Rapid Screening of Lanthipeptide Analogs via In-Colony Removal of Leader Peptides in Escherichia coli

Tong Si, Qiqi Tian, Yuhao Min, Linzixuan Zhang, Jonathan V. Sweedler, Wilfred A. van der Donk and Huimin Zhao

ABSTRACT: Most native producers of ribosomally synthesized and post-translationally modified peptides (RiPPs) utilize N-terminal leader peptides to avoid potential cytotoxicity of mature products to the hosts. Unfortunately, the native machinery of leader peptide removal is often difficult to reconstitute in heterologous hosts. Here we devised a general method to produce bioactive lanthipeptides, a major class of RiPP molecules, in Escherichia coli colonies using synthetic biology principles, where leader peptide removal is programmed temporally by protease compartmentalization and inducible cell autolysis. We demonstrated the method for producing two lantibiotics, haloduracin and lacticin 481, and performed analog screening for haloduracin. This method enables facile, high throughput discovery, characterization, and engineering of RiPPs.

Ribosomally synthesized and post-translationally modified peptides (RiPPs) are a class of natural products with intriguing chemical structures and diverse biological activities.1 Bioinformatic analysis indicates a rich source of unknown RiPP biosynthetic pathways in currently sequenced microbial genomes.2,3 At present, lanthionine-containing peptides (lanthipeptides) represent the largest class of RiPPs.1–3 Lanthipeptides are characterized by thioether cross-links called lanthionine (Lan) and methyllanthionine (MeLan). These modifications are installed post-translationally onto a linear peptide precursor called LanA, which consists of a C-terminal core region and an N-terminal leader region. The leader region is important for precursor recognition by biosynthetic enzymes that process the core peptide into a mature product, from which the leader peptide is typically removed by a protease or the proteolytic domain of a transporter. Synthesized from a ribosomal peptide, lanthipeptide variants can be readily generated by mutagenesis of the precursor genes,4–6 which greatly facilitates structure–activity relationship (SAR) studies and bioengineering efforts.7–10 To avoid challenging genetic manipulation of native producers, it is advantageous to perform such studies in Escherichia coli, but the lack of a general method to remove the leader regions in a heterologous host represents a major challenge.

The common practice for lanthipeptide production in E. coli is to produce a modified precursor peptide (mLanA) with the leader region attached for three reasons—to prevent potential cytotoxicity of the mature product, to avoid heterologous reconstitution of proteolytic activities to cleave leader peptides, and to allow one step purification by attaching an affinity tag to the leader peptide.11–14 To facilitate leader region removal in vitro, various strategies have been devised using precursor peptide engineering, such as incorporating recognition sites for commercial proteases,15,16 and introducing photolabile17 or ester linkers16 via solid-phase synthesis17 or unnatural amino acid incorporation.18 Though effective, these strategies all require separate steps of biosynthesis, mLanA purification, and leader peptide removal, which limits the scale and throughput of bioengineering efforts (for strategies in non E. coli hosts, see the Supporting Information).

We demonstrate herein a generally applicable method for in vivo leader-peptide removal in E. coli colonies (Figure 1). Colony-based assays are widely utilized for analog screening of natural products in high throughput. The main design principle underlying our approach is cellular compartmentalization (Figure 1). The precursor peptide and biosynthetic enzymes are expressed in the cytosol,12 whereas the protease is directed to the periplasmic space by fusion with a secretion signal peptide (Figure 1A). Directing proteolytic activity to a separate cellular compartment prevents both competition for the unmodiﬁed LanA with biosynthetic enzymes and release of a mature product that is potentially cytotoxic to E. coli. Upon
completion of post-translation modifications in the cytosol, autolysis of E. coli cells is induced (Figure 1B), not only initiating contact between mLanA and the protease for leader peptide cleavage (Figure 1C) but also permitting extracellular release of the final product for biological activity analysis (Figure 1D). As such, biosynthesis, leader peptide removal, and product export can be completed in a single E. coli colony to accelerate variant screening. Notably, coordination of production and self-immunity in heterologous hosts is also critical for bioengineering of other RiPPs such as lasso peptidoglycan, and disrupt the outer membrane.27

In this study, to produce Halβ in its mature form in E. coli colonies, the last six amino acid residues of HalA2 immediately upstream to the core peptide were replaced with the recognition sequence (NDVNPE) of LicP (Figure 2). This serine protease is involved in the biosynthesis of the lanthipeptide lichenicidin,23 and we recently demonstrated its soluble expression in E. coli and application for sequence-specific, traceless peptide bond cleavage.24 The native secretion sequence of LicP (residues 1–24)25 was replaced with the signal peptide of the E. coli protein OmpA (SPOmpA),26 which results in periplasmic localization. Also, an N-terminal fusion of maltose-binding protein (MBP) was included in the engineered HalA2 to minimize degradation of mHalA2 by endogenous peptidase activities in E. coli. To also temporarily control leader peptide cleavage, we utilized an engineered system for lysis of E. coli cells, in which SRRz, a lysis gene cassette from bacteriophage λ, was placed under the control of two heat-inducible promoters λ cI857/Pφr.26 Whereas E. coli harboring the corresponding autolytic construct grows normally at 28 °C, >90% of cells are lysed within 4 h upon shifting to 38 °C.26 Cell lysis is achieved through concerted actions of the gene products of the lytic cassette to form micrometer-scale holes in the cytoplasmic membrane, degrade peptidoglycan, and disrupt the outer membrane.27

