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Functional Dissection of Surfactin Synthetase Initiation Module Reveals Insights into the Mechanism of Lipoinitiation

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SUMMARY

Although the N-terminally attached fatty acids are key structural elements of nonribosomally assembled lipopeptide antibiotics, little is known about the mechanism of lipid transfer during the initial step of biosynthesis. In this study, we investigated the activity of the dissected initiation module (C-A_{Glu}-PCP) of surfactin synthetase SrfAA in vitro to gain further insights into the lipoinitiation reaction. The dissected condensation (C) domain catalyzes the transfer of CoA-activated 3-hydroxy fatty acid with high substrate specificity at its donor site to the peptidyl carrier protein (PCP) bound amino acid glutamate (Glu_1). Additionally, biochemical studies on four putative acyl CoA ligases in *Bacillus subtilis* revealed that two of them activate 3-hydroxy fatty acids for surfactin biosynthesis in vitro and that the disruption of corresponding genes has a significant influence on surfactin production.

INTRODUCTION

Natural products are of special importance in pharmaceutical and biotechnological applications and the design of new antibiotic variants from natural scaffolds is a field of intense research, because of upcoming multiresistant bacterial strains. One significant class of antibiotics is the bacterial lipopeptides such as daptomycin or CDA, which are mostly cyclic and contain a fatty acid attached to the N-terminal amino acid (Baltz et al., 2005; Strieker and Marahiel, 2009). The biosynthesis of many lipopeptides and other secondary metabolites is carried out by multienzyme complexes, the so-called nonribosomal peptide synthetases (NRPS) (Mootz et al., 2002). In recent years, a significant amount of information has been collected about the molecular mechanisms of nonribosomal peptide synthesis and about product release by cyclization (Sieber and Marahiel, 2005; Walsh and Fischbach, 2010). However, the detailed mechanism of the lipoinitiation reaction of lipopeptides is still widely unknown. One prominent and well studied member of the class of lipopeptides is the NRPS product surfactin. It is a cyclic lipoheptapeptide lactone produced by *Bacillus subtilis* that contains two acidic amino acids (glutamate and aspartate) beside five nonpolar residues and one 3-hydroxy fatty acid (Figures 1A and 1B) (Kakinuma et al., 1969). The fatty acid moiety varies in chain length between 13 and 15 carbon atoms as well as in branching of the chain (Bonmatin et al., 2003). The peptide backbone also shows divergence at position 7, where the leucine residue was observed to be substituted in some cases by isoleucine or valine. Despite its discovery over 40 years ago and its early primary structure elucidation (Arima et al., 1968; Kakinuma et al., 1969), surfactin is still a subject of intensive research. Due to its excellent properties as a biotenside, it represents an attractive alternative to chemical tensides especially in environmental applications (Mulligan, 2005; Peypoux et al., 1999). Furthermore, it has been shown that surfactin exhibits antibacterial, -viral, -tumoral, and antimycoplasmatic activities, which make it an interesting target for therapeutic applications (Cao et al., 2009; Kameda et al., 1972; Nissen et al., 1997; Tsukagoshi et al., 1970; Vollenbroich et al., 1997). Only its cytotoxicity against erythrocytes, which are lysed by surfactin at concentrations above 40 μM, prevents surfactin from clinical applications (Heerklotz and Seelig, 2007).

The organization of the biosynthetic gene cluster of surfactin was published in the early 1990s by different research groups (Cosmina et al., 1993; Fuma et al., 1993; Nakano et al., 1988). It consists of four biosynthetic genes: three of them encode the nonribosomal peptide synthetases SrfAA, SrfAB, and SrfAC (Figure 1A), while the terminal gene *srfD* encodes a protein with high homology to external thioesterases of type II. It has been reported that SrfD is a repair enzyme, which regenerates with acetyl-CoA or with incorrect amino acids mischarged peptidyl carrier protein (PCP) domains during NRPS assembly (Schwarzer et al., 2002; Yeh et al., 2004). Its positive effect on surfactin production was shown by gene disruption in 1998 (Schneider and Marahiel, 1998) and its NMR structure and reaction mechanism were elucidated recently (Koglin et al., 2008).

The surfactin biosynthesis starts with the acylation of the first amino acid glutamate, which is activated by the three-modular enzyme SrfAA (Menkhaus et al., 1993; Steller et al., 2004). This ten domains comprising synthetase exhibits a special N-terminal condensation (C) domain that is quite unusual for an initiation module. However, NRP-synthetases producing lipopeptides usually feature such N-terminal C domains and that is why they are believed to be involved in the acylation step. The following two leucine residues in surfactin are assembled by the second and the third module of the synthetase SrfAA as well. The third

