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Nisin M is a bioengineered Nisin A variant that retains full induction capacity but has significantly reduced antimicrobial activity.

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Keywords:

Nisin, signal transduction, induction capacity, antimicrobial activity, NICE system, protein expression

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Abstract

Nisin A is a potent antimicrobial with potential as an alternative to traditional antibiotics, and a number of genetically modified variants have been created that target clinically relevant pathogens. In addition to antimicrobial activity, nisin auto-regulates its own production via a signal transduction pathway, a property that has been exploited in a protein expression system termed the Nisin Controlled Gene Expression (NICE) system. Although NICE has become one of the most popular protein expression systems, one drawback is that the inducer peptide, nisin A, also has inhibitory activity. It has already been demonstrated that the N-terminal region of nisin A contributes to antimicrobial activity and signal transduction properties, therefore, we conducted bioengineering of nisin at positions Pro9 and Gly10 within ring B to produce a bank of variants that could potentially be used as alternative induction peptides. One variant, designated nisin M, has threonines at positions 9 and 10 and retains induction capacity comparable to the wild type nisin A, while most of the antimicrobial activity is abolished. Further analysis confirmed that nisin M produces a mix of peptides as a result of different degrees of dehydration of the two threonines. We show that nisin M exhibits potential as a more suitable alternative to nisin A for the expression of proteins that may be difficult to express, or to produce proteins in strains that are sensitive to wild type nisin. Moreover, it may address the increasing demand by industry for optimization of peptide fermentations to increase yields or their production rate.

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43 Importance

44 This study describes the generation of a nisin variant with superior characteristics for use in the
45 NICE protein expression system. The variant, termed nisin M, retains an induction capacity
46 comparable to the wild type nisin A but exhibits significantly reduced antimicrobial activity and
47 can therefore be used at concentrations that are normally toxic to the expression host.

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Introduction

Producing high quantities of proteins of biotechnological and pharmaceutical value from their natural sources can have economic challenges. Although *Escherichia coli* has been the dominant player in the production of recombinant proteins for decades, several issues including the presence of endotoxin or lipopolysaccharide requires expensive and often problematic downstream purification processes (1). The lactic acid bacteria (LAB) *Lactococcus lactis* has gained importance as a host for heterologous protein expression due to its well understood genetics and metabolism, generally regarded as safe (GRAS) status, as well as the availability of a wide range of genetic tools. Indeed, a major advance with regards to protein expression in *L. lactis* was the discovery and use of gene expression systems based on a number of inducible promoters. These include promoters that respond to the environment such as P170, which is upregulated at low pH (2) and zinc-based systems that respond to zinc availability (3). One of the best known and most widely employed expression systems is the nisin-inducible controlled gene expression (NICE) system (4, 5) which stems from the nisin biosynthetic operon (*nisABTCIPRKFEG)* found in some *L. lactis* strains (6). Nisin is a 34 amino acid peptide and is the most extensively studied bacteriocin (ribosomally synthesized, antimicrobial peptides produced by bacteria) (7, 8). It targets a wide range of Gram-positive bacteria, including food pathogens such as *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus* and clostridia (9, 10). Nisin induces its own biosynthesis via a two-component signal transduction pathway NisRK (6) and has led to the development and application of a food grade expression system using *L. lactis* as the host (11). The NICE system encompasses both regulatory elements of the nisin operon, P*nisA*, the nisin-inducible promoter (cloned into several expression vectors) and *nisRK,* the two component histidine kinase response regulator system (harboured by compatible

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plasmids or inserted on the chromosome of a suitable host strain). The system is 'switched on' by the addition of nisin in the nanomolar range which activates the receptor NisK. NisK activates NisR by phosphorylation and the activated NisR induces expression at the nisin A promoter (4). The NICE system has been extensively used to produce proteins in *L. lactis*, such as bacteriophage lysins and metalloendopeptidases to demonstrate their potential in dairy fermentations (12, 13). Moreover, NICE can, under certain conditions and with some modifications to the system components, also be used in other species of LAB and in other Gram-positive bacteria (11, 14). Its numerous advantages include ease of use, exquisitely controlled and efficiently induced expression and amenability to large-scale production processes. As an example, nisin induced fermentations of the antimicrobial lysostaphin have been carried out and even identified areas of the NICE system that need improvement (5). However, for industrial applications, nisin addition remains costly (15). Another drawback of the system is that the inducing peptide is also toxic due to its potent antimicrobial activity. Therefore, a nisin peptide that retains its induction capacity whilst having little to no antimicrobial activity would be highly desirable. The gene-encoded nature of the nisin peptide makes genetic engineering to develop certain characteristics of the molecule an attractive and feasible option. Although the bioengineering of nisin commenced over three decades ago, the majority of studies have largely focused on identifying nisin variants with enhanced antimicrobial activity or an extended-antimicrobial spectrum (10, 16, 17). The importance of the N-terminus rings A and B with respect to induction has been highlighted on a number of occasions (6, 16, 18). These studies involved either combinatorial saturation mutagenesis of rings A and B (18) or the application of alanine scanning approaches to assess the antimicrobial activity and induction properties of various nisin derivatives (16).

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In this study, we carried out a more comprehensive bioengineering approach and created banks of nisin derivatives that have been randomized at positions 9 (P9X) and 10 (G10X) individually and in combination (P9XG10X) and assessed them for antimicrobial activity in conjunction with their ability to induce the nisin promoter using GFP and β-galactosidase reporter systems.

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127 Results

128 Creation and screening nisin derivatives for antimicrobial activity and induction capacity.

129 Previous studies utilising site-directed and alanine scanning mutagenesis of nisin have revealed
130 that the N-terminal ring structures are an important region required to activate NisRK (16, 18). In
131 particular, mutagenesis of ring B has been shown to modulate antimicrobial and induction
132 activity. We selected this location as a suitable target for the generation of variants to screen for
133 our desired activities. In order to fully exploit the potential of the nisin ring B we undertook a
134 complete randomisation of the two amino acids in Pro9 and Gly10, both alone (P9X, G10X) and
135 in combination (P9XG10X) using NNK scanning of both codons in the nisin A structural gene
136 (*nisA*) as previously described (7). A bank consisting of 1,452 individual variants created in *L.*
137 *lactis* NZ9800 pCI372*nisA* (pDF05) were screened for antimicrobial activity using deferred
138 antagonism agar diffusion assays and their ability to induce the nisin promoter, P*nisA* fused to a
139 *gfp* reporter gene. The impact of mutations targeting position nine (proline) on antimicrobial
140 activity was assessed using an overlay assay and resulted in zones ranging from those
141 comparable to the wild type control to those devoid of any observable activity. Analysis of
142 colonies using mass spectrometry and/or DNA sequencing identified 12 different amino acid
143 substitutions corresponding to P9H, P9E, P9S, P9T, P9N, P9A, P9M, P9I, P9V P9L, P9W, and
144 P9F (Figure 1A). Substituting P9 with an alanine (P9A) had no impact on either antimicrobial
145 activity or induction capacity. A number of variants (P9H, P9E, P9W and P9F) displayed a loss
146 of both properties. Several others (P9M, P9L, P9N, P9V and P9I) displayed a significant
147 reduction in antimicrobial activity (between 50-65% of wild type) as well as a reduced ability to
148 induce the *gfp* reporter. Notably, P9T and P9S exhibited a slight reduction in antimicrobial

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activity (70-75%) but retained 100% and 75% induction capacity respectively compared to the wild type control (Figure 1A), which was in agreement with previous studies (16, 18). It is significant that replacement of P9 with either threonine or serine introduces hydroxylated residues which could act as substrates for the lanthionine modification machinery. Indeed, colony mass spectrometry (CMS) of the P9T and P9S producers revealed the presence of masses corresponding to the presence of both unmodified (threonine or serine) and modified residues (dehydrobutyrine (Dhb) or dehydroalanine (Dha)) (Table1).

