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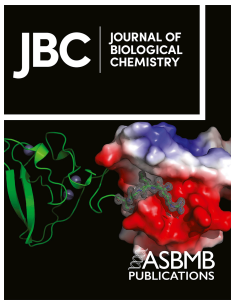
Published in:
The Journal of Biological Chemistry

DOI:
10.1016/j.jbc.2023.104845

Publication date:
2023



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PII: S0021-9258(23)01873-2

DOI: https://doi.org/10.1016/j.jbc.2023.104845

Reference: JBC 104845

To appear in: *Journal of Biological Chemistry*

Received Date: 1 March 2023

Revised Date: 10 May 2023

Accepted Date: 14 May 2023

Please cite this article as: Guo L, Wang C, Broos J, Kuipers OP, Lipidated variants of the antimicrobial peptide nisin produced via incorporation of methionine analogs for click chemistry show improved bioactivity, *Journal of Biological Chemistry* (2023), doi: https://doi.org/10.1016/j.jbc.2023.104845.

Lipidated variants of the antimicrobial peptide nisin produced via incorporation of methionine analogs for click chemistry show improved bioactivity

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Running title: nisin multi-site lipidation

Abstract

The increase in antibiotic resistance calls for accelerated molecular engineering strategies to diversify natural products for drug discovery. Incorporation of non-canonical amino acids (ncAAs) is an elegant strategy for this purpose, offering a diverse pool of building blocks to introduce desired properties into antimicrobial lanthipeptides. We here report an expression system using *Lactococcus lactis* as host, for non-canonical amino acid incorporation with high efficiency and yield. We show that incorporating the more hydrophobic analog ethionine (instead of methionine) into nisin improves its bioactivity against several Gram-positive strains we tested. New-to-nature variants were further created by click chemistry. By azidohomoalanine (Aha) incorporation and subsequent click chemistry, we obtained lipidated variants at different positions in nisin or in truncated nisin variants. Some of them show improved bioactivity and specificity against several pathogenic bacterial strains. These results highlight the ability of this methodology for lanthipeptide multi-site lipidation, to create new-to-nature antimicrobial products with diverse features, and extending the toolbox for (lanthi)peptide drug improvement and discovery.

Keywords: antibiotics, nisin, methionine analogs, click chemistry, multi-site lipidation.

Introduction

Lanthipeptides are a class of ribosomally synthesized and post-translationally modified peptides (RiPPs) that are characterized by the presence of one or more (methyl)lanthionine rings. They are also known to have various unusual amino acids, such as dehydroalanine (Dha) and dehydrobutyrine (Dhb) residues [1]. Lanthipeptides that have antibacterial activity are called lantibiotics [2]. Nisin is the first discovered and the best-studied lanthipeptide to date. Because of its antimicrobial activity and safety, it has been used as a food preservative for many years. Nisin also has great potential for therapeutic applications. It is for instance effective against many Gram-positive antibiotic-resistant organisms, such as vancomycin-resistant *Enterococcus* and methicillin-resistant *Staphylococcus aureus*. Resistance is rare to find because nisin has two inhibition mechanisms: it binds to lipid II, thereby hampering cell wall biosynthesis, and it forms pores in membranes, resulting in leakage of cellular constituents [3]. Compared with polyketides (PKs) and nonribosomal peptides (NRPs), the gene-encoded synthesis and relatively low complexity of the biosynthesis of lantibiotics make them good candidates for further engineering, with the aim to expand the diversity of the antimicrobial activity arsenal [4]. One approach to engineering the properties of nisin is to introduce non-canonical amino acids (ncAAs) during ribosomal peptide synthesis. It has been shown that the ncAAs Dha and Dhb in lantibiotics play an important role in their structural stability and biological activity [5]. Other ncAAs with unique moieties offer a further highly diverse pool of building blocks that can aid the design of novel lantibiotics with enhanced or special properties (e.g. stability, specificity and bioavailability). Among the ncAAs, methionine (Met) analogs are of particular interest. For instance, the Met analog azidohomoalanine (Aha) possesses an azido moiety that can serve as chemical handle for click chemistry with an alkyne-containing compound.

Lipopeptides represent a large class of microbial natural products with important biomedical applications, allowing their use as antibacterial and antifungal drugs. Some of them are known as powerful biosurfactants [6, 7]. A well-known lipopeptide antibiotic is daptomycin. It was approved in 2003 for the non-topical treatment of skin infections caused by Gram-positive pathogens, including methicillin-resistant *Staphylococcus aureus* (MRSA), and in 2006 for the treatment of bacteremia. The daptomycin structure includes a cyclic peptide backbone, similar to internal ring structures of nisin, but with an N-terminal fatty acyl moiety attachment. *In vivo*, these types of compounds are

usually synthesized by non-ribosomal peptide synthetases, giant enzyme complexes that assemble their products in a non–gene-encoded manner. This process also generates heterogeneously acylated peptides, requiring elaborate procedures to get defined compounds [8]. The presence of a lipid chain can provide the lipopeptides with amphiphilic properties that confer versatile functionalities [9]. The antibacterial activity of daptomycin against Gram-positive bacteria is largely dependent on the N-terminal fatty acid moiety [10, 11]. The attachment of lipids to a lanthipeptide has been demonstrated to be a strong and effective strategy to improve their therapeutic potential. However, lipids can only be attached to the C-terminal residue of a peptide using chemical synthesis [12] or attached to hydroxyl containing residues, such as Ser, Thr, or Tyr by the F-family of peptide-prenyltransferases [13].

Here, we describe a methodology for lipidation of lanthipeptides at various positions, by using Aha incorporation into peptides and subsequent *in vitro* click chemistry. Various expression systems have been developed for the incorporation of ncAAs and although the incorporations were modestly successful, the yields were not satisfying, being about 0.1-1 mg per liter bacterial culture, which makes it very hard to perform the click reaction with sufficient yield [14, 15]. In the current study, a new expression system was developed. We tested the new system with different Met analogs (azidohomoalanine, norleucine, and ethionine) incorporation. Tricine-SDS-PAGE gel and mass results showed all the analogs are very efficiently incorporated (>99.5%) at different positions (1, 17, 21, and 35) of nisin or truncated nisin variants. Notably, the yield of nisin variants improved more than 7 times compared to an earlier published expression system [14]. Agar well diffusion assays showed that incorporating the more hydrophobic analog ethionine into nisin can improve its bioactivity against a *L. lactis* strain. Minimal inhibitory concentration (MIC) tests indicated that Aha incorporation does not highly affect the bioactivity against the tested strains. With the Aha analog and subsequent click chemistry, we obtained lipidated nisin variants at different positions of nisin (e.g. at position 1, 17, 21, or 35) or in truncated nisin variants. Some of the lipopeptides show improved bioactivity and specificity against pathogenic strains. These results highlight the suitability of this methodology for lanthipeptide lipidation, to create new products with diverse features such as higher antimicrobial activity, extending the toolbox for peptide drug improvement.