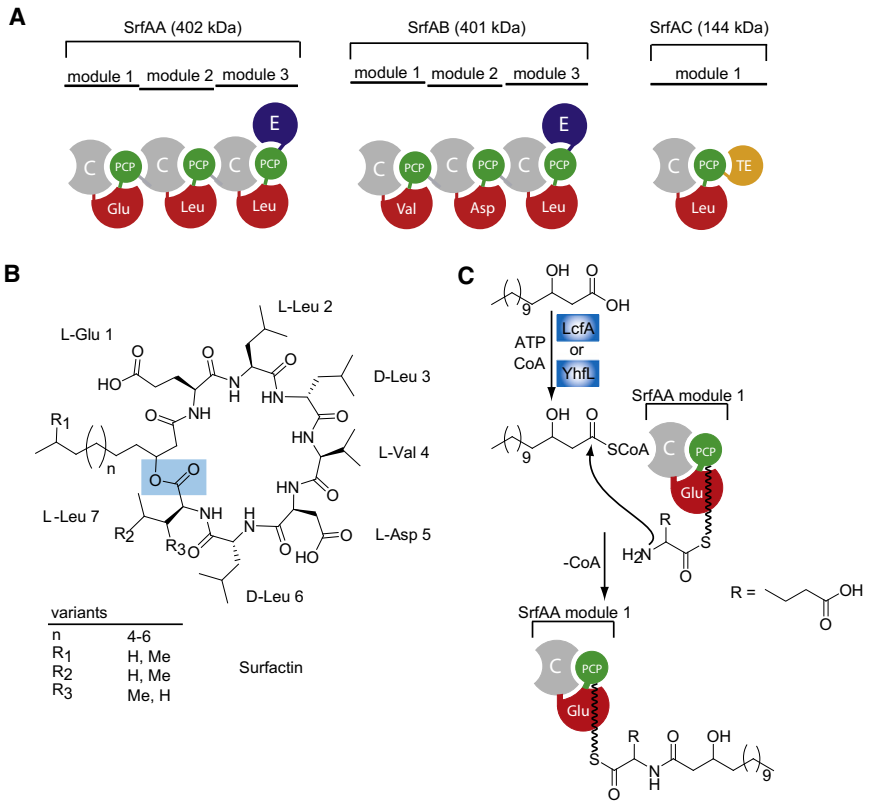

Figure 1. Biosynthesis and Structure of Surfactin

(A) Assembly line of surfactin biosynthesis with condensation domains colored gray, adenylation domains in red, and peptidyl carrier proteins in green. Epimerization domains are shown in blue, the thioesterase domain in orange.

(B) Structure and variants of surfactin. The lactone bond is colored in light blue.

(C) Proposed lipidation reaction during surfactin biosynthesis. The fatty acid is activated by LcfA or YhfL and then recognized by the donor site of the C domain catalyzing the attack of the amino group of PCP-bound glutamate from the acceptor site. Domain coloring is analogous to (A).

module converts L-Leu into the D-configurated isomer by its C-terminal epimerization domain (Figure 1A). SrfAB incorporates the following three amino acids (Val-Asp-D-Leu) while SrfAC is responsible for the activation and incorporation of the last leucine residue and catalysis of product release by cyclization (Cosmina et al., 1993; Tseng et al., 2002).

In this article, we explore the first step of the surfactin biosynthesis, the lipoinitiation reaction (Figure 1C). Fatty acid moieties strongly influence the activity and properties of natural products. Therefore, understanding the mechanism of this reaction is of special interest as it might facilitate the design of new products with improved properties. In an initial reaction, the fatty acid moiety has to be activated before incorporation into the assembly line. Although such activation or transfer enzymes have not been identified within the biosynthetic gene cluster of surfactin, it has been shown that the assembly line machinery starts in the presence of 3-hydroxy fatty acid coenzyme A (CoA) thioester (Menkhaus et al., 1993; Steller et al., 2004). Here, we report on biochemical and gene deletion studies of four putative fatty acyl CoA ligases (FACLs) of *B. subtilis* that reveal their involvement in activation of fatty acids during surfactin synthesis. Furthermore, the results in this study show that the acylation step is carried out by the dissected starter C domain, which was recombinantly produced and shown to catalyze the condensation between the first amino acid glutamate bound to the PCP domain and the CoA-activated fatty acid. Site-directed mutagenesis of the starter C domain clarified the role of two histidine residues of the conserved core motif HHXXXDG.

RESULTS

Activation of 3-Hydroxy Myristic Acid by Fatty Acyl CoA Ligases

It was shown that the assembly line can be initiated in presence of the CoA-activated fatty acid (Steller et al., 2004). Since no acyl CoA ligases are encoded in the surfactin biosynthetic gene cluster (Menkhaus et al., 1993), an in *trans* activation of fatty acids by enzymes encoded in other gene clusters was considered. Four putative fatty acyl CoA ligases were identified, based on homology searches, in the genome of *B. subtilis*: LcfA, YhfL, YngI, and YhfT. The functions of these enzymes were unknown up to now, but LcfA and YhfL were proposed to activate fatty acids for degradation (Matsuoka et al., 2007). To investigate their putative role in surfactin biosynthesis, the corresponding genes were cloned and recombinantly expressed for in vitro studies. Additionally, knockout mutants of FACLs encoding genes were generated to allow in vivo studies. All four FACL enzymes were obtained as His-tagged fusion proteins, whereas YngI and YhfL were cloned into the pBAD102/D-TOPO vector which yielded thioredoxin fusion proteins. LcfA was cloned into the pQE60 vector and YhfT into the pCB28a(+) vector both resulting in C-terminally His-tagged fusions. The proteins were purified by affinity chromatography, yielding 0.8 to 4.8 mg/l of culture. Their purity and identity were confirmed by SDS-PAGE (see Figure S1 available online) and mass spectrometric analysis (data not shown).

To determine the activity of the FACLs, the ligases were incubated with 3-hydroxy myristic acid (HMA), ATP, and CoA as described in Experimental Procedures. The assays were analyzed by HPLC-MS (Figure 2) and it became obvious that LcfA and YhfL catalyze the thioester formation with CoA while YngI showed no activity in this reaction. In the case of YhfT, only the fatty acid adenylate intermediate could be detected under different reaction conditions. Additionally, the substrate specificity of the in vitro active enzymes LcfA and YhfL was tested. 3-Hydroxy acids harboring eight or ten carbon atoms were activated by LcfA as well as by YhfL. Linear fatty acids comprising 12 or 14 carbon atoms without a 3-hydroxy group

