microbial biotechnology

Microbial Biotechnology (2012) **5**(4), 501–508 doi:10.1111/j.1751-7915.2011.00324.x

Bioengineered nisin derivatives with enhanced activity in complex matrices

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Summary

Nisin A is the best known and most extensively characterized lantibiotic. As it is ribosomally synthesized, bioengineering-based strategies can be used to generate variants. We have previously demonstrated that bioengineering of the hinge region of nisin A can result in the generation of variants with enhanced anti-microbial activity against Gram-positive pathogens. Here we created a larger bank of hinge variant producers and screened for producers that exhibit enhanced bioactivity as assessed by agar-based assays against a selection of target strains. Further analysis of 12 'lead' variants reveals that in many cases enhanced bioactivity is not attributable to enhanced specific activity but is instead as a consequence of an enhanced ability to diffuse through complex polymers. In the case of two variants, which contain the residues SVA and NAK, respectively, within the hinge region, we demonstrate that this enhanced trait enables the peptides to dramatically outperform nisin A with respect to controlling *Listeria monocytogenes* in commercially produced chocolate milk that contains carrageenan as a stabilizer.

Introduction

Nisin A is a 3.4 kDa anti-microbial peptide produced by many strains of *Lactococcus lactis* (Lubelski *et al*., 2008; de Juarez *et al*., 2009). As a lantibiotic, nisin undergoes significant post-translational modification crucial to the development of its unique structure and potent antibacterial activity (Cotter *et al*., 2005a; Lubelski *et al*., 2008). There are at least five naturally occurring variants of nisin A. These are nisin Z (Mulders *et al*., 1991), Q (Zendo *et al*., 2003), F (de Kwaadsteniet *et al*., 2008), U and U2 (Wirawan *et al*., 2006), although the description of nisin U and U2 as variants is the subject of some debate (Piper *et al*., 2010).

As a result of their gene encoded nature, lantibiotics, including nisin A and nisin Z, have been the focus of bioengineering with a view to elucidating structure function relationships (Cotter *et al*., 2005b; Lubelski *et al*., 2008; Cortés *et al*., 2009; Field *et al*., 2010b). In recent years, there has been further progress, resulting in the identification of changes that significantly improve specific activity against target cells (Cortés *et al*., 2009; Field *et al*., 2010b). While some enhancements have related to rings A and B of the peptide (Fig. 1; Rink *et al*., 2007), the majority of enhanced peptides have resulted as a consequence of manipulation of the hinge region. The hinge comprises residues 20 (Asn), 21 (Met) and 22 (Lys) (Fig. 1), which are thought to permit the movement of the N- and C-termini relative to one another during pore formation. The first success in this regard related to the creation of nisin derivatives, N20K and M21K, with enhanced anti-microbial activity against Gram-negatives (Yuan *et al*., 2004). Subsequent investigations have further highlighted the benefits of manipulating the hinge and finally resulted in the identification of nisin derivatives, such as nisin N20P, M21V, K22T and K22S, which possess enhanced specific activity against Gram-positive pathogens (Field *et al*., 2008). Indeed, the enhanced specific activity of nisin M21V (or nisin V) against a wide range of food and clinical Gram-positive pathogens has been highlighted (Field *et al*., 2010a).

It should also be noted that, while there has been a particular focus on the identification of nisins with enhanced specific activity in recent years, bioengineering has the potential to also generate peptides that are enhanced in other ways, such as possessing increased stability or solubility (Rollema *et al*., 1995). Here, we carry out a more comprehensive bioengineering of the nisin hinge, through the implementation of saturation mutagenesis at multiple locations, with a view to identifying additional derivatives with enhanced features.

Results

Further mutagenesis of the nisin A hinge

Previously, NNK scanning of the individual residues within the hinge region of nisin A (which naturally consists of the

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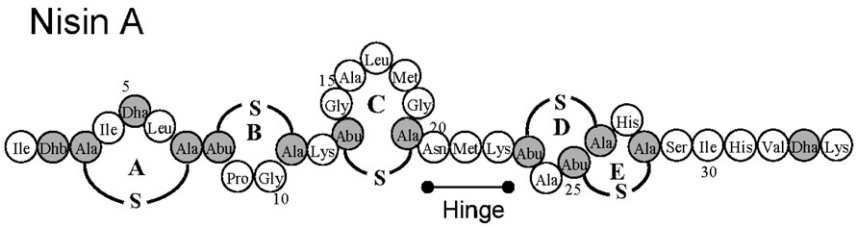

