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Fengycin or plipastatin? A confusing question in Bacilli

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Abstract

According to most of the related literature published since their discovery in 1986, fengycin and plipastatin are very related molecules. These are lipodecapeptides encoded by operons of five synthetase genes. The most important difference between these two molecules lies in the peptide moiety at the position of the D-tyrosine, which is encoded by the second gene *fenB* of fengycin operon and by the fourth gene *ppsD* of plipastatin operon. Here, we aimed to differentiate between fengycin and plipastatin molecules. We designed degenerate primers using the consensus sequence of the epimerization domain responsible for the transformation of L-tyrosine to D-tyrosine from *Bacillus subtilis* 168, *Bacillus amyloliquefaciens* FZB42, and *Bacillus atrophaeus* 1942. These degenerate primers were then used to amplify fragments from *B. amyloliquefaciens* S499, *B. subtilis* ATCC 21332, and *Bacillus cereus*. Alignment of the sequences of the amplified fragments with the sequences from the mentioned strains deposited in GenBank database showed high similarity with 64 *B. subtilis* strains, 24 *B. amyloliquefaciens* strains, seven *B. atrophaeus* strains, one *B. cereus* strain, one *Bacillus sonorensis* strain, two *Bacillus methylotrophicus* strains, and 45 *Bacillus velezensis* strains. The results confirmed that these *Bacillus* strains *harbor* the tyrosine epimerization domain located on the fourth gene of their fengycin or plipastatin operons, which indicated that these strains synthesize plipastatin rather than fengycin.

Key words: fengycin, plipastatin, Bacilli, epimerization domain, NRPS

Introduction

Fengycins and plipastatins are closely related lipodecapeptides produced by various species of *Bacillus*, namely *Bacillus subtilis*, *Bacillus amyloliquefaciens*, *Bacillus globigii*, and *Bacillus cereus* (Schneider et al., 1999; Volpon et al., 2000; Vater et al., 2002; Williams et al., 2002; Bie et al., 2009; Pyoung et al., 2010). These molecules are synthesized by the nonribosomal peptides system (NRPS), which is a multienzyme system consisting of an arrangement of modules. The enzymatic units that reside within a module are called domains. Each module contains three domains: an adenylation (A) domain for substrate recognition, a peptidyl carrier protein (PCP) domain to hold the activated substrate, and a condensation (C) domain to form peptide bonds (Dieckmann et al., 1995; Stachelhaus and Marahiel, 1995; Stachelhaus et al., 1996; Mootz and Marahiel, 1997; Stachelhaus et al., 1998; Ehmann et al., 2000; May et al., 2001; Bergendahl et al., 2002).

Based on the amino acid residue present at position 6, fengycin and plipastatin may have any of the two structures: form A (Ala) or form B (Val). In the structure of these molecules, the peptidic moiety has an internal lactone ring between the carboxyl group of the terminal amino acid (Ile) and the hydroxyl group of the tyrosine residue at position 3. The β-hydroxy fatty acid chains at C14–C18 positions are linked to the N-terminal amino acid residue (Glu) via an amide bond (Vanittanakom et al., 1986; Nishikiori et al., 1986b). The fatty acid chains at C15, C16, and C17 positions are the main representatives and are saturated, except in the case of the lipopeptide isolated from *Bacillus thuringiensis* which has a fatty acid chain with a double bond between C13 and C14 (Kim et al., 2004). The structure of the lipodecapeptide plipastatin from *B. cereus* BMG302-fF67 was first described by Nishikiori et al. (1986a). Based on the structure, plipastatins are divided into two groups: group A and group B. Group A plipastatins have

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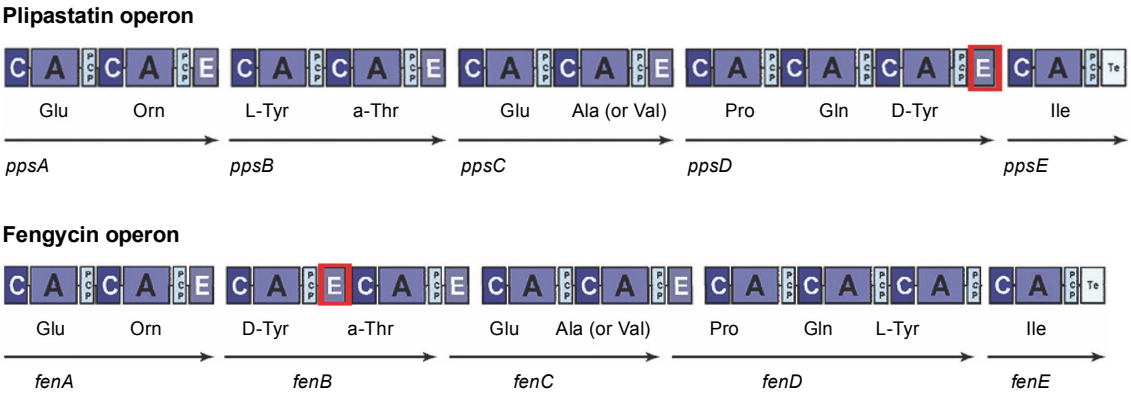

