Short sequence paper

Functional analysis of fengycin synthetase FenD

Tsuey-Pin Lin^a, Chyi-Liang Chen^b, Hui-Chuan Fu^a, Cheng-Yeu Wu^b, Guang-Huey Lin^c,
Shih-Hao Huang^b, Li-Kwan Chang^d, Shih-Tung Liu^{b,*}^aDepartment of Health and Nutrition and Department of Biotechnology, Chia Nan University of Pharmacy and Science, Tainan, 701, Taiwan^bMolecular Genetics Laboratory, Department of Microbiology and Immunology, Chang Gung University, Kwei-Shan, 333, Taiwan^cMicrobial Genetics Laboratory, Institute of Microbiology Immunology and Molecular Medicine, Tzu-Chi University, Hualien, 970, Taiwan^dFaculty of Biological Medicine and Environmental Biology, Kaohsiung Medical University, Kaohsiung, 807, Taiwan

Received 18 September 2004; received in revised form 27 November 2004; accepted 15 February 2005

Available online 2 March 2005

Abstract

Fengycin is a cyclic lipopeptidic antibiotic produced nonribosomally by *Bacillus subtilis*. A fengycin synthetase mutant of *B. subtilis* F29-3 was generated with Tn917lux, which contains a transposon inserted in a 7716-bp gene, *fenD*. The mutation can be genetically complemented by transforming a plasmid carrying a wild-type *fenD*, confirming the participation of the gene in fengycin synthesis. Sequencing and biochemical analysis reveal that this gene encodes an enzyme that includes two amino acid-activating modules, FenD1 and FenD2, which activate L-Tyr and L-Thr, the third and the fourth amino acids in fengycin, respectively.

© 2005 Elsevier B.V. All rights reserved.

Keywords: Fengycin; Peptide synthetase; Nonribosomal peptide synthesis**1. Introduction**

Fengycin is an antifungal antibiotic that comprises a sequence of fatty acids ·L-Glu·D-Orn·L-Tyr·D-allo-Thr·L-Glu·D-Ala/D-Val·L-Pro·L-Glu·D-Tyr·L-Ile with a lactone bond that connects L-Tyr to L-Ile [1]. Our earlier studies characterized three fengycin synthetase genes, *fenC*, *fenE* and *fenB*, and determined the biological functions of the enzymes encoded by these genes [2–4]. As is generally known, peptide synthetases, including fengycin synthetases, generally contain one to several modules, each of which has several domains with functions required for nonribosomal peptide synthesis [5–7]. For instance, a peptide synthetase module typically contains an adenylation domain, which has ATPase activity and can adenylate a specific amino acid [8]. Upstream of the adenylation domain, a peptide synthetase also contains

a condensation domain of approximately 450 amino acids, which is required to form a peptide bond between the two amino acids activated by the two adjacent modules [9]. Meanwhile, a peptide synthetase module contains a thiolation domain of about 100 amino acids downstream of an adenylation domain. After an adenylation domain has adenylated an amino acid, this adenylated amino acid is transferred and covalently linked to the cofactor, 4'-phosphopantetheine, which binds to the thiolation domain, to complete the amino acid activation process [10,11]. In the C-terminal region, peptide synthetases normally contain an epimerization domain, which converts an L-amino acid to a D-amino acid during peptide synthesis [12], explaining why peptides synthesized nonribosomally often include D-amino acids. Finally, the module that terminates the peptide synthesis, rather than having an epimerase domain, contains a thioesterase domain in the C-terminal region to terminate peptide synthesis [13]. This study identifies a fengycin synthetase gene, *fenD*, which encodes a fengycin synthetase that activates the third and the fourth amino acids of fengycin.

* Corresponding author. Tel.: +886 3 2118292.

E-mail address: cgliu@mail.cgu.edu.tw (S.-T. Liu).

2. Materials and methods

2.1. Bacterial strains and plasmid

Escherichia coli BL-SI (Invitrogen) was used as a host to express the proteins that contain the adenylation domains of FenD. Plasmids pDONR201 and pDEST17 (Invitrogen) were used as cloning vectors. *Bacillus subtilis* F29-3 is a wild-type strain that produces fengycin. FX10 is a mutant strain of *B. subtilis* F29-3, which contains a Tn917lux insertion in *fenD*.

