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Recombinant Acylheptapeptide Lichenysin: High Level of Production by *Bacillus subtilis* Cells

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Abstract

Peptide synthetases are multi-domain proteins that catalyze the assembly, from amino acids and amino acid derivatives, of peptides and lipopeptides, some of which exhibit activities (pharmaceutical, surfactant, etc.) of considerable biotechnological importance. Although there is substantial interest in the generation of greater peptide diversity, in order to create new biotechnologically interesting products, attempts reported so far to exchange amino acid-activating minimal modules between enzymes have only yielded hybrid catalysts with poor activities. We report here the replacement of an entire first, L-Glu-, and fifth, L-Asp-incorporating modules of surfactin synthetase, to create a fully active hybrid enzyme that forms a novel peptide in high yields. Whole encoding regions of lichenysin A synthetase modules were introduced into surfactin biosynthesis operon between His140/His1185 of SrfAA and His1183/His2226 of SrfAB, the amino acid residues of a proposed active-site motif (HHXXXDG) of the condensation domains which is involved in the catalysis of nonribosomal peptide bond formation (Stachelhaus *et al.,* 1998). When the lipopeptides produced by the recombinant *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 by their fatty acid profiles.

We thereby demonstrate the utility of whole module swapping for designing novel peptides, for creating peptide diversity, and for redesigning existing peptides produced in performant production strains in high yields to correspond to desired peptides produced in low yields, or from strains unsuitable for production purposes.

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Introduction

Non-ribosomal, enzymatic synthesis of peptides by the multiple carrier thiotemplates of various fungi and bacteria is a fascinating topic of molecular microbiology that has been intensively investigated during the last decade. The hundreds of types of peptides synthesized by this mechanism form a diverse group of bioactive natural products, such as antibiotics, anti-tumor and anti-viral agents, biosurfactants, enzyme inhibitors, immunosupressants and toxins, with important medical and biotechnological applications. The broad spectrum of distinct activities reflects the structural diversity of these ordinarily short, linear, branched or cyclic peptides, most of which contain modified amino acids (there exist some 300 different types of amino acid residues, including acylated, D-, hydroxy-, N-methylated, glycosylated or non-protein amino acids) (Kleinkauf and von Dohren, 1997). There is substantial interest in the generation of greater peptide diversity, in order to find novel bioactive compounds, to improve existing peptides, and to achieve high yields of certain peptides in production strains.

All prokaryotic peptide synthetases that mediate the stepwise assembly of a peptide chain exhibit a multi-modular structure with highly conserved features (Marahiel, 1997). This structural architecture and the autonomous nature of the modules, a coordinated transcription and physical linkage in the multienzyme complex of which are not essential for proper assembly and activity of the peptide synthetases (Guenzi *et al*., 1998), facilitates the creation of new peptide products by the replacement of modules by means of cistron exchange (Stachelhaus *et al*., 1995).

It was recently shown that the L-Leu-activating minimal modules of surfactin synthetase 1 (SrfAA) and surfactin synthetase 3 (SrfAC) can be replaced by the corresponding domains of heterologous synthetases exhibiting different specificities (Stachelhaus *et al*., 1995; Schneider *et al*., 1998). The enzyme could be also modified by moving the carboxyl-terminal intrinsic thioesterase-like region to the end of the internal amino acid-binding domains, thus generating strains that produce new truncated peptides of the predicted sequence and length (de Ferra *et al*., 1997). However, a strong reduction in the rate of lipopeptide biosynthesis was observed for all peptide synthetases so far engineered. In this study, we have therefore explored the possibility that the exchange of entire amino acid-incorporating modules of different origin may conserve principal structural features important for domain-domain interactions, and generate hybrid peptide synthetases with high catalytic activities.

To assess the utility of whole module replacement we employed as model system the surfactin and lichenysin A synthetases, which produce the structurally related lipopeptides, surfactin and lichenysin A, respectively, and have similar modular molecular architectures (Figure1).

Lichenysin A, produced by *B. licheniformis* strains BAS50 and BNP29, is a cyclic lipoheptapeptide

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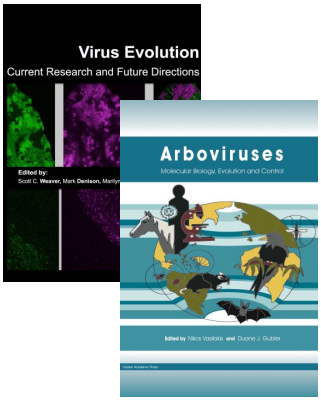

