Engineering of Peptide Synthetases

KEY ROLE OF THE THIOESTERASE-LIKE DOMAIN FOR EFFICIENT PRODUCTION OF RECOMBINANT PEPTIDES

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Peptide synthetases are large enzymatic complexes that catalyze the synthesis of biologically active peptides in microorganisms and fungi and typically have an unusual structure and sequence. Peptide synthetases have recently been engineered to modify the substrate specificity to produce peptides of a new sequence. In this study we show that surfactin synthetase can also be modified by moving the carboxyl-terminal intrinsic thioesterase region to the end of the internal amino acid binding domains, thus generating strains that produce new truncated peptides of the predicted sequence. Omission of the thioesterase domain results in nonproducing strains, thus showing the essential role of this region and the possibility of obtaining peptides of different lengths by genetic engineering. Secretion of the peptides depends on the presence of a functional sfp gene.

Two mechanisms for the biosynthesis of peptides are known to exist in bacteria and fungi, ribosomal synthesis and production by peptide synthetases. These are large enzymatic complexes responsible for the synthesis of hundreds of types of peptides, some of which have immunomodulatory, antibiotic, antifungal, or surfactant properties. Whereas polypeptides produced by ribosomal synthesis typically contain only the amino acids directly specified by the triplets of the genetic code, peptides built on synthetases often contain unusual amino or hydroxy acids as building blocks that are not present in proteins. The amino acids can be modified by peptide synthetases either through methylation, hydroxylation, or enantioomerization. The peptides are typically short (up to about 20 residues) and can be linear, circular, or branched (1, 2). Peptide synthesis proceeds by the "multiple carrier thiotemplate mechanism" (3, 4), and many of the details of these systems remain to be investigated experimentally. According to this mechanism each domain recognizes a specific amino acid (or hydroxy acid) that is covalently bound to the cofactor via a thioester bond after activation to the corresponding acyladenylate derivative. The growth of the polypeptide chain thus occurs through a series of thioester bond cleavages in the simultaneous formation of amide or ester bonds in the peptide. At the end of synthesis of each peptide, the chain is thought to be released from the enzyme by a thioesterase (TE) activity encoded in the synthetase gene (1). Peptide synthetases are interesting not only from a scientific and evolutionary point of view but also for their biotechnological potential. Many enzymatically synthesized peptides are in fact biologically active, and some of them are industrially produced, among which are cyclosporins, surfactin, and fungicides. A growing number of laboratories are involved in applied research projects focused on the isolation and characterization of new peptide synthetases and on the genetic manipulation of peptide synthetase genes to optimize peptide production and to genetically modify the sequence of the peptides produced. In fact, the structural organization of peptide synthetases makes these enzymes particularly amenable to protein engineering. Sequence analysis of the genes encoding peptide synthetases reveals a clear organization of the enzymatic complex in domains associated with diverse functions such as amino acid binding and activation, methylation, or racemization. The order and substrate specificity of repeated homologous regions that are about 1,000 residues long determine the sequence of the peptide product and the type of modification of each single amino acid unit of the peptide. Amino acid binding domains have been shown to functionally exist within the repeated regions, and in these regions, conserved sequence motifs are present homologous to nucleotide binding regions and ATPases; phosphopantetheine cofactor binding sequence motifs have been demonstrated experimentally (3, 10, 11).

Recently it was shown that amino acid binding domains of peptide synthetases can be exchanged among different synthetases to generate hybrid enzymes capable of producing new peptides. In this way, Stachelhaus et al. (12) replaced the last leucine binding domain of Bacillus subtilis surfactin synthetase with domains derived from the Bacillus brevis grs operon and from domains of the fungine 1-α-aminoacidipyl-cysteinyl-D-valine synthetase (accA) gene. These authors demonstrated that the peptides produced by the engineered synthetase carried at the terminal position the amino acid recognized by the replacing domain.