2.2. Isolating fengycin synthesis mutants

B. subtilis F29-3 was transformed with pD917lux [7] with a protoplast transformation method [14]. Transformants were selected on SMM-LBV agar that contained 5 µg/ml of chloramphenicol. The transformants were subsequently cultured in LB broth that contained 0.1 µg/ml of erythromycin to induce transposition. Following induction, pD917lux was cured from the cell by overnight incubation at 45 °C according to a method of Youngman et al. [15]. Colonies that were resistant to chloramphenicol but sensitive to erythromycin were selected. Finally, fengycin synthesis mutants, which cannot inhibit the germination of *Paecilomyces variotii* spores, were identified by spotting the mutant cells on an nHA-spore plate [16]. DNA fragments adjacent to the transposon were subcloned and sequenced as described by Youngman et al. [17].

2.3. Complementation and bioassay

A DNA fragment that contains full-length *fenD* was isolated from pFD200 by *NheI* and *XbaI* digestion. The fragment was then ligated with an *NheI* fragment from pD917lux, which contains the pE194 replicon and an erythromycin-resistance gene. The resulting plasmid, pFD210, was transformed into the FX10 mutant of *B. subtilis* F29-3 [7]. The transformants were selected on SMM-LBV agar that contained 5 µg/ml of chloramphenicol and 7 µg/ml of erythromycin. The transformants were then subcultured in LB broth overnight. The culture (5 µl) was spotted on an nHA-spore plate to examine the anti-fungal activity exhibited by the cells [16].

2.4. Cloning FenD adenylation domains

A DNA fragment that encodes the adenylation domain of FenD1 (AD-FenD1) was amplified using primers attB1-fenD1N(A) (5'-AAAAAGCAGGCTGCATCAACATTGTGCCTACT-3') and attB2-fenD1C(A) (5'-AGAAAAGCTGGGTGTGCCAGTTCCGTTTCCATT-3'). A DNA fragment that encodes the adenylation domain of FenD2 (AD-FenD2) was amplified using primers attB1-fenD2N(A) (5'-AAAAAGCAGGCTTAATGCAATTGC-TATCTGCTCCAG) and attB2-fenD2C(A) (5'-

AGAAAAGCTGGGTGTTCTAGGGCTGATATCATT-CTC). A DNA fragment that encodes the condensation, adenylation and thiolation domains of FenD1 (CATD-FenD1), which includes a sequence from amino acids 1 to 1036 in FenD, was amplified with primers attB1-fenDN (5'-AAAAAGCAGGCTATGACGCAAGCAACAGATCC) and attB2-fenD1C (5'-AGAAAAGCTGGGTTCACAGCGGATAAACATCTCTAAA). PCR was first performed by heating the reaction mixture at 95 °C for 2 min, followed by ten cycles of 94 °C for 30 s, 50 °C for 30 s and 68 °C for 3 min, using pFC6A5 [2] as a template. Two adaptor primers, attB1 (5'-GGGGACAAGTTTGTACAAAAAAGCAGGCT) and attB2 (5'-GGGGACCACTTTGTACAA-GAAAGCTGGGT), were subsequently added to the reaction mixture, which was incubated at 95 °C for 1 min, before undergoing five cycles of PCR at 94 °C for 15 s, 45 °C for 30 s, 68 °C for 3 min and 20 cycles at 94 °C for 15 s, 55 °C for 30 s and 68 °C for 3 min. Finally, the PCR products were inserted into pDONR201 and pDEST17 using a λ insertion kit (Invitrogen).

2.5. Purifying CAT-FenD1 and AD-FenD2

Bacteria (1 l) were cultured to the mid-log phase in an NaCl-free LB broth. The expression of recombinant proteins was induced by adding NaCl to the culture medium to a final concentration of 300 mM. Cells were harvested 3 h after induction. The pellet was suspended in 50 ml of a binding buffer that contained 10 mM imidazole, 300 mM NaCl, and 50 mM NaH₂PO₄ at pH 8.0. Cell lysate was prepared by sonication according to a method described elsewhere [2]. His-tagged recombinant proteins were finally purified with an Ni-NTA column (Qiagen) [2].

