Coordinate Transcription and Physical Linkage of Domains in Surfactin Synthetase Are Not Essential for Proper Assembly and Activity of the Multienzyme Complex*

(Received for publication, February 4, 1998, and in revised form, March 23, 1998)

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Bacterial peptide synthetases have two common features that appear to be strictly conserved. 1) The enzyme subunits are co-regulated at both transcriptional and translational level. 2) The organization of the different enzymatic domains constituting the enzyme fulfills the “colinearity rule” according to which the order of the domains along the chromosome parallels their functional hierarchy. Considering the high degree of conservation of these features, one would expect that mutations such as transcription uncoupling and domain dissociations, deletions, duplications, and reshuffling would result in profound effects on the quality and quantity of synthesized peptides. To start testing this hypothesis, we designed two mutants. In one mutant, the operon structure of surfactin synthetase was destroyed, thus altering the concerted expression of the enzyme subunits. In the other mutant, the thioesterase domain naturally fused to the last amino acid binding domain of surfactin was physically dissociated and independently expressed. When the lipopeptides secreted by the mutant Bacillus subtilis strains were purified and characterized, they appeared to be expressed approximately at the same level of the wild type surfactin and to be identical to it, indicating that specific domain-domain interactions rather than coordinated transcription and translation play the major role in determining the correct assembly and activity of peptide synthetases.

A number of peptides with highly variable structures and a broad range of biological activities are produced in bacteria and fungi as secondary metabolites via a nonribosomal mechanism. The synthesis involves large multienzyme complexes called peptide synthetases that are organized in structural domains and utilize the thiotemplate mechanism first described by Lipmann (1) and recently revised (2).

According to this mechanism, each domain recognizes a specific amino acid that, after activation to the corresponding acyladenylate derivative, is covalently bound via a thioester bond to the phosphopantetheine cofactor present on each domain. The growth of the polypeptide chain occurs through a series of thioester bond cleavages and simultaneous formation of amide bonds. At the end of the synthesis, the peptide is supposed to be released from the enzyme by a thioesterase activity localized within the synthetase complex.

All prokaryotic peptide synthetases share highly conserved features (3, 4). First of all, they are organized in protein subunits consisting of one or more amino acid binding domains. At the DNA level, the order in which these domains are aligned along the chromosome parallels the sequence of the amino acids in the peptide (colinearity rule). For example, if the peptide has the sequence ABC, the domain recognizing amino acid A will be first on the chromosome, followed by the domains binding amino acids B and C. Secondly, the protein subunits coding genes are located within operon-type structures, with their expression coordinated at transcriptional level. Finally, fused at the C-terminal end of the last amino acid binding domain, there is a thioesterase-like domain, the function of which is most probably involved in the release of the peptide from the enzyme.

Although the striking conservation of these structural features would suggest their crucial role in enzyme assembly and functioning, no direct evidence of such a role has been reported so far.

In the present communication, we address two main questions. 1) Is transcription coordination strictly required for proper enzyme assembly and activity? 2) Can the thioesterase domain be physically dissociated from the last amino acid binding domain without impairing peptide synthesis?

To answer these questions we used surfactin synthetase as a model system. This enzyme, produced by Bacillus subtilis (5, 6), is responsible for the synthesis of the seven-amino acid lipopeptide surfactin and is organized in three protein subunits, the first two (srfAORF1 and srfAORF2) carrying three amino acid binding domains each and the third subunit (srfAORF3) having a single amino acid binding domain fused to a thioesterase moiety (TE)1 (7).

We constructed two B. subtilis mutants in which the surfactin synthetase was modified at chromosomal level. In the first mutant, a constitutive promoter was positioned upstream from srfAORF3, encoding the third enzyme subunit, thus making its synthesis transcriptionally dissociated from the other two subunits, which are in turn transcribed from the native, growth-regulated, srf promoter (8–10). In the second construct, the DNA region encoding the thioesterase domain was separated from the upstream region of the leucine binding domain of

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* The work was financed in part by a grant from the European Community, IV Framework Program (to G. Grandi) and in part by the contribution of the “Istituto Pasteur Fondazione Cenci Bolognetti” and the Italian Ministry of University and Scientific and Technological Research (to L. G.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: TE, thioesterase-like domain; HPLC, high pressure liquid chromatography; ORF, open reading frame.
srfAORF3 and expressed independently by adding appropriate regulatory elements. The data presented here demonstrate that the coordinate transcription of enzyme subunits and the physical linkage between amino acid binding and thioesterase modules are not strictly required for the correct assembly of a functional surfactin synthetase.

