

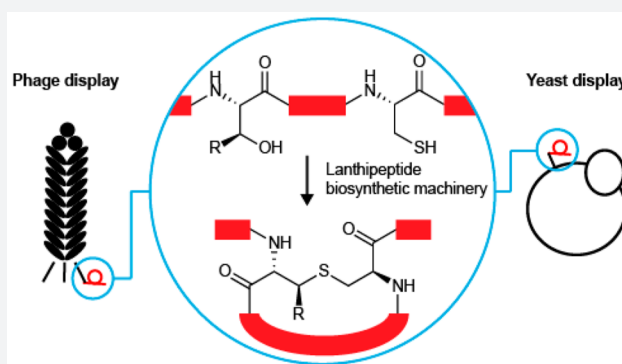
Development and Application of Yeast and Phage Display of Diverse Lanthipeptides

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Supporting Information

ABSTRACT: Peptide display has enabled identification and optimization of ligands to many targets. These ligands are usually linear or disulfide-containing peptides that are vulnerable to proteolysis or reduction. We report yeast surface and phage display of lanthipeptides, macrocyclic ribosomally synthesized and post-translationally modified peptides (RiPPs). Lanthipeptides contain multiple thioether cross-links that bestow their biological activities. We developed C-terminal yeast display of the class II lanthipeptides lactacin 481 and haloduracin β , and randomization of the C-ring of the former was used to select tight binders to $\alpha\beta$ 3 integrin. This represents the first examples of bacterial RiPP production in *Saccharomyces cerevisiae* for identification of variants with new biological activities. We also report N-terminal phage display of the class I lanthipeptide nisin and randomization of its A- and B-rings to enrich binders to a small molecule, lipid II. The successful display and randomization of both class I and II lanthipeptides demonstrates the versatility and potential of RiPP display.



INTRODUCTION

Macrocyclic peptide natural products are a privileged class of compounds used to diagnose and treat disease.^{1,2} A variety of surface display methods such as phage,³ yeast,⁴ bacterial,⁵ and mRNA⁶ display have ubiquitously demonstrated that linear peptides can typically be evolved to bind with high affinity and specificity to nearly any target,^{7,8} but linear peptides are metabolically unstable. In favorable cases, cyclization of the initially evolved peptide can retain activity, but significant optimization is often required. In principle, selection of cyclic peptides directly from displayed libraries would be advantageous. To this end, chemical cyclization strategies of phage or mRNA display libraries have been developed that rely on high yielding transformations.^{9,10} Use of the enzymatic machinery that makes cyclic natural products for display of cyclic peptide libraries has thus far only seen very limited application^{11,12} despite the potential advantages of highly regio- and stereo-selective processes that lead to molecular diversity and stability. The ribosomally synthesized and post-translationally modified peptide (RiPP) class of natural products is particularly promising with respect to engineering new functionality because of a direct link between a gene-encoded precursor peptide and the final macrocyclic compound.¹³ Lanthipeptides are a subgroup of RiPPs and are synthesized from a precursor peptide, generically called LanA, which is split into two regions: an N-terminal leader peptide is involved in recognition of the peptide by the biosynthetic machinery, and a C-terminal core peptide is where the post-translational modifications take

place.¹⁴ Select serine and threonine residues in the core peptide are dehydrated to dehydroalanine and dehydrobutyrine residues, respectively, followed by conjugate additions of cysteine thiols onto the dehydrated residues, resulting in macrocyclization through thioether cross-links that are called lanthionine or methyllanthionine (Figure 1).¹³ Unlike disulfides, these thioether bonds are stable in reducing environments. Because the recognition of the LanA substrate by the post-translational modification enzymes arises from recognition of the leader peptide, many lanthipeptide biosynthetic pathways have been shown to be tolerant of alterations to the core peptide.¹⁵ This substrate tolerance, along with the ability to make large libraries of precursor peptide-encoding genes through DNA synthesis, provides access to a large number of non-natural lanthipeptides that can be screened for new activities.¹⁶ Here we show the feasibility of displaying lanthipeptide libraries on phage and yeast and demonstrate the utility for the selection of cyclic peptides derived from natural products.

RESULTS

Development of a Lanthipeptide Yeast Surface Display System. Techniques have been developed for the directed evolution of disulfide stabilized peptides such as knottins^{17,18} by expressing libraries on the surface of yeast cells.

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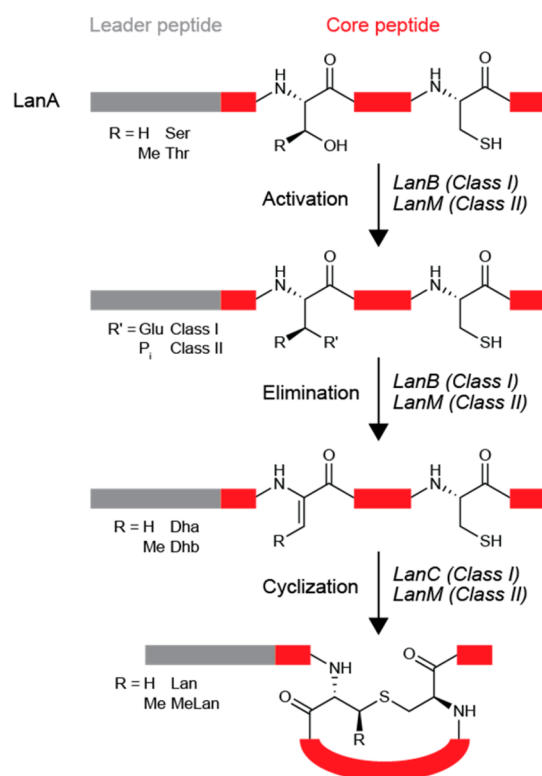


