Structural and functional organization of the fengycin synthetase multienzyme system from *Bacillus subtilis* b213 and A1/3

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Background: *Bacillus subtilis* strains produce a broad spectrum of lipopeptides that are potent biosurfactants and have specific antimicrobial and antiviral activities. The cyclic lipodecapeptide fengycin is one such compound. Although the fengycin biosynthetic genes in *B. subtilis* 168 (*pps* genes) and F29-3 (*fen* genes) have been well characterized, only limited information is available about the biochemical features of the fengycin synthetase multienzyme system.

Results: Five multifunctional peptide synthetases (Fen1–5) that catalyze biosynthesis of the peptide portion of fengycin have been purified from crude extracts of the *B. subtilis* b213 and A1/3 strains. These enzymes activate all fengycin amino-acid components as aminoacyl adenylates or aminoacyl thioesters. Fen1, Fen2 and Fen3 are each ~286 kDa, Fen4 is ~400 kDa and Fen 5 is ~140kDa; each enzyme activates a different set of L-amino acids. A five-gene cluster (*fen*1–5) was detected in the *B. subtilis* A1/3 genome that shows high homology to the *pps* and *fen* genes in *B. subtilis* strains 168 and F29-3. Disruption of *fen4* resulted in a loss of fengycin production. The fengycin synthetase enzymes isolated from *B. subtilis* b213 were assigned to the corresponding A1/3 *fen* genes by their amino-terminal sequences.

Conclusions: The structural and functional organization of the fengycin synthetase system from *B. subtilis* b213 has been characterized in detail and correlated with the corresponding *pps* and *fen* genes in *B. subtilis* strains 168, A1/3 and F29-3. Biosynthesis of the peptide part of fengycin involves five multifunctional modular proteins that assemble the lipopeptide chain using a nonribosomal, multiple carrier thiotemplate mechanism.

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Key words: antifungal antibiotic, *Bacillus subtilis*, fengycin, lipopeptide synthetase, nonribosomal peptide biosynthesis

Received: **17 September 1998**
Revisions requested: **8 October 1998**
Revisions received: **22 October 1998**
Accepted: **23 October 1998**

Published: **21 December 1998**

Chemistry & Biology January 1999, **6**:31–41
http://biomednet.com/elecref/1074552100600031

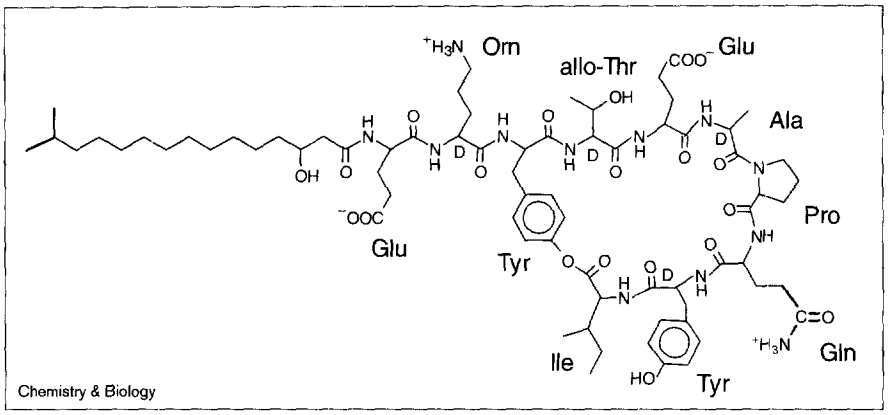
Introduction

Bacillus subtilis strains are a rich source of bioactive peptides with a high potential for biotechnological and pharmaceutical applications. The lipopeptides (surfactin [1–7], fengycin [8] and the members of the iturin family — iturin, mycosubtilin and bacillomycin) are a prominent class of such compounds [9–12]. The plipastatins, which are very similar to fengycin, have been isolated and characterized from *B. cereus* by Umezawa's group [13–17]. All of these agents represent amphiphilic, membrane active biosurfactants and peptide antibiotics with specific antimicrobial activities. Fengycin (Figure 1) is a cyclic lipodecapeptide containing a β-hydroxy fatty acid with a sidechain length of 16–19 carbon atoms. Four D-amino acids and ornithine (a nonproteinogenic residue) have been found in the peptide portion of fengycin. It is specifically active against filamentous fungi [8,18] and inhibits the enzymes phospholipase A2 [13–15] and aromatase [19]. Like the other lipopeptides produced by *B. subtilis*, fengycin appears as a mixture of isoforms that show variations in both the length and branching of the β-hydroxy fatty acid moiety, as well as in the amino-acid composition of the peptide ring [8,14,15]. For example, at position 6 D-alanine (as in fengycin A) can be replaced by D-valine (as in fengycin B).

In the framework of the European *B. subtilis* genome sequencing project, a second large operon structure (*pps*) was detected in the chromosome of *B. subtilis* 168 that encodes a peptide-forming multienzyme system [20,21] in addition to the surfactin biosynthetic operon (*srf*, [22,23]). The *pps* operon is 37.8 kilobases (kb), and contains five open reading frames (ORFs) that comprise ten amino-acid activating modules in total. Because of its organizational features, *pps* was assigned to fengycin biosynthesis, even though *B. subtilis* 168 does not produce this lipopeptide. Chen *et al.* [24] detected a gene cluster (the *fen* genes) in the genome of the fengycin producer *B. subtilis* F29-3 that showed high homology and a similar organization to the corresponding *pps* genes in *B. subtilis* 168. Disruption of one of the *fen* genes prevented fengycin production, providing evidence that these genes are involved in this process [25].

Analysis of the *pps* and *fen* genes revealed a modular structure characteristic of multifunctional peptide synthetases

Figure 1



The structure of fengycin.

that use the multiple carrier thiotemplate mechanism [26,27]. Such multienzymes activate their substrate amino acids using a two-step mechanism, first as aminoacyl adenylates (an ATP-dependent reaction) and then as aminoacyl thioesters. Multiple 4′-phosphopantetheine cofactors, one at each reaction center, function as the thiotemplate sites and manage assembly of the growing peptide chain in a series of transpeptidation reactions. Such multienzymes consist of 1000–1500 amino-acid residue modules that can be divided into three main sections responsible for substrate recognition and binding, as well as aminoacyl adenylate formation; thiolation of the amino-acid substrates; and peptide elongation (in some cases, the third section is also responsible for epimerization). Each module is distinguished by a linear array of highly conserved consensus motifs, which form the cores of multifunctional domains involved in the biosynthetic process. These reactive structural elements are arranged in series along the multifunctional polypeptide chains like assembly points on a production line. Peptide synthetases, therefore, can be regarded as cellular factories.

In contrast to the structure of the fengycin biosynthetic genes, not much is known about the biochemical features of fengycin synthetase at the protein level. In this paper, therefore, we investigated in detail the structural and functional organization of this multienzyme system from the fengycin-producing strains *B. subtilis* b213 and A1/3.

