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Bacilysin within the *Bacillus subtilis* group: gene prevalence *versus* antagonistic activity against Gram-negative foodborne pathogens

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ABSTRACT

The *Bacillus subtilis* group comprises species known for their ability to produce a wide variety of antimicrobial peptides. This work focuses on bacilysin, a broad-spectrum active dipeptide, and its prevalence in the *B. subtilis* group. *In silico* genome analysis of strains from *Bacillus amyloliquefaciens, Bacillus velezensis, Bacillus licheniformis, Bacillus pumilus* and *B. subtilis* subspecies *inaquosorum*, *spizizenii* and *subtilis* revealed that the bacilysin gene cluster is present in all species except for *B. licheniformis*. This observation was corroborated by PCR detection of the bacilysin genetic determinants on a collection of 168 food and environmental strains from the *B. subtilis* group. Phylogenetic analyses also demonstrated that the bacilysin gene cluster sequence showed more than 80 % identity within each species of the *B. subtilis* group. An *in vitro* screening of the strain collection was performed against foodborne pathogens. Twenty-three strains were selected for their ability of their Cell-Free Supernatant to inhibit foodborne pathogens. After an ammonium sulphate precipitation of their supernatant, eight strains, all belonging to *B. velezensis*, exhibited antimicrobial activity against Gram-negative pathogens. Using Ultra High Performance Liquid Chromatography - Mass Spectrometry, the presence of bacilysin was confirmed in these eight precipitates. These findings provide evidence that bacilysin is a major player in the antagonistic activity of *B. velezensis* against Gram-negative foodborne pathogens.

1. Introduction

The *Bacillus subtilis* group includes Gram-positive, ubiquitous, endospore-forming, aerobic bacterial species, with on-going interest in both fundamental and applied microbiology. Their ability to secrete various natural compounds, their high growth rate and their current GRAS (Generally Regarded As Safe) status make them valuable candidates for biopreservation (Schallmey et al., 2004). The four original members of this group, *i.e. Bacillus amyloliquefaciens* (Priest et al., 1987), *Bacillus licheniformis*, *Bacillus pumilus* and *Bacillus subtilis sensu stricto* (*s. s.*), play a major role in many industrial fermentation processes nowadays (Fan et al., 2017), including the production of fungicides, biosurfactants, enzymes, purines, vitamins, as well as poly-γ-glutamic acid and D-ribose, used as flavor enhancers in food (Lahlali et al., 2011; Öztürk et al., 2016; Rane et al., 2017; Rosenberg et al., 2017; Ruiz--Garcia et al., 2005; Shi et al., 2014).

In recent years, the taxonomy of the *B. subtilis* group has become much more complex and numerous phylogenetically and phenetically closely related species and subspecies have been described (reviewed in Caulier et al., 2019). This list contains at least 20 taxa, excluding the heterotypic synonyms such as *B. amyloliquefaciens* subsp. *plantarum*, *Bacillus methylotrophicus* or *Bacillus oryzicola* that were recently proposed as later heterotypic synonyms of *Bacillus velezensis* (Dunlap et al., 2016). It is estimated that 4–5% of the genome of these bacteria are devoted to the synthesis of antimicrobial compounds such as polyketides, bacteriocins or non-ribosomal peptides (NRPs) (Caulier et al., 2019; Stein,

Abbreviations: ACN, acetonitrile; AS, ammonium sulfate; ATPE, aqueous two-phase extraction; CFS, cell-free supernatant; GRAS, generally regarded as safe; KO, knockout; LPLC, low-pressure liquid chromatography; NRP, non-ribosomal peptide; PBS, phosphate buffered saline; SOP, spot-on-plate; *s.s.*, *sensu stricto*; UPLC-MS, ultra-high performance liquid chromatography - mass spectrometry.

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2005). Among these molecules, bacilysin, a 270 Da dipeptide [L-alanyl-(2.3-epoxycyclohexanone-4)-L-alanine], consists of L-alanine and the non-proteinogenic amino acid L-anticapsin (Rogers et al., 1965; Walker and Abraham, 1970). Despite its simple structure, this NRP displays an impressive broad range of antagonistic activity against fungi and bacteria. Bacilysin induces the lysis of the microbial cell wall by inhibiting the glucosamine-6-phosphate synthase and, consequently mannoprotein or peptidoglycan biosynthesis in fungi and bacteria, respectively (Kenig et al., 1976). Bacilysin is also known to be heat stable (15 min at 100 °C) and remains active over a wide range of pH (from 1.4–12.0) (Özcengiz and Alaeddinoglu, 1991). All these properties, make bacilysin a good candidate to be tested for biocontrol purposes in the agro-industry.

The primary metabolic precursor of bacilysin biosynthesis is prephenate that derives from the aromatic amino acid pathway (Hilton et al., 1988). The gene cluster *ywfA-bacABCDE-ywfG-ywfH* has been shown to encompass the core genes involved in bacilysin synthesis (Steinborn et al., 2005). While *bacA*, *bacB* and *bacC* allow the prephenate conversion into anticapsin, *bacD* encodes a L-amino acid ligase catalysing the amide bond between the alanine and anticapsin residues. The *bacE* gene encodes a permease involved in the host self-protection. Finally, *ymfH* and *ymfG* participate to anticapsin synthesis whereas *ymfA* is thought to be involved in the bacilysin transportation across the cytoplasmic membrane (Mahlstedt and Walsh, 2010; Steinborn et al., 2005). It was also recently shown that once transported into fungal pathogens, bacilysin is hydrolysed into anticapsin that, in turn, inhibits the glucosamine-6-phosphate synthase (Wang et al., 2018).

Since its first isolation in 1946 (Foster and Woodruff, 1946; Newton, 1949), bacilysin has generated great interest and has been included in various patents, most of them taking advantage of its antimicrobial properties. Besides these applications, bacilysin has also been detected in Pozol, a Mexican fermented maize dough, used as source of nutrients, but also as poultice to heal wounds (Phister et al., 2004).

Considering the established antimicrobial effect of bacilysin and the widespread use of *B. subtilis* group strains in the agri-food sector, the present study aimed to explore bacilysin prevalence within this bacterial group, both at the genetic and phenotypic levels. The genetic detection of bacilysin gene cluster was first assessed within several reference genomes of the *B. subtilis* group, then within a collection of newly isolated strains. The antimicrobial activity of these strains was screened against a panel of foodborne pathogens. The bacterial strains exhibiting antagonist activities were then evaluated for their bacilysin production.

2. Materials and methods

2.1. Strains of the B. subtilis group: isolation, growth condition and identification

A total of 168 strains belonging to the *B. subtilis* group were used in this work: 87 came from the collection of the Laboratory of Food and Environmental Microbiology (MIAE, UCLouvain), 53 were isolated from various Belgian sites and 28 originated from Vietnamese food samples. A brief overview of the strains is given in Table 1 and their detailed origins are reported in Table S1.

