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Incorporation of non-proteinogenic amino acids in class I and II lantibiotics

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Abstract

Lantibiotics are ribosomally synthesized and post-translationally modified peptide natural products that contain thioether crosslinks formed by lanthionine and methyllanthionine residues. They exert potent antimicrobial activity against Gram-positive bacteria. We herein report production of analogues of two lantibiotics, lacticin 481 and nisin, that contain non-proteinogenic amino acids using two different strategies involving amber stop codon suppression technology. These methods complement recent alternative approaches to incorporate non-proteinogenic amino acids into lantibiotics.

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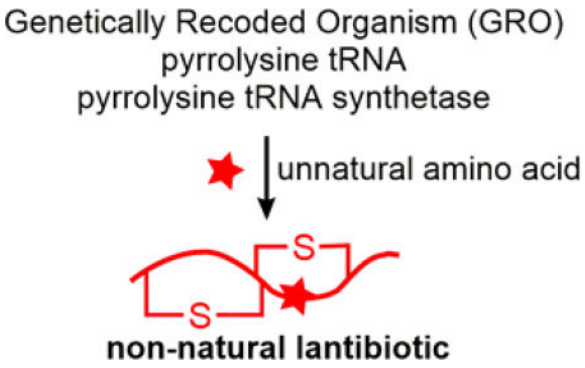

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ASSOCIATED CONTENT

Supporting Information. This information is available free of charge via the Internet at http://pubs.acs.org.

Description of all molecular biology procedures, protein purifications, and supporting figures.

Author Contributions

N.K. and W.A.V. designed the study, N.K. performed all experiments, J.P., W.R.L., and M.J. provided reagents and strains.

The extensive genome sequencing efforts of the past decade have provided unparalleled information regarding the potential capacity of organisms to produce highly diverse biologically active natural products.[1] One large class of such molecules are ribosomally synthesized and post-translationally modified peptides (RiPPs), including the largest group of RiPPs, the lanthipeptides.[2–5] These molecules are characterized by the presence of thioether crosslinks in the bisamino acids lanthionine and methyllanthionine that have given lanthipeptides their name. They are biosynthesized from a linear precursor peptide called LanA, which comprises an N-terminal segment called the leader peptide that is important for recognition by the post-translational modification enzymes, and a C-terminal segment called the core peptide that after processing results in the mature natural product. For class I lanthipeptides, a LanB enzyme first dehydrates select Ser and Thr residues to dehydroalanine (Dha) and dehydrobutyrine (Dhb), respectively, and then a LanC cyclase promotes Michael-type additions of Cys residues to the dehydro amino acids to form the thioether rings (e.g. Figure 1). In class II lanthipeptides, a bifunctional enzyme LanM performs both dehydration and cyclization reactions (e.g. Figure 2). Many lanthipeptides possess antimicrobial activity and are called lantibiotics. These cyclic peptides have garnered increasing attention with respect to engineering for three main reasons. First, they are ribosomally synthesized and hence variants of the precursor peptide can be made with relative ease using mutagenesis. Second, the biosynthetic enzymes have been shown to be inherently promiscuous facilitating analogue formation. And third, the post-translational modifications, by restricting the conformational flexibility, allow for higher chemical stability and tighter and/or more specific binding to the natural target, thereby improving the bioavailability and bioactivity.[1, 4, 6, 7]

Many efforts have been made to increase the diversity of lantibiotics by site-directed mutagenesis to afford variants with substitutions by proteinogenic amino acids.[8–19] More recently, several studies have also shown the potential of expanding the repertoire of substitutions to non-proteinogenic amino acids (or unnatural amino acids, UAAs) for a subset of lanthipeptides.[20–25] Prompted by these recent reports, we here present our investigations of incorporation of UAAs into the class I lanthipeptide nisin and the class II lanthipeptide lacticin 481. Our methods are complementary to previous attempts and illustrate approaches to ensure both incorporation of the UAAs and full post-translational modification.

RESULTS AND DISCUSSION

Lacticin 481 was first isolated in the early 1990s from *Lactococcus lactis* subsp. lactis CNRZ 481 and exhibits inhibitory activity against a range of indicator strains.[26] It is the prototypical member of a large group of natural variants with similar ring topology that includes nukacin ISK-1 and members of the salivaricin family.[27] These compounds exhibit diverse antimicrobial potency, but they all contain the mersacidin-like lipid II binding motif within their A-ring (Figure 2).[10, 28–30] During the biosynthesis of lacticin 481, the lanthipeptide synthetase LctM performs both dehydrations and cyclizations on its precursor peptide LctA (Figure 2).[31] The enzyme first phosphorylates the Ser/Thr targeted for dehydration, and then eliminates the phosphate to produce the dehydro amino acids.[32] A separate active site catalyzes the addition of Cys residues to the dehydro amino acids.[33, 34]

In a previous study, we showed that substituting aromatic residues in the C-ring with UAAs using substrates containing core peptides prepared by solid-phase peptide synthesis (SPPS) could improve the bioactivity of lacticin 481.[35] These findings were a good starting point for biological incorporation of UAAs at those positions. In this study, we aimed to investigate the factors required for successful use of stop codon suppression technology for incorporation on UAAs into lantibiotics.

