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Production of Lantipeptides in *Escherichia coli*

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Supporting Information

ABSTRACT: Lantipeptides are ribosomally synthesized and posttranslationally modified peptides containing thioether cross-links. We describe the preparation of seven different lantipeptides in *Escherichia coli* and demonstrate that this methodology can be used to incorporate nonproteinogenic amino acids.

Lantipeptides are polycyclic peptides with thioether cross-links formed by dehydration of Ser/Thr residues and subsequent Michael additions of Cys residues to the resulting dehydro amino acids as exemplified in Figure 1 for nisin A. These cross-links are introduced by posttranslational modification of a precursor peptide (generically termed LanA) and are called lanthionine (from Ser) or methyllanthionine (from Thr);[1] lantipeptides with antimicrobial activities are called lantibiotics.[2] The posttranslational modifications take place only in the C-terminal core peptide, but they require an N-terminal leader peptide that must be removed by proteolysis for bioactivity of the final product (Figure 1).[3] Engineering of lantipeptides has been very fruitful and has been accomplished by manipulation of the sequence of the precursor peptides in host strains and heterologous expression systems[4] as well as with *in vitro* reconstituted systems.[5] The former methods have the advantage of potential scale-up, whereas the *in vitro* approach allows introduction of nonproteinogenic amino acids. Any improved variants identified by the *in vitro* method could in principle be produced *in vivo* using amber stop-codon suppression technology.[6] Unfortunately, at present this methodology is not available for most lantipeptide expression hosts. To be able to take full advantage of *in vivo* introduction of nonproteinogenic amino acids, production of lantipeptides in *Escherichia coli* would be highly desirable, but only a single such example has been reported to date for a truncated analog of the class II lantibiotic nukacin ISK-1.[7] We present here seven examples of production of mature full-length lantipeptides from different phylogenetic backgrounds in *E. coli*.

We first focused our efforts on the prochlorosins. These lantipeptides were recently discovered as products of the ubiquitous marine cyanobacterium *Prochlorococcus*.[8] The production levels are less than 10 μg of prochlorosin from 20 L of culture of *Prochlorococcus* MIT 9313, insufficient to investigate their function. Prochlorosins are members of the class II lantipeptides that are modified by a single bifunctional lanthionine synthetase that carries out both dehydration and cyclization reactions,[8] but they have unusually large leader peptides (Figure S1). We selected prochlorosin 1.7, 2.11, 3.2, and 3.3 (Figure 2) for investigation because their biosynthesis has been reconstituted *in vitro*.[8] The genes for their precursor peptides and the synthetase ProcM were cloned into the pRSFDUET-1 vector as a biscistronic construct. For rapid purification, an N-terminal hexahistidine tag was encoded at the N-termini of the ProcA peptides, and artificial protease cleavage sites were introduced between the leader and core peptides to allow *in vitro* removal of the leader sequence (Figure S1).[9] *E. coli* BL21 (DE3) cells were transformed with the plasmids, and the cells were grown in Luria−Bertani broth and induced with IPTG. After harvest and lysis of the cells followed by immobilized metal affinity chromatography (IMAC) purification of the ProcA peptides from the soluble protein fraction, the fully modified peptides were isolated in yields ranging from 10 to 35 mg per liter of culture, significantly more than the 1.5 mg/L of modified NukA reported previously using a different expression system.[7] This production level was surprising because when lantipeptide precursor peptides are expressed in the absence of ProcM, they are almost exclusively present in inclusion bodies.[8,10] Hence, posttranslational modifications improve the solubility of the products. The purified peptides were then treated with the appropriate commercial protease (trypsin for ProcA1.7, Lys-C for ProcA 2.11 and 3.3, and

