Engineering novel lantibiotics to target gut pathogens

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Abstract

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Infections with multi-drug resistant bacteria are an urgent health security threat and bacteriocins such as the subclass of lantibiotics represent a promising alternative source to traditional antibiotics. The gut intestinal tract ecosystem has a complex set of bacteria with a huge bacteriogenic potential and it can be explored as a source for new lantibiotic peptides. Using a genome mining approach, a *clos* gene cluster that has the potential to encode for novel lantibiotics was previously discovered in a strain of *Blautia* isolated from the human gut. This *clos* operon may encode two nisin-like peptides.

Several nisin derived *clos*-like sequences were generated, however they did not have improved properties when tested under GI tract conditions. Since we lack the molecular tools to manipulate the original *Blautia obeum* A2-162 gut bacterium that harbours the *clos* operon, the entire *clos* cluster was cloned in *Lactococcus lactis* where it was possible to obtain heterologous expression of the Clos peptides under the control of the nisin inducible NisRK regulatory system. The production of preClosA was demonstrated using an antibody raised against the Clos leader. The biological activity of the Clos peptide was confirmed after treatment with trypsin where the Clos leader was presumably cleaved, releasing the biologically active and mature Clos peptide that was highly active against two clinically important pathogens, *Clostridium perfringens* and *Clostridium difficile* as well as against *L. lactis* MG1614 sensitive indicator strains. Using immunoprecipitation, we obtained pure Clos peptides in solution and after trypsin treatment and alkylation of the cysteine residues, the post-translational modifications and the structure of the preClosA peptides were confirmed. Some of the anticipated dehydrations and ring formations (Rings D and E) were also identified using LC-MS.

The approach taken in this PhD project confirms that genome mining can be used to identify more gut bacteria with potential to produce novel lantibiotics.

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Abbreviations

Abbreviation	Extension
Abu	Aminobutyric acid
Ab	Antibody
Ag	Antigen
AMP	Antimicrobial peptide
Arg	Arginine
Asn	Asparagine
Asp	Aspartate
bp	Base pair
BHI	Brain Heart Infusion
BSA	Bovine Serum Albumin
CAM	Carbaimidomethyl
CE	Cell extract
Cm	Chloramphenicol
CMS	Colony Mass Spectrometry
Cys	Cysteine
Da	Dalton
Dha	Dehydroalanine
Dhb	Dehydrobutyrine
DNA	Deoxyribonucleic acid
Ery	Erythromycin
GI	Gut Intestinal
Glc	Glucose
Gly	Glycine
GRAS	Generally Regarded As Safe
h	Hour
HPLC	High-Performance Liquid Chromatography
IAA	Iodoacetamide
Ile	Isoleucine
IFR	Institute of Food Research
IP	Immunoprecipitation
JIC	John Innes Centre
LAB	Lactic Acid Bacteria
Lan	Lantibiotic
LC-MS	Liquid Chromatography-Mass Spectroscopy
LC-MS/MS	Liquid Chromatography-Tandem Mass Spectrometry
Leu	Leucine
Lys	Lysine
Maldi-ToF	Matrix-Assisted Laser-Desorption Ionisation Time of Flight
(Me)Lan	(Methyl)Lanthionine
MDR	Multi-Drug Resistant
min	Minute
MS	Mass Spectroscopy

m/z	Mass to charge ratio
OD_{600}	Optical density at 600 nm
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
Phe	Phenylalanine
ppm	Parts per million
Pro	Proline
PSM	Peptide Spectra Matches
rDNA	Ribosomal deoxyribonucleic acid
PTM	Post-Translational Modification
Rifr	Rifampicin
rpm	Revolutions per minute
RP-HPLC	Reverse Phase High-Pressure Liquid Chromatography
RT	Room Temperature
qPCR	Quantitative real-time Polymerase Chain Reaction
s	Second
SDS-PAGE	Sodium Dodecyl sulphate PolyAcrylamide Gel Electrophoresis
SEM	Scanning Electron Microscopy
Sm	Streptomycin
Ser	Serine
TA	Temperature for Annealing
TCA	Trichloroacetic acid
TEM	Transmission Electron Microscopy
Thr	Threonine
Tyr	Tyrosine
Tm	Melting temperature
Val	Valine
vs	Versus
UP	Ultra-pure
UV	Ultraviolet

Symbols

±	plus/minus
%	percentage
˚C	degree Celsius
bp	base pair
cm	centimetre
g	gram
h	hour
Hz	hertz
Kb	kilobase
l	litre
µg/ml	micrograms per millilitre
µl	microliter
µmol/g	micromoles per gram
µm	micrometre
µM	micromoles per litre
M	moles per litre
mg	milligram
mg/ml	milligrams per millilitre
MHz	megahertz
min	minute
ml	millilitre
ml/min	millilitre per minute
mm	millimetre
mM	millimoles per litre
ms	milliseconds
ng/ml	nanograms per millilitre
nm	nanometre
pH	potential of hydrogen
ppm	parts per million
rpm	revolutions per minute
s	second
T0	0 hour from inoculation
T12	12 hours post-inoculation
T24	24 hours post-inoculation
g	gravity
w/v	mass per volume

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CHAPTER ONE

1. General introduction

1.1 Antibiotic resistance crisis

Antibiotic resistance is a global health threat and a crisis which has become a major challenge of the 21st century. It is responsible for the rise in the frequency of antibiotic resistance from several classified bacteria which represent a considerable and urgent issue in the health care system. Among the bacteria that are no longer responding to routinely used antibiotics (as listed by the Centers for Disease Control and Prevention), are pathogens like methicillin-resistant *Staphylococcus aureus* (MRSA), *Enterococcus faecalis*, *Mycobacterium tuberculosis* and *Clostridium difficile* [1]. Bacterial infections with these so called superbugs or multi-drug resistant bacteria (MDR) are becoming a serious threat. Treating infections from MDR is a significant burden in the clinical environment and on the finance of the patients, families and the healthcare professionals [1]. The antibiotic crisis has also emerged from the rapid spread of antibiotic resistance, the misuse and the frequency of overused medications which puts into danger the efficacy of currently available antibiotics [2]. In order to control the MDR bacteria at a global level, novel approaches to the treatment of infections from pathogens are needed as well as better diagnosis, a thorough monitoring of prescriptions and prevention of transmitting infections. A greater collaboration between different involved parties to develop and invest into new agents that fight against the antibiotic resistance crisis is highly desirable. The crisis has been more alarming since there has been a decline and even a lack of development of new drugs by the pharmaceutical industries. This is because the process costs of development and production are high and not economically profitable, it is financially risky and the associated requirements for getting a drug in the clinic are very challenging [3, 4].

1.2 Pitfalls and solutions for the crisis of antibiotic resistance

1.2.1 The problem with the rise in MDR bacteria

While infections with MDR bacteria represent a serious threat on the life expectancy of humans, the general trend among clinicians and pharmaceutical industries is to routinely administer and develop broad spectrum antibiotics. As a consequence, a number of antibiotic-associated changes in the commensal population of the gut microbiota have been reported. Cotter *et al.* [5] investigated these changes by high throughput DNA sequencing and pointed asthma, allergies, atopic dermatitis, cholera, candidiasis fungus, and colitis induced by pathogens, eczema, autoimmune encephalitis as emerging antibiotic-associated diseases. Therefore, not only is the development of bacterial resistance a worrying problem associated with antibiotic overuse, but there is also an undefined collateral damage inflicted onto the beneficial microbial populations in the gut which are necessary as they contribute to the human health.

For instance, vancomycin, also known as the antibiotic of last resort, and metronidazole, are the principal antibiotics used for the treatment against infections with the clinically important gut pathogen *C. difficile*, which affects particularly the elderly, as the infections spread in hospitals and in care homes with an alarming increasing carriage rate [1]. It is generally understood that broad-spectrum usage of antibiotics induces diseases and these routinely used therapies result in perturbation of the microbiota, reductions of the functional barrier of the mucus layer, disruption in the intestinal homeostasis and in the integrity of the intestinal defence mechanism. Under normal conditions, the host is being protected against the invasion of pathogens or inflammation, however antibiotics cause additional dysbiosis in the distal colon and disturb the normal function of microbiota [1]. Similarly, antimicrobial resistance is also being triggered by the extensive use of the antibiotics by the farmers to keep the animals alive.

Consequently, a therapeutic agent, a therapy or new available strategies are expected to emerge and to be implemented in order to diminish the negative impact of antibiotics used to tackle the MDR bacterial infections [5].

1.2.2 **Solution for the antibiotic resistance crisis**

Several therapeutic alternatives for treatment of infections with gut intestina (GI) tract pathogens were reviewed by Mathur *et al.* [6]. One therapeutic strategy that could address the rapid emergence of antibiotic resistance of infectious pathogens is quorum quenching [7], which could be used to manipulate the virulence of gene expression and inhibit the process of quorum-sensing. This was successfully demonstrated in *in vivo* infection models with different pathogens such as *S. aureus* [7, 8]. Another possible strategy would be the use of faecal transplantation therapy for the treatment of GI tract diseases particularly from MDR *C. difficile* infections, phage therapy, and the use of prebiotics or probiotics [9, 10].

The discovery of new antibiotics to treat serious infections has been slowed down by a lack of tools used for the possible exploitation of the enormous amount of bacteria from natural environments, which can not be generally cultivated in the laboratory [11].

The best potent alternative to the negative impact and to the damage caused by the extensive overuse of broad spectrum antibiotics is the usage of the group of novel antibiotics, such as the so called class of bacteriocins, a highly diverse family of bacterial toxins. Bacteriocins are small antimicrobial peptides, ribosomally synthesised, which undergo extensive post-translational modification (PTM) and that can be tailored to control various pathogens. Bacteriocins are an attractive therapeutic alternative due to their relatively low toxicity, a narrow spectrum of antimicrobial activity and their efficient mode of action against MDR pathogens. Numerous bacteriocins are very active at nanomolar concentrations and they target a very narrow range of molecules [12, 13].

As an example, the beneficial effect of using of thuricin CD bacteriocin, from the sactibiotics family, was highlighted in a human distal colon model and it was compared to the use of the conventional antibiotics (vancomycin, metronidazole), and lacticin 3147 bacteriocin against infections with *C. difficile* [1, 14]. These infections usually cause fatal diseases and antibiotic induced diarrhoea which often result from the development of spores which are antibiotic-resistant. Interestingly, it was demonstrated that thuricin CD can eliminate *C. difficile* bacteria specifically from trillions of bacteria in a model gut system. Therefore, it should be universally accepted that the narrow spectrum antimicrobials represent the main source for the treatment strategies of the future. Bioengineering of bacteriocins can create new bacteriocins with increased stability, potency and with potentially superior properties of clinical interest [15].

A number of reviews highlight the advances in the research on bacteriocins such as the increasing interest for them to be used as a viable alternative to conventional antibiotics, their use as a novel way to address spore-related problems or the advances in the molecular and bioinformatics tools necessary for the discovery and development of novel bacteriocins as potent therapeutic agents [3, 12, 13, 16, 17].

1.3 Alternative agents to antibiotics - definition of current bacteriocins

The bacteriocin family comprises a diversity of proteins that vary in terms of size, microbial target, mode of action, release and immunity mechanism [18]. Bacteriocins are classified into two main groups: the toxins produced by Gram-negative bacteria and those produced by Gram-positive bacteria. It can be presumed that most bacterial genera produce one or more bacteriocins; the ones produced by Gram-positive bacteria, such as lantibiotics, have been the focus of much attention as a result of their potential commercial applications, including staphylococci and enterococci [19].

Two main features distinguish the majority of bacteriocins from classical antibiotics: they are protein based ribosomally synthesised molecules and they have a relatively narrow spectrum for cell killing [18]. Bacteriocins have unusual amino acids incorporated as a result of extensive post-translational modifications that they undergo. Bacteriocins are ubiquitous in nature and in recent years, they have been targeted for use in human health, veterinary medicine, and food preservation since they can be bioengineered to create pathogen-specific designer drugs [20-22] as it is further described in section 1.6.

1.4 Promising research in the field of bacteriocins

1.4.1 Unexplored resources and novel biotechnologies for the discovery of bacteriocins

Some bacteriocins are produced by bacteria with Generally Regarded As Safe (GRAS) status or food-grade bacteria. In contrast, it was reported that several Gram-positive pathogens are also producing lantibiotics including bacterial strains such as *S. aureus*, *Staphylococcus epidermidis*, *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Streptococcus mutans*, *Streptococcus uberis* and *E. faecalis* [16]. The advantages of using these compounds for the enhancement of virulence of the infectious pathogenic strains was reviewed by Daly *et al.* [19].

The discovery of new lantibiotics is facilitated by exploring unknown environments and by screening of microbes isolated from previously under scrutinized ecological niches. This is not only to tackle an urgent need to address the threat of antibiotic resistance but also because there has been a clear decline in the discovery of novel antimicrobials from the traditional sources. In general, fungi and actinomycetes have been so far the main source of antibiotics which are now clinically used.

Bibb *et al.* [23] have reviewed the work done on manipulating the production of antibiotics in actinomycetes. Actinomycetes are Gram-positive bacteria that are original from the soil or marine sources and they possess huge potential for the production of clinically important antimicrobial peptides. Almost half of all classes of antibiotics that are used clinically, around 50 different compounds, are derived from actinomycetes [23]. The development of genome sequencing facilitated the discovery of an increasing numbers of biosynthetic gene clusters that encode for natural products with useful and interesting structures and functionalities. For instance, microbisporicin is a potent bactericidal lantibiotic variant which is active against a wide range of Gram-positive bacterial pathogens, such as *S. aureus* and *Bacillus megaterium* as it inhibits the peptidoglycan synthesis by binding to lipid II. Microbisporicin is also active against methicillin-resistant and vancomycin-intermediate resistant strains of *S. aureus* and also against some Gram-negative species, which is unusual for a lantibiotic. Foulston *et al.* [24] reported the discovery of the novel biosynthetic gene cluster for microbisporicin produced by the actinomycete *Microbispora coralline* where the genes were identified using a genome scanning approach. The biosynthetic gene cluster was isolated from a *M.*

corallina cosmid library, cloned and characterised in order to elucidate the functions of individual genes and to understand the process of biosynthesis of this lantibiotic. Even if microbisporicin is already commercialised and it has been demonstrated to have superior efficacy in animal models of MDR infections compared with the drugs of last resort (linezolid and vancomycin), the knowledge gained from this study may ultimately enable the development of variants with improved clinical activity [24].

Several candidates of the lantibiotic class of peptides have been successful as novel treatments against infections with MDR: mutacin 1140 (MU1140) and microbisporicin (NAI-107) are in the late pre-clinical trials against Gram-positive bacteria; NVB302 is tested in a Phase I clinical trial for the treatment of infections with *C. difficile*, whereas duramycin has completed Phase II clinical trials to be used for the treatment of cystic fibrosis [25]. Nevertheless, novel methods to identify lantibiotics are needed, as specified below, since the more traditional methods for the discovery of lantibiotics (e.g.: by induction, production and identification) are time-consuming; the traditional methods are also inefficient since they also create a barrier for the rapid detection of novel antimicrobial peptides.

Next generation sequencing is an emerging and revolutionising method of making hundreds of new genome sequences publically available. Genome mining approaches can also facilitate the identification of new natural products and the subsequent elucidation of their biosynthetic pathways [26].

Novel bacteriocin gene clusters from the GI tract microbes have been identified *in silico* as part of the Human Microbiome Project, which reinforces the idea that the human gut microbiota is yet an unexplored but resourceful source for new antimicrobial peptides that could be active against infections from gut pathogens [27]. The human gut microbiota consists of approx. 100 trillion microbial cells and by taking advantage of the bioinformatics approaches and next generation sequencing technologies, bacteriocins could be used as therapeutic tools that would have a meaningful and beneficial influence on various aspects of human physiology such as the immune system, metabolism, absorption of nutrients etc. [27].

Reviews on the use of synthetic biology for bacteriocin discovery and bioengineering of lantibiotic-derived sequences have suggested to combine the genome mining procedures with heterologous expression systems and high-throughput screening which offers a promising strategy to elicit novel bacteriocins [28].

1.4.2 **Applications of bacteriocins**

Apart from their antimicrobial activity and their use to fight against infections with MDR bacteria, bacteriocins have been reported to be used in a number of other applications since they have a positive and beneficial influence on the health of the host organism. Several studies have suggested that bacteriocin producing strains can be used for their potential probiotic traits [29, 30] since they can modulate the gut microbiota [5, 31] and can be explored as promising candidates for future applications as clinical therapeutics [32, 33]. The role of the gut bacteria in the gut tract brain axis and the beneficial effects of bacteriocins have also been considered [34-37]. The use of the antibiotics in agriculture, food preservation and food safety applications has been reviewed [38].

1.4.3 **Bioengineering of bacteriocins**

Many bacterial species have developed antimicrobial defence systems as an ecological advantage to survive against competitors, leading to the production of ribosomally synthesised antimicrobial peptides known as bacteriocins as potential chemotherapeutic agents [39]. The widespread distribution of gene-encoded peptides reflects their importance as a line of defence against microbial attack since they are amenable to being genetically modified [40]. The class II bacteriocins are known to be used as enzyme inhibitors, anti-HIV agents [11, 41-43].

The unique nature of the bacterial cell wall means that it continues to represent an important target when screening for and developing new antimicrobial peptides. As a consequence, significant advances have been made in our understanding of the genetic determinants encoding bacteriocin production, their organisation and molecular mechanisms by which they mediate antibiosis.

In 1925, Gratia identified the first original antimicrobial protein produced by *Escherichia coli* (a Gram-negative bacterium) [44] termed ‘colicin’. Since then, bacteriocins have been one of the most extensively studied microbial defence system [40]. Bacteriocin genes are either chromosomally or plasmid encoded which makes them accessible for genetic manipulation. The resulting toxin protein employs a variety of killing mechanisms: cytoplasmic membrane pore formation, interference with cell wall synthesis, and nuclease activity [45, 46].

It should be noted that unlike antibiotics, bacteriocins are not used for medical purposes, therefore they are more commonly referred to as biological food preservatives. Table 1

summarises the main differences between antibiotics and bacteriocins in an attempt to emphasise that the latter are clearly distinguishable from clinical antibiotics [40].

Characteristic	Bacteriocins	Antibiotics
Application	Food	Clinical
Synthesis	Ribosomal	Secondary metabolite
Activity	Narrow spectrum	Varying spectrum
Host cell immunity	Yes	No
Mechanism of target cell resistance or tolerance	Usually adaptation affecting cell membrane composition	Usually a genetically transferable determinant affecting different sites depending the mode of action
Interaction requirements	Sometimes docking molecules	Specific target
Mode of action	Mostly pore formation, but in a few cases possibly cell wall biosynthesis	Cell membrane or intracellular targets
Toxicity/side effects	None known	Yes

Table 1 - Bacteriocins vs. antibiotics as taken from Cleveland *et al.* [40].

Bacteriocins are natural compounds; some are found in the fermented food available on the market. They are inactivated by enzymes, such as trypsin and pepsin, found in the GI tract and therefore, they do not alter the microbiota of the digestive tract. As a consequence, bacteriocins are not considered antibiotics; otherwise, they may not be used in human food, since the use of antibiotics in food is illegal [12]. The only bacteriocin considered by the Food and Agriculture Organization (FAO) as GRAS is nisin and it is used as a food additive. Nisin is often added to foods such as cheese to prevent the growth of pathogens like *Clostridium* and *Listeria*. Nisin inhibits the outgrowth of *Clostridium botulinum* spores in cheese spreads [40] and it is approved as a food additive in the United States for this purpose and it is also used as a food preservative worldwide including in the United Kingdom.

The search for alternative drugs that inhibit cell wall synthesis has led to the discovery of various lantibiotic peptides which can inhibit bacterial cell wall biosynthesis by binding to the essential precursor of peptidoglycan, lipid II. Interestingly, it has been shown that unlike the docking motif of vancomycin, the lantibiotic nisin can bind to the pyrophosphate group of lipid II, a highly conserved chemical group [15]. As a result of this observation, a number of assumptions have been made: firstly, since lipid II is an essential and invariable region, the development of bacterial resistance is massively decreased; secondly, because it is gene encoded and may be more amenable to

derivatisation, nisin could act as a template for the design of novel drugs with structural features that promote high-affinity binding to lipid II or to other antimicrobial targets [3]. Therefore, by taking advantage of the available tools and knowledge, it is now possible to perform genetic engineering of bacteriocins which have been precisely tailored to target either a wide range or specific number of pathogens that are involved in causing infectious diseases [18].

The defining structural elements of bacteriocins offer not only structural and biological function to these peptides but also they make bacteriocins increasingly resistant to proteolysis from internal proteases as well as they offer tolerance to oxidation [12]. Therefore, the enzyme-mediated post-translationally modifications (PTM) introduced in bacteriocins, allow bacteriocins to particularly exhibit various mechanisms of action compared to currently used chemotherapeutics [15].

Several desirable features in bacteriocins which are introduced by PTM, such as the unusual rings and amino acids, gives them the potential to be used in various applications such as pharmaceutical, biochemical, agricultural, food industries as well as both in human and veterinary medicine. Nevertheless, the subclass of bacteriocins described above, the lantibiotic peptides possess limitations for routine and widespread use. Some examples are the instability and insolubility at physiological pH and the predisposition to being degraded by intestinal proteases which prompted the need to bioengineer lantibiotics with higher potency and stability [47].

1.5 GI tract solution to fix a GI tract problem

Infections with the GI tract pathogen *C. difficile* have increased dramatically in the past years and the efficacy of standard therapies is decreasing. Rea *et al.* [1, 14] have considered the potential of bacteriocins produced by the GI tract bacteria, probiotics as well as phages to function as antimicrobial agents for the treatment of *C. difficile* infections in the human GI tract. O'Conner *et al.* [48] have reported the discovery of a novel nisin H variant produced by a GI tract strain of *Streptococcus hyointestinalis* DPC6484 which inhibits a range of Gram-positive bacteria such as listeria, enterococci, bacilli, streptococci and staphylococci. Marcille *et al.* [49] described the discovery and the analysis of two new lantibiotics, trypsin-dependent, ruminococcin A and ruminococcin C, derived from the commensal bacterium from the human intestinal microbiota, *Ruminococcus gnavus* E1 and which have anti-*Clostridium perfringens* activity [50, 51]. O'Shea *et al.* [52] have also reported the discovery of a novel bacteriocin from a porcine intestinal isolate, *Lactobacillus salivarius* DPC6502, and investigated its biological activity against *S. aureus* and *Listeria monocytogenes* pathogenic species. A similar study on bactoferin A [53] has indicated that it is a broad spectrum bacteriocin and that it could be used to manipulate the gut microbiota to benefit the human health especially as it may have a positive impact on bacterial anaerobic population of *Bacteroides*, *Clostridium* and *Bifidibacterium spp*. Interestingly, *Lactobacillus salivarius* strains isolated both from porcine and human GI tract were shown to produce multiple bacteriocins from a single locus using the same biosynthetic machinery which explains its ability to compete and dominate within the complex ecosystem of the microbiota [54]. The above mentioned studies highlight the diversity and the dynamic environment of the microbiota. The GI tract represents a huge and yet unexplored environment for bioactive antimicrobial peptides. These bacteriocins do not only represent a probiotic trait for the producer intestinal strains, as they fight against infections and signal the immunity system, but they also have the potential to control gut bacterial populations in order to exert a beneficial effect on the human health [53].

1.6 Lantibiotics - gene cluster, structure and biosynthesis

1.6.1 Definition of lantibiotics

Classification of bacteriocins is still under review; their definite classification has proved difficult due to their diversity [55, 56]. While some schemes agree on the two main classes, class I (lantibiotics) and class II (unmodified peptides), other schemes have proposed four classes [57, 58]. Table 2 illustrates the considerable diversity among bacteriocins and in particular among lantibiotics [59].