Figure 1. Lanthipeptide biosynthesis. Select serine and threonine residues in the core peptide of the precursor peptides are activated by glutamylation in a Glu-tRNA^{Glu} dependent manner by a LanB (class I systems) or phosphorylation in an ATP dependent manner by a LanM (class II systems). Elimination of the leaving group results in the production of dehydroalanine (Dha) or dehydrobutyrine (Dhb) residues. Either a LanC (class I) or LanM (class II) catalyzes the cyclization of the peptide.

In one approach to yeast surface display, the protein being evolved is fused to a subunit of the yeast surface protein agglutinin, Aga2.⁴ Aga2 is natively anchored to the Aga1 agglutinin subunit through disulfide bonds, and Aga1 is covalently attached to the yeast cell wall.¹⁹ Therefore, proteins fused to the C-terminus of Aga2 (termed C-terminal display in this study) are presented on the yeast surface where they can be screened for their ability to bind to targets.

We first explored the ability of the lanthipeptide biosynthetic machinery to modify Aga2-LanA fusions. Initially, the biosynthetic machinery of the well-characterized class II lanthipeptide lactacin 481 was tested. This lanthipeptide is modified by a bifunctional enzyme, LctM, that dehydrates Ser/Thr residues in an ATP-dependent manner and also catalyzes the cyclization reactions (Figure 1).²⁰ LctA, the precursor peptide of lactacin 481, was cloned fused to the C-terminus of Aga2, and the fusion protein was coexpressed in *Escherichia coli* with LctM. Aga2-LctA was purified and treated with endoproteinase Lys-N to isolate the core peptide. MALDI-TOF MS analysis showed that the Aga2-LctA had been dehydrated four times, as expected for lactacin 481 (Figures 2a and S1a; for the structure of lactacin 481, see Figure 3a). The fragmentation pattern observed by tandem MS of Lys-N digested Aga2-LctA agreed well with that of lactacin 481 isolated from the native producer (Figure S1b), and its antimicrobial activity against *Lactococcus lactis* CNRZ 117 was similar to that of native lactacin 481 (Figure 2b). Thus, LctM is capable of properly modifying Aga2-LctA in *E. coli*. Similar results were obtained for modification of the fusion of the lanthipeptide precursor HalA2 and Aga2 by the haloduracin β synthetase HalM2,²¹ suggesting that the Aga2 fusion is generally tolerated by class II lanthipeptide synthetases (Figure S2).

Having established the complete modification of the Aga2-LctA fusion, we investigated production of modified LctA on the surface of yeast. It has been noted that the presence of