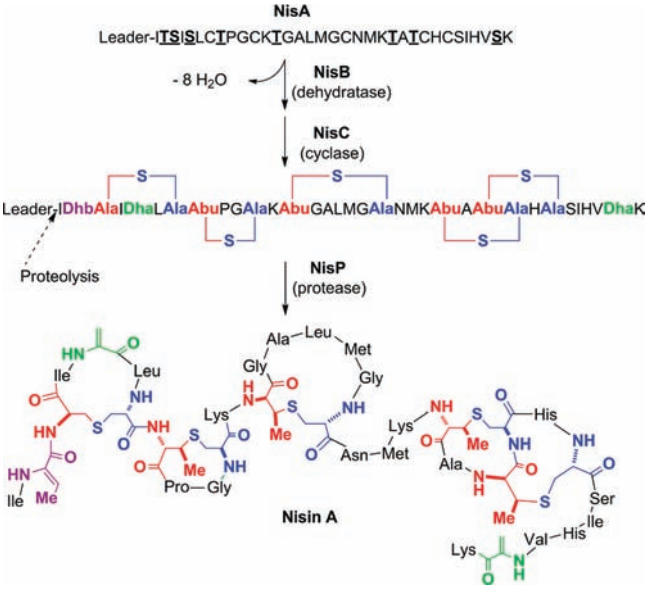

Figure 1. Posttranslational modifications in lantipeptide biosynthesis. Dha, dehydrolalanine; Dhb, dehydrobutyrine; Abu, 2-aminobutyric acid.

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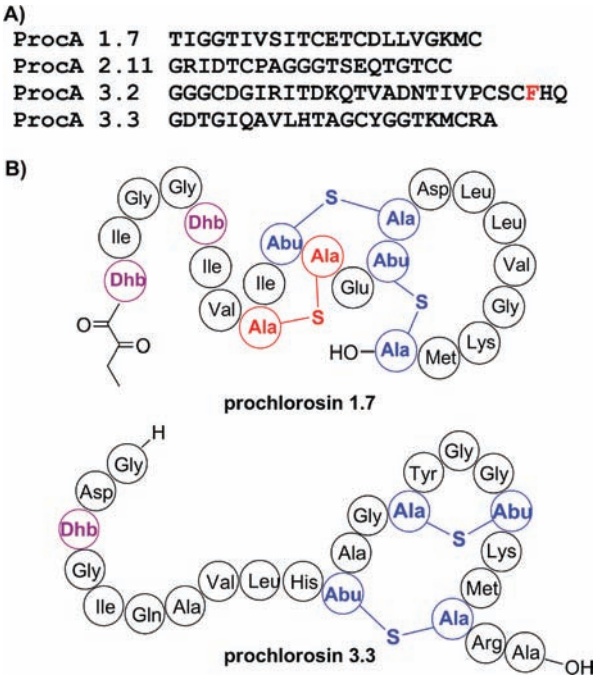

Figure 2. (A) Sequences of the ProcA lantipeptide core peptides used in this study. The residue replaced by *p*Bpa in this work is highlighted in red. For the sequences of the leader peptides, see Figure S1. (B) Representative structures of prochlorosins 1.7 and 3.3. Abu, 2-aminobutyric acid; Dha, dehydroalanine; Dhb, dehydrobutyrine.

TEV protease for ProcA3.2, Figure 3A−C) and purified by HPLC. Although, in principle, the proteolytic step could be carried out *in vivo* by introduction of the gene for the bifunctional protease transporter, we elected to remove the leader peptide *in vitro* to avoid complications often encountered in heterologous expression of proteases and to avoid any potential cytotoxic effects of the final posttranslationally modified products.[11] After HPLC purification, pure prochlorosins were obtained (Figures S2 and S3). The purified, modified peptides were analyzed next by tandem mass spectrometry (MS) displaying the same fragmentation patterns as observed previously[8] (Figure S4) demonstrating that the ring topology of the products is the same as that of the natural products.[12]

As proof of concept of introducing non-natural amino acids into lantipeptides *in vivo*, *procA*3.2 was mutated to introduce an amber stop codon (TAG) at the position of Phe26. The gene product was coexpressed with ProcM and with an orthogonal tRNA/tRNA synthetase pair that incorporates the photo-cross-linking amino acid *p*-benzoyl-L-Phe (*p*Bpa) at the site of the amber stop codon.[13] The resulting peptide containing the nonproteinogenic amino acid *p*Bpa was fully modified resulting in prochlorosin 3.2 containing a photo-cross-linking amino acid (Figure 3D) that will provide a valuable tool for determination of its biological activity.

