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A. Schneider · T. Stachelhaus · M. A. Marahiel

Targeted alteration of the substrate specificity of peptide synthetases by rational module swapping

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Abstract Analysis of the primary structure of peptide synthetases involved in the non-ribosomal synthesis of peptide antibiotics has revealed a highly conserved and ordered modular arrangement. A module contains at least two domains, involved in ATP-dependent substrate activation and thioester formation. The occurrence and arrangement of these functional building blocks is associated with the number and order of the amino acids incorporated in the peptide product. In this study, we present data on the targeted exchange of the leucine-activating module within the three-module surfactin synthetase 1 (SrfA-A) of *Bacillus subtilis*. This was achieved by engineering several hybrid *srfA-A* genes, which were introduced into the surfactin biosynthesis operon by in vivo recombination. We examined the hybrid genes for expression and investigated the enzymatic activities of the resulting recombinant peptide synthetases. For the first time, we demonstrate directly that an individual minimal module, of bacterial or fungal origin, confers its amino acid-specific activity on a multi-modular peptide synthetase. Furthermore, it is shown that directed incorporation of ornithine at the second position of the peptide chain induces a global alteration in the conformation of surfactin and may result in premature cyclization or a branched cyclic structure.

Key words Peptide synthetase · Surfactin · Minimal-module substitution · Amino acid activation

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A. Schneider · T. Stachelhaus · M. A. Marahiel (✉)
Biochemie/Fachbereich Chemie, Philipps-Universität Marburg, Hans-Meerwein-Strasse, D-35032 Marburg, Germany
Fax: +49-6421-282191;
e-mail: marahiel@ps1515.chemie.uni-marburg.de

Introduction

Non-ribosomally synthesized peptides produced by several soil bacteria and fungi belong to a large and diverse family of natural products. They exhibit useful activities and are used as antibiotics, toxins, enzyme inhibitors, anti-viral and anti-tumor agents, immunosuppressants, and as biosurfactants. The linear, cyclic and branched cyclic peptide chains include non-proteinogenic, hydroxy- and D-amino acids and may also be further modified by *N*-methylation, acylation, glycosylation or heterocyclic ring formation. About 300 different residues are known to be incorporated into these peptide secondary metabolites (Kleinkauf and von Döhren 1996; Zuber et al. 1993).

Over the past several decades, a wealth of information concerning non-ribosomal peptide synthesis has been accumulated (for reviews see Zuber et al. 1993; Kleinkauf and von Döhren 1996; Marahiel 1997; Marahiel et al. 1997), which has led to the formulation of a universal model: the multiple carrier thiotemplate mechanism (Stein et al. 1996).

In this template-driven assembly of peptides, multifunctional peptide synthetases show a highly conserved and ordered structure composed of (semi-) autonomous modules, and it is the identity of these modules and the order in which they occur that determines the sequence of the corresponding peptide product (Turgay et al. 1992; Stachelhaus and Marahiel 1995b; Stachelhaus et al. 1995, 1996; Marahiel et al. 1997). The understanding of the structure-function relationship of these enzymes has been enhanced by the elucidation of the primary structures of an increasing number of peptide synthetases (Scott-Craig et al. 1992; Turgay et al. 1992; Cosmina et al. 1993; Fuma et al. 1993; Haese et al. 1993; Weber et al. 1994). Sequence comparisons revealed that the modules, each about 1000–1400 amino acids in length, are themselves composed in a linear arrangement of conserved domains representing the enzyme activities involved in substrate recognition, activation, modification and

condensation (de Crécy-Legard et al. 1995; Stachelhaus and Marahiel 1995a, b; Stachelhaus et al. 1995, 1996; Marahiel et al. 1997). Recent studies clearly indicate that a core fragment of such a module, comprising an adenylation and thiolation domain, retains all catalytic activities for specific activation and covalent binding of the substrate (Stachelhaus and Marahiel 1995a, b). This fragment has been designated a minimal module.

The lipoheptapeptide surfactin is secreted by *Bacillus subtilis* under nutrient depletion, a condition that also leads to the activation of a number of other physiological processes, including sporulation, competence development and the synthesis of degradative enzymes (Losick et al. 1986). Surfactin is made up of seven amino acids (Glu-Leu-DLeu-Val-Asp-DLeu-Leu) linked to a β-hydroxy fatty acid through an amide bond between the carboxyl group of the fatty acid and the amino group of glutamic acid, and an ester bond between the carboxyl group of the last leucine and the β-hydroxy moiety of the fatty acid (Fig. 1B). It is one of the most powerful biosurfactants discovered so far and is synthesized by the gene products of the 27-kb *srfA* operon (Fig. 1A), which encodes the three peptide synthetases SrfA-A, SrfA-B and SrfA-C with molecular masses of 402, 401, and 144 kDa, respectively. These multifunctional enzymes comprise a total of seven amino acid-activating modules that recognize, activate and, in part, epimerize the cognate amino acids of the heptalipopeptide. The fourth open reading frame (ORF) within the *srfA* operon encodes a thioesterase type II-like protein SrfA-TE, whose function in surfactin biosynthesis is still unknown (Cosmina et al. 1993). A small gene called *comS*, located within but out of frame of the *srfA-B* gene (Fig. 1A), is necessary for the development of genetic competence, but not for surfactin biosynthesis (Fuma et al. 1993; Van Sinderen et al. 1993; D'Souza et al. 1994). Another gene, named *sfp*, is located about 4 kb downstream of the *srfA* operon. The gene was found to be essential for surfactin biosynthesis (Nakano et al. 1988) and encodes a protein that belongs to a new protein family, the 4′-phosphopantetheinyl (4′-PP) transferases. These enzymes are responsible for posttranslational modification of 4′-PP-requiring enzymes, including fatty acid and polyketide synthases, as well as several peptide synthetases from different species (Lambalot et al. 1996).

We recently reported on the reprogramming of surfactin synthetase 3, a single-module peptide synthetase, which possesses the simplest structure within the surfactin synthetase complex (Stachelhaus et al. 1995). In this study, we present a generally useful method for substitution of minimal modules within multi-modular surfactin synthetases. The cloning of several regions encoding minimal modules of pro- and eucaryotic origin has enabled the construction of hybrid peptide synthetase genes. These genes have been used for the targeted replacement of the *srfA-A* section that encodes the Leu2-activating minimal module of the surfactin synthetase 1. We have investigated the exchange of minimal modules and the alteration of the substrate specificity of the engineered hybrid synthetases, and studied the production of the modified lipopeptides.