Fig. 1. Structure of nisin A. Modified residues are in grey. Ala-S-Ala, Lanthionine; Abu-S-Ala, β-methyllanthionine; Dha, dehydroalanine; Dhb, dehydrobutyrine. (β-methyl)lanthionine rings are labelled A–E. The location of the hinge region, consisting of Asn-Met-Lys, is also indicated.

residues Asn-Met-Lys) resulted in the identification of amino acid substitutions that bestowed improved specific activity on the resultant peptides compared with the wild-type equivalent. The most notable of the bioengineered peptides created were those in which respective N20P, M21V and K22T changes had been made (Field *et al.*, 2008). In a bid to further explore the flexibility of the hinge region, we initiated a second round of bioengineering of these enhanced derivatives. To this end, the previously created 'enhanced' derivatives were used as the starting point for further saturation mutagenesis of the *nisA* gene, i.e. randomization of two of the residues within the hinge region while retaining the existing, beneficial alteration. Thus, three separate banks of nisin producing variants were made, which were designated the N20PXX, XM21VX and XXK22T banks (where X is any amino acid). The PCR-based mutagenesis approach adopted was carried out successfully in all cases and the modified nisin genes were introduced into *L. lactis* NZ9800, a strain capable of producing the corresponding modified peptides.

Identification of enhanced variants by deferred antagonism assay

The newly created banks of nisin hinge variant producers, as well as 49 variant producers created previously (Field *et al.*, 2008), were screened to identify those with enhanced anti-microbial activity relative to *L. lactis* NZ9700. After screening, 12 'lead' nisin variants, other than N20P, M21V and K22T, were selected on the basis of their activity. Activity was assessed by deferred antagonism anti-microbial activity assays against *Streptococcus mitis* UCC5001, *Streptococcus agalactiae* ATCC 13813, *L. lactis* HP, *L. lactis* UCC90 and *Listeria monocytogenes* UCC35. DNA sequencing, followed by confirmation by mass spectrometric confirmation, revealed that the variants contained the following three amino acid residues within their hinge region: NMA, NMV, NMG, NAK, NGK, SVA, GVK, PTL, PAL, HLT, PMC and PMQ (Table 1) and the results from the deferred antagonism assays are presented in Table 2. These reveal that three variant producers (NMA, SVA and NAK) exhibited enhanced bioactivity against all five indicators, one variant producers (NMV) exhibited enhanced bioactivity against four of the indicators, five (NGK, NMG, PMQ, PMC and GVK) exhibited enhanced bioactivity against three of the indicators, two (PTL and PLT) exhibited enhanced bioactivity against two of the indicators and the activity of HLT was enhanced against *S. mitis* UCC5001 only (Table 2).

Minimum inhibitory concentration (MIC) determinations

Deferred antagonism agar diffusion assays are valuable with respect to rapidly assessing the bioactivity of a strain. However, bioactivity is the result of a combination of factors including the specific activity of the peptide, the levels at which it is produced and, in the case of agar-based assays, its ability to diffuse through agar. In order to determine the basis for the enhanced anti-microbial activity of the 12 nisin variant producers, the corresponding peptides were each purified to facilitate specific activity determination through broth-based MIC assays. The five indicator strains previously employed for agar-based deferred antagonism assays were again used for MIC assays. Surprisingly, and in contrast with the enhanced specific activity of a number of the other peptides previously identified following agar-based assays (Field *et al.*, 2010b), few of the 12 variants demonstrated enhanced specific activity in broth (Table 3). Indeed, only the NMA, NMV and SVA peptides exhibited enhanced specific activity against any of the targets. NMA exhibited enhanced specific activity against *S. agalactiae* ATCC13813 and *L. lactis* UCC90, NMV was enhanced against *L. lactis* UCC90 and SVA was enhanced against both *L. lactis*

Table 1. Sequence and molecular mass (as determined by mass spectrometry) of enhanced nisin variants.