Results

Detection of fengycin and surfactin production of *B. subtilis* b213 and A1/3

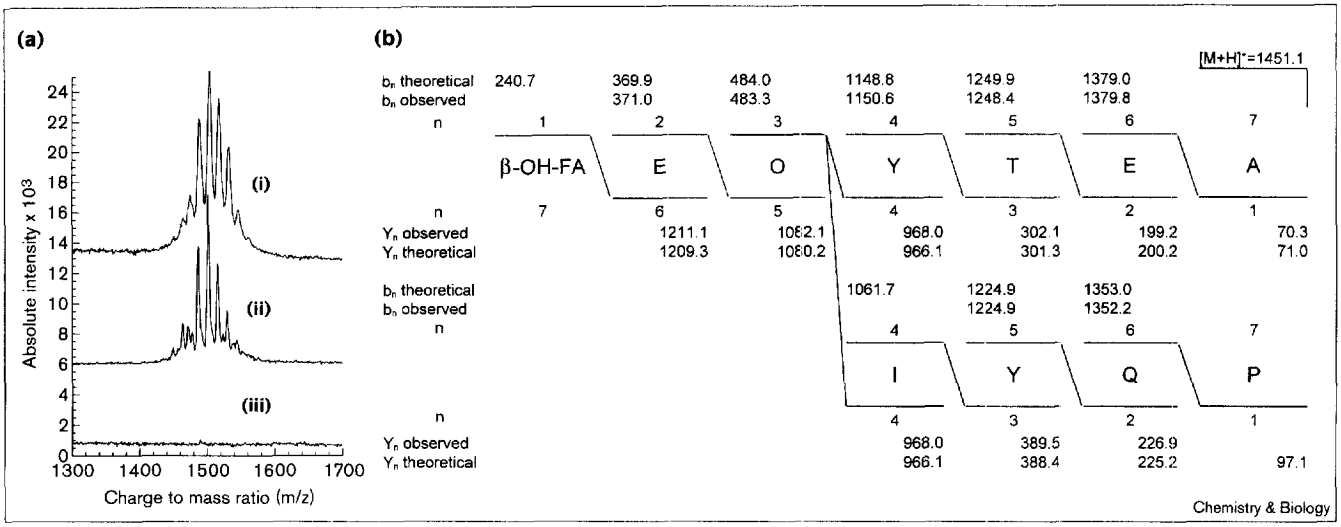
Lipopeptide production in *B. subtilis* strains b213 and A1/3 was investigated using chromatographic procedures and matrix-assisted laser desorption/ionization mass spectroscopy (MALDI–MS). Acid precipitates of the culture supernatants were extracted with methanol and detected by thin layer chromatography (TLC) on silica gel DC 60 plates. Seven prominent spots were visible, by charring with sulfuric acid, in the extracts of both strains. Spots 1–4 had R_f values of 0.07, 0.09, 0.11 and 0.14 and were stained by ninhydrin, whereas spots 5–7 (R_f values of 0.59, 0.75 and 0.8) were ninhydrin negative.

In Figure 2a MALDI–time-of-flight (TOF) mass spectra of the lipopeptide fraction corresponding to spots 1–4 of *B. subtilis* b213 (spectrum i) and A1/3 (spectrum ii) are shown. The mass spectrum of the mutant strain A1/3: SD85 (in which ORF4 of the *pps*-like operon has been disrupted) is also shown (spectrum iii). The mass spectra of spots 1–4 from the two wild-type strains are very similar. In the linear mode we observed a clusters of peaks with mass/charge (m/z) ratios between 1449.6 and 1557.9, which could be attributed to protonated fengycin isoforms and their alkali metal adducts. For example, the peak with a m/z of 1451.1 corresponds to the mass of the $[M+H]^+$ ion of fengycin with a fatty acid chain length of 16 carbon atoms. Structural characterization of this isoform was performed by monitoring and analysing the fragment ions obtained from MALDI–post source decay (PSD) mass spectra (Figure 2b).

Isolation and characterization of fengycin synthetase from a cell free system of *B. subtilis* b213

We studied the structural and functional organization of the fengycin synthetase from *B. subtilis* b213. In order to characterize this multienzyme system, cell-free extracts were prepared by first incubating the cells with lysozyme, and then fractionating the extracts using size-exclusion chromatography on Ultrogel AcA 34. The different fractions were screened for thioester-formation reactions with all fengycin amino-acid components. Three high molecular enzyme fractions were obtained (Figure 3). Fraction I appeared near the exclusion limit of the column, and showed thiolation of L-proline, L-glutamine, and L-tyrosine residues. Fractions II and III eluted at positions that corresponded to lower mass numbers. Fraction II activated L-tyrosine, ornithine, L-glutamate, L-alanine, L-threonine and L-glutamine. Fraction

Figure 2



MALDI–TOF–MS analysis of surfactin and fengycin production by various *B. subtilis* strains. **(a)** MALDI mass spectra of fengycin in the lipopeptide fraction of culture supernatants from (i) *B. subtilis* b213, (ii) *B. subtilis* A1/3 and (iii) the mutant A1/3-sd85, bearing a gene disruption in ORF4 of the *pps*-like operon of *B. subtilis* A1/3. **(b)** Structure of fengycin as determined using MALDI–PSD analysis of the peak at m/z = 1451.1 in the mass spectrum of the *B. subtilis* b213 culture supernatant. After a proline-directed fragmentation, series of amino-terminal (b_n) and carboxy-terminal (Y_n) sequence ions were obtained. In order to confirm the structure of fengycin, we compared the observed m/z values with the theoretical values for the b_n and Y_n sequence ions of a fengycin isoform containing a β-hydroxy fatty acid (β-OH-FA) with 16 carbon atoms. The limit of error of the PSD MS measurements was ± 0.2%. Single-letter amino-acid code is used.

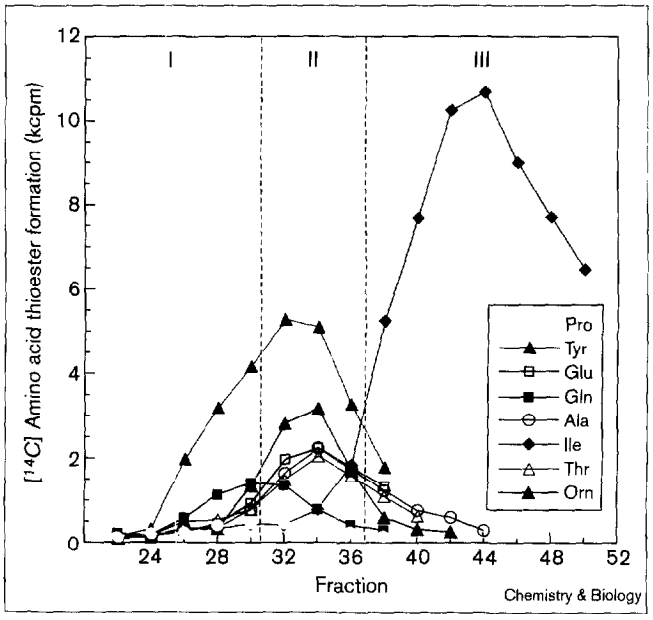
III contained an L-isoleucine-activating enzyme that eluted at a position that corresponded to a molecular mass of ~120–160 kDa.

