Volume 325, number 3, 220–224 FEBS 12627 July 1993
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Analysis of a mutant amino acid-activating domain of surfactin synthetase bearing a serine-to-alanine substitution at the site of carboxylthioester formation

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Received 2 February 1993; revised version received 26 April 1993

The reactive serine of the TGGHSL thioester binding motif of the first amino acid-activating domain of surfactin synthetase was replaced by alanine using site-directed mutagenesis. The multienzyme from cells of the resulting mutant lost its ability for thioester formation with L-Glu and was therefore inactive in surfactin production The thiolation reactions catalyzed by the other amino acid-activating domains of surfactin synthetase were not affected by the mutation. The results show that L-Glu is acativated at the first domain of surfactin synthetase, and give further evidence that a serine residue is essential for substrate amino acid activation at the reaction centers of peptide synthetases

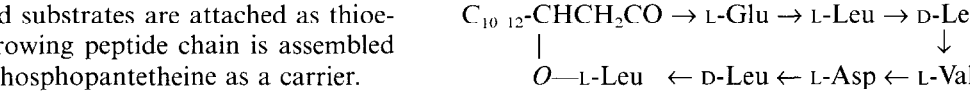
Surfactin synthetase; *Bacillus subtilis*; Site-directed mutagenesis; Thioester binding; Reactive serine; Multiple carrier model

1. INTRODUCTION

The mechanism of non-ribosomal biosynthesis of numerous microbial peptides is compatible with the polyenzyme model suggested by F. Lipmann [1] and the thiotemplate mechanism [2–5]. These models propose that (i) amino acid-activating domains are arranged in series along multifunctional polypeptide chains, (ii) each reaction center of the peptide-synthesizing multienzyme is equipped with a specific sulfhydryl group to which the amino acid substrates are attached as thioesters, and (iii) the growing peptide chain is assembled by the action of 4′-phosphopantetheine as a carrier.

Recent studies of gramicidin S synthetase showed that a serine may be required for covalent attachment of the substrate amino acids to each reaction center of the multienzyme system [6]. These serine residues are part of a LGG H/D S L/I motif which is highly conserved in the C-terminal region of each amino acid-activating domain of the peptide synthetases [7–11]. The H/D S L/I core of this motif has been identified as the 4′-phosphopantetheine binding site of acyl carrier proteins/domains of fatty acid and polyketide synthetases [6]. On the basis of these results, we proposed that there are multiple peripheral 4′-phosphopantetheine carriers forming the thioester activation centers of multifunctional peptide synthetases [6]. The existence of multiple peripheral carriers instead of a central 4′-phosphopantetheine arm, as postulated in the original version of the thiotemplate model, leads to a new concept of the mechanism of non-ribosomal peptide biosynthesis. To begin to test this hypothesis, modification of the site of carboxylthioester formation in the first amino acid-activating domain of surfactin synthetase was undertaken by site-directed mutagenesis.

Surfactin is a cyclic lipopeptide of the following structure:



Ullrich et al. [12] and Menkhaus et al. [13] recently showed that the biosynthesis of surfactin is catalyzed by a multienzyme system consisting of at least 4 enzyme components termed E_{1A}, E_{1B}, E_2 and E_3. Nakano et al. [14] demonstrated that the genes coding for this process are organized into an operon (*srfA*) in the genome of *Bacillus subtilis*. Recent genetic studies ([15], and G. Grandi, personal communications) confirmed that *srfA* encodes the peptide synthetase subunits that catalyze surfactin synthesis. The first gene, *srfAA*, encodes the enzyme E_{1A}, which catalyzes the incorporation of Glu, Leu and D-Leu. The second gene, *srfAB*, encodes E_{1B}, which catalyzes the incorporation of Val, Asp and D-Leu, while the third gene, *srfAC*, encodes E_2, which catalyzes the incorporation of the last amino acid, Leu. E_3 is an acyl transferase catalyzing the binding and transfer of the β-hydroxy fatty acid substrate. The gene coding for this enzyme has not yet been identified. In

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this report the analysis of a mutant *srfA* is presented that had undergone a substitution of the conserved serine for an alanine within the thiolation motif of the first amino acid-activating domain by site-specific mutagenesis.