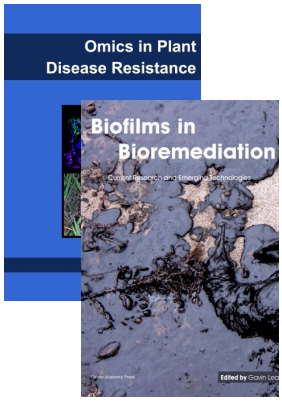

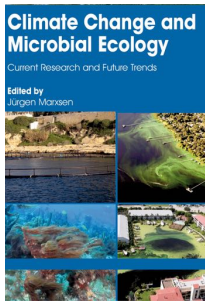

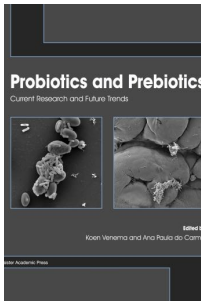

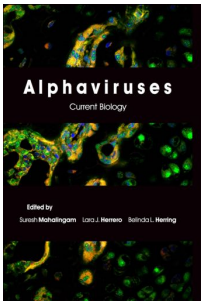

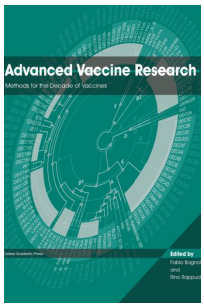

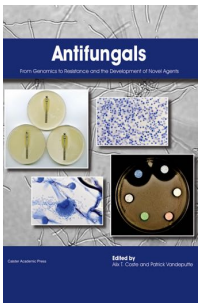

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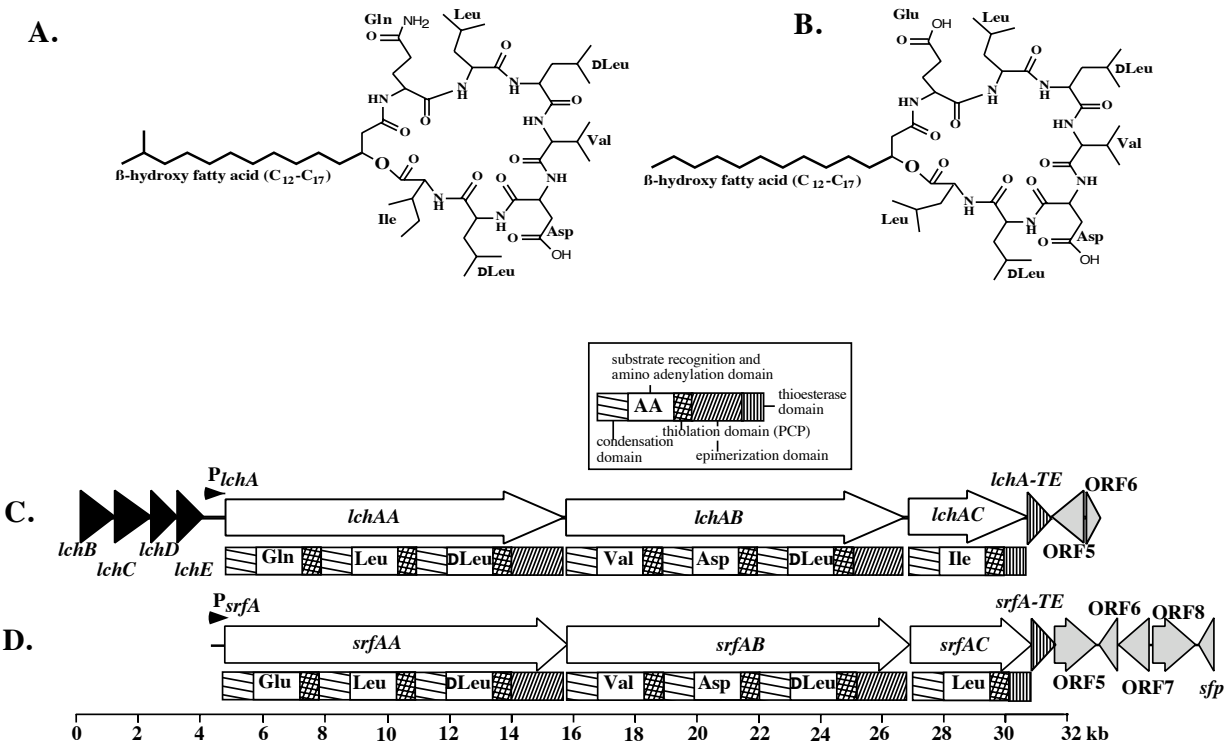


Figure 1. Primary structure of lipoheptapeptides and organisation of their genetic determinants. The primary structure of lichenysin A (A) and surfactin (B), and the structural organization of the chromosomal regions containing the lichenysin A, *lchAA-C* (C) and surfactin, *srfAA-C* (D), synthetase operons are shown, as are the locations of promoters (*P*) and genes associated with these operons (genes encoding ABC-like transporter system; *lchB-E*, thioesterases, *lchA-TE* and *srfA-TE*; 4'-PP transferase, *sfp*; and genes with unknown functions, ORF5-8).

characterized by one of the highest biosurfactant activities thus far reported (Yakimov *et al.*, 1995). It is also halotolerant, which makes it particularly suitable for applications in salt-containing situations, such as tertiary oil recovery (Yakimov *et al.*, 1997). Whereas it is superior to the well known lipopeptide surfactin produced by *B. subtilis*, both in terms of its specific activity and halotolerance, lichenysin A is produced in much lower amounts, more than one order of magnitude lower, than surfactin (Yakimov *et al.*, 1996). Although current knowledge on the expression of peptide synthetases is such that it should be possible to achieve much higher levels of synthesis of lichenysin A by genetic optimization of expression signals (Yakimov and Golyshin, 1997), all attempts to genetically manipulate *B. licheniformis* strains have so far been unsuccessful.