Nisin variant	MW (Da)
NMA (K22A)	3295.24
NMV (K22V)	3324.23
NMG (K22G)	3280.88
NAK (M21A)	3292.50
NGK (M21G)	3278.70
GVK	3262.89
SVA	3235.84
PTL	3289.92
PAL	3259.90
PMC	3309.99
PMQ	3335.58
HLT	3329.94

Table 2. Deferred antagonism agar diffusion based assessment of the bioactivity of the producers of nisin A and nisin variants.

Variant	*S. mitis* UCC5001 (mm)	*S. agalactiae* ATCC 13813 (mm)	*L. lactis* HP (mm)	*L. lactis* 90869 (mm)	*L. monocytogenes* UCC35 (mm)
Nisin A	9 ± 0.32	14.1 ± 0.60	17 ± 0.27	7.5 ± 0.38	12.1 ± 0.57
NMA	**15 ± 0.80**	**18.5 ± 0.50**	**22 ± 0.70**	**13.1 ± 0.81**	**15.7. ± 0.49**
NMV	**17 ± 0.95**	**17.1 ± 0.26**	**20.5 ± 0.50**	6.5 ± 0.15	**14.7 ± 1.59**
NMG	**18.5 ± 0.46**	**18.5 ± 0.55**	**21 ± 0.83**	8.7 ± 0.75	11.9 ± 2.32
NGK	**18 ± 0.06**	**18.4 ± 0.88**	**18.5 ± 0.26**	6.5 ± 0.15	12.1 ± 1.53
NAK	**16.5 ± 0.57**	**18 ± 0.55**	**22.2 ± 1.04**	**11.1 ± 0.82**	**17.4 ± 0.75**
PAL	**16.7 ± 0.61**	12.1 ± 0.26	15 ± 1.30	**11.5 ± 0.65**	12.1 ± 0.93
PMQ	**16 ± 0.90**	13 ± 0.17	**20 ± 0.93**	**8.5 ± 0.25**	11.8 ± 0.87
PTL	**11 ± 0.26**	11 ± 0.55	**18 ± 0.17**	6.5 ± 0.06	7.6 ± 0.36
PMC	**18 ± 0.06**	15.4 ± 0.05	**20 ± 0.35**	**11 ± 0.15**	9.4 ± 1.63
HLT	**10.5 ± 0.11**	15.2 ± 0.60	18 ± 0.95	6.9 ± 0.15	11.3 ± 0.70
GVK	**12 ± 0.65**	**11 ± 0.25**	**21 ± 0.38**	9 ± 0.70	10.7 ± 0.60
SVA	**14.3 ± 0.40**	**17.1 ± 0.80**	**18 ± 0.38**	**13.1 ± 0.85**	**14.5 ± 0.40**

Values correspond to diameter of zone of inhibition and are the average of triplicate experiments. Zones significantly enhanced ($P < 0.05$) relative to those generated by the nisin A producer are in bold.

targets (Table 3). However, even in the case of these three peptides, the previously noted enhanced bioactivity against certain targets was not always matched by enhanced specific activity in broth against the same indicators. Of particular note was the fact that none of the peptides exhibited enhanced specific activity against *L. monocytogenes* UCC35.

Agar and carrageenan-based assays with purified peptide

Further investigations were carried out with a view to determining the basis for the enhanced bioactivity of the 12 variant producers. An alternative explanation for enhanced bioactivity, as determined by deferred antagonism agar diffusion studies, is that the peptides could possess an enhanced ability to diffuse through complex matrices such as agar. To test this hypothesis the activity of equimolar concentrations of purified nisin A and nisin variant peptides against *L. monocytogenes* UCC35 was instead assayed by means of an agar-based assay (Table 4). With the exception of PLT and PAL, all other variants produced zones of inhibition that were significantly larger than those produced by nisin A. It is thus apparent that some of the variant producers exhibit enhanced bioactivity as a consequence of enhanced activity in agar.

To investigate if the peptides that display an enhanced ability to diffuse through agar can also diffuse more successfully through other complex polymer matrices, carrageenan-based assays were carried out. Carrageenan is a polysaccharide extracted from seaweed, which is used in food as a gelling agent. When carrageenan was employed instead of agar in well assays, using *L. monocytogenes* UCC35 as an indicator, NAK, HLT and SVA produced significantly larger zones of inhibition than those generated by nisin A in this matrix (Table 4).

Table 3. Broth-based MICs of nisin A and nisin variants.