Results and discussion

An improved expression system with enhanced efficiency and yield to incorporate Met analogs into nisin

Nisin is a ribosomally synthesized and posttranslational modified peptide, and the production of nisin requires *nisABTC* gene expression, which encode the nisin modification machinery NisBTC and prenisin (modified core nisin with the leader part still attached) (Figure 1, Figure 2A) [16]. In the model nisin expression system (Figure S1), both the *nisA* and *nisBTC* genes were controlled by the P_{nisA} promoter in two separate plasmids [17]. As Met is essential for the expression of NisBTC and falsely incorporating analogs into enzymes may cause a non-functional nisin modification machinery, a cross expression system that allows production of prenisin and NisBTC at different phases was used before and tested for Met analogs incorporation (Figure S1) [14, 15]. The expression of the modification machinery started in a rich medium, after which the medium is replaced by new synthetic medium lacking Met, but containing a Met analog to express prenisin. Thus, the host starts the peptide synthesis in the presence of 19 standard amino acids and the Met analog. After the prenisin is modified by dehydratase NisB and cyclase NisC to form (methyl)lanthionine rings, the prenisin is transported out of the cell by NisT, and then the leader is cleaved off by the extracellular protease NisP to liberate the active peptide (Figure 1). Although the expression of modification enzymes was induced beforehand, no effect on NisBTC production was observed. Using this system, four Met analogs were successfully incorporated at different positions in nisin [14].

Although this approach provided a powerful tool for Met analogs incorporation, the yield and incorporation efficiency were far from satisfying, and it limited further studies and application of the low amounts of peptides produced. Many efforts have been done, following the first low production phase, including optimizing analog concentrations, growth temperatures used, and variable induction times. All these tested conditions did not meet our desired production level. Here, we describe a new expression system with a promoter exchange to express the *nisA* and *nisBTC* genes. We show that in this way the yield and incorporation efficiency can be greatly improved. In the pCZ-nisin system (referring to the old system), *L. lactis* NZ9000 was initially transformed with a plasmid encoding the expression of *nisBTC* under the control of the P_{nisA} promoter and the other plasmid encoding the expression of prenisin derivatives controlled by the P_{czcD} promoter. P_{czcD} is a tight zinc-inducible

promoter used in *L. lactis* that has been cloned from genome of *Streptococcus pneumoniae* [18]. The P_{nisA} promoter is a tight and efficient promoter that has been used for many years with extreme success. From the previous study, it was clear that inside the cells it is not necessary to keep a high concentration of modification enzymes, which shows the high efficiency of these enzymes. In this study, we tried to use the tight but not so strong zinc-inducible promoter to induce the *nisBTC* genes in advance, and then the nisin gene was induced by the efficient promoter P_{nisA} (Figure 3A). We discovered that exchanging the promoters led to 7.5 times higher peptide production with respect to that of wild type nisin, and that the analog (Aha) incorporation efficiency was increased from 88% to above 99.5%, which makes it extremely much easier for subsequent purifications and applications (Figure 3). This system is the most efficient one so far for non-canonical amino acid incorporation in the host strain *L. lactis*. Compared to the Aha incorporation efficiency with the typical strain *Escherichia coli* with a similar incorporation method, Lehner reported incorporated Aha into the well-studied PDZ3 domain of the postsynaptic density protein 95 (PSD-95) with about 94 % incorporation efficiency [19], while in our system using *L. lactis*, the efficiency is above 99.5%. With respect to the reason why *L. lactis* shows higher incorporation efficiency than *E. coil* it has been suggested that the substrate specificity of *L. lactis* aminoacyl-tRNA synthetases (aaRS) is more relaxed than that of *E. coli* aaRS [20]. It should be noted that as the nisin-controlled expression (NICE) system in *L. lactis* is one of the most commonly used systems for inducible expression of not only nisin and other lanthipeptides, but also of proteins [21, 22], it gives the possibility that this efficient incorporation method can be extended to other peptides and proteins. Recently, the NICE system was used to mimic a non-ribosomal peptide (NRP), i.e. brevicidine, resulting in an engineered mimicked peptide displaying a similar antimicrobial activity as the wild-type peptide [23]. The NPRs are among the most promising sources of antibiotics, including more than 20 marketed antibacterial drugs, such as vancomycin, and daptomycin [24]. Non-canonical amino acids, like ornithine and D-amino acids, play important roles in their functioning. The combination of the NICE system with a mimicking strategy and non-canonical amino acid incorporation provides an alternative way for drug modification and discovery. The system's other advantages are that *L. lactis* is typified by rapid growth, ease of genetic manipulation and is generally regarded as safe, making it a versatile system for RiPPs peptide expression and analog incorporation.

Production of nisin variants with various types of ncAAs incorporated

There are two Met residues in wild-type nisin, located at positions 17 and 21, respectively. The expression levels of wild-type nisin in the presence of six Met analogs, including Aha, Nle, Eth, Nva, Pra, and Alg (Figure 4A), are shown in Figure 4B. The protein quantities in the second lane indicate yields when the pCZ-system was used, in the presence of Aha as control. Lanes 3-6 show that the Met, Aha, Nle, and Eth can be incorporated and their production yields are at varying levels. However, when either Nva, Alg or Pra was present in the medium, no production was observed (Figure 4B). The highest production yield was observed when Met was supplemented, yielding 9.5 mg/L pure peptide. The production yield decreased a little bit (12%) compared with Met incorporation in the presence of Aha. A lower production yield was observed in the presence of Nle and Eth, which were 5.3 mg/l and 5 mg/L, respectively (Figure 4C). The analog Nle was successfully incorporated before [14, 25]. The structural property of Nle is similar to that of Met, displaying the same length of the side chain. The only difference is at position 4, where a Met residue has a sulfur atom, while it is a carbon atom at Nle. This illustrates that in this case, a one atom change from sulfur to carbon for the analog does not affect the methionyl-tRNA synthetases to activate the substrate. Interestingly, also the Eth residue can be incorporated into peptides. One more methyl group attached to the side chain of Met constitutes the Eth residue. Here, we also demonstrate that multiple (i.e. 2) non-canonical amino acid incorporation simultaneously is possible, because the analogs could be incorporated at two positions within wild-type nisin at the same time.

To test the effects of single Met replacements with various analogs in bioactive nisin, four single Met mutants, i.e., M17I, M21V, M17IM21V-I1M, M17IM21V-M35 were constructed (Figure 2C). The residues Ile or Val were chosen as substituents to maintain good antimicrobial activity, as both residues share the hydrophobicity of Met and their side chains are quite similar in size [26, 27]. Proteolytic degradation of full-length nisin limits its potential therapeutic applications. However, previous studies have shown that C-terminally truncated nisin mutants lacking rings D and E retain significant antimicrobial activity [28]. To investigate this further, we created four additional truncated nisin mutants, namely nisin(1-21)M17I, nisin(1-21)M21V, nisin(1-22)M17I, and nisin(1-22)M21V, as depicted in Figure 2C. Met analog incorporation in M17I and M21V gave much higher peptide yields

than M17IM21V-M35 and M17IM21V-I1M. Two truncated nisin variants, i.e. nisin(1-21)M17I and nisin(1-21)M21V, showed no visible production when analyzed with a tricine gel. Surprisingly, one additional amino acid, i.e. Lys, attached to the tail of nisin(1-21) made the production yield of nisin(1-22) variants comparable with that of full length nisin M17IM21V-I1M and M17IM21V-M35 mutants (Figure 5). The reason for the varied productions may be due to the levels of (in)tolerance of the modification machinery NisBC and/or the transporter NisT to charge the substrate nisin variants. To assess the efficiency of post-translational modifications and incorporation of ncAAs, all samples were further analyzed by mass spectrometry.