2. MATERIALS AND METHODS

2.1. *Bacterial strains and plasmids*

The *Escherichia coli* strain used for plasmid propagation was AG1574 [*araD139Δ(ara leu)7697 ΔlacX74 galUK r⁻ m⁺ strA recA56 srl*] (from A. Grossman). For M13 phage propagation, *E. coli* strain MV1190 [*Δ(lac proAB) thi supE Δ(srl⁻ recA)306* :: TN10 (Tet^r) F′ *traD36 proAB lacI^q lacZ ΔM15*] was used. NK7085 [*Δ(lac pro) nalA^r*/F′ *lacZYA536 proA⁺ proB⁺ mutS104* :: Tn5 [16]] was used in the mutagenesis procedure as previously described [17]. OKB105 (*pheA sfp*) is a surfactin-producing strain of *B. subtilis* [17]. *Bacillus subtilis* strain LAB848 [*pheA sfp ΔsrfA* :: pNAC14 (Phleo^r)] is a *srfA* deletion mutant. Plasmid pNAC14 was constructed by first inserting a 1.5 kb *Eco*RI fragment of p223-21K into pMMN46 [14], followed by replacement of the *Sal*I fragment with a 0.7 kb *Pvu*II fragment containing the *ble* gene [18] that confers phleomycin resistance. The integration of this plasmid into the chromosome of a Srf$^+$ strain by double-crossover results in the deletion of approximately 17 kb of *srfA* DNA containing the first four amino acid-activating domains. The construction of the mutant was confirmed by Southern hybridization analysis.

2.2. *Culture media*

YT broth (2 ×), LB and DSM agar media, prepared as described previously [19], were used for routine culture of *B. subtilis* and *E. coli* strains. Blood agar solid medium was used for detection of surfactin production. Chloramphenicol resistant (Cm^r) cells were selected as described previously [19]. The surfactin producer strain OKB105 and the non-producing Ser-to-Ala mutant LAB960-7 [*pheA sfp srfAA1* (S to A)] were grown in Landy medium [20] supplemented with 2 mg/l L-Phe and 0.1% yeast extract for surfactin synthetase preparation.

2.3. *Radiochemicals, substrates, and materials for chromatography*

[^{14}C]-Labeled amino acids were purchased from Amersham/Buchler (Braunschweig, Germany). Tetrasodium [^{32}P]pyrophosphate was from DuPon/NEN (Bad Homburg, Germany). The thioester adduct of 3-hydroxytetradecanoic acid with coenzyme A was synthesized as reported [12]. AcA 34 was obtained from Serva (Heidelberg, Germany) and Mono Q HR 5/5 from Pharmacia (Freiburg, Germany).

2.4. *Oligonucleotide-directed mutagenesis*

The mutagenesis was performed by the gapped duplex procedure [21]. The 0.85 kb *Hin*cII–*Rsa*I fragment of pMMN42 [14], containing the TGGHSL motif of the first amino acid-activating domain of *srfA*, was inserted into M13mp9 to yield the plasmid, mCD13, the single-stranded form of which was used for mutagenesis. The oligonucleotide that was used, 5′-TGGCGGACATGCTTTAAAAGCCATGA-3′, contained the amino acylation motif with the alanine codon substituted for the serine codon. The oligonucleotide was designed so that the mutation would result in the introduction of a *Dra*I site (Fig 1). *E. coli* NK7085 was used as the host for propagating the mutated sequence in phage M13. Potential mutant clones were screened by *Dra*I digestion. The complete 0.85 kb fragment was sequenced to verify that the only base changes present were those created using the oligonucleotide.

2.5. *Introduction of the mutation into* B. subtilis

A 0.69 kb *Nde*I–*Sal*I fragment containing the mutation from one of the mutant clones (pCD18) was used to replace the wild-type sequence of pMMN42, a derivative of the integration vector pMMN13 [14] that carries a selectable cat (chloramphenicol acetyl transferase) gene, and the presence of the mutation in the recombinant (pCD20) was once again screened by *Dra*I digestion. The site of the mutation in this construct was too close to the *Sal*I end, and a sufficient length of homology on either side of the mutation site was needed to increase the probability of retention of the mutation at the chromosome following isolation of plasmid-less, Cm^s segregants. To achieve this, a 0.87 kb *Bgl*II–*Sal*I fragment of *srfA* DNA was subcloned upstream of the *Sal*I–*Pst*I fragment of pCD20, and the resultant plasmid (pCD23) was then used to transform OKB105, a surfactin-producing strain of *B subtilis*, followed by selection for chloramphenicol resistance. This integration event occurs by Campbell recombination which results in disruption of *srfA* and a consequent loss of surfactin production in this partial *srfA* diploid strain [LAB855 *pheA sfp srfA* :: pCD23 (Cm^r)].