The strains of the laboratory collection were identified prior this study through 16S rRNA sequencing. Isolation of the strains from the Belgian and Vietnamese samples (hereinafter referred to as CN and HQV, respectively) was carried out by homogenizing 10 g of sample in 90 mL of sterile Ringer solution in a Stomacher® (400 circulator, Seward, UK) for 30 s. After a heat treatment of 10 min at 50 °C, 10-fold dilutions of the samples were spread on Lysogeny Broth (LB) (NaCl, 5 g L^{-1}; yeast extract, 5 g L^{-1}; tryptone, 10 g L^{-1}) agar plates (1.4 % wt/vol agar) and incubated for 24 h at 30 °C. Between one and seven strains were selectively isolated from each sample, based on their colony phenotype. A specific PCR was performed to discriminate the *B. subtilis s. s.* strains from the other members of the *B. subtilis* group (Ashe et al., 2014). The corresponding primers sequences and the PCR parameters are detailed in Table S2. The PCR reactions were performed using GoTaq® Green Master Mix (Promega, Fitchburg, WI, USA) following the manufacturer's recommendations. PCR amplifications were verified by gel electrophoresis and only one positive strain was kept per sample. All the strains were conserved in LB:glycerol (70:30 vol/vol) at −80 °C.

Table 1
Origin of the 168 strains belonging to the *B. subtilis* group used in this work.

Lab strain collection		**87**
	Surface sample	29
	Miscellaneous	24
	Wacobi project [a]	19
	Reference strains	7
	Food sample	6
	Air sample	2
CN strains isolated from Belgian samples		**53**
	Feces, manure and compost	18
	Sewage and wastewater	14
	Miscellaneous	12
	Agricultural soil	9
HQV strains isolated from Vietnamese food samples		**28**
	Fermented chili	22
	Fermented shrimp	4
	Fermented eggplant	2
Total		**168**

[a] Caulier et al., 2018.

Liquid cultures of the *B. subtilis* group strains were prepared after the inoculation of 8 mL of YEN medium (Yeast extract, 45 g L^{-1}; glucose, 17 g L^{-1}; $NaNO_3$ 6.5 g L^{-1}; NH_4Cl 4.1 g L^{-1}; KH_2PO_4, 1 g L^{-1}; K_2HPO_4, 1 g L^{-1}; $MgSO_4.7H_2O$ 0.2 g L^{-1}; $MnSO_4.H_2O$ 0.04 g L^{-1}; pH 7) with a single colony. The inoculated flasks were incubated for 22 h at 37 °C with an agitation of 120 rpm.

Species identification within the *B. subtilis* group was confirmed by *gyrA* sequencing. This gene encodes DNA gyrase subunit A, known as a discriminative phylogenetic marker within the *B. subtilis* group (Chun and Bae, 2000). PCR was performed on fresh colonies resuspended in MilliQ water using GoTaq® G2 Flexi DNA Polymerase (Promega, Fitchburg, WI, USA). The primer sequences and PCR settings are given in Table S2. The PCR amplicons were purified by the GenElute PCR clean up kit (Sigma, Co, USA) and sequenced in both orientations at Macrogen Europe (Amsterdam, The Netherlands). For the tested strains, nucleotide sequences were trimmed, aligned and compared with the BLASTn search tool from GenBank database.

2.2. Screening of the bacilysin gene cluster

The bacilysin gene cluster sequences belonging to the *B. subtilis* group were retrieved from GenBank nucleotide database and multiple alignments were performed with Clustal W (Larkin et al., 2007) on the Clustal Omega Platform using default parameters (EMBL-EBI, Hinxton, UK). Two pairs of degenerated primers (Bac1 F/R and Bac2 F/R) were designed using Primer-BLAST (Ye et al., 2012) after analyzing the conserved regions of the aligned sequences within the *B. subtilis* group. The primer sequences and the PCR parameters are detailed in Table S2. The PCR screening for the prevalence of the bacilysin gene cluster among the strains of the *B. subtilis* group was performed using the GoTaq® G2 Flexi DNA Polymerase as indicated above.

2.3. Growth condition of foodborne pathogenic strains

The pathogenic strains used in this study are listed in Table 2. Bacteria from −80 °C frozen stocks were spread on LB agar plates and grown for 16 h at 37 °C. Liquid cultures were obtained after the inoculation of 8 mL LB medium with a single colony, followed by an incubation at 37 °C for 16 h, 120 rpm.

Table 2
Foodborne pathogens used in this study.

Species	Strain	Origin
	ATCC 8739	ATCC [a]
	ATCC 35150	ATCC
Escherichia coli	LMG 2093	BCCM/LMG [b]
	Si0059	MIAE [c]
	VTEC016	MIAE
	ATCC 35640	ATCC
Salmonella enterica	Poly08	MIAE
	Si0022	MIAE
Listeria ivanovii	Liv005	MIAE
Bacillus cereus	ATCC 14579	ATCC

[a] ATCC: American Type Culture Collection, Manassas, VA, USA.
[b] BCCM/LMG: Belgian Coordinated Collections of Microorganisms, Laboratory of Microbiology, Ghent University, Ghent, Belgium.
[c] MIAE: Laboratory of Food and Environmental Microbiology, UCLouvain, Louvain-la-Neuve, Belgium.

2.4. Antibacterial activity assay

The antagonist activity of culture supernatants against foodborne pathogens was evaluated for the collection of 168 strains belonging to the *B. subtilis* group. For this purpose, overnight liquid cultures of the strains were centrifuged for 20 min at 10,000 rpm, 4 °C and filtered through a 0.22 µm membrane (polyethersulfone, Millex, Millipore, Burlington, MA, USA) to recover the Cell-Free Supernatant (CFS). Eight mL of LB soft agar (0.7 % w/v agar) were inoculated with a foodborne pathogen liquid culture (1% v/v) and overlaid on a LB agar plate. Spot-On-Plate (SOP) assays were achieved to detect the antimicrobial activity of the CFS, supplemented either with 20 mM Ethylene Diamine Tetra-Acetic acid (EDTA) or not. The addition of EDTA is known as a disruptive agent of the outer membrane of Gram-negative bacteria. It has already been identified as able to sensitize them to certain antimicrobial compounds such as bacteriocins (Martin-Visscher et al., 2011). Ten µL of each supernatant were spotted on the foodborne pathogen lawns and the plates were incubated for 16 h at 37 °C before examining the growth inhibition zones.