In a recent report, we showed the genetic incorporation of hydroxy-amino acids into lantibiotics using the pyrrolysine tRNA synthetase and its cognate tRNA.[22] Using that platform, we herein demonstrate the production of several analogs of lacticin 481 and compare their bioactivites with the parent compound. Pyrrolysine tRNA synthetase (PylRS) and its mutants are robust and versatile enzymes that have been employed to charge pyl-tRNA with several lysine and phenylalanine derivatives.[36] We chose the N346A/C346A mutant of the *Methanosarcina mazei* PylRS and its cognate pyl-tRNA to incorporate *o*-Br-Phe, *o*-NO_2-Phe, *m*-Br-Phe, and *m*-CF_3-Phe at three positions (Trp19, Phe21 and Phe23) of lacticin 481.[37–39] pRSFDuet plasmids were prepared encoding N-terminally His-tagged LctA mutants containing the amber stop codon at each of these positions (W19/F21/F23), LctM, and pyltRNA. These plasmids were used together with the previously reported pEVOL plasmid carrying orthogonal translation machinery.[39] Since the mutant PylRS has low selectivity with respect to Phe and its derivatives, and therefore results in background incorporation of Phe in the growing peptide chain, we carried out the co-expressions in synthetic media[40] lacking Phe. Additionally, to improve the solubility of these analogs Asn15 in lacticin 481 was mutated to Arg.[35] Co-expression of His-tagged-LctA/N15R/TAG mutants, LctM, PylRS and pyltRNA was carried out in *E. coli* BL21(DE3) in synthetic media supplemented with the desired non-proteinogenic amino acid. After immobilized metal affinity chromatography (IMAC) purification of the peptide, analysis by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) showed the formation of full-length LctA with four dehydrations and containing the UAA.

The incorporation was complete and well-tolerated at all positions for all four Phe derivatives tested (for a representative example, see Figure 3, all other examples are shown in Figures S1–S11). Thus, the lacticin 481 production system in *E. coli* was not affected by the expression of the additional machinery required for UAA incorporation nor by the need to grow in synthetic media. The yields of the peptides were in the range of 0.5–0.8 mg/L without optimization, which is about a 10-fold decrease compared to previous production in *E. coli* without UAA incorporation (5 mg/L).[41] Hence, although the suppression appears highly efficient in Fig. 3 and Figs. S1–S11, we cannot rule out that the truncated peptide resulting from in-complete stop codon suppression may be degraded in *E. coli*.

Since the first residue of the core peptide is Lys, digestion with endoprotease LysN yielded the lacticin 481 analogs, which were tested for bioactivity against *L. lactis* HP. All mutants were active, with five of the mutants slightly more active than lacticin 481: Trp19*o*-ClPhe, Trp19*m*-BrPhe, Trp19*o*-NO_2Phe, Phe21*o*-NO_2Phe and Phe23*o*-ClPhe (Figure 4), similar to previous observations with aromatic substitutions at these positions prepared with SPPS.[30] Minimal inhibitory concentrations determined in liquid media were 195 nM for Trp19*o*-NO_2Phe, and 390 nM for Trp19*o*-ClPhe, Trp19*m*-BrPhe, Phe21*o*-NO_2Phe and Phe23*o*-

ClPhe and wt-lacticin 481 (Fig. S12). The apparent slightly improved activity on solid medium for the latter four analogs in Fig. 4 may be due to different diffusion rates in agar or minor differences in potency that are not picked up in serial two-fold dilution MIC experiments.

Next, we turned to the class I lantibiotic nisin, which is the best-characterized member of the family. It has been employed in the food industry as a food preservative for the last five decades and significant bacterial resistance is yet to develop.[42] Nisin is biosynthesized by two proteins, the dehydratase NisB and cyclase NisC (Figure 1). The mechanism of dehydration by NisB is fundamentally different from that of the class II dehydratases like LctM that use ATP to phosphorylate the Ser/Thr targeted for dehydration. Instead, NisB uses glutamyl-tRNA to glutamylate the Ser/Thr followed by glutamate elimination to introduce the carbon-carbon double bond.[43, 44] Nisin exerts its antimicrobial activity through a highly potent dual mode of action. The A- and B-rings recognize and sequester the pyrophosphate moiety of lipid II,[45] and the C-, D- and E-rings insert themselves into the membrane creating pores.[46–48] Despite the high antimicrobial potency and non-toxicity to humans, nisin is not employed as a human therapeutic, in part because of its low solubility in water, and degradation at acidic and alkaline pH.[49–53]

We previously reported the heterologous expression of nisin in *E. coli*, which opened up the possibility to investigate generation of nisin analogs using amber stop codon suppression. Taking into account the positions that improved the bioactivity of nisin in previous site-directed mutagenesis studies,[54] as well as the positions at which degradation has been reported,[50] we chose to place amber stop codons at positions encoding Thr2, Ser5 and Met21.