2.6. *Isolation of* Srf$^-$ *segregants of plasmid integrants*

The cells of *B subtilis* strain LAB855 were grown in 2 × YT medium at 37°C to late-log phase and subcultured a number of times. An overnight grown culture was diluted 100 × and chloramphenicol resistance was induced at a concentration of 0.5 μg/ml for 1 h followed by growth at a selective concentration of 5 μg/ml for another hour. The actively growing Cm^r cells were then killed by 2 mM D-cycloserine, a cell wall-synthesis inhibitor. Since chloramphenicol is bacteriostatic the culture was enriched for the chloramphenicol-sensitive (Cm^s) cells. The cells were washed with 2 × YT, appropriately diluted and plated on a non-selective medium such as LB or DSM agar.

2.7. *Assays*

Assays for (i) ATPP/PP$_i$ exchange measurements, (ii) thioester binding of substrate amino acids by surfactin synthetase, as well as (iii) in vitro formation of surfactin were performed as described in [12]. The acyl transferase enzyme E$_3$ of surfactin synthetase was assayed as described [13].

2.8. *Enzyme purification*

The surfactin synthetase multienzyme system from the wild-type strain *B subtilis* OKB105 and the mutant LAB960-7 was prepared as reported [13]. Its components E$_1$, E$_2$, and E$_3$ were fractionated by gel filtration of the crude extracts on an ultrogel AcA 34 column (45 × 2.5 cm). The multienzyme fraction E$_1$ was separated into E$_{1A}$ and E$_{1B}$ by high-resolution anion-exchange FPLC on Pharmacia Mono HR 5/5.

3. RESULTS

3.1. *Mutagenesis of the TGGHSL motif in the first domain of* srfA *and its affect on surfactin production*

In a previous study of the amino acid-activating domains of gramicidin S synthetase of *B. brevis* [6], the LGG H/D S L/I motif was shown to be involved in the covalent attachment of the activated substrate amino acid to the multienzyme (amino acylation). This highly conserved motif among peptide synthetases resembled the 4′-phosphopantetheine binding site of acyl carrier subunits of fatty acid synthetases and polyketide synthetases. Hence, it was hypothesized that its serine residue binds indirectly to the amino acid via a 4′-phosphopantetheine adaptor molecule. In order to determine if the serine is required for amino acylation, the serine of the TGGHSL motif in the first domain of *srfA* was changed to an alanine by site-directed mutagenesis (see section 2). The mutation was introduced into the *B. subtilis* chromosome using an integrative plasmid conferring Cm^r insertion resulting in the formation of a Srf$^-$ mutant (LAB855), which is diploid for the sequence that was subject to the mutagenesis procedure Cm^s segregants were then isolated, some of which were Srf$^+$, hav-