Lichenysin A differs structurally from surfactin in three respects, namely qualitatively, in two constituent amino acids (it has glutamine instead of glutamate as the first residue, and isoleucine instead of leucine as the last), and quantitatively, in the composition of its lipid substituents. It is likely, however, that the Glu/Gln difference is the most relevant to the difference in activities of these two lipopeptides. Thus, we reasoned that if it were possible to substitute the Glu residue of surfactin by the Gln residue of lichenysin A, by exchanging the genetic determinants of the correspondent modules of the peptide synthetase, a new lipopeptide would be generated with properties similar to those of lichenysin A. The construction of *B. subtilis* recombinants containing a hybrid surfactin synthetase which produce high yields of a novel lipopeptide exhibiting the activity level of lichenysin A is present in this work.

Results

Construction of a Hybrid Surfactin Synthetase

The genetic organization of the lichenysin A (*lchA*) and surfactin A (*srfA*) synthetases are shown in Figure 1C, D. The substrate condensation/elongation region present in all amino acid-incorporating modules of surfactin and lichenysin A synthetases is characterized by a high degree of variation compared with other functional domains within the module structure (Marahiel, 1997; Stachelhaus *et al.*, 1998). This specific sequence was therefore assumed to be reasonably tolerant of minor sequence changes, and two encoding regions within SrfA synthetase operon between His140/His1185 of SrfAA and His1183/His2226 of SrfAB were chosen as the sites for the whole module replacements strategy.

The first step in the directed re-programming of surfactin synthetase SrfAA and SrfAB was the deletion of the Glu- and Asp-incorporating modules, respectively. Three types of deletion within the *srfA* operon were generated by using the general strategy shown in Figure 2 and described in detail in Experimental procedure. In the first type of mutants, the 3.16-kb fragment of *srfAA* was replaced with a 1.4-kb fragment carrying the *aphA-3* kanamycin resistance gene (Km^R) (Trieu-Cuot *et al.*, 1985) by the means of the disruption plasmid pSRF-Δ1D. Additionally to this substitution, the imperfect palindromic

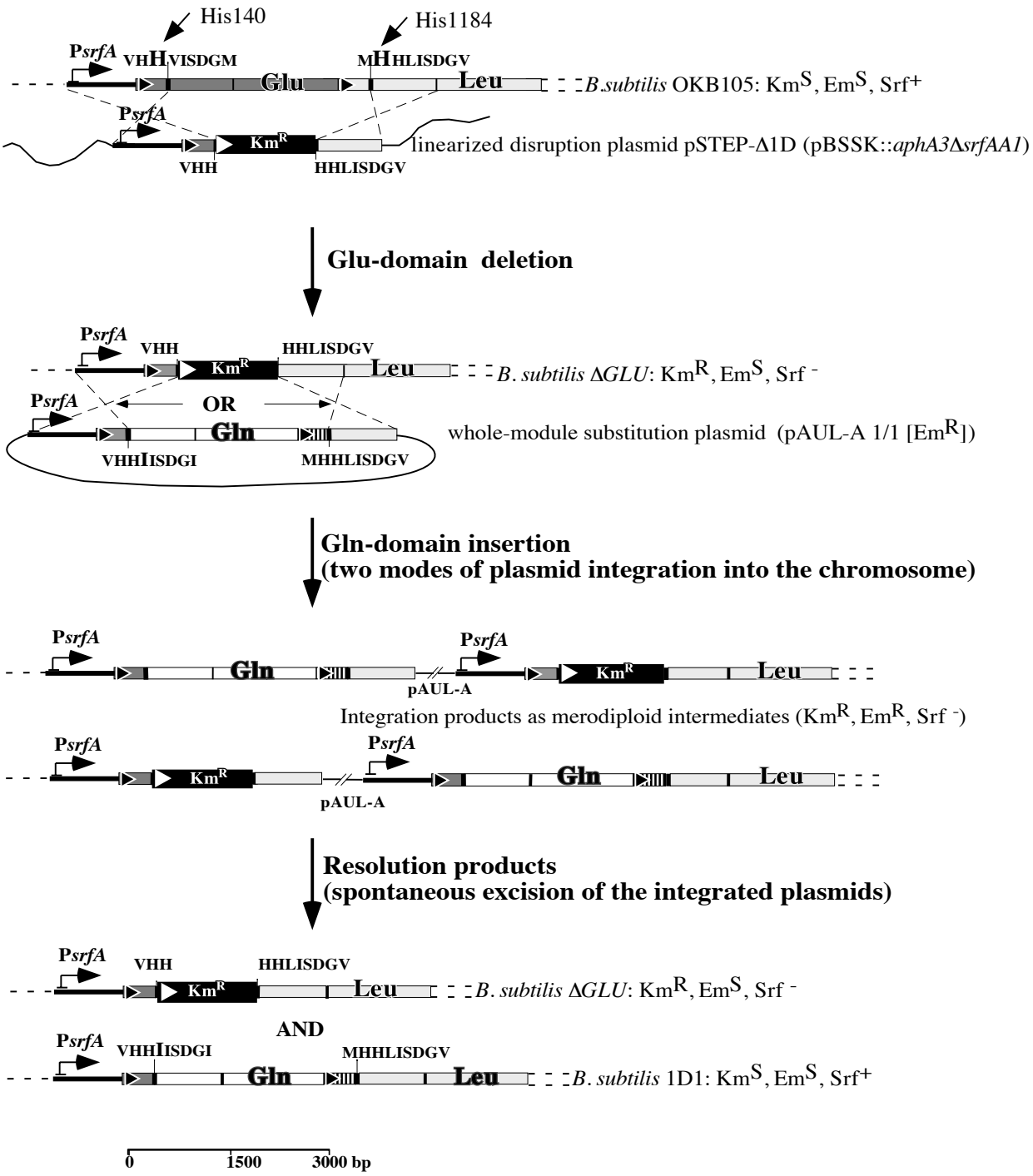


Figure 2. Strategy for whole-module targeted replacement of *srfAA1* in *B. subtilis*.