Variant	*S. mitis* UCC5001 mg l^{-1} (μM)	*S. agalactiae* ATCC13813 mg l^{-1} (μM)	*L. lactis* HP mg l^{-1} (μM)	*L. lactis* UCC90 mg l^{-1} (μM)	*L. monocytogenes* UCC35 mg l^{-1} (μM)
Nisin A	12.57 (3.75)	1.57 (0.467)	0.196 (0.058)	0.392 (0.117)	25.14 (7.5)
NMA	12.57 (3.75)	**0.785 (0.234)**	0.196 (0.058)	**0.196 (0.058)**	>25.14 (>7.5)
NMV	25.14 (7.5)	1.57 (0.467)	0.196 (0.058)	**0.196 (0.058)**	>25.14 (>7.5)
NMG	25.14 (7.5)	3.14 (0.935)	0.196 (0.058)	0.392 (0.117)	>25.14 (>7.5)
NGK	25.14 (7.5)	1.57 (0.467)	1.57 (0.467)	0.785 (0.234)	25.14 (7.5)
NAK	25.14 (7.5)	1.57 (0.467)	0.196 (0.058)	0.392 (0.117)	>25.14 (>7.5)
PAL	25.14 (7.5)	6.28 (1.87)	0.392 (0.117)	0.392 (0.117)	>25.14 (>7.5)
PMQ	12.57 (3.75)	6.28 (1.87)	0.196 (0.058)	0.392 (0.117)	>25.14 (>7.5)
PTL	25.14 (7.5)	6.28 (1.87)	0.392 (0.117)	1.57 (0.467)	>25.14 (>7.5)
PMC	12.57 (3.75)	6.28 (1.87)	0.196 (0.058)	0.785 (0.234)	>25.14 (>7.5)
HLT	12.57 (3.75)	1.57 (0.467)	0.196 (0.058)	0.785 (0.234)	25.14 (7.5)
GVK	25.14 (7.5)	3.14 (0.935)	0.392 (0.117)	0.785 (0.234)	>25.14 (>7.5)
SVA	12.57 (3.75)	1.57 (0.467)	**0.049 (0.014)**	**0.196 (0.058)**	>25.14 (>7.5)

Values enhanced relative to those generated by the nisin A producer are in bold. All results are the average of triplicate data (which in each case was identical).

Table 4. Agar and Carrageenan-based assessments of the activity of nisin A and variants against *L. monocytogenes* UCC 35.

Variant	Agar	Carrageenan
Nisin A	7.9 ± 0.11	8.2 ± 0.21
NMA	8.1 ± 0.17	8.1 ± 0.06
NMV	**9.3 ± 0.06**	9.2 ± 0.11
NMG	**8.9 ± 0.15**	8.5 ± 0.06
NGK	**8.7 ± 0.11**	8.6 ± 0.15
NAK	**9.2 ± 0.26**	**9.2 ± 0.21**
PAL	7.8 ± 0.06	7.7 ± 0.15
PMQ	**8.3 ± 0.15**	8.3 ± 0.10
PTL	7.6 ± 0.15	7.8 ± 0.10
PMC	**8.3 ± 0.15**	8.5 ± 0.21
HLT	**8.5 ± 0.10**	**8.9 ± 0.11**
GVK	**8.3 ± 0.15**	8.6 ± 0.06
SVA	**9.0 ± 0.15**	**9.0 ± 0.15**

Values correspond to diameter of zone of inhibition and are the average of triplicate experiments. Zones significantly enhanced ($P < 0.05$) relative to those generated by the nisin A producer are in bold.

Chocolate milk investigations

To determine if enhanced anti-microbial activity in carrageenan translated to enhanced activity in a carrageenan-containing food, the relative ability of SVA, NAK and nisin A (all at 20 μg ml^{-1}) to control *L. monocytogenes* UCC35 in a commercially available chocolate milk product, which already contains carrageenan as a stabilizer, was assessed. These variants were selected as these were the only two variants that were found to exhibit enhanced anti-microbial activity against UCC35 in all agar and carrageenan-based studies (Tables 2 and 4). In all cases it was noted that the presence of the nisin variants had a rapid impact on the pathogen, in that immediate plating to determine pathogen levels revealed decreases in numbers of UCC35 relative to the amount of the pathogen added.