2.6. ATP-PPi exchange assay

The amino acid activated by CAT-FenD1 and AD-FenD2 was determined by an ATP-PPi exchange assay as described elsewhere [18]. Each reaction mixture contained 1 µCi of [³²P]tetrasodium pyrophosphate (3.03 Ci/mmol NEN, Boston, MA), 2 mM amino acid, 20 mM Hepes–MES at pH 6.5, with 33 µg of CAT-FenD1 or 100 µg of AD-FenD2.

3. Results and discussion

3.1. Inserting Tn917lux into *fenD*

B. subtilis F29-3 was mutagenized using Tn917lux. Colonies that contained a Tn917lux insertion were spotted on nHA-spore plates to isolate the mutants that were defective in fengycin synthesis. Fengycin is the only antibiotic produced by *B. subtilis* F29-3 that inhibits the growth of filamentous fungi [16], therefore, mutants that cannot inhibit the growth of *P. variotii* likely have a Tn917lux insertion into a gene that is involved in fengycin

Fig. 1. Production of fengycins by *B. subtilis* F29-3 and its mutant FX10. *B. subtilis* F29-3 (A), the FX10 mutant (B), and the mutant strain that contains pFD200 (C) were spotted on a nHA-spore plate to assay their capacity to generate fengycins. The zone exhibited by FX10 (B) was attributable to competitive growth between bacteria and fungi.

synthesis. Among about 3000 colonies examined, 72 could not synthesize fengycins and were unable to inhibit the growth of *P. variotii* on bioassay plates. The insertion of a transposon into these mutants was subsequently analyzed by

subcloning and sequencing. Of these mutants, FX10 contained a Tn917lux inserted into *fenD*. This gene was positioned 29 nucleotides downstream of a previously characterized fengycin synthetase gene, *fenC* [2]. Sequencing analysis demonstrated that Tn917lux was inserted 3210 nucleotides downstream of the translation initiation codon of *fenD*. On a bioassay plate, wild-type *B. subtilis* F29-3 produced an inhibition zone surrounded by a white lawn of fungal hyphae (Fig. 1A). However, the FX10 mutant did not establish a similar inhibition zone (Fig. 1B). Although FX10 seemed to yield a smaller zone, this zone actually contained an inner circle in which the bacterial culture was originally applied and an outer ring where agar was covered by bacteria. Such type of inhibition is attributable to competitive growth between bacteria and fungi, rather than from the suppression of fungal growth by an antibiotic produced