To our knowledge, this is the first report describing the feasibility of independent expression of modules and/or subunits in bacterial peptide synthetases. These results might have important implications in the engineering of peptide synthetases.

**EXPERIMENTAL PROCEDURES**

**Enzymes and Reagents**—Restriction enzymes were from N. E. Bio-labs (Schwalbach-Taunus, Germany) and were used according to the manufacturer’s instructions. For polymerase chain reaction amplifications, the recombinant Tt DNA polymerase XL from Perkin-Elmer was used after the protocol recommended by the manufacturer. All chemical reagents were purchased from Sigma. Deoxyoligonucleotides were synthesized using the DNA synthesizer ABI 394 and the reagents from Applied Biosystem.

**Plasmids and Strains**—The Escherichia coli B. subtilis shuttle vector pSM214G, a derivative of pSM214 (11), was used for cloning the fragments amplified from the surfactin synthetase locus of B. subtilis JH642+ strain (7, 12). E. coli XL1 blue strain (Stratagene) was used as the host for intermediate cloning experiments and for plasmid amplification. The suicide plasmid pDIA5304 (13) was used for the construction of the surfactin synthetase mutants of B. subtilis JH642+. Transformation into E. coli and B. subtilis were carried out as already described (14).

**Bacterial Culture Conditions**—LB agar plates supplemented with 5% sheep blood and 5 µg/ml chloramphenicol were used for colony isolation and rapid identification of surfactant-producing B. subtilis colonies. Typically, bacterial cultures were grown at 37 °C in VY liquid medium (25 g/liter Difco veal infusion broth, 5 g/liter yeast extract). For surfactin production, Landy medium was used (20 g/liter glucose, 5 g/liter glutamate, 0.5 g/liter MgSO4, 0.5 g/liter KCl, 1 g/liter KH2PO4, 0.15 g/liter FeSO4·7H2O, 5 mg/liter MnSO4, 0.16 mg/liter CuSO4·5H2O, 0.1% yeast extract), and cells were grown for 24 h at 37 °C.

**Construction of Mutant Strains**—The chromosomal regions spanning position 21327 to 21639 (fragment I) and from position 24496 to 24790 (fragment II) of the srf locus (7) were amplified using the following oligodeoxynucleotides: srfA-FOR (5’-GGGTTACCCGTTTGGTCTT- TCCGTCTCC-3’), srfA-REV (5’-AATGCAAGCCTTCTCGA-TCGC-GATAGG-3’), TE-FOR (5’-CGAATTTCAAGGAAGAAACAATCGC-...
**Fig. 2. Schematic representation of the srfA operon before and after plasmid integration by Campbell-type recombination.** The srfA operon, with its promoter and the three essential ORFs, is represented at the top of the figure. The duplicated sequences due to the Campbell-type recombination are represented by shaded boxes. The position of the constitutive promoter (Pc) as well as the ribosome binding site (RBS) and ATG in pDIA-srfA3 and pDIA-TE are also indicated. Arrows (from A to F) represent the primers used to check the correctness of the chromosomal integrations. P, PstI; Xm, XmaI; E, EcoRI; K, KpnI; Xb, XbaI; Cm, chloramphenicol resistance gene; kb, kilobase pairs; R, racemase.

