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Decreasing the Ring Size of a Cyclic Nonribosomal Peptide Antibiotic by In-Frame Module Deletion in the Biosynthetic Genes

Henning D. Mootz,* Nadine Kessler, Uwe Linne, Katrin Eppelmann, Dirk Schwarzer, and Mohamed A. Marahiel

Philipps-Universität Marburg, Fachbereich Chemie/Biochemie, Hans-Meerwein-Strasse, 35032 Marburg, Germany

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Many natural products of therapeutical and biotechnological importance are nonribosomally synthesized peptides, such as the β-lactam precursor tripeptide δ-aminoadipyl-cysteinyl-D-valine, the immunosuppressive cyclic undecapeptide cyclosporin, and the glyco heptapeptide antibiotic vancomycin.[1] Structural hallmarks of this class of compounds are the occurrence of unusual amino acids, cyclic peptide backbones, and numerous further modifications such as acylation, heterocyclic ring formation, and glycosylation. Because of their structural complexity, chemical synthesis is usually an unattractive route to these molecules. In contrast, genetic engineering of the biosynthetic genes emerges as a potentially powerful approach to the combinatorial biosynthesis of useful analogues of the lead compounds. Nonribosomal peptide synthetases (NRPSs) carry out a sequential multistep assembly and modification of the peptides in a thiotemplate process described by the multiple carrier model.[2] Further tailoring can be achieved by additional enzymes. The modular architecture of NRPSs suggests straightforward methods for the reprogramming of these enzymes by exchange of catalytic subunits.[1] However, many of the reported engineering attempts[3,4] faced low product yields or even inactive hybrid enzymes.[3] Using a new approach to obtain hybrid NRPSs, we show here that the deletion of an entire module in an NRPS assembly line caused the secretion of the predicted peptide antibiotic variant with a decreased ring size. Furthermore, a module exchange resulted in a significantly higher product yield than that observed in previous studies.

Our design of the hybrid NRPSs was guided by refined construction rules which we recently elaborated by using simplified recombinant enzymes;[4b] short nonconserved and flexible linker sequences between elongation modules comprising a condensation (C) domain for peptide bond formation as well as adenylation (A) and thiolation (T) domains for selection, activation, and covalent binding of a substrate amino acid were used as fusion sites.[4b] These linkers are depicted in Figure 1 as thick lines in the corresponding DNA regions. To test if this previous in vitro approach could be extended to other NRPSs and to the actual peptide antibiotic producers to obtain structural variants of the natural products from the growth medium, we chose to manipulate the genes encoding the surfactin NRPS in the chromosome of *Bacillus subtilis* ATCC 21332 (Figure 1A). We further hypothesized that not only exchange of modules would result in an altered sequence of the product, but also that deletion and insertion of modules should be possible to effect more dramatic changes in the backbone of the product. To this end, we attempted to delete the leucine-incorporating SrfA-A2 module of the surfactin NRPS to produce a Δ2-surfactin variant with a decreased ring size.

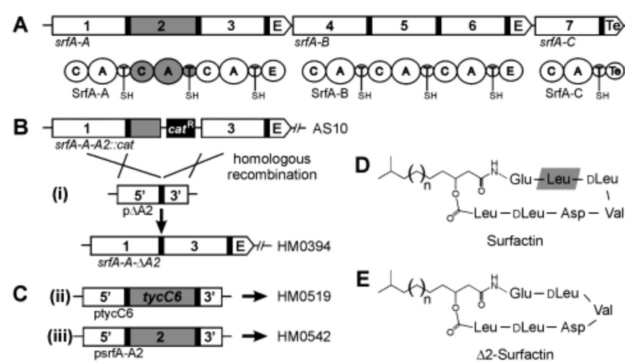

Figure 1. Surfactin biosynthesis genes and enzymes (A). Strategy for in-frame module deletion (B). Constructs used for in-frame module exchange (C). Structures of surfactin (D) and Δ2-surfactin (E).

Surfactin is an *N*-acylated heptapeptide which forms a macrolactone between the C-terminal carboxyl group and the hydroxyl moiety of the (3*R*)-β-hydroxy fatty acid (Figure 1D). Seven modules composed of C, A, and T domains are arranged on three interacting enzymes, SrfA-A, SrfA-B, and SrfA-C (Figure 1A).[5] The C domain of the first module probably transfers the fatty acid to the first amino acid in the initiation reaction. After six elongation steps, the thioesterase (Te) domain of the last module catalyzes macrolactone formation and thereby release of the product from the enzyme.[6] Additional epimerization (E) domains perform the conversion of stereochemistry into the D-isomer on the residues incorporated by modules 3 and 6.

Genetic manipulation of *srfA-A* was carried out in a two-step gene replacement strategy.[3a] A chloramphenicol-resistance cassette (cat^R) was first inserted at the position encoding module 2 to give strain AS10.[3b] Replacement of gene fragments was then accomplished by introducing plasmid DNA which can homologously recombine over identical 5′- and 3′-flanking regions (see Figure 1B). After transformation of AS10 with pΔA2, chloramphenicol-sensitive strain HM0394 could be isolated, whose desired genotype was confirmed by restriction analysis and sequencing of a PCR-fragment amplified from its chromosomal DNA using primers that annealed outside the manipulated region (data not shown). The new gene *srfA-A-ΔA2* encodes an NRPS in which modules 1 and 3 of SrfA-A are directly joined together in the linker sequence. To test whether HM0394 indeed produced a new lipopeptide, we grew cells under conditions for surfactin production and prepared the products from the supernatant.[7] Figure 2A shows a trace of a reversed-phase HPLC for surfactin prepared from the parent strain ATCC 21332. The characteristic multiple peak pattern stems from the variable chain length of the fatty acid attached to the peptide backbone. Analysis of the supernatant from HM0394 revealed a similar peak pattern; however, it appeared at shorter retention times (Figure 2A),

* To whom correspondence should be addressed. Present address: The Rockefeller University, Laboratory of Synthetic Protein Chemistry, 1230 York Avenue, New York, NY 10021. E-mail: mootzh@rockefeller.edu.