Category	Characteristics and subcategories	Bacteriocins (group representatives)
Class I. Lantibiotics	Heat stable peptides, encoded by a structural gene (generically named *lanA*). Ribosomally synthesized peptides that undergo PTM. Molecular weight <10 kDa. Contain lanthionine (Lan) and β-methyl lanthionine (MeLan) bridges that give lantibiotics their characteristic conformations and stability.	
	Type A: Flexible, elongated molecules.	nisin, subtilin, gallidermin, epidermin, microbisporicin
	Type B: Globular molecules with no net charge or net negative charge.	Mersacidin, actagardine, cinnamycin, duramycin
Class II. Small heat stable bacteriocins	Single or two-peptide bacteriocins.	Pediocin PA-1, sakacins A, P, leucocin A, carnobacteriocins, lactococcins G and F, lactacin F, plantaricin EF and JK
Class III. Large heat-labile bacteriocins	Heat-labile proteins. Molecular weight >30 kDa.	Helveticins J and V-1829, acidophilucin A, lactacins A and B
Class IV	Complex bacteriocins carrying lipid or carbohydrate moieties.	Venezuelin

Table 2 - Classification of bacteriocins [56, 59-62].

The alignment of the sequences reveals a number of conserved amino acids stretches that are present both within and across subclasses that may represent functional domains. The name of these subclasses reflects the prototypical lantibiotic in each case and a few examples of lantibiotics are illustrated in Figure 1 [38].

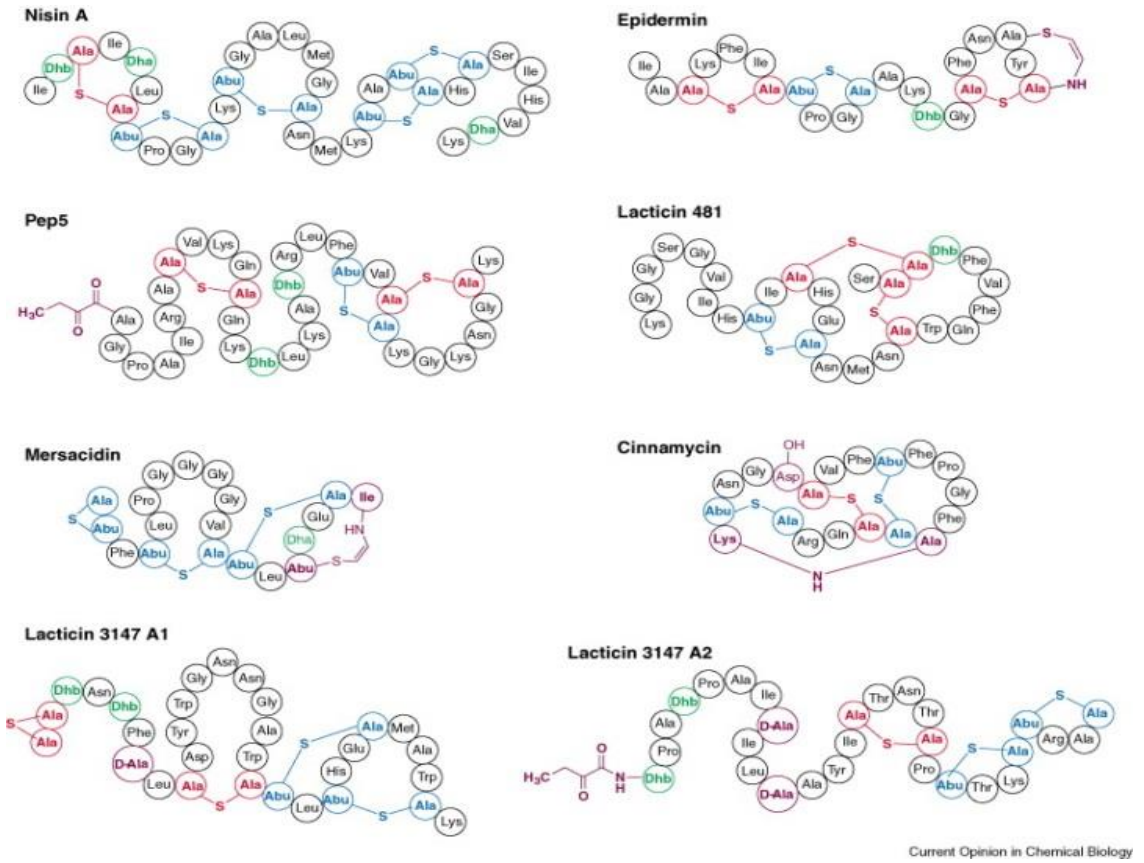


Figure 1 - Structures of representative members of the seven subgroups of lantibiotics taken from Xie *et al.* [63].

Abu, 2-aminobutyric acid; Ala-S-Ala: lanthionine - Lan (red); Abu-S-Ala: 3-methyllanthionine MeLan (blue); Dha, dehydroalanine and Dhb, dehydrobutyrine (green). All other post-translational modifications including Asp-OH, β-hydroxy aspartate, are shown in purple.

According to the classification of bacteriocins, lantibiotics are type A bacteriocins characterised by lanthionine (Lan) and methyl lanthionine ((Me)Lan) bridges that give lantibiotics their characteristic conformations and stability [60]. Lantibiotics are produced by a range of Gram-positive bacteria including lactic acid bacteria (LAB), *Streptomycetes* and *Clostridia*. *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Streptococcus*, and *Carnobacterium* are among several LAB genera, which are capable of producing small antimicrobial peptides that inhibit a broad range of Gram-positive bacteria [40, 57].

Many different types of lantibiotics have been identified and characterised especially the ones produced by LAB [58, 64] (Figure 1). The industrial importance of the LAB is evidenced by their GRAS status, due to their ubiquitous appearance in food and their contribution to the healthy microbiota of human mucosal surfaces. LAB are rod-shaped bacilli or cocci characterised by an increased tolerance to a lower pH range. This aspect partially enables LAB to outcompete other bacteria in a natural fermentation process, as they can withstand the increased acidity from organic acid production (e.g. lactic acid).

Even if lantibiotics have been extensively studied for their application in foods, only a few of them have been used in livestock or considered as viable to treat human infectious diseases [16].

The biosynthesis of lantibiotics from Gram-positive bacteria is self-regulated with dedicated transport mechanisms that facilitate their release [65]. Their most common mode of action involves pore formation in the cell membrane and dissipation of the proton motive force. Lantibiotics can inhibit the growth of Gram-positive bacteria, whereas the Gram-negative bacteria are protected from their lethal effect by the presence of an outer membrane [17, 31]. Gram-negative infectious bacteria responsible for human, animal or plant diseases, such as *Aeromonas*, *Escherichia*, *Xanthomonas*, *Erqinis*, and *Pseudomonas*, can be sensitive to lantibiotics if they are coupled with chelating agents. Consequently, the potential use of lantibiotics and their derivatives is now being extensively investigated in order to inhibit these pathogens.

1.6.2 Structural features of lantibiotics

Lantibiotics have a much higher specific activity compared to the vast class of bacteriocins. Their antimicrobial activity against sensitive strains can be detected at picomolar to nanomolar concentrations, although the action spectra are often narrow at these concentrations [59]. In the context of fermentation, important targets include spoilage organisms such as species of *Clostridium*, *Lactobacilli* and foodborne pathogens including *L. monocytogenes*, *Staphylococcus* spp., *Enterococcus* spp. and *Bacillus* spp. [66]. Some lantibiotics are characterised by a high content of uncommon amino acids which form ring structures also known as thio-ether cross-links (Figure 2) [63]. Enzyme-mediated PTM are responsible for converting the ribosomally synthesized, inactive and immature prepeptides into peptides that contain the characteristic features of lantibiotics. The maturation of lantibiotics takes place via two main reactions to form thio-ether cross-links: serine and threonine amino acids in the core region are dehydrated and sulphur from the cysteine residues are added onto the resulting double bonds of the dehydrated residues [15, 67]. Since the gene clusters involved in the biosynthesis of lantibiotics have been sequenced, the PTM have been the focus of extensive research [63, 68].

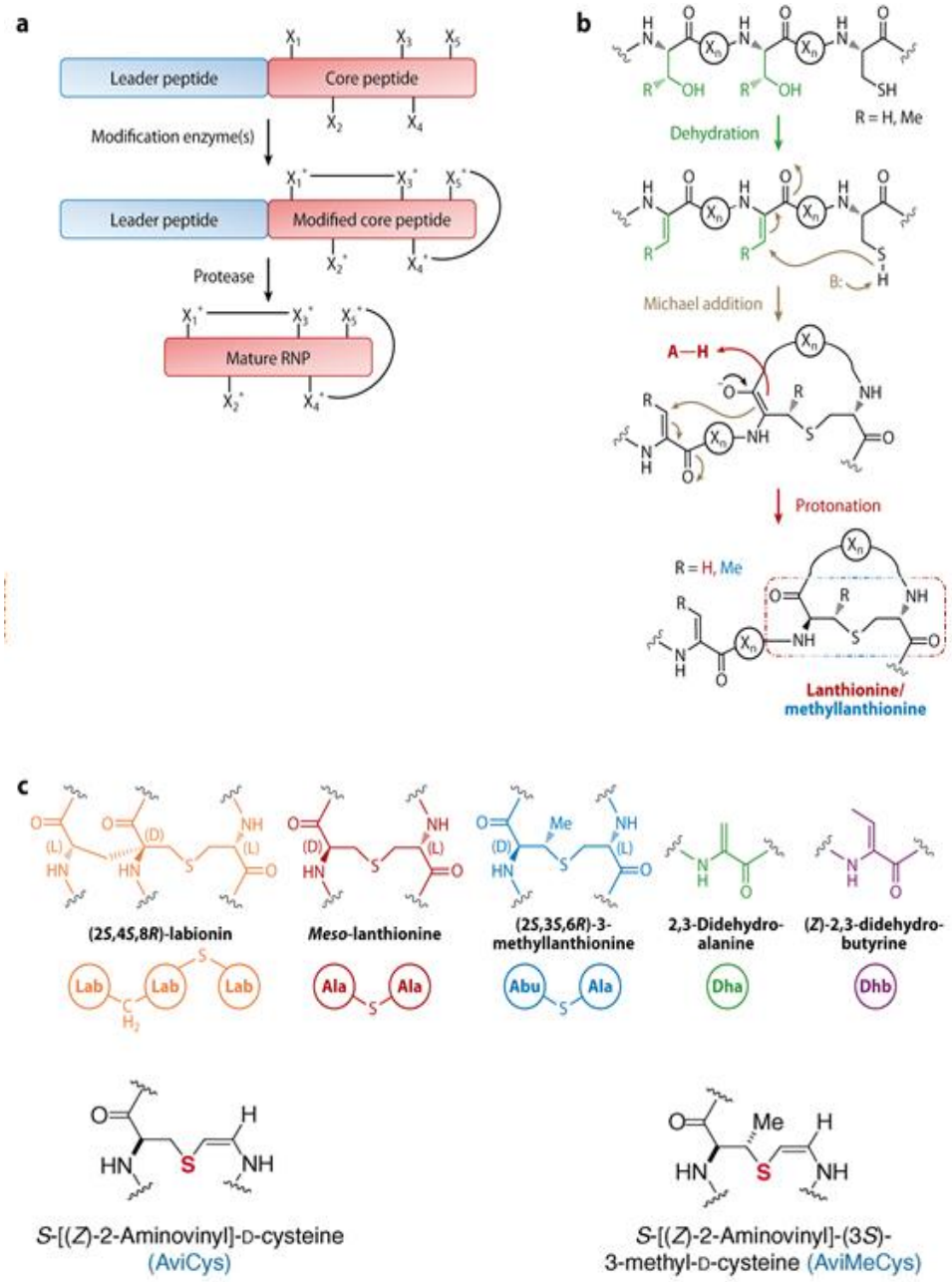


Figure 2 - Post-translational modifications in lantibiotics.

(a) General scheme of the biosynthesis of ribosomally synthesised peptides; Xn* indicates a modified residue, RNP means ribosomally synthesized natural products.

(b) Mechanism of thio-ether cross-links formation.

(c) Examples of structural motifs characteristic to lantibiotics. Abu, 2-aminobutyric acid; Dha, 2,3-didehydroalanine; Dhb, (Z)-2,3-didehydrobutyrine; Lab, labionin. As taken from Xie *et al.* [63], Knerr *et al.* [62].

1.6.3 Lantibiotic gene clusters, biosynthesis and mode of action

All the necessary genes for the biosynthesis of a lantibiotic peptide are located on a gene cluster (operon). A number of genes have been identified, including genes encoding the precursor peptide, enzymes responsible for specific amino acid modifications, proteases able to remove the leader peptide, ABC-superfamily transport proteins involved in lantibiotic translocation, regulatory proteins controlling lantibiotic biosynthesis and immunity proteins that protect the producing strain from the action of its own lantibiotic [63]. Some examples of the gene clusters for lantibiotics are illustrated in Figure 3.

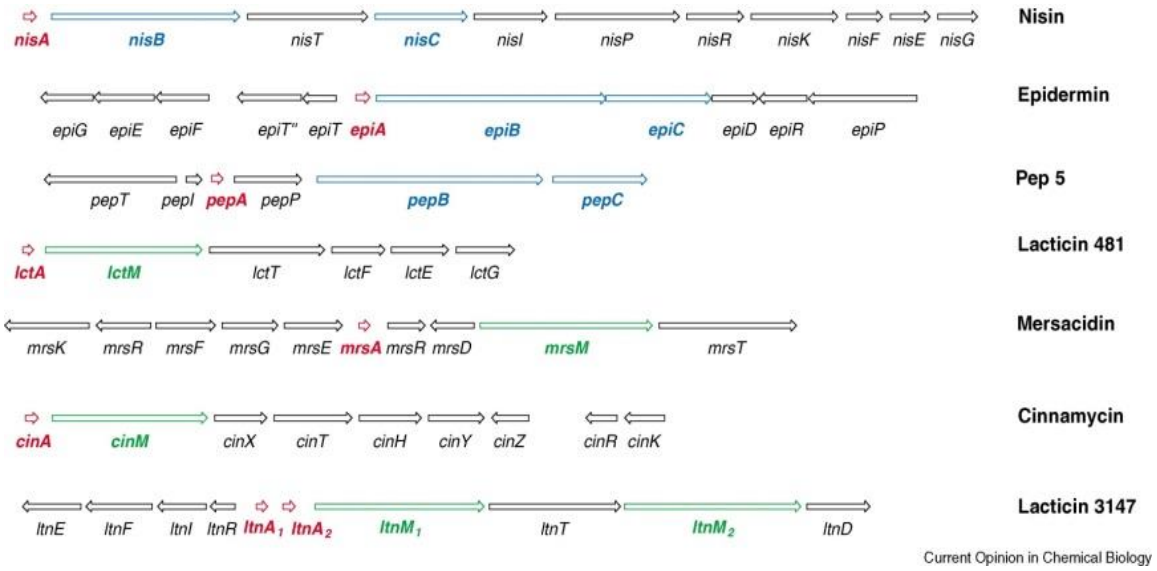


Figure 3 - Biosynthetic gene clusters of the lantibiotics as taken from Xie *et al.* [63].

The genetic markers for lantibiotics have been discussed in great detail in various reviews [56, 58, 60, 69, 70]. The genes for the production of active lantibiotics are usually found on operon clusters and have been well studied. Most characterised lantibiotic operons belong to type A and many lantibiotic homologous genes are found among the sequenced lantibiotic gene clusters [13, 25]. The colours indicate the functions of *lan*.

The biosynthetic gene clusters for these highly modified lantibiotic peptides are localised either on the bacterial chromosome or are encoded on mobile elements such as plasmids or transposons [71-73]. The biosynthetic genes have been given the generic locus symbol *lan* and their products are designated by their functions such as structural (LanA), modification (LanB and LanC or LanM), processing (LanP, LanT), transport (LanT), immunity (LanI, LanH, LanFEG), and regulation (LanR and LanK) systems; the functions are indicated by colours in Figure 3. Some lantibiotics may have extra genes (*lanQ*, *lanX*) that are involved in regulation (Figure 3, Figure 4) [60, 69, 74-76].

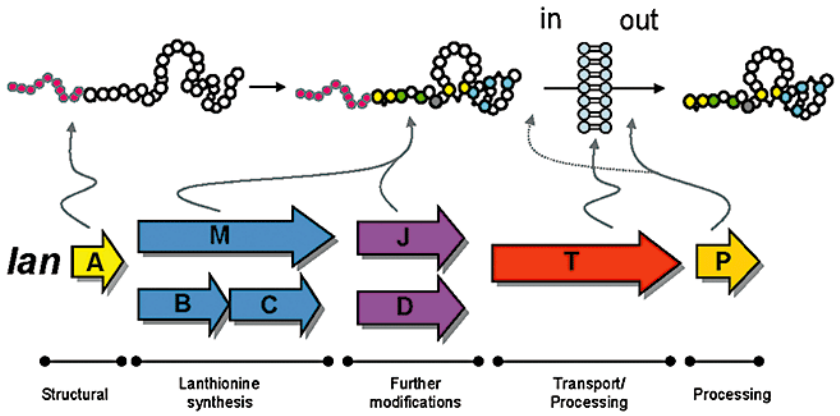


Figure 4 - Lantibiotic biosynthesis taken from Field *et al.* [13, 15].

The precursor peptide (LanA) is ribosomally produced from the structural gene, *lanA*. Serine, threonine and cysteine are three amino acid residues found in the core region that are subject to the modification reactions to form the characteristic structures of lantibiotics: lanthionine (Lan) or methyl lanthionine (MeLan). In the case of nisin, a type A lantibiotic, these modifications are carried out by NisB and NisC enzymes via a two-step route [13, 77] (Figure 5). For type B lantibiotics (e.g. lacticin 481), a bifunctional enzyme, LanM, performs both reactions. Further modifications lead to the formation of other modified amino acids which are present in different lantibiotics. For example, D-alanines (LanJ) or S-aminovinyl-cysteine (LanD) [21]. Type A peptides are then exported out of the cell via a dedicated transporter (LanT), followed by proteolytic removal of the leader sequence by LanP which can be located inside or outside of the cell. The NisP extracellular protease, found in the nisin cluster, removes the leader peptide and activates the lantibiotic followed by the export of the core peptide performed by the ABC-type transporter NisT [13]. In the case of class II lantibiotics, a multifunctional LanT performs both roles even if the cleavage of the leader before or after the export is not fully understood. In both cases the result is the production of the active peptide.

Lantibiotics are synthesised as biologically inactive precursor prepeptides consisting of an N-terminal leader peptide attached to the core region [74]. The leader peptide may function to maintain the peptide in a biologically inactive form during the maturation process until export. It also serves as a recognition sequence for the modifications and transport enzymes by facilitating and promoting the interaction with these precursor proteins [63, 78].

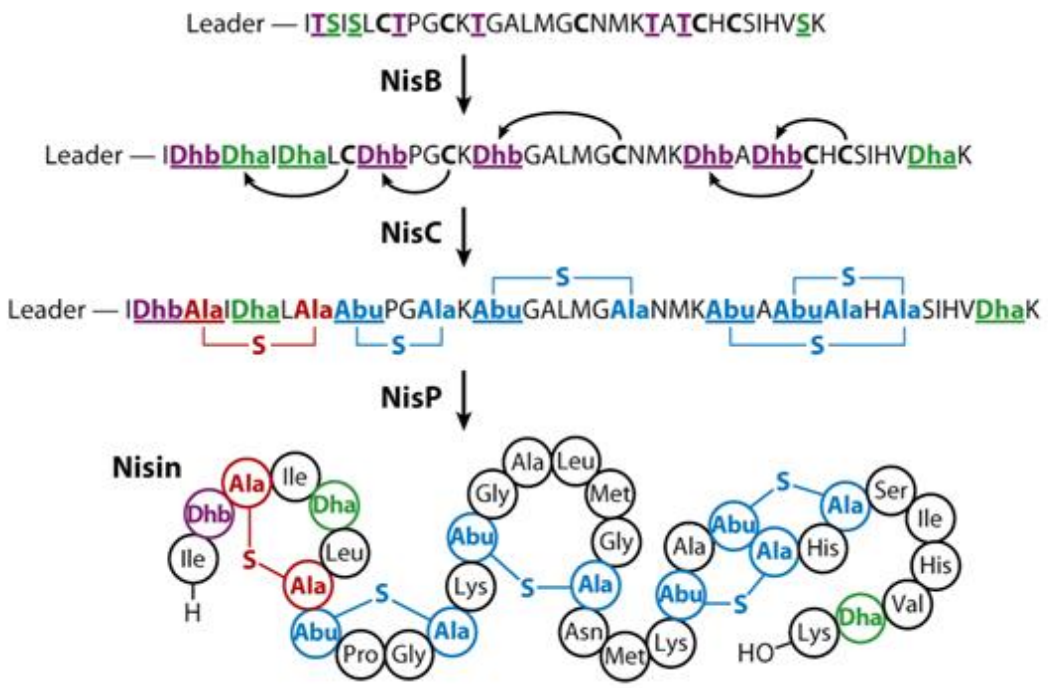


Figure 5 - Illustration of the biosynthesis of nisin as taken from Knerr *et al.* [62].

The inactive precursor peptide contains a leader peptide that guides both the modification and transport machinery. The steps for PTM are highlighted: Ser and Thr dehydration (green) by NisB to give dehydroalanines (Dha) and dehydrobutyrines (Dhb) (orange). The NisC catalysed cyclization of Dha and Dhb with Cys residues in the C-terminus which results in the formation of lanthionine rings (Lan is Ala-S-Ala, MeLan is Abu-S-Ala). Post-translationally modified and mature nisin is exported by NisT outside the cell and the prenisin precursor peptide is activated by NisP, to give leaderless and active nisin. Rings A to E are annotated from the N-terminus and Abu is the aminobutyric acid.

The structural organisation of nisin (Figure 5) allows it to have dual activity by using lipid II as a docking molecule for subsequent pore formation [21]. Transglycosylation, necessary for the formation of peptidoglycan, is inhibited because of the ability of nisin to bind lipid II. Following the binding of lipid II, the second function of nisin is to facilitate pore formation, which results in cell death by the rapid efflux of small cytoplasmic compounds and the dissipation of vital ion gradients [79-81]. It should be noted that lipid II is also the target of the type B lantibiotic mersacidin and actagardine as well as the glycopeptide antibiotics vancomycin, teicoplanin, ramoplanin, and the mannopeptimycins [16, 80]. Early studies indicated that the cationic nature of nisin allows binding to anion phospholipid bilayer through electrostatic interactions [82].

1.6.4 **Immunity and regulation**

In order to maintain a balance between nisin biosynthesis and immunity, the process of nisin production is regulated by a two-component system comprised of *nisRK* genes which encode for a sensor receptor histidine kinase, NisK, and a transcriptional response regulator, NisR.

The mature nisin acts as other peptide pheromone involved in activation of its own biosynthesis via a two-component signal transduction machinery composed of NisK and NisR. Extracellular changes detected by NisK initiate a signal cascade that triggers the response regulator to activate the transcription of the regulated genes. These regulated genes may include the structural gene, the export genes, the immunity genes, and occasionally, the regulatory genes themselves [74]. Some lantibiotic producers autoregulate their own synthesis and operate as quorum sensing molecules allowing cells to sense other organisms in the environment in a cell density-dependent manner [83].

In order for the producing strain to synthesise the antimicrobial peptides, it has to be immune to the action of its own product. An efficient self-protection system is vital for the survival of the lantibiotic-producing bacterial strain against nisin, therefore, immunity is maintained by two systems: a lipoprotein NisI and an ABC transporter, NisFEG which expels nisin from the membrane of the producing strain [76]. As aforementioned, nisin producers also encode transport systems as a second mechanism of immunity; in some cases, the bacteriocin is transported out of the cell, lowering the concentration of the antimicrobial peptide below an inhibitory threshold (Figure 6) [59].