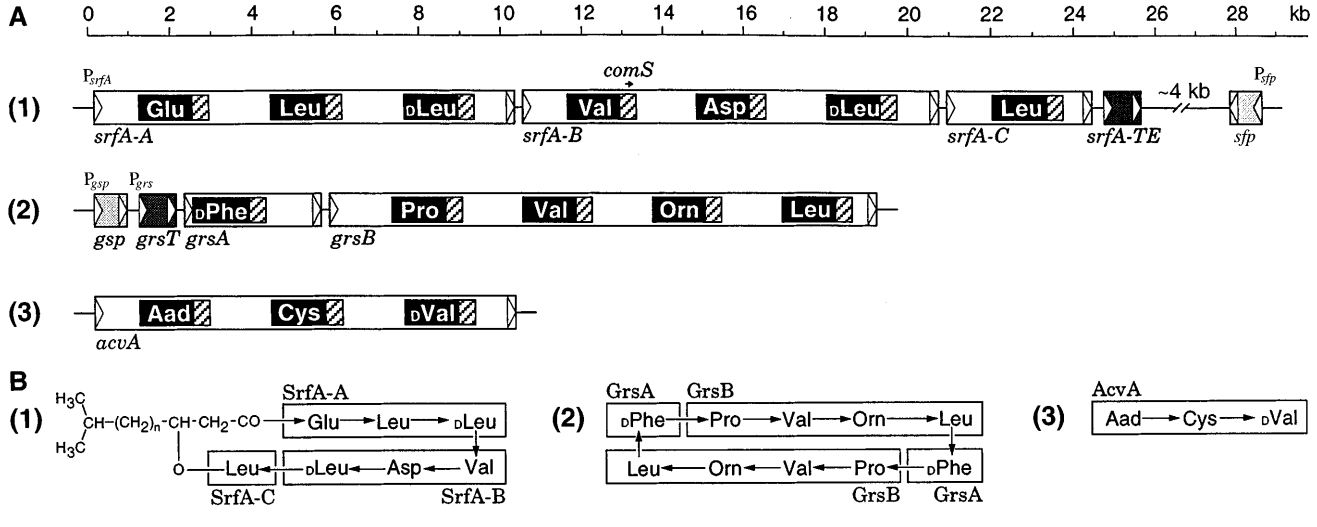


Fig. 1A, B Multimodular structure of peptide synthetases. **A** Schematic diagram of the highly conserved and ordered modular organization of peptide synthetases encoded by the bacterial operons *srfA* (1) and *grs* (2), as well as the fungal gene *acvA* (3). The regions encoding the adenylation and thiolation domains are indicated by *filled* and *hatched boxes*, respectively. The locations of promoters (p) and genes associated with these operons [genes encoding 4′-PP transferases (*sfp* and *gsp*), thioesterases (*srfA-TE* and *grT*) and a competence factor (*comS*)] are shown (Nakano et al. 1992; Cosmina et al. 1993; Borchert et al. 1994; D'Souza et al. 1994; Hamoen et al. 1994; Turgay et al. 1994). **B** Primary structures of the non-ribosomally synthesized peptides surfactin (1), gramicidin S (2) and δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (3), an intermediate in the synthesis of penicillins and cephalosporins (Aad, L-α-aminoadipyl). Residues shown in *boxes* are activated by the corresponding peptide synthetase

Materials and methods

Bacterial strains and plasmids

Bacterial strains and plasmids used in this work are listed in Table 1. *E. coli* cells were grown in 2 × YT medium (Sambrook et al. 1989) and *B. subtilis* cells in either 2 × YT or Difco sporulation medium (Nakano et al. 1988).

Amplification and cloning of DNA fragments

PCR amplification of DNA fragments was used to generate the terminal restriction sites needed for subsequent cloning and was performed using Deep Vent DNA polymerase and 10 × reaction buffer from New England Biolabs (Schwalbach, Germany), and following the enzyme manufacturer's protocol. The primers used in this work are listed in Table 2 and were synthesized by Dr. Michael Krause (Mikrochemische Einheit, FB20, Philipps-Universität Marburg, Germany). Amplified fragments were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany). Standard procedures were used for digestion with restriction enzymes, cloning of DNA fragments and preparation of recombinant plasmid DNA (Sambrook et al. 1989).

Construction of a *srfA-A* disruption and several minimal-module substitution plasmids

After amplification, the 972-bp 5′ *srfA-A′* fragment was digested with *Nde*I and *Nru*I, and ligated into pOSEX5A, digested with the same enzymes. The 1043-bp 3′ *srfA-A′* fragment was ligated into pJLA503, using the *Bam*HI and *Eco*RI restriction sites. The integration vector pJLA/5′-3′-SRFa (Fig. 2) was constructed by subcloning the *Nde*I-*Bam*HI fragment, which contains the 5′ *srfA-A′* fragment and 712 bp of pOSEX5A, into pJLA/3′SRFa digested with the same enzymes.

To obtain pSRFa-ΔLeu, the pOSEX5A fragment was eliminated by digestion with *Nru*I and *Bam*HI and replaced by a 1373-bp *Eco*RV-*Bam*HI fragment, containing the *cat* gene of pDG268 (Fig. 2). Subsequently, the 344-bp P_{srfA} fragment was amplified, digested with *Bam*HI and ligated into the *Bam*HI site of pSRFa-ΔLeu. This resulted in the disruption plasmid pSRFa-ΔLeu-P_{srfA} (Fig. 2).

In order to generate minimal-module substitution plasmids (Fig. 2), several minimal-module coding fragments were amplified, digested at the restriction sites generated by PCR and ligated into pJLA/5′-3′-SRFa digested with *Nru*I and *Bam*HI. The *grsA* fragment encoding the Phe minimal-module was obtained by digestion of pMK21 (Krause and Marahiel 1988; Krätzschmar et al. 1989) with *Pma*CI and *Bgl*II, and ligated into the integration vector.

Expression in *E. coli*

Expression of the hybrid gene fragments was performed as described by Stachelhaus et al. (1995). Expression was checked by SDS-PAGE using the method of Laemmli (1970). After fractionation, the proteins were transferred to a nitrocellulose membrane by the method of Towbin et al. (1979). Detection of heterologous minimal modules was achieved by (cross)reaction with antibodies raised against the adenylation domain of GrsA-Phe or the minimal modules of GrsB-Pro and GrsB-Orn.