We next turned to the two-component lantibiotic haloduracin. Haloduracin consists of two posttranslationally modified peptides, Halα and Hal*β* (Figure 4), that display potent synergistic antimicrobial activity that requires both peptides.[14] Halα and Hal*β* are only produced by *Bacillus halodurans* on solid, rich medium,[9] preventing isotopic labeling of the peptides for NMR studies to investigate their likely interaction with lipid II.[15] If they could be produced in liquid medium in *E. coli*, it would open up a route to isotopically labeled peptides. Thus, the gene for HalA1 with a Factor Xa protease cleavage site between its leader and

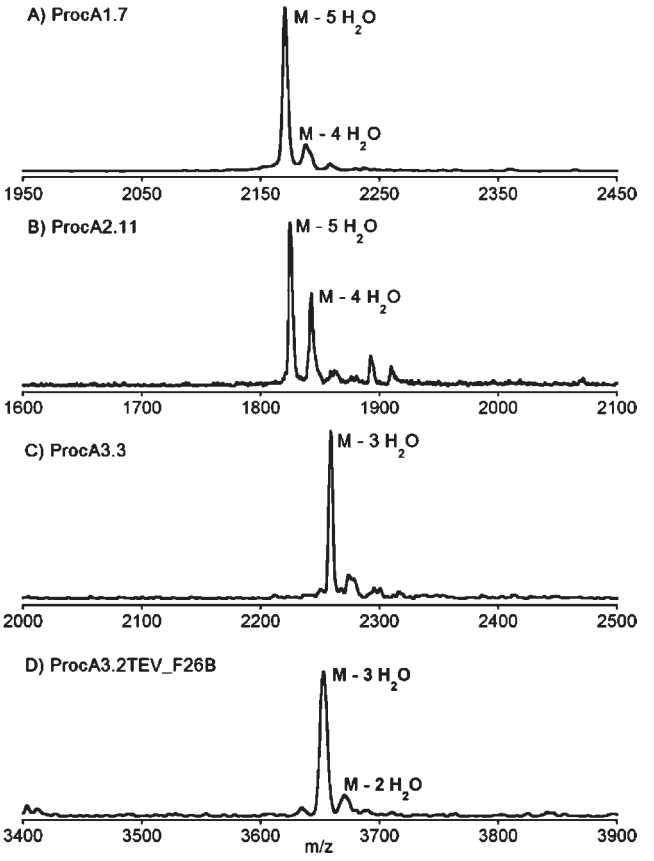

Figure 3. MALDI-MS spectra of precursor peptides modified by ProcM in *E. coli* and treated with a protease to remove the leader peptide. (A) ProcA1.7, (B) ProcA2.11, (C) ProcA3.3, and (D) ProcA3.2 Phe26*p*Bpa.

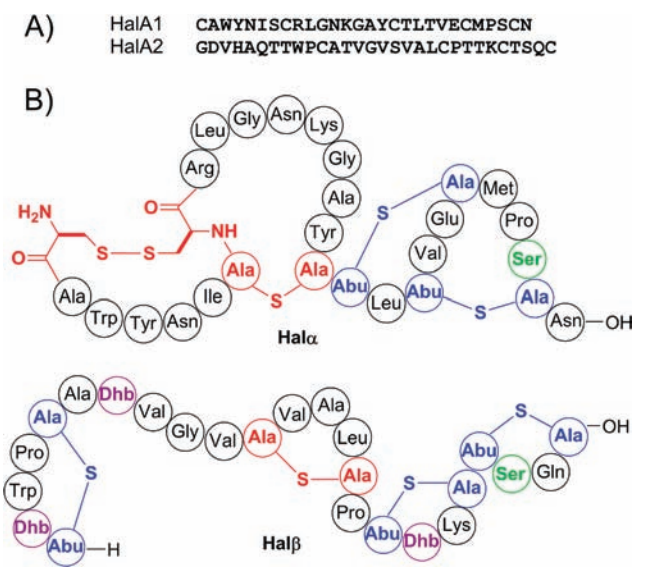

Figure 4. (A) Sequences of the HalA core peptides. For the sequences of the leader peptides, see Figure S1. (B) Structures of haloduracin α and *β*. Abu, 2-aminobutyric acid; Dha, dehydroalanine; Dhb, dehydrobutyrine.