Analysis of 144 variants where position 10 (glycine) was targeted, revealed that the majority of clones exhibited either wild type activity or displayed a complete loss of both antimicrobial activity and induction capacity. Mass Spectrometry (MS) and DNA sequencing determined that almost all of the active variants had retained the original glycine at position 10 (wild type), but we also detected variants corresponding to G10T and G10S (Figure 1B). Here too, CMS identified a mixture of both modified and non-modified residues in the case of G10S (i.e. G10S and G10Dha) but this was not observed when threonine was present at position 10. A variant that displayed little reduction in activity (>50%) had an alanine (G10A) substitution (Figure 1B). A selection of variants that lacked both antimicrobial activity and induction capacity were subjected to DNA sequencing analysis, which identified substitutions corresponding to G10F, G10W and G10L. The inability to detect as wide a range of active variants at this position may arise from the fact that several variants in this position (including G10D, G10N, G10H, G10R, G10L and G10P) have been linked with the loss of threonine dehydration at position 8, meaning that ring B does not undergo cyclization (18).

We then set out to vary both residues 9 and 10 simultaneously. As expected, screening of the doubly randomized P9XG10X bank revealed far fewer bioactive variants (approx. 5.6% of the

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total) of which the majority were wild type (38/64). The remainder exhibited varying degrees of antimicrobial activity ranging from 10-50% with a concomitant loss in induction capacity (data not shown). However, one clone was conspicuous in that despite its apparent lack of antimicrobial activity, it retained an induction capability comparable to the wild type nisin A (Figure 2A). DNA sequencing analysis revealed a variant corresponding to P9T/G10T (both residues replaced with a threonine, Fig 2B). Furthermore, CMS revealed the presence of masses corresponding to the doubly-modified TT peptide (Dhb9Dhb10) but also species with one modified residue to Dhb and a mass close to a peptide with no modified threonine residues (Figure 2A; Table 1). Purification of the derivative P9T/G10T, we termed nisin M, was carried out with our standard nisin purification protocol and subsequent high-performance liquid chromatography (HPLC) evaluation revealed the presence of two major peaks (data not shown). Mass spectrometry analysis of these fractions revealed the presence of one peptide of 3365 Da (consistent with the presence of two Dhb's) and a second peptide of 3383 Da (consistent with a peptide with one threonine and one Dhb). Additionally, a mass in close agreement to a non-modified peptide with threonines in both positions was also observed (data not shown).

Minimum inhibitory concentration (MIC) of nisin M

Following HPLC and freeze-drying of combined fractions to obtain pure peptides, MIC assays were carried out using equimolar concentrations of nisin A and nisin M against a range of Gram positive targets including genera into which the NICE system has been previously introduced (Table 2). The MIC was determined to be the lowest concentration of peptide that resulted in the absence of visible growth of the target strain after 16 hours under the appropriate growth conditions. We established that the MIC of nisin M against a standard laboratory indicator *L.*

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195 *lactis* HP was 2.5 µg mL^{-1}, reflecting a 16-fold increase in MIC compared to wild type nisin A
196 (0.156 µg mL^{-1}). Nisin M displayed a similar decrease in potency against the *L. lactis* NZ9000
197 *gfp* reporter strain and its isogenic equivalent *L. lactis* NZ9000 (Table 2). Several lactobacilli
198 have been used as hosts of the NICE system including *Lactobacillus plantarum*, *Lactobacillus*
199 *helveticus* and *Lactobacillus brevis* (14, 19). When *Lb. plantarum* UCC16 and *Lb. brevis* SA-
200 C12 were assessed, an MIC of >2.5 µg mL^{-1} and 1.25 µg mL^{-1} was observed, demonstrating a
201 >4-fold and 16-fold decrease in antimicrobial activity for nisin M respectively in comparison to
202 wild type peptide. (Table 2).

203

204 Induction capacity of nisin A and nisin M at 10 ng mL^{-1}

205 Determination of the induction capacity of nisin A and nisin M at a concentration of 10 ng mL^{-1}
206 was performed using two reporter systems, by way of measurement of GFP and β-galactosidase
207 production. There was no statistical difference in the dynamics of RLU detection when the GFP
208 reporter strain was induced with nisin A and nisin M (P >.05) (Figure 3A). Similarly, induction
209 of the *βgal*+ reporter strain also revealed no significant difference between nisin A and nisin M
210 at equivalent concentrations (10 ng mL^{-1}) (P >.05) (Figure 3B). Moreover, the rate of expression,
211 and therefore the rate of induction was identical for both nisin A and nisin M under the
212 conditions tested in both GFP and β-galactosidase assays.

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214 Induction capacity and effect on growth of nisin reporter strain at higher induction
215 concentrations

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Next, we employed 50 ng mL^{-1}, 100 ng mL^{-1} and 300 ng mL^{-1} of peptide to determine the effect of higher concentrations of nisin A and M on both protein expression and on growth of the expression host. Fluorescence (RLU) was measured to determine GFP expression and absorbance readings at OD_{595nm} were taken to observe growth of induced strains. For each of these higher concentrations a significant difference between the level of induction by nisin A and nisin M (P <.0005) was noted (Fig. 4A-C). Interestingly, the highest RLU reading attained was from cells induced with nisin M at a final concentration of 50 ng mL^{-1}. At this concentration induction continued for the course of the experiment (18 hrs), whereas at 100 ng mL^{-1} and 300 ng mL^{-1} induction reached maximum and decreased after 10-12 hours. Notably, there was no delay in the rate of GFP expression following induction with nisin M at 50 ng mL^{-1} and 100 ng mL^{-1} (Figure 4A-B) compared to 10 ng mL^{-1} (Figure 3A), where fluorescence intensifies at approximately 6 hours post induction for all experiments; meanwhile, there was a minor delay of 30 minutes in expression with induction at 300 ng mL^{-1} (Figure 4C).