Table 1 Bacterial strains and plasmids

Strain/plasmid	Relevant genotype/description	Reference
E. coli	*recAI endAI gyrA96 thi hsdR17* (r_K^- m_K^+) *supE44 relAI*	Bullock et al. (1987)
XL1-Blue	*lac*[F′ *proAB lacI*^{q}ZΔM15 Tn*10* (Tet^r)]	
BMH71-18	Δ(*lac-proAB*) *supE thi-1 F′* lacI^{q}ZΔM15 *proA*$^+$ *B*$^+$	Rüther et al. (1981)
B. subtilis	Wild-type surfactin producer	Cooper et al. (1981)
ATCC 21332		
AS10	Φ(*srfA-A′*$_1$-*cat*-P_{SRF}-*srfA-A′*$_2$)	This work
AS106	Φ(*srfA-A′*$_1$-*grsB′*(*Phe*)*srfA-A′*$_2$)(Hyb.)	This work
AS112	Φ(*srfA-A′*$_1$-*grsB′*(*Leu*)*srfA-A′*$_2$)(Hyb.)	This work
AS115	Φ(*srfA-A′*$_1$-*grsB′*(*Orn*)*srfA-A′*$_2$)(Hyb.)	This work
AS116	Φ(*srfA-A′*$_1$-*grsB′*(*Pro*)*srfA-A′*$_2$)(Hyb.)	This work
AS122	Φ(*srfA-A′*$_1$-*grsB′*(*Val*)*srfA-A′*$_2$)(Hyb.)	This work
AS103pen	Φ(*srfA-A′*$_1$-*acvA′*(*Cys*)*srfA-A′*$_2$)(Hyb.)	This work
AS122pen	Φ(*srfA-A′*$_1$-*acvA′*(*Val*)*srfA-A′*$_2$)(Hyb.)	This work
B. brevis		
ATCC 9999	Gramicidin S producer	Bacillus Genetic Stock Center[a]
Plasmids		
pDG268	Shuttle vector for *B. subtilis*; *bla*, *cat*	Antoniewski et al. (1990)
pJLA503	Expression vector; *bla*	Schauder et al. (1987)
pNEXT33A	Congression plasmid; Φ(*metD′*-npt-metD′)	Itaya and Tanaka (1990)
pOSEX5A	Expression vector; *bla*	Herbst et al. (1994)
pJLA/5′-3′-SRFa	*srfA-A* Integration vector; *bla*	This work
pSRFa-ΔLEU	*srfA-A* Disruption plasmid; Φ(*srfA-A′*$_1$-*cat*-P_{SRF}-*srfA-A′*$_2$)	This work
srfA-A minimal-module substitution plasmids		
pSRFa-Phe	Φ(*srfA-A′*$_1$-*grsB′*(*Phe*)*srfA-A′*$_2$)(Hyb)	This work
pSRFa-Leu	Φ(*srfA-A′*$_1$-*grsB′*(*Leu*)*srfA-A′*$_2$)(Hyb)	This work
pSRFa-Pro	Φ(*srfA-A′*$_1$-*grsB′*(*Pro*)*srfA-A′*$_2$)(Hyb)	This work
pSRFa-Orn	Φ(*srfA-A′*$_1$-*grsB′*(*Orn*)*srfA-A′*$_2$)(Hyb)	This work
pSRFa-Val	Φ(*srfA-A′*$_1$-*grsB′*(*Val*)*srfA-A′*$_2$)(Hyb)	This work
pSRFa-Cys-pen	Φ(*srfA-A′*$_1$-*acvA′*(*Cys*)*srfA-A′*$_2$)(Hyb)	This work
pSRFa-Val-pen	Φ(*srfA-A′*$_1$-*acvA′*(*Val*)*srfA-A′*$_2$)(Hyb)	This work

[a] Ohio State University, Columbus, Ohio

Table 2 Sequences of the primers used in this work

Primer	Sequence[a]	Location
5′-SRFa-Nde I	5′-GGC TTG AAG AGA ACC GTC ATA TGC TGC-3′	3512[b]
5′-SRFa-Nru I	5′-*CGA TCG CGA* GAT AAG GTG TTC TGC CCG G-3′	4480[b]
3′-SRFa-Bam HI	5′-*CGC GGA TCC* GTG AGC TTG ATG TTG AAA GG-3′	6383[b]
3′-SRFa-Eco RI	5′-*GCG GA*A TTC GAG TAC GAA TGT AAG CCC-3′	7423[b]
5′-Pro-Eco RV	5′-*GCG GAT ATC C*TA AGA CGA TTC ATC AGT TAT T-3′	1440[c]
3′-Pro-Bam HI	5′-*CGG GAT CCC G*AA CAT AAC ACC AGG TAA ATT A-3′	3332[c]
5′-Val-Eco RV	5′-*ATA AG*A TAT CCA CTA TGC AAA CAA TTC-3′	4551[c]
3′-Val-Bgl II	5′-ATA AT*A GAT CT*T TCC TTC TAT AAA CAT TAC AT-3′	6446[c]
5′-Orn-Eco RV	5′-*GCG GAT ATC C*TA AAA TAT TCC ATG AGT TAT TTG-3′	7665[c]
3′-Orn-Bam HI	5′-*CGG GAT CCC G*AG CAT GAT GGA AGG CAT ATT-3′	9577[c]
5′-Leu-Nru I	5′-*ATA TCG C*GA ATC AAA CAA TAC AGG AAT TG-3′	10806[c]
3′-Leu-Bam HI	5′-*TAA G*GA TCC GGA ATA CGT TTC GAT CTG-3′	12696[c]
5′-Cys/pen-Pvu II	5′-*A*TT C*TC* GCG ATG TTT GAA AAC GAA GC-3′	5280[d]
3′-Cys/pen-Bgl II	5′-*T*AG *AG*A *TC*T CAA ATG CAG CGT CGA T-3′	7180[d]
5′-Val/pen-Pma CI	5′-AC*T* A*CA CG*T GAG GAG CAG AAA GTA G-3′	8490[d]
3′-Val/pen-Bgl II	5′-AAC AT*A GA*T CTC TGG CGT TCG GAC-3′	10460[d]
5′-P_{srfA}-Bam HI	5′-*CGG GAT C*CA GCA ATA AAA AAA GAT TGA ACG-3′	–493[b]
3′-P_{srfA}-Bam HI	5′-*CGC GGA* TCC AAT CAA AAA ACA GAT GGC C-3′	–130[b]

[a] Restriction sites are *underlined*, modified sequences are in *italics*
[b] Location of primer in the *srfA* sequence of Cosmina et al. (1993)
[c] Location of primer in the *grs* sequence of Turgay et al. (1992)
[d] Location of primer in the *acvA* sequence of Smith et al. (1990)

Transformation of *B. subtilis*

Transformation of *B. subtilis* was performed according to the method described by Klein et al. (1992). Transformants were plated out on YT plates supplemented with 5 μg/ml chloramphenicol or 10 μg/ml neomycin.

Congression experiments were carried out using pNEXT33A (Itaya and Tanaka 1990). This plasmid contains the gene for neomycin phosphotransferase (*npt*) inserted into the *metD* gene, which enables integration of the *npt* gene into the chromosome of *B. subtilis* by a double cross-over within the flanking *metD′* linkers.

Southern hybridization

Chromosomal DNA was prepared using Genomic Tips (Qiagen) according to the manufacturer's protocol. After digestion, the fragments were separated by agarose gel electrophoresis and transferred to a nylon membrane. Hybridization probes were labelled by standard methods using the Rediprime DNA labelling system (Amersham, Braunschweig, Germany). Southern hybridization reactions were performed as described in Sambrook et al. (1989).