Multienzyme fraction I, obtained by AcA 34 gel filtration, was purified successively using fast protein liquid chromatography (FPLC) MonoQ and SmartQ. The anion-exchange chromatography experiment using the high resolution Pharmacia Smart System is shown in Figure 4. Thioester-formation reactions with the substrate amino acids L-tyrosine, L-glutamine and L-proline were measured in the gradient fractions, showing their maximum at the same position. The specific activities of thioester binding of L-tyrosine and L-glutamine were higher than that of L-proline. Possibly, the proline-dependent activity is more impaired by sodium chloride used as gradient material in ion-exchange chromatography. In these fractions no thioester-binding activity was found with L-glutamate. A protein band with a molecular mass of ~400 kDa is apparent from the SDS–PAGE (Figure 4). Its mobility is comparable with surfactin synthetase 2 (SrfA, 401 kDa), a multienzyme comprised of three amino-acid activating modules.

The enzymes responsible for the amino-acid activation reactions shown by fraction II were additionally purified using high-resolution anion-exchange Pharmacia FPLC on ResourceQ. Three multifunctional protein components of fengycin synthetase were highly purified and finally separated using chromatography on FPLC MonoQ. The enzymes were localized in the elution pattern of the MonoQ column by their thioester-formation reactions with L-alanine, L-tyrosine, L-glutamate, L-threonine, L-valine and L-ornithine (Figure 5). The protein that eluted at the lowest ionic strength shows thioester formation with L-tyrosine and L-threonine, followed by the two other fengycin synthetase components at slightly higher NaCl concentrations. The protein that eluted at the medium position thiolates L-valine, L-alanine and L-glutamic acid, whereas the enzyme that appeared at the higher ionic strength forms thioesters with L-glutamic acid and L-ornithine. All these synthetases have nearly the same mobility in the SDS–polyacrylamide gel, indicating that the molecular mass of each of these enzymes is ~285 kDa (Figure 5).

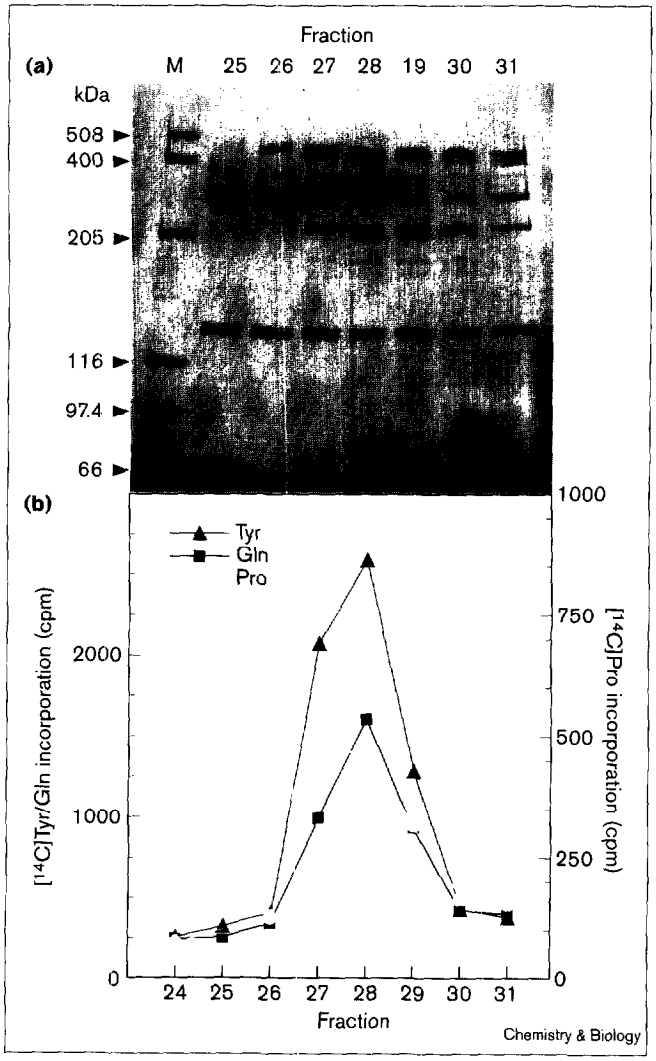
Fraction III, which contains an isoleucine-activating enzyme, was further purified using anion-exchange FPLC on ResourceQ, then size-exclusion chromatography using Pharmacia Sephacryl-200, and finally high-resolution anion-exchange FPLC on Pharmacia MonoQ (Figure 6). This protein eluted from the MonoQ column at lower salt concentrations than all other components of the fengycin multienzyme system. It activates L-isoleucine as a thioester, but none of the other fengycin amino-acid components. It was labeled by thiolation with L-[^{14}C]-Ile. The molecular mass of the labeled enzyme was determined using SDS–PAGE (~140 kDa; Figure 6, lane B). No L-[^{14}C]-Ile incorporation was detected in the absence of ATP.

Figure 3



Gel filtration of a crude cell-free extract of *B. subtilis* b213 on Ultrogel AcA 34. Three enzyme fractions (I–III) of fengycin synthetase were obtained from thioester-binding studies using all the substrate amino acids. The elution buffer was 50 mM Tris–HCl, pH 7.5, 5 mM DTE, 3 mM EDTA containing 10% sucrose. The flow rate was 100 ml/h.

Figure 4



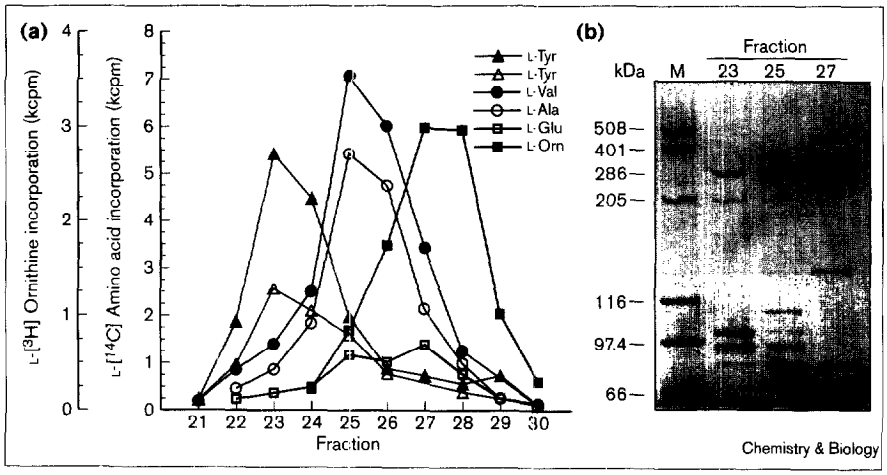
High-resolution anion-exchange FPLC of the fengycin synthetase component in fraction I on Pharmacia SmartQ. This protein, which was purified by AcA 34 gel filtration and anion-exchange FPLC on Pharmacia MonoQ, was rechromatographed on Pharmacia SmartQ. **(a)** Coomassie-stained 5% SDS polyacrylamide slab gel showing the protein composition of fractions tested for substrate amino-acid incorporation. Marker proteins: gramicidin S synthetase 2 (GS2, 508 kDa), SrfB-protein (401 kDa), myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase b (97.4 kDa) and bovine albumin (66 kDa). **(b)** The fractions were tested for covalent incorporation of L-[^{14}C]Tyr, L-[^{14}C]Gln, and L-[^{14}C]Pro as outlined in the Materials and methods section.