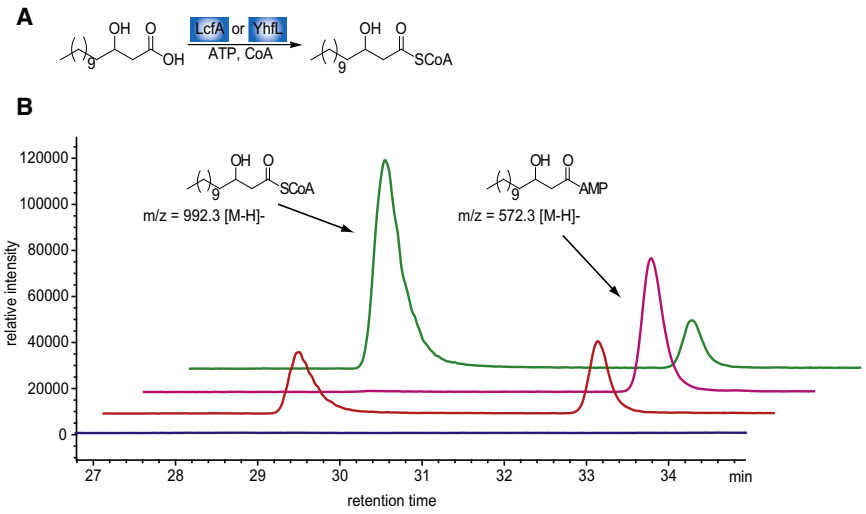

Figure 2. Fatty Acid Activation by FACLs from *B. subtilis*

(A) Reaction scheme of the activation of HMA by LcfA or YhfL.

(B) HPLC-MS analysis of the activation of HMA by LcfA, YhfL, or YhfT. Blue line: control reaction without enzyme; red line: reaction with LcfA; magenta line: reaction with YhfT; green line: reaction with YhfL. The ESI-MS signals were measured in negative single ion mode.

were accepted as well, whereas YhfL showed slightly higher activity than LcfA. The tested fatty acids are listed in Table S1. Notably, some fatty acids, for example 3-hydroxy decanoic acid or *n*-dodecanoic acid, which are not found in native surfactin, were coupled to CoA by LcfA and YhfL.

To confirm that the investigated FACLs are in fact involved in surfactin biosynthesis, knockout mutants of the four genes *lcfA, yhfL, yhfT*, and *yngI* were constructed in *B. subtilis* OKB105 and surfactin production of these mutant strains was monitored. Wild-type and the generated mutant strains were grown in SpIII medium until they reached the stationary phase. After removing cells by centrifugation, surfactin was extracted from the supernatant with butan-1-ol. Product analysis by HPLC-MS showed for all four single mutants that the surfactin production was decreased by 38%–55% compared with the wild-type level (Table 1). In several double and triple knockout mutants, the amount of isolated surfactin was still between 20% and 65% compared with the wild-type. Surprisingly, deletion of all four genes (*lcfA*, *yhfL*, *yhfT*, *yngI*) did not completely abolish surfactin production; however, a significant decrease by 84% compared with the wild-type level was observed.

Transfer of 3-Hydroxy Myristic Acid onto the First Module of SrfAA

After investigation of the fatty acid activation step, the transfer of the fatty acid moiety to the nonribosomal peptide assembly line of surfactin was examined. For these studies, the first module (C-A_{Glu}-PCP) of the synthetase SrfAA was cloned into a pQE60 vector and the resulting His-tagged protein (SrfAA-M1) was purified with a yield of 1.6 mg/l of culture (Figure S2). The 4′-phosphopantethein cofactor of the PCP domain was subsequently transferred from CoA onto the recombinant *apo*-protein by the 4′-phosphopantetheinyl transferase Sfp from *B. subtilis* to generate *holo*-SrfAA-M1 (Mootz et al., 2001). The transfer was confirmed by using fluoresceinyl-*S*-CoA and SDS-PAGE analysis. In presence of Sfp, the emergence of a fluorescent band of the module's PCP domain was observed (Figure S3). The activity of the SrfAA-M1 adenylation (A) domain was verified by ATP/PP_i exchange (Figure S4) (Lee and Lipmann, 1975). To determine whether the fatty acid can be transferred onto the *holo*-module, *holo*-SrfAA-M1 was incubated with glutamate and ATP to load the amino acid onto the PCP domain and subsequently incubated with chemically synthesized HMA-*S*-CoA (Blecher, 1981; Hiramoto et al., 1971). The thioester bond between the product intermediate and the PCP domain was hydrolyzed either by potassium hydroxide or enzymatically by the external type II thioesterase SrfD to detect the acylation product HMA-Glu via HPLC-MS analysis. The HMA-Glu condensation product was only observed in the presence of fatty acid-*S*-CoA but not in the presence of free fatty acid (Figure 3). To explore the enzymatic reaction of the fatty acyl CoA ligases LcfA and YhfL in fatty acid activation and subsequent transfer, coupled assays with free fatty acid, the glutamate-loaded module and the FACLs, in presence of ATP and CoA, were performed. For both ligases the acylation product after thioester hydrolysis could be detected (Figure 3).