The Met analog-containing nisin variants were correctly modified and the analogs were efficiently incorporated

The first Met residue (Met1) in the leader part of prenisin is usually removed by the methionine aminopeptidase [29]. In the pCZ-nisin system, a large part of the peptides produced by this system contained the first Met. For the pNZ-nisin system, the peptides without Met1 showed to be prominent in all variants (Table S4). In most cases, the incorporation of ncAAs into nisin or its (truncated) variants did not affect the dehydration efficiency using this new system, as peptides with fully (8 times or 5 times, respectively) dehydrated residues were prominent. The most affected dehydration of variants was in the M17IM21V-M35 peptide. By introducing Nle into M17IM21V-M35, using the new system, 7 times dehydrated peptides can also be detected. The results also indicated that Nle was hard to incorporate. It may be that the integration speed of Nle during translation is relatively slow which leads to an insufficient modification. The Aha incorporation at the different positions of nisin using the new system always yielded fully dehydrated peptides, while 7 times dehydration of M17IM21V-M35 using the pCZ-nisin system can be detected, suggesting the new system significantly improved the dehydration efficiency when Aha incorporation occurs. Notably, Met can be oxidized, and masses corresponding to oxidized products were also observed (Table S4).

The incorporation efficiency indicates the ratio between the amounts of peptides containing the analogs and the total amount of peptides. For the pCZ-nisin system, the incorporation efficiency was tested by liquid chromatography mass spectrometry (LC-MS). Here we used an easier way to test the incorporation efficiency by using MALDI-TOF [21, 30]. Figure 3D shows that the incorporation

efficiency of wild-type nisin labeled with Aha was 88%, the same as was found with the previous LC-MS analysis. It demonstrates that the MALDI-TOF analysis is a reliable and simple way for checking the analog incorporation. In the peptides M17IM21V-I1M, M17I, M21V, M17IM21V-M35, the Aha incorporation efficiency was 91%, 97%, and 96%, respectively, while with the new system it was always >99.5%, indicating that our new system has a much better incorporation efficiency than the old pCZ-nisin system. The MS data also shows that the incorporation efficiency of Nle and Eth was only 51-88% using the old pCZ-nisin system. Remarkably, using the new system, the incorporation efficiency of Nle and Eth was also >99.5%, as the mass of peptides containing Met were always undetectable. Moreover, in the cases of truncated nisin variants, the incorporation efficiency was also >99.5% for Aha, Nle, and Eth incorporation (Table 1). The reason for the high incorporation efficiency may be due to the rate of nisin expression. When using the efficient promoter P_{nisA} instead of the previously used zinc-inducible promotor, the majority of peptides will complete their expression within less than two hours [31]. If the expression time takes longer, inside the cells more proteins and peptides will be degraded due to the pressure of amino acid starvation and the many active peptidases present. The rate of activation by aminoacyl-tRNA synthetases during translation with normal amino acids is faster than with ncAAs, which finally results in the incorporation efficiency being decreased.

Antimicrobial activity of nisin and its Met or Met-analog containing derivatives

All nisin variants were purified and their antimicrobial activities were tested against *L. lactis* (Figure 6). Only the mutant M21V shows an enhanced antimicrobial activity compared to wild-type nisin. The M21 is located at the hinge region of nisin, which has a profound influence on the antimicrobial activity and host specificity [32, 33] in which the hydrophobic amino acids in nisin play an important role. In the naturally found nisin variants, various hydrophobic amino acids are within the hinge region, i.e. nisin Q-Leu21; nisin U/U2-Pro20/Leu21; nisin J-Phe20/Ala21 [34]. In general, the introduction of hydrophobic amino acids in the hinge region, i.e. Leu (N20L, M21L, K22L), Ile (N20I, M21I), or Met (K22M), resulted in the retention of relatively high levels of bioactivity or improved activity, and M21V was particularly notable for that property [32]. The mutation M17I resulted in the retention of relatively high levels of bioactivity, while the mutants M17IM21V-I1M

and M17IM21V-35M displayed reduced activity to 64% and 76%, respectively. The first two rings of nisin can bind to lipid II by forming a pyrophosphate cage [35]. A previous study showed that the I1W mutant had a twofold reduced activity [15], which was similar to that of the M17IM21V-I1M mutant. This indicates that the first Ile is not crucial for the binding. However, substitution of the first residue of the nisin core peptide has an influence on yield and NisP-cleavage efficiency [36]. The C-terminal amino acid lysine is responsible for the initial interaction of nisin with the target membrane [37]. We guess that the insertion of an additional Met at the end of full length nisin (M17IM21V-35M variant) hampers this interaction and the translocation, thus negatively influencing the activity. The truncated variants showed a dramatic decrease in activity. The activity of nisin(1-21) was completely lost with the peptide concentrations used. One more positively charged amino acid Lys added to the C-terminus of nisin(1-21) significantly enhances its antimicrobial activity and production scale, which is consistent with a previous study [38].

The Aha incorporation into nisin caused the activity to either increase, decrease, or retain activity. The activity of wild-type and M21V labeled with Aha were improved by 30% and 5%, respectively. Incorporation at position M17I did not affect the activity. Incorporation of Aha into M17IM21V-I1M or truncated nisin variants decreased the activity. The incorporation of Nle had a minor influence on the activity (less than 10% change), except for truncated nisin variants. The activity increased by 65% with the nisin(1-22)M17I, while it decreased by 35% with nisin(1-22)M21V. Peptides labeled with Eth had a clear influence on the bioactivity. The activity increased by Eth incorporation in the mutants M17IM21V-I1M, M21V, and nisin(1-22)M21V to 64%, 11%, and 50%, respectively. With the Eth incorporation in M17IM21V-35M, it caused 27% activity loss. It is also interesting to note that for all analogs incorporated in full length nisin, M21V with Met-analog at 17 has increased activity compared to wild-type nisin, indicating the important role of residue 17 located in nisin ring C.

Lipidated nisin variants

To obtain the pure nisin variants with Aha incorporated, we firstly investigated the influence of the analog concentration and induction time on peptide production (Figure S2). The optimal conditions for Aha incorporation were at a final concentration of Aha of 30 mg/L, and 3 hours induction time, for which the highest yield reached 8.3 mg/L pure peptide. With the Aha