**ATAC-3** and TE-REV (5′-CCCGGGGATATGCTACCCAGAAACCGGCCC-3′). The amplification products were purified on MicroSpin S-400 high resolution columns (Amersham Pharmacia Biotech), digested with the appropriate restriction enzymes, and finally inserted into plasmid pSM214 as KpnI-PstI and EcoRI-XmaI fragments, respectively, after further purification on agarose gel using the Quiaex II gel extraction kit (Qiagen). Fragment I was excised from pSM214 by either XbaI-PstI (promoter-carrying fragment) or KpnI-PstI (promoterless fragment) digestions and inserted into the suicide, integrative plasmid pDIA5304, generating plasmids pDIA-srfA3 and pDIA-srfA5, respectively. Analogously, fragment II was isolated from pSM214 by double digestion with either XbaI and XmaI or with EcoRI and XmaI, and the two fragments were cloned in pDIA5304, obtaining the plasmids pDIA-ΔTE and pDIA-TE, respectively. Mutants were isolated by transforming B. subtilis JH642 with the four suicide plasmids and selecting for chloramphenicol-resistant colonies. The correctness of plasmid integrations was verified by polymerase chain reaction using two oligos hybridizing in the chloramphenicol resistance gene (CmI, 5′-ACAATAGCAGG-GAGTTAGTTATCGG-3′ and CmII, 5′-GCCAGTC-TTAGGCTT-ATCTGACAATTCC-3′) and three oligos hybridizing in the srfA region at positions 24459–24477 (S3FOR, 5′-TCCGGTCGACGTCTCT-GGATCACAATCCG-3′) and 20853–20873 (S3REVFOR: 5′-AGATTGTTC-TACGACCATATG-3′) (see Fig. 1).

**Analysis of Surfactin Synthetase Expression**—Total cell proteins were analyzed on SDS-polyacrylamide gel electrophoresis according to Laemmli (15). Briefly, cells from 2-ml culture samples were harvested by centrifugation and lysed by resuspending them in 50 μl of 100 mM Tris-HCl, pH 6.8, 2 mg/ml lysozyme. After incubation at 37 °C for 15 min, an equal volume of gel loading buffer (62.5 mM Tris-HCl, pH 6.8, 5% (v/v) 2-mercaptoethanol, 4% SDS, 10% (v/v) glycerol, 0.025% (w/v) bromphenol blue) was added, and 20 μl of each sample was loaded onto 8% SDS-polyacrylamide after a 10-min heating at 100 °C. Protein separation was carried out at 25 mA for 3 h.

For TLC analysis, surfactin was precipitated from B. subtilis culture supernatants by adding HCl until pH 2.0 was reached. The precipitate was collected by centrifugation and resuspended in 100 mM sodium phosphate buffer, pH 7.6, to a final volume of 1 ml/l. Ano culture before acid precipitation. Samples (1 and 2 μl) were spotted on a silica plate (Silica gel 60, 0.25-mm thickness, Merck), and surfactin was chromatographed using butanol:ethanol:H₂O (4:4:1) as the mobile phase. Surfactin was detected by spraying water on the silica plate.

For HPLC and mass spectroscopy analysis of surfactin, the supernatants of bacterial cultures were concentrated in an Amicon concentrator using a YM 30 membrane (Millipore) and acidified to pH 2 by adding 3 N HCl. The resulting precipitate was separated by centrifugation (8,000 g) and suspended in chloroform, extracted with water, and precipitated by acidification with 3 N HCl. After centrifugation, it was resuspended in a propanol-H₂O 85:15 mixture containing 0.1% trifluoroacetic acid and analyzed by reverse phase HPLC on a C₈ Acquapore RP 300 column (4.6 × 250 mm, 7-μm inner diameter, Applied Biosystems) using a Beckman System Gold 126. The elution was performed using a solvent gradient obtained by mixing solvent A (0.2% trifluoroacetic acid in water) with solvent B (0.1% trifluoroacetic acid in CH₃CN-propanol 4:1 (v/v)). After 1 min of isocratic elution with 30% solvent B, the concentration of solvent B reached 55% in 7 min and then 95% in 15 min. Finally, after 2 min of isocratic elution, the initial conditions were restored. The flow rate was 1.2 ml/min, and the absorbance was monitored at 214 nm. Peaks were manually collected and lyophilized.
RESULTS