sequence (ComA-box 2) located between -74 and -59 from the start of transcription and serving as a binding site for transcriptional activator ComA (Nakano and Zuber, 1993; Yakimov and Golyshin, 1997), was improved in the second type of mutants by the means of the disruption plasmid pSRF-BoxΔ 1D. The third type of mutants has a deletion of *srfAB* fragment from position 3549 to 7753 which encodes Asp-incorporating module. Restriction and sequencing analysis of the relevant PCR-amplified product obtained using the CHECKF and CHECKR primers annealing to the chromosomal regions located upstream and downstream of the integration sites, confirmed the presence of the kanamycin resistance gene at the chromosomal location in Km^R clones deficient in surfactin production (Figure 3).

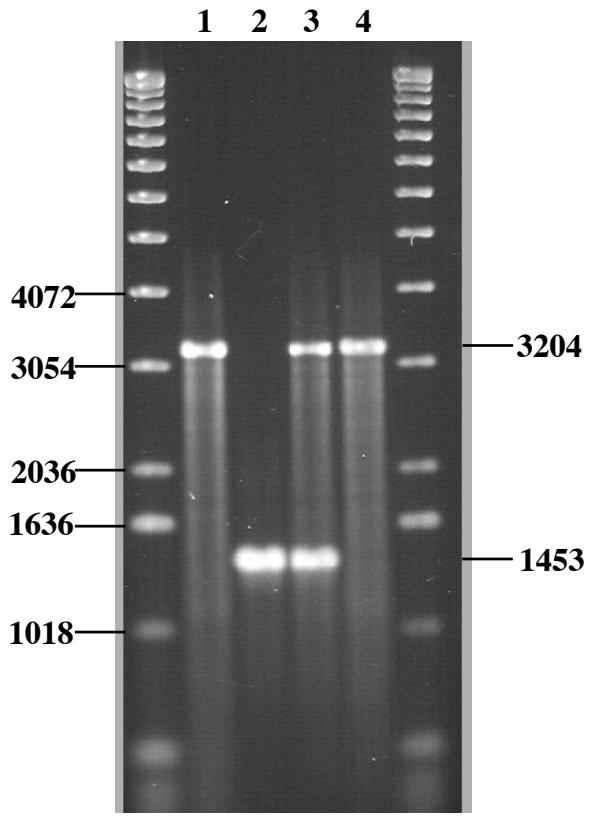


Figure 3. Analysis of the *srfAA1* locus in the constructs. PCR amplification with the CHECKF (389) and CHECKR (3581) primers was carried out with chromosomal DNA isolated from various *B. subtilis* strains used and obtained in this work. Lane A, *B. subtilis* OKB105; lane B, *B. subtilis* ΔGlu (Km^R, Em^S, Srf^-); lane C, *B. subtilis* merodiploid intermediate ΔGlu/Gln (Km^R, Em^R, Srf^-); lane D, *B. subtilis* 1D1 (Km^S, Em^S, Srf^-, "Lch" $^+$).

The second step involved replacement of the *aphA-3* locus with the gene fragments encoding appropriate modules of lichenysin A synthetase by marker exchange. This was achieved by introduction into the deletion mutants of the corresponding module-substitution plasmids, pAUL-A1/1, pAUL-A1B/1 and pAUL-A5/5 by transformation, followed by spontaneous excision of integrated plasmids from the merodiploid intermediates (Em^R, Km^R), by intramolecular homologous recombination (Figure 2) under nonselective conditions. Erythromycin-sensitive clones (Em^S) were selected and screened for kanamycin sensitivity (10% - 17% of Em^S transformants) and Em^S-Km^S clones were tested for production of active surfactant on blood agar plates (hemolytic activity). Halo-positive clones (60% - 89% of Em^S, Km^S transformants) were analyzed for correct in-frame substitution by PCR amplification of chromosomal DNA from the recombinants, using the CHECKF and CHECKR primers and by sequencing the obtained products (Figure 3). This confirmed the expected substitution within the relevant modules of the *srfA* operon in the chromosome of recombinant *B. subtilis* 1D1, 1B1 and 5D5 (for reference see GenBank accession number AF121767 – AF121769).

Physico-Chemical Characterization of the Hybrid Surfactant

The recombinant lipopeptides produced by the hybrid synthetase was extracted from the culture broth of strains, purified, and analysed by TLC using a chloroform : methanol: 28%NH_4OH (65:25:8, v/v/v) solvent system (Yakimov *et al.*, 1995). The mobility index (R_f) of the hybrid lipopeptide 1D1 in the TLC system employed was the same as that of wild-type lichenysin A and distinctly different from that of surfactin (Figure 4A). The negative fast atom bombardment FAB mass spectrometry (FAB^- - MS) ionization spectra of lichenysin A and recombinant lipopeptide were identical and showed deprotonated molecular ions $[M-H]^-$ at *m/z* 991, 1005, 1019, 1033 and 1047 (Figure 4A), while the structurally similar surfactin yielded in the same ionization mode major peaks that are 1 Dalton more, namely at *m/z* 1006, 1020 and 1034, reflecting the molecular mass difference between glutamine and glutamate (Yakimov *et al.*, 1999). These data, together with the lower polarity of recombinant lipopeptide 1D1 compared to that of surfactin, clearly indicate the replacement of the original Glu1 by a Gln1 residue, as deduced from the sequence of lichenysin A. The 1B1 and 5D5 recombinant lipopeptides were also analyzed by TLC and mass-spectrometry and was found to be identical to lipopeptide 1D1 and surfactin, respectively.