Detection and reconstruction of the *fen* gene cluster in *B. subtilis* A1/3

In order to correlate the protein equipment of the fengycin synthetase multienzyme system from *B. subtilis* b213 with the corresponding *fen* (*pps*) genes we used *B. subtilis* A1/3 rather than the well known strain 168, which does not produce fengycin. *B. subtilis* b213 and A1/3 have very similar lipopeptide spectra. Both strains efficiently produce fengycin as demonstrated above, but in contrast to *B. subtilis* b213, the A1/3 strain is transformable and so more amenable to genetic manipulation. We screened the A1/3 genome for the fengycin biosynthetic genes using a polymerase chain reaction (PCR)-based cloning strategy previously used to detect peptide synthetase genes; degenerate primers representing the amino-acid motif 'TGD' (forward primer; using single letter amino-acid code) and 'LGG' (reverse primer), derived from known conserved consensus sequences, were used [28,29]. We were able to amplify and clone a large number of DNA fragments. Sequence comparison revealed that four of the PCR fragments (SD85, SD87, SD292 and SD21) of *B. subtilis* A1/3 DNA exhibited pronounced homology to peptide synthetases encoded by ORF4 (gene *pps4)* and ORF1 (gene *pps1*) of the *pps* operon of *B. subtilis* 168 (EMBL entry Z34883 [22,23]).

Fragment SD85 was ~500 base pairs (bp) long. It was therefore expected to span the core sequences TGD and LGGXS (where X is mostly a histidine or aspartic acid residue) within a single peptide synthetase module. The other PCR fragments, SD87, SD292 and SD21 (Figure 7), were ~4 kb in size. Presumably, in these cases, a piece of DNA was amplified extending from the TGD motif of a peptide synthetase up to the LGGXS motif in either the preceding or the following module. Overlapping sequence regions on fragments SD85, SD87 and SD292 allowed us to localize them to a region spanning ~8 kb of DNA from the 3′ end of ORF3 to about half of ORF4. The SD87

Figure 5

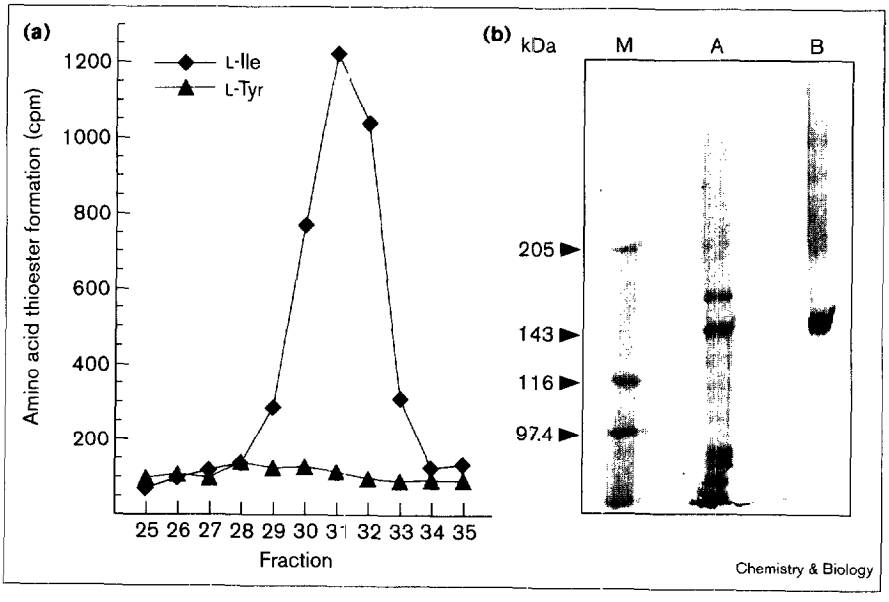


Separation of the fengycin synthetase components in fraction II by anion-exchange FPLC on Pharmacia MonoQ 5/5. These proteins, which were purified by AcA 34 gel filtration and anion-exchange FPLC on Pharmacia ResourceQ, were rechromatographed on Pharmacia MonoQ. They were eluted from the column with a linear gradient of 0–0.5 M NaCl in 50 mM Tris/HCl, pH 7.5, containing 5 mM DTE. The flow rate was 1 ml/min and the fraction size 1 ml. **(a)** All three enzymes were tested for thioester formation with amino acids 1–6 of fengycin: L-alanine, L-tyrosine, L-glutamate, L-threonine, L-valine and L-ornithine. **(b)** A 5% SDS polyacrylamide slab gel of the fractions showing maximal thioester-binding activities with L-tyrosine, L-valine and L-ornithine, respectively. Marker proteins: gramicidin S synthetase 2 (GS2, 508 kDa), SrfB-protein (401 kDa), myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase b (97.4 kDa), and bovine albumin (66 kDa).

fragment should therefore include the region corresponding to the 5′-terminal portion of the *pps4* gene of *B. subtilis* 168. The 5′- and 3′-terminal portions of fragment SD21 extend from one thiolation motif GGXSL to the following and showed the highest homology to *pps1*.

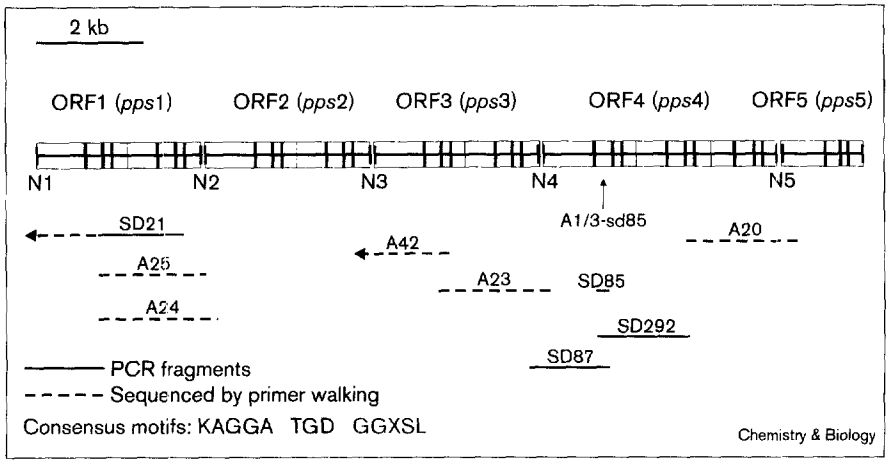
From these initially cloned and sequenced DNA regions we then amplified 'outside' DNA by primer walking using only one specific primer (which apparently functioned as forward as well as reverse primer) for that corresponding DNA region. The amino-terminal region N1 and N2 from gene *pps1* and *pps2*, respectively, were amplified from fragment SD21 in either 5′ or 3′ direction. The N3 region of gene *pps3* was obtained by primer walking from fragment SD87 generating also fragment A42 in 5′ direction. The N4 region was found in fragments SD87 and A23 and the N5 region of gene *pps5* after primer walking from fragment SD292 up in 3′ direction (Figure 7). Knowledge of these DNA fragments allowed us to propose the putative gene organization and module structure of a gene cluster in the genome of *B. subtilis* A1/3, which shows a high homology to the *pps* operon in the 168 reference strain.