Construction of srfA Mutants—Two pairs of constructs in the surfactin synthetase locus were generated by using the strategy shown in Fig. 1 and described in detail under “Experimental Procedures.” In the first pair, srfAORF3 was separated from the rest of the srfA operon by inserting a suicide plasmid at the end of srfAORF2. In the second pair, the suicide plasmid insertion occurred at the end of the leucine binding domain of srfAORF3, thus physically dissociating the thioesterase domain from the rest of the subunit. The constructs of each pair differ for the presence or absence of a constitutive promoter in the suicide plasmid used for integration. Therefore, the integration of the promoter-containing plasmid led to the expression of the srfA region located downstream from the integration site (in one case the region is the entire srfAORF3; in the other the region is the thioesterase domain of srfAORF3), whereas the promoterless plasmid prevented the expression of the same regions. Fig. 2 schematically represents the genetic organization of the srfA locus in the four mutants. The figure also shows the nucleotide sequence of the regions flanking the integration sites in srfA::pDIA-srfA3 and srfA::pDIA-TE, the two mutants obtained with the promoter-harborng plasmid. In srfA::pDIA-srfA3, the translation of srfAORF3 took place from its natural shine-Dalgarano sequence and ATG, whereas in srfA::pDIA-TE the shine-Dalgarano sequence and the start codon for the independent expression of the TE domain were artificially created (see “Experimental Procedures” for details).

The correctness of plasmid integration in the four mutants was confirmed by polymerase chain reaction analysis. Two sets of primers were used, each set having one primer complementary to the vector sequence and the other complementary to the chromosomal region located either upstream or downstream from the integration site (Fig. 2). As judged by gel electrophoresis, the sizes of the fragments generated by polymerase chain reaction were in agreement with the expected theoretical sizes.

Analysis of Surfactin Synthetase Expression—The three surfactin synthetase subunits, srfAORF1, srfAORF2, and srfAORF3, are readily visible by Coomassie Blue staining after separation on SDS-polyacrylamide gels of the total cell proteins from B. subtilis 168, B. subtilis JH642, and mutant strains [16]. Therefore, the expression of the surfactin synthetase subunits in the four srfA mutants can be followed by analyzing the soluble proteins from bacterial cultures grown in rich liquid medium. As shown in Fig. 3A, the three subunits were clearly visible when total proteins from a 24-hour culture of srfA::pDIA-srfA3 were analyzed. On the contrary and as expected, srfAORF3 was absent in srfA::pDIAΔsrfA3 strain. Furthermore, in agreement with the fact that in srfA::pDIAAsrfA3 srfAORF3 transcription is under the control of a constitutive promoter, the expression of this subunit could be detected at the very beginning of the bacterial growth when the other two subunits were still not visible on SDS-polyacrylamide gel electrophoresis (Fig. 3B).

As for the other two mutants, srfA::pDIA-facilitated
FIG. 5. Reverse phase HPLC and mass spectroscopy analysis of surfactin produced by *B. subtilis* JH642+ and the mutant strains. The supernatant of the bacterial cultures were acid-precipitated at pH 2 with 3 N HCl, resuspended in chloroform, extracted with water, and finally acid-precipitated. The pellets were dissolved in propanol:H$_2$O (1:1) containing 0.1% trifluoroacetic acid and loaded on a C8 RP300 column. Elution was followed at 214 nm, and the peaks corresponding to surfactin were collected and analyzed by electron spray mass spectrometry. The mass spectroscopy analysis of the peaks are shown in the small windows of each panel. A, *B. subtilis* JH642+; B, srfA::pDIA-TE; C, srfA::pDIA-srfA3.
srfA::pDIA-TE, plasmid integration in srfA::pDIA-TE, plasmid integration in srfA::pDIA-TE, plasmid integration in srfA::pDIA-TE was confirmed by the disappearance of the protein species migrating with an apparent molecular mass of 144 kDa and corresponding to srfA::pDIA-TE, plasmid integration in srfA::pDIA-TE. However, although plasmid integration in srfA::pDIA-TE should result in the expression of two protein species, one having a molecular mass of 122 kDa and corresponding to the leucine binding domain and the other of 32 kDa carrying the TE activity, under the experimental conditions used, such protein species were not visible on SDS-polyacrylamide gel electrophoresis. This was probably due to the fact that other predominant proteins migrated in the 120- and 30-kDa range, thus making the identification of the two Srf domains impossible.