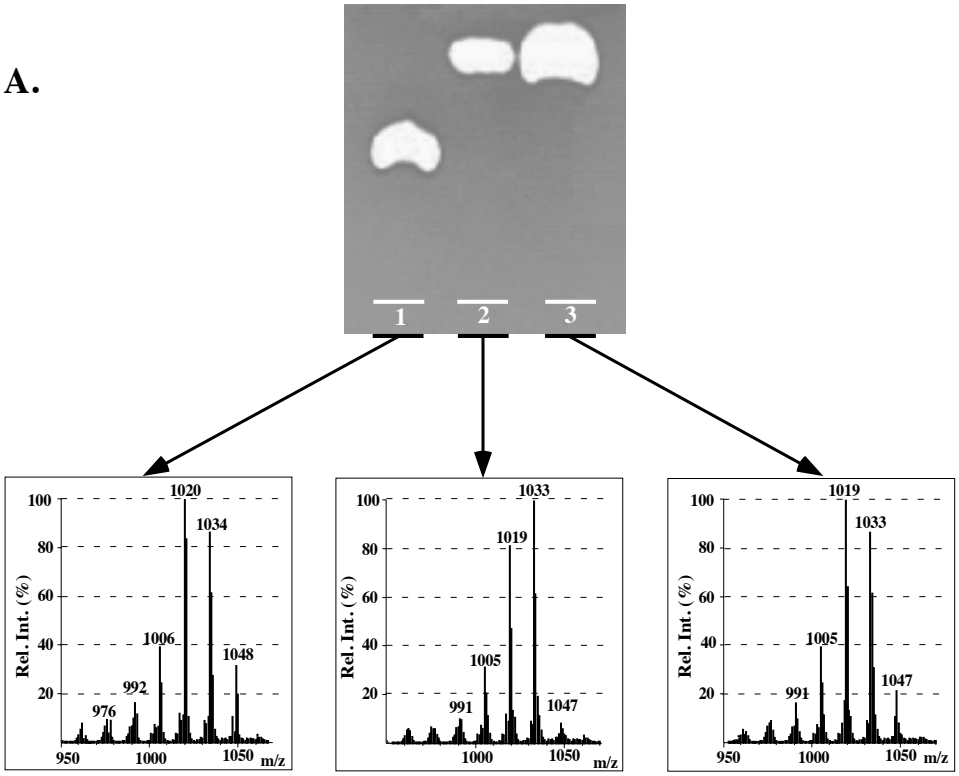
Surface Activity of the Recombinant Lipopeptide

The recombinant lipopeptide 1D1 exhibited a high surface activity, in that it decreased the surface tension of water from 72 mN/m to 25.5 mN/m, and achieved a critical micelle concentration (CMC) at concentrations as low as 13.5 μM, which are similar to that of native lichenysin A (12 μM) and significantly lower than that of surfactin (20 μM). Moreover, in contrast to surfactin whose activity is progressively inhibited by increasing salt concentrations, the recombinant lipopeptide is not inhibited by NaCl concentrations up to 250 g/l (Figure 4B). Such a halotolerance was previously

Table 1. Lipopeptide Production of Parent and Recombinant Bacilli[a]

Strain / description	Dry biomass, g/l	Lipopeptide yield, μM/g
B. subtilis OKB105 / Wild type surfactin	1.92 ± 0.05	792 ± 16
B. licheniformis BNP29/Wild type lichenysin A	1.85 ± 0.04	58 ± 7
Recomb. 5D5 / Φ(*srfAB2-l chAB2[Asp]-srfAB3*)	1.97 ± 0.13	806 ± 37
Recomb. 1B1 / Φ(*PsrfA-lchAA1[Gln]-srfAA2*)	1.83 ± 0.06	818 ± 46
Recomb. 1D1 / Φ(*PsrfA-lchAA1[Gln]-srfAA2*)	1.89 ± 0.03	730 ± 54

[a]Values are means of data obtained from three independent series of biomass determination and lipopeptide isolation procedures after 40 h of cultivation at 30˚C



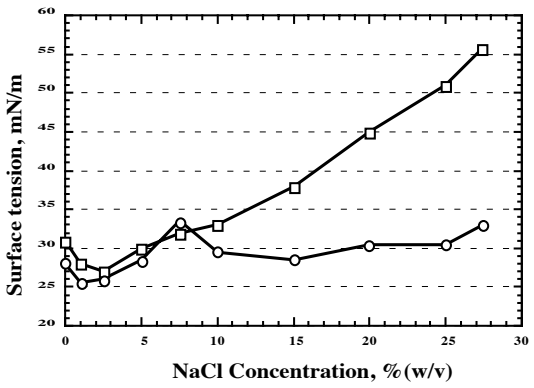


Figure 4. Physico-chemical properties of native and recombinant lipoheptapeptides. (A) TLC of lipopeptides in an alkaline solvent system and their corresponding FAB^- MS spectra. Lane 1, surfactin; lane 2, lichenysin A; lane 3, recombinant lipopeptide 1D1. (B) Surface tension characteristics of surfactin (open squares) and lipopeptide 1D1 (open circles) as function of NaCl concentration.

described for lichenysin A, which is produced by thermo- and halotolerant strain of *B. licheniformis* (Yakimov *et al.*, 1995; 1997).