Figure 6



Anion-exchange FPLC of the fengycin synthetase component in fraction III on Pharmacia MonoQ. This protein was purified by AcA 34 gel filtration, anion-exchange FPLC on Pharmacia ResourceQ, gel filtration on Sephacryl S-200 and finally by high resolution anion exchange chromatography on MonoQ. The protein was eluted from the MonoQ column with a linear gradient of 0–0.5 M NaCl in 50 mM Tris/HCl, pH 7.5, containing 5 mM DTE. The flow rate was 1 ml/min, the fraction size 1 ml. **(a)** The protein was tested for the incorporation of both L-[^{14}C]-Ile and L-[^{14}C]-Tyr. **(b)** A 12.5% SDS polyacrylamide slab gel showing the proteins in fraction 31, which exhibits maximal thioester formation with L-[^{14}C]-Ile (lane A), an autoradiogram of this fraction with L-[^{14}C]-Ile as tracer (lane B) and marker proteins (lane M) myosin (205 kDa), β-galactosidase (116 kDa), and phosphorylase b (97.4 kDa). Details of autoradiography are in the Materials and methods section.

Figure 7



Schematic diagram of the fengycin gene cluster of *B. subtilis* A1/3. The module structures and consensus motifs were deduced from the *pps* operon of *B. subtilis* 168. Sequenced regions are indicated by black or broken lines.

Construction of a *B. subtilis* A1/3 ORF4 (*pps4* gene) disruption mutant

The involvement of the putative *pps* gene cluster of *B. subtilis* A1/3 in fengycin biosynthesis was demonstrated by constructing a *B. subtilis* A1/3 gene disruption mutant. The pMOS vector, which contained the 500 bp SD85 fragment, was ligated to plasmid pE194 (which confers resistance to erythromycin both in *E. coli* and in *B. subtilis).* The resulting plasmid pMOS-SD85-pE194 was transformed into *B. subtilis* A1/3 at 30°C; chromosomal integration of the plasmid was achieved by selecting for erythromycin resistance after repeated rounds of cultivation at 52°C. Integration of the plasmid was confirmed by Southern blot hybridisation with digested chromosomal DNA of the putative integration mutant and the SD85 DNA fragment for a probe. Disruption of the wild-type DNA fragment, presumably in ORF4 of the *B. subtilis* A1/3 *fen* gene cluster was indicated in the mutant A1/3-sd85 by the appearance of new hybridising fragments (data not shown).

The A1/3-sd85 mutant was tested for fengycin production in ammonium citrate/sucrose (ACS) medium using TLC and MALDI–MS analysis in the same manner as performed for the wild-type strain. Fengycin was not detected by the TLC in the mutant strain. Also, in the MALDI mass spectrum, the fengycin cluster was lacking in the lipopeptide extract of the mutant strain (Figure 2a, spectrum iii). These results support the idea that the *pps*-like operon in *B. subtilis* A1/3 encodes the fengycin biosynthetic genes.

Correlation of the fengycin synthetase subunits from *B. subtilis* b213 to the corresponding *pps* and *fen* genes using amino-terminal sequences

All fengycin synthetase components from *B. subtilis* b213 were further purified to SmartQ grade. They were blotted from preparative SDS polyacrylamide gels onto polyvinylidene difluoride membranes for amino-terminal sequencing by Edman degradation. These sequences are summarized in Table 1. At least ten amino acids were sequenced for each of the proteins and revealed nearly 100% identity with the corresponding segments derived from the *fen* genes of *B. subtilis* A1/3. Comparing these protein sequences with the DNA sequences of the *pps* and *fen* genes from *B. subtilis* strains 168 and F29-3 also allowed the purified fengycin synthetase components to be reliably identified (Table 1). The *pps* genes from *B. subtilis* 168 have been sequenced, and the predicted protein sequences of the Pps1–5 products (P39845, P39846 and P39847 in the Swiss-Prot databank [22]; Y13917 in the EMBL databank [23]) are 66, 60, 100, 80 and 58%, respectively, identical with our protein sequences. The *B. subtilis* F29-3, FenE (AF023465; GenbankTM), FenA (AF023464; GenbankTM), and FenB (L42523; EMBL databank [30]) proteins have 100, 80 and 66% identity with the corresponding segments of the fengycin synthetase component enzymes from *B. subtilis* A1/3. The sequence of the Fen3-related genes did not vary between the strains.

Discussion

Bacillus subtilis is one of the most prominent peptide-forming cell factories hitherto known. In particular, its lipopeptide products (the surfactins, fengycins and the members of the iturin family) are valuable, industrially important compounds. They represent potent biosurfactants and show efficient antimicrobial and antiviral properties [31–33]. They are synthesized by large multienzyme systems that have a modular structure [26,27]. Both their commercial exploitation and elucidation of the synthetic mechanism of lipopeptide biosynthesis in *B. subtilis* require knowledge about the sequence and organization of the biosynthetic genes, as well as about the structure and function of the corresponding peptide synthetases.

Table 1

Amino-terminal sequence alignment of fengycin synthetase subunits from various *Bacillus* strains.

Gene product	*B. subtilis* strain	Amino-terminal sequence	Identity (%)	Homology (%)
Fen1	213	MENTVYSLTHAQ		
A1/3orf1	A1/3	MXNTVYSLTHAQRRVWFTELLEPGTSICNL-30	100	100
Pps1	168	MSEHTYSLTHAQRRVWFTELLEPDTSICNL	58	58
Fen2	213	TQATEIQDIY		
A1/3orf2	A1/3	MTQATEIQDIYPLSYMQEGMLFHSLLDSGS-30	100	100
Pps2	168	MTQSAQIQDIYPLSHMQEGMLFHSLMDFSS	60	80
Fen3	213	QQPEIQDIYPLSFMQ		
A1/3orf3	A1/3	MGQQPEIQDIYPLSFMQEGMLFHSLLDHDS-30	100	100
Pps3	168	MPQQPEIQDIYPLSFMQEGMLFHSLYDEQS	100	100
FenE	F29-3	MPQQPEIQDIYPLSFMQEGMLFHSLYDEQS	100	100
Fen4	213	TKKNAIQDIY		
A1/3orf4	A1/3	MTKKNAIQDIYPLSYMQEGMLFHSLLQKES-30	100	100
Pps4	168	MTKANSIQDIYPLSYMQEGMLFHSLLQKDS	80	90
FenA	F29-3	MTKANSIQDIYPLSYMQEGLLFHSLLQKDS	80	90
Fen5	213	MDKTKNIQNIYP		
A1/3orf5	A1/3	MDKTKNIQNIYPLSRMQEGMLFHSFLQKEG-30	100	100
Pps5	168	MNTIKKIKNIYPLSHMQEGMLFHSFLRKEE	58	75
FenB	F29-3	MVKTKKIKNIYPLSHMQEGMLFHSFLHKEE	66	83

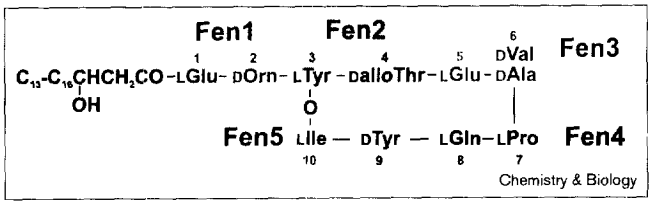
Identical positions in all available sequences are in blue. Accession numbers and references for the published gene sequences: *pps1*, P39845 [22]; *pps2*, P39846 [22]; *pps3*, P39847; *pps4*, Y13917; *pps5*, Y13917 [23]; *fenA*, AF023464; *fenB*, L42523 [30]; *fenE*, AF023465.