Partial purification of the surfactin synthetases

A 400-ml volume of SpII medium without calcium chloride (Cutting and Vander Horn 1990) was inoculated with cells from colonies grown on a TBAB plate and incubated at 30° C and 250 rpm. In order to examine the expression of the different *srfA-A* genes, the OD_{600} of the cultures were monitored. Samples were taken at several times during the period of transition from log to stationary phase (the time of entry into stationary phase is defined as t_0) and cells were treated with lysozyme for 15 min. Total cellular proteins were analyzed by SDS-PAGE and Western blotting as described above.

All purification operations were carried out at 4° C, except for the FPLC, which was performed as rapidly as possible at room temperature. Partial purification of the surfactin synthetases was carried out as described (Menkhaus et al. 1993; Ullrich et al. 1991; steps 1, 2 and 5 only), except that a Superdex 200 HR 16/60 column was used for gel filtration (Pharmacia, Freiburg, Germany). The protein content of the fractions was determinated using the method of Bradford (1976). In order to distinguish between surfactin synthetases 1 and 2, the fractions were investigated for their Leu- and Val-dependent ATP-PPi exchange activities.

Assays

The ATP-PP_i exchange and thioester formation activities of the fractions were measured as described by Stachelhaus and Marahiel (1995a). The tests were performed at a final concentration of the desired amino acid and ATP of 0.2 mM.

Surfactin preparation

B. subtilis strains were incubated in modified SpII medium as described above for the purification of the surfactin synthetases. After 3 days of incubation, the cells were pelleted by centrifugation and the clear supernatant was extracted twice with one volume of n-butanol. The phases were separated by a short centrifugation step ($1000 \times g$ for 5 min) and the butanol phase was evaporated under vacuum. The residue was dissolved in methanol and the resulting solution was decolorized by shaking with charcoal. After centrifugation, the supernatant was concentrated under vacuum.

In order to analyze the hemolytic activity of the surfactin derivatives, the extracted probes were spotted onto blood-agar plates (Nakano et al. 1988) and incubated for 24 h at 37° C.

Incorporation of [2, 3^3H]Orn

A 3-ml aliquot of modified SpII medium was inoculated with a single colony of *B. subtilis* grown on a TBAB plate and incubated as described above. After 5 h (at about t_0) [2, 3^3H]Orn was added at a final concentration and specific activity of 38 pM and 40 Ci/mol, respectively. After incubation of the culture for an additional 2.5 days, the cells were harvested and the supernatant extracted with n-butanol as described above.

Thin-layer chromatography and mass spectrometry

Silica gel SI F plates (Riedel de Haen, Seelze, Germany) were used with the following solvent system: $CHCl_3/CH_3OH/H_2O$

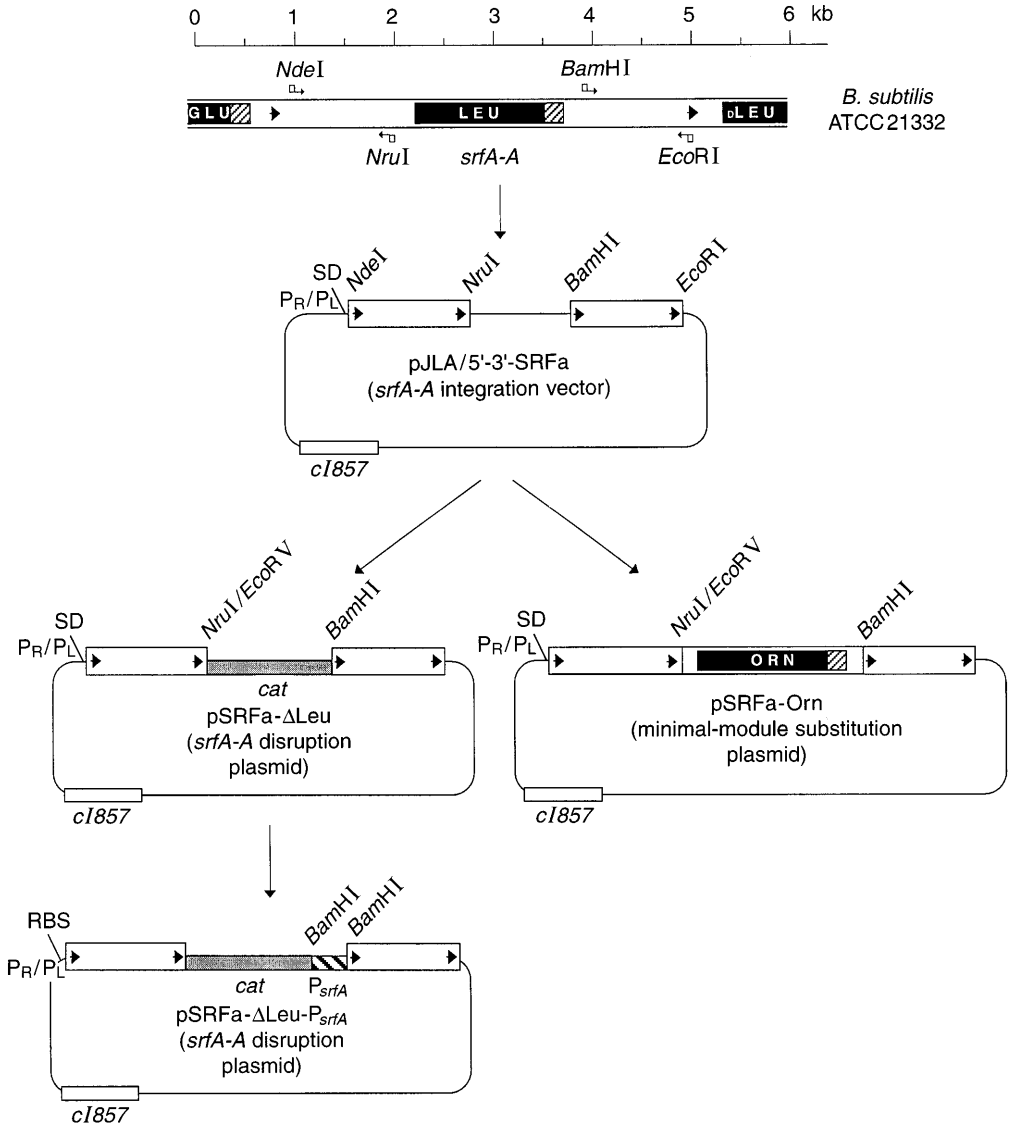


Fig. 2 Construction of a *srfA-A* disruption and minimal-module substitution plasmid. Locations of the primers and the restriction sites generated are indicated. Inserted fragments are under the control of the tandem P_R/P_L promotor, which is regulated by the heat-sensitive λ repressor CI857. Cloning of an internal ATG codon of *srfA-A* at an optimal distance downstream of the Shine-Dalgarno sequence (SD) of pJLA503 allows the expression of gene fragments consisting of an in-frame hybrid of the *srfA-A′* linkers and a heterologous, minimal-module encoding region. For detailed information on the construction of the plasmids, see the text

(65:25:4, v/v) (Ullrich et al. 1991). Radioactive spots were detected by scanning in a Berthold LB2723 thin-layer scanner II. Labelled spots and the surfactin spot were scraped off the plate and eluted from the matrix with methanol. The probes were concentrated and investigated by mass spectrometry. This was carried out using the plasma desorptions technique (PDMS) and was performed by L. Schmidt and H. Jungclas (Abt. Klinische Nuklearmedizin, FB20, Philipps-Universität Marburg, Germany).

