New Antimicrobials to Target Gut Pathogens

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Thesis

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Abstract

Nisin O is an antimicrobial peptide produced by the human gut bacterium *Blautia obeum* A2-162 which has antimicrobial activity against clinically relevant organisms, such as *Clostridium difficile* and *Clostridium perfringens*. The biosynthetic gene cluster of nisin O is unusual when compared to other nisin variants, with four putative structural peptides, two two-component regulatory systems and no leader peptide cleaving protease. This project aimed to investigate the regulation of this cluster and to search for the presence of a protease within the *B. obeum* A2-162 genome.

Various induction conditions previously used for antimicrobial production in *B. obeum* A2-162 were assessed, however no antimicrobial activity was observed. A previously designed heterologous nisin O expression system in *Lactococcus lactis* UKLc10 was optimised for nisin O production. A pan-genome analysis of 2094 *Lachnospiraceae* genomes was performed and identified six nisin O-like clusters across multiple genera, including *Blautia*, *Dorea* and *Ruminococcus*. Additional novel lantibiotic clusters were identified in *Pseudobutryvibrio* and *Ruminococcus* species. The *B. obeum* A2-162 genome was searched for candidate proteases, of which 10 were identified, with six cloned into inducible plasmids, and used in pre-Nisin O leader peptide cleavage assays. None of the proteases tested were able to cleave the NsoA1-3 leader peptide, however leader peptide cleavage and antimicrobial activity was observed in the presence of trypsin. The two two-component regulatory systems were constitutively expressed and used in a *pepI* gene reporter assay to assess their interaction with the predicted promoters. It was shown that the P*nsoR2K2* provided constitutive expression and that the P*nsoA* would only interact with the regulatory system from nisin A when induced with nisin A. No activity was seen with any other promoter-regulatory system interaction.

This project has identified a number of novel nisin O-like clusters. However, no candidate protease or nisin O regulatory system interaction could be elucidated.

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Abbreviations

Abbreviation	Extension
Abu	Aminobutyric acid
Ala	Alanine
Arg	Arginine
Asn	Asparagine
bp	Base pair
BHI + C	Brain Heart Infusion with complements
BPM	Brain Heart Infusion with complements, Postgate C and de Man-Rogosa-Sharpe media (1:1:1 ratio)
BSA	Bovine Serum Albumin
CFE	Cell free extract
CTAB	Hexadecyltrimethyl ammonium bromide
CWE	Cell wall extract
Cys	Cysteine
Dha	Dehydroalanine
Dhb	Dehydrobutyrine
DNA	Deoxyribonucleic acid
Ery	Erythromycin
Gly	Glycine
His	Histidine
hr	Hour
Ile	Isoleucine
IPTG	Isopropylthio-β-galactoside
kb	Kilobase
kDa	Kilodalton
LAB	Lactic acid bacteria
Lan	Lantibiotic
Leu	Leucine
Lys	Lysine
Met	Methionine
min	Minute
MRS	de Man-Rogosa-Sharpe
Nso	Nisin O
OD_{600}	Optical density at 600 nm
PBS	Phosphate buffered saline

PCR	Polymerase chain reaction
Pro	Proline
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT-PCR	Reverse transcription polymerase chain reaction
sec	Second
SEM	Scanning electron microscopy
Ser	Serine
TCA	Trichloroacetic acid
TCS	Two-component regulatory system
TEM	Transmission electron microscopy
Thr	Threonine
Tm	Melting temperature
Val	Valine
vs	Versus
UPH_2O	Ultra-pure water
UV	Ultraviolet

Symbols and Units

Symbol	Meaning
%	Percentage
+	Plus/with
^{o}C	Degrees celsius
bp	Base pairs
cm	Centimetre
g	Gram
hr	Hour
kb	Kilo base
l	Litre
µg/ml	Microgram per millilitre
µl	Microlitre
µm	Micrometre
M	Molar
mg	Milligram
mg/ml	Milligram per millilitre
min	Minute
ml	Millilitre
mm	Millimetre
mM	Nanomolar
ng/ml	Nanogram per millilitre
nmol/mg/min	Nanomole per milligram of protein per minute
nm	Nanometre
pH	Potential of hydrogen
rpm	Revolutions per minute
sec	Second
T0	0 hour timepoint
T24	24 hour timepoint
X g	Gravity
v/v	Volume per volume
w/v	Weight per volume

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Chapter 1.

Introduction

1.1 Antimicrobials and Antimicrobial Resistance

Antimicrobial proteins are produced by all living organisms (Boparai and Sharma 2020). These antagonistic peptides can kill or inhibit the growth of microorganisms and aid in the prevention of infections or competition from other bacteria. With the rise of antimicrobial resistance increasing the burden on worldwide healthcare systems, the identification and production of novel antimicrobials to combat infections is a fundamental part of reversing this current issue (Piddock 2012, Garcia-Gutierrez, Mayer *et al*. 2019). However, the discovery of new antimicrobials is a significant challenge with only a small number of new antimicrobials introduced to the market since the 'Golden Era' of microbial discovery in the 1960s to 1970s (Lewis 2013).

1.2 Bacteriocins

Bacteriocins are ribosomally synthesised antimicrobial peptides produced by various bacterial genera which can undergo post-translational modifications (Cotter, Ross *et al*. 2013). Bacteriocins offer a potential solution to the problem of antimicrobial resistance, with these antimicrobial peptides generally showing many desirable traits for novel antimicrobial applications, including *in vivo* potency against pathogens (Kim, Becattini *et al*. 2019), low toxicity (Maher and McClean 2006), broad- and narrow-spectrum activity *(*Kuwano, Tanaka *et al*. 2005, Inoue, Tomita *et al*. 2006) and the potential to be produced by probiotic bacteria (Flynn, van Sinderen *et al*. 2002). Some bacteriocins are produced by lactic acid bacteria (LAB) and have received the generally regarded as safe (GRAS) designation, leading to their use in food products (Cotter, Hill *et al.* 2005). Based on the most recent update of bacteriocin classification, proposed by Alvarez-Sieiro *et al* (2016), the authors suggested three sub-classes of bacteriocins grouped according to their biosynthesis and activity, which has previously been used in the classification of bacteriocins (Arnison, Bibb *et al*. 2013, Cotter, Ross *et al*. 2013). Both classes I and II are small peptides (<10 kDa) that are heat stable, whereas class III bacteriocins are larger (>10 kDa) and susceptible to heat (Table 1.1). Class I bacteriocins differ from class II bacteriocins as they undergo post-translational modification however class

II peptides remain unmodified (Alvarez-Sieiro, Montalban-Lopez *et al*. 2016). Multiple sub-groups of bacteriocins are encompassed within each class which are grouped as a result of their structure.

Class I bacteriocins are collectively known as ribosomally synthesised post-translationally modified peptides (RiPPs). Modification enzymes interact with these antimicrobial peptides during biosynthesis which alters their final structure, this can include the formation of lanthionine rings, head-to-tail cyclisation and glycosylation (Knerr and van der Donk 2012, Wang, Oman *et al*. 2014). The biosynthesis of some class I bacteriocins have been studied in depth, for example the lantibiotic nisin A (Lubelski, Rink *et al*. 2008). Within this biosynthetic cluster all the genes that are required for the maturation, activation and regulation of nisin A are present with additional immunity genes. Class I bacteriocins are organised into six distinct families (Table 1.1) (Alvarez-Sieiro, Montalban-Lopez *et al*. 2016). Peptides belonging to each of these families were categorised based on the post-translation modification that is performed during biosynthesis.

Group	Examples	Reference
Class I **Heat-stable, <10 kDa, Modified**		
Lanthipeptides	Nisin A, Planosporicin, Marinsedin	(Rogers and Whittier 1928, Castiglione, Cavaletti *et al*. 2007, Repka, Chekan *et al*. 2017, Han, Wang *et al*. 2022)
Cyclised Peptides	Carnocyclin A, Pumilarin	(Martin-Visscher, van Belkum *et al*. 2008, van Heel, Montalban-Lopez *et al*. 2017)
Linear Azol(in)e-containing Peptides	Streptolysin S, Microcin B17	(Molloy, Cotter *et al*. 2011, Collin and Maxwell 2019)
Sactibiotics	Subtilosin A, Thuricin Z	(Babasaki, Takao *et al*. 1985, Mo, Ji *et al*. 2019)
Glycocins	Glycocin F, Enterocin F4-9	(Venugopal, Edwards *et al*. 2011, Maky, Ishibashi *et al*. 2021)

Lasso Peptides	Benenodin-1, Microcin Y	(Zong, Wu *et al*. 2017, Li, Han *et al*. 2021)
Linaridins	Grisemycin	(Claesen and Bibb 2011)
Class II **Heat-stable, <10 kDa, Unmodified**		
Pediocin-like	Pediocin PA-1, Bac43	*(Chikindas, Garcia-Garcera et al. 1993, Todokoro, Tomita et al*. 2006)
Two Peptide	Lactococcin G, Gassericin T	(Nissen-Meyer, Holo *et al*. 1992, Kawai, Saitoh *et al*. 2000)
Cyclic Peptide	Peptide AS-48	(Maqueda, Galvez *et al*. 2004)
Non pediocin-like	Leucocin B	(Wan, Saris *et al*. 2015)
Class III **Heat-labile, >10 kDa**		
Non-bacteriocin Lytic Peptides	Enterolysin A, Microcin S	(Nilsen, Nes *et al*. 2003, Beimfohr 2016)

Table 1.1. Classification of bacteriocins, including sub-groups and examples.

1.3 Lanthipeptides

Lanthipeptides are a group of class I bacteriocins that are characterised by the presence of unusual amino acids such as lanthionine and methyl-lanthionine which form lanthionine rings during post-translational modifications (Arnison, Bibb *et al*. 2013). There are four types of lanthipeptides which are determined by their biosynthetic machinery to produce the mature peptide (Knerr and van der Donk 2012). However, only types I and II have antimicrobial properties and are known as lantibiotics. Therefore, as types III and IV produced by LAB have no known antimicrobial characteristics they cannot be considered as bacteriocins.

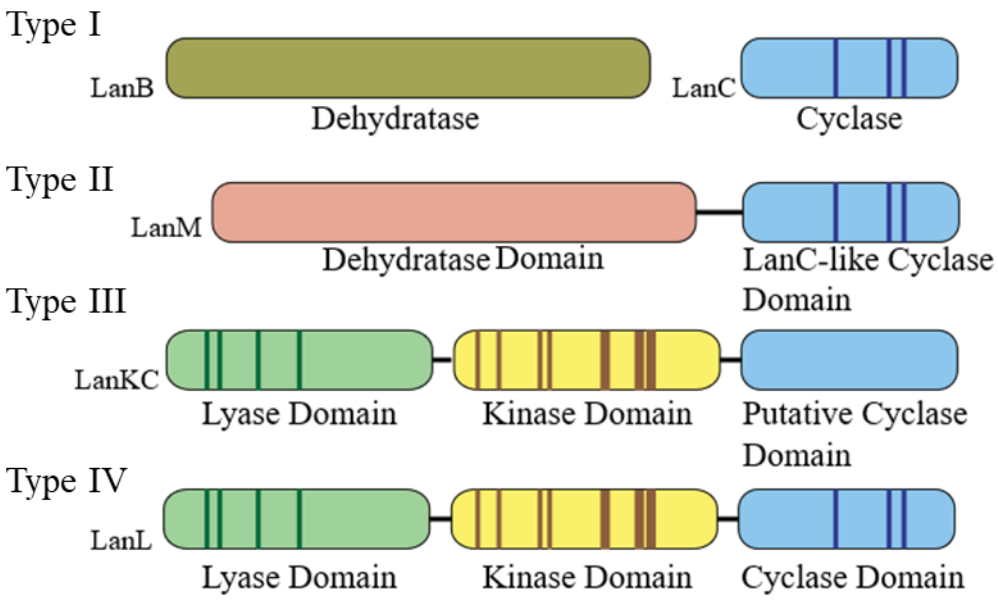


Figure 1.1. Enzymes involved in the construction of lanthionine rings during biosynthesis of type I-IV lanthipeptides. Conserved domains have been highlighted. Adapted from Knerr and van der Donk, 2012.

1.3.1 Type I

Type I lanthipeptides have two separate proteins involved in the formation of lanthionine rings, LanB which dehydrates serine (Ser) and threonine (Thr) residues and LanC which promotes cyclisation and ring formation (Figure 1.1) (Garg, Salazar-Ocampo *et al*. 2013, Yang and van der Donk 2015). Due to the similarity between biosynthetic systems that produce lantibiotics, genes within the cluster are given the generic name of *lan* (de Vos, Kuipers *et al*. 1995). LanB (Dehydratase) is an enzyme which converts Thr and Ser into dehydrobutyrine (Dhb) and dehydroalanine (Dha), respectively. This dehydration process is then followed by cyclisation of these residues with cysteine by LanC (Cyclase) to form lanthionine and methyl-lanthionine rings as part of the mature core peptide (Xie and van der Donk 2004).

1.3.2 Type II

Type II lanthipeptides utilise LanM for dehydration and cyclisation (Figure 1.1). This enzyme is bi-functional with the N-terminus of LanM functioning as a dehydratase which is responsible for the phosphorylation of Ser and Thr followed by removal of the phosphate group. Cyclisation and subsequent ring formation is performed by the C-terminus of LanM, which shares sequence homology with type I lanthipeptide cyclases, including zinc-binding domains (Li, Yu *et al*. 2006, Yu, Zhang *et al*. 2013).

1.3.3 Types III and IV

Both types III and IV lanthipeptides use a single bi-functional enzyme that performs both dehydration and cyclisation to produce mature core peptides. LanKC and LanL (class III and IV, respectively) contain an N-terminal lyase domain, a central kinase domain and a C-terminal cyclase domain (Figure 1.1) (Knerr and van der Donk 2012, Zhang, Yu *et al*. 2012).

1.4 Lantibiotic Operon Structure

The biosynthetic clusters usually contain a structural gene (*lanA*), modification machinery (*lanB* and *lanC*, *lanM* etc.) a transmembrane transporter (*lanT*), immunity proteins (*lanI*, *lanFEG* etc.), a protease (*lanP*) and a regulatory system (*lanRK*) (Dischinger, Basi Chipalu *et al*. 2014). Nisin A is one of the most studied bacteriocins, it is produced by *Lactococcus lactis* and has a broad range of activity against Gram-positive bacteria (Severina, Severin *et al*. 1998, Lubelski, Rink *et al*. 2008, Shin, Ateia *et al*. 2015). The cluster is organised into four operons: *nisABTCIPRK*, *nisI*, *nisRK* and *nisFEG*, each with a separate promoter. The *nisI* and *nisRK* operons are constitutively expressed, therefore providing the producer organism with a constant level of immunity and regulation of the biosynthesis cluster (de Ruyter, Kuipers *et al*. 1996). However, other lanthipeptides, such as the type II lanthipeptide ruminococcin A produced by the strict anaerobic bacterium *Ruminococcus gnavus* E1 have differences in their biosynthetic gene cluster (Dabard, Bridonneau *et al*. 2001). Within the ruminococcin A cluster the first 5 open reading frames (ORFs), *rumFEGHR2*, are arranged in a 3' to 5' direction comparative to the other genes in the cluster. The cluster also contains genes required for

regulation, *rumRK*, three genes encoding core peptides, *rumA1-3*, and the modification and transport machinery, *rumM* and *rumT*, respectively. An additional gene *rumX* is also found at the end of the cluster, however its function has not yet been elucidated (Gomez, Ladire *et al*. 2002). This shows that although the genes involved in modification, immunity and regulation of lantibiotics are present within different biosynthetic gene clusters, the structure of these clusters can be varied.

1.5 Nisin A

The aforementioned nisin A is a type I lantibiotic that is produced by *L. lactis* which was first identified by Rogers and Whittier in 1928 during a study investigating the lactic fermentation of milk cultures (Rogers and Whittier 1928). From 1953 nisin A was marketed as an antimicrobial agent in England and was recognised as a safe food additive by the World Health Organisation and the Joint Food and Agriculture Organisation in 1969 (Cotter, Hill *et al*. 2005, Shin, Gwak *et al*. 2016). Nisin A was added to the European food additive list as E234 in the 1980s and was also approved by the United States of America Food and Drug Agency to be used in selected cheese products (Cotter, Hill *et al*. 2005, Younes, Aggett *et al*. 2017). As this antimicrobial has shown its efficacy to prevent food spoilage and safety over approximately 100 years since its discovery, more than 50 countries use nisin A as a food additive (Shin, Gwak *et al*. 2016).

1.6 Biomedical Applications of Nisin

Not only is nisin A used as a food preservative but studies have shown that it can have potential applications in other fields. Nisin has been studied in the context of dentistry as an antimicrobial agent. In 2015, a study by Shin *et al*. (2015), showed that nisin was able to disrupt biofilms formed by multiple species which were isolated from saliva, additionally, there were no cytotoxic effects on human oral cells (Shin, Ateia *et al*. 2015). Furthermore, a recent study observed that both the use of nisin and the presence of the producer organism *L. lactis* significantly decreased the level of periodontal pathogens, the host inflammatory

response, alveolar bone loss and returned the oral microbiome to a healthy, control state (Gao, Kuraji *et al*. 2022).

Nisin has also been shown in a number of studies to have anti-cancer properties. Nisin has been shown to induce apoptosis of cervical, hepatic and skin cancer cell lines, with minimal cytotoxicity against healthy cell lines (Rana, Sharma *et al*. 2019, Zainodini, Hajizadeh *et al*. 2021, Sadri, Aghaei *et al*. 2022). Nisin and its variants have also been shown to be capable of combating drug resistant pathogens and gastrointestinal infections both individually and in combination with other antimicrobials (Severina, Severin *et al*. 1998, Alves, Albano *et al*. 2020, O'Reilly, O'Connor *et al*. 2022). Although nisin has been used for many decades only now is the true scale of potential applications of nisin being realised.

Additionally, nisin has been use in treatment for mastitis in both humans and dairy cattle (Cao, Wu *et al*. 2007, Fernández, Delgado *et al*. 2008). Fernández *et al* (2008), showed that application of a nisin containing solution to the nipple and mammary areola of women with staphylococcal mastitis significantly lowered the colony forming units compared to the control group and the clinical signs were no longer observed (Fernández, Delgado *et al*. 2008). In a recent study by Huang *et al* (2022), the authors showed that nisin Z inhibited the activation of ERK1/2 and p38 mitogen activated kinase pathway and decreased IL-10 in lipopolysaccharide induced MCF10A cells and promoted the blood milk barrier (Huang, Teng *et al*. 2022).

1.7 Nisin A Biosynthesis

The nisin A biosynthetic pathway is well understood due to its use as a food spoilage prevention molecule. The core peptide is encoded by *nisA* and when expressed forms a pre-peptide containing 57 amino acid residues, including the N-terminal leader peptide which consists of 23 amino acids (Gross and Morell 1971). This pre-nisin molecule is targeted to the modification machinery NisBTC, which recently has been isolated as a complete complex from the cytoplasmic membrane through affinity purification (Chen and Kuipers 2021). NisB, as previously mentioned, is responsible for dehydration of Ser and Thr residues to Dha and Dhb at positions 25, 26, 28, 31, 36, 46, 48 and 56 of pre-nisin (Figure 1.2). Inactivation of

nisB has been shown to give a complete loss of nisin post-translational modification, indicating NisB's fundamental role in the modification of pre-nisin (Koponen, Tolonen *et al*. 2002). Conversely, fourfold overexpression of *nisB* resulted in significantly increased dehydration at the Ser56 (Karakas Sen, Narbad *et al*. 1999). Although both Ser and Thr residues are present in the leader peptide these are not dehydrated, this is most likely due to the leader peptide's function in binding to NisB and therefore preventing interaction with the catalytic site (Lubelski, Rink *et al*. 2008). Once the residues are dehydrated the formation of five lanthionine rings is performed by NisC (Figure 1.2). Chromosomal deletion of *nisC* prevented the formation of these rings, however dehydration still occurred, indicating that the NisB can still function, albeit with a reduced efficiency, even in the absence of NisC from the NisBTC complex (Koponen, Tolonen *et al*. 2002). The leader peptide of pre-nisin is required for interaction with NisC, independent of maturation state. Mutagenesis of the leader peptide identified that phenylalanine (Phe) and leucine (Leu) at positions six and eight of pre-nisin, respectively, are essential for NisC binding (Abts, Montalban-Lopez *et al*. 2013). Following the formation of the lanthionine rings the modified pre-nisin export of the pre-peptide is performed by the ABC half-transporter, NisT (Figure 1.2). NisT only contains two nucleotide binding domains and two transmembrane segments rather than the usual four, thus labelling it an ABC half-transporter, however it is assumed that in order to function correctly NisT forms a homodimer (Lubelski, Rink *et al*. 2008). In deletion experiments to remove *nisT*, the relative abundance of nisin in the extracellular space was reduced and there was an accumulation of nisin within the cells. This subsequently reduced the growth rate of the bacteria by 30% (van den Berg van Saparoea, Bakkes *et al*. 2008). In a recent study by Lagedroste *et al* (2020), an ATP hydrolysis deficient mutant of NisT exported no nisin A and the authors found that NisB and NisC were required for secretion. Furthermore, it was observed that it was the creation of the lanthionine rings by NisC rather than the interaction of the enzyme with NisT that was required for transport, as catalytically inactive NisC mutants reduced the secretion level (Lagedroste, Reiners *et al*. 2020).

Once exported out of the cell mature pre-nisin has the leader peptide cleaved by NisP which leads to mature, active nisin in the extracellular environment (Figure 1.2). NisP contains 682 amino acids (54 kDa) and is a subtilisin-like protease (van der Meer, Polman *et al*. 1993, Siezen, Rollema *et al*. 1995). NisP is anchored to the cell wall by the LPXTG motif after NisP is exported to the extracellular side of the membrane by a Sec-signalling sequence (van der Meer, Polman *et al*. 1993). The specificity of NisP was shown to be slightly promiscuous, however through leader peptide mutagenesis it was shown that the arginine at position one of the leader peptide was crucial for cleavage (Montalban-Lopez, Deng *et al*. 2018). Furthermore, Lagedroste *et al* (2017), found substrate recognition by NisP was reliant on the presence of lanthionine rings within the core peptide (Lagedroste, Smits *et al*. 2017).

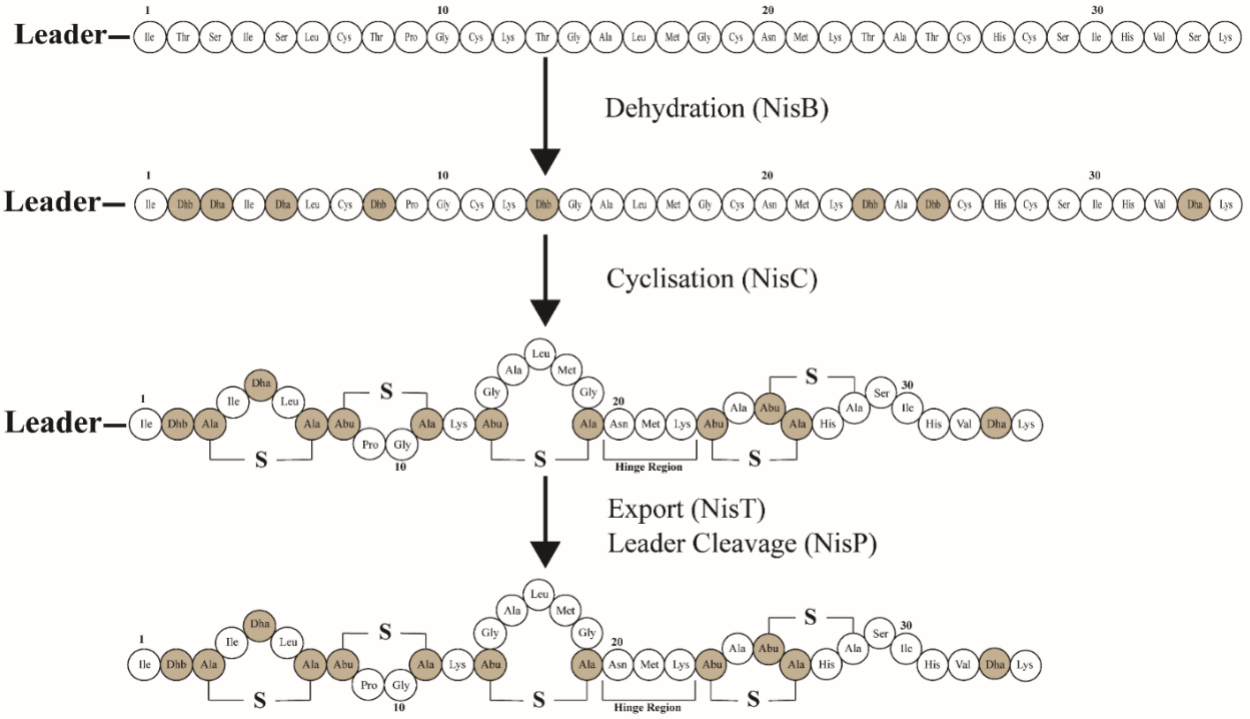


Figure 1.2. Maturation of pre-nisin to active nisin. NisB dehydrates Thr and Ser residues to Dhb and Dha, respectively. Cyclisation by NisC forms lanthionine and methyllanthionine rings to form mature pre-nisin. Secretion (NisT) and leader peptide cleavage (NisP) are responsible for the export and activation of the nisin peptide.

Once cleavage of the leader peptide has occurred nisin A becomes an active antimicrobial molecule. Therefore, to protect itself from the bioactive peptide, the biosynthetic cluster in *L. lactis* contains genes involved in immunity. These immunity genes consist of *nisI* and *nisFEG*

which encode a lipoprotein and ABC transporter, respectively (Figure 1.3) (Siegers and Entian 1995). NisI is located on the outer leaflet of the cytoplasmic membrane and is 25.8 kDa in size (Qiao, Immonen *et al*. 1995). NisI provides immunity through interactions with nisin to form an insoluble complex, whereas NisFEG is an efflux pump which can export any nisin A molecules that enter the cytoplasm (Stein, Heinzmann *et al*. 2003, Hacker, Christ *et al*. 2015). Deletions of either *nisFEG* or *nisI* increased the sensitivity of the producer strain to nisin, with a greater effect observed when *nisI* was absent (Siegers and Entian 1995, Draper, Ross *et al*. 2008).

The regulation of the nisin A cluster relies on the two-component regulatory system (TCS) NisRK (Figure 1.3 and 1.4). The *nisI* and *nisRK* operons are constitutively expressed, providing the producer organism with a constant level of immunity and regulation of the biosynthesis cluster (de Ruyter, Kuipers *et al*. 1996, Li and O'Sullivan 2006). However, both the *nisABTCIPRK* and *nisFEG* are regulated by the NisRK system via the promoters P*nisA* and P*nisF*, respectively (Figure 1.3). NisK is a histidine kinase that auto-phosphorylates when bound to the correct substrate, in this case the N-terminal rings A and B of nisin A (Kuipers, Beerthuyzen *et al*. 1995, Ge, Teng *et al*. 2016). Conserved residues in the extracellular domain of NisK are important for binding of nisin A as Ge *et al* (2017), demonstrated via mutations to key residues in NisK which inhibited binding of nisin A (Ge, Teng *et al*. 2017). After auto-phosphorylation of NisK the response regulator NisR interacts and becomes phosphorylated and then activates transcription of *nisABTCIPRK* and *nisFEG*. Van der Meer *et al* (1993), showed that when *nisR* expression was prevented then the expression of *nisA* was reduced, indicated that this protein was important for the production of nisin A (van der Meer, Polman *et al*. 1993). Therefore, the NisRK system is responsible for a positive feedback loop during the production of nisin A, as through quorum sensing any nisin A present in the extracellular environment will activate both the *nisABTCIPRK* operon to increase *nisA* transcription but also *nisFEG* which will increase immunity of the cell. This will therefore allow the *L. lactis* producer to remain active whilst simultaneously out-competing other bacteria for resources via its antimicrobial activity.

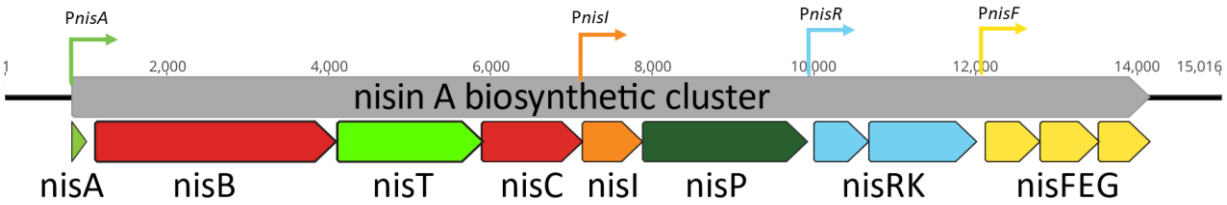


Figure 1.3. Nisin A biosynthetic cluster with promoters indicated above. The structural (Green), maturation and transport (Red and light green, respectively), immunity (Orange and yellow), regulatory (Blue) and leader peptide protease (Dark green) genes are indicated.

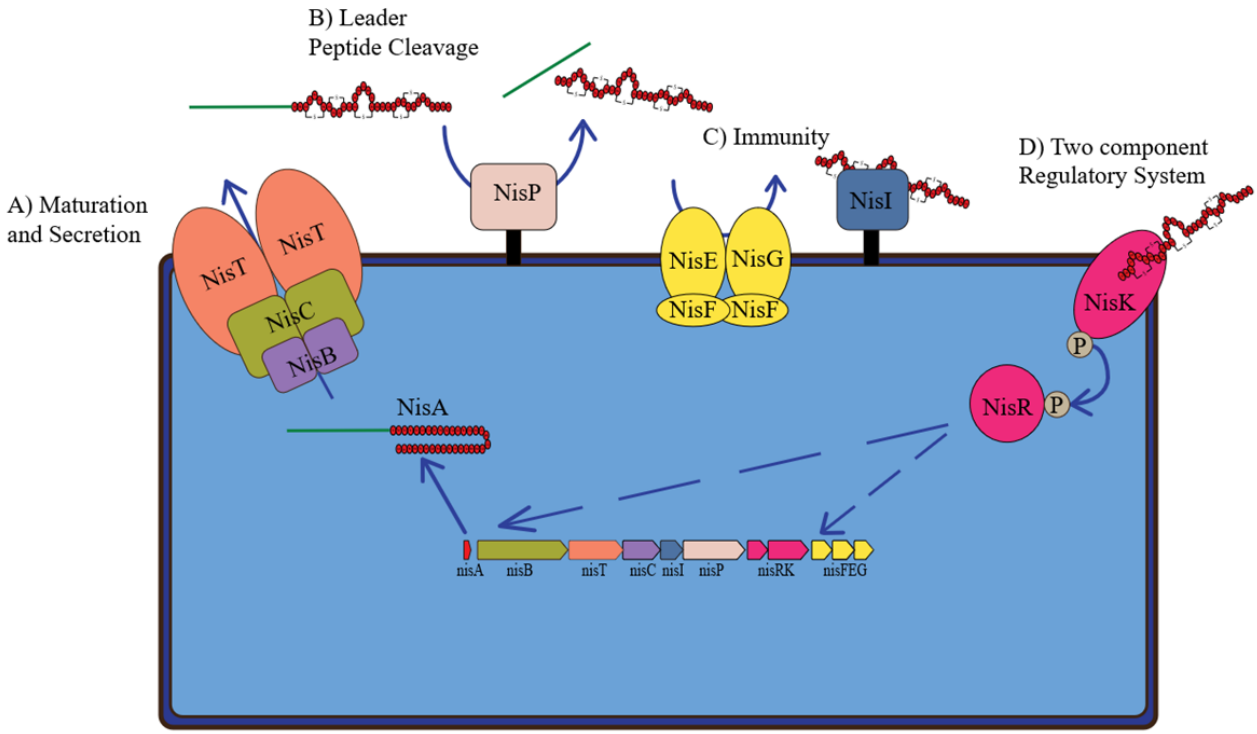


Figure 1.4. The nisin A gene cluster and the biosynthetic pathway to produce mature, active nisin A. Nisin A is initially translated to a pre-peptide which consists of an N-terminal leader peptide and a C-terminal core peptide. A) NisB dehydrates residues on the pre-peptide, followed by cyclisation through NisC and transport across the membrane by NisT; B) Cleavage of the leader peptide by the protease NisP; C) Immunity is provided by NisI, and the efflux pump NisFEG; D) Regulation of nisin A expression is controlled by the histidine kinase NisK and the response regulator NisR. Adapted from Perez, Zendo and Sonomoto (2014).

1.8 Nisin A's Mode of action

Due to nisin A's potent antimicrobial activity against Gram-positive bacteria, such as *Staphylococcus aureus*, *Clostridium difficile*, *Streptococcus pneumoniae* and Enterococci, multiple studies have been carried out to understand the mode of action of this antimicrobial (Goldstein, Wei *et al*. 1998, Severina, Severin *et al*. 1998, Bartoloni, Mantella *et al*. 2004, Piper, Draper *et al*. 2009). Nisin A causes cell death by binding to lipid II, which is vital for cell wall production as a peptidoglycan precursor, and therefore causes pore formation in the cell membrane (Figure 1.5) (Scherer, Spille *et al*. 2015). The pore formation involves four lipid II and eight nisin molecules (Hasper, de Kruijff *et al*. 2004).

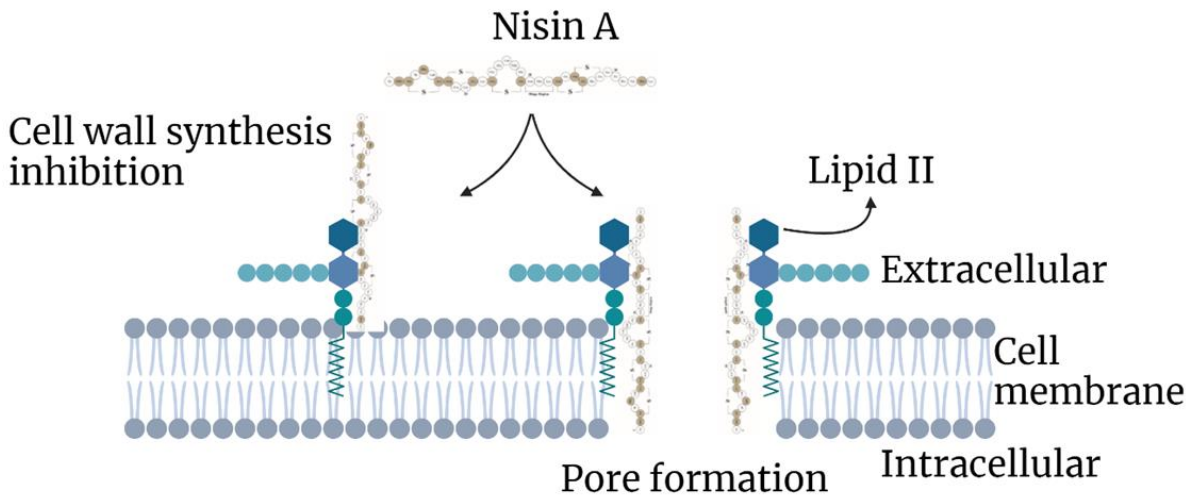


Figure 1.5. Nisin A mode of action either by pore formation or inhibition of cell wall synthesis after binding to lipid II. Adapted from Gharsallaoui *et al*., (2016).

Furthermore, nisin A can also prevent cell wall synthesis and thus disrupt the integrity of the membrane through segregation and loss of lipid II (Figure 1.5) (Reisinger, Seidel *et al*. 1980, Bauer and Dicks 2005). In 2001 a study was performed to investigate nisin A's interaction with lipid II through the creation of nisin A mutants (Wiedemann, Breukink *et al*. 2001). The authors observed that even when mutant nisin did not form pores in the cell membrane antimicrobial effects were still seen, indicating an alternative antimicrobial mode of action of the nisin A molecule. Additionally, the authors also showed that the N-terminus of the nisin A molecule was required for initial binding to lipid II. The C-terminus was assumed to then

translocate across the membrane as a result of the hinge region found within nisin A which was vital for pore formation (Figure 1.5 and 1.6) (Wiedemann, Breukink *et al*. 2001). The mode of action of nisin A to bind and/or disrupt lipid II in the cell membrane leads to increased permeability of the membrane and therefore disruption of membrane potential which causes bactericidal effects (Brotz, Josten *et al*. 1998). In 2016, Prince *et al* (2016), showed a novel mechanism of nisin A binding to lipid II whereby oligomerisation and peptide crowding lead to distortion of the membrane and consequent cell death (Prince, Sandhu *et al*. 2016).

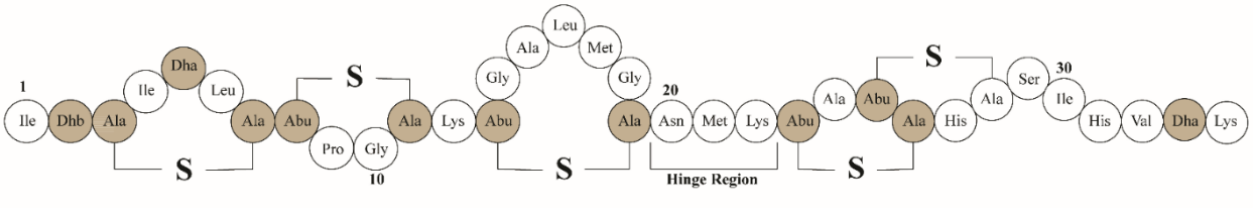


Figure 1.6. Structure of the mature nisin peptide. Highlighted amino acids are involved in the formation of the lanthionine rings. Dha, dehydroalanine; Dhb, dehydrobutyrine; Ala-S-Ala, lanthionine; Abu-S-Ala, β-methyllanthionine.

1.9 Nisin Variants

Since the first description of nisin A in 1928 there have been many nisin variants which share a high homology to that originally discovered nisin, which is still used as a 'standard' when comparing variants and other lantibiotics. Nisin Z is the most similar variant with a single residue change at position 27 of the core peptide from asparagine to a histidine. This variant was isolated from *L. lactis* NIZO22186 by Mulders *et al*., in 1991 and has antimicrobial effects against *Clostridium*, *Listeria* and *Bacillus* spp. (Mulders, Boerrigter *et al*. 1991, de Vos, Mulders *et al*. 1993). *L. lactis* strains also produce nisin F and nisin Q which differ from nisin A by two (positions 27 and 30) and four (positions 15, 21, 27 and 30) residue substitutions of the core peptide, respectively (Zendo, Fukao *et al*. 2003, de Kwaadsteniet, Ten Doeschate *et al*. 2008). However, nisin variants are not limited to *L. lactis* species. A number of nisin variants have been identified from *Streptococcus* species, these include nisin U/U2, nisin H and nisin P which are produced by *S. uberis*, *S. hyointestinalis* and *S. gallolyticus* subsp. *pateuranus*, respectively (Wirawan, Klesse *et al*. 2006, Zhang, Yu *et al*. 2012, O'Connor,

O'Shea *et al*. 2015). A recent study has identified a nisin J produced by *Staphylococcus capitis* APC 2923 which has antimicrobial activity against methicillin resistant *Staphylococcus aureus* (MRSA) and *Cutibacterium acnes*. Furthermore, this cluster does not contain a TCS or the nisin immunity gene *nisI* (O'Sullivan, O'Connor *et al*. 2020). Nisin P which was previously identified by O'Connor *et al*., in the genome of *S. gallolyticus* subsp. *pateuranus* was recently shown to be actively produced by faecal isolate *Streptococcus agalactiae* DPC7040 and had antimicrobial activity, although lower than nisin A and nisin H (O'Connor, O'Shea *et al*. 2015, Garcia-Gutierrez, O'Connor *et al*. 2020).

1.10 Nisin O

In addition to nisin variants being identified in streptococcal and staphylococcal species a nisin variant, nisin O, was identified in human gut bacterium *Blautia obeum* A2-162 (Hatziioanou, Gherghisan-Filip *et al*. 2017). Although the biosynthetic gene cluster consists of 'standard' immunity and modification genes (*nsoF*, *nsoE*, *nsoG*, *nsoI*, *nsoB*, *nsoT*, *nsoC*) observed in other nisin variant clusters (Figure 1.7), this cluster is unusual as it contains four putative structural genes, the first three of sharing very high homology, with the NsoA1 and NsoA2 being identical and NsoA3 having a single G2A substitution. The fourth peptide, NsoA4, is very dissimilar in its amino acid sequence when compared to the others within the cluster and other nisin variants.

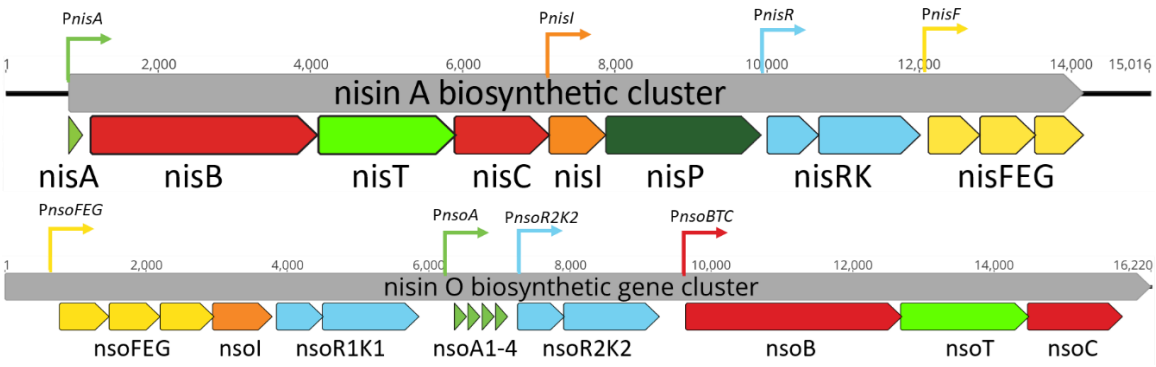


Figure 1.7. Comparison of the nisin A and nisin O biosynthetic clusters. Promoters and predicted promoters are indicated above the nisin A and nisin O clusters, respectively (Gherghisan-Filip 2016). The structural (Green), maturation and transport (Red and light

green, respectively), immunity (Orange and yellow), regulatory (Blue) and leader peptide protease (Dark green) genes are indicated.

Comparisons between nisin variants show that NsoA1-3 have a high level of sequence homology to nisin U (Hatziioanou, Gherghisan-Filip *et al*. 2017). Additionally, unlike nisin A which contains a single TCS or nisin J which completely lacks any regulatory system the nisin O biosynthetic gene cluster contains two TCSs either side of the putative structural genes (Figure 1.7) (Hatziioanou, Gherghisan-Filip *et al*. 2017). Furthermore, the cluster in *B. obeum* A2-162 does not contain a protease which is required for leader peptide cleavage and therefore activation of the mature nisin O molecule. Antimicrobial activity against *Clostridium perfringens*, *C. difficile* and *L. lactis* strains has been observed in both the original producer *B. obeum* A2-162 and a heterologous expression system in *L. lactis* UKLc10. However, activity was only seen when trypsin was used in the solid media as there are trypsin cleavage sites at the end of the NsoA1-3 leader peptide (Hatziioanou, Gherghisan-Filip *et al*. 2017). Although trypsin has the ability to act a protease and remove the leader peptide it may also have an inducing function as observed by Gomez *et al*., in ruminococcin A produced by *R. gnavus* (Gomez, Ladire *et al*. 2002).

An additional study investigating the structure of nisin O demonstrated that fully dehydrated peptides preNsoA1, A2 and A3 were present, and also gave evidence of NsoA4 peptide production (Gherghisan-Filip, Saalbach *et al*. 2018). Furthermore, the authors observed that there was incomplete dehydration of the NsoA1-3 core peptide occurring, but analysis of ring formation showed that all five lanthionine rings were being created. However, experiments analysing the N- and C-termini identified that lanthionine rings A, B and C located at the N-terminus were not formed in the majority of the preNsoA1-3 core peptides. On the other hand, lanthionine rings C and D located at the C-terminus had a formation rate of 22% (Gherghisan-Filip, Saalbach *et al*. 2018). The authors determined that there was reduced efficiency of mature nisin O production due to incomplete dehydration and ring formation, however, the

nisin O molecules that were successfully modified and exported were able to confer an antimicrobial effect (Gherghisan-Filip, Saalbach *et al*. 2018).

In a recent study by Kim *et al* (2019), the authors identified a novel bacteriocin cluster within the genome of *Blautia producta* SCSK (Kim, Becattini *et al*. 2019). This lantibiotic was shown to reduce the growth of vancomycin resistant *Enterococcus faecium* (VRE) yet had minimal bactericidal effects on gut commensal bacteria in murine *in vivo* models. The germ-free mice were given faecal transplants which contained high concentrations of lantibiotic genes, which prevented establishment and subsequent infection from VRE, whereas mice given faecal samples with lower concentrations of this lantibiotic producing strain had a greater risk of VRE colonisation (Kim, Becattini *et al*. 2019). Not only was the cluster found within another *Blautia* species but the cluster to which the lantibiotic belongs is very similar to the biosynthetic gene cluster of nisin O, albeit with slight differences. The *B. producta* SCSK cluster contains five structural genes rather than the four of the nisin O cluster and contains a protease at the start of the cluster (Kim, Becattini *et al*. 2019). This demonstrates that a fully operational cluster with high similarities to the nisin O cluster is present in nature and has the capability to modulate the gut microbiome.

1.11 Human Gut Microbiota

The human gastrointestinal tract is an environment within the host body where dietary, microbial and host factors are able to interact with each other (Thursby and Juge 2017, Heintz-Buschart and Wilmes 2018). The microbial community that resides within the gut differs between hosts and includes bacterial, viral, eukaryotic and archaeal species, which are known collectively as the gut microbiota (Backhed, Ley *et al*. 2005, Passos and Moraes-Filho 2017).

Initial colonisation occurs during birth and alters over time as the diet and environment of the host changes (De Filippo, Cavalieri *et al*. 2010, Koenig, Spor *et al*. 2011, Backhed, Roswall *et al*. 2015, Rodriguez, Murphy *et al*. 2015, Hojsak, Benninga *et al*. 2022). Studies have shown that *Bacteroidetes*, *Firmicutes*, *Proteobacteria* and *Actinobacteria* are the most abundant bacterial phyla in the gut microbiota (Shapira 2016). The gut microbiota is responsible for a

number of functions important to human health including metabolism, growth and development and resistance to infection (Karczewski, Troost *et al*. 2010, LeBlanc, Milani *et al*. 2013, Louis and Flint 2017, Thursby and Juge 2017, Robertson, Manges *et al*. 2019). The gut microbiota have also been shown to influence brain function through production of lipoproteins and lipopolysaccharides which in turn alter autoimmune function, leading to the release of cytokines which can cross the blood brain barrier and hence alter behaviour and mood (Sampson and Mazmanian 2015).

However, if dysbiosis of the gut microbiota occurs there are a number of potential impacts on the host's health. These alterations to the composition of the microbiota have been associated with an increased risk of gastrointestinal diseases such as irritable bowel syndrome and inflammatory bowel disease (Vich Vila, Imhann *et al*. 2018, Glassner, Abraham *et al*. 2020, Staudacher, Scholz *et al*. 2021). Furthermore, the pathophysiology of obesity has been shown to be impacted by the gut microbiota and the microbiota should be considered an important factor in addition to diet, lifestyle and host genomics (Turnbaugh, Ley *et al*. 2006). Conditions such as cardiovascular disease, host vs graft disease, oncological disease and neural diseases such as mood disorders and Alzheimer's disease have also been associated with changes to the microbiota composition (Kostic, Gevers *et al*. 2012, Kelly, Borre *et al*. 2016, Tang, Kitai *et al*. 2017, Fredricks 2019, Qian, Xie *et al*. 2022). Therefore, the gut microbiota is a major factor in human health and disease and the modulation of this microbial composition is fundamental to decrease the risk of dysbiosis and potential negative health effects.

Recent studies have focused on how bacteriocins can perform microbiome-shaping roles (Garcia-Gutierrez, Mayer *et al*. 2019). The production of these antimicrobial peptides have been suggested to aid in facilitating community invasion through clearing niches, preventing invasion from colonising bacteria and promoting community differentiation (Heilbronner, Krismer *et al*. 2021). It has been observed that the human pathogen *Salmonella enterica* serovar Typhimurium strain SL1344, which produces colicin Ib, had a competitive advantage over commensal *Escherichia coli* during an inflammation-inflicted bloom of

Enterobacteriaceae (Nedialkova, Denzler *et al*. 2014). Furthermore, Kim *et al*, (2019) showed that *B. producta* SCSK produced a lantibiotic which provided resistance against VRE. The effects of purified nisin A on *C. difficile* were assessed using a *ex vivo* model of the colon, which showed that *C. difficile* was not viable in the presence of 50-500 μM nisin A (O'Reilly, O'Connor *et al*. 2022). Furthermore, the authors showed that there was a relative increase in the abundance of Gram-negative bacteria (O'Reilly, O'Connor *et al*. 2022). Therefore, not only can the gut microbiome be a source of novel antimicrobials but they can also influence the composition of the microbiota.

1.12 Aims of the Thesis

With the world facing a crisis in the development of new antimicrobials it is important to identify antimicrobials that are active against clinically relevant pathogens. Nisin O has been shown to be active against *C. perfringens* and *C. difficile* therefore further understanding of how this antimicrobial is produced is important to understanding its role in the human gut environment and the potential for this bacteriocin to be produced for clinical use.

This thesis aimed to induce expression of nisin O from *B. obeum* A2-162 and assess under what conditions nisin O has increased expression. Since the original activity of nisin O being observed from *B. obeum* A2-162 there has been multiple attempts to observe antimicrobial activity that have been unsuccessful (Gherghisan-Filip 2016). We hypothesised that through co-culturing *B. obeum* A2-162 with bacteria found within the gut, expression of nisin O will be regained and antimicrobial activity will be observed. To test this, co-culturing assays in an *in vitro* fermentation model, competition assays and nisin A exposure assays were used to attempt induce nisin O production from *B. obeum* A2-162.

As *B. obeum* A2-162 is present in the human gut environment an investigation to study how widespread the biosynthetic gene cluster was in other bacteria was performed, as horizontal gene transfer would be possible in this environment. We hypothesised that there will be other *Blautia* species that contain the nisin O biosynthetic cluster. A pan-genome analysis was used on *Lachnospiraceae* genomes to observe how widespread the nisin O biosynthetic cluster is

found. This allowed us to see whether the cluster is identical in all cases or whether there were any differences between clusters within the family, as seen in the *B. obeum* A2-162 and *B. producta* SCSK lantibiotic clusters (Hatziioanou, Gherghisan-Filip *et al*. 2017, Kim, Becattini *et al*. 2019).

Furthermore, we aimed to investigate the regulation of the nisin O cluster, by looking at the interactions between the two TCSs and the previously identified predicted promoters of the cluster, as these interactions are currently unknown (Gherghisan-Filip 2016). These assays aimed to understand how the cluster is regulated and how this could be manipulated to produce nisin O at a greater level. The investigation's hypothesis was that the NsoR1K1 and NsoR2K2 regulatory systems would interact with at least one of the predicted promoters within the nisin O biosynthetic gene cluster. This will provide information on which of the regulatory systems can increase the expression of nisin O.

The nisin O biosynthetic gene cluster does not contain a protease required for leader peptide cleavage. Therefore, identification of potential candidate proteases within the *B. obeum* A2-162 genome was important to understand how this antimicrobial molecule can become active in the gut environment. In this set of experiments, we hypothesised that *B. obeum* A2-162 contains a protease within its genome capable of cleaving the leader peptide of nisin O. Proteases identified through genome searches were cloned into expression plasmids and their capability to cleave the leader peptide of nisin O was assessed through nisin O cleavage assays and co-expression in the *L. lactis* heterologous expression system.

Additionally, we aimed to assess the effect the effect nisin O has on the composition of the gut microbiota using a human gut model system. We used both *B. obeum* A2-162 and the *L. lactis* heterologous expression system in a MicroMatrix *in vitro* gut model system. We hypothesised that nisin O producing strains will decrease the number of Gram-positive bacteria in the sample.

Chapter 2.

General Materials and Methods

Culture Media

All chemicals were purchased from Sigma unless stated otherwise.

M17 media (Oxoid), Brain Heart Infusion (BHI) (Oxoid), de Man-Rogosa-Sharpe (MRS) (Oxoid) were all made from powder as per manufacturer's instructions. For GM17, glucose was added at 5 g/l to M17 prior to autoclaving. BHI plus complement was made by the addition of 50 mg/l vitamin K, 1 mg/l resazurin, 5 mg/l hemin and 0.5 g/l L-cysteine to BHI media before autoclaving. Cooked Double Meat Broth (Robertson's) was purchased from Southern Group Laboratory, UK. Postgate C media was made as per Zdanowski *et al*., (2017) ($NaC_3H_5O_3$ 6 g/l, Na_2SO_4 4.5 g/l, NH_4Cl 1 g/l, BD yeast extract 1 g/l, K_2HPO_4 0.5 g/l, $HOC(COONa)(CH_2COONa)_2.2H_2O$ 0.3 g/l, $MgSO_4.7H_2O$ 0.06 g/l, $FeSO_4.7H_2O$ 1 ml/l (0.4 g in 100 ml, filter sterilised), $CaCl_2.2H_2O$ 0.04 g/l, cysteine.HCl 0.5 g/l, 0.02% resazurin 4 ml/l). A mixed media consisting of BHI plus complement, Postgate C and MRS (BPM) was created for the growth of humanisation bacterial strains (Becker, Kunath *et al*. 2011). This media was formed in a 1:1:1 ratio of these pre-made media under sterile conditions. Luria (L) Broth (BD tryptone 10 g/l, BD yeast extract 5 g/l, NaCl 5 g/l, D-glucose 1 g/l). For MicroMatrix assays Colon Model Media and Complex Intestinal Media were tested. Colon Model Media was made as per Parmanand *et al* (2019) (Peptone water 2 g/l, yeast extract 2 g/l, NaCl 0.1 g/l, K_2HPO_4 0.04 g/l, KH_2PO_4 0.04 g/l, $MgSO_4.7H_2O$ 0.01 g/l, $CaCl_2.2H_2O$ 0.01 g/l, $NaHCO_3$ 2 g/l, L-cysteine.HCl 0.5 g/l, bile salts 0.5 g/l, tween-80 2 ml/l, hemin 0.02 g/l, vitamin K 10 µl/l, glucose 10 g/l, pH 7.0) and Complex Intestinal Media was made as per Krause *et al* (2020) (Arabinogalactan (Larch Wood) 2 g/l, bile salts 0.5 g/l, $CaCl_2.2H_2O$ 0.01 g/l, casein peptone (pancreatic) 4.3 g/l, K_2HPO_4 0.04 g/l, hemin 0.005 g/l, inulin 1 g/l, L-cysteine.HCl 0.5 g/l, $MgSO_4.7H_2O$ 0.02 g/l, vitamin K3 0.001 g/l, mucin (porcine gastric type II) 4 g/l, pectin 2 g/l, KH_2PO_4 0.04 g/l, NaCl 0.72 g/l, $NaHCO_3$ 2 g/l, starch 5 g/l, xylo-oligosaccharide 2 g/l, yeast extract 2 g/l, pH 6.7). Both media were autoclaved before use. Antibiotics and additional chemicals were supplemented after autoclaving under sterile conditions.

Solid media was made by the addition of 1.5% (w/v) agar to the media prior to autoclaving. When making GM17 plates the stock of 95 ml M17 agar was melted in a 95°C water bath followed by addition of 5 ml sterile 10% glucose, 0.2 g $NaHCO_3$ and relevant antibiotics. For soft agar 0.7% (w/v) agar was added prior to autoclaving. When antibiotics and other chemicals were added to the media, the solid media was melted using a 95°C water bath and allowed to cool to ~40°C before antibiotics were added.

All manipulations requiring anaerobic conditions were carried out in an anaerobic cabinet (Don Whitley, UK) with materials pre-reduced overnight in the cabinet in an atmosphere of 5% CO_2, 10% H_2 in N_2 at 37°C.

Trypsin (Sigma) stocks (10 mg/ml) were made by resuspending the trypsin in 50 mM acetic acid. Aliquots were stored at -20°C.

Nisin A (Merck) stocks were made at a concentration of 1 mg/ml in 50% diluted HCl pH 3.0 and filter sterilised using a 0.25 µm filter (Sartorius). Aliquots of 100 µg/ml were made by diluting in 1 M phosphate buffer pH 6.0. Aliquots were stored at -20°C

Table 2.1. Concentrations and preparation method of chemicals used.

Chemical	**Preparation**	**Final Concentration for *L. lactis* Strains**	**Final Concentration for *E. coli***
Erythromycin (Ery)	Dissolved in ethanol	5 µg/ml	100 µg/ml
Chloramphenicol (Chl)	Dissolved in ethanol	5 µg/ml	15 µg/ml
Ampicillin (Amp)	Dissolved in ultra pure (UP)H_2O; Filter sterilised	N/A	100 µg/ml
Nisin A	Dissolved in 50% HCl pH 3	10 ng/ml (Inducing)	N/A
Trypsin	Dissolved in 50 mM acetic acid	10 µg/ml (Cleaving)	N/A

Organisms used and growth conditions

Table 2.2. Bacterial Strains and Growth Conditions.

Strains	Relevant Characteristics	Storage	Growth Conditions	Reference/Source
B. obeum A2-162	Strain isolated from human GI tract*	40% glycerol	Reduced BHI with complements; 37ºC; Anaerobic.	(Hatziioanou, Gherghisan-Filip *et al*. 2017)
L. lactis MG1614	*L. lactis* subsp *lactis* 712 containing no plasmids or prophage	40% glycerol	GM17; 30ºC; Static.	(Gasson 1984)
L. lactis FI5876	*L. lactis* MG1614 containing nisin A lantibiotic cluster	40% glycerol	GM17; 30ºC; Static.	(Dodd, Horn *et al*. 1990)
L. lactis MG1614 pIL253	MG1614 containing pIl253 (Ery resistance)	40% glycerol	GM17; 30ºC; Static; 5 µg/ml erythromycin	This Study
L. lactis FI5876 pIL253	FI5876 containing pIl253(Ery resistance)	40% glycerol	GM17; 30ºC; Static; 5 µg/ml erythromycin	This Study
L. lactis UKLc10 p*nso*	*nisRK* integrated on the chromosome; Nisin O lantibiotic cluster in pIL253 (Ery resistance)	40% glycerol	GM17; 30ºC; Static; 5 µg/ml erythromycin	(Hatziioanou, Gherghisan-Filip *et al*. 2017)
L. lactis UKLc10 pIL253	*nisRK* integrated on the chromosome; containing pIL253 (Ery resistance)	40% glycerol	GM17; 30ºC; Static; 5 µg/ml erythromycin	(Gherghisan-Filip, Saalbach *et al*. 2018)
C. perfringens NCTC 3110	Indicator Strain	Robertson's Cooked Meat Media	Reduced BHI with complements; 37ºC; Anaerobic.	National Collection of Type Cultures

Escherichia coli MC1022	Shuttle vector cloning strain	40% glycerol	L broth; 37ºC; Shaking.	(Casadaban and Cohen 1980)
E. coli BL21 (DE3)	Chemically competent protein expression strain	40% glycerol	L broth; 37ºC; Shaking.	Invitrogen
Anaerostipes caccae DSM 14662	Potential competitor and humanisation strain	40% glycerol	Reduced BHI with complements/MRS mixed media (1:1); 37ºC; Anaerobic	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ)
Bacteroides thetaiotaomicron DSM 2079	Potential competitor and humanisation strain	40% glycerol	Reduced BHI with complements/MRS mixed media (1:1); 37ºC; Anaerobic	DSMZ
Blautia producta DSM 2950	Potential competitor and humanisation strain	40% glycerol	Reduced BHI with complements/MRS mixed media (1:1); 37ºC; Anaerobic	DSMZ
Lactobacillus plantarum DSM 20174	Potential competitor and humanisation strain	40% glycerol	Reduced BHI with complements/MRS mixed media (1:1); 37ºC; Anaerobic	DSMZ
E. coli K-12 MG1655	Potential competitor and humanisation strain	40% glycerol	Reduced BHI with complements/MRS mixed media (1:1); 37ºC; Anaerobic	DSMZ
Clostridium butyricum DSM 10702	Potential competitor and humanisation strain	40% glycerol	Reduced BHI with complements/MRS mixed media (1:1); 37ºC; Anaerobic	DSMZ
Clostridium ramosum DSM 1402	Potential competitor and humanisation strain	40% glycerol	Reduced BHI with complements/MRS mixed media (1:1); 37ºC; Anaerobic	DSMZ
Bifidobacterium longum DSMZ 20219	Potential competitor and humanisation strain	40% glycerol	Reduced BHI with complements/MRS mixed media (1:1); 37ºC; Anaerobic	DSMZ

*Kindly provided by Prof Harry Flint and Dr Sylvia Duncan, Rowett Institute, University of Aberdeen.