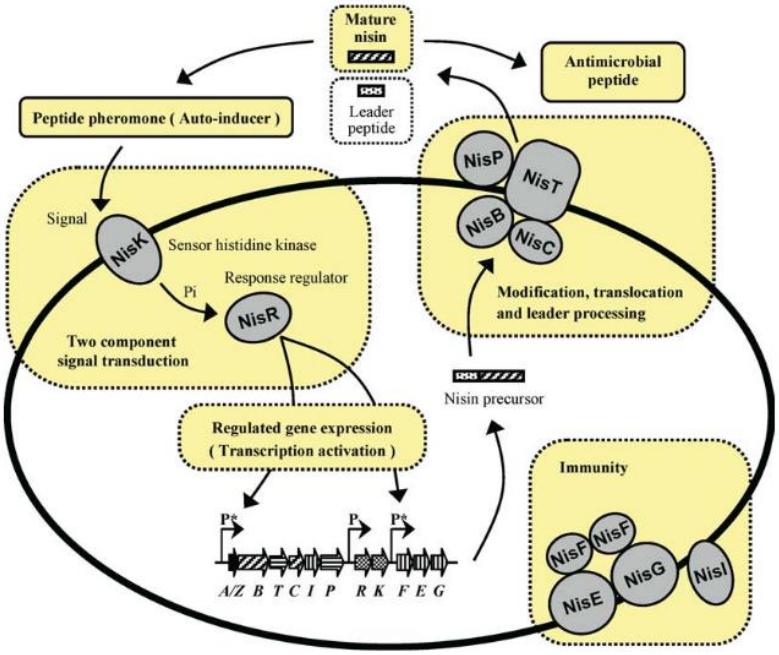


Figure 6 - Schematic representation of nisin biosynthesis and regulation in *L. lactis* taken from Cheigh *et al.* [84].

1.6.5 Resistance to nisin

The main target of nisin in sensitive strains is the cytoplasmic membrane where it binds to the lipid II and gets incorporated to form ion channels or pores through which efflux of ions, ATP and amino acids results in destruction of the membrane potential. It has been shown that nisin-resistant strains are likely to arise. Many Gram-positive bacteria were shown to be resistant to nisin due their ability to synthesise nisin-specific proteases, termed nisinases, which could inactivate nisin [81].

The ability of some pathogens to develop resistance to nisin is clearly an obstacle to the development of new food or therapeutic applications. Nisinases are specific to nisin-resistant strains and are produced by several species, including *Bacillus cereus*, *B. megaterium*, *B. polymyxa*, *S. aureus*, *Streptococcus agalacticae*, *Strep. thermophilus*, *Lactobacillus plantarum* and *E. faecalis*. Although the presence of these enzymes was reported in several bacterial strains, a conclusive study indicating the presence of nisinases in *L. lactis* is still missing [85]. Nisin resistance of these aforementioned strains was reported to be conferred by a specific nisin resistance gene (*nsr*), which is located on a 60-kb lactococcal plasmid, pNP40, and encodes a 35-kDa nisin resistance protein (NSR). These genes have already been cloned and sequenced but their mode of action has not been elucidated yet. An additional mechanism of immunity or resistance of these strains, not genetically linked to production of nisin, was also reported in which a nisin

immunity gene was linked to lactoccocal plasmid DNA and it was associated with phage resistance [85].

It has been shown that resistance to nisin is not dependent on the level of lipid II content in the cell membrane; a number of protoplasts containing different amounts of lipid II in their membrane were used to show that their membranes were equally sensitive to nisin [86]. Therefore, it was assumed that the development of nisin resistance among Gram-positive bacteria was connected to changes in the cell wall structure architecture which is the most plausible explanations for acquired nisin resistance [85].

1.7 Natural nisin variants

Mature nisin is an antimicrobial peptide consisting of 34 amino acids residues of which 13 are post-translationally modified resulting in pentacyclic peptide and it is typically produced by *Lactoccocus lactis* strains [87]. Although many lantibiotics have been shown to be effective against pathogens and spoilage bacteria, nisin is probably the best characterised lantibiotic and is the only antimicrobial peptide approved for use in food in its partially purified form [80]. Nine natural derivatives of nisin (nisin A, nisin Z, nisin Q, nisin F, nisin U and U2, nisin H, nisin P, and a distantly related nisin-like lantibiotic salivaricin D) have so far been identified (Figure 7) [48]. Nisin displays four different activities: 1. it autoinduces its own synthesis, 2. it inhibits the growth of target bacteria by membrane pore formation, 3. it inhibits bacterial growth by interfering with cell wall synthesis, 4. it inhibits the outgrowth of spores [88].

Nisin is amphiphilic in nature: the N-terminal region includes many hydrophilic residues which interact with phospholipid head-groups, whereas the C-terminal region has more hydrophilic residues as shown by NMR studies [89]. The hydrophilic and hydrophobic residues are located on opposite sides of the molecule as a consequence of the conformational restrictions imposed by the lanthionine rings [87].

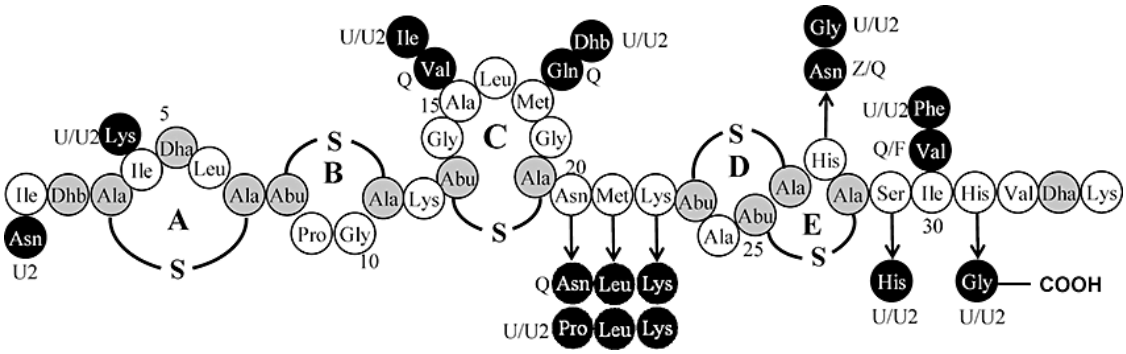


Figure 7 - Structures of natural variants nisin A and nisin Z and putative structures of variant nisins Q, U and U2 as taken from Field *et al.* [87].

In black, amino acid differences between the natural nisin variants. In grey, post-translational modifications. Dha, dehydroalanine; Dhb, dehydrobutyrine; Abu, 2-aminobutyric acid; Ala-S-Ala, lanthionine; Abu-S-Ala, 3-methyllanthionine.

Nisin derivatives are mainly composed of two structural domains: the N-terminal domain (Rings A, B, C) is linked to the C-terminal domain (Rings D, E) by a flexible hinge region (positions 20, 21, 22). The unique mode of action as described by Field *et al.* [87] makes nisin highly potent at nanomolar concentrations against many Gram-positive bacteria. NMR analysis of the nisin-lipid II complex reveals that Rings A and B of nisin bind to the pyrophosphate moiety of lipid II with a hydrogen bond. On the basis of these findings, the residues in the hinge region or the residues in Rings A and B have been mainly targeted for mutagenesis [13].

1.8 Bioengineering of nisin

The biosynthesis of lantibiotics of type A seems to be a complex process. This makes it difficult to make changes in the sequence of the prepeptide (inactive form of the lantibiotic) and to predict the effect on the biosynthetic pathway. Even if some studies have given hints for the function of individual amino acids, there is a lack of knowledge in terms of the effect of individual or multiple mutations in the nisin structure. So far, little is known about the residues important for the interaction with the modification, export and processing systems and in the regulation of expression. *In vitro* bioengineering of novel compounds, in combination with a defined set of guidelines predicting where permissive modifications can be applied, may allow to incorporate thio-ether bridges in addition to or to substitute for more labile cysteine disulphide bridges [16]. These developments could ultimately permit the rational design and assembly of more constrained peptide configurations with enhanced stability in natural or synthetic peptides. In summary, current knowledge of *in vitro* and *in vivo* based technologies may

enable researchers to not only appropriately modify lantibiotics but also introduce amino acids and modifications not typically encountered in nature as part of the efforts to generate peptides with enhanced structural features.

In vivo engineering of lantibiotics readily generates peptides and enables the rapid use of manipulated genes and (host) organisms. In the case of nisin, a mutated *nisA* gene can be expressed in the original producer strain by replacement of the original structural gene. An alternative strategy is the heterologous expression of a biosynthetic gene cluster; this provides a simple approach for constructing and producing the large number of structural analogues required for further studies in the structure-activity relationship of lantibiotics [90]. With the gene replacement systems described for nisin [91-93] and subtilin [83], the only alterations to the genome are the specific mutations introduced in the structural gene. Random mutagenesis also seems a powerful approach for generating lantibiotics with desired properties, provided that an adequate and sensitive phenotypic screening method is available [87].

Dodd *et al.* [94] performed gene replacement experiments for the construction and testing of a parent strain (FI7990 expression host) followed by transformation of a nisin cluster in *L. lactis* strain; this was done to substitute a chromosomal sequence for an equivalent region which resulted in very low levels of expression/ frequency of mutants exhibiting specific nisin antimicrobial activities when mutations were done on a chromosome. In a later study, Dodd *et al.* [95, 96] reported the use of a developed expression-system that allows the exclusive production of novel nisin variants which are encoded by mutated prenisin obtained by the site directed mutagenesis of the *nisA* genes.

The original study indicated the presence of a reduced level of immunity which was explained by the fact that the strains which acquired a *nisA* gene by gene replacement may not have recovered full immunity before the nisin molecule exerted the antimicrobial activity, hence their growth was impeded. To improve this protocol and to recover the nisin immunity, Dodd *et al.* [95] included an addition of nisin (10 pg/ml) to the GM17 agar plates to select for those colonies which were immune to this level of nisin. A similar approach to the one described above was being taken in this PhD project.

Large numbers of nisin mutants have already been generated and characterised [12]. Single or multiple mutations have resulted in different effects on antimicrobial activity, physical properties such as solubility or (thermo)stability when the mutants were tested against a selected and a limited number of target strains [38]. All these studies have

provided a wealth of information on fundamental aspects of nisin biosynthesis, secretion and processing and have started to bring light on the immunity and the effect of *in vivo* and *in vitro* modifications of nisin. In early studies, several nisin mutants were generated by site-directed mutagenesis [13]; the active peptides were present in the supernatant of the cultures where processes such as unusual amino acid formation, transport and processing of the leader peptide were part of the normal biosynthetic pathway. Among the numerous bioengineered nisin variants generated to date, only a few of them have exhibited improved functionality such as higher antimicrobial activity against some indicator strains. Notable examples are: Asn27Lys and His31Lys mutants of nisin Z which have increased solubility at a neutral pH without changing their antimicrobial activity; the Thr2Ser mutant of nisin Z displayed increased activity; Asn20Lys and Met21Lys mutants of nisin Z showed slightly decreased activity against Gram-positive bacteria, but have improved activity against several Gram-negative bacteria [93].

In a more systematic approach, Field *et al.* [13, 16] have taken advantage of *in vivo* engineering to construct and screen for highly potent nisin variants. By using random (site saturation) mutagenesis in the hinge region of nisin A, a number of variants were successfully engineered with enhanced antimicrobial activity: Ans20Pro, Met21Val and Lys22Thr mutants of nisin A which displayed enhanced antimicrobial activity against pathogenic bacteria such as *L. monocyto*genes and *S. aureus* compared to the wild type nisin peptide [16, 88]. Further random-based mutations of these amino acids generated nisin variants with increased antimicrobial activity. In contrast, *in vitro* engineering is not suitable for the generation of libraries containing huge numbers of variants; instead, it can be used for more drastic engineering by combining biological and chemical approaches [90].

Several studies have reported the bioengineering of novel nisin mutants with improved physico-chemical properties and biological activity compared to nisin [15]. Even if nisin has been approved by FDA and it is used as a food preservative worldwide for a number of years (GRAS status), nisin is not stable under physiological conditions [47]. Bioengineering of nisin could help to create a nisin mutant that can be used as a therapeutic agent in a clinical setting in the future. If such a nisin mutant is developed which is effective at physiological pH such as for lacticin 3147 lantibiotic [32], the novel compound could have considerable potential for biomedical applications including the fight against GI tract infections from MDR pathogens.

1.9 Background to previous work on the project

Earlier work in the group involved screening of the gut microbiota to identify and characterise novel gene clusters that may encode bacteriocins with antimicrobial activity. A PCR based genome mining approach to identify a novel gene cluster encoding lantibiotics in a strain of *Blautia obeum* A2-162 isolated from the human GI tract was used previously by Hatziioanou [97]. The predicted ClosA1 peptides were nisin-like lantibiotics with more than one *lanA* gene (type A lantibiotics). All the four *closA* genes have GG type leader peptides typical of type B lantibiotics. Nisin has a PR type leader peptide sequence. However, this is an example of a type A lantibiotic containing type B (GG) leader peptide sequences. So far, the structures of these potential types A lantibiotics and their antimicrobial activity have not been fully characterised (Figure 8).

The *clos* gene cluster encoding putative lantibiotics of the *Blautia obeum* A2-162 strain was sequenced and lantibiotic *clos* gene functions were investigated by heterologous expression in *L. lactis.* Attempts to clone different designed constructs into the nisin inducible pUK200 vector using initially *E. coli* followed by *L. lactis* indicated that the genes may be lethal for *E. coli*. It was also found that members of the *Blautia obeum* A2-162 gene cluster showed some cross-reactivity with the nisin machinery (Hatziioanou *et al.*, in preparation). Attempts to induce antimicrobial production in liquid culture, purify and characterise the active peptides were not successful. Also, attempts to induce antimicrobial production using nisin A in strain *Blautia obeum* A2-162 were unsuccessful after growth in the presence of 100 ng/ml of nisin A.

In her work, Hatziioanou [97] did show that the novel gene cluster can be subcloned and expressed in *L. lactis*; despite complementation with the *closA* gene on an inducible plasmid, the expression of mature biologically active ClosA peptides was not possible. Hatziioanou also tried to express the *clos* gene cluster in *L. lactis* MG1614 and to use the nisin A biosynthetic machinery to modify and to produce active ClosA peptides. Even if cloning of the novel *clos* operon in the nisin system of *L. lactis* was successful, the nisin machinery may have not expressed, modified and released active Clos antimicrobial peptide since none of these steps were demonstrated in Hatziioanou's work [97]. A possible explanation was that the unique ClosA peptides may have not been modified due to the lack of a protease capable of cleaving the leader peptides from the modified and mature core peptides. A protein the size of the calculated ClosA prepeptide which

hybridised to the antiClos leader antibody was however detected but it was not confirmed by MS. This could further explain the absence of a fully active Clos peptide.

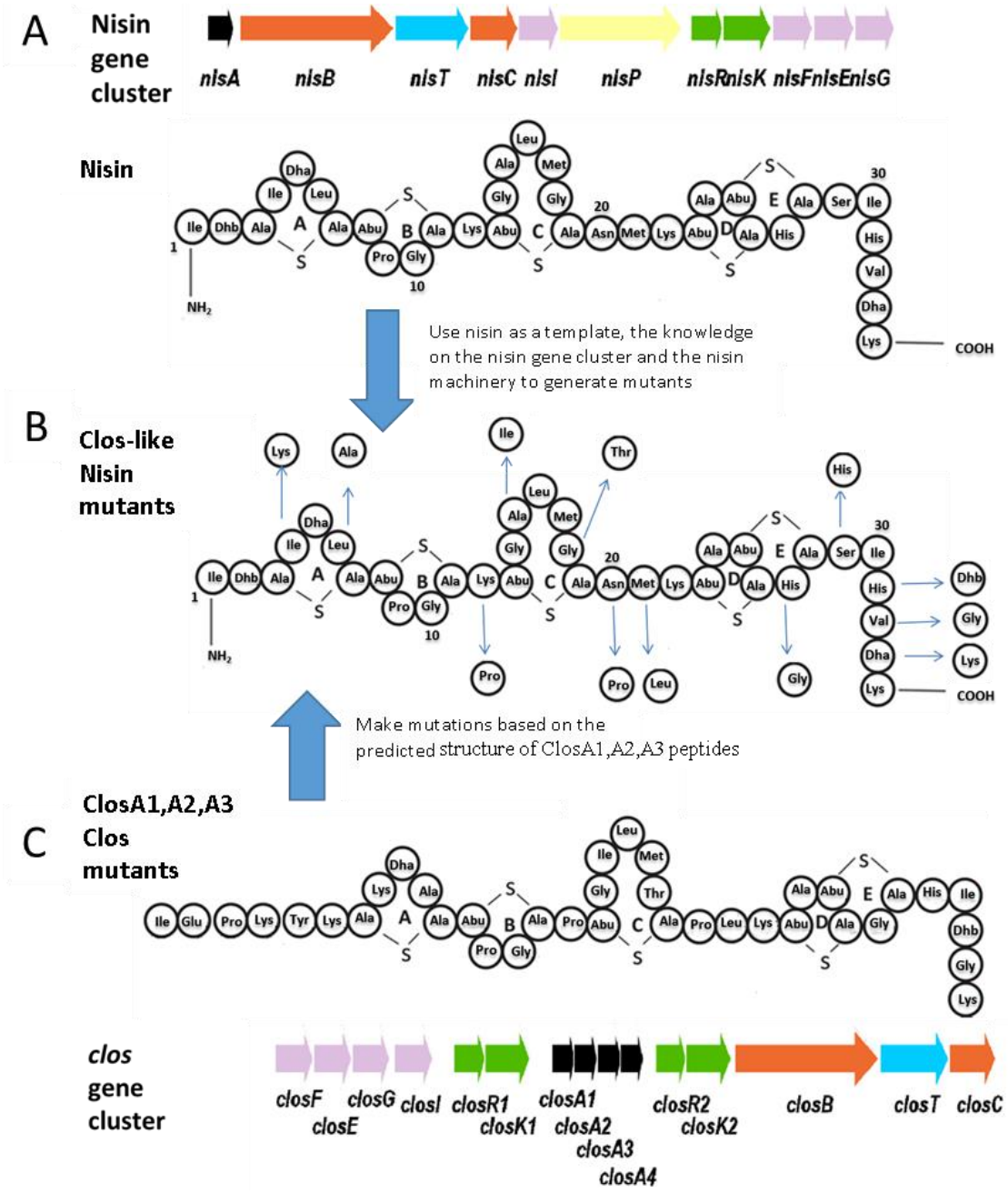


Figure 8 - Nisin and *clos* gene clusters and protein sequences.

The nisin machinery and the original structure of nisin, used as a template, will allow the generation of *clos*-like nisin mutants illustrated in (b); (c) arrangement of the sequenced *clos* lantibiotic cluster highlighting the genes identified from *Blautia obeum* A2-162 strain. The predicted structures of one of the two mature ClosA peptides (ClosA1, A2, A3 are identical) will be used to identify and substitute amino acids in the original nisin sequence (c).

The genes in the *clos* cluster were colour coded: structural genes in black (*closA1-4*), modification genes in orange (*closBC*), transport gene in blue (*closT*), immunity genes in purple (*closFEG*, *closI*), regulatory genes in green (*closR1K1*, *closR2K2*). Abu, Dha and Dhb are unusual amino acids introduced by PTM. Adapted from Hatziioanou [97].

Alignment of nisin (Figure 8, A) and the predicted structure of the ClosA1 peptide (Figure 8, C) indicated the presence of a highly conserved number of amino acids [97]. Using the nisin sequence as a template, amino acid substitutions were introduced to give 12 nisin variants with features of the ClosA1-3 peptide as illustrated in Figure 8, B. The ClosA peptides are identical with one amino acid difference in the leader peptide. The predicted structure of the second putative ClosA4 peptide was illustrated in Figure 20, chapter 4.

The genetic manipulation of the entire *clos* gene cluster from *Blautia obeum* A2-162 producing strain will allow knowledge-based improvements in Clos antimicrobial production and Clos bioactivity as well as the application of genetic engineering to develop novel, potentially improved *clos*-like nisin derivatives. Understanding if and how active lantibiotics from this *clos* novel gene clusters are made could enable the development of *clos*-like nisin variants with improved clinical activity.

1.10 Scope of the PhD thesis

The principle aims of this PhD project were to establish if the novel *clos* cluster identified earlier in a gut bacterium by genome mining was able to produce active Clos antimicrobial peptide as well as to identify nisin mutants with improved bioactivity under GI tract conditions. This is due to the need for new therapies effective against bacterial infections due to antimicrobial resistance. Lantibiotics such as nisin can be used as food preservatives, however they are unstable in the human gut and the *clos* cluster was identified in commensal gut bacteria.

To demonstrate the aims, a number of approaches were taken.

- **Generation and characterisation of Clos-like nisin mutants**

To genetically engineer Clos-like nisin mutants and to determine the stability of purified peptides under gastrointestinal tract conditions (temperature, physiological pH).

- **Identification and functional characterisation of promoters in the *clos* operon**

To genetically engineer a reporter gene promoter system for the identification and assessment of functional promoters in the *clos* cluster, to measure the specific *pepI* activity and to determine which regulatory system switches on which *clos* promoters.

- **Heterologous expression of Clos peptides**

To perform the heterologous expression of the preClosA peptides using the nisin induced clos cluster, to cleave the leader with external proteases such as trypsin and to investigate the biological activity against indicator strains including gut pathogens using the overlay assays.

- **Structure confirmation of the preclosA peptide**

To perform scaled up isolation of immunoprecipitated preClosA peptides, to detect and to investigate PTM introduced by the *clos* cluster machinery and to finally validate the structure of the Clos peptides by comparison with nisin using MS.

CHAPTER TWO

2. Materials and methods

2.1 Microbiology Work

The following material and methods were used throughout the PhD project. Details about bacterial strains and plasmids are in Table 4 to Table 7, buffers and their protocols in Table 3. Consumables were purchased from Sigma Aldrich (Dorset, UK) unless stated otherwise.

2.1.1 Culture media

Trypsin bovine pancreas mix (Sigma-Aldrich) or pure Trypsin (Promega, Mass Spec Grade) were prepared in fresh Trypsin Resuspension Buffer (Promega) or in 50 mM acetic acid. Trypsin was used for digestion of Clos peptides and for the cleavage of the Clos leader to give active antimicrobial peptide.

1) Nisin A (Aplin & Barrett Ltd, Beaminster, UK) was used to induce antimicrobial production and it was prepared in 0.2 μm filter in 50% diluted HCl pH adjusted to 3.0. Aliquots of the stock were stored at -20°C at concentrations of 10 mg/ml and 10 μg/ml.
2) For the induction of nisin mutants and purification in large fermenters, nisin powder (Sigma, 2.5 % sodium chloride and denatured milk solids) was used and prepared as above

Table 3 - List of liquid and solid media used in this thesis.

	Details	State
L medium	10 g/l bacto tryptone, 5 g/l bacto yeast extract, 5 g/l NaCl, 1 g/l glucose.	Liquid, solid*
MRS medium (de Man-Rogosa-Sharpe, modified with glucose addition)	8 g/l lab lemco (Oxoid), 10 g/l peptone (Oxoid), 5 g/l yeast extract (Difco), 5 g/l sodium acetate.$3H_2O$, 2 g/l K_2HPO_4, 2 g/l triammonium citrate, 5.75 mg/l $MgSO_4.7H_2O$, 1.4 mg/l $MgSO_4.4H_2O$, 1 ml Tween 80 and 20 g/l glucose).	Liquid, solid*
BHI + c media	Brain heart infusion, Oxoid + complements: 50 mg/l vitamin K, 5 mg/l hemin, 1 mg/l resazurin, 0.5g/l cysteine).	Liquid, solid*
M17 (Oxoid)	Prepared from ready prepared commercial powder.	Liquid, solid*
Robertson's cooked meat medium	Purchased from Southern Group Laboratories Ltd, UK.	Liquid
GM17	5 g/l glucose to M17 media after autoclaving.	Liquid, solid*
SPY-S*	9 g yeast extract, 9 g peptone, 9 g K_2HPO_4, 1.8 g NaCl, 0.018 g $MgSO_4.7\ H_2O$, 810 ml UPH_2O. Adjust to pH 6.0 with 10N NaOH. Add 90 ml of 30 % sucrose post autoclaving.	Media used in fermenters
SM17 & 3 % glycine	11.175 g M17 broth powder, 51.35 g sucrose, 9 g glycine in 300 ml UPH_2O.	Used for preparation of *L. lactis* (ultra) electrocompetent cells (3 day protocol)
SOC broth	2 % Tryptone, 0.5 % yeast extract 8.56 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose, all diluted in UPH_2O, adjust to pH 7.0	Used for preparation of *E. coli* electrocompetent cells

The solid media was prepared by adding 1.5 % (w/v) agar to the appropriate medium before autoclaving.