Table 1
Comparison of conserved sequences in peptide synthetases and FenD

Domain	Core	Sequence conserved in peptide synthetases ^a	Sequence in FenD	Module in <i>fenD</i>	Positions ^b
Condensation	C1	SxAQxR(L/M)(W/Y)xL	SYMQEGMIFH	D1	14–23
			SFSQKRLYL	D2	1059–1068
	C2	RHExLRTxF	RYDIFRTIF	D1	60–68
			RHESLRTAF	D2	1105–1113
	C3	MHHxISDG(W/V)S	HHHIIMDG	D1	145–154
			MHHIADGIS	D2	1180–1189
	C4	YxD(F/Y)AVW	YSRYIEW	D1	183–189
Adenylation			YKDFAVW	D2	1213–1219
	C5	(I/V)GxFVNT(Q/L)(C/A)xR	VGLFINTVPVR	D1	296–306
			IGMFVNTLAMR	D2	1328–1338
	C6	(H/N)QD(Y/V)PFE	HQDYPFE	D2	1364–1370
	C7	RDxSRNPL	EVNRNPL	D2	1381–1387
	A1	L(T/S)YxEL	RTYRQL	D1	495–500
			LTYRDL	D2	1526–1531
	A2	LKAGxAYL(V/L)P(L/I)D	WKAGGAYLPLD	D1	540–550
			LKAGGAYLPID	D2	1570–1581
	A3	LAYxxYTSG(S/T)TGxPKG	LAYLIYTS GTTGRPKG	D1	609–624
			PAYVIYTS GTTGQPKG	D2	1642–1657
	A4	FDxS	FDGC	D1	659–662
			FDFG	D2	1692–1695
	A5	NxYGPTE	NEYGPTE	D1	753–759
			NMYGITE	D2	1787–1789
	A6	GELxIxGxG(V/L)ARGYL	GELCISGAGLARGYY	D1	802–816
			GELYIGGEGVARGYL	D2	1838–1853
	A7	Y(R/K)TGDL	YRTGDA	D1	838–843
			YRTGDL	D2	1875–1880
	A8	GRxDxQVKIRGxRIELGEIE	GRFDDQVKIRGYRIELSEIE	D1	856–875
			GRIDDQVKVRGHRIELGEIQ	D2	1893–1912
Thiolation	A9	LPxYM(I/V)P	LPSYMIP	D1	924–930
			LPDFMVP	D2	1964–1970
Epimerization	A10	NGK(V/L)DR	SGKLDL	D1	944–949
			NGKLDQ	D2	1984–1989
	T	DxFFxxLGG(H/D)S(L/I)	DDFFQLGGQSL	D1	991–1001
			ANFFELGGDSI	D2	2032–2042
	E1	PIQxWF	PVQQWF	D2	2095–2101
	E2	HHxISDG(W/V)S	HHLVIDAVS	D2	2215–2223
	E3	DxLLxAxG	DILLTALG	D2	2349–2356
	E4	EGHGRE	EGHGRE	D2	2373–2378
	E5	RTVGWFTxxYP(Y/V)PFE	RTVGWFTSIYPVW	D2	2389–2401
	E6	PxxGxGYG	PHKGAGYG	D2	2430–2437
	E7	FNYLG(Q/R)	FNYLGQ	D2	2457–2462

^a From reference [9].

^b Amino acid positions in FenD.

by bacteria since similar zones were also observed when an *E. coli* culture was applied to the plate. More importantly, the capacity to produce fengycin was restored when the mutant was transformed with pFD210 (Fig. 1C), indicating that the mutation can be genetically complemented and *fenD* participates in fengycin synthesis.

3.2. Sequence of *FenD*

In mutant FX10, Tn917*lux* was inserted in *fenD*, which is 7716 nucleotides long (GenBank accession number: AJ011849) and preceded by a Shine-Dalgarno sequence, 5'-TGGA, five nucleotides upstream of the initiation codon of the gene. Sequence analysis predicts that FenD has a molecular mass of 287 kDa and contains two sets of motifs that are commonly found in the condensation, adenylation, and thiolation domains in peptide synthetases (Table 1) [9], indicating that FenD contains two amino acid activation modules — FenD1 and FenD2. Therefore, FenD probably activates two amino acids during fengycin synthesis. Meanwhile, FenD1 includes a condensation domain between amino acids 1 and 444, an adenylation domain between amino acids 445 and 957, and a thiolation domain between amino acids 976 and 1036. These domains in FenD2 are between amino acids 1037 and 1474, 1475 and 2016, and 2017 and 2076, respectively. Additionally, FenD has a C-terminal region that is 496 amino acids long, which includes seven conserved motifs that are typically present in an epimerase domain (Table 1) [9], indicating that this region contains an epimerase domain and the amino acid activated by FenD2 is epimerized during fengycin synthesis. Furthermore, among the seven conserved motifs identified in the condensation domain in FenD2, C6 and C7 are absent from the corresponding domain in FenD1 (Table 1). These two motifs were also found to be absent from the first module of a peptide synthetase in other peptide synthetases, including those involved in fengycin, surfactin and tyrocidine synthesis [2,4,19,20]. The functions of these two motifs are unknown.

