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Rapid screening of lanthipeptide analogs via in-colony removal of leader peptides in *Escherichia coli*

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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

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Author Contributions

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ASSOCIATED CONTENT

Supporting Information

Description of experimental details and additional tables, figures and references. The Supporting Information is available free of charge via Internet at <http://pubs.acs.org>.

Notes

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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,^{11, 15–16} and introducing photolabile¹⁷ or ester linkers¹⁸ via solid-phase synthesis¹⁷ or unnatural amino acid incorporation.¹⁸ While 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 (Fig. 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 (Fig. 1). The precursor peptide and biosynthetic enzymes are expressed in the cytosol,¹¹ while the protease is directed to the periplasmic space by fusion with a secretion signal peptide (Fig. 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 (Fig. 1B), not only initiating contact between mLanA and the protease for leader peptide cleavage (Fig. 1C), but also permitting extracellular release of the final product for biological activity analysis (Fig. 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 peptides.¹⁹

We first validated the design using a two-component class II lanthipeptide, haloduracin.^{15, 20} For these peptides, the LanA precursor is modified by its cognate, bifunctional synthetase LanM, which dehydrates specific Ser and Thr residues and catalyzes subsequent Michael-type additions of Cys thiols to the resulting dehydro amino acids (Fig. 2).²¹ Haloduracin consists of Hal α and Hal β , which are synthesized from the precursor peptides HalA1 and HalA2 by their cognate lanthipeptide synthetase, HalM1 and HalM2, respectively. Recombinant production of mHalA1 and mHalA2 has been achieved in *E. coli* via co-expression of cognate pairs of HalA and HalM.¹¹ In the native producer *B. halodurans*,^{15, 20} leader peptide removal from mHalA1 and mHalA2 is achieved by the protease domain of a single, ATP-binding cassette transporter HalT, which cleaves after a double-Gly recognition motif (Fig. 2). Whereas cleavage by HalT produces the mature Hal α , it results in a Hal β intermediate with six residues at its N-terminus (GDVHAQ).²² N-terminal trimming of GDVHAQ-Hal β in the native producer is performed by an unidentified *B. halodurans* protease.²²

In this study, to produce Hal β 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 (Fig. 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 1–24²⁴) was replaced with the signal peptide of the *E. coli* protein OmpA (SP_{OmpA}),²⁵ which results in periplasmic localization. To also temporally 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/*p_R*.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 micron-scale holes in the cytoplasmic membrane, degrade peptidoglycan, and disrupt the outer membrane.²⁷

A two-plasmid system was utilized to implement the design. One plasmid was derived from the pRSFDUET-1 vector encoding HalM2 and engineered HalA2, similar to what was previously described.¹¹ The engineered HalA2 contains both the NDVNPE sequence before the core peptide and an N-terminal fusion of maltose-binding protein (MBP). The MBP fusion was included to minimize degradation of mHalA2 by endogenous peptidase activities in *E. coli*. In the other plasmid created from pBAD18, the SP_{OmpA}-LicP fusion gene was placed after the P_{BAD} promoter, and the CI857/*p_R*-SRRz cassette was inserted into the backbone. As such, the transcription of HalA2/HalM2, SP_{OmpA}-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 (Fig. S1). *E. coli* BL21 (DE3) cells transformed with both plasmids were first selected on noninducing plates at 28 °C. Then, colonies were picked up by a membrane filter for parallel manipulation of many colonies.²⁸ The colony-containing filter was transferred to inducing plates containing IPTG and L-arabinose to initiate peptide production and modification. Following overnight induction at 18 °C, the filter was incubated at 38 °C for 2 h to induce autolysis, before being overlaid with *Lactococcus lactis* HP, a haloduracin-sensitive indicator strain. As haloduracin functions through the synergistic activities of both Hal α and Hal β ,^{20, 29} Hal α purified from *B. halodurans* was pre-mixed with the *L. lactis* strain at a concentration of 8 nM. For Hal β 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 Hal β can be produced in *E. coli* colonies. When co-transformed with the two plasmids containing HalA2/HalM2, SP_{OmpA}-LicP, and SRRz (Strain 1, 38 °C), *E. coli* cells inhibited *L. lactis* HP growth (Fig. 3A and Fig. S2A) following the devised workflow. In control experiments, inhibition zones were absent when either SP_{OmpA}-LicP (Strain 2) or HalA2/HalM2 (Strain 3) were missing, indicating antibiotic activity was specific to production of mature Hal β . 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 Hal β was produced before 38 °C treatment (Fig. S3). These observations indicate that the stringency of protease compartmentalization or autolytic control needed further improvements. Notably, *E. coli* cells co-expressing wild-type (WT) HalA2, HalM2, and HalT (Strain 4) did not exhibit antibiotic activities under these conditions (Fig. S2A). Moreover, whole *E. coli* colonies were subjected to MALDI-ToF MS analysis, and peaks consistent with the *m/z* value of Hal β were only observed in colonies producing zones of growth inhibition (Fig. 3B and Fig. 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-Hal β , but not of Hal β , were detected (Fig. S2B), consistent with previous observations that HalT alone is not sufficient to produce the final Hal β product.²² Collectively, these results show that mature Hal β was produced in *E. coli* colonies as demonstrated by bioactivity and MS analyses. We further examined the capability of Hal β -producing *E. coli* to inhibit growth of other indicator strains including *L. lactis* 481, *B. subtilis* ATCC 6633, and *Bacillus cereus* ATCC 14579, which are less susceptible to haloduracin relative to *L. lactis* HP (Fig. S2D).²⁹ Assay optimization was necessary, such as to spot crude extracts of liquid cultures instead of single colonies to increase Hal β quantity. Zones of growth inhibition were generated for *L. lactis* 481 and *B. subtilis*, but not for *B. cereus* (Fig. S2C), indicating the method is constrained by the sensitivity of indicator strains to a target antibiotic.

