 100:8453–8463

Flissi A, Dufresne Y, Michalik J, Tonon L, Janot S, Noe L, Jacques P, Leclère V, Pupin M (2016) Norine, the knowledgebase dedicated to nonribosomal peptides, is now open to crowdsourcing. Nucleic Acids Res 44:D1113-D1118

Hathout Y, Ho Y-P, Ryzhov V, Demirev P, Fenselau C (2000) Kurstakins: a new class of lipopeptides isolated from *Bacillus thuringiensis*. J Nat Prod 63:1492–1496

Jacobs A (2007) Transport bactérien en milieux poreux: Expérimentations et Modélisation. Migration de bactéries issues de boues de STEP. Thèse de Doctorat. Université d'Avignon, France

Jacques P (2011) Surfactin and other lipopeptides from *Bacillus* spp. *In* Biosurfactant Microbiology Monographs. *Edited by* Soberon-Chavez G, Springer-Verlag, Berlin Heidelberg 20: pp 57–91

Jullien N (2012) AmplifX 1.7.0. Website http://crn2m.univ-mrs.fr/pub/amplifx-list

Li X-Y, Mao Z-C, Wang Y-H, Wu Y-X, He Y-Q, Long C-L (2012) ESI LC-MS and MS/MS characterization of antifungal cyclic lipopeptides produced by *Bacillus subtilis* XF-1. J Mol Microbiol Biotechnol 22:83–93

Luo C, Liu X, Zhou H, Wang X, Chen Z (2015a) Nonribosomal peptide synthase gene clusters for lipopeptide biosynthesis in *Bacillus subtilis* 916 and their phenotypic functions. Appl Environ Microbiol 81:422–431

Luo C, Liu X, Zhou X, Guo J, Truong J, Wang X, Zhou H, Li X, Chen Z (2015b) Unusual biosynthesis and structure of locillomycins from *Bacillus subtilis* 916. Appl Environ Microbiol 81:6601–6609

Marahiel M-A, Stachelhaus T, Mootz H-D (1997) Modular peptide synthetases involved in nonribosomal peptide synthesis. Chem Rev 97:2651–2674

Mootz H-D, Marahiel M-A (1997)) The tyrocidine biosynthesis operon of *Bacillus brevis*: complete nucleotide sequence and biochemical characterization of functional internal adenylation domains. J Bacteriol 179:6843–6850

Ongena M, Jacques P (2008) *Bacillus* lipopeptides: versatile weapons for plant disease biocontrol. Trends Microbiol 16:115–125

Overbeek R, Olson R, Pusch G-D, Olsen G-J, Davis J-J, Disz T, Edwards R-A, Gerdes S, Parrello B, Shukla M, Vonstein V, Wattam A-R, Xia F, Stevens R (2014) The SEED and the Rapid Annotation of microbial genomes using Subsystems Technology (RAST). Nucleic Acids Res 42:D206-D214

Pathak K-V, Keharia H, Gupta K, Thakur S-S, Balaram P (2012) Lipopeptides from the banyan endophyte, *Bacillus subtilis* K1: mass spectrometric characterization of a library of fengycins. J Am Soc Mass Spectrom 23:1716–1728

Peypoux F, Guinand M, Michel G, Delcambe L, Das B-C, Lederer E (1978) Structure of iturin A, a peptidolipid antibiotic from *Bacillus subtilis*. Biochemistry 17:3992–3996

Raaijmakers J-M, Paulitz C-T, Steinberg C, Alabouvette C, Moënne-Loccoz Y (2009) The rhizosphere: A playground and battlefield for soilborne pathogens and beneficial microorganisms. Plant Soil 321:341–361

Stachelhaus T-H, Mootz H-D, Marahiel M-A (1999) The specificity-conferring code of adenylation domains in nonribosomal peptide synthetases. Chem Biol 6:493–505

Tapi A, Chollet-Imbert M, Scherens B, Jacques P (2010) New approach for the detection of non ribosomal peptide synthetase genes in *Bacillus* strains by polymerase chain reaction. Appl Microbiol Biotechnol 85:1521–1531

Van Beneden S, Pannecoucque J, Debode J, De Backer G, Höfte M (2009) Characterisation of fungal pathogens causing basal rot of lettuce in Belgian greenhouses. Eur J Plant Pathol 124:9–19

Walker J-E, Abraham E-P (1970) The structure of bacilysin and other products of *Bacillus subtilis*. Biochem J 118:563–570

Weber T, Blin K, Duddela S, Krug D, Kim H-U, Bruccoleri R, Lee S-Y, Fischbach M-A, Müller R, Wohlleben W, Breitling R, Takano E, Medema M-H (2015) antiSMASH 3.0 - a comprehensive resource for the genome mining of biosynthetic gene clusters. Nucleic Acids Res 43:W237-W243

Yamamoto S, Harayama S (1995) PCR amplification and direct sequencing of *gyrB* genes with universal primers and their application to the detection and taxonomic analysis of *Pseudomonas putida* strains. Appl Environ Microbiol 61:1104–1109