3.3. Expressing and purifying the proteins that contain the amino acid adenylation domains of *FenD*

A DNA fragment that encodes the region between amino acids 444 and 975 of FenD was amplified by PCR and cloned into pDEST17 to express a recombinant protein that contains the adenylation domain of FenD1 (AD-FenD1) in *E. coli* BL-SI. Following NaCl treatment to induce the expression of the recombinant protein, a cell lysate was prepared from the cells. SDS-polyacrylamide gel electrophoresis revealed that AD-FenD1 was present in the pellet fraction, indicating that the protein was insoluble and could not be used in enzymatic analysis. Meanwhile, a recombinant protein that contains the condensation, adenylation, and thiolation domains of FenD1 (CATD-FenD1) was expressed in *E. coli* BL-SI (Fig. 2, lanes 1 and 2) and was present in

Fig. 2. Expressing and purifying recombinant FenD adenylation domains. Cell lysates were prepared from *E. coli* BL-SI expressing CATD-FenD1 (lanes 1 to 5) and AD-FenD2 (lanes 6 to 10). Cells were lysed before (lanes 1 and 6) and after (lanes 2 and 7) NaCl induction. After induction, cells were homogenized by sonication and lysates were centrifuged to separate the supernatant (lanes 4 and 9) and the pellet (lanes 3 and 8) fractions. Recombinant protein in the soluble fraction was finally purified using an Ni-NTA column (lanes 5 and 10).

both the pellet and the supernatant fraction of the cell lysate (Fig. 2, lanes 3 and 4). Furthermore, the adenylation domain of FenD2 (AD-FenD2), between amino acids 1569 and 2108 in FenD, was also present in the pellet and the supernatant fractions of the cell lysate (Fig. 2, lanes 8 and 9). Hence, CATD-FenD1 and AD-FenD2 were purified from the cell lysate with an Ni-NTA column (Fig. 2, lanes 5 and 10).

3.4. Amino acids activated by *FenD*

The amino acids activated by the adenylation domains of FenD were first analyzed using the eight amino acids in the fengycin molecule as the substrate. ATP-PPi exchange assay demonstrated that adding L-Tyr to a reaction mixture that contained 33 µg of CATD-FenD1 yielded the highest exchange activity, indicating that FenD1 activates L-Tyr, the third amino acid in the fengycin molecule (Fig. 3A). Meanwhile, adding the 13 common amino acids that were absent from the fengycin molecule yielded only background counts (data not shown), indicating that L-Tyr is the only amino acid to be activated by AD-FenD1. According to Marahiel et al. [21], a peptide synthetase can use D-amino acids as a substrate *in vitro* if the condensation domain of the enzyme remains intact. However, in the case of CATD-FenD1, this enzyme exhibited an exchange activity of about tenfold above the background when D-Tyr was used as the substrate, but the activity was substantially lower than that exhibited by the enzyme when L-Tyr was used as the substrate (Fig. 3A), suggesting that FenD1 does not

Fig. 3. ATP-Pi exchange activities of CATD-FenD1 (A) and AD-FenD2 (B).

efficiently use D-Tyr as a substrate. In the case of AD-FenD2, adding L-Thr to a reaction mixture that contained 100 μg of AD-FenD2 yielded the highest exchange activity (Fig. 3B). Neither the other seven amino acids in the fengycin molecule (Fig. 3B) nor the other 13 common amino acids yielded a high level of exchange activity, indicating that AD-FenD2 activates only L-Thr, which is then converted into D-allo-Thr, the fourth amino acid in the fengycin molecule. Indeed, AD-FenD2 does not use D-Thr as a substrate [21,22]. Moreover, D-allo-Thr can only be generated following epimerization.

3.5. Biochemical characterization of CATD-FenD1 and AD-FenD2

The enzymatic activity of CATD-FenD1 and AD-FenD2 was investigated under various reaction conditions. These two recombinant proteins have an optimal pH between 6.5 and 8.0, which resembles the optimal pH range of FenE1 and FenE2 [4] but is much higher than those of FenB, FenC1, and FenC2, which have an optimal pH of 4.5–5.0 [2,3]. This may indicate that fengycin synthetases function in various pH environments in the cells. Meanwhile, CATD-FenC1 and AD-FenD2 also have an optimal activity at 25 $^{\circ}\text{C}$, which is consistent with the optimal temperature for the adenylate activation reactions associated with the activation

domains of FenC and FenB [2,3]. At optimal pH and temperature, CATD-FenD1 has a K_M value of 0.051 mM and a k_{cat} value of 26 s^{-1} for L-Tyr; AD-FenD2 has a K_M value of 0.058 mM and a k_{cat} value of 2.65 s^{-1} for L-Thr.