Catalytic Role of the Starter C Domain SrfAA-C1

To determine the role of the starter C domain of SrfAA in surfactin biosynthesis, the C domain and the PCP domain of the first module SrfAA were cloned independently into pCB28a(+) vectors. The resulting C-terminally His-tagged proteins were purified by affinity chromatography yielding 7.1 and 0.8 mg/l of culture, respectively. The recombinant proteins were identified by SDS-PAGE (Figure S5) and mass spectrometry (data not shown). In a first reaction, it was validated that the PCP domain can be loaded with the cofactor 4′-phosphopantethein using CoA and Sfp and that chemically synthesized glutamate-*S*-CoA is transferred to the *apo*-PCP domain as well. Both reactions showed that Sfp transfers the 4′-phosphopantetheinyl group and the glutamate derivative onto the PCP domain. The next step to be analyzed was the acylation of PCP-bound glutamate with HMA. The glutamate-loaded PCP domain was incubated with the recombinant C domain and either with HMA-*S*-CoA or with the free fatty acid and the ligases LcfA or YhfL in presence of ATP and CoA. After hydrolysis of the thioester bond between the acylated glutamate and the *holo*-PCP domain with the external thioesterase SrfD or KOH, the supernatant was analyzed by HPLC-MS. Control reactions without the C domain or with free glutamate were carried out accordingly. In the presence of the dissected starter C domain the acylation product of glutamate and HMA could be observed

Table 1. Surfactin Production of Some Deletion Mutants of FACLs

Knockout Mutant	OD_{600} before Surfactin Extraction	% of WT[a] Production
ΔlcfA	1.34	45.6
ΔyhfT	1.55	53.0
ΔyhfL	1.34	44.5
ΔyngI	1.63	62.3
ΔyhfLΔyngI	1.55	46.2
ΔlcfAΔyhfTΔyhfL	1.36	36.0
ΔlcfAΔyhfTΔyngI	1.60	20.1
ΔlcfAΔyhfLΔyhfTΔyngI	1.36	16.4

Results from selected mutant strains are listed as exemplary values of surfactin production. All values from the generated mutant strains can be found in Table S2.
[a] WT, wild-type.

in similar amounts compared to the intact module (Figure 4). Fatty acid coupling was observed with the fatty acid CoA thioester as well as with the free fatty acid activated in situ by the ligases LcfA or YhfL. The control reaction without the C domain or with free glutamate instead of PCP-bound glutamate showed no acylation product, indicating that the C domain catalyzes the acylation of PCP-bound glutamate with the fatty acid-*S*-CoA.

To determine the specificity of the C domain at the donor and acceptor sites, different fatty acids as well as different PCP domains loaded with glutamate and other amino acids were investigated for the condensation reaction (Table 2). From the tested fatty acids, only HMA was accepted by the C domain, while the acylation product could be observed with all four foreign PCP domains, originated from various NRP synthetases and loaded with glutamate-*S*-CoA (Table 2). These were TycC-PCP5 and TycC-PCP6 from the tyrocidine biosynthesis cluster of *Bacillus brevis* (Samel et al., 2007), CDAPS2-PCP3 from the CDA biosynthetic gene cluster of *Streptomyces coelicolor* (Strieker et al., 2007), and the stand-alone PCP domain Cgc19 from *Streptomyces ambofaciens* that is thought to be involved in congocidine biosynthesis (Juguet et al., 2009).

To determine the substrate specificity of the acceptor site of the C domain for other amino acids, phenylalanine, lysine, threonine, and asparagine were coupled to CoA and loaded onto SrfAA-PCP1 by Sfp. Phenylalanine, lysine, and threonine were not accepted as acylation substrates, only for asparagine very little amount of acylation product could be detected (Table 2).

Sequence analysis of condensation domains revealed the conserved core motif HHXXXDG, which was predicted to be involved in the catalysis of peptide bond formation (De Crecy-Lagard et al., 1995). Support for this came from site-directed mutagenesis studies (Stachelhaus et al., 1998; Vater et al., 1997). The starter C domain of SrfAA exhibits this motif as well. An alignment of different starter C domains (Figure S6) of the CDA synthetase CDAPS1, the friulimicin synthetase PstB, and of the daptomycin synthetase DptA shows that this motif is highly conserved within initiation condensation domains (Rausch et al., 2007). To determine if the conserved histidine residues of starter C domains are involved in the catalysis of the lipid transfer, as it is the case for peptide bond formation (Roche and Walsh, 2003; Stachelhaus et al., 1998), the histidine motif was point mutated by site-directed mutagenesis. H139A, H140A, and H139A/H140A variants were isolated by purification after recombinant expression. The variants were applied in the acylation reaction analogously to the wild-type C domain. In the case of the variants H140A/H139A and H140A, the acylation product formation was abolished showing the necessity of

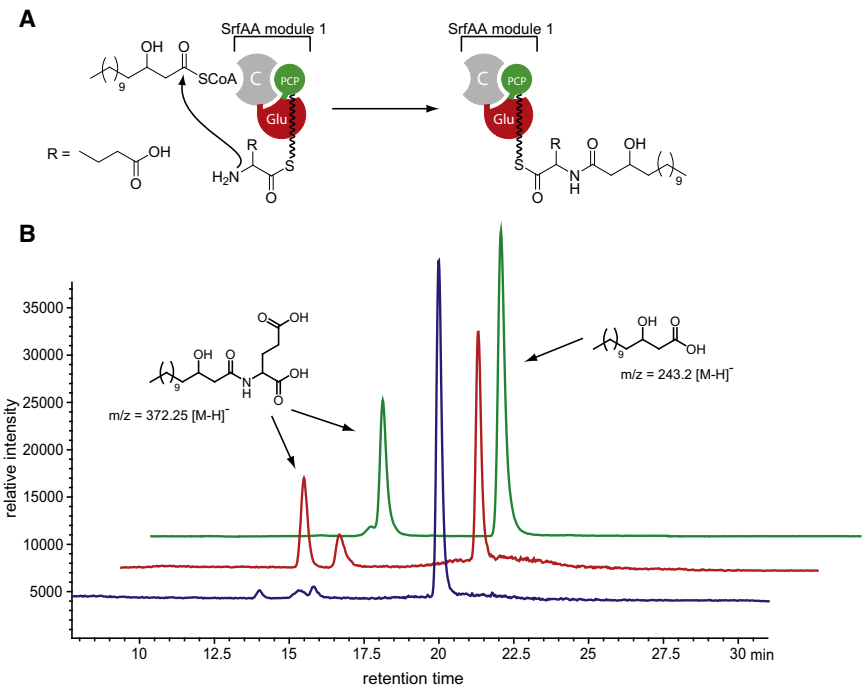

Figure 3. Transfer of HMA onto Glutamate-Loaded Initiation Module of SrfAA
(A) Reaction scheme of the transfer step of HMA. (B) HPLC-MS analysis of the transfer of in situ LcfA activated HMA onto glutamate-loaded SrfAA-M1. Blue line: control reaction without LcfA; red line: reaction with LcfA and free HMA; green line: reaction with HMA-*S*-CoA. Signals were measured in negative single ion mode.