In the course of our investigation of the elements necessary and sufficient for peptide synthesis and in an attempt to further expand the possibilities of engineering peptide synthetases, we have focused our attention on the thioesterase that is responsible for the release of the newly synthesized peptide from the enzyme. Interestingly, in the surfactin synthetase (srfA) and gramicidin synthetase (grs) operons, two regions contain sequences homologous to thioesterases. One encodes a 25-kDa protein (srfAorf4 (5) or the grsT product (13)) homologous to fatty acid synthase thioesterase type II. The other one, present in all peptide synthetases characterized so far, lies
downstream from the sequence of the last amino acid binding domain and is homologous to fatty acid synthase thioesterase type I (6, 7, 14). We have shown that insertional inactivation or deletion of the thioesterase type I region results in a stable but unproductive surfactin synthetase (15), whereas deletion of srfAORF4 has no effect on peptide production (5).

In this study we show that recombinant enzymes obtained by repositioning the integral thioesterase type I domain within surfactin synthetase efficiently synthesize peptides of reduced length as long as the region is properly fused to an amino acid activating domain of the enzyme complex. These data open the way to the synthesis of peptides of desired length and amino acid composition.

EXPERIMENTAL PROCEDURES

Plasmid Constructions—The integrative plasmids used to fuse the thioesterase domain at the end of amino acid activating domains were all derivatives of pJM103 or pJM102 (the difference between the two vectors being the orientation of the multicloning site) and were constructed as follows: part of the srfA fourth and fifth domains corresponding to the 12400-13827 and the 16538-16949 regions of srfA (5) were amplified using JH642 chromosomal DNA as the template and the corresponding primers: 4VAL-FOR 5'-GGATCCAGCCTGCTCTTGGAATGGTGGCAATCA-3' and 4VAL-REV 5'-TTCCCTATGAGCCTCTTCTGAATTTTCGCCGTCA-3'; and 5ASP-FOR 5'-TCA-CGGAATTCAAGAAGCGGTT-3' and 5ASP-REV 5'-CTTTTCTCAGCTCCCAGTTGAGTCGAAATTTGGCGATCAAA-3' encoding EcoRI and SacI sites at the two extremities (underlined). Cloning of the two fragments in pJM103 (16) between these two sites generated plasmids pVAL and pASP. For the construction of plasmids pVAL-TE and pASP-TE, the EcoRI-SacI inserts from pVAL and pASP were introduced between the EcoRI and SacI sites of a pJM103 derived construct pTE. In this construct, the 795-bp pair-long sequence encoding the last 260 residues of srfAORF3 was cloned as a polymerase chain reaction fragment between the SacI and BamHI sites using the couple of oligonucleotides with sequence 5'-CTTTTCTCAGCTCCCAGTTGAGTCGAAATTTGGCGATCAAA-3' and TE-REV 5'-GTGGTGACCCATTTAGAAACCGTTACGGTTTG-3' as primers for amplification of this region from chromosomal DNA.

All plasmids containing fragments derived from polymerase chain reactions were sequenced to confirm the absence of mutations by the dideoxy terminators method (17). The integrative plasmids were introduced into B. subtilis surfactin producing strain JH642+ as described (5).

Cell Culture Conditions for Surfactin and Altered Peptide Production—A single colony from a fresh spore agar plate (containing 8 ml DIFCO nutrient broth, 1 μl FeSO₄, 10 μl MnCl₂, and 1 ml Ca(NO₃)₂) was inoculated in 25 ml DIFCO veal infusion broth, 5 ml yeast extract to yield a turbidity of 0.1 (0.1 A₅₅₀) in a standard 100 ml glass test tube. The culture was grown in a water-jacketed and agitated fermentor, or in a standard shaker, at 37 °C and 200 rpm until a final optical density of 0.8-1.0 at 600 nm was reached. For dideoxy terminators analysis—

The amino acid composition analysis was performed as follows. Strains integrated with pVal-TE: two spots representing all hydrorepellent material were scraped from the plate and the amino acid composition was analyzed (this minor contaminant, a peptide rich in Gly secreted by the parental strain (non-hydrorepellent) of known amino acid composition and corresponding to the material shown in panel A of the HPLC pattern was removed in this way). Strains integrated with pASP-TE: three hydrorepellent spots representing all of the lipopeptides were scraped, eluted with methanol, and analyzed by HPLC; their amino acid composition was determined.