To this end we studied the biosynthesis of fengycin in the *B. subtilis* strains b213 and A1/3. Using MALDI–MS we demonstrated that both strains have a very similar lipopeptide spectrum, fengycin being one of the prominent products. The structural and functional organization of the fengycin-producing multienzyme system was investigated in *B. subtilis* b213. It was demonstrated that five enzymes, Fen1–5, are involved in the assembly of the peptide part of this lipodecapeptide. All of the fengycin amino-acid components are activated as aminoacyl adenylates and aminoacyl thioesters according to the two-step thiotemplate mechanism [26]. Corresponding proteins have also been isolated and purified from *B. subtilis* A1/3. Genetic studies on the biosynthesis of fengycin have been performed with this strain because, in contrast to *B. subtilis* b213, it is transformable and therefore amenable to genetic manipulation. Our gene-sequencing studies and the characterization of the fengycin nonproducing gene-disruption mutant A1/3-sd85 revealed that *B. subtilis* A1/3 contains a complete operon-encoding fengycin production that has high homology to the *pps* operon from *B. subtilis* 168, as well as to the *fen* genes (investigated by Liu *et al.* [24,25] in *B. subtilis* F29-3). The *pps* operon has been completely sequenced in the framework of the European *B. subtilis* genome project [22,23]. The reference strain *B. subtilis* 168 does not produce fengycin, but obviously contains fengycin biosynthetic genes. The F29-3 strain, however, is a fengycin producer.

We identified the fengycin synthetase components purified from cell-free extracts of *B. subtilis* b213 by amino-terminal sequencing and comparison of these results with the gene sequences of the *pps* and *fen* operons from the *B. subtilis* strains 168, A1/3 and F29-3. There is perfect agreement between our proteinchemical data with the amino-terminal sequences derived from the fengycin biosynthetic genes of the A1/3 strain and high homology to the sequences available from the *pps* and *fen* operons in the other two strains.

By correlating the characteristics of fengycin synthetase at the level of both protein and DNA, the structural and functional organization of this multienzyme system becomes apparent (Figure 8). The *pps* and *fen* operons include five open reading frames that comprise ten amino-acid activating modules. The first three genes encode three proteins of similar size (fengycin synthetase 1–3, Fen1–3). Each protein consists of two modules that in turn activate amino-acid components 1–6 of fengycin. Fen1 activates glutamate (position 1) and ornithine (position 2) which form the sidechain of fengycin. Fen2 activates and incorporates tyrosine (position 3) and allo-threonine (position 4). Fen3 incorporates glutamate (position 5) and valine or alanine (position 6). These four amino acids are integrated into its peptide ring. The Fen1–3 genes are followed in the fengycin operon by a larger ORF, which codes for a three-module enzyme (Fen4) that activates proline (position 7), glutamine (position 8) and tyrosine (position 9); the final and smallest ORF in the operon encodes Fen5, which activates isoleucine (position 10). Fen5 is presumably involved in closing the fengycin ring by facilitating lactone formation between the carboxyl functional group of L-isoleucine and the hydroxyl group of L-tyrosine in position 3. Each of

Figure 8



Organization of the fengycin synthetase multienzyme system.

Fen1–4 contains an epimerization domain in their carboxyl-terminal module that catalyzes the conversion of amino acids 2, 4, 6 and 9 into their D-enantiomers. Obviously, the amino-acid activating modules of fengycin synthetase are arranged in the same sequence as the amino-acid components of the fengycin product.

The FenB protein from strain F29-3 has recently been overexpressed in *Escherichia coli* and tested for its adenylation and thioester binding specificity by Lin *et al.* [30]. These experiments revealed that FenB has a high affinity for L-isoleucine and corresponds to the putative *ppsE* gene product from *B. subtilis* 168 and Fen5 from *B. subtilis* b213. FenB contains a thioesterase domain that is usually found at the carboxy-terminal end of the last module of prokaryotic peptide synthetases. These authors therefore suggested that FenB is involved in the terminal event of fengycin biosynthesis, which is the cyclization of the lipopeptide ring by lactone formation [30]. Fengycin formation is initiated by the transfer of the β-hydroxy fatty-acid component to the starting amino acid, L-glutamic acid, by an acyltransferase, in a similar way as in the biosynthesis of surfactin [34]. This acyltransferase has not yet been identified, however.

Our structural analysis of fengycin, using MALDI–MS, in combination with enzymatic studies on the amino-acid activation pattern of fengycin synthetase is consistent with the structures originally reported for fengycin by Vanittanakom and Loeffler [8] and for the plipastatins by Umezawa's group [13–16]. In contrast, the amino-acid sequence of fengycin proposed by Tosato *et al.* [23] (derived from sequence homologies of the amino-acid recognition regions in the *B. subtilis* 168 *pps* genes compared with the gene structures of other peptide synthetases) conflicts with all of these results. Tosato *et al.* [23] argued that PpsE would be responsible for activating L-tyrosine instead of L-isoleucine; in their conclusions, L-isoleucine would be activated by the first module of PpsB. In addition, they proposed that the positions of D-Ala/D-Val (position 6) and D-Tyr (position 9) would be reversed. Our results make the view of Tosato *et al.* [23] improbable, and suggest that it is not yet possible to identify unequivocally which amino acid will be activated by a given peptide synthetase module from sequence homology considerations alone. Furthermore, it is difficult to understand how the fengycin ring could be closed by lactone formation if L-tyrosine in position 3 and L-isoleucine in position 10 were interchanged.

Our results should facilitate the design and construction of recombinant peptide synthetases using module and domain replacements, as well as site-directed mutagenesis, in order to create novel lipopeptide products with improved surfactant, antimicrobial and antiviral activities.

In addition, the lipopeptide-forming multienzymes of *B. subtilis* (including fengycin synthetase) qualify as model systems for investigating the mechanism of peptide biosynthesis in general. *B. subtilis* provides a potential of at least 15 amino-acid activation modules (from its lipopeptide multienzymes) that are combined in different and specific ways during the assembly of the various peptide synthetases. In this context, the fengycin system is of particular interest for investigating important questions such as the acyl-transfer reactions in the initiation process and the mechanism of subunit interactions in the elongation cycle. In *B. subtilis*, analagous modules are used in modified forms for different biosynthetic processes (e.g. the glutamic acid activating units used in surfactin and fengycin biosynthesis, or glutamine- and proline-activating modules in fengycin or iturin biosynthesis), a peculiarity that might be helpful in understanding the structural elements that control subunit interaction in these multienzymes. All of these questions can be approached efficiently using mutational studies in combination with the analysis of peptide intermediates. For such studies the preparation and characterization of the wild-type multienzymes are indispensable prerequisites.