Table 2.3. Plasmids and relevant characteristics

Plasmids	Relevant Characteristics	Reference/Source
pIL253	*Lactococcus* plasmid; Erythromycin resistance	(Simon and Chopin 1988)
p*nso*	Nisin O lantibiotic cluster in pIL253	(Hatziioanou, Gherghisan-Filip *et al*. 2017)
pORI280	P32 promoter-containing plasmid containing *lacZ;* Erythromycin resistance	(Leenhouts, Kok *et al*. 1991)
pUK200	*E. coli*/*Lactococcus* shuttle vector; Chloramphenicol resistance	(Wegmann, Klein *et al*. 1999)
pUK200_P32	Shuttle vector with P32 promoter	This Study
pUK200_P32_*P62*	Candidate protease *P62* insertion and *Lactococcus* promoter P32 in pUK200	This Study
pUK200_P32_*P49*	Candidate protease *P49* insertion and *Lactococcus* promoter P32 in pUK200	This Study
pUK200_P32_*lanP*	*LanP* protease from *B. producta* cluster insertion (Kim, Becattini *et al*. 2019) and *Lactococcus* promoter P32 in pUK200	This Study
pUK200_P32_*P140*	Candidate protease *P140* insertion with *Lactococcus* promoter P32 in pUK200	This Study
pUK200_*R2K2*	*nsoR2K2* insertion in pUK200	(Gherghisan-Filip 2016)
pUK200_P32_*R1K1*	*nsoR1K1* insertion with *Lactococcus* promoter P32 in pUK200	This Study
pUK200_P32_*R2K2*	*nsoR2K2* insertion with *Lactococcus* promoter P32 in pUK200	This Study
pUK200_P32_*nsoA4_S24K*	*nsoA4_S24K* insertion with *Lactococcus* promoter P32 in pUK200	This Study
pTG262	*E. coli*/*Lactococcus* shuttle vector; Chloramphenicol resistance	(Fernandez, Horn *et al*. 2009)
pTG262_P32_*nsoR1A*	Modified *nsoR1* insertion with *Lactococcus* promoter P32 in pTG262	M. Mayer
pTG262_P32_*nsoR1AK1*	Modified *nsoR1* and *nsoK1* insertion with *Lactococcus* promoter P32 in pTG262	M. Mayer
p*PnsoA_pepI*	*pepI* with predicted promotor region P*nsoA* in pIL253	(Gherghisan-Filip 2016)
p*PnsoR2K2_pepI*	*pepI* with predicted promotor region P*nsoR2K2* in pIL253	(Gherghisan-Filip 2016)
p*PnsoBTC_pepI*	*pepI* with predicted promotor region P*nsoBTC* in pIL253	(Gherghisan-Filip 2016)
p*PnsoFEG_pepI*	*pepI* with predicted promotor region P*nsoFEG* in pIL253	(Gherghisan-Filip 2016)
pET15b	Expression vector; Contains N-terminal His-tag	Novogen
pET15b_*P66*	Candidate protease *P66* insertion in pET15b	This Study
pET15b_*P570*	Candidate protease *P570* insertion in pET15b	This Study

pET15b_*P49*	Candidate protease *P49* insertion in pET15b	This Study
pET15b_*P62*	Candidate protease *P62* insertion in pET15b	This Study
pET15b_*lanP*	Candidate protease *lanP* insertion in pET15b	This Study
pET15b_*P140*	Candidate protease *P140* insertion in pET15b	This Study

Table 2.4. Bacterial Transformants created and used in this work

Strains	Relevant Characteristics	Storage	Growth Conditions
L. lactis FI5876 pIL253	*L. lactis* MG1614 containing nisin A lantibiotic cluster containing pIL253 (Ery resistance)	40% glycerol	GM17; 30°C; Static; 5 µg/ml Ery
L. lactis UKLc10 p*nso* pUK200_P32	*nisRK* integrated on the chromosome; nisin O operon in pIL253; containing pUK200 with the P32 promoter (Ery and Chl resistance)	40% glycerol	GM17; 30°C; Static; 5 µg/ml Ery and 5 µg/ml Chl
L. lactis UKLc10 p*nso* pUK200_P32_*P62*	*nisRK* integrated on the chromosome; nisin O operon in pIL253; containing pUK200 with the P32 promoter and candidate protease *P62* (Ery and Chl resistance)	40% glycerol	GM17; 30°C; Static; 5 µg/ml Ery and 5 µg/ml Chl
L. lactis UKLc10 p*nso* pUK200_P32_*P49*	*nisRK* integrated on the chromosome; nisin O operon in pIL253; containing pUK200 with the P32 promoter and candidate protease *P49* (Ery and Chl resistance)	40% glycerol	GM17; 30°C; Static; 5 µg/ml Ery and 5 µg/ml Chl
L. lactis UKLc10 p*nso* pUK200_P32_*P140*	*nisRK* integrated on the chromosome; nisin O operon in pIL253; containing pUK200 with the P32 promoter and candidate protease *P140* (Ery and Chl resistance)	40% glycerol	GM17; 30°C; Static; 5 µg/ml Ery and 5 µg/ml Chl
L. lactis UKLc10 p*nso* pUK200_P32_*lanP*	*nisRK* integrated on the chromosome; nisin O operon in pIL253; containing pUK200 with the P32 promoter and candidate protease *lanP* (Ery and Chl resistance)	40% glycerol	GM17; 30°C; Static; 5 µg/ml Ery and 5 µg/ml Chl
L. lactis UKLc10 p*nso* pUK200_P32_*nsoA4*_S24K	*nisRK* integrated on the chromosome; nisin O operon	40% glycerol	GM17; 30°C; Static; 5

	in pIL253; containing pUK200 with the P32 promoter and candidate protease *P62* (Ery and Chl resistance)		µg/ml Ery and 5 µg/ml Chl
E. coli MC1022 pUK200_P32_R1K1	Shuttle cloning vector containing *nsoR1K1* insertion with *Lactococcus* promoter P32 in pUK200 (Chl resistance)	40% glycerol	L broth; 37ºC; Shaking; 15 µg/ml Chl
E. coli MC1022 pUK200_P32_R2K2	Shuttle cloning vector containing *nsoR2K2* insertion with *Lactococcus* promoter P32 in pUK200 (Chl resistance)	40% glycerol	L broth; 37ºC; Shaking; 15 µg/ml Chl
L. lactis MG1614 pP*closA_pepI* pUK200_P32_R1K1	*pepI* with predicted promotor region P*nsoA* in pIL253 and *nsoR1K1* insertion with *Lactococcus* promoter P32 in pUK200 with no *nisRK* system in the chromosome (Ery and Chl resistance)	40% glycerol	GM17; 30ºC; Static; 5 µg/ml Ery and 5 µg/ml Chl
L. lactis MG1614 pP*closA_pepI* pUK200_P32_R2K2	*pepI* with predicted promotor region P*nsoA* in pIL253 and *nsoR2K2* insertion with *Lactococcus* promoter P32 in pUK200 with no *nisRK* system in the chromosome (Ery and Chl resistance)	40% glycerol	GM17; 30ºC; Static; 5 µg/ml Ery and 5 µg/ml Chl
L. lactis MG1614 pP*closA_pepI* pUK200_P32	*pepI* with predicted promotor region P*nsoA* in pIL253 and *Lactococcus* promoter P32 in pUK200 with no *nisRK* system in the chromosome (Ery and Chl resistance)	40% glycerol	GM17; 30ºC; Static; 5 µg/ml Ery and 5 µg/ml Chl
L. lactis MG1614 pP*closFEG_pepI* pUK200_P32_R1K1	*pepI* with predicted promotor region P*nsoFEG* in pIL253 and *nsoR1K1* insertion with *Lactococcus* promoter P32 in pUK200 with no *nisRK* system in the chromosome (Ery and Chl resistance)	40% glycerol	GM17; 30ºC; Static; 5 µg/ml Ery and 5 µg/ml Chl
L. lactis MG1614 pP*closFEG_pepI* pUK200_P32_R2K2	*pepI* with predicted promotor region P*nsoFEG* in pIL253 and *nsoR2K2* insertion with *Lactococcus* promoter P32 in pUK200 with no *nisRK* system in the chromosome (Ery and Chl resistance)	40% glycerol	GM17; 30ºC; Static; 5 µg/ml Ery and 5 µg/ml Chl
L. lactis MG1614 pP*closFEG_pepI* pUK200_P32	*pepI* with predicted promotor region P*nsoFEG* in pIL253 and *Lactococcus* promoter P32 in pUK200 with no *nisRK* system in the	40% glycerol	GM17; 30ºC; Static; 5 µg/ml Ery and 5 µg/ml Chl

	chromosome (Ery and Chl resistance)		
L. lactis MG1614 pP*closBTC_pepI* pUK200_P32_R1K1	*pepI* with predicted promotor region P*nsoBTC* in pIL253 and *nsoR1K1* insertion with *Lactococcus* promoter P32 in pUK200 with no *nisRK* system in the chromosome (Ery and Chl resistance)	40% glycerol	GM17; 30ºC; Static; 5 µg/ml Ery and 5 µl/ml Chl
L. lactis MG1614 pP*closBTC_pepI* pUK200_P32_R2K2	*pepI* with predicted promotor region P*nsoBTC* in pIL253 and *nsoR2K2* insertion with *Lactococcus* promoter P32 in pUK200 with no *nisRK* system in the chromosome (Ery and Chl resistance)	40% glycerol	GM17; 30ºC; Static; 5 µg/ml Ery and 5 µg/ml Chl
L. lactis MG1614 pP*closBTC_pepI* pUK200_P32	*pepI* with predicted promotor region P*nsoBTC* in pIL253 and *Lactococcus* promoter P32 in pUK200 with no *nisRK* system in the chromosome (Ery and Chl resistance)	40% glycerol	GM17; 30ºC; Static; 5 µg/ml Ery and 5 µg/ml Chl
L. lactis MG1614 pP*closR2K2_pepI* pUK200_P32_R1K1	*pepI* with predicted promotor region P*nsoR2K2* in pIL253 and *nsoR1K1* insertion with *Lactococcus* promoter P32 in pUK200 with no *nisRK* system in the chromosome (Ery and Chl resistance)	40% glycerol	GM17; 30ºC; Static; 5 µg/ml Ery and 5 µg/ml Chl
L. lactis MG1614 pP*closR2K2_pepI* pUK200_P32_R2K2	*pepI* with predicted promotor region P*nsoR2K2* in pIL253 and *nsoR2K2* insertion with *Lactococcus* promoter P32 in pUK200 with no *nisRK* system in the chromosome (Ery and Chl resistance)	40% glycerol	GM17; 30ºC; Static; 5 µg/ml Ery and 5 µg/ml Chl
L. lactis MG1614 pP*closR2K2_pepI* pUK200_P32	*pepI* with predicted promotor region P*nsoR2K2* in pIL253 and *Lactococcus* promoter P32 in pUK200 with no *nisRK* system in the chromosome (Ery and Chl resistance)	40% glycerol	GM17; 30ºC; Static; 5 µg/ml Ery and 5 µg/ml Chl
E. coli BL21 (DE3) pET15b	Expression strain containing empty expression vector	40% glycerol	L broth; 37ºC; Shaking; 100 µg/ml Amp
E. coli BL21 (DE3) pET15b_*P66*	Expression strain containing expression vector with candidate protease *P66*	40% glycerol	L broth; 37ºC; Shaking; 100 µg/ml Amp
E. coli BL21 (DE3) pET15b_*P570*	Expression strain containing expression vector with candidate protease *P570*	40% glycerol	L broth; 37ºC; Shaking; 100 µg/ml Amp

E. coli BL21 (DE3) pET15b_*P49*	Expression strain containing expression vector with candidate protease *P49*	40% glycerol	L broth; 37°C; Shaking; 100 µg/ml Amp
E. coli BL21 (DE3) pET15b_*P62*	Expression strain containing expression vector with candidate protease *P62*	40% glycerol	L broth; 37°C; Shaking; 100 µg/ml Amp
E. coli BL21 (DE3) pET15b_*lanP*	Expression strain containing expression vector with candidate protease *lanP*	40% glycerol	L broth; 37°C; Shaking; 100 µg/ml Amp
E. coli BL21 (DE3) pET15b_*P140*	Expression strain containing expression vector with candidate protease *P140*	40% glycerol	L broth; 37°C; Shaking; 100 µg/ml Amp

Table 2.5. Primers used in this work

Name	Sequence (5' -> 3')
nsoC3_F (Hatziioanou 2011)	TGCGATATTTTAGCTATGACC
nsoF5_R (Hatziioanou 2011)	AATATCAGTTGACATTCACGG
pIL253_F (Hatziioanou 2011)	CGACAATGATTGTATTTGC
pIL253_R (Hatziioanou 2011)	TAGTTCTTGTGGTTACGTGG
Universal (Hatziioanou 2011)	GTTTTCCCAGTCACGACGTTGT
Reverse (Hatziioanou 2011)	AGCGGATAACAATTTCACACAGGA
p54 (Hatziioanou 2011)	CGGCTCTGATTAAATTCTGAAG
p181 (Hatziioanou 2011)	GCGAAGATAACAGTGACTCTA
Amp_F (Baker, Smith *et al*. 2003)	GAGAGTTTGATYCTGGCTCAG
Amp_R (Baker, Smith *et al*. 2003)	AAGGAGGTGATCCARCCGCA
P66NDE_F	GTATACATATGAATCAGCCATTTTATACTGG
P66XHO_R	CTGACTCGAGGATTTCCGTCAATACTTTC
P66CAC_F	AAAAAAATAGCACATGATGACAATGGAC
P66CAC_R	CATTGTCATCATGTGCTATTTTTTTATTT
P570NDE_F	CACCATATGAACGACGAAGAAAAATGG
P570XHO_R	GTAAACTCGAGTTTTTATAAATTCAGTCAGC
T7P2 (Cebeci 2017)	TGAGCGGATAACAATTCCC
T7T(Cebeci 2017)	GCTAGTTATTGCTCAGCGG
P32_F	TCGAGATCTACCAATTCGGTCCT
P32_R	CTTCCATGGCAAAATTCCTCCGAA
pUK_F (pUK200)	TGAGATAATGCCGACTGTACTT
P49_Splice32_F	GAATTTTGCCATGCGAATTGCGGAA
P49_PstI_R	CCCTGCAGTGCCATAATTTTCACT
pUK_BglII_F	CAAGATCTACCAATTCGGTCCTCGG
pUK_Splice49_R	CCGCAATTCGCATGGCAAAATTCCTC
P62_Splice32_F	GGAATTTTGCCATGAAAGCACTTCGT
P62_Splice32Ext_F	GAATTTTGCCATGAAAGCACTTCGTAATTT
P62_PstI_R	CCCTGCAGATACTCTCACCTTATCC
P62_BglIITGA_F	TATGTGCGGGTGATCTGCTGCTTTATTACA
P62_BglIITGA_R	TAATAAAGCATCAGATCACCCGCACATACC

pUK_Splice62_R	GAAGTGCTTTCATGGCAAAATTCCTC
P32_PstI_F	GCCTGCAGGTCGACCA
P32_SpliceR2K2_R	ACAAGTATATTTTCCATTTCAAAATTCCTCCG
R2K2_SpliceP32_F	AGGAATTTTGAAATGGAAAATATACTTGTGATT
P32_SpliceR1K1_R	ACTAACATCCACCATTTCAAAATTCCTCCG
R1K1_SpliceP32_F	AGGAATTTTGAAATGGTGGATGTTAGTAAAC
P32_PstF2	GTAAAACGACGGCCAGTGC
P32_R1_R	CGTGTCCTCTATTCTGTATCAGCAA
P32_K1 _F	CGTTGACGATCATAGGTGGT
P32_R2_R	TGTGCATTGGCATCTAAGTCATA
P32_K2_F	GGGCAAGATGGAGGAATACATT
P32_StuI_F (pUK200)	TCAGATAGGCCTAATGACTGG
P32_NcoI_R (pUK200)	TACCCATGGCAAAATTCCT
P32_R (pUK200)	ATGGCAAAATTCCTCCGAA
LanP_SpliceP32_F	GAGGAATTTTGAAATGCGCAAAAAGACCTAC
P32_BglII_F (pUK200)	CCAAGATCTACCAATTCGGTC
LanP_NcoI_R	TAGACGAGTCCATGGGCT
P32_SpliceLanP_R(2)	TCTTTTTGCGCATGGCAAAATTCCT
P32_BglII_F (pTG262)	GCAGATCTACCAATTCGGTCCT
P140_NcoI_F	TGACCATGGATATAAAAAATTGGAAAC
P140_BamHI_R	GCAGGATCCGCCTTATTTAGAAG
P140_Ext_F	TTCCTGAATCACATTCTGTAT
P140_Ext_R	TTAGCATGGAGAGTATAGTATAAT
P32_SpliceP140_R	ATTTTTTATATCCATGGCAAAATTCCTC
P140_SpliceP32_F	AGGAATTTTGCCATGGATATAAAAAATTGGA
nsoA4_NdeI_F	AGCCATATGGCAAAATTTGATGATT
nsoA4_splice_R	GTGTTGACTTGTAATTTTCGCAGGTATAAT
nsoA4_splice_F	GATATTATACCTGCGAAAATTACAAGTCAA
nsoA4_BamHI_R	CCATTATCGGATCCTTTATGTTTTTACTAACTAAT
nsoA4_NcoI_F	GCGCCATGGCAAAATTTGATGATTTTG
pclosFEG_R	GAAATTATCTCATGGATAAAATAACTC
pclosFEG_F	GTGACTAACACAATTAGTGG
pepI_R	TGCTCTCTGACATTTTCCA
nisR_F (UKLc10)	AGGAGTAAGTTTGCACCATA
nisK_R (UKLc10)	GAACGAAGCGTATGATTTGTA
nsoA1_F (*B. obeum*)	GGGAAAATTTGATGATTTCGATTT
nsoA1_R (*B. obeum*)	TTTACCTGTGATATGGCATC
P49_NdeI_F	TTTCATATGCGAATTGCGGAAAAAGC
P62_NdeI_F	TTTCATATGAAAGCACTTCGTAATTTTCTGATC
P62_SpliceR_NdeKO	CTCTGAATCATAAGCCGGTGTCGTAT
P62_SpliceF_NdeKO	ATACGACACCGGCTTATGATTCAGAG
pUK200_XhoI_R	TGCACTCGAGCTTAAAGACATTACAC
lanP_NdeI_F	TTTCATATGCGCAAAAAGACCTACCT
lanP_XhoI_R	CAGACTCGAGGTACAGGACGAC
P140_NdeI_F	TTTCATATGGATATAAAAAATTGGAAACAAAGTCC

P140_XhoI_R	ATGACTCGAGTACCACTGTACGTTCA
nsoR2_F	CTATGTTGACAATACTAATATTGG
nsoR2_R	CAAATTTCTTCTTAATGCAACT
P32_BglII_F (pUK200)	CCAAGATCTACCAATTCGGTC
P32_SpliceNso1_14_R	CTTCTTTTGTTTTCATGGCAAAATTCCTC
Nso1_14_spliceP32_F	GAGGAATTTTGCCATGAAAACAAAAGAAG
Nso1_14_BamHI_R	GGGGGATCCGTAAATTTGGTC
nsoF_F	ATGGAAACATTACTGAGAACAG
nsoF_R	TTAAATAATCTCCCTTCTATTTCTTTT
nsoE_R	CCTTCCACCTCTCTGAATTTT
nsoG_R	ACTATTCTTCATAATTTTTTCTTCCTTC
nsoI_R	CTAATCTTTTAATATAGCATATACACATTTAT
nsoR1_R	TTACTCCCATTTATATCCTATCCC
nsoK1_R	CTATATAATAATATCTACTTCTGCTCCTC
nsoA1_F	ATGGGAAAATTTGATGATTTCGA
nsoA1/2/3_R	TTATTTACCTGTGATATGGCATC
nsoA4_R	TTATTGACCCTTTAATTTACATGTG
nsoR2_F	ATGGAAAATATACTTGTGATTGACG
nsoR2_R	TTATTTTATATCCCATTTATAACCTATCCC
nsoK2_R	CTATGTGATCGGTAATTTTATGCTT
nsoB_F	ATGAAAAATTTATACTATAATACTCAAAAGTTTA
nsoB_R	TTATTCATGATCTTCATTCCTCTTC
nsoT_R	CTACATGTCCTCTCTTTCATATG
nsoC_R	TTATGCCAATAAAAAAGCTTTCTT
nsoR1_F	ATTAAAATTCTAGTTATAGATGATGAAGAG
nso1_14_F	ATGAAAACAAAAGAAGAACTTCTAC
nso1_14_R	TTACATATCCGTGTCACTTTCT
nsoB_R_Short	ATTTGATCTCTTGAATTTAACGACG
gyrB_F	ATGGGCGGAGAGAATACA
gyrB_R	AATATGTGTACCACCTTCCG

Microbiology

2.1 Antimicrobial Assays

2.1.1 Bacterial Overlay Assay

Producer bacteria were grown overnight in liquid media. 5 µl of overnight culture was spotted onto a dried agar plate and grown overnight. The indicator bacterium was also grown overnight. The following day the strains plated on solid media were killed by exposure to ultra-violet (UV) radiation for 15 mins using a UV Stratalinker 2400 (Stratagene). The plate was then overlaid with soft agar (0.7%) seeded with 2% (v/v) of the indicator bacterium. The indicator strain was grown overnight. The following day the plates were analysed visually for any zone of inhibition caused by the producer strain. When performing antimicrobial assays using nisin A and nisin O producing strains, 10 ng/ml nisin A (Sigma) was used to induce antimicrobial production in liquid media and was used in the base agar of the plates, unless stated otherwise (Hatziioanou, Gherghisan-Filip *et al*. 2017). For pre-NsoA1-3 leader peptide cleavage 10 µg/ml trypsin (Sigma) was added to both the base agar and soft agar.

2.1.2 Supernatant Drop Test

An overnight culture of producer bacteria was centrifuged for 10-15 min at 1,700 x g. The supernatant was removed and filtered through a 0.22 µm filter (Millipore) to remove bacteria and stored at 4˚C. The overnight culture of the indicator strain was diluted 1:100 in sterile PBS and a sterile cotton swab was used to make a lawn of the indicator strain on solid media and allowed to air dry. The plate was divided into sections, one per supernatant, allowing space for spreading. 10 µl of supernatant was pipetted on to plate, air dried and incubated as appropriate for each indicator strain. The zone of inhibition was measured the following day.

2.2 *B. obeum* Growth and Visualisation

2.2.1 Electron Microscopy and Gram Staining of *B. obeum*

For both scanning electron microscopy (SEM) using the Nova NanoSEM (Field Electron and Ion Company) and transmission electron microscopy (TEM) using the Talos F200C TEM

(Fiel Electron and Ion Company), *B. obeum* was either grown over six days on BHI agar or overnight in liquid pre-reduced BHI with complements. A sample of the six-day culture was taken with a sterile toothpick and resuspended in 15 µl PBS. 1 ml of the fresh liquid culture was removed, centrifuged for 1 min at 16,000 x g and the supernatant removed, followed by resuspension of the pellet in 15 µl PBS. Two samples of fresh and 6 day old culture were prepared and sent to the Quadram Institute Biosciences Microscopy laboratory for processing and image capture which were performed by Kathryn Gotts and Dr Catherine Booth.

Gram staining (Remel) was carried out as described in Smith and Hussey (2005). Slides were analysed and images captured using light microscopy with a Nikon Eclipse 50i microscope

2.2.2 *B. obeum* Growth Curve Assay

B. obeum was grown overnight in pre-reduced BHI with complements at 37ºC in an anaerobic cabinet. 25 ml of pre-reduced BHI with complements was inoculated with 1% (v/v) of overnight culture. All sampling and plating occurred within the anaerobic cabinet to minimise exposure of *B. obeum* to oxygen. 1 ml of inoculum was used for optical density measurements at 600nm (OD_{600}) using a 6715 UV/Vis spectrophotometer (Jenway). An additional 100 µl was diluted 1:10 in phosphate buffered saline (PBS) and used to make a serial dilution (10^{-1} to 10^{-6}). OD_{600} was recorded undiluted every hour, until it had reached ≈ 0.4, after which it was diluted 1:10 in BHI with complements. At each timepoint, a 1:10 dilution series of the sample in PBS was prepared. Sampling ceased once the OD_{600} measurements of three consecutive samples indicated that stationary phase had been reached. Three 10 µl samples of each 1:10 dilution (10^{-2} to 10^{-5} before OD_{600} ≈ 0.4; 10^{-3} to 10^{-6} after OD_{600} ≈ 0.4) were spotted on BHI agar plates and incubated overnight in the anaerobic cabinet at 37ºC. Colonies were counted for each serial dilution at each time point where the no confluent growth was seen.

Molecular Microbiology

2.3 *B. obeum* Genomic DNA Extraction

Genomic DNA was extracted by the method of hexadecyltrimethyl ammonium bromide (CTAB) (Sigma) extraction, with the protocol adapted from Wilson (2001). An overnight culture of *B. obeum* was prepared in pre-reduced BHI with complements at 37ºC in the anaerobic cabinet. Pre-reduced BHI with complements was inoculated with 2% (v/v) of overnight culture. Once OD_{600} had reached ≈ 1, the culture was centrifuged at 2000 x g for 15 mins at 4ºC. The supernatant was discarded and the pellet resuspended in 3 ml 10 mM Tris-HCl Buffer pH 8.5 (EB Buffer). The following steps were carried out in triplicate for each sample. The sample was centrifuged at 16,000 x g for 2 mins and the supernatant discarded. The pellet was washed in 400 µl colony wash buffer (100 mM NaCl, 10 mM Tris-HCl pH 7, 1 mM EDTA). The suspension was centrifuged again at 16,000 x g for 2 mins and the supernatant discarded. The pellet was resuspended in 400 µl EB buffer and 20 µl lysozyme (Sigma, 100 mg/ml), 45 µl proteinase K (Roche, 20 mg/ml) and 1 µl RNAse A (Sigma, 10 mg/ml) were added. The mixture was incubated at 37ºC for 15 mins, followed by the addition of 70 µl 10% SDS and the sample mixed by inversion. The solution was incubated at 65ºC for 10 mins. 100 µl 5M NaCl was added, immediately followed by addition of 100 µl CTAB (10% in 0.7 M NaCl). The solution was vortexed until white and was incubated for a further 10 mins at 65ºC. 500 µl phenol/chloroform/isoamyl (25:24:1, Sigma) was added in a fume hood and vortexed for at least 10 s. The solution was centrifuged at 16,000 x g for 5 mins. The clear upper layer was removed and transferred into a new 1.7 ml Eppendorf tube. An equal volume of chloroform was added, the mixture vortexed for 10 sec and centrifuged at 16,000 x g for 5 mins. Again, the clear upper layer was carefully removed and transferred to a new 1.7 ml Eppendorf, followed by addition of 0.1x volume 3 M NaOAc pH 5.2 and 2.5x volumes of ice-cold 100% ethanol. The solution was inverted five times to mix and incubated for 30 mins on ice. The DNA was retrieved from the tube using a closed Pasteur pipette tip. The DNA was soaked in 70% ethanol for 2 mins. The DNA was retrieved and air-dried until no visible drops

of ethanol were left. The DNA was then resuspended in 50 µl EB buffer supplemented with 1 µl RNAse A (10 mg/ml) and incubated at 37ºC for 30 mins. Post incubation, 5 µl 3 M NaOAc pH 5.2 and 100 µl ice-cold ethanol were added and the sample inverted to mix. The Pasteur retrieval procedure was repeated, and the DNA was eluted in 50 µl EB buffer. Concentration and quality of DNA was assessed using a Nanodrop spectrophotometer (ThermoFisher Scientific) and the samples were analysed by agarose gel electrophoresis on a 0.9% agarose gel (Melford) in 0.5x Tris-borate-EDTA (TBE) buffer (Sigma) to ensure that the DNA had not been fragmented during extraction. Samples were stored at 4ºC for use in future experiments.

2.4 Maxwell gDNA Extraction

Cell cultures were harvested by centrifugation (minimum 2 ml) at 1,700 x g for 15 mins at 4ºC using a Centrifuge 5810R (Eppendorf). The pellet was resuspended in 300 µl TE buffer provide by the Maxwell RSC Culture Cells DNA Kit (Promega) with the addition of 100 µl lysozyme (25 mg/ml) (Sigma) and 3 µl mutanolysin (10 U/µl, Sigma). This was incubated for 30 mins at 37ºC. The Cartridges were prepared as per the manufacturer's instructions and placed in the Maxwell RSC 48 Instrument (Promega). Final elutions were quantified using Qubit Broad Range DNA Quantification Kit (Thermo Scientific) and Qubit 3.0 fluorometer (Life Technologies) as per the manufacturer's instructions and stored at -20^{0}C.

2.5 Plasmid Extraction

An overnight culture of the bacterial strain containing the desired plasmid was grown in the appropriate conditions and with the correct antibiotic if the plasmid confers antibiotic resistance. Plasmid extraction and purification were performed using an EZNA kit (Omega Bio-Tek). For *E. coli*, 3 ml of an overnight culture was used. From Gram-positive bacteria an additional lysis step was added. The 10 ml overnight culture was centrifuged at 1,700 x g for 10 mins at 4ºC, the pellet was resuspended in a lysis solution (250 µl EZNA Solution 1 containing 5 mg/ml lysozyme (Sigma) and 3 µl 10 U/µl mutanolysin (Sigma)). The suspension was then incubated for 15 mins at 37ºC in a water bath. The protocol was performed as per

the EZNA kit manufacturer's instructions, following cell lysis. Once eluted in EB buffer, DNA was quantified using a Nanodrop spectrophotometer and analysed by agarose gel electrophoresis. 9 µl of sample is mixed with 1 µl 10x loading buffer (0.15% bromophenol blue, 25 ml glycerol, 5 ml 50x TBE). Electrophoresis used 90 V. Staining used ethidium bromide for 20 min followed by analysis by Alphaimager transilluminator or by using 1 µl of Midori Green Xtra (Nippon Genetics) per 25 ml 0.9% agarose gel for products >200 bp and visualised by a blue light-emitting diode. For products with a length <200 bp Midori Green Direct (Nippon Genetics) was used where 1 µl was directly added to each 10 µl sample prior to loading.

2.6 16S rRNA Gene Polymerase Chain Reaction (PCR)

An overnight culture was grown using appropriate conditions and 150 µl of culture was centrifuged at 16,000 x g for 1 min. The supernatant was removed, the pellet was resuspended in 150 µl of colony wash buffer (100 mM NaCl, 10 mM Tris pH 7, 1 mM EDTA) and centrifuged again at 16,000 x g for 1 min. The supernatant was removed, and a final resuspension made in 15 µl UPH_2O. The sample was heated at 95°C for 5 mins. PCR was performed using a 50 µl PCR mixture, see below. Oligonucleotide primers were provided by Sigma Genosys (Table 2.5).

Master Mix						
5x GoTaq Buffer (Clear) (Promega)	UPH_2O	dNTP mix (Bioline) (100 mM)	Amp_F (20 µM)	Amp_R (20 µM)	Bacterial Template	GoTaq G2 polymerase (Promega)
10 µl	36.35 µl	0.4 µl	1 µl	1 µl	1 µl	0.25 µl

Conditions for 16S rRNA gene PCR were as follows:

95ºC	95ºC	55ºC	72ºC	72ºC
2 mins	30 sec	30 sec	1 min (1.5 kb)	5 mins

X 25

10 µl of PCR product was electrophoresed on an 0.9% agarose gel with 5 µl 1x loading buffer. The samples were then purified using the SureClean Plus kit (Bioline) using 6 µl pink co-precipitate. The manufacturer's protocol was followed with the following modifications; times for incubation, and first and second centrifugation were increased to 30 mins, 30 mins and 5 mins, respectively. Pellets were resuspended in 50 µl EB buffer. Samples were left on ice for 15 mins before DNA quantification by nanodrop.

2.7 Colony PCR for Detection of Transformants

Colonies from agar plates were picked with a sterile toothpick into 10 µl UPH_2O. PCR reaction mixes were assembled as described in Method 2.6, with the buffer being replaced by 5x GoTaq Green Buffer (Promega). PCR reaction conditions were adjusted according to the primer melting temperature (T_m) and expected product sizes according to the manufacturer's protocol (Promega) using the maximum annealing temperature of 3^OC below the lowest primer T_m and a maximum extension time of 1 min/kb. Analysis of the plasmid DNA sequence using CloneManager V9 (Scientific and Educational software) enabled primers design; Universal and Reverse were used for pTG262 and its variants, and p54 and p181 were used for pUK200 (designed by Nikki Horn, Quadram Institute Bioscience).

95ºC	95ºC	55ºC*	72ºC	72ºC
2 mins	30 sec	30 sec	30 sec**	5 mins

X 25

*Adjust according to primer T_m

**Adjust based on predicted product size (1 kb in 1 min)

Successful transformation was indicated by presence of a band at the expected size after gel electrophoresis. Size of the PCR product was predicted using CloneManager and the respective primers for each plasmid.

2.8 Colony PCR Using 96 Well Plates

The method previously described in method 2.7 is followed, however, individual colonies were picked and mixed with 50 µl UPH_2O in a 96 well sterile plate in rows A-G. 10 µl of each sample were mixed in row H in their respective columns. 10 µl of this mix was used as a template, with the volume of UPH_2O in the master mix (Method 2.6) reduced to 26.35 µl per sample. The PCR and gel electrophoresis were performed as standard. When analysing the agarose gel, if a band of the correct size was present, a PCR reaction would be repeated using the sample within each well from that column as the template for the reaction. The final gel electrophoresis showed which of the samples in the original mix had the correct sized band, which was then taken and used to create an overnight culture.

2.9 Splice Overlap Extension PCR

Splice overlap extension was performed following the method of Horton (Horton, Cai *et al*. 1990). Primers were designed (Table 2.5) with a 100% match sequence and a tail region matching that of the other gene or plasmid region that is being spliced. Each gene was amplified using Phusion PCR (New England Biolabs (NEB)) with reactions made as follows:

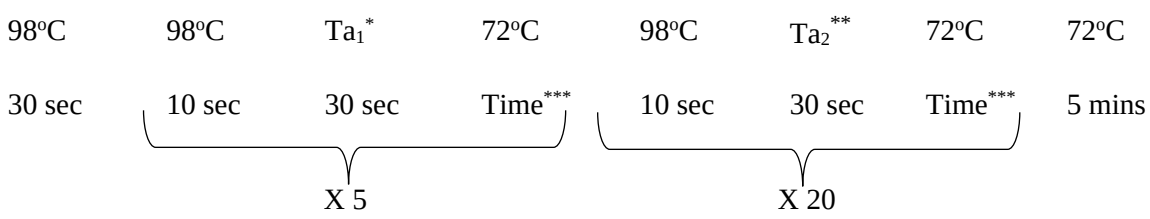
Master Mix						
5x Phusion Buffer (NEB)	UPH_2O	dNTP mix (Bioline) (100 mM)	Forward primer (20 µM)	Reverse primer (20 µM)	Sample DNA Template	Phusion polymerase (NEB)
10 µl	34.7 µl	0.4 µl	1.25 µl	1.25 µl	1 µl	0.4 µl

PCR reaction conditions were adjusted according to the primer melting temperature (T_m) (calculated using Finnzymes calculator, where the annealing temperature is the T_m for primers

<20 nt or T_m +3^{O}C for primers of >20 nt or OligoCalc to give the Nearest Neighbour and expected product sizes).

Finnzymes: https://www.thermofisher.com/uk/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/tm-calculator.html

OligoCalc: http://biotools.nubic.northwestern.edu/OligoCalc.html



5 cycles annealing temperature (Ta_1) Nearest Neighbour of the part of the primer sequence that is a 100% (linear) match to the template*, 20 cycles Ta_2 Nearest Neighbour of the whole primer**. The extension time varied depending on the length of the predicted product, 15-30 secs/kb***)

The products were electrophoresed to confirm that a band of the correct size was present. Products were purified by SureClean Plus (Bioline), as per the manufacturer's instructions, with alterations (See Method 2.6). Templates were dilyted to 1 ng/µl then the outermost primers for both products were used to perform the overlap extension, using the Nearest Neighbour of the overlap sequence or the Finnzymes calculated Ta of the outer primers if it is lower. The final product was analysed on a gel to confirm that the correct size of the product had been produced and the product was purified using SureClean extended method (See Method 2.6).

2.10 Restriction and Ligation

The restriction enzymes selected were based on the restriction sites present in the plasmid that was being manipulated. Additionally, corresponding restriction sites were inserted into the products during the splice overlap extension PCR. Restriction reactions used 1-2 µg of the product and followed manufacturer's instructions (New England Biolabs).

Restriction enzymes were heat denatured as per the NEB recommendation. The restricted products were then purified by the extended SureClean method described in method 2.6. The vector plasmid was prepared using the method 2.5 and restricted as above, with the final volume dephosphorylated with Antarctic phosphatase (NEB) following the manufacturer's instructions with a final heat step of 5 min at 65^{O}C to inactivate the enzyme.

All incubations were performed in a water bath. The restricted vector plasmid was purified by the extended SureClean method (Method 2.6). Ligation was performed after quantification (2:1 molar ratio insert to vector, x and y, respectively) in a final volume of 15 µl using the Fast-Link DNA Ligase (Epicentre Biotechnologies). The ligation reaction was performed overnight at room temperature and the ligation mix was heated for 15 mins at 70^{o}C before transformation.

DNA Insert	x µl
DNA vector	y µl
10x Fast-Link buffer	1.5 µl
ATP	1.5 µl
FastLink ligase	1 µl
H_2O	to 15 µl

2.11 Transformation into *E. coli*

E. coli MC1022 was grown overnight in 10 ml L broth at 37^{o}C with shaking at 250 rpm. 800 µl of overnight culture was added to 40 ml pre-warmed L broth and was grown until an OD_{600} of 0.45-0.55. The culture was split between 2 chilled Oakridge tubes and centrifuged at 3000 x g for 10 mins at 4^{o}C (Avanti J-26 XP, Beckman Coulter, JA17 4.5K). The supernatant was discarded and 1 ml freshly prepared ice-cold 10% glycerol was used to resuspend the pellet, followed by the addition of 19 ml ice-cold 10% glycerol. This centrifugation and resuspension step was repeated. The cell suspension was centrifuged again using the same conditions and the supernatant removed with the final pellet resuspended in 200 µl 10% glycerol. This was aliquoted into 40 µl amounts of the competent cells which were stored at -80^{o}C until use.

100-500 ng of plasmid DNA was added and mixed using the pipette tip. This sample was incubated on ice for 1 min and transferred to a 0.2 cm gap electroporation cuvette (CellProjects). The mixture was pulsed at 2.5 kV, 200 Ω and 25 uF using a Bio-rad GenePulser Xcell. Immediately after, 460 µl SOC broth (2% tryptone (Oxoid), 0.5% yeast extract (BD), 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose, pH 7) was added to the electroporation cuvette. This mixture was transferred to 2 ml screw capped tubes and incubated at 37°C for 2 hrs shaking at 250 rpm. 100 µl of undiluted sample and 10^{-1} dilutions in PBS were inoculated on selective L agar plates and grown at 37°C for two days.

For protein expression, 1-2 µl ligation mixes were transformed into chemically competent *E. coli* TOP10 (Invitrogen) as the manufacturer's instructions. After selective growth, colony PCR and sequence confirmation plasmids were extracted and transformed into chemically competent *E. coli* expression strain BL21(DE3) (Invitrogen) as manufacturer's recommendations.

2.12 *L. lactis* Strain Transformation

The protocol for *L. lactis* transformation was adapted from the method developed by Wells, Wilson and Le Page (1993). The *L. lactis* strain used for generation of electro-competent cells was grown overnight at 30°C in GM17. All solutions used were made fresh using UPH_2O water and filter sterilised then chilled on ice. 3 ml of overnight culture was added to 47.5 ml M17 broth containing 2.5% glycine and 2.4 ml 10% glucose pre-warmed to 30°C and incubated at 30°C until the OD_{600} had reached ~ 0.5. The culture was centrifuged for 10 mins at 3,000 x g at 4°C. The supernatant was discarded, and the pellet resuspended gently in 5 ml of ice-cold electroporation buffer (0.5M sucrose in 10% glycerol), after initial resuspension in 500 µl. Centrifugation and the resuspension step were repeated. The final resuspension of the pellet was made in 500 µl electroporation buffer. The electrocompetent cells were dispensed in 40 µl aliquots and those that were not needed for the current transformation were then flash frozen using dry ice and stored at -80°C.

Electroporation occurred as per method 2.11, however the reanimation media used was 460 µl GM17/0.5 M sucrose containing 20 mM $MgCl_2$ and 2 mM $CaCl_2$ followed by incubation on ice for 10 mins. A water bath was used for 2 hrs at 30ºC for incubation before plating of undiluted and 10-fold diluted aliquots onto selective GM17 agar.

2.13 DNA Quantification

Purified plasmid DNA, gDNA and purified PCR products were quantified using a NanoDrop 1000 (Thermo Scientific). 1 µl of sample was used per reading with the NanoDrop 1000 after initial calibration using UPH_2O followed by blanking with the selected buffer used to resuspend or wash the DNA in the previous assay. The NanoDrop 1000 was cleaned using lint-free Kimchi wipes between each sample. DNA samples were stored at -20ºC.

2.14 Sequencing of PCR Products

Purified PCR products were quantified (See Method 2.13). Forward and reverse amplicon sequencing was carried out at Eurofins Genomics (Germany) using the Mix2Seq kit. The concentration of each sample was normalised to 10 ng/µl with the addition of 5 µl of either forward or reverse primer (20 mM) in separate tubes. The total volume was made to 10 µl using UPH_2O. Sequence results were visualised and quality assessed using FinchTV (version 1.4.0) followed by alignment using Geneious (version 11.1.5, Biomatters, New Zealand) to provide the consensus sequence. Species identification from 16S rDNA PCR products was made with the consensus sequence using the Ribosomal Database Project (http://rdp.cme.msu.edu/).

2.15 RNA extraction

RNA extractions were performed using the RNeasy Mini Kit (Qiagen) following the manufacturer's instructions with alterations. All optional steps were followed, and the on-column DNase treatment was performed twice with an extended incubation time of 30 mins. Additionally, once the RNA had been eluted and quantified using Qubit Broad Range RNA Quantification Kit (Thermo Scientific) and Qubit 3.0 fluorometer (Life Technologies) another

DNase treatment using the Turbo DNase treatment (Thermo Scientific) was performed as per manufacturer's instructions. Quantification using Qubit Broad Range RNA Quantification Kit was repeated. The samples were flash frozen using dry ice and stored at -80ºC.

Protein Extractions and analysis

2.16 Induction and Purification of pre-Nso peptides

L. lactis strains were grown overnight in 10 ml GM17. The following day the culture was used to inoculate 100 ml pre-warmed GM17 to OD_{600} of 0.1 with antibiotic addition to select for the appropriate plasmids. At OD_{600} 0.5-0.6 the cultures were induced with 10 ng/ml nisin A and incubated for 3 hrs at 30ºC. 50 ml portions were centrifuged at 1700 x g for 15 mins at 4ºC. The supernatants were collected and filtered using a 0.45 µm filter. Proteins were precipitated via the addition of 10 ml 100% trichloroacetic acid (TCA) to 40 ml supernatant incubated at 4ºC overnight. The proteins were collected by centrifugation at 1,700 x g for 30 mins at 4ºC. The pellet was washed using $1/5^{th}$ volume ice-cold acetone for 2 hrs at -20ºC, recentrifuged, the supernatant removed and the pellet air dried for 10 mins, followed by resuspension in 800 µl 50 mM sodium acetate pH 5.5.

Additionally, the cell pellets from the initial culture were washed once with 0.3 M Tris-HCl pH 7.4 and soluble protein extracts were produced via bead beating with 500 µl Tris buffer and 200 µl sterile acid-washed 0.1 mm glass beads using a FastPrep FR120 cell disrupter (MP Biomedicals) for 4 x for 30 sec at speed 6 with 5-10 mins on ice between beating. The tubes were centrifuged for 20 mins at 13,000 x g at 4ºC, supernatant removed and stored at 4ºC. Protein concentration was measured using the Bradford reagent (BioRad).

2.17 Protein Extraction from *E. coli*

The strain of *E. coli* BL21 (DE3) was grown overnight in 10 ml L broth with appropriate antibiotic. The following day a fresh 10 ml prewarmed broth with antibiotic was inoculated with 200 µl of the overnight culture. This was grown until OD_{600} 0.4 after which it was induced with 0.5 mM isopropylthio-β-galactoside (IPTG) for 3 hrs or 4 hrs at either 37ºC or 18ºC. The

cells were harvested by centrifugation for 15 min at 1,700 x g at 4ºC. The supernatant was removed and the pellet was resuspended in 500 µl of TN buffer (20 mM Tris-HCl pH 8, 50 mM NaCl). The bacteria were removed to a 2 ml screw cap tube containing ~100 µl sterile acid washed beads (0.1 mm) on ice. A FastPrep FR120 Cell Disruptor was used to bead beat the cells. Beatings consisted of 30 sec at speed 6 repeated 4 times with 5 mins on ice between each repeat. The tubes were centrifuged at 13,000 x g for 30 mins at 4ºC and the supernatant was removed and transferred to a sterile Eppendorf tube and stored at 4ºC.

2.18 SDS Polyacrylamide gel electrophoresis (PAGE)

SDS-PAGE was performed using materials and protocols from Invitrogen. Samples were made to 10 µg total protein in either 10, 15 or 20 µl reaction mixes as follows:

Sample + UPH_2O	6.5	9.75	13
Reducing agent	1	1.5	2
LDS Sample buffer	2.5	3.75	5
	10	15	20

The samples were heated at 70°C for 10 mins followed by chilling on ice for 2 mins. The samples were briefly centrifuged to collect any sample on the sides of the tube. 600 ml of MES running buffer was prepared. A pre-made 4-12% NuPage Novex Bis-Tris 12 well gel (Invitrogen) was rinsed with UPH_2O and loaded with the samples. If the samples were reduced, then 500 µl of NuPage antioxidant (Invitrogen) were added to the inner chamber of the gel tank. The gel was run using 200 V for approximately 35 mins. The gel was washed 3 times for 5 mins in UPH_2O with gentle shaking then strained for 1 hr with 20 ml Simply Blue Safestain (Themo Scientific). The gel was destained in 100 ml UPH_2O for 1 hr with gentle shaking.

2.19 Western Blotting

1 l of 1X NuPage Transfer Buffer was prepared and used to soak blotting pads. The transfer membranes (poly(vinylidene fluroride) 0.2 µm pore size) were prepared as recommended by the manufacturer (Invitrogen). The filter paper was briefly soaked in NuPage Transfer Buffer and placed on top of the gel, this was then turned over, the transfer membrane applied to the

other side of the gel and a second piece of soaked filter paper placed on top. The gel and transfer membrane were placed in between the blotting pads and inserted into the lower buffer chamber of the gel tank. This chamber was filled with 1X NuPage Transfer Buffer and the outer chamber was filled with 650 ml UPH_2O. The transfer used 30 V for 1 hr.

Once completed the transfer membrane was removed and washed twice for 10 mins in Tris-buffered Saline (TBS) buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl) at room temperature with gentle shaking. The membrane was then incubated with 3% Bovine Serum Albumin (BSA) (Sigma) in TBS buffer at 4^{o}C overnight on a rotating tilter. The membrane was washed twice for 10 mins using TBS-Tween/Triton buffer (20 mM Tris-HCl pH 7.5, 500 mM NaCl, 0.05% (v/v) Tween 20 (Sigma), 0.2% (v/v) Triton X-100 (Sigma)) at room temperature, followed by a 10 min wash using TBS buffer. The primary antibody incubation was performed using 2.5 µl (1:2000 dilution) Anti-His-Tag (Novagen) in 5 ml 3% BSA for 1 hr at room temperature on a tilter, this was followed a repeat of the wash steps previously described. Incubation with the secondary antibody was performed using 2.5 µl horse radish peroxidase (HRP) conjugated anti-mouse IgG (1:2000 dilution) in 5 ml 3% BSA in TBS (Sigma) for 1 hr at room temperature. The membrane was washed 4 x for 10 mins using TBS-Tween/Triton buffer at room temperature. The membrane was stained with 5-bromo-4-chloro-3-indoyl phosphate (B-CIP/NBT) tablets (Sigma) for up to 15 mins. The reaction was stopped by washing with UPH_2O twice.

2.20 Pre-Nso Cleavage Assay

Pre-Nso peptides were induced and purified (See Method 2.16) and crude soluble extracts of cultures expressing the candidate proteases were quantified via Bradford reagent (BioRad). An incubation mix of 30 µg pre-Nso and 10 µg crude extract was made to 13 µl using 100 mM Tris HCl pH 6 supplemented with 5 mM $CaCl_2$ and left at 37^{o}C overnight, as recommended by Montalban-Lopez *et al* (2018). TCA precipitated extract from *L. lactis* pIL253 was used as a negative control in combination with the candidate proteases. A positive control using 15 µg/ml trypsin was also used. Samples were loaded in an SDS-PAGE gel

followed by a Western Blot transfer to visualise the results (Methods 2.18 and 2.19). The protocols for these two methods remained the same as described, however, a Clos leader antibody CL-1 (Genscript Corp) (Hatziioanou 2011) (1:200 dilution) was used as the primary antibody (25 µl in 5 ml 3% BSA in TBS) and HRP conjugated anti-rabbit IgG (1:5000 dilution) used as secondary antibody (1 µl in 5 ml 3% BSA in TBS).

Chapter 3.

Identification of the Nisin O-Like Cluster in *Lachnospiraceae*

3.1 Introduction

With four structural genes, two regulatory systems and no protease within the cluster the nisin O biosynthetic gene cluster has a number of distinct features that differentiate it from other nisin variants (Chinachoti, Matsusaki *et al.* 1998, O'Connor, O'Shea *et al.* 2015, Hatziioanou, Gherghisan-Filip *et al*. 2017, M, Field *et al*. 2020, O'Sullivan, O'Connor *et al.* 2020). In a recent study by Kim *et al*., a novel lantibiotic biosynthetic gene cluster was identified which, like the nisin O cluster, contained two regulatory systems and genes involved in its maturation and immunity. However, this cluster contained five structural genes and a protease was present (Kim, Becattini *et al.* 2019). This cluster was identified in *Blautia producta* SCSK which is of the same genus as *B. obeum* A2-162 indicating that similar clusters are present within the same genus in nature.

Blautia is a member of the *Lachnospiraceae* family and is relatively abundant in the human gut, representing between 2-8% of the gut microbiome (Arumugam, Raes *et al.* 2011, Eren, Sogin *et al.* 2015). In recent years the *Blautia* genus has become better studied and has been shown to have roles in carbohydrate degradation and short chain fatty acid production (Zhang, Zhao *et al.* 2015, P, Martin *et al*. 2016). In an investigation looking into the potential of *Blautia* species as probiotic strains, Liu *et al*., analysed 74 *Blautia* strains and found 261 secondary metabolic biosynthetic gene clusters; these included sactipeptides, lanthipeptides, lasso peptides, beta-lactones, trans-AT polyketide synthases and non-ribosomal peptides (Liu, Mao *et al*. 2021). Azevedo *et al*. identified a biosynthetic gene cluster in *Blautia schinkii* DSM 10518 and *Blautia* sp. SF-50 which encodes a sactipeptide (Azevedo, Bento *et al*. 2015).

In this chapter strains within the *B. obeum* species were analysed *in silico* to see whether the nisin O cluster was present. Furthermore, this analysis was widened to include genomes from members of the *Lachnospiraceae* family. The hypothesis of this work was that nisin O-like clusters are found across multiple genera within *Lachnospiraceae*.

3.2 Methods

3.2.1 Pan-genome Analysis of *B. obeum* Strains

To identify proteases that were specific to *B. obeum* strains that contained the nisin O cluster, all available (50) *B. obeum* genomes were downloaded from National Center for Biotechnology Information (NCBI). FastANI was used to ensure that the strains to be used in the pan-genome analysis were correctly assigned as *B. obeum* using *B. obeum* A2-162 as the reference strain (Taxon ID: 657314.3.fna). An Average Nucleotide Identity (ANI) score of 95% was used as the minimum score required for the genome to be included, as this a well-accepted cut-off for ANI similarity (Jain, Rodriguez *et al*. 2018). The five *B. obeum* genomes with an ANI score greater than 95% were used to estimate their pan-genome using ROARY (Sanger, version 3.11.2)*. To identify the presence or absence of the nisin O cluster the FASTA file of each of the *B. obeum* genomes that passed the 95% ANI score threshold was analysed using Geneious R11 (version 11.1.5) and BAGEL4 (van Heel, de Jong *et al*. 2018).

*I would like to acknowledge the contribution of Dr Lizbeth Sayavedra for her guidance with running the ROARY analysis.

3.2.2 BLASTP

Initial searches for proteins with homology to the NsoB amino acid sequence were analysed using BLASTP (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) using standard databases and no specific organisms selected (Johnson, Zaretskaya *et al*. 2008, Hatziioanou 2011). Sequences produced by this computational search were copied into Geneious R11 and further analysed using the method described in 3.2.4.

3.2.3 Identification of Nisin O Clusters in *Lachnospiraceae*

To identify the prevalence of clusters in *Lachnospiraceae* and identify the presence of proteases, 2094 *Lachnospiraceae* genomes were downloaded from the Genome Taxonomy Database (GTDB) (Parks, Chuvochina *et al*. 2020). Genome completeness and quality was assessed using CheckM (Parks, Imelfort *et al*. 2015) and the marker gene set for the

Lachnospiraceae family (version 1.0.18) which excluded 664 genomes which had <95% completeness or > 5% contamination, leaving 1430 *Lachnospiraceae* genomes for further analysis. These genomes were then annotated using Prokka (Seemann 2014). Once annotated the genomes underwent analysis by AntiSmash 5.0 (Bacterial Version) for identification of secondary metabolites (Blin, Shaw *et al*. 2019) and BiG-SCAPE to look at cluster similarity (Navarro-Muñoz, Selem-Mojica *et al*. 2020). Additionally, protein sequences of the 1430 genomes were clustered using OrthoFinder (version 2.5.2) (https://genomebiology.biomedcentral.com/articles/10.1186/s13059-019-1832-y). *nsoB* and *nsoC* were chosen as markers of the nisin O cluster and genomes with proteins that clustered together with one or both of the markers were further investigated for the presence of the nisin O cluster**. All hits from both of the methods above were then downloaded individually and analysed manually using Geneious R11, BAGEL4 (van Heel, de Jong *et al*. 2018) and AntiSmash 5.0 (Bacterial Version) (Blin, Shaw *et al*. 2019) to confirm the presence of the nisin O cluster.

**I would like to acknowledge the contribution of Dr Rebecca Ansorge and Dr Stefano Romano with their assistance developing this method and running the initial pan-genome analysis on the *Lachnospiraceae* genomes.

3.2.4 Homology of Amino Acid Sequences from Nisin O like clusters

To assess the sequence homology of proteins within the identified nisin O-like clusters the genomes of those bacteria which contained the nisin O-like cluster were downloaded into Geneious R11 from the NCBI genome database. The genome was then searched manually using “nisin” as the search criteria to identify the cluster within the annotated genome. The cluster was then extracted, and each gene extracted and translated using the extraction and translation tools within the Geneious R11 software. This was performed for the putative structural genes (NsoA1, NsoA2, NsoA3 and NsoA4) as well as the modification proteins NsoB and NsoC. Each translated sequence from these examples were then aligned using Muscle 3.8.425 (Edgar 2004).

3.2.5 Mauve Alignment of Nisin O-like Clusters

Alignment of the nisin O-like clusters (nucleotide), downloaded as described in method 4.2.4, was performed using the progressive Mauve algorithm (Geneious R11 plugin) using the standard conditions pre-selected as part of the Geneious R11 software (Darling, Mau *et al.* 2004).

3.2.6 Neighbour-Joining Phylogenetic Trees

To determine the similarity of the nisin O-like clusters phylogenetic trees were created using Geneious R11's tree builder. The nucleotide sequences of the concatenated *nsoB-nsoC* genes and the whole clusters were globally aligned with free end gaps and a cost matrix of 65% similarity. Neighbour-joining trees were reconstructed using the Tamura-Nei genetic distance model.

3.3 Results

3.3.1 ROARY Pan-Genome Analysis of *B. obeum* Strains

A ROARY pan-genome analysis of 50 *B. obeum* strain genomes downloaded from NBCI was performed to identify any new nisin O biosynthetic gene clusters that were present in *B. obeum* strains. The *B. obeum* strains underwent a FastANI test using *B. obeum* A2-162 as a reference genome to confirm that the species of these strains was annotated correctly in the database. Out of the 50 genomes only 5 had an ANI score greater than the cut-off of 95, see Table 3.3.1. These 5 strains were then used in a ROARY pan-genome analysis to identify the core and accessory genomes. Manual searches using BAGEL4 and AntiSmash for the nisin O cluster showed that only *B. obeum* A2-162 contained the nisin O cluster from these 5 genomes.

B. obeum Strain	Genome ID (PATRIC)	ANI Score
A2-162	657314.3	99.9998
MGYG-HGUT-00252	40520.1588	98.7192
AM28-23	40520.96	98.6974
AM32-41	40520.89	98.6357
AM32-10AC	40520.93	98.3287

Table 3.3.1. ANI scores and IDs of five *B. obeum* genomes with an ANI score >95%

The remaining genomes were searched using a word search of 'nisin' for the presence of the nisin O biosynthetic cluster and it was found that *B. obeum* TM09-13AC and *B. obeum* AM27-32LB each contained a cluster that included four putative structural peptides. Additionally, 33 of the 50 genomes (66%) contained lantibiotic immunity genes including a *nisI*-like gene and a lantibiotic ABC efflux transporter although no other genes that would normally constitute a biosynthetic gene cluster, such as modification machinery or regulatory systems, were present (Table 3.3.2). Furthermore, manual searches were performed on all 50 genomes to investigate if any other lantibiotic-like clusters were present. Within these genomes 14 (28%) contained a *lanM* gene and a lantibiotic which was similar to plantaricin C (Table 3.3.2). Additionally, in the genomes of *B. obeum* AF35-14AC, *B. obeum* AF14-52, *B. obeum* MGYG-HGUT-00252, *B. obeum* AM28-23, *B. obeum* AF14-23 and *B. obeum* AM29-25AC two *lanM* genes and two plantaricin C-like genes were present (Table 3.3.2).

Strain	Nisin O Cluster Present	Immunity Genes Present	Other Lantibiotic Genes Present	Additional Lantibiotic Details
A2-162	✓	✓	×	N/A
AM22-9LB	×	✓	✓	*lanM* + lantibiotic
AM18-2AC	×	✓	×	N/A
AF39-4	×	×	×	N/A
AF37-6AC	×	×	✓	*lanM* + lantibiotic
AF35-14AC	×	✓	✓	*lanM* + lantibiotic x2
AF29-2BH	×	×	×	N/A
AF25-21	×	✓	×	N/A
AF24-10LB	×	×	×	N/A
AF21-24	×	×	×	N/A
AF14-52	×	✓	✓	*lanM* + lantibiotic x2
AM43-41	×	✓	×	N/A
AM32-10AC	×	×	×	N/A
TM09-13AC	✓	✓	×	N/A
OM06-11A	×	✓	✓	*lanM* + lantibiotic,
OM03-6	×	✓	×	N/A
SSTS_Bg7063	×	✓	×	N/A
ATCC 29174	×	✓	×	Potential Class II
MGYG-HGUT-00212	×	×	×	N/A
MGYG-HGUT-00252	×	✓	✓	*lanM* + lantibiotic x2

2789STDY5608838	×	✓	×	N/A
2789STDY5608837	×	×	×	N/A
2789STDY5834861	×	✓	✓	*lanM* only
2789STDY5834921	×	✓	✓	*lanM* + lantibiotic
2789STDY5834957	×	✓	✓	*lanM* only
MSK.18.40	×	×	✓	*lanM* + lantibiotic, Sactipeptide
MSK.20.67	×	✓	×	N/A
MSK.20.66	×	✓	×	N/A
AM32-10AC	×	×	×	N/A
AM28-23	×	✓	✓	*lanM* + lantibiotic x2
af_0058	×	×	×	N/A
1001271st1_E5	×	×	×	N/A
S11A.meta.bin_7	×	×	×	N/A
LMAG:45	×	×	×	N/A
TF09-10AC	×	✓	✓	*lanM* and lantibiotic
OF03-14	×	✓	×	N/A
AM54-7NE	×	✓	✓	*lanM* and lantibiotic
AF14-23	×	✓	✓	*lanM* + lantibiotic x2
AM41-5	×	✓	×	N/A
AM27-32LB	✓	✓	×	N/A
AM39-5AC	×	×	×	N/A
AM37-4AC	×	×	×	N/A
AM29-25AC	×	✓	✓	*lanM* + lantibiotic x2
AM34-12	×	✓	×	N/A
MSK.14.21	×	✓	×	N/A
MSK.13.37	×	✓	×	N/A
MSK.20.89	×	✓	×	N/A
MSK.17.81	×	✓	×	N/A
MSK.18.90	×	✓	×	N/A
BIOML-A1	×	×	×	N/A

Table 3.3.2. Analysis of the 50 *B. obeum* strains downloaded from NCBI assessing the presence of the nisin O cluster, lantibiotic immunity genes and other lantibiotic genes. Gene presence is indicated by a tick (✓) and gene absence indicated by a cross (×).

3.3.2 BLASTP searches for nisin O-like proteins

To investigate whether there were any other nisin O-like clusters present within bacterial species an initial screening using the NsoB amino acid sequence from *B. obeum* A2-162 was performed. This would give an insight into which bacteria contained this protein and hence whether the cluster has a similar genetic structure or whether other clusters may have contained a potential candidate protease or altered regulatory systems. This initial screening identified many similar proteins however most notably all the bacterial species (*Blautia*, *Dorea* and *Ruminococcus* species) that contained a homologue of NsoB with 100% identity were from the family *Lachnospiraceae* (Table 3.3.3). Manual searches of these genomes using BAGEL4 and AntiSmash identified that these species did indeed contain a nisin O-like cluster. However due to the prevalence of nisin O-like clusters identified via BLAST searches, a pan-genome analysis was performed on *Lachnospiraceae* genomes.