Fig. 1. The organization of plipastatin and fengycin operon and the differences in peptide structures as reported by Ongena and Jacques (2008); D-tyrosine is located on *ppsD* gene at position 9 (amino acid) in plipastatin, whereas it is located on *fenD* gene at position 3 in fengycin

D-Ala at the sixth position of the amino acid sequence from the N-terminus, whereas group B plipastatins have D-Val. The plipastatins numbered ′1′1 (A1 and B1) have 3(R)-hydroxyhexadecanoic acid (B-hydroxypalmitic acid) as the fatty acid residue, whereas those numbered ′2′2 (A2 and B2) have 14(S)-methyl-3(R)-hydroxyhexadecanoic acid (B-hydroxy-anteiso-palmitic acid) as the fatty acid residue.

Electrospray ionization/collision-induced dissociation mass spectrometry was used in a study to analyze the fengycin homologues produced by *B. subtilis* JA (Wang et al., 2004). The L and D forms of tyrosine and the presence of a Gln residue instead of a Glu at position 8 were found to constitute the differences between fengycin and plipastatin. The L and D forms of tyrosine were found at positions 3 and 9 for plipastatins, respectively, whereas they were found at positions 9 and 3 for fengycins (Wang et al., 2004) (Fig. 1). However, Schneider et al. (1999) detected a fengycin molecule with a D-Tyr at position 3 from the supernatant of the culture of *B. subtilis* S499. This result could be correlated with the findings on the structure of the synthetases described for other fengycin- or plipastatin-producing strains.

Bacillus subtilis 168 was the first strain in which the operon encoding fengycin or plipastatin synthetases was described (Tosato et al., 1997); five synthetase-encoding genes (*ppsABCDE*) were reported in this strain. Wu et al. (2007) reported that in *B. subtilis* F29–3 strain five genes encoding fengycin synthetases were present in *fenCDEAB* order. The other plipastatin or fengycin synthetases were described in *B. subtilis* b213 and A1/3 (Steller et al., 2000). The authors reported that the operon was a cluster of five genes (*fen* 1–5) which showed a high homology to the *pps* and *fen* genes from *B. subtilis* strains 168 and F29–3. Finally, in *B. amyloliquefaciens* FZB42, Koumoutsi et al. (2004) reported that the organization and location of fengycin (*fen*) operon were similar to *B. subtilis* 168.

Plipastatins are reported to act as inhibitors of phospholipase A2, an enzyme involved in various important physiological cellular functions such as inflammation, acute hypersensitivity, and blood platelet aggregation (Umezawa et al., 1986). Fengycins are known to act as strong fungitoxic agents against various filamentous fungi such as *Botrytis cinerea*, *Colletotrichum acutatum*, *Monilinia fructicola*, *Monilinia hiemalis*, *Paecilomyces variotii*, *Pyricularia oryzae*, *Rhizoctonia solani*, *Schizophyllum commune*, *Fusarium moniliforme* Sheldon ATCC 38932, and *Colletotrichum gloeosporioides* (Vanittanakom et al., 1986; Sun et al., 2006; Romero et al., 2007; Hu et al., 2007; Bie et al., 2009; Pyoung et al., 2010).

Here, we aimed to differentiate between fengycin and plipastatin based on the position of tyrosine epimerization domain in the second and fourth genes of the operon using degenerate primers for polymerase chain reaction (PCR) and NRPS predictor software for analyzing the multi-domain synthetases involved in the biosynthesis of fengycin and plipastatin from different *Bacillus* strains.

Materials and methods

Bacterial strains

The *Bacillus* species and *Escherichia coli* strains used in this study were grown aerobically in Luria–Bertani medium at 37 °C (Table 1).