*For the overlay assay, the solid agar medium was prepared by adding 0.7 % agar (Oxoid) to M17. For antimicrobial screening of producer strains, 20 mg/l $NaHCO_3$ was added to the solid media before seeding unless stated otherwise. For antimicrobial screening of purified nisin mutants, the pH of medium was adjusted to pH 3.0, 5.0, 5.5, 6.0, 6.5, 7.0 prior to addition of agar and autoclaving.

**The SPY-S special media was used for the scale up production of nisin mutants in 300 ml or 11 l fermenters.

2.1.2 List of bacterial strains and their growth conditions

The (non) genetically modified bacterial organisms and all the plasmids used or generated in this PhD thesis are listed in Table 4 to Table 7. Lists of antibiotic concentrations are in Table 8.

The *L. lactis* strains, listed in Table 6 and Table 7 were obtained from in-house culture collections (IFR, Norwich, UK) and some of the strains were generated in this study. All strains were stored at -80°C in multiple use 1 ml aliquots in 40 % glycerol and grown in media under conditions as in Table 3.

All bacterial strains were grown in aerobic conditions. If anaerobic conditions were necessary, media was pre-reduced in an anaerobic chamber (Don Whitley, UK) overnight in an atmosphere of 5 % CO_2, 10 % H_2 in N_2.

Stocks of *Clostridium* species were stored in Robertson's cooked meat medium (Southern Group Laboratory, Corby). One hundred µl of the stock was used to inoculate pre-reduced BHI (with complements) which was then transferred immediately to the anaerobic cabinet and incubated at 37°C overnight. *C. difficile* and *C. perfringens* were further subcultured before they were used in the experiments.

Cell density was measured with a CECIL CE2041 S/W version R0044 spectrophotometer using bandwidth 4 nm wavelength 600 nm [98].

Table 4 - List of plasmids used in this work.

Plasmid	**Details (reference)**	**Antibiotic Selection***
pIL253	Simon and Chopin [97, 99]	Ery
pUK200	Cloning shuttle vector under the control of the nisin promoter P_{nisA} [100]	Cm
pTG262	Shearman *et al.* [98]	Cm
pNisI	pTG262_P_{nisA} with *nisI*	Cm
pUK200_NisL_ClosA1(IE) pFI2734	Hatziioanou [97]	Cm
pUK200_NisL_ClosA1(YK) pFI2735	Hatziioanou [97]	Cm
pUK200_NisL_ClosA4 pFI2736	Hatziioanou [97]	Cm
pNisL-NisA	pUK200_P_{nisA} with the nisin A leader sequence followed by the *nisA* structural sequence pUK200_P_{nisA}_*nisA*	Cm
pClosCluster	pILClosCluster	Ery
pClosL_*closA34* (pFI2728) pTG262_P_{nisA}_Clos Leader+*closA34*	pFI2728 with *Blautia obeum* A2-162 *lanA* copy 3 and 4 genes pTG262_P_{nisA} with *closA3* followed by strain *Blautia obeum* A2-162 RBS and *closA4* [97]	Cm
pFI2724	pIL252_ClosCluster (17 kp *clos* cluster isolated from *Blautia obeum* A2-162); 6B [97]	Ery
pFI2725	pFI2725 (pIL253 Clos Cluster) with Clos A1A2A3A4 deleted FI7847 pIL253_ClosCluster(Δ*closA*)	Ery
pFI2734	pUK200 with *nisA* leader- *Blautia obeum* A2-162 *closA* (triplicate sequence) structural gene with IE start site.	Cm
pFI2735	pUK200 with *nisA* leader- *Blautia obeum* A2-162 *closA* (triplicate sequence) structural gene with YK start site.	Cm

*Ery is Erythromycin, Cm is chloramphenicol.

Table 5 - List of plasmids constructed in this work.

Plasmid	**Description**	**Antibiotic resistance**
pFI2724	pIL253 with Clos Cluster 6B	Ery
ppTG262_*nisA* or pNisL-NisA b	pTG262_P_{nisA} with nisin leader sequence followed by *nisA* structural gene	Cm
ppIL_NisI	pIL253 with nisin promoter (P_{nisA}) and *nisI* immunity genes	Ery
ppUK200_I4K	pUK200_*nisA*_I4K mutant with nisin leader sequence followed by *nisA* structural gene with the I4K mutation (lysine(K) substituted isoleucine(I) on position 4)	Cm
ppTG262_I4K	pTG262_*nisA*_I4K with nisin leader sequence followed by *nisA* structural gene with the I4K mutation (lysine(K) substituted isoleucine(I) on position 4)	Cm
ppUK200_ L6A	pUK200_*nisA*_L6A mutant	Cm
ppTG262_L6A	pTG262_*nisA*_L6A mutant	Cm
ppTG262_ I4K_L6A	pTG262_*nisA*_ I4K_L6A mutant	Cm
ppTG262_ A15I	pTG262_*nisA*_A15I mutant	Cm
ppUK200_ A15I	pUK200_*nisA*_ A15I mutant	Cm
ppTG262_ G18T	pTG262_*nisA*_G18T mutant	Cm
ppTG262_ A15I_G18T	pTG262_*nisA*_A15I_G18T mutant	Cm
ppTG262_ N20P	pTG262_*nisA*_N20P mutant	Cm
ppTG262_ M21L	pTG262_*nisA*_ M21L mutant	Cm
ppTG262_ N20P_M21L	pTG262_*nisA*_N20P_M21L mutant	Cm
ppTG262_ H27G	pTG262_*nisA*_H27G mutant	Cm
ppTG262_ H27G_S29H_ H31T_V32G_ S33K	pTG262_*nisA*_H27G_S29H_ H31T_V32G_ S33K mutant	Cm
ppTG262_H31T_V32G_ S33K	pTG262_*nisA*_ H31T_V32G_ S33K mutant	Cm
$pP_{ClosA1A4}$_*pepI* pP_{ClosA}_*pepI*	MG1614 or UKLc10 with pIL253_$P_{ClosA1A4}$_*pepI* pIL253 with ClosA1A4 promoter sequence ($P_{ClosA1A4}$) followed by *pepI* reporter gene.	Ery
$pP_{ClosBTC}$_*pepI* $pP_{ClosBTC}$_I	MG1614 or UKLc10 with pIL253_$P_{ClosBTC}$_*pepI* pIL253 with BTC promoter sequence ($P_{ClosBTC}$) followed by *pepI* reporter gene.	Ery
$pP_{ClosFEG}$ _*pepI* $pP_{ClosFEG}$ _I	MG1614 or UKLc10 with pIL253_ $P_{ClosFEG}$ _*pepI* pIL253 with FEG promoter sequence ($P_{ClosFEG}$) followed by *pepI* reporter gene .	Ery
$pP_{ClosR2K2}$_*pepI* $pP_{ClosR2K2}$_I	MG1614 or UKLc10 with pIL253_ $P_{ClosR2K2}$_*pepI* pIL253 with R2K2 promoter sequence ($P_{ClosR2K2}$) followed by *pepI* reporter gene.	Ery
pP_{nisA} _*closR1K1*	pUK200_P_{nisA} with ClosR1K1 two-component regulatory system sequence/gene(s) from the *clos* cluster of *Blautia obeum* A2-162	Cm
pP_{nisA} _*closR2K2*	pUK200_P_{nisA} with ClosR2K2 two-component regulatory system sequence/gene(s) from the *clos* cluster of *Blautia obeum* A2-162	Cm
pP_{nisA} _*closΔR1K1* Frameshift	MG1614 or UKLC10 with pUK200_P_{nisA} with *closR1K1* with mutation/ frameshift in ClosR1.	Cm

pFI2735	MG1614 with pUK200 with *nisA* leader- *Blautia obeum* A2-162 (triplicate sequence) *closA* structural gene with YK start site.	Cm
pNisL_ClosA1(IE_IT)	pTG262 with *nisA* leader- *Blautia obeum* A2-162 (single sequence, A1≡A2≡A3) *closA* structural gene with the start site IT instead of IE.	Cm
pNisL_ClosA1(YK_IT)	pTG262_NisL_closA with the start site IT instead of YK.	Cm
pNisL_ClosA1(IE)	pTG262_NisL_closA with the start site IE.	Cm
pNisL_ClosA1(YK)	pTG262_NisL_closA with the start site YK.	Cm
pNisL_ClosA4	pTG262_NisL_closA4	Cm
pLC6B	pIL253 containing the strain *Blautia obeum* A2-162 lantibiotic cluster from clone 6B	Ery
pLC6BΔA	pLC6B Δ*closA*	Ery

Table 6 - List of organisms and their growth conditions used in this thesis.

Organism	**strain name/ number**	**Media**	**Details on growth conditions and description**	**Antibiotic resistance**
Lactococcus lactis	MG1614	GM17	*L. lactis* MG712 cured of plasmids and prophage, Smr Rifr Gasson [101] 37°C, static	Smr, streptomycin resistance; Rifr, rifampicin resistance.
Lactococcus lactis	FI5876	GM17	MG1614::Tn5301, transconjugant, nisin producer [102] 37°C, static	
Escherichia coli	MC1022	L broth	37°C, 250 rpm Plasmid free [103]	
Bacillus cereus 0880	ATCC 9139	BHI	37°C, 220 rpm	N/A
Bacillus subtilis	ATCC 9139	BHI	37°C, 220 rpm	N/A
*Clostridium difficile***	NCTC11204	BHI+C*	37°C, anaerobic	N/A
*Clostridium perfringens***	NCTC3110	BHI+C*	37°C, anaerobic	N/A
Enterococcus faecalis	ATCC376	MRS(HM)+ glucose	37°C, static	N/A
Enterococcus faecium	ATCC6057	BHI	37°C, static	N/A
L. lactis	MG1614	GM17	30°C, static	N/A
Leuconostoc mesenteroides sub mes. 2205	ATCC8293	MRS(HM)+ glucose	30°C, static	N/A
Listeria innocua	ATCC33090	BHI	37°C, shaken	N/A
Listeria ivanovii	NCTC11007	BHI	37°C, shaken	N/A
Listeria monocytogenes	FI10270	BHI	37°C, shaken	N/A
Micrococcus luteus	FI10640	MRS(HM)+ glucose	37°C, static	N/A
Staphylococcus aureus	FI10139	BHI	37°C, static	N/A
L. lactis	FI5876		MG1614 with nisin biosynthetic operon	N/A

L. lactis	FI7847	GM17	FI5876 Δ*nisA*	N/A
L. lactis	FI8438		FI5876 Δ*nisP*	N/A
L. lactis			pClosCluster + pP_{nisA}_closLeader_*closA34* pIL253 with ClosCluster and pTG262_P_{nisA} and closLeader and *closA34* structural genes	Cm, Ery
L. lactis	FI10899		FI8438 with pFI2734 = pUK200 with *nisA* leader- *Blautia obeum* A2-162 *lanA* (3 sequence) structural gene with IE start site	Cm
L. lactis	FI10900		FI8438 with pFI2735 = pUK200 with *nisA* leader- *Blautia obeum* A2-162 *lanA* (3 sequences) structural gene with YK start site	Cm
L. lactis	NisL_ClosA1(IE)	GM17*	FI7847 with pNisL-ClosA1 with IE start site (in pUK200 or pTG262)	Cm
L. lactis	NisL_ClosA1 (YK)		FI7847 with pNisL-ClosA1 with YK start site (in pUK200 or pTG262)	Cm
L. lactis	NisL_ClosA4		FI7847 with pNisL-ClosA4 (in pUK200 or pTG262)	Cm
L. lactis	NisL_ClosA1 (IE_IT)		FI7847 with pNisL-ClosA1 (in pTG262)	Cm
L. lactis	NisL_ClosA1 (YK_IT)		FI7847 with pNisL-ClosA1 (in pTG262) [97]	Cm
L. lactis	UKLc10	GM17	*nisRK* regulatory genes inserted into chromosome at *pepN* locus	N/A
L. lactis	GH204		pUK200I = pUK200_ P_{nisA} with *pepI* reporter gene from *Lb. delbrueckii*	Cm
L. lactis	FI10235		MG5267 with pIL253 [97]	Er
L. lactis	FI10881		MG1614 with pFI2724 (pIL253 with Clos Cluster 6B) [97]	Er
L. lactis	FI10882		MG1614_pFI2725 (pIL253 Clos Cluster) Δ*closA1-4* [97]	Er
L. lactis	FI10885		MG1614 with pTG262_P_{nisA} _ClosL_*closA34*	Cm

$pP_{ClosBTC}$_*pepI* $pP_{ClosBTC}$_I	MG1614 with pIL253_$P_{ClosBTC}$_*pepI* pIL253 with BTC promoter sequence ($P_{ClosBTC}$) followed by *pepI* reporter gene.	Ery
$pP_{ClosFEG}$ _*pepI* $pP_{ClosFEG}$ _I	UKLc10 with pIL253_ $P_{ClosFEG}$ _*pepI* pIL253 with FEG promoter sequence ($P_{ClosFEG}$) followed by *pepI* reporter gene.	Ery
$pP_{ClosR2K2}$_*pepI* $pP_{ClosR2K2}$_I	UKLc10 with pIL253_ $P_{ClosR2K2}$_*pepI* pIL253 with R2K2 promoter sequence ($P_{ClosR2K2}$) followed by *pepI* reporter gene.	Ery
$pP_{ClosR2K2}$_*pepI* $pP_{ClosR2K2}$_I	MG1614 with pIL253_ $P_{ClosR2K2}$_*pepI* pIL253 with R2K2 promoter sequence ($P_{ClosR2K2}$) followed by *pepI* reporter gene.	Ery

*in pUK200 and in pTG262 plasmids.

Table 7 - List of organisms made and their growth conditions used in this thesis.

Organism	**Strain name/ number**	**Details on growth conditions and description**	**Antibiotic resistance**
L. lactis	UKLc10_CC	UKLC10 with pClosCluster	Er
	UKLc10_CC_*cA34*	UKLC10 with pClosCluster and pP_{nisA}_closLeader_*closA34*	Er, Cm
	FI7369	FI7332+pFI378,pTG262_*nisA* [94]	Cm
	FI7847_CC	FI7847 with pClosCluster FI7847+pILClosCluster	Er
	FI7847_CC_c*A34*	FI7847 with pClosCluster (Δ*closA*) and pP_{nisA}_ closLeader_*closA34* FI7847+pILClosCluster (Δ*closA*) + pTG262_ pP_{nisA}_ClosLeader_ *closA34*	Er, Cm
	CC(ΔClosA) & NisL_ClosA1(IE)	FI7847 with pTG262 *clos* cluster with *closA* structural genes deleted and pTG262_P_{nisA} with nisin leader sequence and ClosA1 (IE start site) structural gene sequence FI7847+pIL253_CLosCluster(Δ*closA*)+ NisL_ClosA1(IE)	Er, Cm
	CC(ΔClosA) & NisL_ClosA1(YK)	FI7847 with pTG262 *clos* cluster with *closA* structural genes deleted and pTG262_P_{nisA} with nisin leader sequence and ClosA1 (YK start site) structural gene sequence FI7847+pIL253_ClosCluster(Δ*closA*)+ NisL_ClosA1(YK)	Er, Cm
	CC(ΔClosA) & NisL_ClosA4	FI7847 with pTG262 *clos* cluster with *closA* structural genes deleted and pTG262_P_{nisA} with nisin leader sequence and *closA4* structural gene sequence FI7847+pIL253_ClosCluster(Δ*closA*)+Nis L_ClosA4	Er, Cm
	CC(ΔClosA) & NisL_ClosA1 (IE_IT)	FI7847 with pTG262 *clos* cluster with *closA* structural genes deleted and pTG262_P_{nisA} with nisin leader sequence and ClosA1 (IT start site instead of IT) structural gene sequence FI7847+pIL253_ClosCluster(Δ*closA*)+ NisL_ClosA1(IE_IT)	Er, Cm
	CC(ΔClosA) & NisL_ClosA1 (YK_IT)	FI7847 with pTG262 *clos* cluster with *closA* structural genes deleted	Er, Cm

		and pTG262_P_{nisA} with nisin leader sequence and ClosA1 (IT start site instead of YK) structural gene sequence FI7847+pIL253_ClosCluster(Δ*closA*)+ NisL_ClosA1(YK_IT)	

Table 8 - The antibiotics used for general genetic modifications and selection studies.

Antibiotic	**Solvent used for preparation**	**Final concentration for *L. lactis***	**Final concentration for *E. coli***
Chloramphenicol (Cm)	Ethanol	5 µg/ml	15 µg/ml
Erythromycin (Er)	Ethanol	5 µg/ml	100 µg/ml

2.1.3 Bioassays for antimicrobial activity

2.1.3.1 *Bacterial overlay assay (deferred antagonism assay) with colonies*

For the overlay assay [16], the nisin derivative or Clos peptide producers were grown overnight in 10 ml liquid cultures. 1 to 5 µl of the overnight culture were spotted onto GM17 agar plates typically containing nisin (0, 10, 100 ng/ml) with appropriate antibiotics (if required), and incubated overnight at 30°C. The plates were irradiated under UV light on AlphaImager (Alpha Innotech) for 15 min prior to being overlaid with soft agar seeded with 1-2 % of the indicator culture and incubated overnight at the optimal temperature for the sensitive strains. Zones of clearing in the indicator lawn were compared against control: nisin producing wild type strain FI5876 [102] and empty vector negative control.

2.1.3.2 *Plate diffusion assay (well diffusion assay) with supernatant*

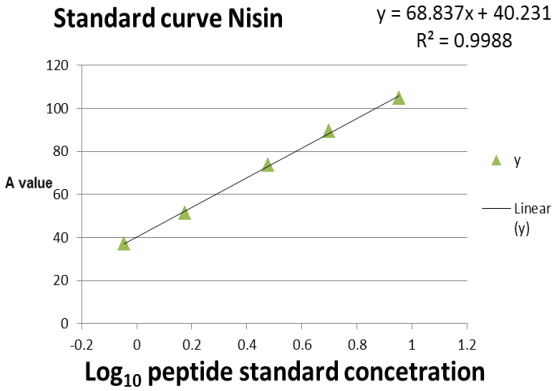
Nisin A (Aplin & Barrett Ltd, Beaminster, UK) was prepared as described in section 2.1.1.

For the large scale production and purification in large fermenters of the nisin mutants, the producing strains were induced with Sigma nisin powder (Sigma, 2.5 %, balance sodium chloride and denatured milk solids) which was used and prepared as above.

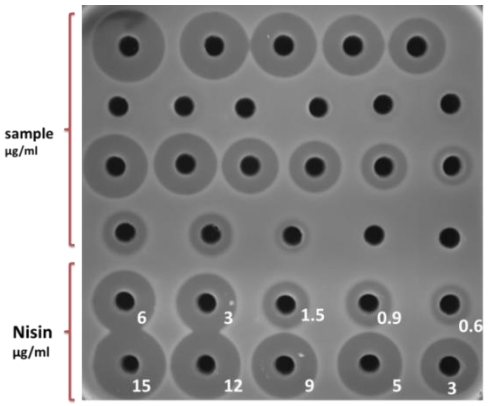
Nisin standards. A stock nisin solution of 1 mg/ml was prepared by adding 1 mg of commercial nisin into 50 % diluted HCl at pH 3.0. Standard nisin solutions of 15, 12, 9, 6, 3, 1.5, 0.9, 0.6, 0.3 µg/ml were prepared using nisin stock solution, either in 50 % diluted HCl pH 3.0 or 1 M phosphate buffer pH 6.0 and used to construct the standard curve (Figure 9).

Nisin bioassay. Based on the method of Olasupo *et al.* [104], the bioassay agar plates were prepared for different indicator strains and optimal media were used for each organism. Typically 95 ml of melted M17 agar at 55°C was mixed with 5 ml of 10 % glucose and 2 ml of 1:1 (1/4) Ringers:Tween 20 and inoculated with 1 ml of a 24 h culture of the corresponding nisin-sensitive strain. 100 ml of aseptic agar was poured into sterile square Petri dishes and allowed to solidify for 2 h or overnight at 4°C. Wells were bored into the agar using a 7-mm outer diameter stainless steel borer. Samples were prepared from 1 ml of acidified overnight culture to pH 3.0, centrifuged at 4000 rpm for 10 min, then the supernatant was filter sterilised with a 0.2 µm filter. 200 µl of sample nisin standards were loaded onto the plates, incubated for 3-5 h at 4°C (to allow for diffusion) then incubated as appropriate for each sensitive strain. Digital callipers were used to measure the diameter of the inhibition zone around each well in triplicate; the diameters were averaged and the well diameter was subtracted from this measurement. Figure 9 has all the equations used to calculate nisin mutant peptide concentrations based on the nisin standard curve [16].

A.



B.



C.

A value (y) is defined as :

$$y = \frac{(Diameter\ of\ \text{Nisin std.andard}\ \ zone - Diameter\ of\ well)^2}{2} \quad (1)$$

x is defined as : Log_{10} Nisin Standard Concentration (2)

$\log_{10}[sample] = x \Rightarrow \log_{10}[Nisin] = x$

A value (y) is defined as :

$$y = \frac{(\text{Diameter of SAMPLE zone} - \text{Diameter of well})^2}{2} \quad (3)$$

Therefore, concentration of peptide in the sample $= 10^x$ (5)

trendline equation: y = 68.837x + 40.231

Where: $x = \frac{y - 40.231}{68.837}$ (4)

Figure 9 - Nisin standard curve from a plate diffusion bioassay using culture supernatants.

For each equation, a number in blue was associated (1-5). The nisin standard curve (A) was plotted by using the measurements taken in the plate diffusion assay (B) and the equations in C. X and y of nisin (in blue, 1-3) were determined from the standard curve, then the real concentrations of nisin or of the nisin mutants (I4K and H27G) were determined based on the same equations (in blue, 3-5). *L. lactis* MG1614 was used as an indicator strain in this assay.

2.1.3.3 *Drop Test with supernatant*

Cultures of overnight indicator strains were diluted 1:10 or 1:100 in phosphate buffer saline (PBS), then lawns were made on solid agar using a sterile cotton swab and air dried. Overnight liquid cultures were centrifuged for 10 min at 4000 rpm, the supernatant was then filtered through a 0.22 µm filter to remove bacteria and stored at 4°C. Aliquots of 5 to 10 µl samples were placed aerobically on the surface of the lawn, air dried and incubated at optimal temperature specific for the sensitive strain. For anaerobic indicator strains, lawns were made aerobically on pre-reduced BHI solid media and air dried anaerobically for at least 5 min.

2.1.4 **Bacterial growth analysis by Scanning and Transmission electron microscopy**

L. lactis strains were grown overnight from glycerol stocks. 1 ml of cell suspension from 16 h cultures was centrifuged at 4000 g, 5 min at RT, and washed with PBS twice then resuspended in 1 ml PBS. Samples were taken over for SEM (Scanning Electron Microscopy) and TEM (Transmission Electron Microscopy) experiments as performed by Kathryn Cross (IFR) using methods described previously [105].

2.2 Molecular Biology Work

Molecular biology protocols were performed by conventional methods according to Sambrook *et al.* [106] and by using kits according to the manufacturer's instructions.

2.2.1 **Oligonucleotides**

Oligonucleotides were synthesized by Sigma-Aldrich (Dorset, UK) and are listed in Table 9.

Table 9 - Oligonucleotides used or designed in this work.