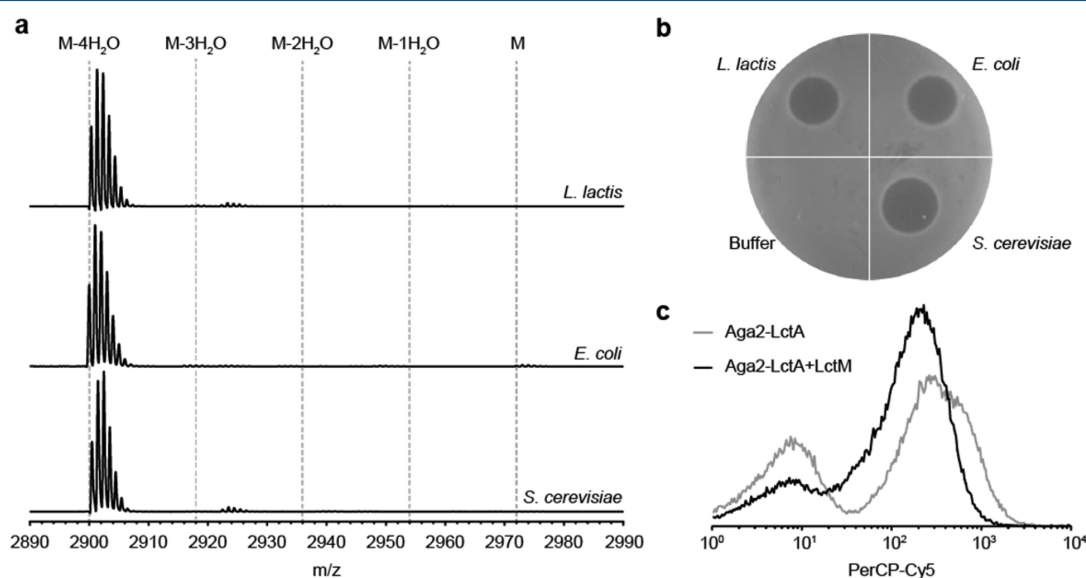


Figure 2. Characterization of Aga2-LctA fusions. (a) MALDI-TOF MS analysis shows that the isolated core peptides of Aga2-LctA from the *E. coli* cytosol and the *S. cerevisiae* surface are 4-fold dehydrated as desired for lactacin 481 based on material isolated from the native producer *L. lactis*.⁴⁹ Lactacin 481 predicted M+H: 2,900.3 Da; observed *m/z* 2,900.3 (*L. lactis*), 2,900.0 (*E. coli*), 2,900.5 (*S. cerevisiae*). (b) Similar zones of growth inhibition are observed for the antimicrobial activity of lacticin 481 produced in *L. lactis* or *E. coli*, or on the surface of yeast against the sensitive strain *L. lactis* CNRZ117. (c) The presence of peptide on the surface of yeast was detected based on immunostaining of the twin strep tag in the Aga2-LctA construct, demonstrating that coexpression of LctM does not reduce the level of Aga2-LctA display.

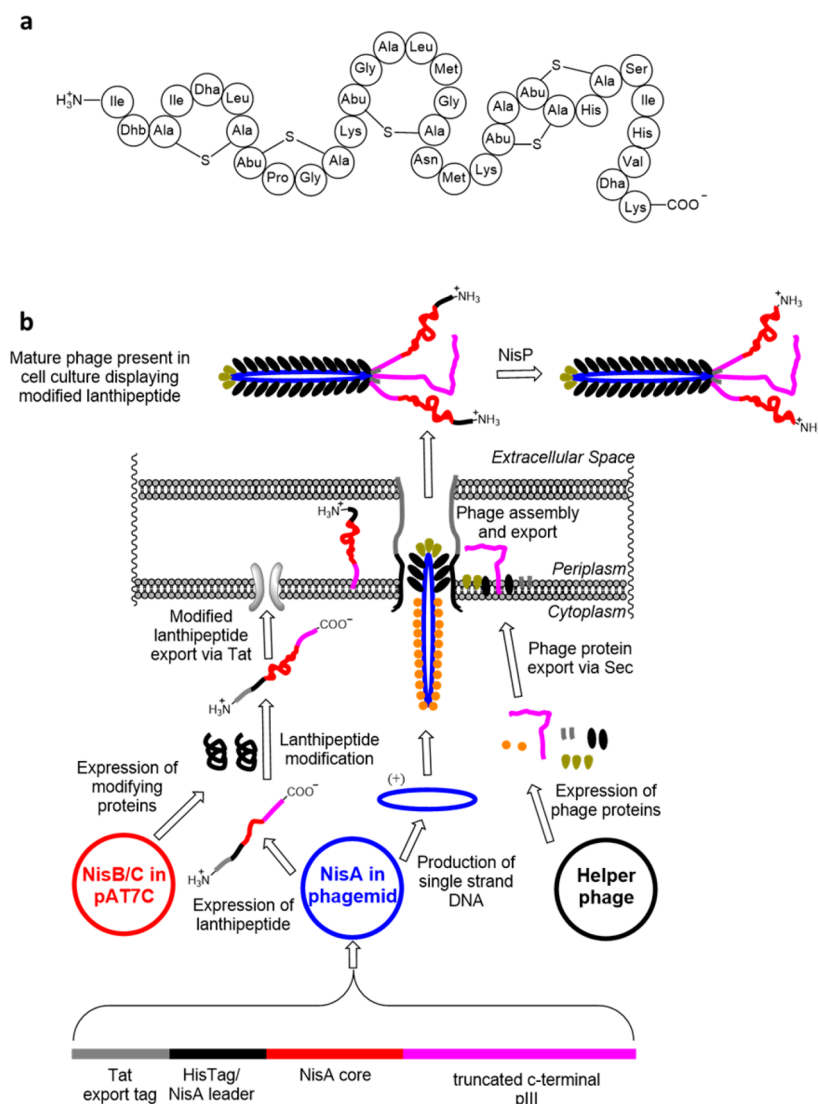


Figure 4. Structure of nisin and schematic of the constructs and process for phage display of nisin. (a) The structure of nisin, demonstrating the position of the post-translationally installed dehydroalanine (Dha), dehydrobutyrine (Dhb), and thioether rings. (b) The NisA construct contains the Tat signal peptide-lanthipeptide-phage protein pIII fusion. NisB/C is expressed from the modification plasmid. After modification, the Tat-HisTag-NisA-pIII is exported to the periplasm, where the signal sequence is proteolytically removed. As in wild type pIII, the modified NisA-pIII then inserts into the inner membrane awaiting phage assembly. The final phage is then assembled and secreted into the culture medium.

proteolytically cleaved with endoproteinase Lys-N, and characterized. MALDI-TOF MS analysis showed the expected 4-fold dehydrated product (Figure 2a) and the tandem MS fragmentation pattern agreed well with that of native lactacin 481 (Figure S1b). Finally, the bioactivity of Aga2-LctA isolated from the yeast surface and treated with Lys-N was similar to that of the native compound (Figure 2b), clearly demonstrating that yeast are capable of displaying properly modified Aga2-LctA on their surface. Yeast was likewise capable of displaying completely modified Aga2-HalA2 on the surface (Figure S3).