incorporation (Figure S3), the MIC results showed that the replacement of Met by Aha generally had a minor effect on antimicrobial activity (Table S1), which make them good candidates for next modifications. The Met analog Aha possesses the unique azide functional group that can react with alkyne substrates, commonly referred to as "click chemistry" (Figure 2B) [39, 40]. Here, 1-undecyne (C11) was chosen for further reactions and was successfully coupled at four different positions (1, 17, 21, and 35) of full length nisin and at two positions of truncated nisin variants (Figure 2C). The yield, which varied from 42% to 100% depending on the click position, is presented in Figure S4 in the supplementary materials. Resulting compounds were purified and characterized by MALDI-TOF (Figure S5). Figure 7 shows that all full length nisin mutants and truncated nisin variants did not show antimicrobial activity against the tested strains, when used at low concentration conditions (0.01 mg/ml of full nisin variants or 0.04 mg/ml of truncated variants). However, the activity of four full length nisin variants significantly improved against *L. lactis* after coupling with C11, while this was not the case for two of the truncated nisin variants. For the mutants M17I and M17IM21V-35M, the lipidated nisin variants show selective activity against *E. feacalis*, but not for other tested strains. Coupling the C11 at position 17 (mutation M21V) made the antimicrobial spectrum broader and also more specific, as it showed bioactivity against *L. monocytogenes*, *E. faecium*, and *E. faecalis*, but did not show activity against *S. aureus*. There is some evidence for the important role of lipids in the activity of lanthipeptides. Zhao *et.al* reported that adding lipid tails to low-active lanthipeptides yielded semisynthetic macrocyclic lipo-lanthipeptides, which showed significant bactericidal activity against a number of Gram-positive and Gram-negative pathogenic bacteria [41]. Different hydrophobic tails have been attached to nisin variants at the C-terminus. Deng *et.al* used the lipid II binding fragments of nisin (nisin 1-12 and nisin 1-20) as the backbone to conjugate of different synthetic polyproline moieties, which yielded active and stable antimicrobials [42]. Attaching lipids to the nisin fragment (nisin 1-12) resulted in lipopeptides that displayed antibacterial activity almost on par with that of the wild-type nisin [12]. We now show that lipids can be attached at different internal positions of peptides by combining the incorporation of the analog Aha and then perform click chemistry, whereas previously, lipids and other interesting moieties could only be attached to the C-terminus of these lanthipeptides in chemical strategies [12], or these lipid attachments are catalyzed by the F superfamily of peptide prenyltransferase enzymes that use 5-carbon (prenylation) or 10-

carbon (geranylation) donors attached on a Ser, Thr, or Tyr residue [13]. We further demonstrate that the best position for further lipid modification is located within ring C of nisin. It is noteworthy that the method described in this study can be applied to conjugate other compatible moieties (e.g. glycans, active peptide moieties, fluorescent moieties) that can give the lanthipeptides desired properties. Future studies will concentrate on coupling more diverse lipids to nisin variants to create novel derivatives with different characteristics (e.g. stability, activity, and/or specificity).

Conclusion

Here, we demonstrate a new expression system with enhanced efficiency and yield to incorporate Met analogs into lanthipeptides in *L. lactis*. Three Met analogs were successfully installed at various positions of the lantibiotic nisin, while peptides containing Met residues were undetectable, which means the incorporation efficiency was always above 99.5%, compared with the previous expression system, where incorporation varied from 51-99.5%. The improved system leads to 7.5 times more modified peptide production, making it easier for further purification, characterization and click-chemistry application. The structural diversity of analogs incorporation resulted in antimicrobial activity against specific bacteria being reduced, retained, or even improved. In addition, this study underlines that the bio-orthogonal reactive groups of ncAAs can serve as a platform for post-biosynthetic modifications via click chemistry. We anticipate that more of these semisynthetic lipopeptides can be made for further optimization and development as novel antimicrobials.

Experimental procedures

Materials

Reagents used for molecular biology experiments were obtained from Thermo Fisher Scientific (Waltham, MA). Unless otherwise noted, all chemicals were acquired from Sigma-Aldrich (St. Louis, MO). The Met analogs L-azidohomoalanine (Aha), L-norvaline (Nva), L-norleucine (Nle) and L-allyglycine (Alg) were obtained from Iris Biotech GmbH (Marktredwitz, Germany). L-ethionine (Eth) was purchased from Alfa Aesar (Karlsruhe, Germany).

Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this study are listed in Table S2 in the supplemental material. All *L. lactis* strains were grown in M17 broth (BD Difco) supplemented with 0.5% (w/v) glucose (GM17) at 30 °C. When appropriate, 5 μg/mL chloramphenicol (Cm) and/or erythromycin (Em) were added to the media. *L. lactis* NZ9000 was used as the host for cloning, plasmid maintenance, and peptide expression. Chemical defined medium lacking tryptone (CDM-P) was used for protein expression and Met analogs incorporation [43].

Molecular biology techniques

The primers used in this study for PCR and sequencing are listed in Table S3. All primers were purchased at Biolegio B.V. (Nijmegen, The Netherlands). Plasmids encoding the mutations were constructed by amplifying the template plasmid using a phosphorylated downstream sense (or upstream antisense) primer and an upstream antisense (or downstream sense) primer [44]. Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific) was used to amplify the DNA. PCR products were first checked by agarose (1%) gel electrophoresis and the correct molecular weight band was cut and purified by NucleoSpin gel and PCR cleanup kit (Bioke, Leiden, the Netherlands). Subsequently, self-ligation of the DNA fragment was carried out with T4 DNA ligase. The ligation product was first desalted and transformed into *L. lactis* NZ9000 as previously described [45] using a Bio-Rad gene pulser (Bio-Rad, Richmond, CA). For the construction of plasmid pTLReBTC, the purified PCR products were mixed and fused using the Gibson assembly master mix (Bioke, Leiden, the Netherlands) according to the manufacturer's instructions. In this procedure, ligase was not essential. The plasmid was isolated and verified by sequencing using the pNZ-sequencing primer (Table S3).

Expression of ncAAs-incorporated prenisin

To check if the ncAAs were successfully incorporated, small scale (20 mL) expression and purification was performed. For the pCZ-nisin expression system, the precursor peptide precipitation procedure was performed as previously described [14]. The expression protocol for the pNZ-nisin system was as follows*: L. lactis* NZ9000 cells harboring the *nisBTC* plasmid were electroporated with the plasmid harboring the *nisA* gene (100 ng), plated on GM17 agar plates supplemented with chloramphenicol (5μg/ml) and erythromycin (5μg/ml), and grown at 30°C overnight. A single colony was picked and added to 4 ml of GM17CmEm medium for growth. 0.5 mL overnight culture was

diluted in 20 mL of the same medium. When the OD_{600} reached about 0.4, 0.5 mM $ZnSO_4$ was added to induce the expression of nisin modification machinery *nisBTC.* After 3 hours, the cells were washed three times with phosphate-buffered saline (pH 7.2) and resuspended in 20 mL of CDM-P lacking Met. After 1-hour starvation time, Met (38 mg/L) or Met analogs (50 mg/L) and 8 ng/mL nisin were added to induce peptide expression. After overnight growth, the supernatant was collected by centrifugation at 8,000 g for 15 min. Prenisin was precipitated with 10% trichloroacetic acid (TCA) on ice for at least 2 h, after then centrifuged at 10,000 g and 4°C for 45 min. The pellets were washed with 10 mL ice-cold acetone to remove TCA. Samples were dried in the fume hood and stored at -20 °C or resuspended in 0.4 mL 0.05% aqueous acetic acid solution for further analysis.

Tricine-SDS-PAGE analysis

The precipitated peptides were analyzed by the Tricine-SDS-PAGE system as described by Schagger [46]. 10 μL of sample was mixed with 8 μL loading dye and loaded on the 16% gel. Coomassie brilliant blue G-250 was used for protein staining.

Mass spectrometry

1 μL of the prenisin was spotted on the target, dried, and washed several times with Milli-Q water. Subsequently, an equal volume of matrix solution (5 mg/mL α-cyano-4-hydroxycinnamic acid dissolved in 50% acetonitrile containing 0.1% trifluoroacetic acid) was spotted on top of the sample. An Applied Biosystems 4800 Plus matrix-assisted laser desorption/ionization time-of-flight analyzer (MALDI-TOF) operating in linear mode using external calibration was used to obtain mass spectra. The analog incorporation efficiency was calculated by measuring the peak areas of the analog-containing peptides and the Met-containing peptides.