Primer name	**Primer sequence (5'-3')**	**Reference**
universal	GTTTTCCCAGTCACGACGTTGT	Oligonucleotides used in this work.
reverse	AGCGGATAACAATTTCACACAGGA	
p54	CGGCTCTGATTAAATTCTGAAG	
p55	AACGGTTGAGCTTTAAATGAAC	
p181	GCGAAGATAACAGTGACTCTA	
pTG262_B_R	TACGAATTCTGCAGCCCG	Oligonucleotides designed in this work.
pTG262_S_F	CGCTATGTACACCCGGTTG	
pTG262_S_R	CGGGCTGCAGAATTCGTA	
SalI_F:	TAAGTCGACGGCCAGTGC	
G18T_F	ACAGGAATTCTGATGACTTGTAACA	
H27G_F	[Phos]CTGATGGGTTGTAACATGAAAACAGCAACTTGTGGTTGTAGTATTCACGTAAGCAAATAAG	
H27G_R	[Phos]GATCCTTATTTGCTTACGTGAATACTACAACCACAAGTTGCTGTTTTCATGTTACAACCCATCAGAGCT	
N20P_F	[Phos]CTGATGGGTTGTCCCATGAAAACAGCAACTTGTCATTGTAGTATTCACGTAAGCAAATAAG	
N20P_R	[Phos]GATCCTTATTTGCTTACGTGAATACTACAATGACAAGTTGCTGTTTTCATGGGACAACCCATCAGAGCT	

M21L_F	[Phos]CTGATGGGTTGTAACTTGAAAACAGCAACTTGTCATTGTAGTATTCACGTAAGCAAATAAG	
M21L_R	Phos]GATCCTTATTTGCTTACGTGAATACTACAATGACAAGTTGCTGTTTTCAAGTTACAACCCATCAGAGCT	
N20P_M21L_F	[Phos]CTGATGGGTTGTCCCTTGAAAACAGCAACTTGTCATTGTAGTATTCACGTAAGCAAATAAG	
N20P_M21L_R	[Phos]GATCCTTATTTGCTTACGTGAATACTACAATGACAAGTTGCTGTTTTCAAGGGACAACCCATCAGAGCT	
H31Dhb_V32G_Dha33K_F	[Phos]CTGATGGGTTGTAACATGAAAACAGCAACTTGTCATTGTAGTATTACCGGAAAAAAATAAG	
H31Dhb_V32G_Dha33K_R	[Phos]GATCCTTATTTTTTTCCGGTAATACTACAATGACAAGTTGCTGTTTTCATGTTACAACCCATCAGAGCT	
H27G_S29H_H31Dhb_V32G_Dha33K_F	[Phos]CTGATGGGTTGTAACATGAAAACAGCAACTTGTGGTTGTCATATTACCGGAAAAAAATAAG	
H27G_S29H_H31Dhb_V32G_Dha33K_R	[Phos]GATCCTTATTTTTTTCCGGTAATATGACAACCACAAGTTGCTGTTTTCATGTTACAACCCATCAGAGCT	
I4K_L6A_F	[Phos]CATGAGTACAAAAGATTTTAACTTGGATTTGGTATCTGTTTCGAAGAAAGATTCAGGTGCATCACCACGCATTACAAGTAAATCGGCATGTACACCCGGTTGTAAAACAGGAGCTCG	
I4K_L6A_R	[Phos]ATCCGAGCTCCTGTTTTACAACCGGGTGTACATGCCGATTTACTTGTAATGCGTGGTGATGCACCTGAATCTTTCTTCGAAACAGATACCAAATCCAAGTTAAAATCTTTTGTACT	
pIL253_For	GGTGTGTGTGTCTTGTTGTTAGT	
pIL253_Rev	AAAAAGCAGTTTAAAATTGTTGCT	
pIL253F:	CGACAATGATTGTATTTGC	
pIL253R:	TAGTTCTTGTGGTTACGTGG	
pIL253_F_Rev	GCGACAATGATTGTATTTGCTTG	
pIL253_F_Rev1	CTGTTGCGACAATGATTGTATTTGC	
pIL_RevPC	GTGACTCTAGAATTCTGCAGCCC	
pepForÂ	CTCTGACATTTTCCAGCTCCTTG	
pepREVÂ Â	CAAGGAGCTGGAAAATGTCAGAG	
*closR1K1*_F_splice	GAAGCCAGTAATGGATGCGACTAC	
*closR1K1*_R_splice	GTAGTCGCATCCATTACTGGCTTC	
closR2K2_F_splice	TATGACATTACACTTAGTTGGCATC	
closR2K2_R_splice	GATGCCAACTAAGTGTAATGTCATA	
ClosR1K1_F	CAACCATGGTGGATGTTAGTAA	
ClosR2K2_F	GCAGACCATGGAAAATATACTTG	
ClosR1K1_R_SalI	GGTGTAGTCGACCAGAGCTAAC	
ClosR1K1_R_BamHI	GACACCCGGATCCTATAAAGTAA	
ClosR2K2_R_SalI	TATATGTCGACTCATATACTCCCG	
ClosR2K2_R_BamHI	TTGGATCCGACAAGTTAGTGT	
PClosBTC-F	TATAAGATCTTGGTGCTGTGGTAAGC	
PBTC-F	TATAACAGATTAGGAGGTAGAAATGCAAATCACAGAAAAA	
PBTC-R	TTTTTCTGTGATTTGCATTTCTACCTCCTAATCTGTTATA	
PClosR2K2-F	GAAGAGATCTTATACCTGCGAGTATTACAAGTC	

PCRK-F	CATAAAGGAGCAGATAATGCAAATCACAGAAAA ATATC	
PCRK-R	GATATTTTTCTGTGATTTGCATTATCTGCTCCTTTA TG	
PFEG-F	AAAAGGAGATGTATAATAATATGCAAATCACAGA AA	
PFEG-R	TTTCTGTGATTTGCATATTATTATACATCTCCTTTT	
PClosFEG-F	TGTAGATCTTTAGAAGGAACG	
pepI-R	GTGACTCGAGAATTCTGCAGCCC	
PClosBTC_F	AAAGATCTTGGTGCTGTGGTAAG	
pepI_R	AGCCTCGAGAACAGTGACTCTAGAA	
PBTC_F	AGGTAGAAATGCAAATCACAGAAA	
PBTC_R	TGATTTGCATTTCTACCTCCTAAT	
PClosR2K2_F	GAAGAGATCTTATACCTGCGAGTA	
PCRK_F	GCAGATAATGCAAATCACAGAAA	
PCRK_R1	TGATTTGCATTATCTGCTCCTT	
PCRK_R2	TGATTTGCATTATCTGCTCCTTT	
PClosA_F	TTACAAGATCTATTGTGACACAGCA	
PCA_F	CACCAAATGCAAATCACAGAAA	
PCA_R	TGATTTGCATTTGGTGCCTCC	
PClosFEG_F	TGTAGATCTTTAGAAGGAACGACC	
PFEG_F	GTATAATAATATGCAAATCACAGAAA	
PFEG_R	TGATTTGCATATTATTATACATCTCCT	
PCfeg_Forward	TGAAGGAGAGAAGAAGAGCGATAATTTATG	
pIL253R_Forward	TGGATTAGTTCTTGTGGTTACGTGGTT	
pIL253_For	CCGATGATAAGCTGTCAAACATGAG	

2.2.2 **Agarose gel electrophoresis of nucleic acids**

DNA was typically loaded on a 1 % or 2 % (w/v, for shorter bands) agarose gel cast in 0.5 x TBE buffer (Fisher Scientific). 5 µl of 1 kb Hyperladder I (Bioline, UK), 100 bp was used as a DNA size marker (NEB) (Figure 10). The amount of DNA in each lane was estimated according to Hyperladder. 1 µl of loading buffer was mixed with samples prior to loading into the gel. 5 to 10 µl of sample mixed with 1 x loading buffer were loaded into the appropriate wells. The gel electrophoresis was performed in 0.5 x TBE buffer until completion. The gels were stained in ethidium bromide for 30 min, rinsed in ultra-pure water UPH_2O and visualised with UV light using the AlphaImager machine.

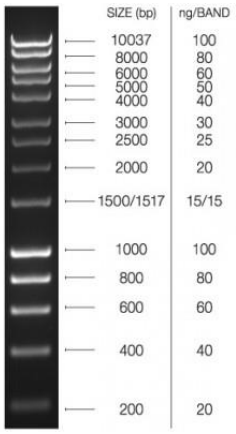


Figure 10 - Size marker: 5 µl of 1 kb Hyperladder I was loaded on a 1 % agarose gel and visualized using ethidium bromide.

2.2.3 **Genomic DNA extraction**

Genomic DNA was extracted from mid exponential phase cells of *L. lactis* using the genomic DNA extraction kit with Genomic Tip 500/G columns (Qiagen) as described by the manufacturer with the following modification. For *L. lactis*, 50 U mutanolysin (Sigma) was added to improve lysis of the cell wall. For the DNA quantification, 1 to 5 µl aliquots of genomic DNA were run on a 1 % agarose gel. Alternatively, a Nanodrop instrument machine software (Thermo Scientific) was used with the DNA peaks being at 260 nm. The extracted DNA was confirmed to be of good quality when the absorbance ratio (260/280 nm is RNA/protein ratio) was 1.7 to 2. The ratio is a measure of protein contamination in the sample.

2.2.4 Plasmid preparation, excision from agarose gel and DNA purification kits

A number of kits were used according to the manufacturers' protocols:
For plasmid preparation: E.Z.N.A Plasmid Mini Kit I (Omega Bio-Tec), QIAprep spin miniprep kit (Qiagen).
For DNA band excision from gel: QIAEXII Gel extraction kit (Qiagen, Sussex, UK).
For the purification of PCR products: Sureclean (Bioline, London, UK).

2.2.4.1 *Plasmid preparation*

For plasmid preparation, a 10 ml overnight culture of *E. coli* or *L. lactis* was centrifuged for 10 min at 4000 rpm, 4°C. For *L. lactis*, the pellet was resuspended in 300 µl P1 buffer from Qiagen plasmid kit and 5 mg/ml of lysozyme powder was added After the mix was removed to a new 1.5 ml Eppendorf tube, 50 U mutanolysin was added (for *L. lactis*) to ensure cell lysis. After 15 min incubation at 37°C, plasmid preparation was continued according to the manufacturers' instructions. For the plasmid preparations from *E. coli* cells, the manufacturers' protocols were followed. The quality of the plasmid DNA was checked on an agarose gel and DNA concentration was assessed by Nanodrop.

2.2.4.2 *Extraction of DNA bands from an agarose gel*

Fragments of DNA were separated in agarose gels and the excised bands from the gel with a clean scalpel and further purified using the instructions from the QIAquick gel extraction kit. In short, the agarose gel slice that contains the DNA band of interest was dissolved in a neutral pH, high salt buffer from the kit, and then applied to a silica-gel membrane mounted in a microcentrifuge tube. The DNA was trapped on the membrane while the unwanted impurities were washed away. The DNA was finally eluted in 30 µl of elution buffer. Quantification of DNA was performed using a 1 % (w/v) agarose gel.

2.2.4.3 *DNA purification using Sure Clean*

For purification of the PCR products, Sure-Clean (Bioline) was used to rapidly remove PCR reaction components like primers, dNTPs, buffer, short DNA fragments and the enzyme. The clean-up was done according to the manufacturer's instructions: DNA was incubated with the dye, and an equal volume of Sure Clean buffer for 30 min at room temperature (RT). The mix was centrifuged for 10 min at 14, 000 g at RT and washed

twice with 70 % ethanol. The air-dried pellet was eluted in 5 to 20 µl of elution buffer and the concentration was read by Nanodrop.

2.2.5 DNA digestion with restriction enzymes (restriction digests)

Restriction enzyme digestion of plasmid or genomic DNA was carried out using the enzyme manufacturer's instructions as described by New England Biolabs (NEB, Herts, UK). For the double digestion of DNA, an optimal buffer was selected from the literature of Roche or NEB. The reaction volume was usually 10 µl per 1 µl of enzyme, and unless otherwise stated the digestion was carried out for 1 hour at 37°C per 1 µl of enzyme. After incubation, the enzymes were heat-inactivated at 80°C and the products were purified with Sure-Clean (Bioline).

To calculate the amount of enzyme to add in the reaction mix, the following formula was used as adapted from NEB. This method of calculation proved sometimes to work better in cloning.

Size of Vector DNA (kb) / size of plasmid to be cut (kb) x

x no of cuts in plasmid / no of cuts in Vector DNA = N Unit/µg (1 fold).

This means that for 1-fold restriction, N units will cut 1 µg of plasmid. 2 to 5-fold excess were typically used for restriction. The size of the vector DNA, where the enzyme is usually defined, and the number of cuts in the Vector DNA were taken from the manufacturer's catalog.

2.2.6 General methods for PCR

PCR Sprint (Thermo Scientific) and Professional Basic (Biometra) thermal cyclers were typically used to perform polymerase chain reaction (PCR). Primers were usually designed with an annealing temperature (TA) of 55 - 60°C, with TA for most applications of 56°C unless it was otherwise stated.

Oligonucleotides and primers were designed and ordered from Sigma Genosys (UK). The melting temperatures were calculated as follows:

- for GoTaq reactions used http://www.oligoevaluator.com/OligoCalcServlet;
- for Phusion PCR used: http://www.thermoscientificbio.com/webtools/tmc/;
- Reverse primers were obtained by swapping the sequence on http://www.basic.northwestern.edu/biotools/oligocalc.html
  (the only exception was with the splice primers).

reaction volumes of 50 µl were prepared in special PCR tubes and the conditions typically used for different applications are listed in Table 10 and Table 11.

Table 10 - Composition of PCR reactions mix. GoTaq polymerase (Promega) (left) and Phusion polymerase (Finnzymes) (right).

GoTaq polymerase		**Phusion polymerase**	
Component	**Quantity**	**Component**	**Quantity**
DNA template	1 µl	DNA template	1 µl (~5 or 50 ng)
5 x GoTaq Reaction Buffer (Promega)	10 µl	5 x Phusion Reaction Buffer (finnzymes)	10 µl
0.2 mM of each dNTP (Bioline)	0.4 µl	0.2 mM of each dNTP (Bioline)	0.4 µl
Forward primer 20 µM (Sigma Genosys)	1 µl	Forward primer 20 µM (Sigma Genosys)	1.25 µl
Reverse primer 20 µM (Sigma Genosys)	1 µl	Reverse primer 20 µM (Sigma Genosys)	1.25 µl
UPH_2O	36.35 µl	UPH_2O	35.7 µl
1.25 U/µl GoTaq DNA polymerase (Promega)	0.25 µl	1 U/µl Phusion Polymerase (finnzymes)	0.4 µl
Total volume	50 µl		

Table 11 - PCR cycling conditions for GoTaq polymerase and Phusion polymerase.

GoTaq polymerase		**Phusion polymerase**	
Temperature & time	**Cycles**	**Temperature**	**Cycles**
95 °C 2 min	x 1 cycle	98 °C 30 sec	x 1 cycle
95 °C 30 s	x 25-30 cycles;	98 °C 10 s	x 25-30 cycles;
55-60 °C* 30 s	x 25-30 cycles;	TA°C** 30 s	x 25-30 cycles;
72 °C 1 min/kb	x 25-30 cycles	72 °C 15-30s/kb	x 25-30 cycles
72 °C 5 min	x 1 cycle	72 °C 5 min	x 1 cycle

*Annealing temperature (TA) is adjusted depending on the primer melting temperature, TA is max Tm-3.

**TA is Tm (or ≤20 bp primers or Tm+3°C for >20 bp primers.

The Phusion kit was used for high-fidelity amplification of genes (genomic/plasmid DNA) for cloning applications such as the construction of a plasmid. The GoTaq PCR kit was used for general analytical PCR with colonies from plates or with ligation products. For colony PCR with GoTaq, individual colonies of *E. coli* or *L. lactis* were picked from the agar plates using a sterile toothpick and were smeared into 9 µl of UPH_2O. 1 µl of this mix was then transferred in the PCR tube before adding the appropriate PCR mix and DNA was amplified using the appropriate conditions as indicated in Table 11.

PCR was also used to screen colonies picked with a sterile toothpick and resuspended in 10 µl of sterile UPH_2O. 1 µl of mixture was added to a 50 µl PCR reaction volume as template DNA (Table 11, Table 12).

2.2.7 Splice overlap extension PCR

Splice overlap extension PCR was used as described previously [107] either for site-directed mutagenesis [108] to introduce mutations in the original structure of nisin (to generate *clos*-like nisin mutants) or to create new plasmids (by joining PCR promoter sequences and the gene sequences of interest). The splice overlap reaction components are listed in Table 12 and the method for splice overlap PCR is illustrated in Figure 11.

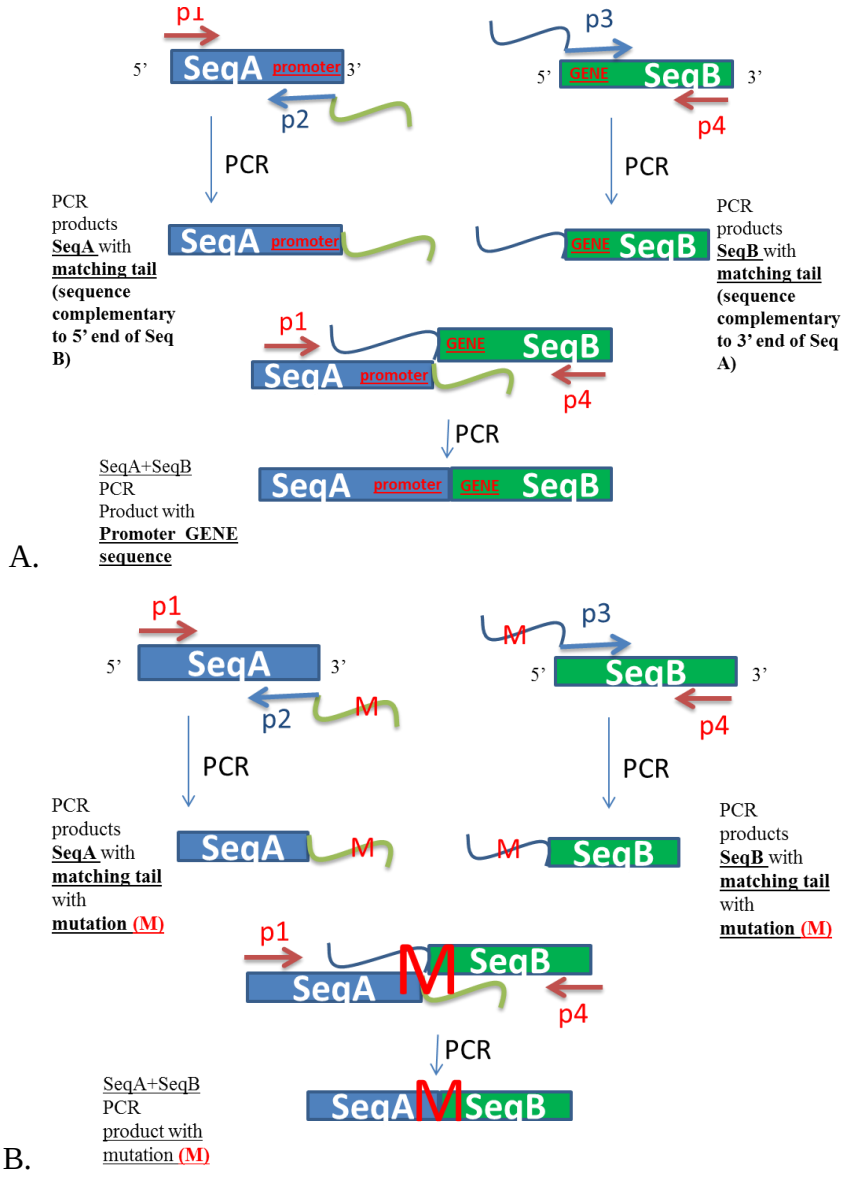


Figure 11 - Diagrams for splice overlap extension PCR.

A. To join/ligate 2 PCR fragments to make a new product eg: SeqA + SeqB have a new promoter region and a gene site. P1, p4 are the outer primers and p3, p2 are the inner primers. P2 was designed to be 100 % match to the right hand end (3' end) of SeqA with a tail complementary to the start of SeqB (5' end). P3 was designed to be 100 % match to the left hand end of SeqB (5' end) with a tail that is complementary to the right of SeqA (3' end). The sequences were fused during PCR, as the overlapping sequences hybridized and extended to make a full-length sequence.

B. To insert a mutation (M). The inner primers, p2 and p3, were designed in a similar fashion as in A however the mutation was added to the matching tail of p2 in the 5' end of Seq B and for p3 in the 3' end of Seq A.

Table 12 - Details for splice overlap extension PCR.

Phusion polymerase			
Component	**Quantity**	**Temperature & time**	**No. of cycles**
DNA template	*1 µl (~1 or 50 ng) **1 µl or 4 µl (1 or 4 ng)	98°C ……30 sec	x 1 cycle
		98°C ……10 sec	x 5 cycles
5 x Phusion reaction buffer (Finnzymes)	10 µl	TA1*……..30 sec	
dNTP (100 mM stock, Bioline)	0.4 µl	72°C 15-30 s/kbs	
Forward primer 20 µM (Sigma Genosys)	1.25 µl	98°C ……10 sec	x 20 cycles
Reverse primer 20 µM (Sigma Genosys)	1.25 µl	TA2** ……..30 sec	
UPH_2O	35.7 µl	72°C 15-30 s/kb	
Phusion Polymerase (Finnzymes)	0.4 µl	72°C 5 min	x 1 cycle
Total volume	50 µl		

*For the first PCR rounds the templates are Seq A or Seq B (- Diagrams for splice overlap extension PCR.). TA1 was calculated as NN of the overlap (approx. 37°C) or if multiple products were seen, TA was raised to TA of whole primer minus the miss match (approx. 50°C).

**For the second round of PCR, the template is SeqA with matching tail and Seq B with matching tail. TA2 was calculated as the lower Tm for ≤20 bp primers or Tm+3°C for >20 bp primers.

Primers for splice overlap PCR for both cases (Figure 11, A and B) were designed using the following rules:

- for p2 and p3 splice primers, the 100 % matching sequence should be approx. 15 bp with a similar length of tail;
- the nearest neighbour (NN) for the overlap is calculated with http://www.basic.northwestern.edu/biotools/ oligocalc.html); NN values should be above 37°C;
- for p1 and p4 end primers with a maximum of 20 bp in length, the annealing temperature (TA) will be the lower Tm; Tm is calculated using http://www.thermoscientificbio.com/webtools/tmc/;
- for p1 and p4 end primers:

TA is or Tm+3°C for >20 bp primers.
TA is the lower Tm for ≤20 bp primers.
For method development the cycle parameters were:

- 5 cycles of the NN of the splice primer 100 % matching sequence (overlap).
- 5 x TA1 was approx. 37°C.

20 cycles of TA the whole outer primer (or TA calculated for the end primer, whichever is lower).

- TA2 = the lower Tm (≤20 bp primers) or
- TA2 = Tm+3°C for >20 bp primers.

If multiple products were seen, TA1 was raised to a max of TA calculated as TA of whole primer minus the miss match.
5 x TA1 was approx. 50°C.
After SeqA and SeqB with matching tails were purified with Sure Clean, 1 ng or 4 ng of each PCR product were used in the splice overlap extension PCR reaction with the end primers' (p1 and p4).
TA was either the NN of the ENTIRE overlap region between the two sequences (SeqA and SeqB) or TA was the lowest TA calculated for the end primers if this TA was lower).
The details for cloning are specific for *L. lactis*.

2.2.8 **Vector DNA dephosphorylation**

In order to remove 5' phosphate groups from restricted plasmid vectors and to prevent self-ligation of vectors, the Antarctic Phosphatase (NEB) was used according to the manufacturer's instructions. Dephosphorylated vector was purified with SureClean to remove the reaction components including the restriction enzyme (Table 13).

Table 13 - Details of dephosphorylation of plasmid vector.