Bacteria	GenBank Assembly Accession
B. obeum A2-162	GCA_000210015.1
B. obeum AM27-32LB	GCA_003469945.1
B. obeum TM09-13AC	GCA_003436075.1
Blautia sp AM47-4	GCA_003479865.1
Ruminococcus sp. AM50-15BH	GCA_003478325.1
Ruminococcus gnavus AF33-12	GCA_003475365.1
Dorea formicigenerans AM37-5	GCA_003467665.1
Dorea logicatena 1001136B-160425	NZ_JADNJR000000000

Table 3.3.3. *Lachnospiraceae* strains with nisin O-like clusters identified through manual searches.

3.3.3 Cluster identification in *Lachnospiraceae*

To help understand the nisin O cluster and its prevalence amongst bacteria, a bioinformatic analysis was performed of the *Lachnospiraceae* family. The 2094 genomes downloaded from GTDB underwent a quality and completeness check using CheckM, after which 1460 genomes were kept for further analysis. An analysis to identify the cluster was performed using AntiSmash and BiG-Scape, however no new clusters were identified via this method using the *nsoA2/3* genes as marker genes. This was due to issues with the assembly of the genome as the structural peptides in the nisin O cluster were very similar and could potentially

be mis-assembled, therefore making the identification of the cluster unlikely. However, using *nsoB* and *nsoC* as reference genes in OrthoFinder, 59 genomes contained either *nsoB, nsoC* or both, with 34 of these containing both genes (57.6%) (Table 3.3.4). All 59 genomes were manually searched using BAGEL4 to determine whether they contained any bacteriocin biosynthetic gene clusters. Only 12 of the genomes did not contain a cluster containing structural bacteriocin genes, however these genomes did contain individual genes involved in bacteriocin maturation. Interestingly, 71.2% (42 of the 59 genomes) contained genes involved in lantibiotic immunity, even in cases where there was no cluster present, such as *Clostridium* sp. AF36-18BH. Furthermore, only 5 genomes (8.5%) were not isolated from animal faecal material, with 48 of the remaining genomes being isolated from the human gut or faeces (Table 3.3.4).

Bacteria	**Strain**	***nsoC*-like gene present**	***nsoB*-like gene present**	**Structural peptides**	**Lantibiotic Immunity Genes**	**Isolation Source**
uncultured *Butyrivibrio* sp.	UMGS920	0	1	Rathipeptide, Thiopeptide, LAP, Zoocin A	No	Human Gut
uncultured *Lachnospiraceae* bacterium	UMGS1368	0	1	Zoocin A	No	Human Gut
Luxibacter massiliensis	Marseille-P5551	0	1	Number of potential clusters and structural peptides	Yes	Human Stool
Dorea formicigenerans	AM42-8	2	1	LanB present but no structural	Yes	Human Faeces
Dorea formicigenerans	**AM37-5**	1	1	4 - Lanthipeptide	Yes	Human Faeces

Blautia obeum	**AM27-32LB**	2	2	4 - Lanthipeptide	Yes	Human Faeces
Dorea longicatena	AM23-13	1	0	Plantaricin A family structural peptide (LanM)	Yes	Human Faeces
[Eubacterium] rectale	AF39-14AC	0	2	No Cluster Present	Yes	Human Faeces
Eubacterium rectale	AF38-24	0	1	1 - Class IIb bacteriocin (lactobin A/cerein 7B family)	Yes	Human Faeces
Ruminococcus gnavus	AF33-12	4	2	4 - Lanthipeptide	Yes	Human Faeces
Clostridium sp.	AF37-7	1	0	No Cluster present	No	Human Faeces
Clostridium sp.	AF36-18BH	0	1	No Cluster present	Yes	Human Faeces
Clostridium sp.	AF34-10BH	0	1	Lasso Peptide Biosynthesis Machinery	Yes	Human Faeces
Firmicutes bacterium	AM59-13	0	1	Colicin, Zoocin A	No	Human Faeces
***Ruminococcus* sp.**	**AM50-15BH**	2	1	3 - Lanthipeptide	Yes	Human Faeces
Firmicutes bacterium	AF22-6AC	0	1	Zoocin A	Yes	Human Faeces
***Blautia* sp.**	**AM47-4**	2	1	4 - Lanthipeptide	Yes	Human Faeces
Clostridium sp.	AM43-3BH	1	1	1 structural peptide and LanB	No	Human Faeces

Firmicutes bacterium	AM43-11BH	0	1	Ranthipeptide, NRPS (Dipeptide Aldehydes)	Yes	Human Faeces
Clostridium sp.	AM34-9AC	0	2	Zoocin A, Lasso Peptide	Yes	Human Faeces
Blautia obeum	**A2-162**	1	2	4 - Lanthipeptide	Yes	European Bioinformatics Institute
Dorea formicigenerans	4_6_53AFAA	3	1	LanB present but no structural	Yes	Gastrointestinal Tract
Lachnospiraceae bacterium MD308	03_02	3	1	1 structural class I lanthipeptide	Yes	Mouse Ceca
Dorea longicatena	AGR2136	1	0	Plantaricin A family structural peptide (lanM) and BlpK-like bacteriocin cluster	Yes	The Hungate 1000. A catalogue of reference genomes from the rumen microbiome.
Clostridium sp.	TF08-15	0	1	Lasso Peptide Biosynthesis Machinery, Ranthipeptide, Zoocin A	Yes	Human Faeces
Lachnospiraceae bacterium	GAM79	1	2	1 structural class I lanthipeptide, lasso peptide	No	Human Faeces
Butyrivibrio sp.	X503	1	0	7 - Type II lanthipeptide (LanM)	No	Buffalo Rumen

Clostridium sp.	E02	1	1	1 structural class I lanthipeptide, 1 class II lanthipeptide	Yes	River Dee Sediment
Dorea longicatena	1001175st1_H1	2	1	No Cluster present	Yes	Human Stool
Dorea longicatena	P3wC11	1	1	No Cluster present	No	Human Faeces
uncultured Clostridium sp.	2789STDY5834873	0	1	Lasso peptide	No	Human Faeces
Clostridium sp.	Marseille-P2538	1	1	1 structural class I lanthipeptide, 1 class II lanthipeptide	Yes	Human Faeces
Pseudobutyrivibrio sp.	**49**	1	1	4 - Lanthipeptide	Yes	The Hungate 1000. A catalogue of reference genomes from the rumen microbiome.
Pseudobutyrivibrio sp.	**UC1225**	1	1	4 - Lanthipeptide	Yes	The Hungate 1000. A catalogue of reference genomes from the rumen microbiome.
Dorea sp.	Marseille-P4042	2	1	1 structural class I lanthipeptide	Yes	Human Stool

uncultured Clostridiales bacterium	UMGS361	0	1	2 class II lanthipeptides (LanM) and 2 structural gallidermin like structural genes	No	Human Gut
Clostridium indicum (firmicutes)	PI-S10-A1B	1	1	1 structural class I lanthipeptide, 2 class II lanthipeptide (lanM)	Yes	Sludge of industrial effluent plant
Clostridium sp.	AM25-23AC	1	0	UviB like structural peptides	No	Human Faeces
Clostridium sp. (firmicutes)	AF28-12	1	1	No cluster present	No	Human Faeces
Clostridiaceae bacterium (firmicutes)	AF02-42	1	1	Ranthipeptide	No	Human Faeces
Clostridiaceae bacterium (firmicutes)	TF01-6	2	1	Partial cluster, no structural genes	No	Human Faeces
***Blautia obeum* (firmicutes)**	**TM09-13AC**	1	1	4 - Lanthipeptide	Yes	Human Faeces
Dorea formicigenerans	TF09-3	0	1	No cluster present	Yes	Human Faeces
Ruminococcus gnavus (firmicutes)	AF27-4BH	3	1	3 class II lantibiotic	Yes	Human Faeces
Eubacterium rectale	AF25-15	0	1	No Cluster present	Yes	Human Faeces

Dorea longicatena	AF17-8AC	1	0	No Cluster present (2 class I structural genes)	Yes	Human Faeces
[Eubacterium] rectale	AF17-27	0	1	3 - bacteriocin structural genes	Yes	Human Faeces
Ruminococcus sp. (firmicutes)	AF32-2AC	1	1	1 class I lanthipeptide	Yes	Human Faeces
Ruminococcus sp. (firmicutes)	AM49-8	1	1	1 class I lanthipeptide	Yes	Human Faeces
Ruminococcus sp. (firmicutes)	AM49-10BH	1	1	1 class I lanthipeptide	Yes	Human Faeces
Ruminococcus sp.	OM04-4AA	0	2	1 class I lanthipeptide	Yes	Human Faeces
[Ruminococcus] gnavus (firmicutes)	AF13-14A	3	1	1 class I lanthipeptide	Yes	Human Faeces
[Eubacterium] rectale	2789STDY 5834884	0	1	Bacteriocin cluster - no structural peptide	No	Human Faeces
Blautia sp. (firmicutes)	YL58	1	1	5 - Lanthipeptide	Yes	Mouse Ceca
Merdimonas faecis (firmicutes)	BR31	2	1	2 class I lanthipeptide (1 with lanM)	Yes	Human Stool
Drancourtella sp. (firmicutes)	An177	1	1	1 class I lanthipeptide	No	Chicken Caecum
Blautia coccoides (firmicutes)	YL58	1	1	5 - Lanthipeptide	Yes	Mouse Ceca
Hungatella sp. (firmicutes)	UBA3048	1	1	1 class I lanthipeptide	No	Wood

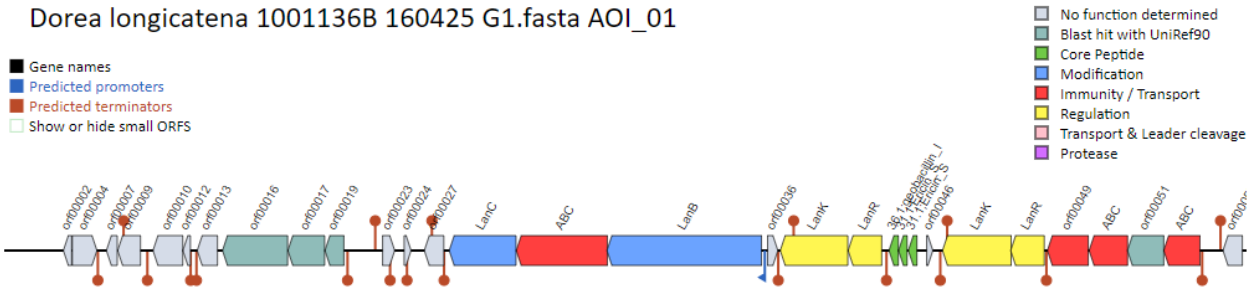
Hungatella xylanolytica (firmicutes)	DSM 3808	1	1	1 class I lanthipeptide	Yes	Sequencing as part of the Genomic Encyclopedia of Archaeal and Bacterial Type Strains, Phase II project

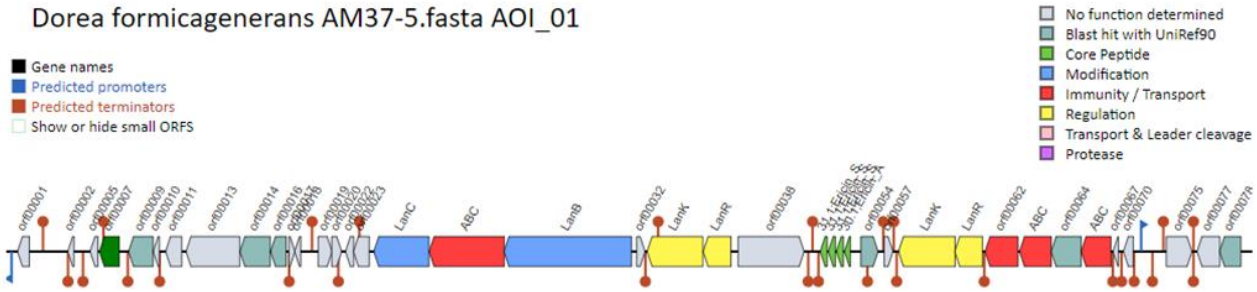
Table 3.3.4. Analysis of the 59 *Lachnospiraceae* genomes that contained either *nsoB* and/or *nsoC*. The presence or absence of the *nsoB* or *nsoC* genes, the bacteriocin biosynthetic gene cluster, presence or absence of immunity genes and isolation source are displayed. Bacterial and strain names in bold and underlined indicate those genomes which contained four putative structural peptides within the biosynthetic gene cluster.

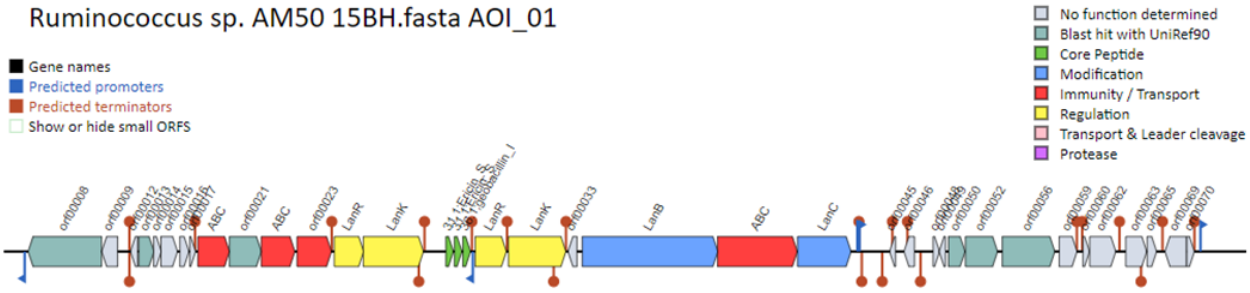
The 34 genomes that contained both *nsoB* and *nsoC* were used to manually search for the presence of the nisin O cluster and an additional two genomes with nisin O-like clusters were found, *Pseudobutyrivibrio* sp. 49 and *Pseudobutyrivibrio* sp. UC1225, based on the presence of four putative structural peptides (Table 3.3.4). However, both clusters were split between two contigs (Figure 3.3.1). This was because the assembly was broken into two scaffolds with partial genes where the annotation pipeline does not correctly recognise the start and the end of the genes, thus splitting the cluster into two. With the addition of these two genomes 10 nisin O-like clusters have been identified in members of the *Lachnospiraceae* family. It was also seen in a number of other nisin gene operons that the clusters appear to be split over two contigs, particularly around the putative structural genes, which was likely due to the repetitive nature of the genes causing a break in the assembly (Figure 3.3.1).

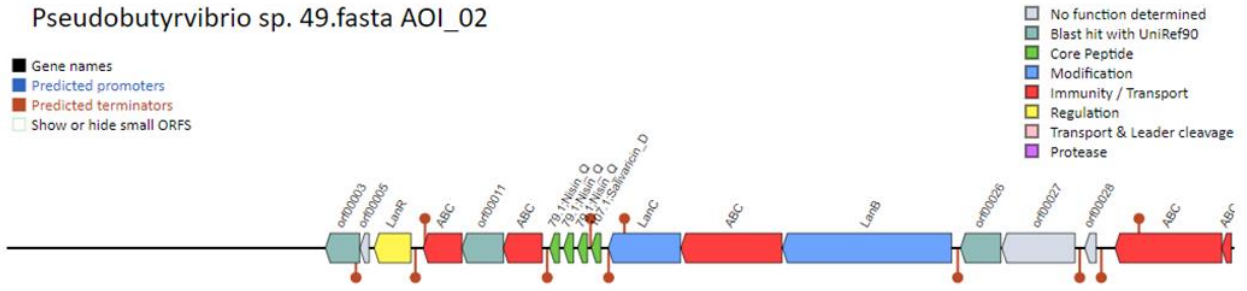
The number of structural peptides differed across the clusters; however, this was most likely an assembly breakage point from the automatic annotation performed in Geneious R11. This can clearly be seen in the BAGEL4 output for *B. obeum* A2-162 which only identifies one structural gene rather than four (Figure 3.3.1). Interestingly, none of the clusters identified

have a protease like that of LanP in *B. producta* SCSK (Kim, Becattini *et al*. 2019). In the case of the cluster found in *R. gnavus* AF33-12 the cluster was identified in AntiSmash, however it was not recognised in BAGEL4. Additionally, the analysis using Orthofinder failed to identify *D. longicatena* 1001136B-160425 as a genome that contained both *nsoB* and *nsoC*.


Dorea longicatena 1001136B 160425 G1.fasta AOI_01
Gene names
Predicted promoters
Predicted terminators
Show or hide small ORFS
No function determined
Blast hit with UniRef90
Core Peptide
Modification
Immunity / Transport
Regulation
Transport & Leader cleavage
Protease


Dorea formicageneransAM37-5.fasta AOI_01
Gene names
Predicted promoters
Predicted terminators
Show or hide small ORFS
No function determined
Blast hit with UniRef90
Core Peptide
Modification
Immunity / Transport
Regulation
Transport & Leader cleavage
Protease


Ruminococcus sp. AM50 15BH.fasta AOI_01
Gene names
Predicted promoters
Predicted terminators
Show or hide small ORFS
No function determined
Blast hit with UniRef90
Core Peptide
Modification
Immunity / Transport
Regulation
Transport & Leader cleavage
Protease


Pseudobutyrvibrio sp. 49.fasta AOI_02
Gene names
Predicted promoters
Predicted terminators
Show or hide small ORFS
No function determined
Blast hit with UniRef90
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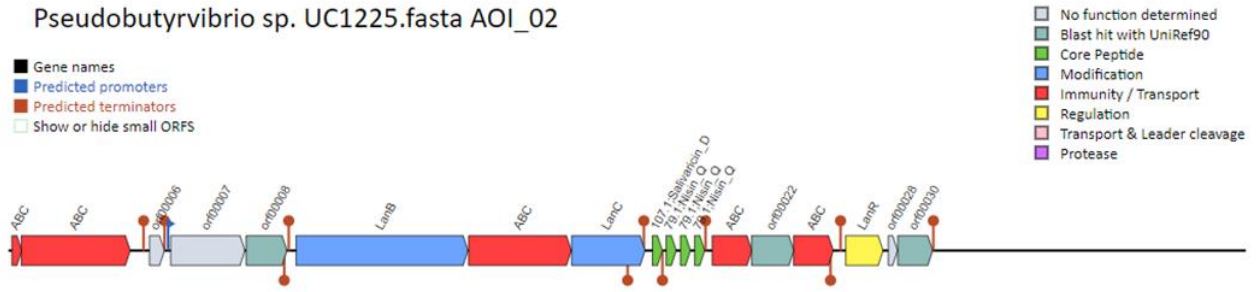
Modification
Immunity / Transport
Regulation
Transport & Leader cleavage
Protease


Pseudobutyrvibrio sp. UC1225.fasta AOI_02
Gene names
Predicted promoters
Predicted terminators
Show or hide small ORFS
No function determined
Blast hit with UniRef90
Core Peptide
Modification
Immunity / Transport
Regulation
Transport & Leader cleavage
Protease

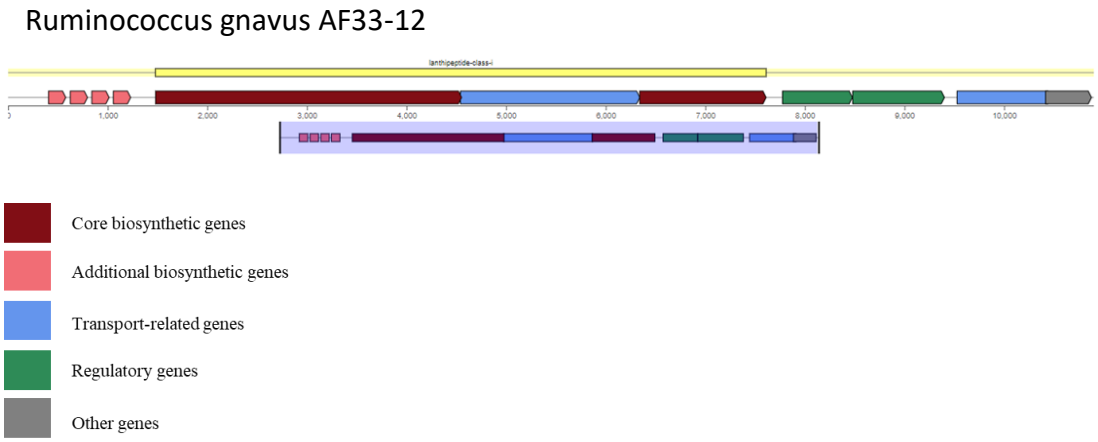


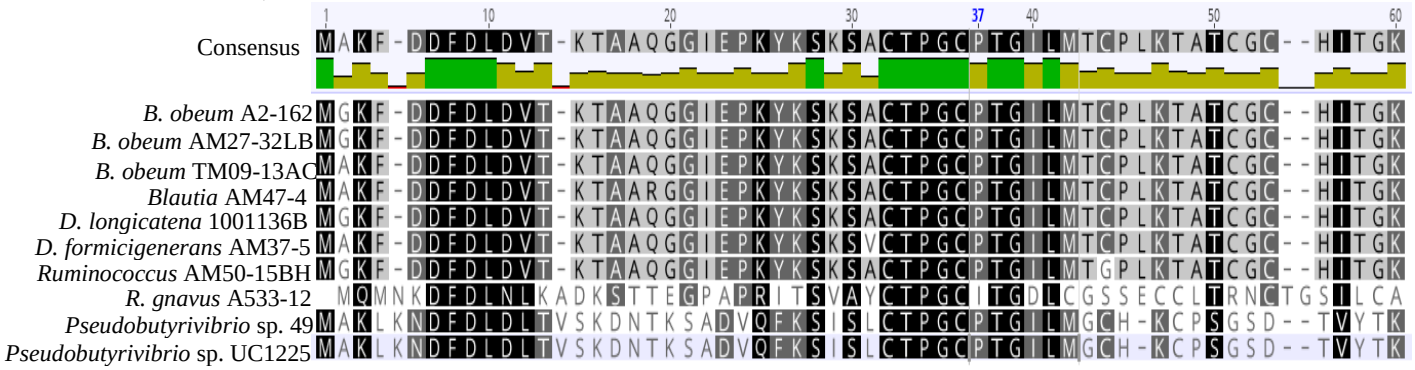
Figure 3.3.1. Nisin O-like clusters found in *Lachnospiraceae*. All clusters are shown using the output from BAGEL4, except the cluster from *Ruminococcus gnavus* AF33-12 which is displayed using AntiSmash output.

Curiously, the clusters identified in *Blautia* sp. AM47-4, *Ruminococcus* sp. AM50-15BH, *D. formicigenerans* AM37-5 and *D. longicatena* 1001136B-160425 had very high synteny with the original nisin O cluster found in *B. obeum* A2-162. The BAGEL4 output for *B. obeum* AM27-32LB and *B. obeum* TM09-13AC identified only half of a nisin O-like cluster, with the cluster further identified through manual analysis of the genome in Geneious R11, which showed similar synteny to the *B. obeum* A2-162 cluster. However, the clusters for the two *Pseudobutyrivibrio* species, although identical to each other in terms of their gene presence and order, differed from the nisin O cluster in *B. obeum* A2-162. This can clearly be observed using the BAGEL4 analysis as although there are four structural genes present the locations of the modification, immunity and regulatory genes are found in different places when compared to the *B. obeum* A2-162 cluster. This is also seen for the cluster identified in *R. gnavus* AF33-12, where the location of the modification and regulatory genes are found in different *loci* compared to the nisin O cluster. From all the clusters identified either directly using the BAGEL4 analysis or further analysis searching the whole genome, two TCSs were present within the biosynthetic gene clusters, except those from the *Pseudobutyrivibrio* strains and *R. gnavus* AF33-12 which only contained one regulatory system. Additionally, in all clusters no putative protease, which is required for leader peptide cleavage and hence activation of the antimicrobial peptide was found within the cluster.

3.3.4 Homology Between NsoA and LanA Peptides from Nisin O-Like Clusters

To investigate whether the amino acid homology between NsoA1-4 and the sequences from the LanA1-4 genes, termed LanA as it was unknown at this stage of the study whether these peptides were indeed homologues of nisin O, were aligned to assess the conserved residues. Overall NsoA1 and the corresponding LanA1 had a similarity of 66.7% across all protein sequences, with a conserved DFDLD region within the leader peptide and CTPGC in the core peptide across all peptides (Figure 3.3.2 A). These residues in the leader and core peptides are also conserved in other nisin variants (Hatziioanou, Gherghisan-Filip *et al*. 2017). However, it was clearly observed that the LanA1 peptides from *R. gnavus* A533-12, *Pseudobutyrivibrio* sp. 49 and *Pseudobutyrivibrio* sp. UC1225 had very different amino acid percentage identity to NsoA1 at 30.51%, 43.10% and 43.10%, respectively (Figure 3.3.2 B). Within the core peptide there was a high level of conservation of serine, cysteine and threonine residues which is important for lanthionine ring formation. This was also seen across the LanA2, LanA3 and LanA4 peptides. However, there was less conservation in the *Pseudobutyrivibrio* species and *R. gnavus* A533-12 when compared to the structural peptide from *B. obeum* A2-162 (Figures 3.3.2-5A).

A)



B)

	NsoA1 - *B. obeum* A2-162	LanA1 - *B. obeum* AM27-32LB	LanA1 - *B. obeum* TM09-13AC	LanA1 - *Blautia* AM47-4	LanA1 - *D. longicatena* 1001136B	LanA1 - *D. formicigenerans* AM37-5	LanA1 - *Ruminococcus* AM50-15BH	LanA1 - *R. gnavus* A533-12	LanA1 - *Pseudobutyrivibrio* sp. 49	LanA1 - *Pseudobutyrivibrio* sp. UC1225
NsoA1 - *B. obeum* A2-162	-	100%	98.21%	96.43%	100%	96.43%	98.21%	30.51%	43.10%	43.10%
LanA1 - *B. obeum* AM27-32LB	100%	-	98.21%	96.43%	100%	96.43%	98.21%	30.51%	43.10%	43.10%
LanA1 - *B. obeum* TM09-13AC	98.21%	98.21%	-	98.21%	98.21%	98.21%	96.43%	30.51%	44.83%	44.83%
LanA1 - *Blautia* AM47-4	96.43%	96.43%	98.21%	-	96.43%	96.43%	94.64%	30.51%	44.83%	44.83%
LanA1 - *D. longicatena* 1001136B	100%	100%	98.21%	96.43%	-	96.43%	98.21%	30.51%	43.10%	43.10%
LanA1 - *D. formicigenerans* AM37-5	96.43%	96.43%	98.21%	96.43%	96.43%	-	94.64%	30.51%	44.83%	44.83%
LanA1 - *Ruminococcus* AM50-15BH	98.21%	98.21%	96.43%	94.64%	98.21%	94.64%	-	30.51%	41.38%	41.38%
LanA1 - *R. gnavus* A533-12	30.51%	30.51%	30.51%	30.51%	30.51%	30.51%	30.51%	-	30.51%	30.51%
LanA1 - *Pseudobutyrivibrio* sp. 49	43.10%	43.10%	44.83%	44.83%	43.10%	44.83%	41.38%	30.51%	-	100%
LanA1 - *Pseudobutyrivibrio* sp. UC1225	43.10%	43.10%	44.83%	44.83%	43.10%	44.83%	41.38%	30.51%	100%	-

Figure 3.3.2. A) A Muscle alignment of LanA1 amino acid sequences from novel nisin O-like clusters compared with NsoA1 from *B. obeum* A2-162. B) Percentage identity heatmap of LanA1 and NsoA1 amino acid sequences compared with each other.

Further analysis of the NsoA2 and NsoA3 homologues, termed LanA2 and LanA3 from those found in the new nisin O-like cluster, respectively, was performed in the same manner as NsoA1. Again, it was found that LanA1 peptides from *R. gnavus* A533-12, *Pseudobutyrivibrio* sp. 49 and *Pseudobutyrivibrio* sp. UC1225 LanA2 and LanA3 peptides shared far less homology to NsoA2 (36.21%, 50.00% and 50.00%, respectively) and NsoA3 (31.58%, 46.55% and 46.55%, respectively) compared with the other LanA2 and LanA3 amino acid sequences from the other bacterial species which were all greater that 98.21% for both LanA2 and LanA3 (Figure 3.3.3 B and Figure 3.3.4 B). The same conserved regions as seen in the NsoA1 comparison were present in this alignment (Figure 3.3.3 A and Figure 3.3.4 A). Curiously, *D. longicatena* 100136B-160425 and *Ruminococcus* AM50-15BH did not contain a *lanA3* in their annotated genome, however, as both NsoA2 and NsoA3 are identical in their amino acid sequence it is possible that the LanA3 was mis-annotated during the assembly of the genome and was therefore missing from the biosynthetic gene cluster. Furthermore, the amino similarities between LanA2 and LanA3 with NsoA2 and NsoA3, respectively, was not an exact match within all clusters with the glutamine residue at position 19 replaced with an

arginine in the LanA2 peptide of *B. obeum* TM09-13AC, *Blautia* AM47-4 and *D. formicigenerans* AM37-5 and the alanine at position 31 in NsoA3 replaced with a valine in *D. formicigenerans* AM37-5 (Figure 3.3.3A). This is unlike what is observed in *B. obeum* A2-162 where the NsoA2 and NsoA3 are identical.

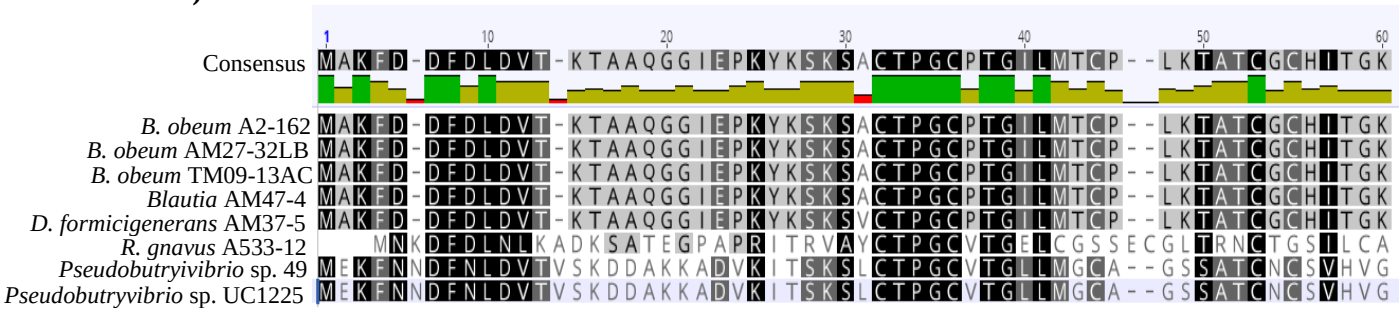
A)

B)

	NsoA2 - *B. obeum* A2-162	LanA2 - *B. obeum* AM27-32LB	LanA2 - *B. obeum* TM09-13AC	LanA2 - *Blautia* AM47-4	LanA2 - *D. longicatena* 1001136B	LanA2 - *D. formicigenerans* AM37-5	LanA2 - *Ruminococcus* AM50-15BH	LanA2 - *R. gnavus* A533-12	LanA2 - *Pseudobutyrivibrio* sp. 49	LanA2 - *Pseudobutyrivibrio* sp. UC1225
NsoA2 - *B. obeum* A2-162	-	100%	98.21%	98.21%	100%	98.21%	100%	36.21%	50.00%	50.00%
LanA2 - *B. obeum* AM27-32LB	100%	-	98.21%	98.21%	100%	98.21%	100%	36.21%	50.00%	50.00%
LanA2 - *B. obeum* TM09-13AC	98.21%	98.21%	-	100%	98.21%	100%	98.21%	36.21%	50.00%	50.00%
LanA2 - *Blautia* AM47-4	98.21%	98.21%	100%	-	98.21%	100%	98.21%	36.21%	50.00%	50.00%
LanA2 - *D. longicatena* 1001136B	100%	100%	98.21%	98.21%	-	98.21%	100%	36.21%	50.00%	50.00%
LanA2 - *D. formicigenerans* AM37-5	98.21%	98.21%	100%	100%	98.21%	-	98.21%	36.21%	50.00%	50.00%
LanA2 - *Ruminococcus* AM50-15BH	100%	100%	98.21%	98.21%	100%	98.21%	-	36.21%	50.00%	50.00%
LanA2 - *R. gnavus* A533-12	36.21%	36.21%	36.21%	36.21%	36.21%	36.21%	36.21%	-	39.66%	39.66%
LanA2 - *Pseudobutyrivibrio* sp. 49	50.00%	50.00%	50.00%	50.00%	50.00%	50.00%	50.00%	39.66%	-	100%
LanA2 - *Pseudobutyrivibrio* sp. UC1225	50.00%	50.00%	50.00%	50.00%	50.00%	50.00%	50.00%	39.66%	100%	-

Figure 3.3.3. A) A Muscle alignment of LanA2 amino acid sequences from novel nisin O-like clusters compared with NsoA2 from *B. obeum* A2-162. B) Percentage identity heatmap of LanA2 and NsoA2 amino acid sequences compared with each other.

A)



B)

	NsoA3 - *B. obeum* A2-162	LanA3 - *B. obeum* AM27-32LB	LanA13 - *B. obeum* TM09-13AC	LanA3 - *Blautia* AM47-4	LanA3 - *D. formicigenerans* AM37-5	LanA3 - *R. gnavus* A533-12	LanA3 - *Pseudobutyrivibrio* sp. 49	LanA3 - *Pseudobutyrivibrio* sp. UC1225
NsoA3 - *B. obeum* A2-162	-	100%	100%	100%	98.21%	31.58%	46.55%	46.55%
LanA3 - *B. obeum* AM27-32LB	100%	-	100%	100%	98.21%	31.58%	46.55%	46.55%
LanA3 - *B. obeum* TM09-13AC	100%	100%	-	100%	98.21%	31.58%	46.55%	46.55%
LanA3 - *Blautia* AM47-4	100%	100%	100%	-	98.21%	31.58%	46.55%	46.55%
LanA3 - *D. formicigenerans* AM37-5	98.21%	98.21%	98.21%	98.21%	-	31.58%	46.55%	46.55%
LanA3 - *R. gnavus* A533-12	31.58%	31.58%	31.58%	31.58%	31.58%	-	35.09%	35.09%
LanA3 - *Pseudobutyrivibrio* sp. 49	46.55%	46.55%	46.55%	46.55%	46.55%	35.09%	-	100%
LanA3 - *Pseudobutyrivibrio* sp. UC1225	46.55%	46.55%	46.55%	46.55%	46.55%	35.09%	100%	-

Figure 3.3.4. A) A Muscle alignment of LanA3 amino acid sequences from novel nisin O-like clusters compared with NsoA3 from *B. obeum* A2-162. B) Percentage identity heatmap of LanA3 and NsoA3 amino acid sequences compared with each other.

The homology to NsoA4 was the lowest of the four structural peptides with an overall identity percentage of 57.3%. The protein sequences from LanA4 from *R. gnavus* A533-12, *Pseudobutyrivibrio* sp. 49 and *Pseudobutyrivibrio* sp. UC1225 as with the other three structural peptides have largely dissimilar sequence identity at 33.93%, 37.29% and 37.29%, respectively, when compared to NsoA4 (Figure 3.3.5 B). Furthermore, the LanA4 of *D. formicigenerans* AM37-5 also has a low LanA4 amino acid percentage identity when compared to NsoA4 at 32.14%. A conserved domain of DFDLD is maintained in the leader peptide and all predicted core peptides begin with ITS (Figure 3.3.5 A), which is also conserved in the core peptides of nisin A, F, Q, Z and U (Hatziioanou, Gherghisan-Filip *et al*. 2017).

A)

B)

	NsoA4 - *B. obeum* A2-162	LanA4 - *B. obeum* AM27-32LB	LanA4 - *B. obeum* TM09-13AC	LanA4 - *Blautia* AM47-4	LanA4 - *D. longicatena* 1001136B	LanA4 - *D. formicigenerans* AM37-5	LanA4 - *Ruminococcus* AM50-15BH	LanA4 - *R. gnavus* A533-12	LanA4 - *Pseudobutyrivibrio* sp. 49	LanA4 - *Pseudobutyrivibrio* sp. UC1225
NsoA4 - *B. obeum* A2-162	-	100%	94.74%	96.49%	100%	32.14%	100%	33.93%	37.29%	37.29%
LanA4 - *B. obeum* AM27-32LB	100%	-	94.74%	96.49%	100%	32.14%	100%	33.93%	37.29%	37.29%
LanA4 - *B. obeum* TM09-13AC	94.74%	94.74%	-	98%	94.74%	32.14%	94.74%	33.33%	35.59%	35.59%
LanA4 - *Blautia* AM47-4	96.49%	96.49%	98%	-	96.49%	33.93%	96.49%	33.33%	37.29%	37.29%
LanA4 - *D. longicatena* 1001136B	100%	100%	94.74%	96.49%	-	32.14%	100%	33.93%	37.29%	37.29%
LanA4 - *D. formicigenerans* AM37-5	32.14%	32.14%	32.14%	33.93%	32.14%	-	32.14%	24.07%	36.84%	36.84%
LanA4 - *Ruminococcus* AM50-15BH	100%	100%	94.74%	96.49%	100%	32.14%	-	33.93%	37.29%	37.29%
LanA4 - *R. gnavus* A533-12	33.93%	33.93%	33.33%	33.33%	33.93%	24.07%	33.93%	-	38.18%	38.18%
LanA4 - *Pseudobutyrivibrio* sp. 49	37.29%	37.29%	35.59%	37.29%	37.29%	36.84%	37.29%	38.18%	-	100%
LanA4 - *Pseudobutyrivibrio* sp. UC1225	37.29%	37.29%	35.59%	37.29%	37.29%	36.84%	37.29%	38.18%	100%	-

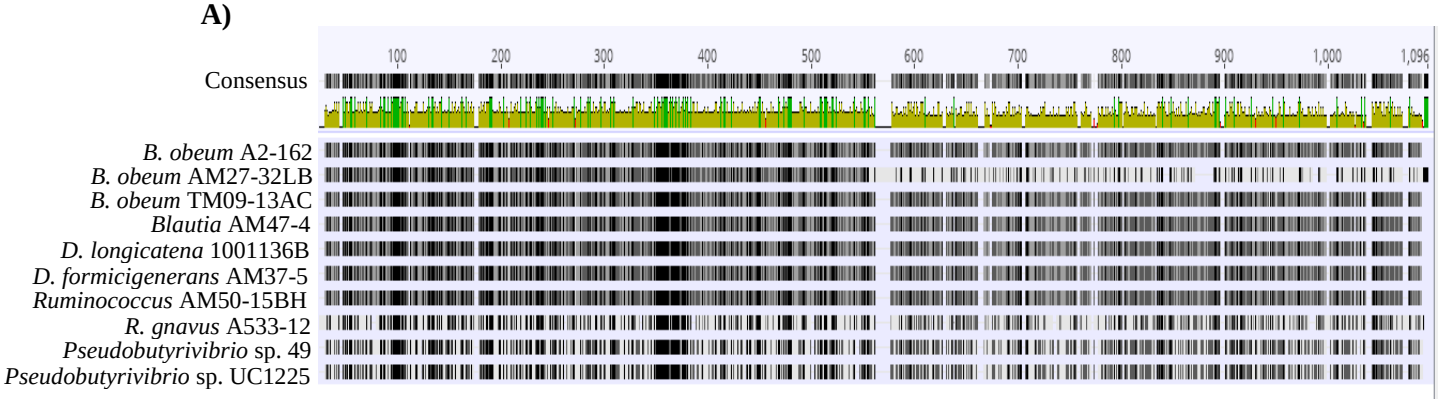
Figure 3.3.5. A) A Muscle alignment of LanA4 amino acid sequences from novel nisin O-like clusters compared with NsoA4 from *B. obeum* A2-162. B) Percentage identity heatmap of LanA4 and NsoA4 amino acid sequences compared with each other.

3.3.5 Homology Between NsoB and NsoC with LanB and LanC from Nisin O-like Clusters

Analysis of the LanB and LanC amino acid sequences from the nisin O-like clusters aligned using NsoB and NsoC as template sequences, respectively, again showed a high level of similarity between the proteins produced by the *B. obeum* A2-162 cluster and those found in *B. obeum* TM09-13AC, *Blautia* sp. AM47-4, *Ruminococcus* sp. AM50-15BH, *D. formicigenerans* AM37-5 and *D. longicatena* 1001136B-160425 (Figure 3.3.6 A). This residue amino acid percentage identity ranged from 98.72% to 100% identity (Figure 3.3.6 B). Interestingly, the sequence for the LanB found in the *B. obeum* AM27-32LB cluster only

had a similarity of 56.70% (Figure 3.3.6 B). Upon inspection of this sequence, it was confirmed that the N-terminus of the LanB was an exact match to NsoB however beyond position 549 of the protein sequence there was very little sequence homology at the C-terminal end of the protein (Figure 3.3.6 A). Furthermore, it was observed that the amino acid sequence identity between *R. gnavus* AF33-12 and the two *Pseudobutyrivibrio* sp. 49 and *Pseudobutyrivibrio* sp. UC1225 had minimal homology to NsoB at 28.27%, 34.51% and 34.70%, respectively (Figure 3.3.6 B).

A)



B)

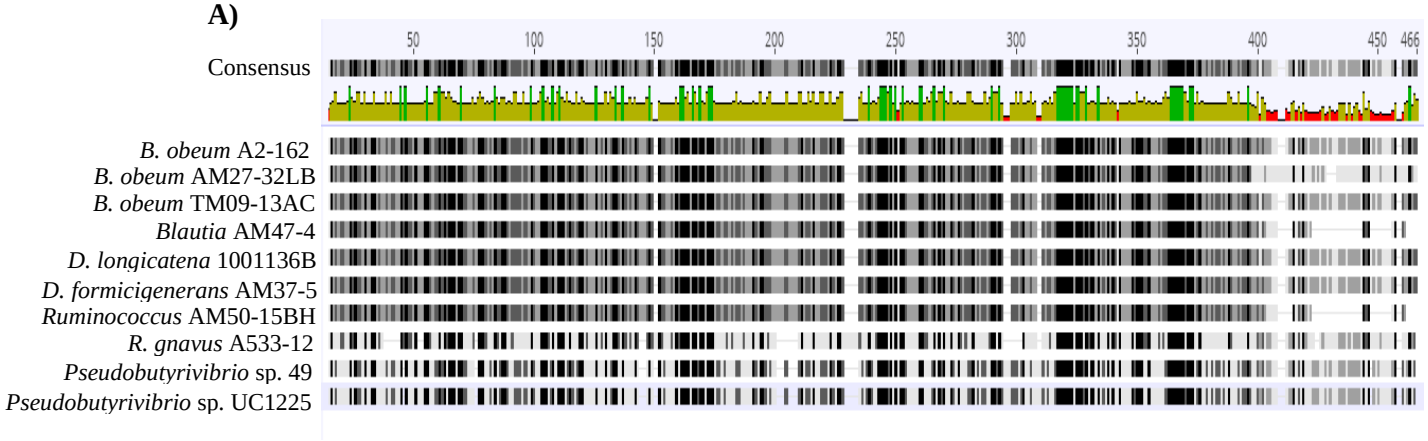
	NsoB - *B. obeum* A2-162	LanB - *B. obeum* AM27-32LB	LanB - *B. obeum* TM09-13AC	LanB - *Blautia* AM47-4	LanB - *D. longicatena* 1001136B	LanB - *D. formicigenerans* AM37-5	LanB - *Ruminococcus* AM50-15BH	LanB - *R. gnavus* A533-12	LanB - *Pseudobutyrivibrio* sp. 49	LanB - *Pseudobutyrivibrio* sp. UC1225
NsoB - *B. obeum* A2-162	-	56.70%	98.85%	98.82%	100%	98.72%	100%	28.27%	34.51%	34.70%
LanB - *B. obeum* AM27-32LB	56.70%	-	56.14%	56.14%	56.70%	56.14%	56.70%	21.01%	22.19%	22.19%
LanB - *B. obeum* TM09-13AC	98.85%	56.14%	-	100%	98.82%	99.90%	98.82%	28.18%	34.51%	34.51%
LanB - *Blautia* AM47-4	98.82%	56.14%	100%	-	98.82%	99.90%	98.82%	28.18%	34.51%	34.51%
LanB - *D. longicatena* 1001136B	100%	56.70%	98.82%	98.82%	-	98.72%	100%	28.27%	34.51%	34.70%
LanB - *D. formicigenerans* AM37-5	98.72%	56.14%	99.90%	99.90%	98.72%	-	98.72%	28.08%	34.51%	34.51%
LanB - *Ruminococcus* AM50-15BH	100%	56.70%	98.82%	98.82%	100%	98.72%	-	28.27%	34.51%	34.70%
LanB - *R. gnavus* A533-12	28.27%	21.01%	28.18%	28.18%	28.27%	28.08%	28.27%	-	25.53%	25.53%
LanB - *Pseudobutyrivibrio* sp. 49	34.51%	22.19%	34.51%	34.51%	34.51%	34.51%	34.51%	25.53%	-	97.51%
LanB - *Pseudobutyrivibrio* sp. UC1225	34.70%	22.19%	34.51%	34.51%	34.70%	34.51%	34.70%	25.53%	97.51%	-

Figure 3.3.6. A) A Muscle alignment of LanB amino acid sequences from novel nisin O-like clusters compared with NsoB from *B. obeum* A2-162. B) Percentage identity heatmap of LanB and NsoB amino acid sequences compared with each other.

Further analysis of the NsoC like proteins from the novel clusters showed that LanC of *B. obeum* AM27-32LB, *B. obeum* TM09-13AC, *Blautia* sp. AM47-4, *Ruminococcus* sp. AM50-15BH, *D. formicigenerans* AM37-5 and *D. longicatena* 1001136B-160425 all had very high

amino acid sequence similarity, ranging from 86.31% to 100% amino acid sequence identity (Figure 3.3.7 A and B). Curiously, from residue 392 of NsoC the similarity of LanC amino acid sequence from the strains *B. obeum* AM27-LB and *Blautia* AM47-4 is greatly reduced, as seen in Figure 3.3.7 A. As previously seen the homology of *R. gnavus* AF33-12 and the two *Pseudobutyrivibrio* sp. 49 and *Pseudobutyrivibrio* sp. UC1225 was poor when compared to NsoB at 23.62%, 30.09% and 30.09%, respectively (Figure 3.3.7 B).

A)



B)

	NsoC - *B. obeum* A2-162	LanC - *B. obeum* AM27-32LB	LanC - *B. obeum* TM09-13AC	LanC - *Blautia* AM47-4	LanC - *D. longicatena* 1001136B	LanC - *D. formicigenerans* AM37-5	LanC - *Ruminococcus* AM50-15BH	LanC - *R. gnavus* A533-12	LanC - *Pseudobutyrivibrio* sp. 49	LanC - *Pseudobutyrivibrio* sp. UC1225
NsoC - *B. obeum* A2-162	-	86.31%	99.11%	87.81%	99.11%	100%	88.71%	23.62%	30.09%	30.09%
LanC - *B. obeum* AM27-32LB	86.31%	-	85.65%	86.26%	85.65%	86.07%	86.94%	19.43%	27.52%	27.52%
LanC - *B. obeum* TM09-13AC	99.11%	85.65%	-	89%	100%	99.09%	87.81%	23.84%	30.09%	30.09%
LanC - *Blautia* AM47-4	87.81%	86.26%	89%	-	88.71%	87.59%	99.02%	20.81%	27.46%	27.46%
LanC - *D. longicatena* 1001136B	99.11%	85.65%	100%	88.71%	-	99.09%	87.81%	23.84%	30.09%	30.09%
LanC - *D. formicigenerans* AM37-5	100%	86.07%	99.09%	87.59%	99.09%	-	88.51%	23.94%	30.09%	30.09%
LanC - *Ruminococcus* AM50-15BH	88.71%	86.94%	87.81%	99.02%	87.81%	88.51%	-	20.58%	27.46%	27.46%
LanC - *R. gnavus* A533-12	23.62%	19.43%	23.84%	20.81%	23.84%	23.94%	20.58%	-	25.74%	25.74%
LanC - *Pseudobutyrivibrio* sp. 49	30.09%	27.52%	30.09%	27.46%	30.09%	30.09%	27.46%	25.74%	-	100%
LanC - *Pseudobutyrivibrio* sp. UC1225	30.09%	27.52%	30.09%	27.46%	30.09%	30.09%	27.46%	25.74%	100%	-

Figure 3.3.7. A) A Muscle alignment of LanC amino acid sequences from novel nisin O-like clusters compared with NsoC from *B. obeum* A2-162. B) Percentage identity heatmap of LanC and NsoC amino acid sequences compared with each other.

To further assess the relatedness of the modification machinery within the nisin O-like clusters the *nsoB* and *nsoC* genes were concatenated and used to make a neighbour-joining phylogenetic tree (Figure 3.3.8). This was in order to understand the similarity of these genes

within the cluster and, when used with the homology data previously described, would allow for a greater understanding of which clusters are more similar when compared to each other. In addition to the nisin O-like clusters the *nisB-nisC* and *nszB-nszC* from the nisin A (*L. lactis* sp. *lactis*) and nisin Z (*L. lactis* IO-1) clusters, respectively, were also included as reference genes. The phylogenetic tree shows that both *Pseudobutyrivibrio* species cluster together as well as both *L. lactis* species. The clustering of *B. obeum* AM27-32LB, *B. obeum* TM09-13AC, *Blautia* sp. AM47-4, *Ruminococcus* sp. AM50-15BH, *D. formicigenerans* AM37-5 and *D. longicatena* 1001136B-160425 all cluster together with a slight sub-clustering of *D. formicigenerans* AM37-5 and *B. obeum* TM09-13AC. Interestingly, *Blautia* AM47-4 has the least similar *lanB-lanC* genes of the clusters that had high percentage identities (Figure 3.3.8).

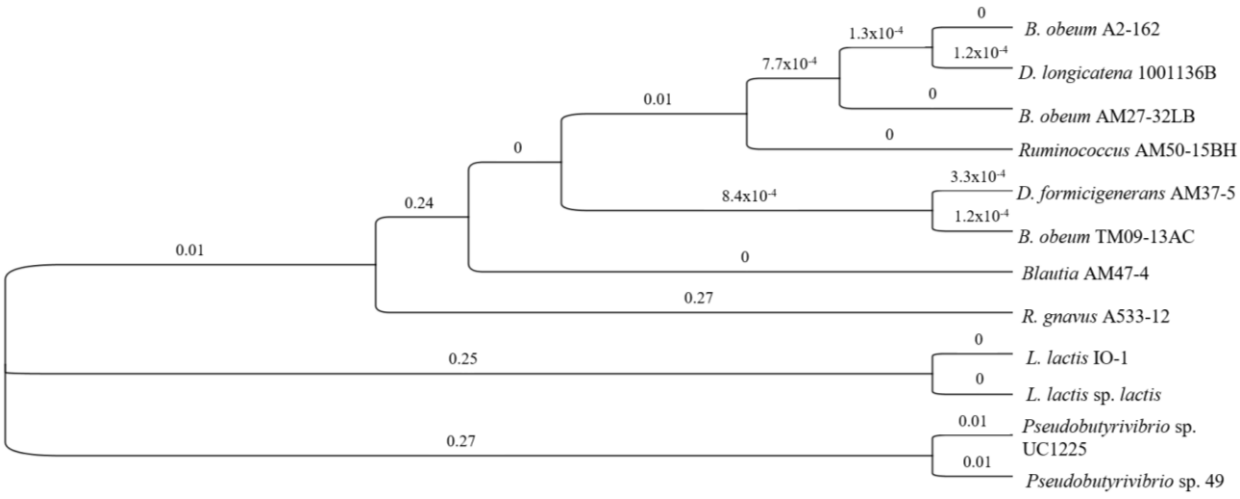


Figure 3.3.8. Neighbour-joining tree of concatenated *lanB-lanC* genes.

An additional neighbour-joining phylogenetic tree was created using all genes within the novel clusters to investigate whether the data seen in figure 3.3.8 was repeatable on a whole cluster comparison. The lantibiotic cluster identified in *B. producta* SCSK was also included as a reference as this cluster has a high similarity to the nisin O cluster in terms of the location of genes within the cluster (Kim, Becattini *et al*. 2019). The nisin O-like clusters form two sub-groups, one containing *B. obeum* A2-162, *B. obeum* AM27-32LB, *Ruminococcus* sp. AM50-15BH and *D. longicatena* 1001136B-160425 and the other cluster containing *B. obeum* TM09-13AC, *Blautia* sp. AM47-4 and *D. formicigenerans* AM37-5 (Figure 3.3.9).

As with the previous tree the two *Pseudobutyrivibrio* species, which cluster together, and the *R. gnavus* A533-12 are the least similar to the nisin O cluster from *B. obeum* A2-126. The lantibiotic cluster in *B. producta* SCSK however is more similar comparatively to the biosynthetic gene cluster of nisin O than these species (Figure 3.3.9).

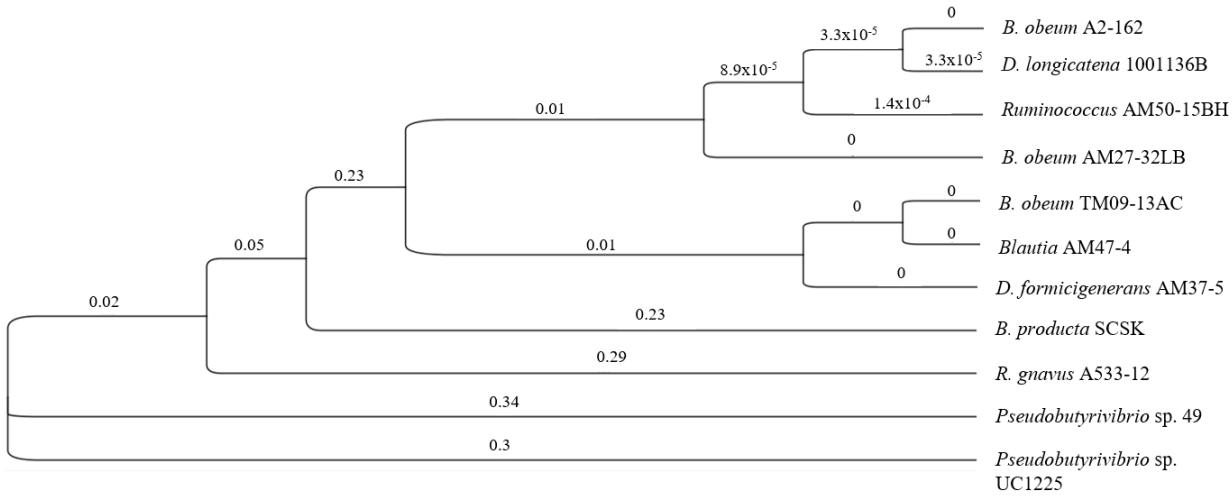


Figure 3.3.9. Neighbour-joining tree of nisin O-like clusters.

Based on the data from both the homology analysis of the LanA1-4, LanB and LanC peptides, as well as the phylogenetic tree using the concatenated *lanB-lanC* and whole clusters, it could be determined that the clusters from both *Pseudobutyrivibrio* species and *R. gnavus* A533-12 were not the nisin O cluster and the remaining novel clusters identified were very likely to be highly similar variants of nisin O. Therefore, to investigate these clusters in more detail a Mauve analysis was used to compare these candidate clusters together to look for similar regions (Figure 3.3.10). Unlike the BAGEL4 analysis, the clusters were manually identified within the genome and the nucleotide sequence extracted prior to the Mauve alignments, therefore selecting all genes within the cluster. All these clusters shared identical synteny, with one exception in *D. formicigenerans* AM37-5 which contained an IS1182 family transposase within the cluster between the structural peptides and the second regulatory system (Figure 3.3.10). This, therefore, led to a total nucleotide pairwise identity of 89.7% across all seven clusters.

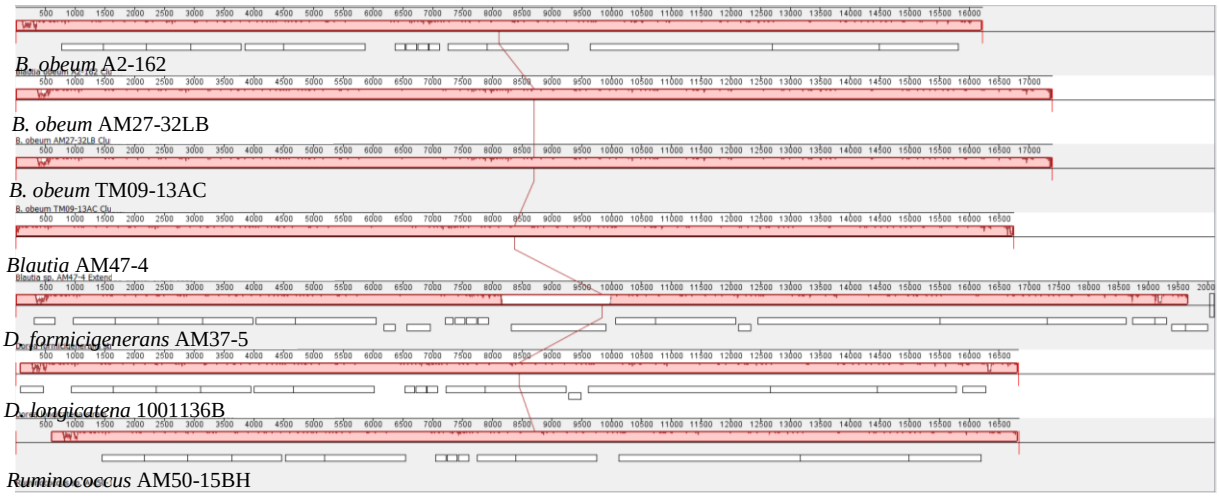


Figure 3.3.10. Mauve alignment of nisin O-like clusters.

3.4 Discussion

The structure of the nisin O cluster is unusual when compared to other nisin variants (Kuipers, Beerthuyzen *et al*. 1993, O'Connor, O'Shea *et al*. 2015, Hatziioanou, Gherghisan-Filip *et al*. 2017). However, the investigation by Kim *et al* (2019), which identified a lantibiotic cluster not only similar to nisin O but also produced by a *Blautia* species from the gut environment showed that there is the potential for this cluster to be more widespread than originally thought and not only present in *B. obeum* A2-162 (Kim, Becattini *et al*. 2019).

An initial search in *B. obeum* strains to investigate whether the cluster was present in any other strains led to the discovery of two further strains of *B. obeum*, *B. obeum* AM27-32LB and *B. obeum* TM09-13AC, which contained a four peptide lantibiotic cluster. This analysis also identified that 66% of the 50 genomes analysed had lantibiotic immunity genes present, many of which did not contain any lantibiotic gene cluster. This is a very interesting result as it suggests that the immunity genes that provide protection against nisin O are more widely found in nature and therefore may provide a competitive advantage to the bacteria that have these immunity genes while not having to bear the fitness cost and energy expenditure of maintaining a full lantibiotic cluster. Additionally, this may imply that the nisin O cluster is found in a greater number of species than those identified in this study considering the number of strains which carry immunity genes is so high. Alternatively, these immunity genes may

provide cross immunity from several nisin variants. This result was also seen in a recent study where the *nisFEG* and *nisI* genes were present in 9% of *L. lactis* ssp. *lactis, L. lactis* ssp. *cremoris* and *L. lactis* ssp. *cremoris* with *lactis* phenotype, however other elements of the cluster were not. These strains were still able to grow in high nisin concentration environments (van Gijtenbeek, Eckhardt *et al*. 2021). Furthermore, 28% of the *B. obeum* genomes contained a type II lantibiotic with the presence of a *lanM* gene and a plantaricin C-like bacteriocin. Plantaricin C is produced by the dairy strain *Lactobacillus plantarum* LL441 and had been shown to inhibit several Gram-positive bacteria (González, Arca *et al*. 1994, Flórez and Mayo 2018). This analysis of the *B. obeum* genomes discovered a potential new group of lantibiotics that may be used by this species within the gut environment. This indicates that within *B. obeum* strains there is a complex production of multiple bacteriocins to maintain a competitive advantage in the gut and that some of these strains can have immunity without the production of antimicrobials, enabling occupation of niches provided by other bacteriocin-producing strains. However, due to the focus on the nisin O cluster these novel type II lantibiotics were not further investigated in this thesis.

Through BLASTP analysis using the nisin O modification proteins NsoB and NsoC as markers seven clusters were identified through manual searches using BAGEL4 and AntiSmash. Intriguingly, not all these *nsoB* and *nsoC* containing bacteria were from the *Blautia* genus but these genes were also found in *Ruminococcus* and *Dorea* species. Due to the spread of this cluster potentially being widespread over many genera a pan-genome analysis was performed using 2094 *Lachnospiraceae* genomes. This distribution of bacteriocins across multiple genera has been seen in glycocins where 220 new putative glycocin biosynthesis clusters were spread across 153 bacterial species across seven phyla (Singh and Rao 2021).