Table 1. Bacterial strains and plasmids used in this study

Bacterial strains	Description	Source
B. subtilis ATCC 21332	wild type	ProBioGEM
B. amyloliquefaciens S499	wild type	ProBioGEM
B. amyloliquefaciens FZB42	wild type	ProBioGEM
B. subtilis 168	trpC^{2}, sfp^{0}	lab stock
B. cereus	wild type	lab stock
E. coli DH5α	F^{-} *endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG purB20* φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, hsdR17($r_K^- m_K^+$), λ^{-}	lab stock
Plasmids	**Description**	**Source**
pGEM-T Easy	cloning vector, Ap^{r}	lab stock
pMG113	1670 bp *fenD* (Ep) fragment of *Bacillus subtilis* ATCC 21332 cloned into pGEM-T Easy	this study
pMG114	1651 bp *fenD* (Ep) fragment of *Bacillus cereus* into pGEM-T Easy	this study
pMG115	1641 bp *fenD* (Ep) fragment of *Bacillus amyloliquefaciens* S499 cloned into pGEM-T Easy	this study
pMG116	1446 bp *fenB* fragment of *Bacillus subtilis* ATCC 21332 cloned into pGEM-T Easy	this study
pMG117	1449 bp *fenB* fragment of *Bacillus amyloliquefaciens* S499 cloned into pGEM-T Easy	this study
pMG118	1447 bp *fenB* fragment of *Bacillus cereus* into pGEM-T Easy	this study

ProBioGEM – Laboratoire des Procédés Biologiques, Génie Enzymatique et Microbien, France

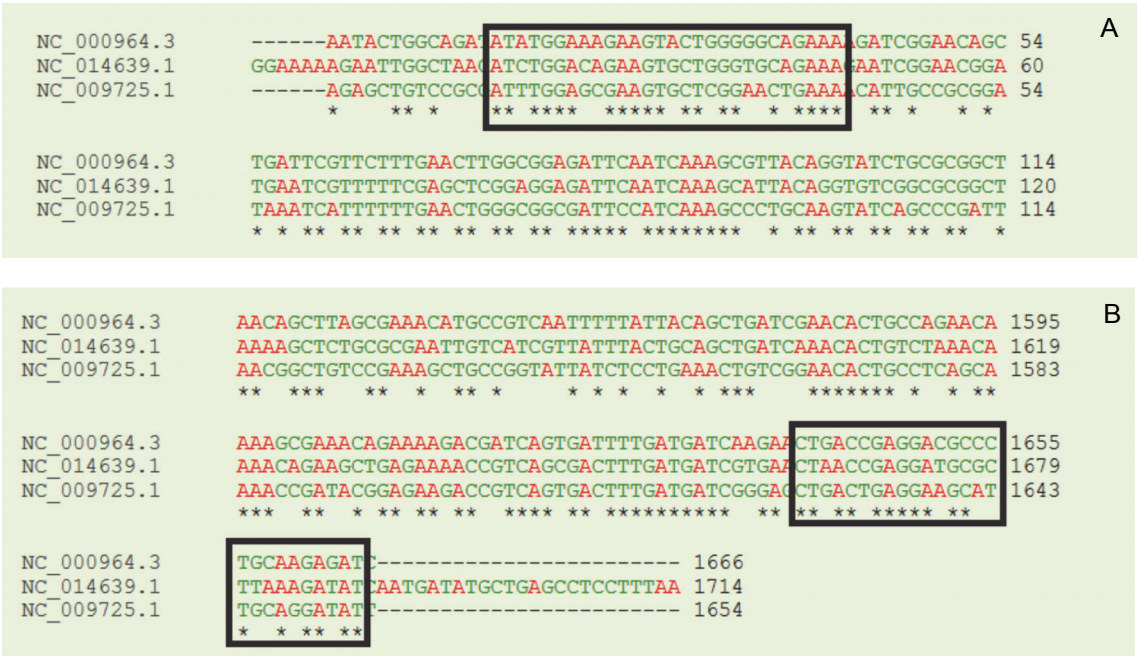

Fig. 2. ClustalW2 multiple-sequence alignment of tyrosine epimerization domain of fengycin (plipastatin) synthetase D of *Bacillus subtilis* 168, *Bacillus atrophaeus* 1942, and *Bacillus amyloliquefaciens* FZB42 (position 9); sequence "A" refers to the consensus sequence used for designing the forward primer, while sequence "B" refers to the consensus sequence used for designing the reverse primer