Antarctic Phosphatase		
Component	**Quantity**	**Temperature & time**
DNA template restricted DNA in any buffer	1 µg (usually in 50 µl PCR reaction mix)	15 min at 37°C for 5' extensions or blunt ends Or 60 min for 3' extensions Heat inactivate at 80°C for 20 min.
10 x Antarctic Phosphatase buffer (NEB),	2.5 µl	
UPH_2O.	1 µl	
5 U/µl Antarctic Phosphatase	1.5 µl	
Total volume	25 µl	

2.2.9 **DNA ligation**

Ligation of DNA sequences was carried out using Fast-Link™ DNA Ligation Kit (Epicentre Biotechnologies) by following the manufacturer's instructions. Typically, 10-20 ng/µl of restriction-digested, dephosphorylated purified vector (150 - 300 ng/µl in final volume) was used in a 1:3 molar ratio with the restriction-digested, purified insert fragment. For blunt-end ligations, a 1:5 molar ratio was suggested by the manufacturer (Table 14).

Table 14 - Details for ligation of insert and plasmid vector.

Fast-Link™ DNA Ligation		
Component	**Quantity**	**Temperature & time**
10 × Fast-Link Ligation Buffer	1.5 µl	Optimal incubation time 2 h to overnight at RT. Heat inactivated at 70°C for 15 min.
10 mM ATP	1.5 µl	
Vector 0.07 pmoles	A µl	
Insert 0.35 pmoles	B µl	
UPH_2O	Up to 15 µl	
Fast-Link DNA Ligase	1 µl	
Total volume	15 µl	

Ligation was initially performed using the manufacturer's protocol regarding the molar ratios (Figure 12). On several occasions, ligation worked better with 0.07 pmoles of vector and 3 or 5-fold molar excess of insert for sticky end and blunt end ligation respectively.

1 µl of the ligation product was amplified with GoTaq polymerase and loaded on a 1 % agarose gel to check the amplified ligation products. The ligation products were then

purified using Sure-Clean to remove the enzyme, then the product was resuspended in 5 µl of elution buffer. Typically, 2.5-5 µl of ligation (100 - 500 ng) or 750 - 1000 ng for bigger inserts (>1500 bp) were used to transform electrocompetent *L. lactis* cells.

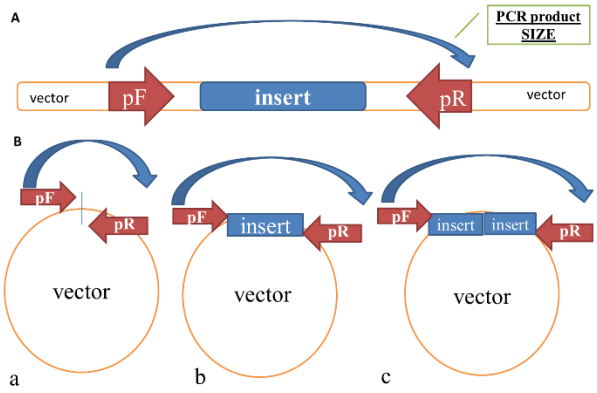


Figure 12 - Illustration of ligation PCR.

A. forward (pF) and reverse primers (pR) are annealing on the new vector template; B. Possible products of the ligation reaction: a) self-ligated vector, b) vector with insert, c) vector with double insert.

2.2.10 **Preparation and transformation of *E. coli***

E. coli MC1022 [103] cells were prepared as electro-competent and transformed with DNA. Briefly, 40 ml pre-warmed L broth (with antibiotic) was inoculated with 800 µl of an overnight culture of *E. coli* and incubated at 37°C shaking (250 rpm) until the optical density at 600 nm (OD_{600}) was 0.5-0.6. The cultures were chilled on ice and processed immediately. Ice-cold liquid cultures were harvested by centrifugation (3000 g, 10 min, 4°C), washed twice in ice-cold 10 % glycerol with resuspension by gentle agitation then centrifuged (10 min, 3000 g, 4°C). Finally, the cell pellet was resuspend in 400 µl of ice-cold 10 % glycerol and 40 µl aliquots were used immediately for transformation or flash frozen in liquid nitrogen or dry ice and stored at -80°C.

Electroporation of the target DNA to the electrocompetent *E. coli* cells was performed with ice cold reagents and glassware. Purified ligation product (100-500 ng) was added to *E. coli* cells, mixed and incubated for 1 min on ice. The mix was pipetted into pre-chilled electroporation cuvettes (Cellprojects, UK) and electroporated at 2.5 kV, 200 Ω resistance and 25 µF using a Gene Pulse Xcell (BioRad) capacitance. 460 µl of SOC broth was added in the cuvette, transferred to a tube then incubated by shaking (225 rpm) for 2 h at 37°C. Next, 100 µl of electroporated cells was plated onto L agar with antibiotic selection. Plates were incubated for 1-2 days at 37°C to allow colonies to grow.

The transformation efficiency for empty vector control was calculated as number of transformants per μg of DNA.

2.2.11 Preparation and transformation of *L. lactis*

2.2.11.1 *Preparation of L. lactis electrocompetent cells*

To prepare *L. lactis* electrocompetent cells, pre-warmed 47.5 ml of M17 supplemented with 2.5 % glycine and 2.5 ml of 10 % glucose were inoculated with 3 ml overnight *L. lactis* culture and incubated at 30°C until OD_{600} was 0.5-0.6. The cultures were chilled on ice and harvested by centrifugation (3000 x g, 10 min, 4°C), washed twice in 1/5 volumes of ice cold electroporation buffer (0.5 M sucrose in 10 % glycerol) with gentle mixing. The pellet was resuspended in 1/100 volumes ice cold electroporation buffer, dispensed in 40 μl aliquots, transformed or flash frozen immediately in dry ice and acetone, then stored at -80°C until ready to use.

2.2.11.2 *Preparation of L. lactis (ultra)electrocompetent cells (3-day protocol)*

To increase the competency of transformation, *L. lactis* (ultra)electrocompetent cells were prepared using a modified 3 day protocol described by Holo & Nes [109]. 300 ml SM17 (51.35 g in 11.174 g M17 broth powder was topped up to 300 ml with UPH_2O) media with 3 % (9 g) glycine was prepared fresh, aliquoted in 10 ml, 50 ml and 200 ml then autoclaved. Prior to inoculation, 0.5 % (w/v) glucose was added to pre-warmed SM17. 10 ml SM17 with 3 % glycine was inoculated with 100 μl of *L. lactis* from a glycerol stock and incubated for 24 h at 30°C. 50 ml SGM17 with 3 % glycine was inoculated with 1 in 10 (v/v) overnight culture and incubated overnight at 30°C. 200 ml pre-warmed SGM17 with 3 % glycine was inoculated with 12.5 % (v/v) of overnight culture and grown at 30°C until the OD_{600} was 0.2-0.3 (to prevent lysis of the cells). The pre-chilled liquid culture was harvested by centrifugation (5000 x g, 10 min, 4°C), the cells were washed once with an equal volume of ice-cold 10 % glycerol/0.5 M sucrose, once with 25 % of 10 % glycerol/0.5 M sucrose/50 mM EDTA, incubated on ice for 15 min then washed with an equal volume of ice-cold 10 % glycerol/0.5 M sucrose. All buffers were prepared fresh, filter sterilized and prechilled, and the cells were gently resuspended and centrifuged at 5000 x g, 10 min, 4°C between washes. 1/100 volume of ice cold 10 %

glycerol/0.5 M sucrose was used to resuspend the pellet, then 40 µl aliquots were used immediately to transform or they were flash frozen in liquid nitrogen and stored at -80°C.

2.2.11.3 *Electroporation of electrocompetent L. lactis*

Electroporation of the target DNA to the (ultra)electrocompetent *L. lactis* cells was performed with ice cold reagents and glassware. Purified ligation product (100-500 ng) was added to a 40 µl cell aliquot which was mixed, incubated for 1 min on ice before the mix was electroporated at 2.5 kV, 200 Ω resistance and 25 µF in a electroporation cuvette using a Gene Pulse Xcell (BioRad). 460 µl or 960 µl transformation buffer (10 ml M17/0.5 M sucrose with 125 µl of 40 % glucose, filter sterilised 200 µl 1 M MgCl2 and 200 µl 0.1 M CaCl2) was added in the cuvette then transferred in a pre-chilled 2 ml screw cap tube and incubated on ice for 10 min. Electroporated cells were further incubated at 30°C for 2 h. 100 µl of electroporated cells (or dilutions) was plated onto GM17 agar with antibiotic selection. Colonies were allowed to grow for 2-3 days at 30°C before they were picked with a sterile toothpick.

2.2.12 **DNA sequencing**

15 µl of purified DNA was sequenced using by Eurofins Genomics, UK. Reagents were added to the tube as follows:

- template DNA: plasmid up to 15 kbp: 50 -100 ng/µl or
- PCR products:150-300 bp: 1 ng/µl;
- 300–1000 bp: 5 ng/µl;
- >1000bp: 10 ng/µl.
- 1 µl of primer with a concentration of 20 pmol/µl (20 µM)
- UPH_2O up to 17 µl total reaction volume.

Analysis of the sequences was performed using Finch TV tool (version 1.4), EditSeq and Seqmatch II (DNAStar) tools.

2.3 Scaled-up production and purification of nisin mutants on Fractogel

Supernatants from *L. lactis* wild type nisin and nisin mutant producing strains were purified on Fractogel TSK Butyl-Toyopearl 650M media (Anachem) as described previously [96, 110, 111] with the following modifications.

Producers of the nisin mutant peptides were grown from glycerol overnight in GM17 at 30°C. Next, 10-14 litres of SPY-S media were inoculated to a starting OD_{600} of 1, and cells were grown with antibiotic and 10 ng/ml nisin (to ensure peptide production and growth) for typically 16-20 h at 30°C with a constant pH 6.0 as monitored with a pH meter. pH was kept to 6.0 by addition of 10 M NaOH to counteract the lactic acid produced by the bacterial strain. Next, the supernatants were harvested by pH adjustment to 2.5 with 50 % HCl and by removing the cells by centrifugation (10, 000 x g, 10 min, 4°C). The cell-free culture supernatant of the producing strains was pH adjusted to 5.0 with 10 M NaOH. After addition of 0.5 M $(NH_4)_2SO_4$ and filtering of the supernatant on 0.45 µm filters (Millipore), the sample was loaded on a pre-treated Fractogel TSK Butyl-Toyopearl 650 M column at 3 ml/min flow rate. As the volume was large, this step was optimally performed in several stages overnight with 10 ml fractions collected. To monitor the protein elution, the absorbance at 220 nm was measured. The column was washed with 10 bed volumes of 0.5 M $(NH_4)_2SO_4$ and subsequently with deionized water until an A_{220} below 0.5 was achieved. The bound antimicrobial peptides were then eluted using 5 mM HCl and assayed in drop tests or plate diffusion assays against *L. lactis* MG1614 [101] as indicator strain.

Active fractions showing indication of antimicrobial activity were pooled, the pH was adjusted to 6.5 with NaOH, re-adjusted to pH 3.5 using glacial acetic acid, lyophilized and the quantified powder was stored at 4°C.

Final purification and analysis of the purified fractions were achieved by C18 reverse-phase semi-preparative high-performance liquid chromatography column (RP-HPLC) (Luna C18 (2), 5 µm, 250 x 21.20 mm) (see section 2.6). Solvents A (0.1 % trifluoroacetic acid, TFA in water) and B (0.1 % TFA in 70 % ACN, HPLC grade acetonitrile (ACN) (Fisher Scientific) and methanol (Fisher Scientific) were used with a grade and purity of 95 % or greater. Solvent 70 % ACN with 0.1 % TFA was prepared by adding 700 ml HPLC grade ACN to a volumetric flask, 300 ml UPH_2O and adding 1 ml of TFA to a final volume of approx. 1 l.

Peptides were eluted with a linear gradient from 5 % to 100 % of B over 40 min at a flow rate of 3 ml/min. The elution was monitored at A_{220}, A_{254}, A_{280}. Fractions from C18 were next tested by drop test (not pH adjusted) against *L. lactis* MG1614 and by Maldi-ToF. Confirmed active fractions were combined, concentrated and the acid was removed by pH adjustment as above using NaOH to get to pH 6.5, and glacial acetic acid to pH 3.5. This was to prevent degradation upon lyophilisation. The lyophilized powder was then stored at -20°C until ready to use. To test the antimicrobial activity of the peptide, the powder was initially dissolved in 50% diluted HCl at pH 3.0. Following an overnight incubation of the resuspended peptide at 4°C, the peptide was tested against different sensitive strains using plate diffusion assays.

2.4 Biochemistry and analysis of proteins

2.4.1 Solutions and buffers

Table 15 - Solutions and buffers used in this study.

Solution/buffer	**Composition and instructions for preparation**	**Method used for**
1 M Tris HCl, pH 7.2, buffer (pH 7.0, 7.5, 8.0)	121 g TRIZMA-Base (Sigma), made up to 1 l with UPH_2O, pH adjusted with 1 M HCl.	Cell extraction
1 M $MgCl_2$	95.21 g $MgCl_2$ (Sigma) made up to 1 litre with UPH_2O	Electrophoresis
0.1 M $CaCl_2$	11.098 g $CaCl_2$ (Sigma) made up to 1 litre with UPH_2O	Electrophoresis
Colony wash buffer	10 mM Tris pH 8.0, 1 mM EDTA pH 8.0, 100 mM NaCl in UPH_2O.	To prepare PCR reaction from colonies
1 M NaCl	58.44 g NaCl (Sigma), made to 1 litre with UPH_2O.	Plate diffusion of mutants at different pH (different buffers)
TBS buffer	10 mM Tris pH 7.5, 150 mM NaCl in UPH_2O	Western blot
TBS-Tween/Triton buffer	20 mM Tris pH 7.5, 500 mM NaCl, 0.05 % (v/v) Tween 20 (Sigma), 0.2 % (v/v) Triton X-100 (Sigma) in UPH_2O.	Western blot
20 × NUPAGE Transfer buffer	Bicine 10.2 g, Bis-Tris 13.1 g, EDTA 0.75 g, made to 125 ml with UPH_2O	Western blot
1 × NUPAGE Transfer buffer	50 ml of 20 × NUPAGE buffer, 1 ml of NUPAGE antioxidant, 100 ml of methanol, 849 ml of UPH_2O.	
Transformation buffer	9.5 ml M17 broth + 0.5 % Sucrose + 125 ul 40 % glycerol, 200 µl of 20 mM $MgCl_2$ (2.03 g $MgCl_2$ in 10 ml UPH_2O, 200 µl $CaCl_2$ (2 mM) (0.13 g $CaCl_2$ in 10 ml of UPH_2O	Used for transformation of *L. lactis* electrocompentent cells
Loading dye	0.015 % bromophenol blue (Sigma), 10 % glycerol (Sigma), 0.5 x TBE buffer.	DNA gels
SDS gel loading buffer (2x)	10 % (w/v) SDS, 40 % (v/v) glycerol, 250 mM Tris HCl (pH 6.8) up to 10 ml total volume.	Protein gel electrophoresis

2.4.2 **Bradford assay to measure protein concentration**

Proteins were quantified using the method of Bradford [112] and Bradford's reagent kit (Biorad) in a colorimetric protein assay which is based on a shift in absorbance of the Coomassie Brilliant Blue G-250 dye; in acidic conditions, the dye's red form is converted into a blue form after it binds to the protein [112]. The BSA assay standards were prepared using commercial bovine serum albumin (BSA) from NEB, prepared in the same buffer as the cell extract, usually 0.1 M Tris HCl, pH 7.4, at concentrations ranging from 0, 20, 40 to 200 µg/ml. A dilution of 1:200 of the sample was applied. 10 µl of each standards and diluted samples were pipetted in duplicate into a sterile, flat-bottomed, 96-well microtitre plate (Sterilin). 190 µl of freshly prepared colorimetric indicator (4 ml Bradford's reagent in 16 ml UPH_2O) were dispensed into each well, then the samples were mixed on the SoftMax plate reader (Molecular Devices), any bubbles were removed and the plates were finally incubated at ambient temperature for 5 min before read at OD_{600}. A mean concentration of the duplicate samples was calculated. The concentration of the proteins was determined by plotting the standard curve and by using the equation generated from that curve as in Figure 13.

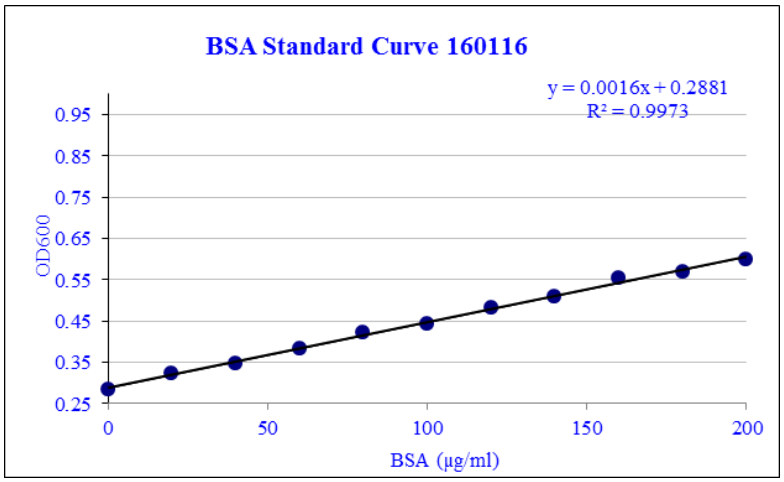


Figure 13 - Example of BSA calibration curve used to calculate protein concentration.

2.4.3 **SDS polyacrylamide gel electrophoresis**

All the sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) materials were purchased from Invitrogen (Paisley, UK). Typically 10 µg of protein was prepared as described in Table 16 where samples were heated, chilled in ice and electrophoresed in 2-(N-morpholino) ethane sulfonic acid (MES) with antioxidant; the gel was finally stained using Safestain. Once heated, the proteins were kept stable in their denatured state by the NuPAGE LDS sample buffer.

Table 16 - Composition of protein samples mix before heating at 70°C for 10 min and loading on the NuPage Novex gels for protein gel or western blot analysis.

Component	**µl**			**µl***
Total volume of sample + H2O (10 µg) or (30 µg)*	6.5	9.75	13	14
Reducing agent	1	1.5	2	
LDS sample buffer	2.5	3.75	5	
SDS gel loading buffer (2x)				6
Total volume (µl)	10	15	20	20

* For band excision from protein gel, the maximum amount of protein was loaded, (approx. 30 µg). The aim was to cut out the bands (gel slice) for the protein of interest, perform alkylation and trypsin digestion and subsequent LC-MS and Maldi-ToF analysis.

10 µl of the pre-stained marker SeeBlue Plus 2 (Figure 14) (Invitrogen) and the heated and chilled samples were loaded on to the protein gel. The protein gel was prepared and run according to the Invitrogen NuPAGE gel instructions. The gel was finally removed from the outer chamber and stained with Simply Blue Safestain or it was used for western blot analysis.

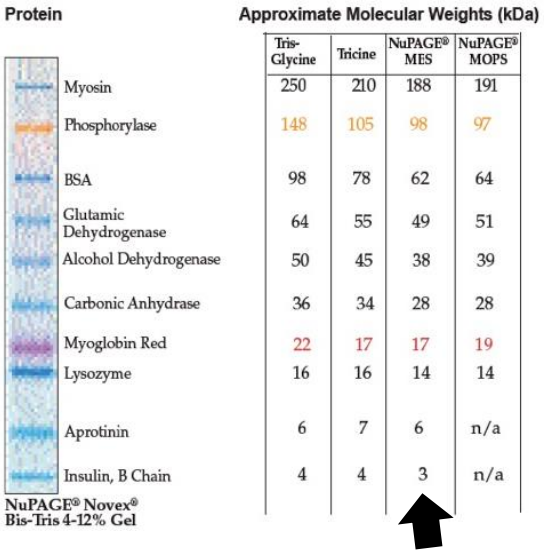


Figure 14 - Protein markers. See Blue Plus prestained ladder (Invitrogen) in MES buffer highlighted by the arrow.

2.4.4 **Western blot analysis**

For western blotting, the Clos containing samples were prepared as described above, run and detected on 12 % Bis-Tris NuPAGE gels in MES (Invitrogen) with polyclonal antiClos leader peptide antibody. For peptide detection, 6.5 µl (sample+UPH_2O) was supplemented with 1 µl Reducing Agent, 2.5 µl LDS sample buffer. For gel extraction from gel slices for trypsin digestion, 6 µl of SDS gel loading buffer (2 x) was added to 14 µl of sample. The samples mixes were heated to 70°C for 10 min, loaded on the gel and ran the gel at 30 V for about 1 h. The electrophoresed proteins were transferred onto a polyvinylidene difluoride membrane (PVDF) using the western transfer methodology and reagents from Invitrogen NuPAGE (Figure 15).

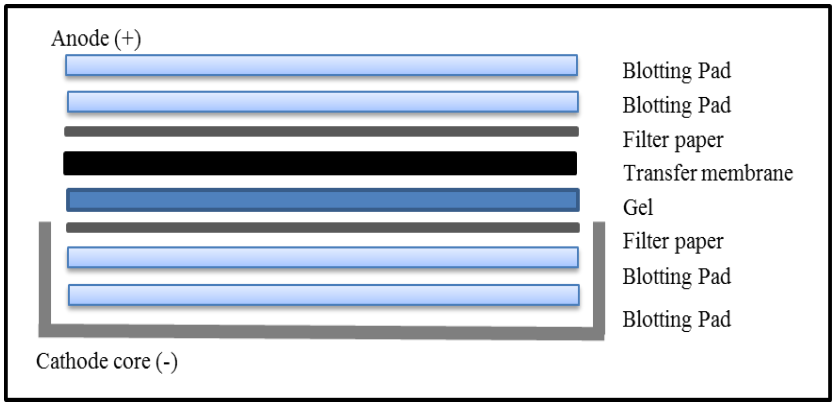


Figure 15 - Western blot assembly. The same buffers as described in Table 15 were used.

The blot module was inserted into the lower buffer chamber of the electrophoresis tank (Invitrogen).

The recipes for all the buffers are detailed in Table 15. After transfer, the PVDF membrane was washed with 2 x TBS buffer for 10 min. To avoid non-specific interactions of the antiClos leader antibody with the membrane, the membrane was then incubated in blocking buffer for 1 h shaking or overnight at 4°C.

The membrane was washed with 2 x 50 ml TBS-Tween/Triton buffer for 10 min (Table 15) and once with TBS buffer. It was then incubated with 1:200 (v/v) primary polyclonal antiClos leader peptide antibody raised against rabbit by Genscript Corp (NJ, USA). The antigen was a synthesized N-terminal acetylated ClosA1-3 leader peptide H2N-AKFDDFDLDVTKTAAQGGC-CONH2 (without the first methionine [113]) in rabbits [97]. 50 µl of antibody in 10 ml 3 % BSA was added and incubated with the membrane for 1 h.

The unbound primary antibody was removed by washing the membrane with 2 x TBS-Tween/Triton buffer for 10 min, with 1 x TBS buffer, then the membrane was exposed to 1:5000 (v/v) anti-Rabbit IgG-alkaline phosphatase secondary antibody (Sigma) for 1 h at RT. After 10 min washes with 4 x TBS-Tween/Triton buffer, colorimetric detection was performed with Sigma Fast BCIP/NBT (5-Bromo-4-chloro-3-indolyl phosphate/ Nitro blue tetrazolium) as a substrate. The tablet was resuspended in 10 ml, incubated with the protein side of membrane without moving for 1-15 min until enough colour development. To stop the colour development reaction, the membrane was washed with 2 x UPH_2O and it was left to dry for visualisation of the bands [113].

2.4.5 **Protein extraction**

L. lactis strains expressing hybrid plasmids were cultured from glycerol stocks in 10 ml GM17 media overnight then subcultured in 100 ml pre-warmed media to a starting OD_{600} of 0.1 with antibiotic addition. At OD_{600} 0.5-0.6 (mid exponential phase), cultures were induced with 10 ng/ml nisin and typically incubated for another 3 h at 30˚C unless otherwise stated. The culture was divided in 50 ml portions and transferred in 50 ml centrifuge tubes (corning) to harvest cells by cell centrifugation (4000 x g, 15 min, 4˚C). The pellet was further processed to prepare cell extracts while the supernatant was treated to obtain the trichloroacetic acid (TCA)/acetone precipitated proteins (Figure 16) [114].