Significance

The research presented in this paper is part of a project to investigate comprehensively the biochemical features of lipopeptide-forming cell factories, as well as the sequence and organization of the corresponding gene clusters in *Bacillus subtilis*. A more comprehensive understanding about these multienzyme systems is highly important to allow us to use lipopeptide compounds in the pharmaceutical and food industries, in plant protection, as well as for the elucidation of the mechanism of lipopeptide biosynthesis.

Here we studied the biosynthesis of the lipodecapeptide fengycin in the *B. subtilis* strains b213 and A1/3. The peptide portion of fengycin is assembled through the cooperation of five multifunctional enzymes (Fen1–5) using a multiple carrier thiotemplate mechanism. The structural and functional organization of this multienzyme system is consistent with the rules derived for prokaryotic peptide synthetases. These proteins were correlated by their amino-terminal sequences with a cluster of five genes (fen1–5) in *B. subtilis* A1/3 that have been cloned and partially sequenced. They show high homology to the *pps*

and *fen* operons in *B. subtilis* 168 and F29-3. The relevance of these genes with respect to fengycin biosynthesis was ascertained by constructing a fengycin-deficient A1/3-sd85 gene-disruption mutant. Our results form the basis for the genetic engineering of this multienzyme system to design novel biosurfactant agents with improved antimicrobial and antiviral activities.

Materials and methods

Materials

L-[U-^{14}C]glutamine (280 mCi/mmol), L-[U-^{14}C]glutamate (249 mCi/mmol), L-[U-^{14}C]threonine (228 mCi/mmol), L-[U-^{14}C]tyrosine (464 mCi/mmol), L-[U-^{14}C]alanine (156 mCi/mmol), L-[U-^{14}C]isoleucine (330 mCi/mmol), L-[U-^{14}C]proline (264 mCi/mmol), and L-[U-^{14}C]valine (262 mCi/mmol) were purchased from Amersham Life Sciences (Braunschweig, Germany). L-[2,3-^{3}H]ornithine (53.4 Ci/mmol) was obtained from American Radiolabeled Chemicals (St. Louis, USA). $Na_4[^{32}P]$pyrophosphate (6.42 Ci/mmol) was a product of DuPont-NEN Life Science (Bad Homburg, Germany). Ultrogel AcA 34 was from BioSepra (Frankfurt a.M., Germany). Anion-exchange chromatography was performed using Q-Sepharose Fast Flow and the FPLC columns MonoQ HR5/5, ResourceQ, and SmartMonoQ PC 1.6/5 from Amersham Pharmacia Biotech (Freiburg, Germany). All other chemicals used were reagent grade.

Strains and plasmids

B. subtilis b213 was selected and characterized by Jacques *et al.* [35]. *B. subtilis* A1/3 has been isolated from greenhouse tomato cultures. It shows a broad spectrum of antibiotic activities against bacteria, fungi and plant viruses (E. Griesbach and A. Habekuß, personal communications). *Escherichia coli* XL1-Blue (New England BioLabs, Schwalbach, Germany) was used as host for PCR fragment cloning.

Plasmids used in this study were pMOS-T vector (Amersham LIFE Sciences), pE194 [36], pMOS-SD85, pMOS-SD87, pMOS-SD292, pMOS-SD21 (pMOS derivatives with inserts of respective PCR fragments that showed homology to distinct peptide synthetase domain regions) and pE194-pMOS-SD85 (shuttle for gene disruption mutagenesis).

Fermentation of Bacillus subtilis *b213*

Bacillus subtilis b213 was cultivated in an optimized sucrose/yeast extract/peptone medium [35] and *Bacillus subtilis* A1/3 in an ammonium citrate medium [37]. Cells of *B. subtilis* b213 used for enzymatic studies were grown in a 300 l fermenter (Biolafitte, France) containing 150 l medium under pH control (pH 7) with an aeration of 1 vvm and an agitation of 150 rpm at 30°C. Cells (2.3 kg) were harvested 23 h after inoculation by centrifugation, when the OD_{600} was 6.27, and stored frozen.

TLC and MALDI-TOF-MS analysis of culture medium extracts from Bacillus subtilis *A1/3 and b213*

Lipopeptides were precipitated from 4 l of culture supernatant after HCl acidification (to pH 2). The precipitate was resuspended in a minimal amount of water, neutralized with NaOH and lyophilized. The lyophilisate was extracted with methanol and the extract applied on a LH-20 size exclusion column (Pharmacia, column size 1500 × 15 cm) using methanol as the eluent. Fractions containing fengycin were visualized using TLC. A mixture of chloroform:MeOH:water (65:25:4) was used as the mobile phase. Silica gel DC 60 plates from Merck (Darmstadt, Germany) were used as the TLC matrix. Fengycin was visualized by treating with ninhydrin and charring with H_2SO_4 and was further purified using reversed phase FPLC and high-performance liquid chromatography (HPLC) on a Pharmacia PepRPC HR 10/10 column and on an ODS Hypersil column (Knauer, Berlin, Germany), respectively. A linear gradient of 20% acetonitrile in 100 mM ammonium acetate (v/v), pH 6.9, and 100% acetonitrile was used for elution at a flow rate of 0.75 ml/min. Samples were scratched out from a nonsprayed TLC plate and eluted from the matrix with MeOH for MALDI-MS analysis. MALDI mass spectra were recorded on a Bruker Reflex MALDI-TOF instrument with a 337 nm nitrogen laser for desorption and ionization. A saturated solution of α-cyano-4-hydroxycinnamic acid in 70% acetonitrile/0.1% trifluoroacetic acid (v/v) was used as matrix. Ions were accelerated with a voltage of 28.5 kV. The positive-ion detection and reflector mode was used.

Enzymatic assays

Substrate amino-acid dependent ATP-PP_i exchange reactions and thioester binding of the amino-acid components of fengycin synthetase were measured using the procedure described for the characterization of surfactin synthetase [34].

Enzyme preparation

All steps were carried out at 4°C. *B. subtilis* b213 cells were suspended in 3 volumes of buffer A (50 mM Tris-HCl, pH 7.5, 5 mM DTE, 3 mM EDTA, 5 mM benzamidine, and 20% sucrose). Protoplasts were generated by lysozyme treatment (4000 U/ml cell suspension, 30°C, 20 min) and passed through a French press at a cell pressure of 18.000 psi. Cell debris was removed by centrifugation (25,000 g, 20 min). Nucleic acids were precipitated by addition of streptomycin sulfate to a final concentration of 1% (w/v) and stirring for 25 min. They were pelleted by centrifugation at 25,000 g for 20 min. The proteins in the supernatant were salted out with ammonium sulfate at 70% saturation, dissolved in a minimum volume of buffer B (50 mM Tris-HCl, pH 7.8, 5 mM DTE, 3 mM EDTA, and 10% sucrose), and dialysed against the same buffer. 40 ml of the crude enzyme extract was loaded on an Ultrogel AcA 34 column (75 × 5 cm) and eluted with buffer B (flow rate 100 ml/h, fraction size 13 ml). Fractions in the high molecular weight range (> 300 kDa) catalyzing thioester formation with proline, glutamine or tyrosine, as well as all fractions in the medium molecular weight range (200–300 kDa) showing thioester formation with glutamic acid, ornithine, tyrosine, threonine and alanine residues were pooled and assigned as enzyme fractions I and II, respectively. All of the fractions in the molecular mass range between 100 and 200 kDa that formed a thioester with isoleucine were pooled and designated as enzyme fraction III.