Results

Construction of hybrid gene fragments for the targeted substitution of the second minimal-module encoding fragment of *srfA-A*

In order to engineer the integration vector pJLA/5′-3′-SRFa (Fig. 2), the 5′ and 3′ linkers of the second SrfA-A minimal-module encoding region were cloned into pJLA503 as described in Materials and methods. Subsequent cloning of the *cat* gene and the P_{srfA} fragment resulted in the disruption plasmid pSRFa-ΔLeu-P_{srfA} (Fig. 2). Several hybrid gene fragments were constructed by cloning the minimal-module encoding fragments of the *grsA* and *grsB* genes of *B. brevis* ATCC 9999 and the *acv* gene of *Penicillium chrysogenum* between the *srfA-A′* linkers. This resulted in several minimal-module substitution plasmids (Table 1), including pSRFa-Orn, comprising several in-frame fusions of various minimal-module encoding fragments to the *srfA-A′*-linkers (Fig. 2).

In order to verify the structure of the constructs, expression of the hybrid gene fragments was examined as described in Materials and methods. Analysis of the total cellular proteins revealed gene products of approximately 145 kDa, which cross-reacted with antibodies raised against the corresponding minimal module. This verified the in-frame ligation of the linkers and the heterologous fragments (data not shown).

Targeted substitution of the second minimal-module encoding fragment of *srfA-A*

The reprogramming of the *srfA-A* gene in the chromosome of *B. subtilis* was accomplished by gene disruption and fragment substitution, and was monitored by using a selective marker. This approach involved two successive steps: marking the chromosomal target site with the *cat* gene by double cross-over and inserting an engineered *srfA-A′* hybrid fragment into the marked chromosome by a second marker exchange.

First, the surfactin producer strain *B. subtilis* ATCC 21332 was transformed with the disruption plasmid pSRFa-ΔLeu (Fig. 2). This generated a strain that had lost the ability to produce surfactin and develop genetic competence. To by-pass the latter defect, we cloned the *sr*fA promoter in the correct orientation between the *cat* gene and the 3′ *srfA-A′* fragment. This allowed the expression of *comS* as well as *srfA-B* and *srfA-C* (data not shown). The resulting strain (*B. subtilis* AS10, Table 1) is deficient in surfactin production, but still develops genetic competence. Correct substitution of the 1.9-kb *srfA-A′* fragment for the 1.7-kb *cat*-P_{srfA} fragment was demonstrated by Southern analysis (Fig. 3A, B).

Next, the *cat*-P_{srfA} fragment was replaced by one of several minimal-module encoding fragments. This was achieved by congression experiments, in which *B. subtilis* was transformed simultaneously with pNEXT33A and the desired minimal-module substitution plasmid, such as pSRFa-Orn.

Neomycin-resistant transformants were selected and screened for chloramphenicol sensitivity. About 0.35% of the transformants had the desired phenotype (Nm^R, Cm^S). Southern blotting analysis revealed the correct substitution of several minimal-module coding fragments for the *cat*-P_{srfA} fragment (Fig. 3A–C).

Successful substitutions were carried out for all five bacterial minimal-module encoding fragments of the *grs* operon of *B. brevis*, resulting in *B. subtilis* strains AS106, 112, 115, 116 and 122 (Table 1), and for two equivalent fragments of the *acvA* gene from the fungus *P. chrysogenum* (AS103pen and 122pen; Table 1).

Expression of the hybrid *srfA-A* genes in *B. subtilis* and partial purification of the hybrid synthetases

In order to investigate the expression of the hybrid *srfA-A* genes, cells were grown in SpIII medium. The total cellular proteins were prepared as described in Materials and methods, and subjected to SDS-PAGE and Western blot analysis. As shown in Fig. 4 for *srfA-A*(Orn), the hybrid *srfA-A* genes are under control of the *srfA* promoter and were expressed over the same period and at the same rate as the natural *srfA-A* gene. Despite the nearly identical molecular masses of SrfA-A and SrfA-B (402 and 401 kDa, respectively), Western blot analysis revealed that the hybrid SrfA-A enzymes contain the desired minimal module.

The surfactin synthetases were partially purified using ammonium sulphate precipitation, gel filtration and anion-exchange chromatography (Fig. 4, lane 9). Substitution of the Leu2-activating minimal module for a heterologous one caused slight differences in the elution behaviour of the hybrid SrfA-A (not shown). Measurement of the Leu- and Val-dependent ATP-PPi exchange activity of fractions from the ion-exchange column enabled us to distinguish between (hybrid) SrfA-A and SrfA-B, and to identify the fractions with the highest activity (Fig. 5).

Biochemical characterization of the hybrid surfactin synthetases

The first two steps of amino acid activation during nonribosomal peptide biosynthesis are ATP-dependent substrate activation as acyladenylate and the covalent binding of this species to the enzyme as a carboxy thioester. In order to examine whether the swapped minimal modules showed the expected enzymatic activities in the heterologous environment and determine whether these modifications had any effect on the neighbouring natural modules, we tested the different SrfA-A proteins for their amino acid-dependent ATP-PPi exchange activity and for covalent binding of the substrates. As shown in Fig. 5A, the parent SrfA-A exhibited Glu- and Leu- but not Orn-dependent ATP-PPi exchange activity. Moreover, the enzyme showed only weak unspecific binding of Orn and Phe (Fig. 5B).

The hybrid SrfA-A enzymes revealed the expected specificities for Glu, Leu and the substrate amino acid activated by the heterologous minimal-modules. For example, SrfA-A(Orn), purified from *B. subtilis* AS115, exhibited strong Glu-, Orn- and Leu-dependent ATP-PPi exchange activity (Fig. 5A). The ratio of Leu- to Glu-dependent activity decreased from 3.8:1 for the parent enzyme to 2.3:1 for SrfA-A(Orn), which is due to the loss of one Leu-specific module. Nevertheless, the basis for the difference between Glu- and Leu-dependent activity remains unclear. This phenomena is also observed for the Asp-dependent ATP-PPi exchange of SrfA-B and is in agreement with data measured by Menkhaus et al. (1993) and Ullrich et al. (1991). Furthermore, SrfA-A(Orn) revealed efficient covalent binding of Orn (Fig. 5B) and, like the wild-type enzyme, it showed weak binding of Phe.