Lipid Composition of Recombinant Lipopeptide

To characterize the lipid moieties of recombinant lipopeptides 1B1, 1D1 and 5D5, the ß-hydroxy fatty acids were released by hydrolysis and analysed as methyl ß-trimethylsilyl (Me/TMS) derivatives by GC-MS. The resulting spectrum was identical for all lipopeptides and showed that four ß-hydroxy acids, namely isomyristic, myristic, 13-methylmyristic and 12-methyltetradecanoic acids, are major residues in the lipid moieties, and make up about 88% of the total fatty acids present in lipopeptide. The fatty acid composition of the recombinant lipopeptide thus corresponds to that of native surfactin, and not of wild-type lichenysin A (Figure 5).

Biosynthesis Levels of the Recombinant Lipopeptide

Analysis of the specific yield (nmol of product per mg of dry biomass) of recombinant lipopeptides from obtained *B. subtilis* strains revealed the yields similar to that of native surfactin produced by *B. subtilis* OKB105 and were around twelve- fold higher than yields of native lichenysin A by strain *B. licheniformis* BNP29 (Table 1).

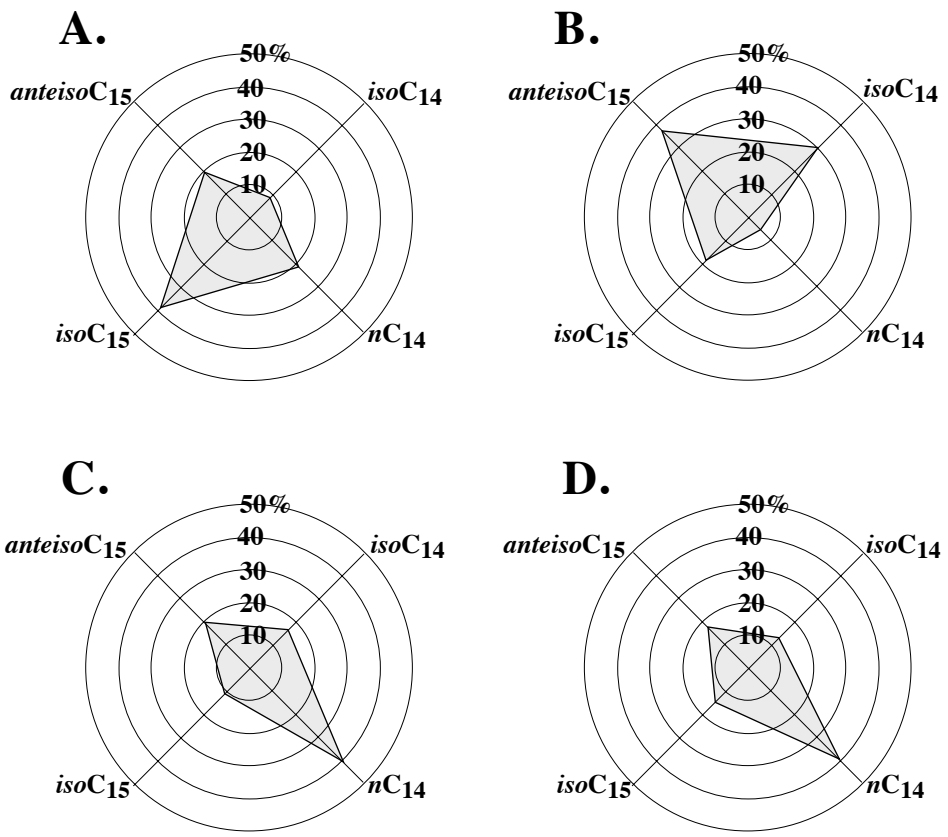


Figure 5. Relative abundances of the four major ß-hydroxy fatty acid series constituting the lipid tails of the lipopeptides: A: native lichenysin A from *B. licheniformis*; B: wild type lichenysin B (Yakimov *et al.*, 1995); C: native surfactin; and D: recombinant lipopeptide 1D1.

Discussion

A general method for the targeted replacement of Leu-activating minimal modules within surfactin synthetase subunits, SrfAA and SrfAC was described recently (Stachelhaus *et al.*, 1995; Schneider *et al.*, 1998). The results presented demonstrate a possibility to exchange minimal modules between multi-modular peptide synthetases of heterologous origin with retaining their activities for specific substrate activation. Nevertheless, swapping of mini-modules in peptide synthetases resulted in a strong reduction in the rate of lipopeptide production. Variation in the primary structure of the resulting lipopeptides can be excluded as a reason for this phenomena, since exchange of mini-modules activating the same amino acid showed the same negative effect (Schneider *et al.*, 1998). These observations suggest that extensive intermodular domain-domain interactions and proper modular assembly of manipulated multienzyme complex might be disturbed. We reasoned that if the manipulations would involve entire enzyme modules, they should not result in the perturbations of intra-molecule interactions critical to enzyme activity, experienced in the targeted exchange of mini-domains, and should therefore result in a hybrid lipopeptides synthesized in *B. subtilis* in amounts similar to those typical of surfactin. The whole module replacement was developed based on strong conservation of core sequence S, HHXXXDG. This motif is located within the substrate condensation/elongation region presenting in all amino acid-incorporating modules of known synthetases and characterizing by highest degree of variations compared with other functional intramodular domains (Marahiel, 1997). Additionally, the cloning strategy minimizing any disordering modifications in polypeptide chain made by point substitution(s) of amino acid due to PCR-generated restriction sites was developed.