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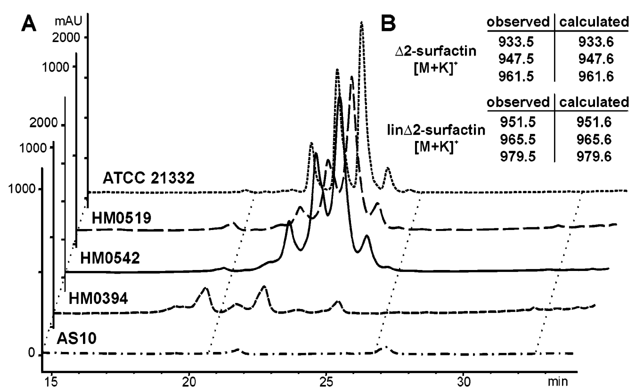

Figure 2. Reversed-phase HPLC analysis of the lipopeptides produced by the parent and the engineered strains (A). Lipopeptides produced by *B. subtilis* strains appear in a multiple peak pattern due to varying chain length of the fatty acid moiety (14 Da difference). For the parent strain ATCC 21332, extracts were applied in a 3-fold dilution as compared to the other strains. Masses of the products of strain HM0394 (B).

indicating a more hydrophilic product, as would be expected as a result of the deletion of a hydrophobic leucine residue. Electrospray ionization mass spectrometry (ESI-MS) analysis confirmed the identity of the predicted cyclic Δ2-surfactin (Figure 1E) with all masses of the products with various fatty acid chain length being reduced by the calculated difference of a leucine residue (113 Da) as compared to those of surfactin (Figure 2B).[7] Interestingly, ESI-MS analysis also revealed the formation of a second set of products, which eluted slightly earlier than but mostly overlapped with that of Δ2-surfactin. Their masses were increased by 18 Da with respect to those of the new cyclic Δ2-surfactin, thus corresponding to the linear variant, linΔ2-surfactin (Figure 2B).[8] No other new products could be detected. Final proof of the identity of Δ2-surfactin was obtained from MSMS peptide sequencing.[7] The new product(s) exhibited slightly reduced bioactivity in an assay for hemolytic properties.[7]

Thus, fusion of modules 1 and 3 in the linker sequences led to an active hybrid NRPS that produced the predicted product. Module 3 and the modules further downstream proved to be of broad enough specificity to process the unnatural substrates. Interestingly, the Te domain was capable of forming the macrolactone with decreased ring size, although about one-third of the flux was directed to the hydrolysis product. These observations are in very good agreement with recent biochemical characterizations of isolated Te domains using peptide *N*-acetylcysteamine thioesters as soluble surrogate substrates.[6a,9] This previous work showed that Te domains are able to cyclize unnatural substrates leading to decreased ring sizes, however, at the expense of higher hydrolysis rates and significantly reduced catalytic efficiency. Te domain-catalyzed formation of the Δ2-surfactin macrolactone with a potentially higher ring strain might therefore be a major determinant of the observed drop in product yield to about 10% as compared to parent strain ATCC 21332.

We next were interested in evaluating the efficiency of our new approach for module exchange experiments. We previously reported a different method which relied on surgery within the domains to achieve exchange of A and T didomain units,[3a,b] which was successfully applied to position 7 in the surfactin NRPS,[3a] although with very low product yields (0.1−0.5% for the exchange of the leucine-specific domains with heterologous domains of the same specificity). However, this previous method failed completely in producing the predicted peptides at position 2, even in the control experiment when the leucine-specific domains were replaced by a heterologous pair of domains of the same specificity.[3b] Therefore, we conducted a similar control experiment and exchanged the SrfA-A2 module for the leucine-specific module TycC6 of the tyrocidine NRPS[10] in an initial proof-of-principle study. TycC6, which is 41% identical to SrfA-A2 in amino acid sequence, processes in its native context a nonapeptide with a C-terminal L-ornithine residue and interacts downstream with a Te domain instead of a C domain, as is the case for SrfA-A2. Except for the same amino acid specificity of the A domain, it is thus very distinct from SrfA-A2 and therefore a good test for the potential of the new approach. Strain HM0519 was created following the same strategy as that described above (see Figure 1C,ii). Indeed, surfactin production was restored, proving the superiority of the new approach (Figure 2A). Furthermore, when compared to the above-mentioned conservative A and T didomain exchange at position 7,[3a] the observed good yields of 19% as compared to the parent strain ATCC 21332 and 31% as compared to the control HM0542 (which resulted from reintroduction of the native SrfA-A2 module,[11] see Figure 1C,iii and Figure 2A) present at least a 40-fold improvement of yield.

We conclude that surgery in the linkers between entire modules seems to be a robust approach to rationally design NRPSs in nonribosomal peptide antibiotic producer strains. Module deletion, exchange, and probably also insertion should provide an almost infinite recombination potential to generate novel peptides.

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Supporting Information Available: Experimental protocols, MS data, and bioactivity assays (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(7) See Supporting Information for experimental details and more results.

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