Since a previously reported nisin mutant (Ile4Val/Ser5Phe/Leu6Gly) is a known inhibitor of *Lactococcus lactis* that overcomes the naturally occurring self-protection mechanism,[12] we used this variant as the back-ground sequence for amber stop codon mutation at Ser5. Plasmids were constructed encoding the His-tagged NisA mutants and NisB (in pRSFDuet), NisC and pyltRNA (in pCDFDuet), and the pylRS mutant and another copy of pyltRNA (in pEVOL). These plasmids were used for coexpression in *E. coli* BL21(DE3) using synthetic media lacking Phe and supplemented with *m*-Br-Phe and *m*-CF_3-Phe. Unfortunately, we observed that the full-length peptide with the unnatural amino acid was only partially dehydrated by NisB, and that truncants of the peptide were observed in sizable amounts due to incomplete amber codon suppression. As a control we also expressed wild type (wt) NisA under these conditions and observed that it also was incompletely dehydrated by NisB (Figure S13), indicating that the incomplete dehydration is not the result of the UAA substitutions. We investigated whether the level of NisB expression was insufficient and switched the media to LB, which previously led to full dehydration,[20] but this resulted in substantial decline in stop codon suppression (Figure 5). Thus, unlike the observations for lacticin 481, where the requirements for stop codon suppression did not affect the lantibiotic processing machinery, for nisin the additional burden placed upon the cell re-quires additional optimization of the post-translational modification process. Similar findings were also recently reported in another study on incorporation of UAAs into nisin.[24]

To improve the activity of NisB in *E. coli* grown in synthetic media, we expressed multiple copies of NisB (up to three) which afforded a mixture of peptides with improved dehydration (Figure S14), but the issue of incomplete stop codon suppression persisted. While amber stop codons have been widely used for genetic incorporation of UAAs, the method in some cases leads to poor incorporation efficiencies and thus lower yield.[55–57] This inherent problem is thought to arise in part because of competition between Release Factor 1 (RF1) and the orthogonal tRNA charged with the UAA during peptide synthesis.[58, 59] In prokaryotes, RF1 is responsible for recognizing the amber stop codon UAG and terminating peptide synthesis.[58, 60] Thus, for an unrestrictive reassignment of the amber codon from a stop signal to an UAA incorporating process, deletion of the *prfA* gene encoding RF1 has been reported by generation of an *E. coli* strain named genomically recoded organism (GRO).[61] In GRO, 321 UAG codons were replaced by synonymous UAA codons, thereby eliminating the basal need for RF1, and the *prfA* gene could be deleted. The GRO provides significant advantages for incorporating UAAs into proteins by amber suppression.[62] To address the problem of truncation of NisA mutants in our studies, we decided to use an even more evolved GRO developed by Jewett and coworkers.[63] This *E. coli* strain (C321.ΔprfA-T7RNAPΔrneΔompTΔlon) has the T7 polymerase gene inserted and the genes for endonuclease (*rne*) and proteases (*ompT* and *lon*) deleted from the genome. These mutations are used commonly in recombinant protein expression to increase mRNA stability and reduce degradation of heterologous proteins, respectively.[64–67] Given the cytosolic expression of the lanthipeptides in this work, we suspected that the *rne* and *lon* mutations could provide benefits. Transformation of this strain with the nisin plasmids overcame the problem of NisA truncation. Moreover, we could switch to LB media and observe fully-modified full-length nisin analogues as the product for UAA incorporation at positions 2, 5, and 21 (Figure 6 and S15–S17), although the growth rate and cell yields were reduced, presumably because of the high antibiotic pressure during cell culture (kanamycin, chloramphenicol and spectinomycin, were required to maintain plasmids), the number of proteins being expressed in this GRO, and the lower tempera-ture at which the strain was employed (30 °C). The UAA-containing nisin variants were produced in the range of 0.2–0.5 mg/L prior to leader peptide removal. This corresponds to about a 50–100-fold decrease compared to production of fully modified NisA in *E. coli* without UAA incorporation.[20] Because of these low yields, we only tested the Ser5*m*-BrPhe mutant for bioactivity after trypsin digestion, and it was found to have lower activity than wt nisin (Figure 7). Very recently, Schultz and coworkers reported a different strategy to improve the dehydration of nisin mutants containing *para*-substitutedPhe and Lys derivatives.[24] In that study, additional copies of *E. coli* $tRNA^{Glu}$/GluRS were expressed, and *Methanocaldococcus jannaschii* $tRNA^{Tyr}$/TyrRS or *Methanosarcina barkeri* pyrrolysine $tRNA^{Pyl}$/PylRS were used for incorporation of the non-proteinogenic amino acid. Our strategy explored a different approach to address the problem of truncation and incomplete dehydration, offering alternative strategies for future studies.