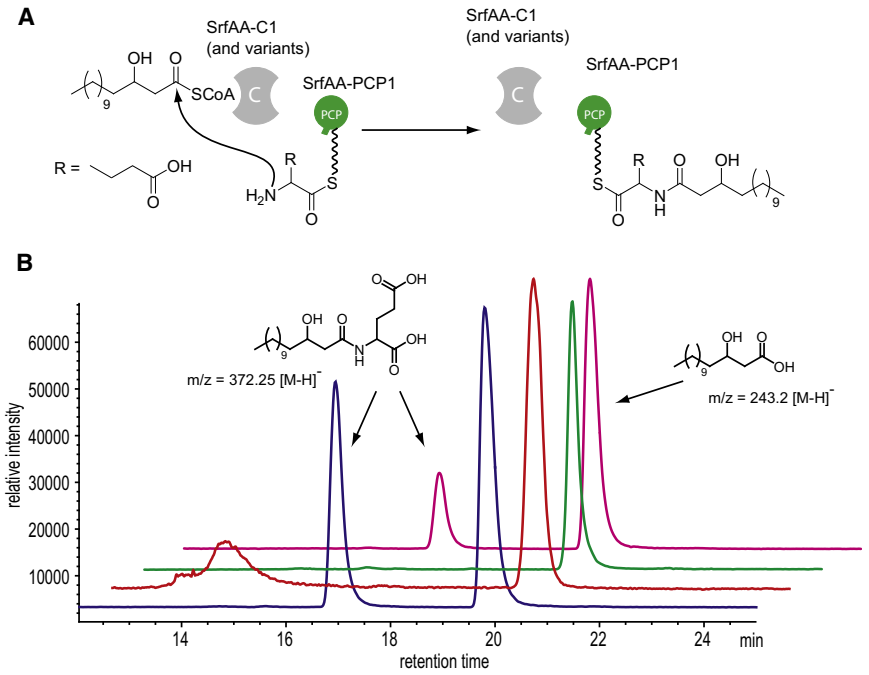

Figure 4. Acylation of PCP-Bound Glutamate Catalyzed In *Trans* by Dissected SrfAA-C1 and Its Variants

(A) Reaction scheme of the acylation step.

(B) HPLC-MS analysis of the fatty acid transfer onto PCP-bound glutamate by the C domain SrfAA-C1 and its variants. Blue line: transfer reaction in presence of wild-type C domain SrfAA-C1; red line: reaction in presence of C domain variant H139AH140A; green line: reaction in presence of C domain variant H140A; magenta line: reaction in presence of C domain variant H139A. Signals were measured in negative single ion mode.

the second histidine residue H140 for catalytic activity (Figure 4). However, the acylation product could be detected in presence of the variant H139A in slightly less amounts than in the case of the wild-type C domain. This shows that the first histidine residue of the core motif HHXXXDG is not essential for catalytic activity while the second one seems to be indispensable.

DISCUSSION

In this study, we investigate the four putative fatty acyl CoA ligases LcfA, YhfL, YhfT, and YngI from *Bacillus subtilis* as possible in *trans* acting enzymes involved in fatty acid-*S*-CoA synthesis during surfactin production. The activation of HMA as thioester with CoA catalyzed by LcfA and YhfL could be shown in vitro. The observed broad tolerance of these ligases for fatty acids with similar chain length is quite usual for this class of enzymes (Black et al., 1997). For YhfT, only the acyl adenylate intermediate and no formation of HMA-*S*-CoA could be observed. However, involvement of YhfT in surfactin biosynthesis by transfer of the acyl AMP derivative onto an ACP, which may in turn transfer the intermediate to the surfactin assembly line, cannot be excluded. ACPs, which can be loaded with the fatty acid moiety for the synthesis of lipopeptides, are found recently in the case of daptomycin or CDA (Wittmann et al., 2008; Powell et al., 2007; Kopp et al., 2008). The ACPs and transfer enzymes are encoded within the corresponding biosynthetic lipopeptide gene clusters, which is not the case in surfactin, but further experiments are still needed to clarify the role of YhfT in surfactin biosynthesis. YngI showed no activity in vitro which might be explained by using the wrong substrates or by the fact that YngI was expressed as a fusion protein with thioredoxin, which could abolish the activity of the protein. In vivo studies with deletion mutants of these FACLs showed decreased surfactin production in almost every mutant strain. Single knockout mutants were not affected in surfactin production (between 45% and 62% of the wild-type level) to a large extent, suggesting that the considered FACLs are at least partially redundant or that they complement each other. Deletion of two or three FACLs coding genes simultaneously led to considerably decreased surfactin production, demonstrating clearly the involvement of these enzymes in surfactin biosynthesis (Table 1). This finding is also supported by the mutant strain missing all four FACLs, as it shows a surfactin production of only 16% compared with the wild-type level. These findings suggest that the cellular pool of CoA-activated 3-hydroxy fatty acids needed for initiation of surfactin synthesis is in fact the result of several CoA ligases all acting in *trans*. Nevertheless, it seems that there are still other pathways providing the fatty

Table 2. Specificity of SrfAA-C1 Domain

Donor Site		Acceptor Site			
Fatty Acid	Reaction	Amino Acid	Reaction	PCP Domain	Reaction
3-Hydroxy tetradecanoic acid (HMA)	+	Glutamate	+	SrfAA-PCP1	+
Octanoic acid	–	Asparagine	(+)	CDA-PCP9	+
Decanoic acid	–	Phenylalanine	–	TycC-PCP5	+
Hexadecanoic acid-*S*-CoA	–	Lysine	–	TycC-PCP6	+
Decanoic acid-*S*-CoA	–	Threonine	–	Cgc19	+
3-Hydroxy butyric acid-*S*-CoA	–				

acid moiety for surfactin production, for example, through transthiolation from ACPs to CoA.