Influence of minimal-module substitutions on surfactin biosynthesis

To examine the influence of minimal-module substitutions on surfactin biosynthesis in vivo, we analysed – as an example – surfactin production by *B. subtilis* AS115. Due to the substitution of the second minimal-module of SrfA-A, we expected that the strain would now incor-

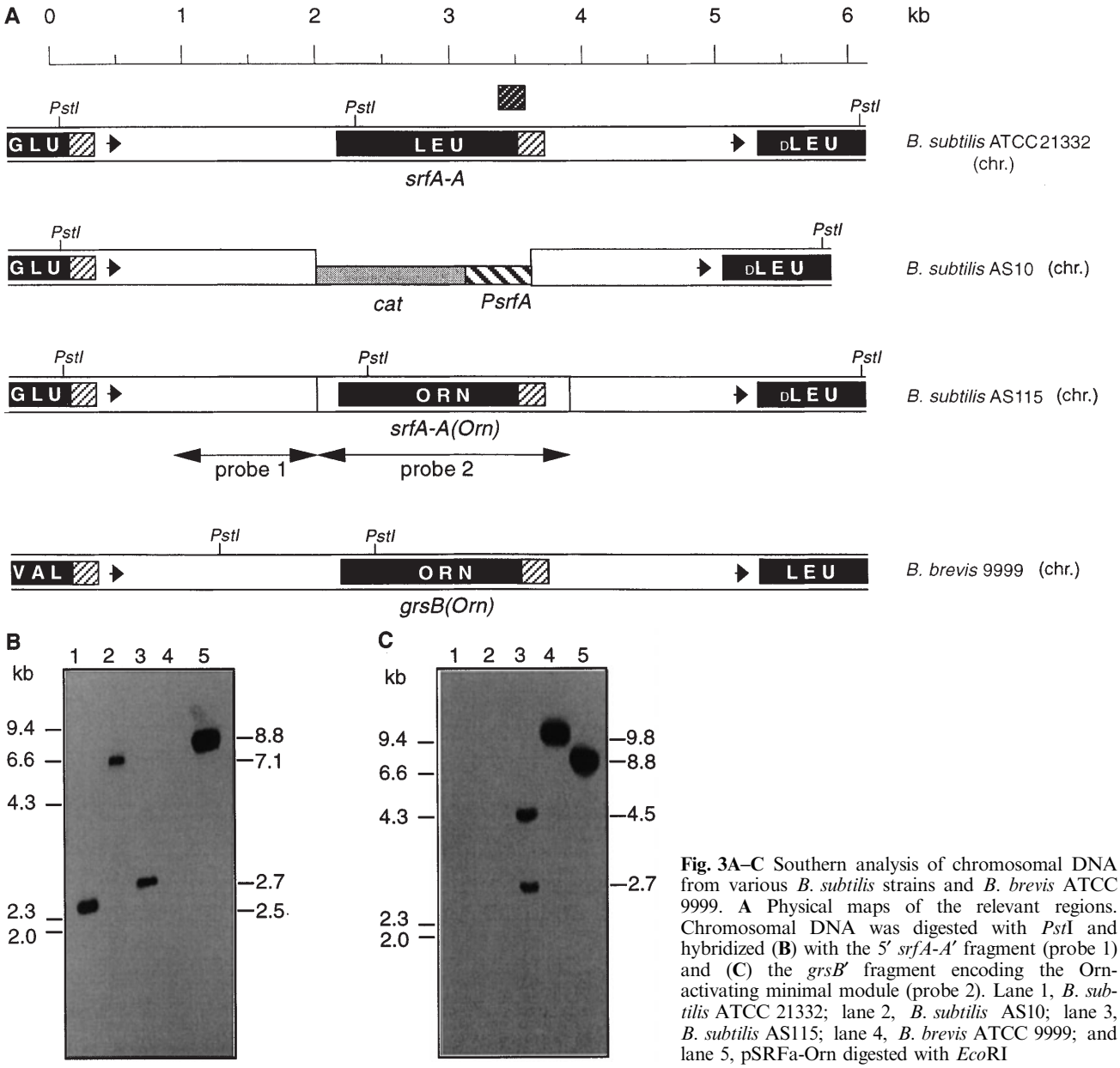


Fig. 3A–C Southern analysis of chromosomal DNA from various *B. subtilis* strains and *B. brevis* ATCC 9999. **A** Physical maps of the relevant regions. Chromosomal DNA was digested with *Pst*I and hybridized (**B**) with the 5′ *srfA-A′* fragment (probe 1) and (**C**) the *grsB′* fragment encoding the Orn-activating minimal module (probe 2). Lane 1, *B. subtilis* ATCC 21332; lane 2, *B. subtilis* AS10; lane 3, *B. subtilis* AS115; lane 4, *B. brevis* ATCC 9999; and lane 5, pSRFa-Orn digested with *Eco*RI

porate the non-proteinogenic amino acid Orn into the second position of the growing peptide chain.

After addition of [2,3^{3}H]Orn to the growing cultures, the lipopeptide was purified as described in Materials and methods. Radioscanning of the TLC plate revealed only one prominent spot for the wild-type probe. The retention (R_f) value of 0.07 was similar to the R_f value for [2,3^{3}H]Orn (Fig. 6). Under UV light, an unlabelled spot with an R_f value of 0.54 was detected. This value corresponds to that previously determined elsewhere for wild-type surfactin (Peypoux et al. 1991, 1994; Ullrich et al. 1991; Menkhaus et al. 1993). Besides non-metabolized Orn, radioscanning of the AS115 probe revealed two additional spots with R_f values of 0.16 and 0.28 (Fig. 6).

The (radioactive) probes were eluted from the matrix and analyzed by mass-spectrometry. This revealed a mixture of surfactin and surfactin derivatives (Table 3). Surfactin extracted from the wild-type strain yielded peaks at a mass-to-charge ratios (m/z) of 995, 1009 and 1023, as expected from the known sequence with slight variations in the length of its β-hydroxy fatty acid moiety (n = 6 to 8). These $[M + H]^+$ peaks were accompanied by corresponding $[M + Na]^+$ signals appearing at m/z 1017, 1031 and 1045.