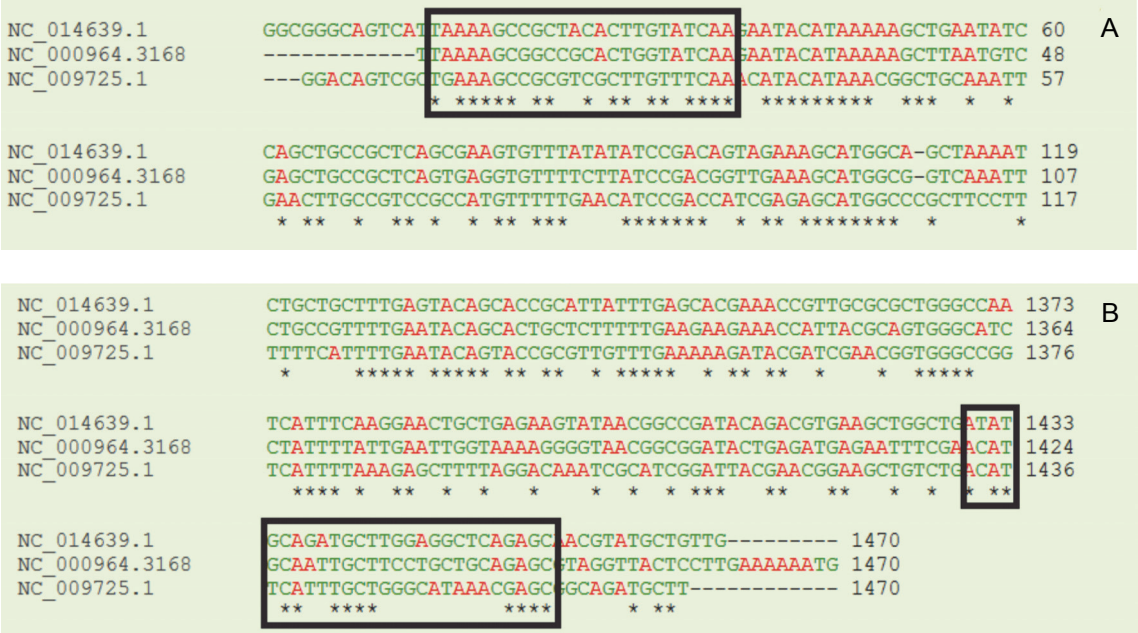

Fig. 3. ClustalW2 multiple-sequence alignment of the tyrosine thiolation domain and the threonine adenylation domain of fengycin (plipastatin) synthetase B of *Bacillus atrophaeus* 1942, *Bacillus subtilis* 168, and *Bacillus amyloliquefaciens* FZB42 (position 3); sequence "A" refers to the consensus sequence used for designing the forward primer, while sequence "B" refers to the consensus sequence used for designing the reverse primer

Table 2. Degenerate primers designed to detect the tyrosine epimerization domain from *Bacillus* species

Primer name	Primer sequence (5′–3′)	Fragment size
Ep fwd	5′–TRAAAGCSGCBDCRCTKGTWTCAA–3′	1641–1670 bp
Ep rev	5′–AYATKCADWTGCTKSSHRBHDMMGAGC–3′	
fenB fwd	5′–ATHTGCAVMGAAGTRCTSGGDRCWGAAA–3′	1443–1449 bp
fenB rev	5′–CTRACYGAGGAHGCVYTKMARGAKAT–3′	

Degenerate primers design and PCR conditions

The sequences used for designing the degenerate primers were taken from GenBank. Among the primers, two pairs were designed manually using the IUPAC symbols for degenerate bases based on the consensus sequence of the epimerization domain. These primers were designed to recognize the tyrosine epimerization domain at position 9 (*fenD* or *ppsD*) but not at position 3 (*fenB* or *ppsB*). The first pair of primers (*Ep*) was designed based on the consensus sequence obtained from the alignment of the sequences of epimerization domain (Ep domain) of *fenD* from *B. amyloliquefaciens* FZB42, *ppsD* from *B. subtilis* 168, and *Bacillus atrophaeus* 1942 (Fig. 2 and Table 2).

The second pair of primers *fenB* was designed based on the consensus sequence obtained from the alignment of the sequences from *B. amyloliquefaciens* FZB42, *ppsB* from *B. subtilis* 168, and *B. atrophaeus* 1942 (Fig. 3). These primers were designed to detect whether there is a tyrosine epimerization domain prior to the adenylation domain of threonine in any of the *Bacillus* strains tested in the study.

The genomic DNA was isolated from the strains using DNA purification kit (Wizard® Genomic, Promega) according to the manufacturer's instructions. The PCR conditions used in this study were as follows: initial denaturation step at 94 °C for 2 min, followed by 35 cycles of denaturation step at 94 °C for 45 sec, annealing step at 50 °C for 30 sec, and elongation step at 72 °C for 1.5 min. Following PCR, the gels were photographed using Gel Doc™ XR+ Gel Documentation System (Bio-Rad).

Extraction of PCR products from agarose gel

Following amplification, the fragments were extracted and purified using Zymoclean® Gel DNA Recovery Kit (Epigenetics Company) according to the manufacturer's instructions.

PCR products ligation

The PCR fragments obtained from strains *B. subtilis* 168 and *B. subtilis* ATCC 21332, and *B. amyloliquefaciens* FZB42 and *B. amyloliquefaciens* S499 were ligated to pGEM-T Easy as described in the technical manual of pGEM®-T and pGEM®-T Easy Vector Systems from QIAGEN.

Cloning and transformation protocol

Cloning and transformation was performed as described in the technical manual of pGEM®-T and pGEM®-T Easy Vector Systems from QIAGEN. The procedure used was as follows: 2 µl of ligation reaction was added to a sterile (17 × 100 mm) polypropylene tube or a 1.5 ml microcentrifuge tube and kept on ice. Another tube with 0.1 ng uncut plasmid was set up on ice to determine the transformation efficiency of the competent *E. coli* DH5α cells prepared in the laboratory (Tang et al., 1994).

Results

Epimerization domain detection by degenerate primers

Using epimerization domain primers (*Ep*) and *fenB* primers, the fragments of expected sizes were amplified by PCR. All strains used for the detection of epimerization domain are listed in Table 2. The epimerization domain primers (*Ep*) amplified one fragment from all tested *Bacillus* strains: a fragment of 1670 bp from *B. subtilis* 168 and *B. subtilis* ATCC 21332 and a fragment of 1641 bp from *B. amyloliquefaciens* FZB42 and *B. amyloliquefaciens* S499; one fragment of 1651 bp was amplified from *B. cereus*.

The *fenB* primers also amplified one fragment from all tested *Bacillus* strains: a fragment of 1446 bp from *B. subtilis* 168 and *B. subtilis* ATCC 21332 and a fragment of 1449 bp from *B. amyloliquefaciens* FZB42 and *B. amyloliquefaciens* S499; one fragment of 1447 bp was amplified from *B. cereus*.

Alignment of the epimerization domain sequence with sequence in GenBank database

The fragments amplified by PCR using degenerate primers were cloned and subjected to sequencing. The sequences of fragments were aligned on BLAST website (NCBI database). Only *B. subtilis* ATCC 21332 fragments submitted to GenBank and obtained these accession numbers (*ppsD* partial gene accession number: KX371639.1 and *ppsB* partial gene accession number: KX371637.1, GenBank). Importantly, the complete genome sequences of *B. amyloliquefaciens* S499 and *B. cereus* were found deposited in GenBank. The results of alignment with sequences from GenBank revealed that the genomes of 144 *Bacillus* strains harbor fengycin operon with the tyrosine epimerization domain at position 9 which has been shown in the literature as encoding plipastatin and not fengycin. The 64 *B. subtilis* strains which harbor tyrosine epimerization domain at position 9 (*fenD* or *ppsD*) are listed in Table 3, and the 80 *Bacillus* strains, including 24 *B. amyloliquefaciens* strains, seven *B. atrophaeus* strains, one *B. cereus* strain, one *Bacillus sonorensis* strain, two *Bacillus methylotrophicus* strains, and 45 *Bacillus velezensis* strains, which harbor the tyrosine epimerization domain at position 9 (*fenD* or *ppsD*) are listed in Table 4.

The epimerization domain catalyzes the epimerization of L-tyrosine of the growing polypeptide chain of fengycin or plipastatin. The NRPS predictor (Brian et al., 2009) identified six important residue positions or sequences of the epimerization domain known as the signature or code. These six sequences were all identified in the protein sequences of the epimerization domain of three strains, namely *B. cereus*, *B. subtilis* ATCC 21332, and *B. amyloliquefaciens* S499 (Fig. S1 in supplementary materials).

The alignment of sequences of the epimerization domain Ep (*fenD*) and *fenB* of *B. subtilis* ATCC 21332 showed 99% similarity with *B. subtilis* 168 and *B. subtilis* NCIB 3610. The sequences of Ep (partial *fenD*) and partial *fenB* from *B. subtilis* ATCC 21332 are shown in Figure S2 in supplementary materials.

The alignment of sequences of the epimerization domain Ep (*fenD*) and *fenB* of *B. cereus* showed 100% similarity with *B. cereus* MBGJa3 strain and 93% similarity with *B. subtilis* 168 strain. The sequences of Ep (partial *fenD*) and partial *fenB* from *B. cereus* are shown in Figure S3 in supplementary materials.

The alignment of sequences of the epimerization domain Ep (partial *fenD*) and partial *fenB* of *B. amyloliquefaciens* S499 revealed 99% similarity with *B. amyloliquefaciens* IT-45 and 97% similarity with *B. amyloliquefaciens*

Table 3. The list of *Bacillus subtilis* strains harboring the tyrosine epimerization domain of fengycin at position 9 based on BLAST alignment

Description	Accession	Description	Accession
Bacillus subtilis subsp. subtilis str. 168	CP019663.1	*Bacillus subtilis strain GQJK2*	CP020367.1
Bacillus subtilis F29–3	AF023464.2	*Bacillus subtilis strain SG6*	CP009796.1
Bacillus subtilis subsp. subtilis strain NCD-2	CP023755.1	*Bacillus subtilis strain TLO3*	CP021169.1
Bacillus subtilis strain SX01705	CP022287.1	*Bacillus subtilis BSn5*	CP002468.1
Bacillus subtilis subsp. subtilis strain BSD-2	CP013654.1	*Bacillus subtilis subsp. subtilis strain D12–5*	CP014858.1
Bacillus subtilis HJ5	CP007173.1	*Bacillus subtilis subsp. subtilis str. BSP1*	CP003695.1
Bacillus subtilis subsp. subtilis str. BAB-1	CP004405.1	*Bacillus subtilis strain BS16045*	CP017112.1
Bacillus subtilis XF-1	CP004019.1	*Bacillus subtilis subsp. subtilis strain SRCM101392*	CP021921.1
Bacillus subtilis strain BJ3–2	CP025941.1	*Bacillus subtilis subsp. inaquosorum strain DE111*	CP013984.1
Bacillus subtilis strain HJ0–6	CP016894.1	*Bacillus subtilis strain CW14*	CP016767.1
Bacillus subtilis subsp. subtilis str. OH 131.1	CP007409.1	*Bacillus subtilis strain ATCC 19217*	CP009749.1
Bacillus subtilis strain VV2	CP017676.1	*Bacillus subtilis subsp. subtilis strain 168G*	CP016852.1
Bacillus subtilis strain ge28	CP021903.1	*Bacillus subtilis subsp. subtilis strain KCTC 3135*	CP015375.1
Bacillus subtilis subsp. subtilis strain SRCM100761	CP021889.1	*Bacillus subtilis subsp. subtilis strain delta6*	CP015975.1
Bacillus subtilis subsp. subtilis strain SRCM100757	CP021499.1	*Bacillus subtilis strain SZMC 6179J*	CP015004.1
Bacillus subtilis subsp. subtilis strain SRCM101444	CP021498.1	*Bacillus subtilis strain ATR2*	CP018133.1
Bacillus subtilis subsp. subtilis RO-NN-1	CP002906.1	*Bacillus subtilis subsp. subtilis strain CU1050*	CP014166.1
Bacillus subtilis strain TLO3	CP023257.1	*Bacillus subtilis strain TO-A JP*	CP011882.1
Bacillus subtilis TOA	CP005997.1	*Bacillus subtilis KCTC 1028*	CP011115.1
Bacillus subtilis subsp. subtilis str. AG1839	CP008698.1	*Bacillus subtilis subsp. subtilis strain 3NA*	CP010314.1
Bacillus subtilis subsp. subtilis str. JH642 substr. AG174	CP007800.1	*Bacillus subtilis strain PS832*	CP010053.1
Bacillus subtilis PY79	CP006881.1	*Bacillus subtilis strain HRBS-10TDI13*	CP015222.1
Bacillus subtilis subsp. subtilis 6051-HGW	CP003329.1	*Bacillus subtilis strain KH2*	CP018184.1
Bacillus subtilis subsp. globigii strain ATCC 49760	CP014840.1	*Bacillus subtilis strain 29R7–12*	CP017763.1
Bacillus subtilis strain UD1022	CP011534.1	*Bacillus subtilis strain Bs-115*	CP020722.1
Bacillus subtilis strain BS38	CP017314.1	*Bacillus subtilis subsp. subtilis strain SRCM100333*	CP021892.1
Bacillus subtilis subsp. natto strain CGMCC 2108	CP014471.1	*Bacillus subtilis strain DKU_NT_02*	CP022890.1
Bacillus subtilis subsp. natto BEST195	AP011541.2	*Bacillus subtilis strain DKU_NT_03*	CP022891.1
Bacillus subtilis strain SR1	CP021985.1	*Bacillus subtilis strain QST713*	DQ011337.1
Bacillus subtilis subsp. subtilis strain QB5412	CP017312.1	*Bacillus subtilis strain MB14*	KF524437.1
Bacillus subtilis strain NCIB 3610	CP020102.1	*Bacillus subtilis strain B-1*	CP009684.1
Bacillus subtilis strain Bs-916	CP009611.1	*Bacillus subtilis strain J-5*	CP018295.1

Table 4. The list of *Bacillus* strains harboring the tyrosine epimerization domain of fengycin at position 9 based on BLAST alignment

Description	Accession	Description	Accession
Bacillus amyloliquefaciens CC178	CP006845.1	*Bacillus velezensis strain SYBC H47*	CP017747.1
Bacillus amyloliquefaciens subsp. plantarum str. FZB42	CP000560.1	*Bacillus velezensis strain YJ11–1-4*	CP011347.1
Bacillus amyloliquefaciens subsp. plantarum UCMB5113	HG328254.1	*Bacillus velezensis SQR9*	CP006890.1
Bacillus amyloliquefaciens subsp. plantarum UCMB5033	HG328253.1	*Bacillus velezensis strain SCGB 1*	CP023320.1
Bacillus amyloliquefaciens subsp. amyloliquefaciens KHG19	CP007242.1	*Bacillus velezensis strain SCDB 291*	CP022654.2
Bacillus amyloliquefaciens strain B15	CP014783.1	*Bacillus velezensis strain 9D-6*	CP020805.1
Bacillus amyloliquefaciens UMAF6639	CP006058.1	*Bacillus velezensis strain CC09*	CP015443.1
Bacillus amyloliquefaciens subsp. plantarum NAU-B3	HG514499.1	*Bacillus velezensis strain SCGB 574*	CP023431.1
Bacillus amyloliquefaciens strain Q-426 fenEDCBA	JQ271536.1	*Bacillus velezensis strain CMT-6*	CP025341.1
Bacillus amyloliquefaciens Y2	CP003332.1	*Bacillus velezensis strain J01*	CP023133.1
Bacillus amyloliquefaciens CC178	CP006845.1	*Bacillus velezensis strain T20E-257*	CP021976.1
Bacillus amyloliquefaciens subsp. plantarum YAU B9601-Y2	HE774679.1	*Bacillus velezensis strain ZL918*	CP021338.1
Bacillus amyloliquefaciens UMAF6614	CP006960.1	*Bacillus velezensis strain GQJK49*	CP021495.1
Bacillus amyloliquefaciens subsp. plantarum UCMB5036	HF563562.1	*Bacillus velezensis strain JTYP2*	CP020375.1
Bacillus amyloliquefaciens LFB112	CP006952.1	*Bacillus velezensis strain sx01604*	CP018007.1
Bacillus amyloliquefaciens strain WS-8	CP018200.1	*Bacillus velezensis strain 157*	CP022341.1
Bacillus amyloliquefaciens strain Y14	CP017953.1	*Bacillus velezensis strain D2–2*	CP014990.1
Bacillus amyloliquefaciens strain S499	CP014700.1	*Bacillus velezensis strain LS69*	CP015911.1
Bacillus amyloliquefaciens strain LM2303	CP018152.1	*Bacillus velezensis strain S3–1*	CP016371.1
Bacillus amyloliquefaciens IT-45	CP004065.1	*Bacillus velezensis strain CBMB205*	CP014838.1
Bacillus amyloliquefaciens strain L-S60	CP011278.1	*Bacillus velezensis NJN-6*	CP007165.1
Bacillus amyloliquefaciens strain L-H15	CP010556.1	*Bacillus velezensis strain GFP-2*	CP021011.1
Bacillus amyloliquefaciens strain MBE128	CP013727.1	*Bacillus velezensis strain GH1–13*	CP019040.1
Bacillus amyloliquefaciens subsp. plantarum CAU B946	HE617159.1	*Bacillus velezensis strain JS25R*	CP009679.1
Bacillus atrophaeus strain NRS 1221A	CP010778.1	*Bacillus velezensis TrigoCor1448*	CP007244.1
Bacillus atrophaeus subsp. globigii strain BSS	CP007640.1	*Bacillus velezensis strain JJ-D34*	CP011346.1
Bacillus atrophaeus 1942	CP002207.1	*Bacillus velezensis strain 10075*	CP025939.1
Bacillus atrophaeus strain SRCM101359	CP021500.1	*Bacillus vallismortis strain NBIF-001*	CP020893.1
Bacillus atrophaeus strain GQJK17	CP022653.1	*Bacillus velezensis strain NKG-1*	CP024203.1
Bacillus atrophaeus strain BA59	CP024051.1	*Bacillus velezensis strain SRCM100072*	CP021888.1
Bacillus atrophaeus UCMB-5137	CP011802.1	*Bacillus velezensis strain Lzh-a42*	CP025308.1
Bacillus cereus strain MBGJa3 c	CP026523.1	*Bacillus velezensis strain CN026*	CP024897.1
Bacillus methylotrophicus strain CBMB205	CP011937.1	*Bacillus velezensis S141*	AP018402.1
Bacillus methylotrophicus strain B25	LN999829.1	*Bacillus velezensis strain OSY-S3*	CP024706.1
Bacillus sonorensis strain SRCM101395	CP021920.1	*Bacillus velezensis strain M75*	CP016395.1
Bacillus velezensis strain 9912D	CP017775.1	*Bacillus velezensis strain AGVL-005*	CP024922.1
Bacillus velezensis strain LABIM40	CP023748.1	*Bacillus velezensis strain TJ02*	CP024797.1
Bacillus velezensis strain SB1216	CP015417.1	*Bacillus velezensis strain L-1*	CP023859.1
Bacillus velezensis AS43.3	CP003838.1	*Bacillus velezensis strain TB1501*	CP022531.1
Bacillus velezensis strain SRCM101413	CP021890.1	*Bacillus velezensis strain DKU_NT_04*	CP026533.1