Analysis of the Lipopeptide Produced by the srfA Mutants—
When the four srfA mutants were grown on blood agar plates, the colonies of srfA::pDIA-srfA3 and srfA::pDIA-TE strains were surrounded by typical lysis halos, indicating that a surfactin-like lipopeptide was secreted. The other two mutants, both obtained with the promoterless suicide plasmid, did not produce halos around their colonies. To quantify the amount of lipopeptide produced by srfA::pDIA-srfA3 and srfA::pDIA-TE, the surfactant was acid-precipitated from the culture supernatants of the two strains as well as from the supernatant of the wild type B. subtilis JH642+ and analyzed by TLC. Since we found that the strain carrying the srfA::pDIA-TE integration grew very poorly in liquid minimal medium, for the quantitative analysis of surfactin production, a two-medium growth was used in which the three strains were first cultured in VY-rich medium until an A600 value of 1.5 was reached, and then the cells were transferred to Landy medium, and the surfactant production analyzed 24 h later. As shown in Fig. 4, srfA::pDIA-srfA3 produced approximately the same amount of surfactant as the wild type strain, whereas in srfA::pDIA-TE, the surfactant was approximately 50% less than the wild type. The fact that the surfactants had the same RF value on TLC strongly suggested that the three strains produced identical products.

To characterize the surfactants produced by the mutant strains, the lipopeptides were isolated from the extracellular material by a procedure involving membrane filtration, precipitation under acidic conditions, and reverse phase HPLC fractionation of redissolved material. The peaks observed in the chromatographic profiles were collected and analyzed by mass spectrometry. As shown in Fig. 5, the extracts from the parent strain JH642+ and from the mutant strains srfA::pDIA-srfA3 and srfA::pDIA-TE show nearly identical chromatographic pro-
files. The cluster of the peaks eluting with retention time between 18 and 21 min was shown to correspond to the known four isoforms of surfactin, which differ in the length of the acyl side chain (MH+ 994, 1008, 1022, 1036), as judged by mass spectroscopy analysis. The results of these analyses provide the clear demonstration that the mutant strains produce the same surfactant as the wild type strain.

**DISCUSSION**

The genetic organization, synthesis, and assembly of bacterial synthetases are schematically represented in Fig. 6A. As shown in this figure, the amino acid binding domains, generally located in more than one subunit and aligned along the chromosome according to the colinearity rule, are tightly coordinated at both the transcriptional and translational level. The highly conserved overall genetic and structural organization could be indicative of the relevant role this organization has in the proper synthesis and assembly of the enzymatic complex.

For example, the colinearity rule, according to which the order of the amino acid binding domains parallels the order of the amino acids in the peptide, might lead to the assumption that by simply changing the order of the domains, different peptides can be generated. This assumption is further sustained by the strong homology existing at the amino acid level among different domains (3, 7, 17, 18).

Furthermore, the coordinated transcription and translation found in bacterial peptide synthetases could suggest that the stoichiometry among subunits needs to be finely regulated. In fact, an excess of a particular subunit in a multisubunit enzyme not only could be energetically disadvantageous but, and more importantly, could result in an incorrect interaction among subunits with the consequent generation of undesired peptides.

With the present work, we started to question to what extent this conserved organization of peptide synthetases is truly indispensable for proper enzyme synthesis and assembly or rather represents the result of peculiar mechanisms of duplication, insertion, and translocation, through which these interesting enzymes have evolved (19, 20).

In particular, we asked ourselves what might happen to the peptide synthesis if 1) subunit transcription is driven by different, independently regulated promoters and, 2) enzymatic domains located on the same protein subunit are physically dissociated and independently expressed (Fig. 6B).

Our mutant strain, pDIA-srfA3, where the srfA operon was interrupted after the ORF2, thus leading ORF3 to be expressed constitutively and independently, was still able to produce wild type surfactin. This demonstrates that the coexpression and coregulation of the different subunits of the surfactin synthetase complex are not a prerequisite for the production of an active enzyme. Moreover, it would suggest that the order by which the amino acid binding domains are aligned at the DNA level is not strictly required for the correct assembly of the multienzyme complex. In fact, since ORF3 appears in pDIA-srfA3 cells well before the other two synthetase subunits (Fig. 3B) and is synthesized about 5 kilobase pairs apart from the rest of the surfactin synthetase operon, the subunit would have equal chances to interact with either ORF1 or ORF2, unless structural constraints exist. The fact that only wild type surfactin could be purified from the supernatant of pDIA-srfA3 and that the fact that its productivity was comparable to the wild type strain would suggest that such structural constraints play the most relevant role in the correct assembly of the enzyme.