To concentrate the active moieties from 40 mL of active CFS, ammonium sulfate (AS) precipitation (90 % of saturation) was performed. After 2 h of stirring at room temperature, the precipitates were centrifuged 20 min at 10,000 rpm at 4 °C. The pellet was resuspended in Phosphate Buffered Saline (PBS, pH 7.4, Sigma tablets) and the activity of this "AS precipitate" was evaluated using the SOP method.

2.5. Low-Pressure Liquid Chromatography (LPLC)

The AS precipitate was loaded into a LPLC column (1,6 × 50 cm) packed with Sephacryl™ High Resolution (GE Healthcare, Chicago, IL, USA) gel filtration media and equilibrated with PBS. The elution was performed at a flow rate of 1 mL min^{-1}, using PBS as eluent and collecting fractions of 1.5 mL. For each fraction, the optical density at 280 nm (OD_{280}) was measured and the antimicrobial activity was tested using the SOP method after being concentrated to one third of the original volume in a modular concentrator (ThermoFisher Scientific, SpeedVac™ system, Waltham, MA, USA). The molecular weight (MW) of the active fractions was assessed according to the supplier recommendations using albumin, glutamine, ribonuclease A and tryptophan as standards.

2.6. Bacilysin extraction

Two Aqueous Two-Phase Extraction (ATPE) methods were applied, based on the bacilysin solubility properties (Newton, 1949). First, the aqueous two-phase system ethanol-$(NH_4)_2SO_4$ was applied (Yuan et al., 2011). For this purpose, 0.5 mL of AS precipitate solution, 0.4 mL of ethanol and 0.1 *g* of $(NH_4)_2SO_4$ salt were mixed, vortexed for 5 min and centrifuged for 5 min at 6000 *g* at 4 °C to complete the separation of the two phases. The upper phase was removed and supplemented with 1 mL of methanol to remove salts by centrifugation. Second, a QuEChERS (Quick, Easy, Cheap, Efficient, Rugged and Safe) derivative technique was implemented (Paraszkiewicz et al., 2017) as follows: 0.5 mL of AS precipitate solution was mixed vigorously with 0.5 mL of acetonitrile (ACN). The tubes were left for 5 min and then centrifuged for 3 min at 5000 *g*, at 4 °C to complete the separation. The upper phase was removed and the ACN extraction procedure was repeated a second time. Prior the chromatography analyses, the samples of both ATPE methods were dried under N_2 at 40 °C. The solid matter was then suspended in 0.4 mL of methanol and centrifuged to avoid any solid residues.

2.7. Ultra high performance liquid chromatography - mass spectrometry analysis

The organic extracts from both ATPE methods were analysed by UPLC-MS as previously described (Chen et al., 2009). In brief, an Agilent chromatography system (Agilent Technologies, 1290 Infinity System) coupled to a heated electrospray ionization source on a triple quadrupole mass spectrometer (Agilent Technologies, 6150 System) was used. First, UPLC separation was performed on a C18 column (Agilent Eclipse Plus C18 Rapid Resolution HD, 2.1 × 50 mm, 1.8 µm). The mobile A and B were water:acetonitrile 95:5 and 5:95 respectively, both supplemented with 0.1 % formic acid. The gradient elution was as follows: 0−5 min 20 % B, 5−10 min from 20 % to 95 % B (linear gradient) and 15−20 min 95 % B. The injection volume was 5 µL and the cycles were performed at a flow rate of 0.2 mL min^{-1} at a temperature of 30 °C. After the UPLC separation, the mass spectra of each peak were acquired using two full-scans positive ion modes: the first one focusing on *m/z* 271 whereas the second one ranging from *m/z* 200 to 120. The MS spectrometer was set with these defined parameters: 2500 V capillary voltage, 1500 V Nozzel voltage, 250 °C drying gas at a flow rate of 8 L min^{-1}, 30 psi nebulizer pressure and 125 V fragmentor voltage.

3. Results

3.1. The bacilysin gene cluster is widespread within the B. subtilis group

In silico detection of the bacilysin gene cluster was performed on 34 genomes retrieved from GenBank database from various species of the *B. subtilis* group (*i.e. B. amyloliquefaciens* (5), *B. licheniformis* (5), *B. pumilus* (4), *B. subtilis s.s.* and its subspecies *inaquosorum*, *spizizenii* and *subtilis* (14) and *B. velezensis* (6)). The eight genes of the bacilysin cluster were detected in all the strains with two exceptions: none of the genes were present in the five *B. licheniformis* strains and *bacE* (involved in the host resistance to bacilysin) was absent from the *B. pumilus* strains.

For the 29 strains in which the bacilysin gene cluster was detected (Fig. 1), the eight gene sequences (seven for the *B. pumilus* strains) were concatenated end-to-end and aligned to identify the most conserved regions. Two pairs of degenerated primers (Bac1 and Bac2, targeting the *bacAB* and *bacBC* genes, respectively - see Table S2) were then designed to assess the presence of the bacilysin gene cluster among a total of 168 strains of the *B. subtilis* group from various origins (Table 1). As reported in Table S1, all PCR were positive, except for the *B. licheniformis* strains.

3.2. Conservation of the bacilysin gene cluster correlates with the B. subtilis group species

The concatenated sequences of the bacilysin gene cluster of the 29 strains were then used for phylogenetic analysis. The dendrogram resulting from the alignment of these sequences revealed a high degree of conservation of the gene cluster sequences within each species of the *B. subtilis* group. As illustrated in Fig. 1, three major clades, characterised by more than 80 % nucleotide sequence identity, were highlighted as being directly linked to the species affiliation:

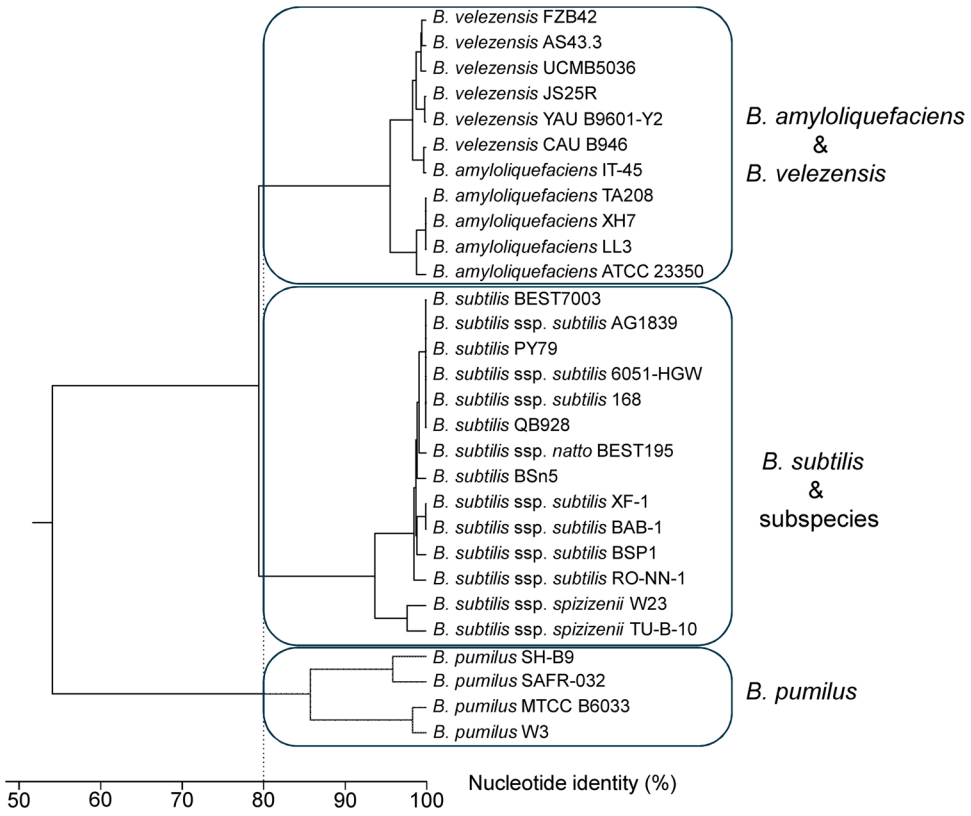

Fig. 1. Similarity dendrogram of nucleotide sequences of the bacilysin gene cluster of 29 representative type strains of the *B. subtilis* group. The DNA sequences of the eight genes from the bacilysin cluster were concatenated and compared using ClustalW.

Table 3
Antimicrobial activity of the cell-free supernatant (CFS) of 23 selected strains against ten foodborne pathogens.

B. subtilis group strains	Number of inhibited pathogens	Gram-negative [a]								Gram-positive	
		Ec ATCC 35150	Ec ATCC 8739	Ec LMG 2093	Ec Si0059	Ec VTEC016	Se ATCC 35640	Se Poly08	Se Si0022	Bc ATCC14579	Li Liv005
CN013	10	+	+	+	+	+	+	+	+	+	+
HQV028	10	+	+	+	+	+	+	+	+	+	+
CN005	9	+	+	+	+	+	+	+	–	+	+
HQV009	9	+	+	+	+	+	+	+	–	+	+
HQV022	7	–	+	+	+	–	+	+	–	+	+
CN009	6	+	+	+	+	–	–	–	–	+	+
CN012	6	+	+	+	+	–	–	–	–	+	+
CN016	6	+	+	+	+	–	–	–	–	+	+
CN022	6	+	+	+	+	–	–	–	–	+	+
HQV014	6	+	+	+	+	–	–	–	–	+	+
CN042	5	+	+	+	+	–	–	–	–	+	–
CN018	5	–	+	+	+	–	–	–	–	+	+
CN019	5	–	+	+	+	–	–	–	–	+	+
CN021	5	–	+	–	+	+	–	–	–	+	+
CN022	5	+	+	–	–	+	–	–	–	+	+
CN026	5	–	+	+	–	+	–	–	–	+	+
HQV004	4	–	–	+	–	+	–	+	–	–	+
HQV021	4	+	–	+	–	+	–	–	–	–	+
CN050	4	–	+	+	–	–	–	–	–	+	+
HQV018	3	+	+	–	–	+	–	–	–	–	–
HQV008	3	–	+	–	+	–	–	–	–	+	–
CN017	2	–	+	–	+	–	–	–	–	–	–
CN028	2	+	+	–	–	–	–	–	–	–	–

Ten μL of CFS supplemented with 20 mM EDTA of the strains from the *B. subtilis* group were tested by the SOP assay to evaluate their ability to inhibit foodborne pathogens. The green "+" indicates an inhibition while "-" refers to the absence of inhibition. The number of pathogens inhibited by each *B. subtilis* group strain is indicated.

[a] The abbreviations are: Ec, *E. coli*; Se, *S. enterica*; Bc, *B. cereus* and Li, *L. ivanovii*.

B. amyloliquefaciens and *B. velezensis*, *B. subtilis* and its subspecies, and *B. pumilus*.

3.3. Bacterial antagonistic activity against foodborne pathogens

The antimicrobial activity of the CFS from the 168 strain collection was assessed by the SOP method against ten distinct (eight Gram-negative and two Gram-positive, Table 2) foodborne pathogens, either in the presence or absence of 20 mM EDTA (Martin-Visscher et al., 2011).

Compared to Gram-positive bacteria, antimicrobial activity exhibited against Gram-negative pathogens was generally less intense and exclusively observed for the strains CFS supplemented with EDTA (data not shown). As shown in Table 3, 23 strains from the collection were therefore selected for the ability of their CFS to inhibit, in the presence of EDTA, at least two out of the eight Gram-negative pathogens. Interestingly, after AS precipitation (90 % saturation) of the CFS, eight strains (CN013, CN016, CN026, HQV004, HQV008, HQV009, HQV021 and HQV028) displayed antagonistic activities against all ten pathogens (data not shown). Sequencing of their *gyrA* gene indicated that they all belong to the *B. velezensis* species (data not shown).

3.4. The antimicrobial active fraction size is between 100 and 300 Da

Size-exclusion column chromatography was performed with the AS precipitated supernatant of the eight selected strains and the *B. subtilis* strain Bs168 as control. The nine resulting LPLC UV chromatograms are provided in Fig. 2. After SpeedVac concentration, the antagonistic activity of each collected fraction was then tested by the SOP technique. For the eight *B. velezensis* strains, antagonistic activities against both Gram-positive and Gram-negative pathogens were detected for the fractions characterized by an elution volume between 100 and 110 mL corresponding to a calculated MW between 100 and 300 Da, compatible with that of bacilysin (270 Da).

3.5. Bacilysin was specifically detected by UPLC-MS

In order to confirm that bacilysin was involved in the antimicrobial activity of the eight *B. velezensis* strains, UPLC-MS analyses were performed on their AS precipitates. The analyses were also performed on four other strains (Bs168, CN054, Sub011 and CN017) randomly selected in our collection of *B. subtilis* group strains.

Based on a previous study (Yazgan et al., 2001), we identified the peak eluted at 6.84 min as fitting the one of bacilysin. As shown in Table 4, the intensity of this peak was variable among the twelve strains with higher intensities for the eight *B. velezensis* strains. For each positive strain, the specific identification of bacilysin was confirmed by mass spectra of this peak using a full-scan positive ion mode, as illustrated by the typical MS spectrum on Fig. 3. A major signal with a *m/z* of 271 was detected and matched to the mass of protonated $[M+H]^+$ bacilysin ion. The minor signal at *m/z* 200 might correspond to the breakdown of bacilysin into anticapsin. These results indicate that bacilysin is the molecule involved in the antimicrobial activity displayed by the *B. velezensis* strains studied in this work.