Five peptide synthetases participate in fengycin synthesis [2–4,6]. Experimental data currently available indicate that the amino acid sequence in fengycin is colinear with the genes in the fengycin synthetase operon [2–4]. As is generally known, *B. subtilis* 168 contains a *pps* operon, which is involved in plipastatin synthesis. This operon comprises five genes that encode enzymes with a sequence that is about 72–80% identical to the sequence of fengycin synthetases [2]. Steller et al. [23] observed that *B. subtilis* b213 produces a fengycin-like antibiotic, which is probably plipastatin, because the eighth amino acid of the antibiotic is glutamine. They purified three peptide synthetases, which have a molecular mass of 286 kDa, as a mixture. Biochemical analysis indicated that these enzymes can activate the six N-terminal amino acids of plipastatin. These findings are consistent with the results concerning the molecular mass and biochemical functions of FenD. Later, Tsuge et al. [24] renamed the *pps* operon *pli* to reflect the fact that the operon produces plipastatin rather than fengycin. They also determined that the expression of the *pli* operon depends on *degQ*. These findings demonstrate that *fen* and *pli* operons are evolutionarily conserved by *Bacillus* spp.; the production of either antibiotic may help the organisms to survive in nature. The results herein constitute a valuable reference for future investigations that involve nonribosomal peptide synthesis and provide further insight into the synthesis of fengycin by *B. subtilis* F29-3.

Acknowledgements

This work was supported by grants from the Chang-gung Memorial Hospital (CMRP1211) and from the National Science Council of the Republic of China (NSC92-2320-B-182-044 and NSC90-2320-B-041-014).

References

- [1] U. Koch, Fengycin: Strukturaufklärung eines mikroheterogenen Lipopeptidantibiotikums. Dissertation, zur Erlangung des Grades eines Doktors, der Naturwissenschaften. Eberhard-Karls-Universität zu Tübingen, Tübingen, Germany, 1988.
- [2] T.P. Lin, C.L. Chen, L.K. Chang, J.S. Tschen, S.T. Liu, Functional and transcriptional analyses of a fengycin synthetase gene, *fenC*, from *Bacillus subtilis*, *J. Bacteriol.* 181 (1999) 5060–5067.
- [3] G.H. Lin, C.L. Chen, J.S. Tschen, S.S. Tsay, Y.S. Chang, S.T. Liu, Molecular cloning and characterization of fengycin synthetase gene *fenB* from *Bacillus subtilis*, *J. Bacteriol.* 180 (1998) 1338–1341.
- [4] H.Y. Shu, G.H. Lin, Y.C. Wu, J.S. Tschen, S.T. Liu, Amino acids activated by fengycin synthetase FenE, *Biochem. Biophys. Res. Commun.* 292 (2002) 789–793.
- [5] H. Kleinkauf, H. Von Döhren, A nonribosomal system of peptide biosynthesis, *Eur. J. Biochem.* 236 (1996) 335–351.
- [6] C.L. Chen, L.K. Chang, Y.S. Chang, S.T. Liu, J.S. Tschen, Trans-