Obviously, a more direct demonstration that the colinear organization of domains in peptide synthetases is not essential for enzyme assembly would be to change the order of domain coding sequences. Such experiments are currently in progress.

The data presented here also suggest that the functional domains of peptide synthetase subunits can be physically dissociated without impairing their subsequent assembly and their concerted activities. Our mutant pDIA-TE, in which the TE domain was separated from the leucine binding domain and expressed under the control of a constitutive promoter, was still able to synthesize surfactin.

Again, as in the case of pDIA-ORF3 mutant, surfactin was the only peptide formed in the supernatant of pDIA-TE culture, indicating that TE cannot proficiently interact with any other amino acid binding domain apart from the leucine binding domain of ORF3.

Therefore, specific protein-protein interactions rather than the ordered position and the progressive expression of the enzyme domains/subunits dictate the way in which peptide synthetases correctly assemble.

This might appear to contradict our recently published results on the engineering of surfactin synthetase in which, when the TE domain was moved next to either the fourth or the fifth domain of the enzyme, functional recombinant peptide synthetases were obtained that led to the synthesis of a four-amino acid and a five-amino acid lipopeptide, respectively (21).

However, in these experiments, for the thioesterase to release the four- and five-amino acid peptides, the C-terminal end of the seventh amino acid binding domain going from the panthetine cofactor binding motif to the beginning of the thioesterase (102 amino acids) had to be included in the thioesterase sequence. This C-terminal end replaced the correspouding regions of the fourth and fifth domains in the reported surfactin synthetase fusions.

Interestingly, it has been recently reported that the thioesterase-like domain found at the end of the six-module polyketide synthetase DEBS of Saccharopolyspora erythraea can be repositioned next to the second module generating a recombinant enzyme that synthesizes a short chain polyketide (22). Also in these experiments, the grafting of the thioesterase domain was carried out maintaining the C-terminal region of the preceding acyl carrier protein domain to which it is naturally fused.

Taken together these data lead us to hypothesize that the thioesterase to function, a specific protein-protein interaction with the preceding amino acid binding domain must be established. The region of the amino acid binding domain involved in the interaction with the thioesterase maps within the sequence spanning from the cofactor binding motif (amino acid position 1015) to the residue next to thioesterase domain (amino acid position 1117). This interaction specificity prevents the thioesterase from binding to other amino acid binding domains when the enzyme is physically separated from the domain to which it is naturally fused.

In all bacterial peptide synthetases so far characterized, why is TE constantly found fused to the C-terminal end of the last amino acid binding domain even though not strictly required for enzyme assembly and activity? The most probable explanation is provided by our finding showing that pDIA-TE mutant in which TE has been dissociated from the last leucine binding domain of surfactin synthetase is severely impaired in growth. This could indicate that when freed from the enzyme, TE can interfere with other important enzymatic functions involved in cell metabolism. The detrimental effect of free TE is supported by our failure in cloning and expressing TE both in *E. coli* and *B. subtilis* using multicopy plasmids (data not shown).

The fact that the specific interaction among domains rather than genetic organization guides the correct assembly of peptide synthetases has emerged from a number of recent observations. First of all, Marahiel and co-workers (23), in their
publication describing the successful engineering of surfactin synthetase, showed that functional enzymes could be obtained when the internal core region of the leucine binding domain was replaced with the corresponding regions of other amino acid binding domains. The sequences flanking the core region, most likely involved in domain-domain interactions, were rigorously kept unchanged in these experiments. Secondly, our findings deliver two messages to those interested in elucidating the structural features of domain interaction.

Our findings deliver two messages to those interested in peptide synthetase engineering. From one side, for the engineered peptide synthetase to function, reconstitution of operon engineering. From one side, for the engineered peptide synthetase to function, reconstitution of operon

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