The experiments reported here show that production of the recombinant lipopeptides by the engineered *Bacillus subtilis* strains was expressed at approximately at the same level as that of wild type surfactin, i.e. a twelve- fourteen-fold higher yield than that of lichenysin A produced by *B. licheniformis* BNP29 (Yakimov *et al.*, 1996). The simultaneous PCR-generated changes within palindromic sequence (ComA-box2), served as promoter regulatory region of surfactin synthetase, results in slight, statistically insufficient increase of lipopeptide production by 1B1 strain compared with that of the strain 1D1. Structural elucidation of recombinant lipopeptides showed that they are identical to lichenysin A by their amino acid content while the fatty acid profile was identical to that of surfactin. Such an origin reflects in functional properties of obtained lipopeptides. Previously we observed that the surfactant activity varied with both the chain length and the branching type; the increasing order was *normal* > *iso* > *anteiso* whereas the activity of nC_{14} was greater than that of *i* or aiC_{15} (Yakimov *et al.*, 1996). This corroborated with the fact that recombinant lipopeptide 1D1 with 40% of ß-hydroxymyristic acid in lipophilic moiety decreases the surface tension of water from 72 mN/m to 25.5 mN/m. Since there are three structural differences between surfactin and lichenysin A, namely Glu/Gln, Leu/Ile and the composition of the fatty acid substituents, it is primarily the Glu/Gln difference is

Table 2. Sequences of the Primers Used in this Work

Primer	Sequence[a]	Location
F-PSrf-1-Kpn I	5'-GCGC**GGTACC**CATAGTCATGTAAAG-3'	16 – 40[b]
F-PSrf-2-Kpn I	5'-GCTGTAAATAAACT**GGTACC***TTGCGG*CATC*CCGCAA*GAAACT-3'	159 - 200[b]
R-SrfAA1-Eco RI	5'-CCCGACA**GAATTC**ATGGACATACCATC-3'	456 - 430[c]
F-SrfAA2-Eco RI/Nsi I	5'-CTG**GAATTC**GAC**ATGCAT**CATTTGATTTCTGACGGTGTATCC-3'	3535 - 3576[c]
R-SrfAA2	5'-CGTATTCGCCGCTTGCTCCTC-3'	4545 - 4525[c]
F-SrfAB1-Kpn I	5'-CAAAAA**GGTACC**GCCTGCACCGCAATCAGAAGCC-3'	13617 - 13650[c]
R-SrfAB2-Eco RI/Pml I	5'-GTCA**GAATTCACGTG**GTGCATATCAATGAGCAGCAGGTGGCG-3'	14337 - 14296[c]
F-SrfAB3-Eco RI	5'-AAG**GAATTC**ATCCGGCCATTCGACTTAAAC-3'	17350 -17379[c]
R-SrfAB3-Xma I	5'-CATCAGAA**CCCGGG**CGAGGCGGTTTGCCTT-3'	18512 - 18540[c]
F-LchAA1-Eco RV	5'-CAT**GATATC**ATTTCCGATGGTATTTCGATGACG-3'	5076 - 5108[d]
R-LchAA2	5'-CGGCTCCGGCAGTTCTTCCC-3'	8309 - 8290[d]
F-LchAB2-Pml I	5'-CAC**CACGTG**ATTACGGACGGAAGCTCCACC-3'	18964 - 18993[d]
R-LchAB3-Nsi I	5'-TATG**ATGCAT**ATCAATCAAAAGCAGATGGC-3'	22108 - 22079[d]
CHECKF-Gln	5'-GAAGTGTGGTTTTACGCAAA-3'	391 - 410[c]
CHECKR-Gln	5'- CTCCTCCAGCATAATGCCA -3'	3594 - 3576[c]
CHECKF-Asp	5'- CCTGCTGCTCATTGATATG -3'	14301 - 14319[c]
CHECKR-Asp	5'- GAGACGCCGTCTGCGATG -3'	17474 - 17457[c]

[a] - Modified sequences are underlined, created restriction sites are in bold, modified palindromic sequence is in italics
[b] – Position of primer in the *srfA* sequence D13262
[c] - Position of primer in the *srfA* sequence X70356-X70359
[d] - Position of primer in the *lchA* sequence AJ005061

responsible for the observed halotolerance of recombinant lipopeptide**.**

The aim of targeted exchange between Asp-incorporating modules SrfAB2 and LchAB2 was to extend the whole module replacement method to the substitution experiments at different locations within *srfA* operon. The results presented demonstrate that in contrast to mini-modular substitution it is also possible to exchange internal entire modules between multi-modular peptide synthetases of heterologous origin without reduction of peptide yields from the engineered enzymes. Although the relatively high homology between exchanged modules of surfactin and lichenysin A synthetases (53% of identity) could diminish an inherent potential of described here re-programming mode of active synthetases, the general high similarity between amino acid-incorporating modules of known peptide synthetases allows to propose it as an universal one. Exploitation of whole module substitution for synthesizing novel peptide compounds is currently under the study.