Purification of Fen1, Fen2 and Fen3

Enzyme fractions I and II were loaded separately on a ResourceQ column that was equilibrated with buffer C (50 mM Tris-HCl, pH 7.5, 5 mM dithioerythritol, DTE). Proteins were eluted from the column with a linear gradient of 0–0.5 M NaCl in buffer C (flow rate 2 ml/min, fraction size 2 ml, total volume 500 ml). Fractions containing enzyme activity were pooled, diluted with 5 volumes of buffer C and loaded on a MonoQ column. Proteins were again eluted with a linear gradient of 0–0.5 M NaCl in buffer C with a flow rate of 1 ml/min and a fraction size of 1 ml (total volume 200 ml).

Purification of Fen4

Fen4 was purified from enzyme fraction I by subsequent anion-exchange FPLC on a Pharmacia MonoQ following the protocol described above. Fractions were tested for thioester binding activities. Active fractions were diluted five times with buffer C. The diluted pool was loaded on a SmartQ column using the Pharmacia Smart System. Fen4 was eluted with a linear gradient of 200–400 mM NaCl in buffer C. The flow rate was 50 µl/min.

Purification and autoradiographic analysis of Fen5

Enzyme fraction III (L-Ile binding activity) was loaded on a ResourceQ column and chromatographed as described above. Fractions showing L-Ile thioester binding activity were pooled and concentrated to a volume of 5 ml using Centriprep concentrators with a cutoff of 100 kDa (Amicon GmbH, Witten, Germany). The concentrated protein was applied to a Superdex-200 gel filtration column (prep grade 16/60, Pharmacia) and eluted with buffer B containing 100 mM NaCl at a flow rate of 0.5 ml/min and a fraction size of 1 ml. Fractions containing enzyme activity were pooled and chromatographed on a MonoQ column as described above. For autoradiography of a SDS gel containing the [^{14}C]-L-Ile-labeled protein 100 µl of protein fraction 34 from the MonoQ separation were incubated in the presence of 0.05 µCi [^{14}C]-L-Ile,

8.3 mM $MgCl_2$, and 12.3 mM ATP in a total volume of 121 µl for 15 min at 37°C. The protein was then precipitated with trichloroacetic acid (TCA) overnight. The pellet was washed with acetone and resuspended in 20 µl Laemmli-SDS–PAGE buffer [38]. The mixture was loaded on a 5% polyacrylamide-SDS slab gel which was stained with Coomassie Blue and dried in a gel dryer. Finally it was subjected to autoradiography by exposure to X-ray film (Konica, Japan) for 4 weeks.

Protein analysis

Protein concentration was measured using the procedure of Bradford [39]. SDS–PAGE was performed according to Laemmli [38]. Molecular weights of denaturated proteins were determined with SDS–PAGE in a mini-gel apparatus (Hoefer, San Francisco, USA). Marker proteins were gramicidin S synthetase 2 (GS2, 512 kDa), surfactin synthetase 3 (SrfB, 405 kDa), and a heigh molecular weight standard mixture (30–200 kDa, Sigma, Deisenhofen, Germany).

For amino-terminal sequence analysis, enzyme fractions obtained from high resolution anion-exchange FPLC on MonoQ were further purified with a Pharmacia Smart System using a SmartQ column. The samples were loaded on the column after dilution with 5 volumes of buffer C. Fen1–3 and Fen 5 were eluted with linear gradients of 200–400 mM NaCl and of 100–300 mM NaCl in buffer C, respectively. The flow rate was 50 µl/min. After preparative SDS–PAGE according to the procedure described in [34] the gels were electroblotted using a polyvinylidene difluoride membrane in a semi-dry Novablot apparatus (LKB, Bromma, Sweden) according to the protocols of the manufacturers. The blotted proteins were stained with a 0.2% (w/v) Ponceau S solution. Bands of the fengycin synthetase components were excised and submitted to Edman degradation analysis using a Procise Sequencer (Perkin Elmer/Applied Biosystems, Weiterstadt, Germany).

DNA manipulations

B. subtilis A1/3 chromosomal DNA was prepared as described by Cutting and van der Horn [40]. Plasmid DNA was isolated from *E. coli* by the alkaline lysis procedure and DNA manipulations were performed according to [41] and [42]. PCR fragments were purified with the QIAEX gel elution kit (QIAGEN, Hilden, Germany) and cloned by the pMOS*Blue*-T vector kit (Amersham Life Sciences). Double-stranded DNA templates for sequencing reactions were purified with the QIAGEN plasmid kit.

Competent cells of *E. coli* were prepared and transformed following the procedure of Hanahan [43]. *B. subtilis* A1/3 was transformed with plasmid DNA by the protoplast method by Chang and Cohen [44].

PCR amplification

To isolate domain fragments of peptide synthetases, PCR amplifications were performed with *B. subtilis* A1/3 genomic DNA using degenerated primers 'TGD' (forward primer) and 'LGG' (reverse primer) as described by Turgay and Marahiel [28]. PCR was performed using *Taq* DNA polymerase and 10× reaction buffer (Boehringer Mannheim, Germany). PCR was run for 35 cycles in a GeneAmp 2400 ThermoCycler apparatus (Perkin Elmer, Überlingen, Germany) using the following protocol: chromosomal DNA 200–500 ng, primer and dNTP concentration 300 nM or 300 µM, respectively; denaturation: 5 min at 94°C in the first cycle and 30 sec at 94°C thereafter; annealing: 30 sec at 55°C; elongation: 1 min at 72°C for amplification of 0.5 kb DNA fragments or 3 min at 72°C for amplification of DNA fragments 3–4 kb in size. For chromosomal walking, the long-range template kit from Boehringer Mannheim was used for PCR amplification.

Sequencing and sequence handling

An automated A.L.F. DNA sequencer (Amersham Pharmacia Biotech, Freiburg, Germany) was used for nucleotide sequencing. Sequences were compared to those in EMBL databases using the BLAST e-mail server [45,46]. In particular, we used BLASTN and BLASTX for homology searches of nucleotide and amino-acid sequence similarities, respectively.

Acknowledgements

We thank Mrs G. Haeselbarth and Professor J. Salnikow for amino-terminal sequence determinations, P. Franke for his instructions and advice in the MALDI-MS measurements, S. König for the help in nucleotide sequencing and B. Fischer for skillful technical assistance. This work was supported by the Commission of the European Communities, grant BIO4CT950176. The work of J.H. and B.C. was supported by grant VV311137 from the BMBF.

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