Agar well diffusion assay

An overnight culture of *L. lactis* MG1363 was added at 0.1% (v/v) to melted GM17 agar at 45 °C and then 30 mL of this solution was poured onto a plate. Once the agar was solid, wells of 8 mm were punched in the agar and filled with 30 μl 1 mg/mL of the lantibiotic solution. When necessary, lantibiotics were activated with 5 μl of NisP added directly to the well. Nisin amounts were determined by HPLC as previous described [43]. The agar plate was incubated at 30 °C overnight, after which the zones of inhibition were measured. Data are representative of three independent experiments. Zone diameters were measured in millimeters and recorded as area of the zone (πr2)

minus the area of the well (πr2) in millimeters.

Optimization of the expression of prenisin labeled with Aha

To achieve maximal production, the Met analog Aha concentration and induction time was investigated in detail. The effect of the Aha concentration was determined by adding 5-50 mg/L Aha into the medium after 3 h *nisBTC* induction. The impact of the induction time on the prenisin production was examined by 1 to 4 h *nisBTC* induction following addition of 30 mg/L Aha to the medium.

Purification of nisin variants labeled with Aha

To obtain larger amounts of nisin variants, experiments were performed at a 2 L scale. The supernatant pH was adjusted to 7.0 and incubated with purified NisP [47] at 37 °C for 3-6 hours to cut off the leader, and then the supernatant was applied to a C18 open column (Spherical C18, 20 g, particle size: 40-75 μm, Sigma-Aldrich). The column was washed with 40 mL of different concentrations (25%, 30%, 35%, 40%, and 60%) of buffer B (buffer A, distilled water with 0.1% TFA; buffer B, acetonitrile with 0.1% TFA). The active fractions were lyophilized and further purified using an Agilent 1200 series HPLC equipped with a C12 column (Jupiter 4 μm Proteo 90A, 250 × 4.6 mm, Phenomenex). The peak with activity and correct molecular weight was collected, lyophilized and stored at 4 °C until further use.

Minimal inhibitory concentration assay

Minimal inhibitory concentration (MIC) values were determined by broth microdilution, according to the standard guidelines using cation-adjusted Mueller-Hinton broth (MHB) [48]. The inoculum was adjusted to approximately $5x10^5$ CFU/mL. The MIC was defined as the lowest concentration of antimicrobial compound with no visible growth after overnight incubation at 37°C (*L. lactis* MG1363 at 30°C).

Lipidation of Aha-labeled nisin with a C11 tail

Stock solutions of copper (100 mM), sodium ascorbate (1 M), and BTTAA (2-(4-((bis((1-tert-butyl-1H-1,2,3-triazol-4-yl)methyl)amino)methyl)-1H-1,2,3-triazol-1-yl)acetic acid, 50 mM) were prepared. Nisin variants labeled with Aha (100 μg) were dissolved in 100 mM phosphate buffer (pH 7.0, final reaction volume: 200 μL), and 2 μL 1-undecyne (C11) was added to the solution. Then, stock solutions $CuSO_4$ (4 μL): BTTAA (40 μL) premix was added followed by the addition of 20 μL

sodium ascorbate. The reaction was performed at 37 °C for 1 h. After completion, the reaction mixture was quenched with 3 mL buffer (H_2O: acetonitrile, 5:95 +0.1% TFA), filtered by a 0.22 μm pore size membrane filter, and purified via HPLC as describe before. Product-containing fractions were lyophilized. 50 μl 0.01 mg/ml of full nisin variants (M17IM21V-I1M, M17I, M21V, M17IM21V-35M) or 0.04 mg/ml of truncated variants (nisin(1-22)M17I, and nisin(1-22)M21V) were used for the agar well diffusion test. A representative image from three independent experiments was presented.

Data availability

All data supporting the findings of this study are available within the paper and its supporting information files. Additional raw data are available from the corresponding author upon reasonable request.

Supporting information

This article contains supporting information.

Acknowledgments

LG and CW were financially supported by the China Scholarship Council (CSC).

Author contributions

OPK and JB conceived the project. CW performed some experimental work on isolating peptides and activity tests. LG carried out the experiments, analyzed data, and wrote the initial manuscript. OPK and JB corrected the manuscript. All authors checked the final version of the manuscript and agreed on it.

Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

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Figure legends

Figure 1 Schematic overview of the force-feeding method for non-canonical amino acid incorporation and the nisin biosynthetic pathway. The expression medium is supplemented with 19 canonical amino acids and a Met analog. Amino acids (AA) or analog are activated by aminoacyl-tRNA synthetases (aaRS) and then transferred of the activated AA or analog to the tRNA to form the aminoacyl-tRNA (aa-tRNA). Elongation factor Tu (EF-Tu) guides the aa-tRNA to the ribosomal aminoacyl-site (A-site). Subsequently, the nascent polypeptide chain (NPC) is transferred from the P-site (peptidyl-site) to aa-tRNA which results in one amino acid extension of the NPC. This cycle of elongation is repeated until the ribosome reaches the stop codon of the mRNA and then triggers the release of the peptide. Precursor nisin (prenisin) is a ribosomally synthesized peptide with a leader part and a core peptide part that is targeted by the nisin modification machinery NisBTC. NisB dehydrates serines and threonines forming dehydroresidues. These dehydroresidues can be coupled to cysteines thus forming (methyl)lanthionine rings catalyzed by NisC. Subsequently, the transporter NisT exports the modified prenisin outside the cells, where the protease NisP removes the leader part, releasing mature nisin. "XXX" indicates the codon or anticodon on the mRNA/tRNA.

Figure 2 (**A**) Structure of nisin A. Dha, dehydroalanine; Dhb, dehydrobutyrine; Ala-S-A, lanthionine; Abu-S-A, methyllanthionine; The functional domains, including lipid II binding site, pore formation domain, and hinge region are indicated. (**B**) Scheme of Aha-labeled nisin attached with lipid C11 catalyzed by copper (Cu^{+})-catalyzed azide-alkyne click chemistry reaction. (**C**) Structure of various nisin constructs used in this study and each labeled with lipid tail produced using click chemistry. In blue, Met replaced by Ile or Val; In red, position of Aha incorporation and subsequent modification by click chemistry.

Figure 3 Two systems for the incorporation of Met analogs into nisin. (**A**) pCZ-nisin system, consisting of the plasmids pIL3eBTC and pCZ-nisA; pNZ-nisin system, consisting of the plasmids pTLReBTC and pNZ-nisA; A major difference between the two systems is the promoters to induce the peptide modification machinery NisBTC and the peptide nisin. the SczA, encoding the repressor of P_{czcD}; P_{czcD}, a zinc inducible promoter; *nisA*, encoding NisA; P_{nisA}, a nisin inducible promoter; *nisB*, *nisT*, and *nisC*, encoding nisin modification machinery NisBTC; *repA* and *repC*, encoding plasmid replication proteins; Cm^{R}, chloramphenicol resistance gene; Em^{R}, erythromycin resistance gene. (**B**)

Tricine-SDS-PAGE analysis of prenisin labeled with Aha using pCZ-nisin and pNZ-nisin expression systems. Each lane contains isolated peptide corresponding to 0.5 mL culture. (**C**) The yield of fully modified nisin labeled with Aha using pCZ-nisin and pNZ-nisin expression systems. The peptide amount was quantified by HPLC according to Schmitt *et al* [43]. (**D**) MALDI-TOF analysis of nisin labeled with Aha using the two systems. The Aha incorporation efficiencies are indicated. -8H_2O, 8 times dehydration (fully dehydrated for nisin). Met1, the first position Met residue in the leader part, usually, it has been cut off. Met in red, the prenisin with Met incorporation.