The data obtained for spots 2 and 3 not only showed the slight differences from the values obtained for surfactin, which were expected to result from the Leu to Orn substitution, but rather pointed to a more extensive modification of the surfactin derivatives. Spot 2 revealed

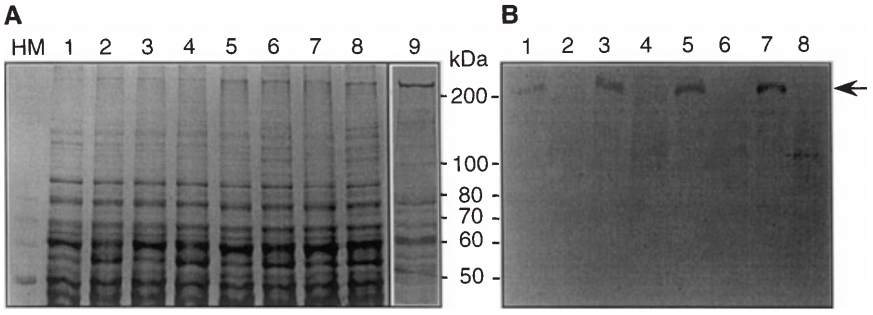


Fig. 4A, B Expression of the hybrid gene *srfA-A*(*Orn*) in *B. subtilis* and partial purification of the corresponding gene product. At the transition between logarithmic and stationary growth phases (the point of entry into stationary phase is defined as t_0), samples of *B. subtilis* ATCC 21332 and *B. subtilis* AS115 cells were taken and treated with lysozyme. **A** SDS-PAGE of total cellular proteins (lane 1–8) and of the pool recovered after anion-exchange chromatography (lane 9), stained with Coomassie blue. **B** Western blot analysis of the gel in **A** with antibodies raised against the Orn minimal-module of GrsB. The following samples were used: *B. subtilis* AS115 30 min before t_0 (lane 1), at t_0 (lane 3), 30 min after t_0 (lane 5) and 1 h after t_0 (lane 7); *B. subtilis* ATCC 21332 30 min before t_0 (lane 2), at t_0 (lane 4), 30 min after t_0 (lane 6) and 1 h after t_0 (lane 8); HM, high molecular weight markers. The *arrow* indicates the position of SrfA-A(Orn) ($\sim$400 kDa)

peaks at *m*/*z* 733 and 747 $[M + H]^+$ as well as 755 and 769 $[M + Na]^+$; spot 3 at 885, 899 and 913 $[M + Na]^+$ (Table 3). These data correspond exactly to those expected for surfactin derivatives that have Leu to Orn substitutions at position 2 but also have shorter peptide chains than wild type [Δ(Leu7-Leu6) or Δ(Val4-Leu3) for spot 2 and ΔLeu7 or ΔVal4 for spot 3] (see Discussion).

In order to investigate the influence of the amino acid substitutions on hemolytic activity, we analyzed the extracted lipopeptides on blood-agar plates. Surfactin derivatives extracted from *B. subtilis* AS106, AS115 and AS122 revealed weak hemolytic activities, whereas no activity was detectable in extracts from the *srfA-A* disruption mutant *B. subtilis* AS10 or from AS112, 116, 103pen and 122pen (not shown).

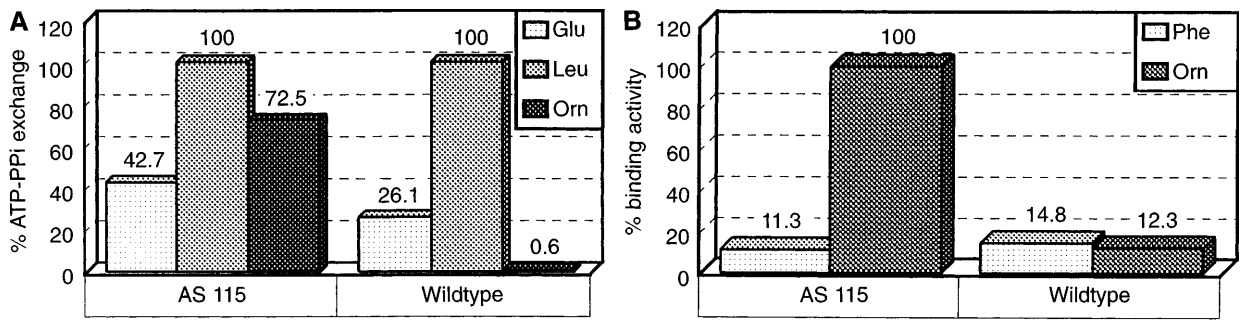
Discussion

Recently, we described a general method for the targeted replacement of an amino acid-activating minimal module within surfactin synthetase SrfA-C (Stachelhaus et al. 1995, 1996). This enzyme contains only one module and therefore represents the simplest peptide synthetase system.

The aim of this study was to extend this method to a multi-modular peptide synthetases, in particular to SrfA-A, which contains three modules. The results presented demonstrate that it is possible to exchange amino acid-activating minimal modules between multi-modular peptide synthetases of heterologous origin. This was achieved by targeted reprogramming of a peptide synthetase gene and was accomplished in two steps. The chromosomal target site was first marked with a selectable marker by double cross-over and an engineered hybrid gene that encodes a peptide synthetase with an altered amino acid specificity was then inserted into the site.

Disruption of the *srf*A operon upstream of the *com*S gene (see Fig. 1) led to a *B. subtilis* strain that was not only deficient in surfactin production, but also unable to develop genetic competence. The latter defect was bypassed by cloning the *srf*A promoter directly upstream of the 3′ linker, which permitted transcription of the residual *srf*A operon, including *com*S. The resulting *srfA-A* disruption mutant was deficient in surfactin

Fig. 5A, B Specific enzymatic activity of SrfA-A and SrfA-A(Orn). MonoQ fractions were examined for their Glu-, Leu- and Orn-dependent ATP-PPi exchange activities (**A**) and covalent binding of Phe and Orn (**B**). The highest activity of each enzyme was defined as 100% and the values obtained for the other amino acids are expressed relative to that



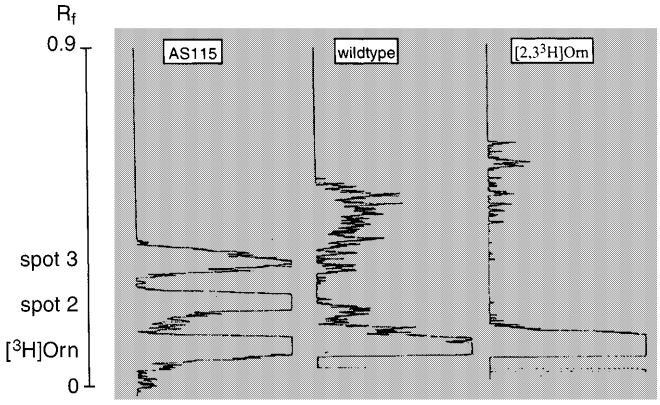


Fig. 6 Determination of [2, 3-^{3}H]Orn incorporation. Butanolic extracts of *B. subtilis* AS115 (AS115) and ATCC 21332 (wild type) were separated by TLC and labelled spots were detected by radioscanning. As a control, pure [2, 3-^{3}H]Orn was used

biosynthesis, but still developed genetic competence. Furthermore, this result confirmed earlier observations that the small region upstream of *srfA* (here the P_{srfA} fragment) is sufficient for the control of *srf*A transcription by the phosphorylated response regulator protein ComA (Nakano and Zuber 1991; Nakano et al. 1991; Roggiani and Dubnau 1993).