Screening Lanthipeptide Libraries by Yeast Surface Display. Having demonstrated that fully modified lanthipeptides can be displayed on the surface of yeast, we turned our attention to generating and screening libraries of lanthipeptides to identify members with new activities. To test the tolerance of the biosynthetic machinery and illustrate the potential of selecting for new activities, we focused on identifying lanthipeptides capable of binding to the $\alpha v \beta 3$ integrin. This integrin binds to peptides containing an RGD amino acid

motif,^{25,26} and ligands are useful for imaging tumors.²⁷ We assembled a library by encoding the C-ring of lactacin 481 with the NDT degenerate codon (N = A, T, G, or C and D = A, G, or T) to ensure that a portion of the library would contain the RGD motif (Figure 3a). This library encodes 12 amino acids (RNDCGHILFSYV), and with six positions that are randomized, its theoretical diversity is 3×10^6 members; deep sequencing of the constructed plasmid library revealed that it comprised 6×10^5 unique members (Figure S4). The library of C-ring mutants of lactacin 481 was displayed on the surface of yeast and screened for integrin binding by two rounds of fluorescence activated cell sorting (FACS) and subjected to deep sequencing after each round. After the second round of FACS, approximately 95% of the library-encoded peptides contained an RGD motif, suggesting that the library was being enriched in integrin binding peptides (Figure 3b). Examining the change in frequency of individual residues at each position in the lactacin 481 C-ring reveals that Gly increases at all positions, Asp is more prevalent at positions 3 to 5, and