In addition, the transfer of the activated fatty acid onto the assembly line was investigated. It could be shown by experiments with the recombinant dissected C domain interacting with the glutamate-loaded PCP domain and fatty acid-*S*-CoA that this step is unambiguously catalyzed by the starter C domain SrfAA-C1. The native activity of the dissected C domain in *trans* allowed detailed studies to explore its substrate specificity and the catalytic role in the lipoinitiation reaction. It has been shown for some elongation C domains that noncognate substrates are accepted at the donor site (Belshaw et al., 1999; Doekel and Marahiel, 2000; Linne et al., 2003; Marshall et al., 2001). However, our present study revealed a distinct specificity at the donor site of the starter C domain, accepting only cognate fatty acids. The fatty acid CoA derivatives for surfactin biosynthesis seem to be provided by enzymes of the primary metabolism of *Bacillus subtilis*. The cell contains many different fatty acid-*S*-CoA substrates which are not all incorporated in the product surfactin. This requires a higher specificity of the starter C domain at its donor site than in elongation C domains where the incorporation of substrates is additionally controlled by A domain specificity.

Not only the donor site of the starter C domain shows a distinct specificity, but also the acceptor site recognizes beside the native amino acid glutamate only the PCP-bound amino acid asparagine that is not unusual for the acceptor sites of condensation domains (Belshaw et al., 1999; Lautru and Challis, 2004; Linne and Marahiel, 2000). However, the PCP domain does not seem to have a higher impact on recognition by the C domain, since the acylation product could be obtained with all tested *holo*-PCP domains loaded with glutamate. The interactions between the C domain and the PCP domain are either not so strong or they might be formed by three-dimensional structural elements which are conserved between the different PCP domains.

Site-directed mutagenesis of the catalytic core motif HHXXXDG of the condensation domain showed the necessity of the second histidine residue (H140) for the catalytic activity in the acylation step. This result is in agreement with earlier studies of elongation C domains of the gramicidin and surfactin synthetases or of the dissected elongation C domain of EntF, where the catalytic role of the second histidine had been shown (Roche and Walsh, 2003; Stachelhaus et al., 1998; Vater et al., 1997). Stachelhaus et al. (1998) proposed a mechanism of peptide bond formation in nonribosomal peptide synthesis analogous to the reaction catalyzed by some acyltransferase subfamilies exhibiting the His-motif as well. Accordingly, the second histidine acts as a catalytic base abstracting a proton from the α-amino group of the amino acid to promote the nucleophilic attack on the carbonyl group of the previous amino acid of the growing peptide chain. Recently, a PCP-C bidomain structure from the tyrocidine synthetase TycC has been solved by Samel et al. (2007) where the second histidine residue is proposed to serve rather as electrostatic stabilization mediator of the putative, zwitterionic reaction intermediate than as general acid/base catalyst. In the case of the starter C domain of SrfAA, the second histidine residue of the core motif HHXXXDG (H140) seems to have a similar relevance for amide bond formation between the fatty acid moiety and the amino acid glutamate. The first histidine residue H139 seems not to be directly involved in the catalytic mechanism as the activity of the C domain variant H139A is not abolished; the amount of acylation product is only slightly decreased. These findings are in agreement with previous studies of the PCP-C bidomain structure, which revealed that the second histidine is pointing toward the active site canyon of the V-shaped condensation domain while the first histidine is turned away from the active site canyon (Samel et al., 2007).

In conlusion, the in vivo and in vitro studies revealed clearly the involvement of the FACLs LcfA, YhfL, YngI, and YhfT in the activation step of the fatty acid during surfactin biosynthesis. However, the residual surfactin production of the quadruple deletion mutant indicates that small amounts of activated fatty acid still can be provided for surfactin production by other pathways. Furthermore, it could be shown that the acylation step of the N-terminal glutamate is executed by the starter C domain with a very distinct specificity for the chain length of 3-hydroxy fatty acids and the PCP-bound amino acid glutamate. This amide bond formation between a fatty and an amino acid is another example for the versatility of reactions catalyzed by C domains, which is also shown by an ester bond forming C domain or dual condensation/epimerization domains (Lin et al., 2009; Balibar et al., 2005).

Based on the results presented in this study, we propose the mechanism shown in Figure 1C for the initiation of the biosynthesis of surfactin. HMA is first activated by a FACL as CoA thioester and then recognized by the donor site of the C domain which catalyzes the nucleophilic attack of the α-amino group of the PCP-bound glutamate at the thioester bond of the fatty acid moiety. The resulting acylated glutamate is then accepted by the next C domain and the peptide assembly can continue, leading to product formation and release.