4. Discussion & conclusions

In the current context of bio-preservation, many *Bacillus* spp. have attracted increasing industrial interest, especially for their wide antagonistic potential against a large spectrum of microbial pathogens. In

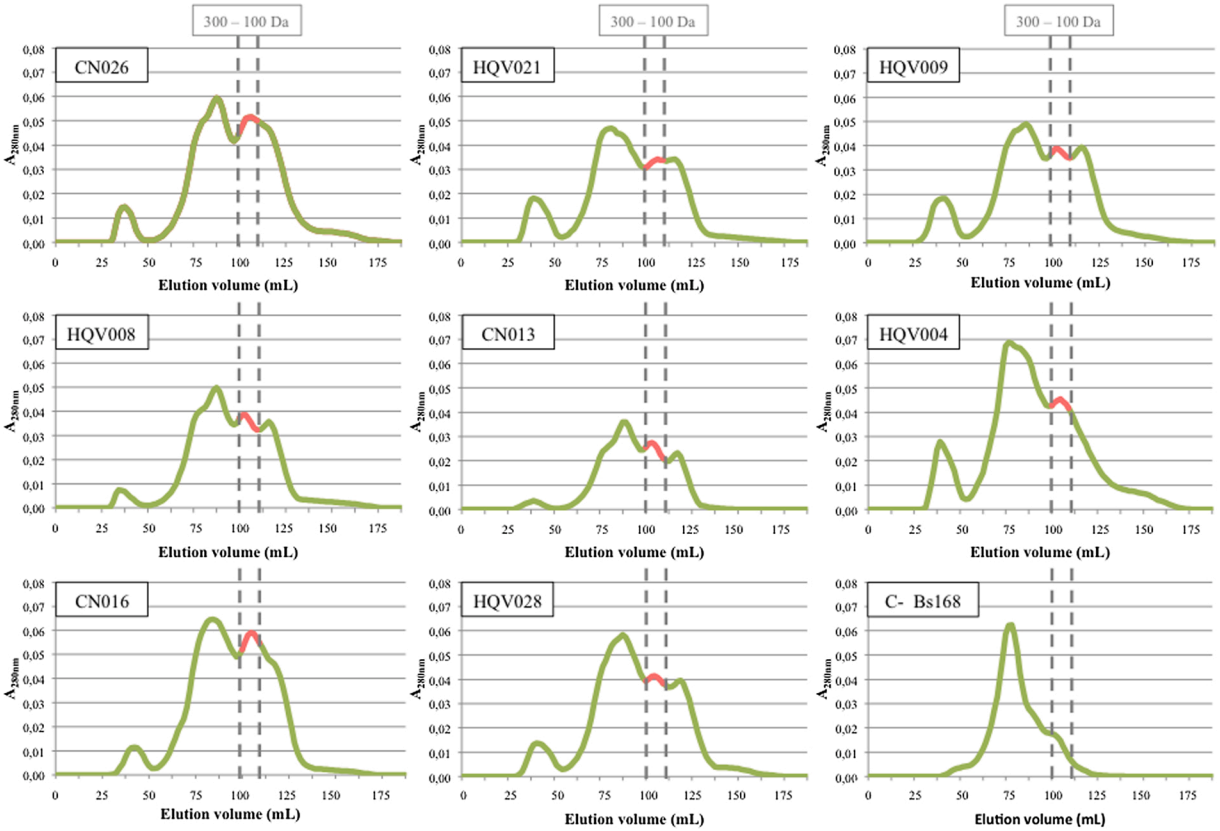

Fig. 2. LPLC UV chromatograms for the eight *B. velezensis* strains selected after the AS precipitation step. Strain Bs168 was used as negative control. The absorbance at 280 nm was spotted for each fraction of 1.5 mL (green line). The fractions exhibiting activity against the foodborne pathogens after SpeedVac concentration are represented in red. The MW of these fractions was estimated between 100 and 300 Da (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Table 4
Intensity of the peak corresponding to bacilysin obtained by UPLC separation.

Strain		Species	Intensity of the peak at 6.84 min (mAU) [a]	
			Extraction ACN	Extraction EtOH
Eight strains exhibiting antimicrobial activity after AS precipitation	CN016	*B. velezensis*	886,356	1,485,174
	CN026	*B. velezensis*	2,539,665	1,667,604
	CN013	*B. velezensis*	2,144,911	1,129,164
	HQV004	*B. velezensis*	2,509,912	1,226,666
	HQV008	*B. velezensis*	537,872	358,020
	HQV009	*B. velezensis*	240,222	357,494
	HQV021	*B. velezensis*	1,378,670	1,333,165
	HQV028	*B. velezensis*	203,310	105,772
Four strains without activity after AS precipitation	CN017	*B. subtilis*	18,237	23,905
	Sub011	*B. subtilis*	0	0
	Bs168	*B. subtilis*	0	0
	CN054	*B. subtilis*	0	0
Negative control (YEN)			0	0

[a] For both NRPs extraction method, the peak intensity is arbitrarily quantified in milli Arbitrary Unit (mAU) are given for 12 strains AS precipitate and for YEN used as negative control.

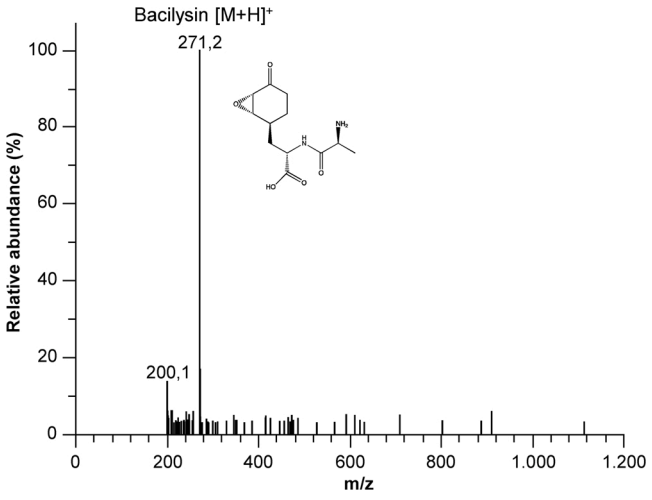

Fig. 3. High-resolution mass spectrum of strain CN026 resulting from the peak eluted after 6.84 min through the UPLC separation. The major signal at *m/z* 271 fits with the mass of protonated $[M+H]^+$ bacilysin ion whereas the minor one at *m/z* 200 could correspond to the anticapsin moiety of bacilysin.

particular, the *B. subtilis* group is currently used for many applications such as processing of fermented food (*e.g.* Japanese fermented soybean Natto, Hosoi and Kiuchi, 2003), probiotics (Pereira et al., 2019), as well as the production of a wide range of active biomolecules to control foodborne (Lv et al., 2020; Wu et al., 2013) or plant pathogens (Chung et al., 2008; Fira et al., 2018; Li et al., 2020; Vinodkumar et al., 2017). Among these, the dipeptide bacilysin is of major interest. Despite its early discovery in 1946 and the numerous potential applications of this NRP (Özcengiz and Öğülür, 2015), little is known about the distribution the bacilysin genetic determinants within the *B. subtilis* group.