Figure 4 (**A**) Structures of Met and its analogs. Met, methionine; Aha, azidohomoalanine; Nle, norleucine; Eth, ethionine; Nva, norvaline; Alg, allyglycine; Pra, propargylglycine. The side chain difference is indicated in blue/red. (**B**) Tricine-SDS-PAGE analysis of prenisin in the presence of Met or analogs using pCZ-nisin and pNZ-nisin expression systems. Each lane contains isolated peptide corresponding to 0.5 mL culture. (**C**) The yield of fully modified nisin labeled with Met or analogs using pNZ-nisin expression system. The peptide amount was quantified by HPLC according to Schmitt *et al* [43].

Figure 5 Tricine-SDS-PAGE analysis of the expression of nisin variants using either pCZ-nisin or pNZ-nisin system in the presence of Met/Met analogs. Each lane contains peptide isolated from 0.5 mL supernatant.

Figure 6 Antimicrobial activity of nisin and nisin variants labeled with Met/Met analogs against strain *L. lactis* MG1363. In red: activity that is improved in comparison to the nisin variants labeled with Met; In grey: lost activity under tested concentrations. Data are representative of three independent experiments.

Figure 7 Antimicrobial activity screening of nisin variants with or without lipid C11 attached against various microorganisms. A representative image from three independent experiments was presented. Red arrow: activity that are improved in comparison to nisin variants without lipid attached. C11: 1-undecyne. VRE: vancomycin resistant. MRSA: methicillin resistant.

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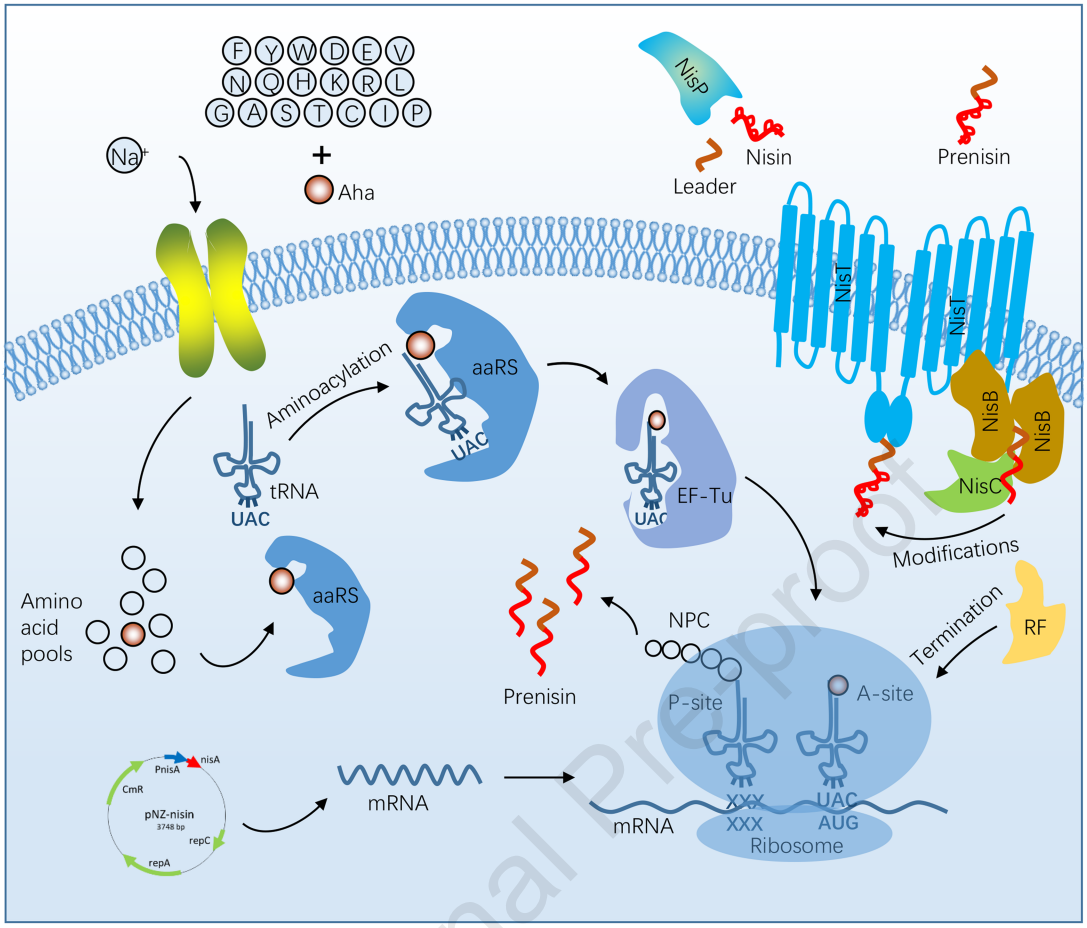
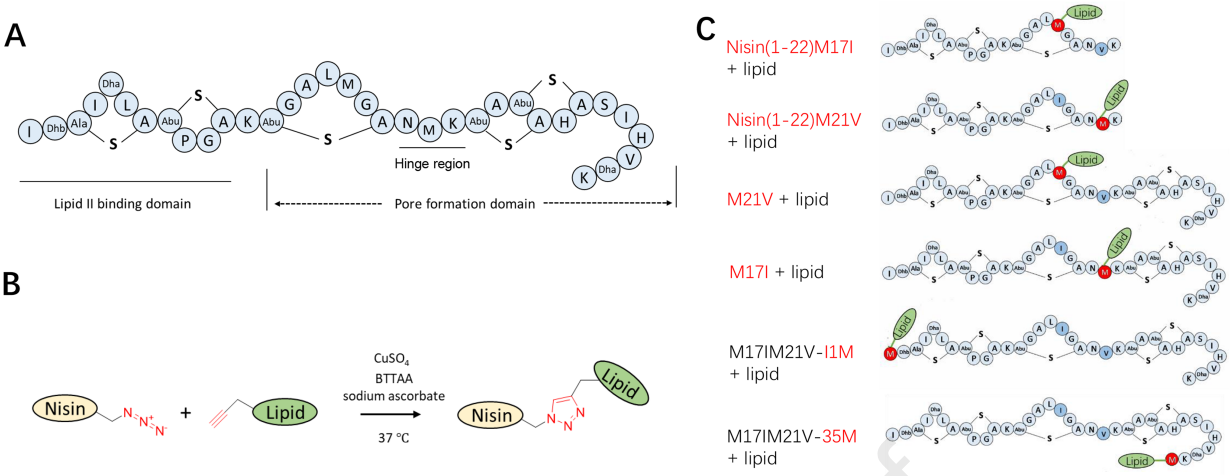
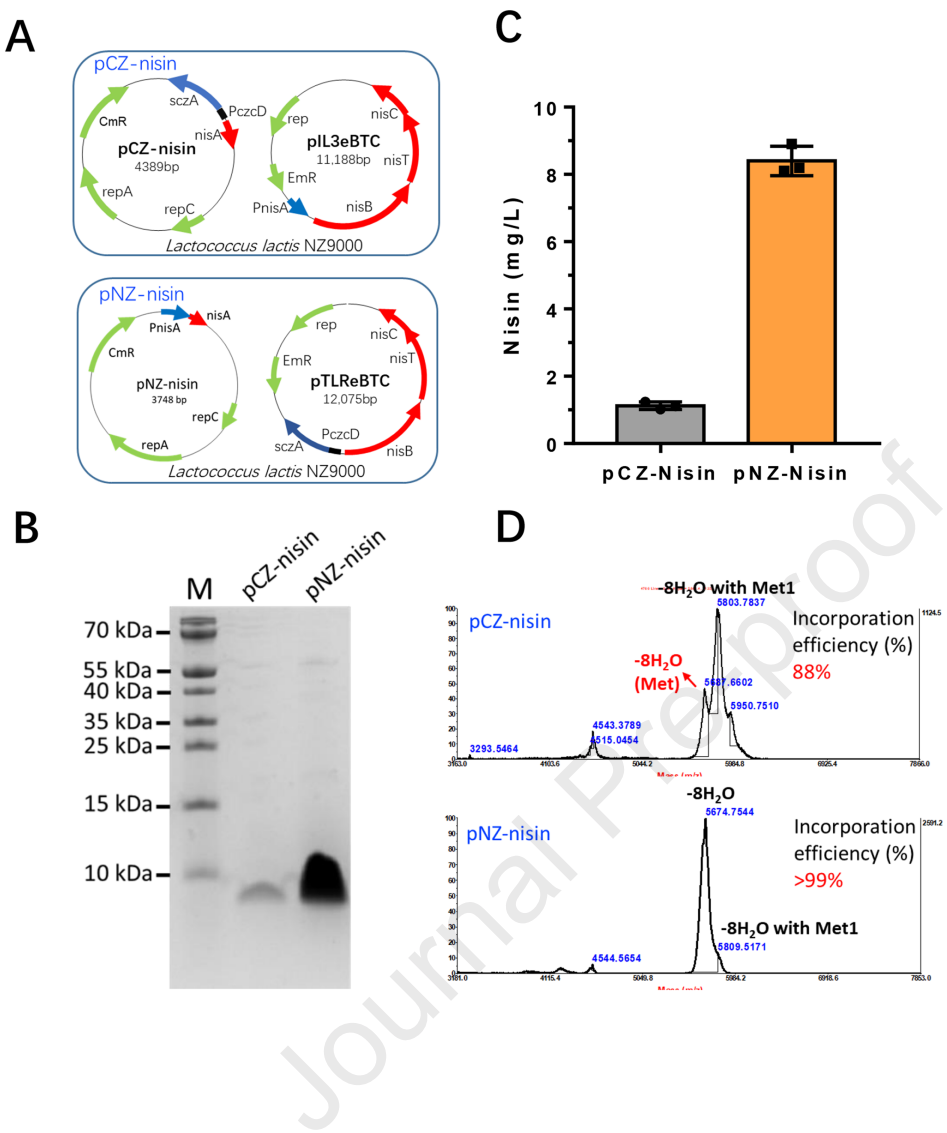
Table 1 Incorporation efficiency of Met analogs in nisin and its variants