RESULTS

srfA Gene Manipulation—As we have previously shown, 3'-deletions of various length in the surfactin synthetase gene (srfA) result in the synthesis of truncated enzymes that are able to recognize and bind amino acids but are unable to produce the corresponding peptides (15). Since experimental evidence indicated that the integrity of the thioesterase type I-like domain (designated the TE region) is necessary for production of surfactin (5), we have examined the productivity of truncated forms of the enzyme after fusion to the TE region at the carboxyl-terminal boundary of amino acid-activating domains. The extent and degree of homology of the TE region to several thioesters is shown in Fig. 1.

To obtain strains in which the TE region is fused to the truncated surfactin synthetate, integrative plasmids containing the appropriate constructs were integrated in the B. subtilis chromosome by recombination. The TE region (comprising the carboxyl-terminal 260 amino acids of srfAORF3) was amplified from the chromosome of the surfactin-producing strain JH642+ (5) as a SacI-BamHI 785-base-pair-long fragment. This fragment was fused to genes encoding carboxyl-terminal portions of the fourth or fifth domains of surfactin synthetase that were amplified as EcoRI-SacI fragments and cloned in the integrative plasmid pJM103 (16), thus generating plasmids pVAL-TE and pASP-TE. Two plasmids (pVAL and pASP) containing the same inserts but lacking the TE domain were also constructed and inserted in the chromosome (see Fig. 2 and “Experimental Procedures” for details).

Transformation of the surfactin-producing JH642+ strain
with the integrative plasmids pVAL-TE and pASP-TE results in the insertion of the plasmid into two possible regions of srfA, depending on the point where Campbell recombination ensues. Recombination in the fourth or fifth amino acid-activating domains generates hybrid surfactin synthetases in which one or the other of these domains is fused to the TE region, whereas recombination at the level of the srfAORF3 TE region results in strains in which the srfA operon preserves the wild-type configuration up to the end of the ORF3. Interruption of the srfA operon after this point by JMI03 integration has no effect on surfactin production (5). Transformation of JH642 cells with plasmids pVAL and pASP are predicted to result in plasmid integration and srfA transcription interruption at the level of the “junction” (5) downstream of the fourth amino acid binding domain (VAL) and a few residues downstream of the cofactor attachment site of the fifth (ASP) domain with pVAL and pASP, respectively. To discriminate between the possible alternative regions of integration in the case of plasmids pVAL-TE and pASP-TE and to confirm the insertion point in the case of plasmids pVAL and pASP, the protein and DNA pattern of the recombinant strains were analyzed.

**Protein Analysis**—Transformation with pVAL-TE and pASP-TE resulted in the selection on chloramphenicol of two classes of transformants as detected on spo plates, halo producers or no-halo transformants. Production of surfactin can be visualized on spo agar plates as a clear halo around the colony. Thus, the protein pattern of several colonies from each class was further examined.

We have previously shown (15) that the srfAORF1, srfAORF2, and srfAORF3 subunits of wild-type surfactin synthetase are readily visible on SDS-polyacrylamide gels by Coomassie Blue staining. Representative patterns of strains in which the srfA gene is interrupted by integration are shown in Fig. 3 lanes 3–6 where they are compared with the srfA wild-type pattern (lane 7) and with the pattern of a mutant strain in which the srfA gene is not transcribed (15). The interruption of srfAORF2 in all halo negative strains could be identified by the disappearance of the corresponding wild-type ORF2 band. The strains were named dom4::pASP, dom5::pASP-TE, dom5::pASP-TE, and dom5::pASP-TE, respectively, which denotes the region of integration and the name of the integrative plasmid (Fig. 2). Halo-positive colonies in which integration occurred at the level of the TE region have a protein pattern identical to the wild-type strain on these gels and are not shown in this figure. The appearance of new bands (Fig. 3, asterisks) can be seen in strains in which integration occurred in the fifth domain (lanes 5 and 6), which indicates the presence of new hybrid proteins migrating to the expected position on the polyacrylamide gel. In the class of colonies in which integration happened at the carboxyl end of the fourth domain, the band that corresponds to srfAORF2 disappears, but no new protein species could be detected by this method. Analysis of DNA (by polymerase chain reaction or Southern blotting techniques) of representative colonies from each class confirmed plasmid integration in the expected regions (data not shown).