SIGNIFICANCE

The nonribosomal lipoheptapeptide surfactin is an important biotenside and is used in some biotechnological applications (Mulligan, 2005). Despite its interesting antiviral, antitumoral and antibacterial properties, the therapeutic applicability is difficult due to the hemolytic effect of surfactin on erythrocytes (Heerklotz and Seelig, 2007). Since the bioactivity is strongly influenced by the fatty acid moiety, complete biochemical characterization and understanding of the lipoinitiation might lead to the design of new products with improved antibacterial properties and less toxicity to eukaryotic cells.

The involvement of the four investigated FACLs in the surfactin biosynthesis found by biochemical and gene disruption studies confirms the suggestion that the activated fatty acids for surfactin production are provided in *trans* by enzymes from biological pathways of the primary metabolism.

This study reveals the important role of the starter condensation domain in the lipid transfer, making it an interesting target for natural product engineering. By changing the specificity for fatty or even for amino acids, new surfactin variants could be produced and important insights into the lipoinitiation reaction could be gained. The fact that

other PCP domains are accepted at the acceptor site in the acylation reaction is a good basis for various approaches of creating hybrid enzymes to synthesize new natural products. Such initiation C domains could be introduced into foreign NRPSs to generate novel lipopeptides by domain swapping (Fischbach et al., 2007; Suo, 2005).

EXPERIMENTAL PROCEDURES

Bacterial Strains and General Methods

Escherichia coli NEB 10-beta was purchased from New England Biolabs and used for cloning procedures of site-directed mutagenesis purposes. *Escherichia coli* TOP10 was used for cloning procedures and were purchased from Invitrogen. For heterologous expression *Escherichia coli* BL21(DE3) was used and purchased from Novagen. Applied and generated strains from *Bacillus subtilis* are listed in Table S2. Oligonucleotides were purchased from Sigma-Aldrich. DNA dideoxy sequencing by GATC Biotech confirmed the identity of constructed plasmids.

Cloning Procedure and Site-Directed Mutagenesis

Phusion High-Fidelity DNA-Polymerase (New England Biolabs) was used for PCR amplification of the genes *yhfL*, *yhfT*, *lcfA*, *yngI*, *srfAA*-M1, *srfAA*-C1, and *srfAA*-PCP1 from genomic DNA of *Bacillus subtilis* and site-directed mutagenesis following the instructions of the manufacturer. The generated constructs and employed oligonucleotides are shown in Table S3. The inserts amplified by PCR were digested with appropriate restriction enzymes and cloned into the vectors pCB28a(+) (*yhfT*, *srfAA*-C1, *srfAA*-PCP1), pBAD102/D-TOPO (*yhfL*, *yngI*), or pQE60 (*srfAA*-M1, *lcfA*). The construct pCB28a(+)_*srfAA*-C1 was used as template for site-directed mutagenesis. After PCR (oligonucleotides, see Table S3), the template DNA was digested with *Dpn*I and *E. coli* NEB 10-beta cells were transformed with the mutagenized plasmids. After selection on LB-agar plates containing the appropriate antibiotics over night, plasmids were isolated from transformants and analyzed by sequencing.

Heterologous Production and Purification of Recombinant Proteins

For heterologous expression of the recombinant proteins, *E. coli* BL21(DE3) cells were transformed with the corresponding plasmids. Cells were grown in LB medium containing the appropriate antibiotic overnight at 37°C. The culture was then used to inoculate 5 liters LB medium (1:100) portioned in 500 ml. IPTG was added at an OD_{600} of 0.6 in a final concentration of 0.1 mM. The cells were harvested after further incubation for 16 hr at 18°C by centrifugation (5000 rpm, 20 min, 4°C). The pellet was suspended in buffer A (50 mM HEPES [pH 8.0], 300 mM NaCl) and the cells were disrupted using a French press (SLM Aminco; Thermo French press). The lysate was centrifuged to remove cell debris (17,000 rpm, 30 min, 4°C), and the His-tagged fusion proteins were purified using Ni^{2+}-NTA superflow resin from QIAGEN. The affinity chromatography was performed using a FPLC system (Amersham Pharmacia Biotechnology) or an Äkta Prime system employing gradient elution with increasing imidazole concentration. Designated protein fractions were identified by SDS-PAGE, pooled and subjected to buffer exchange (25 mM HEPES buffer [pH 7.0], with 50 mM NaCl) using HiTrap desalting columns (GE Healthcare Europe GmbH). The recombinant protein fractions were then concentrated by membrane-based Amicon Ultra-15 concentrators (Millipore GmbH), and the concentration was calculated with the help of NanoDrop spectrophotometer ND-1000 (PeqLab Biotechnologie GmbH) measurements using calculated extinction coefficients. The purified proteins were stored at −80°C.

Generation of Deletion Mutants in *B. subtilis* OKB105

The deletion mutants of long-chain fatty acyl CoA ligases were generated by exchanging the corresponding gene with a resistance cassette by homologous recombination in *B. subtilis* following the method reported by Kuwayama et al. (2002). In brief, the resistance cassette is fused with DNA fragments, which are flanking the target gene in the chromosomal DNA, by fusion PCR (for utilized oligonucleotides, see Table S4) and then used to transform *B. subtilis* OKB105, using the method reported by Klein et al. (1992). In the case of the *yhfL* deletion mutant, the resistance cassette fused to the flanking regions was first transformed into the strain MR168. Subsequently, the chromosomal DNA was prepared, and, after confirmation of the deletion, used for transformation of the strain OKB105. After transformation, the cells were incubated on LB-agar plates containing the corresponding selection marker overnight at 37°C. The colonies were lysed in 50 μl lysis buffer (1% Triton X-100; 20 mM Tris-HCl [pH 8.0]; 2 mM EDTA), and after centrifugation (10 min, 13,000 rpm) 4 μl of supernatant was used for PCR confirmation of the deletion in chromosomal DNA. Multiple deletion mutants were generated by using different resistance cassettes for each target gene (*lcfA::mls, yhfT::spc, yngI::tet, yhfL::kan*) fused to the corresponding up- and downstream DNA fragments.