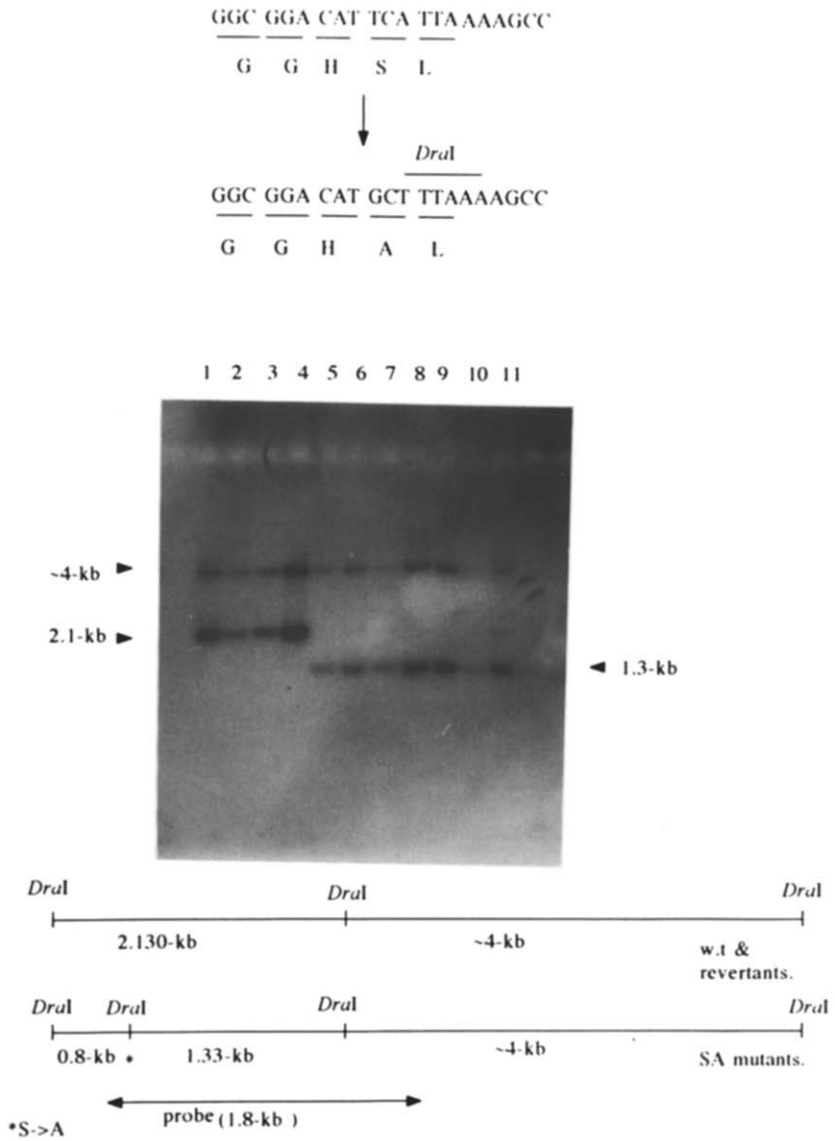

Fig. 1. The Ser-to-Ala mutation results in a *Dra*I recognition site. Southern hybridization analysis of *Dra*I-digested chromosomal DNA from the Ser-to-Ala mutants, Srf^+ segregants and wild-type cells was performed. Lane 1, wild-type; lanes 2–4, Srf^+ segregants; lanes 5–11, mutants. Below the photograph is a map showing the positions of the *Dra*I sites in the chromosome of the wild-type, Srf^+ segregants, and the Ser-to-Ala mutants. Also shown is the location of the *srfA* region (1.8 kb) used as the hybridization probe.

ing retained the wild-type *srfA* sequence, and some of which were Srf^-, having acquired the Ser-to-Ala mutation. The latter was identified by Southern hybridization (Fig. 1). Chromosomal DNA from OKB105, putative revertants and mutants were digested with *Dra*I. A radiolabeled RNA probe was synthesized using T7 RNA polymerase and pMMN42 linearized with *Sal*I as template. A 2.13 kb fragment was observed in the wild-type and in the Srf^+ segregants. As seen in Fig. 1, the mutation indicated by an asterisk results in two *Dra*I fragments (0.8 kb and 1.33 kb), of which only the 1.33 kb fragment could be detected by the probe, probably due to greater overlap of their nucleotide sequences. These results confirmed the presence of the mutation in the Srf^- strains and its absence in the wild-type and in the Srf^+ segregants. The loss of surfactin production in the mutants strongly suggests that the serine residue is required for surfactin biosynthesis.

3.2. *Characterization of the surfactin multienzyme system from the Ser-to-Ala mutant LAB960-7*

Surfactin synthetase was isolated from the surfactin producer, OKB105, and the non-producing mutant, LAB960-7, bearing the Ser-to-Ala substitution in the amino acylation site of the first amino acid-activating domain of *srfA*. In Fig. 2 this multienzyme system obtained from the mutant strain was separated by AcA 34 gel filtration into 3 enzyme fractions, E_1, E_2, and E_3, as demonstrated recently for the wild-type [13]. The thioester incorporation of the amino acid substrates, Leu,