- poson mutagenesis and cloning of the genes encoding the enzymes of fengycin biosynthesis in *Bacillus subtilis*, Mol. Gen. Genet. 248 (1995) 121–125.
- [7] L.K. Chang, C.L. Chen, Y.S. Chang, J.S. Tschen, Y.M. Chen, S.T. Liu, Construction of Tn917*acI*, a transposon useful for mutagenesis and cloning of *Bacillus subtilis* genes, Gene 150 (1994) 129–134.
 - [8] T. Stachelhaus, M.A. Marahiel, Modular structure of peptide synthetases revealed by dissection of the multifunctional enzyme GrsA, J. Biol. Chem. 270 (1995) 6163–6169.
 - [9] M.A. Marahiel, T. Stachelhaus, H.D. Mootz, Modular peptide synthetases involved in nonribosomal peptide synthesis, Chem. Rev. 97 (1997) 2651–2674.
 - [10] T. Stein, J. Vater, V. Kruff, A. Otto, B. Wittmann-Liebold, P. Franke, M. Panico, R. McDowell, H.R. Morris, The multiple carrier model of nonribosomal peptide biosynthesis at modular multienzymatic templates, J. Biol. Chem. 271 (1996) 15428–15435.
 - [11] M.R. Mofid, R. Finking, L.O. Essen, M.A. Marahiel, Structure-based mutational analysis of the 4'-phosphopantetheinyl transferases Sfp from *Bacillus subtilis*: carrier protein recognition and reaction mechanism, Biochemistry 43 (2004) 4128–4136.
 - [12] V. De Crécy-Lagard, P. Marliere, W. Saurin, Multienzymatic non ribosomal peptide biosynthesis: identification of the functional domains catalysing peptide elongation and epimerisation, C. R. Acad. Sci., Ser. III 318 (1995) 927–936.
 - [13] A. Schneider, M.A. Marahiel, Genetic evidence for a role of thioesterase domains, integrated in or associated with peptide synthetases, in non-ribosomal peptide biosynthesis in *Bacillus subtilis*, Arch. Microbiol. 169 (1998) 404–410.
 - [14] T. Imanaka, M. Fujii, I. Aramori, S. Aiba, Transformation of *Bacillus stearothermophilus* with plasmid DNA and characterization of shuttle vector plasmids between *Bacillus stearothermophilus* and *Bacillus subtilis*, J. Bacteriol. 149 (1982) 824–830.
 - [15] P.J. Youngman, J.B. Perkins, R. Losick, Genetic transposition and insertional mutagenesis in *Bacillus subtilis* with *Streptococcus faecalis* transposon Tn917, Proc. Natl. Acad. Sci. U. S. A. 80 (1983) 2305–2309.
 - [16] W. Loeffler, J.S.M. Tschen, N. Vanittanakom, M. Kugler, E. Knorr, T.G. Wu, Antifungal effects of bacilysin and fengycin from *Bacillus subtilis* F29-3. A comparison with activities of other *Bacillus* antibiotics, J. Phytopathol. 115 (1986) 204–213.
 - [17] P. Youngman, J.B. Perkins, R. Losick, Construction of a cloning site near one end of Tn917 into which foreign DNA may be inserted without affecting transposition in *Bacillus subtilis* or expression of the transposon-borne erm gene, Plasmid 12 (1984) 1–9.
 - [18] S.G. Lee, F. Lipmann, Tyrocidine synthetase system, Methods Enzymol. 43 (1975) 585–602.
 - [19] P. Cosmina, F. Rodríguez, F. de Ferra, G. Grandi, M. Perego, G. Venema, D. van Sinderen, Sequence and analysis of the genetic locus responsible for surfactin synthesis in *Bacillus subtilis*, Mol. Microbiol. 8 (1993) 821–831.
 - [20] H.D. Mootz, M.A. Marahiel, The tyrocidine biosynthesis operon of *Bacillus brevis*: complete nucleotide sequence and biochemical characterization of functional internal adenylation domains, J. Bacteriol. 179 (1997) 6843–6850.
 - [21] U. Linne, M.A. Marahiel, Control of directionality in nonribosomal peptide synthesis: role of the condensation domain in preventing misinitiation and timing of epimerization, Biochemistry 39 (2000) 10439–10447.
 - [22] S. Doekel, M.A. Marahiel, Dipeptide formation on engineered hybrid peptide synthetases, Chem. Biol. 7 (2000) 373–384.
 - [23] S. Steller, D. Vollenbroich, F. Leenders, T. Stein, B. Conrad, J. Hofemeister, P. Jacques, P. Thonart, J. Vater, Structural and functional organization of the fengycin synthetase multienzyme system from *Bacillus subtilis* b213 and A1/3, Chem. Biol. 6 (1999) 31–41.
 - [24] K. Tsuge, T. Ano, M. Hirai, Y. Nakamura, M. Shoda, The genes *degQ*, *pps*, and *lpa-8* (*sfp*) are responsible for conversion of *Bacillus subtilis* 168 to plipastatin production, Antimicrob. Agents Chemother. 43 (1999) 2183–2192.