In this study, an *in silico* search indicated that the bacilysin related gene cluster *ywfA-bacABCDE-ywfG-ywfH* is present in all the sequenced genomes of *B. amyloliquefaciens, B. velezensis* and *B. subtilis* subspecies. However, the *B. licheniformis* strains did not possess any of these genes, and *bacE* was not detected in the *B. pumilus* genomes. To extend these observations, a set of 168 *B. subtilis* group strains isolated from various environments (*e.g.* fermented food, soil or faeces) was tested by PCR for the presence for the bacilysin genetic determinants. The results indicated their ubiquitous presence among *B. amyloliquefaciens, B. velezensis* and *B. subtilis s.s.* and subspecies. Similar observations have been made for individual strains of *B. velezensis* (Chen et al., 2019), *B. amyloliquefaciens* (Lv et al., 2020) or *B. subtilis* (Bóka et al., 2019). For *B. licheniformis* and *B. pumilus*, the bacilysin gene cluster was absent or incomplete, respectively. In the latter case, it is plausible that another permease could act in lieu of BacE in *B. pumilus* for exporting the active compound and ensuring the intrinsic resistance of its producer. The existence of yet another detoxifying mechanism cannot be excluded.

Based on this collection of 168 isolates, 23 were selected for the ability of their CFS to inhibit both Gram-positive (*i.e. B. cereus* and/or *L. ivanovii*) and Gram-negative (*i.e. E. coli* and/or *Salmonella enterica*) foodborne pathogens. After an AS precipitation step, eight out of these 23 strains were still exhibiting antagonistic activities against Gram-negative pathogens. The presence of bacilysin in their supernatant was suggested by LPLC cycles and specifically confirmed by UPLC-MS. The *gyrA* gene of these eight strains indicated their affiliation to the *B. velezensis* species. Furthermore, four other strains previously identified by *gyrA* sequencing (*B. subtilis* Bs168, CN054, Sub011 and CN017) were also tested by UPLC-MS. While all of them possess the bacilysin gene cluster, bacilysin was only detected in the supernatant of the CN017 strain, at a much lower intensity compared to the *B. velezensis* strains.

This variation in bacilysin synthesis among those strains might be explained by several, non-exclusive, phenomena. First, the genetic regulation system that triggers bacilysin biosynthesis has been shown to depend on a complex regulation system, which controls several metabolic processes such as peptide antibiotic production, sporulation, biofilm formation and development of cell competence. Interestingly, the pleiotropic effects of bacilysin have recently been confirmed using a *B. subtilis* strain silenced for its bacilysin operon (Ertekin et al., 2020). Variations of this genetic regulatory system among the different strains may account for some of the differences observed in the level of bacilysin synthesis.

Second, this complex regulation pathway is controlled by quorum sensing based on signalling peptides present in the medium. It has indeed been shown that the composition of the growth medium is important for bacilysin biosynthesis (Özcengiz and Öğülür, 2015). For instance, carbon sources other than glucose and sucrose were shown to decrease the yields of bacilysin production (Özcengiz and Alaeddinoglu, 1991). Besides, the nitrogen source has a great impact on bacilysin biosynthesis (Özcengiz et al., 1990; Wu et al., 2015). It has notably been reported that the strain ability to produce bacilysin is mediated by the concentration of yeast extract. In particular, the *B. amyloliquefaciens* strain GSB272 was able to produce bacilysin at high yeast extract concentrations. However, this was not observed for some *B. subtilis* strains whose bacilysin production ability was inhibited at such yeast extract levels (Steinborn et al., 2005). In the present work, the YEN medium used was rich in yeast extract (45 g L^{-1}). Based on the variability previously reported, the YEN composition might not have been optimal to stimulate bacilysin production for all tested strains.

Third, the potential production and diversity of other antimicrobial compounds associated with the different active *Bacillus* spp. strains (Caulier et al., 2018) might also influence, and potentially interfere, with the production of bacilysin.

In conclusion, this study highlights the positive correlation between bacilysin production and the antagonistic activity exhibited against Gram-negative foodborne pathogens for eight *B. velezensis* strains. Yet, this species is known for its ability to produce diverse biologically active molecules (and not only bacilysin). A knockout (KO) of the bacilysin genetic determinants should help to better define and understand its specific contribution to the observed antimicrobial activity. To this end, bacilysin KO-mutant of strain CN026, the most promising strain for both its antimicrobial activity and absence of antibiotic resistance (Nannan et al., 2018), should clarify the potential role of bacilysin and its possible synergistic or antagonistic effects with other antimicrobial compounds.

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CRediT authorship contribution statement

Catherine Nannan: Conceptualization, Investigation, Methodology, Validation, Writing - original draft, Writing - review & editing. **Huong Quynh Vu:** Methodology, Validation. **Annika Gillis:** Methodology, Validation, Writing - review & editing. **Simon Caulier:** Methodology, Validation, Writing - review & editing. **Thuy Thanh Thi Nguyen:** Funding acquisition, Project administration, Resources. **Jacques Mahillon:** Conceptualization, Methodology, Supervision, Validation, Writing - review & editing.

Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jbiotec.2020.12.017.

References

Ashe, S., Maji, U., Sen, R., Mohanty, S., Maiti, N., 2014. Specific oligonucleotide primers for detection of endoglucanase positive *Bacillus subtilis* by PCR. 3 Biotech 4, 461–465. https://doi.org/10.1007/s13205-013-0177-6.

Bóka, B., Manczinger, L., Kocsubé, S., Shine, K., Alharbi, N.S., Khaled, J.M., Münsterkötter, M., Vágvölgyi, C., Kredics, L., 2019. Genome analysis of a *Bacillus subtilis* strain reveals genetic mutations determining biocontrol properties. World J. Microbiol. Biotechnol. 35, 52. https://doi.org/10.1007/s11274-019-2625-x.

Caulier, S., Gillis, A., Colau, G., Licciardi, F., Liépin, M., Desoignies, N., Modrie, P., Legrève, A., Mahillon, J., Bragard, C., 2018. Versatile antagonistic activities of soil-borne *Bacillus* spp. and *Pseudomonas* spp. against *Phytophthora infestans* and other potato pathogens. Front. Microbiol. 9, 143. https://doi.org/10.3389/fmicb.2018.00143.