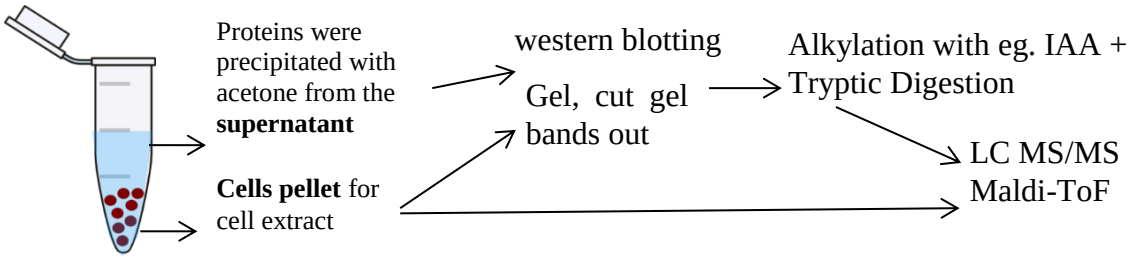


Figure 16 - Processing of liquid cultures: TCA/acetone precipitation of proteins from the supernatant and cell extraction from cell pellet of the original culture.

2.4.5.1 ***Bead beating extraction of crude proteins***

The collected pelleted cells were washed once with 0.025-0.2 M Tris HCl pH 7.4, transferred to 2 ml tubes and resuspended in 1 ml buffer, centrifuged to remove the buffer, flash frozen in dry ice and stored at 4°C. Soluble protein extracts were produced by bead beating with 500 µl buffer and 200 µl glass beads using a FastPrep FR120 cell disrupter, 4 x 30 sec speed 6 with 5 to 10 min on ice between beatings [115]. The tubes were centrifuged for 5 min (10,000 x g, 4°C), the cell extract was removed to fresh 0.5 ml tube, spun again for 30 min to remove the glass beads. The supernatant containing concentrated crude protein/cell extracts was transferred to a new tube and stored at -20˚C until ready to use. Protein concentration was quantified by Bradford assay as 1:200 dilution in Tris HCl buffer, pH 7.4 buffer.

2.4.5.2 *TCA protein precipitation from culture supernatant*

Culture supernatants were collected by centrifugation and filtered with a 0.45 μm filter. Supernatant total proteins were precipitated and concentrated from the filtered supernatant by addition of 1 g/ml cold TCA (25 %, v/v) and incubation at 4˚C for 2 h or overnight. Concentrated and precipitated proteins were pelleted by centrifugation (4000 x g, 30 minutes, 4˚C), washed with 25 % ice cold acetone, dried at RT or by incubating at 50˚C until acetone was completely evaporated. Pellet was stored at -20˚C and resuspended in 50 mM sodium acetate (pH 5.5) until fully dissolved and used in western blot. Proteins of expected mass were identified by SDS-PAGE electrophoresis and immunoblotting as described above [116].

2.4.5.3 *Colony Mass Spectrometry*

Colony Mass Spectrometry (CMS) using isopropanol for extraction of nisin mutants from inside the cells, is an adapted method from Field *et al.* [16]. Prior to the MS analysis, cultures grown overnight were plated on agar. The samples prepared with this method were: 1) 1 ml of liquid culture (fresh or stored at 4˚C) was spun and washed with 1 ml of UPH_2O or 2) single colonies picked with a sterile loop from overnight incubated plates. The resulting cell pellet or the colony were resuspended thoroughly in 50 μl of 70 % isopropanol adjusted to pH 2.0 with 1.5 % formic acid, then sonicated for 10 to 15 min in a water bath. The suspension was vortexed and centrifuged for 2 min at 13000 x g, and the supernatant was removed for Maldi-ToF analysis. Extracts were diluted 1 in 2 with 0.1 % TFA and spotted on pre-coated matrix.

2.4.6 **Protein Immunoprecipitation**

Protein immunoprecipitation (IP) of Clos was carried out using an optimised method based on the Pierce Crosslink Immunoprecipitation Kits (26147, 20423, Thermo Fisher Scientific) with the following modifications. The IP procedure was used to affinity purify Clos on small or large scale IP reactions. More specific details on buffers and reagents volumes and concentrations were added in chapter 6.

Figure 17 is a diagram of antibody binding to Protein A/G agarose resin, the basis for traditional immunoprecipitation and co-immunoprecipitation methods.

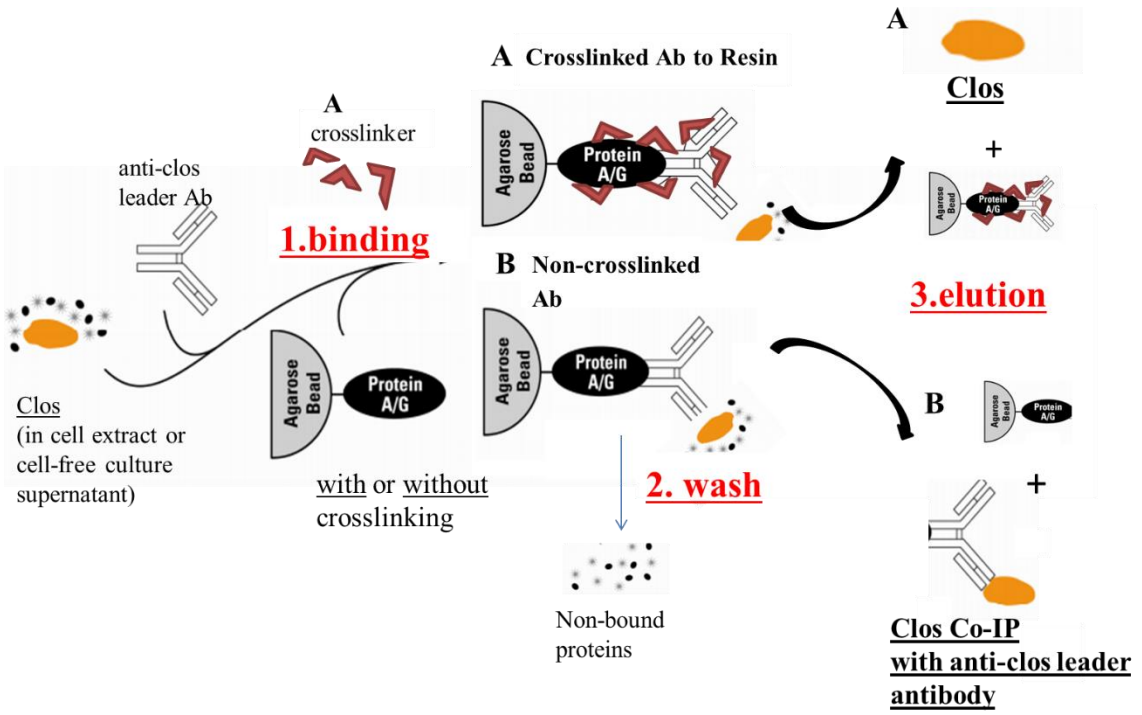


Figure 17 - Diagram to summarise purification of Clos from a mix of proteins using two immunoprecipitation (IP) methods.

A) IP using crosslinker and B) co-IP as adapted from the manufacturer's technical guide.

1. Binding step: Clos-containing sample, antiClos leader antibody (Ab) and beaded agarose affinity beads (eg: Protein A/G) were allowed to bind. 2. Wash step: non-bound components of the sample were washed away; 3. Elution step: conditioning buffer disrupted the binding interactions to give A) pure Clos peptide or B) a complex of Clos co-IP with antiClos leader antibody.

All washes were done by centrifugations at 1000 x g for 1 min in the small column provided by the Pierce Crosslink IP Kit. For the scale up IP, incubations were done in 50 ml centrifuge tubes and the recovery of the supernatants/resin in the small column or 10 ml column was done by centrifugation and gravity flow, respectively. The bulk of supernatant was run through the column a few times. Fresh buffer was used for the wash steps. All the flow through of the remaining samples and washes were recovered for further analysis by western blot. In order to remove contamination with detergent from the reagents, IP Lyis/Wash buffer was replaced with 0.025 mM Tris HCl buffer, pH 7.4. In one scaled up IP reaction, 0.025 mM TrisHCl buffer, pH 7.4 buffer was used to obtain cell extraction and for subsequent wash steps. All the reagents were left at RT prior to being used as in the manufacturer's instructions.

Samples used were typically 1. cell extracts in Tris buffer (0.025 mM) or in IP Lysis/Wash buffer, both in the presence/absence of a protease inhibitor cocktail or 2. cell-free culture supernatant. Based on the WB, the best option was to use cell extract in 0.025 mM Tris HCl buffer, pH 7.4.

Two methods were used for the assembly of the immobilized protein complex: 1) sequential or 2) in one step (Figure 18, Figure 19). In the sequential method (used for method development), 2 ml antibody (approx.70 µg) and Clos cell lysate (extract in Tris buffer) were incubated overnight. One ml of slurry (500 µl affinity beads) was then added to calculate optimal Ab:Ag ratio and to capture the antiClos leader antibody and Clos antigen complex by incubation for 6 h at 4°C on a vortex mixer. Aliquots were collected at every stage of the purification process and visualised by western blotting.

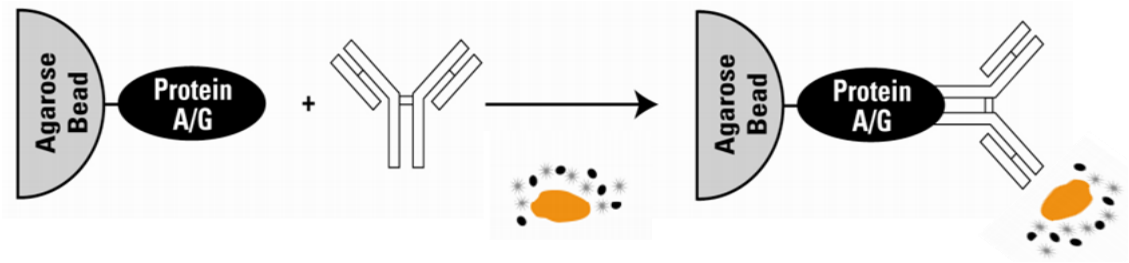


Figure 18 - Diagram of the sequential method to immunoprecipitate the antigen - Clos peptide.

Figure 18 is an illustration of the antiClos leader antibody that binds to Protein A/G agarose resin, as adapted from Thermo Scientific Pierce IP Kit. In short, the antiClos leader antibody was immobilized on a beaded support through the Protein A, IgG binding protein, which in turn was bound on the agarose resin. A non-crosslinked antibody was used. The Clos peptide was co-immunoprecipitated (IP) with the antibody as illustrated in Figure 17.

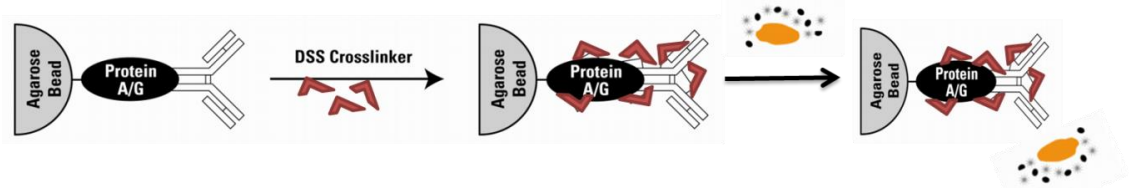


Figure 19 - Diagram of the one step IP method.

AntiClos leader antibody immobilization method to Protein A/G agarose resin was adapted from Thermo Scientific Pierce IP Kit. DSS crosslinker was used to covalently attach the antibody to Protein A/G beaded agarose resin. In comparison to the traditional sequential IP methods, the one step method allows the elution of Clos without contamination from antiClos leader antibody.

In the one step method (scaled up IP/larger scale of IP), the antiClos leader antibody was first incubated with 1 ml of slurry (500 µl resin), 0.5 ml 20 x coupling buffer, 2 ml (approx. 70 µg) of antiClos leader antibody and 6.5 ml UPH_2O. The 50 ml centrifuge tube containing the mix was incubated for 2 h at 4°C on a vortex mixer. Next, the mix was

transferred in a 10 ml column, the resin was washed with 2 x 3 ml elution buffer (to quench DSS) and with 2 x 3 ml Tris HCl, pH 7.4 buffer. The antibody was crosslinked with 1 ml of 2.5 mM DSS, 3.75 ml UPH_2O, and 250 µl of 20 x coupling buffer by vortexing for 1 h at RT. After the bulk of supernatant was run through the column for a few times, it was then recovered by gravity flow and stored for later analysis. The resin was washed once with 3 ml elution buffer and twice with 3 ml Tris HCl, pH 7.4 buffer, then the crosslinked resin and Clos cell extracted in Tris HCl, pH 7.4 buffer were incubated overnight at 4°C on a vortex mixer (in 50 ml centrifuge tube). After the binding of Clos, antibody and support, the beads were recovered by centrifugation and washed extensively with 3 x 3 ml Tris HCl, pH 7.4 buffer and once with 1 ml of 1 x conditioning buffer.

Elution of Clos from the beaded support was performed with 0.5 ml elution buffer by incubation for 5 minutes with vortexing at room temperature. After centrifugation, the eluate was collected in fresh tubes where 100 µl of 1 M Tris HCl, pH 9.5 was previously added to neutralize the buffer. The elution was repeated several times to give a series of fractions (F1-F4) that were frozen in dry ice immediately and stored at -20°C. The resins were regenerated and stored in 1 x coupling buffer at 4°C. Finally, to remove contamination with detergent that would interfere with Maldi-ToF and LC-MS, the eluates were further purified typically on a C8 cartridges/column (HyperSep). Clos was eluted with a linear gradient of 10 %, 15 % to 60 % acetonitrile in 0.1 % formic acid. (see section 2.5)

In previous attempts to test the best purification method, a high spin column, C4 or C8 ZipTip (HypersSep) protein purification methods were also used (see section 2.4.8). The new re-purified eluates in acetonitrile and formic acid had the concentrated buffer removed by drying under vacuum and the samples were resuspended in 5 % formic acid buffer for liquid chromatography–mass spectrometry (LC-MS) or matrix-assisted laser-desorption ionisation time of flight (Maldi-ToF) mass spectrometry. For further structure characterisation, the re-purified eluates were also taken on Orbitrap and (IAA treated) and/or trypsin digested as in section 2.5.

2.4.7 **Preparation of gel slices for in–gel tryptic digestion**

Trypsin digestion of Clos containing gel slices was carried out using a method adapted from Saalbach *et al.* (JIC). Incubation time was 15 min unless otherwise noted and the 1 ml washes were removed by 1 min centrifugation at 5, 000 g. The defrosted gel slices were each washed twice with 30 % ethanol at 65°C, twice in 50 % acetonitrile in 50 mM TEAB. After buffer was removed, either 10 mM dithiothreitol (DTT) or 10 mM (tris(2-carboxyethyl) phosphine in 50 mM triethyl bicarbonate (TEAB) was added and the gel slices were incubated vortexing for 30 min at 55°C or at RT respectively. Alkylation was achieved by vortexing for 1 hour in the dark with one of the freshly prepared agents: 10 mM iodoacetamide (IAA) [117-120]. The gel was then washed once in 50 mM TEAB, once in 50 % acetonitrile, once in 50 mM TEAB. After the buffer was removed from every step, single slices were placed into a petri dish, measured and cut into small pieces (1x1 mm) using scalpels. The small pieces were transferred with one drop of TEAB into fresh low protein collection binding tubes (Thermo Fisher). Finally, the pieces were washed once in 50 % acetonitrile in 50 mM TEAB, once in 50 % acetonitrile, once in 100 % acetonitrile until gels shrunk and became hard and white. After acetonitrile was completely removed in a speed vac for 30 min, the samples were stored at -20°C ready for trypsin digestion, Maldi-ToF MS and LC-MS as performed by Gerhard Saalbach (see section 2.5).

2.4.8 **Protein concentration-ZipTip and C4 column**

Removal of detergent contamination from antibody antigen purification (see section 2.4.6) from the eluates was carried out on C4 and C8 ZipTip (Millipore) first, then on a C4 cartridge or C4 column (HyperSep). In an attempt to test which column was the best, C4 and C8 were compared and it was concluded that Clos did not bind C4 very strongly and had a good elution profile.

For the ZipTip, the eluate was aspirated, dispensed through ZipTip to bind, wash, and elute pure Clos. The recovered repurified Clos was contaminant-free and eluted in 5 µl for direct transfer to MS.

For a scaled purification, a C4 cartridge or C4 column was used. All washes and elutions were done by gravity flow and the flow through was collected at each step. The column was equilibrated in 3 ml of ACN, then it was washed twice in 3 ml of 0.1 % TFA. The eluates (E1-E4) from the antibody antigen purification (see section 2.4.6) were treated

with approx. 25 µl of 10 % TFA, until the pH was 3. Each eluate was loaded one after the other and ran by gravity. The column was washed with 1 ml of 0.1 % TFA, washed once with 3 ml 0.1 TFA, then twice with 3 ml 0.1 % formic acid and finally air dried. The peptide was eluted with a linear gradient: twice with 10 %, 1x with 15 %, 1x 20 %, 1x 25 %, 1x 30 %. 1x 40 %, 1x 50 %, 1x 60 % of acetonitrile (v/v) in 0.1 % (v/v) formic acid. Eluent samples could also be concentrated by drying under vacuum and samples resuspended in 5 % formic acid for Maldi-ToF MS or LC-MS (see section 2.5).

2.5 Mass Spectrometry

Examples of typical samples used for MS are listed below:

1. Liquid cultures prepared as isopropanol cell extracts from cell pellet, isopropanol resuspended pellets or cell free culture supernatants.
2. Colony picked from agar plates extracted by isopropanol method;
3. Liquid cultures prepared as cell extracts from bead beaten pellet, C4 column purified eluates originally collected from the antibody-antigen kit.

2.5.1 Matrix-Assisted Laser-Desorption Ionisation Time of Flight (Maldi-ToF)

The samples (1 µl) were spotted onto a PAC plate (Prespotted AnchorChipTM MALDI target plate, Bruker Daltonics, Coventry, UK), left for 3-5 min, and then the spots were washed shortly with 10 mM ammonium phosphate, 0.1 % TFA according to the manufacturer. Alternatively, samples were spotted onto polished steel plates (Bruker) and left until dry. After drying they were overlaid with 1 µl of a solution of α-cyano-4-hydroxycinnamic acid (Sigma-Aldrich, Dorset, England) (5 mg/ml) in 50 % acetonitrile, 0.1 % TFA. The samples were analysed by Maldi-ToF on a Bruker Autoflex Speed TOF/TOF. The instrument was calibrated using the pre-spotted standards of the PAC plate or SpheriCal® calibrants (Polymer Factory, Stockholm, Sweden). Samples were analysed using a method optimised for peptide analysis in the mass range 1000-7000 and spectra were summed from ca. 10 x 500 laser shots. Data were processed in FlexAnalysis (Bruker) to generate annotated spectra.

2.5.2 Liquid chromatography-mass spectrometry (LC-MS)

2.5.2.1 *LC-MS - Orbitrap Fusion Method*

Gel pieces were washed, treated with trypsin, and extracted according to standard procedures adapted from Shevchenko *et al.* [121]. Aliquots were analyzed by nanoLC-MSMS on an Orbitrap Fusion™ Tribrid™ Mass Spectrometer coupled to an UltiMate® 3000 RSLCnano LC system (Thermo Scientific, Hemel Hempstead, UK). The sample was separated on a PepMap™ 100 C18 LC Column (C18, 2 µm, 500 x 0.75 mm, Thermo) using a gradient of 0.75 % min-1 acetonitrile from 6 % to 40 % in water/0.1 % formic acid at a flow rate of 0.3 µl min-1 and infused directly into the mass spectrometer. The mass spectrometer was run in positive ion mode with quad isolation at 120K resolution over the mass range 400-1600 (m/z) for the precursor scans (orbitrap). One microscan of 50 ms with an AGC target of 2e5 was used. MS2 threshold was set to 2e4 and precursors fragmented by both CID and HCD with CE=30 and an isolation window of 1.6 Da (quadrupole) using the automatic maximum speed option with ion injection for all available parallelizable time. Dynamic exclusion was set to 1 count and 40 s. Recalibrated peaklists were generated using MaxQuant 1.5.3.30 (www.MaxQuant.org) and the database search was performed with the merged HCD and CID peaklists using Mascot 2.4 (Matrixscience, London, UK). The search was performed on a *Lactobacillus lactis* protein sequence database (uniprot) to which the *clos* peptide sequences had been added with a precursor tolerance of 6 ppm and a fragment tolerance of 0.6 Da. The enzyme was set to trypsin/P with a maximum of 2 allowed missed cleavages. All used modifications (Carbamidomethyl (C), oxidation (M), acetylation (Protein N-term), dehydro (T,S)) were set as variable modifications. The Mascot search results were imported into Scaffold 4.4.1.1 (www.proteomsoftware.com) using identification probabilities of 99 % and 95 % for proteins and peptides [121, 122]. More specific details were also added in chapter 6.

2.5.2.2 *LC-MS - Synapt Method*

Typical samples were from:

1) Liquid culture prepared as isopropanol cell extracts from cell pellet, isopropanol resuspended pellets or cell free culture supernatants.
2) Colony picked from agar plates extracted by isopropanol method;
3) Liquid cultures prepared as cell extracts from bead beaten pellet, C4 column purified eluates originally collected from the antibody-antigen kit.

The samples were analysed by LCMS on a Synapt G2-Si mass spectrometer coupled to an Acquity UPLC system (Waters, Manchester, UK). Aliquots of the samples were injected onto an Aeris WIDEPORE 3.6µ C4 column (Phenomenex, Macclesfield, UK) and eluted with a gradient of 1-95 % (Acquity curve 7) in 14 min with a flow rate of 0.2 ml min-1. The mass spectrometer was controlled by the Masslynx 4.1 software (Waters) and operated in positive Maldi-ToF and resolution mode with a capillary voltage of 2.5 kV and a cone voltage of 40 V in the m/z range of 50-1600. Leu-enkephalin peptide (1 ng per ml-1, Waters) was infused at 10 µl per min-1 as a lock mass and measured every 30 s. More specific details were also added in chapter 6.

2.6 Analytical High-Pressure Liquid Chromatography (HPLC)

Lyophilised nisin mutants (collected from Fractogel, see section 2.3) were re-suspended in Buffer A and were further purified on a C18 RP-HPLC column. HPLC was carried out on a HP Agilent 1200 series semi-preparative system, consisting of two 1260 Infinity preparative pumps, a 1290 Infinity dual loop autosampler, a 1290 Infinity preparative fraction collector, and a 1260 Infinity diode array detector. Both auto sampler and fraction collector were equipped with thermostatic controllers. A Luna C18 (2) column with dimensions 5 µm, 250 x 10 mm was used. A 2 ml injection of sample was loaded onto the column and eluted at a 3 ml per min-1 flow rate, using a water/acetonitrile gradient containing 0.1 % TFA UV spectroscopy data were collected at 220 nm, 254 nm and 280 nm. Fractions were collected as indicated by absorption at 220 nm.

CHAPTER THREE

3. Generation and characterisation of Clos-like nisin mutants

3.1 INTRODUCTION

The development of new antimicrobial agents either as an alternative to antibiotics or as a food preservative is a matter of considerable importance; this is due to the rise in MDR pathogenic bacteria and the undesirable side-effects that many chemical preservatives may have. Consequently, there has been a great interest in developing bacteriocins into drugs for the treatment of pathogen-driven infections or into additives for the preservation of food and animal feed. The availability of molecular tools greatly facilitates the generation and characterisation of new bacteriocins and their derivatives.

At present, nisin is the only bacteriocin that is widely used as a biopreservative and approved in over 40 countries for use as a food additive [40]. Genetic engineering of nisin as antimicrobial peptides with enhanced characteristics, which are more potent against a wider number of pathogenic strains, has been the focus of extensive research in the field of antimicrobial peptides [13, 87]. However, the use of nisin and its derivatives in a clinical setting is problematic as it is unstable and it has low solubility at physiological pH (pH 7.0) [47]. It was previously [47] shown that by using site-directed mutagenesis to introduce lysine into nisin Z, the solubility at pH 7.0 was enhanced and, more importantly, the novel bacteriocin had comparable antimicrobial activity to native nisin Z [123]. This indicated that an improved version of nisin may enable the use of the FDA-approved nisin as a therapeutic agent.