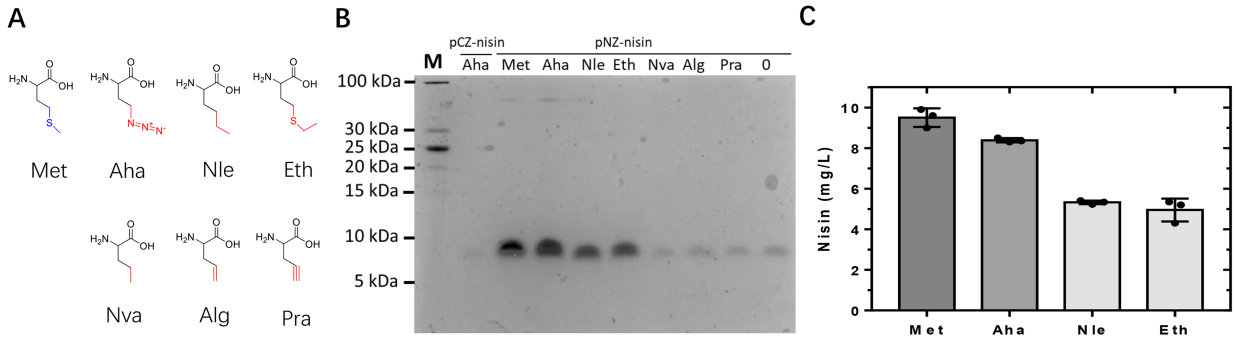
Peptide	Analog	Incorporation efficiency	
		pCZ-nisin	pNZ-nisin
Wild type	Aha	88%	>99.5%
	Nle	77%[a]	>99.5%
	Eth	56%[a]	>99.5%
M17IM21V-I1M	Aha	91%	>99.5%
	Nle	88%[a]	>99.5%
	Eth	71%[a]	>99.5%
M17I	Aha	97%	>99.5%
	Nle	88%[a]	>99.5%
	Eth	71%[a]	>99.5%
M21V	Aha	96%	>99.5%
	Nle	88%[a]	>99.5%
	Eth	73%[a]	>99.5%
M17IM21V-35M	Aha	>99.5%	>99.5%
	Nle	51%[a]	>99.5%
	Eth	71%[a]	>99.5%
nisin(1-21)M17I	Aha	*n.d*	>99.5%
	Nle	*n.d*	>99.5%
	Eth	*n.d*	>99.5%
nisin(1-21)M21V	Aha	*n.d*	>99.5%
	Nle	*n.d*	>99.5%
	Eth	*n.d*	>99.5%
nisin(1-22)M17I	Aha	*n.d*	>99.5%
	Nle	*n.d*	>99.5%
	Eth	*n.d*	>99.5%
nisin(1-22)M21V	Aha	*n.d*	>99.5%
	Nle	*n.d*	>99.5%
	Eth	*n.d*	>99.5%