A two-plasmid system was utilized to implement the design (see Supporting Information for detailed configurations). The transcription of MBP-HalA2/HalM2, SPOmpA-LicP, and SRRz genes was under the inducible control of isopropyl β-D-1-thiogalactopyranoside (IPTG), l-arabinose, and temperature shift, respectively. A workflow was devised to perform successive steps of peptide production and modification, leader peptide removal, bioactivity screening, and mass spectrometry (MS) analysis (Figure S1). E. coli BL21 (DE3) cells transformed with both plasmids were transferred among various plates and temperature conditions on a membrane filter for parallel manipulation of many colonies (Figure S1).28 Lactococcus lactis HP was selected as the sensitive indicator strain. As haloduracin functions through the synergistic activities of both Halα and Halβ,20,29 Halα purified from B. halodurans was premixed with the L. lactis strain at a concentration of 8 nM. For Halβ molecules produced in a single E. coli colony, the size of the zone of growth inhibition was used to estimate antibiotic activity. Also, whole-colony matrix-assisted laser desorption/ionization (MALDI) time-of-flight (ToF) MS provided molecular weight analysis.28

We first examined whether mature Halβ can be produced in E. coli colonies. When cotransformed with the two plasmids containing HalA2/HalM2, SPOmpA-LicP, and SRRz (Strain 1, 38 °C), E. coli cells inhibited L. lactis HP growth (Figure 3A and Figure S2A) following the devised workflow. In control experiments, inhibition zones were absent when either SPOmpA-LicP (Strain 2) or HalA2/HalM2 (Strain 3) were missing, indicating antibiotic activity was specific to production of mature Halβ. However, at 28 °C, Strain 1 also produced zones of growth inhibition, although their sizes were substantially smaller than those exposed to a temperature shift to 38 °C. Liquid chromatographic analysis estimated 45% of Halβ was
produced before 38 °C treatment (Figure S3). These observations indicate that the stringency of protease compartmentalization or autolytic control needed further improvements. Notably, *E. coli* cells coexpressing wild-type (WT) HalA2, HalM2, and HalT (Strain 4) did not exhibit antibiotic activity under these conditions (Figure S2A).

Moreover, whole *E. coli* colonies were subjected to MALDI-ToF MS analysis, and peaks consistent with the *m/z* value of Halβ were only observed in colonies producing zones of growth inhibition (Figure 3B and Figure S2B, Strain 1), indicating biosynthetic modifications and leader cleavage were successful. For Strain 4 harboring WT HalA2/HalM2/HalT, peaks corresponding to the *m/z* value of GDVHAQ-Halβ, but not of Halβ, were detected (Figure 3B), consistent with previous observations that HalT alone is not sufficient to produce the final Halβ product. Collectively, these results show that mature Halβ was produced in *E. coli* colonies as demonstrated by bioactivity and MS analyses. We further examined the capability of Halβ-producing *E. coli* to inhibit growth of other indicator strains (Figure S2C and Figure S2D, see Supporting Information), and the results indicate that the method is constrained by the sensitivity of indicator strains to a target antibiotic.

We proceeded to perform activity mapping of Halβ variants produced in *E. coli* colonies. We targeted all 16 amino acid residues in the HalA2 core peptide that are not involved in thioether ring formation (Figure 2), as ring topology is critical for antimicrobial activity. Site-saturation mutagenesis of each residue was performed using degenerate codon (NNK)-containing primers. For the library of each residue, more than 172 colonies were collectively screened on bioactivity plates (Figure S3) to achieve a >95% probability of full coverage of the NNK library. Around 20–50 *E. coli* colonies producing various sizes of the zones of growth inhibition were analyzed using MALDI-ToF MS to assign mutations based on predicted mass shifts (Figure S4). When amino acid substitutions could not be reliably deduced from mass spectra (for possible reasons see Supporting Information), DNA sequencing was performed, and the assignment strategy for each analog is summarized in Figure S4.