faciens FZB42. The sequences of Ep (partial *fenD*) and partial *fenB* from *B. amyloliquefaciens* S499 are shown in Figure S4 in supplementary materials.

Discussion

An analysis of differences in the structure and biological activities revealed the distinction between the two lipodecapeptides fengycin and plipastatin (Jacques, 2011). To date, only a few studies have shown the differences between these two compounds (Nishikiori et al., 1986b; Vanittanakom et al., 1986; Wang et al., 2004). Only two differences were reported in their structure: 1) the presence of glutamine instead of glutamic acid at position 8 (this difference has not been mentioned in any other studies); and 2) the presence of L and D forms of tyrosine at positions 3 and 9 in plipastatin and positions 9 and 3 in fengycin, respectively (Wang et al., 2004). In this study, we aimed to differentiate plipastatin and fengycin via detecting the presence of the epimerization domain at position 3 or 9 using degenerate primers for PCR. The degenerate primers *Ep* (*fenD*) and *fenB* designed for the detection of position of the epimerization domain in the five *Bacillus* strains, namely *B. subtilis* 168 and *B. subtilis* ATCC 21332, *B. cereus*, and *B. amyloliquefaciens* FZB42 and *B. amyloliquefaciens* S499, revealed the presence of the tyrosine epimerization domain at position 9 (*fenD* or *ppsD* gene). These findings indicate that plipastatin is synthesized by all the *Bacillus* strains tested herein.

The alignment of five sequences of the fragments amplified by PCR using degenerate primers from *B. subtilis* ATCC 21332, *B. cereus*, and *B. amyloliquefaciens* S499 on BLAST website (NCBI database) revealed the presence of the epimerization domain at position 9 (*ppsD* gene). These results are in agreement with the results shown by Hussein et al. (2011) for *B. subtilis* 168 and *B. subtilis* ATCC 21332. The authors stated that the organization of the operon from *B. subtilis* ATCC 21332 is similar to that of plipastatin operon from *B. subtilis* 168. Schneider et al. (1999) suggested that understanding the organization of fengycin operon from *B. subtilis* S499 can remove the barriers to differentiating fengycin and plipastatin. They also confirmed the existence of fengycin molecules (with D-tyrosine at position 3) in the tested bacterial strain. When studying the organization of fengycin operon in *B. subtilis* S499, Hussein et al. (2011) confirmed that this strain belongs to *B. amyloliquefaciens* species. The fengycin operon of *B. amyloliquefaciens* S499 was also studied by the authors, but the results did not confirm whether the molecule harbored by this strain was fengycin or plipastatin (Hussein et al., 2011). However, in our study, we confirmed that this strain harbors plipastatin.

The sequence of fengycin operon of *B. amyloliquefaciens* S499 is now available in GenBank, which confirms our investigations about the presence of the epimerization domain at position 9, indicating that the molecule synthesized by this strain is plipastatin and not fengycin (Puopolo et al., 2016).

Conclusions

This work confirms the presence of plipastatin in 144 *Bacillus* strains, most of which were thought to harbor fengycin. The search for the position of tyrosine epimerization domain using degenerate primers revealed that fengycin was never detected in any *Bacillus* s train or mentioned in any database.

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