The pan-genome analysis of *Lachnospiraceae* genoems identified 59 genomes that contained both *nsoB, nsoC* or both. The marker genes chosen were *nsoB* and *nsoC* as these were the most specific for the nisin O cluster as the *nsoA1-4* genes were deemed too small and could

be mis-matched during the analysis. Additionally, the immunity genes and regulatory genes were not selected as these were shown to be present in bacteria that did not contain the full biosynthetic gene cluster. Each of these 59 genomes were then searched manually using key word searches, BAGEL4 and AntiSmash for the presence of the nisin O biosynthetic cluster, after which two new clusters containing four structural gene were identified in *Pseudobutyrivibrio* sp. 49 and *Pseudobutyrivibrio* sp. UC1225. This resulted in 9 novel nisin O-like clusters being identified from a combination of bioinformatic techniques, including BLASTP searches and pan-genome analysis for further investigation. All the bacteria that contained a novel nisin O-like cluster were isolated from the human gut or faeces suggesting that this bacteriocin may play a role within this environment. The gut environment has previously been suggested to be a source for novel antimicrobials as these bacteriocins would co-evolve with both commensal microbes and the human host and therefore could control pathogens with minor disturbance to the commensal gut organisms (Garcia-Gutierrez, Mayer *et al*. 2019). Furthermore, many other bacteriocins which use modification proteins as part of their biosynthesis were identified as part of the manual curation of these genomes, including zoocin A, lasso family peptides and ranthipeptides. This demonstrates the diversity of bacteriocins present within the family of *Lachnospiraceae* that just contain *nsoB*-like and/or *nsoC*-like genes. Not only is the variety of bacteriocins diverse but also single strains can contain multiple bacteriocin core peptides ranging from just a singular copy to seven type II lantibiotic peptides in the case of *Butyrivibrio* sp. X503, which was isolated from buffalo rumen.

Analysis of the amino acid sequence homology of the LanA1-4, LanB and LanC peptides to their equivalents from the nisin O cluster in *B. obeum* A2-162 showed that the amino acid sequences from *B. obeum* AM27-32LB, *B. obeum* TM09-13AC, *Blautia* sp. AM47-4, *Ruminococcus* sp. AM50-15BH, *D. formicigenerans* AM37-5 and *D. longicatena* 1001136B-160425 were indeed nisin O as they had consistently high percentage identities across all residue sequences analysed. However, *R. gnavus* A533-12 had consistently low sequence homology to the reference amino acid sequence of nisin O and could not therefore be deemed

as a nisin O cluster. Furthermore, the homology and percentage identity from both *Pseudobutyrivibrio* sp. P49 and *Pseudobutyrivibrio* sp. UC1225 were again consistently low, yet slightly higher than *R. gnavus* A533-12. Intriguingly, the clusters from these *Pseudobutyrivibrio* strains were nearly identical both in terms of the gene synteny and the amino acid sequence of the proteins tested. This therefore indicates that even if a cluster is identified that contains a similar gene synteny and the same number of structural peptides it is important to analyse the data on a deeper level to avoid the risk of incorrectly assuming that the cluster of origin, in this case the nisin O cluster, is present. It was observed that for the LanB sequence of *B. obeum* AM27-32LB and the LanC sequence of *B. obeum* AM27-32LB, *Blautia* AM47-4 and *Ruminococcus* AM50-15BH the C-terminal region had a reduced homology to NsoB and NsoC, respectively. It has been shown in a study analysing the function of the NisBTC complex and the degradation of NisB that there is a potential leader peptide binding domain in the C-terminal part of NisB (Chen and Kuipers 2021). This could therefore explain the difference seen in the C-terminus of the LanB from *B. obeum* AM27-32LB as this binding domain may have been altered yet remains functional at the same level or has a great efficiency. Interestingly, the leader peptides for the NsoA1-4-like proteins are identical in *B. obeum* AM27-32-LB and NsoA1-4 from *B. obeum* A2-162. This may indicate that the binding of the structural peptide to the modification machinery relies on the conserved residues within the leader peptide and that alterations to the C-terminus may have minimal effect on leader peptide binding.

Final confirmation that the novel clusters were indeed related to nisin O was performed using neighbour-joining phylogenetic trees. By using concatenated *lanB-lanC* genes to initially test whether there would be clustering of the sequences with higher homology it was observed that the clustering occurred as expected with those with higher homology clustering together. This method was then used on the whole cluster with similar results, indicating that the clusters found in *B. obeum* AM27-32LB, *B. obeum* TM09-13AC, *Blautia* sp. AM47-4, *Ruminococcus* sp. AM50-15BH, *D. formicigenerans* AM37-5 and *D. longicatena* 1001136B-160425 were indeed nisin O. This result was also confirmed using a whole cluster Mauve alignment.

Interestingly, a transposase element was present in the middle of the *D. formicigenerans* AM37-5 cluster which may suggest that these genes can undergo horizontal gene transfer (HGT), especially as it was observed that many of the strains identified in this work contained the immunity genes without the presence of the whole cluster. The human gut microbiome is a complex environment comprising of host, bacterial, viral, archaeal and fungal factors (Schmidt, Raes *et al*. 2018). Within this environment bacterial have the capability to exchange genetic information through HGT (Moralez, Szenkiel *et al*. 2021). HGT could be occurring which would cause the spread of the nisin O cluster in the bacteria isolated from the human gut. This would explain why nisin O-like clusters were identified within different genera which were all isolated from the human gut environment.

Furthermore, of the six nisin O-like clusters identified all contained two TCSs and all lacked a protease within the biosynthetic gene cluster. This is the same gene structure as the nisin O cluster, which is unusual for nisin variant clusters. The absence of a protease is also seen in the ruminococcin A biosynthetic gene cluster which requires trypsin both to induce expression as well as to cleave the leader peptide due the lack of this protein, however this cluster only contains one regulatory system (Gomez, Ladire *et al*. 2002, Ongey, Giessmann *et al*. 2018). Intriguingly, as with *B. obeum* A2-162 the producer of ruminococcin A, *R. gnavus* E1, is also a member of the *Lachnospiraceae* family and was isolated from the human gut. Chapters five and six will investigate the candidate proteases within the *B. obeum* A2-162 genome and the function of the regulatory systems, respectively.

In these sets of *in silico* analyses six nisin O clusters have been identified across the *Blautia*, *Dorea* and *Ruminococcus* genera, confirming the hypothesis that this biosynthetic gene cluster is present across multiple genera. Additionally, two novel bacteriocin clusters have been identified in *R. gnavus* and in the two *Pseudobutyrivibrio* species. This shows that not only can the same bacteriocin cluster be found in multiple genera but that other elements of the gene cluster are also present, indicating that HGT may play a role in the dissemination of bacteriocin gene clusters and cluster elements between bacteria within the human gut.

Chapter 4.

Inducing Nisin O production in *B. obeum* A2-162 and the *L. lactis* heterologous gene expression system

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4.1 Introduction

The expression of genes within the nisin A biosynthetic gene clusters has been widely studied due to its importance as a food preservative and therefore understanding the regulation and induction of the cluster is important for efficient mass production of this antimicrobial peptide. Production of nisin A has been observed to be correlated with increases in cell-density of the producer organism (Kuipers, Beerthuyzen *et al*. 1995, de Ruyter, Kuipers *et al*. 1996). Nisin A is expressed in both monocistronic and polycistronic mRNA transcripts with the former being transcribed at a higher level due to a terminator sequence downstream of *nisA* (Figure 4.1.1) (van der Meer, Polman *et al*. 1993, Kuipers, Beerthuyzen *et al*. 1995). In a study by Kuipers *et al*., a four base pair deletion was made in the structural sequence of *nisA*, which the authors termed Δ*nisA*, which resulted in the abolition of *nisA* transcription. However, through addition of sub-inhibitory concentrations of nisin A to the extracellular medium transcription was restored. This proved that nisin A acted as an autoinducer for its own biosynthetic gene cluster and thus acted not only as an antimicrobial peptide but also as a signalling molecule (Kuipers, Beerthuyzen *et al*. 1995). The authors also observed that mutation of *nisK* led to the elimination of transcription of the Δ*nisA* strain even with supplemented, unmodified nisin A in the medium indicating an interactive relationship between unmodified NisA and NisK (Kuipers, Beerthuyzen *et al*. 1995).

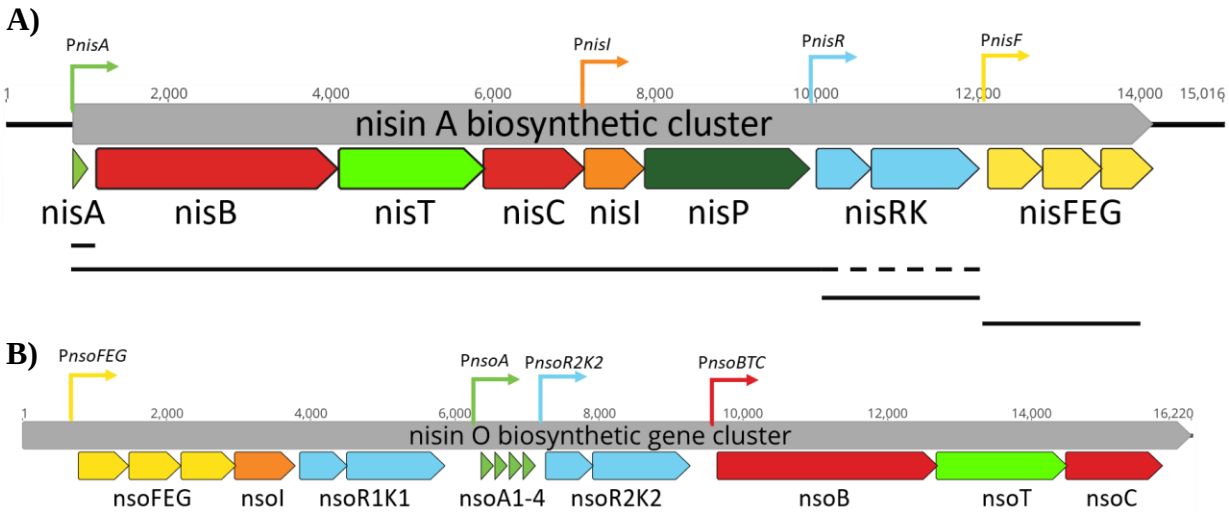


Figure 4.1.1. Organisation of the nisin A (A) and nisin O (B) biosynthetic gene clusters. The structural (Green), maturation and transport (Red and light green, respectively), immunity (Orange and yellow), regulatory (Blue) and leader peptide protease (Dark green) genes are indicated. Promoters are indicated by arrows above the clusters, in the case of nisin O these are predicted promoters (de Ruyter, Kuipers *et al*. 1996, Gherghisan-Filip 2016). Nisin A transcripts derived from these promoters are indicated in the lines below the cluster.

The production of nisin on a commercial scale relies on increasing the efficacy of production by *L. lactis*. The purification procedure itself is costly and therefore maximising nisin production while maintaining biomass is important for production (Khelissa, Chihib *et al*. 2021). It has been shown that nisin production occurs during the exponential phase of growth and plateaus during stationary phase (De Vuyst and Vandamme 1992, Chinachoti, Matsusaki *et al*. 1998, Mitra, Chakrabartty *et al*. 2005). As previously mentioned nisin can act as an autoinducing molecule for its expression within the biosynthetic gene cluster, however studies have shown that other factors may impact the production of nisin. These factors included alterations to the carbon source used and the concentration, which is supplemented to the growth media (De Vuyst and Vandamme 1992). In a recent study investigating nisin Z production from *L. lactis* strains isolated from fermented sausages different carbon sources affected production, with sucrose and fructose being the most efficient for three and one isolate, respectively, whereas glucose supplementation yielded poor nisin Z production (Saraiva, Birri *et al*. 2020). Additionally, pH, nitrogen source, and temperature are all extracellular factors that have been identified to influence the production of nisin (Pongtharangkul and Demirci 2006, Liu, Zheng *et al*. 2010, Liu, Zhou *et al*. 2017, Papiran and Hamedi 2021).

Studies have also shown that co-culturing nisin producing strains can increase the production of the antimicrobial peptide. A co-culture is defined as an incubation of different specified microbial strains in anaerobic or aerobic conditions (Bader, Mast-Gerlach *et al*. 2010). In the case of *L. lactis* and nisin A production a study by Ariana and Hamedi (2017) used *Yarrowia*

lipolytica and *L. lactis* co-cultured in a medium derived from sugar beet molasses where *L. lactis* growth and nisin production were increased by 49% and 50%, respectively (Ariana and Hamedi 2017). Furthermore, in a recent investigation by Qiao *et al* (2022), nisin yields were improved by 85% when *L. lactis* was grown in co-culture with *Enterobacter* sp. (Qiao, Qiao *et al*. 2022).

The activity of nisin O was first described in *B. obeum* A2-162 where it was sub-cultured twice in liquid medium then plated on solid medium with and without trypsin and incubated for 1 to 7 days before its use in antimicrobial assays (Hatziioanou, Gherghisan-Filip *et al*. 2017). Through induction experiments the authors determined that nisin A could be used to induce the expression of the *nsoA* genes and therefore that there is a degree of promiscuity across the regulatory systems of nisin variants (Wirawan, Klesse *et al*. 2006, Hatziioanou, Gherghisan-Filip *et al*. 2017). Additionally, ruminococcin A has the capability of being induced by trypsin and therefore trypsin may act as an additional inducing factor as well as a leader peptide cleavage protein in the case of nisin O (Marcille, Gomez et al. 2002, Hatziioanou, Gherghisan-Filip et al. 2017, Gherghisan-Filip, Saalbach et al. 2018).

The aim of this work was to test further inducing conditions that might increase the production of nisin O in *B. obeum* A2-162, as repeating the induction conditions established in Hatziioanou *et al* (2017) did not show any antimicrobial activity (Hatziioanou, Gherghisan-Filip *et al*. 2017).

4.2 Methods

4.2.1 *B. obeum* A2-162 Nisin Exposure Assay

B. obeum A2-162 was grown overnight in BHI with complements. This was followed with an increase in the concentration of nisin A (Sigma) every alternate sub-culture. The concentrations used were 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90 and 100 ng/ml nisin A. When 100 ng/ml nisin A was used to induce the culture an antimicrobial overlay assay was used to assess antimicrobial activity from the culture. In addition to the 100 ng/ml culture both an uninduced culture and a *B. obeum* A2-162 culture induced with 15 ng/ml nisin A were used

in the assay. *C. perfringens* was used as an indicator organism and the method described in Method 2.1.1 was followed with an adjustment of a 4 day growth period on solid media as per the original antimicrobial activity method developed by Hatziioanou *et al*., (Hatziioanou, Gherghisan-Filip *et al*. 2017). Cultures induced with 15, 30, 60 and 100 ng/ml nisin A had 1 ml of culture aspirated, centrifuged, supernatant removed, and the pellet treated with RNAlater for future transcriptomic analysis.

4.2.2 *B. obeum* Co-culture Induction Assay

Overnight cultures of the eight humanisation strains (*Anaerostipes caccae* DSM 14662, *Bacteroides thetaiotaomicron* DSM 2079, *Blautia producta* DSM 2950, *Lactobacillus plantarum* DSM 20174, *E. coli* K-12 MG1655, *Clostridium butyricum* DSM 10702, *Clostridium ramosum* DSM 1402 and *Bifidobacterium longum* DSMZ 20219) were grown in BPM media in anaerobic conditions. These strains were selected as they had previously been used in animal models as a simplified human intestinal microbiota that could be used in germfree rats and thus provide a quick and efficient way to assess the effect of microbiome associated bacteria on the expression of nisin O in *B. obeum* A2-162 (Becker, Kunath et al. 2011). *B. obeum* A2-162 was also grown overnight in BPM media in anaerobic conditions. 200 µl of each of the humanisation strains were mixed in the base of an Eppendorf tube and a 0.22 µm filter insert (ThermoFisher Scientific) was added to the top of the Eppendorf (Figure 4.2.1). The volume of the humanisation cultures was required to surround the base of the filter insert prior to addition of 200 µl of *B. obeum* A2-162 within the insert. The culture was incubated overnight after which the *B. obeum* A2-162 culture was aspirated from the insert tube and centrifuged at 13’000 x g for 2 mins, the supernatant removed and stored in a sterile Eppendorf, and the pellet stored in 500 µl RNAlater at -80ºC. The supernatant was filter-sterilised using a 0.22 µm filter and used in an antimicrobial drop test (Method 2.1.2).

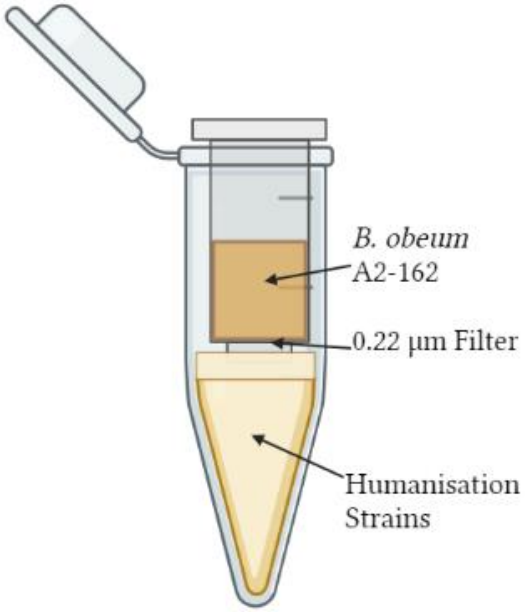


Figure 4.2.1. Schematic diagram of the co-culture of humanisation strains in the bottom of the Eppendorf and *B. obeum* A2-162 in the within filter insert. The humanisation strain mixed culture was required to surround the base of the filter insert to allow for exchange of potential inducing molecules.

4.2.3 *B. obeum* A2-162 and *Bifidobacterium longum* DSMZ 20219 Competition Assay

Both *B. obeum* A2-162 and *B. longum* DSMZ 20219 were grown in standard conditions, with *B. obeum* A2-162 induced with 15 ng/ml nisin A and used to streak a solid agar BHI with complements plate using a sterile inoculation loop (Camlab). The cultures were plated as close to each other as possible without touching (~5 mm) and incubated overnight. The strains were killed using 15 mins UV exposure using a UV Stratalinker 2400 (Stratagene) followed by overlay with soft BHI with complements agar inoculated with 2% *C. perfringens* as the indicator strain. The plate was incubated overnight using standard growth conditions. The growth of the plate was imaged using a Canon EOS 200D and a Leica M165c Stereo Microscope.

4.2.4 Reverse Transcription (RT) PCR

RT PCR was performed using the SuperScript IV One-Step RT-PCR System (ThermoFisher) as per the manufacturer's instructions. RNA extracted using method 2.15 was thawed on ice and 2 µl of sample was used in the reaction mix including 25 µl 2X Platinum SuperFi RT-PCR Master Mix, 2.5 µl each of Forward (nsoA1_F and gyrB_F) and Reverse (nsoA4_R and gyrB_R) primers (10 µM) (See table 2.5) and made to 50 µl using Nuclease Free H_2O. The reverse transcription temperature of 50ºC was selected based on the manufacturer's recommendations. The T_m was selected based on the primers described above. The amplification time was selected based on the product size of *gyrB* (861 bp).

50ºC	98ºC	98ºC	59ºC	72ºC	72ºC
10 min	2 mins	10 sec	10 sec	26 sec	5 mins
		X 45			

Resulting products were electrophoresed on a 0.9% agarose gel stained with Midori Green Xtra as per method 2.5.

4.3 Results

4.3.1 Growth of *B. obeum* A2-162

To ensure that *B. obeum* A2-162 could grow within a time period suitable for induction experiments a growth curve was determined in duplicate. Stationary phase was reached at 7 hrs, with exponential phase beginning at 3 hrs. The peak OD_{600} at stationary phase was ~ 4.0 for both repeats (Figure 4.3.1). In addition to OD_{600} measurements, the number of colony forming units (cfu) was measured and used in conjunction with the OD_{600} at each time point. This provided a growth curve where the cfu/ml could be estimated based on a specific OD_{600} (Figure 4.3.2).

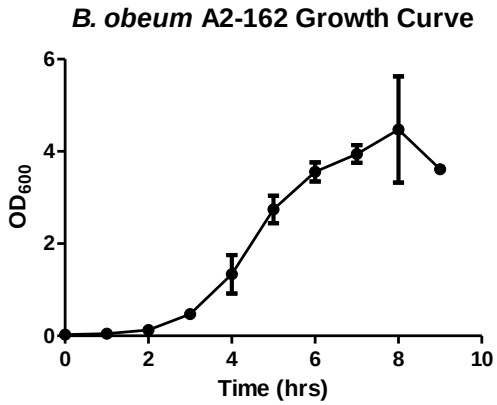


Figure 4.3.1. Mean OD_{600} of *B. obeum* A2-162 over 9 hrs growth in BHI with complements liquid media. Results are in duplicate. Errors bars are from standard error of the mean.

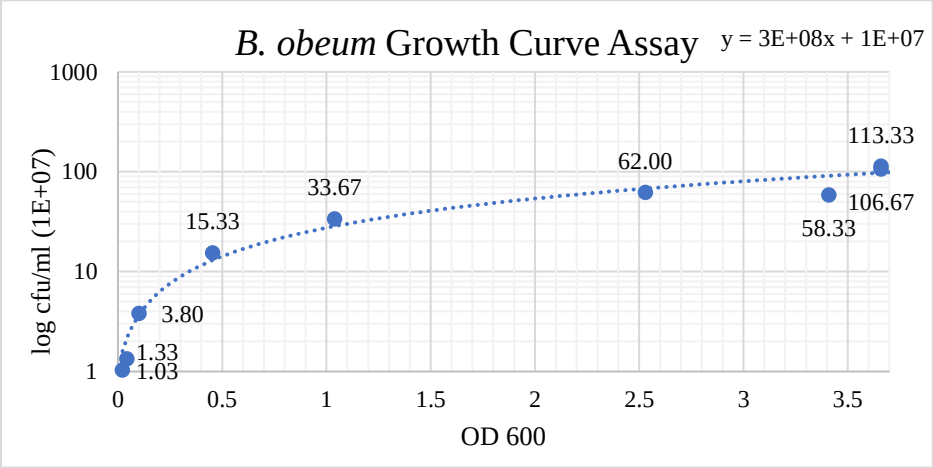


Figure 4.3.2. Log cfu/ml growth curve versus OD_{600} of *B. obeum* A2-162.

4.3.2 Imaging of *B. obeum* A2-162

To confirm that *B. obeum* A2-162 was indeed a Gram-positive bacterium, to analyse if there were any unknown cell surface or structural features and to assess whether this bacterium was capable of forming spores a range of imaging techniques were used. Gram staining confirmed that these bacteria were Gram-positive (Figure 4.3.3). A range of bacterial shapes was also seen, including long and shorter ovoid rods and chain forming structures. Further analysis by SEM of an overnight *B. obeum* A2-162 culture, showed a rough surface morphology, with the majority of bacteria being rod-shaped with tapered ends and unusual striations on the cell surface. SEM image analysis of the six-day old culture showed no clear evidence of spore

formation although there was an increase in extracellular debris, possibly from cell lysis (Figure 4.3.4).

TEM analysis of the overnight grown culture showed that small nodes were present on the cell surface. Interestingly, *B. obeum* A2162 had polar regions that appeared to contain vesicles (Figure 4.3.5 B). These features were also seen in bacteria from the six-day old culture, however, cellular material appeared more condensed and greater separation between the cell wall and the cytoplasm was observed.

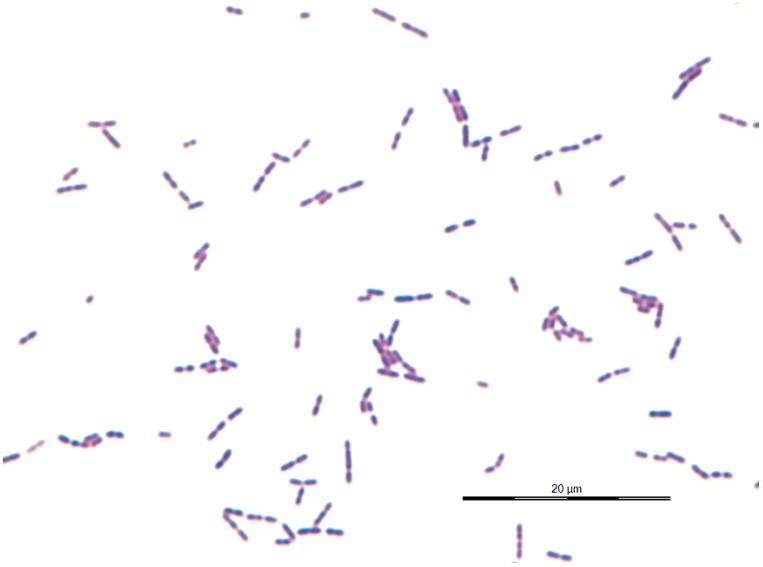


Figure 4.3.3. Gram staining of fresh *B. obeum* A2-162 culture. Bacteria present are Gram-positive, bacillus shaped and, in some cases, form chains of bacteria.

SEM

B. obeum one-day culture

B. obeum six-day culture

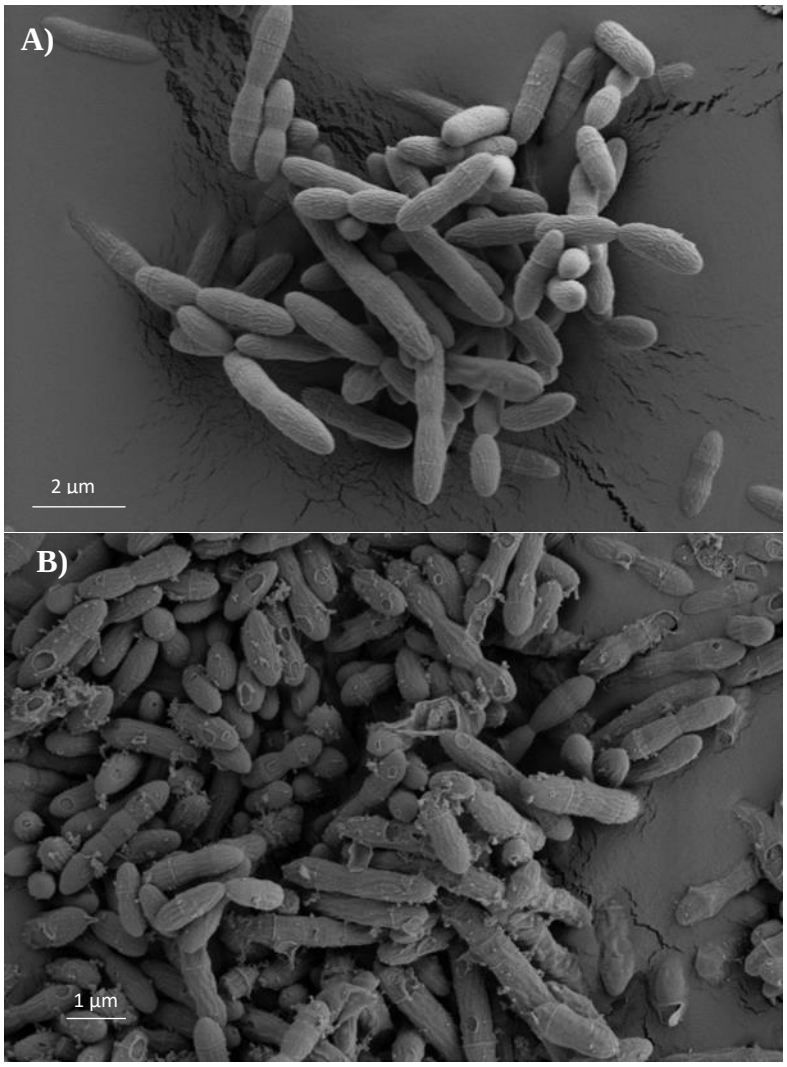
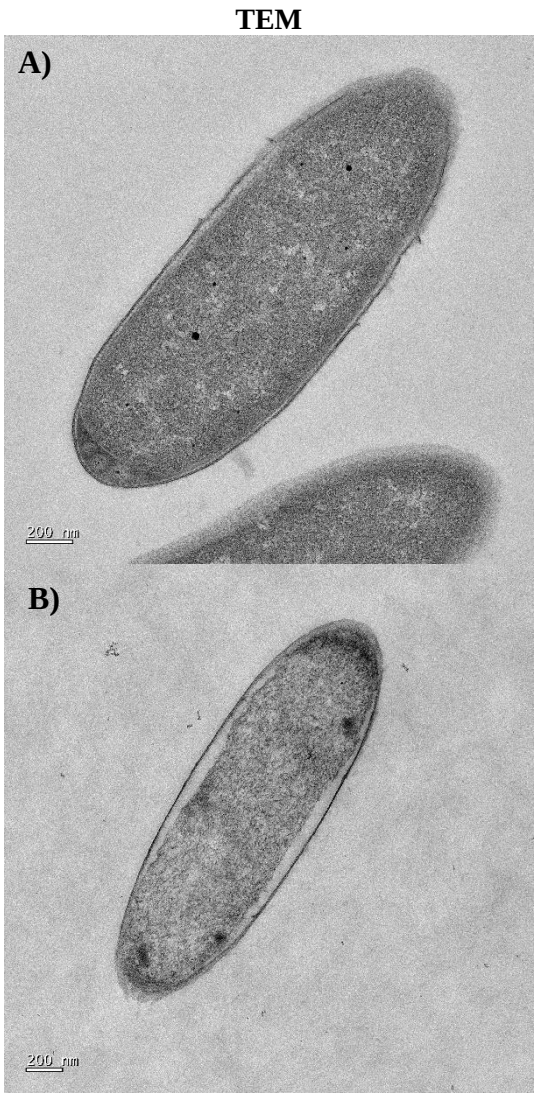


Figure 4.3.4. A) SEM image of overnight grown *B. obeum* culture. B) SEM image of six-day old *B. obeum* culture.



B. obeum one-day culture

B. obeum six-day culture

Figure 4.3.5. A) TEM image of overnight grown *B. obeum* A2-162 culture. B) TEM image of six-day old *B. obeum* A2-162 culture.

4.3.3 *B. obeum* Nisin O Exposure Assay

An antimicrobial overlay assay using *C. perfringens* as the indicator organism was used to assess the production of nisin O by *B. obeum* A2-162 following exposure to an increasing concentration of nisin A as an inducing molecule. The cultures of *B. obeum* A2-162 were able to grow overnight as the concentration of nisin A was increased in the BHI with complements liquid media. Additionally, the cultures were able to grow on solid BHI with complements medium overnight and were left on the plate as per the technique used in Hatziioanou *et al*., (Hatziioanou, Gherghisan-Filip *et al*. 2017). However, for control samples without induction with nisin A, 15 ng/ml and 100 ng/ml, no antimicrobial activity was seen against *C.*

perfringens (Figure 4.3.6). This result occurred on plates where trypsin was both present and absent in the agar.

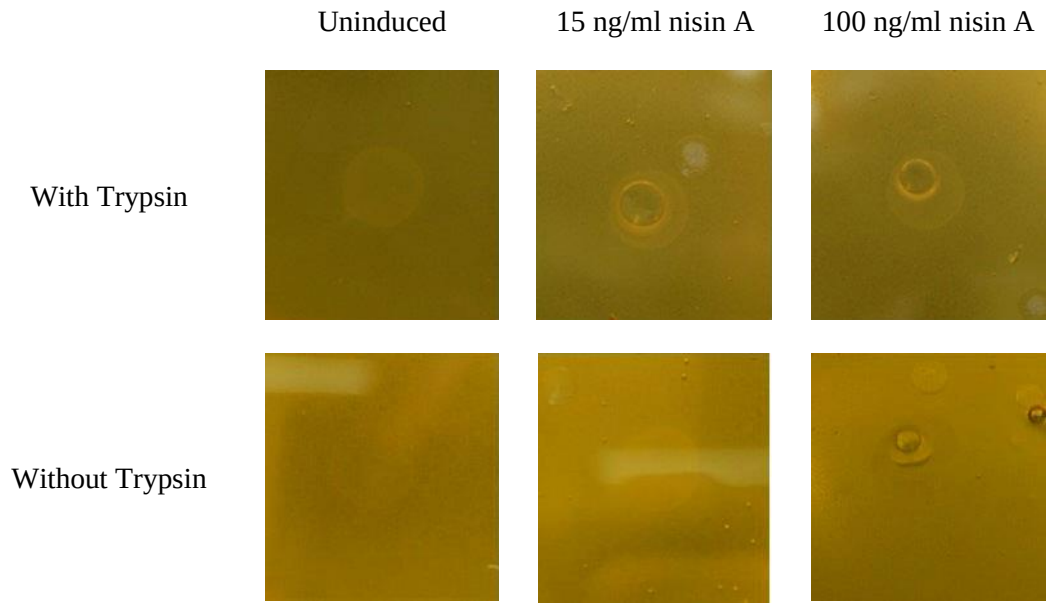
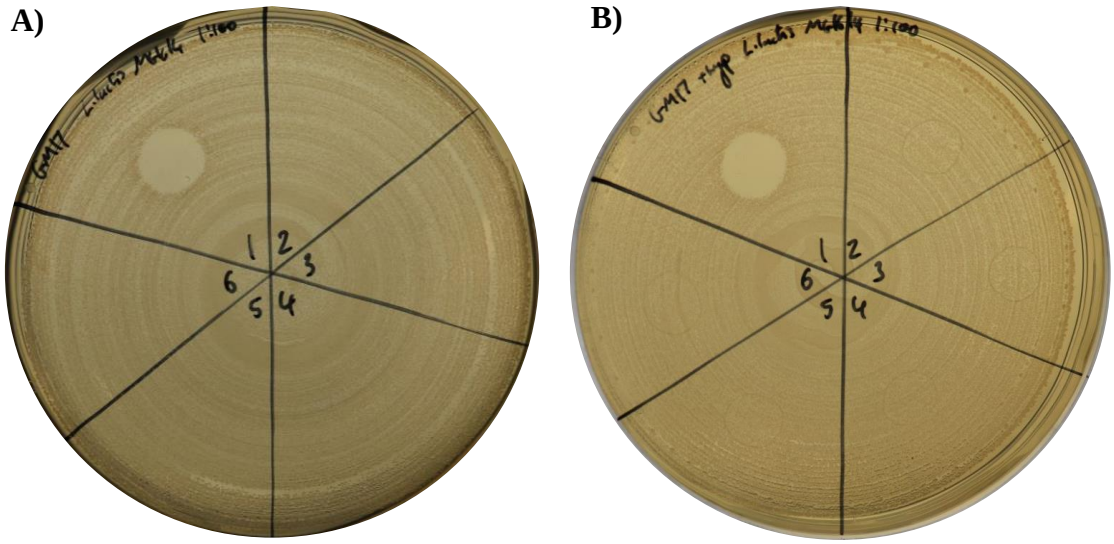


Figure 4.3.6. Antimicrobial activity overlay assay of *B. obeum* A2-162 when uninduced or induced with 15 ng/ml or 100 ng/ml nisin A with and without the presence of 15 µg/ml trypsin using *C. perfringens* as an indicator organism.

4.3.4 Co-culture *B. obeum* A2-162 and Humanisation strains

The co-culture of *B. obeum* A2-162 and humanisation strains (Becker, Kunath *et al*. 2011) was performed to investigate if the presence of additional bacteria could induce nisin O expression and could lead to the cleavage of nisin O without the presence of trypsin via general bacterial proteases. The cell free supernatants of each of the co-culture and mono-culture strains were used in a spot test using a 1:100 dilution *L. lactis* MG1614 in PBS as the indicator organism. However, no zones of inhibition were seen for any of the cultures containing *B. obeum* A2-162 (Figure 4.3.7, sections 2-6). Additionally, no zones of inhibition were seen from supernatants that were taken from the samples with 200 µg/ml trypsin added. A zone of inhibition was observed from the supernatant of *L. lactis* FI5876 which is a known nisin A producer (Figure 4.3.7, section 1) and was used a positive control. The results were seen on both plates containing 15 µg/ml trypsin and without.



1. *L. lactis* FI5876
2. Humanisation strains (Becker, Kunath *et al*. 2011) + *B. obeum* A2-162 + 200 µg/ml trypsin
3. Humanisation strains + 200 µg/ml trypsin
4. Humanisation strains + *B. obeum* A2-162
5. Humanisation strains
6. *B. obeum* A2-162

Figure 4.3.7. A) A supernatant spot test to assess the effect of co-culture on nisin O expression. Plate contained $NaHCO_3$. B) Supernatant spots were in the same order as plate A, however the base plate contains $NaHCO_3$ and 10 µg/ml trypsin in the overlay agar. *L. lactis* MG1614 was used as the indicator strain

4.3.5 RT PCR Analysis of Nisin O Expression in *B. obeum A2-162*

An RT-PCR assay was used to assess whether there was expression of the *nsoA1-4* genes within the nisin O cluster from the various *B. obeum* A2-162 induction experiments tested. Quantification of the RNA extractions from the nisin A increasing exposure induction assay, humanisation co-culture assay and samples from a MicroMatrix experiment in combination with human faecal samples (Described in detail in Chapter 5) showed that there were very low

concentrations of RNA within the samples being tested, however, the RT PCR was continued. The primers nsoA1_F and nsoA4_R (Table 2.5) were used to assess if there was expression of the nisin O structural genes under any of these conditions, however there were no bands present from any of these conditions apart from the positive control of an RNA extraction from *L. lactis* UKLc10 p*nso* (Heterologous expression system) (Figure 4.3.8). Furthermore, primers were designed for the *gyrB* gene in *B. obeum* A2-162 (gyrB_F and gyrB_R (Table 2.5)) as this would act as a housekeeping gene and should be present in all the samples. However, only *B. obeum* A2-162 induced with 100 ng/ml nisin A and RNA extracted from the *B. obeum* A2-162 co-cultured with the humanisation strains with the addition of trypsin contained bands of the correct size for the *gyrB* product (Figure 4.3.9).

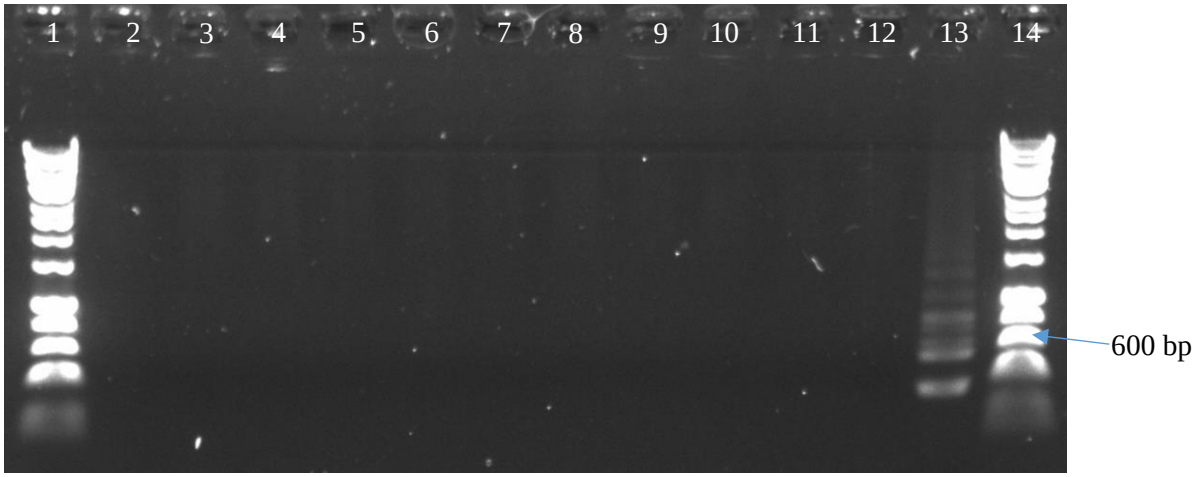


Figure 4.3.8. A gel image of the RT PCR reaction investigating the expression of *nsoA1-4* within *B. obeum* A2-162 under different induction conditions.

1. 1 kb ladder
2. Negative Control
3. No induction (Nisin A evolution assay)
4. 25 ng/ml nisin A (Nisin A evolution assay)
5. 100 ng/ml nisin A (Nisin A evolution assay)
6. 5 µg TCA precipitated *L. lactis* UKLc10 p*nso* supernatant (MicroMatrix)
7. No induction (MicroMatrix)
8. Faecal Water (MicroMatrix)
9. Faecal Water and trypsin (MicroMatrix)
10. Humanisation strains (Co-culture)
11. Humanisation strains and trypsin (Co-culture)
12. No induction (Co-culture)
13. *L. lactis* UKLc10 p*nso* (Positive Control)
14. 1 kb ladder

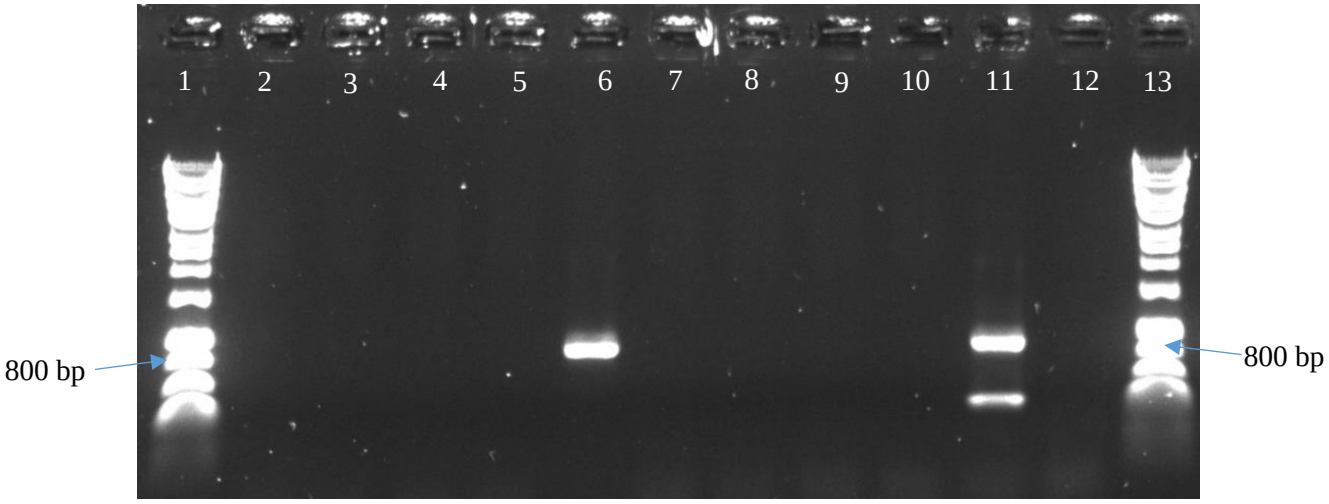


Figure 4.3.9. A gel image of the RT PCR reaction investigating the expression of *gyrB* within *B. obeum* A2-162 under different induction conditions.

1. 1 kb ladder
2. Negative Control
3. 5 µg TCA precipitated *L. lactis* UKLc10 p*nso* supernatant (MicroMatrix)
4. No induction (Nisin A evolution assay)
5. 25 ng/ml nisin A (Nisin A evolution assay)
6. 100 ng/ml nisin A (Nisin A evolution assay)
7. No induction (MicroMatrix)
8. Faecal Water (MicroMatrix)
9. Faecal Water and trypsin (MicroMatrix)
10. Humanisation strains (Co-culture)
11. Humanisation strains and trypsin (Co-culture)
12. No induction (Co-culture)
13. 1 kb ladder

4.3.5 *B. obeum* A2-162 and *B. longum* DSMZ 20219 Competition assay

B. obeum A2-162 and *B. longum* DSMZ 20219 were grown together on BHI agar containing 10 µg/ml trypsin to investigate if competition was required for nisin expression. Following growth for 6 days the streaks were overlaid with *C. perfringens*. No antimicrobial activity was seen caused by *B. obeum* A2-162 (Figure 4.3.10 A). However, the growth of both *B. obeum* A2-162 and *B. longum* DSMZ 20219 was altered in areas that were adjacent to each other with a reduced outer colony 'roughness' (Figure 4.3.10 B).

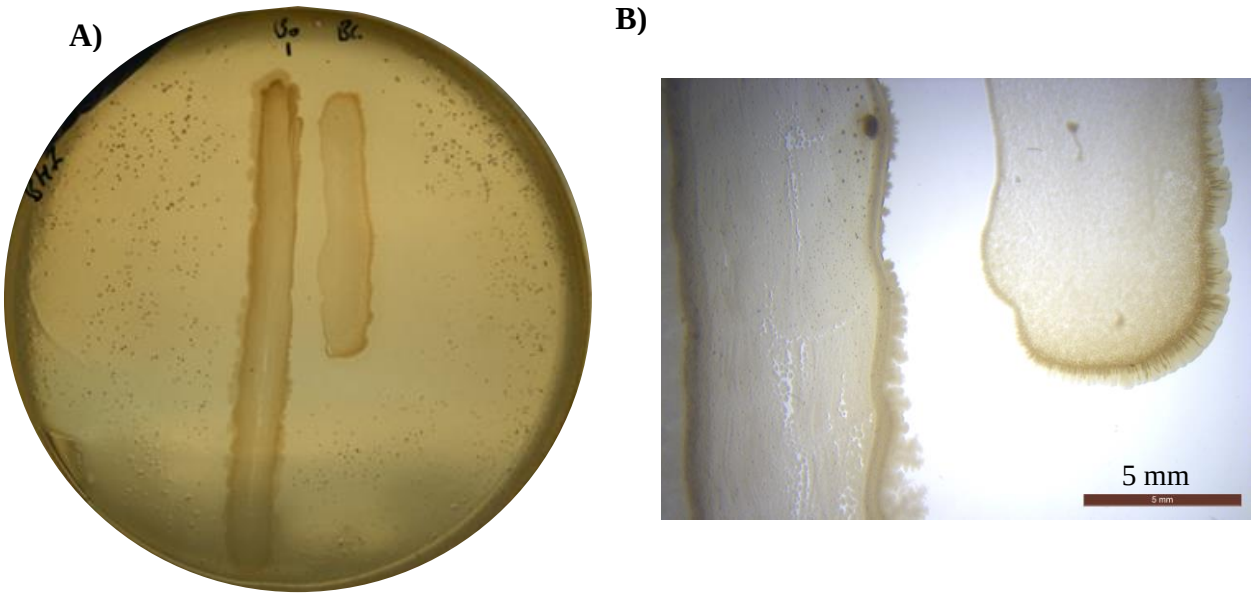


Figure 4.3.10. A) Overlay assay using *B. obeum* (Left) and *B. longum* (Right) using *C. perfringens* as an indicator organism showing no antimicrobial activity; B) Stereo microscopy of *B. obeum* (Left) and *B. longum* (Right) showing altered outer colony morphology at adjacent areas.

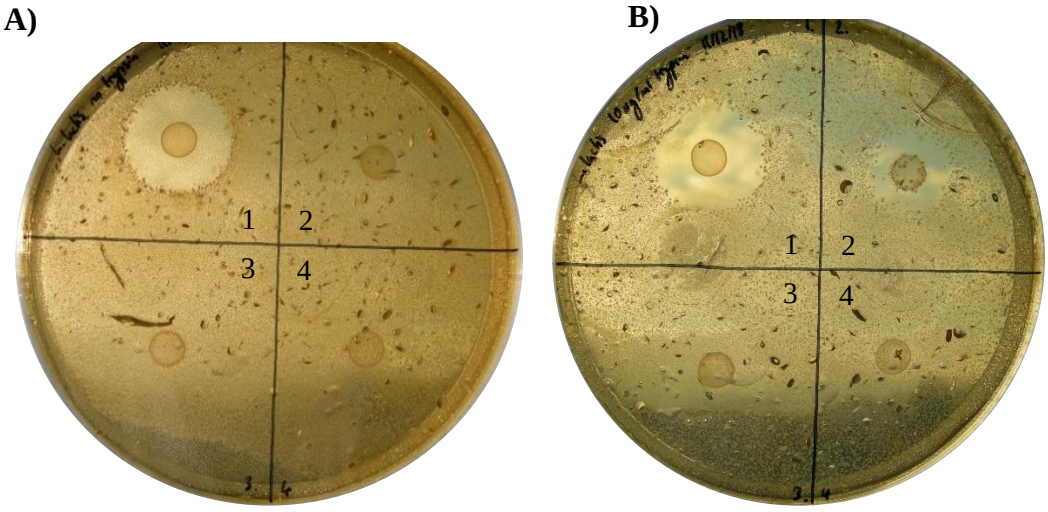
4.3.6 Optimisation of Nisin O production from the *L. lactis* heterologous expression system

Overlay assays using *L. lactis* UKLc10 *pnso* (strain carrying the nisin A *nisRK* genes on the chromosome, plasmid pIL253 containing the entire nisin O cluster from *B. obeum* A2-162 (Hatziioanou, Gherghisan-Filip *et al*. 2017)) with the addition of trypsin showed antimicrobial activity against both *C. perfringens* and *L. lactis* as indicator strains (Figure 4.3.11), confirming the results produced by Hatziioanou *et al* (2017). As previously demonstrated, when no trypsin was added no activity was observed. As trypsin has multiple cleavage sites within modified pre-nisin it was important to understand the correct concentrations to use to have optimal pre-NsoA1-3 leader cleavage with minimal degradation of the core peptide. The concentrations of trypsin (10 and 15 µg/ml) used by the authors were tested and showed no difference in the zone of inhibition caused by the nisin-producing strain using *C. perfringens* as the indicator strain (Figure 4.3.11 A-C). This assay confirmed that the antimicrobial activity observed in *L. lactis* UKLc10 p*nso* was dependent on the presence of trypsin (Hatziioanou,

Gherghisan-Filip et al. 2017). Following this, a concentration of 15 µg/ml trypsin was used for future experiments.

Experiments were performed to investigate the best method to use trypsin to provide maximum antimicrobial activity. When *L. lactis* MG1614 pIL253 was used as an indicator strain, the presence of trypsin in either the base agar or the overlay agar had no effect on the size of the zone of inhibition (Figure 4.3.12 A-D), although a clearer edge to the zone of inhibition was seen when trypsin was present in the base agar.

To try to maximise the expression from p*nso* erythromycin (Ery) was used in the agar to maintain the nisin O gene cluster-containing plasmid and this consequently resulted in improved antimicrobial activity. The use of Ery was made possible through transformation of *L. lactis* MG1614 with pIL253, conferring Ery resistance to the indicator strain. This enabled Ery to be used within the base agar of the overlay assay and act as a selecting agent to maintain the presence of p*nso* within *L. lactis* UKLc10. Again, overlay assays showed that trypsin was required for antimicrobial activity to occur, however the addition of Ery gave more defined zones of inhibition (Figure 4.3.12 A-D).



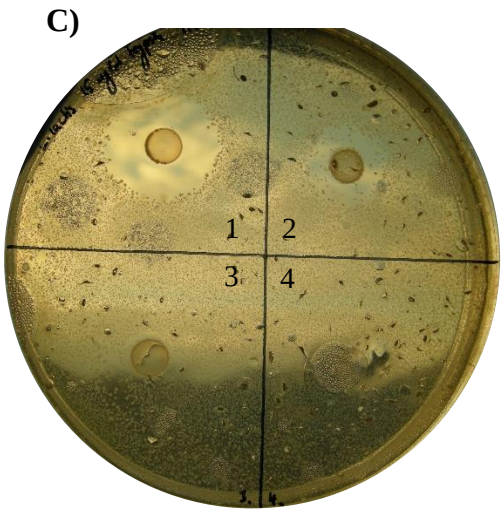


Figure 4.3.11. Overlay assay to test various concentrations of trypsin in the overlay agar on the antimicrobial activity of *L. lactis* strains using *C. perfringens* as the indicator strain. *L. lactis* FI5876, *L. lactis* UKLc10 p*nso*, *L. lactis* UKLc10 pIL253 and *L. lactis* MG1614 p*nso* were spotted in sections 1-4, respectively. A) No trypsin in overlay agar; B) 10 µg/ml trypsin in overlay agar; C) 15 µg/ml trypsin in overlay agar.

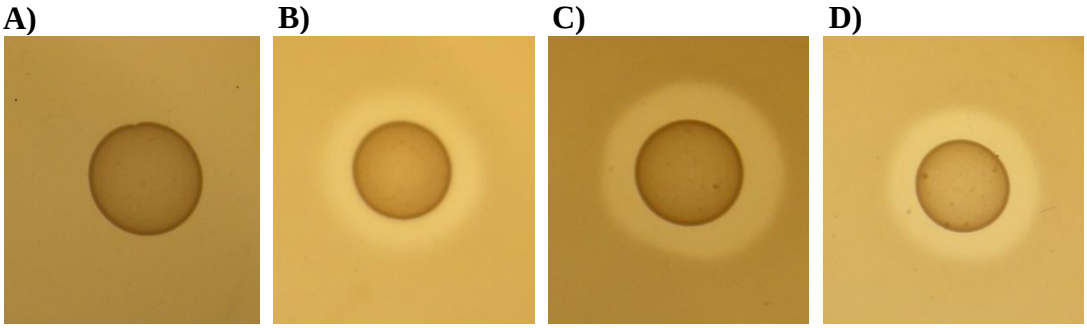


Figure 4.3.12. Overlay assay of *L. lactis* UKLc10 *pnso* using *L. lactis* MG1614 *pIL253* as the indicator organism to test for inducing conditions for maximum antimicrobial activity. A) No trypsin in either base or overlay agar and no Ery in base agar; B) No trypsin and 5 µg/ml Ery in base agar with 10 µg/ml trypsin overlay agar; C) 10 µg/ml trypsin and 5 µg/ml Ery in base agar with no trypsin in overlay agar; D) 10 µg/ml trypsin and 5 µg/ml Ery in base agar with 10 µg/ml trypsin in overlay agar.

4.3.7 Addition of Trypsin Cleavage Site into *nsoA4* at Position 24

In order to assess the antimicrobial and inductive potential of NsoA4, which naturally does not contain a trypsin cleavage site, primers (nsoA4_splice_R; nsoA4_splice_F; nsoA4_BamHI_R; nsoA4_NcoI_F. See Table 2.5 for details) were designed to change the serine (S) at position 24 to a lysine (K). Splice overlap extension PCR was successful, followed by restriction and ligation in pUK200_P32 to express the peptide from the strong constitutive promoter P32. Transformation was performed into *E. coli* MC1022 as a production vector. Purification of the pUK200_P32_*nsoA4_S24K* was performed followed by successful transformation into *L. lactis* UKLc10 p*nso* and confirmed via sequencing. Assessment of the antimicrobial activity of constitutively expression *nsoA4_S24K* showed no zone of inhibition against *L. lactis* MG1614 pIL253 with trypsin present in the base agar. However, zones of inhibition were seen for both the nisin A and nisin O producing strains of *L. lactis* FI5876 and *L. lactis* UKLc10 p*nso*, respectively (Figure 4.3.13). Both negative controls of *L. lactis* UKLc10 pIL253 and *L. lactis* MG1614 pIL253 showed no zones of inhibition (Figure 4.3.13).

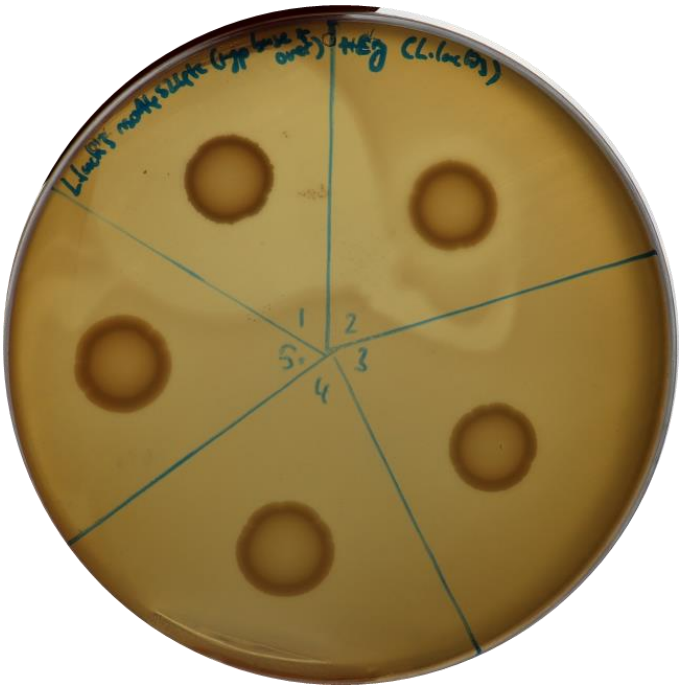


Figure 4.3.13. Overlay assay to test various the antimicrobial activity of modified NsoA4 which contains a trypsin cleavage site at position 24 of the amino acid chain. *L. lactis* FI5876 pIL253, *L. lactis* UKLc10 p*nso*, *L. lactis* UKLc10 p*nso* pUK200_P32_*nsoA4_S24K*, *L. lactis* UKLc10 pIL253 and *L. lactis* MG1614 pIL253 were spotted in sections 1-5, respectively. *L. lactis* MG1614 pIL253 was used as the indicator strain. The plate consisted of GM17 supplement with 15 µg/ml trypsin in the base and overlay agar and 5 µg/ml erythromycin in the base agar.

4.4 Discussion

The production of nisin O was previously described by Hatziioanou *et al*., from a heterologous expression system using nisin A to induce nisin O biosynthesis. The authors also tested several other induction techniques, however none of these resulted in antimicrobial activity from *B. obeum* A2-162 (Hatziioanou 2011, Hatziioanou, Gherghisan-Filip *et al*. 2017). However, since that initial discovery the production of antimicrobial activity from *B. obeum* A2-162 has been unable to be repeated. Therefore, new induction techniques were tested to attempt to observe production of antimicrobial activity from the original producer organism.

Analysis of the growth rate of *B. obeum* A2-162 demonstrated that this bacterium could reach stationary phase by ~7 hrs. This result was important as many of the induction experiments relied on the sub-culturing of *B. obeum* A2-162 and therefore by understanding the growth rate and cell number it could be determined at which stage to induce *B. obeum* A2-162. Previous work on inducing nisin A production in *L. lactis* strains have determined that nisin production occurs at exponential and stationary phase and therefore induction of *B. obeum* A2-162 could occur during an overnight culture (Buchman, Banerjee *et al*. 1988, Chinachoti, Matsusaki *et al*. 1998, Mitra, Chakrabartty *et al*. 2005). Furthermore, microscopy image analysis of *B. obeum* A2-162 indicated that these bacteria were indeed Gram-positive and showed several different cell morphologies, ranging from bacillus-shaped to long chains of bacteria. TEM analysis indicated a region at the poles of the cell which appeared to contain

vesicles and was more condensed, however the function of these regions is unknown. Interestingly, in a recent study by Hill the author demonstrated that the biosynthetic machinery proteins involved in nisin A maturation NisBC were located at the "old" pole of the dividing cell in *L. lactis* (Hill 2020). SEM analysis indicated that there was no spore formation after 6 days in liquid culture, which is consistent with other *Blautia* species (Durand, Pham *et al*. 2017, Lu, Yang *et al*. 2021, Wang, Abdugheni *et al*. 2021).

Induction of nisin O from *B. obeum* A2-162 using increasing concentrations of nisin A to induce over several subcultures resulted in no antimicrobial activity being seen. The addition of trypsin to agar plates did not alter the experimental outcome. Similar results were seen when *B. obeum* was co-cultured with humanisation strains (Becker, Kunath *et al*. 2011). Again, this suggests that nisin O production was not occurring as a result of competition or sensing from other bacteria found within the human microbiota. However, nisin O may be being produced at low concentrations that do not provide noticeable antimicrobial activity. In recent studies the production of other nisin variants have been increased through co-culture with other bacterial species (Ariana and Hamedi 2017, Gao, Fan *et al.* 2021, Qiao, Qiao *et al.* 2022). During the competition assay using *B. longum* as an antagonist organism, no antimicrobial activity was observed from *B. obeum* A2-162. However, interestingly there was an alteration to the colony surface morphology of both *B. obeum* and *B. longum*. This indicates that there is some effect of interaction between these two organisms however the nature of this is still unknown, and no bactericidal effects were recorded. In a recent study by Stincone *et al*., it was shown that nisin is able to have an influence on the cell surface proteins, down-regulating virulence proteins of *Listeria monocytogenes* at sub-lethal concentrations (Stincone, Miyamoto *et al.* 2020). Therefore, it could be possible that *B. obeum* is producing nisin O at sub-lethal concentrations which would explain the lack of antimicrobial activity against *B. longum*, however, this does not show the same level of activity seen against *C. difficile* and *C. perfringens* seen from the initial discovery of nisin O where, using very similar induction conditions, nisin O was produced at concentrations that produced an antimicrobial effect

(Hatziioanou, Gherghisan-Filip *et al.* 2017). The results from the RT PCR analysis indicated that there was no expression of *nsoA1-4* from *B. obeum* A2-162, however, in all but two samples tested there was no product for the housekeeping *gyrB* gene. This indicates that the concentration of RNA extracted from these assays was too low for the RT PCR to occur and therefore could not provide conclusive evidence that *nsoA1-4* were not being expressed. However, the two samples which contained the correct band for *gyrB* and no *nsoA1-4* band suggest that the two induction methods used, in this case the nisin A evolution assay using 100 ng/ml nisin A and the co-culture with humanisation strains with trypsin, did not induce *nsoA1-4* expression in *B. obeum* A2-162. Additionally, as the positive control from the heterologous expression system provided bands for the RT PCR for *nsoA1-4* it indicates that the assay would be able to amplify the *nsoA1-4* transcripts. Interestingly, multiple bands were seen in the *L. lactis* UKLc10 p*nso* RT PCR sample. This most likely due to non-specific binding of the nsoA1_F primer and nsoA4_R primer to the four structural genes within the cluster.