The second step in the procedure was achieved by a congression experiment. *B. subtilis* AS10 was transformed simultaneously with pNEXT33A and a given minimal-module substitution plasmid, which allowed for positive selection of the desired transformants. The marker exchanges led to the formation of the desired hybrid *srfA-A* genes encoding hybrid SrfA-A proteins.

Partial purification of different SrfA-A derivatives revealed that their expression rates are similar and that the fusions between the *srfA-A′* linkers and heterologous minimal-module encoding fragments are in frame. Enzymatic assays demonstrated that the heterologous minimal modules retain their activities for specific substrate activation and that the neighbouring minimal modules were not affected by the substitution of the central minimal module. Similar domain substitutions were reported recently for type I polyketide synthases (Oliynik et al. 1996; Kuhstoss et al. 1996). In both experiments, replacement of an acyltransferase domain or of a loading module by a heterologous counterpart resulted in hybrid polyketide synthases, in which the heterologous domains still retains their specific enzymatic activities. Furthermore, this led to the predicted alterations in the structure of the polyketides synthesized.

Nevertheless, swapping of minimal modules in peptide synthetases resulted in a strong reduction in the rate of lipopeptide biosynthesis, which suggests that intermodular interactions might be disturbed. The same observation has been made for substitutions of the seventh minimal module of the surfactin synthetase complex (Stachelhaus et al. 1995). In both cases, variation in the primary structure of the resulting lipopeptide can be excluded as a reason for this phenomena, since exchange of Leu minimal modules showed the same effect (Stachelhaus et al. 1995).

In contrast to substitutions of the seventh amino acid residue in the cyclic peptide chain, exchanges at position 2 resulted in additional modifications. Mass-to-charge ratios obtained for the [2, 3-^{3}H]Orn-labelled lipopeptides, for example, are best explained by a Leu to Orn substitution at position 2 and a concomitant lack of one or two amino acid residues. The reason for this failure to incorporate the other amino acids is not fully understood; however, one likely explanation takes into consideration the complex structure of surfactin. Although its exact conformation has not been precisely determined, two very similar models exist, which describe a ''saddle'' topology for the cyclic backbone (Bonmatin et al. 1992, 1994). There seem to be extensive intramolecular interactions which play an essential role in maintaining the structure of surfactin. In particular, a hydrogen bond between the Leu2 carbonyl and the Asp5 amide groups stabilizes the β-turn that is characteristic for the structure of surfactin (Bonmatin et al. 1992). Several natural amino acid substitutions within the peptide chains of surfactin, as well as other non-ribosomally synthesized peptide antibiotics, have been described, which are due to a reduction in the substrate specificity of certain modules (Peypoux et al. 1994). In particular, the natural occurrence of a Val to Ala substitution at position 4 (Peypoux et al. 1994) and a Leu to Ile or Val exchange at position 7 (Baumgart et al. 1991; Peypoux et al. 1991, Oka et al. 1993) indicate that these positions are not essential for the topology of surfactin

Table 3 Molecular masses of samples eluted from spots 2 and 3 and from purified surfactin

Sample[a]	Methyl groups[b]					
	n7		n8		n9	
	$[M+H]^+$	$[M+Na]^+$	$[M+H]^+$	$[M+Na]^+$	$[M+H]^+$	$[M+Na]^+$
Surfactin	1009	1031	1023	1045	1037	1059
Spot 2	733[b]	755[b]	747[b]	769[b]		
Spot 3		885[b]		899[b]		913[b]

[a] Samples were recovered from the chromatograms shown in Fig. 6 and subjected to mass spectrometry

[b] The number of methyl groups is indicated by n, and was calculated for [Δ(Val4)Orn2] or [Δ(Val4-Leu3)Orn2]surfactin. In [Δ(Leu7)Orn2] or [Δ(Leu7-Leu6)Orn2]surfactin, the β-hydroxy fatty acid moiety would have one further methyl group

and can be altered without further effects on the structure of the lipopeptide (Stachelhaus et al. 1995, 1996). A previous comparison of the structures of iturin A and mycosubtilin showed that a substitution of only one residue in the cyclic peptide sequence induced important changes in the conformation and biological properties of the lipopeptides (Marion et al. 1986; Peypoux et al. 1986; Genest et al. 1987).

Taking these observations together, one must suppose that a substitution of the second amino acid residue in surfactin causes a more substantial modification in the conformation of the peptide product. For example, a Leu to Orn substitution could induce a nucleophilic attack of the side-chain amino group of Orn2 on the Asp5 carboxyl group. This would result in premature circularization, resulting in a cyclic branched conformation and the loss of the fourth or both the third and fourth amino acid residues (Fig. 7). Alternatively, the substitution could induce the formation of an ester bond between the carboxyl group of Asp5 or Leu6 and the hydroxy group of the β-hydroxy fatty acid moiety (Fig. 7). A peptide bond between the side-chain amide group of Orn2 and the side-chain carboxyl group of Asp5 is also conceivable and could result in a double cyclic conformation. This would cause the additional loss of 18 Da (theoretical loss of one H_2O), which, however, can be excluded by mass spectrometry (Table 3).

In conclusion, our study shows that it is possible to alter the substrate specificity of multi-modular peptide synthetases by targeted substitution of minimal-module encoding regions within synthetase genes. The resulting hybrid synthetases comprise minimal modules of different origins, which retain their specific enzymatic activities. However, the fact that such a modification of the synthetase complex may induce more extensive modification of the peptide-product must be taken into consideration.

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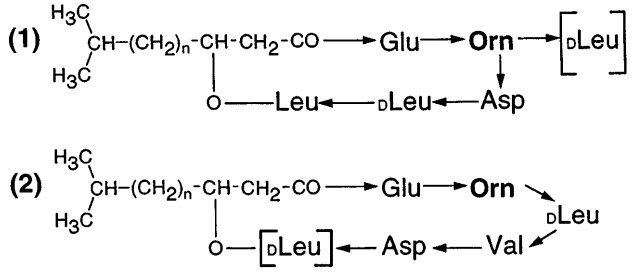

Fig. 7 Putative primary structures of surfactin derivatives. (1) Branched cyclic structure of [Δ(Val4-Leu3)Orn2] and [Δ(Val4)Orn2]surfactin; (2) cyclic structure of [Δ(Leu7-Leu6)Orn2] and [Δ(Leu7)Orn2]surfactin, respectively; n indicates the number of methyl groups in the variable β-hydroxy fatty acid moiety

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