CONCLUSION

In summary, we report two different approaches for the production of lantibiotic variants containing UAAs depending on the demands of the biosynthetic machinery. This study

provides a platform for further exploration of bioactivities and stabilities of various lantibiotics including nisin. This work also adds to the growing list of examples of incorporation of UAAs into RiPPs via stop codon repression methods.[20, 23, 24, 68–70]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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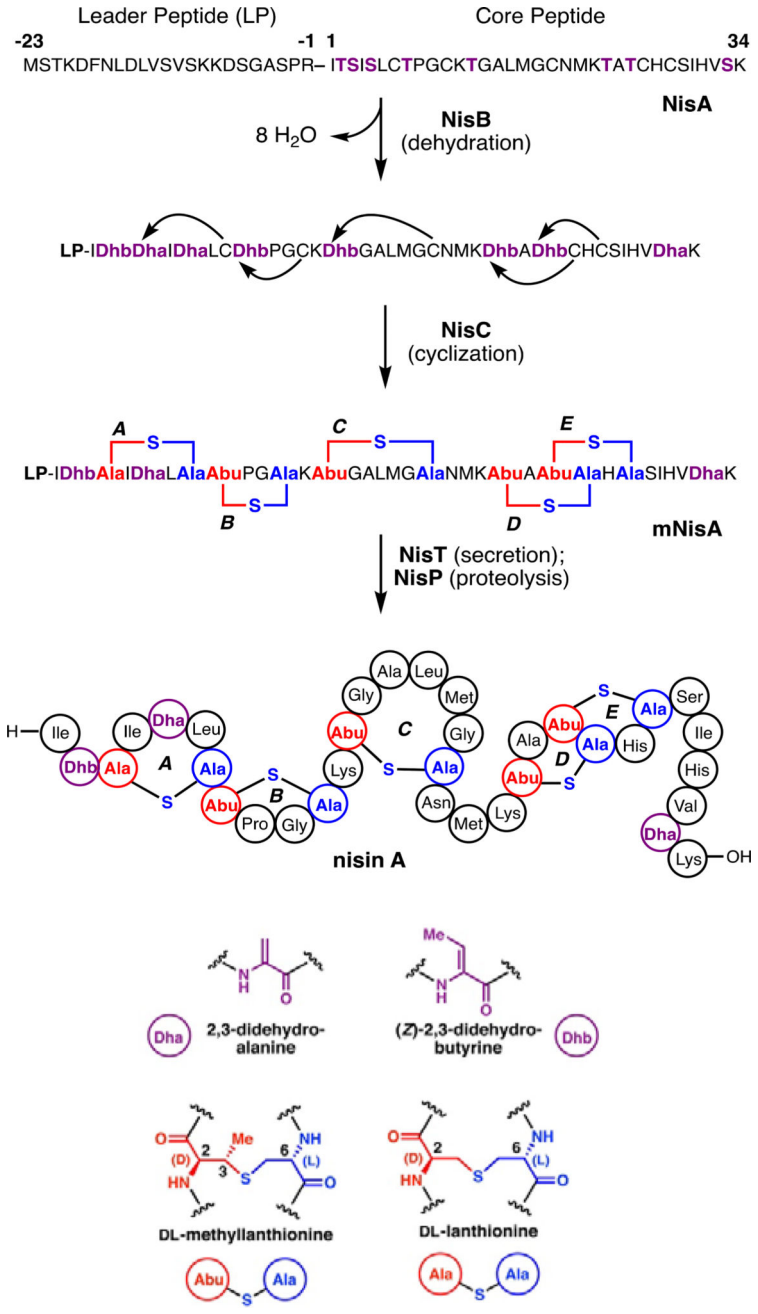

Figure 1.

Biosynthesis of the class I lantibiotic nisin A, and the post-translational modifications commonly associated with this class of antimicrobial peptides.[2]