When the effects on growth were analysed, no significant impact on the growth of the GFP reporter strain was observed following induction with nisin M at 50 ng mL^{-1} (P> .05), 100 ng mL^{-1} (P> .05) and 300 ng mL^{-1} (P> .05). The OD_{595} of both non-induced cells and cells induced with nisin M increased approximately 5 hours post induction. However, induction with nisin A at the same concentrations resulted in a significant lag-time in growth. An increase in OD_{595} was not observed until 7.5, 8 and 10 hours post induction at 50 ng mL^{-1} (P< .05 for comparison of nisin M to WT, P< .0005 for comparison of WT to uninduced samples), 100 ng mL^{-1} (P< .0005) and 300 ng mL^{-1} (P< .0005), respectively. It is worth noting that although growth was observed by samples at these times following induction with WT nisin, there was no fluorescence detected from the same samples until 12, 16 and 18 hours, respectively (Figure 4A-C & 5A-C).

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241 Discussion

242 Any new technological advancements to improve the production of protein biopharmaceuticals
243 and industrial enzymes by microorganisms is highly desirable. Potential methods to optimize the
244 efficiency of an inducible gene expression system may involve adjustment of inducer dosage
245 and/or the timing of inducer addition. The Gram-positive NICE system is somewhat unusual in
246 that the inducer peptide also has the capacity to kill the expression host, and thus induction and
247 killing capacity must be balanced. The generation of a nisin derivative that retained its induction
248 properties but with reduced antimicrobial properties would represent a significant improvement
249 to the NICE system that could be applied to more sensitive strains.

250 Previous work, where the focus has been on the nisin peptide itself, involved randomised
251 mutagenesis of rings A and B (18) and described mutants that retained considerable auto-
252 induction abilities but with lower antimicrobial properties (and vice- versa). Similarly, Ge and
253 co-workers (2016) applied a complete alanine scanning mutagenesis approach and reported that
254 the N-terminal ring structures (ring A and ring B) in nisin were involved in activating NisK to
255 act as an inducing molecule (16). In this study we focused our attention on ring B with a more
256 systematic mutagenesis approach to identify novel derivatives with altered activity/induction
257 properties. This proved to be successful in that we identified a nisin variant that retains induction
258 capacity that is comparable to the wild type peptide but exhibits significantly less antimicrobial
259 activity.

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Notably, another lantibiotic, subtilin is structurally closely related to nisin and contains the same lanthionine ring structure but does not induce P*nisA*. Indeed, in the study by Steiß, Korn, Kötter and Entian (2015) the failure of subtilin to induce the histidine kinase NisK was shown to mostly depend on the presence of an N-terminal tryptophan, as its replacement with the aliphatic amino acid residues isoleucine, leucine, and valine led to activation of NisK (20). This suggests further bioengineering at position 1 and indeed other amino acid locations in the nisin M background could potentially enhance induction and reduce antimicrobial activity even further.

Although this study highlighted ring B of nisin as a critical region in our quest to separate antimicrobial activity from induction/pheromone activity, more residue positions could and should be targeted. Studies with the natural variant nisin Z have revealed that derivatives corresponding to T2S and M17W exhibited an 11-fold and 2-fold increase in induction capacity relative to the parent peptide, respectively, while derivatives S5T and S3T had significantly reduced induction capacity (7).

A computational approach evaluating the antimicrobial activity, induction capacity, production levels and immunity/sensor kinase components of natural and bioengineered nisin derivatives could provide a blueprint for the design of more efficient peptide inducers. For example, the ability of nisin P, A and H to activate P*nisA* fused to a *gfp* reporter was assessed and found to differ (21). The promoter was more sensitive to nisin A (1 ng mL^{-1} – 1 µg mL^{-1}) than nisin H (10 ng mL^{-1} – 1 µg mL^{-1}) and nisin P (100 ng mL^{-1} – 10 µg mL^{-1}). Higher concentrations of nisin P were required to activate the promotor, but it continued to induce promoter activity at higher concentrations (10 µg mL^{-1}) whereas nisin A and H were capable of inducing the promoter only up to 1 µg mL^{-1} concentrations of peptides. The ability to use higher concentrations of nisin P is most likely due to its decreased antimicrobial activity as compared with nisin A and nisin H.

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283 While this might advocate for the use of nisin P as an alternative inducer to nisin A, the peptide
284 does not induce at the lower and commonly used inducing concentration (10 ng mL^{-1}). Notably,
285 the nisin M mutant generated in this study induces at both low and high concentrations. While
286 no significant difference in growth of the induced strain compared to the un-induced control was
287 observed, even at the maximum concentration applied (300 ng mL^{-1}), further evaluation with
288 even higher concentrations of nisin M are necessary and with a variety of expression host strains.
289 However, the practicality of using such high concentrations in terms of industrial applications
290 would need to be considered, given that cell free supernatant from a nisin M producer would be
291 the most likely option for induction (rather than expensive purified nisin peptides); though a
292 fermentate analogous to nisaplin (2.5% nisin A) would enable a range of concentrations to be
293 applied irrespective of the sensitivity of host strains (e.g. induction levels above 10 ng mL^{-1} nisin
294 A results in inhibitory effects on the expression strain *L. lactis* NZ9000) (22).

295 Additionally, the natural variant nisin Q also displays similar antimicrobial capabilities to that of
296 nisin A but differs in its ability to induce the *nisA* promoter (23). Directed mutagenesis and
297 analysis of the four amino acids which differ between nisin A and nisin Q (A15V, M21L, H27N,
298 I30V) may help us to more completely understand the pheromone activity of nisin. Remarkably,
299 in the study by Ge and co-workers the derivatives L16D, L16A, L16H, L16V, M21A, M21D,
300 and M21N all exhibited enhanced induction properties when assessed by β-Galactosidase assays,
301 with L16D being particularly notable given it also displays a significant reduction in
302 antimicrobial activity (16). Other regions of nisin subjected to bioengineering approaches and
303 shown to impact on induction activity include the C-terminus and in particular serine and
304 isoleucine at positions 29 and 30, respectively (24). Although the specifics of the interaction
305 between the nisin peptide and NisK have yet to be fully elucidated, a recent study has provided

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some insight through mutational analysis of NisK. Mutagenesis of conserved residues in the extracellular region of NisK revealed that several hydrophobic residues including two aromatic residues (Tyr113 and Phe133) are crucial for NisK in sensing nisin and regulating nisin biosynthesis (25).