When added to chocolate milk spiked with 10^5 cfu ml^{-1} *L. monocytogenes* UCC35 and stored at 22°C (i.e. representative of a temperature abuse scenario), the level of the pathogen present after 24 h was almost 3 log units lower in the SVA-containing milk than in the nisin A-containing milk. A more modest, but significant, difference was also apparent after 48 h (Fig. 2). The corresponding study with NAK established that this variant also significantly reduced pathogen levels at several time points (Fig. 2).

The benefits of incorporating the nisin variants were more apparent when the chocolate milk was stored at 4°C. In situations where the milk was spiked with 10^4 cfu ml^{-1} *L. monocytogenes* UCC35, the pathogen could not be detected on days 1–3 of the trial, whereas UCC35 numbers increased gradually in the nisin A-containing and non-nisin-containing controls. Although not as dramatic, Nisin NAK also provided significantly enhanced protection relative to the controls in these circumstances. Finally, the significantly enhanced protection

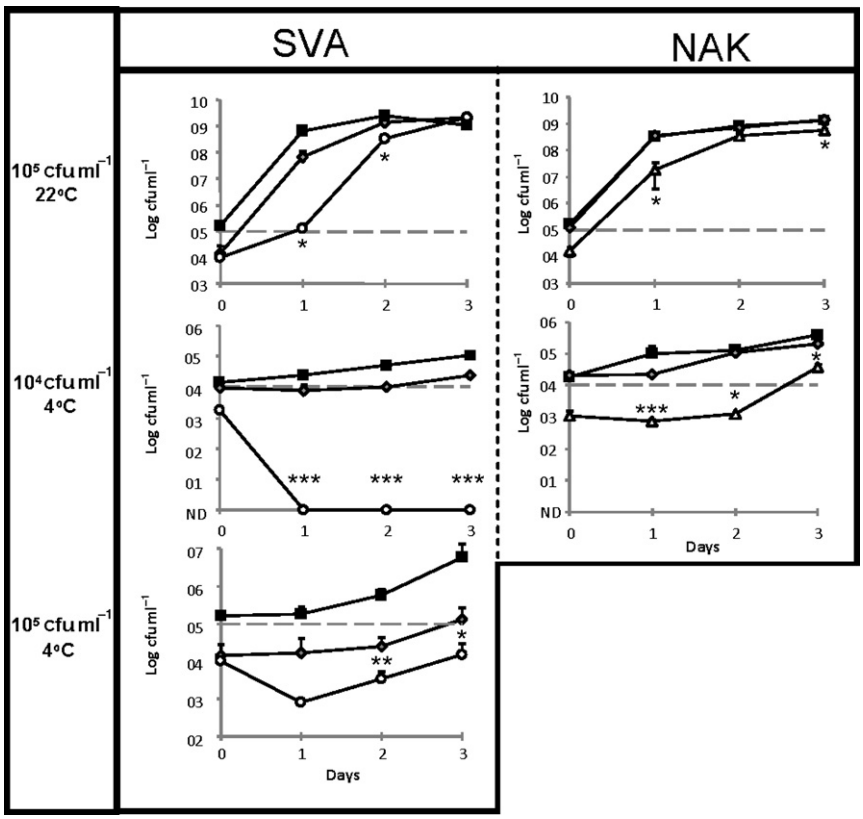

Fig. 2. Impact of nisin A (grey diamonds) and the nisin variants, SVA (white circle) and NAK (white triangle), relative to a non-nisin-containing control (black squares) on the growth/survival of *L. monocytogenes* UCC 35 (spiked at a level of 10^5 cfu ml^{-1} or 10^4 cfu ml^{-1}) in chocolate milk at 22°C and 4°C. Data points are the average of triplicate experiments. Asterix indicate significant differences (*$P < 0.05$, **$P < 0.005$, **$P < 0.001$; two-tailed Student *t*-test) in UCC 35 numbers between the variant and nisin A-treated samples (ND, not detected).

provided by SVA, relative to the nisin A-containing and non-nisin-containing controls at 4°C, was also apparent at multiple time points when the milk was spiked with *L. monocytogenes* UCC35 at 10^5 cfu ml^{-1} (Fig. 2). Notably, the enhanced ability of the variants to control UCC35 occurred despite the fact that these peptides did not display enhanced activity when assessed through broth-based MIC assays.