**Peptide Production by the Mutant Strains**—Peptide production by the mutant synthetases was investigated by growing the mutant strains in conditions that allow surfactin production and by looking for the presence of lipopeptides in the culture medium. The culture supernatants were acidified, and the precipitate was extracted with organic solvents and analyzed by TLC and reverse phase HPLC.

Analysis of the products obtained by TLC shows the appearance of slower migrating lipopeptides in the dom4::pVAL-TE and dom5::pASP-TE integrants in which the thioesterase is fused at the end of the domains, whereas cells in which the TE domain is missing do not secrete appreciable amounts of lipopeptides (Fig. 4).

Interestingly, the Rf of the new peptides does not show any alteration after treatment with NaOH (data not shown) by which the lactone bond of surfactin is hydrolyzed, and a much slower migrating species is detected.

Fig. 5 shows the HPLC profiles of peptides purified from strains in which the srfA gene has been inactivated by promoter deletion (15) (panel A) and from the recombinant strains carrying the TE region fused at the end of the fourth (panel B) and fifth (panel C) domain. Panel A shows the presence of relatively small amounts of a peptide species secreted by the JH642 parental strain together with surfactin (note the difference in scale of the y axes). Panel D indicates the pattern of surfactin similarly purified from JH642 cells. The patterns of the supernatants of strains derived from integration of pVAL and pASP are identical to panel A and are not shown. These results indicate that the recombinant strains, in which the TE region is fused at the end of amino acid-activating domains, produce new lipopeptide species that can be resolved into three major peaks by reverse phase HPLC. In these conditions wild-type surfactins are detected as a group of three main peaks representing fatty acid length polymorphism. The material secreted by strain dom5::pASP-TE (panel C) appears to be less hydrophobic than surfactin and the peptides secreted by strain dom4::pVAL-TE (panel B). The lipopeptides purified from TE::pVAL-TE and TE::pASP-TE had an elution profile similar to surfactin (data not shown).

**Amino Acid Analysis of the Peptides**—The amino acid composition of the peptides produced by the strains containing the TE region fused to amino acid-activating domains four and five and was analyzed as described under “Experimental Procedures.”

The acid-precipitable material was further purified by TLC and HPLC, and amino acid analysis of the different fractions showed that the peptide composition is the same for all fractions. Table I reports the measured relative values corresponding to the amino acids present in surfactin and in the lipopeptides produced by strains dom4::pVAL-TE and dom5::pASP-TE. The results are consistent with the production of tetrapeptides of sequence Glu-Leu-U-Leu-Val by strain dom4::pVAL-TE and pentapeptides containing Glu, Leu, t-Leu, Val, and Asp by strain dom5::pASP-TE. For both strains it is clear that the tetra- and pentalipeptides, respectively, are the major, if not the only, components of the secreted lipopeptide material showing heterogeneity in the lipid moiety as is the
case with surfactins (21). We estimated from HPLC and amino acid analysis that the amount of lipotetrapeptide produced is about 48.3 mg/liter, whereas the pentapeptide is about 18.9 mg/liter, representing about 23 and 9% of surfactin production by weight, respectively, in laboratory conditions.

Production of the Peptides is sfp Dependent—The presence of an intact sfp gene is known to be necessary for surfactin production, although the step involved remains unclear. To investigate whether the synthesis of the shorter peptide is dependent on the presence of an intact sfp gene, an sfp' strain (5) was transformed with plasmid pVAL-TE. Three independent colonies in which the protein pattern indicated that the TE region was fused to the valine domain were analyzed for peptide production by HPLC and TLC. None of the strains produced measurable amounts of lipopeptides, indicating the dependence of peptide production on sfp.