Caulier, S., Nannan, C., Gillis, A., Licciardi, F., Bragard, C., Mahillon, J., 2019. Overview of the antimicrobial compounds produced by members of the *Bacillus subtilis* group. Front. Microbiol. 10, 302. https://doi.org/10.3389/fmicb.2019.00302.

Chen, X.-H., Scholz, R., Borriss, M., Junge, H., Mögel, G., Kunz, S., Borriss, R., 2009. Difficidin and bacilysin produced by plant-associated *Bacillus amyloliquefaciens* are efficient in controlling fire blight disease. J. Biotechnol. 140, 38–44. https://doi.org/10.1016/j.jbiotec.2008.10.015.

Chen, L., Shi, H., Heng, J., Wang, D., Bian, K., 2019. Antimicrobial, plant growth-promoting and genomic properties of the peanut endophyte *Bacillus velezensis* LDO2. Microbiol. Res. 218, 41–48. https://doi.org/10.1016/j.micres.2018.10.002.

Chun, J., Bae, K.S., 2000. Phylogenetic analysis of *Bacillus subtilis* and related taxa based on partial *gyrA* gene sequences. Ant. van Leeuwenhoek 78, 123–127. https://doi.org/10.1023/a:1026555830014.

Chung, S., Kong, H., Buyer, J.S., Lakshman, D.K., Lydon, J., Kim, S.-D., Roberts, D.P., 2008. Isolation and partial characterization of *Bacillus subtilis* ME488 for suppression of soilborne pathogens of cucumber and pepper. Appl. Microbiol. Biotechnol. 80, 115–123. https://doi.org/10.1007/s00253-008-1520-4.

Dunlap, C.A., Kim, S.-J., Kwon, S.-W., Rooney, A.P., 2016. *Bacillus velezensis* is not a later heterotypic synonym of *Bacillus amyloliquefaciens*; *Bacillus methylotrophicus*, *Bacillus amyloliquefaciens* subsp. *plantarum* and' *Bacillus oryzicola*' are later heterotypic synonyms of *Bacillus velezensis* based on phylogenomics. Int. J. Syst. Evol. Microbiol. 66, 1212–1217. https://doi.org/10.1099/ijsem.0.000858.

Ertekin, O., Kutnu, M., Taşkin, A.A., Demir, M., Karataş, A.Y., Özcengiz, G., 2020. Analysis of a *bac* operon-silenced strain suggests pleiotropic effects of Bacilysin in *Bacillus subtilis*. J. Microbiol. 58, 297–313. https://doi.org/10.1007/s12275-020-9064-0.

Fan, B., Blom, J., Klenk, H.-P., Borriss, R., 2017. *Bacillus amyloliquefaciens*, *Bacillus velezensis* and *Bacillus siamensis* form an 'operational group *B. amyloliquefaciens*' within the *B. subtilis* species complex. Front. Microbiol. 8, 22. https://doi.org/10.3389/fmicb.2017.00022.

Fira, D., Dimkić, I., Berić, T., Lozo, J., Stanković, S., 2018. Biological control of plant pathogens by *Bacillus* species. J. Biotechnol. 285, 44–55. https://doi.org/10.1016/j.jbiotec.2018.07.044.

Foster, J.W., Woodruff, H.B., 1946. Bacillin, a new antibiotic substance from a soil isolate of *Bacillus subtilis*. J. Bacteriol. 51, 363–369. http://www.ncbi.nlm.nih.gov/pmc/articles/pmc518064/.

Hilton, M., Alaeddinoglu, N., Demain, A., 1988. Synthesis of bacilysin by *Bacillus subtilis* branches from prephenate of the aromatic amino acid pathway. J. Bacteriol. 170, 482–484. https://doi.org/10.1128/jb.170.1.482-484.1988.

Hosoi, T., Kiuchi, K., 2003. In: Farnworth, E. (Ed.), Natto - A Food Made by Fermenting Cooked Soybeans With *Bacillus subtilis* (Natto). Handbook of Fermented Functional Foods. CRC Press, Boca Raton, FL.

Kenig, M., Vandamme, E., Abraham, E.P., 1976. The mode of action of bacilysin and anticapsin and biochemical properties of bacilysin-resistant mutants. J. Gen. Microbiol. 94, 46–54. https://doi.org/10.1099/00221287-94-1-46.

Lahlali, R., Peng, G., McGregor, L., Gossen, B., Hwang, S., McDonald, M., 2011. Mechanisms of the biofungicide Serenade (*Bacillus subtilis* QST713) in suppressing clubroot. Biocontr. Sc. Technol. 21, 1351–1362. https://doi.org/10.1080/09583157.2011.618263.

Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J., Higgins, D.G., 2007. Clustal W and Clustal X version 2.0. Bioinform 23, 2947–2948. https://doi.org/10.1093/bioinformatics/btm404.

Li, Q., Liao, S., Wei, J., Xing, D., Xiao, Y., Yang, Q., 2020. Isolation of *Bacillus subtilis* strain SEM-2 from silkworm excrement and characterisation of its antagonistic effect against *Fusarium* spp. Can. J. Microbiol. 11, 1–12. https://doi.org/10.1139/cjm-2019-0621.

Lv, J., Da, R., Cheng, Y., Tuo, X., Wei, J., Jiang, K., Monisayo, A.O., Han, B., 2020. Mechanism of antibacterial activity of *Bacillus amyloliquefaciens* C-1 lipopeptide toward anaerobic *Clostridium difficile*. Biomed Res. Int. 2020, 3104613 https://doi.org/10.1155/2020/3104613.

Mahlstedt, S.A., Walsh, C.T., 2010. Investigation of anticapsin biosynthesis reveals a four-enzyme pathway to tetrahydrotyrosine in *Bacillus subtilis*. Biochem. 49, 912–923. https://doi.org/10.1021/bi9021186.

Martin-Visscher, L.A., Yoganathan, S., Sit, C.S., Lohans, C.T., Vederas, J.C., 2011. The activity of bacteriocins from *Carnobacterium maltaromaticum* UAL307 against Gram-negative bacteria in combination with EDTA treatment. FEMS Microbiol. Lett. 317, 152–159. https://doi.org/10.1111/j.1574-6968.2011.02223.x.

Nannan, C., Gillis, A., Caulier, S., Mahillon, J., 2018. Complete genome sequence of *Bacillus velezensis* CN026 strain exhibiting antagonistic activity against Gram-negative foodborne pathogens. Genome Announc. 6, e01543–17. https://doi.org/10.1128/genomea.01543-17.

Newton, G., 1949. Antibiotics from a strain of *B. subtilis*: bacilipin A and B and bacilysin. Brit. J. Exper. Pathol. 30, 306–319. http://www.ncbi.nlm.nih.gov/pmc/articles/pmc2073260/.