We proceeded to perform activity mapping of Hal β 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 (Fig. 2), as ring topology is critical for antimicrobial activity.²² 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 (Fig. S3) to achieve a >95% probability of full coverage of the NNK library.³⁰ 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 (Fig. S4). MALDI-ToF MS cannot differentiate amino acid substitutions that are either isobaric (Leu and Ile), or result in a mass difference indistinguishable to a typical ToF mass analyzer (Glu and Lys²⁸). A small portion of colonies generated mass spectra with low signal-to-noise ratios for various reasons including low production levels or codon substitution by the UAG stop codon.²⁸ In this case, DNA sequencing can be used to identify mutations that could not be deduced from mass spectra. The assignment strategy for each analog is summarized in Fig. S4.

A heat map was generated to visualize relative antimicrobial activities of Hal β mutants towards *L. lactis* using the size of the zone of growth inhibition (Fig. 4). Whereas most mutations reduced antibiotic activity, amino acid substitutions with similar chemical properties to the WT residues were less detrimental to Hal β production and/or activity. In addition, the impact on antimicrobial activities was dependent on residue positions. Whereas many Hal β variants mutated at Pro4 retained antimicrobial activities, 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 Hal β . 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 Hal β is fully conserved (Fig. S4). Our results suggest evolutionary conservation of this Pro may be because it is essential to Hal β 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 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 Hal β 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.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 (Fig. S6). After 18 h, however, only Val12Leu was confirmed to improve Hal β bioactivity with a reduced minimal inhibition concentration (MIC, 50 nM) relative to WT (75 nM, consistent with previous studies²⁹) (Fig. S6). Additionally, modified precursor peptides were purified to investigate the impact of mutations on leader peptide cleavage at Thr2, which is close to the LicP recognition site. Six variants that resulted in smaller or no zone of growth inhibition (Fig. 4) were selected with diverse side-chain chemistry (Gly, Ala, Leu, Ser, Arg, and Glu). By monitoring *in vitro* proteolytic reactions using MALDI-ToF MS (Fig. S7A), no substantial reduction in leader cleavage relative to WT was observed with these mutants (Fig. S7B). To understand the origin of weaker growth inhibition, Hal β analogs produced in proteolytic reactions were treated with iodoacetamide to alkylate free Cys thiols. WT Hal β contains four thioether rings and no reactive Cys thiols (Fig. 2).¹⁵ For Thr2Gly and Thr2Ser that retained activity, no alkylated products were found, suggesting “wild-type-like” ring

topology. For Ala, Leu, Arg and Glu variants that abolished activity, one reactive Cys was observed, indicating disruption of one thioether ring (Fig. S7D). These results are consistent with previous reports that cyclization is essential to Hal β activity.²² 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, lactacin 481, in *E. coli* colonies (Fig. 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.³¹ Following the same workflow, *E. coli* cells that co-expressed LctA/LctM, SP_{ompA}-LctT150, and SRRz exhibited antibiotic activities towards *L. lactis* HP (Fig. S8B). Zones of growth inhibition were absent without temperature shift, suggesting protease activities were compartmentalized as designed before inducible autolysis at 38 °C. MALDI-ToF MS revealed peaks with *m/z* values corresponding to lactacin 481 in associated strains (Fig. S8C), indicating leader peptide cleavage was successful. Moreover, no zones of growth inhibition were observed when the Cys25Ala mutation was introduced into LctA (Fig. S8B), which disrupts formation of the B ring³² and abolishes bioactivity. These results indicate that the observed growth inhibition was dependent on production of mature lactacin 481. However, N-terminally degraded lactacin 481 molecules were also detected from colonies (Fig. S8C), possibly generated by *E. coli* peptidases. Suppression of unspecific host proteolytic activities may therefore be beneficial to improve in-colony lanthipeptide production.

In summary, we produced analogs of haloduracin and lactacin 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.

Supplementary Material

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Figure 1. Synthetic biology design to produce mature class II lanthipeptides in *E. coli* colonies. (A) Periplasmic compartmentalization of protease. (B) Induction of autolytic protein expression by temperature change. (C) Leader peptide removal after autolysis. (D) Structure-activity screening of lanthipeptide variants in colonies using agar diffusion assay and MALDI-ToF MS analysis.

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Figure 3.

Analysis of Hal β 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 Fig. S2).

Figure 4.

Summary of antimicrobial activities against *L. lactis* by Hal β analogs produced in *E. coli* colonies. WT residues are contoured with red dashed lines. Both Leu and Ile substitutions are labeled as L.