Extraction of Surfactin

The extraction of surfactin from wild-type or mutated *B. subtilis* OKB105 or MR168 strains was done in accordance to the method of Nakano, et al., (1988). 20 ml SpIII medium were inoculated with an overnight culture of each strain (1:40, v/v) and grown at 37°C until stationary phase (OD_{600} ~1.5) was reached. 2 ml of each culture was taken and centrifuged for 5 min at 13,000 rpm. The supernatant was extracted three times with 500 μl butan-1-ol, the combined organic extracts were evaporated to dryness at 45°C using a SpeedVac (Eppendorf) and the remnant was dissolved in 50 μl methanol (70%, v/v) for HPLC-MS analysis. A 250/3 Nucleosil 120-3 C8 column was used at a temperature of 45°C with a linear gradient from 70% buffer B up to 100% buffer B in 30 min with a flow rate of 0.3 ml/min to separate surfactin variants (buffer A: 0.05% formic acid in water; buffer B: 0.045% formic acid in methanol). The detection was carried out by UV light at 214 nm and coupled ESI-MS analysis (Agilent 1100 MSD) in positive scan mode ranging from 450 to 1200 Da (Figure S7).

Activity Assay of FACLs

The four FACLs from *B. subtilis* LcfA, YhfL, YhfT, and YngI were tested in an activation reaction of long-chain fatty acids, which are considered to be substrates for surfactin biosynthesis, as CoA thioesters. In a typical reaction mixture (100 μl) 25 mM HEPES (pH 7.0), 50 mM NaCl, 1 mM $MgCl_2$, 1 mM ATP, 10 mM CoA, 100 μM, or 500 μM HMA and 1% dimethylsulfoxide were incubated with 2 μM enzyme at 37°C for 30 min. The reactions were stopped with 30 μl formic acid. For investigation of the specificity of the active enzymes LcfA and YhfL in vitro, the same assay composition was used with different fatty acids as substrates (Table S1). Product formation was analyzed by HPLC-MS. Separation of starting materials and products was achieved on a 125/3 Nucleodur 100-3 C8 ec column (Macherey & Nagel GmbH & Co.KG) at a column temperature of 30°C by applying the following gradient at a flow rate of 0.2 ml/min (buffer A: 2 mM triethylamine/water, buffer B: 2 mM triethylamine/acetonitrile): 5% buffer B (5 min), 5%–95% buffer B (30 min), and then 95% buffer B for further 5 min. The product was identified by UV detection at 215 nm coupled to ESI-MS analysis (Agilent 1100 MSD) in negative single ion mode (SIM).

Transfer of 3-Hydroxy Myristic Acid onto SrfAA-M1

The transfer of the fatty acid onto the first module of the synthetase SrfAA was performed either with CoA-activated fatty acid, which was chemically synthesized (Hiramoto et al., 1971; Blecher, 1981) or with free fatty acid in presence of one of the FACLs LcfA, YhfL, YngI, or YhfT to activate the acid in situ. In a typical reaction mixture (100 μl) 50 mM HEPES buffer (pH 7.0), 25 mM NaCl, 10 mM $MgCl_2$, 2 mM DTT, 1 mM CoA, and 10 μM SrfAA-M1 were incubated at 37°C for 30 min with 1 μM of the 4′-phosphopantetheine transferase Sfp to generate the *holo*-form of the PCP domain. Glutamate (1 mM) and ATP (1 mM) (final concentrations) were then added to load the PCP domain with the amino acid by A-domain catalysis. After incubation for 30 min at 37°C, 500 μM HMA, 1% dimethylsulfoxide, and 2 μM of a FACL were added and incubated for another 30 min. Alternatively, 500 μM HMA-*S*-CoA was added in the last step instead of free fatty acid and activation enzymes. After incubation time, the thioester bond between product and PCP domain was hydrolyzed either by precipitation with 10% TCA followed by treatment of the pellet with 100 μl 0.1 M KOH at 70°C or by addition of the external thioesterase TEII (SrfD) to the reaction mixture for the last 10 min. The proteins were then removed by precipitation with methanol and the supernatant was analyzed

by HPLC-MS with the same method as for fatty acid activation assays (see above).

Transfer of 3-Hydroxy Myristic Acid onto PCP-Bound Glutamate by Catalysis of the Stand-Alone C Domain SrfAA-C1

20 μM *apo*-PCP domain were loaded with glutamate using 1 μM Sfp and 5-fold excess of glutamate-*S*-CoA (Glu-*S*-CoA) instead of free CoA. Reaction conditions were the same as described for the priming step of the module (see above). Glu-*S*-CoA was synthesized chemically with the method reported by Strieker et al. (2007). After loading of the PCP domain, 40 μM SrfAA-C1 (or variants) and 100 μM HMA-*S*-CoA or 100 μM free HMA, 1% dimethylsulfoxide and 2 μM of a FACL were added to the reaction mixture. The thioester bond between PCP domain and product was hydrolyzed either by KOH or by SrfD analogously to SrfAA-M1 assays (see above). Product identification was achieved by HPLC-MS analysis after precipitation of the proteins with methanol (see above).

To test the specificity of the donor site of the C domain, different fatty acids were used for the transfer step in the same way. The specificity of the acceptor site was tested either by using different PCP domains loaded with Glu-*S*-CoA or by using different amino acids coupled to CoA (synthesis, see Strieker et al., 2007) and then loaded onto the SrfAA-PCP1 with Sfp. The reaction conditions and product analysis were the same as with the native system.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and four tables and can be found with this article online at doi:10.1016/j.chembiol.2010.06.015.

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