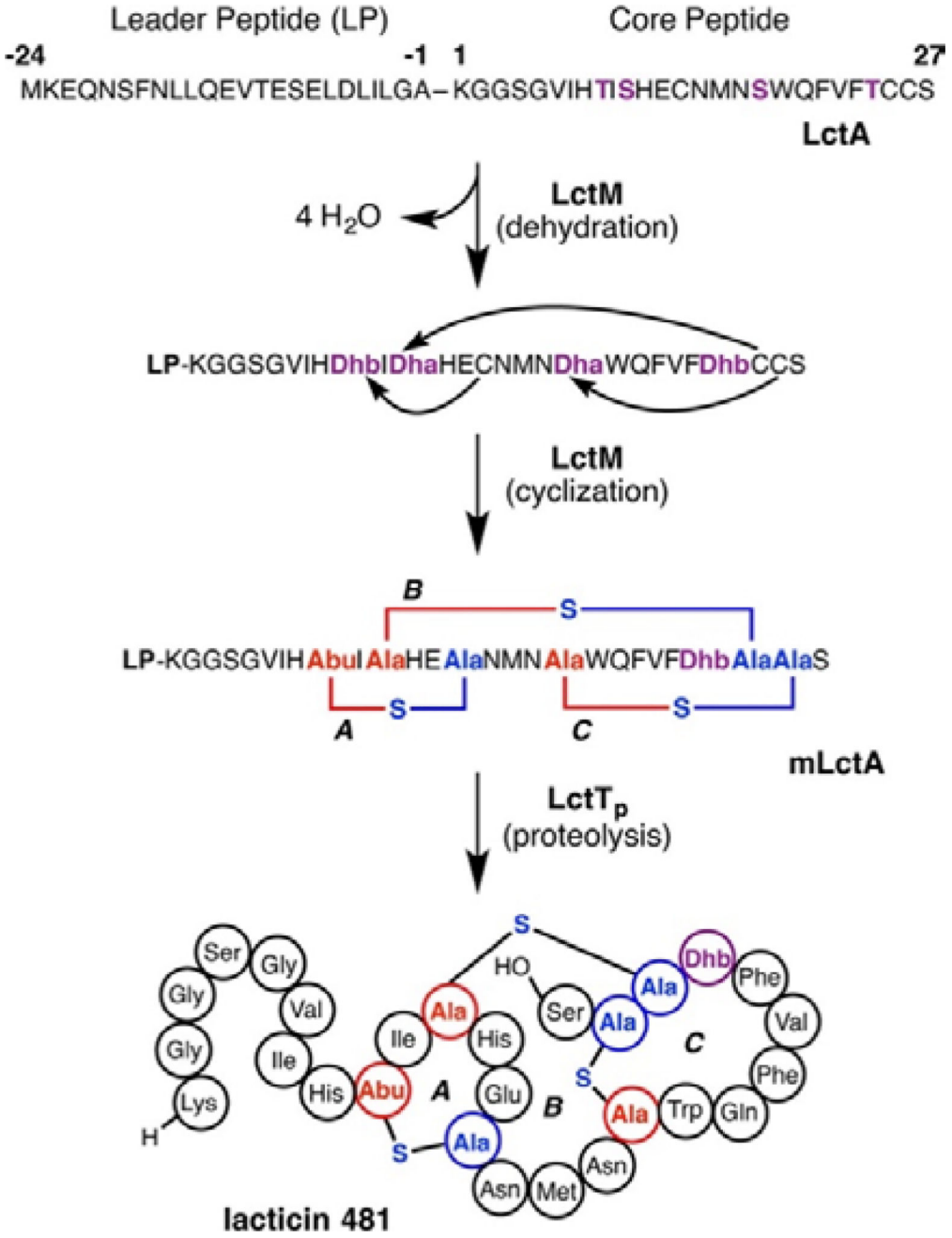

Figure 2.
Biosynthesis of the class II lantibiotic lacticin 481.[2]