Elimination of the antimicrobial activity of nisin is a priority when aiming to improve the nisin peptide in terms of its suitability as a peptide inducer, such as in the NICE system. For example, Reunanen & Saris (2003) developed a method for the quantification of nisin in food samples, through the construction of a non-nisin producing *L. lactis* strain (LAC240), with a plasmid containing a *gfp* gene under the control of the *nisF* promoter and the constituent genes of the nisin two-component regulatory system, *nisRK*. It was reported that upon the addition of nisin peptide concentrations greater than 20 ng mL^{-1}, the LAC240 cells became stressed resulting in a reduction in the quantity of GFP produced and the signal reached the background level when the concentration of nisin was approximately 60 ng mL^{-1} (26). Moreover, in a study that aimed to improve the response of *L. lactis* to freezing damage through expression of an antifreeze peptide (SF-P), the recombinant strain *L. lactis* NZ3900 SF-P was incubated with different concentrations of nisin (25, 50, or 100 ng mL^{-1}) and at various pH and growth temperature values (27). Notably, maximal expression was observed at 25 ng mL^{-1}, with a much lower level of expression at 50 ng mL^{-1} and virtually no expression at 100 ng mL^{-1}, most likely due to the inhibitory effects of nisin A, though pH and temperature values were also a factor (27). In another study that sought to optimize the NICE system for the expression of lysostaphin for both laboratory (1 L) and industrial-scale (3000 L) applications and at high cell densities, the authors noted that the addition of too much nisin was detrimental for product formation. Notably, when the culture was induced at higher cell densities, 160 mg L^{-1} lysostaphin was formed with 20 ng

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mL^{-1} nisin and 220 mg L^{-1} lysostaphin was produced when 40 ng mL^{-1} nisin was used for induction, indicative of a clear correlation between the cell density at induction and the amount of nisin that is needed for maximal induction (5). While this group reported that maximum protein yield in the NICE system is achieved by induction carried out at a cell density of OD_{600} = 5 with a final concentration of 40 ng mL^{-1} of nisin, we suggest that Nisin M provides for a greater flexibility with respect to inducer concentration by virtue of the attenuated antimicrobial activity of the peptides and the application of high concentrations of inducer peptide is not now a limiting factor.

To date, a multitude of peptides, enzymes and vaccines of clinical and biotechnological interest have been overexpressed using nisin, including the anti-bacterial protein lysostaphin (5), a haemagglutinin of the H5N1 influenza virus (28) and Rotavirus VP6 Protein (29), to name but a few. Though several improvements have been made to the NICE system, further improvements are possible. For example, streamlined-genome mutants of *L. lactis* NZ9000 were generated by deletion of four large nonessential DNA regions accounting for 2.83% of the genome and evaluated as microbial cell factories for recombinant protein production. Indeed, following nisin induction, not only was the transcriptional efficiency improved but also the production levels of the expressed reporters were approximately three to fourfold enhanced compared with the wild strain (30). Additionally, expression from the Δ*lac*F host-strain *L. lactis* NZ3900 (a strain unable to utilize lactose), enabled food-grade, lactose-based plasmid selection and induction (31) whilst deletion of a specific proteinase gene (NZ9000 Δ*htrA*) led to increased stability of heterologous-secreted proteins (32).

While the aforementioned studies focused on improving the host strain for expression of proteins, this study focuses on potential improvements that can be made to the inducing peptide

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via mutagenesis of ring B, which has already been reported as playing an important role in induction capacity (16, 18). This study has demonstrated that a nisin A variant with modifications to ring B retained comparable induction capacity to the wild type nisin A peptide yet exhibited less inhibitory effects on the growth of the strain *L. lactis* NZ9000 when applied at concentrations as high as 300 ng mL^{-1} (0.09 μM). It was also determined this combination has between >4 and >16 fold less activity against various genera and species of bacteria into which the NICE system has been introduced, therefore supporting the claim that nisin M exhibits potential as a suitable alternative to nisin A for use in the NICE system.

This study confirms that random mutagenesis experiments continue to be beneficial with a view to enhancing the functional properties of the nisin peptide for specific applications and provide novel nisin variants that exhibit potential for future applications in the pharmaceutical, biotechnological and industrial fields.

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376 Materials and Methods:

377 Bacterial strains and plasmids

378 Bacterial strains and plasmids used in this study are listed in Table 3.

379 Creation and analysis of a bank of nisin A ring-B derivatives

380 Mutagenesis of the *nis*A gene was carried out as described previously (7). Briefly, saturation
381 mutagenesis was carried out using pDF05 (pCI372-*nisA*) as template and using oligonucleotides
382 as listed in (Table 4) containing an NNK codon in place of each native codon. PCR amplification
383 was performed in a total volume of 50 µL with 0.5 ng of target DNA (pCI372-*nisA*), 1 unit
384 Phusion High-Fidelity DNA polymerase (Finnzymes, Finland), 1 mM dNTPs and 500 ng each of
385 the appropriate forward and reverse oligonucleotides. The reaction was pre-heated at 98°C for
386 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,
387 and then finished by incubating at 72°C for 3 min 30 s. Amplified products were treated with
388 Dpn1 (Stratagene) for 60 min at 37°C to digest template DNA and purified using the QIAquick
389 PCR purification kit. Following transformation of *E. coli* Top 10 cells plasmid DNA was isolated
390 and sequenced using primers pCI372FOR and pCI372REV (Table 4) to verify that mutagenesis
391 had taken place. The purified products were subsequently introduced by electroporation into the
392 strain *L. lactis* NZ9800 which has all the genes necessary for nisin production. Approximately
393 150 transformants were chosen at random for each single position (P9X and G10X) and 1152

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394 transformants for the randomised P9XG10X bank. Isolated colonies were inoculated into 96-well
395 plates containing GM17 Cm^{10}, incubated overnight and stored at −20°C after addition of 80%
396 glycerol. Deferred antagonism assays were performed by replicating strains on GM17 agar plates
397 and allowing them to grow overnight before overlaying with GM17 agar (0.75% w/v agar)
398 seeded with the *L. lactis* HP indicator strain. Induction assays were carried out by replicating
399 strains from each 96 well plate into a fresh 96 well plate containing GM17 broth pre-inoculated
400 with *L. lactis* NZ9000 pNZ8150*gfp+,* in which GFP acts as a reporter of expression from a nisin
401 inducible promoter (24). Induction of GFP was monitored over 20 hours in terms of relative
402 fluorescence units (RFU) using a TECAN Genios Fluorescence, Absorbance and Luminescence
403 Reader using excitation and emission spectra of 485nm and 535nm, respectively.

404

405 MALDI TOF Mass Spectrometry

406 For Colony Mass Spectrometry (CMS), bacterial colonies of P9X and G10X mutants were
407 collected with sterile plastic loops and mixed with 50 µL of 70% IPA containing 0.1%
408 Trifluoroacetic acid (TFA). The suspension was vortexed, the cells centrifuged in a benchtop
409 centrifuge at 8260 *g* for 2 min and the supernatant was removed for analysis. For MALDI TOF
410 Mass Spectrometry of nisin M cell free supernatant (CFS) was purified prepared as follows; a
411 1% inoculum of nisin mutant producing strains were grown overnight in 50 mL clarified TY
412 broth and incubated overnight at 30°C. Following incubation cells were centrifuged at 5000rpm
413 for 20 mins at 4°C. Cell free supernatant (CFS) was removed and passed through a 1 g (6 mL)
414 Strata C-18 E column (Phenomenex) pre-equilibrated with 6 mL methanol (Fisher Scientific,
415 UK) and 6 mL HPLC grade H_2O. The column was washed with 12 mL 30% ethanol and nisin
416 eluted using 5 mL 70% isopropanol – 0.1% TFA. Mass Spectrometry in all cases was performed