Discussion

It has become apparent in recent years that, as a consequence of the gene-encoded nature of nisin A, the creation of enhanced peptides is a very realistic goal. This study was initiated to build on previous investigations that highlighted the merits of bioengineering the 'hinge' region of nisin A (Field *et al*., 2008; 2010a) but differed in that the collection of nisin variant producers screened was expanded considerably to include strains producing peptides in which one or more of the hinge residues were randomized. Screening of this bank using the deferred antagonism agar-based assay revealed hundreds of producers that exhibited enhanced bioactivity against at least one target strain. Although only 12 strains were selected for further investigation on the basis of enhanced bioactivity against multiple strains, it is anticipated that this extended bank of variant producers will prove to be a valuable resource in the future with respect to the identification of variants with enhanced anti-microbial activity against specific targets or with other desirable traits. Broth-based investigations with purified peptide revealed that relatively few of the peptides possessed enhanced specific activity against the selection of strains employed. This prompted further assays, which revealed that enhanced bioactivity in agar was in many cases as a consequence of the enhanced ability of the associated peptide to act in complex matrices. In addition to the initial implementation of an agar-based screening, the selection of such peptides was probably as a consequence of the focus on identifying peptides with enhanced bioactivity against multiple targets. It should be noted, however, that the previous use of such an approach did result in the identification of peptides with enhanced specific activity against multiple targets (Field *et al*., 2008; 2010a; Carroll *et al*., 2010).

Agar is a favourable matrix for molecular diffusion owing to its negative charge and disorganized structure compared with other polymers like agarose (which has a dense structure and neutral charge) (Sebti *et al*., 2004; Carnet-Ripoche *et al*., 2006). To determine if the enhanced ability to diffuse through agar also translated to enhanced ability to diffuse through another polymer matrix, carrageenan was employed. In addition to being a polysaccharide similar to agar and which has the same negative charge, carrageenan-based assays were also deemed to be of value due to its frequent use in foods. It was established that the peptides exhibited enhanced ability in carrageenan-based assays. While this was evident from assays involving carrageenan coupled with laboratory media, more importantly, SVA and NAK also showed an enhanced ability to control *L. monocytogenes* in a carrageenan-containing food. The ability of these peptides to control the pathogen in chocolate milk is particularly notable as a chocolate milk product has previously been associated with outbreaks of listeriosis (Proctor *et al*., 1995; Dalton *et al*., 1997). Given that carrageenan is an ingredient of many foods, including processed meats, dairy products, low-fat products, desserts and UHT milk, the use of SVA and/or NAK as an alternative to nisin A in such foods should be considered in the future. For the same reason, further investigations to assess the protection provided by such variants in foods containing similar structural polymers such as pectin, gelatin, guar gum and other polysaccharides, will be carried out in the future.

Experimental procedures

Bacterial cultures and strains

The bacterial strains and plasmids used in this study are listed in Table 5. *Lactococcus lactis* NZ9800 harbouring the plasmid pCI372-*nisA* (pDF05), or a derivative thereof, were grown in M17 broth (Oxoid) supplemented with 0.5% glucose (GM17) and chloramphenicol (10 μg ml^{-1}). The nisin A pro-

Table 5. Strains used in this study.

Strains	Characteristic	Reference
Nisin producing strains		
NZ9700	Wildtype nisin A producer	Kuipers *et al*. (1993)
NZ9800pDF05	NZ9700Δ*nisA* pCI372-*nisA*	Field *et al*. (2008)
Indicators		
Streptococcus mitis UCC5001	Nisin sensitive indicator	UCC culture collection
Lactococcus lactis UCC90	Nisin sensitive indicator	UCC culture collection
Lactococcus lactis HP	Nisin sensitive indicator	UCC culture collection
Listeria monocytogenes UCC35	Nisin tolerant indicator	UCC culture collection
Streptococcus agalactiae ATCC13813	Nisin sensitive indicator	American Type Culture Collection

ducing strain, NZ9700, was grown in GM17 broth at 30°C. *Lactococcus lactis* indicator strains were grown at 30°C in GM17 broth or GM17 agar. *Listeria monocytogenes* strains were grown at 37°C in brain heart infusion broth and agar (Oxoid). Streptococci were grown at 37°C in tryptic soy broth and tryptic soy broth agar (Merck). Solid media were prepared by the addition of 1.5% agar to the appropriate broth, or 0.75% agar in the case of overlay media. Anaerobic conditions were established using Anaerocult A gas packs (Merck). Serial dilutions were prepared using sterile quarter strength Ringers solution (Merck). All cultures used in this study were stored as frozen stocks in 40% glycerol at −20°C or −80°C and were propagated twice in broth for 16 h before experimental use.