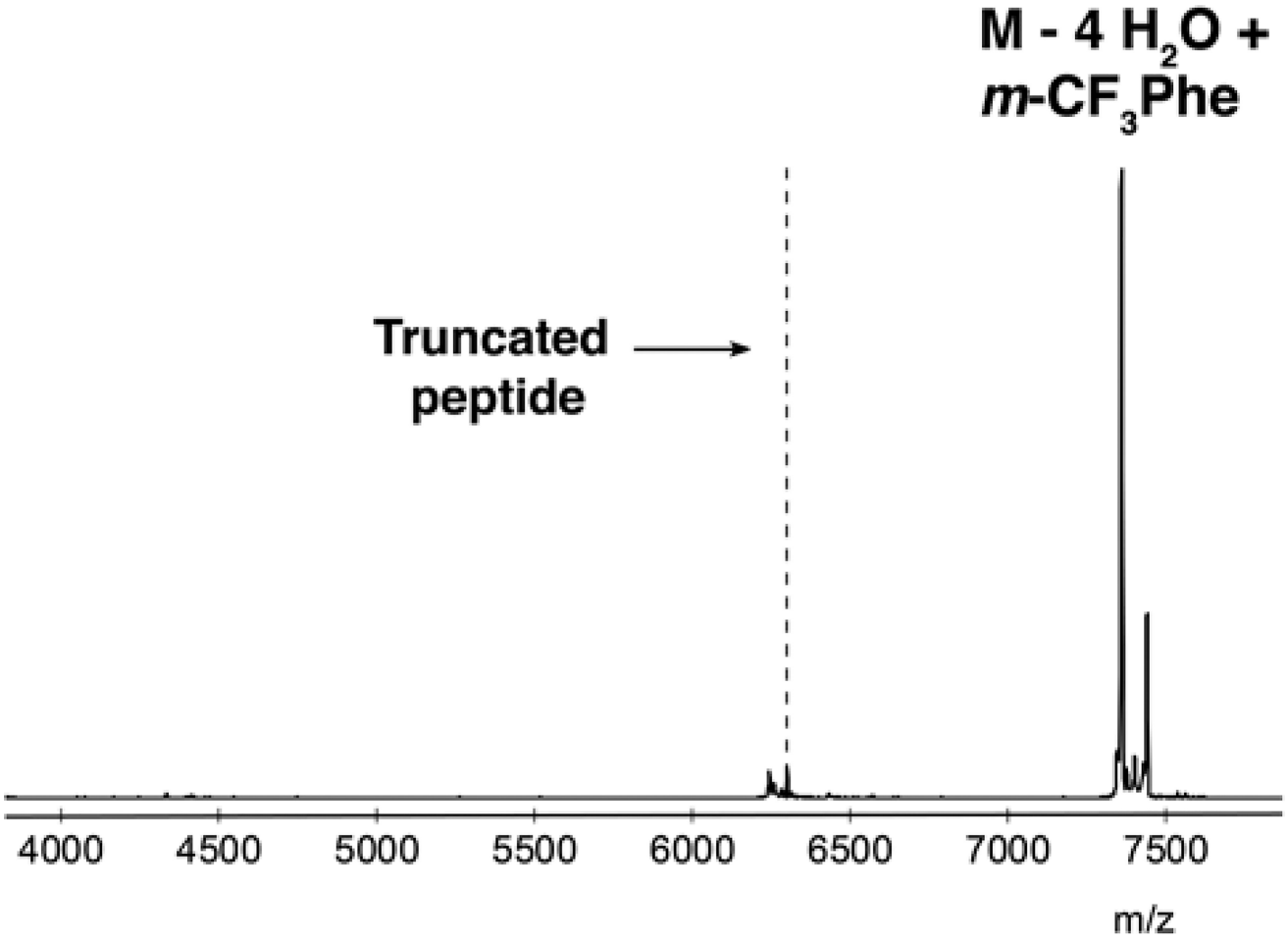

Figure 3.

MALDI-TOF mass spectrum of fully modified LctA-N15R/Trp19*m*-CF_3Phe produced by LctM in *E. coli* BL21(DE3). The dashed line shows the position of the expected peptide if stop codon suppression did not occur.

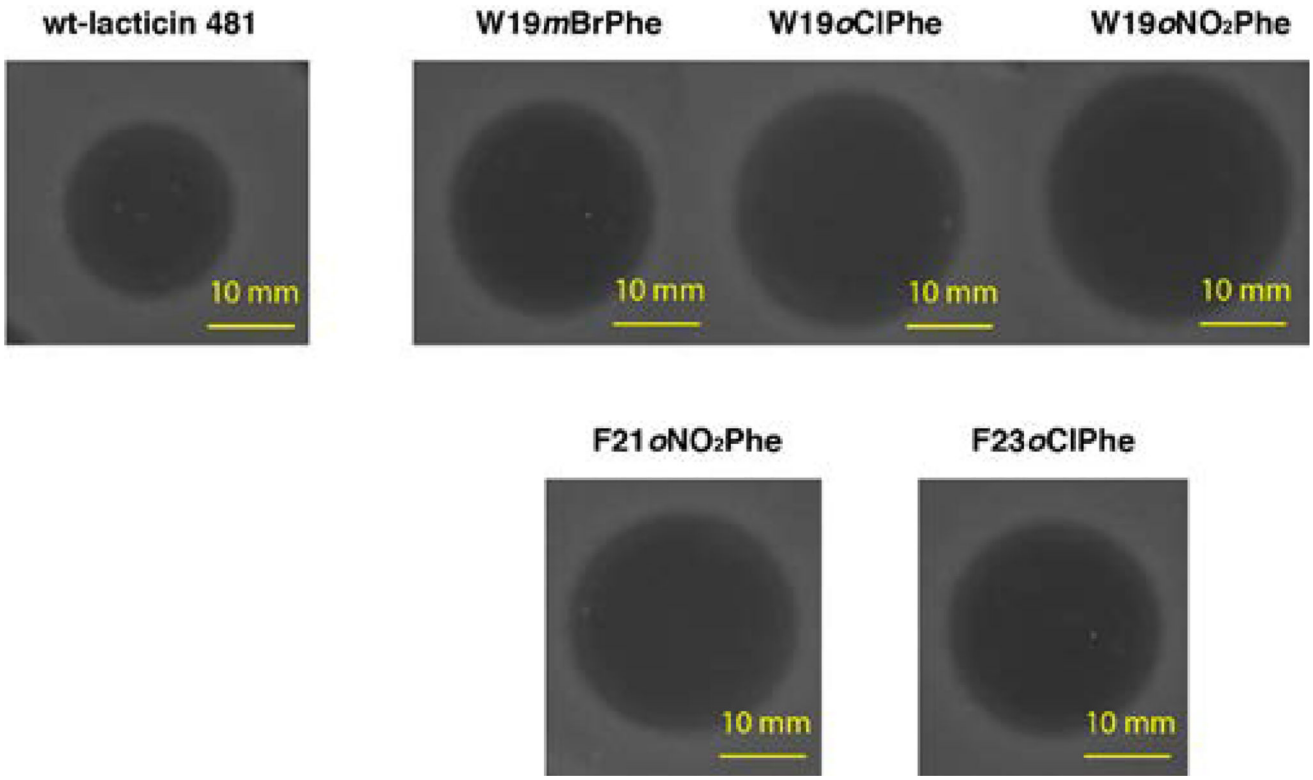

Figure 4.
Agar diffusion assay of wt-lacticin 481 and its mutants against *L. lactis* HP.