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417 with an Axima TOF^2 MALDI TOF mass spectrometer (Shimadzu Biotech, Manchester, UK). A
418 0.5 µL aliquot of matrix solution (alpha-cyano-4-hydroxycinnamic acid (CHCA), 10 mg mL^{-1} in
419 50% acetonitrile-0.1% (v/v) TFA) was placed onto the target and left for 1-2 min before being
420 removed. The residual solution was then air dried and the sample solution (re-suspended
421 lyophilised powder or CMS supernatant) was positioned onto the pre-coated sample spot. Matrix
422 solution (0.5 µL) was added to the sample and allowed to air-dry. The sample was subsequently
423 analysed in a positive-ion linear mode.
424
425 Purification of nisin A and nisin M
426 Purifications of nisin A and variant, nisin M were carried out as per a previously employed (33)
427 with modifications. Briefly, overnight cultures of *L. lactis* NZ9800 pDF05*nisM* (APC3920)
428 and *L. lactis* NZ9700 were inoculated at 0.5% into separate purified tryptone-yeast extract (TY)
429 broth (2 × 900 mL) supplemented with 20% glucose and 20% β-glycerophosphate, and incubated
430 at 30°C overnight. Following incubation, the cultures were centrifuged at 6500*g* at 4°C for 15
431 min. The supernatant was passed through a column containing ~70g Amberlite XAD-16 beads
432 and subsequently washed with 500 mL of 30% ethanol. The nisin was eluted from the column
433 using 70% isopropanol containing 0.1% trifluoroacetic acid (TFA). Simultaneously, bacterial
434 cell pellets were resuspended in 300 mL 70% isopropanol – 0.1% TFA and stirred at room
435 temperature for 3 h. This cell suspension was then centrifuged at 5000*g* at 4°C for 10 min and
436 the supernatant was retained. The column eluant was pooled with the post-centrifugation
437 supernatant and isopropanol evaporated using a rotary evaporator (BÜCHI Rotavapor R-205,
438 Switzerland). The pH of the sample was adjusted to pH 4.0 and was subsequently passed through
439 a 10 g (60 mL) Strata C-18 E column (Phenomenex) pre-equilibrated with 60 mL methanol

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(Fisher Scientific, UK) and 60 mL HPLC grade H_2O. After applying 120 mL 30% ethanol, nisin was eluted from the column using 60 ml 70% isopropanol – 0.1% TFA. For HPLC purification 12 mL volumes were concentrated to a volume of 2 mL by rotary evaporation and applied to a Phenomenex C12 reverse-phase (RP-HPLC) column (Jupiter 4 µm proteo 90 Å, 250 mm × 10.0 mm, 4 µm) previously equilibrated with 25% acetonitrile-0.1% TFA. Nisin was eluted via a gradient of 25–50% acetonitrile-0.1% TFA that was developed from 10–40 min at a flow rate of 3.2 mL min^{-1}. Nisin containing fractions were pooled and acetonitrile removed by rotary evaporation. The purified peptides were lyophilised and stored at -20°C.

Minimum Inhibitory Concentration (MIC) Assays

MIC's were also carried out on strains into which the NICE system was reported to have been introduced including, *Lactobacillus plantarum* (*Lb. plantarum*) and *Lactobacillus brevis* (*Lb. brevis*) in order to determine the potential of nisin M as an alternative to nisin A in the NICE system.

Minimum inhibitory concentration determinations for strains were carried out in triplicate in 96 well microtitre plates (Sarstedt) as described previously (34). Plates were pre-treated with bovine serum albumin (BSA) prior to addition of the peptides. Briefly, to each well of the microtitre plate 200 µL of phosphate buffered saline (PBS) containing 1% (w/v) bovine serum albumin (BSA) was added and incubated at 37°C for 30 min. The wells were washed with 200 µL PBS and allowed to dry. Target strains, *L. lactis* spp. cremoris HP, *L. lactis* NZ9000 pNZ8150*gfp*+ were grown overnight in M17 broth (Sigma) supplemented with glucose (0.5%) at 30°C. *Lb. plantarum* and *Lb. brevis* were grown overnight in MRS broth (Oxoid) at 30°C. Strains were

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sub-cultured into fresh broth and allowed to grow to an OD_{600} of ~0.5, diluted to a final concentration of 10^5 cfu mL^{-1} in a volume of 0.2 mL. Nisin A and nisin M peptides were adjusted to a 750 nM starting concentration and 2-fold serial dilutions of each peptide was added to the target strain. After incubation for 16 h at 30°C the MIC was read as the lowest peptide concentration causing inhibition of visible growth.

Comparison of nisin A and nisin M induction capacity using beta-galactosidase activity

β-galactosidase activity assay was performed as previously employed (35) with modifications. Cultures of *L. lactis* NZ9000 pPTPL*βgal*+ were inoculated in M17 broth (Sigma), supplemented with glucose at 0.5% (GM17) and tetracycline (10 µg mL^{-1}), and incubated at 30˚C overnight. Following incubation, a 1% inoculum of each replicate was sub-cultured into fresh GM17 medium and incubated at 30˚C until an OD_{600} of 0.2-0.3 was reached. Cells were then treated separately with nisin A and nisin M purified peptides to a final concentration of 10 ng mL^{-1}. Every hour 1 mL samples of each test were transferred to an eppendorf and centrifuged at 13,000 rpm for 2 minutes (Sorvall Legend Micro 17 centrifuge, Thermo Scientific) to harvest cells. Cells were re-suspended in 1 mL lacZ buffer and 0.5 mL of this was treated with 12.5 µL of 0.1% SDS and 25 µL of chloroform and incubated at 30°C for 5 minutes to dissolve cell membranes. Following incubation 100 µL of 2-Nitrophenyl-β-D-galactopyranoside (ONPG) (4 mg mL^{-1}) (Sigma-Aldrich) was added to each sample and incubated at 37°C until a yellow colour developed. To stop the reaction samples were treated with 250 µL of a 1 M sodium carbonate solution and centrifuged at 8000rpm for 5 minutes (Thermo Scientific). Absorbance readings of supernatant were read at OD_{420} and OD_{550} (SpectraMax M3 spectrophotometer, Molecular

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Devices, Sunnyvale, California, USA). Measurement of β-galactosidase activity of samples was calculated as $1000 \times (OD_{420} - [1.75 \times OD_{550}])/ (t \times v \times OD_{600})$ as previously described (16).

Assessment of purified nisin A and nisin M induction capacity using a green fluorescent protein reporter system.