Experimental Procedures

The bacteria employed in this study were the surfactin-producing *B. subtilis* strain OKB105 and its isogenic Δ*srfAA1* (*aphA-3*), Δ*srfAA1Box* (*aphA-3*), Δ*srfAB2* (*aphA-3*), Δ*srfAA1* (*lchAA1*), Δ*srfAA1Box* (*lchAA1*) and Δ*srfAB2* (*lchAB2*) mutants, which were routinely grown at 30°C in CMM minimal medium (Yakimov *et al*., 1995) or Luria-Bertani (Sambrook *et al*., 1989) agar, supplemented where appropriate with 100 μg of kanamycin (Km) and 20 μg of erythromycin (Em) per ml.

PCR amplification of DNA fragments was used to generate the terminal restriction sites needed for subsequent cloning, and was performed using *B. subtilis* OKB105 and *B. licheniformis* BNP29 chromosomal DNA as the template, and TaqPlus Long (Stratagene) polymerase, according to the protocol supplied by the manufacturer. The sequences of the oligonucleotide primers were as listed in Table 2. The PCR reaction was carried out in a Perkin-Elmer Thermal Cycler 480 (Ueberlingen, Germany), and products were purified with QIAquick-spin PCR purification kit, as described by the manufacturer. Standard procedures were used for DNA digestion with restriction enzymes, cloning of DNA fragments and preparation of recombinant plasmid DNA (Sambrook *et al*., 1989).

Several steps were used to disrupt the region within *srfA* operon encoding the first and fifth amino acid-incorporating modules. After amplification with following couples of primers, F-PSrf-1-Kpn I + R-SrfAA1-Eco RI, F-PSrf-2-Kpn I + R-SrfAA1-Eco RI and F-SrfAB1-Kpn I + R-SrfAB2-Eco RI/Pml I, obtained P*srfA*-5'*srfAA1* and 5'-*srfAB1* fragments were digested with *Kpn* I and *Eco* RI, and ligated into pBluescript II SK+ (Clontech, Palo Alto, CA), digested with the same enzymes. Resulting plasmids were further digested with *Eco* RI and *Xma* I and accordingly ligated with the 3'-*srfAA2* and 3'-*srfAB3* fragments previously digested with the same enzymes and obtained after amplification of OKB105 chromosomal DNA with F-SrfAA2-Eco RI/Nsi I + R-SrfAA2 and F-SrfAB3-Eco RI + R-SrfAB3-Xma I, respectively. The obtained plasmids, designed as pSRF-1D, pSRF-Box1D and pSRF-5D were digested with *Pml* I and *Nsi* I and ligated with an amplified *aphA-3* gene fragment (Km^R) (Trieu-Cuot *et al*., 1985) flanked by PCR-generated *Pml* I and *Nsi* I sites at the 5'- and the 3'-termini, respectively. This produced the desruption plasmids pSRF-Δ1D, pSRF-BoxΔ1D and pSRF-Δ5D. In order to generate module-substitution plasmids, two 3.1-kb DNA fragments accordingly encoding LchAA1 and LchAB2 modules were amplified, digested with corresponding restriction enzymes and ligated into the same sites of pSRF-1D, pSRF-Box1D and pSRF-5D to produce pSRF-1D1, pSRF1B1 and pSRF-5D5 plasmids, respectively. These were subsequently digested with *Kpn* I and *Xma* I, and the ca. 5.0-kb fragments generated were introduced between *Kpn* I and *Xma* I sites of the temperature-sensitive suicide shuttle vector pAUL-A (Em^R) (Schaferkordt and Chakraborty, 1995) to produce the plasmids pAUL-A1/1, pAUL-A1B/1 and pAUL-A5/5. All intermediate plasmids containing fragments generated by PCR amplification were sequenced, using universal, *srfA*- and *lchA*-derived primers, to confirm the integrity of the sequences. The pAUL-A1/1, pAUL-A1B/1 and pAUL-A5/5 plasmids were checked for in-frame module replacement by sequencing of the PCR products generated by primers CHECKF-Gln + CHECKR-Gln and CHECKF-Asp + CHECKR-Asp, respectively.

Transformation of *B. subtilis* cells was performed as described previously (Yakimov and Golyshin, 1997). Homologous recombination and integration of the pAUL-A derivatives into the chromosome of the strain, previously transformed with the linear form of disruption plasmid, and subsequent spontaneous excision of the integrated plasmid through

intramolecular homologous recombination, was carried out as described previously (Guzman *et al*., 1995). Lipopeptide purification, thin-layer (TLC), and high performance liquid chromatography (HPLC), mass-spectrometry (MS) and protein sequencing was performed as described previously (Yakimov *et al*., 1995). The chemical composition of recombinant lipopeptide was established after total hydrolysis of the product and subsequent gas chromatography-mass spectrometry (GC-MS) analysis of the ß-hydroxy fatty and amino acids as corresponding *C*-methyl ß-trimethylsilyl (FAMe/TMS) and *N*-trifluoroacetyl *C*-*n*-butyl esters, respectively.

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