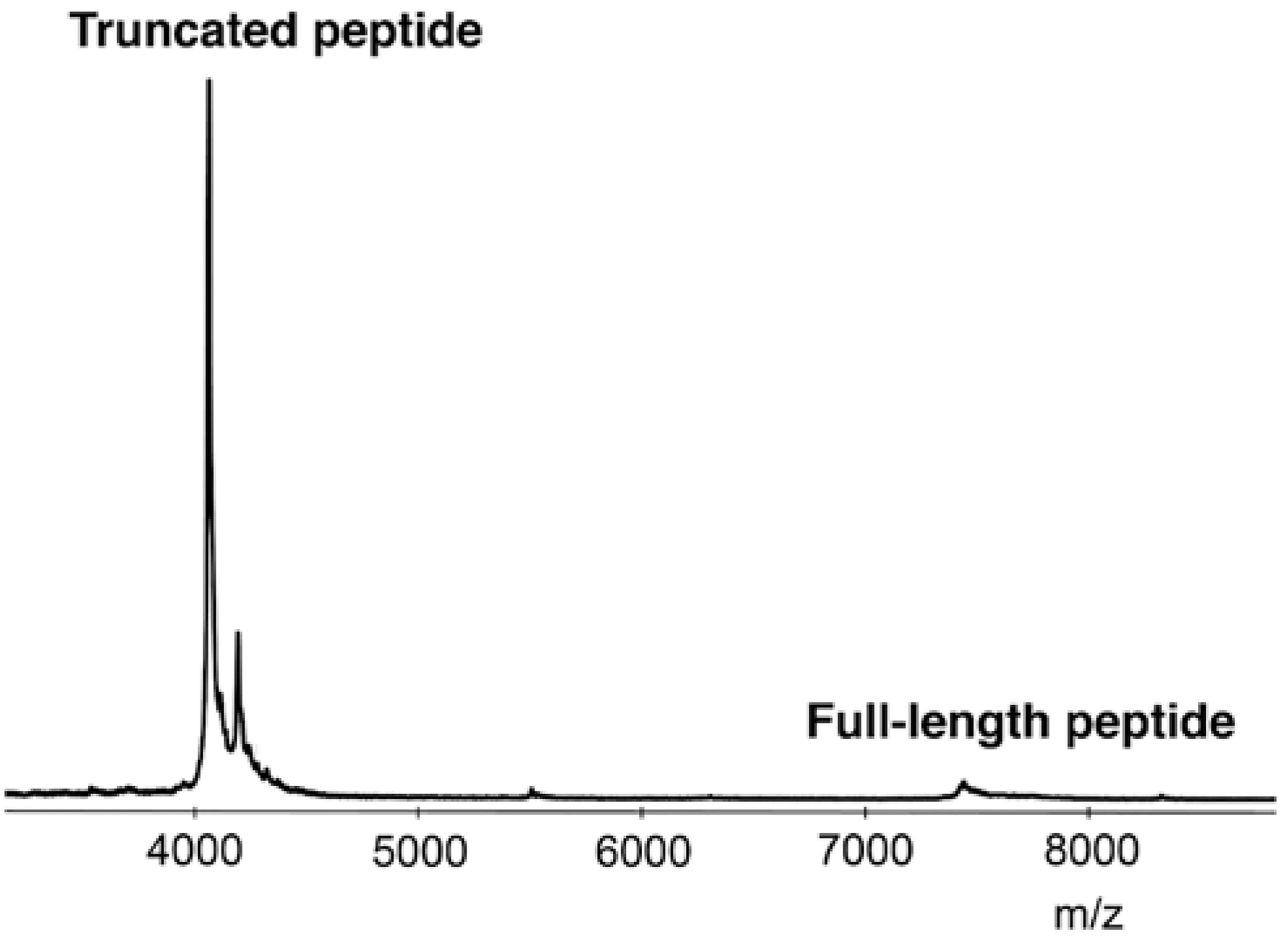

Figure 5.
MALDI-TOF mass spectrum of His_6-NisA-Thr2*m*-BrPhe co-expressed with NisB and NisC in *E. coli* BL21(DE3) grown in LB.

