Discovery and molecular characterisation of novel bacteriocins produced by Gram positive bacteria

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Doctor of Philosophy

by

Paula M O'Connor, MSc

Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland

School of Microbiology, University College Cork, Ireland

APC Microbiome Ireland, University College Cork, Ireland

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Research supervisors: Professor R Paul Ross and Professor Colin Hill

Head of School: Professor Paul O'Toole

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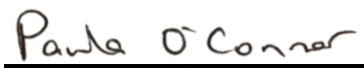
Declaration

This is to certify that the work I am submitting is my own and has not been submitted for another degree, either at University College Cork or elsewhere with the exception of Chapters 2.2, 3, 6.1 and 6.2. All external sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism.



Paula M O'Connor
Student no. 85154873

8th January, 2021

Date

Author Contributions

Paula M O'Connor was involved in study design, guidance with experiments, and interpretation of results for **Chapters 2.2**, **6.1** and **6.2**. She carried out bacteriocin purification, MALDI-TOF MS and antimicrobial assays for all chapters in addition to alkylation of actifenisin for **Chapter 6.2**. She also helped in writing the manuscripts, took part in reviewing the manuscripts and approved the final manuscripts.

Abstract

Bacteriocins are ribosomally synthesised antimicrobial peptides, produced by many bacterial genera that display potent activity against closely (narrow spectrum) or distantly related (broad-spectrum) bacteria. Bacteriocins, produced by Lactic Acid Bacteria (LAB) that are natural constituents of fermented foods, are ideal natural preservatives to control food spoilage/pathogenic bacteria in minimally processed foods. In addition to their role as food preservatives, bacteriocins have potent activity against medically significant pathogens and are considered attractive alternatives or adjuncts to antibiotics, due to their inherent heat stability, potency at nanomolar scale, resistance to proteases and low levels of acquired resistance in commercial applications. Overall, bacteriocins are versatile antimicrobials with huge potential for use as biopreservatives, antibiotic alternatives, health promoting gut modulators and animal growth promotors. The aim of this thesis was to identify, purify and characterise novel bacteriocins from microorganisms isolated from a wide range of niches, with a view to expanding the number of bacteriocins currently available and exploring novel structures and activities.

In this respect, **Chapter 2.1** describes the discovery a novel nisin A variant, nisin H, produced by a porcine gut isolate *Streptococcus hyointestinalis* DPC6484. Nisin H differs from nisin A at five amino acid positions and is an intermediate between naturally occurring nisins of lactococcal and streptococcal origin. The operon encoding nisin H is noteworthy by virtue of the absence of an equivalent of *nisI* that encodes an immunity protein that protects the cell from its own bacteriocin. This is the first report of natural nisin variant production by an intestinal isolate of streptococcal origin and may confer an advantage to the strain by allowing it to

dominate its environment, fight infection or signal the immune system of the host. In a subsequent chapter another natural variant is characterised in the form of nisin J, produced by a human skin isolate *Staphylococcus capitis* APC2923. Nisin J is more dissimilar to nisin A than nisin H with nine amino acid changes, six of which are unique, and an extra amino acid making it the first nisin variant to contain 35 amino acids. Interestingly, the operon lacks both *nisI* (immunity) and *nisRK* (regulatory) equivalents. Nisin J, like nisin A and H, displays activity against a wide number of genera and represents the first natural nisin variant from staphylococci and the first nisin producer from human skin, suggesting a role in competitive colonization for producing organisms.

The natural nisin variants described above (nisin H and J), in addition to nisin P produced by *Streptococcus agalactiae* DPC7040, are all produced by non GRAS strains and are therefore limited in their potential industrial applications. The recent increase in the prevalence of antibiotic resistant pathogens makes it important that all bacteriocins regardless of the producing organism are explored as antibiotic alternatives. As these lantibiotics are gene encoded, bioengineering (**Chapter 3.1**) was used to enable recombinant expression of peptides naturally expressed by non-GRAS organisms in a host derived from safe origins. Specifically, the Nisin A promotor and nisin A leader sequence were fused to nisin H, J or P structural genes and successfully expressed in the GRAS strain *L. lactis* NZ9700, demonstrating that the *L. lactis* production, transport and modification machinery can produce fully functional nisin variants from significantly different genetic backgrounds.

In **Chapter 4**, Bactofencin A produced by *Streptococcus salivarius* DPC6502 was discovered following a porcine gut mining study. It is a 22 amino acid, class IId bacteriocin that displays activity against *Staphylococcus aureus* and *Listeria*

monocytogenes. Structurally, it consists of a positively charged N terminus that we propose could bind to the negatively charged cell surface. The small bacteriocin cluster also encodes a DltB homologue that may well be responsible for immunity through D-alanylation of teichoic acids. In order to probe structure/function relationships in bactofencin A, a library of synthetic bactofencin A peptide variants were synthesized. Substituting cysteine residues significantly reduced activity confirming the importance of the disulphide while sequential removal of the positively charged N terminal resulted in a decreasingly active peptide. Substituting each amino acid for alanine revealed that residues 9-17 within the loop were more affected by substitution, suggesting this region contributes significantly to the potency of the bacteriocin.

In **Chapter 5**, bactofencin A was shown to enhance nisin bactericidal activity and reduce the overall frequency of resistance. Interestingly, these studies highlighted the relatively slow or delayed mode of action of bactofencin A.

The last two chapters (**Chapters 6.1 and 6.2**) again focus on the discovery of two novel bacteriocins, namely formicin and actifensin. The first of these, formicin, is a novel bacteriocin that extends the class of two peptide lantibiotics. It was purified from *Bacillus paralicheniformis* APC1576, a mackerel intestine isolate. Compared with other two component lantibiotics, formicin is most similar to haloduracin and consists of a very hydrophilic Alpha peptide with a charge of +2 whereas the Beta peptide is negatively charged. Formicin displays activity against a broad range of Gram-positive bacteria including clinically relevant pathogens. The second bacteriocin is actifensin a 4091 Da, broad spectrum, Class IId bacteriocin containing three disulphide bridges with more than 50% similarity to eukaryotic defensins that we propose represents a new subclass of bacteriocins. It is produced by *Actinomyces ruminicola,* isolated from sheep feces. A pangenomic screen of available *Actinomyces* spp. revealed the presence

of very diverse actifensin homologues in 29% of genomes examined, suggesting that production of actifensin like bacteriocins is a common trait. This new class of bacteriocins may provide a template to design new broad-spectrum antimicrobials for treatment of human and animal infections.

The developments described in this thesis can be used to contribute to increased commercialisation of bacteriocins in both food systems and human and animal medical treatments.

Publications generated from this thesis

1. **O'Connor PM**, Ross RP, Hill C & Cotter PD (2015) Antimicrobial antagonists against food pathogens: a bacteriocin perspective. *Current Opinion in Food Science* **2**, 51-57.

2. **O'Connor PM,** Kuniyoshi TM, Oliveira RPS, Hill C, Ross RP & Cotter PD (2020) Antimicrobials for food and feed; a bacteriocin perspective. *Curr Opin Biotechnol* **61**, 160-167.

3. **O'Connor PM,** O'Shea EF, Guinane CM, O'Sullivan O, Cotter PD, Ross RP & Hill C (2015) Nisin H is a new nisin variant produced by the gut-derived strain *Streptococcus hyointestinalis* DPC6484. *Appl Environ Microbiol* **81**, 3953-3960.

4. O'Sullivan, JN, **O'Connor PM,** Rea MC, O'Sullivan O, Walsh C, Healy B, Mathur H, Field D, Hill C & Ross RP **(2020)** Nisin J, a novel natural nisin variant, is produced by *Staphylococcus capitis* sourced from the human skin microbiota. *J Bacteriol* **202**, doi:10.1128/JB.00639-19.

5. O'Sullivan JN, **O'Connor PM,** Rea MC, Field D, Hill C & Ross RP **(2020)** Nisin variants from *Streptococcus* and *Staphylococcus* successfully express in *Lactococcus lactis* NZ9800. **Accepted by Journal of Microbiology.**

6. **O'Connor PM,** O'Shea EF, Cotter PD, Hill C & Ross RP **(2018)** The potency of the broad-spectrum bacteriocin, bactofencin A, against staphylococci is highly dependent on primary structure, N-terminal charge and disulphide formation. *Scientific Reports* **8**, doi:Artn 11833 10.1038/S41598-018-30271-6.

7. Collins FW, **O'Connor PM,** O'Sullivan O, Rea MC, Hill C & Ross RP **(2016)** Formicin - a novel broad-spectrum two-component lantibiotic produced by *Bacillus paralicheniformis* APC 1576. *Microbiology* **162**, 1662-1671.

8. Sugrue I, **O'Connor PM,** Hill C, Stanton C & Ross RP **(2020)** Actinomyces produces defensin-like bacteriocins (Actifensins) with a highly degenerate structure and broad antimicrobial activity. *J Bacteriol* **202**, doi:10.1128/JB.00529-19.

Abbreviations

AGP	Antibiotic Growth Promoters
α	Alpha
Å	Angstrom
AMPs	Antimicrobial Peptides
AMR	Antimicrobial Resistance
APC	APC Microbiome Ireland Culture Collection
ATCC	American Type Culture Collection
BAGEL	Bayesian Analysis of Gene Essentiality
BCCM/LMG	Belgium Co-ordinated Collections of Microorganisms
β	Beta
BF	Bactofencin
BHI	Brain Heart Infusion
BLAST	Basic Local Alignment Search Tool
CFS	Cell Free Supernatant
cfu ml^{-1}	Colony Forming Units per millilitre
CHEF	Contour-clamped Homogenous Electric Field
Cm	Chloramphenicol
Da	Dalton
DA	Dopamine
DFM	Direct Fed Microbials
Dha	2, 3-Didehydroalanine
Dhb	2, 3-Didehydrobutyrine
DLT	D-Alanlyl Lipoteichoic Acid
DMF	Dimethylformamide
DNA	Deoxyribonucleic Acid
DPC	Dairy Products Research Centre
EDTA	Ethylenediaminetetra-acetic Acid
EFSA	European Food Safety Authority
ESI/LC/MS	Electrospray Ionization Liquid Chromatography Mass Spectrometry
FDA	Food and Drug Administration
FIC	Fractional Inhibitory Concentration
FMT	Fecal Microbiota Transplantation

GI	Gastrointestinal
GLASS	Global Antimicrobial Resistance Surveillance System
GM17	Glucose M17 media
GRAS	Generally Regarded As Safe
5-HT	5-Hydroxytryptamine
LAB	Lactic Acid Bacteria
LB	Luria-Bertani media
LMG	Laboratorium voor Microbiologie, Universteit Gent
LTA	Lipoteichoic acids
MALDI-	Matrix Assisted Laser Desorption Ionization-
TOF MS	Time of Flight Mass Spectrometry
MDR	Multidrug Resistant
MDRSA	Multidrug Resistant *Staphylococcus aureus*
µl	microliters
µm	micrometers
MIC	Minimum Inhibitory Concentration
min	minute
mm	millimetres
mRCM	modified Reinforced *Clostridium* Medium
MRD	Maximum Recovery Diluent
MRS	de Man Rogosa Sharpe media
MRSA	Methicillin Resistant *Staphylococcus aureus*
MS	Mass Spectrometry
MUSCLE	Multiple Sequence Comparison by Log Expectation
MW-SPPS	Microwave-assisted Solid Phase Peptide Synthesis
NaCl	Sodium Chloride
NICE	Nisin Controlled Gene Expression
NisA	Nisin A
NSR	Nisin Resistance Protein
NZ	Nizo
OD	Optical Density
ORFs	Open Reading Frames
PCR	Polymerase Chain Reaction
PFGE	Pulse Field Gel Electrophoresis

PI	Propidium Orange
PIF	Powdered Infant Formula
QPS	Qualitative Presumption of Safety
RCA	Reinforced *Clostridium* Agar
RepA	Replication protein A
RiPPs	Ribosomally Synthesised and Post-Translationally Modified Peptides
RP-HPLC	Reversed Phase High Performance Liquid Chromatography
RTE	Ready to Eat
SAR	Structure Activity Relationship
SPE	Solid Phase Extraction
SPPS	Solid Phase Peptide Synthesis
TFA	Trifluoroacetic Acid
TO	Thiazole Orange
TSB	Tryptic Soy Broth
VGS	Viridans Group streptococci
Vol/vol	Volume per volume
VRE	Vancomycin Resistant *Enterococcus faecalis*
WDA	Well Diffusion Assay
WHO	World Health Organization
WSLC	Weihenstephan *Listeria* Collection
WTA	Wall Teichoic Acids
Wt/vol	Weight per volume
XAD-BHI	BHI media clarified with Amberlite XAD prior to autoclaving

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Chapter 1.1

Antimicrobial antagonists against food pathogens: a bacteriocin perspective

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PM O'Connor, RP Ross, C Hill and PD Cotter

1.1.1 Abstract

Efforts are continuing to find novel bacteriocins with enhanced specificity and potency. Traditional plating techniques are still being used for bacteriocin screening studies; however, the availability of ever more bacterial genome sequences and the use of *in silico* gene mining tools have revealed novel bacteriocin gene clusters that would otherwise have been overlooked. Furthermore, synthetic biology and bioengineering-based approaches are allowing scientists to harness existing and novel bacteriocin gene clusters through expression in different hosts and by enhancing functionalities. The same principles apply to bacteriocin-producing probiotic cultures and their application to control pathogens in the gut. We can expect that the recent developments on bacteriocins from Lactic Acid Bacteria (LAB) described here will contribute greatly to increased commercialisation of bacteriocins in food systems.

1.1.2 Introduction

Consumer awareness of the effect of diet on health has led to a demand for minimally processed foods in which chemical preservatives are replaced by more natural alternatives. Traditionally foods were preserved by LAB, natural constituents of fermented foods, which confer their preservative effects by the production of lactic acid, hydrogen peroxide and small peptides known as bacteriocins. Bacteriocins are active against a number of genera (broad spectrum) or particular species (narrow spectrum) (1-3) and are very diverse, varying in size, structure and specificity. The fact that many bacteriocins are produced by food-grade LAB and possess potent antimicrobial activity means that they are ideally suited to controlling food spoilage and pathogenic bacteria (4-6).

Bacteriocins can be broadly divided into two classes: class I, of which the lantibiotics

(post-translationally modified peptides containing unusual amino acids) are the best-known example and class II, containing unmodified peptides (7). Their mode of action is likely driven by the primary structure of the bacteriocin with membrane permeabilisation being a very common theme. The producing culture is protected by the production of specific immunity proteins and the low levels of resistance detected so far makes them desirable alternatives to antibiotics (6). Their main advantage over chemical preservatives is their ability to preserve without affecting the sensory qualities of the food while adhering to the demand for natural preservatives. The ideal bacteriocin should be potent at low concentrations, active against a range of spoilage and pathogenic organisms, innocuous to the host and economical to produce (8). These antimicrobials can be introduced into a food through incorporation of the bacteriocin-producing strain into the food product (most commonly in fermented foods), the generation and use of a bacteriocin-containing fermentate or as a more concentrated bacteriocin-containing food preservative. Currently only two bacteriocins are being used commercially as food preservatives: nisin produced by *Lactococcus lactis*, (marketed as Nisaplin and under other brand names), has been used commercially for 50 years (9) and carnocyclin A (marketed as Micocin) a circular bacteriocin produced by *Carnobacterium maltaromaticum* UAL307 is an approved biopreservative in the US and Canada developed to inhibit *Listeria monocytogenes* in ready-to-eat (RTE) meat products (10). This review focuses predominantly on bacteriocins as antimicrobial antagonists and efforts to develop them as viable food biopreservatives. (See Figure 1)

1.1.3 The continuing search for novel bacteriocins

A primary focus of bacteriocin research is identifying novel bacteriocins and

bacteriocin-producing strains for specific applications. The general consensus is that the bacteriocin/bacteriocin-producer that is best suited to controlling a problematic spoilage/pathogenic microorganism will often be one that is found in the same environmental niche. This is based on the expectation that bacteriocins provide an advantage to competitors fighting for scarce resources in a particular environment. A prime example relates to *Weissella hellenica* QU 13, isolated from a barrel in which Japanese pickles were fermented, which was found to produce two leaderless bacteriocins, weissellicin Y, homologous to the class IId Enterocin L50A and L50B, and weisselicin M. In the latter case, it is notable that this novel broad-spectrum class IId antimicrobial is effective against *Bacillus coagulans*, a known contaminant of pickle fermentations. Thus, strain QU 13 is a good example of a fermentation-associated isolate which has the potential to be employed to control an undesirable microbial contaminant (11). *Lactococcus garvieae* is a pathogen affecting farmed and fresh fish from marine and freshwaters and is also considered an emerging zoonotic pathogen. Garvicin A, a novel class IIb bacteriocin produced by the human isolate *L. garvieae* 21881, inhibits other *L. garvieae* stains and has potential to treat or prevent *L. garvieae* infections. More specifically, it is suggested that the purified bacteriocin in combination with probiotic LAB would be useful in the fight against *L. garvieae* infections (12). Another *L. garvieae* strain, a fermented pork sausage isolate *L. garvieae* BCC 43578, produces garvieacin Q, a novel class IId bacteriocin active against other *L. garvieae* and *L. monocytogenes* (13). The ability to control *L. monocytogenes* is a particularly highly sought-after trait and it is thus notable that enterocin W, a two component lantibiotic produced by *Enterococcus faecalis* NKR-4-1 isolated from *pla-ra* Thai fermented fish (14), exhibits activity against this pathogen. Given that *Staphylococcus aureus* is also a major concern for the food industry, it is

interesting that bactofencin A, a cationic disulphide bond-containing bacteriocin similar to eukaryotic defensins, is active against *S. aureus*. In addition to the unusual nature of this bacteriocin, it is notable that its producer, the porcine isolate *Lactobacillus salivarius* DPC6502, does not contain a classical immunity-like gene, but instead encodes a *dltB* homologue that confers resistance (15). While the examples provided above relate to strains that produce a single bacteriocin, it should be noted that the production of multiple bacteriocins by a single strain can be advantageous as the various bacteriocins are likely to have different modes of action, thereby extending the spectrum of inhibition and reducing the likelihood of development of resistance. The genome of *Enterococcus faecium* NKR-5-3, isolated from *pla-ra* Thai fermented fish, encodes 5 enterocins, NKR-5-3 –A, B, C, D and Z and produces at least four of them, that is NKR-5-3 –A, B, C and D. Enterocin NKR-5-3C was confirmed to be a class IIa bacteriocin which exhibits potent antilisterial activity. The other bacteriocins are proposed to represent different classes but further investigations are required to establish this definitively (16, 17).

1.1.4 The particular expansion in numbers of circular bacteriocins

Although previously regarded as being rare, the discovery of circular bacteriocins has become more common in recent years. This is notable as these bacteriocins are thought by some to have the potential to form the next generation of biopreservatives as a consequence of their stability and activity. Indeed, gassericin A, garvicin ML, lactocyclin Q and leucocyclin Q produced by LAB inhibit a range of Gram-positive bacteria including food spoilage bacteria and food pathogens (18). The remarkable stability and activity of these bacteriocins is attributed to their head to tail cyclisation which confers the bacteriocins with increased protease and heat resistance (19, 20).

Garvicin ML is a recently discovered circular bacteriocin produced by *L. garvieae* DCC43 isolated from a Mallard duck which inhibits *L. garvieae* (21). Leucocyclin Q, produced by a Japanese pickle isolate *Leuconostoc mesenteroides* TK41401, is particularly active against *B. coagulans* which, as noted above, is a major pickle food spoilage organism (22). Studies relating to the mode of action of these, and indeed other, bacteriocins continue to also attract attention. Notably, in this regard, Liu *et al* (23) recently noted that sublethal doses of carnocyclin A induced an adaptation response in *L. monocytogenes* 08-5923 by affecting genes responsible for cell wall biosynthesis and metabolic function maintenance.

1.1.5 New studies relating to the use of bacteriocins as part of a hurdle approach to preservation

Bacteriocins can become more effective biopreservatives when used in combination with other antimicrobial hurdles such as organic acids, chelating agents or essential oils. These additive or synergistic phenomena act by reducing the levels of bacteriocin required for target inhibition and, in some instances, can even extend the spectrum of inhibition of bacteriocins to include Gram-negative microorganisms (2). *Cronobacter sakazakii* DPC6445 is an opportunistic Gram-negative pathogen associated with powdered infant formula (PIF) milk which has been associated with meningitis, septicaemia and necrotizing enterocolitis in premature and immunocompromised babies. Producing PIF that could be reconstituted at 40-50°C without risk of *C. sakazakii* infection is of interest to the food industry. Significantly, it has recently been established that nisin or lacticin 3147 when combined with the lactoperoxidase system inhibited *C. sakazakii* outgrowth for 8 hours, thereby providing an excellent example of a combinatory approach to improving the safety of PIF (24).

It has also been frequently demonstrated that using bacteriocins in combination with chelators such as ethylenediaminetetraacetic acid (EDTA) can expand the antimicrobial spectrum of a bacteriocin. Indeed, although carnocyclin A is not effective against *Escherichia coli*, *Pseudomonas aeroginosa* or *Salmonella* Typhimurium when tested alone, it can inhibit *E. coli* and *P. aeruginosa* when combined with 40 mM EDTA. Anti-*E. coli* and *S.* Typhimurium activity could be improved even further when nisin, rather than carnocyclin A, was combined with 40 mM EDTA (10).

Bacteriocins can also be utilised by applying them to a food surface. Due to lower concentrations being sufficient for efficacy in these circumstances, production costs are reduced. The use of immobilised bacteriocins, such as nisin, as components of antimicrobial packaging has been the focus of increasing levels of research, though it is important to appreciate that understanding the mode of action of specific bacteriocins is important to ensuring further progress in the area. In one instance, nisin was absorbed on both hydrophobic and hydrophilic food films and the effectiveness of the active surface against *L. monocytogenes*, *Bacillus cereus* and *S. aureus* was compared. It was established that the hydrophilic surfaces were more bioactive and absorbed higher quantities of nisin than the hydrophobic surfaces and that *S. aureus* was most sensitive to the nisin functionalised films (25). Class IIb lactocin 705 and the pediocin-like class IIa lactocin CL705 also possess potential in this regard. These *Lactobacillus curvatus* CRL705-produced bacteriocins are active against spoilage LAB and *Listeria* and have been incorporated into wheat gluten films to assess their ability to inhibit *L. monocytogenes* in meat products. The bacteriocin-containing gluten film, made at pilot scale, retained antimicrobial activity for 50 days which, importantly, is the shelf life of RTE meat products such as cooked sausages (26). More

specifically, the film reduced *L. monocytogenes* levels in Wiener sausages at day 45 by 2.5 log cycles relative to controls (27). In addition to food surfaces, the surfaces of equipment can also serve as a site for the contamination of food by food spoilers and pathogens such as *L. monocytogenes*. Many such bacteria can colonise surfaces such as stainless steel and form biofilms. Biocides are routinely used to clean processing equipment but biofilms can be particularly difficult to remove. It has recently been established that combining sub-inhibitory concentrations of the class IIc enterocin AS-48 with concentrations of biocides 4-10 fold lower than their MICs inhibited the growth of planktonic (non-biofilm) *L. monocytogenes*. Unsurprisingly, higher concentrations of both bacteriocin and biocide were required to inhibit sessile cells though synergy was still observed (28). Proteomic analysis of the exposure of *L. monocytogenes* to enterocin AS-48 revealed that planktonic and sessile cells respond differently upon exposure to the bacteriocin. Planktonic cells may compensate for changes in cytoplasmic permeability by reinforcing carbohydrate transport and metabolism while sessile cells shift carbohydrate metabolism and reinforce protein synthesis. Both cells states also exhibit a differing response to stress (29).

1.1.6 Bacteriocin engineering

Bacteriocins are ribosomally synthesised and therefore are amenable to genetic manipulation through engineering, which is defined as modifying the amino acid sequence of a protein to change its structure and function (30). Bioengineering (engineering inside the cell) and the use of synthetic biology-based (*in vitro* engineering) approaches have contributed significantly to our understanding of the roles specific amino acids play in structure and activity and resulted in the production of bacteriocins which have extended bioactivity against selected pathogens (31). The

structure-activity relationship of nisin has been extensively studied through bioengineering and this has enabled researchers to design variants with enhanced activity against specific targets. Nisin S29G, with enhanced activity against *S. aureus* SA113, was found by screening a bank of nisin A variants following site-directed mutagenesis specifically targeted against this residue. This resulted in the generation of a number of variants with improved activity against both Gram-positive and Gram-negative pathogens. Indeed, this is the first instance upon which bioengineering of a bacteriocin has led to enhanced activity of this kind (32). Saturation mutagenesis at another location in nisin, lysine 12, resulted in the finding that a K12A derivative displays increased specific activity against food pathogens such as *B. cereus*, *S. aureus* and *S. agalactieae* but not against *L. monocytogenes* (33). Another region of the nisin peptide, the three amino acid 'hinge' region, is particularly amenable to change and bioengineering of this region has had beneficial consequences (34). Indeed, Rouse *et al*. (35) created a bank of hinge mutants and found that nisin peptides containing hinges consisting of SVA or NAK (rather than the original NMK) displayed an enhanced ability to diffuse through complex polymers, a trait which enabled the variants to outcompete nisin A controlling *L. monocytogenes* in commercially produced chocolate milk containing the stabiliser carrageenan. Furthermore, Healy *et al.* (36) used site-directed mutagenesis of the hinge region to create a novel bank of nisin derivatives and found that AAK, NAI and SLS had enhanced activity towards some microorganisms. On the basis of the observation that the incorporation of small, chiral amino acids at this location generally has positive consequences, AAA-containing and SAA-containing 'hinge' derivatives were designed, created and ultimately became the first example of enhanced nisin derivatives to be generated through rational design.

In the case of another lantibiotic, actagardine A, saturation mutagenesis was employed

to engineer each amino acid, with the exception of those involved in bridge formation, in turn through using saturation mutagenesis. Through this approach it was established that the V15F variant demonstrates enhanced activity against *Clostridium difficile*, *E. faecium* and *E. faecalis* (37). The ribosomal nature of bacteriocins also allows for more dramatic changes. To highlight this point, the anti-Gram-negative microcin V was combined, through asymmetrical PCR, with the anti-Gram-positive enterocin 35 to generate the chimeric bacteriocin Ent35-MccV which is active against both Gram-positive and Gram-negative pathogens and thus could be of value to the food or pharmaceutical industries (38). Finally, it is now possible to bioengineer circular peptides by introducing a covalent bond between the N and C termini using advances in molecular biology and protein engineering techniques (30). Theoretically these techniques could allow the generation of more stable bacteriocins with extended applications that could be employed by the food industry. Synthetic biology, considered complementary to bioengineering, is another promising area that provides insights into structure-stability relationships and the mechanism of action of bacteriocins (39, 40). In one instance, Solid Phase Peptide Synthesis (SPPS) has been used to synthesise and modify lantibiotics such as lacticin 481. Using this approach, the role of lanthionine and methyllanthionine residues was investigated by replacing them with diastereoisomers. In this case it was established that activity was lost, suggesting that the 3D structures were modified (41). Synthetic biology also inspired Kong *et al* (42) to clone the nisin biosynthesis pathway from *Lactococcus lactis* K9 into a plasmid and express it in a nisin-deficient strain. They also overexpressed nisin A using constitutive promoters and further optimised yield by integrating the structural peptide determinant *nisA*, overexpression cassettes and the recombinant pathway into a single circuit enabling the strain to produce 6 fold higher levels of nisin. This could

potentially reduce the cost of nisin production for the food industry and also provides a means via which novel bacteriocin clusters identified through genome mining (see below) could be harnessed. Further efforts to increase bacteriocin yield have led to the use of synthetic genes encoding bacteriocins being cloned into and expressed in yeasts. A synthetic gene designed using adapted codon usage from the amino acid sequence of enterocin A from *E. faecium* T136 was cloned into *Pichia pastoris* X-33EAS and production levels increased 21.4 fold and antimicrobial activity against a number of listeria strains increased 4-603 fold when compared to the natural producer (43).

1.1.7 Genome mining

In the past, bacteriocin-producing strains have been identified primarily on the basis of culture-based approaches. However, traditional plating techniques will reveal bacteriocin-producing cultures only if the culture produces the bacteriocin under the conditions used for laboratory growth and only if it is effective against the target organism chosen for the overlay. Recently there has been a move to supplement traditional mining techniques with exploring the genomes of microorganisms from under-exploited environments which could be a reservoir of novel bacteriocins. The number of genome sequences being deposited in public databases is continually increasing as a consequence of significant developments in next generation sequencing technologies. This information is often freely available through online databases and provides an opportunity for screening a wide number of microorganisms to identify those which have the potential to produce bacteriocins (44, 45). This is seen as the dawn of a new era in which *in silico* and bioengineering based approaches can complement, and potentially supersede, culture based methods (45). Despite this potential, finding bacteriocin genomes can be a challenge due to the small size of the

structural peptides and diversity of their operons. BAGEL 3 is a fast genome mining tool that can identify putative bacteriocins based on conserved domains in structural, biosynthetic, transport and immunity genes (46). In addition the BACTIBASE database is a manually curated repository of bacteriocin sequences that can also be helpful. (47). Mass spectrometry is also being used more often in the quest for novel bacteriocins. Natural Product Peptidogenomics is a mass spectrometry based genome mining approach that connects chemotypes with biosynthetic gene clusters, the objective being to match a series of mass shifts from MS^n spectrum of a putative bacteriocin to the genes responsible for production (48). Zendo and co-workers (49, 50) developed a rapid screening method using electrospray ionisation liquid chromatography/mass spectrometry (ESI/LC/MS) coupled with statistical analysis of antimicrobial spectra to accelerate the discovery of novel bacteriocins isolated from various sources. An example of a novel lantibiotic that has recently been discovered using a genome mining and PCR approach is the broad-spectrum cerecidin A1 and cerecidin A7 from *B. cereus* strain As 1.1846 isolated from spoiled soya milk. The *cer* locus differs from other class II lantibiotics in that it contains seven tandem precursor *cerA* genes and the cerecidins are notably active against multidrug resistant *S. aureus* (MDRSA) and vancomycin resistant *E. faecalis* (VRE) (51).

1.1.8 Probiotics

Finally, over the last few years there has been growing evidence that bacteriocin production confers a number of advantages on probiotic strains. It is proposed that the ability to produce bacteriocins may help a strain to establish itself in a new niche, inhibit competitors and pathogens, alter the composition of the microbiota and even modulate the host immune system (52). A recent study of the gut microbiota of elderly

Irish subjects revealed *Enterococcus* strains with anti-listerial activity, which merit closer attention with a view to investigating their use as probiotic strains. In addition, a *Lactobacillus gasseri* strain producing gassericin T was isolated during the same screening programme (53). Notably, *Lb. gasseri* bacteriocins are very active against Gram-positive pathogens and have potential as food preservatives due to their heat stability and pH stability. *Lb. gasseri* have been evaluated as probiotics and these investigations have also highlighted its tolerance of low pH environments, resistance to bile salts, ability to adhere to the host epithelium and modulate the innate and adaptive immune system (54). There have also been a number of recent studies that have highlighted the impact of the Abp118 bacteriocin by *Lactobacillus salivarius* UCC118 on the overall composition of the gut microbiota and on the host epithelium (55-57). Finally, a study of LAB associated with fish for human consumption showed that bacteriocin activity against fish pathogens is a widespread probiotic property. Indeed LAB active against lactococcosis were common among LAB isolated from edible fish, further supporting the theory that the best place to find antimicrobials against a specific pathogen is in the niche the pathogen proliferates (58).

1.1.9 Conclusion

In conclusion, there is a continued drive to find novel bacteriocins that can control food pathogens more effectively. Novel LAB bacteriocins continue to be discovered and the use of LAB that produce multiple bacteriocins is receiving renewed attention. These screening programmes are being aided by the use of genome mining and mass spectrometry to find and characterise new bacteriocins while new engineering-based approaches are being used in parallel to improve previously identified bacteriocins for particular applications /targets. There is great potential to carry out investigations that

would assess the impact of bacteriocins on entire food microbial consortia as has been done previously to assess the impact of bacteriocins on gut microbial populations (56, 59).

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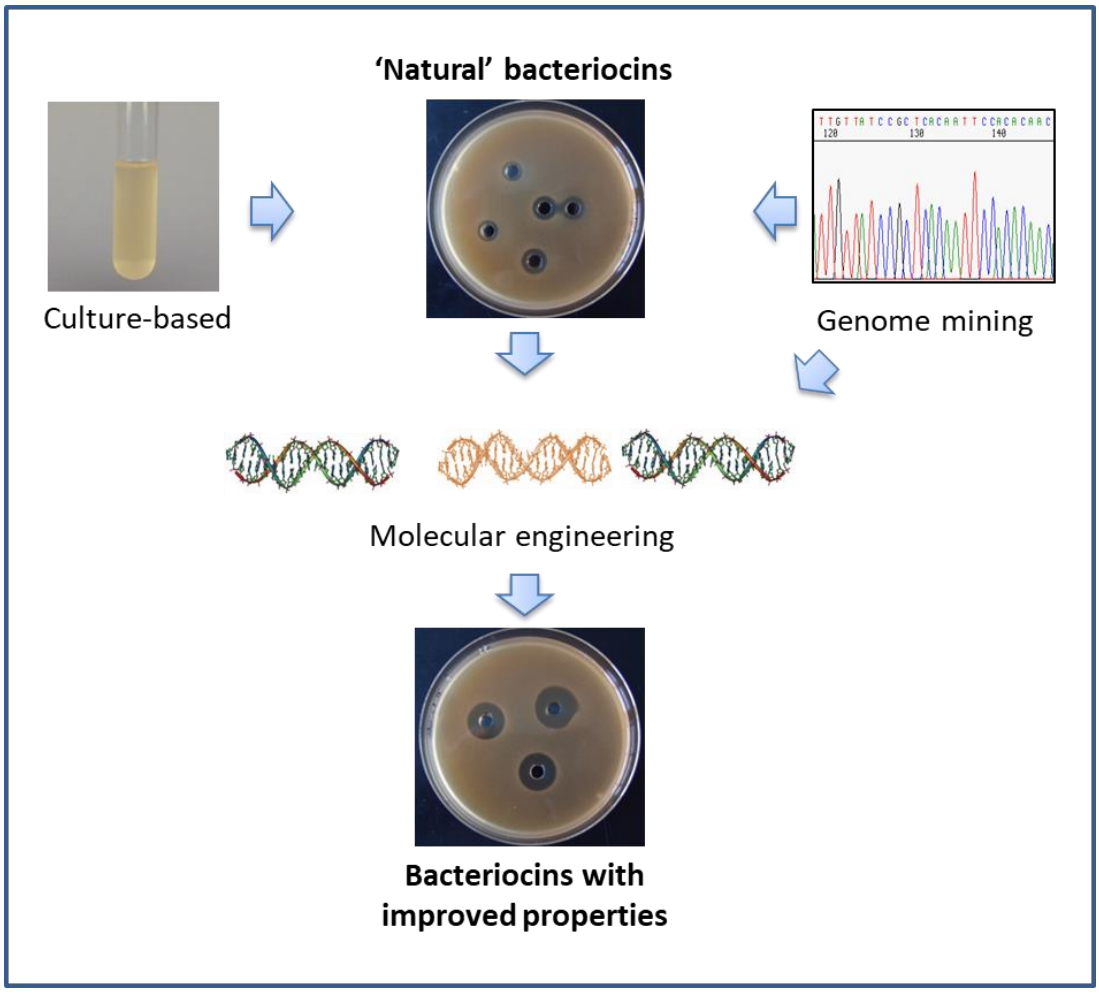
Figure 1 Enhancement of bacteriocin functionality using genome mining and molecular engineering techniques.



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Chapter 1.2

Antimicrobials for food and feed: a bacteriocin perspective

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PM O'Connor†, TM Kuniyoshi†, RPS Oliveira, PD Cotter, C Hill and RP Ross.

† Joint first authorship

1.2.1 Abstract

Bacteriocins are natural antimicrobials that have been consumed via fermented foods for millennia and have been the focus of renewed efforts to identify novel bacteriocins, and their producing microorganisms, for use as food biopreservatives and other applications. Bioengineering bacteriocins or combining bacteriocins with multiple modes of action (hurdle approach) can enhance their preservative effect and reduce the incidence of antimicrobial resistance. In addition to their role as food biopreservatives, bacteriocins are gaining credibility as health modulators, due to their ability to regulate the gut microbiota, which is strongly associated with human wellbeing. Indeed the strengthening link between the gut microbiota and obesity make bacteriocins ideal alternatives to Antibiotic Growth Promoters (AGP) in animal feed also. Here we review recent advances in bacteriocin research that will contribute to the development of functional foods and feeds as a consequence of their roles in food biopreservation and human/animal health.

1.2.2 Introduction

Fermented foods have been part of the human diet for thousands of years and evolved through the need to extend shelf life and improve food safety via the inhibition of food spoilage/pathogenic microorganisms (1, 2). Lactic acid bacteria (LAB) are natural constituents of many fermented foods and contribute greatly to food biopreservation. LAB exert their preservative effects through the production of antimicrobial metabolites including organic acids, diacetyl, ethanol, hydrogen peroxide and bacteriocins. Bacteriocins are a heterogeneous group of ribosomally-synthesised antimicrobial peptides with the ability to kill closely-related (narrow spectrum) or a diverse range of (broad spectrum) microorganisms (3). Bacteriocins are frequently

very potent, being active at nanomolar concentrations, and exert their killing effect predominantly through membrane permeabilisation. They are broadly divided into two classes with Class I containing Ribosomally synthesised and Post-translationally modified Peptides (RiPPs) and Class II containing predominantly unmodified peptides (4). Bacteriocins are produced by Generally Regarded as Safe (GRAS) or Qualitative Presumption of Safety (QPS) organisms and are often sensitive to human proteases. Bacteriocins are now the focus of increased attention i) due to consumer requirements for minimally processed foods free from chemical additives (5) ii) due to their potential as natural alternatives to antibiotics due to increasing concerns about the emerging problem of antimicrobial resistance (4, 6) iii) as modulators of the human microbiome and, therefore, potential to address complex metabolic conditions such as diabetes and inflammatory bowel disease (7), and iv) as bacteriocin-producing probiotic cultures for inclusion in animal feed to promote growth, improve animal health and/or reduce infection (8) (Figure 1).

1.2.3 Bacteriocins as food biopreservatives

Bacteriocins with optimal potential as biopreservatives are safe for human consumption, have minimal effects on the human microbiota and are effective against food pathogens/spoilage microorganisms. They are also stable in the food matrix in which they are employed, which may require resistance to heat, pH and food associated enzymes (9). Bacteriocins can be added to foods in three ways; i) as a pure bacteriocin preparation ii) as bacteriocin-containing fermentates or iii) as bacteriocin-producing cultures (4, 10).

Nisin A is a broad-spectrum Class I lantibiotic, produced by *Lactococcus lactis*, characterized by five intermolecular lanthionine rings that confer inherent heat and

protease stability. It is the most studied bacteriocin and it is the only commercially produced bacteriocin approved as a food additive by regulatory agencies including the World Health Organization (WHO)/Food and Drug Administration (FDA) in the USA and the European Food Safety Authority (EFSA) in Europe (11). It was first produced in England in the 1950s (1) and is now available as, for example, Nisaplin (2.5% nisin) (www.dupontnutritionandsciences.com)(11). Nisin Z, a His27Asn variant of nisin A, with greater solubility at higher pH thereby extending its usefulness for food applications, is also commercially available as, for example, Nisin Z®P ultrapure nisin (>95% nisin) (www.handary.com). The ability to bioengineer nisin has led to a number of nisin variants with improved capabilities, perhaps the most notable of these being nisin V, a Met21Val variant, which has improved activity against a variety of foodborne pathogens, including *Listeria monocytogenes* and *Bacillus cereus* (12). Nisin variants can be bioengineered through food-grade techniques involving double crossover mutagenesis which do not introduce exogenous DNA or antibiotic resistance markers. When made in this way, the producing strains are not regarded as genetically modified microorganisms by the EFSA under contained use legislation. This opens the possibility to custom design nisin for specific applications by increasing yield, increasing potency against specific targets or expanding its spectrum of inhibition thereby increasing its commercial potential as food biopreservatives (13).

Bacteriocin containing food-grade fermentates are also commercially available and widely used in the food industry. These include the FDA approved MicroGARD™ range from Danisco and ALTA 2431 from Quest International and both contain pediocin PA-1 produced by *Pediococcus acidilactici* (5).

Bacteriocin-producing cultures used as starter cultures or as adjunct cultures serve a dual purpose as they can contribute to both flavour and food safety, providing

fermentation and preservation simultaneously. This is more cost effective than using pure peptide and is subject to less regulatory control (9). Examples include the Bactoferm™ range (www.chr-hansen.com), containing pediocin and sakacin producing strains, used to make fermented sausages and dry cured meat and HOLDBAC® protective cultures (www.dupontnutritionandsciences.com) containing a mix of bacteriocin-producing strains used to protect seafood, meat and dairy products from *Listeria*, yeasts and moulds (9). Micocin® is a specifically designed protective culture with potent activity against food spoilage and pathogenic microorganisms in ready to eat meat products and approved for use in the US and Canada (14). It contains *Carnobacterium maltoaromaticum* which produces piscicolin 126 and carnobacterium BM1 and the circular bacteriocin, carnocyclin A, that is particularly potent against *L. monocytogenes* (15). Including Micocin® as a feed additive in the diet of Grimaud rabbits resulted in reduced levels of *L. monocytogenes* in ground meat during storage indicating that including a protective culture in animal diets resulted in safer food products (16).

Recently discovered novel bacteriocins with potential as food preservatives include plantaricyclin A, a circular bacteriocin produced by the olive isolate *Lactobacillus plantarum* NI326, with activity against the beverage spoilage bacterium *Alicyclobacillus acidoterrestris*, which causes significant economic losses to the food industry every year (17). *Enterococcus mundtii* CRL35, a non-virulent, non-antibiotic resistant strain, also shows promise as an adjunct culture. It reduces *L. monocytogenes* during meat fermentation, both *in vitro* and in a beaker sausage model in the presence of curing agents, due to production of enterocin CRL35, a class IIa bacteriocin. Bacteriocin-producing strains are adversely affected by the presence of curing salts so the ability of *E. mundtii* to grow and exert a higher protective effect in fermented meats

is particularly advantageous (18). Gómez-Sala et al (2016) found that the use of the multibacteriocinogenic strain *Lactobacillus curvatus* BCS35 as a protective culture, and of its cell free supernatant used as a food ingredient during refrigerated storage, significantly reduced bacterial counts on fresh fish, thereby increasing the both the quality and commercial value of the product (19). Another exciting development in recent years is the use of antimicrobial-containing edible films and coatings, composed of layers of biopolymers that protect the food from the environment, to improve food safety by inhibiting food pathogens during handling, transportation and storage of food products (5, 20).

1.2.4 Antimicrobial resistance

A recent WHO report highlights concerns about the lack of progress in the search for new antimicrobial classes and calls for increased investment in drug discovery to combat the threat of antimicrobial resistance (6, 21). Bacteriocins are considered promising alternatives due to their stability (especially in the case of modified peptides such as the lantibiotics), low toxicity, frequently excellent potency and potential for target specificity. Many bacteriocins interact electrostatically with the cell membrane and introduce permeabilisation through interaction with receptor or docking molecules. Resistance can occur due to innate mechanisms, including the ability to produce degradation enzymes or the presence of immunity proteins, while acquired resistance occurs due to horizontal gene transfer or gene mutations that alter the cell membrane, binding receptors or transport systems (22, 23). Previously described resistance mechanisms include specific adaptations such as the loss of a receptor, as seen in resistance to class IIa bacteriocins like pediocin, or non-specific adaptations that alter the cell envelope, as seen in the case of resistance to Class I lantibiotics such

as nisin (10, 22, 24, 25). Radical adaptations requiring high energy costs that reduce the fitness of the cell may limit the ability of resistant mutants to compete in established niches, possibly explaining why bacteriocin resistance is rarer than antibiotic resistance (24, 26, 27). Knowledge of a bacteriocin's mode of action (4) and how it acquires resistance facilitates the development of methodologies to minimise resistance occurrence (27). Strategies successfully used to reduce resistance include combining bacteriocins with other bacteriocins with different modes of action (23, 28, 29), other antimicrobials (21, 25), or phages or, generating peptides with increased antimicrobial resistance through bioengineering (30). These hurdle (combinatorial) approaches have the added advantages of broadening the antimicrobial spectra while reducing costs and toxicity (25). Indeed, Perales et al (2018) found that a combination of enterocin AS-48 and nisin A acted synergistically to kill antibiotic resistant staphylococci, a common contaminant in processed food, in fresh goat milk cheese potentially improving its shelf life and safety. Using multiple bacteriocins reduces the bacteriocin dose and prevents the regrowth of bacteriocin resistant/adapted cells (21). Mills et al (2017) also used a multibacteriocin approach to develop a cheese starter system producing both nisin A and lacticin 3147. The use of these, in combination with a *Lactobacillus plantarum* Class II plantaricin producer, reduced *Listeria* numbers in lab scale cheese more effectively than when individual bacteriocin producers were used singly. The concurrent production of nisin A and lacticin 3147 reduces the likelihood of incidence of bacteriocin resistance and this approach shows great potential for food safety applications (31).

A bioengineering approach was used to overcome the efficacy of nisin resistance protein (NSR), expressed by some microorganisms, that cleaves nisin between residue 28, involved in ring E formation, and serine 29, resulting in a truncated nisin 1-28 with

significantly reduced activity (30). A screening study located a nisin Ser29Pro variant with 20 fold increased activity against a NSR^+ strain and a similarly resistant nisin PV variant that was less affected by oxidation (30). Recently, a survey of 182 *Lactobacillus* strains revealed a high level of intrinsic antimicrobial resistance genes, with resistance to kanamycin, vancomycin and trimethoprim being most prevalent (32). Eighty eight per cent of the strains surveyed would fail EFSA regulatory guidelines, despite them being species widely used in foods for human and animal consumption, as the presence of antimicrobial resistance genes impacts on their use in food applications. These findings led the authors to call for revision of EFSA regulatory guidelines for lactobacilli entering the food chain and highlight that a more thorough understanding of antimicrobial resistance and its spread within microorganisms is required (32). Overall, the general consensus is that bacteriocins, like antibiotics, should be used exiguously to avoid selection of resistant phenotypes that may compromise their potential role as biopreservatives (22,24,25).

1.2.5 Bacteriocin-producing probiotic strains as gut microbiome modulators

The role of the gut microbiota in human health is of increasing interest as the links between a balanced, healthy gut microbiota and disease prevention become more apparent (23, 33). Broad-spectrum antibiotics indiscriminately affect the entire microbiota, leading to imbalances that could potentially predispose to conditions such as obesity, diabetes, immune disorders and neurodegenerative disease (29, 34, 35). Bacteriocin-producing LAB are antibiotic alternatives that have the potential to enhance gut health through their ability to survive the gut environment, inhibit pathogens and competitors, modulate the immune system and prevent inflammation and oxidative stress (33, 34, 36, 37). Considerable efforts are being made to understand

the contribution of bacteriocins produced by LAB to gut modulation, pathogen inhibition and their role in the maintenance of host health. To this end, an *in vitro* faecal fermentation system that mimics the anaerobicity of the colon was used to assess the effect of bactofencin A, a class IId bacteriocin produced by the porcine gut isolate *Lactobacillus salivarius*, on the human faecal microbiota. The study found subtle but positive differences in taxonomic profiles between the bactofencin A^+ producing culture and its bactofencin A^- mutant, while more drastic effects in taxonomy were detected in the presence of pure peptide (38). Similarly, *in vivo* studies using mice fed with a *L. salivarius* UCC118 bacteriocin producer, Bac^+, or its bacteriocin-negative, Bac^-, derivative over 8 weeks resulted in slight changes in the gut microbiota at the Phylum level. Whereas at the genus level, the Bac^+ treatment resulted in a significant increase in *Bacteroides* spp. and decrease in *Bifidobacterium* spp. in comparison with the Bac^- group (39). Efforts to assess if bacteriocin production *in vivo* inhibits gut pathogens without negatively impacting beneficial populations require rigorous experimental methods to provide meaningful results (40). Bauer et al (2017) describe a generic method, using compositional 16S rDNA combined with bioinformatics, to compare the effect of bacteriocin producers to their isogenic non-producing equivalents on microbiota composition in a mouse model allowing impacts on the gut microbiota to be measured in a live animal model (36). This model was used in a mouse feeding trial, where five Class II bacteriocin-producing LAB were compared with isogenic non-producing equivalents. The trial showed that while the overall diversity was unchanged, advantageous changes relating to pathogen inhibition and increased LAB levels were seen briefly, suggesting that bacteriocin production facilitated favourable changes without collateral damage to the gut microbiota (41). These studies provide further evidence that bacteriocin production provides subtle positive changes

at lower taxonomic levels that maintain a desirable gut microbiota and are beneficial to the host (7).

Bacteriocins, unlike antibiotics, are often very specific and can kill pathogens without causing detrimental imbalances to the host microbiota. Vancomycin resistant enterococci (VRE) are gut inhabitants that can cause fatal infections, particularly in a hospital environment. A recent report by Kim et al (2019) describes the ability of gut commensals to increase the resistance of the host to vancomycin resistant *Enterococcus faecium* (VREf). They found that one constituent of a four strain cocktail, *Blautia producta* BP_{SCSK}, a nisin A variant producer, was responsible for reduced colonisation by VRE. A direct correlation was found between the amount of the lantibiotic gene and VRE reduction in germ free mice containing patient feces, thus demonstrating the potential of bacteriocins as antibiotic alternatives (42).

The gut microbiota also enables the gastrointestinal tract and the brain to communicate through the gut brain axis, which is described as a neuroendocrine signalling system that transmits information through endocrine signals, neurons and the immune system (34). A recent microbiota-gut-brain–axis study demonstrated that nisin increased duodenal levels of the neurotransmitter serotonin (5-hydroxytryptamine, 5-HT) and dopamine (DA) in a bacterial diarrhoea mouse model induced by pathogenic *E. coli* O_1. In addition, nisin increased the bacterial diversity in the mouse cecum samples by increasing beneficial *Lactobacillus, Bacteroides* and *Bifidobacterium* species while inhibiting pathogenic *E. coli* and *Enterococcus* spp. Taken together, the results show a positive correlation between nisin, the gut microbiota and stress reduction triggered by *E. coli* induced diarrhoea in mice, suggesting that probiotics can both regulate the gut microbiota and affect the expression of neurotransmitters in the brain (43).

1.2.6 Genome mining studies reveal that the gut microbiota is a rich source of bacteriocin genes

In silico genome mining techniques are routinely used to identify bacteriocin gene clusters in bacteria from numerous sources including the commensal mammalian microbiota. An in-depth look at human commensal metagenomic sequences found that the number of putative bacteriocin genes varied according to body site, with a higher proportion found in the mouth, airway and vagina and lowest in the gut (44). However, analysis of genomes specifically from the human gut revealed that almost half, predominantly from LAB, encoded putative bacteriocins and are proposed to aid diversity through establishment of commensal relationships with the host and aid host defence by inhibiting pathogens (45). *In silico* analysis of genomes from rumen bacteria found numerous novel sactipeptide and lanthipeptide bacteriocin gene clusters suggesting that the rumen is a rich source of novel antimicrobial peptides with potential as food preservatives and use in animal production (46). A more recent metagenomic functional screening of the rumen metagenome identified 181 previously unidentified antimicrobial peptides, three of which (Lynronne-1, 2 and 3) were shown to have activity against methicillin resistant *Staphylococcus aureus* (MRSA) and other pathogens. Interestingly, MRSA did not produce resistant mutants when subcultured in sub MIC levels of these peptides over 25 days (47).

Shotgun sequencing of the gut microbiome allows identification of microorganisms to species or strain level and even detects genes related to antibiotic resistance, vitamin production or short chain fatty acid production. However, advances in sequencing-based microbiome profiling methods, such as metatranscriptomics, can go further to assess gene expression, thus providing an accurate method to determine which genes are expressed by the microbiome. This technique is capable of generating large-scale

profiles of complex microbiomes and is expected to improve our understanding of the role that bacteriocins play in gut ecology (48).

1.2.7 Bacteriocin-producing probiotic cultures as antimicrobials in animal feed

Since the 1930s, antibiotics have been used in animal husbandry to treat infections, prevent disease and improve feed efficiency (49). Antibiotic use in global food production is increasing worldwide to meet the growing demand for animal protein and now surpasses human consumption. Indeed, in some countries, it has been reported that 80% of antibiotics important for human medicine are consumed by healthy animals to promote growth (50, 51). Concerns about increases in drug resistance in animal pathogens and its potential transfer from livestock to humans, leading to untreatable infections, has led to the WHO introducing a Global Action Plan on Antimicrobial Resistance highlighting the need for a concerted international approach from consumers, environmentalists, agriculture, human and veterinary medicine to combat this growing crisis (51-53). In 2006, the European Union banned the use of animal growth promoters (AGP) in animal feed, creating a need for a new antimicrobial strategy. A pioneering study by Corr et al (2007) found that production of Abp118 by *L. salivarius* UCC118 protected mice *in vivo* from the food pathogen *L. monocytogenes*, thus confirming the antimicrobial potential of bacteriocin-producing probiotic cultures (54). Since then, there have been numerous studies, predominantly *in vitro*, providing evidence that bacteriocins are potential alternatives to antibiotics in animal production (53). Recently, Hu et al (2018) showed that a faecal microbiota transplantation (FMT) from diarrhoea resistant to diarrhoea susceptible pigs protected against early weaning diarrhoea induced by stress, a huge problem in the swine industry. Further investigation revealed that the protective effect was attributable to

the presence of *Lactobacillus gasseri* and *Lactobacillus frumenti.* More specifically, this effect was due to their ability to produce the circular bacteriocin, gassericin A, which binds to the pig's intestinal epithelial membrane, thus preventing diarrhoea onset and providing further evidence that probiotic cultures have potential as antibiotic alternatives for diarrhoea prevention in mammals (55).

The ability of AGP to improve growth and body weight gain of animals is tentatively attributed to modulation of the gut microbiota as it plays an important role in obesity (33). Direct Fed Microbials (DFM) such as probiotic LAB are associated with weight gain in animals (8) and tentative links are being established between feed efficiency in pigs and the intestinal microbiota (56). This makes it tempting to suggest that bacteriocin-producing LAB can be used to modulate the gut microbiota in a way that improves feed efficiency. Nisin has been proposed as a feed supplement for broiler chickens as preliminary experiments suggest that it improves body weight gain in a dose-related manner, an effect that may be due to gut microbiota modulation (57).

One of the challenges for bacteriocins in feed applications is the ability to pass through the gastrointestinal tract without digestion by proteolytic enzymes. This can be achieved through encapsulation, a protective technique that ensures successful delivery to the target site without loss of bioactivity where they can be released in a controlled fashion (20). Both nisin A and bactofencin A were recently successfully encapsulated in mesoporous matrices, with nisin A being protected from degradation by pepsin and bactofencin A by trypsin (58, 59). Bioactive intact nisin and nisin fractions were detected in the feces of mice pellets following feeding with nisin encapsulated in starch-based matrices, therefore achieving the aim of delivering intact nisin to the gut by oral means (60). The amount of nisin detected in the feces varied with starch matrix, highlighting that optimum delivery requires examination of a range

of substrates and conditions. These preliminary studies show that bacteriocins are effective in a gut environment.

1.2.8 Conclusions

Current research is strengthening the view of bacteriocins as being versatile antimicrobials with considerable potential for use as biopreservatives, antibiotic alternatives, health-promoting gut modulators and animal growth promoters. Excessive use of antibiotics, and especially of broad-spectrum antibiotics, in medicine and food production has been recognised as a cause of microbiome disruption and select for accumulation and transfer of resistance genes within the microbial population of the human gut (35). Overall, though bacteriocins are likely expressed at low levels in the gut, it is considered that their production by gut commensals enables a healthy and stable microbiome by preventing invasion by undesirable species (44) and the establishment of desirable microbes.

The use of DFM as alternatives to AGP is a relatively new area of research that shows promise for bacteriocin-producing LAB as initial studies show that bacteriocins are also effective in the animal gut. While obtaining approval from FDA and EFSA or other agencies to utilise bacteriocins within feed additives for animal nutrition is a lengthy process (61), their potential to play a role beyond that of biopreservative is notable, with a number of studies describing them as versatile health promoter molecules (11, 26).

The incorporation of bacteriocin-producing probiotics into foods and feeds as well as assuring their activity during processing and subsequent passage through the host's gastrointestinal tract are challenges that are being addressed through the discovery and development of new bacteriocin-producing strains and novel encapsulation techniques

(58-60, 62). Commercial scale bacteriocin production is still hampered by high costs and low peptide yield but cost efficiency is being improved through optimisation of fermentation processes and bioengineering strains for maximum bacteriocin production (63). It should also be noted that further studies are also required to establish dosage levels and to further improve effective delivery to target sites.

Overall, the expanding potential role of bacteriocins in food preservation, gut modulation, antimicrobial resistance reduction and animal feed suggest that, if the hurdles described are overcome, there are considerable opportunities for widespread bacteriocin-based applications in the food and feed industries.

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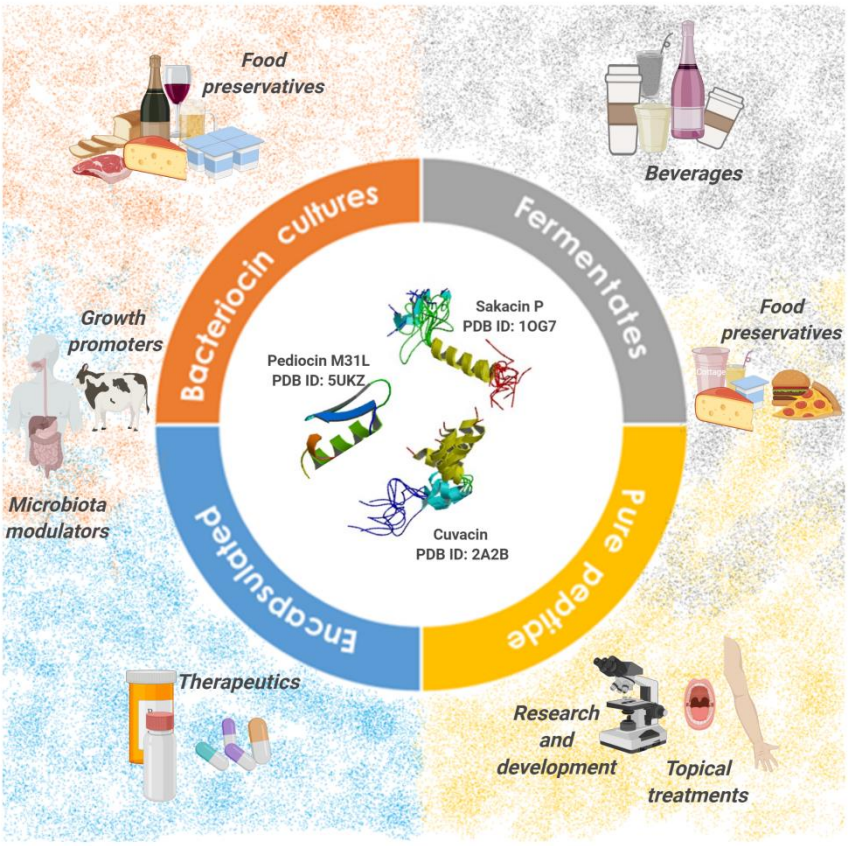
Figure 1 Potential applications of i) bacteriocin-producing cultures, ii) bacteriocin-containing fermentates, iii) purified bacteriocins and iv) encapsulated bacteriocins as food preservatives, gut modulators, feed additives and therapeutics.
Created with BioRender.com.PDB ID 2A2B (10.2210/pdb2a2b/pdb), PDB ID 1OG7 (10.2210/pdb1OG7/pdb), PDB ID 5UKZ (10.2210/pdb5UKZ/pdb).



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Chapter 2.1

Nisin H is a new nisin variant produced by the gut-derived strain *Streptococcus hyointestinalis* DPC6484

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PM O'Connor, EF O'Shea, CM Guinane, Ó O'Sullivan,

PD Cotter, C Hill and RP Ross.

2.1.1 Abstract

Accumulating evidence suggests that bacteriocin production represents a probiotic trait for intestinal strains to promote dominance, fight infection, and even signal the immune system. In this respect, in a previous study, we isolated from the porcine intestine a strain of *Streptococcus hyointestinalis* DPC6484 that displays antimicrobial activity against a wide range of Gram-positive bacteria and produces a bacteriocin with a mass of 3,453 Da. Interestingly, the strain was also found to be immune to a nisin-producing strain. Genome sequencing revealed the genetic determinants responsible for a novel version of nisin, designated nisin H, consisting of the *nshABTCPRKGEF* genes, with transposases encoded between *nshP* and *nshR* and between *nshK* and *nshG*. A similar gene cluster is also found in *S. hyointestinalis* LMG14581. Notably, the cluster lacks an equivalent of the nisin immunity gene, *nisI*. Nisin H is proposed to have the same structure as the prototypical nisin A but differs at 5 amino acid positions Ile1Phe (i.e., at position 1, nisin A has Ile while nisin H has Phe), Leu6Met, Gly18Dhb (threonine dehydrated to dehydrobutyrine), Met21Tyr, and His31Lys and appears to represent an intermediate between the lactococcal nisin A and the streptococcal nisin U variant of nisin. Purified nisin H inhibits a wide range of Gram-positive bacteria, including staphylococci, streptococci, *Listeria* spp., bacilli, and enterococci. It represents the first example of a natural nisin variant produced by an intestinal isolate of streptococcal origin.

2.1.2 Introduction

Bacteriocins of lactic acid bacteria have received extensive attention in recent years given their structural diversity and activity and their potential as biopreservatives and anti-infectives. Indeed, the production of bacteriocins by intestinal bacteria is

considered a probiotic trait and has been shown to be associated with strain dominance, infection control, and host cell signaling (1). One of the oldest and undoubtedly the most extensively characterized bacteriocins is nisin A, which was discovered by Rogers in 1928 (2). Nisin A is produced by many strains of *Lactococcus lactis*, a species widely used for cheese manufacture. It has a broad antimicrobial spectrum against a wide range of Gram-positive genera, including staphylococci, streptococci, *Listeria* spp., bacilli, and enterococci (3). Nisin A has been used in the food industry as a biopreservative for more than 50 years without inducing widespread microbial resistance (4, 5). The bacteriocin has multiple antimicrobial actions; it binds to the precursor of peptidoglycan, lipid II, to inhibit cell wall biosynthesis and then forms pores in the cell membrane, leading to the release of essential ions and, ultimately, cell death (6–8).

The nisin gene cluster in *L. lactis* is associated with a conjugative transposon and consists of *nisABTCIPRKFEG*, where *nisA* encodes the nisin prepropeptide. Immunity to nisin is provided by a specific immunity protein, NisI, and a specialized ABC transporter, NisFEG (9). The lipoprotein, NisI, most probably orients to the outside of the cytoplasmic membrane and binds nisin, preventing it from binding to lipid II and forming pores in the cell membrane (10, 11). NisFEG are thought to transport nisin from the cytoplasmic membrane to the external environment, thus preventing the accumulation of the high number of nisin molecules necessary for pore formation (12, 13). The extent to which nisin is produced is affected by the level of immunity of the producing microorganism. For maximal nisin immunity, both the lipoprotein and the nisin transporters are required (12, 14–16).

To date, eight natural nisin variants have been discovered (Figure. 1). These include nisins Z, F, and Q, which have been isolated from lactococci, nisins U and U2, from

Streptococcus uberis, and nisin P, which is encoded on nisin operons present in both *Streptococcus gallolyticus* subsp. *pasteurianus* (17) and *Streptococcus suis* (18). Nisin Z producers are very common, and the amino acid sequence differs from that of nisin A at a single position (His27Asn) (19, 20), a change that improves the solubility of the peptide at a neutral pH (21). The operon encoding nisin F was found on a plasmid in *L. lactis* F10, isolated from the intestinal tract of a freshwater catfish in South Africa. Nisin F differs from nisin A at 2 amino acid positions: His27Asn, as seen in nisin Z, and Ile30Val (22). Nisin Q is produced by *L. lactis* 61-14, isolated from a river in Japan, and differs from nisin A at 4 amino acid positions, i.e., those observed in nisin F as well as Ala15Val and Met21Leu (23). The *Streptococcus*-associated variants differ more considerably from nisin A. The *S. uberis* producers of nisin U and U2 were isolated in cases of bovine mastitis in the United States. Nisin U differs from nisin A at 9 positions—Ile4Lys, Ala15Ile, Gly18Thr, Asn20Pro, Met21Leu, His27Gly, Ser29His, Ile30Phe, and His31Gly—and also lacks the three C-terminal amino acids of nisin A. In addition to these changes, nisin U2 contains a further Ile1Val change (24). Finally, and most recently, a phylogenetic study of lanthipeptide synthetases by Zhang et al. (2012) (17) revealed an *S. gallolyticus* subsp. *pasteurianus* strain that encodes a structural gene with the potential to produce a new nisin analogue, nisin P. Nisin P is closely related to nisin U2, differing with respect to just 2 amino acids: Phe20 and Leu21 in nisin U2 are changed to Ala20 and Ile21 in nisin P. The more distantly related nisin-like lantibiotic salivaricin D, isolated from *Streptococcus salivarius* 5M6c, a human isolate, differs from nisin A at 17 positions, with most differences seen at the C-terminal end of the molecule (25).

In this study, we have identified a new nisin variant, designated nisin H, produced by a strain of *Streptococcus hyointestinalis* isolated from the porcine intestine. The name *S.*

hyointestinalis was first employed in 1988 to reassign a number of strains that had previously been classified as *Streptococcus salivarius* (26) and was derived from the Greek noun *hyos*, meaning pig, and the Latin adjective *intestinalis*, which reflects the association of the strains with the porcine intestine. Previously an *S. hyointestinalis* isolate producing a broad-spectrum antimicrobial that inhibits bifidobacteria, lactobacilli, *Leuconostoc* spp., *Listeria* spp., *Staphylococcus aureus*, and *Streptococcus agalactiae* was isolated as part of a mammalian-gut-mining study by O'Shea et al. (2009) (27). Since this represented the first report of an *S. hyointestinalis* strain that produces an antimicrobial, we sequenced and analyzed the genome of this strain. Ultimately, this led to the isolation, characterization, and identification of a novel nisin variant, which we designate nisin H, produced by a gut-derived strain.

2.1.3 Materials and Methods

Bacterial strains and culture conditions

The bacterial strains used in this study are listed in Table 1. Anaerobic conditions were generated through the use of anaerobic jars containing Anaerocult A gas packs (Merck, Darmstadt, Germany). Agar (Oxoid Ltd., Basingstoke, Hampshire, United Kingdom) was added (1%, wt/vol) to broth media when agar plates were required.

Isolation of DNA for PCR analysis

DNA was extracted from culture cell pellets for PCR analysis with a GenElute bacterial genomic DNA kit (Sigma-Aldrich, Co. Wicklow, Ireland) and molecular manipulation techniques from the work of Sambrook and Russell (2001) (28) were used when required. Oligonucleotide primers were synthesized by Sigma-Genosys (Poole, Dorset, United Kingdom), and purified PCR amplicons were sequenced by Beckman

Coulter Genomics (Essex, United Kingdom).

DNA was amplified with MyTaq DNA polymerase (Bioline, London, United Kingdom) according to the manufacturer's instructions. PCRs were carried out in a Techne TC-512 thermal cycler (Bibby Scientific, Staffordshire, United Kingdom).

Genome sequencing and analysis of the nisin H gene cluster.

The sequence of the genomic DNA extracted from *S. hyointestinalis* DPC6484 was determined by 454 pyrosequencing (Beckman Coulter Genomics, USA). The resulting sequence reads were assembled into contigs using the Newbler package. Coding regions in the draft genome assembly were predicted using GLIMMER, version 2.0 (29), and annotation was subsequently determined using the GAMOLA software package (30). Sequence similarity analyses were performed using the gapped BLASTp algorithm and the nonredundant database provided by the NCBI (ftp://ftp.ncbi.nih .gov/blast/db) (31). By using the ARTEMIS genome viewer (32), components of the nisin H gene cluster were identified on two distinct contigs. PCR with the primer pair comprising 5' GTTGACTTATTGAGCGAGG 3' and 5' GCCAACTTATTACGTT CTTCAC 3', designed to be specific to the sequences flanking the 3 and 5 termini of the respective contigs, confirmed the contiguous nature of this gene cluster. The annotation of the gene cluster was then verified manually. The sequence data were aligned and analyzed by using LASERGENE software (DNAStar Inc., Madison, WI). The genome was searched for the presence of a *nisI* immunity gene equivalent with primers designed to be specific to the *nisI* immunity gene of *L. lactis* NZ9700 and the *nsuI* immunity gene of *S. uberis* 42 (Table 2).

Purification of the antimicrobial produced by *S. hyointestinalis* DPC6484.

The antimicrobial was purified from the cell-free supernatant (CFS) of a 2-liter culture of *S. hyointestinalis* DPC6484 grown in tryptic soy broth (TSB) at 37°C overnight. The culture supernatant was applied to a 90-ml SP Sepharose column (GE Healthcare, Uppsala, Sweden) pre-equilibrated with 50 mM sodium acetate buffer, pH 4.4 (buffer A). The column was washed with 300 ml of buffer A, and the antimicrobial activity was eluted in 300 ml of buffer A containing 1 M NaCl. This eluent was then applied to a 5-g, 20-ml Strata C18-E solid-phase extraction (SPE) column (Phenomenex, Cheshire, United Kingdom) preequilibrated with methanol and water. The column was washed with 20 ml of 25% ethanol, and the antimicrobial activity was eluted with 20 ml of 70% 2-propanol 0.1% trifluoroacetic acid (TFA). The antimicrobial activities of cell-free supernatants and eluents from purification protocols were determined via the agar well diffusion assay described by Ryan et al. (1996) (33). *Lactobacillus delbrueckii* subsp. *bulgaricus* LMG6901 was used as the indicator strain, and bioactivity was assessed following aerobic incubation of plates overnight at 37°C. The 2-propanol was removed by rotary evaporation, and the sample was applied to a Jupiter Proteo reversed-phase high-performance liquid chromatography (RP-HPLC, Phenomenex, Cheshire, UK) column (length, 10 mm; inside diameter, 250 mm; particle size, 4 µm, pore size, 90 Å) running a 25-to-45% acetonitrile– 0.1% TFA gradient over 35 min at 2.5 ml/min. The resultant eluent was monitored at 214 nm, and fractions were collected at 1-min intervals. Fractions were assayed for antimicrobial activity by a well diffusion assay with *L. delbrueckii* subsp. *bulgaricus* LMG6901 as the indicator strain, and those containing antimicrobial activity were analyzed via matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) to determine the molecular mass of the antimicrobial peptide and to assess peptide purity. HPLC fractions deemed pure by MALDI-TOF MS were combined and were lyophilized in a Genevac (Suffolk,

United Kingdom) lyophilizer. MALDI-TOF mass spectrometry was performed with an Axima TOF^2 MALDI-TOF mass spectrometer in positive-ion reflectron mode (Shimadzu Biotech, Manchester, United Kingdom). N-terminal sequencing (Edman degradation) of purified nisin H was performed by Abingdon Health Laboratory Services (Birmingham, United Kingdom).

Pure nisin A peptide was prepared from *L. lactis* NZ9700 as described for nisin H.

Cross immunity of *S. hyointestinalis* DPC6484 to other nisin-producing cultures

The cross immunity of *S. hyointestinalis* DPC6484 to the bacteriocins produced by *L. lactis* NZ9700 (nisin A), *S. uberis* strain 42 (nisin U), *L. lactis* DPC3251 (lacticin 3147) and to *L. delbrueckii* subsp. *bulgaricus* LMG6901, a non-bacteriocin producer used as a bacteriocin-sensitive strain, was determined by spotting 50-µl aliquots of cell free culture supernatants onto indicator plates seeded with 1% (vol/vol) of each of these strains. *L. delbrueckii* subsp. *bulgaricus* LMG6901, known to be sensitive to each of these nisin variants and to lacticin 3147, was used as an indicator strain to confirm the production of nisins A, H, and U and lacticin 3147 by the respective strains.

Comparison of the inhibitory activities of pure nisin A and nisin H peptides

Purified nisin A and H peptides were resuspended at 0.22 mg/ml in 35% 2-propanol for optimum solubility. Aliquots (50 µl) of each peptide were tested for antimicrobial activity by well diffusion using the indicator strains listed in Table 4. The culture media and incubation conditions are outlined in Table 1. A 50-µl aliquot of 35% 2-propanol was assayed against each strain to ensure that it did not inhibit any of the test strains.

Genomic profiles of *S. hyointestinalis* strains

Molecular fingerprinting of *S. hyointestinalis* isolates was performed by pulsed-field gel electrophoresis (PFGE) as described by Simpson et al. (2002) (34) using SmaI restriction endonucleases and DNA molecular weight markers (9.42 to 242.50 kb; New England BioLabs, Beverly, MA). DNA fragments were resolved with a CHEF (contour-clamped homogeneous electric field) DRIII pulsed-field system (Bio-Rad Laboratories) at 6 V/cm for 18 h with a 1- to 30-s linear ramp time to resolve bands.

Assessment of the distributions of *nshA*, *nshF*, *nshR*, and *nshT* in *S. hyointestinalis* strains

The presence of the *nshA*, *nshF*, *nshR*, and *nshT* genes in *S. hyointestinalis* DPC6484 and *S. hyointestinalis* strains obtained from the BCCM/LMG culture collection were checked using gene-specific primer pairs. The primer pairs and the sizes of the expected gene products are given in Table 2.

Nucleotide sequence accession number

The sequence of the nisin H gene cluster of *Streptococcus hyointestinalis* DPC6484 is available from GenBank/EMBL under accession number KP793707.

2.1.4 Results

Genome sequencing of *S. hyointestinalis* DPC6484 reveals a nisin-like gene cluster

In a previous study, which involved the screening of mammalian samples from the gastrointestinal tracts of humans, pigs, and cows, we identified *S. hyointestinalis* DPC6484, a strain that inhibits bifidobacteria, lactobacilli, *Leuconostoc* spp., *Listeria* spp., *S. aureus*, and *S. agalactiae* (27). Given that antimicrobial production has not been

attributed to an *S. hyointestinalis* strain previously, the genome of DPC6484 was sequenced with a view to the identification of the gene cluster responsible for this phenotype. Analysis of the draft genome revealed the presence of a nisin homologue and of associated biosynthesis genes on two contiguous sequence regions. The assembled gene cluster, of ~15.8 kb (Figure. 2), was found to contain a putative nisin variant-encoding structural gene designated *nshA* (*nsh* for nisin from *S. hyointestinalis*, or nisin H) followed by homologues of *nisBTCP* (designated *nshBTCP*), a region encoding a streptococcal transposase, the equivalents of *nisRK* (designated *nshRK*), another region encoding a streptococcal transposase, and *nisFEG*-like genes (designated *nshFEG*). A notable feature was the absence of an equivalent of the *nisI* immunity gene. Further investigation of the *S. hyointestinalis* strain via a BLAST analysis on the draft genome sequence and PCR-based approaches suggested the absence of an obvious NisI homologue (data not shown).

The protein sequences ranged from 54% identity with the lactococcal equivalent for NshG to 82% identity for NshA. The predicted product of *nshA* is a 57-amino-acid peptide that, on the basis of comparison with other nisin peptides, is likely to consist of a 23-amino acid leader and a 34-amino acid propeptide. The putative propeptide differs from the corresponding nisin A peptide at five positions: Ile1Phe, Leu6Met, Gly18Thr, Met21Tyr, and His31Lys (Figure. 1).

Purification and predicted structure of nisin H

The nisin H peptide was purified using SP Sepharose cation-exchange SPE, C18 SPE, and reversed-phase HPLC. The HPLC chromatogram (Figure. 3) shows a dominant peak corresponding to a fraction that inhibited the indicator strain, *L. delbrueckii* subsp. *bulgaricus* LMG6901. This purification strategy typically yielded 0.15 mg/

liter, which is lower than the 0.50 mg/liter of nisin A recovered from a corresponding starting volume by using the nisin A producer *L. lactis* NZ9700.

The first 10 amino acids of the predicted NshA propeptide are FTSISMCTPG (Figure. 2). Lantibiotics can be difficult to sequence using Edman degradation, because the dehydrated amino acids and ring structures are not compatible with this technique. Nonetheless, Edman sequencing of the newly purified antimicrobial revealed a sequence consisting of F-X-X-X-X-M-X-X-P-G. This sequence conforms to the gene predictions of the identifiable residues at positions 1, 6, 9, and 10 and is consistent with the predicted presence of modified residues at positions 2, 3, 5, 7, and 8. MALDI-TOF MS analysis revealed a molecular mass of 3,453 Da, which is consistent with a modified form of the NshA peptide. MALDI-TOF MS also showed a difference of 101 Da between the molecular mass of nisin A (3,352 Da) and that of the antimicrobial produced by DPC6484 (3,453 Da) (data not shown). This difference precisely matches the molecular mass differences expected from the predicted amino acid changes and the likely dehydration of the additional threonine residue. Converting Ile to Phe, Leu to Met, Gly to Thr, and Met to Tyr results in 34-, 18-, 44-, and 32-Da increases, respectively, and results in a total peptide mass of 3,480 Da. In addition, the alteration of His to Lys results in a 9-Da loss, giving a mass of 3,471 Da, 18 Da higher than the 3,453-Da mass for nisin H. However, in nisin molecules, threonine is always dehydrated to dehydrobutyrine (Dhb), thus accounting for this 18 Da. It is thus apparent that the purified antimicrobial, referred to below as nisin H, represents a modified form of the NshA peptide with 5 substitutions. The proposed structure of nisin H, modeled on known structures of nisin variants, is shown in Figure. 4.

Nisin A- and nisin H-producing strains are cross immune

The nisin H producer was tested to assess its cross immunity to CFSs from producers

of nisins A and U. CFSs from nisin A-, H-, and U-producing cultures inhibited the growth of the indicator strain *L. delbrueckii* subsp. *bulgaricus* LMG6901, as expected (Table 3). Nisin A- and U-containing CFSs did not inhibit *S. hyointestinalis* DPC6484 or *L. lactis* NZ9700. However, CFSs from the nisin A and H producers inhibited the nisin U-producing *S. uberis* strain 42, suggesting that *S. uberis* is not cross immune to nisin A or nisin H. Further analysis with purified nisins revealed that at the concentrations used (0.22 mg ml^{-1}), the immunity mechanisms are overwhelmed, in that purified nisin A and nisin H generate zones of inhibition with areas of 0.32 cm^2 against the strains that produce these peptides and zones that are twice as large (0.69 cm^2) against the opposing producer. Notably, however, these peptides produce significantly larger zones, with areas of 3.39 and 2.38 cm^2, respectively, against *S. uberis* strain 42 (Table 4).

The activities of purified nisin A and nisin H peptides against *Escherichia coli* DPC6912, *Bacillus cereus* 9139, *L. delbrueckii* subsp. *bulgaricus* LMG6901, *Lactococcus lactis* subsp. *cremoris* HP, *Enterococcus faecalis* 6307, *S. agalactiae* ATCC 13813, *S. agalactiae* DPC5338, *Streptococcus bovis* DPC6491, *S. gallolyticus* DPC6501, *Listeria innocua* DPC3572, *Listeria monocytogenes* 1042, *S. aureus* ATCC 25923, *S. aureus* DPC5245, *S. hyointestinalis* DPC6484, and *S. uberis* strain 42 were assessed (Table 4). In the majority of instances, nisin A was more inhibitory than nisin H. However, nisin H was more effective than nisin A against one of the *S. aureus* strains tested, DPC5245, and the two peptides were equally effective against *L. innocua* DPC3572.

Not all *S. hyointestinalis* strains produce nisin H

PFGE of *S. hyointestinalis* DPC6484 and seven *S. hyointestinalis* strains from the

BCCM/LMG culture collection confirm different banding patterns; thus, these strains are not clonal (Figure. 5A). Efforts to amplify each of the genes *nshA*, *nshF*, *nshR*, and *nshT* by PCR using specific primers (Table 2) resulted in the generation of amplicons of the appropriate sizes (Figure. 5Bi) for the nisin H producer *S. hyointestinalis* DPC6484 (positive control) and *S. hyointestinalis* LMG14581 but not for the other S. *hyointestinalis* strains. DNA sequencing of the *S. hyointestinalis* LMG14581 amplicons confirmed the presence of *nsh* equivalents in this strain. Although *S. hyointestinalis* LMG14581 contains *nsh* gene equivalents, this strain did not produce a zone of inhibition against *L. delbrueckii* subsp. *bulgaricus* LMG6901 (Figure. 5Bii).

2.1.5 Discussion

Nisin H is of interest for several reasons. First, it is notable by virtue of being a nisin-like bacteriocin that is produced by an intestinal strain. As such, production of the bacteriocin has the potential to give the strain a competitive advantage in the gut environment, either by directly inhibiting competitor bacteria or by facilitating communication with other strains or even the host. In addition, nisin H may also have a role in signaling to the host, since bacteriocin production has been associated with immunomodulatory effects often mediated through cytokine responses (35–37). Second, nisin H seems to represent an evolutionary link between lactococcal and streptococcal nisins in that, while it is quite different from nisin A by virtue of having five separate substitutions, it retains key features of the lactococcal peptides, including the three C-terminal amino acids, which are absent from the *Streptococcus*-associated nisin U. Third, the nisin H gene cluster is the only nisin gene cluster to lack an equivalent of the *nisI* immunity gene.

The lactococcal nisin A gene cluster (Figure. 2) encodes nisin production genes in the order *nisABTCIPRKFEG*, and this gene order is conserved in the corresponding nisin

Z and nisin Q clusters (38). The gene order is different in streptococcal nisin gene clusters. In the nisin U gene cluster, *nsuPRKFEG* are at the start of the gene cluster, i.e., before the *nsuABTCI* genes, suggesting a rearrangement of *nsuABTCI* and *nsuPRKFEG* in *S. uberis* (24). Gene clusters containing structural genes for nisin analogues have also been identified in *S. gallolyticus* subsp. *pasteurianus* (17, 39), *S. agalactiae* (40), and, most recently, *S. suis* (18). The gene order in these gene clusters is identical to that for nisin U. It would seem most likely that nisin-like clusters have moved between streptococci by horizontal gene transfer, as proposed by Richards et al. (2011) (40). Interestingly, the nisin H gene cluster differs from the *Lactococcus* and *Streptococcus* nisin clusters identified previously. Although the order of *nshABTCP* is the same as that for lactococci, the absence of a *nisI* gene between *nshC* and *nshP* is notable. Attempts to amplify a *nisI* gene equivalent using primers designed to be specific to the *nisI* gene of *L. lactis* and the *nsuI* gene of *S. uberis* were unsuccessful. The absence of an obvious *nisI* gene in the remainder of the draft genome was confirmed by a comprehensive BLAST search; however, the presence of a novel immunity-like gene elsewhere in the genome cannot be ruled out. In addition, the nisin H gene cluster exhibits a number of other significant differences from previously described nisin clusters in gene order and orientation, most likely due to gene rearrangements brought about by the action of transposases.

The lack of an equivalent to the immunity protein, NisI, can have negative implications for bacteriocin production by the producing cell (41, 42). The yield of nisin H from the culture supernatant is low relative to that of nisin A, a finding initially attributed to the lack of a *nisI* immunity gene. However, further investigations revealed that poor bacteriocin production is most likely due to low cell numbers following 16 h of growth in TSB, typically 6×10^7 cfu/ml for *S. hyointestinalis* DPC6484 compared to 4×10^8

cfu/ml for *L. lactis* NZ9700. *S. hyointestinalis* strain LMG14587, which does not contain the nisin H gene cluster, grew to levels similar to those of DPC6484, suggesting that the absence of *nisI* was not responsible for a growth defect in DPC6484. In addition, previous *nisI* knockout studies have shown that a specific immunity gene is not necessary to confer full immunity (10), and Stein et al. (2003) reported that either *nisI* or *nisFEG* were able to confer immunity on nisin-sensitive *Bacillus subtilis* host cells (12). In this study, it would appear that the ABC transporter genes *nisFEG* are sufficient for self-protection in *S. hyointestinalis* DPC6484. Of note, other lantibiotic gene clusters, such as those of mersacidin and lacticin 481, also lack a specific immunity protein but possess NisFEG equivalents (13, 43).

The molecular mass difference of 101 Da between nisin A and nisin H can be accounted for by the amino acid differences. Given the highly conserved nature of nisin structures, we propose that nisin H has the same ring structure as nisins A, Z, F, and Q. It is more similar in structure to the lactococcal nisins in that it contains the three terminal amino acids that are missing from the streptococcal nisins U, U2, and P.

Natural lantibiotic variants are likely to arise from point mutations in structural genes; by definition, they should have few amino acid differences and the same ring pattern, and the associated producers should exhibit cross immunity to other variants (44). Nisin H fits this definition in that it differs from nisin A with respect to five amino acids, is likely to have an identical ring pattern and is immune to nisin A.

The lower antimicrobial activity of the CFS of DPC6484 than that of the control nisin A producer used in this study is not related exclusively to poor peptide production levels, since, at equal concentrations and purity, nisin A is more effective than nisin H against many of the target microorganisms investigated. Nisin has been extensively bioengineered in a quest to generate more active peptides and this strategy has provided

information on the effects of specific amino acid changes (45). It has been stated that an unusual feature of nisin is the absence of aromatic residues and that, to date, any bioengineered nisins containing aromatic residues display reduced activity (46–49). Indeed, Field et al. (2008) have shown that the bioactivity of a derivative of nisin A with Met21Tyr, a change that occurs naturally in nisin H, is reduced to 70% of that of the parental strain against *S. aureus* strains ST528 and DPC5245, and to 65% against *S. agalactiae* ATCC 13813. In addition, nisin H also contains a second aromatic residue, Phe1; thus, this is the first report of the presence of such aromatic amino acids in a natural nisin variant. Interestingly, salivaricin D also contains a phenylalanine at position 1. The presence of two aromatic residues may contribute to the potency of nisin H being lower than that of nisin A. It is also notable that the introduction of positively charged amino acids into nisin Z has had a beneficial impact on activity, in that the bioengineered Asn20Lys and Met21Lys variants were more active against the Gram-negative genera *Shigella*, *Pseudomonas*, and *Salmonella* (48). Although nisin H has a histidine-to-lysine change at position 32, this did not confer enhanced antimicrobial activity against *Escherichia coli*. However, since histidine is positively charged at low pHs, the addition of a lysine at this location does not constitute a significant change from a charge perspective. The Leu6Met amino acid change seen in nisin H is the first amino acid change at this position reported for a natural variant. However, the specific impact of this change on activity will require further investigation.

Notably, nisin H appears as an intermediate between nisins of lactococcal and streptococcal origins, in that it retains the three terminal amino acids found in nisins A, F, Q, and Z while possessing other features, such as a Dhb at position 18, that are associated with nisins U, U2, and P. Alignment of the amino acid sequences of nisins

A, Z, F, Q, H, U, U2, and P in Figure 1 shows an increasing number of amino acid changes from the prototypical nisin A through to nisin P. Overall, nisin H aligns more closely with lactococcal than with streptococcal nisins, a fact that is reflected by amino acid identity.

Very few *S. hyointestinalis* strains have been deposited in culture collections. Since *S. hyointestinalis* was designated a new species in 1988, it is possible that isolates were previously catalogued as *S. salivarius*. PFGE analysis revealed that the seven strains of *S. hyointestinalis* obtained from the BCCM/LMG culture collection differ in their PFGE patterns (Figure 5A). *S. hyointestinalis* LMG14581 conclusively contained the *nshA*, *nshF*, *nshR*, and *nshT* genes (Figure 5Bii), but this strain did not display a bacteriocin- producing phenotype (Figure 5Bi). A thorough analysis of the nisin H gene cluster in this strain would have to be carried out in order to determine the basis for this phenomenon.

In conclusion, we describe nisin H, a novel natural nisin variant produced by an *S. hyointestinalis* strain of porcine origin. The production of nisin H by a gut strain lends further support to accumulating observations suggesting that bacteriocin production may represent a potential probiotic trait for intestinal strains.

2.1.6 Acknowledgments

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Table 1 Bacterial strains and culture conditions.

Strain	Growth Media	Incubation Temperature/Atmosphere
Streptococcus hyointestinalis DPC6484	GM17*/TSB**	37°C Anaerobic
Streptococcus hyointestinalis LMG14579	GM17/TSB	37°C Anaerobic
Streptococcus hyointestinalis LMG14581	GM17/TSB	37°C Anaerobic
Streptococcus hyointestinalis LMG14582	GM17/TSB	37°C Anaerobic
Streptococcus hyointestinalis LMG14583	GM17/TSB	37°C Anaerobic
Streptococcus hyointestinalis LMG14585	GM17/TSB	37°C Anaerobic
Streptococcus hyointestinalis LMG14586	GM17/TSB	37°C Anaerobic
Streptococcus hyointestinalis LMG14587	GM17/TSB	37°C Anaerobic
Lactococcus lactis NZ9700	GM17	30°C Aerobic
Streptococcus uberis strain 42	GM17	37°C Anaerobic
Lactococcus lactis DPC3251	GM17	30°C Aerobic
Escherichia coli DPC6912	LB~	37°C Aerobic
Bacillus cereus 9139	BHI***	37°C Aerobic
Lactococcus lactis subsp. *cremoris* HP	GM17	30°C Aerobic
Enterococcus faecalis 6307	LB	37°C Aerobic
Streptococcus agalactiae ATCC13813	BHI	37°C Anaerobic
Streptococcus agalactiae DPC5338	BHI	37°C Anaerobic
Streptococcus bovis DPC6491	BHI	37°C Anaerobic
Streptococcus gallolyticus DPC6501	BHI	37°C Anaerobic
Listeria innocua DPC3572	BHI	37°C Aerobic
Listeria monocytogenes 1042	BHI	37°C Aerobic
Staphylococcus aureus ATCC 25923	BHI	37°C Aerobic
Staphylococcus aureus DPC5245	BHI	37°C Aerobic
Lactobacillus delbrueckii subsp. *bulgaricus* LMG6901	MRS~~	37°C Aerobic

*M17 with 5 g/L Glucose added (Difco Laboratories, Detroit, MI), **Tryptic Soy Broth (Difco Laboratories, Detroit, MI), ***Brain Heart Infusion (Merck, Darmstadt, Germany), ~ Luria-Bertani or Lysogeny Broth (Merck, Darmstadt, Germany), ~~ deMan, Rogosa, Sharp (Difco Laboratories, Detroit, MI).

Table 2 List of primers used in this study.

Gene	Primer pair sequence	Product size (bp)	Annealing temp (°C)
nshH gap	5' GTTGACTTATTGAGCGAGG 3'	2,267	60
	5' GCCAACTTATTACGTTCTTCAC 3'		
nisI	5' GGAATAAGTGGCTGTATACTGG 3'		
	5' GAGAGTAACTGTTGTGAATTTG 3'	903	50
nsuI	5' TAGTTGCATGTAGATTGGTAG 3'		
	5' ATACGTGCTATTCTATATTCA 3'	619	48
nshT	5' CTCTCCTCTTGTTTATTATCCC 3'		
	5' GTAGAGAGCCATTAGATTGG 3'	702	53
nshA	5' CTACTATTAGCTAAACAGATTG 3'		
	5' GTTGTCCATCTTCATATG 3'	481	50
nshF	5' GCTAGTCAGAATCGCCATAG 3'		
	5' GTCTGGCACTGTATGCGG 3'	324	60
nshR	5' TAGAGGAAAGAAGTGTATGTG 3'		
	5' TTGGGTTTACTTCATATGTAG 3'	177	53

Table 3 Cross immunity of *Streptococcus hyointestinalis* DPC6484 to other nisin producing strains.

	Bacteriocin Cell Free Supernatant			
Target Organism	**A**	**H**	**U**	**Lacticin 3147**
L. delbrueckii subsp. *bulgaricus* LMG6901	11.41 ± 0.64	8.01 ± 0.00	8.17 ± 0.28	2.66 ± 0.00
L. lactis NZ9700	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.66 ± 0.14
S. hyointestinalis DPC6484	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.75 ± 0.30
S. uberis strain 42	0.32 ± 0.00	0.69 ± 0.00	0.00 ± 0.00	0.97 ± 0.24
L. lactis DPC3251	0.32 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Area of zone of inhibition (cm^2) calculated as $(\pi R_1^2)-(\pi R_2^2)$ where R_1 is the radius of zone and R_2 is the radius of well in centimetres.

Table 4 Spectrum of inhibition of purified nisin A and nisin H peptides against a range of strains.

Target microorganisms	Nisin A	Nisin H
Escherichia coli DPC6912	0.00 ± 0.00	0.00 ± 0.00
Bacillus cereus 9139	1.42 ± 0.14	1.03 ± 0.12
Lactobacillus delbruckii subsp. *bulgaricus* LMG6901	23.83 ± 0.89	16.64 ± 0.38
Lactococcus lactis subsp. *cremoris* HP	9.47 ± 0.51	6.52 ± 0.25
Enterococcus faecalis 6307	6.24 ± 0.00	3.94 ± 0.00
Streptococcus agalactiae ATCC13813	6.10 ± 0.25	2.66 ± 0.17
Streptococcus agalactiae DPC5338	4.06 ± 0.21	2.56 ± 0.17
Streptococcus bovis DPC6491	3.50 ± 0.38	1.75 ± 0.29
Streptococcus gallolyticus DPC6501	5.82 ± 0.00	3.94 ± 0.00
Listeria innocua DPC3572	1.50 ± 0.14	1.50 ± 0.14
Listeria monocytogenes 1042	2.10 ± 0.00	1.58 ± 0.00
Staphylococcus aureus ATCC25923	3.28 ± 0.00	2.76 ± 0.17
Staphylococcus aureus DPC5245	3.39 ± 0.19	4.78 ± 0.21
Lactococcus lactis NZ9700	0.32 ± 0.00	0.69 ± 0.00
Streptococcus hyointestinalis DPC6484	0.69 ± 0.00	0.32 ± 0.00
Streptococcus uberis strain 42	3.39 ± 0.19	2.38 ± 0.28

Area of zone of inhibition (cm^2) calculated as $(\pi R_1^2)-(\pi R_2^2)$ where R_1 = radius of zone and R_2 = radius of well in centimetres. Purified nisin A and nisin H were assayed at a concentration of 0.22 mg/ml.

Figure 1 Alignment of natural nisin variants, with amino acid changes in bold face. Asterisks mark conserved amino acid residues.

```
A          ITSISLCTPGCKTGALMGCNMKTATCHCSIHVSK      3352 Da
Z          ITSISLCTPGCKTGALMGCNMKTATCNCSIHVSK      3331 Da
F          ITSISLCTPGCKTGALMGCNMKTATCNCSVHVSK      3315 Da
Q          ITSISLCTPGCKTGVLMGCNLKTATCNCSVHVSK      3327 Da
H          FTSISMCTPGCKTGALMTCNYKTATCHCSIKVSK      3453 Da
U          ITSKSLCTPGCKTGILMTCPLKTATCGCHFG         3029 Da
U2         VTSKSLCTPGCKTGILMTCPLKTATCGCHFG         3015 Da
P          VTSKSLCTPGCKTGILMTCAIKTATCGCHFG         2989 Da
SalD       FTSHSLCTPGCITGVLMGCHIQSIGCNVHIHISK      3468 Da
            ** * *****  ** **  *   *****  *
```

Figure 2 Representation of the bacteriocin-encoding *nshA* gene cluster as revealed by genome sequencing (center), compared with the *nisA* (top) and *nsuA* (bottom) gene clusters. The *nshA* gene cluster contains the nisin production genes *nshABTCP*, a gap region encoding a transposase, *nshRK*, a region encoding a second transposase, and *nshFEG*. For each gene, the percentage of amino acid identity to the protein encoded by the corresponding nisin A-associated gene is presented. The amino acid sequence of the unmodified NshA peptide is shown below the gene cluster. Residues predicted to be within the leader peptide are shown in gray, and those thought to correspond to the structural peptide are shown in black.

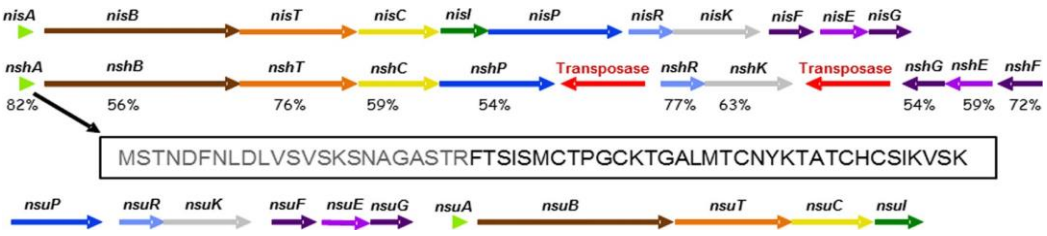


Figure 3 Purification of nisin H from *Streptococcus hyointestinalis* DPC6484 grown in TSB broth. (A) RP-HPLC chromatogram; (B) MALDI-TOF MS of the active fraction; (C) zone of inhibition of an aliquot of the HPLC fraction on a *Lactobacillus delbrueckii* subsp. *bulgaricus* LMG6901 indicator plate.

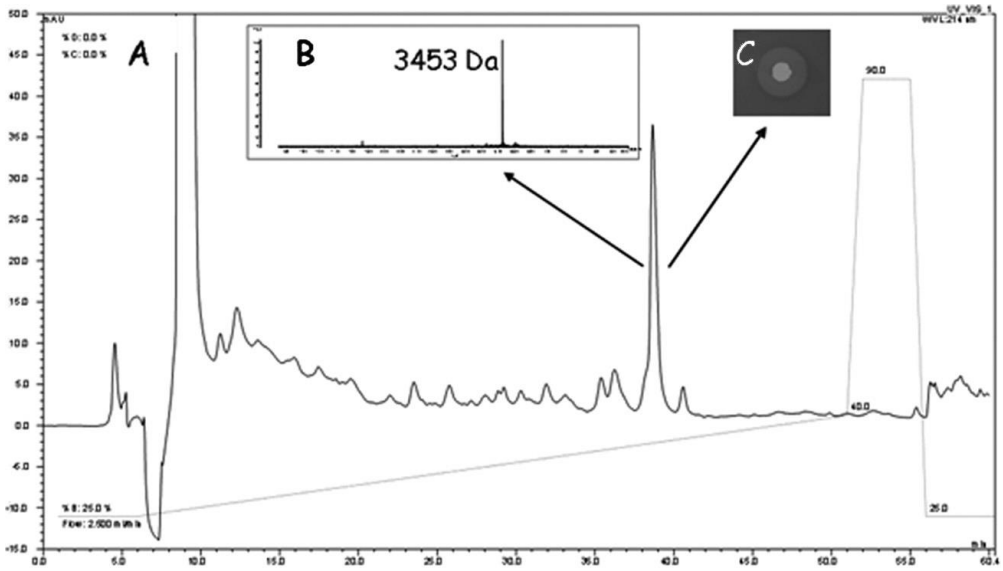


Figure 4 Proposed structure of the new natural nisin variant nisin H.

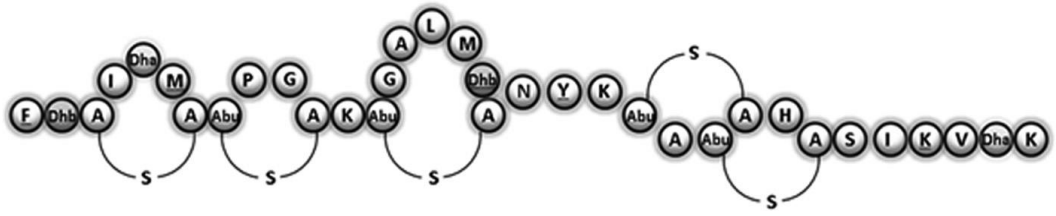
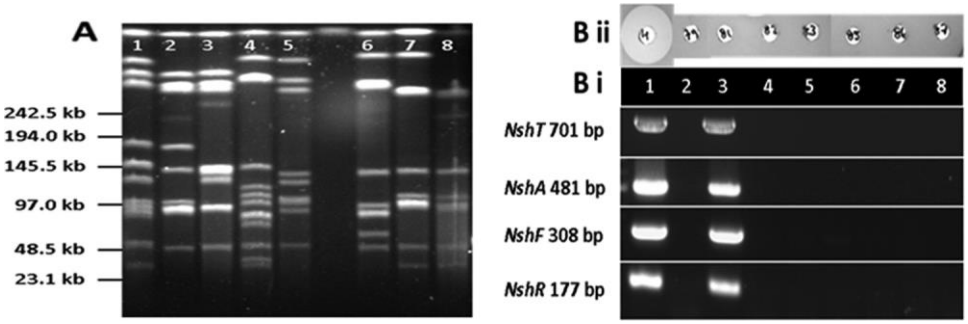


Figure 5 (A) PFGE macrorestriction patterns of *Streptococcus hyointestinalis* strains restricted with SmaI. Lane 1, *S. hyointestinalis* DPC6484; lane 2, *S. hyointestinalis* LMG14579; lane 3, *S. hyointestinalis* LMG14581; lane 4, *S. hyointestinalis* LMG14582; lane 5, *S. hyointestinalis* LMG14583; lane 6, *S. hyointestinalis* LMG14585; lane 7, *S. hyointestinalis* LMG14586; lane 8, *S. hyointestinalis* LMG14587. (B) (i) PCR amplification of strains of *S. hyointestinalis* template DNA with *nshT*-, *nshH*-, *nshF*-, and *nshR*-specific primers. Lanes correspond to those in panel A. (ii) Comparison of antimicrobial activities of *S. hyointesinalis* strains DPC6484 (H), LMG14579, LMG14581, LMG14582, LMG14583, LMG14585, LMG14586, and LMG14587 against the indicator strain, *L. delbrueckii* subsp. *bulgaricus* LMG6901. Wells are labeled with H (for nisin H) and with the last two digits of the strain designation for the other strains.



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Chapter 2.2

Nisin J, a novel nisin variant, is produced by *Staphylococcus capitis* sourced from human skin microbiota

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JN O'Sullivan, **PM O'Connor**, MC Rea, Ó O'Sullivan, CJ Walsh, B Healy,

H Mathur, D Field, C Hill and RP Ross.

CHAPTER CONTRIBUTIONS

Paula M O'Connor purified nisin J, performed MALDI TOF MS, advised on experiments and significantly contributed to manuscript preparation.

Julie N O'Sullivan isolated the strain, identified the genes on the nisin J contig and performed antimicrobial assays, designed experiments and significantly contributed to manuscript preparation.

Órla O'Sullivan performed bioinformatic analysis.

2.2.1 Abstract

The skin microbiota is thought to play a key role in host protection from infection. Nisin J is a novel nisin variant produced by *Staphylococcus capitis* APC 2923, a strain isolated from the toe web space area in a screening study performed on the human skin microbiota. Whole-genome sequencing and MALDI TOF mass spectrometry of the purified peptide confirmed that *S. capitis* APC 2923 produces a 3,458-Da bacteriocin, designated nisin J, which exhibited antimicrobial activity against a range of Gram-positive pathogens, including methicillin-resistant *Staphylococcus aureus* (MRSA) and *Cutibacterium acnes*. The gene order in the nisin J gene cluster (*nsjFEGBTCJP*) differs from that of other nisin variants in that it is lacking the nisin regulatory genes, *nisRK*, as well as the nisin immunity gene *nisI*. Nisin J has nine amino acid changes compared to prototypical nisin A, with eight amino acid substitutions, six of which are not present in other nisin variants (Ile4Lys, Met17Gln, Gly18Thr, Asn20Phe, Met21Ala, Ile30Gly, Val33His, and Lys34Thr), and an extra amino acid close to the C terminus, rendering nisin J the only nisin variant to contain thrity five amino acids. This is the first report of a nisin variant produced by a *Staphylococcus* species and the first nisin producer isolated from human skin.

2.2.2 Importance

This study describes the characterization of nisin J, the first example of a natural nisin variant, produced by a human skin isolate of staphylococcal origin. Nisin J displays inhibitory activity against a wide range of bacterial targets, including MRSA. This work demonstrates the potential of human commensals as a source for novel antimicrobials that could form part of the solution to antibiotic resistance across a broad range of bacterial pathogens.

2.2.3 Introduction

The human skin microbiome is home to ~10^{12} bacteria (1), and interest in the potential of skin bacteria to produce antimicrobials is growing, given the spread of antibiotic resistance (AR). *Staphylococcus capitis* is a member of the resident skin microbiota. First isolated from human skin in 1975, it has since been regarded as an opportunistic pathogen and has been associated with sepsis in neonates, meningitis, and endocarditis (2). Little is known about the inhibitory nature or antimicrobial activity of *S. capitis*, with only one report of *S. capitis* EPK-1 producing the glycylglycine endopeptidase ALE-1, an enzyme that targets the cell wall of *Staphylococcus aureus* (3). More recently, genomic analysis of an *S. capitis* strain isolated from the skin of a human toe revealed the presence of gene clusters capable of encoding gallidermin, epidermin, and phenol soluble modulins, highlighting its potential to produce antimicrobial peptides (AMPs) (4).

In a recent study, our group detected antimicrobial activity by a number of *S. capitis* strains isolated from different areas of the human skin (5) and highlighted the potential for *S. capitis* species to produce bacteriocins (small ribosomally synthesized peptides produced by a range of bacteria which kill other bacteria). Interestingly, bacteriocin production is considered to be a probiotic trait in that bacteriocins function in helping the producer strain to become established in a niche, by killing off competitors and interacting with the immune system. Although the impact of nisin on immune systems has not yet been completely elucidated, this peptide stimulates a wide array of effects, and it influences various populations of cells involved in immunity (6–12).

One of the oldest known and most intensively studied bacteriocins is nisin, which was first described in this journal by Rogers and Whittier in 1928 (13). Nisin has been used

in food preservation since 1953 (14) and was granted Generally Regarded As Safe (GRAS) status in 1988 by the Food and Drug Administration (FDA). It is also approved by the World Health Organization (WHO) as a food additive and has been assigned the E number E234. Since the discovery of nisin, interest in bacteriocins has grown rapidly. Nisin A, composed of 34 amino acids, is produced by several strains of *Lactococcus lactis* (15). Nisin is a lantibiotic and thus a member of the class I bacteriocins (16). Lantibiotics are small peptides (<5 kDa) and are produced by many Gram-positive bacteria to inhibit or kill other Gram-positive bacteria (17). Production of other lantibiotics is common among commensal coagulase-negative staphylococci. For example, *Staphylococcus gallinarum*, *Staphylococcus epidermidis*, and *Staphylococcus hominis* produce the lantibiotics gallidermin, epidermin, and hominicin, respectively (18–20). Class I bacteriocins consist of post-translationally modified bacteriocins which are subdivided into 4 classes, as follows: class Ia, lanthipeptides (of which nisin is the most prominent member); class Ib, head-to-tail cyclized peptides; class Ic, sactibiotics; and class Id, linearazol(in)e-containing peptides (8, 21). Lantibiotics are characterized by the presence of lanthionine/þ-methyllanthionine residues and are produced through the dehydration of serine and threonine residues to form dehydroalanines and dehydrobutyrines respectively. These dehydrated residues in turn react with cysteine thiols, forming lanthionine bridges (22, 23). The lantibiotics are subdivided based on the enzymes catalyzing the formation of lanthionines. Subclass I requires two distinct enzymes, LanB and LanC, whereas subclass II is modified by a single enzyme, LanM. Subclass III has no associated antimicrobial activity and is modified by a single enzyme, LanKC, while subclass IV is modified by LanL (24). Studies have revealed that nisin and other structurally related lantibiotics use the membrane-bound peptidoglycan precursor lipid II as a

docking molecule, which consequently promotes two bactericidal activities, pore formation and inhibition of peptidoglycan biosynthesis (25). Significantly, lantibiotics have been shown to possess activity against antibiotic-resistant targets such as vancomycin-resistant enterococci (VRE) and methicillin-resistant *Staphylococcus aureus* (MRSA) and may have the potential to mitigate the looming global AR crisis (26).

A number of nisin variants have been discovered since the original nisin A was characterized (Figure 1A). Nisin variants of lactococcal origin are more similar to each other than to variants from other genera such as *Streptococcus* (Figure 1B). Nisin Z is the most closely related nisin variant to nisin A, with only a single amino acid substitution, His27Asn. Nisin U, U_2 and P each contain 31 amino acids, nisins $O_{1–3}$ contain 33 amino acids, and nisin O_4 contains 32 amino acids, making them shorter than other previously described nisin variants. Here, we describe nisin J, produced by the *S. capitis* strain APC 2923, isolated in a screening study of the human skin microbiota. At 35 amino acids, nisin J is the longest nisin variant identified to date and has antimicrobial activity against significant human pathogens, including staphylococci, streptococci, and *Cutibacterium acnes*.

2.2.4 Materials and Methods

The antimicrobial-producing strain *S. capitis* APC 2923 was isolated in a previous screening study of the human skin microbiota by our group (5).

Bacterial strains and culture conditions

The growth conditions of the bacterial strains used in this study are listed in Table 4. Anaerobic conditions, where appropriate, were attained using anaerobic jars and

Anaerocult A gas packs (Merck, Darmstadt, Germany).

Draft genome sequence of *S. capitis* APC 2923 and *in silico* analysis of the nisin J gene cluster

Bacterial DNA was extracted using the GenElute kit, as described by the manufacturer (Sigma-Aldrich Ireland Limited, Co. Wicklow, Ireland), and was prepared for sequencing following the Nextera XT DNA library prep reference guide (Illumina, Inc.). A Qubit 3.0 fluorometer (Thermo Fisher Scientific, MA) was used for DNA quantification. Sequencing was performed at the Teagasc/APC Microbiome Ireland Sequencing facility, Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland. In total, 94 contigs, including 16 large contigs, were revealed by *de novo* assembly using SPAdes (version 3.10.0). A total of 2,453 open reading frames (ORFs) and 60 tRNAs were detected and subsequently annotated using Prokka (version 1.11). The online tools BActeriocin GEnome mining tooL (BAGEL4) and antiSMASH 3.0 were employed to identify bacteriocin operons/gene clusters in the genomes of interest, and by combining these software programs with the ARTEMIS genome viewer, the presence of the nisin J gene cluster was confirmed.

Evolutionary links between natural nisin variants

The European Bioinformatics Institute toolkit (https://www.ebi.ac.uk/services) was used to investigate the evolutionary relationships between the nisin structural variants. A multiple-sequence alignment was generated using MUSCLE (version 3.8) and visualized on a neighbor-joining tree without distance corrections. This tree was visualized using the ggtree package (version 1.10.5) in R (version 3.4.4).

Purification of the antimicrobial produced by *S. capitis* APC 2923

To purify the antimicrobial produced by *S. capitis* APC 2923, the culture was grown in a shaking 37°C incubator overnight in 1,800 ml of brain heart infusion (BHI) which had been passed through a column containing Amberlite XAD 16N to remove hydrophobic peptides before autoclaving (XAD-BHI). The culture supernatant was applied to an Econo-Column containing 60 g Amberlite XAD-16N beads (Sigma Aldrich, Co. Wicklow, Ireland). The column was then washed with 350 ml of 30% ethanol, and the antimicrobial activity was eluted with 70% propan-2-ol (IPA) containing 0.1% trifluoroacetic acid (TFA) (Sigma Aldrich). The IPA was removed from the active column eluent and the pH adjusted to 4.4 with 7.5 N NaOH. The sample was then applied to an Econo-Column containing 90 ml SP Sepharose beads preequilibrated with 20 mM sodium acetate buffer (pH 4.4) (buffer A). The column was washed with 50 ml of buffer A and the antimicrobial activity eluted in 250 ml buffer A containing 1 M NaCl. The salt-containing eluent was applied to a 60 ml, 10-g C18 solid-phase extraction (SPE) column (Phenomenex, Cheshire, United Kingdom) preequilibrated with methanol and water. The column was washed with 60 ml of 25% ethanol, and nisin was eluted in 60 ml IPA (0.1% TFA), which was subjected to reversed-phase high-performance liquid chromatography (RP-HPLC). The sample was applied to a semi-preparative Jupiter Proteo (250 mm [length] by 10 mm [inside diameter], 90 Å [pore size], 4 µm [particle size]) RP-HPLC column (Phenomenex, Cheshire, UK) running a gradient of 25 to 40% acetonitrile and 0.1% TFA, where buffer A was 0.1% TFA and buffer B was 90% acetonitrile and 0.1% TFA. The resulting eluent was monitored at 214 nm, and fractions were collected at 1-min intervals.

Column eluents and HPLC fractions were assayed for antimicrobial activity by well diffusion assays (WDAs), according to the method of Parente and Hill (41), using *L. delbrueckii* subsp. *bulgaricus* LMG 6901 as the target organism. Column eluents and HPLC fractions displaying antimicrobial activity were assayed for the nisin J molecular mass by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) on an Axima TOF^2 MALDI-TOF MS in positive-ion reflectron mode (Shimadzu Biotech, Manchester, United Kingdom). Fractions containing pure nisin J were pooled and lyophilized in a Genevac lyophilizer (Suffolk, United Kingdom). Pure nisin A peptide was prepared from *L. lactis* NZ9700 as described for nisin J but excluding the SP Sepharose step. Nisin Z pure peptide was sourced from Handary (Fleurus, Belgium).

Comparison of the inhibitory spectra of nisins A, Z, and J

Pure nisins A, Z, and J were resuspended in RNase-free water to a final concentration of 1 mg/ml and subsequently assayed by WDA against a range of target indicator strains (Table 2). Zone diameters were measured in millimeters using Vernier calipers (DML-Digital Micrometers Ltd., Sheffield, United Kingdom) and recorded in Table 2 as area of the zone (πr^2) minus the area of the well (πr^2) in millimeters.

MIC determinations

MICs were determined in triplicate from pure nisins A, Z, and J against approximately 1×10^5 cfu/ml of the target indicator strain *Lactococcus lactis* subsp. *cremoris* HP using 96-well microtiter plates (Sarstedt, Co. Wexford, Ireland) and using a Libra S2 colorimeter (Biochrom Ltd., Cambridge, United Kingdom) to measure the optical density at 600 nm (OD_{600}) of the indicator strains. Peptide concentrations of 4x the

test concentration (2,048 nM) were prepared in 400 µl RNase-free and DNase-free water. One hundred microliters of growth medium was added to all wells of the 96-well plate. One hundred microliters of 4x concentration was added to the first well, and subsequently, 2-fold serial dilutions were carried out. MIC readings were taken after 16 h at 30°C. The MIC was recorded as the lowest concentration of lantipeptide where no growth of the indicator was observed (42).

Cross-immunity of nisin J-producing *S. capitis* APC 2923 to other nisin-producing strains

To investigate if the nisin J-producing *S. capitis* APC 2923 strain was immune to other nisin-producing cultures (*L. lactis* NZ9700 producing nisin A, *Streptococcus hyointestinalis* DPC 6484 producing nisin H, and *S. uberis* strain 42 producing nisin U), cross-immunity assays were performed based on the WDA method, whereby each strain was tested as an indicator and a producer (43).

Determining if the nisin J structural gene is unique to *S. capitis* APC 2923

To determine if the nisin J structural gene was present in other *S. capitis* strains isolated from the study by O'Sullivan et al. (5), oligonucleotide primers designed to specifically amplify the nisin J structural gene (*nisJ* F, 5'-ACTT TATAACTAAGATTAGC-3', and *nisJ* R, 5'-TCGCTTTATTATTTAGTAT GCACG-3') were used in a PCR under the following conditions: initial denaturation, 94°C for 5 min; 35 cycles of 94°C for 40 s, 52°C for 30 s, and 72°C for 1 min; and a final extension 72°C for 10 min. Sequencing was conducted by Genewiz (Essex, United Kingdom). Sequencing data were analyzed employing the Lasergene 8 software (DNAStar, Inc., Madison, WI) and subsequently input into

the ExPASy online translate tool (https://web.expasy.org/translate/) to translate the nucleotides into amino acid sequences.

Sequence analysis of the nisin J plasmid pJOS01

To confirm that the nisin J gene cluster was plasmid associated, the plasmid DNA of *S. capitis* APC 2923 was extracted using the Plasmid maxi kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions following an adapted user-developed protocol specific to staphylococcal species (https://www.qiagen.com /ie/resources/resourcedetail ?id=82ddd661-fbab-4d35819c-defd6269fc64&lang=en), using lysostaphin (Sigma-Aldrich Ireland Limited, Co. Wicklow, Ireland). The resulting DNA extract was sequenced by Illumina MiSeq technology (2 x 250-bp paired-end reads; GenProbio, Parma, Italy). *De novo* sequence assemblies and automated gene calling were performed using the MEGAnnotator pipeline (44) and assessed for predicted tRNA genes via transcend-SE version 1.2.1 (45). Predicted open reading frames (ORFs) were determined via Prodigal version 2.6 and Genemark.hmm (46). A BLASTP (47) analysis was performed to assign functional annotations to the predicted ORFs (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (Table 1). PlasmidFinder (version 2.0) was employed to confirm that the generated assembled contigs were plasmid sequences based on the identification of Rep proteins. SnapGene version 2.3.2 was employed to generate a map of the plasmid harboring the *nisJ* gene cluster (designated pJOS01 here). In addition to the sequence data analysis to confirm the plasmid association of the nisin J cluster, PCR-based analysis was undertaken using the plasmid DNA extract as the template. Oligonucleotide primers designed to specifically amplify the nisin J structural gene (*nisJ* F, 5'-ACTTTATAACTAAGATTAGC-3', and *nisJ* R, 5'-TCGCTTTATTATT

TAGTATGCACG-3') were used in a PCR using Phusion Green Hot Start II high-fidelity PCR master mix with the following conditions: initial denaturation, 98°C for 5 min; 30 cycles of 98°C for 10 s, 52°C for 30 s, and 72°C for 15 s; and a final extension of 72°C for 10 min. Validation of the amplicon was performed by Sanger sequencing of the generated product (Source BioScience, Waterford, Ireland). Furthermore, restriction digestion of the plasmid DNA was carried out using EcoRI in 10x CutSmart buffer (New England BioLabs, Herts, United Kingdom).

Investigation for the presence of nisin-resistant determinants in *S. capitis* APC 2923

To determine if the gene encoding the nisin resistance protein (NSR) was present in *S. capitis* APC 2923 and the 7 other *S. capitis* isolates previously identified from the O'Sullivan et al. study (5), PCR was employed using the primers and reaction conditions described by Simões et al. (38). To determine if the nisin J-producing *S. capitis* strain APC 2923 was cross-immune or sensitive to NSR-producing strains, bioassays were carried out by spotting 10 µl of the nisin J overnight culture onto 1.5% BHI agar (Merck, Darmstadt, Germany). Following overnight incubation at 37°C, the plates were then overlaid with soft (0.75%) GM17 agar (BD Difco Trafalgar Scientific Ltd., Leicester, United Kingdom) seeded with 0.25% of an overnight culture of the NSR-positive strain *L. lactis* subsp. *diacetylactis* DRC3. To directly compare the resistance levels of nisin A and nisin J to NSR, WDAs were carried out as previously described (43), employing *L. lactis* MG1614/pNP40 (NSR-positive strain) and *L. lactis* MG1614 (NSR-negative strain) as target indicators. All lactococcal NSR indicator strains were grown aerobically overnight at 30°C. Agarose assays were subsequently performed as outlined in reference 42. Data obtained from the agarose assays were

subjected to normality tests prior to statistical analysis using the GraphPad Prism software (version 8.2.1). *P* values were calculated using an unpaired *t* test.

Data availability

The plasmid map of pJOS01 has been deposited in GenBank under accession number MN602039. This whole-genome shotgun project has been deposited at DDBJ/ENA/GenBank under the accession number WHVU00000000. The version described in this paper is version WHVU01000000.1.

2.2.5 Results

A nisin-like gene cluster exists within the *S. capitis* APC 2923 genome

S. capitis APC 2923 was previously isolated from the toe web space area in a screening study of the human skin microbiota that sought to identify novel antimicrobial-producing strains (5). This strain was of particular interest due to its potent activity against the indicator strain *Lactobacillus delbrueckii* subsp. *bulgaricus* LMG 6901 and its broad inhibitory spectrum against a panel of *Staphylococcus*, *Streptococcus*, and *Corynebacterium* species and against *Cutibacterium acnes*. Whole-genome sequencing of this strain revealed a nisin gene cluster of ~9.78 kb compared to ~13.3 kb for nisin A. The structural gene *nisJ* encodes a peptide with the following eight amino acid variations compared to nisin A: Ile4Lys, Met17Gln, Gly18Thr, Asn20Phe, Met21Ala, Ile30Gly, Val33His, and Lys34Thr. Nisin J also contains an extra amino acid at the C terminus, making nisin J the longest nisin variant identified to date (Figure 1A). A dendrogram of the natural nisin variants (Figure 1B) demonstrates that peptides which have a closer common ancestor are more similar than are peptides that have

more distant branching points. Lactococcal nisin variants are structurally distinct from all other nisin variants. Staphylococcal nisin J groups in the middle of the tree and appears to be more similar to streptococcal nisin than to lactococcal nisins. Nisins of *Blautia* origin appear to be more phylogenetically distinct due to longer branching. Streptococcal nisins H and J are more closely related to lactococcal nisins than to other streptococcal nisins, U, U_2 and P. The gene order of the nisin J cluster (*FEGBTCJP*) also differs from that of the nisin A in that it contains eight as opposed to the 11 genes within the cluster (Figure 2). The BAGEL4 bacteriocin genome mining tool predicted that the nisin J prepeptide is composed of 61 amino acids with a leader sequence consisting of 26 amino acids. Overall, the nisin J mature peptide has 62.5% identity to the nisin H structural peptide produced by *Streptococcus hyointestinalis* (27). The identity and function of features of the nisin J operon are listed in Table 1.

Other genes contained in the *S. capitis* APC 2923 draft genome

In addition to the nisin J cluster, BAGEL4 and antiSMASH3.0 also highlighted a small gene cluster containing the *lanB* and *lanC* genes and a gene encoding a peptide with 93% identity to the gallidermin family in *S. capitis* APC 2923. These were located on a different contig from that of the nisin J gene cluster, and this mass was not detected from either the colony or purified cell free supernatants.

Purification and predicted structure of nisin J

Nisin J was purified in four steps using Amberlite XAD-16N solid-phase extraction (SPE), SP Sepharose cation exchange, C18 SPE, and reversed-phase high-performance liquid chromatography (HPLC). Antimicrobial activity

correlated with the most dominant peak eluting at 24.5 min in the HPLC chromatogram, and matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) revealed that the corresponding fractions had a mass of 3,458 Da (Figure 3). This correlates with the predicted mass of the putative nisin J bacteriocin (following subsequent dehydration and ring formation reactions) as calculated from the draft genome sequence. Fractions deemed pure by MALDI-TOF MS were combined and lyophilized to give a yield of 3.00 mg liter^{-1}. Given that nisin J is a natural nisin variant with demonstrable conservation between key structural amino acids common to all natural nisin variants, it is predicted that the structure will be in line with those of other lactococcal nisins, as shown in Figure 4.

Comparing the activities of purified nisins A, Z, and J

The spectrum of activity of pure nisin A, nisin Z, and nisin J, by means of a well diffusion assay (WDA), was performed on several target indicator strains. Nisin J was more active than nisin A against 12 of the 13 strains tested, while nisin J was more active than nisin Z for 7 of the target strains tested, including *Corynebacterium xerosis*, MRSA, *Streptococcus uberis*, and *S. aureus* (Table 2). However, in an MIC assay using *L. lactis* HP as the indicator, no difference was observed between nisins A, Z, and J, with all exhibiting MICs of 32 nM.

The nisin J-producing strain is cross-immune to nisin A and H but not to nisin U producers

Cross-immunity assays were performed to investigate whether the nisin J-, A-, H- and U-producing strains were cross-immune to one another (Table 3). No zones were

observed between nisins A, H and J, indicating that these producing strains are all cross-immune. However, a zone was observed from the nisin J-producing strain against the nisin U producer (*S. uberis* strain 42), demonstrating that the strain is sensitive to nisin J.

Not all *S. capitis* strains contain a nisin-like gene cluster

The *nisJ* structural gene was amplified from nine antimicrobial-producing *S. capitis* strains isolated from human skin in a previous study by our group (5). Two of the nine *S. capitis* strains (APC 2918 and APC 2934) did not contain the *nisJ* structural gene. The other seven *S. capitis* strains tested positive for the *nisJ* structural gene, correlating with findings from our earlier study which found these strains to be cross-immune and to possess the same pulsotype, indicating that they were the same strain or very closely related strains and were therefore most likely producing the same bacteriocin (5). These 7 strains were isolated from 4 different subjects, indicating that the same pulsotype is shared across a number of individuals, implying that the ability to produce nisin J may be a dominant feature and thus an ecological advantage for this *S. capitis* strain.

The nisin J gene cluster resides on a plasmid

Analysis of the *S. capitis* APC 2923 contig harboring the nisin J gene cluster identified the presence of a plasmid replication protein A (RepA) and other plasmid replication-associated proteins, suggesting that it was of plasmid origin. Plasmid DNA was readily obtained from *S. capitis* APC 2923 using a commercially available plasmid maxi kit (data not shown). Short-read sequencing was performed on the plasmid DNA using the Illumina MiSeq platform to approximately 200-fold

coverage. *De novo* assembly resulted in four contigs (Figure 5), with a combined size of 49,951 bp. A plasmid map of pJOS01 (GenBank accession number MN602039) shows all of the genes encoding immunity and the biosynthetic machinery for nisin J (*nsjFEG*, *nsjB*, *nsjT*, *nsjC*, *nisJ*, and *nsjP*) reside on one of the contigs, supporting the plasmid association of the nisin J gene cluster (Figure 5). Furthermore, three genes encoding plasmid replication functions (RepA and RepB) as well as genes encoding other nonessential plasmid-associated roles were present on the other contigs (Figure 5 and Table 1). Restriction digestion with EcoRI yielded a profile comparable to the virtual digestion of the generated plasmid sequence, supporting the predicted size of ~50 kb (data not shown). Subsequent analysis revealed a GC content of ~28%, which is considerably lower than that of *S. capitis* chromosomal DNA (32 to 33%), a characteristic that has been observed for plasmids of many Gram-positive species (28).

Nisin J exhibits resistance to NSR

Deferred antagonism assays using *L. lactis* subsp. *diacetylactis* DRC3 (nisin resistance protein positive [NSR^+]) as a target indicator strain revealed that nisin J is partially resistant to NSR (result not shown). To establish if nisin J had increased inhibitory activity against NSR compared to that of nisin A, further WDAs were conducted using the NSR^+ and NSR^- strains *L. lactis* MG1614/pNP40 and *L. lactis* MG1614, respectively. While the inhibition zone of the nisin J producer is slightly decreased against the NSR-positive strain compared to the NSR- negative strain, it appears that nisin J is more active than nisin A and may be less susceptible to the proteolytic effects of NSR (Figure 6A), which was also demonstrated in agarose assays (Figure 6B). The analysis revealed a significant difference in the zones of inhibition between nisin A

and nisin J against an NSR^+ strain (MG1614/pNP40), with a *P* value of 0.0001 compared to zone sizes against an NSR^- strain (MG1614), where no statistical difference ($P = 0.1701$) was observed (these data support Figure 6).

2.2.6 Discussion

As the burden of antibiotic resistance increases globally, there is an urgent need for novel therapeutic options. In addition to the well-established use of nisin as a food preservative, many studies have focused on using nisin against drug–resistant pathogens in clinical or veterinary settings due to its high potency and multiple mechanisms of action (10 –12). Nisin J is a novel nisin variant and the first such variant reported from a *Staphylococcus* species. A combination of whole-genome sequencing of *S. capitis* APC 2923 and peptide purification resulted in the identification of this broad-spectrum lantibiotic. The nisin J-producing *S. capitis* strain was isolated from the toe web space, an area associated with high microbial load. This suggests that the production of a broad-spectrum bacteriocin confers an advantage on this strain over competing commensal skin flora, as was also observed by O'Sullivan and colleagues (5) when four of the twenty subjects screened in the study exhibited the same pulsotype. The residence of the nisin J gene cluster on a plasmid is significant in that it may facilitate its dissemination to other skin microbes.

As mentioned previously, nisin J has eight amino acid changes and one extra amino acid near the C-terminal end compared to nisin A. Interestingly, six of the eight changes are unique compared to natural nisin variants. Natural nisin variants are tolerant to some amino acid changes at the N terminus, with Ile4 being the most commonly substituted amino acid. Nisin J contains an Ile4Lys substitution which

is also seen in nisins P, U, U_2, and $O_{1–3}$ but remains unchanged in lactococcal nisins (A, Z, F, and Q) and nisin H. Nisin J differs most from other natural nisin variants in the center and at the C terminus of the peptide, which could be key to nisin J's enhanced activity. At the center of nisin J, amino acid positions 17 to 21, there are 4 amino acids that differ compared to nisin A. It contains a Met17Gln substitution which is unique, as all other natural nisin variants that demonstrate antimicrobial activity have Met at this position. The Gly18Thr change is also interesting, as it is observed in nisins H, U, U_2, P, and $O_{1–3}$ and is proposed to be modified to dehydrobutyrine (Dhb), in light of the dehydration observed in a M17Q-G18T derivative of nisin Z (29). At position 20, nisin J has a highly hydrophobic residue, phenylalanine, compared to the polar asparagine in nisin A. Li et al. (30) found that extending the C terminus of nisin improves both its ability to permeate membranes and its inhibitory potential against Gram-negative bacteria. Therefore, nisin J's longer C terminus (compared to other nisin variants) could be more attracted to negatively charged cell membranes resulting in enhanced membrane insertion, which may be responsible for its broader host range. The skin origin of this nisin J producer suggests that its exposure to many competitors from the external environment may be responsible for the greater variation in the structure of nisin J.

Analysis of the nisin J gene cluster identified several key features associated with bacteriocin operons. These include a structural gene (*nisJ*), 2 genes associated with enzymatic modification (*nsjB* and *nsjC*), a gene involved in transport (*nsjT*), and immunity genes (*nsjFEG*) (Table 1 lists the identity and functions of features of the nisin J gene cluster). The arrangement of genes in the nisin J gene cluster differs from that of other nisin operons. Interestingly, the only conservation of gene order

throughout all operons of natural nisin variants is *lanBTC*. Similarities in the structural peptides of different nisin variants from different origins indicate the possibility that an evolutionary link exists between lactococcal, streptococcal, *Blautia*, and now, staphylococcal species, a link previously mentioned by O'Connor et al. (27) with reference to streptococcal and lactococcal species. A dendrogram based on the primary structures of all known natural variants highlights the genetic relatedness between the nisin-producing species and further suggests the likelihood of this evolutionary link. The FEG locus is present in lantibiotic systems other than nisin, including subtilin (31) and epidermin (32), and has been linked to transport, immunity, and defense (33). Inactivation of these genes in the nisin A gene cluster decreased nisin production and immunity, confirming their role in immunity (34). Although the *nsjFEG* genes are present in the nisin J gene cluster, the absence of a specific immunity gene, *nsjI*, as well as the absence of an expression regulatory system, *nsjRK*, could explain why nisin J immunity mechanisms appear to be less able to protect the cell. It also further supports the finding that the producing strain was more sensitive to its own purified nisin J peptide than was a nisin A producer with a specific nisin immunity determinant.

The production of lantibiotics such as gallidermin and epidermin is associated with increased release of lipids and ATP and protein excretion, which are indicators of cell membrane damage (35). Thus, the production of these lantibiotics has been deemed a "burden" to staphylococci that produce them; therefore, the incomplete lantibiotic gene cluster, having only the *lanB* and *lanC* genes present, may be either an evolutionary feature of *S. capitis* genomes or may be an incomplete cluster of lantibiotic biosynthetic genes previously shown to occur in many microbes (35). As previously discussed, the nisin J gene cluster resides on a plasmid, inviting the

speculation that *S. capitis* acquired its antimicrobial ability through horizontal gene transfer. Indeed, residence on mobile genetic elements is a feature of natural nisin variants, as observed with nisins A and H, and may explain their presence in many different species.

Purification of nisin J resulted in a peptide with a mass of 3,458 Da. The mass of nisin J was predicted to be 3,622 Da, where the difference between predicted and observed masses can be accounted for by 9 dehydration reactions (-18 Da per loss of water residue) involved in the formation of lanthionine and β-methyl-lanthionine bridges (36). The predicted peptide structure was based on the nisin A template, with a lanthionine bridge likely to occur between Ser3 and Cys7 and four β-methyl-lanthionine bridges between Thr8 and Cys11, Thr13 and Cys19, Thr23 and Cys26, and Thr25 and Cys28.

True to all nisin variants, nisin J is a broad-spectrum lantibiotic with inhibitory activity similar to that of nisins A and Z, as can be seen in Table 2, inhibiting a wide range of bacterial genera with greater inhibition of staphylococcal targets than with nisins A and Z. This suggests that the nisin J-producing *S. capitis* strain may have naturally evolved to produce a nisin peptide with enhanced activity against other staphylococci in the skin microbiota (Table 2). Nisin J-, A- and H-producing strains are immune to nisin peptides J, A, H, and U; however, the nisin U-producing strain is not immune to nisin J (Table 3). This may be due to the lack of the *nsjI* immunity gene in the nisin J cluster.

The nisin resistance protein (NSR) is a protease which cleaves nisin A at Ser29, significantly reducing the activity of the peptide. Employing a bioengineering strategy, Field et al. (37) demonstrated that the substitution of residues 29 and 30 with proline and valine respectively (derivative designated S29PV), rendered the peptide resistant

to proteolytic digestion by NSR. In this study, we found that the nisin J producer displays a higher resistance to NSR proteolytic enzymes than does nisin A, which is possibly due to a glycine residue at position 30 instead of the isoleucine as found in nisin A. Interestingly, a study carried out by Simões et al. (38) involving a multidrug-resistant *S. capitis* clone, NRCS-A, a major pathogen involved in sepsis in preterm neonates, demonstrated the presence of an NSR-encoding gene. PCR analysis failed to detect the presence of any *nsr* gene in any nisin J-producing *S. capitis* strain from our previous study (5).

Nisin J may have evolved to be more potent against specific competing organisms in a particular niche environment such as the skin. Employing a bioengineering strategy, Rink et al. (39) demonstrated that the replacement of residues I, S, and L at positions 4, 5, and 6 in nisin A with the residues K, S, and I, respectively resulted in enhanced bioactivity. Notably, the residues K-S-L are naturally present in nisin J at the same positions. In a separate bioengineering study, Kuipers et al. (29) generated a novel nisin variant (M17Q/G18T) exhibiting enhanced bioactivity. It is interesting that both of these mutations are naturally present in nisin J. Furthermore, Field et al. (40) reported that a nisin A derivative, M21A, demonstrated enhanced bioactivity. Remarkably, alanine is naturally present at position 21 in nisin J.

In conclusion, we have identified a new natural nisin variant, nisin J, produced by *S. capitis* APC 2923, which was isolated from the human skin microbiota. Nisin J represents the first nisin variant isolated from *Staphylococcus* species and the first to demonstrate partial recalcitrance to NSR. Indeed, the enhanced activity of nisin J compared to that of nisin A and Z as observed against all staphylococcal strains utilized in this study is notable. The production of bacteriocins such as nisin J from skin bacteria highlights the potential of bacterial strains of skin origin to be used as live

biotherapeutics.

2.2.7 Acknowledgments

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Table 1 Identity and function of features of the draft pJOS01 plasmid sequence[a].

Feature name	Position of: Start codon	Stop Codon	Size (aa)	E value	Putative function (conserved domain)	% identity to best match
J1	3	518	172	3E—124	DDE_Tnp_IS*240* superfamily; Rve transposase	100 to IS*6* family transposase of *S. epidermidis*
J2	562	1221	220	6E—149	ABC2_membrane superfamily; NosY ABC-type transport system involved in multicopper enzyme maturation, permease component	100 to ABC transporter permease subunit of *S. capitis*
J3	1749	1874	125	3E—17	DUF2648 superfamily; unknown function	100 to multiple species; DUF2648 domain- containing protein
J4	1886	3385	500	0	NADB_Rossmann superfamily; MqO malate: quinone oxidoreductase	100 to multiple species; malate dehydrogenase (quinone) (*Staphylococcus*)
J5	3446	5050	535	0	L-Lactate permease superfamily (energy production and conversion)	100 to *L. lactate* permease (*Staphylococcus*)
J6	5085	5789	235	6E—171	Alpha-acetolactate decarboxylase superfamily (secondary metabolite biosynthesis, transport, and catabolism)	100 to alpha-acetolactate decarboxylase
J7	5823	7487	555	0	Acetolactate synthase superfamily (P RK08617) (amino acid transport and metabolism, coenzyme transport, and metabolism)	100 to acetolactate synthase
J8	8213	8413	67	8E—39	CspA family (transcription) DNA binding domain	100 to cold shock protein (*Staphylococcus*)
CdR	8807	9424	206	1E—139	Cadmium resistance transporter superfamily; CadD protein, predicted permease (inorganic ion transport and metabolism)	100 to cadmium resistance transporter (*Mycobacteroides abscessus* subsp. *massiliense*)
J10	9442	9789	116	4E—74	Arsenical resistance operon repressor family; DNA-binding transcriptional regulator (transcription)	100 to HTH transcriptional regulator (*Staphylococcus*)
J11	10002	10610	203	2E—144	Serine recombinase family	100 to recombinase family protein (*Staphylococcus*)
J12	10716	11276	187	1E—124	None detected	100 to hypothetical protein (*Staphylococcus*)
J13	11884	12369	162	2E—112	None detected	100 to hypothetical protein (*Staphylococcus*)
J14	12632	13309	226	5E—166	NlpC/P60 family; the function of this domain is unknown; it is found in several lipoproteins	100 to hypothetical protein (*Staphylococcus*)
PSM	13578	13712	45	1E—22	*Staphylococcus* hemolytic protein	100 to beta class phenol-soluble modulin
J16	13944	14054	37	4E—17	DUF2648 superfamily; protein of unknown function	100 to multiple species; DUF2648 domain- containing protein (*Staphylococcus*)

Feature name	Position of: Start codon	Stop Codon	Size (aa)	E value	Putative function (conserved domain)	% identity to best match
J17	14064	15560	499	0	NADB_Rossmann superfamily; MqO malate: quinone oxidoreductase	100 to malate dehydrogenase: quinone (*S. capitis*)
J18	15780	16118	113	6E—75	DNA binding transcription regulator	100 to transcriptional regulator HXIR family (*Staphylococcus caprae*)
RepA	17825	18760	312	0	Replication initiator protein A (RepA) N terminus family; DNA replication initiator in plasmids	100 to replication initiator protein A (Staphylococcus)
J20	19190	19957	256	1E—178	Polar chromosomal segregation protein	100 to DUF536 binding domain (*Staphylococcus*)
J21	20132	20734	201	2E—140	NADB Rossmann superfamily; PRK07578 short-chain dehydrogenase	100 to short-chain dehydrogenase (bacteria)
J22	21220	21894	225	7E—165	DDE_Tnp_IS240 superfamily; Rve transposase	100 to IS*6*-like element IS*257* family transposase
nsjF	22148	22855	236	6.0E—119	ABC-type multidrug transport system, ATPase component (defense mechanisms)	75 to Lan protection ABC transporter ATP binding subunit in *Staphylococcus succinus*
nsjE	22857	23603	249	4E—85	Lantibiotic protection ABC transporter permease subunit, MutE/EpiE family; ABC-2 membrane superfamily	61.29 to hypothetical protein BU069_09230 in *S. succinus*
nsjG	23600	24337	246	1E—73	Lantibiotic protection ABC transporter permease subunit, MutG family; ABC-2 membrane superfamily	52.92 to hypothetical protein in *S. succinus*
nsjB	24362	27277	972	8E—90	Lantibiotic dehydratase C-terminal, thiopeptide-type bacteriocin biosynthesis domain	30.11 to lantibiotic dehydratase *Lactobacillus bombicola*
nsjT	27450	29000	517	2E—120	MdlB: ABC-type multidrug transport system, ATPase and permease component (defense mechanisms)	40.95 to ABC transporter ATP-binding protein *L. bombicola*
nsjC	28993	30222	410	2E—40	LanC is the cyclase enzyme of lanthionine synthetase; LanC-like superfamily	29.31 to lanthionine synthetase family protein (*Bacillus nakamurai*)
nisJ	30263	30445	61	1E—09	Structural gene; lantibiotic precursor in gallidermin/nisin family	62.5 to nisin H structural protein (*Streptococcus hyointestinalis*)
nsjP	30565	31905	447	2E—58	Peptidase S8 family domain in lantibiotic-specific proteases	32.58 to peptidase S8 (*Bacillus endophyticus*)
J31	31962	32357	132	7E—88	None detected	99.24 to hypothetical protein (*S. epidermidis*)
J32	32449	33057	203	4E—144	Serine recombinase revolvase invertase superfamily; PinE	100 to multiple species; recombinase family protein (*Staphylococcus*)
J33	33277	33477	67	1E—39	Predicted transcriptional regulator; COG3905 superfamily	100 to plasmid replication-associated protein (*S. epidermidis*)
ParA	33483	34277	265	0	ParA family chromosomal segregation and plasmid partition: cellulose biosynthesis protein BcsQ	99.62 to ParA family protein (*S. epidermidis*)
J35	34343	34882	180			No significant similarity found

Feature name	Position of: Start codon	Stop Codon	Size (aa)	E value	Putative function (conserved domain)	% identity to best match
RepA	35097	36089	331	0	DNA replication initiator of plasmids; HTH superfamily	99.7 to replication initiator protein A (S. capitis)
J37	36119	36820	234	1E—173	Putative transposase (InsQ) DNA-binding domain; OrfB_Zn_ribbon superfamily	100 to transposase (S. capitis)
J38	36827	37102	92	8E—60	None detected	100 to hypothetical protein EQ811_12225 (S. capitis)
J39	37705	37857	51	1E—27	None detected	100 to transposase (S. aureus)
RepB	380 75	38932	286	0	COG5527 superfamily	99.65 to RepB family plasmid initiator protein (Staphylococcus)
J41	39215	39664	150	2E—99	None detected	100 to hypothetical protein (Staphylococcus)
J42	39867	40565	233	3E—169	None detected	98.28 to hypothetical protein (*Staphylococcus*)
J43	40667	41023	119	5E—80	None detected	100 to hypothetical protein (*Staphylococcus*
J44	41124	41618	165	1E—107	Asp_carb_tr superfamily; pyrimidine biosynthesis	99.38 to aspartate carbamoyltransferase (*S. epidermidis*)
J45	41674	41847	58	2E—22	None detected	93.48 to molybdopterin biosynthesis protein MoaB
HTH	41985	42665	227	3E—161	HTH superfamily	99.12 to “winged” HTH transcription regulator (*S. epidermidis*)
J47	42777	44150	458	0	Multidrug resistance MFS family permease; transport and metabolism	99.78 to MFS transporter (*S. epidermidis*)
J48	44846	45340	165	3E—109	None detected	98.78 to hypothetical protein (*S. epidermidis*)
J49	45337	46101	255	6E—176	None detected	100 to hypothetical protein (*S. epidermidis*)
HTH	46186	46455	90	2E—58	HTH XRE superfamily	100 to HTH transcription regulator (*Auricoccus indicus*)
J51	47042	47155	38	1E—15	None detected	100 to hypothetical protein UF66_0802 (*Staphylococcus cohnii* subsp. *cohnii*)
J52	48210	48476	89	8E—57	None detected	98.86 to multispecies hypothetical protein (*Staphylococcus*)
J53	48623	49843	407	0	None detected	100 to hypothetical protein (*S. capitis*)

aa, amino acid. HTH, helix-turn-helix; XRE, xenobiotic response element; MFS, major facilitator superfamily.

Table 2 Inhibition spectra of purified peptides of nisins A, Z, and J against indicator strains using well diffusion assays and expressed as the area of the zone of inhibition

		Area of inhibition zone (mm^2)*		
Target Microorganism		**Nisin A**	**Nisin Z**	**Nisin J**
Corynebacterium xerosis	DPC 5629	51.5	66.2	133.8
Cutibacterium acnes	LMG 16711	537.0	587.5	469.0
Lactobacillus delbrueckii subsp. *bulgaricus*	LMG 6901	555.7	672.7	651.4
Lactococcus lactis subsp. *cremoris*	HP	241.9	325.8	363.0
Listeria monocytogenes	WSLC 112	60.0	73.0	37.0
Enterococcus faecium	APC 852	93.2	120.6	170.9
Enterococcus faecalis	ATCC 19433	101.2	120.8	103.0
Methicillin-resistant *Staphylococcus aureus*	DPC 5645	77.0	115.9	135.8
Staphylococcus aureus	DPC 7016	109.4	143.4	153.1
Staphylococcus epidermidis	DPC 5990	136.8	180.3	159.5
Staphylococcus simulans	APC 3482	148.7	197.1	395.5
Streptococcus agalactiae	ATCC 13813	174.4	221.7	136.8
Streptococcus uberis	DPC 5344	98.5	153.9	248.8

*Calculated as the area of zone of inhibition (Πr^2) — area of well (Πr^2) in millimeters. Assays were carried out in duplicate; mean zone areas shown.

Table 3 Cross-immunity of nisin A-, U-, H and J-producing strains using well diffusion assays and expressed as the area of the zone of inhibition.

Target organism	Strain	Nisin produced	Area of zone of inhibition[a] (mm^2) against nisin:			
			A	U	H	J
Lactococcus lactis	NZ9700	A	0	0	0	0
Streptococcus uberis	42	U	0	0	0	85
Streptococcus hyointestinalis	DPC 6484	H	0	0	0	0
Staphylococcus capitis	APC 2923	J	0	0	0	0

[a]Calculated as the area of zone of inhibition ($\Pi r^{2)}$ area of well (Πr^2) in millimeters. Values are the means from triplicate assays. 0, no zone observed.

Table 4 Growth conditions of the bacterial strains used in this study.

		Growth conditions		
Species	**Strain**	**Temp (°C)**	**Atmosphere**	**Growth media**
Corynebacterium xerosis	DPC 5629	37	Aerobic	BHI
Cutibacterium acnes	LMG 16711	37	Anaerobic*	mRCM&RCA
Enterococcus faecalis	ATCC 19433	37	Anaerobic*	MRS
Enterococcus faecium	APC 852	37	Anaerobic*	MRS
Lactobacillus delbrueckii subsp. bulgaricus	LMG 6901	37	Anaerobic*	MRS
Lactococcus lactis	NZ9700	30	Aerobic	GM17
Lactococcus lactis subsp. cremoris	HP	30	Aerobic	GM17
Lactococcus lactis	MG1614	30	Aerobic	GM17
Lactococcus lactis	MG1614 pNP40	30	Aerobic	GM17
Lactococcus lactis subsp. diacetylactis	DRC3	30	Aerobic	GM17
Listeria monocytogenes	WSLC 1211	37	Aerobic	BHI
MRSA	DPC 5645	37	Aerobic	BHI
Staphylococcus aureus	DPC 7016	37	Aerobic	BHI
Staphylococcus capitis	APC 2923	37	Aerobic	BHI
Staphylococcus epidermidis	DPC 5990	37	Aerobic	BHI
Staphylococcus simulans	APC 3482	37	Aerobic	BHI
Streptococcus agalactiae	ATCC 13813	37	Aerobic	BHI
Streptococcus hyointestinalis	DPC 6484	37	Anaerobic*	GM17
Streptococcus uberis	DPC 5344	37	Aerobic	BHI
Streptococcus uberis	strain 42	37	Anaerobic*	GM17

[a]ATCC, American Type Culture Collection; APC, APC Microbiome Ireland Culture Collection; DPC, Teagasc Culture Collection; WSLC, Weihenstephan *Listeria* Collection; LMG, Laboratorium voor Microbiologie.

[b]Anaerobic conditions, where appropriate, were achieved through the use of anaerobic jars and Anaerocult A gas packs (Merck, Darmstadt, Germany).

[c]MRS, de Man-Rogosa-Sharpe; mRCM, modified reinforced *Clostridium* medium (made following the ATCC medium: 2107 modified reinforced clostridial agar/broth [prereduced] protocol); RCA, reinforced *Clostridium* agar; BHI, brain heart infusion; GM17, 0.5% glucose added to M17 agar.

[d]MRSA, methicillin-resistant *S. aureus*.

Figure 1 (A) Visualization of the multiple-sequence alignment from MUSCLE (plotted using http://msa.biojs.net/app/) of all natural nisin (nis) variants aligned with strain origin. The total height of the sequence logo at each position reflects the degree of conservation at that position in the alignment, while the height of each letter in that position is proportional to the observed frequency of the corresponding amino acid at that position. Nisin A (13), nisin Z (48), nisin F (49), nisin Q (50), nisin H (27), nisin J (5), nisins U and U_2 (51), nisin P (52, 53), and nisins O_1 to O_4 (54) are shown. *L.*, *Lactococcus*; *S.*, *Staphylococcus*; *B.*, *Blautia*; *St.*, *Streptococcus*. (B) Dendrogram showing phylogenetic relatedness in primary structures of all known natural nisin variants, suggesting the possible existence of an evolutionary link between the nisin-producing species. The order in which they branch shows the relatedness between them and the branch length represents phylogenetic distance (0.05 represents a scale for the phylogenetic distance).

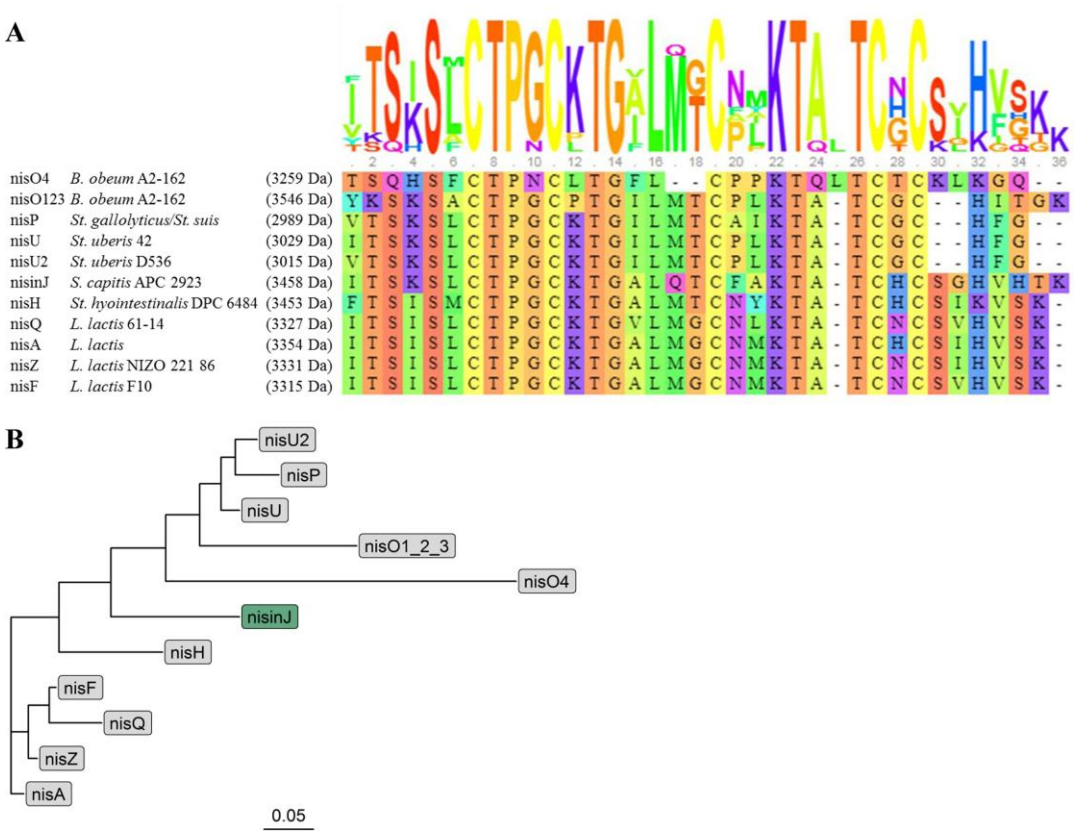


Figure 2 Comparison of bacteriocin gene clusters of different nisin variants.

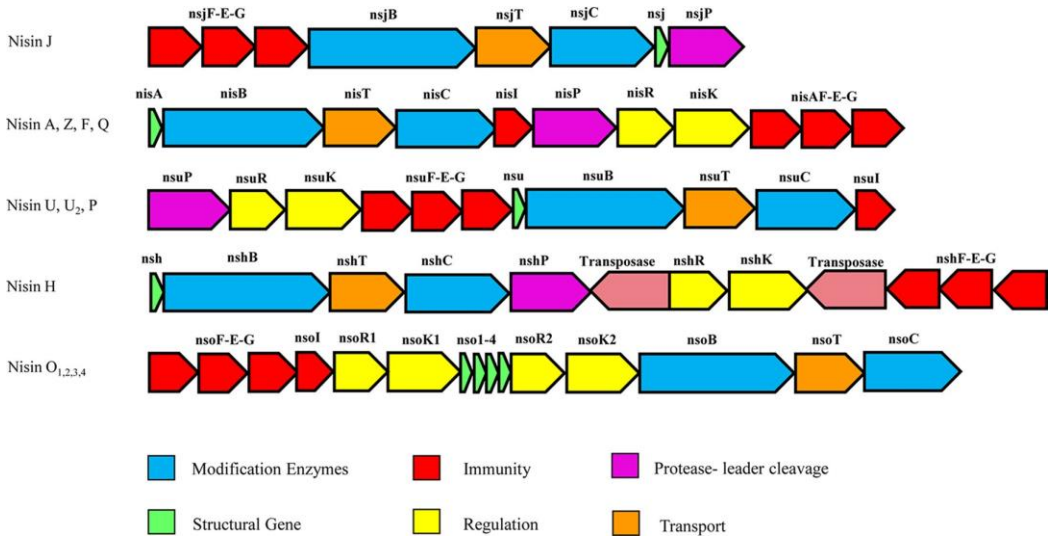


Figure 3 Purification of nisin J from *S. capitis* APC 2923 grown in XAD-BHI. (A) Original overlay plate where antimicrobial activity of the *S. capitis* APC 2923 strain was detected. (B) The RP-HPLC profile shows a peak at HPLC-active fraction of 24.5 minutes, which correlates with where pure nisin J elutes. (C) MALDI-TOF MS of active fraction. mAu, milli-arbitrary units.

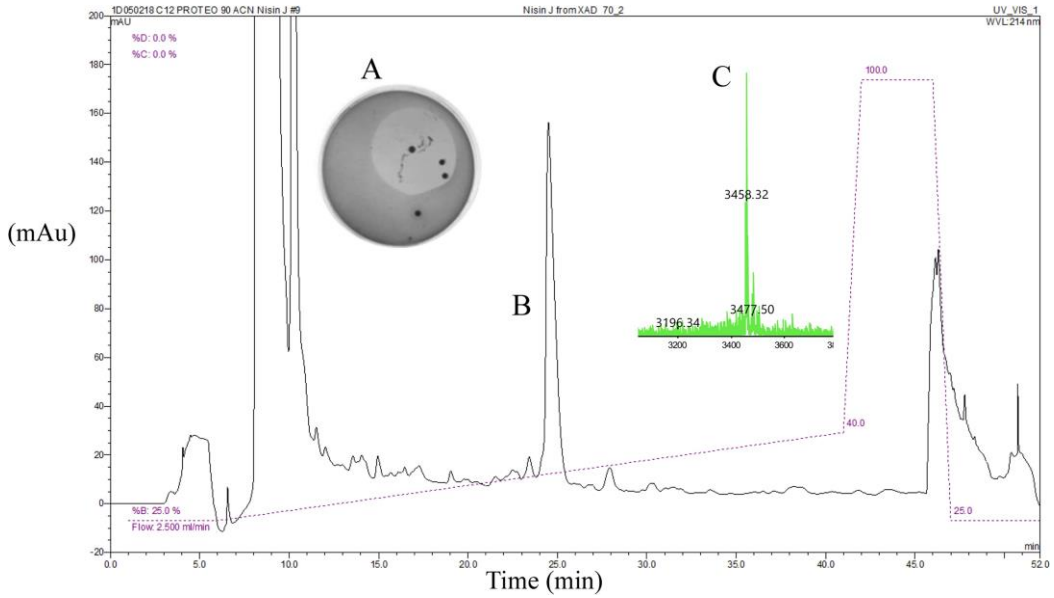


Figure 4 Proposed structure of the novel nisin variant nisin J. Residues are represented by the single-letter code. Post-translational modifications are indicated as follows: Dha, dehydroalanine; Dhb, dehydrobutyrine; Abu, 2-aminobutyric acid; Abu-S-Ala, 3-methyllanthionine.

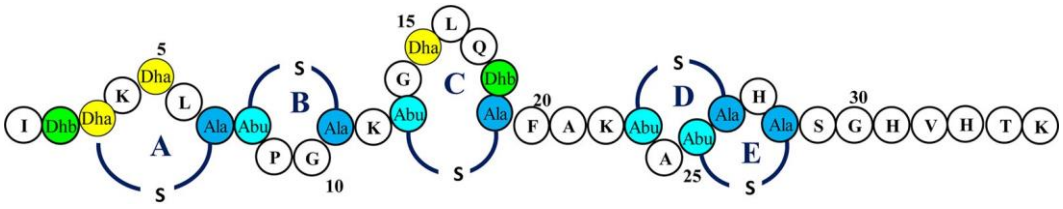


Figure 5 Plasmid map of pJOS01 draft sequence created on SnapGene version 2.3.2 (GenBank accession number MN602039).

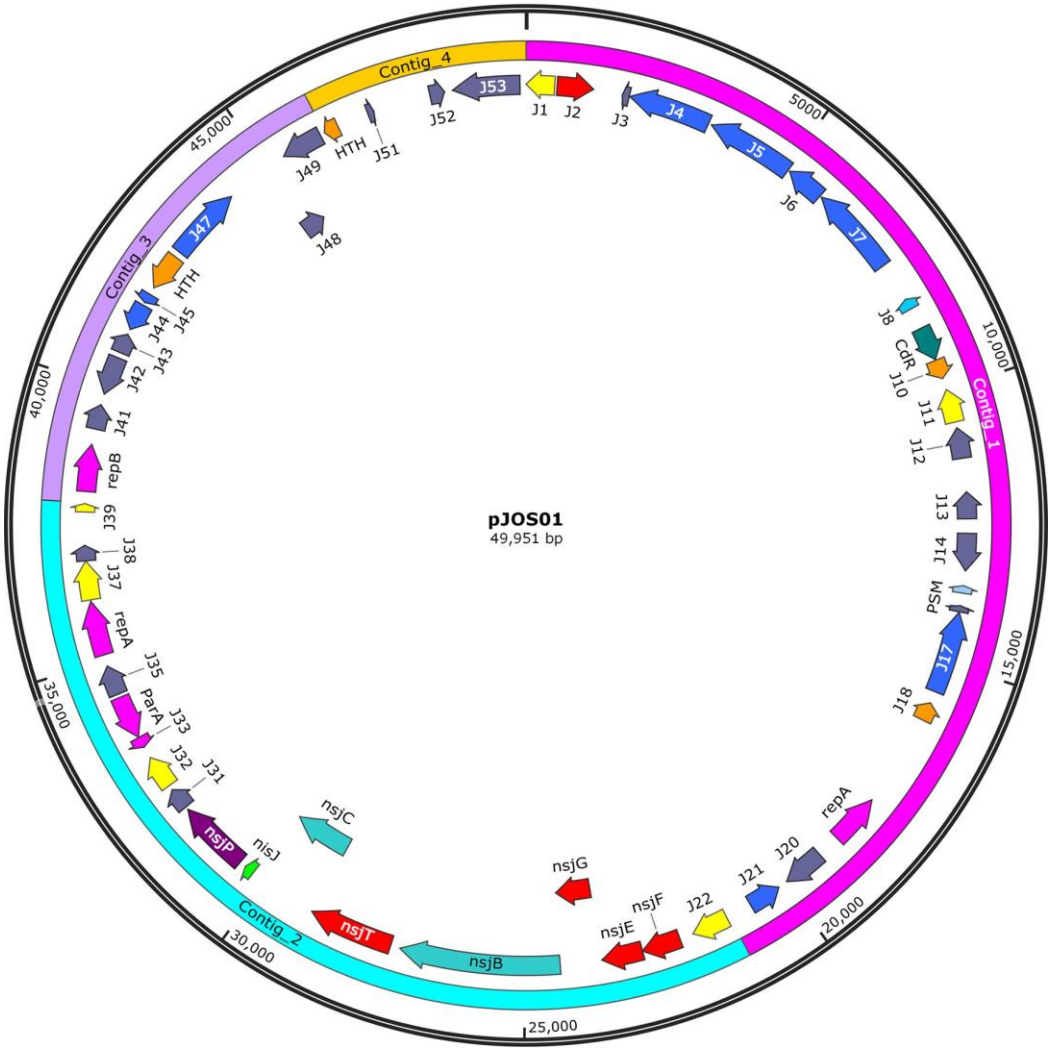
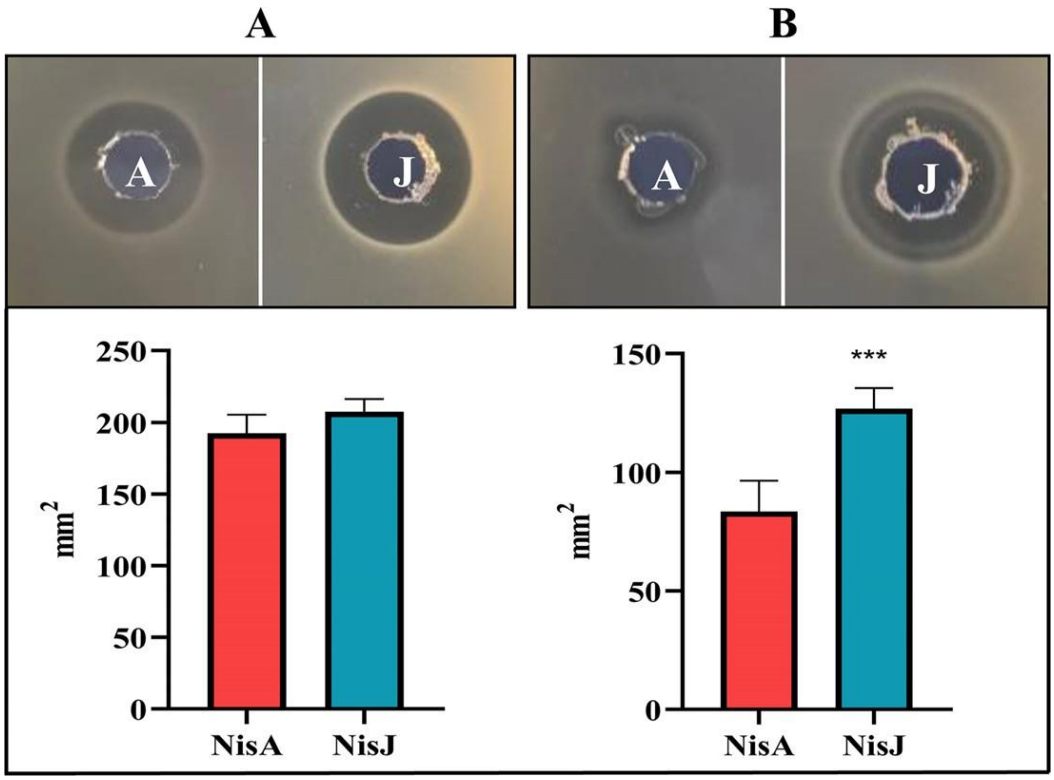


Figure 6 Activity of cell-free supernatant of nisin J- and A-producing strains as observed in WDA against MG1614 (NSR^-) (A) and MG1614/pNP40 (NSR^+) (B). The assay results are representative of triplicate experiments. The results reveal no significant differences in zones of inhibition against NSR^- (*P* value of 0.1701) (bar graph in panel A) but show a significant difference (***) against NSR^+ (*P* value of 0.0001) (bar graph in panel B).



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Chapter 3.1

Nisin variants from *Streptococcus* and *Staphylococcus* successfully express in *Lactococcus lactis* NZ9800

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JN O'Sullivan†, **PM O'Connor**†, MC Rea, D Field, C Hill and RP Ross

† Joint first authorship

3.1.1 Abstract

Nisin A and its natural variants, like other lantibiotics, are Class I bacteriocins encoded by gene clusters which typically include a structural peptide, modification enzymes, a protease, regulatory, immunity and transporter proteins. Many of the more recently discovered nisin variants, such as nisin H, nisin J and nisin P, are produced by non-GRAS strains. Growing concerns regarding the increases in antimicrobial resistance (AMR) has meant that the antimicrobial potential of lantibiotics is now being investigated irrespective of the nature of the producing organism. Bioengineering and synthetic biology approaches may offer a solution by permitting the recombinant expression of peptides naturally expressed by non-GRAS organisms in well characterized hosts derived from safe strain lineages.

This study involved cloning the nisin promoter and nisin A leader sequence fused to either nisin H, nisin J or nisin P structural gene sequences originally produced by *Streptococcus hyointestinalis* DPC 6484, *Staphylococcus capitis* APC 2923, and *Streptococcus agalactiae* DPC 7040 respectively. This resulted in their expression in *Lactococcus lactis* NZ9800, a genetically modified strain that does not produce nisin A. Antimicrobial activity of nisin H, nisin J and nisin P was observed following induction of the nisin-controlled gene expression system demonstrating that these 3 nisin variants could be acted on by Nisin A machinery provided by the host strain. This study describes the first successful heterologous production of natural nisin variants by a GRAS strain, and demonstrates how such systems could be harnessed not only for lantibiotic production, but also in the expansion of their structural diversity and development for use as future biotherapeutics.

3.1.2 Introduction

Resistance to antibiotics has increased the risks associated with many infectious bacterial strains, resulting in higher mortality and increased costs on health care services (1). A combination of overuse and/or misuse of antibiotics has played a detrimental role in the spread of antimicrobial resistance (2). Proliferation in the number of multidrug resistant (MDR) bacteria combined with an alarming paucity of new antibiotics means the discovery and development of novel antimicrobial therapies is vital (3, 4). Antimicrobial peptides (AMPs) are produced by a wide array of organisms, forming part of the innate response in plants, animals, bacteria and fungi (5). Due to an enhanced understanding of their properties and functions, many AMPs, and especially bacteriocins, are now under consideration as suitable alternatives to antibiotics with potential applications in both animal and human health (6, 7). Bacteriocins are small ribosomally-synthesised heat stable peptides produced by bacteria and can have either a broad or narrow antimicrobial inhibition spectrum. As such, they are increasingly attractive alternatives to antibiotics for some applications (8-11). Lantibiotics (lanthionine containing antibiotics) make up the largest class (class I) of bacteriocins and are generally associated with low levels of resistance (9,12). The presence of lanthionine (Lan) and/or B-methyllanthionine (MeLan) residues are characteristic features of lantibiotics and are the result of the dehydration of serine and threonine to form 2,3 dehydroalanine (Dha) and 2,3 dehydrobutyrine (Dhb), respectively. These dehydrated residues subsequently react with the thiols of cysteine molecules forming Lan/MeLan bridges (13-16).

Discovered by Rogers in 1928 (17), nisin A, a 34 amino acid peptide produced by *Lactococcus lactis* (*L. lactis*), is the oldest and most extensively characterised lantibiotic. It is synthesised by a typical nisin gene cluster that encodes the modification enzymes NisB and NisC responsible for catalysing dehydration and

cyclisation reactions respectively, a transporter (NisT), a regulatory system (NisRK), a protease involved in cleaving the pre-peptide (NisP) and immunity proteins NisI and NisFEG (18, 19). Structurally, nisin A consists of one Lan and four MeLan rings and three dehydrated residues, two Dha and one Dhb (20). Nisin A binds to Lipid II, a bactoprenol-bound peptidoglycan precursor, essential for peptidoglycan synthesis. The N-terminal of nisin A sequesters the pyrophosphate moiety of Lipid II resulting in the formation of a pyrophosphate cage complex. This complex induces a conformational change at the C-terminal of nisin enabling it to translocate into the cell membrane thus interfering with membrane integrity causing pore formation and cell death (21). While nisin has been widely used as a safe food preservative for decades (22), the increased incidence of multidrug-resistant bacterial infections has resulted in a revival in interest surrounding its potential use in life-threatening infections. Many studies have highlighted the *in vitro* potency of nisin against human pathogens (the reader is directed to comprehensive reviews (23, 24)).

While nisin A is Food and Drug Administration (FDA) approved as a food ingredient due to its production by Generally Recognised as Safe (GRAS) strains of *L. lactis*, several recently characterised nisin variants have been identified in rare and/or emerging pathogenic bacteria. It is becoming apparent that nisin production has a broader distribution than first thought and variants have been found in a number of genera including lactococci, streptococci, staphylococci and Blautia species. In addition, the nisin variants, such as the three used here, have many amino acid substitutions compared to nisin A. For example, Nisin H is produced by *St. hyointestinalis*, a member of the viridans streptococci group (VGS) that have the pathogenic potential to cause a variety of infections. The nisin H peptide has 5 amino acid substitutions in comparison to nisin A specifically, Ile1Phe (i.e., at position 1,

nisin A has Ile while nisin H has Phe), Leu6Met, Gly18Dhb, Met21Tyr, and His31Lys (25). Nisin J, produced by *Staphylococcus capitis* (*S. capitis*) (also an emerging opportunistic pathogen), has 9 alterations with respect to nisin A, comprising 8 amino acid changes (Ile4Lys, Met17Gln, Gly18Thr, Asn20Phe, Met21Ala, Ile30Gly, Val33His and Lys34Thr) and an extra amino acid (Lys35) at the C- terminus, making it the longest natural nisin variant (26). Nisin P, produced by *Streptococcus agalactiae* (*St. agalactiae*) (group B *Streptococcus*) (27) is the shortest natural nisin variant, consisting of just 31 amino acids, and has 10 amino acid differences with respect to prototypical nisin A (Ile1Val, Ile4Lys, Ala15Ile, Gly18Thr, Asn20Ala, Met21Ile, His27Gly, Ser29His, Ile30Phe, His31Gly) (28,29) (Figure 1). All nisin variants are predicted to have a similar structure and mode of action to nisin A.

Nisin has been the subject of extensive bioengineering strategies in a bid to improve and enhance its antimicrobial activity, heat stability, solubility, diffusion, and protease sensitivity (30–34). To that end, the development of novel expression systems has been necessary. As multiple genes are required for nisin synthesis and immunity, it is often effective to produce bioengineered nisin variants against the background of the original producer (31, 35–37). Moreover, it has been demonstrated that a *L. lactis* strain expressing *nisABTC* is sufficient for the production and export of not only fully modified nisin but also a non-lantibiotic fusion of leader peptide with dehydrated angiotensin (38). This broad substrate specificity of the nisin dehydrating and transport machinery suggests that lantibiotic enzymes could be utilized for the synthesis of a wide range of novel dehydro residue-containing peptides or novel lantibiotic structures (39–41).

The development or utilization of heterologous bacteriocin expression systems offers advantages over natural producers including: i) the potential to improve yield as

production levels in their original hosts can be very low (42) ii) improved efficiency of purification protocols as many producing cultures require complex broth for growth, making purification time consuming and expensive and iii) production in a GRAS organism in instances where the original producer may be pathogenic with limited industrial application (43). The looming antimicrobial resistance crisis makes it inevitable that the creation of such heterologous systems will become more important to produce novel alternative antimicrobials at industrial scale for both food safety and healthcare applications.

The aim of this study was to develop a heterologous expression system for the production of the natural nisin variants nisin H, nisin J and nisin P, in a GRAS host, namely *L. lactis* NZ9800, by adopting a synthetic biology approach.

3.1.3 Materials and Methods

Bacterial strains, culture conditions and plasmids

Bacterial strains, their growth conditions and plasmids used in this study are listed in Table 1. Anaerobic conditions for bacterial strains, where applicable, were achieved using anaerobic jars and Anaerocult A gas packs (Merck, Darmstadt, Germany). *L. lactis* strains were grown in M17 broth or agar (Sigma-Aldrich, (Merck, Darmstadt, Germany), supplemented with 0.5% glucose (GM17) at 30°C. *E. coli* was grown in Luria-Bertani (LB) agar or broth with vigorous shaking at 37°C. Antibiotics were used, where indicated, at the following concentrations: Chloramphenicol (Cm) at 5 and 10 μg mL^{-1} respectively for *L. lactis* and *E. coli* (31).

Synthetic gene hybrid design

To express nisin H, nisin J and nisin P into a lactococcal background, synthetic gene hybrids containing the full NisA leader sequence ($nisA^L$), followed by each of the nisin H, nisin J and nisin P structural gene sequences were designed (Table 2). Hybrid genes containing the NisA promoter, the full NisA leader sequence ($nisA^L$), followed by each of the structural sequences, nisin H, nisin J and nisin P, were synthesised by IDT (1710 Commercial Park, Coralville, Iowa, 52241, USA).

Molecular cloning procedures

The *E. coli/L. lactis* shuttle vector pCI372 (64) was extracted from *E. coli* HB101 cells using a NucleoSpin plasmid kit (Macherey-Nagel, Duren, Germany). The $nisA^L$–nisH/nisJ/nisP gene strands (100 ng) were amplified by polymerase chain reaction (PCR) set up as follows: templates (100 ng) pUCIDT-KAN-*nis*J, pUCIDT-KAN-*nis*H and pUCIDT-KAN-*nis*P; Forward and Reverse oligos (0.3 µM) (including EcoRI and XbaI (NEB) restriction sites), (see Table 3, (Sigma-Aldrich, Merck, Darmstadt, Germany); 0.2 mM dNTPs; 1.5 mM Mg^{2+} and 2 units of KOD polymerase (Novagen) in a final volume of 50 µL. PCR reactions were carried out in a MJ Research PTC-200 thermocycler using the following conditions: initial denaturation at 98°C x 2 minutes, cycling conditions: 30 cycles of 95°C x 20 seconds, 50°C x 10 seconds, 72°C x 10 seconds each, followed by a final extension at 72°C x 10 minutes. PCR products were purified using NucleoSpin Gel and PCR clean-up (Macherey-Nagel, Duren, Germany) and (~3000 ng) digested with EcoRI and XbaI restriction enzymes (20 U) in CutSmart 10X buffer (New England Biolabs, Ipswich, UK) in a 40 µL reaction. The plasmid pCI372 (14.76 µg) was also digested in a 70 µL reaction, with EcoRI and XbaI (40 U). The digested plasmid and insert were purified using the Thermo Scientific GeneJET PCR purification kit and the products ligated in a reaction set up as follows: T4 ligase

(800 U) (New England Biolabs, Ipswich, UK); final volume of nisJ and nisP ligation reactions = 20 µL, final volume of nisH ligation reaction = 30 µL; insert concentration = 1 µg; plasmid concentration = 60 ng/µL. The ligation reactions were transformed into *E. coli* Top10 competent cells (Invitrogen, Dublin, Ireland) as per manufacturer's instructions. Plasmid DNA was extracted and the *nisH/nisJ/nisP* genes were amplified, and products sequenced (Source BioScience, Waterford, Ireland) to ensure integrity and subsequently introduced by electroporation into *L. lactis* NZ9800, a non-producing derivative of the nisin A producer *L. lactis* NZ9700, as previously described (65).

Nisin induction experiments

Production of nisin H, nisin J and nisin P was achieved through induction via the addition of 1 µL of nisin A cell-free supernatant (CFS) per mL to each culture broth, corresponding to approximately 10 ng/mL nisin A. Single colonies from each nisin producer were inoculated into 5 mL GM17 broth (Difco laboratories Inc, Omagh, UK) with and without 5 µL of nisin A CFS. Cultures were incubated at 30°C overnight and the presence of antimicrobial activity in CFS assessed by deferred antagonism assays, as outlined by Parente and Hill (1992), using *L. delbrueckii* subspecies (ssp) *bulgaricus* LMG 6901 as the target organism (66).

Purification of nisin A, nisin H, nisin J and nisin P

NZ9800 transformants containing pCI-*nis*H, pCI-*nis*J and pCI-*nis*P plasmids were streaked on GM17 agar plates (Difco Laboratories Inc., Omagh, UK) containing 10 µg/ mL chloramphenicol (Cm10). Colonies of each culture were subsequently grown in liquid media supplemented with Cm10 and 1 µL of nisin A per ml of broth.

Specifically, a single colony was inoculated into 5 ml GM17 (0.5% glucose) broth and grown aerobically overnight at 30°C. This was then used to inoculate 25 mL GM17 which, in turn, was used to inoculate 2 Litres of TY broth. The nisin A producer was grown as described above but without the addition of Cm10 or nisin A supernatant. Cultures were centrifuged (8000 rpm, 20 mins, 10°C), and cell free supernatant (CFS) retained for purification of nisin variants.

The Activity Units per ml (AU/mL) of each CFS was determined as follows: 100 µL aliquots of nisin A, nisin H, nisin J and nisin P CFS were 2 fold serially diluted in Milli Q water and 50 µL aliquots of each dilution assayed by well diffusion assays (WDA) against *L. delbrueckii* ssp. *bulgaricus* LMG 6901. AU/mL is taken as the reciprocal of the highest dilution showing activity against the indicator strain corrected per mL of culture.

Two Litres of culture supernatant were passed through an Econo column containing 60 g Amberlite XAD16N beads (Sigma Aldrich, Co. Wicklow, Ireland), prewashed with Milli Q water. The column was washed with 400 mL of 35% ethanol (30% ethanol for nisin J as it is more hydrophilic) and antimicrobial peptide eluted in 400 mL of 70% 2 propanol 0.1% TFA (IPA). To allow interaction with a C18 SPE column, the IPA was removed from the XAD IPA eluent by rotary evaporation (Buchi, Flawil, Switzerland) and sample concentrated to approximately 80 mL. The concentrated sample was then applied to a 5 g, 20 mL Strata-E C18 SPE column (Phenomenex, Cheshire, UK) pre-equilibrated with methanol and water. The column was washed with 30 mL 25% ethanol (20% ethanol for nisin J) and antimicrobial activity eluted with 30 mL IPA.

IPA was removed from the previously eluted C18 SPE IPA eluent and the resulting sample (2 ml) applied to a semi-preparative Jupiter Proteo (10 x 250 mm, 90Å, 4 µm)

RP-HPLC column (Phenomenex, Cheshire, UK) running a 25-50% acetonitrile gradient 0.1% TFA where buffer A is 0.1% TFA and B is 90% acetonitrile 0.1% TFA. Eluent was monitored at 214 nm and fractions were collected at 1-minute intervals (27). Fifty µl aliquots of HPLC fractions were assayed for antimicrobial activity against the indicator organism, *L. delbrueckii* ssp. *bulgaricus* LMG 6901, and active fractions checked by MALDI TOF mass spectrometry to assess fraction purity.

MALDI TOF Mass Spectrometry

MALDI TOF Mass Spectrometry was performed with an Axima TOF^2 MALDI TOF mass spectrometer (Shimadzu Biotech, Manchester, UK) as described by Field et al., 2015 (67).The instrument was operated in positive ion linear mode and masses reported as average values.

***In silico* comparison of nisin H/J/P biosynthetic machinery to nisin A**

Each gene sequence of each segment of the nisin H/J/P bacteriocin gene cluster was inputted into NCBI BLASTP suite and aligned to the corresponding section of nisin A gene cluster so as to obtain the percentage identity.

3.1.4 Results

Cloning nisA^L- nisin H nisin J, and nisin P pre-peptides into *L. lactis* NZ9800

Gene constructs containing the nisin A leader (nisA^L) fused to the nisin H, nisin J and nisin P structural genes were amplified and subcloned from pUCIDT-KAN into the *Escherichia coli*-(*E. coli*)- *L. lactis* shuttle vector pCI372 (31, 44), to generate pcI-*nis*H, pcI-*nis*J and pcI-*nis*P plasmids. DNA sequence analysis confirmed the integrity of each synthetic hybrid gene. These plasmids were purified and subsequently

introduced into *L. lactis* NZ9800 following the cloning strategy outlined in Figure 2. *L. lactis* NZ9800 was chosen as it is derived from the wild type nisin A producing strain NZ9700. A 4bp deletion from the structural gene *nis*A means this strain does not produce nisin, and the transcription of the nisin operon is blocked. This was one of the early host strains of the nisin-controlled gene expression (NICE) system since it provides the necessary regulatory (*nisK* and *nisR*) and immunity (*nis*I, *nis*FEG) genes. Importantly, it contains genes for the nisin biosynthetic machinery (*nisBCTP*) thus making it a potentially suitable strain in which to express nisin H, nisin J and nisin P genes cloned under the control of the nisin promoter (36), see Table 1.

Production of nisin H, nisin J and nisin P by *L. lactis* NZ9800

Initially, transformants of pCI-*nis*H, pCI-*nis*J and pCI-*nis*P failed to produce zones of inhibition against the indicator strain, *Lactobacillus delbreuckii* ssp. *bulgaricus* LMG 6901, when assayed by deferred antagonism assays on solid agar (data not shown). Similarly, when cell free supernatant (CFS) from overnight cultures was assessed using well diffusion assays (WDA), no antimicrobial activity was observed when compared to the nisin A control (Figure 3A). However, the addition of nisin A CFS at 1 µL/mL (approx 10 ng nisin A) resulted in bioactivity against the indicator strain *L. delbreuckii* ssp. *bulgaricus* LMG 6901 (Figure 3B). Addition of exogenous nisin A CFS to growing transformant cultures was essential for antimicrobial production, presumably because it was necessary to induce the system.

Purification of nisin A, nisin H, nisin J and nisin P from *L. lactis* NZ9800 grown in TY broth

The ability of the transformants to produce fully modified and active nisin H, nisin J and nisin P, in comparison to the nisin A producer, was assessed by growing the producing strains in equal volumes of TY broth and, purifying the peptides from CFS using Amberlite XAD16N, C18 SPE and Reversed Phase HPLC. The cell pellet is not typically a good source of nisin A and so is routinely discarded. The cell pellets of the nisin H, J and P producers were assayed for antimicrobial activity by WDA and potential masses of interest by MALDI TOF MS. Both assays suggested that activity was low from cells as evidenced by low AU/mL in WDA of cell extract and failure to detect nisin H, nisin J and nisin P masses by MALDI TOF MS (data not shown).

Determination of the AU/mL of each of the culture supernatants revealed that the nisin A producer, *L. lactis* NZ9700, produces the most activity (10,240 AU/mL) when compared to nisin H (80 AU/mL), J (80 AU/mL) and P (160 AU/mL). The HPLC chromatogram for nisin A shows that it elutes at 47 minutes, the corresponding fraction is active against *L. delbrueckii* ssp. *bulgaricus* LMG 6901 and a 3354 Da mass was detected in this fraction which agrees with the 3352 Da theoretical mass (Figure 4a). Nisin H and P are similarly hydrophobic and elute at 47 and 48 minutes, respectively. The corresponding fractions are active and masses of 3453 Da (expected mass 3453 Da) and 2989 Da (expected mass 2989 Da) were detected for nisin H and P respectively. Nisin J is a more hydrophilic peptide and elutes earlier in the gradient in fraction 31.

The HPLC chromatograms also show that the mV response is low for nisin H (Figure 4b), J (Figure 4c) and P (Figure 4d) compared to nisin A (Figure 4a). This is expected as the initial WDA assay of TY supernatants show that the AU/mL was much lower for nisin H, J and P than nisin A.

The purification of nisin H, J and P from transformants confirms the successful expression of nisin H, J and P in *L. lactis* NZ9800, albeit with a low purification yield for the three nisin variants (< 0.01 mg/L for H, J and P compared to 2.5 mg/L for nisin A). HPLC and MALDI TOF MS confirm the functioning of the NICE system as peptides of the correct mass for each of the nisin variants are being produced. Low production levels could be the result of impaired processing, given the low percentage identities between the nisin A biosynthetic genes and the nisin H, J and P counterparts, or may result from the inability of the cell to export the unprocessed peptide due to incompatibility with the transporter peptide or codon usage. Production could potentially be improved by substituting cognate modification genes and optimising codon usage, but successful production of variants confirms redundancy in the nisin biosynthetic and/or immunity machinery.

Of the three natural nisin variants used in this study, nisin P (structural gene plus leader sequence) is most similar to nisin A with a percentage identity of 90.91%, while nisin H and nisin J have percentage identities of 82.00% and 52.56% respectively. It is interesting to note that nisin P (160 AU/mL) CFS has higher activity as measured in AU/mL in TY broth compared to nisin H (80 AU/mL) and nisin J (80 AU/mL). The percentage identity of the nisin J biosynthetic machinery compared to nisin A is considerably lower than that of nisin H and P, see Figure 5. Overall, the successful production of nisin H, J and P demonstrates the promiscuity of the nisin A biosynthetic/modification machinery.

These results demonstrate the potential of heterologous expression and suggest that, with further optimisation, this system could be utilised to express and harness nisin variants produced by non-GRAS approved strains.

3.1.5 Discussion

Bacteriocins have major potential for applications in food preservation, food safety and both animal and human healthcare (45–47). Although many novel lantibiotics, (Class I RiPPs), have been discovered, production of these peptides at high levels from their original hosts can be challenging and costly due to the natural producer not being a GRAS approved strain or difficulties growing the host strain. The increasing threat of an AMR crisis means that potentially potent lantibiotics should not be overlooked and attempts should be made to harness them through heterologous expression in alternative hosts. Their gene encoded nature allows for easier modification resulting in the development of novel lantibiotics with improved properties, giving them an advantage over other antimicrobial classes (48). Previous studies have heterologously expressed bacteriocins for applications in food or medicine (49–51). The aim of this study was to express the natural nisin variants H, J and P in a GRAS strain, *L. lactis* NZ9800, under the control of the nisin A promoter. The main advantage of this approach is that it does not require potentially pathogenic host strains for the production of antimicrobial peptides that possess potent activity against food spoilage and pathogenic strains.

Nisin is the prototypical lantibiotic that exhibits potent broad-spectrum antimicrobial activity against a wide array of pathogens. Several bioengineering studies of wild type nisin A have been carried out to improve its antimicrobial properties and functions (52–55). Numerous elegant studies have established that the nisin A biosynthetic/modification machinery demonstrates tolerance to a broad range of substrates (40, 53, 56). Majchrzykiewicz et al., (2010) effectively used the nisin modification proteins to produce, modify and secrete entirely unrelated putative lantibiotics, identified from *in silico* screening, via the nisin expression system (40).

Specifically, novel two component lantibiotic pneumococcin, from *Streptococcus pneumoniae* R6, was selected as the substrate for the nisin enzymes. Introduction of a fusion peptide, consisting of nisin leader and mature peptide, into an *L. lactis* host that overproduces NisBTC, resulted in modification of the peptides and inhibition of *Micrococcus flavus.*

Another noteworthy example involved using the nisin expression/modification system to effectively dehydrate, secrete and, in a few cases, form ring structures, in derivatives of a variety of different peptide hormones that mediate a myriad of physiological functions including enkephalin, luteinizing hormone, angiotensin and erythropoietin (56, 57). Moreover, the modified angiotensin derivatives exhibited increased resistance to proteases and enhanced biological activity relative to their linear counterparts (57, 58). Fusion of the wild type nisin leader sequence to 54 genes encoding novel lantibiotics resulted in the successful expression of 30 peptides using the prototypical nisin A machinery system, five of which exhibited good antimicrobial activity (41).

Induction of the transformants with nisin A supernatant was required for production of antimicrobial activity in the nisA^{L}-nisH/J/P supernatants demonstrating that the expression of these natural nisin variants in *L. lactis* is dependent on induction of the NisRK system. Induction by the antimicrobial of interest is a requirement for production that switches on the NICE system in clones, thereby stimulating the nisin A biosynthetic machinery to produce peptides of interest (31, 59).

Purification of nisin H, nisin J and nisin P from the *L. lactis* host was performed following detection of antimicrobial activity from the clones though yields were low compared to nisin A. The low production yield may be attributed to the low percentage identity between the biosynthetic machinery (LanB and LanC) of nisin H, nisin J, nisin

P to nisin A, with *lanB* and *lanC* of nisin H/ J / P~58% , ~25%, ~ 90% similar to that of nisin A. Also, the transporters of nisin H, J, P are ~76%, ~33% and ~59% similar to the nisin A LanT, while the nisin A protease is ~54%, ~31%, ~30% similar to those of nisin H, J and P respectively. Thus, the nisin A biosynthetic machinery, transporters and protease may not cleave and export the nisin H, nisin J and nisin P variants efficiently. Indeed, in the study by Lagedroste and co-workers (60) a systematic characterization of position one variants of nisin A expressed in the nisin A background revealed an extensive variation in the ability of the protease NisP to cleave the leader peptide and release the active nisin derivative. The greatest impact was observed when charged (His, Arg, Lys, Asp, Glu) and aromatic amino acids (Trp, Phe, Tyr) were substituted in place of the normally present valine. Notably, I1F which corresponds to the first amino acid in nisin H displayed approximately 13% cleavage compared to the wild type peptide. While this highlights one of the reasons for the relatively poor production of nisin H in our study, nisin P contains valine at position one and so should not impact on NisP activity. Furthermore, a study by Piper and colleagues (44), demonstrated that, although the heterologous expression of the natural nisin variants NisF, NisZ and NisQ facilitated the production of sufficient peptides for direct specific activity comparisons with NisA, no Nisin U and U2 production was observed in the nisin A background.

MALDI TOF mass spectrometry of active fractions revealed the presence of the expected masses for nisin H, nisin J and nisin P confirming that the correct fully processed nisin variants are being produced in the GRAS strain. Codon usage may also be a factor in the poor expression levels as this was not considered in the design of the *nisA*^{L}*-nisH/J/P* genes. However, the use of *L. lactis* codon utilization preferences when *nisU* and *nisU2* genes incorporated with the nisin A leader by Piper et al., (2011) did

not result in successful production of these peptides even though nisin A was added exogenously to ensure that induction of the P*nis* promoter was not a concern (44). This suggests that, although heterologous peptide production was successful using this system, poor efficiency is possibly due to the low similarity of the transporters and machinery of the nisin variants to wild type nisin A. Low percentage identity leading to the failure of one or more of the biosynthetic (NisBC), leader removal (NisP) or export (NisT) proteins to recognize the encoding peptides was also suggested by Piper *et al.* (2011) (44).

Further optimisation of this system is required for increased production of nisin variants and their further characterisation. Indeed, a recent study involving synthetic biology approaches describes the development of a genetic system whereby cloning an entire nisin biosynthesis pathway from a nisin-producing strain facilitates significant overproduction of nisin (61). Furthermore, extra copies of *nis*FEG, *nis*RK and *nis*I cloned under strong constitutive promoters has been shown to improve production levels and is an approach that may be employed in future studies to improve the heterologous production of nisin H, nisin J and nisin P (62, 63).

3.1.6 Conclusion

This is the first description of the heterologous production of different naturally-occurring nisins in *L. lactis*. From this, it is apparent that there is considerable redundancy in the nisin modification and transport machinery which can be employed to process different nisin structures. Furthermore, the system represents a powerful tool that could facilitate the production of novel nisin derivatives as well as aid in the understanding of structure-activity relationships in nisin A and its variants.

3.1.7 Acknowledgements

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Table 1 Bacterial strains, their growth conditions and plasmids used in this study.

		Growth conditions		
Species	Strain	**Temp (°C)**	**Atmosphere**	**Growth media**
E. coli	TOP10	37	Aerobic*	LB
Lactobacillus delbrueckii subsp. *bulgaricus*	LMG 6901	37	Anaerobic**	MRS
Lactococcus lactis	NZ9800	30	Aerobic	GM17
Lactococcus lactis	NZ9700	30	Aerobic	GM17
Lactococcus lactis	NZ9000	30	Aerobic	GM17
Lactococcus lactis subsp. *cremoris HP*		30	Aerobic	GM17
Plasmid vectors				
pCI372 plasmid vector		30		
pcI- J		30		
pcI-H		30		
pcI-P		30		

LMG Laboratorium voor Microbiologie, Universteit Gent, Belgium; NZ= NIZO

* Vigorous shaking required

** Anaerobic conditions were achieved through the use of anaerobic jars and Anaerocult A gas packs (Merck, Darmstadt, Germany).

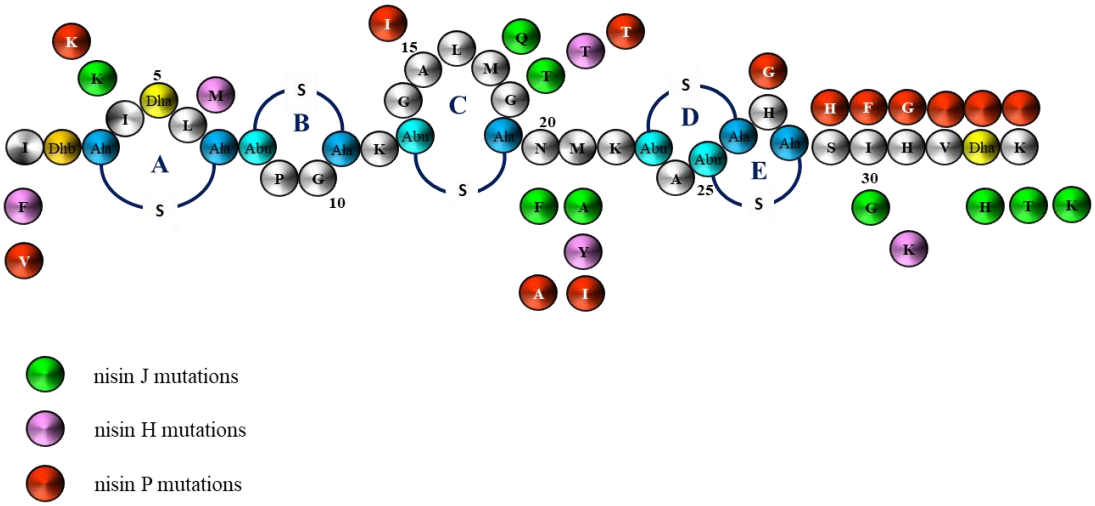
Table 2 Gene hybrid synthesis design.

Blue = nisin A promoter, Red = nisin A leader sequence, yellow highlight= nisin J structural gene, blue highlight= nisin H structural gene, green highlight= nisin P structural gene, pink highlighted ‘taa’= stop codon.

Nisin J	Tagtcttataactatactgacaatagaaacattaacaaatctaaaacagtcttaattctatcttgagaaagtattggtaataatattattgtcgat aacgcgagcataataaacggctctgattaaattctgaagtttgttagatacaatgatttcgttcgaaggaactacaaaataaattataaggag gcactcaaa**atgagtacaaaagattttaacttggatttggtatctgtttcgaagaaagattcaggtgcatcaccacgcatta ctagtaaatcactttgtacaccaggatgtaaaactggagcactacaaacatgctttgctaaaactgcaacttgccactgttctgg acacgtgcatactaaataa**
Nisin H	tagtcttataactatactgacaatagaaacattaacaaatctaaaacagtcttaattctatcttgagaaagtattggtaataatattattgtcgat aacgcgagcataataaacggctctgattaaattctgaagtttgttagatacaatgatttcgttcgaaggaactacaaaataaattataaggag gcactcaaa**atgagtacaaaagattttaacttggatttggtatctgtttcgaagaaagattcaggtgcatcaccacgctttacaagt atttcgatgtgtacaccgggatgtaagactggtgcattaatgacgtgtaattataaaactgctacatgtcactgtagtatcaaggtt tcaaaataa**
Nisin P	tagtcttataactatactgacaatagaaacattaacaaatctaaaacagtcttaattctatcttgagaaagtattggtaataatattattgtcgat aacgcgagcataataaacggctctgattaaattctgaagtttgttagatacaatgatttcgttcgaaggaactacaaaataaattataaggag gcactcaaa**atgagtacaaaagattttaacttggatttggtatctgtttcgaagaaagattcaggtgcatcaccacgcgtaactagt aaatcattatgtactcctggatgtaagacgggtattttgatgacctgtgcaatcaaaactgcaacttgtggttgccattttggataa**

Figure 1 Graphical representation (A) of nisin A with nisin H, nisin J and nisin P amino acid substitutions highlighted; and sequence alignment (B) of nisin A with nisin H, nisin J and nisin P variants used in this study.

A)



B)

A	ITSISLCTPGCKTGALMGCNMKTATCHCSIHVSK	3354 Da	*L. lactis* NZ9700
H	**F**TSIS**M**CTPGCKTGALM**T**CN**Y**KTATCHCSI**K**VSK	3453 Da	*St. hyointestinalis* DPC 6484
J	ITS**K**SLCTPGCKTGAL**QT**C**FA**KTATCHCS**G**HV**HT**K	3459 Da	*S. capitis* APC 2923
P	**V**TS**K**SLCTPGCKTG**I**LM**T**C**AI**KTATC**G**C**HFG**	2989 Da	*St. agalactiae* DPC 7040

Figure 2 Description of cloning strategy adapted for this study. Synthetic genes consisting of nisA promoter and leader fused to nisH/J/P were separately amplified by PCR from pUCIDT-KAN-*nis*J, pUCIDT-KAN-*nis*H and pUCIDT-KAN-*nis*P, digested with *Eco*RI and *XbaI*, and subsequently ligated with pCI372 of each construct. The constructs were transformed into *L. lactis* NZ9800 following sequencing to confirm integrity.

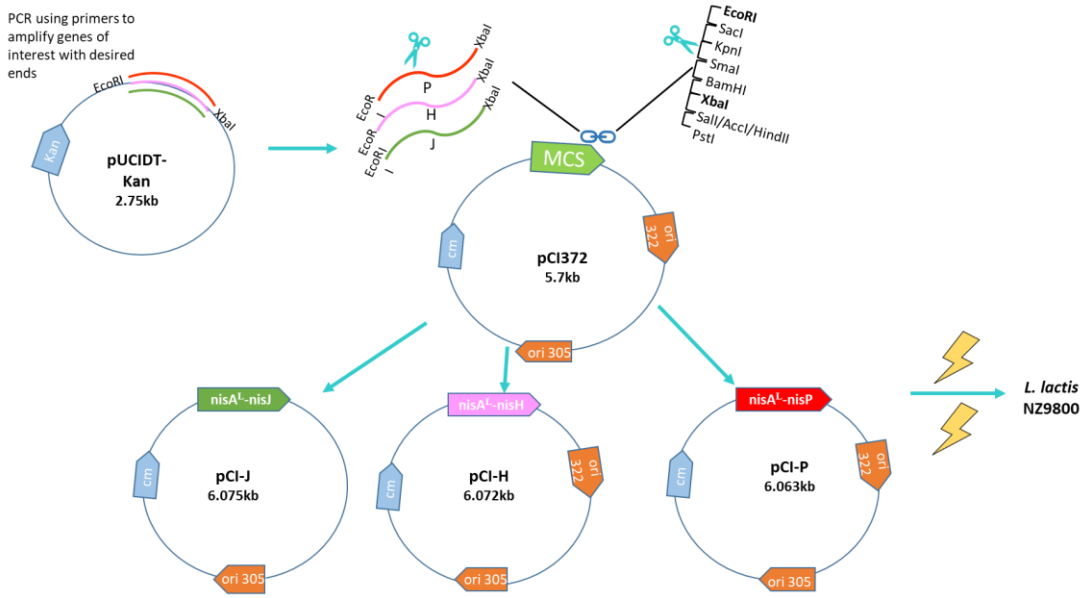


Figure 3 Well diffusion assays of nisin A, nisin H, nisin J and nisin P WDA of cell free supernatants (CFS) of uninduced (top row) and induced (bottom row), overnight, cultures of nisin A (A), H (H), J (J) and P (P) with *L. delbrueckii* ssp. *bulgaricus* LMG 6901 as the target indicator. GM17 media (M) and GM17 containing 1 µL/ mL nisin A CFS (Mi) were included as controls.

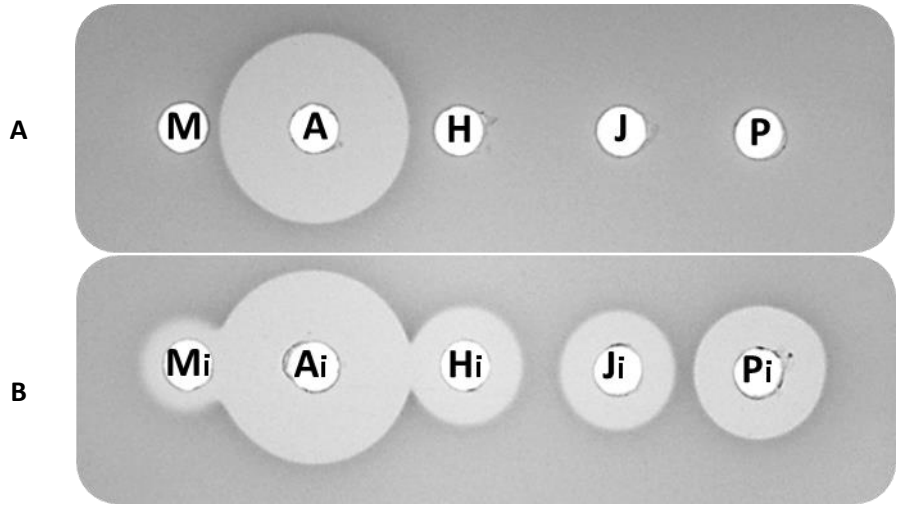


Figure 4 Purification of nisin A, nisin H, nisin J, and nisin P from their respective producing strains. HPLC chromatogram (mAU vs time in minutes) acquired at 214 nm, MALDI TOF mass spectra (% intensity of mass to charge ratio (m/z)) and zone of inhibition for active fractions are shown for nisin A (4a) nisin H (4b), nisin J (4c) and nisin P (4d).

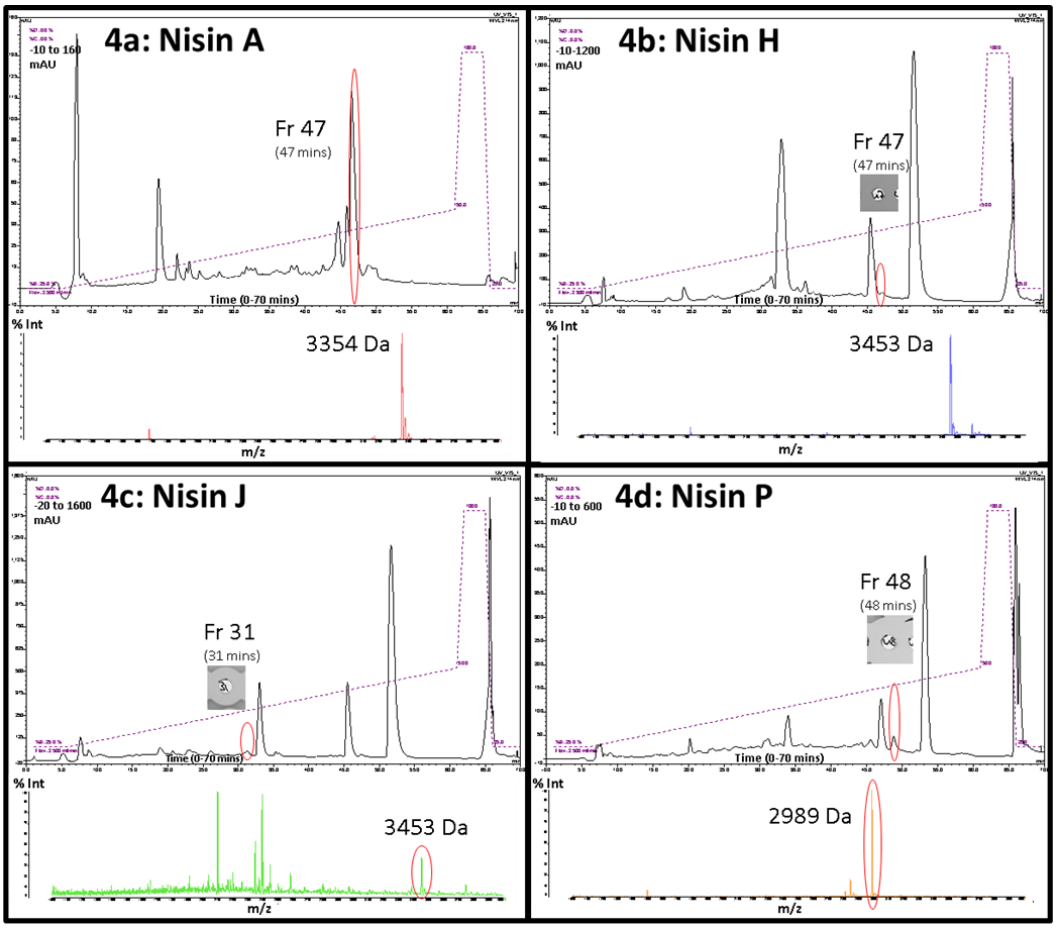
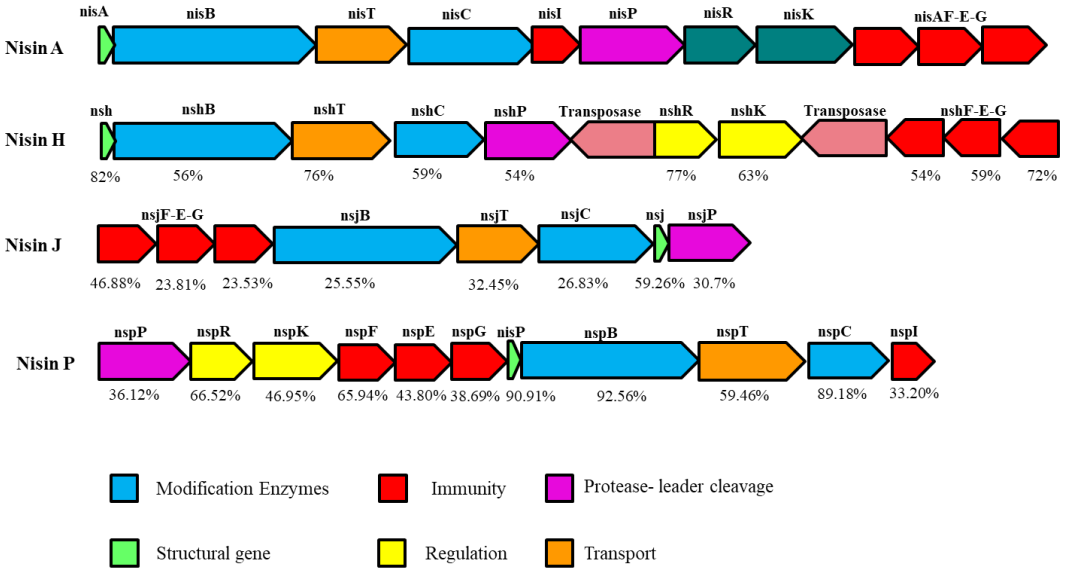


Figure 5 Percentage identity comparison of the nisin H/J/P to nisin A biosynthetic machinery.



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Chapter 4.1

The potency of the broad-spectrum bacteriocin, bactofencin A, against staphylococci is highly dependent on primary structure, N-terminal charge and disulphide formation

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PM O'Connor, EF O'Shea, PD Cotter, C Hill and RP Ross.

4.1.1 Abstract

Bactofencin A is a novel class IId bacteriocin, produced by the intestinal isolate *Lactobacillus salivarius* DPC6502, which has potent activity against medically significant pathogens including *Staphylococcus aureus*. This bacteriocin is unusual in that it has a highly cationic N terminus and a single disulfide bond between Cys7 and Cys22, resulting in a large C terminal loop. In this study, a library of synthetic bactofencin A variants was screened against the mastitis isolate, *S. aureus* DPC5246, to identify key residues responsible for activity. It was apparent that substituting either cysteine of the disulfide bond with either serine or alanine significantly reduced the activity of the bacteriocin, confirming the importance of the C terminal loop. Substituting N terminal amino acids with alanine had no effect on activity, whereas sequential removal of the N terminal positively charged residues resulted in an increasingly inactive peptide. A complete (synthetic) alanine scanning analysis revealed that the residues between Val9 and Gly17 were most affected by substitution, suggesting that this area has a major influence on the potency of the bacteriocin. Substituting residues in the loop region between Cys7 and Cys22 for D-amino acid equivalents had a more detrimental effect on activity than L-alanine substitutions. Specifically Y10A, N11A, P15A and T16A are active at 4, 16, 1 and 16 μM respectively while their D equivalents were inactive at 1000 μM, the highest concentration tested. Ultimately, this study identifies the critical features in the primary structure of the bacteriocin which gives it such potent activity against pathogenic staphylococci.

4.1.2 Introduction

Concerns about the increased incidence of antimicrobial resistance (AMR) against

human pathogens have led to calls for global efforts to combat this worrying phenomenon. If left untackled, once treatable infections will again become incurable (1). The Global Antimicrobial Resistance Surveillance System (GLASS) was set up in 2015 to standardise the collection and sharing of data on AMR at a global level and to promote coordinated action. In support of this initiative, the WHO recently surveyed the development of new antibiotics in the clinical pipeline against priority pathogens and found that there is particular need for new classes of antimicrobial to abate the threat of AMR. Specifically, they found that most antibiotics are derived from existing antibiotic classes and these are only considered a temporary solution to AMR as they will be quickly rendered ineffective by existing resistance mechanisms. Expressly, they state that more investment is needed in fundamental drug discovery to discover more innovative antimicrobials against priority pathogens including *Mycobacterium tuberculosis*, *Clostridium difficile* and *Staphylococcus aureus* (2).

One class of antimicrobial that is receiving increased attention is the bacteriocins. These are stable peptides naturally produced by many bacteria and have potent activity against other bacteria including antimicrobial resistant pathogens (3). Bacteriocin production by intestinal strains is considered a desirable probiotic trait that could potentially mediate an effect in three different ways; it may allow the producing strain to compete in the crowded gut ecosystem, it could provide protection to the host against pathogens and could potentially signal the immune system in a similar fashion to host antimicrobial peptides (AMPs) (4-7).

Recently, we described bactofencin A, a small, positively charged bacteriocin produced by the porcine gut isolate *L. salivarius* DPC6502. Structurally, it consists of a positively charged N terminal attached to a C terminal loop formed via a disulfide bond between Cys7 and Cys22. Bactofencin A is highly cationic and has been

compared to eukaryotic defensins which contain a high Lys/Arg molar ratio, considered essential for bactericidal activity (8, 9) Another unusual feature of bactofencin A is that immunity is mediated through a *dltB* homologue proposed to decrease the negative charge of the cell wall thereby reducing attraction between bacteriocin and cell, rather than a specific immunity protein. Bactofencin A displays activity against *S. aureus* and *Listeria* spp. and has been shown to subtly modify gut populations (10, 11).

The rational design of novel antimicrobials is rapidly evolving via the use of bioengineering to generate novel bacteriocin variants with enhanced functionality. This has been realised through the recent generation of both one and two peptide bacteriocins with greater activity against foodborne and medically significant Gram-positive and Gram-negative pathogens (12-17). Indeed, nisin V, a single Met21Val substitution variant of the well characterised commercial bacteriocin nisin A generated in our laboratory, has greater *in vivo* efficacy against *Listeria monocytogenes* when compared with the native peptide (18). In many cases, the identification of enhanced derivatives has been realised following initial studies in which saturation or scanning mutagenesis have been employed to reveal key important residues and structures within the peptide (12).

The aim of this study was to determine the importance of specific residues and regions within bactofencin A to its anti-*S. aureus* activity. Bactofencin A is a relatively short Class IId peptide in which a disulfide bond naturally forms, making it especially amenable to peptide synthesis as a means of carrying out such structure-function investigations. Specifically, a saturation approach was undertaken as follows; firstly, both cysteines were substituted with serine and alanine, individually and in tandem, to elucidate their role in peptide structure. Secondly, deleted variants were synthesised

to assess the function of the positively charged N terminal. Thirdly, alanine was substituted for each amino acid (alanine scanning) and finally, a series of D-amino acid variants specific to the loop were synthesised to determine if chiral interactions with a receptor were likely to be involved in activity.

4.1.3 Materials and methods

Peptide synthesis

Bactofencin A and variants were synthesised from the C terminus to the N terminus using microwave-assisted solid phase peptide synthesis (MW-SPPS) on a Liberty Blue microwave peptide synthesizer (CEM Corporation. Mathews, North Carolina, USA). Peptides with a C terminal cysteine were synthesized on an H-Cys(Trt)-HMBP pre-loaded resin, bactofencin C22A on an H-Ala-HMBP resin and bactofencin C22Cd on HMBP resin where the initial D-Cys was manually added to the resin (PCAS BioMatrix Inc., Quebec, Canada). The amino acid attached to the resin and the following two amino acids were deprotected conventionally at 25°C, 0 W for 900 seconds in 5% piperizine in DMF to limit the formation of an undesirable 51 Da modification commonly seen in C terminal cysteine peptides. Following deprotection, the exposed amino group is coupled conventionally with the carbonyl group of the next amino-protected amino acid at 75°C, 0 W for 3600 seconds in the presence of the activator, 0.5 M N,N′-diisopropylcarbodiimide in DMF, and activator base, 1.1 M hydroxybenzotriazole in DMF. The fourth and subsequent amino acids were added using microwave deprotection at 75 °C, 60 W for 600 seconds and microwave coupling at 75 °C, 35 W for 600 seconds. Arginines were double coupled at 75 °C, 35 W, 300 seconds and histidine and cysteine coupled at 25°C, 0 W, 300 seconds and then 50°C, 35 W for 900 seconds. Following synthesis, the peptide was cleaved from the resin by

adding a cleavage mix containing 9.25 ml trifluoroacetic acid (TFA), 250 µl water, 250 µl 2′2-(ethylenedioxy)-diethanethiol and 500 µl triisopropylsilane. This mixture was then heated at 37 °C for 1 hour to cleave the peptide from the resin. Resin was removed from the cleavage mix using an Accent Cleavage system (CEM, Corporation. Mathews, North Carolina, USA) and the TFA evaporated by bubbling with nitrogen. Peptide was precipitated from the remaining solution by adding 45 ml of diethyl ether pre-cooled to −20°C and centrifuging at 1000 g for 3 minutes. The precipitated peptide was washed free of scavengers by resuspending in a second aliquot of 45 ml ice cold diethyl ether and the centrifugation step repeated.

Purification of synthetic peptides

Crude peptide was purified using Reversed Phase-HPLC on a semi preparative Jupiter Proteo (10 × 250 mm, 4 µ, 90 Å) column (Phenomenex, Cheshire, UK) running an 11–45% acetonitrile 0.1% TFA gradient over 40 minutes where buffer A is Milli Q water containing 0.1% TFA and buffer B is 90% acetonitrile containing 0.1% TFA. Fractions with the desired molecular mass were identified using matrix assisted laser deionisation -time of flight-mass spectrometry (MALDI-TOF-MS) on an Axima TOF^2 MALDI TOF mass spectrometer (Shimadzu Biotech, Manchester, UK) operating in positive ion reflectron mode and were pooled and lyophilized on a Genevac HT 4X lyophilizer (Genevac Ltd., Ipswich, UK). Peptides were resuspended in 50 mM sodium phosphate buffer pH 6.8 at approximately 1000 µM and kept at room temperature for 24–48 hours until the disulfide bond between Cys7 and Cys22 fully formed as monitored by MALDI-TOF-MS. Peptides were then further purified by a second HPLC run as described above except the gradient used was 15–30% acetonitrile 0.1% TFA gradient over 30 minutes. Again fractions containing pure

bactofencin A were identified and lyophilised for specific activity experiments.

Purification of natural bactofencin A

Bactofencin A was purified from MRS culture media as described by O'Shea *et al*. (10). Briefly peptide was purified from an overnight culture of *L. salivarius* grown in 1 litre of MRS media using SP sepharose Cation Exchange, C18 Solid Phase Extraction (SPE) and Reversed Phase HPLC.

Specific activity of bactofencin A variants

Peptides were resuspended at 1000 µM in 50 mM sodium phosphate buffer pH 6.8 and checked for purity by MALDI-TOF-MS. Peptides were serially diluted 1 in 4 to give a dilution series of 250, 62.50, 15.63, 3.91, 0.98 and 0.24 µM and assayed by the agar well diffusion assay described by Ryan *et al*. (19). Briefly, 50 µl aliquots of each peptide concentration were plated on a *S. aureus* DPC5246 indicator plate and the plate incubated at 37 °C. Peptide activity (Minimum Inhibitory Concentration (MIC)) was taken as the lowest concentration of peptide to give a zone of inhibition. All assays were performed in triplicate. The dilution series values were rounded to the nearest whole number to give 1000, 250, 63, 16, 4, 1 and 0.25 µM and data is colour coded for ease of interpretation.

Bactofencin variants synthesised for specific activity studies

Bactofencin A, KRKKHRCRVYNNGMPTGMYRWC, is a 22 amino acid bacteriocin with a disulfide bond between Cys7 and Cys22. Bactofencin variants are labelled according to the amino acid position number using the one letter code followed by the change it undergoes e.g. a lysine at position 1 to alanine change is labelled K1A.

Bactofencin Cys7 and Cys22 were substituted with serine both individually and in tandem to give bactofencin C7S, bactofencin C22S and bactofencin C7S-C22S. These amino acids were also substituted with alanine to give bactofencin C7A, bactofencin C22A and bactofencin C7A-C22A.

To assess the importance of the positively charged N terminal, the following deletion variants were synthesised; bactofencin R2-C22, K3-C22, K4-C22, H5-C22, R6-C22 and C7-C22.

Each amino acid in bactofencin A was changed to alanine to give a library of alanine scanning variants, these are specifically; K1A, R2A, K3A, K4A, H5A, R6A, C7A, R8A, V9A, Y10A, N11A, N12A, G13A, M14A, P15A,T16A, G17A, M18A, Y19A, R20A, W21A and C22A. Bactofencin C7A and C22A were also used to assess the role of cysteine in activity as described above.

Each amino acid in the loop from Cys7 to Cys22 was substituted for a D-amino acid equivalent to give a series of variants, namely bactofencin C7Cd, R8Rd, V9Vd, Y10Yd, N11Nd, N12Nd, M14Md, P15Pd, T16Td, M18Md, Y19Yd, R20Rd, W21Wd and C22Cd. An all D-amino acid variant for a preliminary MIC_{50} assay was synthesised by Alta Bioscience (Birmingham, UK).

Comparison of bactofencin A and bactofencin R8Q

Bactofencin R8Q and bactofencin R8K were synthesised as described above and their activity compared to bactofencin A against *S. aureus* DPC5246, *Listeria innocua* DPC3572 and *L. monocytogenes* ATCC 23074.

4.1.4 Results

Formation of the disulfide bond in synthetic bactofencin A

To investigate how specific residues and domains within bactofencin A contribute to its potency against staphylococci, a series of bactofencin variants were synthesized, purified and assayed. Natural bactofencin A is encoded on a four gene operon that includes an accessory protein that ensures the correct formation of the disulfide bond (10). Although synthetic peptides are initially synthesized without a disulfide bond, this bond appears to form naturally, given that we can detect it by MALDI TOF MS. Synthetic bactofencin A (2784 Da) in the reduced form was resuspended in sodium phosphate buffer pH 6.8 at 1000 µM, and bond formation (i.e. presence of peptide at 2782 Da) monitored over time by MALDI TOF MS analysis (data not shown). For subsequent investigations, synthetic peptides were HPLC purified, resuspended in sodium phosphate buffer until disulfide bond formation occurred (where appropriate), as confirmed by MALDI TOF MS, and then HPLC purified for a second time to obtain pure peptide with an intact disulfide bond.

Comparison of natural and synthetic bactofencin A

The yield of natural bactofencin A following purification from *L. salivarius* DPC6502 culture media is generally very low (<0.3 mg/L) making it difficult to generate sufficient peptide for structure/function experiments and making genetic approaches to generating peptide variants impractical. Bactofencin A, being a small 22 amino acid peptide with a single disulfide, is well suited to peptide synthesis and this approach generated milligram quantities of peptide variants for specific activity studies. Comparison of activity of natural bactofencin A (2782 Da) with synthetic reduced bactofencin A (2784 Da) and synthetic oxidised bactofencin A (2782 Da) showed that all peptides were equally active against *S. aureus* DPC5246 (Figure 1)

The role of cysteines in bactofencin A

To assess the contribution of the cysteines to the activity of bactofencin A, Cys7 and Cys22 were replaced with serine. The results (Figure 2) show that replacing Cys7 with serine results in a 63 fold reduction in activity and replacing Cys22 caused an even more detrimental 250 fold reduction in activity. Notably, replacing both Cys7 and Cys22 with a serine residue resulted in activity comparable to the C7S change alone. Replacing individual cysteine residues with alanine had less of a negative effect than serine substitution as a C7A change reduces activity 16 fold and C22A is reduced 63 fold. The C7A-C22A variant is, like the serine equivalent, comparable with the single C7A variant being 16 fold reduced. Overall, it was apparent that substituting Cys22 with either serine or alanine resulted in peptides with lower activity than those generated containing Cys7-Cys22 substitutions.

The role of the N terminal positively charged tail

Bactofencin is characterized by a cationic N terminal, KRKKHR, where 5 out of 6 amino acids are positively charged at neutral pH. To determine how this feature contributes to the activity of the peptide, a series of deletion variants were synthesized including R2-C22, K3-C22, K4-C22, H5-C22, R6-C22 and C7-C22. The specific activity of each peptide against *S. aureus* was determined and reveals that deleting the first two amino acids has no effect on activity. The further exclusion of Lys3 (K4-C22), Lys4 (H5-C22) and His5 (R6-C22) resulted in sequential 4 fold reductions in activity resulting in MICs of 4 µM, 16 µM and 63 µM, respectively. Deleting the entire positively charged N terminal region resulted in a looped peptide for which no activity could be detected (MIC >1000 µM; Figure 3).

Alanine Scanning

A series of alanine scanning variants, where each individual amino acid was replaced by alanine, were synthesized and their activity assessed. The results show that changing individual amino acids to alanine within the N terminal region had no effect on activity. This was also the case for the R8A, P15A, M18A, Y19A, R20A and W21A containing peptides. As noted previously, the C7A and C22A variants were 16 fold and 63 fold less active respectively. Substitutions between V9A and G17A show reduced activity with V9A, Y10A, N12A and G17A being 4 fold less active and N11A, G13A, M14A and T16A being 16 fold less active (Figure 4), thereby highlighting that this region is highly important for the antimicrobial activity of the peptide.

D-amino acid substitution of the loop

The generation of peptides containing D-amino acids provides an insight into the importance of chirality across the whole peptide or within specific regions. As preliminary experiments showed that an all D-variant with every amino acid changed was inactive (MIC50 >20 µM; data not shown), a series of D-amino acid variants from C7-C22 were synthesized. The D-amino acid substitutions (Figure 5) were all found to be detrimental to activity – even more so than the equivalent alanine substitutions (with the exception of C7 and N12 which are equally detrimental at 16 and 4 µM, respectively). In the case of C22, the Ala substitution (MIC = 63 µM) is much less active than the C22Cd equivalent (MIC = 4 µM). As for the alanine-containing variants, the substitution of amino acids within the R8-Y19 region of the peptide has a particularly deleterious impact as bactofencin R8Rd, V9Vd, M14Md and Y19Yd are 63 fold less active than wild type and Y10Yd, N11Nd, P15Pd and T16Td are inactive at 1000 µM, the highest concentration tested.

Comparison of bactofencin A with bactofencin R8Q (plantaricin ST31), a potential natural variant

Todorov *et al*. (1999) previously described plantaricin ST31, a bacteriocin with an amino acid sequence determined to be KRKKHRXQVYNNGMPTGMYR, produced by a sourdough isolate *Lactobacillus plantarum* ST31, with a reported mass of 2755 +/− 0.3 Da (20). Substitution of Cys for X results in a peptide with a mass of 2468 Da. However, if tryptophan and cysteine, the C terminal amino acids of bactofencin A are included, a mass of 2757 Da is obtained and subsequent oxidation of cysteines gives the published mass, 2755 Da. This suggests that plantaricin ST31 is likely to be a variant of bactofencin A with an arginine to glutamine change at position 8. Although plantaricin ST31 was reported as inactive against *Listeria* spp., bactofencin A is active against *Listeria* at high concentrations. For this reason, it was decided to directly compare the activity of bactofencin R8Q (i.e. plantaricin ST31) and bactofencin A against *S. aureus* DPC5246, *L. innocua* DPC3572 and *L. monocytogenes* ATCC 23074. Although bactofencin A and bactofencin R8Q (plantaricin ST31) were equally active at 1 µM against *S. aureus* DPC5246, it was established that bactofencin A is indeed more active (63 µM) than bactofencin R8Q (plantaricin ST31; 250 µM) against *L. innocua* DPC3572 and *L. monocytogenes* ATCC 23074. The reduced activity of R8Q was not evident after a substitution that retained the charge at position 8, i.e. R8K (Figure 6).

4.1.5 Discussion

There is an urgent need for new antimicrobials to combat infection and bactofencin is a clear candidate. Although it is produced in small amounts by the producing strain, the lack of post-translational modifications makes it very accessible to synthesis

strategies. With this in mind, we generated a bank of synthetic variants which included a number of amino acid substitution and deletion variants. Preliminary experiments showed that the disulfide bond forms naturally over time and that synthetic bactofencin with an intact disulfide is as active as the native peptide making this a valid approach (Figure 1). The synthetic peptide with reduced cysteines was also found to be as active as natural bactofencin suggesting that the disulfide bond is not essential for full activity (Figure 1).

Substituting either cysteine with serine resulted in peptide variants with lower activity than alanine equivalents proving that serine is not a good substitute for cysteine in bactofencin A (Figure 2). Cysteines and serines differ in both chemical and physical properties as cysteines are often found in the interior of a molecule especially when involved in disulfide bond formation while comparatively hydrophilic serines are typically exposed (21, 22). Alanine, however, is hydrophobic so it is possible that hydrophobic interactions between C7A and C22A are stronger than serine equivalents and so can better maintain structural conformation. This was seen when Cys9 and Cys14 residues of the Class IIa leucocin A were replaced with hydrophobic equivalents (23). It appears that single C22 changes to either serine or alanine have a greater negative impact on activity than the corresponding C7 and C7-C22 changes. The possibility here is that substituting C22 alone with serine or alanine may introduce steric hindrance, thus preventing any semblance of loop conformation resulting in a less active peptide. Disulfides have a very distinct role in stabilizing protein structure (22) and the results of this study suggest that the presence of both cysteines play a key role in maintaining peptide structure and are required for full activity of bactofencin A.

Sequential deletion of the positively charged N terminal from Lys3 to Arg6 resulted

in a series of variants with decreasing activity against *S. aureus* DPC5246, while removal of the entire N terminal region prior to the loop resulted in an inactive peptide (Figure 3). Interestingly, the C7-C22 variant contained an intact disulfide bond as determined by MALDI TOF MS. The N terminal, KRKKHR, with a charge of + 5 at neutral pH is unique among known Class II bacteriocin sequences and invites conjecture as to its function. Lysine and arginine play an important role in the interaction with negatively charged phospholipid membranes. Indeed, arginine is more effective than lysine in this regard as it forms more extensive H bonding, thereby stabilizing arginine-phosphate clusters enabling enhanced interfacial binding leading to membrane disruptions (24). In addition, a high positive charge allows bacteriocins and AMPs to insert further into membranes (25). It may be that KRKKHR plays a role in binding to anionic lipids in cell membranes and that a charge of at least +3 at the N terminus is required for full activity, given that variants with a lower charge were significantly less active (Figure 3). The highly positive charge of bactofencin A may also have played a role in the evolution of the unique bacteriocin immunity associated with the producer, *L. salivarius* DPC6502. In this respect, immunity is mediated through a homologue of *DltB*, a protein which results in the reduction in the charge of teichoic acids in the cell wall. Thus the mechanism mediating immunity could be through reducing the affinity of the positively charged bactofencin A to the producer surface (10).

Alanine scanning mutagenesis approaches have been successfully used to study the lantibiotic, lacticin 3147 (26) and the Class IIa bacteriocin, durancin GL (27). In bactofencin A, replacing the amino acids of the N terminal with alanine did not lead to a reduction of activity when compared to the native peptide. This correlates with the results from the deletion experiment as it is expected that the loss of a single positive

charge would not adversely affect activity. Changing Cys7 to alanine does have an effect but this is expected due to possible structural changes in the peptide as speculated earlier. Changes to Arg8 and the C terminal side of the loop were also well tolerated as R8A, P15A, M18A, Y19A, R20A and W21A are as active as bactofencin A. However, when residues between Val9 and Gly17 are changed to alanine activity is significantly reduced, suggesting that this part of the loop makes an important contribution to activity (Figure 4).

D-amino acid substitutions were used to investigate the importance of stereochemistry for target interaction as introduction of D-amino acids typically disrupts the helicity of AMPs (28). The retention of significant levels of activity in an all D variant of AMPs suggests the natural peptide functions by interacting with the lipid membrane, rather than a specific receptor, whereas a significant reduction in activity among such variants suggests that a stereospecific target, such as a membrane receptor, is involved in activity (29). The latter proved to be the case for bactofencin A. Substituting the amino acids of the loop for D equivalents was detrimental to activity in every case but most particularly in peptides with substitutions between Arg8 and Tyr19. Indeed, Y10Yd, N11Nd, P15Pd and T16Td were totally inactive at 1000 μM further suggesting a chiral interaction between bactofencin A and a specific receptor or that the introduction of a D residue disrupted structural conformation within this region. The inactivity of the P15Pd peptide is particularly notable and is in stark contrast to the activity observed with the P15A variant, suggesting that kinking the molecule in the opposite direction leads to detrimental structural changes. Taken together, the results from the N-Terminal deletion, alanine scanning and D amino acid variant studies make it tempting to suggest that bactofencin A interacts with the cell membrane through initial electrostatic interaction with the N terminal

and then disrupts the cell through binding with a putative receptor to amino acids in the interior of the loop.

The existence of an apparent natural variant of bactofencin A is interesting and provides an opportunity to compare the activities of the two peptides, especially in light of the absence of activity in cell free supernatants of plantaricin ST31-producing *L plantarum* ST31 against *L. innocua* and *L. monocytogenes* (20). This natural substitution did indeed result in reduced activity against *Listeria* as evidenced by our studies with bactofencin R8Q (plantaricin ST31). Substituting glutamine for lysine, the other positively charged amino acid restored activity suggesting that a positive charge is necessary in this position for activity against *Listeria*.

None of the bactofencin A variants investigated in this study resulted in enhanced anti-staphylococcal activity with respect to the native bactofencin suggesting that wild type bactofencin A has close to maximal antimicrobial activity. However, the reduction in activity against *Listeria* spp. due to an R8Q change and its restoration with an R8K change suggests that there is potential to change the spectrum of activity of bacteriocins. Ultimately, it will now be possible to build on this blueprint to further investigate the fundamental biology underlying the activity of bactofencin and, in turn, enhance the spectrum and activity of this cationic bacteriocin.

4.1.6 Acknowledgements

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Figure 1 Activity (µM) of oxidized synthetic bactofencin A (BFo), reduced bactofencin A (BFr) and natural bactofencin A (BFn) against *S. aureus* DPC5246 (**A**) and molecular mass of oxidized synthetic bactofencin A (Top), reduced bactofencin A (Middle) and natural bactofencin A (Bottom) (**B**).

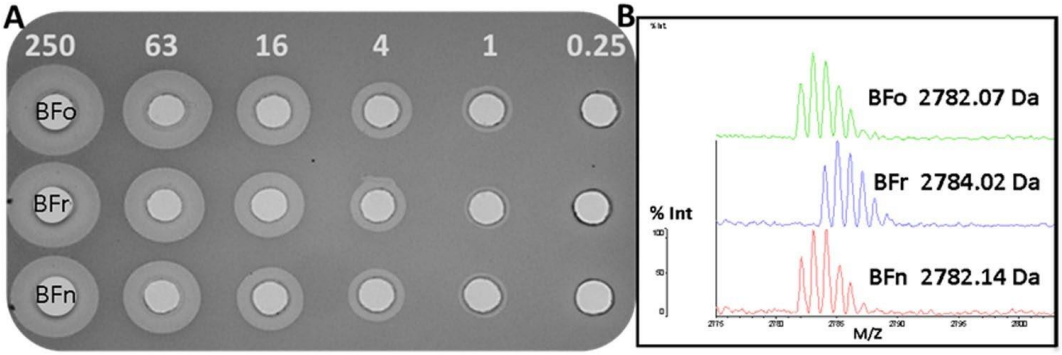
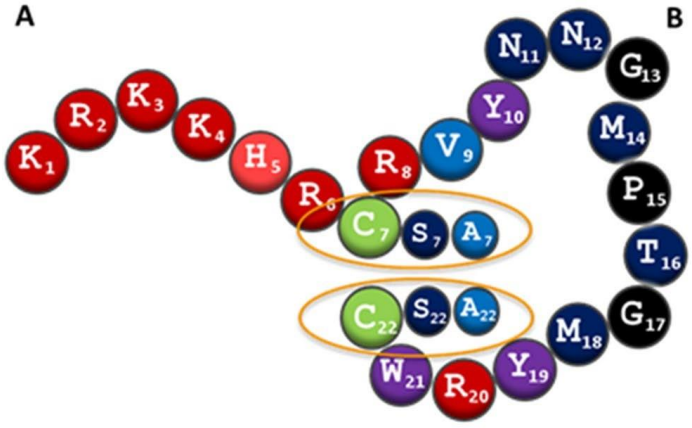


Figure 2 (**A**) Cys7 and Cys22 were substituted individually and in tandem with serine and alanine to give bactofencin variants C7S, C22S, C7S-C22S, C7A, C22A, C7A-C22A. Activity (µM) of bactofencin A compared to bactofencin cysteine variants C7S, C22S, C7S-C22S, C7A, C22A and C7A-C22A is presented in (**B**).

A



B

Peptide	Activity µM
A	1
C7S	63
C22S	250
C7S C22S	63
C7A	16
C22A	63
C7A C22A	16

Figure 3 (**A**) Activity (µM) of bactofencin A and deletion variants, R2-C22, K3-C22, K4-C22, H5-C22, R6-C22 and C7-C22 against *S. aureus* DPC5246. (**B**) Activity (µM) of bactofencin A and deletion variants against *S. aureus* DPC5246. Activity is colour coded with green being most active and red inactive.

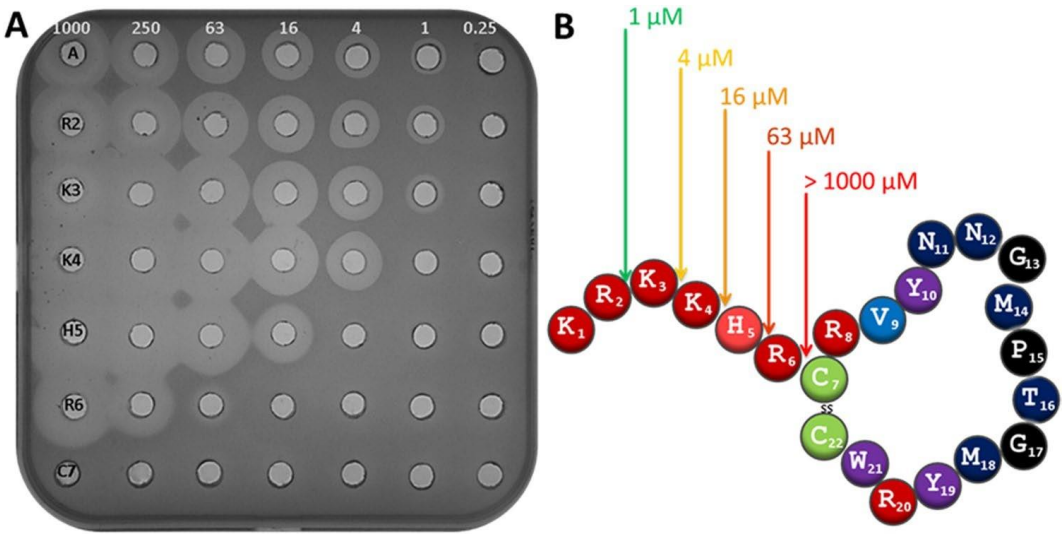
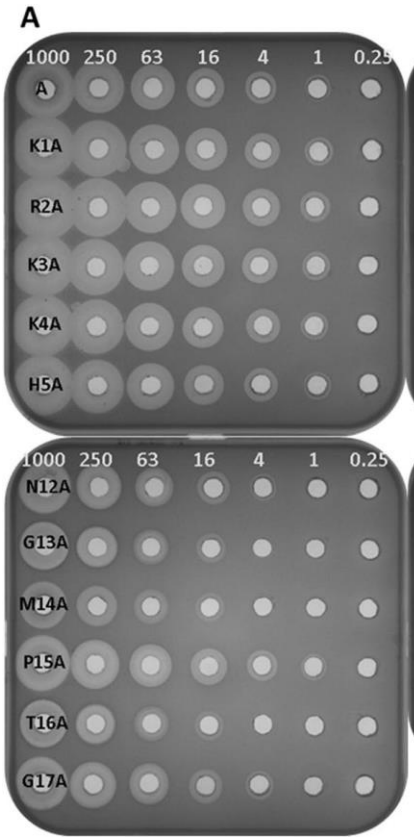


Figure 4 (**A**) Serial dilutions of each alanine variant plated on *S. aureus* DPC5246 indicator plates and (**B**) shows activity (µM) of bactofencin alanine variants against *S. aureus* DPC5246. Activity is colour coded with green being most active and red least active.

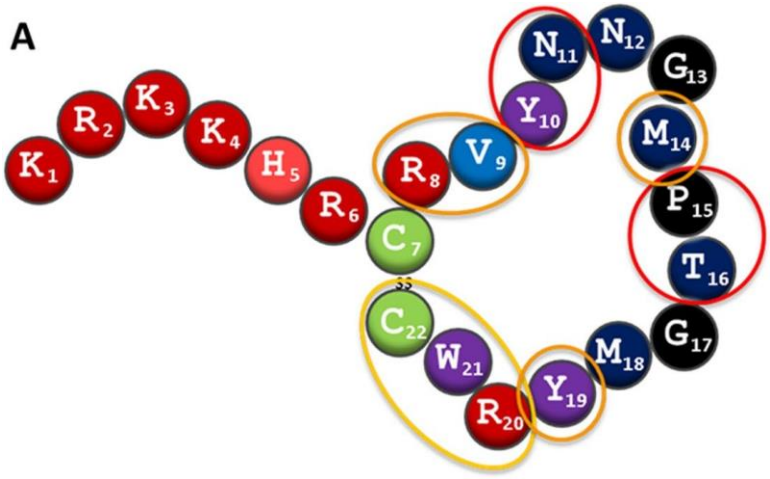


B

Peptide	Activity µM
A	1
K1A	1
R2A	1
K3A	1
K4A	1
H5A	1
R6A	1
C7A	16
R8A	1
V9A	4
Y10A	4
N11A	16
N12A	4
G13A	16
M14A	16
P15A	1
T16A	16
G17A	4
M18A	1
Y19A	1
R20A	1
W21A	1
C22A	63

Figure 5 Activity (µM) of bactofencin D-variants C7Cd-C22Cd against *S. aureus* DPC5246. Activity is colour coded with green being most active and red inactive. Amino acids most affected by D-substitution are circled in (A) and activities presented in (B).

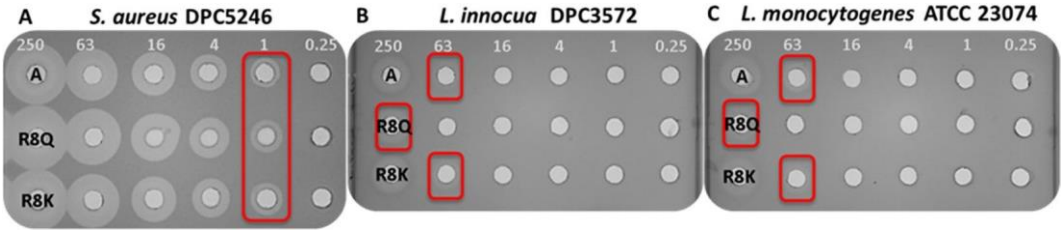
A



B

Peptide	Activity µM
A	1
C7Cd	16
R8Rd	63
V9Vd	63
Y10Yd	> 1000
N11Nd	> 1000
N12Nd	4
M14Md	63
P15Pd	> 1000
T16Td	1000
M18Md	16
Y19Yd	63
R20Rd	4
W21Wd	4
C22Cd	4

Figure 6 Activity (µM) of bactofencin A (**A**) bactofencin R8Q (plantaricin ST 1) and bactofencin R8K against *S. aureus* DPC5246 (**A**) *L. innocua* DPC3572 (**B**) and *L. monocytogenes* ATCC 23074 (**C**).



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Chapter 5.1

Bactofencin A displays a delayed killing effect on staphylococci which is greatly accelerated in the presence of nisin

In preparation for submission

PM O'Connor, PD Cotter, C Hill and RP Ross

5.1.1 Abstract

Bacteriocins can be considered a novel source of natural alternatives to antibiotics with the potential to fight against antimicrobial resistance in some instances. Commercialized as food preservatives, they also have the potential to treat drug resistant clinical pathogens and recent research shows that they play a role in immune modulation. To achieve their full potential an understanding of their mode of action and resistance mechanisms is required. We report that bactofencin A displays delayed inhibition against the mastitis pathogen, *Staphylococcus aureus* DPC5246, suggesting that it employs an unusual mode of action. This characteristic was clearly visible on plate media where formation of inhibition zones against the staphylococcal strain was very much delayed when compared to zones resulting from the action of nisin. This delayed killing and injury was also evident using flow cytometry where damage was evident four hours after bacteriocin addition. In particular, treatment with 2 μM bactofencin A resulted in approximately 20 fold higher injured and 50 fold higher dead cells. Combining bactofencin A with the nisin A resulted in faster killing at lower bacteriocin concentrations. When combined in an equal ratio, the combination exhibited a four-fold increase in inhibition compared to nisin A alone. These results demonstrate that the combination may be very effective in therapeutic applications against pathogenic staphylococci.

5.1.2 Introduction

Antimicrobial resistance (AMR) is a worsening global public health crisis that has been associated with overuse and misuse of antibiotics in both human and animal welfare. The emergence of multidrug-resistant pathogens threatens to undo a century of medical advances, placing anticancer treatments and routine surgeries at risk. At

present, at least 700,000 people die each year from superbugs that arise due to AMR and it has been predicted that this could lead to 10 million deaths by 2050 unless action is taken (1, 2). It is also expected that health expenditure will have to increase by $300 billion to $1 trillion per year to deal with this problem as patients are sick for longer, require more healthcare interventions and more expensive drugs to combat their illness (3, 4). The cost of AMR to the economy is also significant and, when combined with healthcare costs, could rise to $100-$200 trillion per year by 2050 (4, 5). In 2017, the WHO published a report highlighting the lack of new antimicrobials in development against priority pathogens and the need for new classes of antimicrobials to combat the crisis (6). Recently, the WHO launched an AMR Action Fund in collaboration with financial investors and the pharmaceutical industry to ensure a sustainable pipeline of new antibiotics effective against superbugs, with a specific aim of developing two to four new antimicrobial treatments for patients by 2030 (7). Interestingly, alternative therapeutic avenues using antibodies, probiotics, vaccines and antimicrobial peptides are now attracting increasing attention in the fight against AMR (8).

Bacteriocins are ribosomally-synthesised antimicrobial peptides, produced by most genera of bacteria, and can have a broad or narrow-spectrum of inhibition (9). The production of bacteriocins by Generally Regarded As Safe (GRAS) strains makes them of particular interest to the food industry where they (mainly nisin) have been used as food biopreservatives (10). The increase in AMR has meant that new antimicrobials are required which is why bacteriocins are being increasingly looked at as an alternative for certain applications. In this respect their stability, low toxicity, target specificity or activity against a broad range of bacteria including pathogens that have acquired resistance are all distinct advantages (11). They are also ribosomally synthesised and can be bioengineered, an approach that has been effective for nisin in

producing variants with improved activity against certain pathogens (12). Antimicrobial efficacy and AMR can also be improved through combinations with other bacteriocins including those with different modes of action (13). In addition, recent studies suggest that commensal bacteriocin producers are modulators of the human microbiome with potential to play a role in treating intestinal infections (14, 15).

Bactofencin A, produced by the porcine gut isolate *Lactobacillus salivarius* DPC6502, is a Class IId bacteriocin that is particularly potent against *Staphylococcus aureus*. A DltB homologue that may increase dealanylation of teichoic acids thereby reducing the negative charge on the cell wall and preventing binding of cationic bactofencin A, is proposed to provide immunity to the producing strain. Bactofencin A is a twenty two amino acid bacteriocin with a positively charged N terminal containing a series of positively-charged amino acids (KRKKHR) and a C terminal loop formed via a disulphide bond between Cys7 and Cys22 (16). Charge and structure play a significant role in its potency and its proposed mode of action is through an initial attraction to the cell membrane via the cationic N terminal, with inhibition occurring through interaction of the loop with a putative receptor. Bactofencin A has also been shown to effect subtle changes in the microbiome with potential to inhibit anaerobic inhabitants such as *Clostridium* and *Bacteroides* (17).

Nisin A is a Class I lantibiotic produced by strains of *Lactococcus lactis* that displays broad-spectrum activity against most Gram-positive microorganisms (18). It has been used as a food preservative by the food industry since the 1950s (9) and, more recently, its use has been extended to biomedical applications including inhibition of drug-resistant pathogens such as methicillin resistant *Staphylococcus aureus* (MRSA), enterococci and *Clostridioides difficile* (19). Nisin A is a thirty four amino acid peptide

containing five lanthionine rings (20) that are responsible for its intrinsic stability and potent activity that is often reported at nanomolar concentrations (21-22). Nisin exerts its antimicrobial effect by way of multiple modes of action including, blocking of cell wall biosynthesis through lipid II binding, pore formation and most recently, DNA condensation (23-25). It is noteworthy that significant spontaneous nisin resistance rarely occurs in nature despite widespread use in the food industry and this is attributed to its multiple modes of action (19).

Combining bacteriocins with alternative antimicrobials, referred to as antimicrobial combinatorial therapy, has the dual advantage of improving potency while reducing the incidence of AMR. Furthermore, these synergistic combinations can result in cheaper treatments and reduced toxicity to the host due to lower concentrations of antimicrobial required for effective treatment (26). Recently, this approach has been used to improve potential treatments against the foodborne clinical pathogen, *S. aureus*. Specifically, the lantibiotic nisin has shown promising results when assessed in combination with other antimicrobial compounds including citric acid (27) essential oils (28) antibiotics (29-31), phage endolysins (32) and other bacteriocins (33).

The aim of this study was to assess the effectiveness of bactofencin A and nisin A, both alone and in combination, at killing the mastitis isolate *S. aureus* DPC5246.

5.1.3 Materials and Methods

Staphylococcus aureus DPC5246 culture conditions

S. aureus DPC5246 (34), a mastitis clinical isolate, was grown aerobically in BHI broth at 37°C.

Bactofencin A synthesis and purification

Bactofencin A, KRKKHRCRVYNNGMPTGMYRWC, was synthesised using microwave-assisted solid phase peptide synthesis (MW-SPPS) on a Liberty Blue microwave peptide synthesizer (CEM Corporation. Mathews, North Carolina, USA) and purified by Reversed Phase HPLC according to the method described by O'Connor et al (2018) (35).

Purification of nisin A

Nisin A was purified from nisinA®P provided by Handary SA (Brussels, Belgium) by Reversed Phase HPLC. Specifically, 60 mg of nisinA®P was resuspended at 10 mg ml^{-1} in Milli Q water and 2 ml aliquots run on a semi preparative, Jupiter Proteo (10 x 250 mm, 4µ, 90Å), Reversed Phase HPLC column (Phenomenex, Cheshire, UK) running a 25-45% acetonitrile gradient, over 40 minutes, where buffer A is 0.1% trifluoroacetic acid (TFA) and buffer B is 100% acetonitrile 0.1% TFA. Eluent was monitored at 214 nm and fractions collected at 30 second intervals. Fractions containing nisin A were assayed to confirm the nisin A molecular mass (3352 Da) by MALDI TOF mass spectrometry and those deemed pure were pooled and lyophilised.

Preparation of peptides for activity assays

Bactofencin A was resuspended in 50 mM sodium phosphate buffer pH 6.8 at 1000 µM while nisin A was resuspended in Milli Q water at 1000 µM. Resuspended peptides were assessed for purity before use by analytical Reversed Phase HPLC. Fifteen µl of bactofencin A was added to 135 µl of Milli Q water and a 100 µl aliquot run on an analytical Aeris Peptide (4.6 x 250 mm, 5µ, 100Å) Reversed Phase HPLC column (Phenomenex, Cheshire, UK) running a 10-30% gradient over 40 minutes where buffer A is 0.1% TFA and buffer B is 100% acetonitrile 0.1% TFA. Eluent was monitored at

214 nm and fractions collected at approximately 1 minute intervals. Bactofencin A eluted as a single peak and the bactofencin A containing fraction was assessed for the bactofencin A mass (2782 Da) by MALDI TOF mass spectrometry.

Nisin A (3352 Da) was assessed as described for bactofencin A except a 20-50% acetonitrile 0.1% TFA gradient was used.

MALDI TOF mass spectrometry

MALDI TOF mass spectrometry was performed on HPLC fractions of interest from purification protocols and resuspended pure peptides using an Axima TOF^2 MALDI TOF mass spectrometer (Shimadzu Biotech, Manchester, UK). 0.5-µl aliquot of matrix solution (α - cyano 4-hydroxy cinnamic acid), 10 mg/ml in 50% acetonitrile-0.1% TFA) was deposited onto the target and left for 20 seconds before being removed. The residual solution was allowed to air-dry and 0.5 µl sample solution was deposited onto the pre-coated sample spot. 0.5µl of matrix solution was added to the deposited sample and allowed to air-dry. The sample was subsequently analysed in positive-ion linear or reflectron mode.

Effect of bactofencin A on the growth of *S. aureus* DPC5246

An overnight culture of *S. aureus* DPC5246 was diluted 200 fold in BHI broth to give a 0.5% inoculum containing ~ 1 x 10^6 colony forming units/ml (cfu ml^{-1}). A 100 µM stock solution of bactofencin A was serially diluted twofold in 100 µl aliquots of BHI broth and 80 µl aliquots of each dilution added to 3920 µl of inoculum to give 4 ml samples containing 2, 1, 0.5, 0.25, 0.125 and 0.063 µM bactofencin A. Four ml of the inoculum without bactofencin A was included as a control. Samples were prepared in duplicate and incubated in a 37°C water bath and growth measured

spectrophotometrically (Jenway 6300 spectrophotometer, Staffordshire, UK) via optical density at 600 nm (OD_{600}) at hourly intervals. Viable cells were enumerated by determining cfu ml^{-1} at 0, 2, 4, 6, 8, 10 and 23 hours. Specifically, 100 µl aliquots of sample were 10 fold serially diluted in 900 µl Maximum Recovery Diluent (MRD) and 10 µl aliquots of each dilution spotted onto BHI agar plates and allowed to dry. Plates were incubated overnight at 37°C and the cfu ml^{-1} calculated at each time point.

Assessment of cell viability via flow cytometry

The proportion of live, injured and dead cells in *S. aureus* DPC5246 cultures grown in the presence of bactofencin A, nisin A and bactofencin A/nisin A combinations was assessed by flow cytometry at 4, 9 and 23 hours. Cells were stained with a BD™ Cell Viability Kit which uses thiazole orange (TO) and propidium iodide (PI) to distinguish live and dead cell populations. Cultures for assay were diluted to ~ 10^6 cells ml^{-1} in staining buffer which is phosphate buffered saline containing 0.01% Tween 80 and 1 mmol/L EDTA. Two µl of each dye (TO and PI) was added to 200 µl of diluted sample and analysed on a BD Accuri™ C6 flow cytometer (Becton, Dickinson and Company, BD Biosciences, San Jose, CA 95131, USA). Gates, to distinguish between live and dead cells, were assigned using the BD Accuri's associated software in line with the manufacturer's guidelines.

Inhibition of *S. aureus* DPC5246 by bactofencin A and nisin A assayed by agar well diffusion

Inhibition of *S. aureus* DPC5246 by bactofencin A and nisin A was demonstrated initially by the agar well diffusion assay described by Ryan et al. (1996) (36). An indicator plate containing *S. aureus* DPC5246 was prepared by adding 225 µl of an

overnight culture to 45 ml of molten BHI agar (0.5% inoculum) which was allowed to solidify in a 120 mm square petri dish. Fifty µl aliquots of bactofencin A were placed close to 50 µl of nisin A in pre-bored wells and the plate incubated at 37°C. The plate was photographed at 6, 8, 10 and 23 hours.

Inhibition studies with bactofencin A and nisin A

Four ml samples were prepared by adding aliquots of 10 µM bactofencin A and/or 10 µM nisin A to the *S. aureus* DPC5246 inoculum containing ~ 1 x 10^6 cfu ml^{-1} to give the required bacteriocin concentration. Four ml of the inoculum without bactofencin A or nisin A was included as a control. Samples were prepared in duplicate and incubated in a 37°C water bath. OD_{600} was recorded every hour. Viable cells were enumerated, where required, by determining cfu ml^{-1} at 0, 2, 4, 6, 8, 10 and 23 hours as described above.

5.1.4 Results

In previous studies we had shown that bactofencin A is particularly effective against *S. aureus* including MRSA strains. In this study, we tested the effectiveness of bactofencin A alone and when combined with nisin A against a strain that was isolated from the milk of a cow with mastitis (inflammation of the udder).

Purification of peptides for activity assays

The stock solutions of bactofencin A and nisin A were assessed for purity prior to use in activity assays by analytical HPLC and MALDI TOF Mass Spectrometry. Bactofencin A eluted as a single peak at 29 minutes on the HPLC chromatogram and MALDI TOF mass spectrometry of the bactofencin A containing fraction detected a

mass of 2782 Da (Figure 1A) which is within the expected mass range (2782 +/- 1 Da) for bactofencin A. Nisin A also eluted as a single peak at 31 minutes and MALDI TOF mass spectrometry detected a mass of 3353 Da in the nisin A containing fraction which is also within the expected range (3352 Da +/- 1 Da) (Figure 1B).

The delayed inhibition displayed by bactofencin A and its inability to fully lyse the target culture required an alternative approach to the traditional 96 well assay procedures for assessment of inhibition, minimal inhibitory concentration (MIC) and fractional inhibitory concentration (FIC) measurements. Consequently, assays were carried out at 4 ml scale to provide a sufficient volume for cell number enumeration at intervals throughout the growth time period.

The inhibitory effect of increasing concentrations of bactofencin A on *S. aureus* DPC5246

Initially, the effect of a wide range of two-fold increasing concentrations of bactofencin A (0.063, 0.125, 0.250, 0.5, 1 and 2 µM) on the OD_{600} and viable cell numbers of a growing culture of *S. aureus* DPC5246 was measured. The optical density readings (Figure 2A) suggest that bactofencin A had little or no effect on growth for the first 3-4 hours regardless of concentration. After 4 hours, the growth of bactofencin A treated cultures slowed down compared to the control; this became more apparent from five hours onwards when the OD_{600} decreased suggesting the cells were starting to lyse. Following seven hours incubation, the cultures appeared to recover in a concentration dependent manner with samples containing lower bactofencin A concentrations recovering before those containing higher concentrations. Interestingly, after 23 hours the OD_{600} for higher bactofencin A concentrations (0.5, 1 and 2 µM)

was found to be significantly lower than the control, suggesting that bactofencin A was still having an effect on the culture at these concentrations.

The viable count results (Figure 2B) showed a similar delayed response with no significant difference in viability after 2 hours, while at 4 hours a 0.4 log reduction in cell numbers for 2 µM bactofencin A, the highest concentration tested, was detected. At six hours, all bactofencin A concentrations showed a ten-fold reduction compared to the control with the exception of 0.063 µM which had a slightly less effect at 0.7 log, while at 8 hours the maximum effect is achieved with bactofencin A 0.5-2 µM showing a 1.5 log reduction compared to the control. Taken together, the OD_{600} and viable plate count results suggest a delayed action of the bacteriocin on the culture which is first slowed down and then killed.

Flow cytometry analysis of *S. aureus* DPC5246 grown in the presence of 0, 0.2 and 2 µM bactofencin A

Flow cytometry can be used to study the real time effect of bacteriocins on cell membranes through the use of fluorescent dyes such as thiazole orange (TO) and propidium iodide (PI) (37). TO can enter and label live cells whereas PI can only label cells with compromised membranes thereby allowing distinction between live (green), injured (orange) and dead (red) cells. The separation of each cell type through gating optimisation results in a reliable assay that can quantify the number of each cell type. Here, we used flow cytometry to study the effect of bactofencin A on *S. aureus* DPC5246 cells.

S. aureus DPC5246 was grown in BHI broth in the presence of 0, 0.2 and 2 µM bactofencin A and growth assessed hourly by OD_{600} (Figure 3A). The OD_{600} results

showed that 0.2 and 2 µM bactofencin A inhibited the culture compared to untreated control with reductions in OD_{600} following a dose response behavior.

Comparison of cell numbers by traditional plating and flow cytometry at 4, 9 and 23 hours compared very well (Figure 3B) demonstrating that flow cytometry is a fast, reliable method to enumerate viable cells for this strain. The OD_{600} and cell enumeration results again suggest that bactofencin A has minimal effect on growth in the first 4 hours of exposure with maximum killing occurring at eight hours, followed by a limited recovery of the culture at 23 hours for both concentrations tested.

In addition to enumerating live cells (green), the flow cytometry results, which were corrected to exclude background debris (black), show the number of dead (red) and injured (orange) cells (Figure 3C) at 4, 9 and 23 hours. At four hours, the untreated control contains 2 x 10^8 live cells and about 100-fold fewer injured cells (1.2 x 10^6) and dead cells (3 x 10^6) which is indicative of a "healthy" log phase culture (Figure 3C 1a). In comparison, the 0.2 µM (Figure 3C 1b) and 2 µM bactofencin A (Figure 3C 1c) treated cultures contained live cells numbers slightly lower than the untreated control (1.3 x 10^8 and 1.0 x 10^8) but approximately 20-fold higher levels of injured (2.3 x 10^7 and 2.6 x 10^7) and 50-fold higher numbers of dead cells (1.3 x 10^7 and 1.7 x 10^7). At nine hours the effect of bactofencin A on live cells is apparent as the untreated control sample contained 8.8 x 10^8 (Figure 3C 2a) compared to a 1.3-1.6 log reduction (5.6 x 10^7 and 2.6 x 10^7) detected for 0.2 µM (Figure 3C 2b) and 2 µM (Figure 3C 2c) bactofencin A respectively. However, the number of injured (3.4 x 10^6, and 3.3 x 10^6) and dead cells (3.4 x 10^6 and 4.3 x 10^6) were now just three-fold higher than the control sample (1.0 x 10^6). At 23 hours, we again observed that bactofencin A had a slight inhibitory effect on the culture as the untreated control contained 1.1 x 10^9 live cells (Figure 3C 3a) while 0.2 µM bactofencin A (Figure 3C 3b) contained 6.4

x 10^8 and 0.2 µM bactofencin A (Figure 3C 3c) contained 3.4 x 10^8 live cells. Notably, at this stage the numbers of injured and dead cells are comparable to the untreated control.

In summary, flow cytometry suggests that bactofencin A is causing considerable damage to the cell within four hours of exposure suggesting that the antimicrobial effect is delayed.

Antimicrobial interaction between bactofencin A and nisin A on an *S. aureus* DPC5246 indicator plate

Inhibition of *S. aureus* DPC5246 by bactofencin A and nisin A was assessed by well diffusion assay. The indicator plate (Figure 4) showed that the zone of inhibition for bactofencin A appeared gradually between eight and 23 hours, reflecting the growth curve results (Figure 2A) again suggesting a delayed action by the bacteriocin. In contrast, the zone of inhibition for nisin A was already apparent after six hours, confirming the lytic nature and rapid killing effect of nisin. Interestingly, a small enhancement of activity was observed at the point where the two zones of clearing intersect, suggesting the possibility of synergism between bactofencin A and nisin A.

Inhibitory effect of bactofencin A and nisin A alone on growth of *S. aureus* DPC5246

The observation that bactofencin activity is enhanced by nisin prompted us to investigate whether this phenomenon could be observed in liquid media. The effect of a narrow range of concentrations of bactofencin A at 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5 µM and nisin A at 10 fold lower concentrations 0.005, 0.01, 0.02, 0.03, 0.04 and 0.05 µM on *S. aureus* DPC5246 was assessed with a view to selecting optimum bacteriocin

concentrations for combinatorial studies. The OD_{600} results (Figure 5A) for the bactofencin A concentration range again showed a delayed inhibition curve for all bactofencin concentrations tested in line with the trends seen for the wider bactofencin A concentration range in Figures 2A and 3A. The plate count results (Figure 5B) showed no significant reduction in viable cell counts compared to the control at four hours and optimum killing at eight hours, so again a delayed killing effect was observed.

Nisin A was assayed at a ten-fold lower concentration range (0.05-0.005 µM) than bactofencin A given its greater potency at earlier stages of growth. One thing to note is that the higher nisin A concentrations inhibit the cultures immediately (Figure 5C) and also that the delay in recovery is concentration dependent. At 23 hours, all nisin A treated cultures grew to the same extent as the untreated control, regardless of nisin A concentration, suggesting that nisin A is no longer effective against the culture at these concentrations. The plate count results (Figure 5D) show a similar trend in terms of viability where the higher concentrations reduced the viability of the culture for the first four hours which then recovered in a dose response fashion.

Given that nisin appeared to enhance the inhibitory effect of bactofencin, we then evaluated the effect of different bactofencin A/nisin A concentrations where nisin A was present in ten-fold lower concentrations.

Inhibitory effect of bactofencin A/nisin A combinations

Initially, bactofencin A concentrations (0.05-0.5 µM) were combined with ten-fold less nisin A concentrations (0.05-0.005 µM) to assess their effect on *S. aureus* DPC5246 growth (Figure 6). The highest bacteriocin concentrations alone (bactofencin A 0.4 µM and nisin A 0.04 µM) were included as controls.

The optical density results (Figure 6A) showed that the untreated control is fully grown at six hours, 0.4 µM bactofencin A showed delayed inhibition while 0.04 µM nisin inhibited the culture for seven hours after which it recovered to levels comparable to the untreated control. Interestingly, all bactofencin A/nisin A combinations suppressed growth for eight hours. The two lowest combinations, bactofencin A 0.05 µM/nisin A 0.005 µM and bactofencin A 0.1 µM/nisin A 0.01 µM, inhibited *S. aureus* DPC5246 for nine hours (Figure 6). This is noteworthy considering that nisin A alone at these concentrations has no significant effect on growth (see Figure 5C) while equivalent bactofencin A concentrations are recovering (Figure 5A), suggesting possible additive or synergistic action between the two bacteriocins. At 23 hours, the inhibitory effects of the bacteriocin combinations are dose dependent with the OD_{600} for the highest combination tested comparable to the OD_{600} at T0, suggesting complete suppression of culture growth.

The plate count results (Figure 6B) confirmed that the delayed killing effect of bactofencin A no longer occurs at all bactofencin A/nisin A combinations including the lowest concentrations tested. The lower combinations are equivalent to nisin A 0.04 µM alone while the higher combinations are significantly better with the highest combination resulting in complete killing of the culture. Overall, we see that both killing and regrowth occur in a dose response manner.

Flow cytometry was used to assess the effect of the lower bactofencin A/nisin A combinations on live, dead and injured cell numbers at 4 hours. The untreated control at 4 hours (Figure 6C a) was similar to that shown in Figure 3C with 2×10^8 live cells, and 3.0×10^7 injured cells, again commensurate with a control untreated culture. The numbers of live, dead and injured cells are comparable for bactofencin A alone at 0.1 (Figure 6C b) and 0.2 (Figure 6C e) µM and it is interesting to note the high proportion

of injured cells even at the lower bactofencin A concentration. The number of live and injured cells in the presence of nisin A 0.01 µM alone was similar to the control though more injured cells were detected (5.8×10^7 vs 3.0×10^7) while nisin 0.02 µM resulted in an almost 2 log reduction in live cells compared to the control in agreement with Figure 5D. Notably, both combinations resulted in a 3-4 log reduction in cell numbers demonstrating the effectiveness of the combinations compared to either bacteriocin alone.

The effect of decreasing nisin A concentrations in combination with 0.4 µM bactofencin A and decreasing bactofencin A concentrations in combination with 0.04 µM nisin A on *S. aureus* DPC5246

The effect of decreasing nisin A concentrations in relation to bactofencin A (Figure 7A) and decreasing bactofencin A in relation to nisin A (Figure 7B) was assessed to determine the contribution each bacteriocin makes to activity. In both experiments (Figure 7A and 7B) the control samples (untreated control, bactofencin A alone and nisin A alone) are similar to those shown in Figure 6A. Treatment of *S. aureus* DPC5462 with 0.04 µM bactofencin A and decreasing nisin A concentrations resulted in inhibition of culture for seven hours at all combinations tested (Figure 7A). Recovery was in a dose response manner as evidenced by bactofencin A 0.4 µM/nisin A 0.005 µM and bactofencin A 0.4 µM/nisin A 0.01 µM starting to recover at eight and nine hours respectively while the three higher nisin A concentrations were still inhibitory at eleven hours. At 23 hours there was a reduction in OD_{600} at all bactofencin A/nisin A combinations assayed compared to the untreated control while bactofencin A 0.4 µM/nisin A 0.04 µM was totally inhibited. Overall, the results suggest that while

the cultures are significantly inhibited at all combinations tested, decreasing the nisin A concentration results in less inhibition.

In contrast to the constant bactofencin A and decreasing nisin A concentrations, the OD_{600} does not recover in samples with constant nisin A 0.04 µM and decreasing bactofencin A concentrations (Figure 7B) as inhibition is maintained for 23 hours, suggesting that bactofencin A levels can be reduced once sufficient nisin A is present. As it appeared that the bactofencin A concentration could be reduced in relation to nisin A and bactofencin A 0.05µM/nisin A 0.04 µM was inhibitory for up to 23 hours, it was decided to assess the effectiveness of the bacteriocins in a 1:1 ratio against *S. aureus* DPC5246.

Assessment of a 1:1 ratio of bactofencin A and nisin A on *S. aureus* DPC5246

The effect of bactofencin A (0.0025, 0.005, 0.01 and 0.02 µM) and nisin A (0.0025, 0.005, 0.01 and 0.02 µM) both alone and in a 1:1 ratio on the OD_{600} of a growing culture of *S. aureus* DPC5246 was determined. Interestingly, at eight hours the culture was inhibited by bactofencin A 0.02 µM/nisin A 0.02µM which is particularly impressive as the equivalent controls were almost fully grown at this time point (Figure 8).

Assessment of the FIC of bactofencin A/nisin A combinations against *S. aureus* DPC5246

As bactofencin A 0.02µM/nisin A 0.02 µM inhibited the culture for eight hours, it was decided to see if 1x, 0.5x and 0.25x combinations had an effect on efficacy at this time point. None of the single bacteriocin controls were inhibitory at eight hours (Figure 9A) and bactofencin A 0.02 µM/nisin A 0.02 µM was inhibitory at eight hours as

expected, though reducing the nisin A concentration to 0.01 and 0.005 µM resulted in less inhibition (Figure 9D). Reducing the bactofencin A concentration to 0.005 µM reduced the effectiveness of the combinations (Figure 9B), while bactofencin A 0.01 µM nisin A 0.02 µM was inhibitory at eight hours (Figure 9C) suggesting that there may be potential to reduce the bactofencin A concentration even further. In a subsequent experiment (not shown) we observed that nisin A 0.08 µM was as inhibitory as bactofencin A 0.02 µM/nisin A 0.02 µM at eight hours suggesting that the combination is four times more effective than nisin A alone. Bactofencin A 0.08 µM alone resulted in a ~0.6 OD_{600} reduction compared to the control.

Finally, the effect of a wide range of two-fold increasing concentrations of bactofencin A (0.015, 0.031, 0.063, 0.125, 0.250, 0.5, 1 and 2 µM) and nisin A (0.015, 0.031, 0.063, 0.125, 0.250, 0.5, 1 and 2 µM) both alone and in a 1:1 ratio on the OD_{600} of a growing culture of *S. aureus* DPC5246 was measured every hour for 23 hours. The results (data not shown) showed that the MIC of nisin A was 0.125 µM compared to 0.031 µM bactofencin A/nisin A at eight hours, a 4 fold improvement in efficacy compared to nisin A alone, while at 23 hours it required 2 µM nisin A to inhibit the culture compared to 0.25 µM bactofencin A/nisin A, an 8 fold improvement indicative of a synergistic effect.

5.1.5 Discussion

Bactofencin A is a novel bacteriocin with potential to fight infection as, in addition to its potency against *S. aureus*, its small size and lack of post-translational modifications make it amenable to peptide synthesis. Synthetic bactofencin A is as active as the naturally-produced peptide and the disulphide bonds form naturally over time making it a suitable source of peptide for characterization studies (35). Interestingly, the studies

presented in this chapter demonstrate that the bacteriocin has a delayed action when compared to nisin and probably has a very different mechanism. Initially, stock solutions of bactofencin A and nisin A were assessed for peptide purity by Reversed Phase HPLC and MALDI TOF mass spectrometry and the presence of a single HPLC peak containing the correct peptide mass was taken as evidence of sufficient purity for inhibition studies (Figure 1).

Class II bacteriocins typically act through an initial electrostatic interaction with negatively charged components of the cell membrane, and in some cases (class IIa in particular), bind to a cell receptor, resulting in a loss of ion gradients, membrane integrity and cell death (38-39). It has been tentatively proposed that bactofencin A interacts with the cell wall via the positively charged N terminal and also binds to an unknown receptor by interaction with amino acids found in the C terminal half of the peptide given that some of these are essential for activity.

Teichoic acids, including lipoteichoic acids attached to the cell membrane (LTA) and wall teichoic acids (WTA) attached to peptidoglycan, are major components of Gram-positive cell walls that play a role in adhesion, growth, virulence and biofilm formation (40-42). The addition of D-alanine esters to teichoic acids via the D-alanyl lipoteichoic acid (DLT) pathway reduces the overall negative charge on the cell wall making the cell more resistant to cationic peptides (41). In *S. aureus,* the DLT pathway proteins are encoded on the *dlt* operon, *dltABCD,* with DltA catalyzing the alanylation of D-alanine in the cytoplasm and transferring it to DltC, a D-alanyl carrier protein (41, 42). Activated DltC forms a tight complex with DltB, a channel/funnel forming acyltransferase that moves the activated D-alanine across the membrane where DltD, which is located outside the cell membrane, transfers it to the teichoic acids (43). Interestingly, the DLT pathway, and DltB in particular, have been proposed as targets

for drug resistant *S. aureus* infections (44). Indeed, numerous Gram-positive bacteria with mutated *dlt* genes have a higher negative charge on the cell wall making them more susceptible to cationic AMPs (45). Furthermore, wild type *S. aureus* strains with extra copies of the *dlt* operon result in teichoic acid with increased D alanylation making the cell more positively charged and consequently more resistant to cationic antimicrobial peptides (46). The delayed action of bactofencin A on *S. aureus* DPC5246 (Figure 2), in addition to the cell damage observed at 4 hours by flow cytometry (Figure 3) and the absence of significant cell lysis, suggest that bactofencin A executes its antimicrobial action via a mechanism that takes considerable time. Cell disruption by bactofencin A may simply be due to the strong interaction between the bacteriocin and the cell surface due to its strong positive charge (+7 at neutral pH) and an as yet unknown receptor; further research is required to identify this possible receptor.

The ability of ten-fold less nisin A to bactofencin A (Figure 5) to completely inhibit the culture may be attributable to the different modes of action as pore forming bacteriocins often act at nanomolar concentrations while cell wall disrupters require higher peptide concentrations to exert an effect (47). Combining bactofencin A with ten-fold less nisin A resulted in increased killing compared to either bacteriocin alone (Figure 6), again suggesting that the two different modes of action are complementary to each other.

Lowering the bactofencin A concentration in relation to nisin A resulted in greater inhibition compared to lowering the nisin A concentration in relation to bactofencin A (Figure 7); further investigation revealed that combining the bacteriocins in a 1:1 ratio (Figures 8 and 9) could effectively inhibit *S. aureus* DPC5246.

In summary, bactofencin A is very effective against *S. aureus* when combined with nisin A - a phenomenon that is most likely due to acting synergistically through two different modes of action.

5.1.6 Acknowledgements

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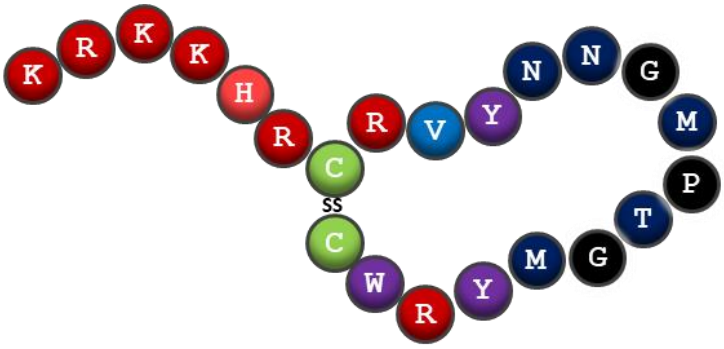
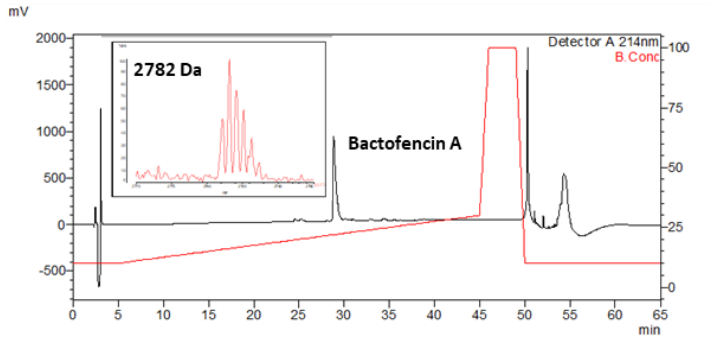
Figure 1 Assessment of peptide purity of bactofencin A (A) and nisin A (B) stock solutions by Reversed Phase HPLC and MALDI TOF mass spectrometry (inset).

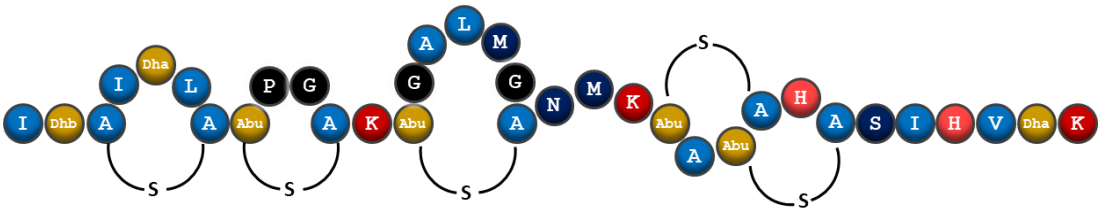
(Ai) Bactofencin A



(Aii) Bactofencin A HPLC and MALDI TOF MS



(Bi) Nisin A



(Bii) Nisin A HPLC and MALDI TOF MS

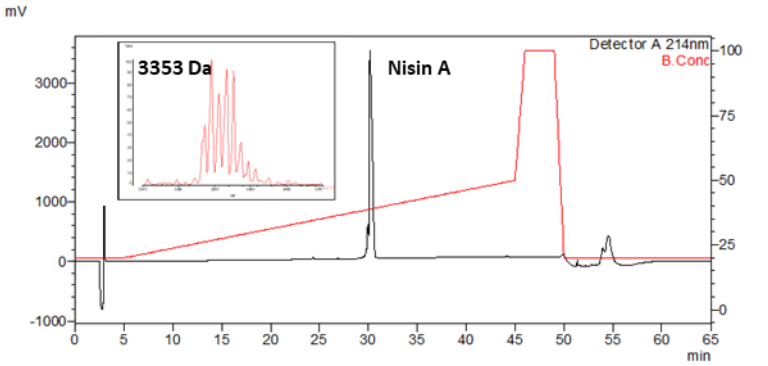


Figure 2 Inhibitory effect of 0.063, 0.125, 0.25, 0.50, 1 and 2 µM bactofencin A on *S. aureus* DPC5246 in BHI broth at 37°C as measured by OD_{600} (A) and viable cell counts (cfu ml^{-1}) (B).

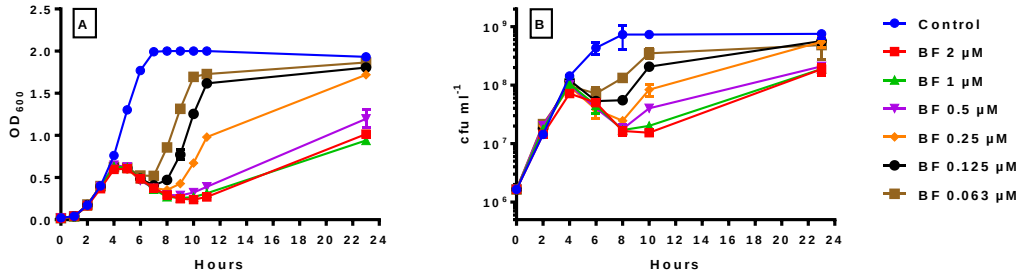


Figure 3 The effect of 0 (Control), 0.2 and 2 µM bactofencin A on cell viability of *S. aureus* DPC5246 at 4, 9 and 23 hours as measured by OD_{600} (A), cell numbers by flow cytometry (FC Live) and conventional plating (plating) (B) and flow cytometry (C).

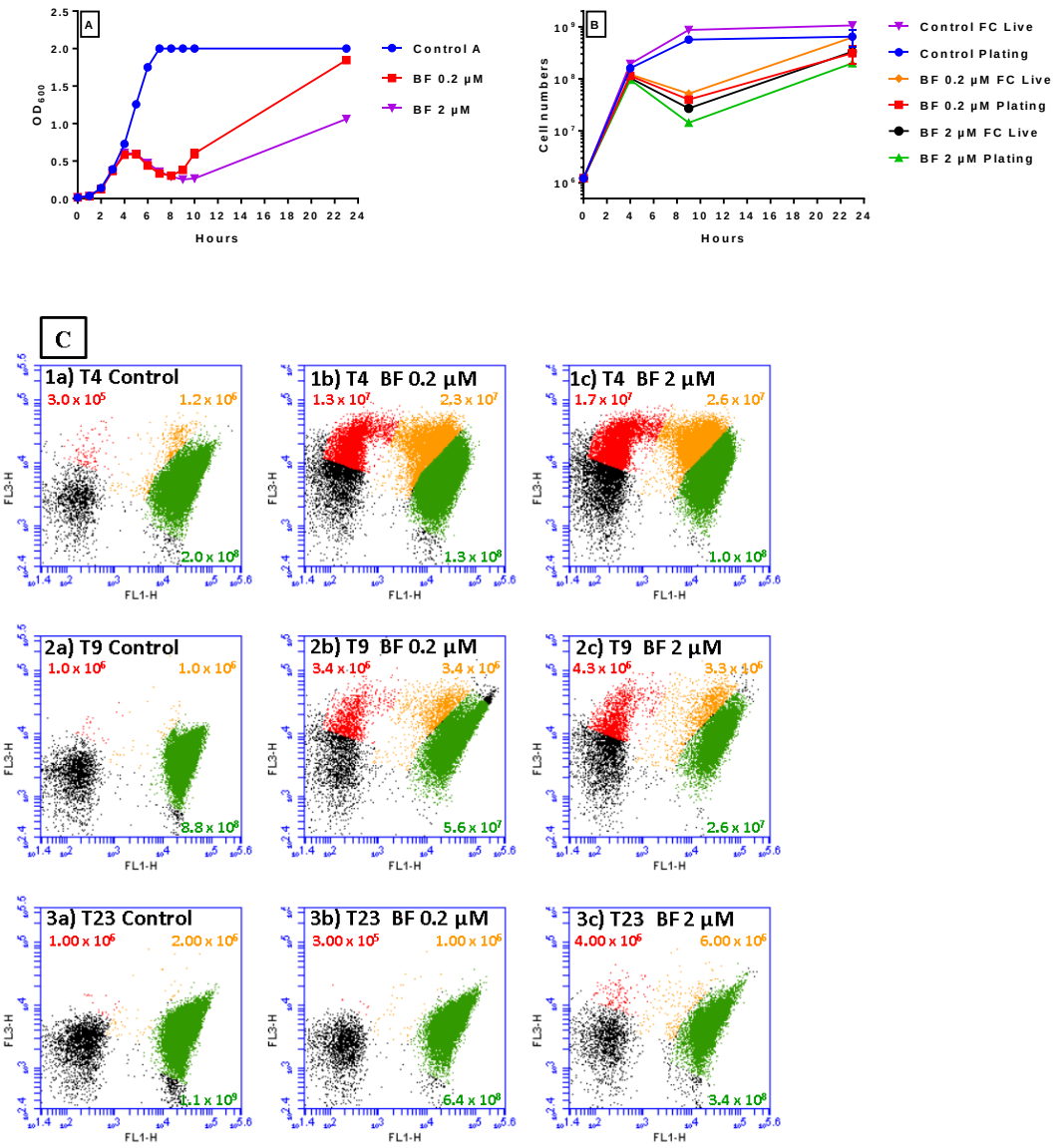


Figure 4 Antimicrobial interaction between 10 μM bactofencin A (B) and 10 μM nisin A (N) against *S. aureus* DPC5246 at 6, 8, 10 and 23 Hours.

Figure 5 Inhibitory effect of 0.05-1 µM bactofencin A on OD_{600} (A) and cfu ml^{-1} (B) and 0.005-0.05 µM nisin A on OD_{600} (C) and cfu ml^{-1} (D) of *S. aureus* DPC5246 in BHI broth at 37°C.

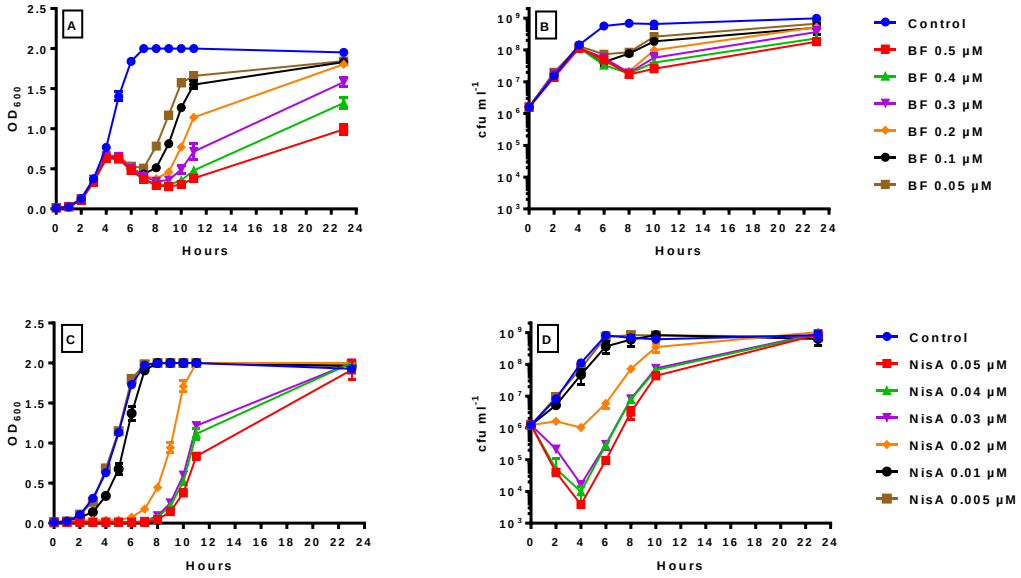


Figure 6 The effect of bactofencin A/nisin A combinations on *S. aureus* DPC5246 as measured by OD_{600} (A) and cfu ml^{-1} (B) and flow cytometry of bactofencin A 0.1 or 0.2 µM alone, nisin A 0.01 or 0.02 µM alone and bactofencin A/nisin A 0.1/0.01 or 0.2/0.02 µM combinations at 4 hours (C). The flow cytometry results at 4 hours shown in Figure 6C were generated from a subsequent growth experiment (OD_{600} data not shown).

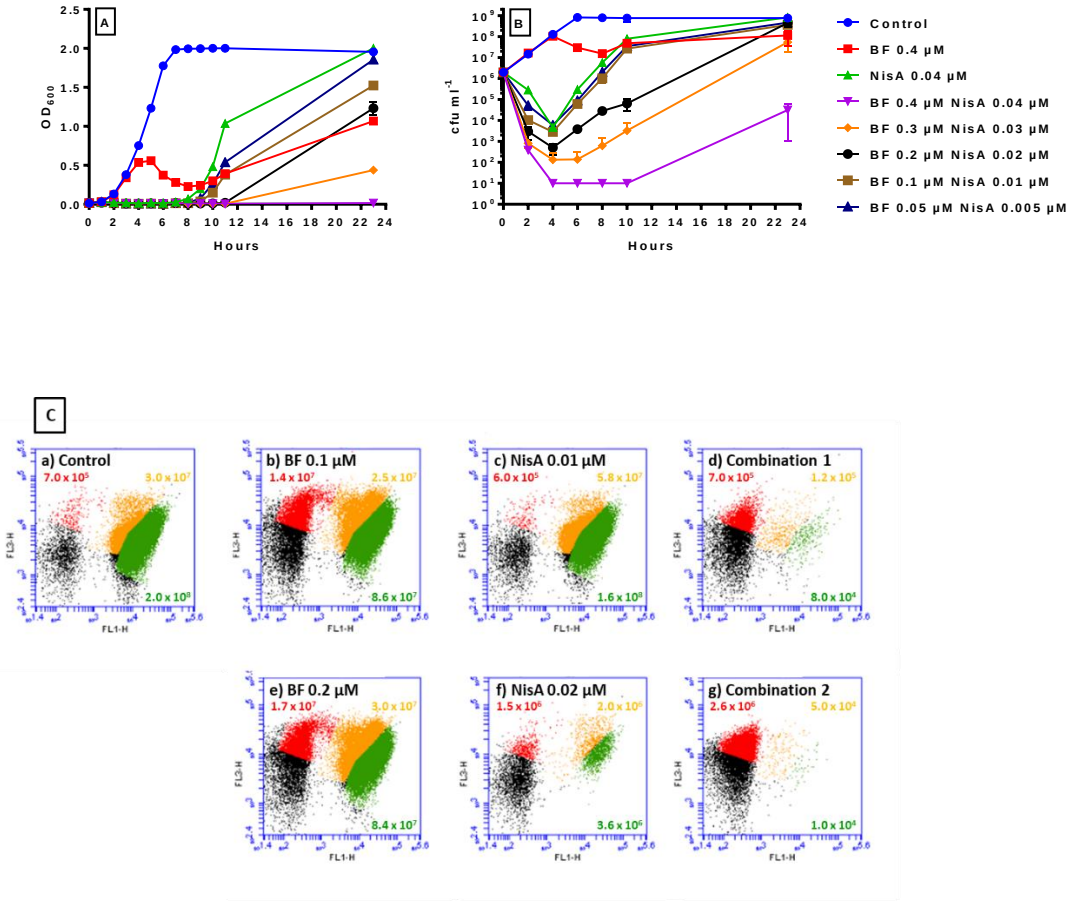


Figure 7 Inhibitory effect of decreasing concentrations of nisin A (0.05-0.005 µM) in the presence of 0.4 µM bactofencin A on OD_{600} (A) and decreasing concentrations of bactofencin A (0.5-0.05 µM) in the presence of 0.04 µM nisin A on OD_{600} (B) on growth of *S. aureus* DPC5246 in BHI broth at 37°C.

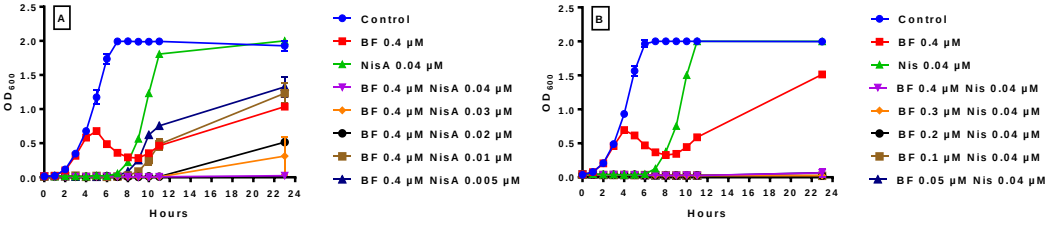


Figure 8 Inhibitory effect of bactofencin A (0.02-0.0025µM) and nisin A (0.02-0.0025µM) combined at 1:1 ratio on growth of *S. aureus* DPC5246 in BHI broth at 37°C.

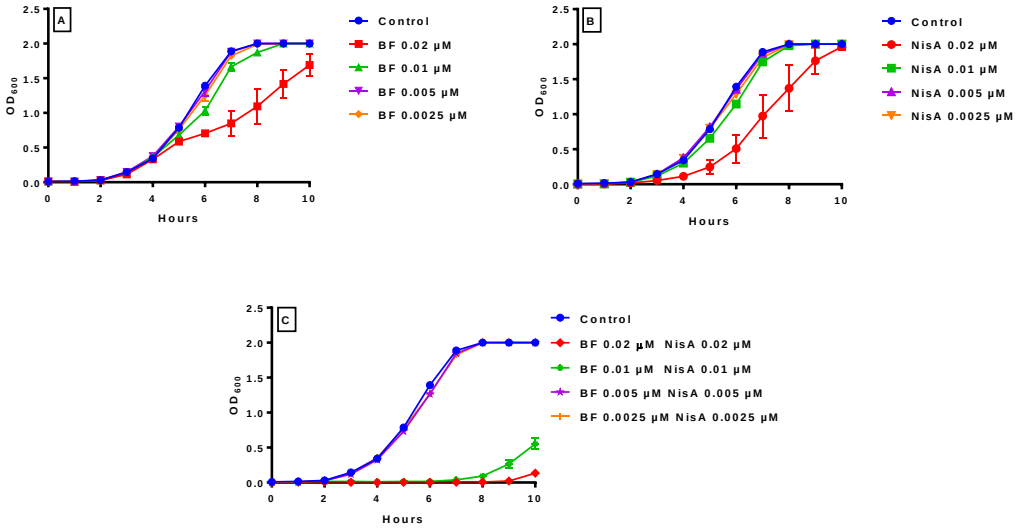
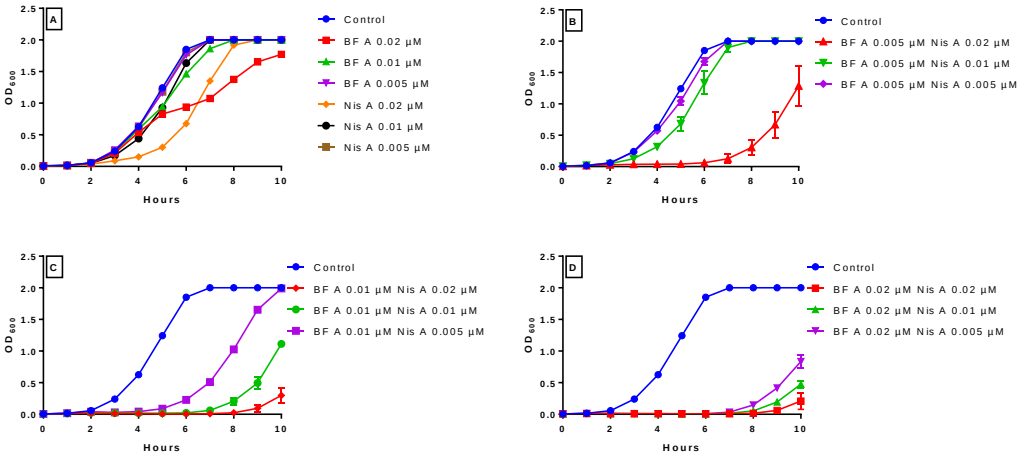


Figure 9 Growth (OD_{600}) of *S. aureus* DPC5246 in bactofencin A 1x, 0.5x and 0.25x in relation to nisin A 0.02µM and nisin A 1x, 0.5x and 0.25x in relation to bactofencin A 0.02µM in BHI broth at 37°C.



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Chapter 6.1

Formicin – a novel broad-spectrum two-component lantibiotic produced by *Bacillus paralicheniformis* APC 1576

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FWJ Collins, **PM O'Connor**, Ó O'Sullivan, MC Rea,

C Hill and RP Ross

CHAPTER CONTRIBUTIONS

Paula M O'Connor purified formicin, performed MALDI TOF MS, advised on experiments and significantly contributed to manuscript preparation.
Fergus WJ Collins isolated the strain and performed antimicrobial assays designed experiments and significantly contributed to manuscript preparation.
Órla O'Sullivan performed bioinformatic analysis.

6.1.1 Abstract

Bacteriocins represent a rather underutilized class of antimicrobials despite often displaying activity against many drug-resistant pathogens. Lantibiotics are a post-translationally modified class of bacteriocins, characterized by the presence of lanthionine and methyllanthionine bridges. In this study, a novel two-peptide lantibiotic was isolated and characterized. Formicin was isolated from *Bacillus paralicheniformis* APC 1576, an antimicrobial-producing strain originally isolated from the intestine of a mackerel. Genome sequencing allowed for the detection of the formicin operon and, from this, the formicin structural genes were identified, along with those involved in lantibiotic modification, transport and immunity. The identified bacteriocin was subsequently purified from the bacterial supernatant. Despite the degree of conservation seen amongst the entire class of two-peptide lantibiotics, the formicin peptides are unique in many respects. The formicin α peptide is far less hydrophobic than any of the equivalent lantibiotics, and with a charge of plus two, it is one of the most positively charged α peptides. The β peptide is unique in that it is the only such peptide with a negative charge due to the presence of an aspartic acid residue in the C-terminus, possibly indicating a slight variation to the mode of action of the bacteriocin. Formicin also displays a broad-spectrum of inhibition against Gram-positive strains, inhibiting many clinically relevant pathogens such as *Staphylococcus aureus*, *Clostridium difficile* and *Listeria monocytogenes*. The range of inhibition displayed against many important pathogens indicates a potential therapeutic use against such strains where antibiotic resistance is such a growing concern.

6.1.2 Introduction

With the increased prevalence of many drug-resistant bacterial strains, the development of new antimicrobials is becoming a growing necessity. One such class of antimicrobials that appear to be underrepresented in clinical applications are bacteriocins (Cotter *et al.*, 2013). Unlike traditional antibiotics, bacteriocins are gene-encoded, ribosomally synthesized peptides, making them suitable for genetic manipulation, with the potential for novel and specialized drug design (Gillor *et al.*, 2005). The spectrum of inhibition of bacteriocins can range from broad to narrow, the latter may allow for highly targeted antibacterial therapies that may reduce the collateral damage associated with the use of broad-spectrum antibiotics (Rea *et al.*, 2011).

The lantibiotics (*lan*thionine-containing *antibiotics*) comprise a well-studied class of bacteriocins, the most notable of which is nisin (Rogers, 1928), which is commonly used as a food preservative. Lantibiotics are classified based on the presence of lanthionine or methyllanthionine bridges. In these peptides, serine and threonine residues are post-translationally modified and dehydrated to form 2,3-didehydroalanine (Dha) and 2,3-didehydrobutyrine (Dhb) residues. The thiol group of a cysteine residue subsequently reacts with the Dha or Dhb residues resulting in the formation of lanthionine or methyllanthionine thioether crosslinks (Xie & van der Donk, 2004).

The lantibiotic gene cluster encodes an array of genes required for modification, regulation and transport of the bacteriocin. Lantibiotics are divided into classes depending on the mechanism by which they are synthesized. Class I lantibiotics encode the enzymes LanB and LanC within the bacteriocin operon where LanB catalyses the dehydration of the serine and threonine residues, whilst LanC catalyses the cyclization of the lanthionine rings. In Class II lantibiotics, LanM

alone catalyses both dehydration and cyclization of the lantibiotics (Willey & van der Donk, 2007). LanR and LanK play key roles in the regulation of lantibiotic production (Lee *et al.*, 2011). Once the mature lantibiotic is produced, its cleavage and transport are carried out by LanP and LanT respectively (Escano *et al.*, 2015). In some cases, LanT can carry out both leader sequence cleavage and peptide secretion functions (Furgerson Ihnken *et al.*, 2008). Immunity to lantibiotics can be afforded by immunity proteins such as the lipoprotein LanI that likely binds the secreted lantibiotic and the ABC transporter LanFEG that transports bacteriocin peptides from the membrane to the extracellular medium. Here LanF binds and hydrolyses ATP that provides the energy required for the transport of the bacteriocin through the LanEG membrane complex (Stein *et al.*, 2005; Takala *et al.*, 2004; Alkhatib *et al.*, 2012). For a review on this class of bacteriocins, see Willey & van der Donk (2007).

Within the lantibiotic class of bacteriocins exist a small subgroup of two-peptide lantibiotics. Such bacteriocins are produced by an array of genera, including *Staphylococcus* and *Lactobacillus* (Navaratna *et al.*, 1998; Holo *et al.*, 2001). Interestingly, of the few two-component lantibiotics that have been described, two of these bacteriocins identified prior to this study are produced by *Bacillus* species. *Bacillus* species are known to produce a vast range of antimicrobials, whether antibiotics (e.g. gramicidin, bacitracin) or bacteriocins (e.g. thuricin CD, mersacidin) (Katz & Demain, 1977; Rea *et al.*, 2010; Chatterjee *et al.*, 1992). The currently identified two-component lantibiotics include lacticin 3147 (*Lactococcus lactis*) (Ryan *et al.*, 1996), lichenicidin (*Bacillus licheniformis*) (Begley *et al.*, 2009; Dischinger *et al.*, 2009), haloduracin (*Bacillus halodurans*) (McClerren *et al.*, 2006), enterocin W (*Enterococcus faecalis*) (Sawa *et al.*, 2012), plantaricin W

(*Lactobacillus plantarum*) (Holo *et al.*, 2001), BHT (*Streptococcus rattus*) (Hyink *et al.*, 2005), Smb (*Streptococcus mutans*) (Yonezawa & Kuramitsu, 2005) and staphylococcin C55 (*Staph. aureus*) (Navaratna *et al.*, 1998). In this subclass of bacteriocins, the two peptides produced tend to act synergistically and usually display negligible antimicrobial activity on their own.

The mode of action of lacticin 3147 identifies a likely model for the mode of action of similarly structured lantibiotics. The α peptide of lacticin 3147 (Ltnα) resembles the globular lantibiotic mersacidin, mirroring its activity by binding to lipid II that acts as an important docking molecule. Binding to lipid II results in a conformational change of Ltnα, which presents a site to which the β peptide (Ltnβ) can then bind. Ltnβ resembles an elongated lantibiotic, which, once recruited by Ltnα, inserts itself into the target membrane inducing pore formation resulting in cell death. Here the cooperative activity of both peptides is necessary for optimal antimicrobial activity, as the stability of the total bacteriocin–lipid II complex is important for both pore formation and the inhibition of cell wall biosynthesis (Martin *et al.*, 2004; Wiedemann *et al.*, 2006).

In this study, we extend the class of two-peptide lantibiotics by identifying a novel bacteriocin known as formicin that is produced by a marine isolate, *Bacillus paralicheniformis* APC 1576. Whilst this lantibiotic resembles the previously described two-peptide lantibiotics, it contains a number of features that differentiate it from the rest of the class.

6.1.3 Methods

Isolation of bacteria from fish samples

Marine fish were caught off the coast of Ireland and stored on ice prior to analysis. The intestinal contents of the fish and a sample of the skin and gills were aseptically removed. Samples were suspended in maximum recovery diluent (Oxoid), serial dilutions were then plated on brain–heart infusion (BHI) agar (Merck) and marine media 2216 (Difco Laboratories) and were incubated aerobically at 30℃ for 3 days. Colonies were isolated from these plates and analysed for antimicrobial activity using deferred antagonism assays, whereby spots of the bacterial cultures were overlaid with 10 ml de Man, Rogosa and Sharpe agar (Difco Laboratories) seeded with 25 µl of a *Lactobacillus delbrueckii* subsp. *bulgaricus* LMG 6901 overnight culture. Colonies that displayed significant zones of inhibition were further characterized.

In this study, the strain of interest, *B. paralicheniformis* APC 1576, was isolated from the intestinal tract of a mackerel (*Scomber scombrus*) and grown on BHI aerobically at 37°C. The strain was identified by 16S rRNA sequencing using the UniF (5'-AGAGTTTGATCCTGGCTCAGG-3') and UniR (5'-ACGGCAACCTTGTTAC GAGT-3') primers to amplify the sequence. PCR products were cleaned using an illustra GFX PCR DNA and Gel Band Purification kit (GE Healthcare) and subsequent sequencing was completed by Cogenics (Essex).

Colony MS

Colony MALDI-TOF MS (Axima TOF^2 MALDI-TOF mass spectrometer, Shimadzu Biotech) was used to determine the molecular mass of the peptides produced as follows: cells were first mixed with 70% 2-propanol/0.1% TFA (IPA) and vortexed, the sample was separated by centrifugation and the supernatant was subsequently used for analysis. A MALDI target plate was precoated with CHCA

matrix solution, 0.5 µl of the supernatant from the cell extract was then placed on the target and a final layer of matrix solution was added. Positive-ion reflectron mode was used to identify the peptide masses. The masses detected were then compared to those of known bacteriocins.

Draft genome sequencing

Genomic DNA was extracted using the GenElute bacterial genomic kit (Sigma-Aldrich) and the Nextera XT DNA kit (Illumina) was used for library preparation. The DNA was quantified using a Qubit 2.0 fluorometer. Sequencing was performed using Illumina's MiSeq platform using paired-end 2×300 base pair reads in the Teagasc Sequencing Centre, Teagasc Food Research Centre, Moorepark. Reads were assembled *de novo*, using SPADES (version 3.1.1), resulting in 70 contigs. ORFs were identified and annotated using Prokka (version 1.1). Further manual annotation was implemented with ARTEMIS and Artemis Comparison Tool. Genomic data are available from GenBank/EMBL under accession no. LXPD00000000.

Bacteriocin identification

The bacteriocin mining tool BAGEL3 was used to identify the bacteriocin operons encoded in the genome (van Heel *et al.*, 2013). BAGEL3 scans small ORFs to identify potential bacteriocin-encoding genes. The surrounding genes were then analysed for other bacteriocin-related components such as transporters and immunity proteins, thus allowing the entire bacteriocin operon to be identified (de Jong *et al.*, 2006). The program antiSMASH was also used to identify antibiotic and secondary metabolite encoding genes within the genome, as these compounds

are often associated with the *Bacillus* genus (Medema *et al.*, 2011). Sequence alignments of the bacteriocin were performed using the Clustal Omega software.

Bacteriocin purification

Cultures of *B. paralicheniformis* APC 1576 were grown statically overnight in 400 ml volumes of BHI broth aerobically at 37℃. The cell-free supernatant (CFS) was passed through a column containing 30 g of Amberlite XAD-16N beads (Sigma-Aldrich). The column was washed with 250 ml of 35% ethanol and antimicrobial activity eluted with 250 ml of IPA. The IPA was removed via rotary evaporation and the sample was then applied to a 10 g, 60 ml Strata C18-E solid-phase extraction (SPE) column (Phenomenex). The SPE column was washed with 90 ml of 35% ethanol and 90 ml of IPA. The IPA was once again removed via rotary evaporation from the eluent and the sample applied to a semiprep Jupiter Proteo HPLC column (10 x 250 mm, 90 Å, 4 µm) running a 27.5–65 % acetonitrile/0.1 % TFA gradient where buffer A was 0.1 % TFA and buffer B was 90% acetonitrile/0.1% TFA. Fractions were collected at 1 min intervals and were subsequently analysed with MALDI-TOF MS and agar well diffusion assays as described below using *Lb. delbrueckii* subsp. *bulgaricus* LMG 6901 as the target organism to identify active fractions containing peptides of interest.

Antimicrobial assays

The antimicrobial activity of the isolated peptides was analysed using well diffusion assays against a range of indicator organisms (Table 1). Briefly, this involved seeding 20 ml of the appropriate agar with 50 µl of an overnight indicator culture; the agar was allowed to cool and 7 mm wide wells were then bored in the agar.

The purified bacteriocin peptides were lyophilized and diluted separately in potassium phosphate buffer (pH 6.8) to a concentration of 50 µM. The combination of these peptides in a 1:1 ratio thus gave a total bacteriocin concentration of 25 µM for each peptide. Fifty microlitres of this solution was then placed in wells in the indicator plate, and these were subsequently incubated overnight under the appropriate growth conditions as outlined in Table 1.

Peptide stability

The stability of the bacteriocin was determined using purified peptides. To determine the active temperature range of the lantibiotic, we treated 25 µM aliquots of the bacteriocin at 60, 70, 80, 90 and 100°C for 30 min; a sample was also treated at 121°C for 15 min. These samples were then tested for inhibitory activity against *Lb. delbrueckii* subsp. *bulgaricus* LMG 6901 in well diffusion assays as previously described. To determine the susceptibility of the bacteriocin to proteases, we treated 5 µM aliquots of the α and β peptides separately with proteinase K and α-chymotrypsin each at a concentration of 10 mg ml^{-1} (Sigma-Aldrich). Samples were incubated at 37°C for 3 h followed by treatment at 100°C for 10 min to inactivate these proteases. Both bacteriocin peptides were then combined post-treatment to give a final total concentration of 2.5 µM; these were then screened against *Lb. delbrueckii* subsp. *bulgaricus* LMG 6901 in well diffusion assays to determine the antimicrobial activity.

6.1.4 Results

Isolation of *B. paralicheniformis* APC 1576

B. paralicheniformis APC 1576 was isolated from the intestinal microbiota of a

freshly caught mackerel. In an initial screen for bacteriocin producers, the strain was found to inhibit *Lb. delbrueckii* subsp. *bulgaricus* LMG 6901 in an overlay assay (Figure 1a). In addition, CFSs also inhibited *Lb. delbrueckii* subsp. *bulgaricus* LMG 6901 in a well diffusion assay, indicating that the antimicrobial substance was secreted by the cells into the media (Figure 1b). Colony MS was used to determine the molecular masses of the peptides produced by the cell; however, the detected peptide masses (Figure 1c) failed to match any previously characterized bacteriocin, including lichenicidin, a bacteriocin produced by *B. licheniformis* (Begley *et al.*, 2009). Moreover, more than one source of antimicrobial activity was found following purification of the antimicrobial peptides. MALDI-TOF MS identified a molecule with a mass of 1422.54 Da, which displayed activity against *Lb. delbrueckii* subsp. *bulgaricus* LMG 6901 once purified; this mass correlates closely with that of bacitracin, which is encoded on the genome. The production of more than one antimicrobial from *Bacillus* species is not unexpected. Therefore, in order to identify all potential antimicrobials with activity against *Lb. delbrueckii* subsp. *bulgaricus* LMG 6901, we sequenced the genome of *B. paralicheniformis* APC 1576.

Identification of a novel two-peptide lantibiotic operon

Once the draft genome was obtained, the sequence was analysed with BAGEL3 and antiSMASH to identify the antimicrobials encoded. Gene clusters encoding the antibiotics bacitracin, surfactin and fengycin were found within the genome. The strain likely produces at least one of these antimicrobials, as antifungal activity was also observed against *Aspergillus niger* in overlay assays (data not shown).

A novel lantibiotic operon was also identified within the genome of the strain (Figure 2). This operon spans approximately 17 kb and was located on a single contig of the draft bacterial genome. Two putative lantibiotic-encoding structural genes were identified on this operon. ORF1 (*frcA1*) encodes a 66-amino-acid peptide and ORF3 (*frcA2*) encodes a 71-amino-acid peptide. Analysis of the prepropeptides (including the bacteriocin leader sequence) of these lantibiotics shows that the formicin A1 prepropeptide displayed 47.8% amino acid identity with that of the unmodified haloduracin A1 equivalent and 35.9% identity with that of the lantibiotic mersacidin. As the putative bacteriocin appears to be a two-peptide bacteriocin, two lantibiotic modification enzymes should be present. The order of the genes in the operon would suggest that ORF2 (*frcM1*) is the modification enzyme associated with *frcA1*. Upon analysis, this ORF displayed 38.7% identity with that of the haloduracin HalM1 modification enzyme. The second lantibiotic gene, ORF3 (*frcA2*), appears to resemble the elongated β peptides of the other two-peptide lantibiotics that are involved in membrane insertion (Wiedemann *et al.*, 2006). Upon analysis, formicin A2 revealed 42.4% identity with the unmodified lichenicidin LchA2 prepropeptide. ORF4 (*frcM2*) encodes the modification enzyme, which follows this structural peptide, and displayed 33.6% identity with that of the lichenicidin LchM2 modification enzyme.

ORF5 located downstream of LchM2 is predicted to encode a lantibiotic transporter, displaying 52.5% identity with that of the haloduracin transporter, HalT. In addition to its function in bacteriocin transport, a sequence encoding a C39 peptidase domain (cd02425) can also be found within the gene; this is likely involved in the cleavage of the leader sequence from the prebacteriocin. BLAST

analysis of ORF6 identified the gene as encoding a hypothetical protein; the sequence, however, did show 28.4% identity with that of LanY encoded within the lichenicidin operon (Begley *et al.*, 2009). ORF7, ORF8 and ORF9 all encode ABC transporter-related peptides, as do ORF11, ORF12 and ORF13. These are likely to be involved in bacteriocin immunity. ORF7 and ORF11 both encode domains resembling that of the ABC-binding cassette domain of the bacitracin resistance transporter (cd03268) and displayed 44.5% identity with that of each other. Instead of the common Q-loop motif found in the nucleotide-binding domains of such transporters, both these proteins instead encode an E-loop motif that is indicative of lantibiotic immunity proteins (Okuda *et al.*, 2010; Alkhatib *et al.*, 2012). Each of the other components encodes ABC-2-type transporter domains (cl21474). The presence of these gene clusters may suggest a dual mechanism of bacteriocin immunity. Immunity to the lichenicidin bacteriocin is thought to follow a similar mechanism, with two transporters being encoded, with one showing homology to the bacitracin transporter (Dischinger *et al.*, 2009). Such mechanisms, however, do not confer a general immunity against all two-peptide lantibiotics, as the producers of both lichenicidin (*B. licheniformis* ATCC 14580) and lacticin 3147 (*Lc. lactis* subsp. *lactis* DPC 3147) displayed sensitivity to formicin (Table 1).

ORF10 (*frcR*) that splits the transporter clusters encodes a LanR-equivalent transcriptional regulator. This gene encodes helix–turn–helix XRE family domains, crucial for binding DNA and regulating gene expression. This LanRtype protein displayed 49.4% and 60.3% identity with those of the regulators found within the lichenicidin and haloduracin operons, respectively. ORF14 (*frcP*) encodes a lanthionine-specific protease displaying 29.8% identity with that of LicP

found in the lichenicidin operon. As in lichenicidin, the LanT-like ORF (*frcT*) likely cleaves the N-terminal glycine leader sequence from both propeptides upon transport, whilst the LanP-like protease (*frcP*) possibly cleaves the six newly exposed N-terminal amino acids from the β peptide to generate the mature bacteriocin (Tang *et al*., 2015). The final ORF found in the gene cluster encodes a DNA damage-inducible protein.

Bacteriocin structure prediction and analysis

The spectrum of activity and characteristics of the bacteriocin could not be determined from the crude bacteriocin supernatant alone due to the interference from other antimicrobials produced by the strain. Thus, it was necessary to purify the bacteriocin from the CFS in order to determine the activity of formicin. With the use of the predicted masses of the lantibiotic structural peptides identified from genomic data, it was possible to determine if the formicin peptides were present in active HPLC-derived fractions using MALDI-TOF MS.

From the purified peptides, masses of 3254.34 and 2472.06 Da were detected for the α and β peptides respectively. The predicted mass of the Frcα peptide based on the amino acid sequence from the genome is 3310.80 Da; the difference between the predicted and observed masses correlates with the loss of three water residues, which is most likely associated with the formation of lanthionine and methyllanthionine bridges, as well as also the possible formation of one disulfide bond, resulting in a predicted mass of 3254.80 Da. Due to the similarities between the two, the structure of Halα was used as a basis for the prediction of the structure of Frcα. Based on the Halα template, the formation of a lanthionine bridge may occur between Ser-7 and Cys-17, whilst methyllanthionine bridges could form

between Thr-18 and Cys-23, as well as between Thr-20 and Cys-27, whilst Ser-26 remains unaltered. In addition, a disulfide bridge is also likely to form between Cys-1 and Cys-8 (Figure 3).

The second mass determined by MALDI-TOF MS relates to the β peptide of the bacteriocin. Due to the presence of the extra LanP serine protease encoded in the bacteriocin operon and the similarity formicin displays to haloduracin and lichenicidin, it is likely that the first six amino acids following the lantibiotic leader sequence are also cleaved from the formicin peptide. Once these amino acids are discounted, the predicted mass of the peptide is 2614.95 Da, a difference of 142.89 Da from the mass detected by MALDI-TOF MS. This mass difference corresponds closely with the loss of 144 Da, which would be associated with eight dehydration reactions. Using the β peptides of lichenicidin and lacticin 3147 as templates, we predicted that the peptide is most likely to form bridges between Thr-1 and Cys-8, Thr13 and Cys-17, Ser-19 and Cys-22, and Thr-23 and Cys-26. This would result in Thr-2, Ser-4, Ser-5 and Thr-10 being dehydrated to their respective Dha and Dhb residues, whilst Ser-24 remains unaltered (Figure 3).

The purified peptides were screened against a range of indicator organisms to determine the spectrum of inhibition (Table 1). Purified formicin inhibited 29 of the 35 indicator strains screened, exhibiting a broad-spectrum of activity against a range of bacterial genera including lactobacilli and enterococci, as well as notable pathogens such as *Staph. aureus, Strep. mutans, Ls. monocytogenes, C. difficile* and *B. subtilis*. The Frcα peptide alone at a concentration of 50 µM also displayed antimicrobial activity against a number of indicators, whilst Frcβ alone displayed no detectable antimicrobial activity.

In terms of thermostability, the bacteriocin retained a high degree of activity after

treatment at 100°C for 30 min, displaying a reduction in the size of the zone of inhibition of approximately 28%. Activity was, however, lost after treatment for 15 min at 121°C. The bacteriocin was also found to be susceptible to digestion by *a*-chymotrypsin and proteinase K, indicating its proteinaceous nature.

Homology between bacteriocins

The previously described two-peptide lantibiotics all display a degree of homology with certain conserved residues found throughout. As a result, sequence comparisons of these structural peptides were carried out with formicin to determine if this conservation extended to the new bacteriocin (Figure 4). The results indicate that formicin complies with the conservation that is seen amongst the other bacteriocins. The mersacidin-like α peptides display the greatest levels of conservation and this reflects the shared mode of action in specifically binding to lipid II. This homology, especially in the lanthionine and methyllanthionine bridgeforming regions, confers a structural similarity in each of the peptides. The broader role of the β peptides in membrane insertion is reflected in a greater degree of divergence in the composition of these peptides. The regions of conservation that are seen amongst the β peptides extend to Frcβ also, with the C-terminus of the peptides showing a relatively conserved pattern of lanthionine and methyllanthionine bridge formation. The N-terminus of the β peptides displays a much lower degree of conservation amongst the bacteriocins; despite this, these N-terminal regions are rich in hydrophobic amino acids, which likely play an important role in membrane insertion and pore formation.

6.1.5 Discussion

Formicin represents a novel member of the class of two-peptide lantibiotics. This class of bacteriocins are themselves unusual given that the lipid II-binding and pore-forming activities of the bacteriocin are performed by two separate peptides, whilst certain lantibiotics such as nisin and subtilin have the ability to carry out both functions on a single peptide. It is unclear as to whether these two-component lantibiotics have evolved due to a divergence of a nisin-like lantibiotic into two separate genes due to a duplication event or whether they have come about due to the convergence of a mersacidin-like lipid II-binding lantibiotic and a pore-forming lantibiotic. If the latter is the case, it is interesting as to how such different peptides would have evolved to depend on each other for antibacterial activity, and in some cases, lose the activity each would have shown on its own.

Sequencing of *B. paralicheniformis* APC 1576 allowed for the elucidation of the formicin bacteriocin operon (Figure 2). Analysis of the bacteriocin operon identified two lantibiotic structural genes (*frcA1* and *frcA2*) and two modification enzymes (*frcM1* and *frcM2*) that convert the formicin structural peptides into the mature lantibiotics. Transport and leader cleavage are likely to be carried out by *frcT*, whilst *frcP* may act as a further protease, cleaving six N-terminal amino acids from Frcβ. ORF7, ORF8 and ORF9 and ORF11, ORF12 and ORF13 all predict to encode ABC transporters that are likely to comprise the strain's immunity mechanism, protecting itself from attack by its own bacteriocin. Comparative analysis of the bacteriocin structural genes allows for the homology between bacteriocins to be determined (Figure 4). In the case of both Frcα and Frcβ, the closest homologues are the haloduracin α and β mature peptides, displaying 71% and 39% identity respectively. Such homology reflects the close relationship of the two producers, both belonging to the *Bacillus* genus. The differences between the

formicin and lichenicidin peptides are surprisingly large, given that both are produced from related species, with the α peptides displaying 46% identity and the β peptides displaying 36% identity. This would suggest that both strains may have acquired these operons independently. The layout of the formicin operon itself differs from that of the previously characterized two-peptide lantibiotics, and transcription of the formicin operon would appear to be unidirectional whereby the genes for the structural peptides are separated by those encoding the LanM modification enzymes, an arrangement that seems to be unique to formicin. Both the haloduracin and lichenicidin structural genes (Figure 2) would likely be transcribed in opposite directions, possibly indicating that gene inversion may have taken place. Such differences again display the evolutionary divergence seen between this class of bacteriocins.

Analysis of the primary structure of these peptides indicates that some key differences exist between the formicin peptides and other members of the class despite such strong regions of homology found throughout. The α peptide of formicin, for example, contains only five hydrophobic amino acids, whilst others in the class contain an average of nine. Whilst hydrophobic residues are crucial for membrane activity in certain bacteriocins, it has been suggested that it is the charged residues of these lantibiotics that control binding to lipid II as opposed to hydrophobic interactions. This indicates that binding of formicin to lipid II is not compromised despite its lower hydrophobicity, a fact that is supported by the activity of the α peptide independent of the β peptide (Hsu *et al.*, 2003; Fimland *et al.*, 2006). As with the α peptides from enterocin W and plantaricin W, the α peptide of formicin contains six charged amino acids, with an overall positive charge of plus two, rendering them amongst the most highly charged in the class.

Not only do these charged residues affect the structure of the peptide but also the higher positive charge may lead to an increased affinity for the anionic bacterial membrane. The formicin β peptide differs most when compared to other lantibiotic β peptides with regard to charge. As is common in this class, the N-terminal tails of the β peptides are composed largely of hydrophobic residues, crucial for membrane insertion and pore formation. Whilst the previously described β peptides all contain a positively charged C-terminus, containing Lys and Arg residues, formicin is unique in that it encodes a negatively charged β peptide. The lone charged residue found in the peptide is the penultimate C-terminal Asp residue. This portion of the peptide is believed to be involved in the interaction between the α and β peptides (Wiedemann *et al.*, 2006); thus, this negative residue may suggest an increased affinity for the positively charged α peptide, possibly representing a stronger complex compared to previously described pairs.

The tertiary structure of these peptides has an important functional role in the antimicrobial activity of these lantibiotics. Analysis of the N-terminus of Frcα suggests the formation of a disulfide bridge between Cys-1 and Cys-8. Whilst this has been shown to be inessential for antimicrobial activity, it may reduce the degradation of the peptide once secreted (Cooper *et al.*, 2008). Of the lantibiotic rings believed to be formed in Frcα, only the C ring is thought to be essential, with alterations abolishing all activity completely in both haloduracin and lacticin 3147 (Cooper *et al.*, 2008; Cotter *et al.*, 2006). The B ring found in these α peptides has been shown to be unnecessary, which is unusual given the high degree of conservation amongst such bacteriocins, including mersacidin. Disruption of the A ring in haloduracin has been shown to reduce but not eliminate activity, thus showing that this region is important but not essential for the antibacterial activity

of the bacteriocin (Cooper *et al.*, 2008). As per analysis of the haloduracin β peptide, the A ring of the peptide has been found to be dispensable, whilst loss of the C and D rings led to a reduction in activity but not total elimination. Disruption of the B ring could not be achieved without disruption of the other ring structures (Cooper *et al.*, 2008).

6.1.6 Conclusion

In this study, formicin, a novel member of the class of two peptide lantibiotics has been identified. Key regions of homology, primarily those involved in lanthionine and methyllanthionine bridge formation, seen throughout this class have been shown to be extended to formicin. Such homology is expected to confer a similar mode of action to all lantibiotics in this class, with the α peptide of the bacteriocin binding to lipid II and subsequently recruiting the β peptide for membrane insertion and pore formation. Whilst formicin likely conforms to such mechanisms, there are certain key variations differentiating it from the rest of the class. The reduction of hydrophobicity of Frcα and the unusual negative charge of Frcβ make formicin a unique member of the two-peptide lantibiotics. Further studies are required to determine the effects of such changes on the activity of the bacteriocins, as it is recognized that charge and hydrophobicity play a central role in the activity of these lantibiotics and in bacteriocins in general. Formicin itself displays a broad range of inhibition, inhibiting several clinically relevant Gram-positive pathogens, such as *C. difficile*, *Staph. aureus*, *Strep. mutans* and *Ls. monocytogenes*. With the continued progression of antibiotic resistance in pathogenic bacteria, the discovery of novel therapies against such agents is a priority and since the bacteriocin is produced by a species long associated with

biotechnology applications, a straightforward route towards large-scale processing of the readily purified peptides is anticipated. Thus, formicin represents a potential novel antimicrobial therapy against a range of pathogenic bacteria.

6.1.7 Acknowledgements

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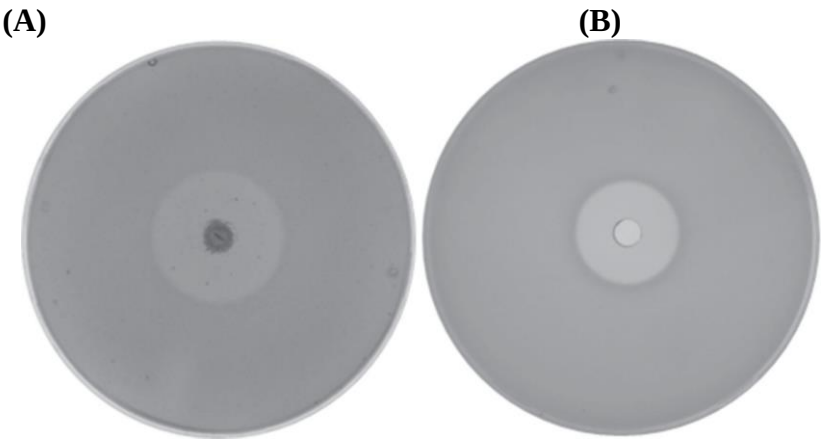
Table 1. Growth conditions of indicator strains and inhibition spectrum of formicin pure peptides following well diffusion assays.

		Growth conditions			
Species	**Strain**	**Temp. (°C)**	**Atmosphere**	**Growth media**	**Inhibition**
Lactobacillus delbrueckii subsp. *bulgaricus*	LMG 6901	37	Anaerobic	MRS	+++
Lactobacillus delbrueckii subsp. *lactis*	LMG 7942	37	Anaerobic	MRS	+++
Lactobacillus amylovorus	LMG 9496	37	Anaerobic	MRS	+
Lactobacillus fermentum	LMG 6902	37	Anaerobic	MRS	++
Lactobacillus agilis	LMG 9186	37	Anaerobic	MRS	++
Lactobacillus casei	LMG 6904	37	Anaerobic	MRS	++
Lactobacillus amylophilus	DSM 20533	37	Anaerobic	MRS	+++
Lactobacillus acidophilus	LMG 9433	37	Anaerobic	MRS	-
Lactobacillus buchneri	DSM 20057	37	Anaerobic	MRS	-
Enterococcus faecium	DPC 4898	37	Anaerobic	MRS	++
Enterococcus faecalis	LMG7397	37	Anaerobic	MRS	++
Enterococcus saccharolyticus	DPC 4902	37	Anaerobic	MRS	++
Enterococcus mundtii	LMG 10748	37	Anaerobic	MRS	+
Lactococcus lactis	HP	30	Aerobic	LM17	+++
Lactococcus lactis subsp. *lactis*	DPC 3147	30	Aerobic	LM17	+++
Micrococcus luteus	DPC 6275	30	Aerobic	BHI	+++
Listeria innocua	DPC 3572	37	Aerobic	BHI	++
Listeria monocytogenes	DPC 5788	37	Aerobic	BHI	+
Listeria monocytogenes	DPC 6893	37	Aerobic	BHI	+
Listeria monocytogenes	DPC 6894	37	Aerobic	BHI	+
Bacillus cereus	DPC 6087	37	Aerobic	BHI	+
Bacillus subtilis	DPC 6551	37	Aerobic	BHI	++
Bacillus subtilis	LMG 8198	37	Aerobic	BHI	+
Bacillus licheniformis	DSM 13	37	Aerobic	BHI	++
Pseudomonas aeruginosa	APC 2064	37	Aerobic	BHI	-
Staphylococcus chromogenes	APC 82	37	Aerobic	BHI	-
Staphylococcusaureus	C55	37	Aerobic	BHI	-
Staphylococcusaureus	R963	37	Aerobic	BHI	+
Streptococcusmutans	APC 1076	37	Aerobic	BHI	+++
Clostridium indolis	DPC 6345	37	Anaerobic	RCM	++
Clostridium histolyticum	DPC 6344	37	Anaerobic	RCM	++
Clostridium sporogenes	DPC 6341	37	Anaerobic	RCM	++
Clostridium difficile	ATCC 1382	37	Anaerobic	RCM	+
Clostridium perfringens	LMG 11264	37	Anaerobic	RCM	++
Salmonella enterica	APC 174	37	Aerobic	BHI	-

MRS, de Man, Rogosa and Sharpe; BHI, brain–heart infusion; RCM, reinforced clostridial media. -, No activity; +, 0.5–1.5 mm inhibition zone; ++, 2-3.5 mm inhibition zone, +++, ≥ 4 mm inhibition zone

Figure 1 Formicin identification and activity. **(A)** Deferred antagonism assay against *Lb. delbrueckii* subsp. *bulgaricus* LMG 6901 identified *B. licheniformis* APC 1576 as an antimicrobial producer. **(B)** Antibacterial activity of the *B. licheniformis* APC 1576 CFS against *Lb. delbrueckii* subsp. *bulgaricus* LMG 6901 in a well diffusion assay. **(C)** Colony MALDI-TOF MS displaying the masses of the peptides produced by *B. licheniformis* APC 1576, allowing identification of the antimicrobials produced (3255.92 Da = Frcα (formicin); Frcβ is not seen using colony MALDI-TOF MS; 1423.94 Da = bacitracin)



(C)

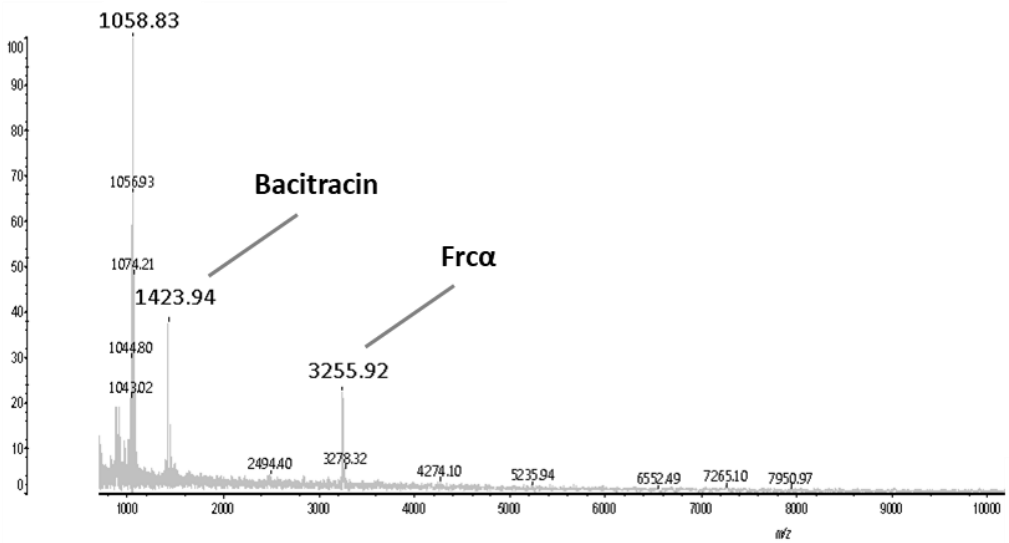


Figure 2 Formicin operon and visualization of the formicin, lichenicidin and haloduracin bacteriocin gene clusters. Clear bacteriocin homologues are identified using the accepted nomenclature for describing lantibiotics. For formicin, *frcA1* and *frcA2* encode the putative bacteriocins, *frcM1* and *frcM2* encode the accompanying modification enzymes and *frcT* and *frcP* are involved in bacteriocin transport and leader cleavage. Similar nomenclature is used for lichenicidin (*lic*) and haloduracin (*hal*) genes. Genes are colour-coded as per BAGEL3, indicating the putative role of each protein.

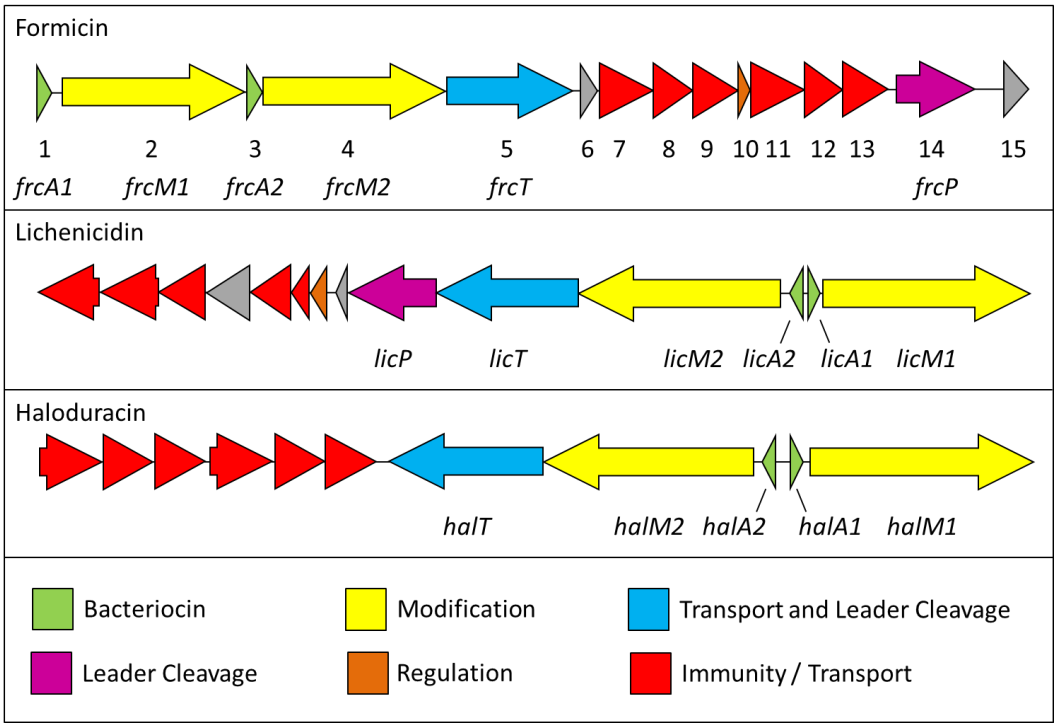
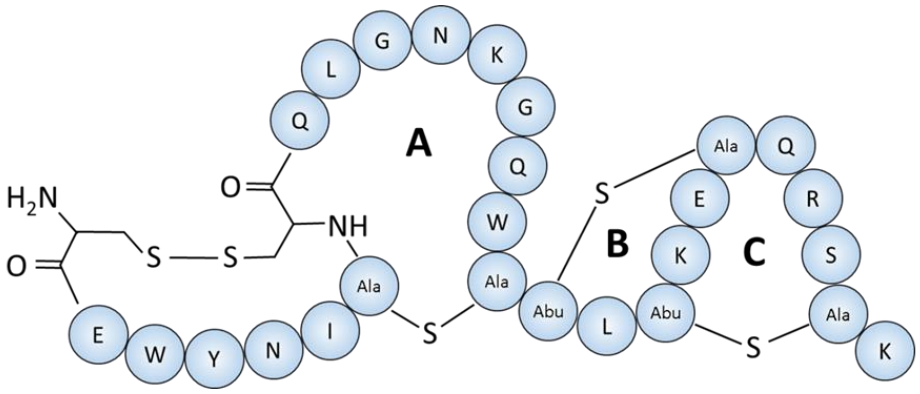


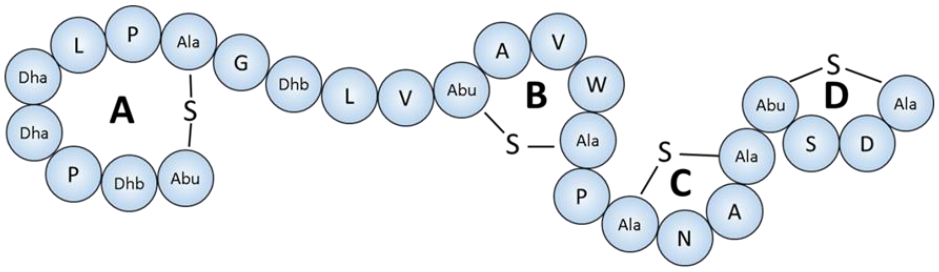
Figure 3 Lantibiotic structure prediction. The structures of the formicin α and β peptides were predicted using the Halα and Licβ peptides, respectively, as templates. The conservation of key amino acids suggests a structural homology between the peptides. The rings formed from lanthionine and methyllanthionine bridges are labelled alphabetically, with the N-terminal ring of Frcα excluded as it is predicted to be formed via a disulfide bond. The bacteriocin prepropeptides are shown below each structure, with likely dehydrated serine and threonine residues indicated in red.



MSKIEAWKNPVARMNSQIVSPAGDLMDELSDSEMEMLAGGCEWYNISCQLGNKGQWCTLTKECQRSCK

Leader Cleavage

Frcα

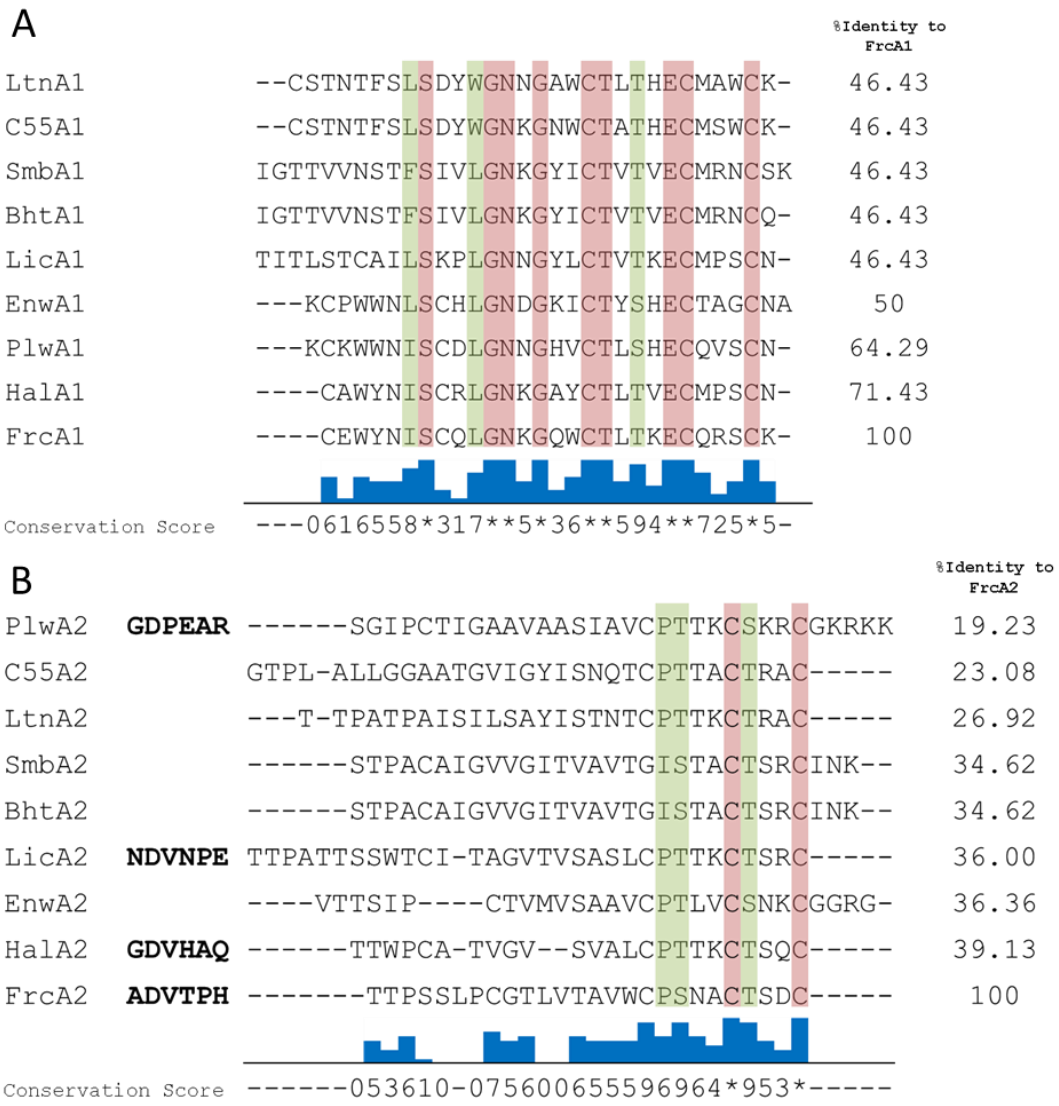


MSHREMAAIYRDANKRANLEFSNPVGEVNEEELKNLAGAADVTPHTTPSSLPCGTLVTAVWCPSNACTSDC

Leader Cleavage

Frcβ

Figure 4 Sequence alignment of formicin structural peptides. Using Clustal Omega, the formicin peptides FrcA1 (top image) and FrcA2 (bottom image) were aligned against the previously described two-component bacteriocins. The percentage amino acid identities of each peptide with the formicin peptides are shown. The conservation scores between the peptides were calculated with Clustal Omega for the alignments containing less than 25% gaps; asterisk (*) represents a score of 10. The sequences in bold face represent the six amino acids cleaved from the N-terminus of these peptides by LanP proteases.



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Chapter 6.2

Actinomyces produces defensin-like bacteriocins (actifensins) with a highly degenerate structure and broad antimicrobial activity

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I Sugrue, **PM O'Connor,** C Hill, C Stanton and RP Ross

Chapter Contributions

Paula M O'Connor purified actifensin, performed MALDI TOF MS, alkylated actifensin, advised on experiments and significantly contributed to manuscript preparation.

Ivan Sugrue performed *in silico* screen, isolated the bacteriocin producer, performed whole genome sequencing, performed antimicrobial assays, identified and characterised the homologue, designed experiments and significantly contributed to manuscript preparation.

6.2.1 Abstract

We identified a strain of *Actinomyces ruminicola* which produces a potent bacteriocin with activity against a broad range of Gram-positive bacteria, many of which are pathogenic to animals and humans. The bacteriocin was purified and found to have a mass of 4,091 ± 1 Da with a sequence of GFGCNLITSNPYQCSNHCKSVGYRGGYCKLRTVCTCY containing three disulfide bridges. Surprisingly, near relatives of actifensin were found to be a series of related eukaryotic defensins displaying greater than 50% identity to the bacteriocin. A pangenomic screen further revealed that production of actifensin-related bacteriocins is a common trait within the genus, with 47 being encoded in 161 genomes. Furthermore, these bacteriocins displayed a remarkable level of diversity with a mean amino acid identity of only 52% between strains/species. This level of redundancy suggests that this new class of bacteriocins may provide a very broad structural basis on which to deliver and design new broad-spectrum antimicrobials for treatment of animal and human infections.

6.2.2 Importance

Bacteriocins (ribosomally-produced antimicrobial peptides) are potential alternatives to current antimicrobials given the global challenge of antimicrobial resistance. We identified a novel bacteriocin from *Actinomyces ruminicola* with no previously characterized antimicrobial activity. Using publicly available genomic data, we found a highly conserved yet divergent family of previously unidentified homologous peptide sequences within the genus *Actinomyces* with striking similarity to eukaryotic defensins. These actifensins may provide a potent line of antimicrobial defense/offense, and the machinery to produce them could be

used for the design of new antimicrobials given the degeneracy that exists naturally in their structure.

6.2.3 Introduction

Novel antimicrobial compounds are increasingly important in the food, agriculture and medical fields due to decreasing efficacies of current antimicrobial treatments. Bacteriocins are ribosomally-synthesized antimicrobial peptides produced by bacteria which can target another bacterium of the same species (narrow spectrum) or bacteria of other species/genera (broad spectrum) (1). Bacteriocin producers are self-protected through the production of specific immunity proteins and, as bacteriocins are gene encoded, they can be genetically modified. Bacteriocins produced by Gram-positive bacteria have been grouped according to their primary structure into class I (post-translationally modified bacteriocins) and class II (unmodified or cyclic bacteriocins) (2). Class II is split into several subgroups, including the class IId bacteriocins, which are a heterogenous group of linear, unmodified, nonpediocin-like peptides (3). Defensins are antimicrobial peptides ubiquitous among eukaryotes which play a role in innate immunity but have also been found to act as signaling peptides, toxins, enzyme inhibitors and abiotic stress responders, and to have anticancer properties. Defensins are small (<10 kDa) cysteine-rich (forming three to six disulfide bonds) peptides with low amino acid identity, and the two superfamilies are thought to have evolved convergently (4). Only two expressed defensin-like bacteriocins have been described; the laterosporulins were previously identified among prokaryotes and contain disulfide bonds in positions homologous to those in eukaryotic defensins (5, 6). Other disulfide bond-containing bacteriocins, such as bactofencin, have been compared with eukaryotic defensins due to their highly cationic nature (7, 8).

Laterosporulin and its homolog laterosporulin10 are class IId bacteriocins produced by *Brevibacillus* spp. which have been described as broad-spectrum antimicrobials against both Gram-negative and Gram-positive bacteria. The two peptides are 5.6 kDa and 6.0 kDa and share only 57.6% amino acid sequence identity but have conserved cysteines, which are characteristic of eukaryotic defensins (6).

Actinomyces spp. are a heterogenous group of high-GC-content, Gram-positive non-spore-forming facultative or obligate anaerobes that belong to the *Actinomycetaceae* family within the phylum *Actinobacteria* (9). In humans, a number of species are known colonizers of hard surfaces in the oral cavity, where they play a key role in plaque biofilm formation (10, 11). They have been identified as core members of the oral bacteriome, present in moderate abundance (>0.1% to >2.0%) among geographically diverse populations (10, 12–15). *Actinomyces* spp. have been implicated in oral health as being associated in greater abundance in individuals with dental caries, one of the most prevalent chronic oral diseases worldwide (14, 15). Most characterized strains are clinical isolates of human origin, while some opportunistically pathogenic species such as *Actinomyces israelii* and *Actinomyces gerencseriae* are known to cause the uncommon infectious disease actinomycosis (16). Though *Actinomyces* spp. are abundant in the oral cavity, little is known about their presence in the gut, probably due to their low abundance (<0.1%) (10). Many *Actinomyces* spp. have been isolated from fecal material and from the gastrointestinal tracts of different animals, indicating a propensity for gastric transit survival, and their presence has also been noted in the urogenital tract (17–24). Here, we identify a new group of bacteriocins using a pangenomic *in silico* approach paired with functional screening. Many *in silico* genome mining tools have been developed for the successful detection of novel

antimicrobial producing operons (25, 26). Obviously, these methods rely on relationships with previously known genes and therefore, functional screening is crucial for the identification of unrelated antimicrobials. In this study, we isolated a potent bacteriocin-producing strain of *Actinomyces ruminicola* from sheep feces; the bacteriocin produced resembled eukaryotic defensins, having three characteristic disulfide bridges. A subsequent pangenus *Actinomyces* analysis revealed that such bacteriocins are widely distributed in these bacteria, albeit with a highly variable structure.

6.2.4 Materials and Methods

Isolation of bacteria and identification of bacteriocin production

Samples of raw milk, unpasteurized cheeses, sheep feces and honey were serially diluted in maximum recovery diluent (MRD) (Oxoid) and plated on several medium types for the isolation of bacteriocin-producing bacteria: *Streptococcus thermophilus* selective agar (tryptone, 10.0 g liter^{-1}; sucrose, 10.0 g liter^{-1}; yeast extract, 5.0 g liter^{-1}; K_2HPO_4, 2.0 g liter^{-1}; bromocresol purple, 0.03 g liter^{-1}; agar, 15.0 g liter^{-1}) incubated aerobically at 42°C; M17 (Merck) supplemented with 10% (wt/vol) lactose incubated at 30°C aerobically; de Man, Rogosa, and Sharpe (MRS; Difco) agar supplemented with 30 μg ml^{-1} L-vancomycin hydrochloride incubated at 37°C; MRS adjusted to pH 5.4 incubated at 42°C anaerobically; *Lactobacillus* selective agar (LBS) incubated at 30°C anaerobically; and TOS (transgalactosylated oligosaccharide) agar supplemented with 50 μg ml^{-1} lithium mupirocin incubated at 37°C anaerobically.

Isolates were subject to an initial bacteriocin production screen by overlaying with 10 ml “sloppy” MRS agar (7.5 g liter^{-1} agar) tempered to 50°C and seeded with an

overnight culture of *Lactobacillus delbrueckii* subsp. *bulgaricus* LMG 6901 (0.25% [vol/vol]). Cultures which were found to produce distinct zones of inhibition in the agar overlay were cultured in broth for well diffusion assays. For well diffusion assays, 20 ml of sloppy MRS agar seeded with *L. delbrueckii* subsp. *bulgaricus* LMG 6901, as described above, was poured and allowed to set, in which 6-mm-wide wells were then bored. Fifty microliters of cell-free supernatant was added to each well, and plates were incubated at 37°C overnight. Zones of inhibition were indicative of antimicrobial activity.

Bacterial strains, media, reagents

Strains used in this study and their incubation conditions are listed in Table S3 in the supplemental material. *A. ruminicola* DPC 7226 was routinely maintained in brain heart infusion (BHI) broth (Oxoid) anaerobically at 37°C. Medium reagents were sourced from Sigma Aldrich (Co. Wicklow, Ireland) unless stated otherwise.

Purification of actifensin

A. ruminicola DPC 7226 was grown anaerobically and statically at 37°C in 500-ml volumes of BHI broth for 48 h. Following centrifugation, cell-free supernatant was applied to an Econo column containing 30 g Amberlite XAD beads prewashed with Milli-Q water. The column was washed with 300 ml 30% ethanol and 300 ml 2-propanol–0.1% trifluoroacetic acid (TFA) (IPA). IPA was removed by rotary evaporation, and the sample was applied to a 60-ml 10-g Strata-E C18 SPE column (Phenomenex, Cheshire, UK) preequilibrated with methanol and water. The column was washed with 60 ml 25% ethanol and then 60 ml IPA.

Centrifuged cells were combined with 100 ml IPA and stirred at room temperature

for 3 to 4 h. The resulting suspension was centrifuged, and the cell extract and purified CFS were assayed by MALDI-TOF mass spectrometry to determine the molecular mass of antimicrobial compounds (Axima TOF^2 MALDI-TOF mass spectrometer; Shimadzu Biotech, Manchester, UK). A MALDI target plate was precoated with a-cyano-4-hydroxycinnamic acid (CHCA) matrix solution, 0.5 µl of the supernatant from the cell extract was then placed on the target, and a final layer of matrix solution was added. Positive-ion linear or reflectron mode was used to detect peptide masses.

Actifensin characterization

Characterization was performed using purified bacteriocin. To test protease susceptibility, 100 µl aliquots of 50 µg ml^{-1} were subjected to treatment with 20 mg ml^{-1} proteinase K (Sigma-Aldrich) and a-chymotrypsin (Sigma-Aldrich) at 37°C for 3 h, followed by a 10-min incubation at 100°C to denature the enzymes. Fifty-microliter aliquots were assayed on *L. delbrueckii* subsp. *bulgaricus* LMG 6901 indicator plates. Heat stability was determined by 30 min incubations at 60°C, 70°C, 80°C, 90°C, and 100°C and by autoclaving at 121°C for 15 min.

For spectrum of activity, a well diffusion assay was carried out as described above with the strains in the appropriate medium. Fifty microliters of purified bacteriocin at a concentration of 50 µg ml^{-1} was added to a well. Following overnight incubation under the appropriate conditions, zones of activity were measured and categorized as no inhibition, weak inhibition (0.5 mm to 2 mm), strong inhibition (2.5 mm to 5 mm), and very strong inhibition (>5 mm). MIC against selected pathogens was assayed as described above, starting at 100 µg ml^{-1} peptide solution and serially diluted 1:2 to 0.78 µg ml^{-1}.

Draft genome sequencing

DNA was extracted using a GenElute bacterial genomic DNA kit (Sigma) and prepared for sequencing using a Nextera XT kit (Illumina) for library preparation. DNA was quantified using a Qubit 2.0 fluorometer. Sequencing was carried out using an Illumina MiSeq platform with paired-end 2 x 300-bp reads by the Teagasc Sequencing Centre, Teagasc Food Research Centre, Moorepark, Fermoy, Ireland. Assembly was performed using tools available on the public server at https://usegalaxy.org (30). Assembly was performed *de novo* using SPADES (version 3.0.0) and resulted in 116 contigs. Contigs were aligned to a reference genome using Mauve (version 20150226, build 10), followed by annotation with RAST (version 2.0). The annotated genome was analyzed for predicted bacteriocin and secondary metabolite production clusters using BAGEL4 (37), and any further annotation was carried out using Artemis genome browser (version 16.0.0).

BAGEL screen and phylogenetic analysis of *Actinomyces* species

GenBank and FASTA assemblies of the genus *Actinomyces* were acquired from the NCBI assembly database and screened using BAGEL4 (37). Where available, corresponding 16S rRNA sequences were acquired from the RDP database (38) and, where unavailable, *Actinomyces* sp. genomes were subject to analysis using RNAmmer (32). 16S rRNA sequences were aligned using MUSCLE (33), and a phylogram was generated using iTOL (39). The phylogram was then overlaid with the BAGEL screen data.

Reverse bacteriocin identification, peptide and structure prediction, and

homology

Two hundred micrograms freeze-dried purified peptide was sent for N-terminal amino acid sequencing (AltaBioscience, UK). The resulting 15-residue sequence, GFGXNLITSNPYQXS, was used to search for a bacteriocin structural gene with Artemis genome browser. Following identification of the structural gene, other genomes were searched for genes homologous to the active propeptide using BLASTp; genes on contigs consisting of less than 5 kbp were excluded. Additional actifensin homologs were identified from the study by Dash et al. (27) among 147 nonredundant bacterial CSaþ peptide sequences (27). Alignments were generated using Clustal Omega (40) and visualized with Jalview (41). Structural modeling was performed using SWISSMODEL (42) online software, and structural images were generated using PyMOL (43).

Data availability

Genomic data analyzed in this study were deposited in GenBank/EMBL under accession number SPKK00000000 and are publicly available from the NCBI database at https://www.ncbi.nlm.nih.gov/.

6.2.5 Results

Identification of a novel bacteriocin-producing *Actinomyces* sp.

Actinomyces ruminicola DPC 7226 was isolated from sheep feces. During an initial screen of >10,000 colonies for bacteriocin producers, this strain was found to produce a large zone of inhibition when overlaid with an acid-tolerant indicator species, *Lactobacillus delbrueckii* subsp. *bulgaricus* LMG 6901 (Figure 1a). The neutralized cell-free supernatant (CFS) was also found to produce a zone of

inhibition against *L. delbrueckii* subsp. *bulgaricus* LMG 6901, indicating production of a soluble antimicrobial molecule (Figure 1b). This activity was eliminated when the supernatant was treated with proteinase K, demonstrating that the antimicrobial is proteinaceous in nature (data not shown).

Antimicrobial activity was purified from pelleted bacterial cells (C18 SPE; reversed phase high-performance liquid chromatography [HPLC]) and CFS (Amberlite XAD16N, C18 SPE; reversed-phase HPLC), and matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) of active peaks detected a mass of 4,091 ± 1 Da (Figure 2a and 2b). The mass was also detected by colony MS (Figure 2c). The activity of the HPLC-purified fraction from CFS was assayed against *L. delbrueckii* subsp. *bulgaricus* LMG 6901 and found to be active at <1 µg ml^{-1} (Figure 2d). The antimicrobial peptide was found to be heat stable, retaining almost all activity after treatment for 30 min at 100°C, but was completely lost after treatment at 121°C for 15 min.

Spectrum of inhibition

A range of indicator organisms was tested against the purified antimicrobial to determine the spectrum of inhibition. The antimicrobial was active against a broad range of genera, with 22 of the 27 strains screened inhibited to various degrees, including species of the genera *Lactococcus*, *Enterococcus*, *Lactobacillus*, *Streptococcus*, *Pediococcus*, *Bacillus*, *Staphylococcus*, other *Actinomyces* spp., and *Clostridium* spp. (Figure 3). No inhibition against the Gram-negative species *Salmonella enterica* or *Escherichia coli* was observed. *Listeria* spp. and *Bacillus* spp. were inhibited weakly or not at all (Figure 3). Inhibition against other *Actinomyces* spp. was found, and activity was particularly strong against *Staphylococcus aureus* and

Clostridium difficile.

MICs were determined against *Enterococcus faecium* APC1031, *E. faecium* NCDO0942, *S. aureus* R693, *Streptococcus agalactiae* APC1055, and *C. difficile* DPC6534 (see Figure S1). Enterococci were inhibited at 3.05 to 6.10 µM. *S. aureus* was inhibited at 3.05 µM. *S. agalactiae* and *C. difficile* were inhibited at 0.76 µM (Figure S1).

Distribution of genes encoding bacteriocins in the genus *Actinomyces*

As the active mass could not be matched to any previously known antimicrobial peptide and no antimicrobial compounds were previously described within the species, the genome of *A. ruminicola* DPC 7226 was sequenced. Following genome annotation, the draft genome was analyzed using BAGEL4 to search for potential antimicrobial-encoding operons. Gene clusters were identified containing putative genes for thiopeptide production (data not shown), but the masses predicted, 2,195.4 Da and 1,152.5 Da, did not correspond with the mass detected in the antimicrobial HPLC fraction.

In conjunction with screening of the genome of *A. ruminicola* DPC 7226, we also set out to characterize the antimicrobial potential of the genus. One hundred and sixty one *Actinomyces* species genomes in various stages of assembly were screened using BAGEL4. The isolates were obtained from humans (78.2%) or other animals (16.1%) or were of unknown origin (4.9%), while one was an environmental isolate (0.6%). One hundred and six areas of interest were revealed in 76 strains, covering 18 species. Ninety areas of interest contained complete operons for antimicrobial production. Twenty-nine were predicted to encode class I bacteriocins, including 7 LanBC modified lantibiotics, 16 LanM modified lantibiotics, 1 single-peptide sactibiotic, 3 lasso

peptides, and 2 thiopeptides. Thirteen operons were predicted to encode class IId bacteriocins, and a further 48 operons were predicted to encode bacteriolysins. A phylogenetic tree was generated from the 16S rRNA sequences of 142 *Actinomyces* genomes with *Bacteroides fragilis* ATCC 25285 as the root and overlaid with operon type and strain source (Figure 4). Bacteriocin production was widely distributed across the *Actinomyces* pangenome, though bacteriolysin production was found exclusively among human isolates (Figure 4)

Genetic and molecular characterization of the actifensin determinant

To identify the gene encoding the 4,091 ± 1 Da peptide within the genome of *A. ruminicola* DPC 7226, pure peptide was subjected to N-terminal sequencing, which revealed a primary sequence consisting of Gly-Phe-Gly-X-Asn-Leu-Ile-Thr-Ser-Asn-Pro-Tyr-Glu-XSer, with blanks at residue positions 4 and 14 denoted as probable cysteines (Figure 5a). This 15-amino-acid sequence was matched to a 69-residue small open reading frame in the draft genome, capable of encoding a 37-amino-acid mature peptide (hereafter referred to as actifensin) with a predicted mass of 4,097.7 Da preceded by a 32-residue leader sequence (Figure 5a).

The genetic locus encoding actifensin is shown in Figure 5b, where *afnA* encodes actifensin. Within an approximately 6.5-kbp upstream region of *afnA*, genes encoding an ABC transporter permease (*afnJ*), an ATP binding ABC transporter (*afnK*), and another ABC transporter permease (*afnL*) were identified as being present. Downstream of *afnA*, three hypothetical genes of unknown function (*afnG* to *afnI*) were found, followed by genes encoding another ATP binding ABC transporter (*afnF*), a predicted α/β hydrolase superfamily protein (*afnE*), another protein of unknown function, a subtilisin-like protease, and a LuxR family

transcription factor (*afnD*, *afnC*, and *afnB*, respectively). Within *afnE* is a predicted RHO-independent transcription terminator, and upstream of the structural gene are four predicted promoters. A putative ribosome binding site was also identified nine base pairs upstream of the ATG start codon for the peptide consisting of a purine rich sequence, 5'-GAAAGG-3' (Figure 5a).

The leaderless structural peptide was found to have a predicted mass of 4,097.7 Da. This mass was approximately 6 Da higher than detected by MALDI-TOF MS. The difference between predicted and observed masses most likely corresponds to the loss of six hydrogen atoms during the formation of disulfide bonds between the six cysteines. Short peptides with numerous disulfides in specific positions are characteristic of the defensin peptide families (4). To confirm the presence of disulfide bonds in actifensin, pure peptide was reduced and alkylated to break open the disulfide bonds and then subjected to trypsin digestion and peptide mass fingerprint analysis by MALDI-TOF MS. Reduction and alkylation of actifensin resulted in a 4,440-Da mass, which correlates with the expected increase in mass of 58 Da for each cysteine. MALDI-TOF MS analysis of the subsequent trypsin digest detected a mass of 2,257.02 Da, which corresponds to the first 19 amino acids of the peptide (Gly-1 to Lys-19) containing three alkylated cysteine residues. Three other predicted masses for Ser-20 to Arg-24, Gly-25 to Arg-31, and Thr-32 to Tyr-37 (predicted and alkylated masses of 581.30 Da, 584.25 Da, and 803.31 Da, respectively) were not detected.

Discovery of actifensin homologs

BLASTp analysis with AfnA found homologous open reading frames (ORFs) within the fungal genera *Blastomyces*, *Emmonsia*, and *Emergomyces*, *Helicocarpus griseus*,

and a defensin from the mollusk species *Ruditapes philippinarum* (58%, 58%, 55%, 52%, and 61% identity, respectively) (see Figure S2). Characteristic conserved cysteines were noted, though low sequence identity was observed between the mature actifensin peptide and eukaryotic defensins. The same was found when AfnA was compared with known previously characterized arthropod, ascomycete, and mollusk defensins (Figure 6a) with conserved secondary structures (Figure 6b). BLASTp analysis using the 69-residue AfnA sequence identified 37 homologous structural genes within the genus *Actinomyces* and one homolog from a *Corynebacterium* sp. sequence (Figure 7a). Further analysis indicated that the homologs were present in 15 operons from 14 strains, in addition to conserved genes for transport, transcription regulation, and proteolytic activity (Figure 7b). *Actinomyces* sp. strain 2119, *Actinomyces oris* S64C, *Actinomyces succiniciruminis* AM4, *A. oris* CCUG34286, *Actinomyces* sp. strain F0337, *Actinomyces* sp. strain HMSC075C01, and *A. oris* MMRCO6-1 had at least two actifensin homologs, while *Actinomyces* sp. F0337 contained an operon with seven copies, the most observed within one genome (Figure 7b). The genome of *A. oris* MMRCO6-1 contained six encoded actifensin homologs detectable over two contigs, but only one (contig 50) contained the other conserved ORFs (*afnB-I* and *afnJ-K*) present in the actifensin operon. Twelve of 14 operons had a highly conserved arrangement of *afnB-I*, all of which also had ABC transporter genes directly upstream of the bacteriocin ORF. The mean amino acid identity between all structural genes was 52%. The highest identity observed between actifensin and a homolog was 77% identity with *afnA* in *Actinomyces* sp. strain CTC72, though higher identities were observed between other peptides (see Figure S3). We proceeded to characterize ten predicted cysteine-stabilized aþ (CSaþ) peptides predicted by Dash et al. (27). The peptides are present

in five *Actinomyces* genomes bringing the total number of peptides to 47 homologous structural genes in 19 strains. *Actinomyces oris* S24V, *Actinomyces denticolens* PA, *Actinomyces* sp. strain Chiba-101, *Actinomyces johnsonii* F0542, and *Actinomyces* sp. strain F0330 have genes which were not identified using BLASTp and the actifensin propeptide sequence (27). Strains S24V, PA, and Chiba-101 display the conserved *afnB* to *afnI* ORFs following *afnA*, which are absent in strains F0330 and F0542 (Figure 7b).

The propeptide contains a conserved G-X-E motif prior to the start of the mature peptide (Figure. 7a). In 36 of the peptides, an alanine residue is present after the glycine, which may be involved in secretion and cleavage. This putative GA cleavage signal is replaced by a TS motif in 8 of the 49 peptides (*A. oris* S64C *afnA5*, *A. oris* CCUG34286 *afnA7*, *A. oris* MMRCO6-1 contig 75 *afnA2*, *Actinomyces* sp. F0337 *afnA4*, *Actinomyces* sp. HMSC075C01 *afnA4*, *A. oris* MMRCO6-1 contig 50 *afnA4* and *afnA3*, and *A. oris* S24V *afnA5*). A conserved Pro residue was noted following the first conserved Cys in addition to a conserved G-Y-X-G-G-X-C sequence at positions 56 to 62 of the propeptide (22 to 28 in the active peptide) (Figure 7a).

6.2.6 Discussion

We describe a novel group of bacteriocins with broad-spectrum inhibitory activity within the *Actinomyces* genus. Actifensin is the first such bacteriocin to be discovered, which is produced by a strain of *Actinomyces ruminicola*.

Actifensin inhibited a broad range of Gram-positive species, including notable pathogens such as vancomycin-resistant *Enterococcus* and methicillin-resistant *Staphylococcus*. Given the global challenge of the increase in antibiotic resistance, there is an urgent need for new classes of antimicrobials. Bacteriocins have been

suggested as an alternative to conventional antibiotics due to their effectiveness at low concentrations and their potential to be genetically modified (2). Class II bacteriocins are diverse in sequence and structure whose mechanism of action is through interaction with the cell membrane, causing permeabilization and pore formation and dissipating the membrane potential (3). The defensin-like bacteriocin laterosporulin10 has been found to act on the cell membrane of *S. aureus* Mtb H37Rv, disrupting cellular homeostasis (6). Plectasin and eurocin, fungal C6 defensins, are known to bind lipid II, inhibiting bacterial cell wall biosynthesis (44, 45). Actifensin possesses an N-terminal loop extension which, in other defensin peptides, has been implicated in membrane disruptive capability (31). The loop consists of nine residues between Cys-4 and Cys-14 beginning with an Asn. In most of the other peptide sequences identified, the N loop is six residues long, beginning with a Pro (except in AfnA from *Actinomyces* sp. strain F0588 or *A. naeslundii* S44D, which has an eight-residue N loop with a serine or arginine in the first position, respectively, followed by a Pro) (Figure 7a).

Actifensin also inhibited the growth of *C. difficile* and *Clostridium sporogenes*. Clostridia are known colonizers of the rumen and, as *A. ruminicola* DPC7226 was isolated from the feces of a ruminant, actifensin production may provide a competitive advantage in the gut microbiome. *Actinomyces neuii* and *Actinomyces radingae* were both inhibited by actifensin; however, it would be interesting to see if cross-resistance between actifensin and other actifensin-like producers exists.

A pangenus *in silico* screen revealed that the genus *Actinomyces* (Figure 4) is a rich source of antimicrobials and has genes for bacteriolysin and lantibiotic production (48/90 and 29/90 operons, respectively). Thirteen class II bacteriocins were predicted by BAGEL, but neither the actifensin operon nor its homologs were detected due

to lack of similarity with known systems. One previous study described odontolycin, a bacteriocin produced by an *Actinomyces odontolyticus* dental plaque isolate, though no further research on the peptide was reported (34). Interestingly, in our study, no operons for bacteriocin production were found among five *A. odontolyticus* genomes screened (Figure 4).

The actifensin structural gene encodes a 37-amino-acid mature peptide preceded by a 32-amino-acid leader sequence (Figure 5). A GA motif at positions -3 and -2 was identified, which is a known cleavage signal used in ABC transporter-mediated secretion (36). Indeed, there are a number of predicted ABC transporter genes within the actifensin operon. ABC transporter genes could also play a role in self-immunity to the actifensin peptide. Unusually, an additional glutamic acid residue is present at position-1 before the mature peptide. As the purified peptide was subjected to N-terminal sequencing, we can be certain that the mature peptide begins with a glycine residue. Therefore, the additional glutamic acid residue at position-1 is most likely subject to exopeptidase cleavage prior to activity, and indeed, there are genes present with predicted protease activities (Figure 5).

The GA cleavage motif is present in 36 of the homolog structural genes, with TS replacing the motif in eight instances, GT and GG in two cases, and GS, SA, and DA in one each (Figure 7a). A double glycine is the most commonly found motif for ABC transporter-mediated cleavage among bacteriocins, though GA and GS have also been observed (36). It will be interesting to see if the peptides bearing other residues at this location are indeed subject to ABC-mediated transport. We note that each operon containing a gene with a nontraditional TS/GT/SA/DA signal contains at least one more structural gene than those with a GG/GA sequence. This could indicate potential diversification of a repertoire of bacteriocins enabling improved ability to combat

multiple competitors. It was also surprising that an actifensin homolog was found in a distantly related *Corynebacterium* sp., though many of the conserved genes in the *Actinomyces* sp. operons were not present (Figure 7b). As such, this may be nonfunctional, as ABC transporter-related genes are missing upstream of the structural gene and the conserved *afnB* to *afnI* pattern is absent. The genera *Corynebacterium* and *Actinomyces* are distantly related members within the phylum *Actinobacteria*, and some species are known members of plaque biofilms, providing an opportunity for horizontal gene transfer (16). However, given the dissimilarity of the operons, they may have been acquired independently at some stage.

As stated above, the laterosporulins produced by *Brevibacillus* spp. are two structurally defensin-like bacteriocins with broad-spectrum inhibitory activity (5, 6). Their amino acid sequences are 57.6% similar, which is comparable to that for actifensin and its predicted homologs, but share the conserved cysteine residues which form disulfide bridges. Conserved disulfides are characteristic of defensins and are present in vertebrate, invertebrate, plant, fungal defensins, and defensin-like peptides (4). Actifensin has a predicted mass of 4,097.7 Da, but the actual mass is 4,091 ± 1 Da by MALDI-TOF MS. The same discrepancy in predicted and observed masses was noted with laterosporulin, where six hydrogen atoms are lost in the formation of disulfide bonds. We hypothesize that bonds in actifensin likely form in the 1-4, 2-5, and 3-6 formations, similar to that in ascomycete and arthropod C6 defensins (Figure 6), as the amino acid motifs (C-$X_{5–12}$-C-X_3-C-$X_{9–10}$-C-$X_{4–5}$-C-X-C) are conserved (5). The structure of laterosporulin10 has been determined to be architecturally similar to human a-defensin, though its disulfide connectivity is homologous to that of β-defensins (Figure 8) (6). The overall architecture and disulfide connectivity of actifensin are likely to be homologous to those of C6 defensins, consisting of an N-terminal α-

helix followed by a two-stranded antiparallel β-sheet stabilized by disulfide bridges (Figure 8). Interestingly, an actifensin homolog we identify as AfnA from *Actinomyces* sp. oral taxon 171 strain F0337 has had its three-dimensional (3D) structure determined and is publicly available under PDB accession number 2RU0. The peptide labeled actinomycesin is strikingly similar to C6 fungal and arthropod defensins, which have also been characterized (Figure 6); however, no published material is available regarding its activity, antimicrobial or otherwise. Indeed, two antiparallel beta sheets stabilized by disulfide bonds with an interposed short turn region, previously described as the μ-core motif, are a ubiquitous feature of antimicrobial peptides (35). Actifensin exhibits the highly conserved GXC (positions 26 to 28 in the mature peptide) as do all of its homologs.

CSaβ peptides comprise one of the most widespread families of defensins and defensin-like peptides. A recent publication identified a number of CSaβ sequences in bacterial genomes with potential for antimicrobial, toxin, or signaling activity (27). Of 58 peptides identified within the phylum *Actinobacteria* by Dash et al. (27), 34 were of the genus *Actinomyces*, 24 of which we identified using BLAST with the actifensin propeptide sequence (see Table S2 in the supplemental material). A further 113 bacterial peptide sequences identified by Dash et al. (27) remain to be characterized from a functional perspective and may be a potent source for antimicrobials. Interestingly, a bacterial defensin-like peptide, AdDLP, identified *in silico* was synthesized and recombinantly expressed, and the peptide was found to have anti-*Plasmodium* activity (28). The bacterial CSaþ peptides may be an untapped source of potential applications and have been proposed as the ancestral evolutionary origin of eukaryotic defensins (29).

In the search for novel antimicrobials for application in health and food, genomic and pangenomic approaches are becoming increasingly common (25, 26). These approaches are advantageous in that large amounts of genetic data can be analyzed to identify novel antimicrobials/bacteriocins and can even allow one to "reincarnate" otherwise "dormant" genes (46). However, such analyses are dependent on the ability of programs to predict based on databases of previously identified sequences, and so peptides with novel structures and operons may not be detected. Though a number of bacteriocin operons were found in the *Actinomyces* spp. genomes using BAGEL, actifensin was not identified by genome sequence alone, which highlights the importance of functional screening for antimicrobial compounds in addition to *in silico* screening. By using BLAST, 37 structural genes with homology to actifensin were found in *Actinomyces* spp. along with a single structural gene from a *Corynebacterium* sp. As some CSaβ peptides function as toxins, future applications will require any potential cytotoxic effects to be assayed. We propose that actifensins and the laterosporulins may constitute a new subgroup of class II bacteriocins: the defensin-like bacteriocins. These bacteriocins share only moderate identity to each other but contain highly conserved cysteine residues and are structurally related to eukaryotic defensins.

6.2.7 Conclusion

A series of novel defensin-like bacteriocins within the genus *Actinomyces* were identified using an *in silico* pangenomic approach coupled with a functional screen. The bacteriocins represent a potential new class of antimicrobial peptides, defensin-like bacteriocins, which may have widespread applications as antimicrobials in food and human health.

6.2.8 Acknowledgments

We thank Daragh Hill for technical assistance during the screen for bacteriocin-producing isolates. This work was supported by funding from JPI Food Processing for Health Longlife Project and Science Foundation Ireland (SFI) under grant number SFI/12/RC/2273 in APC Microbiome Ireland.

Figure 1 Antimicrobial activity of *Actinomyces ruminicola* DPC 7226 from colonies overlaid with *L. delbrueckii* subsp. *bulgaricus* LMG 6901 in sloppy MRS (a) and in well diffusion with neutralized CFS (b).

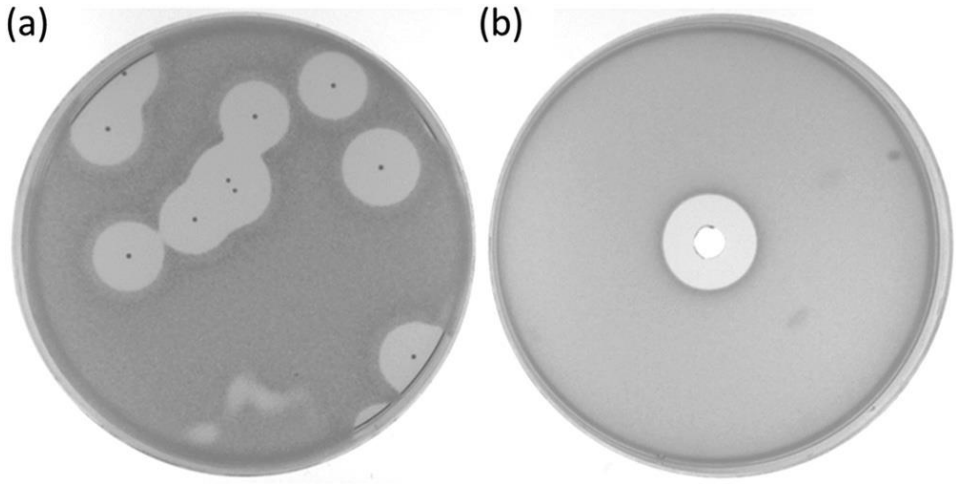


Figure 2 Detection of actifensin 4,091 Da ± 1 Da (indicated by arrows) by MALDI-TOF MS from cell-free supernatant (a), cell extract (b), and colonies on a plate (c). (d) The 4,091 (±1)-Da compound when purified was active to <1 µg ml^{-1}; indicator, *L. bulgaricus* LMG 6901.

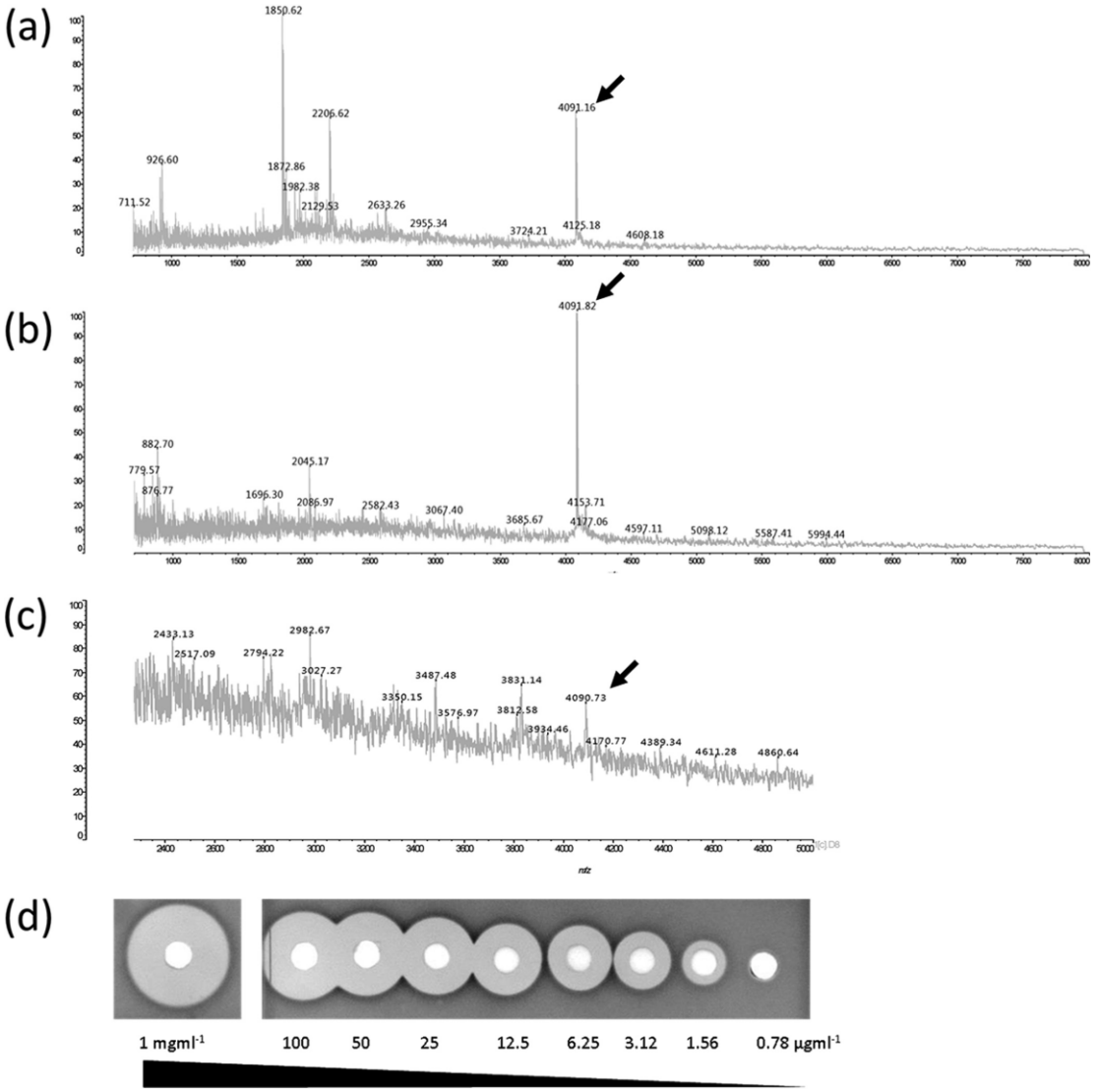


Figure 3 Inhibition of actifensin against a broad-spectrum of indicator species. Weak inhibition, 0.5- to 3-mm zone; strong inhibition, 3- to 5-mm zone; very strong inhibition, >5-mm zone. VRE, vancomycin-resistant *Enterococcus*; MRSA, methicillin-resistant *Staphylococcus aureus*.

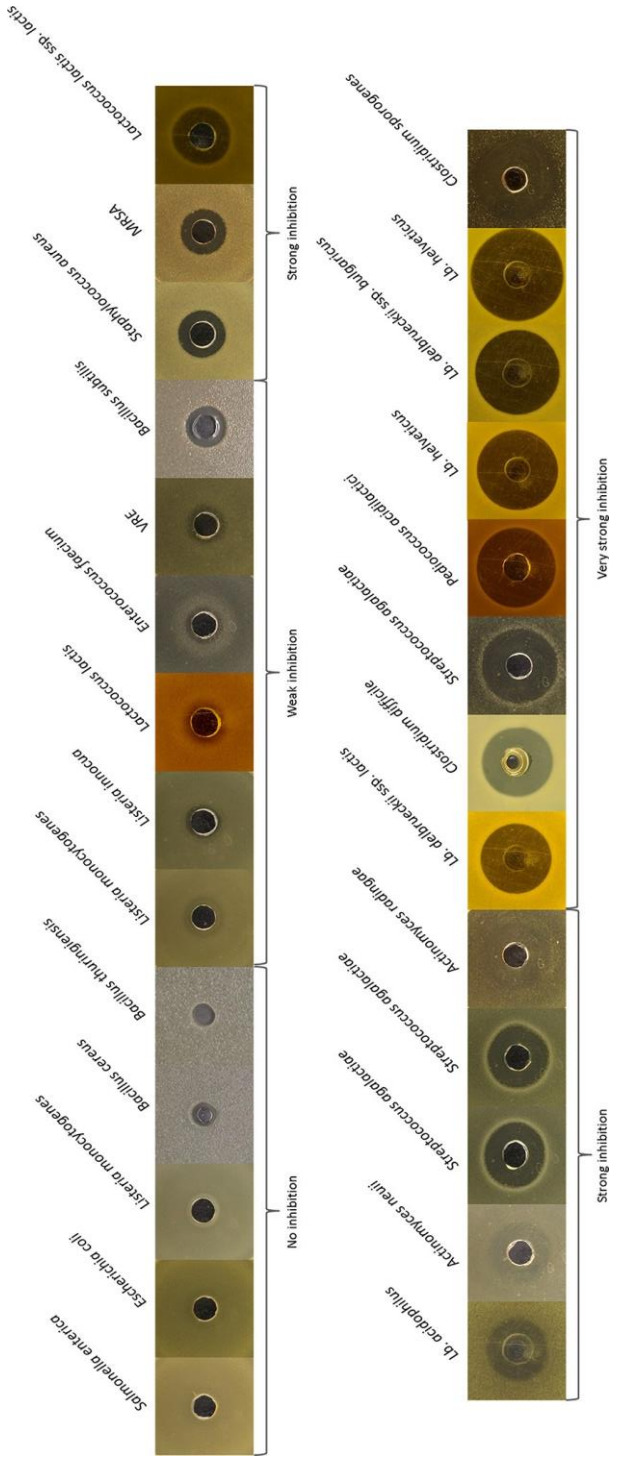


Figure 4 Phylogram of *Actinomyces* genomes using 16S sequences overlaid with BAGEL4 predictions, strain source, and presence of actifensin or predicted homolog operon.

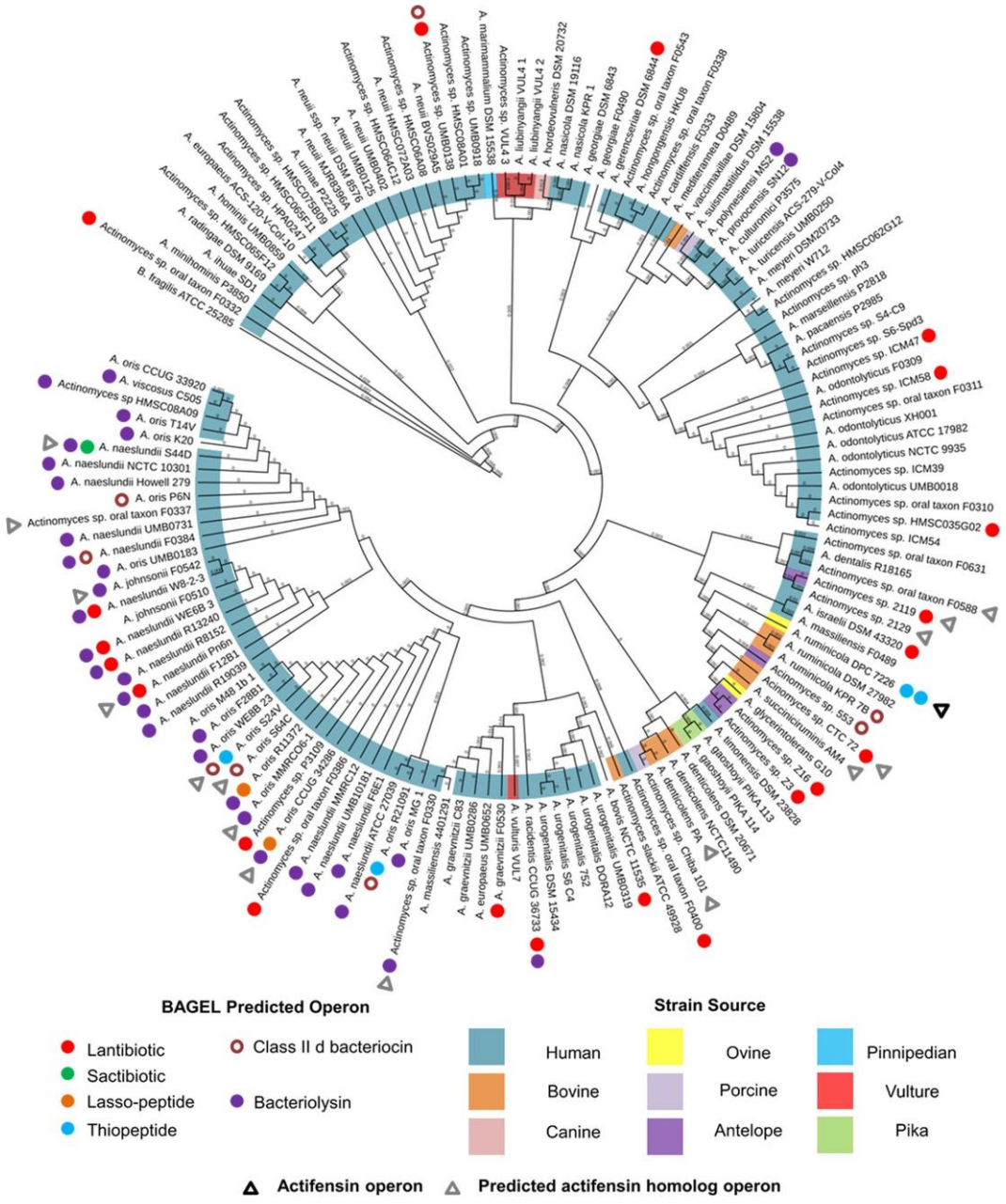


Figure 5 (a) Sixty-nine-residue propeptide identified following genome analysis using the 15-amino-acid sequence (underlined) determined by N-terminal amino acid sequencing. RBS, putative ribosome binding site highlighted 8 bp upstream of the start codon. (b) Genetic vicinity of structural gene containing nearby genes for transport, hypothetical and proteolytic proteins, and a transcription factor.

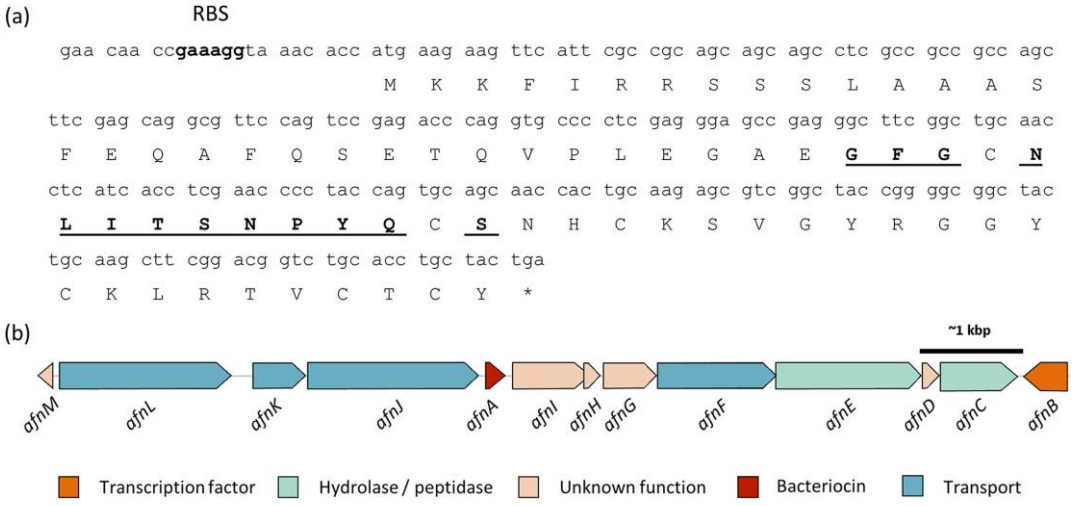


Figure 6 (a) Mature peptide sequence alignment of AfnA with characterized defensin family peptides from different phyla. Known disulfide connectivities are outlined above highlighted cysteine residues. (b) Available 3D structures of sequences in panel a. Alpha helices are colored red, and beta sheets are shown in blue. Protein data bank accession numbers shown below the structures (in parentheses).

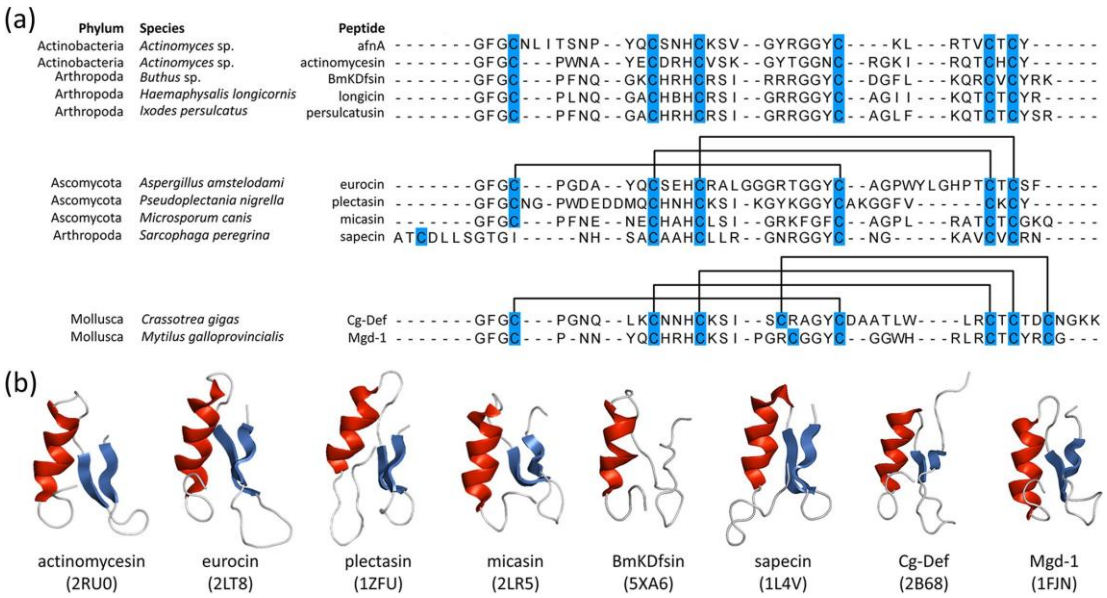


Figure 7 (a) Sequence aligment of actifensin propeptide sequence (boxed) with structural genes predicted for *Actinomyces* sp. peptides. Amino acids with greater than 80% conservation are colored, and leader sequences and mature active peptides are indicated at the top. Putative disulfide connectivity between conserved cysteines of the mature peptide is indicated at the bottom right, and putative cleavage sites are indicated at the bottom center. (b) Diagrams of actifensin homolog production operons. Multiple bacteriocin genes within one operon are denoted *afnA1* to *afnA7* where present.

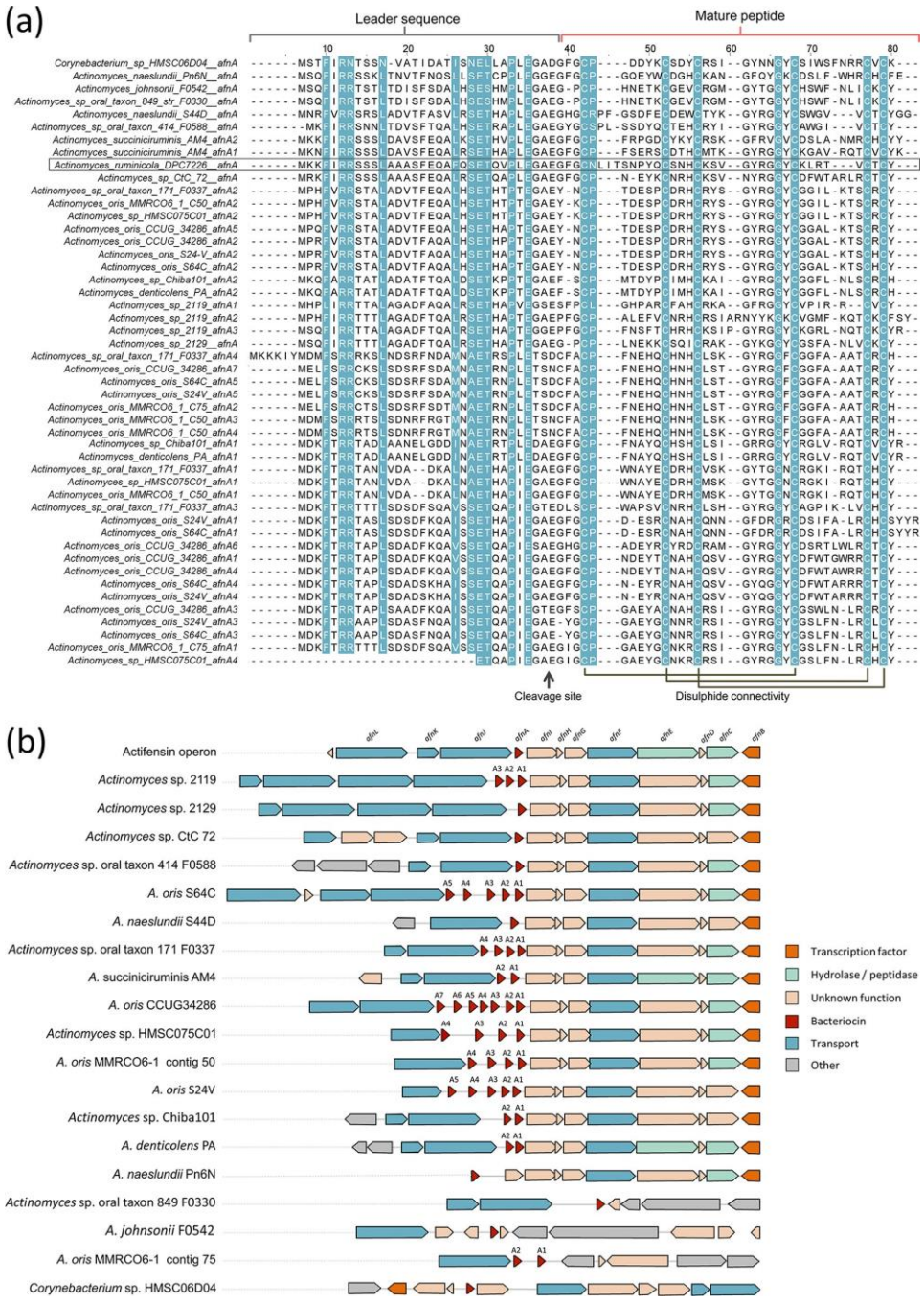
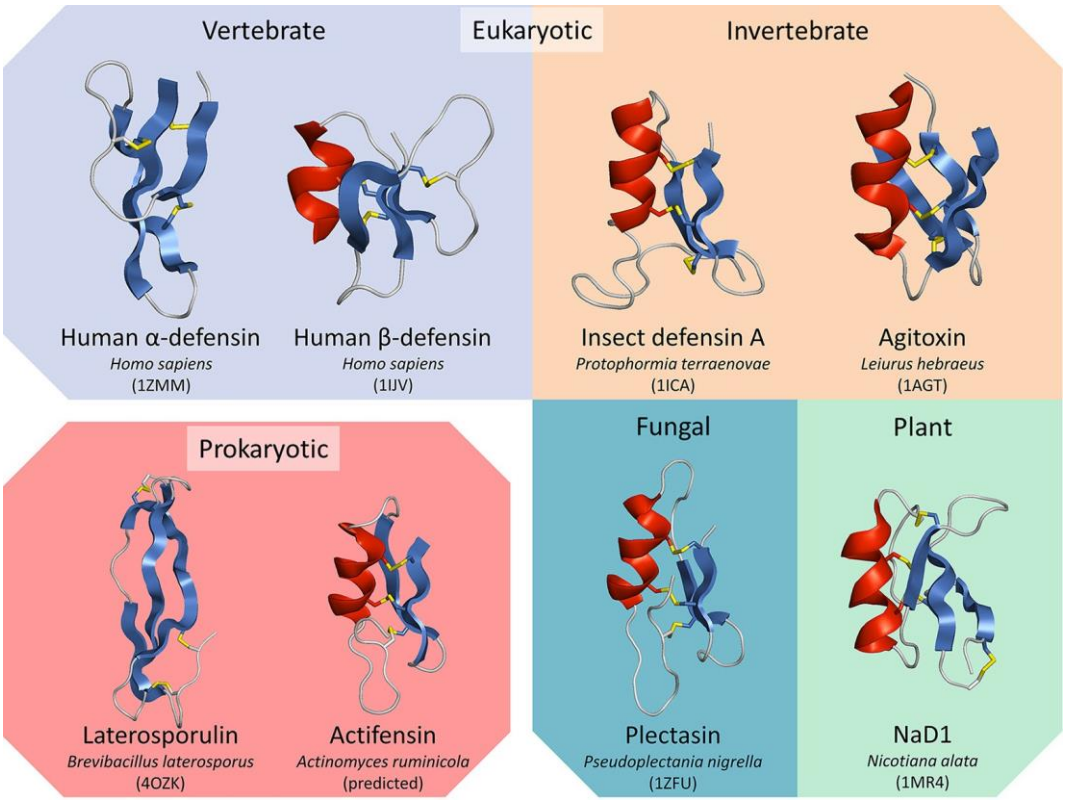


Figure 8 Conserved structures of the defensin peptide superfamily and defensin-like bacteriocins, laterosporulin and actifensin. β sheets are colored blue, α helices are colored red, and disulfide bonds are shown in yellow.

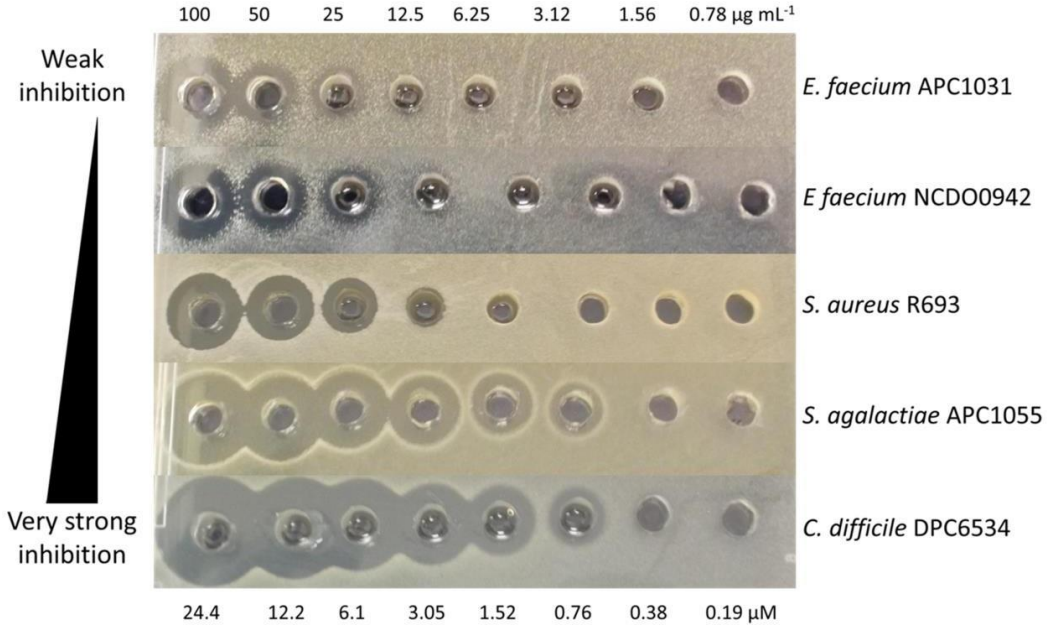


Suplemental material with the exception of Table S3 and Figure S1 are available online at https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6989792/

Table S3 Bacterial strains and culture conditions.

Indicator Species+B2:D23	**Growth Medium**	**Incubation Conditions**
Actinomyces neuii LMG 19524t	BHI	37°C, O_2^-
Actinomyces radingae LMG 15960t	BHI	37°C, O_2^-
Bacillus cereus NCIMB700577	BHI	37°C, O_2^+
Bacillus subtilus S249	BHI	37°C, O_2^+
Bacillus thuringiensis DPC643*1*	BHI	37°C, O_2^+
Clostridium difficile DPC6534	RCM	37°C, O_2^-
Clostridium sporogenes LMG10143	RCM	37°C, O_2^-
Enterococcus faecium APC1031	TSY	37°C, O_2^-
Enterococcus faecium NCDO942	TSY	37°C, O_2^-
Escherichia coli DPC6054	BHI	37°C, O_2^+
Lactobacillus acidophilus DPC5377	MRS	37°C, O_2^-
Lactobacillus delbrueckii ssp. *bulgaricus* LMG6901	MRS	37°C, O_2^-
Lactobacillus delbrueckii ssp. *lactis* DPC5387	MRS	37°C, O_2^-
Lactobacillus helveticus DPC5353	MRS	37°C, O_2^-
Lactobacillus helveticus DPC5385	MRS	37°C, O_2^-
Lactococcus lactis ATCC11454	GM17	30°C, O_2^+
Lactococcus lactis ssp. lactis DPC3147	GM17	30°C, O_2^+
Listeria innocua DPC1768	BHI	37°C, O_2^+
Listeria monocytogenes DPC3572	BHI	37°C, O_2^+
Listeria monocytogenes DPC6893	BHI	37°C, O_2^+
Pediococcus acidilactici LMG2351	MRS	30°C, O_2^+
Salmonella enterica ser. Typhimurium DPC6046	BHI	37°C, O_2^+
Staphylococcus aureus DPC5645	BHI	37°C, O_2^+
Staphylococcus aureus R963	BHI	37°C, O_2^+
Streptococcus agalactiae APC1055	BHI	37°C, O_2^+

Figure S1 Minimum inhibitory concentration of actifensin peptide against Gram-positive pathogens determined by well diffusion assay.



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Conclusions

Bacteriocin research has expanded greatly over the last decade, from an explosion in the number of newly discovered novel bacteriocins with potential as antimicrobial alternatives, to their use as gut microbiota modulators and signal molecules that play a role in the well-being of humans and animals. As bacteriocins are such a heterogeneous group of molecules with regard to size, charge, hydrophobicity, specificity and mode of action, they have potential for development as bespoke molecules to target specific microbial challenges for the food, pharma and veterinary industry. Indeed, it is envisaged that bacteriocins will be developed as a molecular arsenal produced by live bio-therapeutic strains to control food spoilage/pathogenic microorganisms, to reduce AMR with a particular emphasis on multidrug-resistant clinical pathogens and as microbiome modulators to fight disease and promote well-being. For example, narrow-spectrum bacteriocins, such as Thuricin CD which is potent against *Clostridioides difficle* (1), have considerable potential as vancomycin alternatives in some instances, as they target specific pathogens without inducing substantial collateral damage to the microbiome (2).

To date, bacteriocins have been discovered from every conceivable niche including fermented foods and beverages, animal gastrointestinal (GI) tract, human GI tract from all ages and states of health, human skin, soil and aquaculture. Indeed the bacteriocins described herein were isolated from a variety of habitats including nisin H from the porcine intestine (3), nisin J from human skin (4), nisin P: a human faecal isolate (5); bactofencin A: a porcine faecal isolate (6); formicin: a mackerel intestine isolate (7) and actifensin: a sheep faecal isolate (8). Interestingly, all were isolated initially from screening using solid media followed by DNA sequencing while peptide purification and MALDI TOF mass spectrometry were used to confirm the identity of the

bacteriocin. In contrast, novel bacteriocins produced by *Lactobacillus* spp. (9) and *Actinomyces* spp. were discovered using an *in silico* approach (8). It is generally accepted using that both approaches in parallel will be more successful, given that bacteriocin gene clusters could be "switched off" or incomplete, or that the bacteriocin produced may not kill any given indicator used in wetlab experiments. Specifically, traditional plate assays may miss bacteriocin producers due to incorrect media selection to support its growth or incorrect microbial target selection, while genome mining will detect bacteriocin gene clusters but does not establish if the bacteriocin is being produced.

The prototypical bacteriocin, Nisin A (E234), produced by *Lactococcus lactis,* is a lantibiotic approved by regulatory agencies for use as a commercial food preservative and has been used by the food industry for over 65 years. Nisin A, is the first described natural nisin produced by *L. lactis* (10) and is now one of 15 natural nisin variants from a wide variety of organisms (*L. lactis* spp., *Streptococcus* spp., *Staphylococcus capitis*, *Blautia* spp. and *Apilactibacillus*) and habitats (catfish GI tract, porcine and human GI tract, pig GI tract, a river, human skin, bee), making nisin production a ubiquitous trait in many genera and many habitats.

In addition to the diversity in the range of producing organisms and habitats, there is considerable heterogeneity within the structural gene in naturally-occurring nisin variants with 1-14 amino acid changes from the prototypical nisin A, for example: five for nisin H **(Chapter 2.1)**, eight for nisin J **(Chapter 2.2)** and ten for nisin P (**Chapter 3.1**). Furthermore, there are interesting differences within the nisin gene clusters with nisin A encoded by eleven genes whereas H lacks a *nisI*, equivalent, nisin J lacks *nisI* and *nisRK* equivalents (differences also common to the newest nisin variant kunkecin A 11) while nisin P contains the full complement of eleven genes, though in a different

gene order. The widespread abundance of nisin and its variants in nature suggest that production of the bacteriocin is a very useful trait for bacteria to have in different microbiome niches, either for reasons of competition or communication. Moreover, the fact that the nisin gene cluster can often be found on mobile gene clusters (transposon for nisA and plasmid for nisin J) suggests that horizontal gene transfer plays a significant role in dissemination of nisin genes in different environments. Interestingly, of the fifteen natural nisin variants reported to date, Teagasc/APC researchers discovered and characterised three with two more, currently unreported, natural nisin variants in the pipeline.

Following discovery and identification, the emphasis turns to increasing their potential. Their gene-encoded nature makes bacteriocins, and lantibiotics in particular, amenable to bioengineering, resulting in "designer bacteriocins" possibly with improved physiochemical characteristics, including increased specificity and potency (12, 13). Indeed, using this approach the contribution that every amino acid residue makes to nisin A bioactivity has been examined by individually changing it to the nineteen other possible amino acids. In addition to increasing the understanding of the significance of each residue, post-translational modification and internal ring structure, this work has resulted in the availability of an arsenal of nisin variants for assessment against particular targets, thereby expanding the variety and scope of the application of nisin (14). Notable successes include nisin variants with increased activity against Gram-negative (15) and both food (16) and clinically (17, 18) significant Gram-positive pathogens and variants that can overcome nisin resistance mechanisms (19).

As the natural nisin variants, H, J and P, are produced by non GRAS strains, they are consequently of limited use for food or medical applications. Therefore, it was decided to improve their commercial potential **(Chapter 3)** by expressing the bacteriocins in a

Lactococcus lactis GRAS strain. This was achieved by fusing the Nisin A promotor and nisin A leader sequence to their respective structural genes, and using the nisin A biosynthetic machinery to produce the peptide. Successful production of the fully post-translationally modified nisin variants confirmed the ability of the nisin A gene cluster, to produce nisin variants from genetically different backgrounds. While activity was low there is potential for improvement. The nisin A gene cluster (*nisABTCIPRKFEG*) is encoded on a transposon (20) and consists of four operons under the control of an inducible promotor in the case of *nisABTC* and three constitutive promotors for *nisIP*, *nisRK*, *nisFEG* (21). As the percentage identities differ so much between nisin A *nisBTC*, *nisP*, *nisRK* and *nisFEG* and nisin H, J and P equivalents, it would be interesting to clone the entire gene cluster from nisin H, J and P into *L. lactis*, as a first step, to improve production. If this was unsuccessful, it may be worth attempting to clone the nisin variant structural gene on *nisBTC* with, *nisI, nisP, nisRK* and *nisFEG* equivalents from the producer and assess their relevance for production as it is becoming apparent that the nisin encoding gene clusters differ with regard to gene content. It also would be interesting to mix and match the nisin biosynthetic genes from different backgrounds to see if improved generic gene clusters could be generated which are capable of producing significant quantities of peptide regardless of background. Reiners et al (2020) recently reported the cloning of the nisin H structural gene into a lactococcal background and, while their attempt was successful, they did find that *nisP* cleaves nisin H inefficiently due to the presence of a phenylalanine residue at position 1. They overcame this hurdle by changing this phenylalanine to isoleucine, the first amino acid residue of nisin A, and found that the peptide was then cleaved efficiently (22). Overall, bioengineering is a powerful tool used to improve functionality of and add value to bacteriocins.

Structure-Activity relationships can be used to predict the effect that chemical/physical structure has on biological activity. Alanine scanning, where each amino acid is sequentially changed to alanine, has been successfully used to identify the importance of particular amino acid residues to activity. This can be either achieved genetically, as described for lacticin 3147 (23) and durancin GL (24), or by peptide synthesis, as for Class II bacteriocins, and as described in **Chapter 4.1** for bactofencin A. Crystal structure and site directed mutagenesis were used to show that cationic and aromatic residues were responsible for bioactivity of the broad spectrum, circular bacteriocin, plantacyclin B21AG (25). NMR has also been used to determine bacteriocin structure and provide insightful information into functionality. In the case of the Class IIb, two peptide plantaricin S, NMR and site directed mutagenesis, were used to show that the peptides formed an α helix between amino residues 7-24 and that the GxxxG motif was important for activity (26). Bactofencin A is structurally noteworthy due to the exceptionally positively charged N terminal (+7), and its C terminal loop resulting from the presence of a disulphide bond between Cys7 and Cys22. A series of peptide synthesis variants revealed that these three features are essential for full activity of this unusual bacteriocin.

To achieve the full potential of any bacteriocin in practical applications, it is desirable to decipher their activity, spectrum of inhibition, production and mode of action. In **Chapter 5.1**, bactofencin A displayed delayed killing against *S. aureus* DPC5246, suggesting a mode of action that acts predominantly through cell wall inhibition. Combining bactofencin A with the pore forming nisin A resulted in faster killing at lower bacteriocin concentrations, most likely due to the combination of bacteriocins with different modes of action. This is known as a "hurdle approach" or antimicrobial combinatorial therapy and is commonly used to reduce the quantity of bacteriocin

required for efficacy, making a more cost effective treatment with lower toxicity potential (27). This combinatorial approach has been particularly effective in the case of nisin, which has been successfully combined with a range of antimicrobials including essential oils (28), antibiotics (29) and other bacteriocins (30). A combinatorial approach is also used to reduce the incidence of AMR and, in this instance, resistance to bactofencin A did not occur at low bactofencin A concentrations. Overall, the effectiveness of bactofencin A to treat *S. aureus* DPC5246 was significantly improved when combined with nisin A and this interesting result warrants further investigation to discover the mechanisms underpinning this synergy. Future studies may also include assessing the effectiveness of bactofencin A/nisin A combinations against other *S. aureus* strains including MRSA and other genera such as *Listeria monocytogenes,* while the interaction between bactofencin A and other lantibiotics such as lacticin 3147 against different targets is also worth investigating.

The search for new, novel bacteriocins is as relevant today as it was at the beginning of bacteriocin research. Mining studies from all conceivable habitats are ongoing and these endeavours are very successful as all bacteriocin classes have been expanded significantly and new subclasses discovered since the commencement of this thesis. Indeed, the work described herein contributes significantly to novel bacteriocin discovery as, in addition to the novel lantibiotics, nisin H, nisin J and nisin P described in **Chapters 2.1, 2.2** and **3.1**, **Chapter 6.1** describes formicin, a novel two peptide lantibiotic and actifensin, a potential new subclass of antimicrobial. Since the discovery of formicin, the most recent addition to the two component lantibiotics is roseocin, discovered through *in silico* mining and described as the first two-component lantibiotic in an actinomycete, *Streptomyces roseosporus* NRRL 113789 (31). The peptides were heterologously expressed in *Escherichia coli* and found to act

synergistically when plated in close proximity. The peptides are less similar to those previously described, with the alpha peptide likely to contain a different ring structure. The actifensins **(Chapter 6.2)** are broad-spectrum single peptide bacteriocins, produced another by *Actinomyces* genera represent an exciting new type of bacteriocin due to the presence of multiple disulphide bonds potentially giving the bacteriocins enhanced stability. As these show a high redundancy in sequence and lack post-translational modifications, there is potential to assess their efficacy by cloning the structural genes into a host capable of expressing them at high concentrations thus allowing selection of the most effective sequences for further development.

The ultimate goal for bacteriocin research is the development of bacteriocins into viable antimicrobial products that are available for commercial use. Currently, the number and range of bacteriocin sequences, and our increased understanding of how best to use them, provide a good foundation for development of antimicrobial products available for uptake by industry. However, commercialisation is challenging as bacteriocins are often produced in low amounts, are peptide based and therefore susceptible to digestive enzymes and they require complex media for production making them expensive to produce. Another difficulty associated with commercialisation is the scarcity of information on safety and toxicity required for regulatory approval that can be a time consuming, lengthy process (32). However, these are issues that can be overcome once industry commits to invest in these fascinating molecules that have such potential as antimicrobials and microbiome modulators. It is hoped that the bacteriocins described in this thesis will be developed for commercial use one day.

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