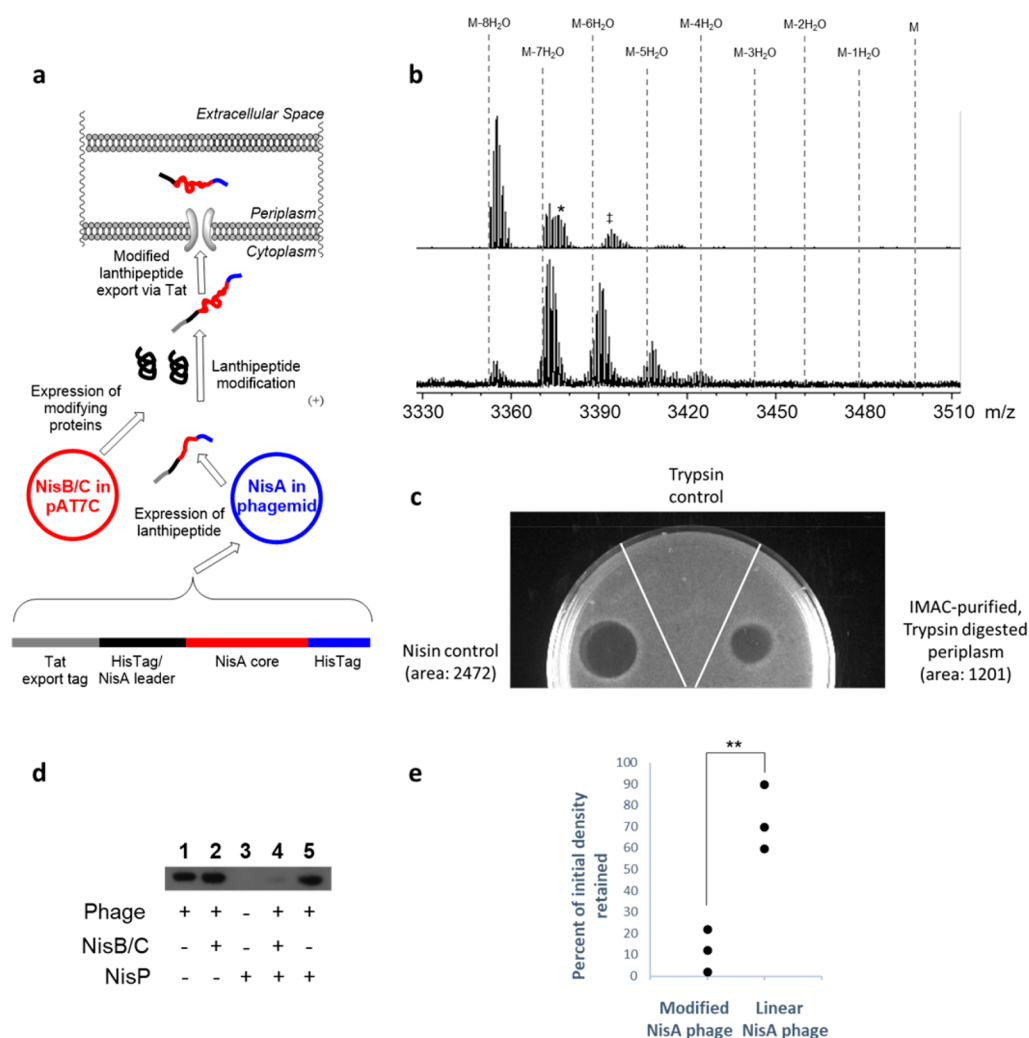


Figure 5. Characterization of periplasmic and phage displayed NisA. (a) The HisTag-NisA-HisTag gene construct is coexpressed with the modification plasmid, yielding export of modified NisA into the periplasmic space via the Tat pathway. Purification of periplasmic proteins is accomplished through osmotic shock coupled with IMAC. (b) MALDI-TOF MS of authentic nisin compared to the IMAC-purified, trypsin-digested periplasmic isolate demonstrates that the Tat pathway yields primarily 7-fold dehydrated NisA, although some fully dehydrated NisA is also observed (predicted mass of nisin, 3352.5 Da; observed mass, 3353.1; * indicates a nisin + Na adduct; ‡ indicates an M - 7H₂O + Na adduct). (c) Bioactivity of the trypsin-digested periplasmic isolate demonstrates that cyclization has occurred in the exported peptide. (d) Representative Western blot of linear NisA phage before and after digestion with NisP (lanes 1 and 5, respectively), modified NisA phage before and after digestion with NisP (lanes 2 and 4, respectively), and a control of NisP alone (lane 3). (e) Quantification of the percent of initial density retained in Western blot analysis. ** represents $p = 0.004$, $n = 3$; two-tailed t test.

correspondingly Arg increases at positions 2 and 3 (Figure 3c). The increased frequency of Cys at positions 1 and 2 is likely due to off-pathway cyclizations occurring via disulfide bonds, suggesting that construction of libraries using a degenerate codon that does not encode Cys may be useful in the future. The 100 most abundant sequences following the second round of sorting account for 98% of the library, and from these sequences 36 were enriched at least 2-fold after both the first and second sorts. From these sequences, 28 have the RGD starting at the second or third positions of the lactacin 481 C-ring (Figure 3d).

To further characterize the hits, we cloned a selection of the most highly enriched peptides for production in *E. coli*. Coexpression with LctM and subsequent purification and analysis by MALDI-TOF MS showed that the peptides were dehydrated 3-fold as desired (a Thr that is normally dehydrated in the C-ring was removed in the library because of the use of the NDT codon), and the fragmentation patterns observed by

tandem mass spectrometry were consistent with the overlapping ring structure of lactacin 481 (Figures S5 and S6). To demonstrate the ability of these peptides to bind to $\alpha v\beta 3$ integrin, a fluorescence polarization competition assay with fluorescein labeled c(RDGyK)²⁸ was performed (Figure S7). All tested lanthipeptide hits competed with c(RDGyK) with K_i s in the low nanomolar range, whereas no competition was observed with wild type lactacin 481 (Figures 3e and 3f). The K_i s for the five lanthipeptides tested were within about an order of magnitude of those of cilengitide,²⁹ the tightest binding cyclic pentapeptide identified for integrins, and echistatin,³⁰ an integrin-binding peptide from snake venom. The inhibition constants of the lanthipeptides are comparable with those of other engineered integrin-binding peptides, for example a rationally designed lasso peptide (IC₅₀ 20-fold higher than that of cilengitide),³¹ rationally designed cyclotides (IC₅₀ 20-fold higher than cilengitide),³² and evolved cysteine knot peptides (IC₅₀ 2- to 5-fold higher than echistatin).^{17,33}