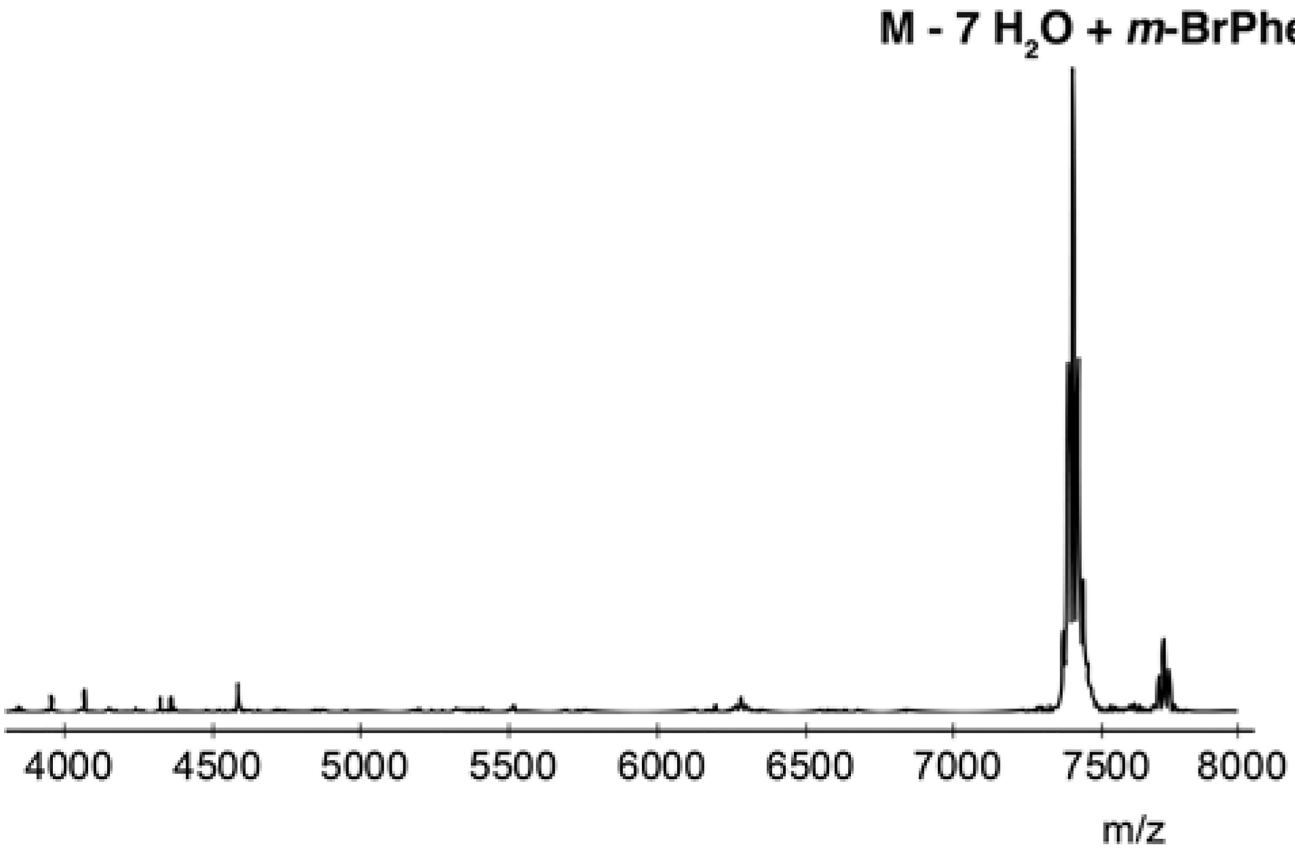

Figure 6.
MALDI-TOF mass spectrum of NisA-Thr2*m*-BrPhe modified by NisB and NisC in GRO expressing a single copy of *nisB* grown in LB medium.

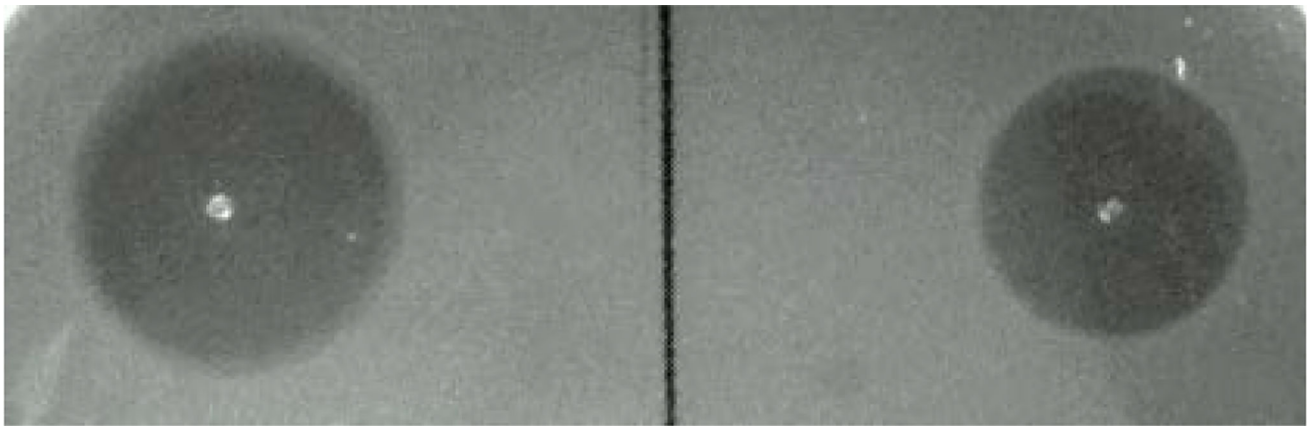

Figure 7.
Agar diffusion assay of wt nisin A (left) and nisin-I4V/Ser5*m*-BrPhe/L6G (right) against *L. lactis* HP.