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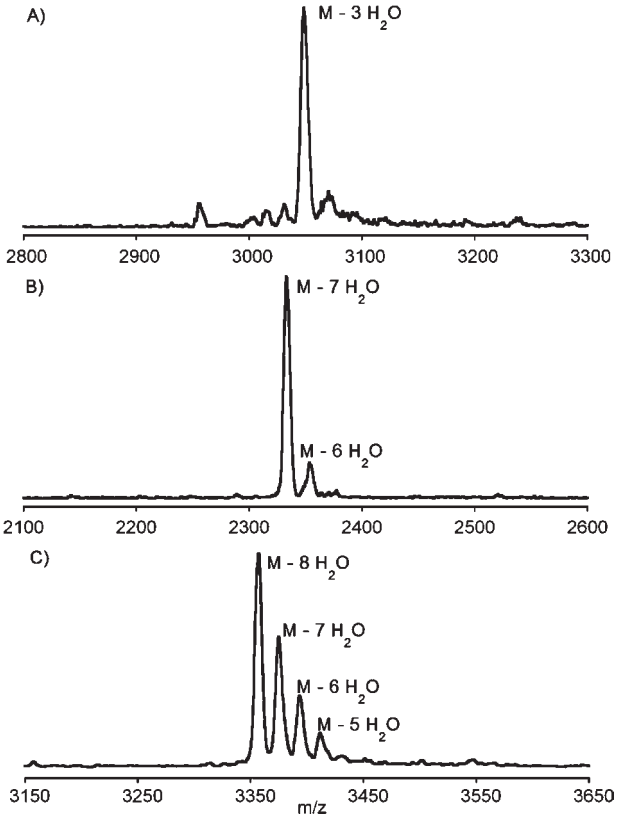

Figure 5. MALDI-MS spectra of precursor peptides modified in *E. coli* and treated with a protease to remove the leader peptide. (A) HalA1, (B) HalA2, and (C) NisA.

core peptide was inserted into pRSFDUET-1 along with its bifunctional modification enzyme HalM1 that both dehydrates and cyclizes the peptide.[12] *E. coli* BL21 (DE3) cells were transformed, grown at 37 °C, and induced as described above. After harvest, lysis, IMAC purification, and proteolysis with Factor Xa to remove the leader peptide, the modified HalA1 peptide (termed Halα)[9] was obtained (Figure 5A). Similar results were obtained for HalA2 (Figure 5B) showing that the methodology extends to class II lantibiotics from bacilli with prototypical leader peptides for this class.[16] The purified Halα thus obtained was combined with Hal*β* for bioactivity assays and demonstrated very similar antimicrobial activity against *Lactococcus lactis* as an authentic sample of these peptides obtained from the producing organism (Figure 6).[12] Repeating the expression in minimal medium required for producing isotopically labeled product for NMR studies was also successful albeit the yield was reduced (~1 mg of pure HalA1 per liter of culture).

Lastly we turned to the class I lantibiotic nisin, which is assembled by two dedicated proteins, NisB for dehydration and NisC for cyclization (Figure 1). To date, *in vitro* reconstitution of a purified LanB dehydratase has not been achieved, as LanB proteins that were heterologously expressed in *E. coli* did not display any *in vitro* activity.[17] Thus, production of nisin in *E. coli* presented the most challenging system. The genes encoding His_6-NisA and NisB were inserted into the pRSFDUET-1 vector, and the *nis*C gene was inserted into a pACYCDUET-1 plasmid. *E. coli* BL21 (DE3) cells were transformed with both plasmids, grown at 37 °C on LB medium, and induced with IPTG. After harvesting the cells, lysis, and IMAC purification, 24 mg of

Figure 6. Antimicrobial assays against *L. lactis* HP. (1) authentic nisin; (2) nisin produced in *E. coli*; (3) Halα produced in *E. coli* combined with authentic Hal*β*; (4) authentic Halα combined with Hal*β* produced in *E. coli*; (5) authentic Halα and Hal*β*; (6) Halα and Hal*β* both produced in *E. coli*.

fully modified NisA were obtained per liter of culture. The leader peptide was then removed using trypsin,[18] and the final product was analyzed by bioassays and tandem MS (Figures 6, S6, and S7) demonstrating the formation of authentic nisin. These experiments demonstrate that active NisB can be expressed in *E. coli* opening up the potential of future mechanistic studies.

In summary, we demonstrate that three very different types of lantipeptides can be produced in *E. coli*. This methodology may be useful for cryptic lantipeptides that cannot be isolated from the producer strain, for compounds that are produced in very low abundancies, or for preparing these compounds in isotopically labeled forms. Furthermore, this methodology may open up investigations of the LanB proteins and introduction of non-proteinogenic amino acids into lantipeptides using stop-codon suppression technology.

■ ASSOCIATED CONTENT

Supporting Information. Experimental procedures, mass spectral characterization of all lantipeptides, and description and results of bioactivity assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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