Özcengiz, G., Alaeddinoglu, N., 1991. Bacilysin production by *Bacillus subtilis*: effects of bacilysin, pH and temperature. Folia Microbiol. (Praha) 36, 522–526. https://doi.org/10.1007/bf02884030.

Özcengiz, G., Öğülür, İ., 2015. Biochemistry, genetics and regulation of bacilysin biosynthesis and its significance more than an antibiotic. New Biotechnol. 32, 612–619. https://doi.org/10.1016/j.nbt.2015.01.006.

Özcengiz, G., Alaeddinoglu, N.G., Demain, A.L., 1990. Regulation of biosynthesis of bacilysin by *Bacillus subtilis*. J. Ind. Microbiol. 6, 91–100. https://doi.org/10.1007/bf01576428.

Öztürk, S., Çalık, P., Özdamar, T.H., 2016. Fed-batch biomolecule production by *Bacillus subtilis*: a state of the art review. Trends Biotechnol. 34, 329–345. https://doi.org/10.1016/j.tibtech.2015.12.008.

Paraszkiewicz, K., Bernat, P., Siewiera, P., Moryl, M., Paszt, L.S., Trzciński, P., Jałowiecki, Ł., Płaza, G., 2017. Agricultural potential of rhizospheric *Bacillus subtilis* strains exhibiting varied efficiency of surfactin production. Sc. Horticult. 225, 802–809. https://doi.org/10.1016/j.scienta.2017.07.034.

Pereira, J.Q., Ritter, A.C., Cibulski, S., Brandelli, A., 2019. Functional genome annotation depicts probiotic properties of *Bacillus velezensis* FTC01. Gene 713, 143971. https://doi.org/10.1016/j.gene.2019.143971.

Phister, T.G., O'Sullivan, D.J., 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. Microbiol. 70, 631–634. https://doi.org/10.1128/aem.70.1.631-634.2004.

Priest, F., Goodfellow, M., Shute, L., Berkeley, R., 1987. *Bacillus amyloliquefaciens* sp. nov., nom. rev. Int. J. Syst. Evol. Microbiol. 37, 69–71. https://doi.org/10.1099/00207713-37-1-69.

Rane, A.N., Baikar, V.V., Ravi Kumar, V., Deopurkar, R.L., 2017. Agro-industrial wastes for production of biosurfactant by *Bacillus subtilis* ANR 88 and its application in synthesis of silver and gold nanoparticles. Front. Microbiol. 8, 492. https://doi.org/10.3389/fmicb.2017.00492.

Rogers, H.J., Newton, G., Abraham, E., 1965. Production and purification of bacilysin. Biochem. J. 97, 573–578. https://doi.org/10.1042/bj0970573.

Rosenberg, J., Ischebeck, T., Commichau, F.M., 2017. Vitamin B6 metabolism in microbes and approaches for fermentative production. Biotechnol. Adv. 35, 31–40. https://doi.org/10.1016/j.biotechadv.2016.11.004.

Ruiz-Garcia, C., Bejar, V., Martinez-Checa, F., Llamas, I., Quesada, E., 2005. *Bacillus velezensis* sp. nov., a surfactant-producing bacterium isolated from the river Velez in Malaga, southern Spain. Int. J. Syst. Evol. Microbiol. 55, 191–195. https://doi.org/10.1099/ijs.0.63310-0.

Schallmey, M., Singh, A., Ward, O.P., 2004. Developments in the use of *Bacillus* species for industrial production. Can. J. Microbiol. 50, 1–17. https://doi.org/10.1139/w03-076.

Shi, T., Wang, Y., Wang, Z., Wang, G., Liu, D., Fu, J., Chen, T., Zhao, X., 2014. Deregulation of purine pathway in *Bacillus subtilis* and its use in riboflavin biosynthesis. Microb. Cell Fact. 13, 101. https://doi.org/10.1186/s12934-014-0101-8.

Stein, T., 2005. *Bacillus subtilis* antibiotics: structures, syntheses and specific functions. Mol. Microbiol. 56, 845–857. https://doi.org/10.1111/j.1365-2958.2005.04587.x.

Steinborn, G., Hajirezaei, M.-R., Hofemeister, J., 2005. *Bac* genes for recombinant bacilysin and anticapsin production in *Bacillus* host strains. Arch. Microbiol. 183, 71–79. https://doi.org/10.1007/s00203-004-0743-8.

Vinodkumar, S., Nakkeeran, S., Renukadevi, P., Malathi, V., 2017. Biocontrol potentials of antimicrobial peptide producing *Bacillus* species: multifaceted antagonists for the management of stem rot of carnation caused by *Sclerotinia sclerotiorum*. Front. Microbiol. 8, 446. https://doi.org/10.3389/fmicb.2017.00446.

Walker, J., Abraham, E., 1970. The structure of bacilysin and other products of *Bacillus subtilis*. Biochem. J. 118, 563–570. https://doi.org/10.1042/bj1180563.

Wang, T., Liu, X.H., Wu, M.B., Ge, S., 2018. Molecular insights into the antifungal mechanism of Bacilysin. J. Mol. Model. 24, 118. https://doi.org/10.1007/s00894-018-3645-4.

Wu, W.-J., Park, S.-M., Ahn, B.-Y., 2013. Isolation and characterization of an antimicrobial substance from *Bacillus subtilis* BY08 antagonistic to *Bacillus cereus* and *Listeria monocytogenes*. Food Sc. Biotechnol. 22, 433–440. https://doi.org/10.1007/s10068-013-0098-5.

Wu, L., Wu, H., Chen, L., Lin, L., Borriss, R., Gao, X., 2015. Bacilysin overproduction in *Bacillus amyloliquefaciens* FZB42 markerless derivative strains FZBREP and FZBSPA enhances antibacterial activity. Appl. Microbiol. Biotechnol. 99, 4255–4263. https://doi.org/10.1007/s00253-014-6251-0.

Yazgan, A., Özcengiz, G., Özcengiz, E., Kılınc, K., Marahiel, M.A., Alaeddinoglu, N.G., 2001. Bacilysin biosynthesis by a partially purified enzyme fraction from *Bacillus subtilis*. Enz. Microb. Technol. 29, 400–406. https://doi.org/10.1016/S0141-229(01)00401-X.

Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S., Madden, T.L., 2012. Primer-BLAST: a tool to design target- specific primers for polymerase chain reaction. BMC Bioinform. 13, 134. https://doi.org/10.1186/1471-2105-13-134.

Yuan, J., Raza, W., Huang, Q., Shen, Q., 2011. Quantification of the antifungal lipopeptide iturin A by high performance liquid chromatography coupled with aqueous two-phase extraction. J. Chromat. B 879, 2746–2750. https://doi.org/10.1016/j.jchromb.2011.07.041.