The heterologous expression system in *L. lactis* was optimised for nisin O expression. Not only was it found that nisin O required trypsin for antimicrobial activity to be observed, confirming the results from Hatziioanou and Gherghisan-Filip, but also that erythromycin provided increased consistency of antimicrobial activity from *L. lactis* UKLc10 p*nso* (Hatziioanou, Gherghisan-Filip *et al.* 2017, Gherghisan-Filip, Saalbach *et al.* 2018). Minor variations in the concentration of trypsin (10 or 15 µg/ml) had no effect on the zone of inhibition of nisin O, therefore indicating that 10 µg/ml is sufficient to cleave the leader peptide to form active nisin O. Importantly, no antimicrobial activity was seen without the presence of trypsin. Trypsin is able to cleave the pre-NsoA1-3 leader peptide due to a lysine found at position 24 of the nisin O peptide, however in regulation of ruminococcin A trypsin can act as an inducer of ruminococcin A expression (Gomez, Ladire *et al.* 2002). Therefore, this may also be the case for the *L. lactis* heterologous system. On the other hand, in the *B.*

obeum A2-162 co-culture with the humanisation strains 200 µg/ml trypsin was added in addition and no antimicrobial activity was seen.

Creation of a modified gene for the NsoA4 peptide that changed a serine to lysine providing a trypsin cleavage site for leader removal was successful, and it was inserted into a plasmid with a constitutive promoter and transformed into *L. lactis* UKLc10 p*nso*. Upon testing using an antimicrobial overlay assay there was no activity from this strain, indicating that NsoA4 produced by the heterologous expression system did not have antimicrobial potential against the indicator organism of *L. lactis* MG1614 pIL253. Intriguingly, as there was activity for the positive control of *L. lactis* UKLc10 p*nso*, but no activity for the *L. lactis* UKLc10 p*nso* producing NsoA4_S24K on a second plasmid, this peptide might be a regulatory peptide which down-regulates the production of NsoA1-3. In a study by Straume *et al*., the authors showed that by truncating the gene regulator *plnC* produced *by Lactobacillus plantarum* C11 the bacteriocin production was repressed (Straume, Kjos *et al*. 2007).This effect might be occurring when NsoA4 is cleaved by trypsin therefore allowing it to have this gene repressing effect. Additionally, it is important to note that the use of a second plasmid may affect expression levels as a study has shown that the addition of multiple plasmids within a single bacterium can result in a larger fitness cost and therefore reduce the level of expression for both plasmids (San Millan and MacLean 2017). Within the core peptide of NsoA4 there are three trypsin cleavage sites present which may indicate that there is an increased level of peptide degradation and therefore a reduction in antimicrobial activity (Gherghisan-Filip, Saalbach *et al*. 2018).

Therefore, it was observed that *B. obeum* A2-162 did not produce an antimicrobial effect when induced either with increasing concentrations of nisin A, co-culture with humanisation strains or with competition with *B. longum*. The *L. lactis* heterologous expression system was able to produce an antimicrobial effect only when trypsin was present, which was made more reliable with the presence of erythromycin to maintain the p*nso* plasmid. Additionally, leader-cleaved

NsoA4 did not produce an antimicrobial effect but might play a role in the regulation of the nisin O biosynthetic gene cluster.

Chapter 5.

Identification and Testing of Candidate Proteases Within the *B. obeum* A2-162 Genome

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5.1 Introduction

The protease NisP is an important protein in the biosynthesis of nisin A - it cleaves the leader peptide, resulting in the mature nisin A and enabling antimicrobial activity (Lubelski, Rink *et al*. 2008). This protease is composed of 682 amino acids and provides a signal peptidase I-mediated interaction with the modified pre-nisin peptide after transport across the cell membrane (van der Meer, Polman *et al*. 1993, Siezen, Rollema *et al*. 1995). The interaction between NisP and the pre-nisin occurs extracellularly, with NisP translocated across the membrane via the Sec pathway and anchored in the cell wall based on the LPXTG sequence located at the C-terminus of the protein (van der Meer, Polman *et al*. 1993, Schneewind and Missiakas 2014, Lagedroste, Smits *et al*. 2017). A study investigating the substrate specificity of NisP using mutations in the leader peptide showed that NisP has a greater tolerance to alterations in the leader peptide than originally thought, although the exact motif of recognition has not yet been elucidated (Montalban-Lopez, Deng *et al*. 2018). Interestingly single alanine mutations in the (D)FNLD box did not prevent NisP cleavage of the leader peptide, however, multiple mutations of this site were not made and therefore it could not be stated whether multiple mutations in this conserved motif would prevent recognition (Montalban-Lopez, Deng *et al*. 2018). A study by Lagedroste *et al*, showed that the presence of at least one lanthionine ring is crucial for recognition of the substrate and therefore subsequent cleavage of the leader peptide (Lagedroste, Smits *et al*. 2017).

The nisin variants P, U, U2, J, H, Z, F, Q and H contain a protease within their biosynthetic clusters which are thought to function in the same way as NisP (O'Sullivan, O'Connor *et al*. 2020). However, within the nisin O cluster no protease is present (Hatziioanou, Gherghisan-Filip *et al*. 2017). This is unusual as the proteolytic function of the protease is important for leader peptide cleavage and hence providing antimicrobial activity (Kuipers, Rollema *et al*. 1993). Interestingly, the biosynthetic gene cluster identified in *B. producta* SCSK by Kim *et al*, contains a protease. However, unlike the larger protease found in the nisin A cluster this protease is much smaller with a 146 amino acid sequence length compared to 682 amino acids

(van der Meer, Polman *et al*. 1993, Kim, Becattini *et al*. 2019). However, the nisin O cluster from *B. obeum* A2-162 is not unique in its lack of a protease within its biosynthetic gene cluster. Subtilin is a lantibiotic produced by *Bacillus subtilis* ATCC 6633 which also does not contain a protease within its biosynthetic gene cluster (Gross, Kiltz *et al*. 1973). In a study which used the protease inhibitor PMSF, an accumulation of pre-cursor subtilin was found in the supernatant, indicating that leader peptide cleavage was occurring extracellularly (Stein and Entian 2002). A study by Corvey *et al*, identified three proteases within the *B. subtilis* genome (AprE, WprA and Vpr) that were capable of leader peptide cleavage of subtilin although were not directly associated with its biosynthetic gene cluster (Corvey, Stein *et al*. 2003). Furthermore, ruminococcin A produced by *R. gnavus* E1 also does not contain a specific protease as part of its biosynthetic gene cluster, however it has been shown that leader peptide cleavage can occur due to a cleavage domain within the transport protein RumT, therefore providing a bi-functional role in the biosynthesis of ruminococcin A (Ongey, Giessmann *et al*. 2018). Originally it was thought that trypsin was required for leader peptide cleavage, however it was subsequently shown that trypsin is required for the regulation of RumA production (Ramare, Nicoli *et al*. 1993, Gomez, Ladire *et al*. 2002). Trypsin is a serine protease and cleaves between the carboxyl group of arginine or lysine and the amino group of the subsequent adjacent amino acid (Simpson 2006). Although a trypsin site is present within the core peptide of RumA at position six, no cleavage occurs here due to the post-translational modifications made to the neighbouring threonine residue (Dabard, Bridonneau *et al*. 2001). Trypsin has been previously shown to cleave the leader from the NsoA1-3 pre-peptides and produce antimicrobial activity, but can also cut within the amino acid sequence of the mature core peptide, resulting in a family of products (Hatziioanou, Gherghisan-Filip *et al*. 2017, Gherghisan-Filip, Saalbach *et al*. 2018). Furthermore, work described in chapter 3 of this thesis identified six nisin O-like clusters in the genera *Blautia*, *Ruminococcus* and *Dorea*, all of which did not contain a protease within the biosynthetic gene cluster. This indicates that the lack of a protease within the cluster is a shared feature across bacterial strains that contain

this antimicrobial gene cluster and therefore identification of candidate proteases would require analysis of the whole genome of *B. obeum* A2-162.

In this chapter the genome of *B. obeum* A2-162 was interrogated for candidate proteases, and those identified were cloned into plasmids to assess their ability to cleave the leader peptides of pre-NsoA1-4 peptides. Additionally, the candidate proteases identified were co-expressed with the nisin O biosynthetic gene cluster within the *L. lactis* heterologous expression system to observe any antimicrobial activity in the absence of trypsin. A MicroMatrix fermentation assay was performed to observe any induction or leader peptide cleavage of nisin O due to the presence of gut bacterial communities, and whether nisin O has an influence on the composition of the gut microbiota.

The hypothesis of this work is that the *B. obeum* A2-162 genome contains a protease which will be capable of cleaving the leader peptide/s of nisin O and provide antimicrobial activity in the absence of trypsin. We also hypothesise that the bacterial composition within the fermentation assay will change to predominantly Gram-negative due to the antimicrobial activity of nisin O.

I would like to thank Professor Eric Pamer and Dr Zhenrun Zhang, University of Chicago for providing the *lanP* gene from *B. producta* SCSK for use in this work.

5.2 Methods

5.2.1 BLAST Analysis within the *B. obeum* A2-162 Genome

Candidate protease and regulatory amino acid sequence analysis was performed using BLAST (Altschul, Madden *et al*. 1997) to compare known sequences of *nisP* from *L. lactis* and *lanP* from *B. producta* to the genome of *B. obeum* A2-162 (Gao, Lu *et al*. 2011, Kim, Becattini *et al*. 2019). Amino acid sequence alignment was performed on Geneious R11. Conserved regions of the candidate proteases were examined using CD Batch Search (Lu, Wang *et al*. 2020). Predictive modelling of the transmembrane domains using Transmembrane Helices; Hidden Markov Model (TMHMM) online software was used to assess the structural similarity

between the candidate proteases and those known to cleave the leader peptide of pre-nisin (Sonnhammer, von Heijne *et al*. 1998, Drozdetskiy, Cole *et al*. 2015).

5.2.2 Manual Searching for Proteases using LanP Conserved Domains

New candidate proteases were identified using a CD batch search (Lu, Wang *et al*. 2020) using the amino acid sequence of LanP from *B. producta* to look for conserved domains within this protease (Kim, Becattini *et al*. 2019). The conserved domain S26_SPase_1 was identified and the Pfam domain (PF10502) for this region was used in Uniprot (UniProt-Consortium 2021). The proteins found via this method were then used to search in the *B. obeum* A2-162 genome in Geneious R11. Predictive modelling was also used to assess the similarity between the candidate proteases and those known to cleave the leader peptide of pre-nisin. This was performed using TMHMM (Sonnhammer, von Heijne *et al*. 1998, Drozdetskiy, Cole *et al*. 2015).

5.2.3 Cloning of Candidate Proteases

Cloning of the candidate proteases identified through bioinformatic searches was performed using the methods described in methods 2.6, 2.9, 2.10, 2.11 and 2.12. Candidate proteases were cloned into pET15b to insert them behind a His-tag which were then transformed into an *E. coli* BL21 (DE3) expression strain and others were cloned into pUK200 after the P32 promoter and they were then transformed into the *L. lactis* heterologous expression strain (van der Vossen, van der Lelie *et al*. 1987). Primer names and reaction conditions for each PCR and splice overlap PCR are given below (Table 5.2.1) (See Table 2.5 for primer details). See example below of splice overlap PCR conditions with five cycles at a lower annealing temperature followed by 20 cycles using a higher annealing temperature.

98ºC	98ºC	59ºC	72ºC	98ºC	61ºC	72ºC	72ºC
30 sec	10 sec	30 sec	4 sec	10 sec	30 sec	4 sec	5 mins
	X 5			X 20			

	Forward Primer	Reverse Primer	Product Size (bp)	Annealing Temperature (ºC) (First 5 cycles)	Annealing Temperature (ºC) (Next 20 cycles)	Extension Time (Sec)
Cloning of *p66* into pET15b	P66NDE_F	P66CAC_R	150	59	61	4
	P66CAC_F	P66XHO_R	783	58	63	23
	P66NDE_F	P66XHO_R	943	51	62	27
Cloning of *p570* into pET15b	P570NDE_F	P570XHO_R	1534	55	65	46
Cloning of *p62* into pET15b	P62_NdeI_F	P62_spliceR_NdeKO	336	66	68	11
	P62_spliceF_NdeKO	pUK_XhoI_R	403	69	69	11
	P62_NdeI_F	pUK_XhoI_R	714	59	68	22
Cloning of p49 into pET15b	P49_NdeI_F	pUK_XhoI_R	725	66	69	22
Cloning of *lanP* into pET15b	lanP_NdeI_F	lanP_XhoI_R	444	66	69	14
Cloning of *p140* into pET15b	P140_NdeI_F	P140_XhoI_R	625	56	68	19
Cloning of *p62* into pUK200_P32	P62_Splice32_F	P62_BglIITGA_R	158	39	67	5
	P62_BglIITGA_F	P62_PstI_R	430	39	66	13
	P62_splice32_F	P62_PstI_R	558	39	66	17
	pUK_BglII_F	pUK_Splice62_R	200	40	65	7
	pUK_BglII_F	P62_PstI_R	735	59	66	22
Cloning of *p49* into pUK200_P32	P49_Splice32_F	P49_PstI_R	530	44	65	17
	pUK_BglII_F	pUKSplice49_R	200	44	65	7
	pUK_BglII_F	P49_PstI_R	707	57	65	21
Cloning of *p140* into pUK200_P32	P140_spliceP32_F	P140_BamHI_R	815	53	67	24
	P32_StuI_F	P32_spliceP140_R	320	52	64	9
	P32_StuI_F	P140_BamHI_R	1093	52	64	33
Cloning of *lanP* into pUK200_P32	LanP_Splice32_F	lanP_NcoI_R	517	58	62	16
	P32_BglII_F	P32_SplicelanP_R(2)	206	50	64	7
	P32_BglII_F	LanP_NcoI_R	683	55	62	21

Table 5.2.1. PCR conditions and primers used for cloning of candidate proteases into expression plasmids used in this work.

5.2.4 MicroMatrix Fermentation

Faecal samples used in the colon model experiments were obtained from participants recruited onto the QIB Colon Model study. For experiments three donors were used who each provided a single faecal sample, however due to COVID-19 infections for each of the donors' repeats were unable to be performed due to revised QIB health and safety criteria for the use of the MicroMatrix. Men and women aged 18 years or older who live or work within 10 miles of the Norwich Research Park were recruited onto the QIB Colon Model study if they satisfied the following criteria. The study was approved by the Quadram Institute Bioscience (formally Institute of Food Research) Human Research Governance committee (IFR01/2015), and London-Westminster Research Ethics Committee (15/LO/2169). Participants who were assessed to have a normal bowel habit, regular defecation between three times a day and three times a week, with an average stool type of 3–5 on the Bristol Stool Chart, and no diagnosed chronic gastrointestinal health problems, such as irritable bowel syndrome, inflammatory bowel disease, or coeliac disease were deemed eligible. Demographic information was gathered, and a brief health questionnaire was completed as part of the eligibility screening. Participants were asked additional questions immediately prior to donating a stool sample to confirm that they had not taken antibiotics or probiotics within the last four weeks, had not experienced a gastrointestinal complaint, such as vomiting or diarrhoea, within the last 72 h, were not currently pregnant or breast-feeding, had not recently had an operation requiring general anaesthetic.

The preparation of the human faecal slurry was prepared as per the method described by Parmanand *et al* (2019).The faecal slurry was prepared by weighing 20 g faeces and mixing with PBS until a total mass of 200 g, followed by homogenisation using a stomacher 400 (Seward, UK) and filtration. The samples were used immediately and made into a 10% stock solution using PBS. Using a MicroMatrix 24-well cassette (Applikon Biotechnology) 600 µl of faecal slurry was used in the wells which required its presence as performed in the method described by Garcia-Gutierrez *et al* (2020). For *B. obeum* A2-162 and *L. lactis* UKLc10 p*nso*

300 µl of culture was used. Where TCA precipitated pre-NsoA1-4 and trypsin were added to the wells a concentration of 10 µg/ml and 400 µg/ml trypsin were added, respectively, in appropriate wells. Cassette loading occurred in anaerobic conditions and an initial 1 ml of sample was taken before fermentation as a T0 timepoint. Fermentation took place for 24 hrs at 37ºC at pH 6.8 using Colon Model Media (Described in Chapter 2) in a MicroMatrix Fermenter (Applikon Biotechnology). Samples were stored at -80ºC for further analysis. DNA and RNA extractions were performed as per Methods 2.4 and 2.15, respectively.

5.2.5 Analysis of DNA from MicroMatrix Fermentation Assays

Genomic DNA was normalised to 0.5ng/µl with EB (10mM Tris-HCl). Library construction and sequencing were performed by Dr David Baker and Rhiannon Evans described in the following method. 0.9 µl of TD Tagment DNA Buffer (Illumina) was mixed with 0.09 µl TDE1, Tagment DNA Enzyme (Illumina) and 2 µl PCR grade water in a master mix and 3ul added to a chilled 96 well plate. 2 µl of normalised DNA (1ng total) was pipette mixed with the 3 µl of the Tagmentation mix and heated to 55 ºC for 10 minutes in a PCR block. A PCR master mix was made up using 4 ul kapa2G buffer, 0.4 µl dNTP's, 0.08 µl Polymerase and 6.52 µl PCR grade water, contained in the Kap2G Robust PCR kit (Sigma) per sample and 11 µl added to each well in a 96-well plate. 2 µl of each P7 and P5 of Nextera XT Index Kit v2 index primers (Illumina) were added to each well. Finally, the 5 µl of Tagmentation mix was added to each well and mixed. The PCR was run at 72ºC for 3 mins, 95ºC for 1 min, 14 cycles of 95ºC for 10 secs, 55ºC for 20 secs and 72ºC for 3 mins. Following the PCR the libraries were quantified using the Quant-iT dsDNA Assay Kit, high sensitivity kit and run on a FLUOstar Optima plate reader. Libraries were pooled following quantification in equal quantities. The final pool was double-SPRI size selected between 0.5 and 0.7X bead volumes using KAPA Pure Beads (Roche). The final pool was quantified on a Qubit 3.0 instrument and run on a High Sensitivity D1000 ScreenTape (Agilent) using the Agilent Tapestation 4200 to calculate the final library pool molarity.

The pool was run at a final concentration of 1.8 pM on an Illumina Nextseq500 instrument using a Mid Output Flowcell (NSQ® 500 Mid Output KT v2(300 CYS) Illumina Catalogue FC-404-2003) following the Illumina recommended denaturation and loading recommendations which included a 1% PhiX spike in (PhiX Control v3 Illumina Catalogue FC-110-3001). Data was uploaded to Basespace (www. basespace.illumina.com) where the raw data was converted to 8 FASTQ files for each sample.

The sequence data was analysed using the Galaxy (release_19.05) pipeline QC_module_wf_1.0_nohost created by Dr Rebecca Ansorge (Afgan, Baker *et al*. 2018, Matthews, Bristow *et al*. 2018). RNA was used in an RT-PCR as described in Method 3.2.4.

5.3 Results

5.3.1 Identifying Candidate Proteases Within the *B. obeum* A2-162 Genome

5.3.1.1 BLAST Searches Using *nisP* From *L. lactis*

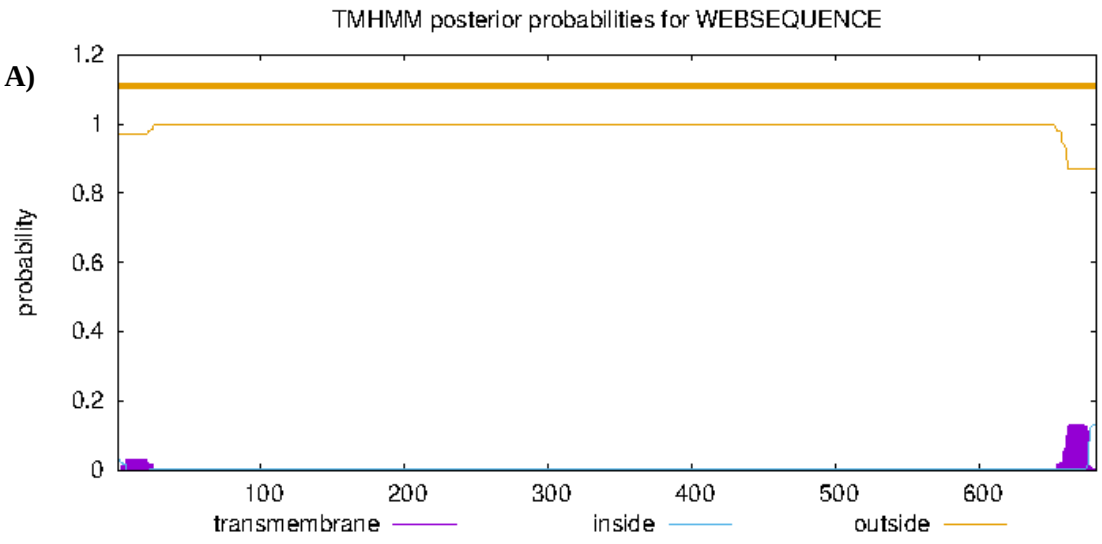
Bioinformatic analysis was used to investigate and identify candidate proteases. Six proteases were identified in the *B. obeum* A2-162 genome using Geneious R11 software, with annotations identifying them as subtilisin-like or serine proteases. These were subsequently aligned with NisP from *L. lactis* (Table 5.3.1).

Geneious ID	Length (bp)	Protein ID	% Similarity with NisP
Trypsin-like serine proteases, typically periplasmic, contain C-terminal PDZ domain CDS (P712)	1,509	CBL22712.1	14.8
Trypsin-like serine proteases, typically periplasmic, contain C-terminal PDZ domain CDS (P570)	1,509	CBL24570.1	14.6
Trypsin-like serine proteases, typically periplasmic, contain C-terminal PDZ domain CDS (P954)	1,332	CBL21954.1	12.7
Subtilisin-like serine proteases CDS (P31)	1,722	CBL23031.1	16.1
Subtilisin-like serine proteases CDS (P66)	837	CBL24066.1	18.0
Subtilase family. CDS (P550)	1,689	CBL23550.1	13.8

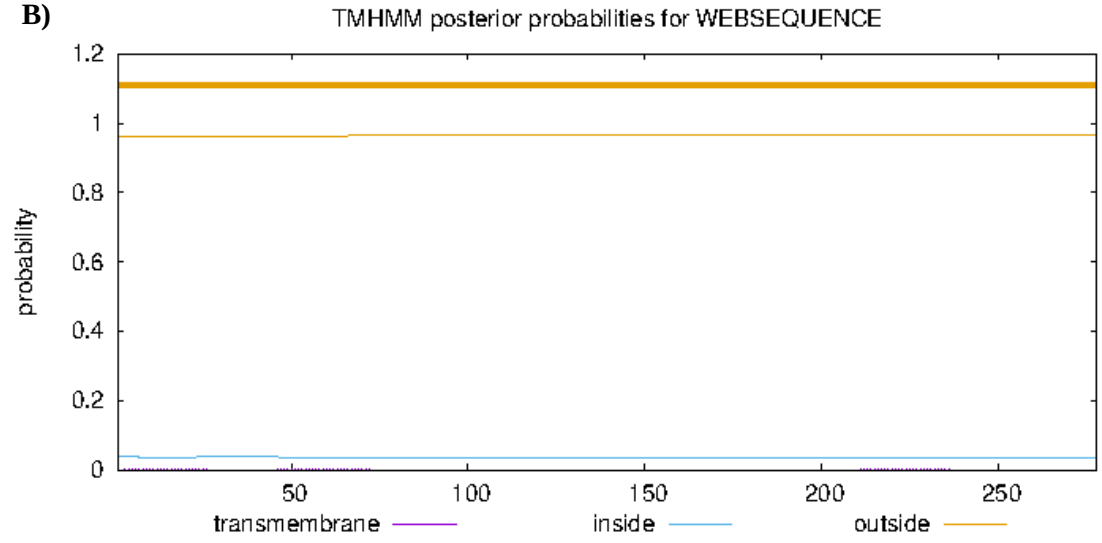
Table 5.3.1. Candidate proteases from *B. obeum* A2-162 using *nisP* as the reference sequence. Protein names, first column in brackets, were based on the last three digits of the Protein ID.

p66 and *p570* were selected for further investigation due to the percentage similarity when compared to the amino acid sequence of NisP of 18.0% and 14.6%, respectively, across 100% of the protein sequence of NisP. *p570* was chosen ahead of *p31* and *p712* due to the latter both having restriction sites within their nucleotide sequence which would require removal before insertion into the pET15b and pUK200_P32 plasmids. Although a restriction site was present within *p66*, due to the increased percentage amino acid similarity with NisP it was selected to be cloned into *E. coli* TOP10. When the predicted transmembrane domains of NisP, P66 and P570 were analysed (Figure 5.3.1), for both NisP and P66 there was a low probability of a transmembrane domain occurring, although a slightly increased probability at the C-terminus of NisP. However, P570 had a very high probability of a transmembrane domain approximately between residues 50-80. Additionally, the nucleotide sequences of *nisP* and *P570* were identical in length at 1,509, whereas the length of *p66* was shorter at 837 bp (Table 5.3.1).

A)


TMHMM posterior probabilities for WEBSEQUENCE
probability
1.2
1
0.8
0.6
0.4
0.2
0
100
200
300
400
500
600
transmembrane
inside
outside

B)


TMHMM posterior probabilities for WEBSEQUENCE
probability
1.2
1
0.8
0.6
0.4
0.2
0
50
100
150
200
250
transmembrane
inside
outside

C)

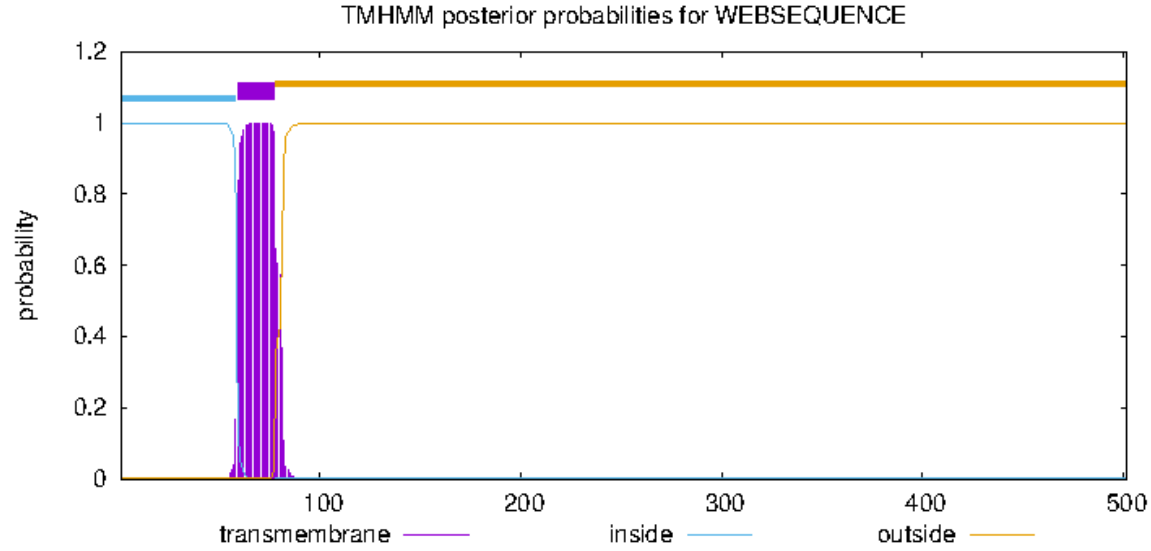

TMHMM posterior probabilities for WEBSEQUENCE
probability
1.2
1
0.8
0.6
0.4
0.2
0
100
200
300
400
500
transmembrane
inside
outside

Figure 5.3.1. A) TMHMM analysis of NisP from *L. lactis* cluster, B) P66 from *B. obeum* A2-162. C) P570 from *B. obeum* A2-162.

Furthermore, analysis of the conserved domains within NisP, P66 and P570 identified that both NisP and P66 had similar conserved domains, particularly Peptidase_S8. P66 also contained predicted catalytic sites in the same pattern as NisP (Figure 5.3.2). P570 on the other hand although identical in size to NisP, had no conserved domains shared between them (Figure 5.3.2).

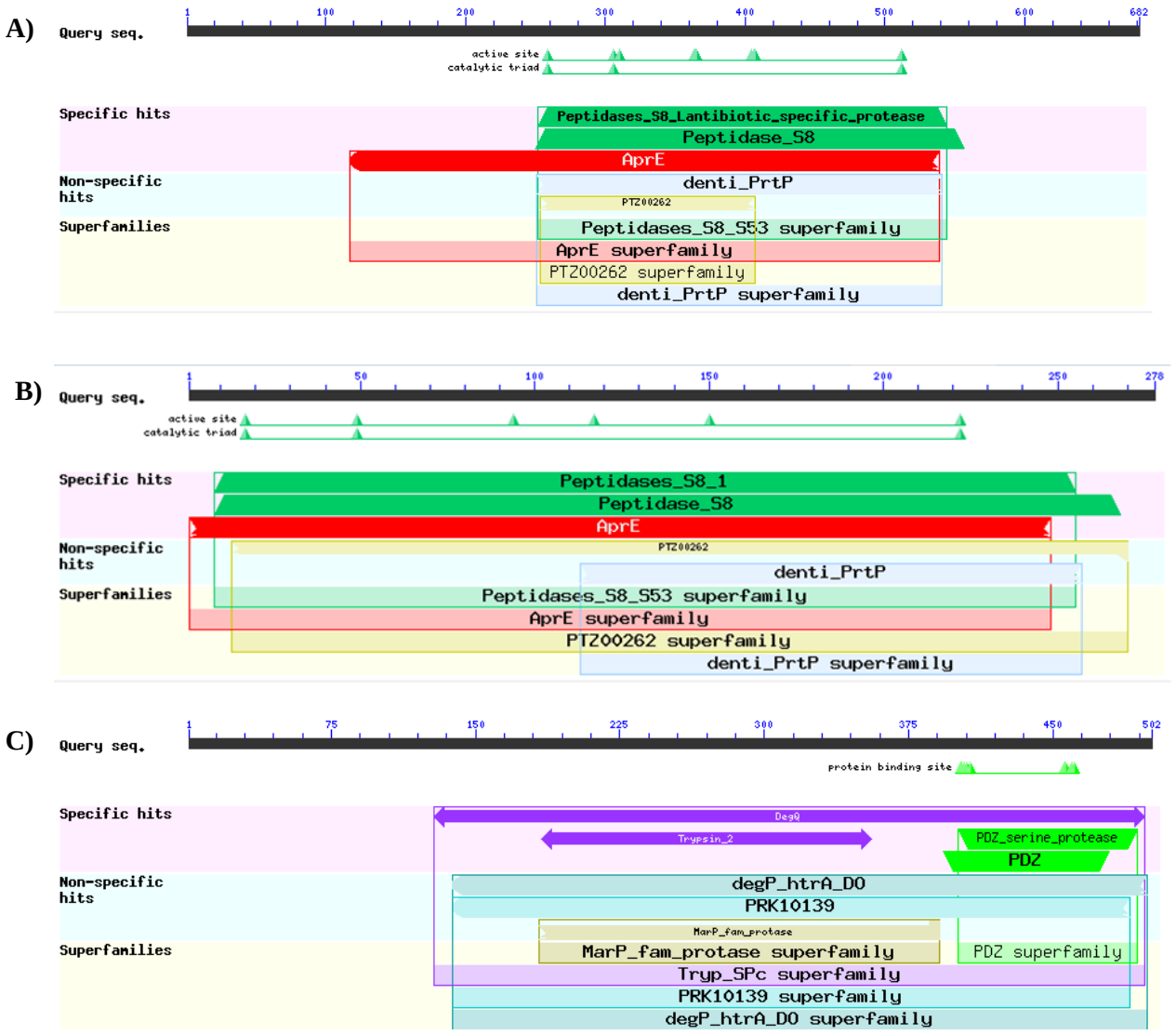


Figure 5.3.2. CD Batch search of amino acid sequences of A) NisP from *L. lactis*, B) P66 and C) P570 both found within the *B. obeum* genome. Predicted catalytic sites are indicated by green arrows beneath the query sequence.

5.3.1.2 BLAST Searches Using *lanP* From *B. producta* SCSK

BLAST analysis identified two novel candidate proteases in *B. obeum* A2-162 when *lanP* from *B. producta* SCSK was used as the reference sequence (Kim, Becattini *et al*. 2019). Proteases P62 and P49 were identified and have amino acid similarities of 25.6% and 17.8%, respectively, across 100% of the amino acid sequence of LanP (Table 5.3.2).

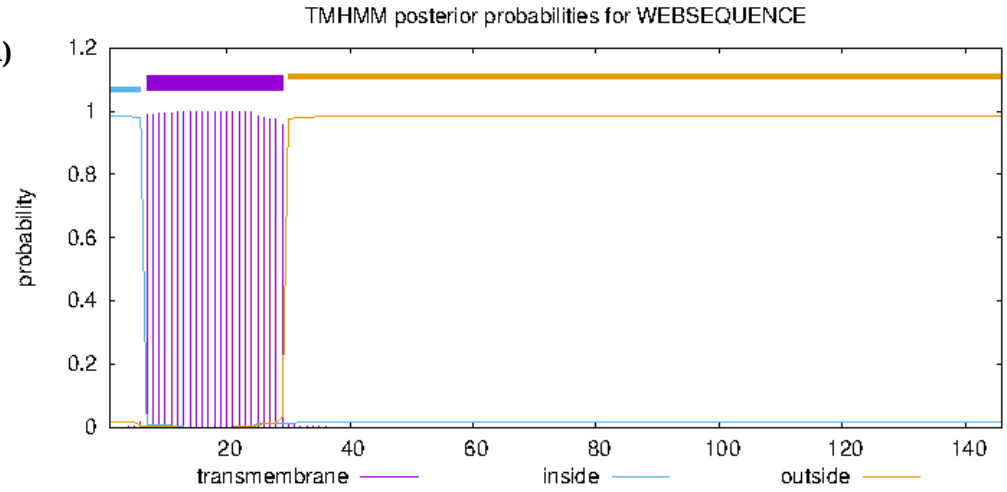
Geneious ID	Length (bp)	Protein ID	% Similarity with LanP
Signal peptidase I, bacterial type CDS (P62)	486	CBL24462.1	25.6
Signal peptidase I archael type CDS (P49)	486	CBL24349.1	17.8

Table 5.3.2. Candidate proteases from *B. obeum* A2-162 using *lanP* as the reference sequence. Protein names, first column in brackets, were based on the last two digits of the Protein ID.

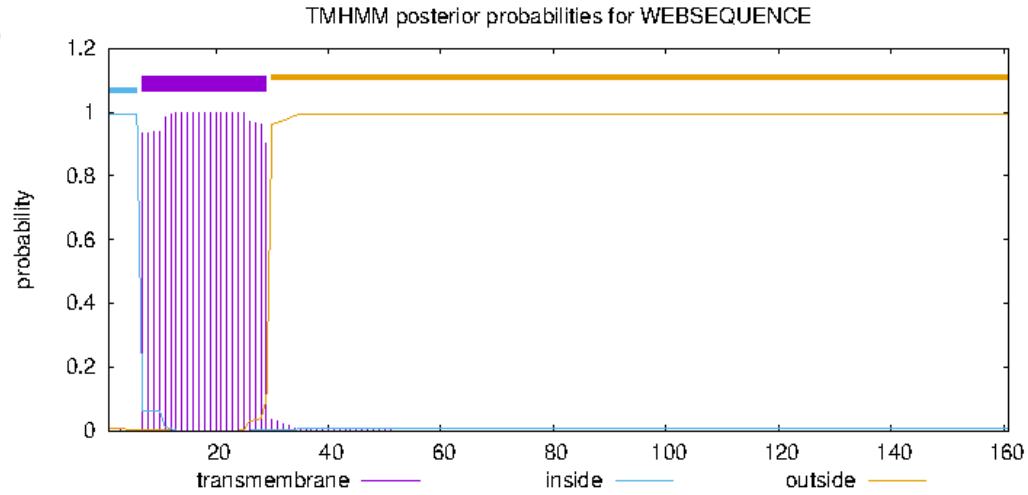
P62 and P49 were investigated further due to their similarity with LanP. Conserved regions within the amino acid sequence were of interest. CD Batch searches and TMHMM analysis were used to predict and compare conserved regions and transmembrane domains, respectively (Figure 5.3.3 and 5.3.4). The predicted transmembrane domains of LanP and P62 were identical (Figure 5.3.3) with a transmembrane domain predicted at the N-terminus followed by an extracellular domain. Interestingly, P49 although also containing a transmembrane domain at the N-terminus followed by an extracellular domain, also contained a transmembrane domain at the C-terminus (Figure 5.3.3). This analysis showed that P62 had greater similarity to LanP compared to P49 in terms of their shared conserved regions with both LepB-like and 26_SPase_I-like domains identified across the majority of the peptide. P49 also contained 26_SPase_I-like and LepB-like domains however they were only found in

a considerably smaller proportion of the candidate protease. However, there were no predicted catalytic sites for P62 whereas catalytic sites were predicted in P49 (Figure 5.3.4).

A)



B)



C)

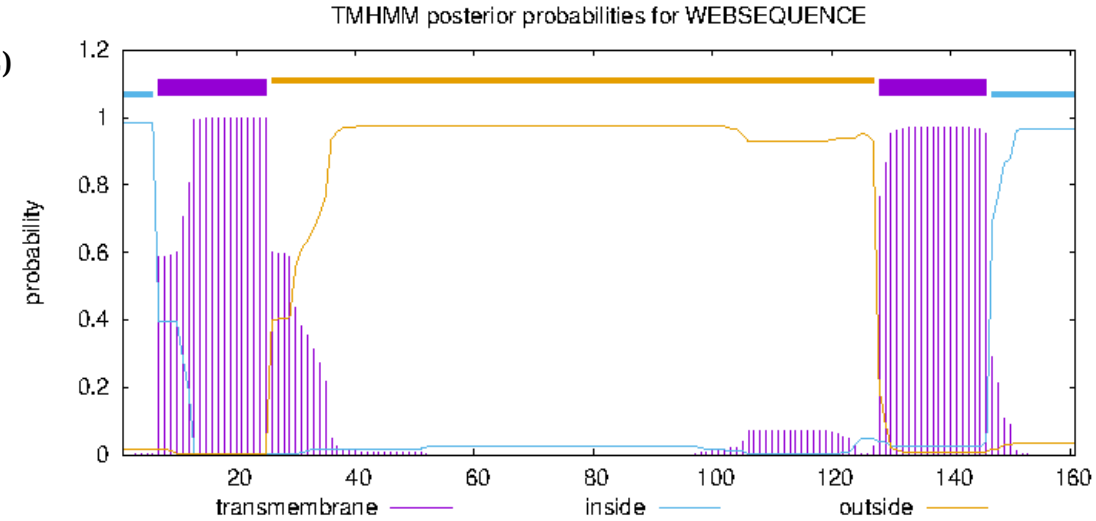


Figure 5.3.3. A) TMHMM analysis of LanP from *B. producta* SCSK cluster showing a single predicted transmembrane domain and a large extracellular region. B) P62 from *B. obeum* with a predicted single transmembrane and extracellular domain. C) P49 from *B. obeum* with two predicted transmembrane domains with a large extracellular region in between.

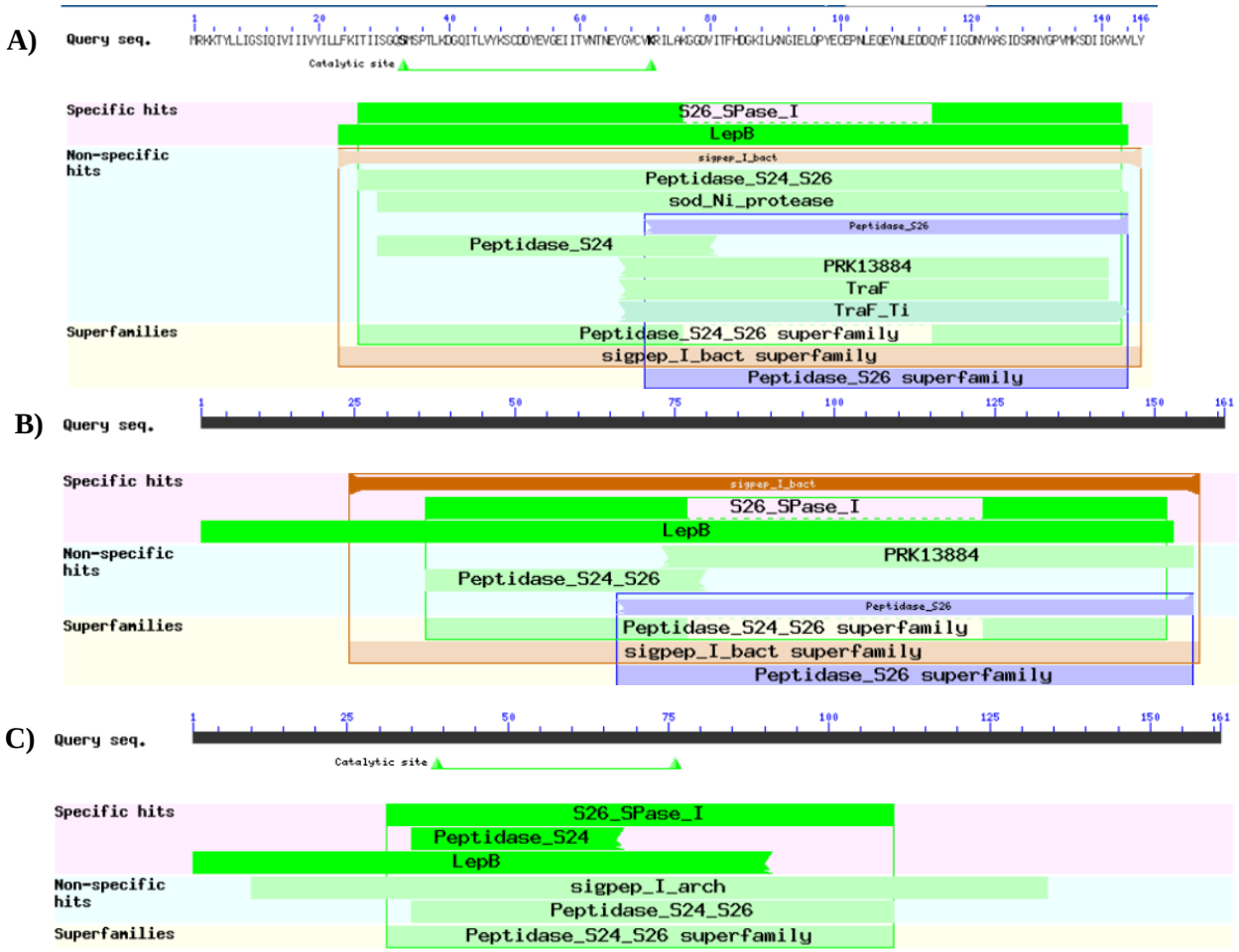


Figure 5.3.4. A) CD Batch search of amino acid sequences of LanP from *B. producta*, B) P62 and C) P49 both found within the *B. obeum* genome. Predicted catalytic sites are indicated by green arrows beneath the sequence.

5.3.1.3 Bioinformatic Analysis of New Candidate Proteases Using Conserved Domains in LanP

From the bioinformatic analysis of the LanP, P62 and P49 amino acid sequences the 26_SPase_I domain was conserved in all these candidates, therefore this conserved domain

was used to search the *B. obeum* A2-162 genome. Using the Pfam domain of S26_SPase_1 (PF10502) to search, three proteases were found to contain this domain (Table 5.3.1). This domain is conserved in a family of serine signal peptidases that can process newly synthesised peptides. Three candidate proteases were identified using this method, including the previously identified P62. P928 was not selected for further investigation due to the presence of an internal NcoI site which would have affected the Splice Overlap PCR with P32. However, P140 had a greater percentage similarity with LanP and also had similar predicted transmembrane domains, conserved domains and predicted catalytic sites (Figures 5.3.5 and 5.3.6).

Candidate Name	Length (bp)	Protein ID	% Similarity with LanP
Signal peptidase I. Serine peptidase. MEROPS family S26A CDS (P928)	558	CBL23928.1	22.6
Signal peptidase I, bacterial type CDS (P140)	594	CBL24140.1	23.8
Signal peptidase I, bacterial type CDS (P62)	486	CBL24462.1	25.6

Table 5.3.3. Candidate proteases identified via presence of S26_SPase_1 in *B. obeum* A2-162.

P140 was selected due to its greatest percentage amino acid similarity with LanP of the new candidates, as P62 had already been identified from the BLAST search using LanP as the reference sequence.

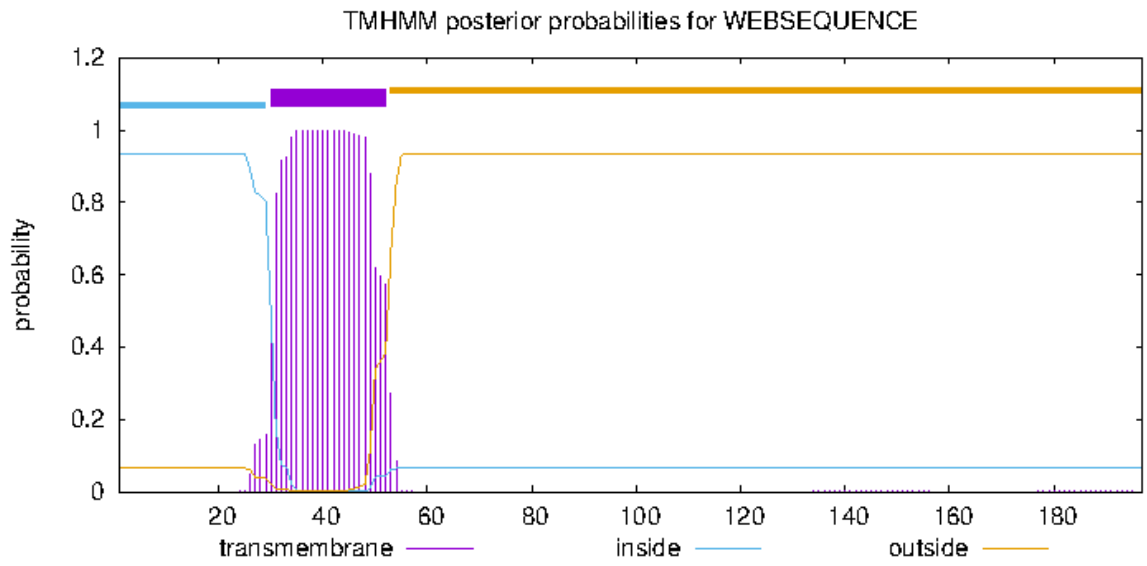


Figure 5.3.5. TMHMM analysis of P140 showing a single predicted transmembrane domain and a large extracellular region.

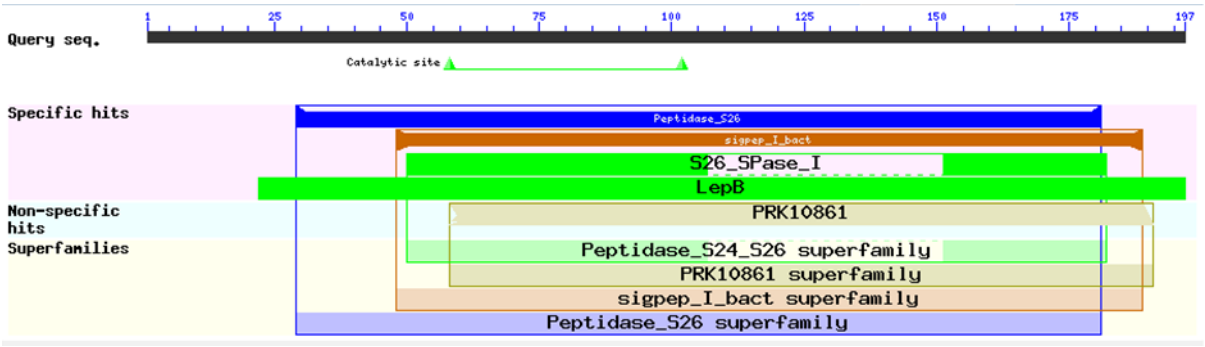


Figure 5.3.6. CD Batch search of amino acid sequence of P140 found within the *B. obeum* A2-162 genome. Predicted catalytic sites are indicated by green arrows beneath the sequence.

5.3.2 ROARY Pan-Genome Analysis of *B. obeum* Strains

The ROARY pan-genome analysis of 50 *B. obeum* strain genomes downloaded from NBCI, previously described in chapter 3, was also used to identify any new proteases that were only present in *B. obeum* strains that contained the nisin O cluster or similar cluster. The 5 genomes that had a FastANI score greater than 95% were analysed for the nisin O cluster, of which only *B. obeum* A2-162 contained the cluster (Table 3.3.1). Therefore, only proteins that were present in *B. obeum* A2-162 and not present in the other four genomes were studied through

a manual search. However, none of the proteins that were found only in *B. obeum* A2-162 had any similarity to known nisin proteases/signal peptidases or were annotated as such (Table 3.3.1).

5.3.3 Splice Overlap PCR of Candidate Proteases and Transformation into *E. coli* BL21(DE3)

For expression of the candidate proteases identified within the *B. obeum* A2-162 genome, splice overlap PCR was used to prepare these candidates for cloning into vector plasmids, described in Method 2.9, 2.10, 2.11 and 2.12. All candidate proteases were successfully incorporated into pET15b with the addition of an N-terminal His-tag. For *p66* insertion, primers P66NDE_F and P66XHO_R were used with the *p66* internal NdeI restriction site removed using P66CAC_F and P66CAC_R (Table 2.5). For P570 insertion, the P570NDE_F and P570XHO_R primers were used (Table 2.5). *p62* required the removal of an internal NdeI restriction site using the primers P62_NdeI_F, P62_SpliceR_NdeKO, P62_SpliceF_NdeKO, pUK200_XhoI_R to remove this and for insertion into pET15b. For *p49* the primers P49_NdeI_F and pUK200_XhoI_R were used (Table 2.5). Both *p62* and *p49* were inserted into pUK200_P32 prior to insertion into pET15b. Both *lanP* and *P140* were also successfully cloned into pET15b using the primers lanP_NdeI_F, lanP_XhoI_R, P140_NdeI_F and P140_XhoI_R, respectively (Table 2.5). The modified plasmids were transformed into *E. coli* BL21(DE3) in preparation for candidate protease expression and analysis.

5.3.4 Splice Overlap PCR of *p62* and *p49* and Transformation into *E. coli* MC1022

Both *p62* and *p49* were successfully cloned into pUK200. As *p62* contained a BglII restriction site within the sequence it was removed through an additional splice PCR using primers P62_BglIITGA_F and P62_BglIITGA_R. Both *p62* (P62_Splice32_F, P62_PstI_R, pUK_BglII_F and pUK_Splice62_R) and *p49* (P49_Splice32_F, P49_PstI_R, pUK_BglII_F

and pUK_Splice49_R) were spliced with the constitutive promoter P32 to increase the expression of the candidate proteases (van der Vossen, van der Lelie *et al*. 1987).

Each candidate protease was first transformed into *E. coli* MC1022. This would enable increased plasmid production, and therefore, increase the transformation rate in *L. lactis* UKLc10 p*nso*. All candidate proteases were successfully transformed into *E. coli*. Positive colonies were analysed on a 0.9% agarose gel to check for the presence of a band at the correct predicted size based on CloneManager prediction. Figure 5.3.7A shows the results from a 96-well colony PCR where columns 1, 2, 3 and 12 contained a band of the correct size (821 bp). The samples from column 1 underwent colony PCR individually (Figure 5.3.7B) where one sample produced a band at the correct size. Transformations involving *P62* did not require multiwell PCR (Figure 5.3.8).

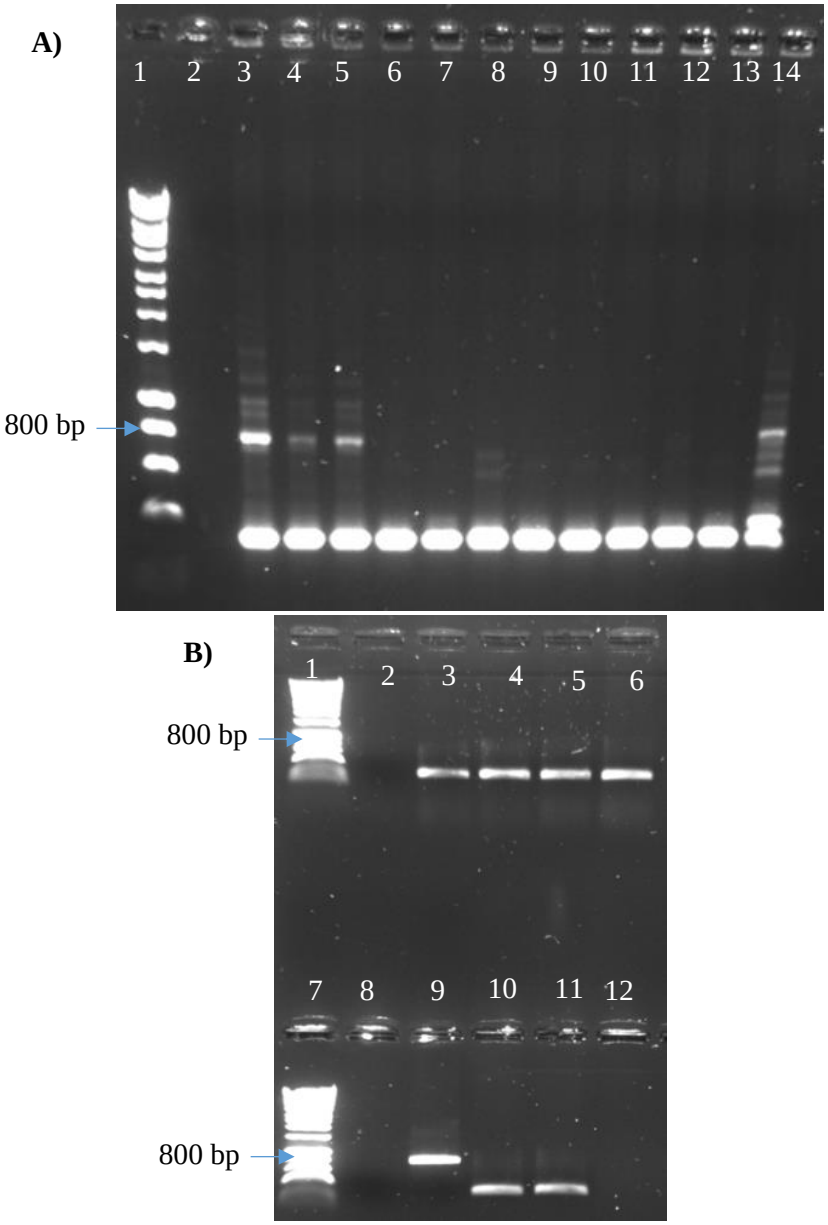


Figure 5.3.7. A) A multiwell plate colony PCR gel loaded with a 1 kb ladder (Lane 1), negative control (Lane 2) and lanes 3-14 corresponding to mixed samples of *E. coli* pUK200_P32_*p49* candidates from columns 1-12, respectively. B) Individual colony PCR of samples that made the mix for column one with one sample, lane 9, containing a band of the correct predicted size (821 bp).

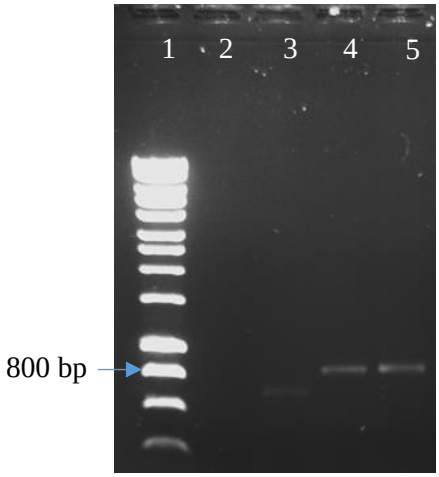


Figure 5.3.8. A gel of a colony PCR for 3 potential positive candidates of pUK200_P32_*p62*. Gel loaded with 1 kb ladder, negative control and *p62* candidates 1, 2 and 3 in lanes 1-5, respectively (823 bp).

5.3.5 Transformation of pUK200_P32_*P62* and pUK200_P32_*P49* into *L. lactis* UKLc10 p*nso*

Both pUK200_P32_*p62* and pUK200_P32_*p49* were purified from *E. coli* MC1022 cultures and transformed into *L. lactis* UKLc10 p*nso* and MG1614; transformations were all successful, as confirmed by sequencing. The transformations into *L. lactis* MG1614 were performed initially to ensure that the plasmid containing the new proteases would be able to function and replicate within an *L. lactis* strain.

Sequencing found that there was insertion of a thymine residue within the P32 promoter sequence of both candidate protease *p62* and *p49*. However, as this insertion did not affect the binding site of the promoter region the planned assays went ahead with these proteases.

5.3.6 Creation and Transformation of pUK200_P32_*p140* and pUK200_P32_*lanP* into *E. coli* MC1022 and *L. lactis* UKLc10 p*nso*

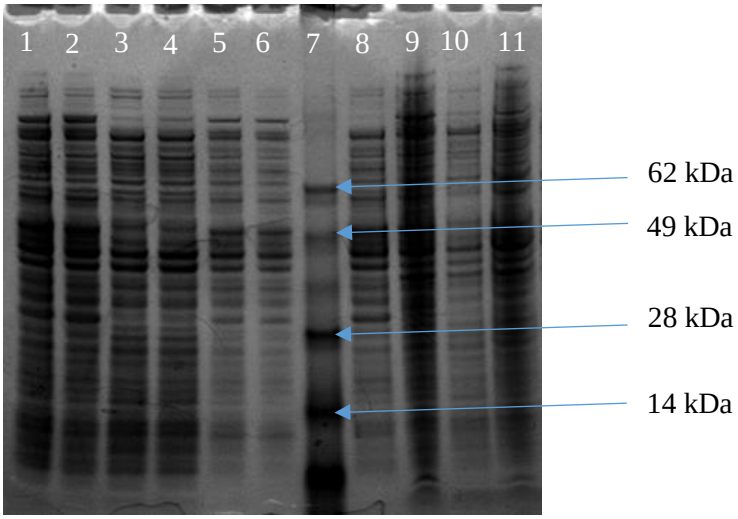
To test the activity of both P140 and LanP, both genes were amplified. *p140* was amplified from *B. obeum* A2-162 gDNA, whereas *lanP* was amplified from a plasmid provided by the Pamer group, Chicago. Both *lanP* (LanP_SpliceP32_F, P32_BglII_F (pUK200), LanP_NcoI_R and P32_SpliceLanP_R(2)) and *p140* (P140_NcoI_F, P140_BamHI_R,

P32_SpliceP140_R and P140_SpliceP32_F) were successfully cloned into pUK200_P32, which would provide constitutive expression of both these proteases due to the presence of the P32 promoter. Each protease was transformed into *E. coli* MC1022, to enable increased plasmid replication and purification before transformation into the *L. lactis* heterologous expression system.

The proteases were purified from *E. coli* cultures and successfully transformed into *L. lactis* UKLc10 p*nso* and *L. lactis* UKLc10 pIL253. Confirmation that the correct plasmid had been transformed was performed by sequencing of colony PCR products.

5.3.4 SDS-PAGE Analysis of P66 and P570

The production and cellular location of candidate proteases was analysed using SDS-PAGE gels. Both the crude cell free extract (CFE) and crude cell wall extract (CWE) were used to determine, in conjunction with western blot analysis using antibodies to detect the His tag, the presence of the candidate proteases P66 and P570. Both proteases were expressed in *E. coli* BL21(DE3) and were His-tagged. As we expected the lantibiotic cleavage proteases to be located at the cell wall, we tested different induction times and temperatures to try to maximise the protein yield in the soluble fraction. Although different induction times (3 hrs and 4 hrs) were used, there was no clear increase in production of these proteases across these induction times. His-tagged P66 and His-tagged P570 were predicted to be 31.8 kDa and 55.1 kDa, respectively. The soluble fractions for the candidate proteases P66 did appear to show a strong band at the correct size after both 3 hrs and 4 hrs induction with IPTG induction (Figure 5.3.9). Furthermore, due to smearing of the CWEs for both proteases it was unclear if the final position of the proteases is within the cell wall (Figure 5.3.9, lanes 9 and 11).



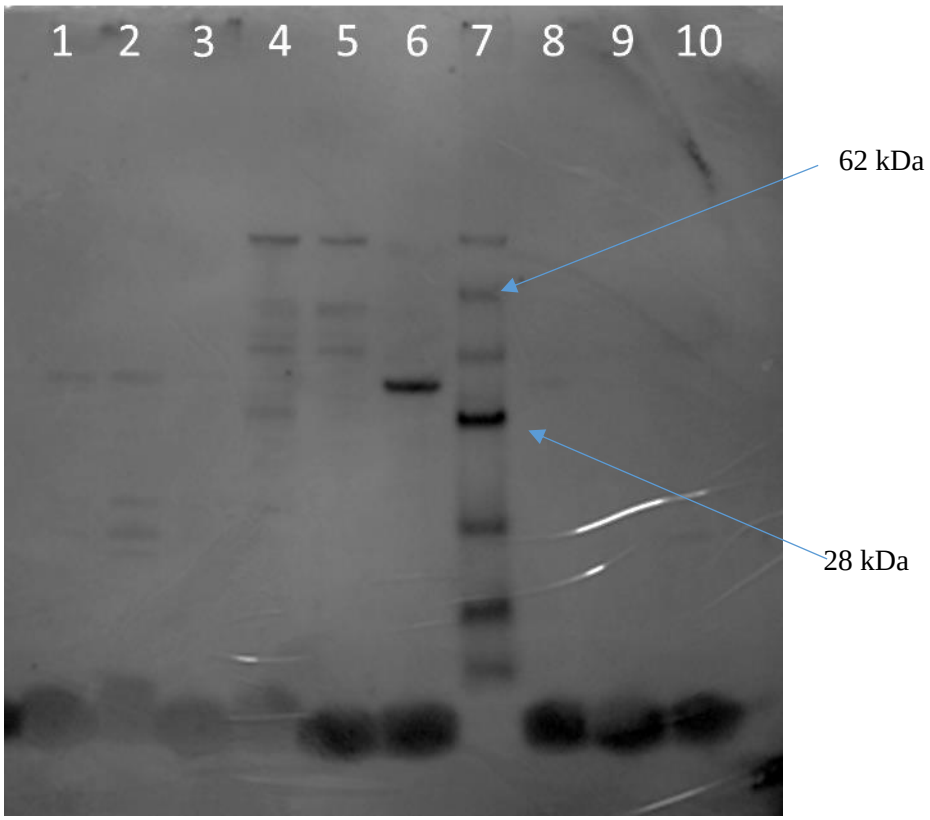
1. pET15b_*p66* 3 hr Cell Free Extract (CFE)
2. pET15b_*p66* 4 hr CFE
3. pET15b 3 hr CFE
4. pET15b 4 hr CFE
5. pET15b_*p570* 3 hr CFE
6. pET15b_*p570* 4 hr CFE
7. SeeBlue™ Plus2 Pre-stained Protein Standard
8. pET15b_*p66* Uninduced Cell Wall Extract (CWE)
9. pET15b_*p66* CWE
10. pET15b CWE
11. pET15b_*p570* CWE

Figure 5.3.9. An SDS-PAGE gel of the crude cell free extracts (CFE) and cell wall extracts (CWE) of *E. coli* BL21(DE3) pET15b_*p66*, *E. coli* BL21(DE3) pET15b and *E. coli* BL21(DE3) pET15b_*p570* at 3 and 4 hrs induction using IPTG for each culture.