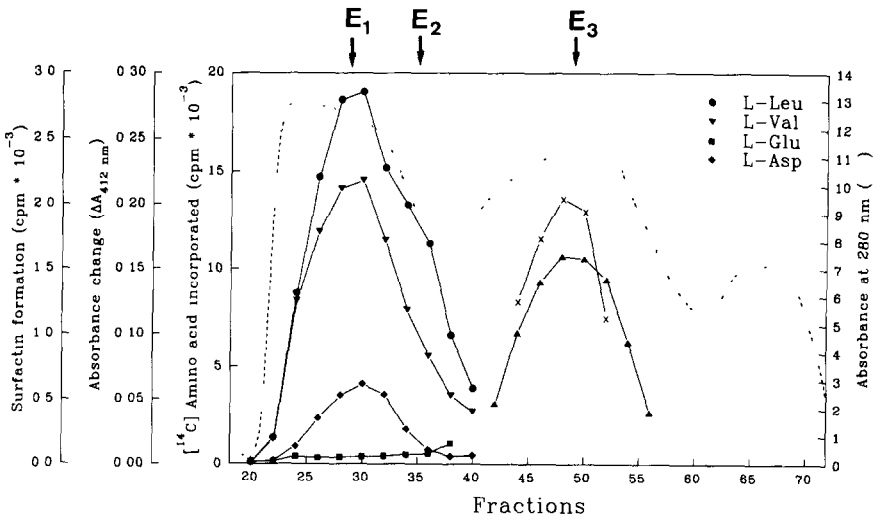

Fig. 2. AcA 34 gel filtration of the crude extracts from the Ser-to-Ala mutant strain *B. subtilis* LAB960-7. The multienzyme fraction E_1 of surfactin synthetase was detected by the thioester incorporation of ^{14}C-labeled substrate amino acids (Leu ●; Val ▼; Asp ◆ and Glu ■). The acyl transferase component, E_3, was monitored by the DTNB assay (▲) and by its activity in surfactin formation (×).

Val, Asp, and Glu, into E_1 was examined. Evidently, E_1 had lost the ability to incorporate Glu as a consequence of the Ser-to-Ala mutation in the first domain of surfactin synthetase. Thioester formation with the other E_1 substrate amino acids was not affected by the mutation as they showed the pattern of amino acid incorporation of the wild-type multienzyme [12]. The leucine-activating enzyme, E_2, appeared as a shoulder at the right border of the E_1 peak. All components of surfactin synthetase were tested for their activities in the biosynthesis of surfactin using the complementary enzymes prepared from the OKB105 reference strain. E_1 was completely inactive, while E_2 and E_3 showed similar activities as the corresponding enzymes from the wild-type strain, as demonstrated in Fig. 2 for the acyl transferase enzyme E_3.

In a recent report [13] it was demonstrated that E_1 consists of two multifunctional enzymes, E_{1A} and E_{1B}, which can be separated by high-resolution anion-exchange FPLC, as shown in Fig. 3A–D. E_{1A}, which appears at higher ionic strength, incorporates Leu and Glu as thioesters in a molar ratio of approximately 2:1, while E_{1B} incorporates Leu, Val, and Asp. In Fig. 3A and B the thioester formation activities of both enzymes prepared from the surfactin producer OKB105 (B) and as well as from the mutant strain, *B. subtilis* LAB960-7 (A), were compared. Mutant E_{1A} is completely lacking in thioester formation with Glu. In either case E_{1B} shows similar amino acid incorporation effects. In Fig. 3C it is shown that mutant E_{1A} is also deficient in the Glu-dependent ATP–PP_i exchange. In Fig. 3D the activities of E_{1A} and E_{1B} from the mutant strain were tested for surfactin synthesis using assay mixtures containing the complementary enzymes prepared from the wild-type *B. subtilis* OKB105. E_{1B} from the mutant is active in product formation, while mutant E_{1A} is inactive.

4. DISCUSSION

The aim of this study was to obtain further evidence for the participation of the reactive serines in substrate amino acid activation at the reaction centers of peptide synthetases by protein engineering techniques investigating surfactin synthetase from *B. subtilis*. The TGG HSL thioester site motif [6,11] in the first amino acid-activating domain of the *srfA* operon was mutationally altered by substituting the conserved serine for an alanine. The components E_{1B}, E_2, and E_3 of surfactin synthetase prepared from the Ser-to-Ala mutant *B. subtilis* LAB960-7, showed the same reaction pattern and similar activities as the corresponding proteins from the wild-type strain, OKB105. Mutant E_{1A}, however, lost the ability to thioesterify Glu and was therefore inactive in surfactin production, consistent with the complete loss of lipopeptide formation observed in vivo investigating whole mutant cells. In addition, the Glu-dependent ATP–PP_i exchange in the primary aminoacyl adenylate activation disappeared as a consequence of the Ser-to-Ala mutation. The Ser-to-Ala mutation, evidently, leads to a defect in the activation of Glu, possibly by inducing secondary effects on the sites functioning in primary adenylate formation. These results confirm that the serine in the LGG H/D S L/I motif plays a crucial role in substrate amino acid activation.