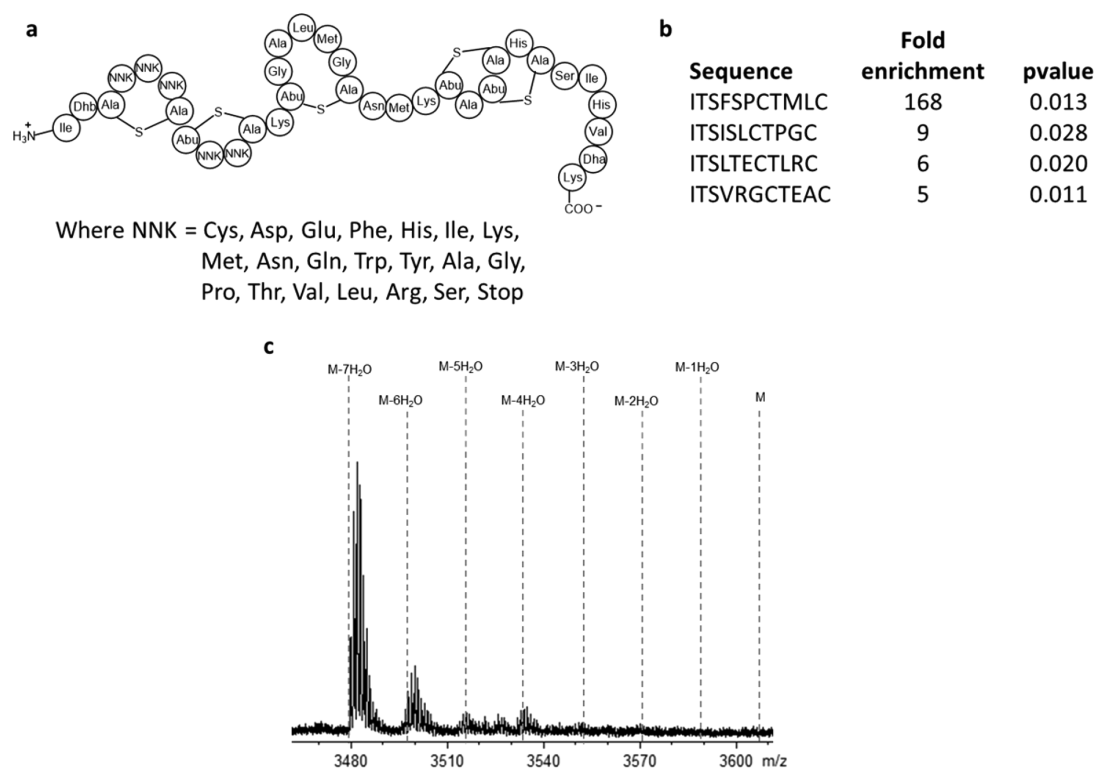


Figure 6. Phage library creation and selection results. (a) Library generation strategy showing the residues of rings A and B of nisin that were altered using the NNK codon. (b) Sequences that were enriched more than 2-fold after one round of selection. Sequences that were also enriched in the control selection that lacked lipid II are not included. *P*-values were determined by a two-tailed *t* test of the frequencies of the variant in the selected library ($n = 3$) to the frequency of the variants in the unselected library ($n = 3$). (c) MALDI-TOF MS characterization of the NisA-14F/L6P/P9M/G10L variant coexpressed with NisB/C in *E. coli*. Calculated mass of 7-fold dehydrated variant: 3478.6 Da. Observed mass: 3479.7.

Development of a Phage Display System. Having established that the class II lanthipeptides lactacin 481 and haloduracin β can be successfully displayed on yeast and used for selections of protein binders, we next decided to investigate phage display. We chose the class I lanthipeptide nisin (Figure 4a) to investigate three aspects of lanthipeptide display not addressed in the yeast display study. First, we wanted to investigate the breadth of systems that can be displayed, as the biosynthetic machinery for class I lanthipeptides is very different from that of class II (see below). Second, we sought to expand the type of targets that can be used for selections, since nisin binds to the small molecular weight metabolite lipid II.³⁴ And third, we wanted to explore N-terminal display rather than the C-terminal yeast display presented above.

Class I lanthipeptide precursor peptides undergo the same net transformations as those discussed for class II lanthipeptides (i.e., Ser/Thr dehydration and subsequent Lan formation) (Figure 1). However, the enzymatic machinery features a LanB enzyme that activates Ser/Thr residues in the substrate peptide through glutamylation, using glutamyl-tRNA as the glutamyl source.³⁵ As such, these compounds are not suited for yeast display, as glutamyl-tRNA would not be present in the yeast ER. LanB then also catalyzes elimination of the glutamyl group from the intermediate to yield the dehydroalanine/dehydrobutyrine residues. An independent enzyme, LanC, then installs the thioether linkages. Proteolysis of the leader peptide by a LanP enzyme liberates the mature, active product. The action of NisB, NisC, and NisP on the precursor peptide NisA thus yields the mature product nisin A³⁶ that targets the diphosphate moiety of the cell wall building block lipid II.³⁴ Since nisin analogues that are extended at the N-terminus, even only by a

single Ala,³⁷ have been shown to be inactive,^{38,39} presumably because of interference with lipid II binding, C-terminal display as in the yeast display method would likely not be useful to select for variants that bind to lipid II.

Thus, we aimed to display nisin on phage by genetically fusing the C-terminus of the *nisA* gene to a gene that encodes an N-terminally truncated phage protein pIII (residues 205–406) of the filamentous phage M13 (Figure 4). Filamentous phage assemblies in the periplasm of *E. coli*, and the required proteins must therefore be exported into the periplasmic space. It was previously demonstrated that the Sec-mediated translocation of nisin cannot export fully modified nisin precursor peptide,²² and therefore the Tat pathway, which is known to translocate cytoplasmically folded proteins,⁴⁰ was chosen for export in this study. The desired *Tat-HisTag-NisA-truncpIII* construct was assembled into a phagemid: a plasmid containing both an *E. coli* origin and a phage origin, which ensures that it is packaged in the mature phage. The modification enzymes NisB and NisC were encoded on a separate modification plasmid. Helper phage M13KO7 was used to supply the remaining required phage proteins, and expression of these components was envisioned to yield phage displaying modified NisA. Addition of the leader peptidase NisP *in vitro* was then anticipated to remove the leader peptide, yielding phage displaying nisin A and containing the associated *nisA* genetic material.