5.3.5 Western Blot Analysis to Detect P66 and P570

Further analysis of the candidate proteases P66 and P570 showed that P66 had a greater proportion of the expressed protein present in the cell wall compared to the cytoplasm (Figure 5.3.10 Lane 7). However, optimisation of the conditions using an incubation time of 4 hrs at 18^{o}C increased the proportion of P66 present in the CFE (Figure 5.3.10 Lanes 2 and 3). In the figure presented, a further cell wall extraction of *E. coli* pET15b_*p66* (Lane 10) showed no presence of the protease, which was contrary to previous experimental results, but loading cell wall samples was problematic due to viscosity. Conversely, P570 did not show a presence in the cell wall however there was a band at the correct size in the cytoplasmic fraction of the

expression vector. Increasing the IPTG induction time from 3 hrs to 4 hrs showed a marginal increase in the expression of both proteases, yet changes to the induction temperature to 18ºC had no effect on the amount of protein in the soluble fraction for the expression of *p570* whereas this change in temperature increased the amount of *p66* expressed.



1. *E. coli* pET15b_*p66* incubated for 3 hrs at 18ºC Cell free extract
2. *E. coli* pET15b_*p66* incubated for 4 hrs at 37ºC (CFE)
3. *E. coli* pET15b incubated for 4 hrs at 18ºC (CFE)
4. *E. coli* pET15b_*p570* incubated for 3 hrs at 18ºC (CFE)
5. Positive *E. coli* pET15b_*p570* sample (4 hrs at 37ºC) (CFE)
6. Positive *E. coli* pET15b_*p66* sample Cell wall extract (4 hrs at 37ºC) (CWE)
7. SeeBlue™ Plus2 Pre-stained Protein Standard
8. *E. coli* pET15b_*p66* incubated at for 3 hrs at 18ºC (CWE)
9. *E. coli* pET15b_*p66* incubated for 4 hrs at 37ºC (CWE)
10. *E. coli* pET15b_*p570* incubated for 3 hrs at 18ºC (CWE)

Figure 5.3.10. A western blot showing the cellular distribution of proteases P66 and P570 produced by *E. coli* using alterations in temperature and induction time to assess the optimal expression conditions. Crude cell free extract (CFE) and crude cell wall extract (CWE) were used. Positive controls for both P66 and P570 were produced in a previous assay assessing

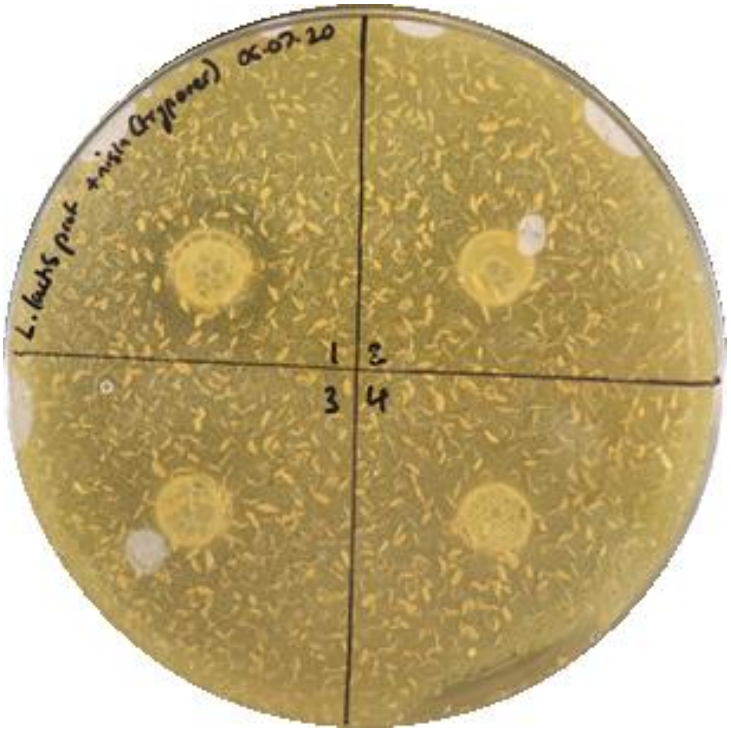
whether P66 or P570 could be detected in the CFE or CWE when inducing for 3 hrs with IPTG at 37ºC.

5.3.6 Overlay Assays using *L. lactis* Strains with Co-Expression of *P62* and *P49*

Overlay assays were used to assess the antimicrobial activity of a variety of created strains to test the efficacy of the candidate proteases. For all overlay assays *C. perfringens* was used as an indicator organism.

P62 and P49 were used to attempt cleavage of the leader peptides of nisin O by heterologous expression in *L. lactis*. These candidate proteases were selected as they had the highest percentage similarity to the LanP from the *B. producta* SCSK biosynthetic gene cluster. There was no zone of inhibition seen surrounding either *p62* or *p49* expressing *L. lactis* strain, either when trypsin was present in the overlay agar or absent (Figure 5.3.11A and B). A zone of inhibition was only observed for the positive control of *L. lactis* UKLc10 p*nso* when trypsin was present in the overlay agar (5.3.11A). No trypsin was used in the base or overlay agar for these overlay assays. Therefore, due to the lack of antimicrobial activity seen by both the *p62* and *p49* co-expressing strains without the presence of trypsin it was determined that these candidate proteases could not cleave the leader peptide or have an efficiency such that no antimicrobial activity could be seen. Additionally, it must be noted that no antimicrobial activity was observed for the *p62* and *p49* expression *L. lactis* strains even when trypsin was present. This should have occurred as the *nsoA1-4* genes should still be expressed and therefore provide a zone of inhibition. This may have occurred due to the constitutive expression of the two candidate proteases within these heterologous expression systems causing a downregulation of the *nsoA1-4* genes and hence not providing the antimicrobial activity expected.

A)



B)

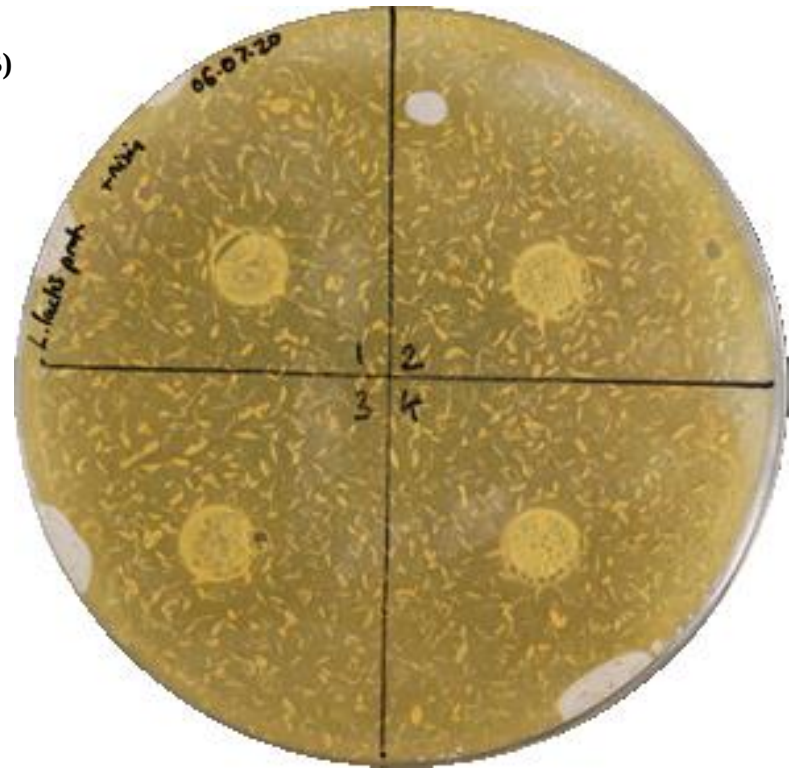


Figure 5.3.11. A) An overlay assay to assess the effect of candidate proteases on nisin O leader cleavage using, *L. lactis* UKLc10 p*nso, L. lactis* UKLc10 p*nso* pUK200_P32*_p62*, *L. lactis* UKLc10 p*nso* pUK200_P32*_p49*, *L. lactis* MG1614 pIL253 in section 1-4, respectively,

with trypsin present only in the overlay agar. B) Strains are spotted in the same order as A, however there is no trypsin present in the base or overlay agar.

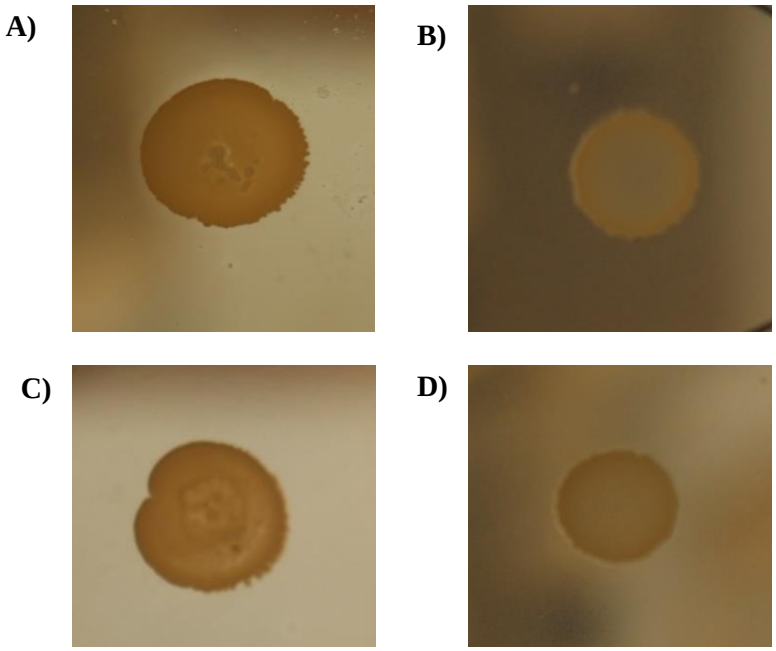


Figure 5.3.12. A) An antimicrobial overlay assay using *L. lactis* UKLc10 p*nso* and 10 µg/ml cell wall extract (CWE) from *E. coli* BL21 (DE3) pET15b_*p570* incubated overnight at 37ºC with no trypsin present in the base or overlay agar. B) *L. lactis* UKLc10 p*nso* and 10 µg/ml CWE from *E. coli* BL21 (DE3) pET15b_*p570* incubated overnight at 37ºC with trypsin present in the overlay agar. C) *L. lactis* UKLc10 p*nso* and 10 µg/ml CWE from *E. coli* BL21 (DE3) pET15b_*p66* incubated overnight at 37ºC with no trypsin present in the base or overlay agar. D) *L. lactis* UKLc10 p*nso* and 10 µg/ml CWE from *E. coli* BL21 (DE3) pET15b_*p66* incubated overnight at 37ºC with trypsin present in the overlay agar. *C. perfringens* was used as the indicator organism.

Overlay assays were also used for both P570 and P66 crude CWEs after being incubated for 3 hrs with IPTG at 37ºC, with the *E. coli* crude extract mixed with *L. lactis* UKLc10 p*nso* and incubated overnight at 37ºC before the latter was grown and overlaid. However, no zones of inhibition were observed for either P66 or P570 samples in the presence or absence of trypsin

(Figure 5.3.12). Again, this was not expected as in the presence of trypsin antimicrobial activity should occur, and as there is no co-expressing of the candidate proteases, the CWEs might be interacting with either *L. lactis* or trypsin to prevent the cleavage and therefore activity of pre-NsoA1-4.

5.3.7 Overlay Assays using *L. lactis* Heterologous Expression System with *LanP* and *P140*

Overlay assays were used to assess whether cleavage of the leader peptides of NsoA1-4 were occurring with the co-expression of *lanP* or *p140*, using *C. perfringens* as an indicator organism. However, there was no zone of inhibition around either *lanP* or *p140* co-expressing strains without the presence of trypsin (Figure 5.3.13B). This suggests that neither of these candidate proteases are able to cleave the leader peptide of NsoA1-4 or cannot cleave nisin O efficiently to provide an antimicrobial zone of inhibition. On both plates, the nisin A producing positive control (*L. lactis* FI5876 pIL253) provided a zone of inhibition and the nisin O producing strain (*L. lactis* UKLc10 p*nso*) was only observed to have a zone of inhibition when trypsin was present in the overlay agar. However, as with the previous overlay assays, no antimicrobial activity was observed for the *p140* and *lanP* producing strains in the presence of trypsin, which was unexpected. This again may be due to reduced expression of the nisin O peptides to a level below that for an observable antimicrobial effect due to the constitutive expression of the two candidate proteases within the strain.

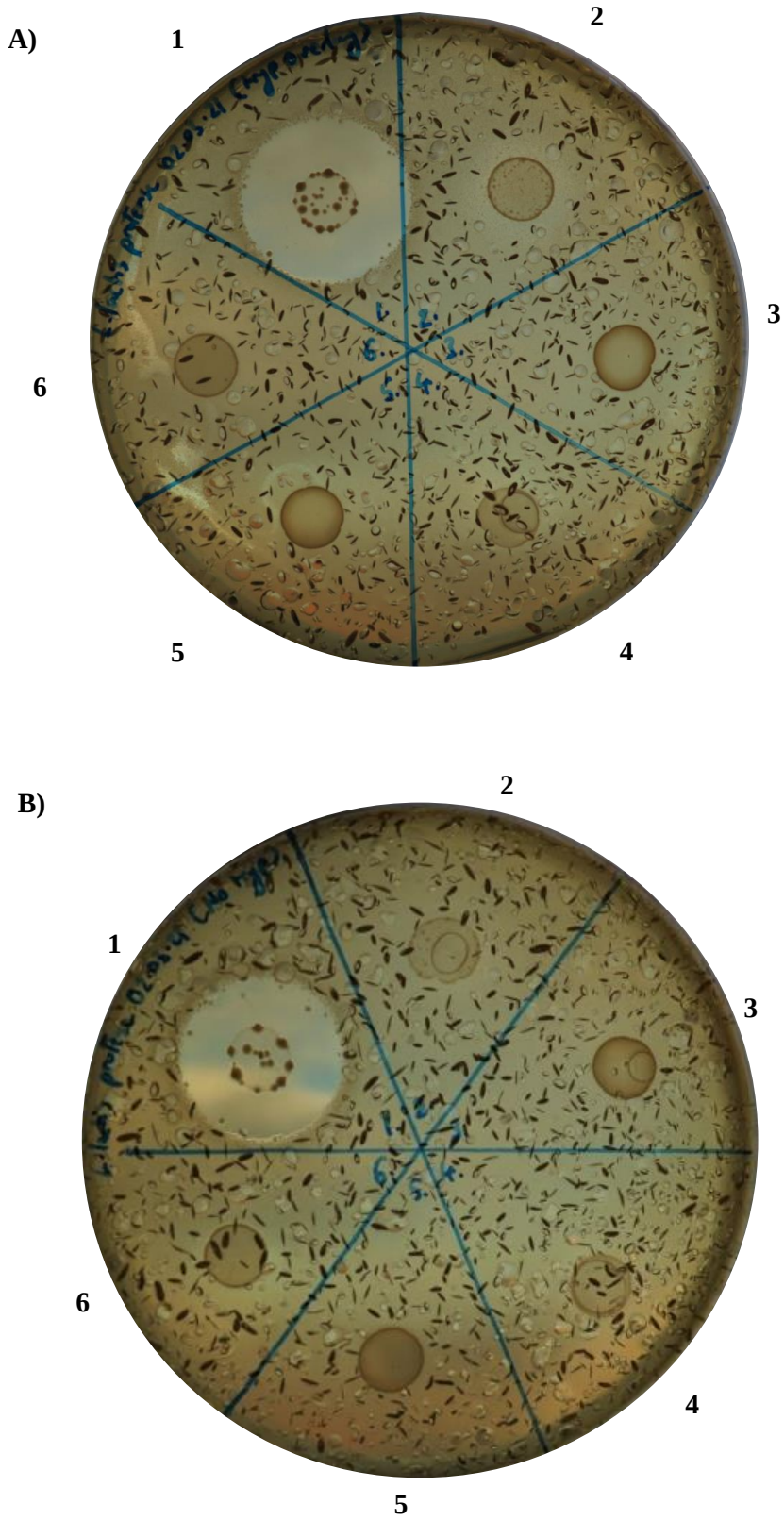


Figure 5.3.13. A) An overlay assay to assess the effect of candidate proteases on nisin O leader cleavage using, *L. lactis* FI5876 pIL253, *L. lactis* UKLc10 p*nso*, *L. lactis* UKLc10 pIL253 pUK200_P32_*p140*, *L. lactis* UKLc10 p*nso* pUK200_P32_*p140*, *L. lactis* UKLc10 pIL253 pUK200_P32_*lanP*, and *L. lactis* UKLc10 p*nso* pUK200_P32_*lanP*, as spots 1-6,

respectively. Plate contained 15 ng/ml nisin A in the base agar and $NaHCO_3$ and 10 µg/ml trypsin in the overlay agar. B) Bacteria spots were in the same order as plate A, however the base plate contains 15 ng/ml nisin and $NaHCO_3$, with no trypsin used in the overlay agar. *C. perfringens* was used as an indicator strain

5.3.8 pre-nso Leader Cleavage Assay using P570, P66, P62, P49, P140 and LanP

To investigate whether the candidate proteases were capable of cleaving the NsoA1-3 pre-peptides, a protease incubation assay using pre-Nso (5.8 kDa) as the substrate was performed followed by a Western Blot with an antibody for the NsoA1-3 leader peptide to check for leader peptide cleavage. No notable leader cleavage of pre-Nso was seen for any candidate protease as bands are visible in all incubations with candidate proteases at approximately 6 kDa (Fig 5.3.14). Crude CWEs were used for all candidate proteases, and CFEs were also used for P140 and LanP strains. The CWEs were selected as NisP is found anchored in the cell wall and most of the candidate proteases assessed contained predicted transmembrane domains, indicating that they are bound on the cell surface. Interestingly, the non-specific bands seen in the P49 and P62 western were absent in the P140 and LanP Western Blot.

The CWEs of the empty vector pET15b, *p570* and *p66* expressing strains were used in an incubation with TCA precipitated pre-NsoA1-4 (Figure 5.3.14A). Bands were clearly in all wells that contained the CWEs from the *E. coli* strains at the correct size of 5.8 kDa. This therefore indicates that neither P66 or P570 are able to cleave the leader peptide of pre-NsoA1-3 and hence cannot activate the peptide. A positive control using 15 µg/ml trypsin and 15 µg/ml trypsin that had been adjusted to pH 6.8 was used and no bands were observed on the Western Blot transfer membrane after antibody treatment. This confirms that trypsin is capable of cleaving the leader peptide of pre-NsoA1-3 (Figure 5.3.14A).

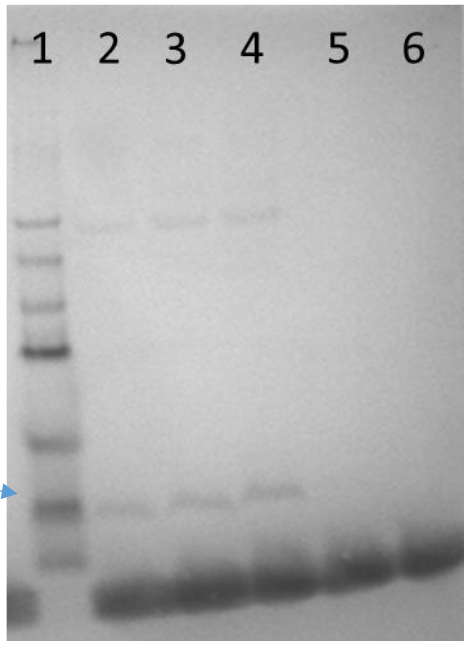
With the results of this experiment established, the *p49* and *p62* producing strains were induced using IPTG with the conditions optimised previously (4 hrs incubation at 18^{o}C). These crude CWEs were used in an overnight incubation with the TCA precipitated pre-

NsoA1-4 and the precipitated supernatant from an empty pIL253 vector strain to act as a negative control. No bands were observed in the pIL253 control lanes as expected as there were no pre-NsoA1-3 peptides for the leader binding antibody to attach to (Figure 5.3.14B). Furthermore, clear bands were observed for P49, P62 and pET15b CWEs after the incubation indicating that no leader peptide cleavage was occurring in the presence of these proteases (Figure 5.3.14B). Additionally, the positive trypsin control showed no bands in the pIL253 precipitated supernatant or that of the pre-NsoA1-4 supernatant, indicating that trypsin is able to cleave the leader peptide of pre-NsoA1-3 (Figure 5.3.14B).

Both LanP and P140 were assessed for their ability to cleave the leader peptide from pre-NsoA1-3. Both the CFEs as well as the CWEs was used in the overnight incubation with the TCA precipitated pre-NsoA1-4 supernatant. However, clear bands at approximately 6 kDa were observed for all the extracts, again indicating that the pre-NsoA1-3 leader peptide was not cleaved by these candidate proteases (Figure 5.3.14C). In this assay the CWEs from both *B. obeum* and the humanisation strain co-culture in addition to the mix of humanisation strains were used in order to observe whether any proteases on the cell surfaces of these bacteria were capable of cleaving the leader peptide. However, bands were observed using these CWEs of mixed culture, which means that there was no protease found attached to the cell wall of these bacteria that was able to cleave the leader peptide of pre-NsoA1-3 (Figure 5.3.14C). Furthermore, the CWEs and CFEs of *L. lactis* MG1614 was tested to ensure that there was no protein capable of cleaving the leader from *L. lactis*, therefore confirming that the antimicrobial activity seen in the heterologous expression system was indeed activated by trypsin. A band was observed when the *L. lactis* MG1614 CFE was used indicating that no leader peptide cleavage occurred. However, the *L. lactis* MG1614 the CWEs, along with the trypsin positive control did not have a band within their respective lanes (Figure 5.3.14C). This was likely due to poor transfer as when this assay was repeated using *L. lactis* MG1614 CWE and CFE clear bands were visible using both extracts (Figure 5.3.14D). These results further confirm that none of the candidate proteases selected for cloning were able to cleave

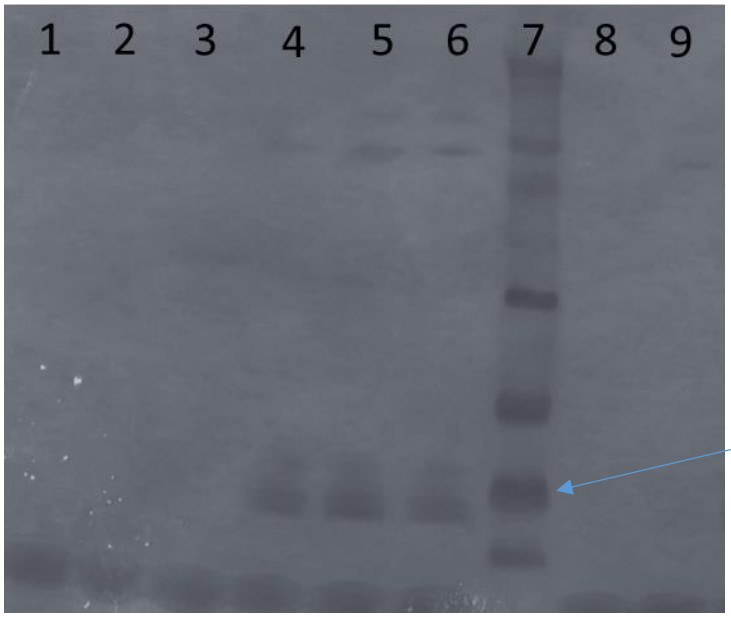
the leader peptide of pre-NsoA1-3 and furthermore, trypsin has been shown to cleave the leader peptide resulting in no bands being observed as the fragment remaining (2.65 kDa) after leader peptide cleavage is too small and would either be electrophoresed off the gel or the product was within the dye front and therefore was not observable.

A)



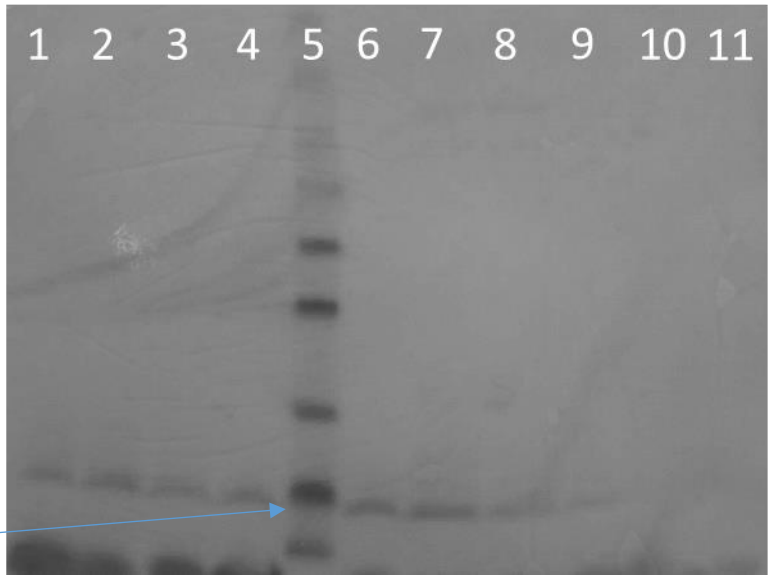
1. SeeBlue™ Plus2 Standard
2. Pre-nso with pET15b CWE
3. Pre-nso with P570 CWE
4. Pre-nso with P66 CWE
5. Pre-nso with trypsin
6. Pre-nso with trypsin (pH adjusted)

B)



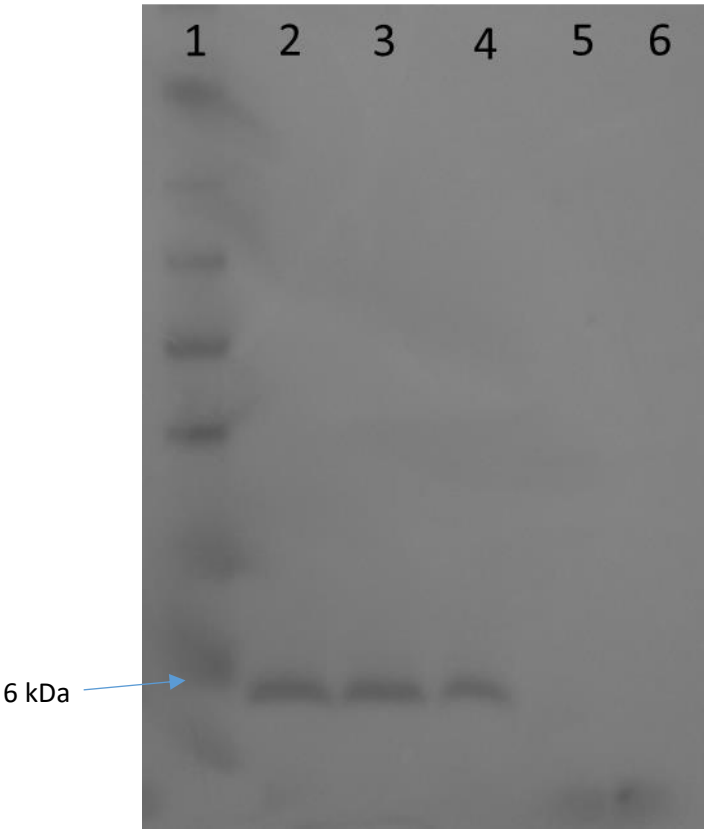
1. pIL253 with P62 CWE
2. pIL253 with P49 CWE
3. pIL253 with pET15b CWE
4. Pre-nso with P62 CWE
5. Pre-nso with P49 CWE
6. Pre-nso with pET15b CWE
7. SeeBlue™ Plus2 Standard
8. pIL253 with trypsin
9. Pre-nso with trypsin

C)



1. Pre-nso with P140 CFE
2. Pre-nso with LanP CFE
3. Pre-nso with P140 CWE
4. Pre-nso with LanP CWE
5. SeeBlue™ Plus2 Standard
6. Pre-nso
7. Pre-nso with Hu + *B. obeum* CWE
8. Pre-nso with Hu CWE
9. Pre-nso with MG1614 CFE
10. Pre-nso with MG1614 CWE
11. Pre-nso with 10 µg/ml trypsin

D)



1. SeeBlue™ Plus2 Standard
2. Pre-nso
3. Pre-nso with MG1614 CFE
4. Pre-nso with MG1614 CWE
5. Pre-nso with 10 µg/ml trypsin
6. Pre-nso with 50 µg/ml trypsin

Figure 5.3.14 A) Western Blot of candidate proteases P570 and P66 crude cell wall extracts (CWE) using pre-NsoA1-4 TCA-precipitated proteins as the substrate. B) Western Blot of candidate proteases P49 and P62 CWE using pre-NsoA1-4 or pIL253 TCA-precipitated proteins as substrates. C) Western Blot of candidate proteases P140 and LanP crude cell free extracts (CFE) and crude CWEs using pre-NsoA1-4 TCA-precipitated proteins as substrate.

CWE of the co-culture of *B. obeum* A2-162 and humanisation strains (Hu) were also used. *L. lactis* MG1614 was used as a negative control and trypsin was used as a positive control. D) Western blot of the CFE and CWE of *L. lactis* MG1614 incubated with TCA precipitated pre-NsoA1-4. Trypsin was used a positive control for all assays.

5.3.9 MicroMatrix Fermentation Assay

A MicroMatrix fermentation experiment was performed to assess whether mature active nisin O could be produced within a simulated gut environment and whether the levels of production were sufficient for antimicrobial activity or changes in the microbiome profile to be observed. Both *L. lactis* UKLc10 p*nso* and *B. obeum* A2-162 were used in combination with human faecal slurry which would enable any antimicrobial effect on the microbial composition to be observed. Additionally, trypsin was added to a subset of samples to replicate the concentrations found in the gut and to ensure that the leader peptide of nisin O was cleaved within the fermentation model (Goldberg, Campbell *et al*. 1969). Samples were taken at timepoints 0 hrs and 24 hrs and the samples were pelleted and prepared for DNA and RNA extraction. DNA samples were sequenced using Illumina metagenomic shotgun sequencing with the data received being applied to the QC_module_wf_1.0_nohost pipeline on Galaxy to assess the bacterial composition (Figure 5.3.15). In all samples when comparing the T0 and T24 timepoint samples there was an increased proportion of *Escherichia coli* present within the samples after 24 hrs, indicated in pink in figure 5.3.15. However, the increase in reads being annotated as *E. coli* were also observed in the *L. lactis* UKLc10 p*nso* only control wells. This was very unexpected as the samples were treated under aseptic conditions prior to the experiment preparation and sampling. This result was also seen in a repeat of this experiment using the same donor. This increase in *E. coli* reads may have been due to the cassettes for the MicroMatrix being sterilised and re-used for multiple experiments, therefore it is possible that some strains, in this case *E. coli*, were able to survive the sterilisation of the cassettes and therefore contaminate the growth medium including that of single strain only wells. Because of this likely contamination there was no indication that the presence of nisin O producing

strains in the presence or absence of trypsin caused a change in the bacterial composition. It can also be stated that the *E. coli* growth was not due to the nisin O antimicrobial activity as the faecal slurry only wells also had an increase in *E. coli* mapped reads. Furthermore, *B. obeum* A2-162 was not identified within the key produced by the QC_module_wf_1.0_nohost Galaxy pipeline. This was likely due to the *B. obeum* A2-162 reads being mis-annotated by the pipeline, therefore explaining its absence from the key. Only one *Blautia* species, *Blautia hansenii* was present within the analysed data, of which it consisted of just 0.5%, 0.01%, 0.5% and 0.02% of the *B. obeum* A2-162 + Faecal T0, *B. obeum* A2-162 + Faecal T24, *B. obeum* A2-162 + Faecal + 400 µg/ml trypsin T0 and *B. obeum* A2-162 + Faecal + 400 µg/ml trypsin T24 samples. This shows that *B. obeum* A2-162 was not mis-annotated as another *Blautia* species as the *L. lactis* samples contained a very high percentage of *L. lactis* reads at T0, between 31% to 90%. Additionally, the presence of trypsin also did not appear to influence the growth of *E. coli* between the different conditions. The extracted DNA was used in a PCR to observe if the nisin O cluster was present in the sample using the nsoA1_F and nsoA1/2/3_R primers. In all samples which contained either *B. obeum* A2-162 or the *L. lactis* heterologous expression system, bands were present which indicated the presence of the *nsoA1-3* genes within the samples (Figure 5.3.16A and B).

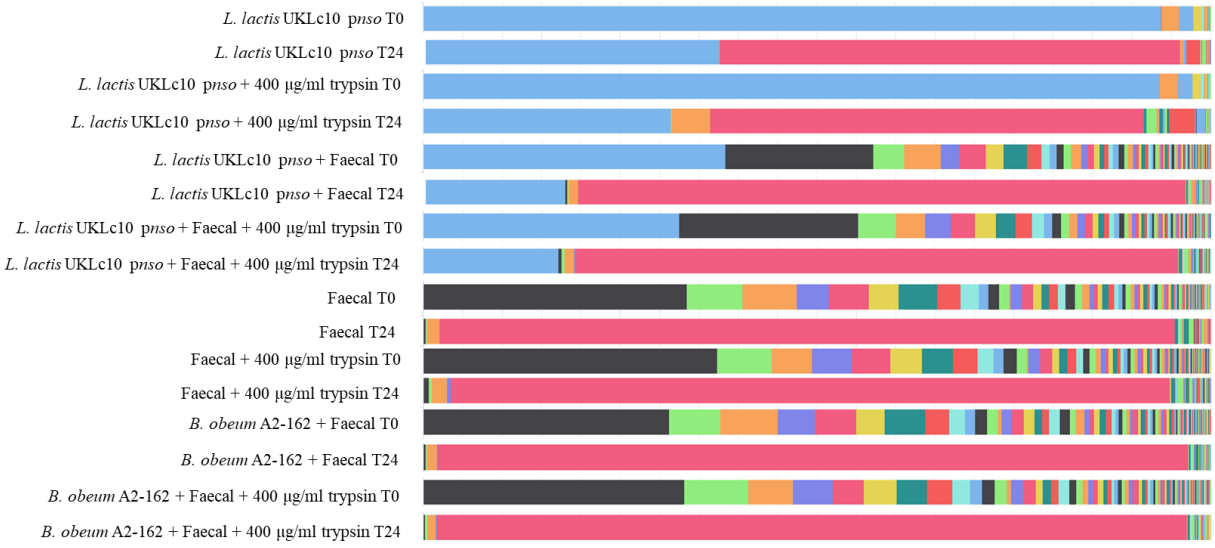
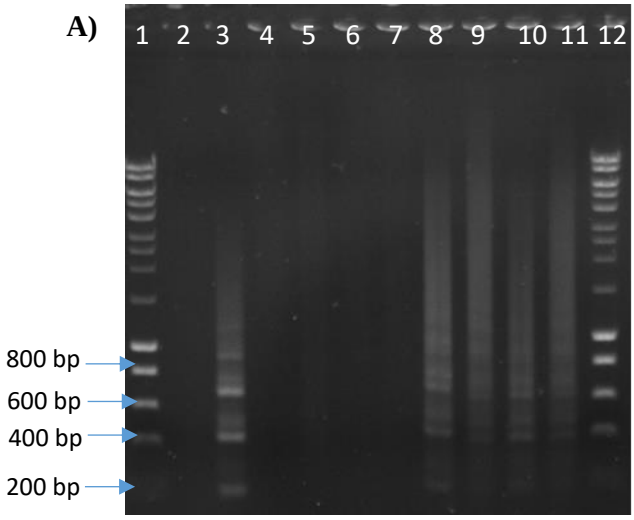
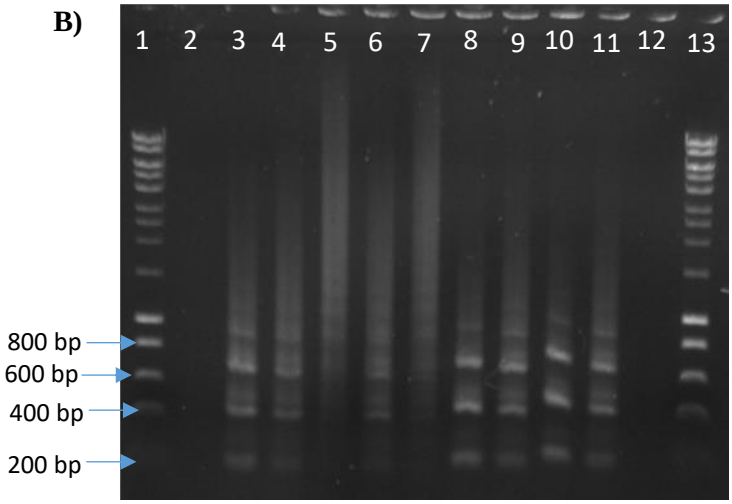


Figure 5.3.15. Shotgun metagenomic sequencing output from the QC_module_wf_1.0_nohost Galaxy pipeline showing bacterial composition of samples from timepoint 0 hrs (T0) and timepoint 24 hrs (T24).



1. 1 kB ladder
2. Negative
3. *B. obeum* gDNA
4. Faecal slurry T0
5. Faecal slurry T24
6. Faecal slurry + trypsin T0
7. Faecal slurry + trypsin T24
8. Faecal slurry + *L. lactis* pnso T0
9. Faecal slurry + *L. lactis* pnso T24
10. Faecal slurry + *L. lactis* pnso + trypsin T0
11. Faecal slurry + *L. lactis* pnso + trypsin T24
12. 1 kB ladder

B)



1. 1 kB ladder
2. Negative
3. *B. obeum* gDNA
4. *L. lactis pnso T0*
5. *L. lactis pnso T24*
6. *L. lactis pnso + trypsin T0*
7. *L. lactis pnso + trypsin T24*
8. Faecal slurry + *B. obeum* T0
9. Faecal slurry + *B. obeum* T24
10. Faecal slurry + *B. obeum* + trypsin T0
11. Faecal slurry + *B. obeum* + trypsin T24
12. Kitome negative control
13. 1 kB ladder

Figure 5.3.16. A) An agarose gel of the extracted DNA from a MicroMatrix fermentation experiment (lanes 4-11). B) An agarose gel of the extracted DNA from a MicroMatrix fermentation experiment (lanes 4-11).

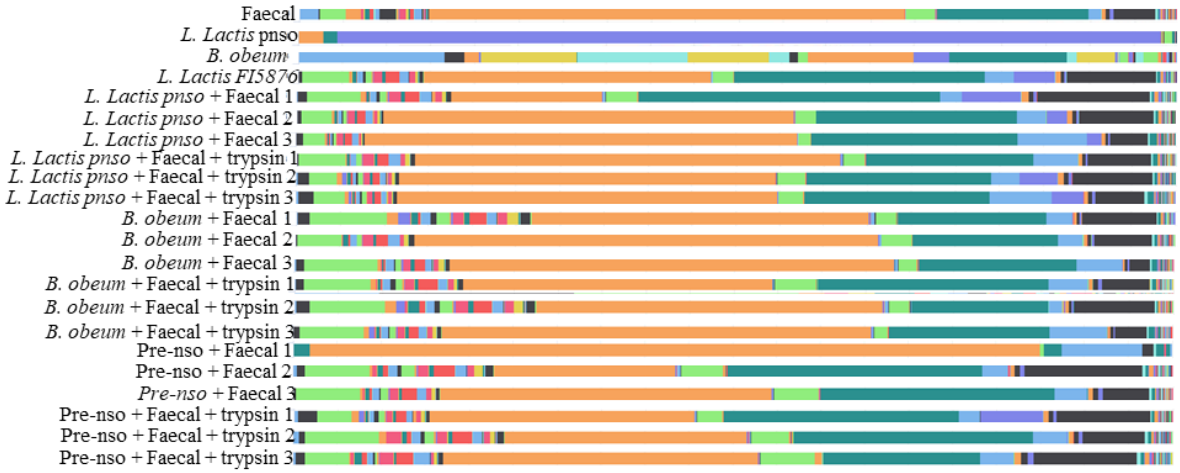
A repeat of the assay was performed using a new donor (as the original donor became infected with SARS_CoV_19), with an additional test sample of 25 µg/ml TCA-precipitated *L. lactis* UKLc10 p*nso* supernatant in the presence and absence of trypsin. Again, as with the previous assay, there was a large increase in the abundance of specific bacteria, in this case *E. coli* (Dark Green) and *Enterobacter cloacae* (Orange) when comparing T0 and T24 samples. This result was seen in all samples that contained the faecal slurry, including the faecal slurry only control. In this experiment a new MicroMatrix cassette was used and aseptic conditions were observed through well loading and transfer to the MicroMatrix fermenter. However, the fact that this pattern was also seen in the Faecal slurry only control indicated that this was not due to the presence or action of nisin O (Figure 5.3.17A and B). The conditions of *L. lactis* UKLc10 p*nso* + faecal, *L. lactis* UKLc10 p*nso* + faecal + trypsin, *B. obeum* + faecal, *B. obeum* + faecal + trypsin, Pre-nso + faecal and Pre-nso + faecal + trypsin were all performed in triplicate in the same cassette. There was no observable difference in the percentage of *B. salanitronis* and *E. cloacae* reads present between each of the conditions (Figure 5.3.17A and B). Furthermore, the *B. obeum* A2-162 sample, as with the previous assay, did not align to the *B. obeum* A2-162 genome, instead members of the *Terrabacteria* group and *Proteobacteria*

were present in the sample. However, in this case the composition remained the same between T0 and T24 indicating that this potentially was *B. obeum* A2-162 but the reads had been mis-annotated to the database used by the Galaxy pipeline. In the case of the *L. lactis* UKLc10 p*nso* only well, there was an observed maintainance of the strain across the 24 hr experiment, with the percentage of *L. lactis* mapped reads within the samples dropping only 1% from 91% to 90% (Figure 5.3.17A and B). Due to this apparent absence of contamination in the single strain only wells and overgrowth of *E. coli* and *E. cloacae* occuring only in wells containing the faecal slurry, it lead to the possibility that these strains were outcompeting the other strains within the donor sample and therefore that nisin O did not have an observable effect because of this growth.

An antimicrobial assay was performed using the supernatants of each of the conditions both at the T0 and T24 timepoints (Figure 5.3.18A) but no zones of inhibition were seen when using *C. perfringens* as the indicator. Additionally the T24 supernatant samples were concentrated and used in a supernatant drop test assay; again no antimicrobial activity was observed except from the positive control, nisin A producing strain *L. lactis* FI876 (Figure 5.3.18B). This indicates that there was no expression of an antimicrobial either by the nisin O containing strains or by those within the faecal slurry to a level that would produce observable antimicrobial activity.

A)

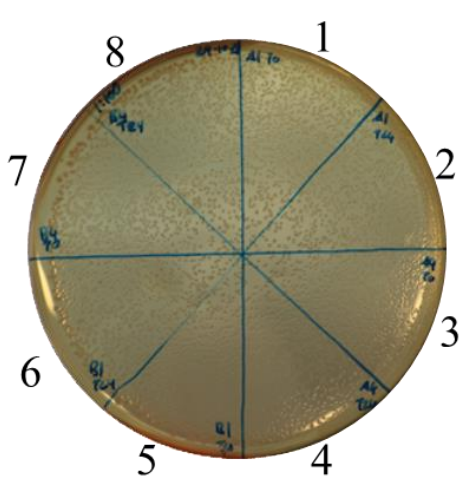
B)


Faecal
L. Lactis pnso
B. obeum
L. Lactis FI5876
L. Lactis pnso + Faecal 1
L. Lactis pnso + Faecal 2
L. Lactis pnso + Faecal 3
L. Lactis pnso + Faecal + trypsin 1
L. Lactis pnso + Faecal + trypsin 2
L. Lactis pnso + Faecal + trypsin 3
B. obeum + Faecal 1
B. obeum + Faecal 2
B. obeum + Faecal 3
B. obeum + Faecal + trypsin 1
B. obeum + Faecal + trypsin 2
B. obeum + Faecal + trypsin 3
Pre-nso + Faecal 1
Pre-nso + Faecal 2
Pre-nso + Faecal 3
Pre-nso + Faecal + trypsin 1
Pre-nso + Faecal + trypsin 2
Pre-nso + Faecal + trypsin 3

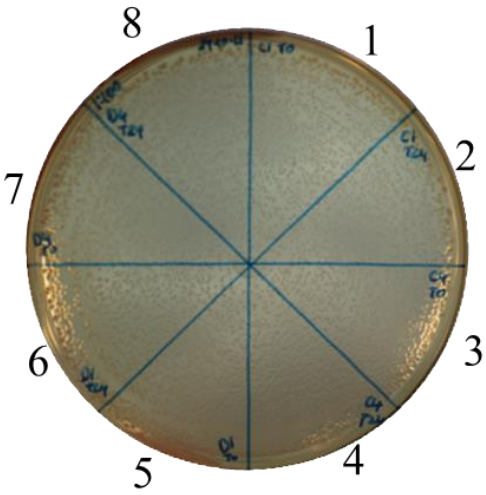
Faecalibacterium prausnitzii
[Eubacterium] rectale
Bacteroides vulgatus
[Eubacterium] eligens
Lactobacillus plantarum
Streptococcus salivarius
Roseburia hominis
Bifidobacterium longum
Bifidobacterium adolescentis
Mordavella sp. Marseille-P3756
Bacteroides dorei
Ruminococcus bicirculans
Bacteroides cellulosilyticus
Streptococcus thermophilus
Odoribacter splanchnicus
Parabacteroides sp. CT06
[Eubacterium] hallii
Bacteroides fragilis
Bacteroides thetaiotaomicron
Faecalitalea cylindroides
Parabacteroides distasonis
Anaerostipes hadrus
Clostridioides difficile
Enterococcus faecium
Blautia hansenii
Bacteroides caccae
Alistipes shahii
Burkholderia cenocepacia
Streptococcus parasanguinis
[Clostridium] bolteae
Streptococcus equinus
Bacteroides ovatus
Collinsella aerofaciens
Enterobacter cloacae
Bacteroides caecimuris
Streptococcus sp. A12
Blautia sp. YL58
Haemophilus parainfluenzae
Bifidobacterium catenulatum
Streptococcus infantarius
Alistipes finegoldii
Prevotella intermedia
Klebsiella oxytoca
Veillonella parvula
Bifidobacterium breve
Streptococcus sp. FDAARGOS_192
Intestinimonas butyriciproducens
Bacteroides salanitronis
Streptococcus australis
Ruminiclostridium sp. KB18
Acutalibacter muris
Campylobacter coli
Lachnoclostridium phocaeense
Bifidobacterium kashiwanohense
Clostridium sp. SY8519
Lachnoclostridium sp. YL32
Streptococcus sp. I-G2
Rothia mucilaginosa
Streptococcus sp. I-P16
Streptococcus acidominimus
Gordonibacter pamelaeae
Lactobacillus rhamnosus
Campylobacter jejuni
Bifidobacterium angulatum
Christensenella massiliensis
Flavonifractor plautii
Streptococcus mitis
Escherichia coli
Turicibacter sp. H121
Ruminococcus champanellensis
Cronobacter sakazakii
[Clostridium] saccharolyticum
Adlercreutzia equolifaciens
Streptococcus agalactiae
Lactococcus lactis
Streptococcus pneumoniae
Eggerthella lenta
Lactobacillus paracasei
Streptococcus suis
Streptococcus lutetiensis
Bifidobacterium bifidum
Streptococcus pasteurianus
Burkholderia cepacia
[Eubacterium] cellulosolvens
Bifidobacterium pseudocatenulatum
Streptococcus constellatus
Lactobacillus curvatus
Treponema denticola
Streptococcus gordonii
Bacteroides helcogenes
Prevotella denticola
Acidaminococcus intestini
Streptococcus sanguinis
Ornithobacterium rhinotracheale
Barnesiella viscericola
[Clostridium] innocuum
Streptococcus oralis
Herbinix luporum
Enterococcus cecorum
Clostridium chauvoei
Erysipelotrichaceae bacterium I46
Fusobacterium periodonticum
Pediococcus claussenii
Klebsiella michiganensis
Streptococcus sp. oral taxon 431
Prevotella enoeca
Streptococcus intermedius
Muribaculum intestinale
Streptococcus anginosus
Gordonibacter urolithinfaciens
Haemophilus influenzae
Klebsiella pneumoniae
Clostridium perfringens
Leuconostoc citreum
Klebsiella sp. M5al
Phoenicibacter massiliensis
Bacteroides zoogleoformans
Aggregatibacter aphrophilus
Oscillibacter valericigenes
Bacteroides heparinolyticus
Monoglobus pectinilyticus
Murdochiella vaginalis
Clostridium baratii
Prevotella scopos
Lactobacillus acidipiscis
Lactobacillus brevis
Lactobacillus paracollinoides
Prevotella melaninogenica
Burkholderia multivorans
Butyrivibrio fibrisolvens
Lactobacillus delbrueckii
Citrobacter freundii
Eggerthella sp. YY7918
Lactobacillus backii
Streptococcus cristatus
Eubacterium limosum
Prevotella ruminicola
Lactobacillus fermentum
Streptococcus gallolyticus
Enterobacter asburiae
Burkholderia contaminans
Filifactor alocis
Leifsonia xyli
Bifidobacterium pseudolongum
Butyrivibrio hungatei
Haemophilus pittmaniae
Prevotella fusca
Cronobacter universalis
Streptococcus pseudopneumoniae
Streptococcus sp. oral taxon 064
Myroides odoratimimus
Lactobacillus ruminis
Lachnoclostridium phytofermentans
Lactobacillus amylovorus
Streptococcus sp. NPS 308
Lactobacillus sakei
Mogibacterium diversum
Prevotella dentalis
Lachnospiraceae bacterium oral taxon 500
Lactobacillus pentosus
Streptococcus macedonicus
Paraburkholderia phytofirmans
Aggregatibacter segnis
Treponema sp. OMZ 838
Paeniclostridium sordellii
Lactobacillus coryniformis
Rothia dentocariosa
Bifidobacterium thermophilum
Pediococcus acidilactici
Ruminococcaceae bacterium CPB6
Anaerotignum propionicum
Treponema putidum
Treponema succinifaciens
Citrobacter freundii complex sp. CFNIH4
Salmonella enterica
Enterobacter hormaechei
Alkaliphilus oremlandii
Cronobacter malonaticus
Enterobacter cancerogenus
Klebsiella sp. LTGPAF-6F
Enterobacter cloacae complex bacterium
Enterobacter cloacae complex 'Hoffmann cluster IV'
Citrobacter werkmanii
Enterobacter bugandensis
Enterobacter cloacae complex sp. ECNIH7
Citrobacter youngae
Citrobacter freundii complex sp. CFNIH9
Lelliottia nimipressuralis
Serratia marcescens
Citrobacter freundii complex sp. CFNIH3
Cronobacter dublinensis
Klebsiella aerogenes
Citrobacter braakii
Klebsiella variicola
Enterobacter sp. HK169
Aeromonas hydrophila
Cronobacter turicensis
Leclercia sp. LSNIH3
Lelliottia sp. WB101
Citrobacter koseri
Citrobacter amalonaticus
Shigella dysenteriae
Enterobacter xiangfangensis
Citrobacter sp. FDAARGOS_156
Clostridium argentinense
Klebsiella quasipneumoniae
Raoultella ornithinolytica
Enterobacter cloacae complex 'Hoffmann cluster III'
Escherichia albertii
Kosakonia oryzae
Enterobacter sp. E20
Lelliottia amnigena
Cupriavidus gilardii
Leclercia adecarboxylata
Clostridium sp. DL-VIII
Desulfovibrio piger
Escherichia fergusonii
Citrobacter farmeri
Clostridium taeniosporum
Enterobacter kobei
Shigella flexneri
Cedecea neteri
Citrobacter sp. CRE-46
Enterobacter sp. Crenshaw
Citrobacter sp. CFNIH10
Enterobacter sp. FY-07
Klebsiella quasivariicola
Leclercia sp. LSNIH1
Shewanella sp. ANA-3
Enterobacter cloacae complex sp. FDA-CDC-AR_0164
Kosakonia sacchari
Pluralibacter gergoviae
Janthinobacterium sp. B9-8
Enterobacter sp. SA187
Clostridium acetobutylicum
Enterobacteriaceae bacterium ENNIH2
Clostridium estertheticum
[Enterobacter] lignolyticus
Cronobacter condimenti
Enterobacter sp. CRENT-193
Enterobacteriaceae bacterium strain FGI 57
Porphyromonas gingivalis
Providencia stuartii
Cronobacter muytjensii
Enterobacter sp. 638
Enterobacteriaceae bacterium ENNIH1
Riemerella anatipestifer
Salmonella bongori
Clostridium bornimense
Desulfovibrio vulgaris
Enterobacter sp. ODB01
Kosakonia cowanii
Cupriavidus pinatubonensis
Enterobacteriaceae bacterium ENNIH3
Xylella fastidiosa
Actinomyces meyeri
Actinomyces oris
Actinomyces pacaensis
Anaerococcus mediterraneensis
Bacillus cereus
Bacillus coagulans
Bacillus kruiwichiae
Bacillus thuringiensis
Buchnera aphidicola
Burkholderia ambifaria
Burkholderia anthina
Burkholderia lata
Burkholderia pyrrocinia
Burkholderia sp. LA-2-3-30-S1-D2
Burkholderia sp. MSMB0856
Burkholderia sp. NRF60-BP8
Burkholderia stabilis
Burkholderia ubonensis
Burkholderia vietnamiensis
Burkholderiales bacterium YL45
Butyrivibrio proteoclasticus
Campylobacter concisus
Candidatus Sodalis pierantonius
Candidatus Symbiobacter mobilis
Carnobacterium divergens
Chryseobacterium gallinarum
Citrobacter freundii complex sp. CFNIH2
Citrobacter rodentium
Citrobacter sp. 86
Citrobacter sp. 92
Cloacibacillus porcorum
Clostridium butyricum
Clostridium cellulovorans
Clostridium formicaceticum
Clostridium kluyveri
Clostridium pasteurianum
Cutibacterium acnes
Desulfitobacterium metallireducens
Desulfotomaculum gibsoniae
Desulfovibrio desulfuricans
Desulfovibrio fairfieldensis
Dickeya dadantii
Dickeya solani
Edwardsiella sp. EA181011
Edwardsiella tarda
Enterobacter cloacae complex sp. FDA-CDC-AR_0132
Enterobacter ludwigii
Enterobacter sp. R4-368
Enterobacteria phage fiAA91-ss
Enterobacterial phage mEp390
Enterococcus casseliflavus
Enterococcus durans
Enterococcus faecalis
Enterococcus hirae
Enterococcus sp. CR-Ec1
Enterococcus sp. FDAARGOS_375
Erysipelothrix rhusiopathiae
Escherichia marmotae
Escherichia phage TL-2011b
Fastidiosipila sanguinis
Fermentimonas caenicola
Finegoldia magna
Fusobacterium gonidiaformans
Fusobacterium hwasookii
Fusobacterium nucleatum
Fusobacterium sp. oral taxon 203
Gemella morbillorum
Gibbsiella quercinecans
Haemophilus haemolyticus
Haemophilus sp. oral taxon 036
Jeotgalibaca sp. PTS2502
Klebsiella phage phiKO2
Kosakonia radicincitans
Lactobacillus alimentarius
Lactobacillus amylophilus
Lactobacillus helveticus
Lactobacillus hokkaidonensis
Lactobacillus kefiranofaciens
Lactobacillus paraplantarum
Lactobacillus sanfranciscensis
Lactobacillus sp. D1501
Lactococcus garvieae
Lactococcus phage 98201
Lactococcus phage BK5-T
Lactococcus phage P335 sensu lato
Lactococcus phage bIL286
Lactococcus phage bIL310
Lactococcus piscium
Lactococcus raffinolactis
Lactococcus virus bIL67
Lactococcus virus c2
Lawsonia intracellularis
Lelliottia jeotgali
Leuconostoc gelidum
Leuconostoc mesenteroides
Libanicoccus massiliensis
Limnobaculum parvum
Mageeibacillus indolicus
Martelella mediterranea
Megasphaera elsdenii
Moraxella osloensis
Neisseria elongata
Novibacillus thermophilus
Olsenella sp. oral taxon 807
Paenibacillus glucanolyticus
Paenibacillus terrae
Pantoea sp. PSNIH2
Pantoea vagans
Parascardovia denticolens
Pectobacterium atrosepticum
Pectobacterium carotovorum
Pectobacterium parmentieri
Pectobacterium wasabiae
Pediococcus pentosaceus
Peptoniphilus sp. ING2-D1G
Peptostreptococcaceae bacterium oral taxon 929
Plautia stali symbiont
Plesiomonas shigelloides
Prevotella sp. oral taxon 299
Proteus mirabilis
Pseudomonas aeruginosa
Pseudomonas putida
Pseudomonas sp. JY-Q
Rahnella sp. WMR42
Raoultella planticola
Ruminococcus albus
Serratia fonticola
Serratia plymuthica
Serratia sp. FGI94
Shewanella putrefaciens
Shigella boydii
Shigella sonnei
Staphylococcus aureus
Staphylococcus epidermidis
Staphylococcus saprophyticus
Streptococcus equi
Streptococcus mutans
Streptococcus parauberis
Streptococcus pyogenes
Streptococcus sobrinus
Syntrophobotulus glycolicus
Syntrophomonas wolfei
Tannerella forsythia
Tannerella sp. oral taxon HOT-286
Veillonella rodentium
Weissella cibaria
Xenorhabdus doucetiae
Yersinia enterocolitica
Yersinia pestis
Yersinia virus L413C
[Clostridium] cellulolyticum
[Clostridium] sphenoides
[Eubacterium] sulci
[Haemophilus] ducreyi

Figure 5.3.17. A) Bacterial composition of the T0 sample from the MicroMatrix assay using a new donor. B) Bacterial composition of the T24 sample from the MicroMatrix assay using a new donor.

A)

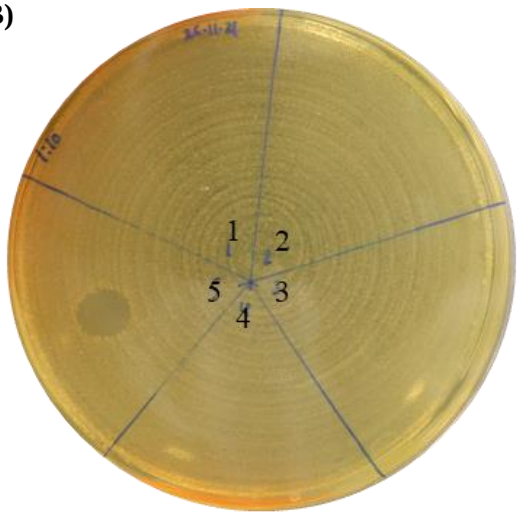


1. Faecal slurry T0
2. Faecal slurry T24
3. Faecal slurry + Trypsin T0
4. Faecal slurry + Trypsin T24
5. Faecal slurry + *L. lactis* pnso T0
6. Faecal slurry + *L. lactis* pnso T24
7. Faecal slurry + *L. lactis* pnso +Trypsin T0
8. Faecal slurry + *L. lactis* pnso +Trypsin T24



1. *L. lactis* pnso T0
2. *L. lactis* pnso T24
3. *L. lactis* pnso + Trypsin T0
4. *L. lactis* pnso + Trypsin T24
5. Faecal slurry + *B. obeum* T0
6. Faecal slurry + *B. obeum* T24
7. Faecal slurry + *B. obeum* +Trypsin T0
8. Faecal slurry + *B. obeum* +Trypsin T24

B)



1. Faecal slurry
2. Faecal slurry + trypsin
3. Faecal slurry + *L. lactis* pnso
4. Faecal slurry + *L. lactis* pnso + trypsin
5. *L. lactis* FI5876

1. *L. lactis* pnso
2. *L. lactis* pnso + trypsin
3. Faecal slurry + *B. obeum*
4. Faecal slurry + *B. obeum* + trypsin
5. BHI + C

Figure 5.3.18. A) Supernatant drop test assay of the supernatants from the MicroMatrix fermentation assay at T0 and T24. B) Supernatant drop test assay of the concentrated supernatants from the MicroMatrix fermentation assay at T24. *C. perfringens* was used as the indicator strain in both assays.