A heat map was generated to visualize relative antimicrobial activities of Halβ mutants toward *L. lactis* using the size of the zone of growth inhibition (Figure 4). Whereas most mutations reduced antibiotic activity, amino acid substitutions with similar chemical properties to the WT residues were less detrimental to Halβ production and/or activity. Notably, whereas many Halβ variants mutated at Pro4 retained *L. lactis* growth inhibition, most amino acid substitutions at Pro16 led to loss of *L. lactis* growth inhibition, even though seven dehydrations were still observed in the resultant mutant peptides as in WT Halβ. This observation is in agreement with an alignment analysis of the core region of β-peptides of reported two-component lantibiotics, which indicates Pro at position 16 in Halβ is fully conserved (Figure S5). Our results suggest evolutionary conservation of this Pro may be because it is essential to Halβ function.

One limitation of colony-based approaches is that the size of zone of growth inhibition is affected by several factors including production level, leader peptide cleavage, and

![Figure 3. Analysis of Halβ molecules produced from *E. coli* colonies via agar diffusion assay (A) and MALDI-ToF MS (B). Strain background is listed in Table S1. Mass spectra were normalized using ion intensities of an unidentified *E. coli* peptide with an *m/z* value of 2833.5 Da (see Figure S2).](image)

![Figure 4. Summary of antimicrobial activities against *L. lactis* by Halβ analogs produced in *E. coli* colonies. WT residues are contoured with red dashed lines. Both Leu and Ile substitutions are labeled as L.](image)
antimicrobial potency. Thus, further analysis is required to rule out hits that are “false positive” (low activity but high production) or “false negative” (high activity but low production). To measure true specific activity, three Halβ analogs (Ala6Val, Val12Leu, and Gln23Arg) producing larger zones of growth inhibition than WT against L. lactis HP were purified using reported approaches, whereby the modified precursor peptide with an N-terminal affinity tag was isolated followed by leader peptide removal in vitro and HPLC purification.\textsuperscript{11} Under current assay conditions, all three purified analogs showed stronger growth inhibition of L. lactis HP during the first 12 h of the time course (Figure S6). After 18 h, however, only Val12Leu was confirmed to improve Halβ bioactivity with a reduced minimal inhibition concentration (MIC, 50 nM) relative to WT (75 nM, consistent with previous studies\textsuperscript{20,23} (Figure S6)). Additionally, modified precursor peptides were purified to investigate the impact of mutations at Thr2 on leader peptide cleavage and ring topology (Figure S7 and Supporting Information), demonstrating the tolerance of LicP to variation at the P2’ site. Taken together, integration of rapid, colony-based screening with detailed, in vitro biochemical characterization provides complementary capabilities for SAR studies.

To demonstrate the versatility of the method, we examined production of another class II lanthipeptide, lacticin 481, in E. coli colonies (Figure S8). The genes of the LctA precursor and LctM synthetase were cloned into the pRSFDUET-1 vector, and LctA was engineered with an N-terminal MBP fusion as for HalA2. Different from the design for haloduracin, the protease domain (1–150) of the LctT transporter was utilized to replace LicP, as we previously demonstrated that LctT150 can cleave the leader region of modified LctA.\textsuperscript{31} Following the same workflow, production of mature lacticin 481 in E. coli colonies was confirmed via bioactivity and MALDI-ToF MS analyses (Figure S8 and Supporting Information). However, N-terminally degraded lacticin 481 molecules were also detected from colonies (Figure S8C), possibly generated by E. coli peptidases. Suppression of unspecified host proteolytic activities may therefore be beneficial to improve in-colony lanthipeptide production.

In summary, we produced analogs of haloduracin and lacticin 481 in E. coli colonies via spatial and temporal control of proteolytic activities for leader peptide removal. Compared with methods requiring separate steps of peptide modification, purification, and digestion, our synthetic biology design enables parallel biosynthesis of lanthipeptide mutants for activity screening. We envision applications for bioengineering and genome mining of lanthipeptides and other classes of RiPPs using this general methodology. This strategy fully capitalizes on the gene-encoded nature of RiPPs by allowing large scale analog generation and activity testing using the advantages of E. coli for genetic manipulation.

**ASSOCIATED CONTENT**

* Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.8b05544.

Description of experimental details and additional tables, figures and references (PDF)

**AUTHOR INFORMATION**

**Corresponding Authors**

*zhao5@illinois.edu*

*yvdonk@illinois.edu*

**ORCID**

Tong Si: 0000-0003-2985-9014

Jonathan V. Sweedler: 0000-0003-3107-9922

Wilfred A. van der Donk: 0000-0002-5467-7071

Huimin Zhao: 0000-0002-9069-6739

**Author Contributions**

*These authors contributed equally.

**Notes**

The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

This work was supported by the U.S. National Institutes of Health (GM077596 to H.Z. and GM088822 to W.A.V.) and the U.S. Department of Energy (DE-SC00018420 to H.Z. and J.V.S.). T.S. acknowledges postdoctoral fellowship support from the Carl R. Woese Institute for Genomic Biology (UIUC). We thank Z. Lin at Tsinghua University for kindly providing E. coli autolytic constructs.

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