To verify that this system indeed functioned as desired, we first determined whether export via the Tat pathway delivered modified NisA into the periplasmic space of *E. coli* (Figure 5a). Since the pIII protein inserts into the membrane following export into the periplasm,⁴¹ we replaced the pIII gene with a

hexahistidine tag (HisTag) to facilitate detection. This approach allows for isolation of the HisTag-NisA-HisTag fusion peptide from the periplasmic space by osmotic shock followed by IMAC purification. The resulting peptide was digested with trypsin to remove the leader peptide and the C-terminal HisTag, and MALDI-TOF MS analysis showed that the Tat pathway does allow export of fully dehydrated NisA (8-fold dehydrated), although the predominant product was a 7-fold dehydrated intermediate (Figure 5b). We note that authentic nisin A also consists of a mixture of 7- and 8-fold dehydrated nisin. Bioactivity of the peptide against *Bacillus subtilis* ATCC 6633 demonstrated that the periplasmic isolate is cyclized since installation of the thioether cross-links is required for bioactivity (Figure 5c). Comparison of the zone of growth inhibition displayed by the periplasmic isolate to that of authentic nisin A was used to determine the approximate amount of nisin that is exported to the periplasm. Correlation of this value to the number of virions produced from a parallel culture suggests that about 20 molecules of nisin are produced in the periplasmic space for each phage. Since each phage contains 3–5 copies of pIII, these data show that sufficient nisin export is achieved to ensure that each virion produced displays nisin.

We next investigated whether the modified Tat-HisTag-NisA-truncated pIII fusion peptide indeed assembles on the phage. Unlike the yeast display system, where sufficient amounts of material could be liberated from the surface, with phage display mass spectrometric analysis proved not possible. We therefore produced two varieties of phage: the first prepared with coexpression of the modifying enzymes NisB/NisC, termed modified-NisA phage, and the second produced without the expression of the modifying enzymes, termed linear NisA phage. The precursor peptide includes a His₆-tag at the N-terminus of the NisA leader peptide, and thus SDS-PAGE of the phage samples followed by Western blotting with an anti-His-tag antibody showed the same band in both samples prior to digestion with NisP (Figure 5d), suggesting that the modified and linear constructs are assembled on the phage. Next, we incubated both phage samples with the mature NisP protease. The activity of NisP is severely diminished for substrates without the thioether rings installed,^{42,43} predicting that NisP treatment would result in disappearance of the HisTag band in the Western blot of modified NisA phage, whereas the band should be largely retained in the linear NisA phage band. Densitometric analysis for both modified NisA phage and linear NisA phage demonstrated that the band retained $70 \pm 20\%$ ($n = 3$) of the initial density upon NisP treatment for linear NisA phage, whereas the modified NisA phage maintained only $12 \pm 9\%$ ($n = 3$) of the initial density following NisP treatment (Figure 5e). Thus, this data shows a statistically significant difference between the two samples ($p = 0.004$) that suggests that modified phage displays a significant fraction of cyclized NisA. This conclusion is further supported by the binding of the modified phage to immobilized lipid II (see below).

Screening Lanthipeptides by Phage Display. We next sought to establish that a library of nisin variants could be screened for a specific activity. We created a library of variants of the A- and B-rings of nisin through randomizing of the five residues within these rings using the NNK codon ($K = G/C$; Figure 6a) that can encode all 20 amino acids. Deep sequencing revealed that the library encoded 1.2×10^6 variants out of a theoretically possible 3.2×10^6 (20^5) (Figure S8). Constructs

encoding nisin A were present in the library, suggesting that a successful lipid II binding selection should result in enrichment of this library member. To determine whether this was indeed the case, we produced phage bearing this library of variants and digested the phage with NisP to remove the leader peptide. We then incubated the phage library with biotinylated lipid II, pulled down the bound variants using Strep-Tactin resin, washed the resin to remove unbound phage, and released the bound variants through incubation with nisin A. The eluted phage were used to infect *E. coli*, and the plasmids were isolated and subjected to deep sequencing. As a control, the experiment was repeated without the addition of biotinylated lipid II. Both the selection and control were performed in triplicate to allow comparison of enrichment relative to the initial library prior to selection.

Following a single round of selection, comparison of the variant frequencies in the lipid II binding selection to the initial variant frequencies in the library prior to selection revealed that while the vast majority of variants were not enriched or were depleted, four variants were more than 2-fold enriched and did not appear in the control selection lacking lipid II. The nisin A-bearing phage was the second most enriched of these variants (9-fold enriched; $p = 0.03$) when compared to the initial library, whereas in the control selection, nisin A-bearing phage was not significantly enriched. The enrichment of nisin from the library demonstrates that the selection successfully isolated a lipid II binding molecule.

Three other variants were also enriched in the selection, one of which was enriched more than nisin A (Figure 6b). This variant, I4F/L6P/P9M/G10L, was chosen for further characterization. It was produced in *E. coli* through coexpression of the corresponding His₆-tagged NisA variant with NisB and NisC, purified and digested with NisP, and characterized via MALDI-TOF MS (Figure 6c). The mutations were well-tolerated by the processing enzymes, yielding a primarily 7-fold dehydrated species. Moreover, reaction of the processed peptide with *N*-ethylmaleimide did not result in any adducts, indicating the Cys residues are engaged in thioethers, and the peptide was successfully digested by the endoprotease NisP, suggesting that the rings are formed correctly. Indeed, tandem mass spectrometry analysis of the variant revealed a very similar fragmentation pattern as nisin A (Figure S9). Bioactivity assays demonstrated that the product did not possess any antimicrobial activity against *Lactococcus lactis* NZ9000 (Figure S9c). It is therefore likely that the simultaneous variation of four of the five residues in the A- and B-rings disrupts the correct assembly of a pore-forming lipid II:nisin complex in a membrane environment that is required for activity.^{44,45}