Induction assays were performed previously described (24) with modifications. Briefly, cultures of *L. lactis* NZ9000 pNZ8150*gfp*+ were inoculated in M17 broth (Sigma), supplemented with glucose at 0.5% (GM17) with chloramphenicol (10 µg mL^{-1}) and incubated at 30˚C overnight. Following incubation, a 1% inoculum of each replicate was sub-cultured into fresh GM17 medium and incubated at 30˚C until an OD_{600} of ~0.5 was reached. Cells were then diluted to a final concentration of 10^5 cfu mL^{-1} and treated with nisin A and nisin M at final concentrations of 10 ng mL^{-1}, 50 ng mL^{-1}, 100 ng mL^{-1} and 300 ng mL^{-1}. Subsequently, 2 mL was transferred to black, 24 well microtitre plates (PerkinElmer) for induction and 200 µL into a 96 well plate (Sarstedt) for absorbance readings. Fluorescence was detected using a SpectraMax M3 spectrophotometer (Molecular Devices, Sunnyvale, California, USA) where excitation and emission parameters were set to 485nm and 528nm respectively for fluorescence, while absorbance readings were taken at OD_{595} using a Multiskan FC microplate photometer v1.01.14 (Thermo Scientific, Waltham, Massachusetts, USA). Baseline absorbance of un-cultured GM17 was subtracted from the fluorescence and absorbance readings of all test samples using SoftMax Pro v6.3 and SkanIt RE v4.1 software, respectively. Fluorescence was reported as relative light units (RLU) and absorbance as OD_{595nm}. Tests were carried out in triplicate.

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509 Statistical analysis

510 Statistical analysis was carried out with SPSS Statistics v2. A test of normality was performed to
511 determine data for each test was normally distributed. For normally distributed data a Repeated
512 Measures ANOVA was performed. For data not normally distributed a Levene's test of
513 homogeneity was performed, where if equal variances were assumed the Repeated Measures
514 ANOVA was carried out; and if equal variances were not assumed the non-parametric Friedman
515 test was performed to determine if differences between the two nisin variants induction capacity,
516 and between the growth of the strains when induced with the peptides at higher concentrations
517 compared to an un-induced control were significant. For ANOVA/Friedman's results with a
518 significant difference between groups (P <.05) a post hoc test was performed. Post hoc tests for
519 normally distributed/equal variances assumed samples was the Bonferroni test, and for non-
520 normally distributed/equal variances not assumed samples Dunnett's T3 test was performed. The
521 significance threshold for all ANOVA's and non-parametric tests performed was set at .05.

522

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530

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638 Table 1. Mass spectrometry analysis of selected derivatives

Ring B Derivative	Predicted Mass (Da)	Actual Mass (Da)	Dehydrations		Ref
			Observed	Lacking	
P9A	3328	3327.87	8	0	This study (16)
P9T	3357	3356.67	8	1	This study
		3339.62	9	0	
P9S	3343	3342.73	8	1	This study
		3324.69			
G10A	3366	3367.14	8	0	This study (18)
G10T	3398	3397.76	8	1	This study (18)
G10S	3384	3384.57	8	1	This study
		3367.23	9	0	(18)
P9T/G10T (nisin M)	3402	3399.86	8	2	This study
		3382.91	9	1	
		3365.33	10	0	
Nisin A	3354	3353.44	8	0	

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641 Table 2: MIC of nisin A and nisin M against standard indicator strains (including those reported
642 to have had the NICE system introduced).

Indicator organism	Nisin A µg mL^{-1} (µM)	Nisin M µg mL^{-1} (µM)	Fold decrease in activity
Lb. plantarum UCC16	0.625 (0.1875)	>2.5 (>0.750)	>4
L. lactis NZ9000 pNZ8150*gfp*+	0.156 (0.046)	>2.5 (>0.750)	>16

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L. lactis NZ9000 pNZ8150	0.156 (0.046)	2.5 (0.750)	16
Lb. brevis SA-C12	0.078 (0.0234)	1.25 (0.375)	16
L. lactis spp. *cremoris* HP	0.156 (0.0468)	2.5 (0.750)	16

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645 Table 3: Bacterial strains and plasmids used in this study

Strain or Plasmid	Characteristic	Reference
L. lactis NZ9000	MG1363 derivative, *NisRK* integrated into *pepN* gene (*pepN*-). Most commonly used host of the NICE system.	(22) (4)
L. lactis NZ9000 pNZ8150	NZ9000 strain harbouring pNZ8150. pNZ8150: *Sca*I site for translational fusions, standard vector for NICE system, Cm^R.	(4)
L. lactis NZ9000 pNZ8150*gfp*+	NZ9000 strain harbouring pNZ8150 *gfp*+ under P*nisA* promoter. Cm^R.	(24)
L. lactis NZ9000 pPTPL*βgal*+	NZ9000 strain harbouring low copy plasmid pPTPL with β-galactosidase expressing gene under the control of the P*nisA* promoter. Tet^R	(7)
L. lactis NZ9800	Derivative of NZ9700 with 4bp deletion rendering an inactive nisin operon (Δ*nis*A), except *nisRK* genes. Host of the NICE system.	(22, 36)
L. lactis NZ9800 pDF05	NZ9800 harbouring pDF05 (pCI372 with *nisA* under its own promoter). Wild type nisin A producer, Cm^R.	(22, 36) (7)

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L. lactis NZ9800 pDF05*nisM*	pDF05 where codons 9 and 10 of *nis*A have been randomized. Nisin M producer, Cm^R.	This work UCC Culture Collection (APC3920)
Lb. plantarum UCC16	Nisin sensitive indicator Species in which NICE system has been utilized.	UCC Culture Collection (4, 19)
Lb. brevis SA-C12	Nisin sensitive indicator Species in which NICE system has been utilized.	UCC Culture Collection (4, 37)
L. lactis ssp. *cremoris* HP	Nisin sensitive indicator strain	UCC Culture Collection

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648 Table 4. Oligonucleotides utilised in this study.

Primer name	Sequence
NisP9degFOR	5' CTA TGT ACA NNK GGT TGT AAA ACA GGA GCT CTG ATG GGT 3'
NisP9degREV	5' TTT ACA ACC MNN TGT ACA TAG CGA AAT ACT TGT AAT GCG 3'
NisG10degFOR	5' TGT ACA CCC NNK TGT AAA ACA GGA GCT CTG ATG GGT TGT 3'
NisG10degREV	5' TGT TTT ACA MNN GGG TGT ACA TAG CGA AAT ACT TGT AAT 3'
NisP9G10degFOR	5' CTA TGT ACA NNK NNK TGT AAA ACA GGA GCT CTG ATG GGT 3'
NisP9G10degREV	5' TTT ACA MNN MNN TGT ACA TAG CGA AAT ACT TGT AAT

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	GCG 3’
pCI372For	5’ CGGGAAGCTAGAGTAAGTAG 3'
pCI372Rev	5’ ACCTCTCGGTTATGAGTTAG 3’

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663 Figure 1. Induction and antimicrobial activity analyses of nisin mutants with substitutions at (A)

664 position 9 and (B) position 10. Induction capacity (red) and antimicrobial activity (green) is

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shown as percentages (%) and ordered from highest to lowest based on biological activity of the variants.