Isolation of enhanced nisin variants

Banks of producers of nisin hinge variants were generated by saturation and combinatorial saturation/site-directed mutagenesis as previously described (Field *et al*., 2008). Three banks of variants in which each of the three residues in the hinge were randomized, respectively, have been generated previously (Field *et al*., 2008). Simultaneous saturation mutagenesis of two of the three hinge residues was carried out using pDF05 as template and using oligonucleotides designed to introduce a specific codon change (i.e. N20P or M21V or K22T) in combination with an NNK codon (site-saturation; N = GATC and K = GT) at the two other locations (Table 6). PCR amplification was performed in a 50 μl reaction containing approximately 0.5 ng of target DNA (pDF05), 1 unit Phusion High-Fidelity DNA polymerase (Finnzymes, Finland), 1 mM dNTPs and 500 ng each of the appropriate forward and reverse oligonucleotide. The reaction was preheated at 98°C for 2 min, and then incubated for 29 cycles at 98°C for 30 s, 55°C for 15 s and 72°C for 3 min 30 s, and then finished by incubating at 72°C for 3 min 30 s. Amplified products were treated with DpnI (Stratagene) for 60 min at 37°C to digest template DNA and purified using the QIAquick PCR purification kit. Following transformation of *Escherichia coli* Top 10 cells, plasmid DNA was isolated and sequenced using the primers pCI372 For and pCI372 Rev to verify that mutagenesis had taken place. Engineered plasmid DNA was then introduced into *L. lactis* NZ9800 by electroporation (Holo and Nes, 1995) to generate banks of nisin variant producers.

Table 6. Oligonucleotides used in this study.

Primer name	Sequence
N20PXX For	5′ TG ATG GGT TGT **CCT NNK NNK** ACA GCA ACT TGT CAT TGT AGT 3′
N20PXX Rev	5′ CA AGT TGC TGT **MNN MNN AGG** ACA ACC CAT CAG AGC TCC TGT 3′
XM21VX For	5′ TG ATG GGT TGT **NNK GTT NNK** ACA GCA ACT TGT CAT TGT AGT 3′
XM21VX Rev	5′ CA AGT TGC TGT **MNN CAA MNN** ACA ACC CAT CAG AGC TCC TGT 3′
XXK22T For	5′ TG ATG GGT TGT **NNK NNK ACT** ACA GCA ACT TGT CAT TGT AGT 3′
XXK22T Rev	5′ CA AGT TGC TGT **AGT MNN MNN** ACA ACC CAT CAG AGC TCC TGT 3′
pCI372FOR	5′ CGG GAA GCT AGA GTA AGT AG 3′
pCI372REV	5′ ACC TCT CGG TTA TGA GTT AG 3′

Deferred antagonism activity assays

Banks of nisin derivative producers were screened to identify strains exhibiting enhanced bioactivity relative to NZ9700, the wild-type nisin A producer (which has a level of activity corresponding to that of NZ9800 pCI372 *nisA*), using the agar overlay assay. Strains from the banks were spotted (5 μl) on GM17 agar and incubated at 30°C for 16 h. All spots were irradiated under UV light for 15 min before being overlaid with soft agar seeded with 1% of the indicator culture and incubated at the appropriate temperature overnight. Zones of clearing in the indicator lawn were measured in diameter and the diameter of the bacterial spot subtracted from this measurement.

Identification of changes occurring in nisin variants

Nisin variant producers of interest were streaked onto GM17 agar before being subjected to colony mass spectrometric analysis as described previously (Field *et al*., 2008) with an Axima TOF^2 mass spectrometer (Shimadzu Biotech, Manchester, UK) and analysed in positive-ion reflectron mode. The DNA sequence of variant *nisA* genes was also determined. To facilitate this, plasmid DNA was isolated from overnight cultures of the nisin variant producers of interest using the QIAprep Spin Miniprep kit (Qiagen, Gmbh, Hilden, Germany), following an initial treatment of the lactococcal cells with lysis buffer (Anderson and McKay, 1983), The purified plasmid served as a template for the amplification of the variant *nisA* gene by PCR as described by Field *et al*., 2008. PCR products were purified using the QIAquik Purification Kit (Qiagen, Hilden, Germany) and DNA sequencing reactions were performed by MWG Biotech, Germany.