Because of the low solubility of this variant in aqueous media, we were unable to investigate its interaction with lipid II using common techniques including thin-layer chromatography⁴⁶ or isothermal titration calorimetry.⁴⁷ We therefore assessed the interaction using ³¹P NMR spectroscopy in dimethyl sulfoxide. Previous ³¹P NMR characterization of nisin's interaction with lipid II in this solvent revealed that the pyrophosphate peaks shift upfield upon nisin binding.⁴⁸ We performed similar experiments by mixing either nisin A or the nisin variant with lipid II and acquiring ³¹P NMR spectra. Two new ³¹P NMR resonances were observed for both nisin and the variant confirming that both compounds bind to lipid II (Figure S10). Consistent with the previous study,⁴⁸ both new resonances arising in the nisin/lipid II spectra were shifted upfield. However, only one of the new resonances arising for the variant

was shifted upfield, whereas the other was shifted downfield (Figure S10). This suggests that the variant indeed forms a complex with lipid II but in a different manner from nisin, possibly explaining the lack of bioactivity.

DISCUSSION

Phage and yeast display techniques have been powerful tools for identification of peptides and proteins that can bind to specific targets. In this work, we have expanded these platforms for the display of enzymatically cyclized peptides, which often have desirable characteristics that their linear congeners do not possess, such as protease resistance and defined three-dimensional structure. We demonstrate the generation of two class II lanthipeptides on the surface of yeast and a class I lanthipeptide on phage. To the best of our knowledge, these studies represent the first examples of heterologous expression of bacterial RiPPs in yeast and the first examples of utilizing a post-translational modification not native to yeast for yeast surface display. Our strategy of targeting the post-translational modification machinery to the ER would likely allow for the installation of post-translational modifications from other classes of RiPPs, as long as the cosubstrates/cofactors for those enzymes are available in the ER. This system therefore lays the groundwork to allow directed evolution to produce RiPPs with new activities.

By displaying a library of lanthipeptides on the surface of yeast, we were able to identify lanthipeptides with a new activity, namely, binding to the $\alpha\beta$ integrin. Deep sequencing of the libraries allowed identification of peptides that were enriched over 1,000-fold after only two rounds of sorting and that bound the $\alpha\beta$ integrin with low nanomolar affinities whereas the parent compound did not bind. Our characterization of individual hits in this screen revealed that the peptides were fully dehydrated and cyclized as desired, highlighting the capability of class II lanthipeptide biosynthetic machinery to modify variants of the core peptides when the leader peptide remains constant. We note that all of the hit peptides in Figure 3 have all six positions altered compared to the sequence of the C-ring of native lactacin 481, demonstrating the high tolerance of the biosynthetic enzymes. Also of note is that the peptides that are most highly enriched have the RGD motif in positions 2–4 or 3–5, suggesting that having this motif too close to the residues that form the ring may be deleterious.

We were also able to achieve phage display of nisin A, a class I lanthipeptide with a more demanding biosynthetic pathway since it requires dedicated dehydratase and cyclase enzymes with the former having an unusual requirement of glutamyl-tRNA as cosubstrate. In our system, we used the Tat export pathway and we demonstrated that NisBC could correctly and efficiently modify a NisA fusion peptide inside the cytoplasm prior to export into the periplasm. This finding contrasts with a very recent report on attempts at N-terminal phage display of nisin using the Sec pathway that resulted in unmodified NisA being exported too efficiently into the periplasm.¹² In the latter report by Urban et al., the authors then moved to C-terminal display, which was demonstrated to result in successful export of modified peptides into the periplasm.¹² Since nisin analogues with amino acid or peptide extensions at the N-terminus have been shown to be inactive,^{37–39} C-terminal display, while very powerful to select binders to non-native targets, likely cannot be used for selecting lipid II binders. With the ability to remove the N-terminal leader peptide with NisP thus exposing the native nisin N-terminus, our N-terminal phage display method

of nisin using the Tat export pathway is complementary with the recently reported C-terminal display using the Sec pathway. The latter pathway was also used to C-terminally display libraries of the prochlorosin family of lanthipeptides that were made by the highly substrate tolerant class II ProcM enzyme,¹² which we have not investigated. These displayed prochlorosin variants were then selected for binders to urokinase plasminogen activator and streptavidin.¹² Together with the results reported in this work, this brings the total number of lanthipeptide biosynthetic enzyme systems that have been demonstrated to be amenable for display of lanthipeptides by N- or C-terminal phage display and yeast display to four (LctM, HalM2, ProcM, and NisBC), strongly suggesting that the methods will be generally applicable. Collectively, these two studies provide a blueprint for display of RiPPs on either yeast or phage. Such display methods allow full exploration of the remarkable substrate tolerance of RiPP biosynthetic enzymes well beyond previous site saturation mutagenesis studies.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscentsci.7b00581.

Experimental methods, Tables S1–S4 (calculated and observed m/z values for all ions detected by mass spectrometry), S5 (sequences of oligonucleotide primers), S6 (sequences of gBlocks), and S7 (plasmids and phagemids used or constructed in this study), and Figures S1–S10 (PDF)

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Notes

The authors declare no competing financial interest.

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