Figure 2. A: (Top) Biological activity of nisin A and nisin M as determined by deferred antagonism assays and assessment of induction capacity following induction of a *L. lactis* strain containing *gfp*+ under control of the nisin promoter. (Bottom) Colony Mass Spectrometry of the wild type nisin A producer (3353.44 Da) and nisin M comprising of a combination of unmodified peptide, single dehydration or two dehydrations at P9T/G10T (3399.86 Da, 3382.91 Da, and 3365.33 Da respectively). B: Structure of nisin A where amino acids are represented by their single letter codes and modified residues are indicated as follows; Dha: dehydroalanine, Dhb: dehydrobutyrine, Abu: 2-aminoabutyric acid, Ala-S-Ala: lanthionine, Abu-S-Ala: methyllanthionine. Residues in orange and pink show amino acid substitutions for nisin M, producing 4 possible forms of the peptide.

Figure 3. Induction capacity of nisin A (red/circle) and nisin M (blue/square) determined by expression of (A) GFP and (B) β-galactosidase reporter genes under the control of the P*nisA* promoter in *L. lactis* NZ9000 pNZ8150*gfp*+ and *L. lactis* NZ9000 pPTPL*βgal*+ respectively when induced at a final concentration of 10 ng mL^{-1}. Negative controls (green/triangle) are uninduced test strains. Statistical analysis shows there is no significant difference between the induction capacities of nisin M and nisin A in both methods tested ($P > .05$).

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686 Figure 4. Comparison of induction capacities of nisin A (red/circle), nisin M (blue/square)
687 determined by expression of GFP under the control of the P*nisA* promoter in *L. lactis*
688 pNZ8150*gfp*+ induced at final concentrations of (A) 50 ng mL^{-1}, (B) 100 ng mL^{-1} and (C) 300
689 ng mL^{-1}. Statistical analysis demonstrates a significant difference between induction capacity of
690 the two peptides at all concentrations tested, ($P< .0005$) Negative control in this assay is
691 uninduced *L. lactis* NZ9000 pNZ8150*gfp*+ (green/triangle).

692

693 Figure 5. Effects of nisin A (red/circle) and nisin M (blue/square) on growth of *L. lactis* NZ9000
694 pNZ8150*gfp*+ induced at concentrations of (A) 50 ng mL^{-1}, (B) 100 ng mL^{-1} and (C) 300 ng mL^{-1}
695 compared to an un-induced control (green/triangle) determined by absorbance at OD_{595nm}.
696 Results show no significant difference between growth of the uninduced control and cells
697 induced with nisin M at all concentrations tested ($P> .05$); while there is a significant difference
698 between the growth of cells induced with WT nisin compared to both the uninduced control (50
699 ng mL^{-1}: $P< .0005$; 100 ng mL^{-1}: $P< .0005$; 300 ng mL^{-1}: $P< .0005$), and samples induced with
700 nisin M (50 ng mL^{-1}: $P< .05$; 100 ng mL^{-1}: $P< .0005$; 300 ng mL^{-1}: $P< .0005$).