5.4 Discussion

The nisin O cluster in *B. obeum* A2-162 is unusual as it does not contain a protease which is required in other nisin variant clusters to cleave the leader peptide and therefore activate the antimicrobial characteristic of the molecule (Lubelski, Rink *et al*. 2008, Hatziioanou, Gherghisan-Filip *et al*. 2017). Analysis of the *B. obeum* A2-162 genome using the *nisP* or *lanP* sequences from *L. lactis* and *B. producta* SCSK (Kim, Becattini *et al*. 2019), respectively, indicated a number of candidates with the *B. obeum* A2-162 genome that had homology to the amino acid sequence of these two reference proteins (P66, P570, P62 and P49). It was decided to look at the entire *B. obeum* A2-162 genome, rather than upstream and downstream of the cluster as there are examples of proteases capable of cleaving the leader peptide of some lantibiotics which are not found within the biosynthetic gene cluster, such as

the AprE, WprA and Vpr proteins which can cleave the leader peptide of subtilin (Corvey, Stein *et al*. 2003, Hatziioanou, Gherghisan-Filip *et al*. 2017). Furthermore, when looking at the conserved domains and predicted structure of *B. producta* SCSK LanP, candidates were also identified which shared these domains and structures (P140). The candidate proteases P66, P570, P49, P62, P140 and LanP were selected as they either had the greatest percentage homology to NisP, LanP or shared conserved domains with LanP. All the candidate protease genes selected were successfully inserted into inducible plasmids and transformed into *E. coli* BL21(DE3) and the signal peptide-like proteases *p62*, *p49*, *p140* and *lanP* were cloned into a pUK200_P32 plasmid which enabled constitutive expression of the candidate proteases in a co-expression system in *L. lactis* UKLc10 p*nso*. However, no antimicrobial activity was observed when the nisin O gene cluster was co-expressed with the candidate proteases. Interestingly, activity was observed only when trypsin was present in the *L. lactis* UKLc10 p*nso* strain, yet not when also expressing the candidate proteases. This may be because the heterologous expression system has an increased energy expenditure to maintain and express genes from two plasmids rather than only one, therefore the concentration of nisin O produced may be lower leading to a lack of observable antimicrobial activity. Although transcription itself is not considered a major fitness cost it is thought that the energy expenditure mostly arises from translation, with highly expressed foreign genes being a major factor in the use of host bacterium tRNA (Plotkin and Kudla 2011, San Millan and MacLean 2017).

Additionally, a nisin O cleavage assay was performed for all candidate proteases using the crude CWEs in an incubation with TCA-precipitated supernatant from induced *L. lactis* UKLc10 p*nso*. The pre-Nso peptide was detected using the NsoA1-3 leader antibody, with loss of the 6kDa band indicating cleavage. In all cases there was no evidence of leader peptide cleavage when incubated with crude CWEs of *E. coli* expressing candidate proteases tested, or the crude CWEs from co-culture humanisation strains (Becker, Kunath *et al*. 2011). This indicates that the candidate proteases identified and selected through BLAST searching are not able to cleave the leader peptides of NsoA1-3. However, it is important to note that only

the crude CWEs and CFEs were used and that purification of the His-tagged proteins would have been more desirable, but due to the problems producing the candidate proteases in the CFE for nickel column chromatography this line of enquiry was stopped. Additionally, it shows that there are no extracellular proteases expressed by the humanisation strains that can cleave the NsoA1-3 leader peptides. However, it must be stated that not all candidate proteases from *B. obeum* were tested; the *B. producta* LanP protease is a very different protein to the NisP and this cluster may use a protease with no homology to these enzymes. Conversely, the crude CWEs of *B. obeum* A2-162 was used in the incubation with pre-NsoA1-3 in the TCA precipitated supernatant and no cleavage was observed, implying that there are no cell surface proteases expressed under the growth conditions used that can cleave NsoA1-2 under the conditions tested in this assay. However, cleavage of nisin O was observed when trypsin was used. This confirms the results of Hatziioanou *et al* (2017), where antimicrobial activity of nisin O was only observed when trypsin was present. This differs slightly from the work performed on ruminococcin A, which also does not contain a protease, which originally stated that trypsin performed leader peptide cleavage but further work indicated its role as an inducing molecule (Dabard, Bridonneau *et al*. 2001, Gomez, Ladire *et al*. 2002, Ongey, Giessmann *et al*. 2018).

A MicroMatrix Fermentation assay was performed to investigated whether nisin O could be induced and cleaved into its functional form when in an environment which mimics the human gut with multiple species present. The data from this assay showed that, even though the nisin O genes were present in the DNA extracted from the samples, there was no alteration in the bacterial composition specifically due to the production of this lantibiotic. This was observed as the faecal slurry only negative control changed composition in highly similar way as the samples which contained a nisin O producing organism, indicating that these alterations in microbial composition were due to specific bacteria (*E. coli* in Donor 1 and *E. coli* and *E. cloacae* in Donor 2) being a contaminant or out-competing others. Additionally, the supernatants of these samples showed no antimicrobial activity against *C. perfringens*,

implying that they either are not expressing nisin O or not at high enough levels to inhibit the growth of the indicator strain. Interestingly, the addition of trypsin had no effect on the microbial profile or expression of antimicrobial activity of nisin O. This implies that trypsin does not act as an inducing molecule at the concentrations tested for the expression of nisin O. In a recent study, nisin A was used in a range of concentrations (0-500 µM) in a MicroMatrix fermentation assay to assess its activity against *C. difficile* (O'Reilly, O'Connor *et al*. 2022). The authors found that no viable *C. difficile* was detected between 50 and 500 µM and that the diversity of the microbiota decreased as the concentration of nisin increased, with an increase in the relative abundance of Gram-negative bacteria at higher nisin concentrations (O'Reilly, O'Connor *et al*. 2022). Additionally, another nisin variant, nisin P, was used in a MicroMatrix assay along with nisin A and nisin H. In this study, an analysis of the total live bacteria was performed and showed that nisin A had the greatest increase on cell mortality whereas nisin P and nisin H had minimal decreases in the total proportion of dead cells (Garcia-Gutierrez, O'Connor *et al*. 2020). These experiments not only indicate that nisin variants can have an effect on the human gut microbiome composition, but also can be used for more targeted approaches due to their specificity to Gram-positive bacteria. This indicates that further work can be performed using nisin O and the strains in which it is expressed in order to understand the role that this antimicrobial has in the human gut microbiome.

Trypsin has a higher concentration within the intestinal tract compared to the gastric tract, 143.0 µg/ml to 19.3 µg/ml, respectively (Metheny, Stewart *et al*. 1997). Trypsin cleaves between either a lysine or arginine residue and the following amino acid in the sequence, with the rate of cleavage slower when the lysine or arginine are followed either by a cysteine or an acidic amino acid (Simpson 2006). Furthermore, trypsin cleavage does not occur when proline follows either arginine or lysine (Simpson 2006). NsoA1-3 contain six trypsin cleavage sites within its pre-peptide, two of which are at the end of the leader peptide, however there are two sites within the core peptide (Gherghisan-Filip, Saalbach *et al*. 2018). However, in a recent study investigating *sapT* in an *E. coli* expression system it was shown that the lanthionine

rings of the lantibiotic are able to provide protection of the core peptide from proteolytic degradation (Sarksian, Hegemann *et al*. 2022). This might explain what is occurring with nisin O in an environment with high concentrations of trypsin. Additionally, a study has recently shown the trypsin degrading characteristics of commensals in the large intestinal, which would in turn reduce the concentration of trypsin in the gut and therefore reducing the risk of the core peptide being cleaved due to its further protection through its post-translational modifications while allowing the leader peptide cleavage to occur (Li, Watanabe *et al*. 2022).

This work has identified candidate lantibiotic proteases from the *B. obeum* A2-162 genome and following insertion to inducible and constitutive expression plasmids, showed no leader peptide cleavage in pre-NsoA1-3 incubation assays or co-expression assays. Additionally, no antimicrobial activity was observed in the MicroMatrix fermentation assays and any changes in bacterial composition were likely due to overgrowth or out-competing other strains within the system.

Chapter 6.

Investigating the Interactions of Nisin O Regulatory Systems with Predicted Nisin O Promoters

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6.1 Introduction

In order to increase expression of antimicrobial peptides produced by bacteria it is important to understand how these biosynthetic gene clusters are regulated. There are four operons which comprise the nisin A biosynthetic gene cluster, *nisABTCIPRK*, *nisI*, *nisRK* and *nisFEG* with each operon being preceded by its own promoter (de Ruyter, Kuipers *et al*. 1996). However, interestingly the *nisI* and *nisRK* promoters act constitutively and therefore provide a constant provision of immunity and regulatory capabilities within *L. lactis* (de Ruyter, Kuipers *et al*. 1996). The regulation of the operons of *nisABTCIPRK* and *nisFEG* are controlled by the TCS NisRK. TCSs are highly prevalent in bacteria and are used to sense changes in the extracellular environment through ligand binding with a sensor histidine kinase which auto-phosphorylates which in turn phosphorylates a response regulator allowing it to bind to target DNA and hence alter gene expression levels (Tierney and Rather 2019). In the case of nisin A regulation, NisK is a histidine kinase which recognises the N-terminal A and B lanthionine rings of nisin A and auto-phosphorylates (Kuipers, Beerthuyzen *et al*. 1995, Ge, Teng *et al*. 2016). This is followed by phosphorylation of NisR, the response regulator, which then activates the transcription of the genes within the nisin A biosynthetic gene cluster by binding to the specific region of the operon (de Ruyter, Kuipers *et al*. 1996). In this regard nisin A acts as its own inducer, increasing its own expression levels (Kuipers, Beerthuyzen *et al*. 1995). As a result of this method of regulation within the cluster, the Nisin-controlled gene expression system was first developed by de Ruyter *et al* (1996) which uses the NisRK system and the nisin A promoter (P*nisA*) cloned upstream of a gene of interest to provide an inducible expression of the gene upon the addition of nisin, the subsequent protein can then accumulate inside the cell or be transported into the extracellular space dependent on the signal sequence (Mierau, Leij *et al*. 2005, Zhou, Li *et al*. 2006).

Although nisin A is regarded as the standard used for the regulation of lantibiotics other antimicrobials have been identified that have alternative regulatory pathways. For example, mutacin I, found in *Streptococcus mutans*, is an interesting example of a type I lantibiotic

which has multiple pathways of regulation. In the biosynthetic cluster this lantibiotic contains *mutR*, which was predicted to be a positive regulator for the cluster due to similarities with the mutacin II and III operons, type II and I lanthipeptides respectively (Qi, Chen *et al*. 1999, Qi, Chen *et al*. 1999, Qi, Chen *et al*. 2000). Furthermore, mutacin I has undergone detailed study via construction of mutagenesis libraries to identify both positive and negative regulators of the gene cluster (Tsang, Merritt *et al*. 2005, Nguyen, Zhang *et al*. 2009). As a result of these studies 25 genes were identified that were involved in a positive regulation of mutacin I. These genes encoded TCS, stress response, energy metabolism and other cellular processes (Tsang, Merritt *et al*. 2005). Whereas 17 genes were identified as negative regulators involved in surface binding, cell wall metabolism, sugar transport, peptide hydrolysis and amino acid/nucleotide synthesis (Nguyen, Zhang *et al*. 2009). Additionally, LuxS and CiaH have been reported to be required for mutacin I production, although these were not identified as part of the mutagenesis library described previously. Merritt *et al* (2005), described that mutacin I expression increased at a high cell density and that deletion of *luxS*, involved in interspecies signalling, abolished both *mutR* expression and the expression of the entire operon itself. It was also showed that constitutively expressing 'inducible repressor of virulence', *irvA*, supressed expression of *mutA*. Therefore, it was concluded that LuxS is a negative regulator of *irvA*, which in turn is a repressor of *mutR* (Merritt, Kreth *et al*. 2005). An additional study by Qi *et al*., demonstrated that disruption of histidine kinase *ciaH*, part of the TCS CiaRH, abolished mutacin I production, however this effect was not seen when response regulator *ciaR* was inactivated. It is important to note that inactivation of *ciaH* also altered biofilm formation and reduced acid tolerance, indicating that this TCS is involved in multiple regulatory pathways (Qi, Merritt *et al*. 2004). Additionally, quorum sensing by *Streptococcus pneumoniae* can also regulate the regulation of lantibiotic expression (Hoover, Perez *et al*. 2015). In this study the authors observed that the Phr peptide quorum sensing system induced the expression of the lantibiotic in the presence of galactose at high cell density, indicating that expression is influenced by cell density nutritional signals (Hoover, Perez *et al*. 2015).

Furthermore, a recent study by Meng et al (2021), showed that the presence of acetate activated the kinase activity of PlnB, SppK and HpK3 which in turn increased the yields of their respective bacteriocins of plantaricin EF, sakacin A and rhamnosin B (Meng, Zhao et al. 2021). This result was further verified through antimicrobial activity analysis against *Staphylococcus aureus* where the cultures treated with acetate increased their antimicrobial activity by 298% (plantaricin EF), 198% (sakacin A) and 289% (rhamnosin B) (Meng, Zhao et al. 2021). This indicates that environmental factors can influence the expression of bacteriocins.

The nisin O cluster in *B. obeum* A2-162 contains two TCSs unlike the single TCS found in the nisin A biosynthetic gene cluster (Hatziioanou, Gherghisan-Filip *et al*. 2017). Additionally, there were four *in silico* predicted promoters within the nisin O biosynthetic gene cluster, preceding *nsoF*, *nsoA1*, *nsoR2* and *nsoB* (Gherghisan-Filip 2016). These promoters were analysed for interaction using a *pepI* gene reporter assay, however, the two TCSs from the nisin O cluster were inserted into a plasmid under the control of the P*nisA* promoter and required the presence of NisRK into order to express the NsoR1K1 and NsoR2K2 systems. However, activity was observed from an interaction between NisRK and P*nsoA* thus any result from this assay may have been due to interactions from this system and the predicted promoters from the nisin O cluster rather than the nisin O TCSs (Gherghisan-Filip 2016). Interestingly, the biosynthetic gene cluster identified by Kim *et al,* (2019) in *B. producta* SCSK also contained two TCSs, however the function of these systems was not characterised. In unpublished work performed by Romano *et al*, the RNA from the nisin O heterologous expression system in *L. lactis* UKLc10 induced with nisin A was extracted and used in an Oxford Nanopore long read sequencing assay. The results of this sequencing were mapped back to the cluster sequence to determine which genes were being expressed under this induction condition (Figure 6.1.1). It was clear that there were high levels of expression of the *nsoA1-4* and *nsoR2K2* genes with a slight increase in expression of *nsoBTC*. There was limited expression for *nsoFEG*, *nsoI* and *nsoR1K1*. However most interesting was the level of

expression of a previously unidentified gene between *nsoK2* and *nsoB*, named *nso1.14*, which had greatly increased levels of expression and the function of which has yet to be determined (Romano *et al*., Unpublished) (Figure 6.1.1).

Figure 6.1.1. Mapped reads of cDNA from nisin A induced *L. lactis* UKLc10 p*nso* created from long strand RNA using Nanopore sequencing. Courtesy of Romano *et al*, (unpublished) (2022).

Understanding the regulation of antimicrobial peptides is important as this provides valuable information into how these naturally derived products not only are regulated within their original niches but also how over-expression can be achieved to help to further study the effect of these bacteriocins. In this work the relationship between the predicted promoters and the nisin O regulatory systems was investigated and RT-PCR was used to ascertain whether co-transcription across promoters is occurring within the nisin O cluster.

The hypothesis of this study is that either NsoR1K1 or NsoR2K2, or both, interact with the predicted promoters when induced with trypsinated pre-NsoA1-4. Furthermore, co-transcription will occur within the nisin O biosynthetic gene cluster.

I would like to acknowledge Dr Stefano Romano and his co-authors for allowing me to use their unpublished data in this chapter.

6.2 Methods

6.2.1 Cloning and Transformation of Nisin O regulatory systems

Methods used for the splice overlap PCR, restriction, ligation and transformation into *E. coli* and *L. lactis* strains and reverse transcription PCR were performed as described in methods 2.9, 2.10, 2.11, 2.12 and 3.2.4. Reaction conditions and primers used (See table 2.5 for primer details) for each of the PCRs performed are described below in table 6.2.1. Below are examples of two step PCR (used in splice overlay PCRs), colony PCRs and RT-PCRs

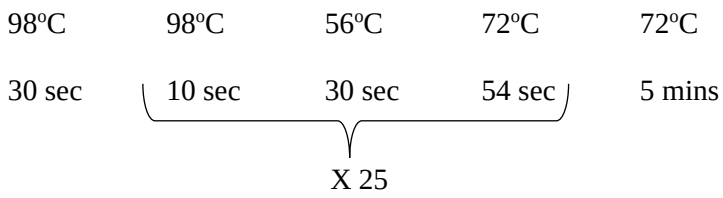
6.2.1.1 Two step PCR:

98°C	98°C	43°C	72°C	98°C	59°C	72°C	72°C
30 sec	10 sec	30 sec	1 min 7 sec	10 sec	30 sec	1 min 7 sec	5 mins
	X 5			X 20			

	Forward Primer	Reverse Primer	Product Size (bp)	Annealing Temperature (°C) (First 5 cycles)	Annealing Temperature (°C) (Next 20 cycles)	Extension Time (Sec)
Cloning of *nsoR2K2* into pUK200_P32	R2K2_SpliceP32_F	p181	2257	43	59	67
	P32_PstIF2	P32_SpliceR2K2_R	250	54	64	7
	P32_PstIF2	p181	2518	53	63	73
Cloning of *nsoR1K1* into pUK200_P32	R1K1_SpliceP32_F	p181	2164	55	60	65
	P32_PstIF2	P32_SpliceR1K1_R	250	54	64	7
	P32_PstIF2	p181	2288	54	63	67
Cloning *nso1.14* into pUK200_P32	Nso1_14_spliceP32_F	Nso1_14_BamHI_R	252	57	66	8
	P32_BglII_F	P32_SpliceNso1_14_R	191	37	65	6
	P32_BglII_F	Nso1_14_BamHI_R	431	40	64	14
	pUK_F	p181	552	58	58	17

Table 6.2.1. PCR conditions and primers used for cloning of the *nso* regulatory systems and *nso1.14* into expression plasmids used in this work.

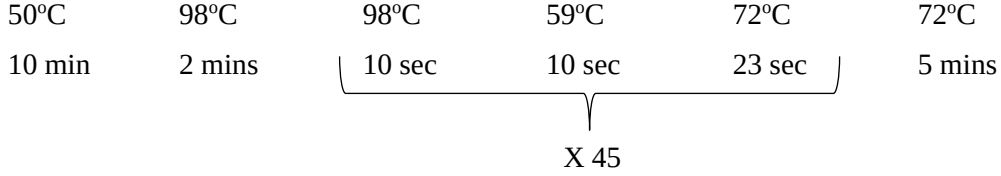
6.2.1.2 Colony PCR:



	Forward Primer	Reverse Primer	Product Size (bp)	Annealing Temperature (ºC) (25 cycles)	Extension Time (Sec)
***pPnsoA1-4_pepI* promoter confirmation**	pIL253_F	pIL253_R	1815	56	54
***pPnsoR2K2_pepI* promoter confirmation**	pIL253_F	pIL253_R	1498	56	45
***pPnsoBTC_pepI* promoter confirmation**	pIL253_F	pIL253_R	1645	56	50
***nsoR1* system confirmation**	P32_F	P32_R1_R	585	59	18
***nsoR2* system confirmation**	P32_F	P32_R2_R	739	59	22
***nsoK1* system confirmation**	P32_K1_F	p181	706	59	22
***nsoK2* system confirmation**	P32_K2_	p181	755	59	22

Table 6.2.2. PCR conditions and primers used for confirmation of the predicted promoters and *nso* regulatory systems in the *L. lactis* pepI reporter strains used in this work.

6.2.1.3 RT-PCR:



	Forward Primer	Reverse Primer	Product Size (bp)	Annealing Temperature (ºC) (45 cycles)	Extension Time (Sec)
***nsoA1-4* expression**	nsoA1_F	nsoA4_R	752	59	23
***nsoR2* expression**	nsoR2_F	nsoR2_R	666	59	23

***nsoF* expression**	nsoF_F	nsoF_R	705	59	23
***nsoB* expression**	nsoB_F	nsoB_R	3060	59	90
***nsoR1-nsoA1-4* co-expression**	nsoR1_F	nsoA4_R	3269	59	98
***nsoA1-4-nsoK2* co-expression**	nsoA1_F	nsoK2_R	2903	59	87
***nsoR2-nsoB* co-expression**	nsoR2_F	nsoB_R_Short	3084	59	93

Table 6.2.3. RT-PCR conditions and primers used for investigating the expression of nso genes in the *L. lactis* heterologous expression system in this work.

6.2.1 PepI Reporter Assay

L. lactis strains containing the regulatory systems and *pepI* with *nso* promoter plasmids were grown overnight using appropriate conditions. A 2% inoculum was used to inoculate 10 ml GM17 and grown until the OD_{600} reached 0.5. The cultures were normalised to 10 OD units and harvested by centrifugation at 10,000 x g for 15 mins at 4ºC. The supernatant was discarded, and the pellets washed with 5 ml 50 mM Tris HCl (pH 7.4) and centrifuged. The pellets were resuspended in 250 µl 50 mM Tris HCl (pH 7.4) and transferred to a screw cap tube with 100 µl acid washed beads. The samples were bead beaten using a FastPrep FR120 cell disrupter for 4 x for 30 secs at speed 6 with 5-10 mins on ice between beating. A final centrifugation at 14,000 x g for 30 mins at 4ºC was performed followed by removal of the crude cell free extract. Dilutions of 10x and 100x were made in 50 mM Tris HCl (pH 7.4). Using a flat-bottomed clear plate (Greiner) 50 µl of undiluted or diluted cell free samples with 0.7 mM L-proline p-nitroanilide trifluoroacetate salt was loaded and PepI activity measured using a Benchmark Plus plate reader (Biorad) at A_{405} at 32ºC for 15 mins with readings every 30s. The protein quantification was performed using the Bradfords reagent (Biorad). Data were analysed *in silico* and the rate of activity was calculated in nmol/mg/min.

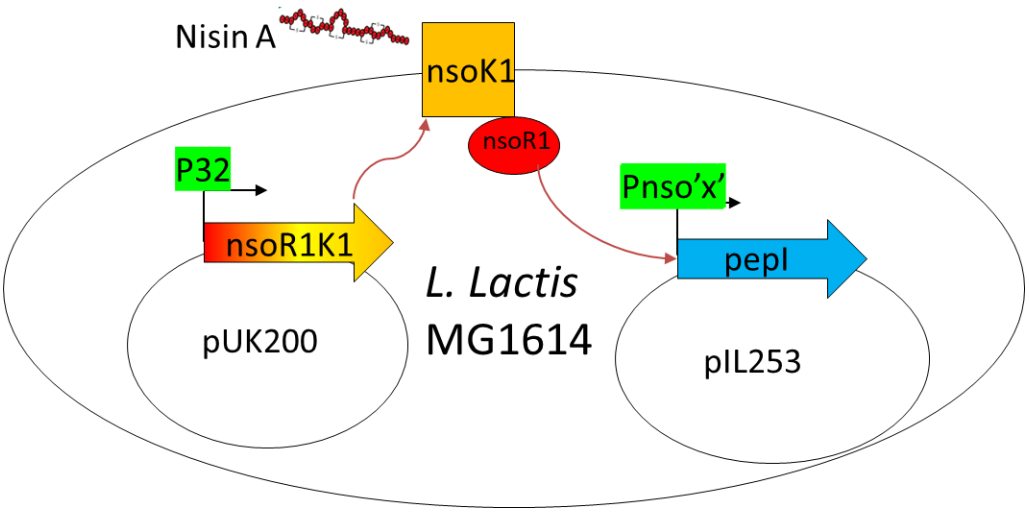


Figure 6.2.1. A schematic diagram of the process by which the *pepI* reporter assay functions. Where Pnso'x' represents one of the four predicted promoters within the nisin O cluster (Gherghisan-Filip 2016).

6.2.2 AlphaFold2 Analysis of Nso1.14

To analyse the predicted tertiary of Nso1.14 an AlphaFold2_mmseqs2 analysis was performed (Jumper, Evans *et al*. 2021). The amino acid sequence of Nso1.14 was used at the input and programme was performed using: https://colab.research.google.com/github/sokrypton/ColabFold/blob/main/AlphaFold2.ipynb

6.2.3 *In silico* Analysis of Predicted Promoter Positions in Nisin O-like clusters

To predict the promoter positions within the nisin O-like clusters identified in chapter 3 Bprom:(http://www.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb) was used across the whole of the clusters and the positions of the predicted promoters within each cluster were compared to those previous described by Gherghisan-Filip (2016).

6.3 Results

6.3.1 Co-expression of *nsoRK* Systems in *L. lactis*

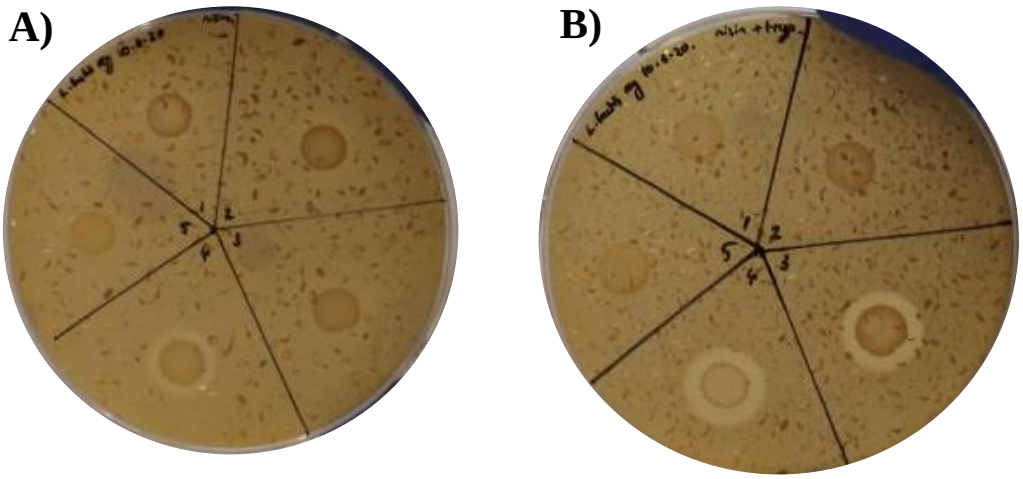
6.3.1.1 Transformation of Nisin O Regulatory Systems into *L. lactis* UKLc10 p*nso*

Transformation of pUK200_*nsoR2K2* in *L. lactis* UKLc10 p*nso* was successful which provided a range of the receptor-kinase systems found in *B. obeum* being co-expressed in *L. lactis,* as *nsoR1A* (R1 with 5' extension to match the start site of similar regulators identified by BLASTP, Mayer unpublished) and *nsoR1AK1* had previously been transformed. However, unlike *nsoR2K2*, these two modified *nsoR1K1* elements had been cloned into pTG262 rather than pUK200. When transforming into *L. lactis* UKLc10 p*nso* both the plasmid containing the regulatory system and the plasmid containing the nisin O cluster were checked to ensure that they were maintained in the bacterium. All candidates maintained both the p*nso* plasmid and had successful transformation of pUK200_*nsoR2K2*.

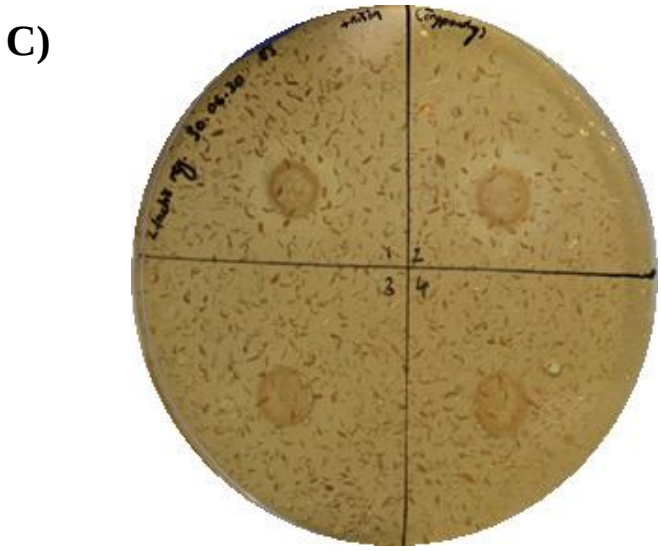
6.3.1.2 Co-expression of *nsoR1A*, *nsoR1AK1* and *nsoR2K2* in *L. lactis* UKLc10 p*nso*

To investigate whether nisin O expression could be increased by overexpression of the regulatory systems *nsoR2K2* and the 5' extended version of *nsoR1K1*, *nsoR1AK1*, both regulatory systems were transformed into the *L. lactis* UKLc10 heterologous expression system. The transformations were successful, and an overlay assay was performed (Figure 6.3.1). Although the positive control did not work, a clear zone of inhibition was seen with the addition of the *nsoR2K2* systems in the presence of trypsin (Figure 6.3.1A and B). Interestingly, the *nsoR2K2* expressing strain with no nisin A induction during growth only showed inhibition when trypsin was present in both the overlay and base agar. However, when there was nisin A induction during growth of the overnight culture, inhibition was seen on both plates, even when trypsin was absent in the overlay agar (Figure 6.3.1A and B). This indicates that nisin A induction is required for antimicrobial activity from this strain. However, in this assay the positive control did not produce a zone of inhibition so no comparison could

be made regarding the size of the zone of inhibition. Furthermore, a faint zone of inhibition was observed when *nsoR1AK1* was expressed however no zone of inhibition was observed for the *nsoR2K2* expressing strain in this repeat of the assay. In this assay all overnight cultures were induced with nisin A prior to inoculation on the agar. The positive control of the nisin O heterologous expression system with no additional regulatory system also produced a zone of inhibition. The zone of inhibition for NsoR1AK1 expressing strains was not any greater than that of the positive control indicating that this TCS does not increase the level of nisin O expression more than previously observed (Figure 6.3.1C).



1. *L. lactis* UKLc10 p*nso*
2. *L. lactis* UKLc10 p*nso* pTG262_P32_*R1AK1*
3. *L. lactis* UKLc10 p*nso* pUK200_*R2K2* (without nisin)
4. *L. lactis* UKLc10 p*nso* pUK200_*R2K2* (with nisin)
5. *L. lactis* MG1614 pTG262

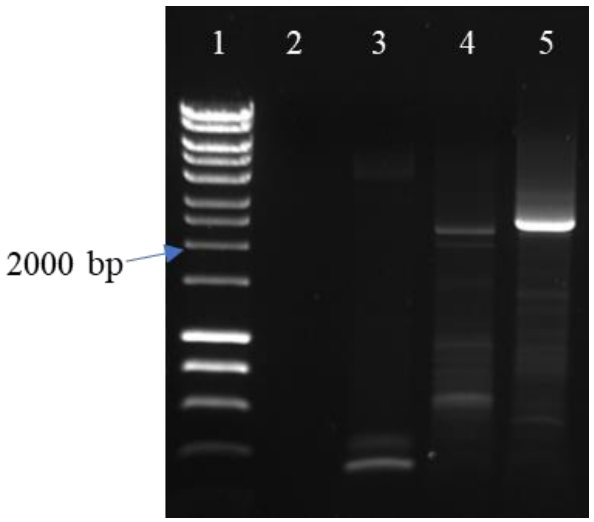


1. *L. lactis* UKLc10 p*nso*
2. *L. lactis* UKLc10 p*nso* pTG262_P32_*nsoR1AK1*
3. *L. lactis* UKLc10 p*nso* pUK200_*nsoR2K2*
4. *L. lactis* MG1614 pIL253

Figure 6.3.1 A) An overlay assay to assess the effect of NsoR1AK1 and NsoR2K2 on nisin O expression. The base agar contained $NaHCO_3$ and 10 µg/ml trypsin, with no trypsin in the overlay agar. B) *L. lactis* strain spots were in the same order as plate A, however the base plate contained $NaHCO_3$ and 10 µg/ml trypsin, with 10 µg/ml trypsin in the overlay agar. C) An *L. lactis* overlay assay with the base agar containing $NaHCO_3$ and 10 µg/ml trypsin, with 10 µg/ml trypsin in the overlay agar. *C. perfringens* was used as the indicator strain for all assays.

6.3.2 Creation of pUK200_P32_*nsoR1K1* and pUK200_P32_*nsoR2K2*

Both pUK200_P32_*nsoR1K1* and pUK200_P32_*nsoR2K2* plasmids were successfully created using splice overlap PCR, followed by restriction and ligation into pUK200 and transformed into *E. coli* MC1022 (Figure 6.3.2).

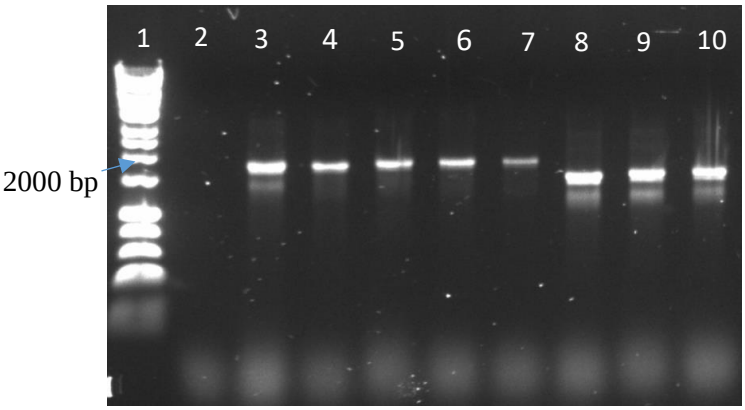


1. 1 kB ladder
2. Negative
3. pUK200_P32 control
4. pUK200_P32_*nsoR1K1* (2288 bp)
5. pUK200_P32_*nsoR2K2* (2518 bp)

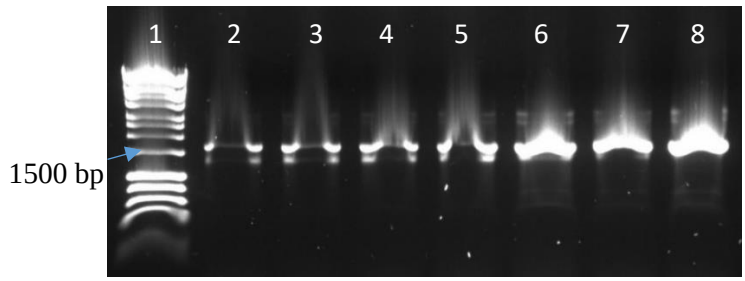
Figure 6.3.2. Gel image of pUK200_P32_*nsoR1K1* and pUK200_P32_*nsoR2K2* colony PCR from *E. coli* MC1022.

6.3.3 Transformation of pP*nsoA1-4_pepI*, pP*nsoR2K2_pepI* and pP*nsoBTC_pepI* into *L. lactis* MG1614

In order to remove the effect of the nisin A *nisRK* system found on the chromosome of *L. lactis* UKLc10, which was thought to have interfered with the *pepI* reporter assay shown in previous work (Gherghisan-Filip 2016), transformation of previously made plasmids containing the promoters of nisin O upstream of *pepI* were co-expressed with the *nsoRK* genes under the control of a constitutive promoter instead of the nisin A promoter P*nisA*. The plasmid which contains the predicted promoter preceding the *pepI* gene was pIL253, which will be further referenced in this chapter as the 'p' prior to the predicted promoter. The pP*nsoA1-4_pepI*, pP*nsoR2K2_pepI* (Figure 6.3.3A) and pP*nsoBTC_pepI* plasmids (Figure 6.3.3B) were purified and transformed successfully into *L. lactis* MG1614, which does not have a *nisRK* system on its chromosome. The pP*nsoFEG_pepI* had previously been transformed into *L. lactis* MG1614 and was used in this work (Gherghisan-Filip 2016).



1. 1 kB ladder
2. Negative
3. pP*nsoA1-4_pepI* 1 (1815 bp)
4. pP*nsoA1-4_pepI* 2
5. pP*nsoA1-4_pepI* 3
6. pP*nsoA1-4_pepI* 4
7. pP*nsoA1-4_pepI* 5
8. pP*nsoR2K2_pepI* 1 (1498 bp)
9. pP*nsoR2K2_pepI* 2
10. pP*nsoR2K2_pepI* 3



1. 1 kB ladder
2. pP*nsoBTC_pepI* 1 (1645 bp)
3. pP*nsoBTC_pepI* 2
4. pP*nsoBTC_pepI* 3
5. pP*nsoBTC_pepI* 4
6. pP*nsoBTC_pepI* 5
7. pP*nsoBTC_pepI* 6
8. pP*nsoBTC_pepI* 7

Figure 6.3.3. A) Gel image of pP*nsoA1-4_pepI* and pP*nsoR2K2_pepI* colony PCR in *L. lactis* MG1614. B) Gel image of pP*nsoBTC_pepI* colony PCR in *L. lactis* MG1614.

6.3.4 Transformation of pUK200_P32_*nsoR1K1* and pUK200_P32_*nsoR2K2* into *L. lactis* MG1614 Strains

Electrocompetent cells were made for the *L. lactis* MG1614 nisin O promoter strains and pUK200_P32_*nsoR1K1*, pUK200_P32_*nsoR2K2* were successfully transformed into *L. lactis* MG1614 pP*nsoA1-4_pepI*, *L. lactis* MG1614 pP*nsoR2K2_pepI* and *L. lactis* MG1614 pP*nsoBTC_pepI*. In each case the *nsoR* and *nsoK* genes from each regulatory system were amplified and their sequences confirmed via sequencing.

6.3.5 Identification of promoters from Nisin O-like clusters

The pan-genome analysis described in chapter 3 identified six nisin O-like clusters within *B. obeum* AM27-32LB, *B. obeum* TM09-13AC, *Blautia* AM47-4, *D. longicatena* 1001136B, *D. formicigenerans* AM37-5 and *Ruminococcus* AM50-15BH. *In silico* analysis of the clusters identified that all of these clusters contained two TCSs with the nucleotide percentage identity across all nucleotide sequences of *nsoR1*, *nsoK1*, *nsoR2* and *nsoK2* being 99.4%, 99.5%, 99.5% and 99.5%, respectively. Analysis of the regions upstream of all the genes within the nisin O cluster identified the same promoter positions predicted in previous work using Bprom (Softberry) (Gherghisan-Filip 2016). The predicted promoters from this analysis contain the same -10 and -35 boxes upstream of the genes (Table 6.3.1), additionally the scores predicted, based on the weighted matrices of the prediction model, were exactly the same between all seven nisin O-like clusters, including from *B. obeum* A2-162.

Promoter (nucleotide upstream of start site)	-10 box			-35 box		
	Promoter position upstream of start site		Score	Promoter position upstream of start site		Score
P*nsoFEG* (57)	72	TTTTAATCT	55	90	TTTACA	47
P*nsoA1-4* (28)	44	TTTTATATT	66	54	TTGATA	58
P*nsoR2K2* (22)	37	TGTTATTAT	79	56	GTGATT	20
P*nsoBTC* (33)	48	TTTGATAAT	50	69	TTCAAA	40

Table 6.3.1. Predicted promoter regions of the nisin O-like biosynthetic gene clusters. The scores for the -10 and -35 boxes represents the weighted matrices of different conserved promoter motifs combined with linear discriminant analysis (Yona, Alm *et al*. 2018).

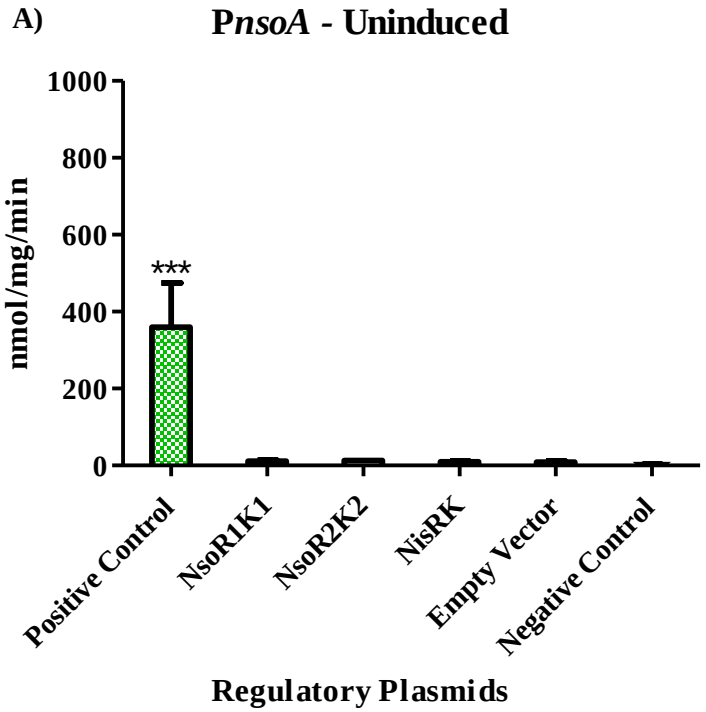
6.3.6 PepI Reporter Assays

To investigate the relationship between the regulatory systems found in the nisin O clusters and which of the four predicted promoters identified by Bprom (Gherghisan-Filip 2016) they interact with, a *pepI* reporter assay was performed. Using *L. lactis* UKLc10 pUK200_P*nisA_pepI* in a strain with the *nisRK* system on the chromosome as a positive control for all assays and *L. lactis* MIG1614 pUK200_P32 negative control an assessment could be made of which regulatory systems interact with individual promoters based on the rate of activity of PepI in the presence of the substrate. All experiments had three technical and two biological replicates. The work performed by Romano *et al*, indicated that the start site of *nsoR1K1* was indeed correct and therefore the *nsoR1AK1* strains were not used in this analysis.

6.3.6.1 Interactions Between P*nsoA1-4* and Regulatory Systems from the Nisin O and Nisin A Biosynthetic Gene Clusters

When uninduced no PepI activity was observed with any regulatory system, with background activity from the regulatory system containing strains ranging from 9.2 to 11.3 nmol/mg/min (Figure 6.3.4A). The positive control resulted in significantly higher PepI activity (P-value < 0.001) when compared to any other regulatory system. When nisin A was used to induce the culture prior to bead beating there was an increase in both the level of activity seen in the positive control, from 359.9 to 883.4 nmol/mg/min, and *L. lactis* UKLc10 strain which contained the *nisRK* system on its chromosome from the nisin A cluster to 448.0 nmol/mg/min. The increase in PepI activity observed when the *nisRK* was expressed was significantly different to the other regulatory systems, the empty vector control and the negative control with a P-value of < 0.05 (Figure 6.3.4B). A significant difference (P-value <

0.05) was seen between the positive control, where the *nisRK* system can interact with the P*nisA* promoter from the nisin A cluster, and the *nisRK* system interacting with the P*nsoA* promoter from the nisin O cluster. However, neither of the two regulatory systems from the nisin O cluster showed a significant increase in PepI activity, indicating that the level of expression was very low and therefore there was no interaction between NsoR1K1 or NsoR2K2 and the P*nsoA1-4* promoter. Additionally, when the trypsinated TCA-precipitated supernatant of a nisin A induced *L. lactis* UKLc10 p*nso* culture, further referred to as trypsinated pre-NsoA1-4, was used to induce the cultures there was minimal activity from PepI observed, with only the *nisRK* expressing strain increasing its level of PepI activity to 44.5 nmol/mg/min, which was not significant compared to any other strain. Again, the positive control resulted in an increased level of activity of 539.6 nmol/mg/ml which was significant when compared to all the other strains tested (Figure 6.3.4C).



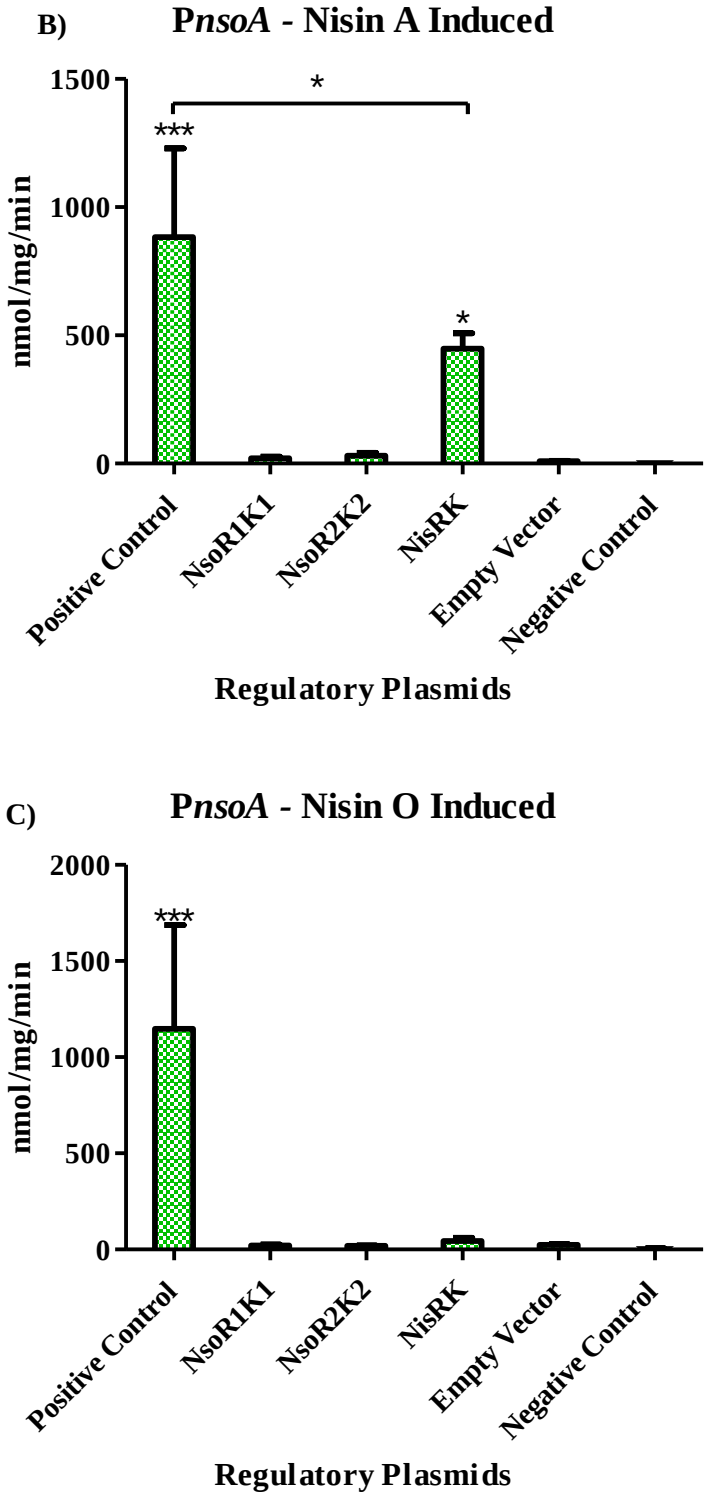
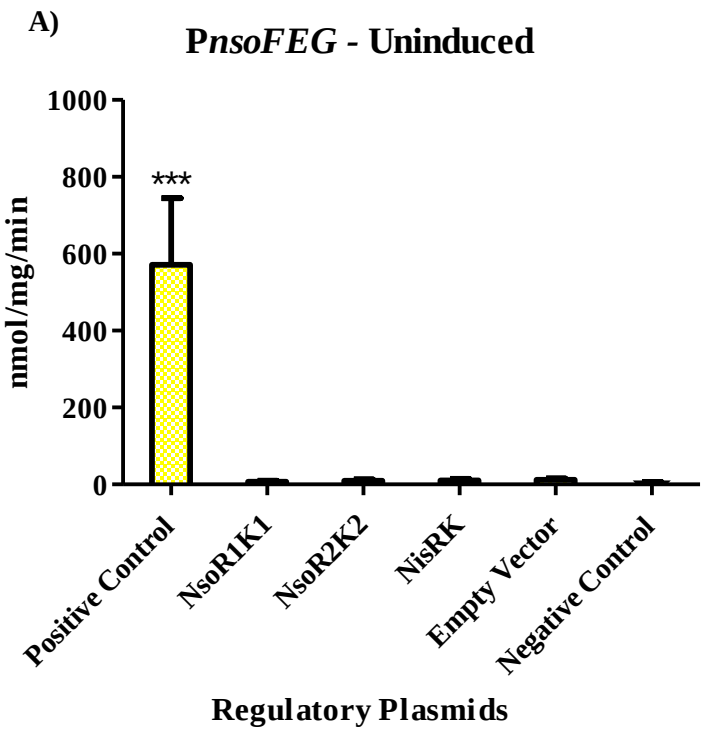


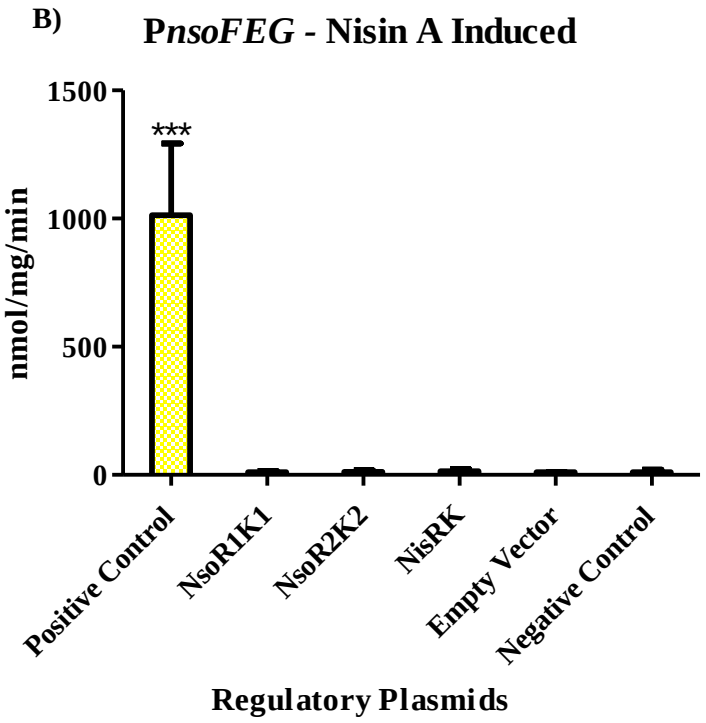
Figure 6.3.4. A) The rates of activity of PepI expressed by p*PnsoA1-4_pepI* containing strains which also contained the regulatory systems from the nisin O cluster (NsoR1K1 and NsoR2K2) and the nisin A cluster (NisRK) when uninduced. B) Induced with nisin A. C) Induced with trypsinated pre-NsoA1-4. The empty vector control in all assays contained

pUK200_P32 with no regulatory system. The positive control was a *L. lactis* UKLc10 strain with the *nisRK* on the chromosome with the promoter for nisin A preceding the *pepI* gene. The negative control was *L. lactis* MG1614 pIL253. Significance was determined by a One-way ANOVA followed by a Tukey Multiple Comparison Test. A P-value of < 0.05, < 0.01 and < 0.001 are indicated by '*', '**' and '***', respectively. The asterisks directly above each column indicate the significance compared to all other conditions unless indicated otherwise. Error bars were made using the standard deviation of the mean. Each induction condition was repeated in triplicate.

6.3.6.2 Interactions Between P*nsoFEG* and Regulatory Systems from the Nisin O and Nisin A Biosynthetic Gene Clusters

When uninduced no PepI activity was seen for any strain except for the positive control, which showed an activity level of 571.4 nmol.mg/min. The PepI activity of the three regulatory systems tested ranged from 7.3 to 10.4 nmol/mg/min which was not significantly different to either the empty vector or the negative control (Figure 6.3.5A). This result was also seen when using nisin A to induce the cultures, however the positive control had an increased level of activity at 1012.9 nmol/mg/min, yet no activity seen for any strain containing a regulatory system, ranging from 11.2 to 13.8 nmol/mg/min (Figure 6.3.5B). When nisin O was used to induce the cultures there was no significant increase in the level of PepI activity from any of the regulatory systems. However, there was a slight PepI activity increase seen in the *nisRK* expressing strain, 34.9 nmol/mg/min, although this was not significantly different to any other strain except the positive control. Again, the level of activity observed for the *nsoR1K1* and *nsoR2K2* expressing strains was similar to the uninduced and nisin A induced levels of activity seen, at 9.12 and 11.7 nmol/mg/min, respectively (Figure 6.3.5C). Interestingly, there was a slight increase in the empty vector (pUK200_P32) PepI activity at 17.5 nmol/mg/min when compared to uninduced and nisin A induced, and a decrease in the activity of the positive control at 618.2 nmol/mg/min when compared to the nisin A induced assay condition (Figure 6.3.5C).


A)
P*nsoFEG* - Uninduced
1000
800
600
400
200
0
nmol/mg/min
***
Positive Control
NsoR1K1
NsoR2K2
NisRK
Empty Vector
Negative Control
Regulatory Plasmids


B)
P*nsoFEG* - Nisin A Induced
1500
1000
500
0
nmol/mg/min
***
Positive Control
NsoR1K1
NsoR2K2
NisRK
Empty Vector
Negative Control
Regulatory Plasmids

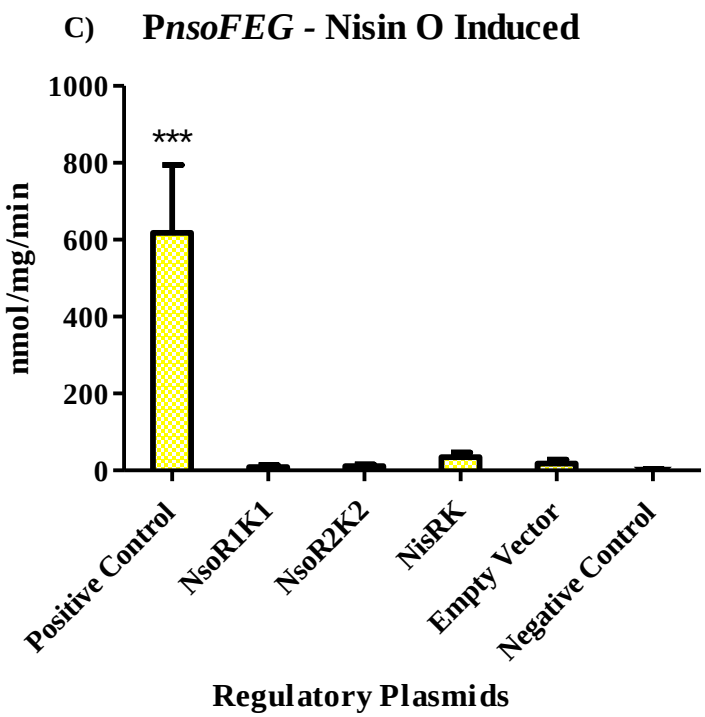
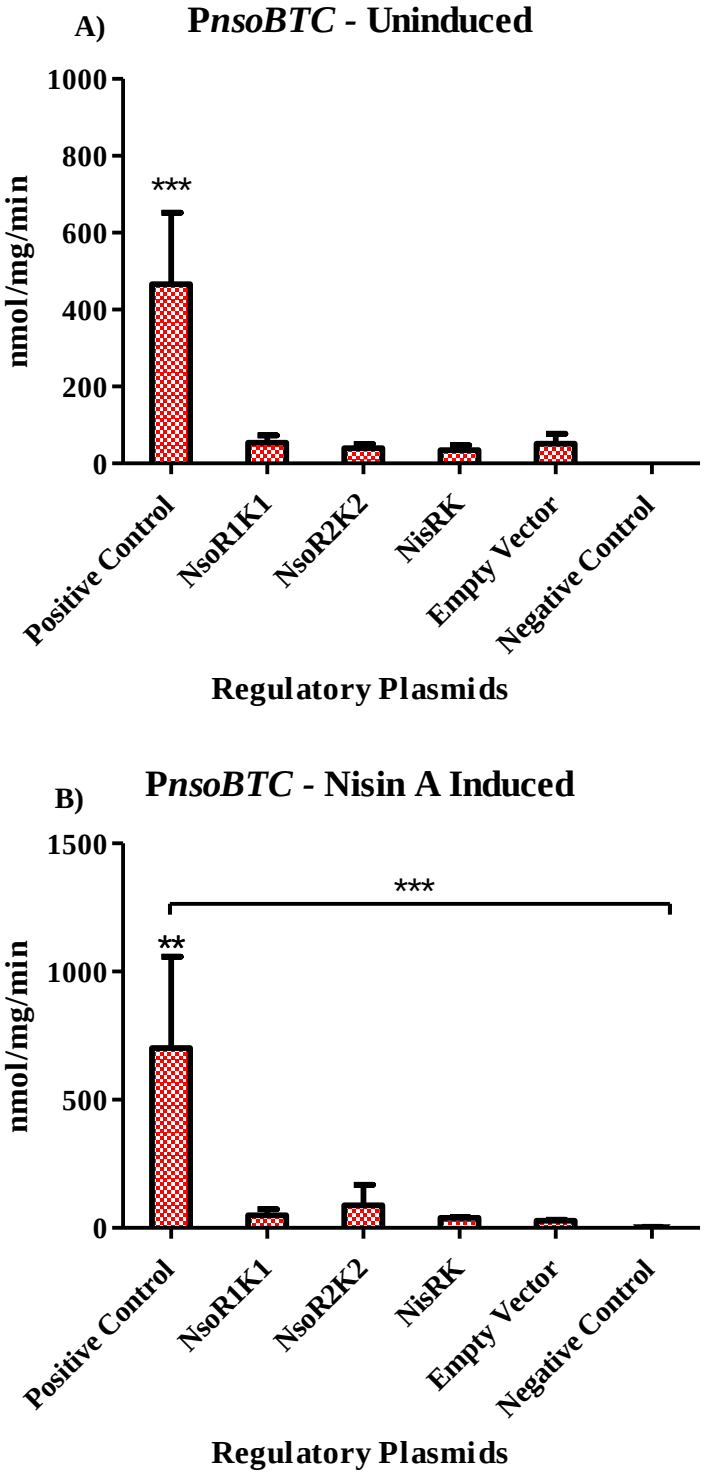


Figure 6.3.5. A) The rates of activity of PepI expressed by pP*nsoFEG_pepI* containing strains which also contained the regulatory systems from the nisin O cluster (NsoR1K1 and NsoR2K2) and the nisin A cluster (NisRK) when uninduced. B) Induced with nisin A. C) Induced with trypsinated pre-NsoA1-4. The empty vector control in all assays contained pUK200_P32 with no regulatory system. The positive control was a *L. lactis* UKLc10 strain with the *nisRK* on the chromosome with the promoter for nisin A preceding the *pepI* gene. The negative control was *L. lactis* MG1614 pIL253. Significance was determined by a One-way ANOVA followed by a Tukey Multiple Comparison Test. A P-value of < 0.05, < 0.01 and < 0.001 are indicated by '*', '**' and '***', respectively. The asterisks directly above each column indicate the significance compared to all other conditions unless indicated otherwise. Error bars were made using the standard deviation of the mean. Each induction condition was repeated in triplicate.

6.3.6.3 Interactions Between P*nsoBTC* and Regulatory Systems from the Nisin O and Nisin A Biosynthetic Gene Clusters

The interactions between the *nsoBTC* promoter and the regulatory systems in the nisin O and nisin A cluster showed a greater level of PepI activity when uninduced when compared to that of the *nsoA1-4* and *nsoFEG* promoters. Although not significantly different from either the negative or empty vector controls the NsoR1K1, NsoR2K2 and NisRK systems had an increased level of activity than those assays previously described, with PepI activity levels of 54.0, 39.6 and 35.0 nmol/mg/min, respectively. Also of note is the activity levels seen in the empty vector control were also increased at 51.6 nmol/mg/min. As previously seen the positive control was significantly different when compared to all other conditions (P-value < 0.001) at 466.1 nmol/m/min (Figure 6.3.6A). The assay using nisin A to induce resulted in a similar pattern of results seen for the uninduced conditions, however most interestingly, the P-value of the positive control when compared to the *nsoR1K1*, *nsoR2K2*, *nisRK* and empty vector containing strains was reduced to < 0.01. The activity rates of these strains were 48.9, 87.63, 40.0 and 28.1 nmol/mg/min for *nsoR1K1*, *nsoR2K2*, *nisRK* and empty vector containing strains, respectively (Figure 6.3.6B). However, it must be noted that the standard deviation for the positive control was larger than previously recorded using other induction conditions, at 355.3 nmol/mg/min. Nisin O induction again showed higher rates of PepI activity for *nsoR1K1*, *nsoR2K2*, *nisRK* and empty vector containing strains at 87.8, 49.2, 74.1 and 26.4 nmol/mg/min, respectively, however there was no significant difference between in the PepI activity rate and the negative control (Figure 6.3.6C).