DISCUSSION

Peptide synthetases are codified by genes organized in a modular structure in which repeated domains are associated to specific functions. This organization in structurally and functionally separated regions suggests the possibility of altering the order and type of building units genetically to create new enzymes of novel specificity and to produce peptides that may contain many of the unusual amino acids that are present in these secondary metabolites and not in proteins.

We have previously shown that for efficient surfactin production it is necessary to maintain in the enzyme complex the function(s) associated with the carboxyl-terminal region (TE) fused to the last amino acid activation domain. Deletion of this region impairs peptide production even in the presence of normal amounts of the truncated enzyme. We show here that the fusion of the TE domain to the carboxyl terminus of different amino acid-activating domains in strains dom5::pASP-TE and dom5::pASP. Integrated plasmid vector sequences inserted in the srfA gene are indicated with a dotted line.
to thioesterases type I, especially in the N-terminal half. SrfAORF4, together with the protein encoded by grsT, shares homology with thioesterases belonging to class II. We have shown previously that inactivation of this gene in B. subtilis has no influence on the levels of surfactin production. All of the integrations in srfA described in this work also disrupt transcription of the srfAORF4 gene. It would thus appear that the thioesterase codified by the srfAORF4 gene is not needed either for the release of the peptide or for whatever role one could presume for this protein. However, the presence in the cell of other proteins with a similar role that might replace them cannot be excluded. One could predict, for example, that the recently cloned pps synthetase of B. subtilis (23) might be associated with a protein homologue of srfA and grsT.

Many similarities exist between the structural organization of the peptide synthetase or polyketide synthase enzymatic complexes, and the engineering of polyketide synthase has resulted in the possibility of producing new polyketides by domain replacement or rearrangement (24). In particular the carboxyl-terminal portion of deoxyerythronolide B synthase, encoding the thioesterase-cyclase function that is responsible for the detachment of deoxyerythronolide B synthase, was repositioned in an internal region of the complex to generate a shorter triketide lactone (25).

The experiments reported here also show that the production of the linear tetra- and pentapeptides is dependent, like surfactin, on the presence of a wild-type copy of the product of the sfp gene. The function of this gene, necessary for surfactin production, is still unclear. sfp strains accumulate normal levels of the structural components of the surfactin synthetase complex, and the enzyme is active in vitro as measured by the pyrophosphate exchange assay (15). Since the product of sfp (and of its grs homologue, gsp) shares sequence similarity and can functionally replace the product of the entD gene involved in iron transport, it was suggested that wild-type sfp might be needed for peptide secretion or iron acquisition (26, 27). The sfp dependence on peptide production shown in the present study only indicates that the step inhibited in sfp mutant strains is not dependent on either peptide sequence or peptide conformation.

Nothing is known about the mechanism of secretion of surfactin or of lipopeptides in general. However, our experiments indirectly show that the mechanism might not be very sequence specific, and that the linear and shorter peptides can be transported equally well even with surfactin variants that carry internal deletions.2

Engineering of peptide synthetases is of great industrial interest for at least two reasons; it implies the possibility of non-ribosomal synthesis of commercially valuable natural compounds in hosts that are better suited for industrial production; it opens the way to the biological synthesis of new molecules, analogues, and substituted versions of biologically active peptides as an alternative to the chemical synthesis that is often difficult and costly especially in the case of non-linear and modified peptides. We have determined that insertion of the thioesterase region at the end of amino acid binding domains resulted in the possibility of producing new polyketides by domain replacement or rearrangement (24). In particular the carboxyl-terminal portion of deoxyerythronolide B synthase, encoding the thioesterase-cyclase function that is responsible for the detachment of deoxyerythronolide B synthase, was repositioned in an internal region of the complex to generate a shorter triketide lactone (25).

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ulation are at hand (this study and Ref. 12), many aspects of the thiotemplate mechanism of peptide production are still unclear and need to be investigated to better exploit this system for the efficient synthesis of new peptides by engineered microorganisms.

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