Purification of nisin hinge variants

The nisin A producing strain, *L. lactis* NZ9700, or the producers of hinge variants, were subcultured (1%) into 2 l tryptone yeast broth and incubated overnight at 30°C. The culture was then centrifuged at 7000 r.p.m. for 15 min and both the cell pellet and supernatant were retained. The culture supernatant was passed through 60 g beads (Amberlite XAD16; Sigma-Aldrich Chemie, Steinheim, Germany), prewashed with 1 l of distilled H_2O. The column was washed with 500 ml of 30% ethanol and the bacteriocin was eluted in 500 ml of 70% isopropanol 0.1% TFA. The cell pellet was resuspended in 300 ml of 70% isopropanol 0.1% TFA and stirred at room temperature for approximately 3 h. This was then centrifuged at 7000 r.p.m. for 15 min and the supernatant retained. The cell pellet and supernatant preparations were combined and underwent rotary evaporation using a Buchi, Postfach (Switzerland) before being applied to a 10 g (60 ml) Phenomenex SPE C-18 column pre-equilibrated with methanol (60 ml) and then water (60 ml). Six millilitres of the final preparation was concentrated to 2 ml by rotary evaporation and this was subsequently applied to a Phenomenex C12 reverse phase

(RP)-HPLC column (Jupiter 4 μ proteo 90 Å, 250 × 10.0 mm) previously equilibrated with 25% acetonitrile, 0.1% TFA. The column was developed in a gradient of 30–50% acetonitrile containing 0.1% TFA. Fractions containing the anti-microbial peptide were pooled and lyophilized following rotary evaporation of acetonitrile. For mass spectrometric analysis, purified peptide was resuspended in 70% isopropanol and the molecular mass was determined using an Axima TOF^2 mass spectrometer (Shimadzu Biotech, Manchester, UK).

Minimum inhibitory concentration determination

Minimum inhibitory concentration assays were completed in 96-well microtitre plates as described by Wiedemann *et al*. (Wiedemann *et al*., 2006) and performed in triplicate. Before peptide was added, the plates were treated with 1% BSA in 200 μl of PBS and incubated at 37°C for 30 min. The solution was then removed and the plates washed with 200 μl PBS before being air dried in a laminar flow hood. Serial twofold dilutions of the peptide were made in the appropriate medium, at a starting concentration of 7.5 μM. Indicator strains grown overnight were subcultured and grown to OD_{600} 0.5 before being diluted to give a final inoculum of 10^5 cfu ml^{-1} in 200 μl. The plates were incubated under the following conditions: 16 h at 30°C for *L. lactis* HP and *L. lactis* UCC90 and 16h at 37°C for *S. mitis* UCC5001, *S. agalactiae* ATCC13813 and *L. monocytogenes* UCC35. The MIC value was recorded as the lowest peptide concentration required for inhibition of visible growth.

Agar and carrageenan-based assays with purified peptide

To assess specific activity in agar and carrageenan-based matrices, the relevant matrix was added to GM17 broth at a concentration of 1.5% and, after autoclaving, was inoculated with 0.2% *L. monocytogenes* UCC35 before being poured. Wells were generated in the solidified media, into which the purified nisin A and nisin variants were added. The plates were incubated at 37°C for 16 h after which time the zone of inhibition was recorded. Results represent the average of triplicate experiments.

Chocolate milk studies

A commercially available chocolate milk product, which contains carrageenan, was streaked on *Listeria* Selective Agar (LSA) (Oxoid) to check for the presence of *Listeria*. An overnight culture of *L. monocytogenes* UCC35 was diluted and inoculated into the chocolate milk at a final concentration of 10^4 or 10^5 cfu ml^{-1}. To this, nisin or a nisin variant was added at a concentration of 20 μg ml^{-1}. Chocolate milk, to which only the pathogen was added or to which neither pathogen nor peptide was introduced, served as a control. The milk was incubated at 4°C or 22°C and assessed over 3 days (including one reading immediately after the introduction of the pathogen). Pathogen levels were determined through serial dilution and plating on LSA. Experiments were carried out in triplicate.

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