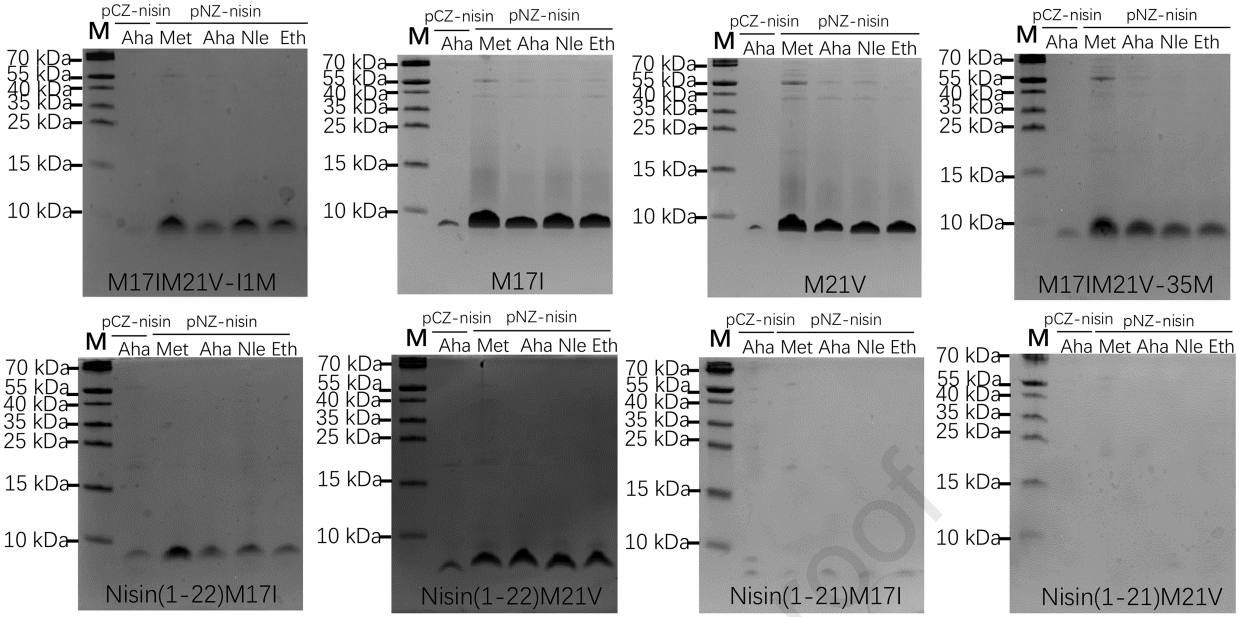
>99.5% means the mass of the peptides containing Met was undetectable. *n.d*, not detected. a, results from reference [14].











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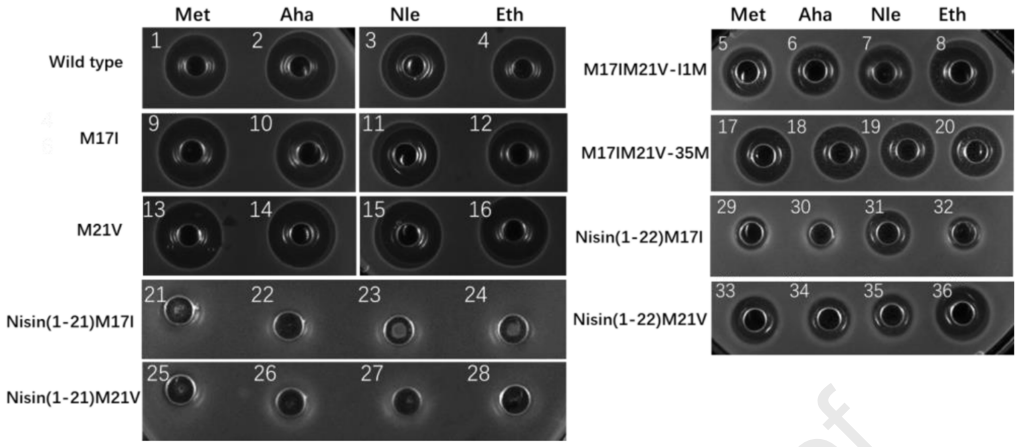
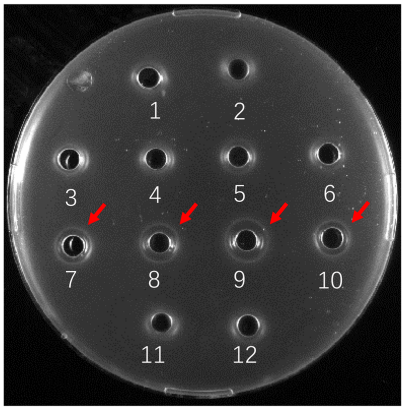


B

Peptide	Area (mm²)			
	Met	Aha	Nle	Eth
Wild type	295,95	383,28	312,63	279,66
M17IM21V-I1M	190,17	163,48	204,10	312,63
M17I	295,95	295,95	263,76	248,26
M21V	312,63	329,70	312,63	347,17
M17IM21V-35M	224,27	209,78	204,10	163,48
Nisin(1-21)M17I	0,00	0,00	0,00	0,00
Nisin(1-21)M21V	0,00	0,00	0,00	0,00
Nisin(1-22)M17I	62,80	28,26	103,62	53,58
Nisin(1-22)M21V	126,39	82,43	82,43	190,17

Lactococcus lactis



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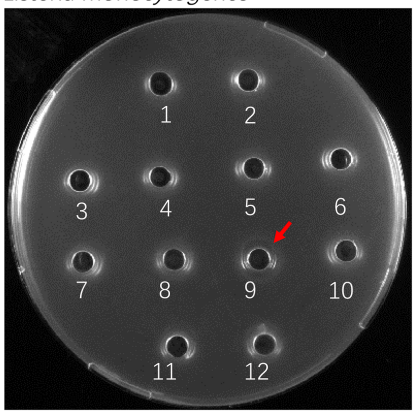

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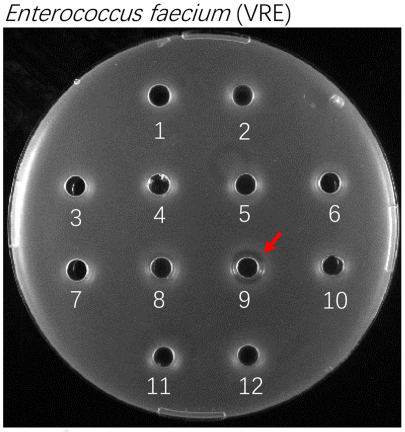
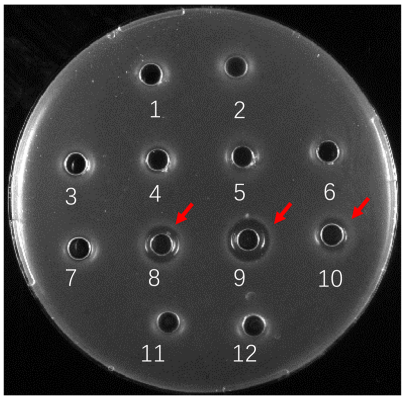
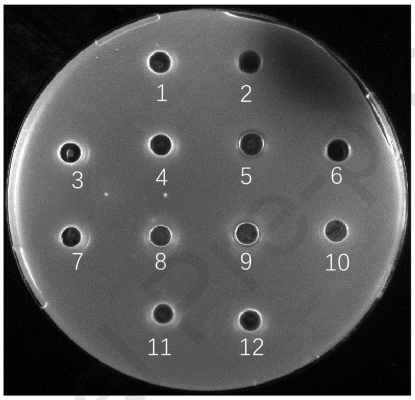


Enterococcus faecalis (VRE)



Staphylococcus aureus (MRSA)



1 Nisin(M17IM21V-I1M)
2 Nisin(M17I)
3 Nisin(M21V)
4 Nisin(M17IM21V-35M)
5 Nisin(1-22)M17I
6 Nisin(1-22)M21V

7 Nisin(M17IM21V-I1M)+C11
8 Nisin(M17I)+C11
9 Nisin(M21V)+C11
10 Nisin(M17IM21V-35M)+C11
11 Nisin(1-22)M17I+C11
12 Nisin(1-22)M21V+C11

CRediT author statement

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: