Discovery of a novel lantibiotic nisin O from *Blautia obeum* A2-162, isolated from the human gastrointestinal tract

Diane Hatziioanou[1†], Cristina Gherghisan-Filip[1], Gerhard Saalbach[2], Nikki Horn[1], Udo Wegmann[1], Sylvia H. Duncan[3], Harry J. Flint[3], Melinda J. Mayer[1*], Arjan Narbad[1].

[1]Gut Health and Food Safety Institute Strategic Programme, Institute of Food Research, Colney, Norwich, NR4 7UA, UK

[2]John Innes Centre, Norwich Research Park, Norwich, NR4 7UA, UK

[3]Gut Health Group, Rowett Institute, University of Aberdeen, Foresterhill, Aberdeen, UK

[†]present address: Oxford Biomedica (UK) Ltd Windrush Court, Transport Way, Oxford OX46LT, UK

*****For correspondence: Email: melinda.mayer@ifr.ac.uk; Tel. +44 1603 255284

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Abbreviations NCTC, National Collection of Type Cultures; OD, optical density; SEM, scanning electron microscopy; SSC, saline sodium citrate; TCA, trichloroacetic acid; TEM, transmission electron microscopy.

Abstract

A novel *lanC*-like sequence was identified from the dominant human gut bacterium *Blautia obeum* strain A2-162. This sequence was extended to reveal putative biosynthetic and transport genes, two sets of regulatory genes, immunity genes, three copies of a nisin–like *lanA* gene (*nsoA1, 2, 3*) with an unusual leader peptide and a fourth putative *lanA* gene (*nsoA4*). Comparison with other nisin clusters showed the closest relationship to nisin U.

B. obeum A2-162 demonstrated antimicrobial activity against *Clostridium perfringens* when grown on solid media in the presence of trypsin. Fusions of the predicted *nsoA* structural sequences with the nisin A leader were expressed in *Lactococcus lactis* containing the nisin A cluster without *nisA*. Expression of the *nisA* leader sequence fused to the predicted structural *nsoA1* produced a growth defect in *L. lactis* which was dependent upon the presence of biosynthetic genes, but failed to produce antimicrobial activity. Insertion of the *nso* cluster into *L. lactis* MG1614 gave an increased immunity to nisin A but this was not replicated by the expression of *nsoI*.

Nisin A induction of *L. lactis* containing the *nso* cluster and *nisRK* genes allowed detection of the NsoA1 pre-peptide by Western hybridisation. When this heterologous producer was grown with nisin induction on solid media, antimicrobial activity was demonstrated in the presence of trypsin against *C. perfringens*, *Clostridium difficile* and *L. lactis*. This research adds to evidence that lantibiotic production may be an important trait of gut bacteria and could lead to the development of novel treatments for intestinal diseases.

INTRODUCTION

Lantibiotics are small amphiphilic lanthipeptides produced by Gram-positive bacteria and commonly have antimicrobial activity against a wide range of mostly Gram-positive bacteria (Arnison *et al.*, 2013; Cotter *et al.*, 2013). While their potential in applications has so far mostly been seen as preservatives or as probiotics (Hegarty *et al.*, 2016), they have been of increasing interest since novel therapeutic applications have been discovered (Dischinger *et al.*, 2014; Hammami *et al.*, 2012; Malik *et al.*, 2012). Lantibiotics are gene encoded and the genes involved in their biosynthesis, regulation and immunity are usually clustered. Following synthesis of the precursor peptides on the ribosome, they undergo a series of post-translational modifications such as serine and threonine dehydration and lanthionine bridge formation to produce the characteristic lanthionine and methyllanthionine rings which contribute to their stability (Chatterjee *et al.*, 2005). Their mode of synthesis makes genetic engineering a powerful tool to create improved peptides and to study biosynthesis (Field *et al.*, 2012; Montalban-Lopez *et al.*, 2017).

Nisin A is the best characterized example of the type A lantibiotics; it is the only bacteriocin to date to be authorized for use as a food preservative, under the name Nisaplin, and is also in use for prevention of bovine mastitis (Dischinger *et al.*, 2014). It is a highly stable peptide with antimicrobial activity against a wide range of Gram-positive organisms including food spoilage and food pathogenic bacteria from genera such as *Clostridium*, *Listeria*, *Staphylococcus* and *Bacillus* (Shin *et al.*, 2016). The nisin A biosynthetic cluster is located within a 70 kb transposon named Tn5307 which has been shown to be transferrable by conjugation (Horn *et al.*, 1991). To date, eight natural forms of nisin have been identified from either

lactococci (A, Z, F, Q) or streptococci (U, U2, P, H), with nisin H representing the first example from a gastrointestinal tract bacterium (O'Connor *et al.*, 2015). A related nisin homologue has also been identified in the thermophilic bacterium *Geobacillus thermodenitrificans* (Garg et al., 2012). Beside the natural forms of nisin, both random and targeted mutation studies have created libraries of nisin derivatives, the most notable being a nisin A derivative with an S29G substitution, with enhanced antimicrobial activity against both Gram-positive and Gram negative pathogens (Field *et al.*, 2012). Hinge region variants such as N20P, M21V (Field *et al.*, 2008), K22T (Field *et al.*, 2010) and N20K, M21K (Yuan *et al.*, 2004) have also led to increases in bioactivity against a range of bacteria.

In addition to the pre-peptide gene *nisA*, nisin gene clusters typically encode NisB, an enzyme which dehydrates serines and threonines and NisC, a zinc-dependent metalloprotein which cyclises dehydrated residues to cysteines, both of which have been shown to be essential for nisin production (Ra *et al.*, 1999). In order for these biosynthetic modifications to take place, it is important that the appropriate leader peptide is attached to the bacteriocin precursor. In the case of nisin A, the leader peptide is removed by the protease NisP following transport across the cell membrane by NisT, an ABC-transporter which forms a membrane-associated complex with NisB and NisC. Once modified, the mature nisins all contain three dehydrated amino acids and five thioether bridges. NisFEG and NisI control self-immunity, while the two component sensor-histidine kinase system NisRK allows self-induction of the *nisA* promoter by the mature nisin product (Chatterjee *et al.*, 2005). The modified nisin A and U molecules have been shown to have the capacity to act as inducers of their own and each other's promoters even when attached to their leader peptides (Wirawan *et al.*, 2006), while cross-immunity has also been demonstrated between nisins A and Z (de Vos *et al.*, 1993) and A and H (O'Connor *et al.*, 2015).

Heterologous expression of lantibiotic clusters and genes, especially when their inducing conditions are not known, has been a powerful aid in the sequencing and characterization of several lantibiotic clusters such as those of epicidin 280 (Heidrich *et al.*, 1998), enterocin A (O'Keeffe *et al.*, 1999) and nukacin ISK-1 (Aso *et al.*, 2004). The nisin A biosynthetic cluster has already been expressed successfully in *L. lactis* and *Enterococcus* sp (Li and O'Sullivan, 2002); furthermore, the nisin biosynthetic machinery has been shown to be capable of modifying other non-nisin peptides (Rink *et al.*, 2005), while the nisin promoter elements have been used extensively for inducible expression of cloned genes (Mierau and Kleerebezem, 2005). The extreme robustness of the biosynthetic machinery was demonstrated by Majchrzykiewicz *et al.* (Majchrzykiewicz *et al.*, 2010), who successfully expressed a fully modified and biologically active two-component class II lantibiotic from *Streptococcus pneumoniae* using the nisin A biosynthetic machinery, while several other studies have demonstrated that the lantibiotic biosynthetic machinery is able to recognize alternative peptides containing the peptide leader sequence of their own *lanA* gene and in some cases modify them (Kluskens *et al.*, 2005; Rink *et al.*, 2007).

The human gut harbours a large number of bacteria, reaching 10^{12} bacteria per gram of intestinal content, that are diverse in their composition and contain many unknown species; other species, such as *Blautia obeum*, are recognized as being dominant in the human colon (Flint *et al.*, 2012). It is a rich potential source of novel antimicrobials which have evolved to function in the challenging conditions of the gastrointestinal

tract, and recent research suggests bacteriocin production is widespread (Birri *et al.*, 2013; Walsh *et al.*, 2015). Using genome mining of human gut bacteria, new lantibiotic sequences sharing considerable sequence homology with the Class AI lantibiotics and especially nisin U were discovered from anaerobic bacterium *Blautia obeum* A2-162. The novel lantibiotic cluster was cloned into *L. lactis* and evidence of antimicrobial activity and cross-immunity with nisin A was shown.

METHODS

Strains, plasmids and growth conditions. *Lactococcus lactis* strains and plasmids used in this study are listed in Table 1 and primers in Table S1. *Ruminococcus obeum* A2-162 was isolated previously from human faeces from an adult female consuming a Western style diet (Barcenilla, 1999; Dabek *et al.*, 2008; Flint *et al.*, 2007) and its genome was sequenced as part of the MetaHIT project. *R. obeum* has subsequently been re-classified as *Blautia obeum* (Lawson and Finegold, 2015). *B. obeum*, *C. perfringens* NCTC 3110 and *C. difficile* NCTC 11204 were cultured in pre-reduced brain heart infusion broth (BHI, Oxoid) with complements (50 mg/l vitamin K, 5 mg/l hemin, 1 mg/l resazurin, 0.5 g/l L-cysteine) at 37˚C in an atmosphere of 5% CO_2, 10% H_2 in N_2; *L. lactis* strains were cultured in M17 medium (Oxoid) supplemented with 5 g/l glucose (GM17) at 30˚C; *E. coli* MC1022 was cultured in L media (Oxoid) at 37˚C with shaking. For plasmid selection erythromycin and chloramphenicol were used at 5 μg/ml for *L. lactis* or at 100 μg/ml and 15 μg/ml respectively for *E. coli* and ampicillin was used at 100 μg/ml.

Degenerate Oligonucleotide Primer design and screening for *lanC* genes. Genomic DNA from *B. obeum* A2-162 was extracted using the Qiagen Genomic-tip kit. Degenerate AT-rich primers lanC340 and rlanC460 were designed from the WCYG region (position 294 in SpaC) and the GLIxG region (position 403 in SpaC) of an alignment of the following LanC sequences from the NCBI database: CAA74351 (EciC), AAF99580 (Mut C), CAA48383 (NisC), CAA90026 (PepC) P33115 (SpaC), BAB08164 (SrtC) P30196 (EpiC), as described previously (Mayer *et al.*, 2006). Degenerate oligonucleotide primer PCR used GoTaq (Promega) with 10 μM of each degenerate primer lanC340 and rlanC460. PCR products were electrophoresed, bands of 200-300 bp were excised and extracted from agarose gels (Qiaex II Gel extraction kit, Qiagen), purified using Sureclean (Bioline), ligated into vector pCR2.1 and transformed into chemically competent *E. coli* TOP10 (TA Cloning Kit, Life Technologies). Positive colonies were identified by colony PCR using GoTaq with universal and reverse primers and confirmed by sequencing. Sequence was extended using the DNA Walking SpeedUp™ Premix kit (Seegene) and genomic DNA.

DNA library construction and lantibiotic cluster sequencing. Genomic DNA from *B. obeum* A2-162 was used to construct a DNA library (Lucigen Corp, Middleton, WI, USA) using the *E. coli* vector pJAZZ-OC. For hybridisation analysis of the library, a 736 bp DNA probe comprising bases 15292 to 16027 of the lantibiotic cluster sequence (accession number KY914474), which includes the C-terminus of the *nsoC* gene and downstream sequence, was prepared by PCR using primers 5PrA2162280 and 3PrA2162c and purified. Hybridisation of the probe to filter membranes arrayed with the library was performed by pretreating the

membranes for 2 h with gentle shaking in 5 x SSC (saline sodium citrate buffer (Sambrook *et al.*, 1989)), 0.5% SDS, 1 mM EDTA (pH 8.0) at 42°C, scraping with wet paper towels and rinsing in 2 x SSC then hybridisation using the ECL hybridisation kit (GE Healthcare). Positive clones identified from the DNA library were cultured and sequenced using primers pJAZZf and nzrevcpJAZZ. The known sequence was extended from the library clones using primer walking and the gaps were filled by PCR until the lantibiotic cluster had been fully sequenced in both directions.

Bioinformatic analysis. Genomic DNA sequences were assembled with the Phred-Phrap program and contigs were assembled in SeqMan (DNASTAR). ORFs were determined by Artemis (Rutherford, 2000). Start sites were selected on best match to the consensus ribosome binding site AGGAGG where present and to homologous sequences identified using BLASTp and tBLASTx searches (Altschul *et al.*, 1997) using the UniProtKB/TrEMBL database. Amino acid alignments were performed using the Clustal W algorithm in Vector NTI (Invitrogen) and edited in Genedoc; the average distance tree was generated using BLOSUM62 from the Clustal WS alignment of secondary structure prediction of the peptide sequences (Larkin *et al.*, 2007). Pairwise cluster comparisons were performed using BLASTn and tBLASTx from BLASTall vs 2.2.26. The clusters were aligned using mega-cc 7 with the Neighbor Joining method (MUSCLE) and a tree was made using RAxML vs 8.2.9 with the BS and ML recommended settings. Cluster comparisons were visualized using the tBLASTx comparison files and RAxML tree in R vs 3.3.2 using the genoPlotR package.

Expression of the Nisin O cluster in *L. lactis*. A 17,438 bp sequence containing the novel lantibiotic cluster was restricted from the identified pJAZZ-OC clone with ClaI and PstI (NEB) then ligated into vector pIL253 (MspI, PstI restricted and dephosphorylated (Antarctic Phosphatase, NEB)) using Fastlink DNA ligase (Epicentre) to create p*nso.* The construct was transformed into electrocompetent *L. lactis* MG1614 using a Gene Pulse Xcell (BioRad, (Mayer *et al.*, 2008)). Plasmid DNA was extracted using the QIAprep Spin Miniprep Kit (Qiagen) with an additional 15 min at 37˚C with 5 mg/ml lysozyme and 30 U mutanolysin (Sigma) at the lysis stage and the insert was confirmed by sequencing with primers pIL253F and pIL253R. The region containing the four *nsoA* genes was deleted from p*nso* by splice overlap extension PCR (Horton *et al.*, 1989) using Phusion (Finnzymes). Sequences surrounding the *nsoA* region were amplified at the 5' end using primers splA1 and splA2 and at the 3' end with primers splA3 and splA4. These products were spliced and amplified with primers splA1 and splA4, giving an amplicon of 4,818 bp which was digested with BsaI and StuI and ligated to restricted, dephosphorylated plasmid p*nso* to produce plasmid p*nso*Δ*nsoA*, which was transformed into *L. lactis* MG1614. Both p*nso* and p*nso*Δ*nsoA* were also transformed into *L. lactis* UKLc10.

The *nsoA* genes were cloned separately into a nisin-inducible expression vector. Each *nsoA* gene was amplified using primer combinations pTGA13 with a13AleI for gene *nsoA1* and pTGA23 with a13AleI for gene *nsoA2* and pTGA23 with a43AleI for genes *nsoA3-nsoA4* to make amplicons *nsoA1Ale, nsoA2Ale* and *nsoA3nsoA4Ale* respectively. The region of plasmid pFI2596 (Fernandez *et al.*, 2009) containing the nisin promoter P_{nisA} was amplified using primers pTG262-F with a15pTG to make amplicon *nisPa* and pTG262-F

with a25pTG to make amplicon *nisPb.* The P_{nisA} and *nsoA* amplicons were used as DNA templates in splice overlap extension PCR by combining templates *nisPa* with *nsoA1Ale* using primers pTG262-F with a13AleI, *nisPb* with *nsoA2Ale* using primers pTG262-F and a13AleI and finally *nisPb* with *nsoA3nsoA4Ale* using primers pTG262-F and a43AleI. The P_{nisA}-*IL12* region of pFI2596 was then replaced with the spliced amplicons containing the *nsoA* genes by SmaI and AleI digestion and ligation to digested, dephosphorylated plasmid pFI2596. Ligation products were transformed into *L. lactis* MG1614. Clones with P_{nisA}-*nsoA1* (pTG*nsoA1*), P_{nisA}-*nsoA2* (pTG*nsoA2*) and P_{nisA}-*nsoA3*-*nsoA4* (pTG*nsoA3*-*nsoA4*) were identified as described previously using primers p54 and p181 and transformed into electrocompetent MG1614-p*nso*, MG1614-p*nso*Δ*nsoA*, UKLc10-p*nso* and UKLc10-p*nso*Δ*nsoA*. The *nisA* gene was also subcloned into pTG262 as a positive control; *nisA* was amplified from *L. lactis* FI5876 genomic DNA by PCR using primers NisA-BspHF and NisA-BspHR, restricted with BspHI and ligated into NcoI-restricted pUK200. After transformation into *E. coli* MC1022 and sequence confirmation the insert was then excised with SspI and EcoRI and cloned into HindIII-EcoRI restricted pTG262.

Cloning of hybrid $nisA^L$ -*nsoA* genes into the nisin A biosynthetic system. Hybrid $nisA^L$-*nsoA* pre-peptides were designed to contain the full NisA leader sequence MSTKDFNLDLVSVSKKDSGASPR ($nisA^L$) followed by the predicted NsoA structural peptides of *nsoA1* with possible cleavage sites: *nsoA1*IE: IEPKYKSKSACTPGCPTGILMTCPLKTATCGCHITGK, *nsoA1YK*: YKSKSACTPGCPTGILMTCPLKTATCGCHITGK or *nsoA4*: ITSQHSFCTPNCLTGFLCPPKTQLTCTCKLKGQ. The 69 bp $nisA^L$ DNA was amplified from *L. lactis* FI5876 genomic DNA using primer pr1 combined with each primer 1pr2ie, 1pr2yk or 4pr2 to make $nisA^L$*IE*, $nisA^L$*YK* and $nisA^L$*4* amplicons respectively and the *nsoA1* and *nsoA4* structural genes were amplified from plasmid DNA containing the full lantibiotic cluster using primer 1Pr4 combined with 1Pr3IE, 1Pr3YK or 4Pr3 to make amplicons *nsoA1IE*, *nsoA1YK* and *nsoA4* respectively. Each hybrid $nisA^L$-*nsoA* was prepared by splice overlap extension PCR using template sets $nisA^L$*IE* with *nsoA1IE* and $nisA^L$*YK* with *nsoA1YK* with primers pr1 and lanA14 to make $nisA^L$-*nsoA1IE* and $nisA^L$-*nsoA1YK* respectively and templates $nisA^L$*4* with *nsoA4* with primers pr1 and LanA44 to make $nisA^L$-*nsoA4*. Purified PCR products were digested with BspHI and XbaI and ligated into NcoI and XbaI restricted, dephosphorylated pUK200 and products transformed into electrocompetent *E. coli* MC1022. After sequence confirmation using primers p54 and p181, plasmid DNA from positive clones and the pUK200 vector control was transformed into *L. lactis* strains FI5876Δ*nisA*, FI5876Δ*nisP*, FI5876Δ*nisB*, FI5876Δ*nisC* and FI5876Δ*nisCP*.

Construction of *nsoI* and *nisI* expression vectors. The *nsoI* gene was amplified from p*nso* using primers pTGI3 with iAleIb and the nisin promoter region of plasmid pFI2596 was amplified with primers i5pTG and pTG262-F. Splice overlap extension PCR was performed using pTG262-F with iAleIb, the product was ligated into SmaI and AleI digested, dephosphorylated pFI2596 and transformed into *L. lactis* MG1614. Inserts were confirmed by sequencing using primers pTG262-F and pTG262-R. The *nisI* gene was amplified from *L. lactis* FI5876 genomic DNA using primers spI5 and splI3AleI and the nisin promoter region of

plasmid pFI2596 was prepared using primers splA1 and pTG262-F. These two amplicons were spliced and amplified using pTG262-F with iAleIb and ligated into vector pFI2596 as described for *nsoI*.

Measurement of bacterial growth, viability and phenotype. *L. lactis* FI5876Δ*nisA* p*nisA*^{L}*-nsoA1YK*, *L. lactis* FI5876Δ*nisA* pUK200 and *L. lactis* FI5876Δ*nisP* p*nisA*^{L}*-nsoA1YK* were subcultured from overnight cultures in selective media and at 2 h were induced with 10 ng/ml nisin A. After 18 h growth the cultured cells were prepared for scanning and transmission electron microscopy (SEM and TEM) analysis as described previously (Dertli *et al.*, 2013; Pitino *et al.*, 2012). Samples were examined and imaged in a FEI Tecnai G2 20 Twin transmission electron microscope at 200 kV. Bacterial growth was measured using a Labsystems Bioscreen C (Labsystems Oy). Test cultures were subcultured twice from glycerol stocks and induced appropriately overnight for 16 h. Cells were pelleted by centrifugation (10,000 g, 10 min), resuspended in 1 ml PBS, pelleted again and resuspended to an optical density (OD_{600}) of 3.0 in selective media. Bioscreen plates (honeycomb, Thermo Fisher Scientific) were prepared with 300 µl media per well seeded with 1% of the prepared inoculum in triplicate and grown at 30˚C. To measure viability, stationary phase *L. lactis* strains were washed and resuspended in PBS then diluted 500-fold in filter sterilized PBS with 1 µl each of propidium iodide and FM 4-64FX (Life Technologies) then analyzed on a Cytomics FC500 MPL (Beckman Coulter). Flow cytometry data were analysed using Flowjo (Treestar).

Preparation and analysis of protein extracts. Pre-warmed media was inoculated with 1:100 v/v overnight culture of *L. lactis* strains expressing hybrid plasmids. At mid exponential phase cultures were induced with 10 ng/ml nisin and incubated from 2 h to overnight. Cells were harvested by centrifugation (4000 g, 40 min, 4˚C) and frozen while total protein from filtered (0.45 µm) culture supernatants was precipitated by adding 1 g/ml (nisin leader hybrids) or 20% (*nsoA* pre-peptides) cold trichloroacetic acid (TCA) and incubating overnight at 4˚C. Precipitated proteins were pelleted by centrifugation (13000 g, 30 min, 4˚C), washed with ice cold acetone and resuspended in 0.05 volumes 50 mM sodium acetate (pH 5.5). Cells were resuspended in 50 mM sodium acetate (pH 5.5) (MG1614) or 0.2 M Tris HCl pH 7.4 (UKLc10) and soluble protein extracts produced by bead beating (Mayer *et al.*, 2008). Proteins were analysed by SDS-PAGE electrophoresis and Western blotting as described previously (Mayer *et al.*, 2008) using 12% or 4-12% Bis-Tris NuPAGE gels in MES SDS buffer (Invitrogen) and an antibody (at 1/100 dilution) raised against the nisin A leader peptide or a polyclonal anti-leader peptide antibody raised by Genscript Corp (NJ, USA) from synthesized N-terminal acetylated NsoA1 leader peptide H_2N-AKFDDFDLDVTKTAAQGGC-$CONH_2$ with anti-Rabbit IgG-alkaline phosphatase secondary antibody (Sigma).

Antimicrobial activity and testing of different media and inducing agents. To measure antimicrobial activity using drop tests, strains were cultured from glycerol stocks in the appropriate media overnight and subcultured twice before inoculating the appropriate media. For overlay assays, *B. obeum* A2-162 was sub-cultured twice in liquid media before plating or streaking on solid media or solid media supplemented with 50 µg/ml trypsin. After 1 to 7 d incubation cultures were killed using chloroform treatment, overlaid with

soft media (0.7 % w/v agar) seeded with indicator bacteria and incubated overnight. For heterologous production, *L. lactis* strains were sub-cultured with 10 ng/ml nisin A then 5 µl overnight culture spotted on solid agar with 20 mg/l $NaHCO_3$ and 10 ng/ml nisin and grown overnight. Bacterial growth was killed by irradiation with UV light for 15 min then overlaid with soft medium containing 1-2% of an overnight culture of the indicator strain, with or without trypsin (sequencing grade modified trypsin, Promega, 1 or 5 µg/ml or trypsin Tpck treated from bovine pancreas Sigma at 1, 5, 10 or 15 µg/ml). Plates were incubated overnight in the conditions preferred by the indicator strain.

To attempt to induce antimicrobial production, *B. obeum* A2-162 and *L. lactis* MG1614 and FI5876 were cultured from glycerol stocks in selective media overnight then subcultured in a range of test media (BHI pH 5.0, 6.0 and 7.0, BHI with 5 mg/l hemin, YCFA medium, PYGS medium, de Man Rogosa Sharp medium, Reinforced Clostridial Medium, Luria broth, lysogeny broth, Rogosa, GM17, M17 supplemented with 5% of each: lactose, mannitol, cellobiose, mannose, sorbitol, galactose, xylose or inulin, all with and without 50 µg/ml trypsin) and inducing agents. Inducing agents included 5 g/l yeast extract, a mixture of 2 g/l glucose, 1 g/l soluble starch, 2 g/l cellobiose, a mixture of 2 g/l xylose, cellobiose and sorbitol, 2 g/l inulin, 2 g/l sodium acetate 3-hydrate, 0.31% volatile fatty acids mix (33 mM acetic acid, 9 mM propionic acid, 1 mM n-valeric acid, 1 mM isovaleric acid and 1 mM isobutyric acid), 50 µg/ml trypsin, heat killed *C. perfringens* culture, 10 to 1000 ng/ml nisin A and combined trypsin and nisin. Filtered (0.22 µm) stationary phase culture supernatants from *L. lactis* strains MG1614-p*nso*, MG1614-pIL253 or FI5876 with and without nisin (1:20 v/v) and *L. lactis* cell extracts in 50 mM NaOAc or 50 mM NaOAc, 8 M Urea buffer and supernatant TCA-extracted proteins were also tested as inducers of activity in *B. obeum* A2-162; and spent culture of *B. obeum* was similarly tested as an additive to nisin-induced MG1614-p*nso* culture. Samples were tested for antimicrobial activity using well diffusion, drop tests and overlay assays (Ryan *et al.*, 1996).

To remove leader peptides, soluble cell extracts (2 µg) and TCA precipitated culture supernatant extracts from *B. obeum* A2-162 and *L. lactis* strains were digested with 0.5 mg/ml trypsin for 1 h at 37˚C in 50 mM sodium acetate buffer (pH 5.5) (Cheng *et al.*, 2007), or subcultured to media containing 2.5 µg/ml nisin A and 1:40 v/v filter sterilized culture supernatant of *Bacillus subtilis*. Samples were assayed for antimicrobial activity using well diffusion assays. Additionally, *L. lactis* MG1614-p*nso* and its derivative strains were cultured with 100 ng/ml of inducing nisin A and cross-streaked with *B. subtilis*, grown overnight then overlaid with *C. perfringens*.

RESULTS

B. obeum A2-162 contains a lantibiotic-like gene cluster

Previous studies have shown novel lantibiotic genes can be identified by PCR using degenerate primers designed from conserved regions of lantibiotic cluster genes (Mayer *et al.*, 2006; Wirawan *et al.*, 2006). Here, AT-rich degenerate primers were designed and used to screen a bacterium previously isolated from the human GI tract for *lanC*-like sequences. We identified a 180 bp DNA sequence from *B. obeum* A2-162, whose translated product aligned with other LanC proteins. The sequence was extended in both directions and a full *lanC*-like gene as well as part of a *lanT*-like gene indicated that the genes belonged to a lantibiotic

cluster. A DNA library in the *E. coli* linear vector pJAZZ-OC was prepared and the surrounding genes were sequenced in both directions by primer walking, identifying a c. 15 kb lantibiotic cluster within a c. 19 kb insert. Comparison to clusters of other nisins and subtilin suggested that the full cluster had been identified. Later publication of the whole draft genome (GenBank FP929054, A. Pajon, K. Turner, J. Parkhill, S. Duncan and H.,Flint, 2015, unpublished) confirmed this.

Computational analysis of the cluster identified 15 probable ORFs encoding lantibiotic–associated genes (abbreviated with *nso* here), whose functions were predicted by BLASTp analysis (Figure 1a, Table S2). The genes all had the same orientation and included four *nsoA* genes, with the first three coding for identical proteins with only one amino acid difference in the leader peptide (Figure 1b), *nsoB* and *nsoC* biosynthetic genes, an *nsoT* ABC transporter, two sets of *nsoRK* two-component regulator system genes and *nsoI* and *nsoFEG* genes presumed to be involved in immunity. No probable protease genes capable of cleaving the leader sequence were identified; the predicted NsoT protein showed similarity to other NisT type lantibiotic ABC transporter ATP-binding proteins and did not contain the N-terminal protease domain responsible for leader cleavage in dual-function ABC transporters which is frequently found in lantibiotic clusters which do not contain a *lanP* gene. A 506 bp region between the end of *nsoK1* and the start of *nsoA1* genes displayed no homologies to other known genes commonly found in lantibiotic clusters.

The predicted mature protein sequence of *nsoA1* was found to be very similar to other NisA proteins, with conservation of the positions of the serine, threonine and cysteine residues; the predicted mature protein sequence of *nsoA4* showed a lower similarity but had the majority of serine, threonine and cysteine residues in similar positions to other nisin analogues (Figure 1b). The spaces separating the *nsoA* genes were 22, 23 and 19 bp with the first two gaps exhibiting 95% identity. BLASTp searches categorized the three NsoA1-3 pre-peptides as part of the gallidermin/nisin family with 63% identity to the nisin U precursor peptide. The N-terminal region of the NsoA1-3 pre-peptides contained an FDLD motif followed by a GG motif and a further PK motif. The most likely leader peptide cleavage sites were therefore presumed to be following either the GG or the PK motif and the two predicted structural peptides are referred to as NsoA1IE and NsoA1YK respectively. These exhibited 82% and 90% sequence identity to nisin U respectively (Figure 1b). Only an FDLD leader peptide motif was identified in the N-terminal region of the NsoA4 pre-peptide and an ITS amino acid sequence resembling that of the start of the active nisin A was found in a similar region of the pre-peptide. The predicted NsoA4 structural peptide is one amino acid shorter than nisin A and showed the best similarity to geobacillin I (59% identity).

BLASTp analysis of an ORF at the 5’ end of the cluster (*orf1*) showed homology to the second half of a transcriptional regulator from *B. obeum* (Figure 1a, Table S2); the preceding nucleotide sequence encoded the earlier part of the protein but contained frame shifts. At the 3’ end of the cluster, *orf2* showed homology to hypothetical proteins from several Clostridiales species and to a transposase from an uncultured faecal bacterium (AMP50088, $2e^{-52}$). This was followed by sequences which matched short regions of database proteins with the sequence interrupted by frameshifts – *orf3* and *orf4* had similarity to consecutive regions from a transposase from *Blautia wexlerae* and to other transposases from a range of other Clostridiales bacteria, while *orf5* and *orf6* matched consecutive regions of a putative transcriptional regulator. The first

seemingly complete protein is *orf7*, which shows high homologies to DNA binding response regulators; *orf8* is truncated by the end of the clone and has similarity to ATP binding proteins/sensory histidine kinases.

The nisin O cluster may have evolved from the nisin U cluster

BLASTn comparison of the full nisin O cluster to those of the other nisins did not show considerable sequence conservation, but tBLASTx showed a high similarity between the nisin O cluster and nisins U, A and Q (Figure 1c). It was interesting to note that the GC percentage of the three highly similar *nsoA* genes was higher than that of the fourth structural gene and the rest of the cluster, whose average GC content was 31% in comparison to 41.6% for the producing organism *B. obeum* A2-162.

Comparison of all nisin and *B. obeum* A2-162 clusters showed the clusters of nisins Q and A were highly conserved, while there appeared to be an inversion event between the nisin A and nisin U clusters which was also present in the nisin O cluster. The 3' end of the nisin U cluster containing *nsuA*, *nsuB*, *nsuT* and *nsuC* was conserved in the nisin O cluster while the 5' end of the cluster containing genes *nsuP*, *nsuRK* and *nsuFEG* showed evidence of a few translocations of genes between the clusters. BLASTn search of the region between *nsuA* and *nsuG* in the nisin U cluster showed the presence of a ISSmu4-like putative transposase sequence (DQ368682) within the region. However, BLASTp analysis shows that the Nso translated proteins share the best homologies with proteins from other Clostridiales and Bacillales bacteria, frequently from faecal sources, so any evolution from *Streptococcus uberis* would appear to be ancient.

***B. obeum* A2-162 exhibits trypsin-induced antimicrobial production on solid media**

A range of growth conditions, culture media and media additives which included supernatants or cell extracts from spent cultures were tested for their ability to induce antimicrobial production in *B. obeum* A2-162. From those, only cultures grown for at least 4 days in liquid culture then plated on solid media supplemented with 50 μg/ml trypsin before overlaying reproducibly showed evidence of antimicrobial production against the indicator strain *C. perfringens* (Figure 2); no antimicrobial activity was detectable from culture supernatants by drop tests.

Hybrid NisA^{L}-NsoA peptides alter the phenotype of *L. lactis* when they are expressed in the presence of the nisin modification machinery

To investigate whether the nisin A biosynthetic cluster could modify and produce active NsoA peptides, the predicted leader of each NsoA pre-peptide was replaced with that of nisin A (*nisA*^{L}) and the hybrid *nisA*^{L}-*nsoA1IE*, *nisA*^{L}-*nsoA1YK* and *nisA*^{L}-*nsoA4* genes were expressed from vector pUK200 in *L. lactis* strains FI5876Δ*nisA*, FI5876Δ*nisP*, FI5876Δ*nisB*, FI5876Δ*nisC* and FI5876Δ*nisCP*.

Although plasmids expressing *nisA*^{L}-*nsoA1IE* and *nisA*^{L}-*nsoA4* had no effect on growth, strain FI5876Δ*nisA* p*nisA*^{L}-*nsoA1YK* exhibited a longer lag phase and reached lower maximum OD_{600} values (Figure 3a). This strain displayed an aggregated phenotype in liquid culture, and TEM and SEM revealed loss of cell shape, extensive aggregation and less defined cell membranes (Figure 3b), suggesting problems with membrane synthesis or stability. The nisin biosynthetic gene knockout strains FI5876Δ*nisB*, FI5876Δ*nisC* and

FI5876Δ*nisP* containing the hybrid plasmid p*nisA*^{L}-*nsoA1YK* were not phenotypically different from their empty vector control counterparts and showed similar growth rates, suggesting that the nisin biosynthetic genes were necessary for the slow growth phenotype (Figure 3c). These results were supported by flow cytometry analysis of stationary phase cells, showing that FI5876Δ*nisA* p*nisA*^{L}-*nsoA1YK* had an increased percentage of PI-positive cells (Figure 3d).

Despite the altered growth and phenotype, strains expressing p*nisA*^{L}-*nsoA* hybrids showed no evidence of antimicrobial activity using a number of different antimicrobial detection tests, which included the use of trypsin or filtered culture supernatant as inducers (data not shown). However, using an antibody to the nisin A leader, Western analysis of FI5876Δ*nisA* containing p*nisA*^{L}-*nsoA1IE*, p*nisA*^{L}-*nsoA1YK*, p*nisA*^{L}-*nsoA4* or pUK200 identified a band at c. 6 kDa only in the FI5876Δ*nisA* p*nisA*^{L}-*nsoA1YK* samples (Figure 4a). The absence of this band in the other strains suggests that NisA^{L}-NsoA1IE or NisA^{L}-NsoA4 are either not produced or not modified, causing instability and rapid degradation of produced pre-peptides. Examination of nisin biosynthetic gene knockout strains demonstrated that deletions in *nisB, nisC* or *nisCP* prevented accumulation of the NisA^{L}-NsoA1YK pre-peptide (Figure 4b). Western analysis did not detect any cleaved nisin A leader, but it did show the presence of the prepeptide in TCA-precipitated culture supernatants (Figure 4a), suggesting that the prepeptide was either exported or released from damaged or lysed cells during culture.

NsoA production in the presence of nisin O biosynthetic machinery in *L. lactis*

We inserted a 17,438 bp sequence containing the lantibiotic cluster into pIL253 to create plasmid p*nso* and transformed it into the non-nisin producer *L. lactis* MG1614. Initial antimicrobial testing of this strain using deferred antagonism tests did not identify any antimicrobial activity. However, a high level of resistance to nisin A was observed in both MG1614-p*nso* and in a strain where the four *nsoA* genes had been deleted (MG1614-p*nso*Δ*nsoA*) (Figure 5a). The putative *nsoI* gene and the nisin immunity gene *nisI* were expressed in MG1614 separately; although pTG*nisI* increased the immunity of MG1614 to nisin A, pTG*nsoI* did not (data not shown). Nisin A was detrimental to *B. obeum* A2-162 at concentrations above 100 ng/ml, suggesting that the immunity systems conferring resistance to the MG1614-p*nso* strain were not being expressed.

We hypothesised that functional similarities between nisin O and nisin A clusters might allow one of the *nsoRK* systems to interact with nisin A to induce expression via the nisin A promoter. Genes *nsoA1, nsoA2* and *nsoA3-nsoA4* were inserted into vector pTG262Pn under the control of the nisin A promoter and co-expressed in MG1614-p*nso* and MG1614-p*nso*Δ*nsoA* with nisin A induction. Western blot analysis with a peptide antibody made to the NsoA1 leader showed hybridisation at c. 6 kDa to the MG1614-p*nso* pTG*nsoA3-nsoA4* samples (Figure 6). There was also faint hybridisation to extracts from MG1614-p*nso* containing pTG262Pn. The 6 kDa band was not detectable in any of the strains expressing just the *A1* or *A2* sequences, MG1614-p*nso*Δ*nsoA* samples, the original producer *B. obeum* A2-162 or nisin producer FI5876. It was not possible to identify a cleaved leader at c. 2 kDa (cleaving at GG/IE) or c. 2.5 kDa (cleaving at PK) in these cell extracts, possibly due to high background hybridisation in this size range or instability of the

cleaved leader peptide. None of these cell extracts produced antimicrobial activity. Attempts to cleave the leader peptide and release an active antimicrobial from these strains using treatment of culture supernatants with trypsin, or culture supernatant from *B. subtilis*, which is known to produce extracellular proteases, or by co-culturing with *B. subtilis*, failed to produce an antimicrobial activity against *C. perfringens* (data not shown).

Antimicrobial activity after nisin A induction and trypsin treatment

To investigate whether nisin A could act as a heterologous inducer of the *nso* pre-peptides, p*nso* or p*nso*Δ*nsoA* were expressed in *L. lactis* UKLc10, which has the *nisRK* genes integrated in the chromosome, and cultures were induced with 10 ng/ul nisin. Western analysis showed improved production of the pre-peptide in both cell extracts and TCA-precipitated supernatants from cultures after nisin induction (Figure 7). Culture supernatants from these strains did not exhibit antimicrobial activity. However, when strains were grown on solid media containing nisin A and then overlaid with indicator strains in soft agar containing trypsin, clear activity was seen against *C. perfringens*, *C. difficile* and *L. lactis* (Figure 8). These zones of inhibition were absent when trypsin was not added to the soft agar but were evident in the presence of 1, 5, 10 and 15 ng/ul trypsin from either source. The activity seen from the positive nisin A control strain FI5876Δ*nisA* pTG*nisA* was maintained in the presence of trypsin.

DISCUSSION

In this work a novel Type A lantibiotic cluster with a unique gene arrangement was discovered in the genome of *B. obeum* A2-162 and heterologous production of the structural peptides in *L. lactis* was investigated using either the native or the nisin A biosynthetic machinery. According to Sahl *et al.* (Sahl *et al.*, 1995), lantibiotic natural variants can be defined as having only a few amino acid substitutions, essentially the same ring pattern, and cross immunity between producing strains. The novel cluster contained a triplicate structural peptide which showed close sequence similarity to other nisins and conservation of the predicted ring positions, while the lantibiotic cluster provided immunity to exogenous nisin A. Consequently, the predicted lantibiotic was regarded as a member of the nisin group and named nisin O. However, it was interesting to note that the native producer *B. obeum* showed sensitivity to nisin A, suggesting that the immunity system may require induction. O'Connor *et al.* (O'Connor *et al.*, 2015) also found that the native nisin U producing strain *S. uberis* was inhibited by supernatant from a nisin A producer.

The nisin O cluster is unusual in that it is the first nisin cluster to have more than one copy of a nisin-like structural gene, two sets of *lanRK* genes and no identifiable protease. Differences in nisin cluster gene arrangements have been described before (O'Connor *et al.*, 2015; Richards *et al.*, 2011; Wirawan *et al.*, 2006) and have been proposed to be a consequence of horizontal gene transfer, but up to now only nisin H has been found to be different in its gene content with the absence of a detectable *nisI* (O'Connor *et al.*, 2015). At the pre-peptide level, the *nsoA* genes deviated from the conserved leader peptide and cleavage sequences found in other *nisA* genes. Class I lantibiotic leader peptides share conserved F(N/D)LD boxes and C-terminal PQ or PR amino acid sequences while Class II lantibiotic leader peptides contain the motif

ELXXBXG (B= V,L or I) and usually end in a GG motif (Plat *et al.*, 2011); only the F(N/D)LD box was present in all the NsoA leader peptides.

This is also the first report of a nisin-like cluster in the genus *Blautia*. *B. obeum*-like organisms can make up a significant percentage of the faecal microbiome (Lawson and Finegold, 2015). Increased levels of *Blautia* in the human gut have been associated with a reduced risk of death from graft-versus-host disease (Jenq *et al.*, 2015), as well as good cognition and reduced inflammation (Bajaj *et al.*, 2012), while decreased levels have been associated with the occurrence of type I diabetes in children (Murri *et al.*, 2013) and increased risk of colorectal cancer (Chen *et al.*, 2012). The amount of influence and mechanisms which lie behind the associations of intestinal *Blautia* to these conditions, and whether lantibiotic production is important to their ecology, is currently unknown.

The nisins discovered to date are produced by *L. lactis* (A, Z, F, Q), *Streptococcus uberis* (U), *Streptococcus agalactiae* (U2), *Streptococcus gallolyticus* and *Streptococcus suis* (P) and more recently another gut-derived strain, *Streptococcus hyointestinales* (H) (O'Connor *et al.*, 2015). An *in silico* study of the genomes of gut bacteria from the Human Microbiome Project identified lantibiotic-associated genes from a range of genera, including *R. obeum* A2-162 (Walsh *et al.*, 2015). Other Clostridiales have been shown to produce the bacteriocins albusin B, a type III bacteriocin (Chen *et al.*, 2004) and the lantibiotics ruminococcin A and ruminococcin C (Crost *et al.*, 2011; Dabard *et al.*, 2001), but these were not found to have any sequence similarities to the nisin O cluster. The discrepancy between the GC content of the structural gene region, the remaining cluster and the producer organism and the presence of transposase-like sequences at the 3' end of the cluster could signify that some ORFs have been acquired by horizontal gene transfer. The high gene and intergenic sequence similarity between the *nsoA1* genes suggests that the triplication occurred by consecutive duplication events. This is not unprecedented - ruminococcin A, also found in the gut and induced by trypsin, contains three *rumA* genes in its cluster which code for the same peptide (Marcille *et al.*, 2002). Two-component lantibiotics which contain two active structural genes are not uncommon (Lawton *et al.*, 2007). McAuliffe *et al.* (2000) observed that in most cases the sequence of two pre-peptides in two-component lantibiotics is c. 25% conserved, while many contain different enzymes for post-translational modification of each peptide. It is not known whether *nsoA4* encodes a functional lantibiotic peptide which is active on its own or in combination with *nsoA123* peptides - further work in heterologous systems or the original host is required to determine its contribution.

Several lantibiotics have been successfully produced using the nisin A biosynthetic machinery (Majchrzykiewicz *et al.*, 2010; Piper *et al.*, 2011). Slow growth, an altered phenotype and reduced viability effects in nisin leader hybrid expressing strains suggest that the NsoA1YK peptide can be stably expressed, but this is detrimental to *L. lactis* in the presence of the nisin biosynthetic machinery. These effects, combined with the visualisation of hybrid pre-peptides, suggest that the YK site is the correct start of the mature NsoA1. However, despite extensive experimentation using extracts from *B. obeum* A2-162 and hybrid $NisA^{L}$-NsoA producing strains, we did not identify any inducing agents able to produce antimicrobial activity in liquid culture. This suggests that the prepeptides are produced but not cleaved to the active product. As with subtilin (Corvey *et al.*, 2003; Stein and Entian, 2002) and mutacin I (Qi *et al.*, 2001), an

extracellular protease encoded elsewhere in the genome might be necessary for cleavage of the NsoA leader peptides to activate the *B. obeum* A2-162 lantibiotics, and under the culture conditions used this protease was either not expressed from the native strain or was not effective. The differences in the NsoA leader peptides and starts of the active peptides compared to other nisin analogues support the hypothesis that processing uses a different type of protease. Experiments using trypsin, filter sterilized *B. subtilis* spent culture supernatants or co-culturing with *B. subtilis* strains before overlaying with *C. perfringens* did not show reliable evidence of antimicrobial activity. However, antimicrobial activity against *C. perfringens* was observed when *B. obeum* A2-16 was cultured on solid media with trypsin. Lantibiotic regulation by trypsin has been seen before with ruminococcin A, a response that suggests adaption to its environment in the gut (Gomez *et al.*, 2002). Given the presence of two *nsoRK* systems and the low antimicrobial production it could be that a further inducing factor is involved in regulation in the native host. This factor and/or the antimicrobial itself may be expressed in low quantities by *B. obeum* A2-162 and could be concentrated around the culture in solid media but would be too dilute in liquid media, explaining our inability to detect antimicrobial activity from culture supernatants. The yield of nisin H in culture supernatants from gut bacterium *S. hyointestinalis* was also found to be low compared to that of nisin A (O'Connor *et al.*, 2015). Production of mutacin I, the *Bifidobacterium longum* DJO10A lantibiotic and the two component haloduracin from *Bacillus halodurans* were also only seen on solid media (Lee *et al.*, 2011; McClerren *et al.*, 2006; Qi *et al.*, 2001) and it has been proposed that the dense colonization necessary for mutacin I production is reminiscent of a biofilm condition (Qi *et al.*, 2001). Alternatively, the mechanism of trypsin may rely on pre-peptide cleavage rather than induction; *in vitro* biosynthesis of nisin using just *nisABC* successfully produced active nisin after treatment with trypsin (Cheng *et al.*, 2007). Trypsin is known to cleave after arginine or lysine residues and there is a lysine immediately before the proposed NsoA1,2,3 YK peptides, so trypsin activity could be generating the mature peptide in the absence of a suitable host protease, as appeared to be the case where trypsin was included in the soft agar of overlay assays of the *nso* cluster in *L. lactis*. In either case, trypsin could be a useful tool to identify novel lantibiotic activity from gut bacteria. The *nso* cluster was able to confer immunity to nisin A in *L. lactis* MG1614-p*nso*, and the use of nisin A to induce coexpression of *nsoA* genes allowed visualisation of bands which hybridized to the NsoA1 leader antibody. As nisin variants have been shown to induce the production of alternative nisin genes (Wirawan *et al.*, 2006), we investigated whether nisin A was able to induce *nso* gene expression using a strain with the NisRK two-component regulatory system integrated into the chromosome. This increased production to levels high enough to identify antimicrobial activity, as long as trypsin was present in the overlaying agar, presumably to release the active peptide from the leader sequence. Given that the full peptide is expected to be only a small fraction of the peptides generated by trypsin digestion, the resultant activity is impressive and suggests that further understanding and production of this lantibiotic could provide a novel weapon against clostridial pathogens. Future production of mature peptides may allow us to test whether, like nisin A, the system is self-regulating and can be induced in the original host strain to produce the native modified peptide.

In this work screening of gut bacterial isolates for lantibiotic biosynthetic genes revealed a novel lantibiotic cluster from *B. obeum* with four novel structural peptides and an unusual leader peptide sequence. Cross-immunity of the nisin O cluster to nisin A was demonstrated and heterologous expression of the novel cluster with the structural peptides on a nisin A inducible system showed evidence of antimicrobial activity against pathogens *C. perfringens* and *C. difficile* in the presence of trypsin. Further work on the regulation of this novel cluster and its spectrum of antimicrobial activity will expand our understanding of the evolution of type I lantibiotics and may lead to the development of novel antimicrobials to target gut pathogens.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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735

736 Table 1. Strains and plasmids used in this study

Strains	**Relevant characteristics**	**Reference/ source**
L. lactis MG1614	*L. lactis* subsp. lactis 712 cured of plasmids and prophage	(Gasson, 1984)
L. lactis FI5876	MG1614 with the nisin A biosynthetic cluster	(Dodd *et al.*, 1990)
L. lactis FI5876 *ΔnisA*	Part of *nisA* deleted (FI7847)	(Dodd *et al.*, 1996)
L. lactis FI5876 *ΔnisP*	*nisP* deleted (FI8438)	A. Narbad
L. lactis FI5876 *ΔnisC*	*nisC* deleted (FI8531)	A. Narbad
L. lactis FI5876 *ΔnisCP*	*nisC* and *nisP* deleted (FI8532)	A. Narbad
L. lactis FI5876 *ΔnisB*	*nisB* deleted (FI8620)	(Sen *et al.*, 1999)
L. lactis UKLc10	*nisRK* genes integrated on the chromosome	(Wegmann *et al.*, 1999)
B. obeum A2-162	Genome mining strain isolated from human GI tract	S. Duncan
C. perfringens NCTC 3110	Indicator strain	National Collection of Type Cultures
C. difficile NCTC 11204	Indicator strain	National Collection of Type Cultures
E. coli MC1022	Shuttle vector cloning strain	(Casadaban and Cohen, 1980)
Plasmids		
pIL253	Erythromycin resistance	(Simon and Chopin, 1988)
pJAZZ-OC	Chloramphenicol resistance	(Lucigen Corp, USA)
pUK200	Chloramphenicol resistance	(Wegmann *et al.*, 1999)
p*nisA*^{L}*-nsoA4*	pUK200 with the nisin leader peptide DNA sequence followed by the *nsoA4* DNA sequence under the control of the *nisA* promoter	This study
p*nisA*^{L}*-nsoA1IE*	pUK200 with the nisin leader peptide DNA sequence followed by the *nsoA1IE* DNA sequence under the control of the *nisA* promoter	This study
p*nisA*^{L}*-nsoA1YK*	pUK200 with the nisin leader peptide DNA sequence followed by the *nsoA1YK* DNA	This study

	sequence under the control of the *nisA* promoter	
p*nso*	nisin O lantibiotic cluster in pIL253	This study
p*nsoΔnsoA*	nisin O lantibiotic cluster in pIL253 with the *nsoA* genes deleted	This study
pFI2596	Nisin inducible vector based on pTG262 engineered to contain the *nisA* promoter and RBS sequences followed by genes encoding the mIL-12 p40 and p35 subunits	(Fernandez *et al*., 2009)
pTG262Pn	pFI2596 with mIL-12 removed, empty control vector	This study
pTG*nsoA1*	pTG262Pn with *nsoA1* under the control of the *nisA* promoter	This study
pTG*nsoA2*	pTG262Pn with under the control of the *nisA* promoter	This study
pTG*nsoA3-nsoA4*	pTG262Pn with *nsoA3* and *nsoA4* under the control of the *nisA* promoter	This study
pTG*nsoI*	pTG262Pn with *nsoI* under the control of the *nisA* promoter	This study
pTG*nisI*	pTG262Pn with *nisI* under the control of the *nisA* promoter	This study
pTG*nisA*	pTG262Pn with *nisA* under the control of the *nisA* promoter	This study

Figure legends

Figure 1 (a) Organisation of the nisin O lantibiotic cluster and surrounding ORFs from the library clone; (b) Amino acid sequence alignment of the translated *nsoA1*, *nsoA2*, *nsoA3* and *nsoA4* genes to subtilin (SpaA, P10946), nisin Z (CAA79467), nisin U (ABA00878), nisin U2 (ADB43138), nisin F (ABU45463), nisin A (AAA25188), nisin Q (BAG71479), nisin P (BAK30164), and nisin H (AKB95119). Blue, mid blue, light blue and white correspond to conservation of 100%, 80%, 60% and <40% respectively; (c) Similarity of the nisin O lantibiotic cluster and with other lantibiotic clusters from nisin U (DQ146939), nisin A (HM219853), nisin Q (AB362350) and nisin H (KP793707); *, transposase or insertion element sequence.

Figure 2 Antimicrobial activity. Overlay assays of *B. obeum* A2-162 after 6 d (top) or 7 d growth (bottom), grown on solid medium or solid media supplemented with 50 μg/ml trypsin and overlaid with *C. perfringens*.

Figure 3 Effect of hybrid genes on growth and phenotype. **(**a) Effect of hybrid constructs on the growth of *L. lactis*. X, FI5876, ●, FI5876Δ*nisA* p*nisA*^{L}*-nsoA1IE*, ▲, FI5876Δ*nisA* p*nisA*^{L}*-nsoA1YK*, ♦, FI5876Δ*nisA* p*nisA*^{L}*-nsoA4*, □, FI5876Δ*nisA* pUK200. Results are the mean of triplicate measurements +/- SD. (b) SEM (left) and TEM (right) analysis of the effect of hybrid construct expression on cell phenotype; bar, 1 µm; (c) Growth of FI5876Δ*nisA* (▲), FI5876Δ*nisP* (●), FI5876Δ*nisC* (♦) and FI5876Δ*nisB* (■) containing p*nisA*^{L}*-nsoA1YK* (closed symbols) or pUK200 (open symbols); X, FI5876. (d) Viability of stationary phase *L. lactis* FI5876 and knockout strains containing p*nisA*^{L}*-nsoA1YK* (red) or pUK200 (blue).

Figure 4 Expression of nisin leader hybrids in *L. lactis.* SDS-PAGE electrophoresis (top) and Western hybridisation (bottom) using the nisin A leader antibody. (a) Extracts from cells (lanes 1, 2, 4, 5) and TCA-precipitated culture supernatant (lane 3) from FI5876Δ*nisA* containing p*nisA*^{L}*-nsoA1IE* (lane 1), p*nisA*^{L}*-nsoA1YK* (lanes 2, 3), p*nisA*^{L}*-nsoA4* (lane 4) or pUK200 (lane 5); (b) Extracts from cells (lanes 1-4, 8) and TCA-precipitated culture supernatant (lanes 5-7) from FI5876 biosynthetic gene knockout strains containing either p*nisA*^{L}*-nsoA1YK* (lanes 1-7) or pUK200 (lane 8). Lanes 1 and 8, FI5876Δ*nisA*, lanes 2 and 5, FI5876Δ*nisB*, lanes 3 and 6, FI5876Δ*nisC*, lanes 4 and 7, FI5876Δ*nisCP*. M, marker.

Figure 5 Effect of nisin O genes on immunity to nisin. Growth of *L. lactis* strains FI5876 (X), and MG1614 with plasmids pIL253 (□), p*nso* (▲) or p*nso*Δ*nsoA* (●) in selective media supplemented with 1 µg/ml nisin A. Results are the mean of triplicate samples +/- SD

Figure 6 Heterologous expression of the *nso* cluster. SDS-PAGE analysis (top) and Western hybridisation (bottom) using the NsoA1 leader antibody. Comparison of *L. lactis* TCA-precipitated culture supernatant extracts from MG1614-p*nso*Δ*nsoA* (lanes 1 to 5) or MG1614-p*nso* (lanes 6-10) containing plasmids pTG262Pn (1, 6), pTG*nsoA1* (2, 7) pTG*nsoA2* (3, 8), pTG*nsoA3-nsoA4* (4, 5, 9, 10) and cell extracts from *B. obeum* A2-162 (11) and FI5876 (12); M, marker.

Figure 7 Western hybridisation using the NsoA1 leader antibody to detect pre-peptide production in UKLc10. Comparison of *L. lactis* TCA-precipitated culture supernatant extracts (lanes 1-5) or cell extracts (lanes 6-11) from UKLc10 (lanes 1, 2, 4-8, 10, 11) or MG1614 (lanes 3,9) containing plasmids p*nsoA*Δ*A* (lanes 1, 11), p*nsoA* (lanes 2, 6, 10), p*nsoA*Δ*A*, pTG*nsoA3-nsoA4* (lanes 3, 9), pIL253 (lanes 4, 8), p*nsoA* pTG*nsoA3-nsoA4* (lanes 5, 7). Samples were induced with nisin for 3 h except lane 6 (2 h). M, marker.

Figure 8 Antimicrobial activity in the presence of trypsin. Overlay assays of *L. lactis* strains grown on solid agar with $NaHCO_3$ and 10 ng/ml nisin, then overlaid with soft agar with or without trypsin and the indicator strain.