From our data we conclude that the first amino acid-activating domain of surfactin synthetase bears the activation site for Glu consistent with our previous finding that the acyl transferase, E_3, initiates surfactin formation by transfer of the β-hydroxy fatty acid substrate to

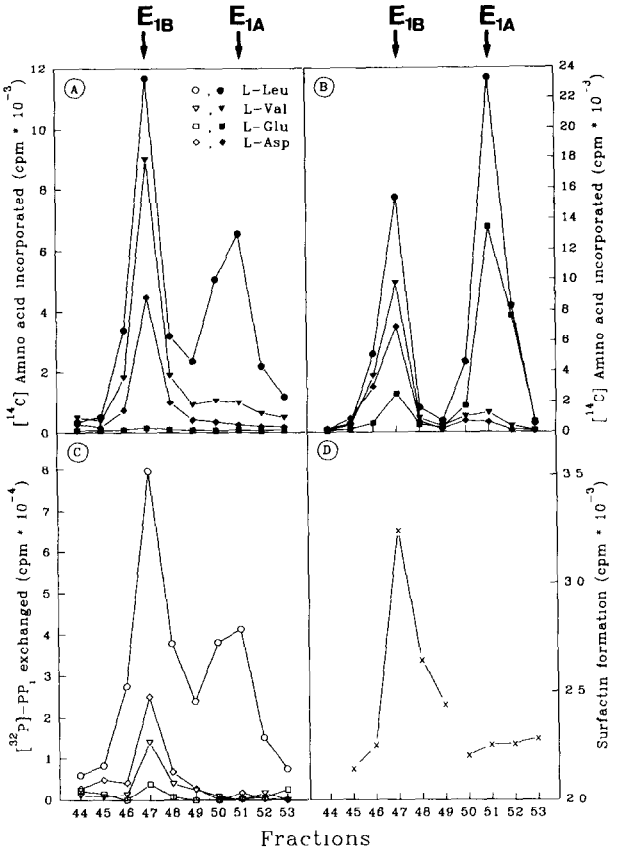

Fig. 3. Separation of the E_1 complex of surfactin synthetase into its components E_{1A} and E_{1B} by high-resolution anion-exchange FPLC on Pharmacia Mono Q HR 5/5. The proteins were eluted with a linear gradient of 0–0.5 M NaCl in 50 mM Tris-HCl buffer, pH 7.5 containing 5 mM dithioerythritol. Flow rate, 1 ml/min; fraction size, 1 ml. (A and B) Comparison of the thioester formation reactions of E_{1A} and E_{1B} prepared from the Ser-to-Ala mutant strain *B. subtilis* LAB960-7 (A), and the wild-type strain *B. subtilis* OKB105 (B) with Leu (●), Val (▼), Asp (◆), and Glu (■). (C) E_{1A} and E_{1B} from the mutant strain were tested for substrate amino acid-dependent ATP–PP_i exchange reactions (Leu ○; Val ▽; Asp ◇ and Glu □). (D) Both enzymes obtained from the mutant cells were detected by covalent incorporation of Leu. Thereafter their activities in the biosynthesis of surfactin (×) were determined using assay mixtures containing the complementary enzymes from the wild-type OKB105.

E_{1A}, forming β-hydroxyacyl glutamic acid as the initiation product [13]. Considering this result and the activation pattern of E_{1A}, E_{1B} and E_2, the following sequence of events in the biosynthesis of surfactin can be inferred: E_{1A} (*srfAA*) presumably elongates the hydroxyacyl glutamate intermediate forming the lipotripeptide β-hydroxyacyl–Glu–Leu–D-Leu, which is transferred to E_{1B} (*srfAB*), where the lipohexapeptide β-hydroxyacyl–Glu–Leu–D-Leu–Val–Asp–D-Leu is assembled. The addition of Leu into position 7 and the cyclization of the complete chain by lactone formation between the carboxyl group of the terminal leucine and the hydroxyl function of the β-hydroxy fatty acid in the first position would be catalyzed by the enzyme E_2 (*srfAC*). This sequence of the intermediate steps in the biosynthesis of surfactin has to be confirmed by an analysis of the elongation and termination processes.

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