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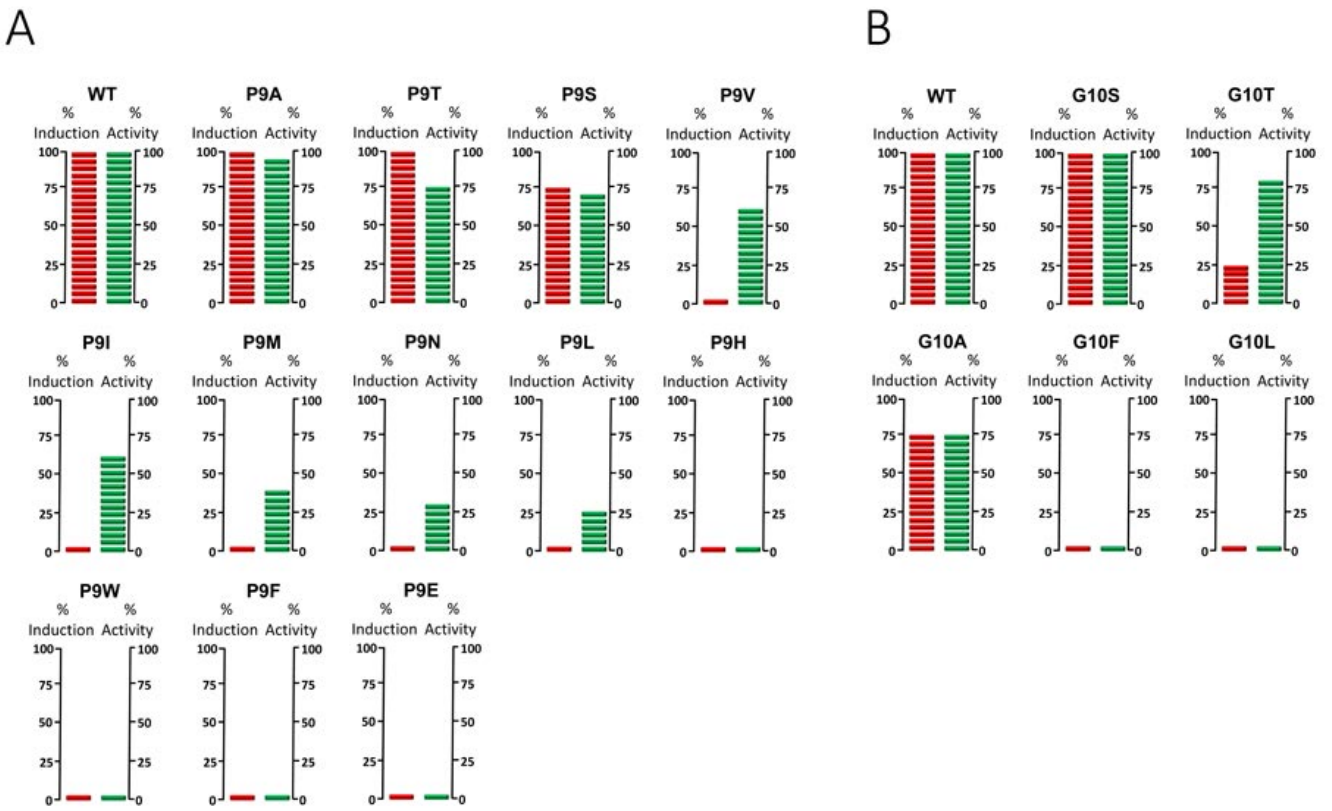
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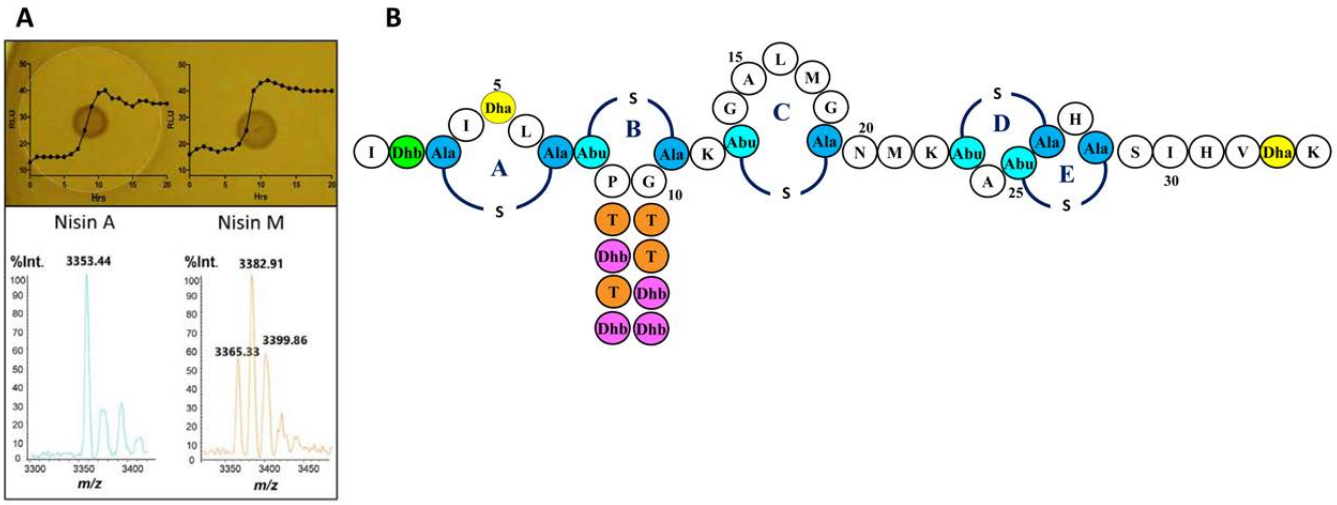
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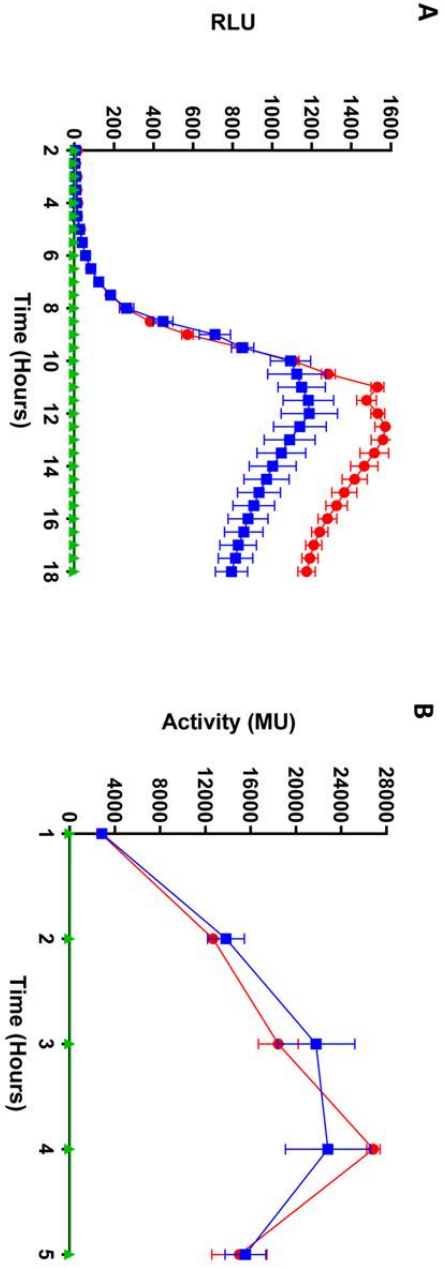
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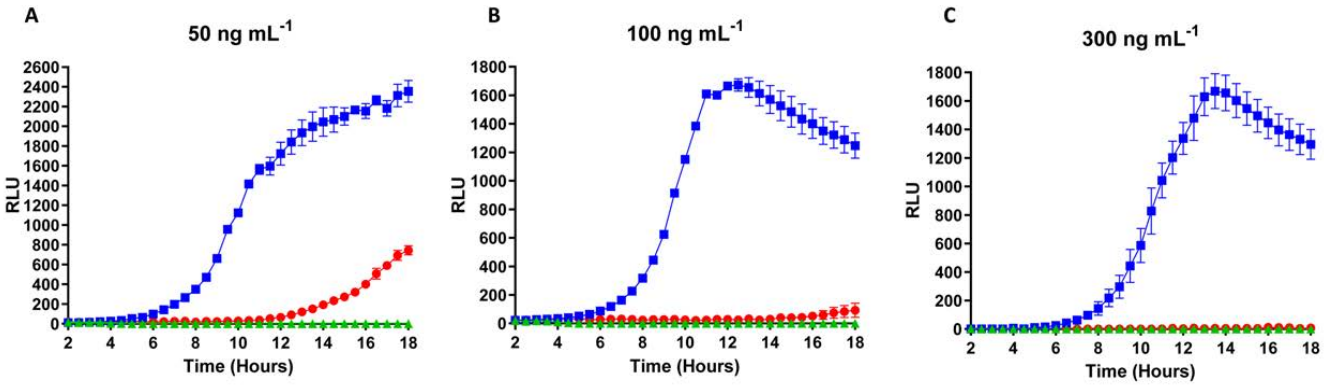
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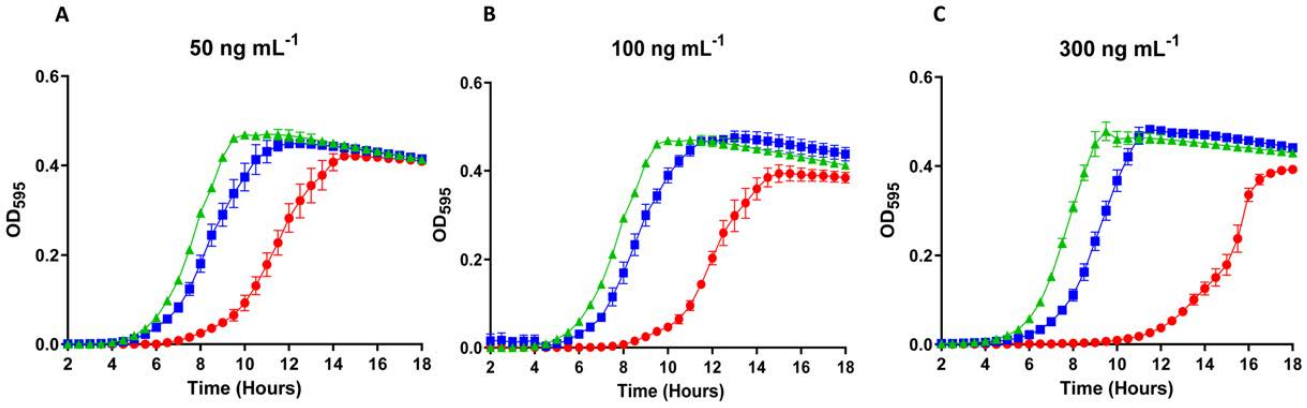
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