A)
P*nsoBTC* - Uninduced
1000
800
600
400
200
0
nmol/mg/min
***
Positive Control
NsoR1K1
NsoR2K2
NisRK
Empty Vector
Negative Control
Regulatory Plasmids
B)
P*nsoBTC* - Nisin A Induced
1500
1000
500
0
nmol/mg/min
***
**
Positive Control
NsoR1K1
NsoR2K2
NisRK
Empty Vector
Negative Control
Regulatory Plasmids

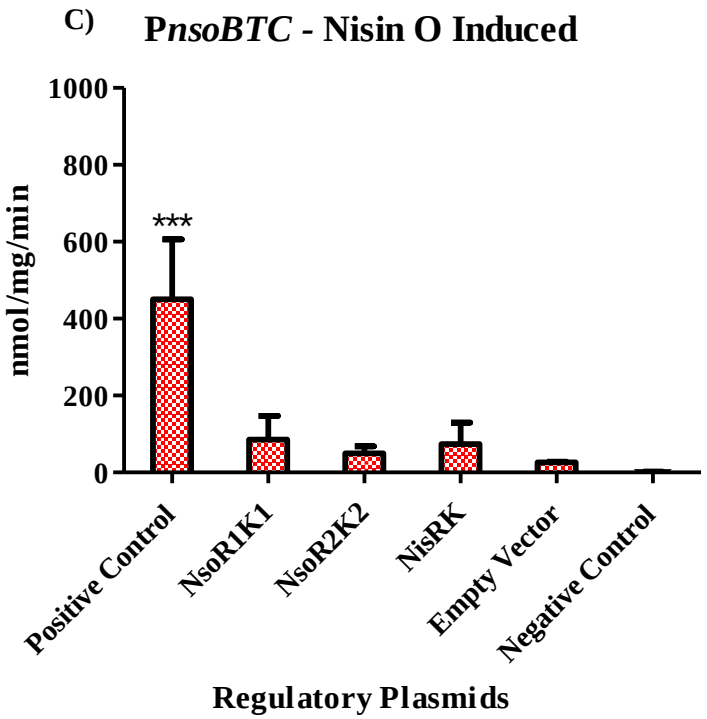
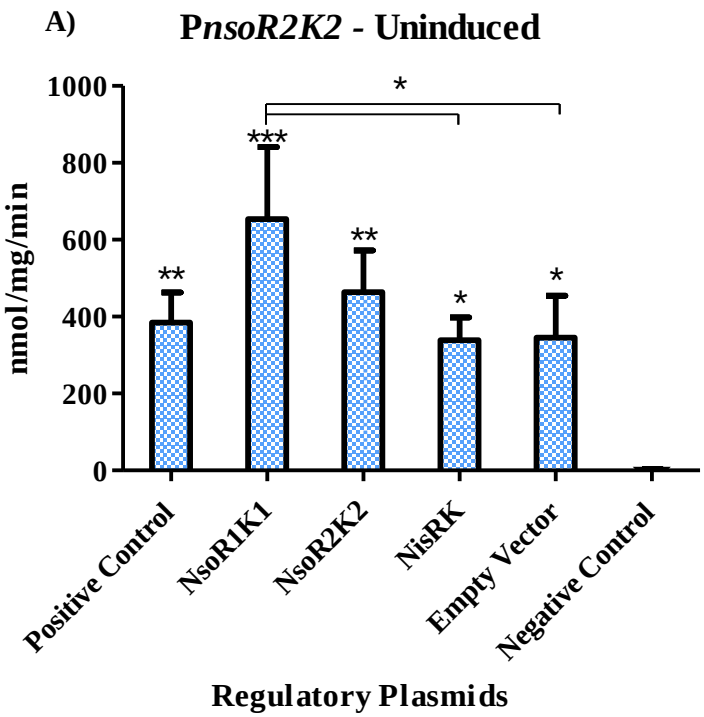
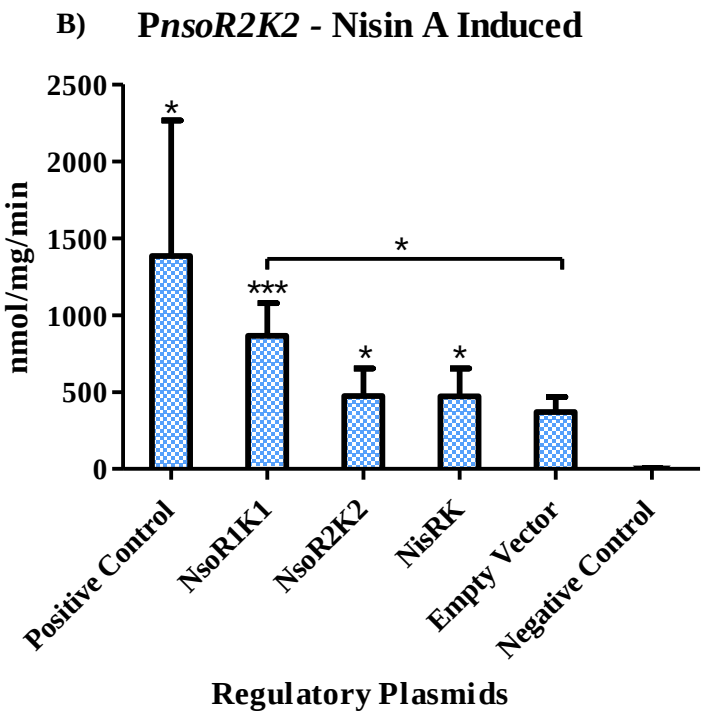


Figure 6.3.6. A) The rates of activity of PepI expressed by pP*nsoBTC_pepI* containing strains which also contained the regulatory systems from the nisin O cluster (NsoR1K1 and NsoR2K2) and the nisin A cluster (NisRK) when uninduced. B) Induced with nisin A. C) Induced with trypsinated pre-NsoA1-4. The empty vector control in all assays contained pUK200_P32 with no regulatory system. The positive control was a *L. lactis* UKLc10 strain with the *nisRK* on the chromosome with the promoter for nisin A preceding the *pepI* gene. The negative control was *L. lactis* MG1614 pIL253. Significance was determined by a One-way ANOVA followed by a Tukey Multiple Comparison Test. A P-value of < 0.05, < 0.01 and < 0.001 are indicated by '*', '**' and '***', respectively. The asterisks directly above each column indicate the significance compared to all other conditions unless indicated otherwise. Error bars were made using the standard deviation of the mean. Each induction condition was repeated in triplicate.

6.3.6.4 Interactions Between P*nsoR2K2* and Regulatory Systems from the Nisin O and Nisin A Biosynthetic Gene Clusters

When using the *nsoR2K2* promoter in the *pepI* reporter assay a much greater level of activity was seen for all strains which expressed a regulatory system. The rate of PepI activity was greatest in the strain expressing *nsoR1K1* (653.4 nmol/mg/min) when uninduced which was significantly higher than the negative control (P-value < 0.001) and the NisRK and empty vector strains (P-value < 0.05). Both the strains expressing *nsoR2K2* and *nisRK* had significantly greater activity rates when compared to the negative control at P-value < 0.01 and < 0.05, respectively. Most interesting were the values observed for the empty vector control, which did not express any regulatory system, which produced a PepI activity rate of 338.8 nmol/mg/min, which was significantly different compared to the negative control (P value < 0.05) (Figure 6.3.7A). This pattern of results was seen when the cultures were induced with nisin A and nisin O. However, when these nisin variants were used to induce the cultures the level of PepI activity was increased for the positive control at 1384.9 and 1436.6 nmol/mg/min for nisin A and nisin O induction, respectively (Figure 6.3.7B and C). The significance of these results was lower than expected due to the variability in activity seen in during the repeats of the assays which lead to larger error bars. Furthermore, the strain expressing *nsoR1K1* had an increased level of activity when induced with both nisin A and nisin O (864.6 and 1067.5 nmol/mg/min, respectively) when compared to the strains expressing *nsoR2K2* and *nisRK*, however this difference was only significantly greater when induced with nisin O (Figure 6.3.7B and C). Again, the empty vector controls showed a high level of PepI activity under both induction conditions producing 369.1 and 645.5 nmol/mg/min during nisin A and nisin O induction, respectively (Figure 6.3.7B and C).


A)
PnsoR2K2 - Uninduced
1000
800
600
400
200
0
nmol/mg/min
*
***
**
**
*
*
Positive Control
NsoR1K1
NsoR2K2
NisRK
Empty Vector
Negative Control
Regulatory Plasmids


B)
PnsoR2K2 - Nisin A Induced
2500
2000
1500
1000
500
0
nmol/mg/min
*
*
***
*
*
Positive Control
NsoR1K1
NsoR2K2
NisRK
Empty Vector
Negative Control
Regulatory Plasmids

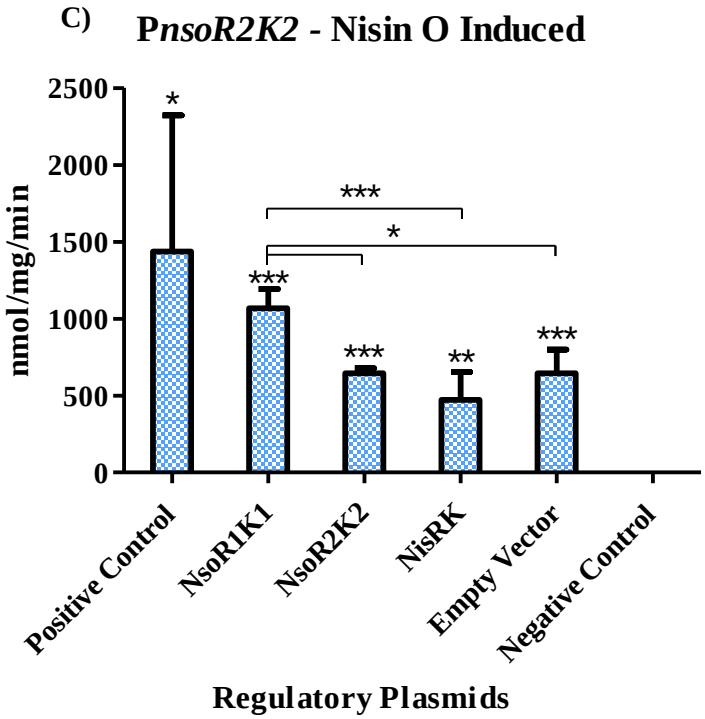
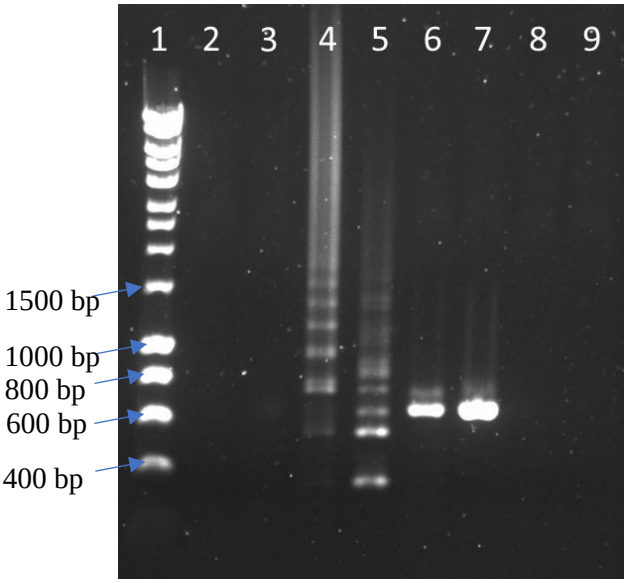


Figure 6.3.7. A) The rates of activity of PepI expressed by pP*nsoR2K2_pepI* containing strains which also contained the regulatory systems from the nisin O cluster (NsoR1K1 and NsoR2K2) and the nisin A cluster (NisRK) when uninduced. B) Induced with nisin A. C) Induced with trypsinated pre-NsoA1-4. The empty vector control in all assays contained pUK200_P32 with no regulatory system. The positive control was a *L. lactis* UKLc10 strain with the *nisRK* on the chromosome with the promoter for nisin A preceding the *pepI* gene. The negative control was *L. lactis* MG1614 Pil253. Significance was determined by a One-way ANOVA followed by a Tukey Multiple Comparison Test. A P-value of < 0.05, < 0.01 and < 0.001 are indicated by '*', '**' and '***', respectively. The asterisks directly above each column indicate the significance compared to the negative control unless indicated otherwise. Error bars were made using the standard deviation of the mean. Each induction condition was repeated in triplicate.

6.3.7 Analysis of RNA extracted from *L. lactis* UKLc10 p*nso*

To assess whether any co-transcription of operons occurs within the nisin O cluster the RNA from a nisin A induced *L. lactis* UKLc10 p*nso* culture was performed and used in a RT-PCR.

Both the *nsoA1-4* and *nsoR2K2* genes were clearly amplified, indicating that these genes were being expressed (Figure 6.3.8). Interestingly, multiple bands were observed for the *nsoA1-4* PCR products, which were expected to be 171 bp, 364 bp, 558 bp and 751 bp for *nsoA1*, *nsoA1-2*, *nsoA1-3* and *nsoA1-4*, respectively, ranging from approximately 300 bp to 1500 bp in size. This could potentially be due to non-specific binding of the primers. However, no bands were clearly present for the *nsoF* and *nsoB* products, suggesting that no or very minimal expression of these genes was occurring using nisin A induction of the heterologous expression system. This assay was performed using RNA extracted as part of work from this project and by Dr Stefano Romano (SR) as part of a separate project, however, in both experiments the bands observed after RT-PCR and gel electrophoresis were the same, with only slight differences product sizes for the *nsoA1-4* product.



1. Ladder
2. *nsoF* – SR (705bp)
3. *nsoF*
4. *nsoA1* – *nsoA4* – SR (752bp)
5. *nsoA1* – *nsoA4*
6. *nsoR2* – SR (666bp)
7. *nsoR2*
8. *nsoB* – SR (3060bp)
9. *nsoB*

Figure 6.3.8. A gel electrophoresis of RT-PCR products derived from RNA extracted from nisin A induced *L. lactis* UKLc10 p*nso*. RNA extracted by Dr Stefano Romano is termed SR.

To assess whether co-transcription of the predicted operons within the nisin O cluster was occurring, the RNA extracted from the *L. lactis* heterologous expression system was used in a RT-PCR, however in this reaction primers were used in various combinations using a forward primer from the end of one operon and a reverse primer from the beginning of the

next (Figure 6.3.9). Therefore, if a product of the correct size is present this would indicate that the RNA transcript spans genes found in both operons. The RT-PCR was also repeated for first genes within each operon and analysed via gel electrophoresis. The repeats of the RT-PCR, seen in lanes 2, 3, 4 and 5 of figure 6.3.10, show the same product sizes produced by the previous reaction observed in figure 6.3.8. However, a band is present for the *nsoB* product at approximately 1500 bp which is considerably smaller than the product size expected of 3060 bp. No bands were visible for the products spanning the *nsoR1-nsoA4* containing operons and the *nsoA1-nsoK2* containing operons. Most notably, a product of the correct size was observed for the *nsoR2-nsoB* containing operons, which indicates that a transcript is expressed within the *L. lactis* heterologous expression system when induced with nisin A (Figure 6.3.10).

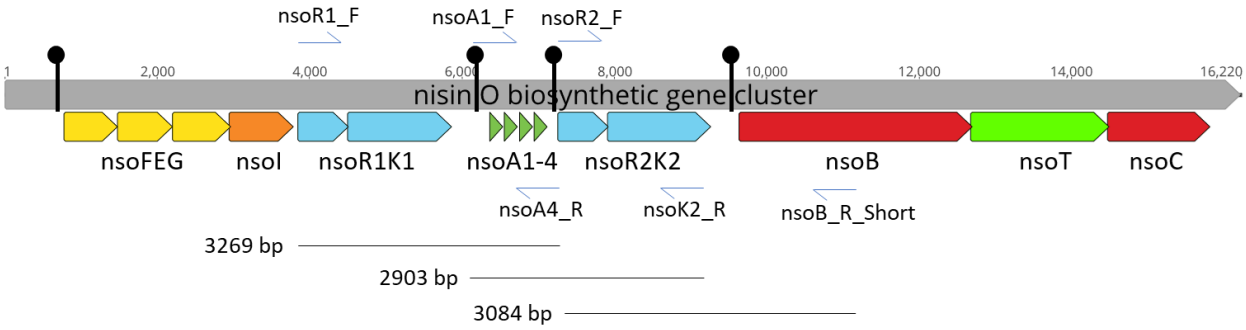


Figure 6.3.9. A schematic diagram of the nisin O cluster and the primers used to assess whether co-transcription was taking place through the use of RT-PCR. Black circles indicate position of promoters. Primers used are indicated by arrows above and below the cluster (labelled). The three predicted co-expressed transcripts are indicated by the lines at the bottom of the figure with the product size given.

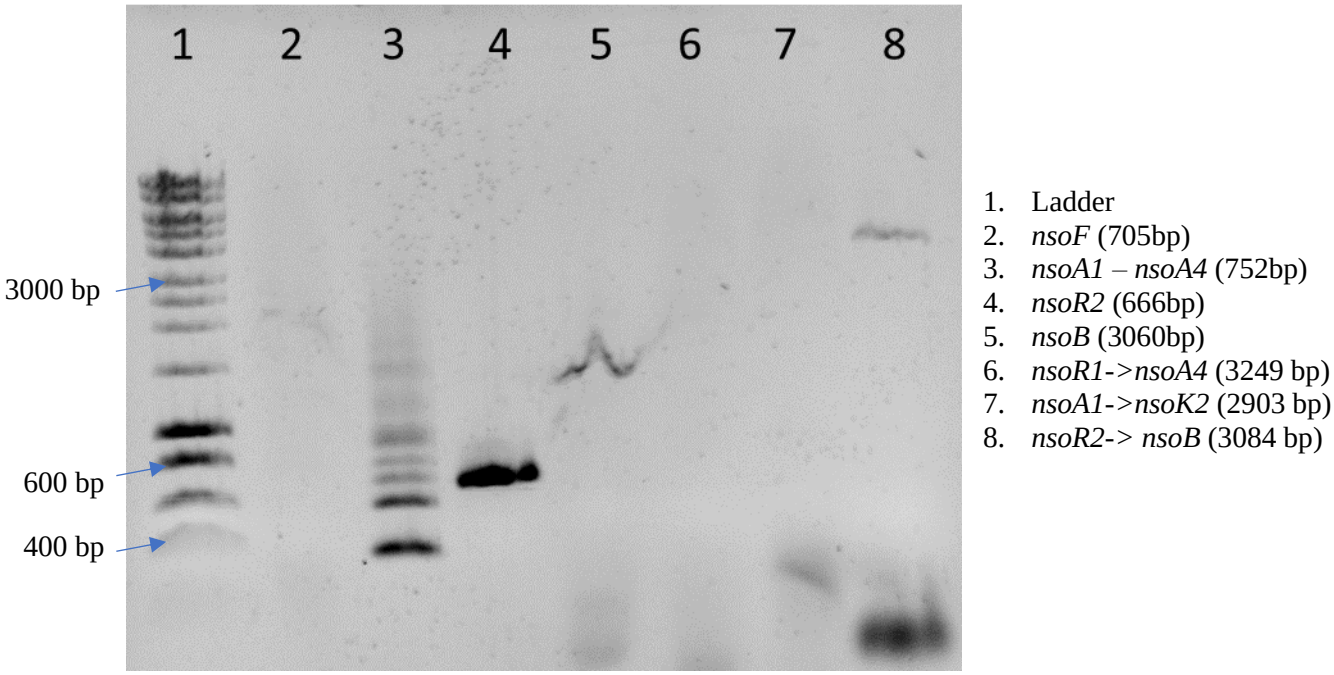


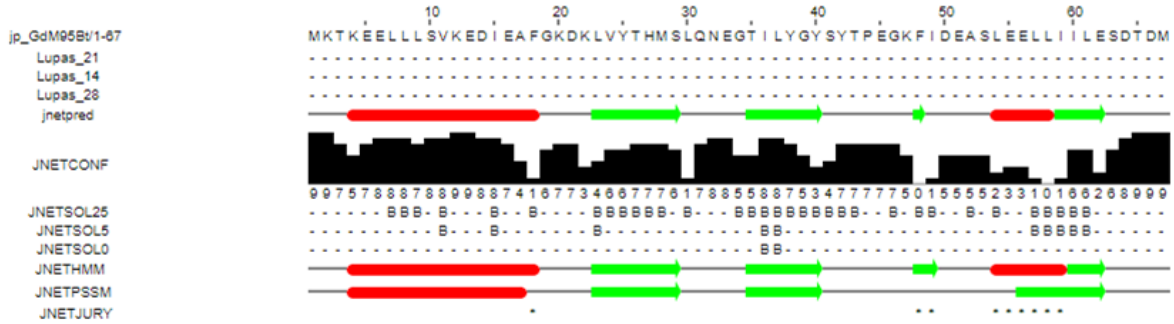
Figure 6.3.10. A gel electrophoresis of RT-PCR products of the nisin O cluster and products which span across multiple predicted operons derived from RNA extracted from nisin A induced *L. lactis* UKLc10 p*nso*.

6.3.8 Identification of a Novel Gene Within the Nisin O Biosynthetic Gene Cluster

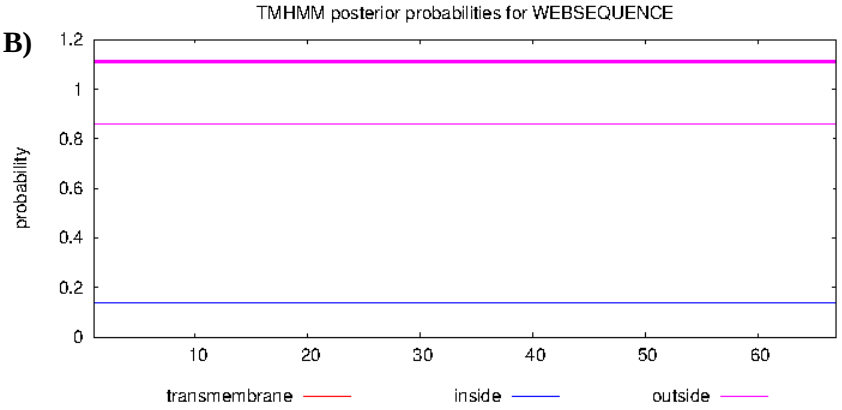
In a project performed by Romano *et al* (unpublished), using Oxford Nanopore sequencing techniques to analyse the transcription of bacterial gene clusters, the authors were able to identify a gene within the nisin O biosynthetic operon which had not been characterised that was highly expressed (Figure 6.1.1). This gene was termed *nso1.14* as it was the fourteenth gene within the nisin O biosynthetic gene cluster and is found on the reverse strand of the cluster. Further *in silico* anlaysis of the protein derived from *nso1.14* indicated that it was 67 amino acids in length and Jpred analysis predicted a helix-turn-helix structure for this protein (Figure 6.3.11A). TMHMM analysis predicted that Nso1.14 did not contain any transmembrane domains and had a high probability of being extracellular in nature (Figure 6.3.11B). Furthermore, using AlphaFold2 to predict the tertiary structure and folding of Nso1.14 correlated with the results predicted by Jpred of containing a helix-turn-helix domain.

It must be noted that the confidence of this model was a maximum confidence of 68 predicted local distance difference test (plDDT) at the N-terminus and reached as low as 41 plDDT at the C-terminus and the middle of the protein, both of which are below the lower acceptable cut-off of 70 plDDT (Figure 6.3.11C) (Tunyasuvunakool, Adler *et al*. 2021). BLAST searches of the amino acid sequence yielded no positive hits and a search for the conserved domains using CD Batch Search (Lu, Wang *et al*. 2020) indicated that there was no conserved domain family present within the amino acid sequence. Analysis of the six nisin O-like clusters showed that this gene was also present there, although not annotated, with an overall percentage identity of 99.7% when compared to the *B. obeum* A2-162 *nso1.14*.

A)



B)



C)

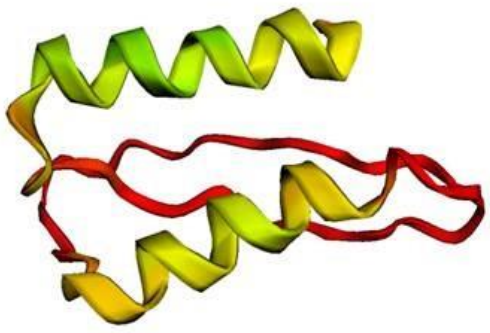


Figure 6.3.11. Outputs from *in silico* analysis of Nso1.14 A) Jpred analysis of Nso1.14. Red indicates the predicted helices and green indicates predicted β-pleated sheets. B) TMHMM analysis of Nso1.14. C) Alphafold2 output of the predicted tertiary structure of Nso1.14.

As a helix-turn-helix structure is a common structure of DNA binding transcription factors it was decided to clone *nso1.14* into a plasmid which would provide constitutive expression followed by transformation into *L. lactis* MG1614 pP*nsoA1-4_pepI* to investigate if this protein had any interaction with the promoter of the *nsoA1-4* genes and therefore potential regulation of the antimicrobial activity observed in the *L. lactis* heterologous expression system.

The *nso1.14* gene was sucessfully inserted into pUK200 with the the P32 promoter via splice overlap PCR. The insertion of *nso1.14* was confirmed via sequencing the purified product.This was followed by sucessful transformation in *L. lactis* MG1614 pP*nsoA1-4_pepI*, which enabled the reporter assay using uninduced, nisin A induced and trypsinated pre-NsoA1-4 induced conditions to be assessed to observe any alterations in PepI activity, therefore indicating whether this protein was able to affect the level of *nsoA1-4* expression.

The *pepI* reporter assay using the *nsoA1-4* promoter preceding the *pepI* gene and the constitutively expressed *nso1.14* gene was performed in triplicate. The *nso1.14* containing strains were analysed after either remaining uninduced or being induced with nisin A or trypsinated pre-NsoA1-4. Furthermore, all the controls in this experiment were induced using trypsinated pre-NsoA1-4. Analysis of the data from this assay determined that there was very low PepI activity from the the strains expressing *nso1.14* in any induction condition at 24.1, 19.6 and 21.8 nmol/mg/min for uninduced, nisin A induced and trypsinated pre-NsoA1-4 induced, respectively (Figure 6.3.12).

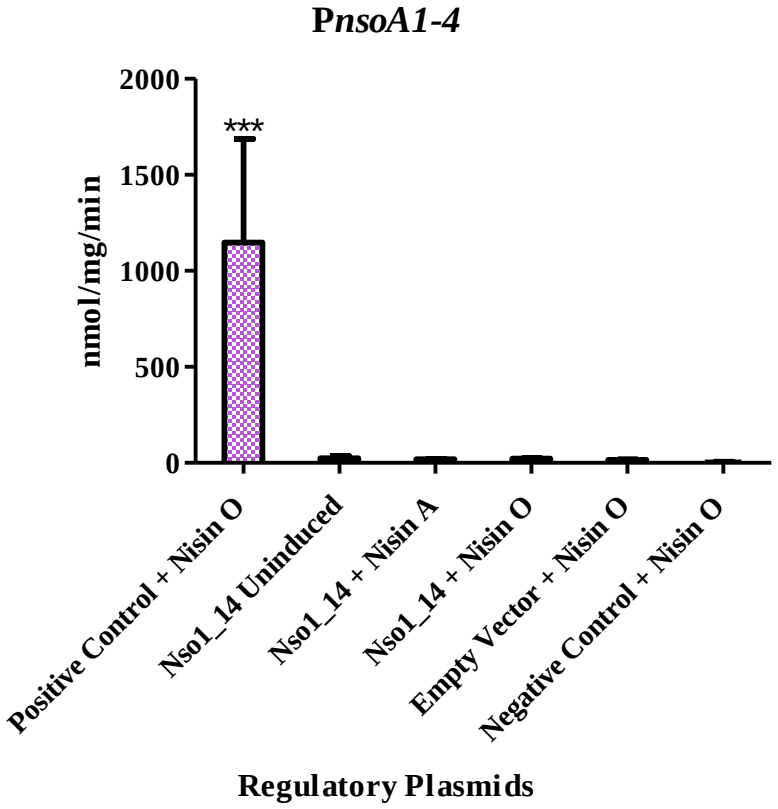


Figure 6.3.12. The rates of activity of PepI expressed by pP*nsoA1-4_pepI* containing strains which also contained a plasmid which constitutively expressed nso1.14 when uninduced, induced with nisin A and induced with trypsinated pre-NsoA1-4. The empty vector control in all assays contained pUK200_P32 with no regulatory system. The positive control was a *L. lactis* UKLc10 strain with the *nisRK* on the chromosome with the promoter for nisin A preceding the *pepI* gene. The negative control was *L. lactis* MG1614 pIL253. All controls were induced with trypsinated pre-NsoA1-4. Significance was determined by a One-way ANOVA followed by a Tukey Multiple Comparison Test. A P-value of < 0.05, < 0.01 and < 0.001 are indicated by '*', '**' and '***', respectively. The asterisks directly above each column indicate the significance compared to all other strains unless indicated otherwise. Error bars were made using the standard deviation of the mean. Each induction condition was repeated in triplicate.

6.4 Discussion

Understanding the regulation of antimicrobial biosynthetic gene clusters is important to maximise their potential as part of industries such as healthcare and food preservation. This work aimed to investigate the regulation of the nisin O biosynthetic gene cluster through analysis of the two TCSs found within the cluster.

The lantibiotic cluster discovered in *B. producta* SCSK by Kim *et al* (2019), contained two TCSs as seen in the nisin O biosynthetic gene cluster in *B. obeum* A2-162 (Hatziioanou, Gherghisan-Filip *et al*. 2017, Kim, Becattini *et al*. 2019). This differed from the nisin A gene cluster which only contains one TCS, NisRK, which has previously been shown to interact with the promoters P*nisA* and P*nisFEG* (de Ruyter, Kuipers *et al*. 1996). However, due to the presence of an additional regulatory system in the *B. obeum* A2-162 cluster and also that of the *B. producta* SCSK cluster further study of these regulatory systems was required to assess how the cluster is regulated. This additional regulatory system was also observed in the nisin O-like clusters found in *B. obeum* AM27-32LB, *B. obeum* TM09-13AC, *Blautia* sp. AM47-4, *Ruminococcus* sp. AM50-15BH, *D. formicigenerans* AM37-5 and *D. longicatena* 1001136B-160425, indicating that the presence of these two regulatory systems is conserved within these biosynthetic gene clusters. Although the presence of two regulatory systems is not seen in many bacteriocin biosynthetic gene clusters there are examples of multiple regulatory systems found within a single biosynthetic gene cluster (Azevedo, Bento *et al*. 2015). Mersacidin, produced by *Bacillus* sp. HIL Y-85,54728, is a lantibiotic that has three regulatory genes within its biosynthetic gene cluster (Chatterjee, Chatterjee *et al*. 1992). A TCS, MrsR2/K2 is primarily involved in immunity and the induction of mersacidin biosynthesis in the presence of mersacidin (Schmitz, Hoffmann *et al*. 2006). Furthermore, MrsR1, which does not have a histidine kinase gene up- or downstream was found to be essential for mersacidin expression (Guder, Schmitter *et al*. 2002). A lantibiotic cluster found within *Bifidobacterium longum* DJO10A, has also been identified having three genes involved in regulation, with two forming a LanR2K system and a third *lanR1* gene, with the authors suggesting that LanR1 may act as

a repressor of *lanA* transcription (Lee, Li *et al*. 2011). This therefore suggests that multiple regulatory genes can not only be present within a biosynthetic gene cluster but also can be functional in regard to the expression of genes within the cluster.

To assess whether the nisin O regulatory systems could increase the expression of nisin O and therefore increase the antimicrobial activity observed, NsoR2K2 and NsoR1AK1, which had a 5' extended version of *nsoR1*, were included in this assay. This *nsoR1A* regulatory system was selected as this had a greater nucleotide sequence length and was more similar to the *nisR* at 666 bp and 690 bp, respectively, whereas the *nsoR1* nucleotide sequence was only 561 bp. Activity was observed when both regulatory systems were present, although this only occurred on separate plates indicating that antimicrobial activity was not consistently observed during these assays. Furthermore, the NsoR2K2 strains showed activity when the nisin A was used to induce. This indicates that the nisin A could potential interact with these regulatory systems and enable interaction with the P*nsoA1-4* promoter to increase expression of the antimicrobial peptide. However, it must be noted that the *nsoR1AK1* genes were being constitutively expressed whereas the *nsoR2K2* genes were under the expression of the P*nisA* promoter. It should also be taken into account that in this co-expression assay in the *L. lactis* heterologous expression system the *nisRK* system from the nisin A biosynthetic gene cluster was present on the chromosome and therefore this regulatory system may have been interacting with the promoters of the nisin O system thus causing the antimicrobial effect. The interaction between NisRK and P*nsoA1-4* has previously been demonstrated in a *pepI* reporter assay, indicating that the antimicrobial activity from this system is at least partially due to this regulatory system (Gherghisan-Filip 2016). However, *B. obeum* A2-162 was shown to produce an antimicrobial effect indicating that the regulation of these antimicrobial peptides can occur in the native host without the presence of NisRK (Hatziioanou, Gherghisan-Filip *et al*. 2017).

During this study, work performed by Romano *et al* (unpublished) to investigate the potential of nanopore technology for long read RNA sequencing, using the nisin O cluster in the *L. lactis* heterologous expression system with nisin A induction, indicated that there were high

levels of *nsoA1-4* and *nsoR2K2* expression (Romano, Ansorge *et al*. 2022). This study also indicated that the start site for the *nsoR1* gene was as originally published and not extended. Therefore to assess the function of the NsoR1K1 and NsoR2K2 regulatory systems these systems were successfully cloned into pUK200 with the P32 promoter (van der Vossen, van der Lelie *et al*. 1987) which would provide constitutive expression. Additionally, the pIL253 plasmids containing the P*nsoA1-4*, P*nsoFEG*, P*nsoBTC* and P*nsoR2K2* promoters were transformed into *L. lactis* MG1614, which did not contain the *nisRK* system on the chromosome. The pUK200_P32_*nsoR1K1* and pUK200_P32_*nsoR2K2* were then successfully transformed into the nisin O promoter strains in *L. lactis* MG1614 and the *pepI* reporter assays were performed. There was no activity seen using P*nsoFEG* with any regulatory system including the NisRK system. This was surprising as the immunity of nisin A is regulated by the NisRK system within its biosynthetic gene cluster and is important to prevent cell death caused by production of nisin A (de Ruyter, Kuipers *et al*. 1996, AlKhatib, Lagedroste *et al*. 2014). This may be due to the predicted promoter position being incorrect, although as there are transcripts observed by Romano *et al* (unpublished), there may be very low constitutive expression of the *nsoFEG* genes which was not detectable during the reporter assay. Furthermore, initial analysis of the nisin O cluster identified half a transcriptional regulator upstream of *nsoF* with frameshifts, therefore it is possible that a promoter upstream from this gene is responsible for expression of *nsoFEG* (Hatziioanou, Gherghisan-Filip *et al*. 2017). A greater level of PepI activity was seen in the pP*nsoBTC_pepI* strains in all conditions, including the empty vector control, however these were not significantly different when compared to the negative control. Again, this was surprising as the NisRK system in *L. lactis* can regulate the expression of these genes in the nisin A cluster and therefore it was expected that either NsoR1K1 or NsoR2K2 would perform this role. However, due to the low level of activity seen in the empty vector control it suggests a very low level of expression occurring in the absence of any inductive conditions or regulatory systems. This is also supported by the mapped reads from the work performed by Romano *et al* (unpublished), which also shows an

increased level of reads mapped to the *nsoBTC* genes compared to the *nsoFEG* genes. This may indicate that the modification machinery is expressed at low levels even without induction, which can be increased under the correct inducing conditions to maximise nisin O maturation.

Interestingly, neither the NsoR1K1 nor NsoR2K2 systems interacted with the P*nsoA1-4* promoter in any induction conditions tested. This indicates that these TCSs do not increase the expression of the NsoA1-4 peptides when induced with nisin A or nisin O. However, when the NisRK system was present an increase in PepI activity was observed only when induced with nisin A indicating that the regulatory system from the nisin A cluster is able to interact with a promoter from a different nisin variant cluster. This explains the antimicrobial activity seen in the *L. lactis* UKLc10 p*nso* strain when induced with nisin A. Cross induction of nisin promoters has been shown before in a reporter assay using green fluorescent protein downstream of the nisin A promoter (Garcia-Gutierrez, O'Connor *et al*. 2020). The authors used nisin A, nisin H and nisin P to induce the *L. lactis* reporter strain and reported that the promoter was more sensitive to nisin A induction (1 ng/ml - 1 µg/ml) compared to that of nisin H (10 ng/ml – 1 µg/ml) and nisin P (100 ng/ml – 10 µg/ml) (Garcia-Gutierrez, O'Connor *et al*. 2020). This therefore indicates that different nisin variants are capable of interacting with regulatory systems and inducing activity from promoters from other nisin variants. This data also not only confirms the results produced by Gherghisan-Filip (2016) but demonstrates that it is the nisin A-activated *nisRK* system acting on the nsoA1-4 promoter and not the nisin A acting via either of the *nsoRK* systems. The lack of activity produced by the NsoR1K1 and NsoR2K2 systems may suggest that other inducing factors or ligands may be required to interact with these regulatory systems and therefore interact with the P*nsoA1-4* promoter, however further work is required to investigate this using the *pepI* reporter system designed as part of this study.

Finally, the promoter preceding *nsoR2K2* indicated a high level of activity when present with all the regulatory systems tested. Furthermore, activity was also observed in the empty vector

control, indicating that this promoter acts constitutively. When the NsoR1K1 system is present in all inducing conditions a significant increase in PepI activity was observed compared to the empty vector control, indicating that this TCS is able to interact with the P*nsoR2K2* promoter and increase the expression of the genes downstream. Similar results are also seen in the nisin A system where the *nisRK* promoter is also constitutively expressed, this suggested that there are some genes within the clusters which are important to express at a high level (de Ruyter, Kuipers *et al*. 1996). This data was also observed by Romano *et al*, where there was a high level read mapping against the *nsoR2K2* genes, indicating that the genes are indeed being expressed (Romano *et al*, unpublished). However, unlike the NisRK system, the NsoR2K2 system did not show any interaction with any of the predicted promoters using the conditions tested. This again indicates that the inducing factor or factors required for nisin O expression are still to be discovered. Additionally, further work must be performed to confirm the position of the promoters, particularly those that of P*nsoFEG*. It must also be noted that the nisin O regulatory systems may be repressing expression of the nisin O cluster and require a specific agonist to activate expression. This has been observed in the regulation of the subtilin biosynthetic gene cluster, where expression is controlled by the TCS SpaRK, however *spaRK* expression itself is regulated by sigma H (Stein, Borchert *et al*. 2002). Therefore, there may be other regulatory systems or sigma factors within *B. obeum* A21-162 that may influence the level of expression of genes within the nisin O cluster.

The nisin O cluster was also analysed for co-expression of the operons within the cluster using RT-PCR. Using various primer combinations which would provide a product that overlapped each operon, it was found that only the *nsoR2K2BTC* genes were co-expressed. This data also helps to confirm the results produced by Romano *et al* (unpublished), further explaining why a greater number of reads were mapped to *nsoBTC*, indicating a higher level of expression. Furthermore, due to the lack of a clear terminator between *nisP* and *nisRK* in the nisin A cluster, the two operons on which these genes are found may also be co-expressed (de Ruyter, Kuipers *et al*. 1996, Kleerebezem 2004). This assay also showed that there was very low

expression of *nsoFEG*. This is again surprising as it is expected that expression of these genes is required to provide host immunity against the antimicrobial effects of nisin O.

The work undertaken by Romano *et al* (unpublished), also indicated a novel gene within the nisin O biosynthetic cluster that is highly expressed, termed *nso1.14*. This protein was predicted to have a helix-turn-helix domain and did not have any recognised conserved domains. Interestingly, helix-turn-helix domains are a common structure found within both global and specific transcriptional regulators in all organisms (Aravind, Anantharaman *et al*. 2005). This gene was successfully cloned into the pUK200_P32 plasmid and transformed into the *L. lactis* MG1614 p*PnsoA1-4_pepI* strains to elucidate whether this protein can interact with the promoter for the nisin O structural genes. However, no PepI activity was observed when this small protein was present therefore showing that this protein did not activate with P*nsoA1-4* using these inducing conditions. Although the thought that this protein could be repressing the expression of *nsoA1-4* was considered the evidence provided by the RT-PCR and from Romano *et al* (unpublished), clearly showed that *nsoA1-4* was being expressed when there were high expression levels of *nso1.14*. Furthermore, this gene was identified in all six nisin O-like clusters implying that there must be a function performed in order to conserve this gene across these clusters. Alternatively, this sequence may be a small non-coding RNA, which have been shown previously to increase the expression of nisin A in the *L. lactis* F44 using s015 (Qi, Caiyin *et al*. 2017). Further work needs to be performed to ascertain the function of this sequence in the context of the nisin O cluster.

This work has shown that the nisin O regulatory systems did not activate with P*nsoFEG*, P*nsoBTC* or P*nsoA1-4* using either nisin A or nisin O induction, although the NisRK system does interact with P*nsoA1-4* when induced with nisin A, which disagrees with the original hypothesis that one of the two TCSs from the nisin O cluster would activate the predicted promoters. Furthermore, there is constitutive expression of the P*nsoR2K2* promoter with an increase in expression levels when NsoR1K1 is present. Moreover, analysis of the RNA transcripts of the nisin O cluster identified that *nsoA1-4* and *nsoR2K2* are highly expressed in

the *L. lactis* heterologous expression system and that there is co-expression of the *nsoR2K2* and *nsoBTC* containing operons, which was predicted in the hypothesis. A novel highly expressed sequence, *nso1.14*, was found in all six nisin O-like clusters, however, there was no positive interaction between the Nso1.14 and P*nsoA1-4* and its function remains to be determined.

Chapter 7.

Discussion

Dysbiosis of the gut microbiome has been linked with a number of human diseases, including irritable bowel syndrome, inflammatory bowel disease, cardiovascular disease, host vs graft disease and Alzheimer's (Kostic, Gevers *et al*. 2012, Tang, Kitai *et al*. 2017, Vich Vila, Imhann *et al*. 2018, Fredricks 2019, Glassner, Abraham *et al*. 2020, Staudacher, Scholz *et al*. 2021, Qian, Xie *et al*. 2022). This therefore implies that maintenance of a health gut microbiome is important in preventing a number of diseases. Bacteriocins are a family of antimicrobial peptides that show promise for being used in human health, including the potential to modulate the gut microbiome and provide targeted antimicrobial effects with minimal disturbance of commensal bacteria (Cotter, Ross *et al*. 2013, Kim, Becattini *et al*. 2019, O'Reilly, O'Connor *et al*. 2022). The gut microbiome has also been shown to be a source of novel antimicrobials, with the work in this thesis particularly interested in the nisin O biosynthetic cluster identified in the human gut bacterium *B. obeum* A2-162 which has been shown to have antimicrobial activity against human pathogens *C. difficile* and *C. perfringens* (Hatziioanou, Gherghisan-Filip *et al*. 2017, Garcia-Gutierrez, Mayer *et al*. 2019).

This thesis aimed to ascertain the inducing conditions necessary for nisin O production from *B. obeum* A2-162, to identify nisin O-like biosynthetic gene clusters present within the *Lachnospiraceae* family, detect candidate proteases that would be able to cleave the leader peptides from the pre-NsoA1-4 peptides and to elucidate the whether the two TCSs in the nisin O cluster can activate gene expression by binding to the predicted promoters within the cluster as part of a *pepI* reporter assay.

In chapter 3 attempts were made to identify the presence of homologues of the nisin O cluster in *Lachnospiraceae*. Due to nisin O's unusual cluster structure and the publication of a cluster identified in *B. producta* SCSK with a very similar structure to that of nisin O, it was decided to determine how widely the cluster is distributed among bacterial genera within the *Lachnospiraceae* family (Kim, Becattini *et al*. 2019). Initial BLASTP analyses using NsoB and NsoC identified nisin O-like clusters present not only in *Blautia* species but also in *Dorea* and *Ruminococcus* species. Due to this result a pan-genome analysis of 2094 *Lachnospiraceae*

genomes was performed which identified 59 genomes with *nsoB*, *nsoC* or both. After manual searches of each of these genomes clusters additional clusters were found in *Dorea* and *Pseudobutyrivibrio* species. Further analysis of the proteins encoded within these clusters identified that *B. obeum* AM27-32LB, *B. obeum* TM09-13AC, *Blautia* sp. AM47-4, *Ruminococcus* sp. AM50-15BH, *D. formicigenerans* AM37-5 and *D. longicatena* 1001136B-160425 did indeed carry nisin O-like clusters with high percentage identities to each of the NsoA1-4 peptides as well as NsoB and NsoC. Additionally, all nisin O-like clusters also contained two TCSs and no protease within their biosynthetic gene clusters. Interestingly, all of the bacteria were initially found within human faecal samples. This not only shows that the very similar clusters are identified within multiple genera but also that the human gut can be a major source of novel antimicrobials. This area of study is becoming increasingly significant as antibiotic resistance continues to be a major global issue, with recent studies investigating how the gut microbiota can be not only a source of bacteriocins but also how they can play a role in modulating the human gut microbiome (Piddock 2012, Garcia-Gutierrez, Mayer *et al*. 2019, Kim, Becattini *et al*. 2019, Wang, Yu *et al*. 2019, O'Reilly, O'Connor *et al*. 2022, Teng, Huang *et al*. 2022).

Furthermore, this work has identified a number of novel lantibiotic clusters within the family *Lachnospiraceae*, with in depth analysis of the clusters identified in *Pseudobutyrivibrio* sp. P49, *Pseudobutyrivibrio* sp. UC1225 and *R. gnavus* A533-12. This shows that genome analysis of bacteria is an efficient process by which to identify novel biosynthetic gene clusters, however, it must be considered that functional analysis and demonstration of antimicrobial activity must occur. Curiously, the fact that the nisin O cluster is found in multiple bacterial species across genera could indicate the possibility that this cluster might be disseminated via horizontal gene transfer. A recent study has shown that phages are able encode biosynthetic gene clusters and can transfer a fully functional cluster between *B. subtilis* species which provided a competitive advantage when compared to the ancestor species (Dragoš, Andersen *et al*. 2021). Analysis of 50 *B. obeum* genomes identified the presence of

lantibiotic immunity genes which were present in 66% of genomes even in genomes where no lantibiotic cluster was identified. This shows the potential of the immunity genes to be transferred by horizontal gene transfer across different strains to provide immunity to antimicrobials produced by a few bacterial species which produce the lantibiotic. This has been shown in studies investigating the dissemination of nisin A immunity genes in *L. lactis* strains and also cross-immunity assays using nisin A, nisin U, nisin H and nisin P producing bacteria which showed that some of these bacteria were resistant to other nisin variants (Garcia-Gutierrez, O'Connor *et al*. 2020, van Gijtenbeek, Eckhardt *et al*. 2021). This suggests that in a complex environment such as the human gut microbiome there is scope for the potential of the nisin O biosynthetic gene cluster or the immunity genes being able to be transferred between bacteria to provide a competitive advantage. In chapter 4, attempts were made to repeat the antimicrobial activity produced by the original nisin O producing organism *B. obeum* A2-162 (Hatziioanou, Gherghisan-Filip *et al*. 2017). However, repeating the conditions tested by Hatziioanou *et al*, performing an increasing nisin A exposure assay and co-culturing with humanisation strains resulted in no antimicrobial activity being observed from *B. obeum* A2-162. It may be possible that due to the storage and sub-culturing of *B. obeum* A2-162 the pathways that were active in samples taken from fresh faeces have been switched off over time and use in *in vitro* experiments. However, interestingly, in a competition assay using a *B. longum* strain grown adjacent to *B. obeum* A2-162 there were observable changes to the cell surface morphology on both cultures. This may indicate that nisin O may be being produced at a sub-lethal concentration and therefore affects the growth and morphology of neighbouring bacteria. Alternatively, the observed phenotype may be due to a different signalling system between the two strains. It has previously been shown that nisin A is able to down-regulate virulence proteins at sub-lethal concentrations, thus changing the protein composition of the cell surface (Stincone, Miyamoto *et al*. 2020).

Previous work investigating the nisin O cluster showed antimicrobial activity using the *L. lactis* heterologous expression system where trypsin was used to cleave the leader peptide of

NsoA1-3 (Hatziioanou, Gherghisan-Filip *et al*. 2017, Gherghisan-Filip, Saalbach *et al*. 2018). NsoA4 does not have a trypsin cleavage site upstream of the core peptide and therefore the antimicrobial activity of the NsoA4 could not be assessed. A *nsoA4* mutant was created which added a trypsin cleavage site at the end of the leader peptide and was then tested for potential antimicrobial activity, however, no antimicrobial activity was seen when this peptide was produced in the *L. lactis* heterologous expression system. This indicates that NsoA4_S24K has no antimicrobial activity when the leader peptide is cleaved as activity was seen in the positive control demonstrating the trypsin was functioning correctly to cleave the leader peptides of NsoA1-3. However, as no zone of inhibition was seen for the strain expression NsoA4_S24K this may suggest that this peptide may act as a repressor in the nisin O cluster. Additionally, the presence of two plasmids both expressing genes may have led to a decreased level of expression of the antimicrobial NsoA1-3 peptides, therefore not producing an antimicrobial affect (San Millan and MacLean 2017). It must be noted that the use of trypsin for leader peptide cleavage might lead to the degradation of the core peptide due to trypsin cleavage sites present in the core peptide, this in turn would then affect the potential antimicrobial activity.

Microscopy of *B. obeum* A2-162 revealed that this bacterium is found as both bacillus-shaped individuals and as part of longer chains, with no evidence that this strain produces spores after 6 days of growth. Furthermore, condensed vesicle-like regions were present at the poles of the cell. Intriguingly, recent studies have illustrated that the nisin A modification machinery, NisBTC, are found at the 'old' dividing pole of the cell which might explain a reason for the location of these condensed regions within *B. obeum* A2-162 (Chen, van Heel *et al*. 2020, Hill 2020).

The expression of nisin O from the *L. lactis* heterologous expression system was optimised to consistently produce an antimicrobial effect. This required the use of 15 ng/ml nisin A, 10 µg/ml trypsin and 5 µg/ml erythromycin in the base agar and 10 µg/ml trypsin added to the overlay agar. The use of erythromycin was important as this helped to maintain the pIL253

plasmid which contained the nisin O biosynthetic gene cluster and conferred erythromycin resistance. Furthermore, trypsin was added in order to cleave the nisin O leader peptide due to there being a trypsin cleavage site present at the C-terminus of the leader peptide (Hatziioanou, Gherghisan-Filip *et al*. 2017, Gherghisan-Filip, Saalbach *et al*. 2018). However, trypsin can also act as an inducing molecule in the case of ruminococcin A expression (Gomez, Ladire *et al*. 2002).

This work was able to demonstrate that optimisation of lantibiotic expression systems can be performed and can potentially help to increase expression of the antimicrobial peptides. However, further work should be invested into understanding the conditions under which *B. obeum* A2-162 can express nisin O and therefore those which correlate to conditions found in the human gut. This work also shows that antimicrobial activity of structural peptides should not be assumed, and full characterisation assays should be performed.

The nisin O cluster is unusual as it does not contain a protease which is required for leader peptide cleavage and thus activation of the antimicrobial peptide (Hatziioanou, Gherghisan-Filip *et al*. 2017). In chapter 5 identification and testing of candidate proteases found in the *B. obeum* A2-162 genome was performed. In all other known nisin variant clusters, a protease is always present, including the cluster within *B. producta* SCSK, although this protease is of a different type to other NisP proteins (de Vos, Mulders *et al*. 1993, O'Connor, O'Shea *et al*. 2015, Kim, Becattini *et al*. 2019, Garcia-Gutierrez, O'Connor *et al*. 2020, M, Field *et al*. 2020, O'Sullivan, O'Connor *et al*. 2020). Therefore the *B. obeum* A2-162 genome was analysed for candidate proteases using both NisP from the nisin A cluster and LanP from the *B. producta* SCSK lantibiotic cluster to search. This led to the identification of 10 candidate protease of which the six with the greatest homology to NisP or LanP or shared conserved domains were chosen for further analysis.

These candidate proteases were used in co-expression antimicrobial assays which did not result in any antimicrobial activity in the absence of trypsin for any candidate protease.

Furthermore, the leader peptide cleavage ability of these candidate proteases was assessed using an incubation assay using pre-NsoA1-4 as a substrate. This assay showed that there was no leader peptide cleavage of pre-NsoA1-3 by any candidate protease or indeed the cell wall fractions of the humanisation strains (Becker, Kunath *et al*. 2011). Cleavage of the leader peptide was only observed when trypsin was used within the assay, confirming the results of Hatziioanou *et al* and Gherghisan-Filip *et al* (Hatziioanou, Gherghisan-Filip *et al*. 2017, Gherghisan-Filip, Saalbach *et al*. 2018). Trypsin is present within the human gut and therefore could be acting in place of a protease within the *B. obeum* A2-162 genome to provide leader peptide cleavage (Metheny, Stewart *et al*. 1997, Koshikawa, Hasegawa *et al*. 1998). Additionally, the results of the pan-genome analysis identified six nisin O-like cluster which all did not contain a protease within their cluster. However, it must also be noted that trypsin can also act as an inducing molecule as seen in the expression regulation of ruminococcin A, which incidentally also does not contain a protease within its cluster (Dabard, Bridonneau *et al*. 2001, Gomez, Ladire *et al*. 2002). However, there are two trypsin cleavage sites with the core peptide of NsoA1-3 which, although the lanthionine rings may offer some protection against degradation, could lead to the cleavage of the core peptide and hence a decrease in observable antimicrobial activity.

MicroMatrix fermentation assays using the nisin O producing bacteria, *B. obeum* A2-162 and *L. lactis* UKLc10 p*nso*, did not show an alteration in the composition of the bacterial composition when compared with the faecal slurry only control. Additionally, the addition of human gut concentrations of trypsin to either of the nisin O producers also had no notable influence on bacterial composition when compared to the faecal slurry control. Additionally, in repeats of the assays there appeared to be a disproportionate growth of a small group of bacteria which was also seen the faecal only control. This indicated that the protocol needed greater development to prevent either contamination or overgrowth of specific bacteria. It can be stated that the effects seen are not due to the antimicrobial activity of nisin O due to the composition of faecal slurry only wells following the same pattern as those with the nisin O

producers. However, it would be interesting to repeat this work as recent studies using nisin A and nisin P in MicroMatrix fermentation models have shown how these antimicrobial peptides can influence the composition of the human gut microbiota (Garcia-Gutierrez, O'Connor *et al*. 2020, O'Reilly, O'Connor *et al*. 2022). The hypothesis of this work stated that a protease would be present within the *B. obeum* A2-162 genome that could cleave pre-NsoA1-3 and produce an antimicrobial effect. None of the proteases tested were able to cleave the leader peptide and no antimicrobial effect was observed.

There are two TCSs within the nisin O cluster in *B. obeum* A2-162 (Hatziioanou, Gherghisan-Filip *et al*. 2017). In chapter 6 the interactions between these two TCS and the predicted promoters in the nisin O biosynthetic gene cluster were investigated. These two regulatory systems were also identified within the six nisin O-like clusters identified in chapter 3 and additionally in the lantibiotic cluster within *B. producta* SCSK (Kim, Becattini *et al*. 2019). Co-expression of plasmid expressed NsoR1AK1, a 5' extended version of NsoR1K1, and NsoR2K2 with the nisin O cluster demonstrated activity could occur in their presence. However, no increased antimicrobial activity was observed when these regulatory systems were overexpressed in the heterologous expression system when compared to the heterologous expression system without the *nsoR1AK1* and *nsoR2K2* expressing plasmids.

To assess whether the NsoR1K1 or NsoR2K2 regulatory systems interact with the predicted promoters from the nisin O cluster, which are also the same within the six nisin O-like clusters, a *pepI* reporter assay was used. NsoR1K1 was used instead of NsoR1AK1 as unpublished data from Romano *et al*, demonstrated that the cDNA produced from RNA extracted from a nisin A induced *L. lactis* UKLc10 p*nso* culture, mapped to the *nsoR1K1* sequence. No interaction was observed between the two nisin O regulatory systems and the promoters P*nsoFEG*, P*nsoA1-4* and P*nsoBTC* when uninduced or induced with nisin A or nisin O. This differs from the regulation of the nisin A cluster where nisin A is able to interact with the NisRK system and therefore activate expression of the cluster (de Ruyter, Kuipers *et al*. 1996). Activity was only seen for the P*nsoA1-4* promoter when it interacted with NisRK when induced with nisin

A. This explains why nisin A could be used to induce the heterologous expression system (Hatziioanou, Gherghisan-Filip *et al*. 2017) and that there is a level of promiscuity between the regulatory system and promoters from different nisin clusters. Interestingly, the P*nsoR2K2* promoter provided constitutive expression of *pepI* as activity of PepI was observed even when a regulatory system was not being expressed. This has been reported in previous literature which showed that the P*nisRK* promoter is also constitutive (de Ruyter, Kuipers *et al*. 1996). Furthermore, the results from these assays using P*nsoR2K2* indicated that NsoR1K1 was able to significantly increase expression of *pepI* when compared to the empty vector control for all induction conditions. Significant increases in PepI activity were also seen when NsoR1K1 was present when compared to NisRK when uninduced and induced with trypsinated pre-NsoA1-4 and NsoR2K2 only when induced trypsinated pre-NsoA1-4. This indicates that NsoR1K1 is able to activate P*nsoR2K2* and is induced by both nisin A and trypsinated pre-NsoA1-4, which shows that this regulatory system may be more promiscuous than previously though in terms of its inducing factors and that one regulatory system interacts with the promoter of the other. Further work could look to ascertain the exact function of this system and how it relates to the expression of other genes within the nisin O cluster.

RT-PCR from a nisin A induced *L lactis* UKLc10 p*nso* culture confirmed that the *nsoA1-4* as well as *nsoR2K2* genes were being expressed. Co-expression of the operons containing *nsoR2K2* and *nsoBTC* was also observed. This confirms that the results from the *pepI* reporter assay and from the data obtained by Romano *et al* on the nisin O transcripts (Romano, Ansorge *et al*. 2022). Interestingly, the immunity genes and the first regulatory system did not appear to be expressed, or at least not at detectable levels. This was not expected as in the nisin A cluster the *nisFEG* genes are regulated by NisRK and *nisI* is preceded by a constitutive promoter to provide immunity against nisin A (de Ruyter, Kuipers *et al*. 1996).

A novel sequence that was highly expressed in the nisin O cluster, termed *nso1.14*, was identified by Romano *et al*, which encoded a protein that was predicted to have a helix-turn-helix domain (Romano, Ansorge *et al*. 2022). This domain is characteristic of transcriptional

regulators and therefore this gene was cloned into a plasmid to provide constitutive expression. However, when used in a *pepI* reporter assay with P*nsoA1-4* no activity was seen. It was determined that this protein was not a repressor of P*nsoA1-4* as there was still a high level of expression even when *nso1.14* was expressed (Romano, Ansorge *et al*. 2022). However, this protein could still interact with other promoters within the nisin O cluster and repress expression, but further work would be required to investigate this.

The work performed in this chapter has investigated the interactions between the nisin O regulatory systems and the predicted promoters within the cluster. However, neither of these systems indicated any interaction with the P*nsoA1-4* promoter which is in contrast to the hypothesis. Furthermore, it was shown that the P*nsoR2K2* promoter produced constitutive expression. The only activity from P*nsoA1-4* was observed when NisRK was induced with nisin A. These results were confirmed by RT-PCR, which also show co-expression of the *nsoR2K2BTC* genes. Additionally, *nso1.14* was identified however the function of this protein is yet to be discovered which could be a target for further study. Further work can also use the *pepI* reporter assay system to determine which inducing conditions are required for interaction between the nisin O regulatory systems and the predicted promoters of the nisin O cluster.

It has been demonstrated in this thesis that a lantibiotic biosynthetic gene cluster with very high similarity to the nisin O cluster is found across multiple genera. Optimisation of the *L. lactis* heterologous expression was performed, showing that this antimicrobial does have biological activity against gut pathogens. Additionally, candidate proteases were identified from the *B. obeum* A2-162 genome, however, these demonstrated no cleavage function against the leader peptide of pre-NsoA1-3, although trypsin was able to cleave the leader peptide. Furthermore, the regulation of the cluster was assessed, and it was found that there was no interaction between the NsoR1K1 and NsoR2K2 systems and the P*nsoFEG*, P*nsoA1-4* and P*nsoBTC* promoters using the induction conditions tested. However, constitutive expression was observed from the P*nsoR2K2* promoter. RNA analysis identified that *nsoA1-4* and

nsoR2K2 were expressed as well as co-expression between the *nsoR2K2* and *nsoBTC* containing operons. Finally, a novel, highly expressed sequence was identified within the nisin O biosynthetic cluster however the function was not established.

The work presented in this thesis provides an opportunity for future work to be performed. It would be prudent to perform further experiments to identify the inducing factors required for nisin O expression from *B. obeum* A2-162. This could either be performed through testing a wider range of inducing molecules and conditions and assessing antimicrobial activity, but also the use of the reporter assay could determine exactly which TCS is responsible for the increase in expression. The conditions to use could be using a greater array of sugars (Such as fructose, xylose and sucrose), pH conditions and metabolites in the growth media as well as investigating the role that quorum sensing could have in the regulation of nisin O expression (Chandrapati and O'Sullivan 1999, Pongtharangkul and Demirci 2006, Liu, Zheng *et al*. 2010, Hoover, Perez *et al*. 2015, Girgin Ersoy, Kayıhan *et al*. 2020, Zhang, Han *et al.* 2020). It would also be important to understand which protease is required for the leader peptide cleavage of nisin O. This could involve transcriptomic analysis of *B. obeum* A2-162 once the inducing condition has been determined and therefore assess which genes are also upregulated, if any, when *nsoA1-4* is being expressed. Furthermore, as nisin O is active against clinically relevant pathogens such as *C. difficile*, it would be interesting to understand how the presence of this strain can influence the human gut microbiota. This work could be performed *in vitro* using fermentation assays or potentially *in vivo* using murine models. This would provide evidence on whether nisin O can act as an important antimicrobial in the human gut and additionally, whether *B. obeum* A2-162 could act as a probiotic strain to aid in human health and preventative medicine.

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