Nisin is a ribosomally synthesised peptide with antimicrobial activity [124]. In a more systematic approach, Field *et al.* [87] took advantage of an *in vivo* engineering approach to construct and screen for highly potent nisin variants. By using random (site saturation) mutagenesis in the hinge region of nisin A, a number of variants were successfully engineered by amino acid substitutions: the Asn20Pro, Met21Val and Lys22Thr mutants of nisin A displayed enhanced antimicrobial activity against pathogenic bacteria such as *Listeria monocytogenes* and *Staphylococcus aureus* [13, 88, 90, 91].

Nisin is a polycyclic lantibiotic peptide that contains unusual amino acids and it specifically binds and targets lipid II, a component of the bacterial cell wall, to form pores. Therefore, the action mode of nisin is bactericidal since it lyses the cell [125].

Previous work on the screening of gut microbiota strains involved the use of a PCR based genome mining approach to identify a novel gene cluster (Figure 20) possibly encoding lantibiotics in a strain of *Blautia obeum* A2-162 isolated from the human GI tract [97].

The predicted ClosA peptides are nisin-like peptides: three are exactly identical (ClosA1, ClosA2, ClosA3) and the fourth (ClosA4) has a different sequence (Figure 20). Compared to nisin, there are 12 amino acids different in ClosA1-3. The structures of these peptides and their antimicrobial activity have not been fully characterised. However, the *Blautia* strain showed variable and inconsistent antimicrobial activity against *C. perfringens* [97].

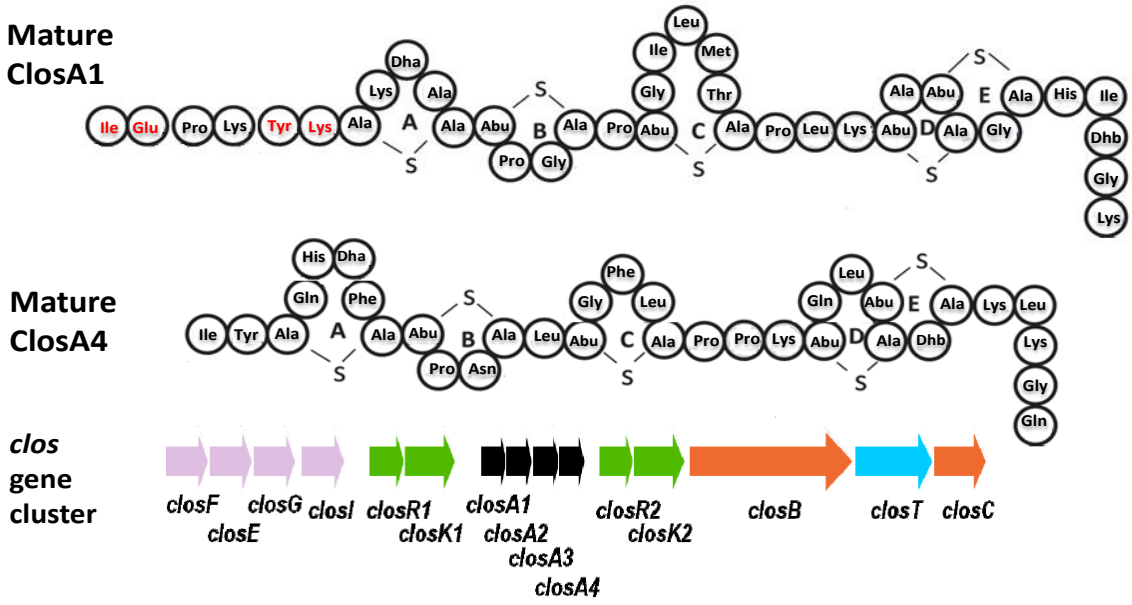


Figure 20 – Arrangement of the sequenced genes in the *clos* cluster identified from *Blautia obeum* A2-162 strain.

The predicted structure of mature ClosA1 and ClosA4 peptides with alternative start sites in red for ClosA1. The genes in the *clos* cluster are colour coded: structural genes in black (*closA1-4*), modification genes in orange (*closB*, *closC*), transport gene in blue (*closT*), immunity genes in purple (*closFEG*, *closI*), regulatory genes in green (*closR1K1*, *closR2K2*). Abu, Dha and Dhb are unusual amino acids introduced by ring formation and dehydration, respectively. The predicted Clos peptides differ from the structure of nisin by 12 amino acids. Adapted from Hatziioanou [97].

Our hypothesis is that amino acid substitutions can be introduced in the nisin template to create functional *clos*-like nisin molecules that are expected to be active under GI tract conditions.

The objective of this work was to use the nisin machinery and the genes identified in the novel *clos* cluster from *Blautia obeum* A2-162 to make a variety of engineered clos-like nisin variants. This will enable the investigation and the production of novel lantibiotics by expression in a lactococcal host strain. The ultimate aim was to tackle the great need for an improved treatment against GI tract diseases such as infections from GI tract pathogens.

A nisin construct was made containing the *nisA* gene. This construct was subsequently used as a template to incorporate amino acid substitutions based on the ClosA1 peptide sequence. The *nisA* gene was subcloned into two expression vectors and transformed in

L. lactis MG1614 host strain. To test for nisin expression, these constructs were transformed into the *L. lactis* FI7847 (Δ*nisA*) strain. This host strain has the entire nisin gene cluster and a 20 bp deletion in *nisA* which created the Δ*nisA* phenotype and caused the structural peptide to be out of frame [95, 96]. After the expression of *nisA*, the highly active nisin construct was selected for further subcloning as it produced similar levels of nisin as the wild type *L. lactis* FI5876 nisin producing strain.

A screening approach was then used to assess the antimicrobial production and nisin induction/self-induction of the mutant peptides using bioassays. The activity spectrum of these variants was also tested against different indicator strains aiming to detect mutants with similar or higher antimicrobial activities compared to the *nisA* construct. Colony Mass Spectrometry (CMS) allowed the successful detection of the mutant peptides and the intracellular processing of nisin such as effective proteolytic cleavage and full dehydration by NisB. Selected nisin mutants were purified and their specific activity and stability were tested under GI tract conditions. The results revealed that the purified I4K and H2G are novel bacteriocins, however their antimicrobial activity, stability and host range were not improved compared to the native nisin A.

3.2 MATERIALS AND METHODS

3.2.1 Generation of nisin derivatives

Site-directed mutagenesis of *nisA* [94, 111, 126] was achieved by PCR and by designing oligomers for cloning (Tables 10-12). Splice overlap extension PCR is a straightforward and reliable method that facilitates the engineering of genes [127]. PCR was performed as described in sections 2.2.6 and 2.2.7 [16].

3.2.1.1 *Selection of vectors for expression of modified nisin*

The insert containing the *nisA* structural gene was amplified from genomic or plasmid DNA from *L. lactis* strain FI5876 (nisin positive control) using the methods described in sections 2.2.3 and 2.2.4. Genomic DNA from *L. lactis* strain MG1614 (nisin negative control) was used as a negative control. The *nisA* wild type gene from bacterial genomic DNA was amplified with NisA_BspHF/NisA_BspHR primers to introduce the *BspH*I restriction site using the high fidelity Phusion DNA polymerase as described in section 2.2.6.

In order to optimise the cloning process, three different methods were used; the best cloning strategy was to use a *Nco*I, dephosphorylated pUK200 expression vector and an insert (PCR product with *BspH*I sites) cut with *BspH*I (section 3.3.1). The *Nco*I and *BspH*I restriction enzymes produce compatible cohesive ends (5' TCATGA 3') which allowed for cloning. The plasmid was initially cloned in *L. lactis* MG1614, then it was transformed in *L. lactis* FI7847 (Δ*nisA*) to allow for expression of the nisin peptide as described in section 2.2.

The antimicrobial activity of the pUK200_*nisA* construct was low compared to the wild type positive control so a smaller pTG262 vector was further selected for cloning. The pUK200_*nisA* construct was double digested with *Ssp*I and *EcoR*I restriction enzymes to create one blunt end/one sticky end insert which was cloned into the *Hind*III cut, end filled and *EcoR*I digested pTG262 vector (section 3.3.1.1).

The ligation product was used to transform electrocompetent *L. lactis* MG1614 strain. The transformants and controls were plated, and any positive controls were picked using toothpicks and grown in GM17 media with antibiotic selection. After sequence confirmation using primer NisA_BspHR (chapter 2), the plasmid was transformed in *L. lactis* FI7847 (Δ*nisA*) strain.

3.2.1.2 *Generation of novel clos-like nisin variants by site directed mutagenesis*

Splice overlap extension PCR was used to introduce I4K, L6A and A15I mutations to first make two PCR products using primer pairs which had matching tail with the amino acid substitution in. For I4K, the primer pair was *nisA*_I4K_F & pUK_R and pUK_F & *nisA*_I4K_R. The two PCR products were then overlapped and amplified using outer primers pUK_F & pUK_R as described in chapter 2. A similar splice overlap extension PCR cloning strategy was used to introduce I4K, L6A and A15I mutations in the *nisA* gene. The splice overlap extension PCR product was then digested with *Bgl*II and *Xho*I enzymes and cloned in pUK200 vector.

The three nisin mutants and the *nisA* as a positive control were cloned in the lactoccocal vectors pUK200, then they were all transferred in pTG262. To introduce the other mutations in the nisin template, only the pTG262 vector was used for cloning. All the plasmids were initially made in *L. lactis* MG1614, then they were finally transformed in *L. lactis* FI7847 to allow for expression and further characterisation of the mutant peptides. All the subcloning steps (cloning and transformation in *L. lactis*) were performed as described in section 2.2.

To introduce mutations in Ring C and in the C-terminus of nisin, a new cloning strategy that involved the use of a *Sac*I restriction site (at amino acid position 15) was used. Table 17 has details about the designed oligomer pairs used for cloning.

Within the *nisA* gene there is a *Sac*I restriction site and mutations were introduced by using oligonucleotides and cloning was organised in two ways: mutations between residues Ile1 to Ala15 were designed with the *Sac*I site at the C-terminal of the oligonucleotide, whereas mutations between Leu16 to Lys34 were designed with *Sac*I site at the N-terminus part of the oligonucleotide.

Mutations in Ile1 to Ala15 of *nisA* (N-terminal to the *Sac*I). This involved a two-step cloning strategy. A pUK200_I4K_L6A construct was first created using splice overlap extension PCR. The full region of nisin containing the mutation was then excised, and cloned in the pTG262 vector.

Mutations in Leu16 to Lys34 of *nisA* (C-terminal to the *Sac*I). The *Sac*I restriction site at position 15 in nisin was also used to generate the following mutants: G18T, N20P, M21L, H27G, H27G_S29H_ H31T_V32G_ S33K, H31T_V32G_ S33K.

Table 17 - Details for splice overlap extension PCR fragments and oligomers to make nisin mutants.

Mutation	Oligomer pair	Insert size *
I4K_L6A	I4K_L6A_F & I4K_L6A_R	123 bp cloned as *BspHI*/*BamH*I (1st part of nisin)
G18T	G18T_F & G18T_R	73 bp cloned as *Sac*I/*BamH*I (2nd part of nisin)
N20P	N20P_F & N20P_R	73 bp cloned as *Sac*I/*BamH*I (2nd part of nisin)
M21L	M21L_F & M21L_R	
N20P_M21L	N20P_M21L_F & N20P_M21L_R	
H27G		
H27G_S29H_ H31T_V32G_ S33K	H27G_S29H_H31Dhb_V32G_Dha33K_F & H27G_S29H_H31Dhb_V32G_Dha33K_R	
H31T_V32G_ S33K	H31Dhb_V32G_Dha33K_F & H31Dhb_V32G_Dha33K_R	
Mutation	Primer pair	Insert size**
I4K	*nisA*_I4K_F & pUK_R pUK_F & *nisA*_I4K_R	575 bp cloned as *Bgl*II/*Xho*I
L6A	*nisA*_L6A_F & pUK_R pUK_F & *nisA*_L6A_R	
A15I	*nisA*_A15I_F & pUK_R pUK_F & *nisA*_ A15I _R	
A15I_G18T***	G18T_F & pUK_R pUK_F & A15I_R	

*The mutations were introduced by designing two oligonucleotide sequences (forward and reverse). Annealing was done with equimolar concentrations at 90-100°C for 5 minutes, with slow cooling to RT for 60 minutes. A double stranded insert with restriction sites at each end was made and finally cloned in the pTG262 vector.

**The mutations were introduced by splice overlap extension PCR in a pUK200 vector. The nisin fragment with the mutations in was then excised and cloned in pTG262 vector.

*** The template was pUK200_*nisA*_A15I.

3.2.2 **Production and characterisation of nisin mutants**

The nisin mutant and control strains were grown from glycerol in 10 ml of GM17 with 10 µg/ml of chloramphenicol, subcultured in fresh media with antibiotic and induced with 10 µg/ml of nisin (DuPond, UK). Liquid culture was either dropped on plates (overlay assay) or the cultures were acidified to pH 3.0 with HCl diluted to 50 %, centrifuged for 10 min at 4000 x g then dropped on plate (drop test) or loaded in wells on a *L. lactis* strain seeded plated (plate diffusion assay) unless specified otherwise.

3.2.2.1 *Testing the antimicrobial activity of the engineered nisin variants*

To investigate the bioactivity, each nisin variant-producing strain was grown on GM17 agar plates containing 10 µg/ml of chloramphenicol and a nisin concentration suitable for induction. The mixture was prepared by adding a final concentration of 10 ng/ml of native nisin to a pre-warmed 100 ml GM17 agar bottle prior to pouring into plates. 1 to 3 µl of an overnight liquid culture from each of the 12 nisin mutant producing strains were spotted onto the plates, which were then incubated at 30°C for 12 h as described in section 2.1. Colonies grown overnight (drop of liquid culture) were rendered non-viable as they were inactivated under UV and antimicrobial activity was detected by overlaying strains directly with soft agar seeded with a sensitive indicator strain and by identifying zones of growth inhibition. Plates were incubated overnight at the optimal temperature of the indicator strain (e.g. typically, plates were overlaid with *L. lactis* MG1614 and were incubated at 30°C) as described in chapter 2, including the pTG262_*nisA* positive control and the pTG262 empty vector control. CMS was used to determine the relative molecular mass of the 12 nisin variants as described in section 2.5.

3.2.2.2 *Screening of nisin mutant producer strains for their spectrum of activity*

The same methods were used as described in chapter 2 with the following modifications. Nisin A (DuPont, UK) was used to induce antimicrobial production and it was prepared in filter sterile 50 diluted HCl adjusted to pH 3.0. Aliquots of the stock were stored at -20°C at concentrations of 10 mg/ml and 10 µg/ml.

3.2.2.2.1 **Colony overlay assay**

The UV treated plates were each overlaid with soft agar on an appropriate plate seeded with one of the indicator strains: *Micrococcus luteus, Bacillus cereus, Bacillus subtilis, Clostridium difficile, Clostridium perfringens, Enterococcus faecalis, Enterococcus faecium, Leuconostoc mesenteroides* sub., *Listeria innocua, Listeria ivanovii,* and *Staphylococcus aureus;* then, they were incubated overnight at the optimal temperature for the growth of the indicator organism.

3.2.2.2.2 **Drop test with culture supernatant**

Indicator strains grown overnight (listed in previous section) were diluted 1:10 or 1:100 in PBS, then lawns of sensitive strains were made on solid agar suitable for each strain using cotton swabs. 12 overnight cultures of nisin mutant producing strains were acidified

to pH 3.0 with HCl diluted to 50 % and centrifuged for 10 min at 4000 x g. 5 to 10 µl aliquots of each cell free acidified supernatant were placed on the surface of each lawn, air dried and incubated overnight at the optimal temperature in aerobic conditions. A negative control with 50% diluted HCl alone was also included in the test.

3.2.2.3 *Testing for self-induction capacity of nisin mutants*

Bioassay plates were seeded with *L. lactis* MG1614 nisin-sensitive indicator strain as described in 2.1 with modifications as described below. The bioassay was used for the detection of nisin mutant production in liquid cultures, which were previously induced with different concentrations of native nisin.

L. lactis producer strains for 12 nisin variants were grown from glycerol in individual 10 ml of GM17 with 10 µg/ml of chloramphenicol and subcultured (1:100) in fresh media with 10 ng/ml of nisin for induction in *L. lactis* FI7847 (Δ*nisA*) host strain. After overnight incubation with subinhibitory amount of nisin to allow for transcription initiation of mutated *nisA* sequence, the liquid cultures were subcultured with 0, 10, 100 ng/ml of nisin. The induction capacity of each mutant was determined in relation to native nisin. The culture supernatants were assayed directly from an overnight culture, prepared by acidifying liquid cultures to pH 3.0, centrifuged to remove cells and filter sterilised. Plates were incubated at 4°C for 3-5 h, prior to overnight incubation at 30°C, to allow maximum diffusion of sample into the agar before the growth of the *L. lactis* MG1614 indicator organism. Zones of inhibition for the induced mutants were compared to vector only negative control (pTG262 in FI7847).

The zones of inhibition were measured for nisin standards over a suitable series of concentrations and were plotted against the logarithm of nisin standard concentrations. The nisin standard curve was then used to estimate the relative activity in the tested samples (see 2.1.3.2).

3.2.2.4 *TEM of nisin mutant producer strains*

To investigate any change in phenotype of the cell morphology of nisin mutants H27G_S29H_ H31T_V32G_ S33K and L6A, nisin and the nisin mutant producer strains were cultured from glycerol in selective media, subcultured and induced with 10 ng/ml nisin. After strains were grown overnight, the cultured cells were analysed by transmission electron microscopy (TEM) by Kathryn Cross (IFR).

3.2.3 Purification and assessment of bioactivity of nisin mutants

3.2.3.1 *Optimising the amount of nisin used for induction*

Nisin induces its own production [74] however, I4K, L6A and H27G nisin mutants are not self-inducers based on section 3.2.2. In order to optimise the amount of nisin needed to induce nisin variants for the purpose of large scale peptide production, liquid cultures for I4K, L6A, H27G, pTG262_*nisA* (positive control) were grown with increasing concentrations of external nisin and bioactivity was assessed in plate diffusion assay.

The four *L. lactis* bacterial strains were grown overnight from glycerol in 10 ml GM17, then they were subcultured with 10 ng/ml of nisin and grown overnight at 30˚C. Strains were subcultured with 0, 3, 30, 100 and 200 ng/ml of native nisin and incubated overnight. In this experiment, and for the scaled up production of nisin mutants in large fermenters, a nisin stock of 2.5 % pure nisin was purchased from Sigma. Nisin powder formulation contains 75 % NaCl and 22.5 % denatured milk solids. Nisin stock concentrations were prepared at 1 mg/ml in 50% diluted HCl to pH 3.0. In this study, the Sigma nisin concentrations were based on the pure nisin concentration not on weight of the formulation.

3.2.3.2 *Purification of nisin mutants*

In order to maximize the scaled up peptide production in the 11-14 L fermenters, pH was maintained at 6.0. The nisin mutant-producer strains were grown from glycerol in 600 ml of GM17 with antibiotic then the overnight liquid cultures were subcultured in 1:100 in SPY-S media, or until initial OD_{600} reached 0.1. The liquid cultures were induced with 100 ng/ml nisin powder (Sigma) (for H27G, nisin and L6A) or 200 ng/ml nisin (for I4K); 10 µg/ml of chloramphenicol was also added for plasmid selection. The protocol described in chapter 2, section 2.3 was used.

After the overnight liquid culture was harvested, the cell-free supernatant of the nisin mutants adjusted to pH 6.0 was purified using a two-step process. Culture supernatant was subjected to a first purification step involving hydrophobic interaction chromatography on Fractogel TSK Butyl 650-S. The second step involved purification of lyophilised and resuspended fractions on a C18 RP semi-preparative HPLC column. The purity of the Fractogel fractions was assessed on an analytical C18 HPLC column before it was further purified on semi-preparative RP-HPLC. The bioactivity of the peptide was

monitored via plate diffusion assays against *L. lactis* MG1614 as indicator strain and the extent and nature of the amino acid substitution was identified by Maldi-ToF.

3.2.4 Characterisation of purified nisin mutants

3.2.4.1 *Determining the specific activity of I4K and H27G nisin mutants*

A range of concentrations for the purified I4K, H27G and wild type nisin A were tested for their ability to inhibit growth of *L. lactis* MG1614 by plate diffusion assays (chapter 2, section 2.1.3).

The aim was to select an equivalent concentration for I4K and H27G that would give similar antimicrobial activity as pure nisin. The antimicrobial activity was reported in nisin units: 1 unit of nisin was set as 100 % activity detected from 9 µg/ml of nisin.

A stock solution of 1.5 mg/ml of nisin A, H27G and I4K was prepared by resuspending 1.5 mg of pure nisin A (Du Pont, UK), pure H27G or pure I4K into diluted HCl to 50 % adjusted to pH 3.0. Standard solutions for each peptide were prepared in buffer as follows:

For nisin: 15, 12, 9, 6, 3, 1.5, 0.9 and 0.6 µg/ml.

For I4K: 50, 40, 30, 20, 10, 5, 2.5 µg/ml and 15, 12, 9, 5, 3 and 1.5 µg/ml.

For H7G: 50, 40, 30, 20, 10, 5, 2.5, 0.9, 0.6 µg/ml and

15, 12, 9, 5, 3, 1.5, 0.9, 0.6 and 0.3 µg/ml.

9 µg of H27G, 40 µg of I4K and 9 µg of nisin were each resuspended in 1 ml of buffer as described in section 2.1.3.2. The three sets of samples and 1 set of buffer only were incubated at 37°C for 7 days and triple aliquots were removed at set intervals and tested in plate diffusion assay against *L. lactis* MG1614 indicator strain.

3.2.4.2 *Activity of purified nisin mutants under GI tract conditions*

The pH of the GI tract varies from 2.0 to 8.0 [128]. To test the bioactivity and the stability of purified nisin mutant peptides under GI tract conditions, peptides were incubated at pH values and temperature relevant to the GI tract.

Initial stocks of purified H27G, I4K and pure nisin (Aplin & Barret) were redissolved in diluted HCl to 50 % adjusted to pH 3.0. 9 µg of H27G, 9 µg of nisin and 40 µg of I4K were dissolved in 1 ml of buffer at different pHs following the method described by Rollema *et al.* [47]. Solutions at pH 3.0, 4.0, 5.5, 6.0, 6.5 and 7.0 were prepared and incubated at 37°C. For pH 3.0, no other buffer was used. For pH 4.0 to 5.5, and for pH range of 6.0, 6.5 and 7.0, 20 mM sodium acetate buffer and 20 mM sodium phosphate buffer were used, respectively. All peptide solutions contained 0.1 M NaCl and the

samples were incubated at 37°C for 7 days. Aliquots were collected at regular time intervals and 200 µl (approx. 1.8 µg for H27G and nisin, 8 µg for I4K) of peptide was loaded in each well in duplicate plates. Aliquots were collected at time zero, and after 24 h, 48 h, 68 h and 7 days of being incubated at 37°C. Peptide stability and biological activity was determined by plated diffusion assay against *L. lactis* MG1614 incubated at 30°C.

3.2.4.3 *Antimicrobial spectrum of activity of nisin mutants*

Solutions of 12, 9, 5, 3, 1.5, 0.9, 0.6 µg/ml of H27G and nisin A were prepared in 20 mM sodium phosphate at pH 6.0 and tested in plate diffusion assays. In order to mimic the GI tract conditions (colon for pH 6.0), both the peptide solutions as well as the seeded agar were prepared at pH 6.0 and plates were incubated at 37°C overnight. The solutions were loaded on seeded plates with agar that was pH adjusted to 6 prior to loading the well. The selected indicator strains were grown in pH 6.0 adjusted medium: *B. subtilis, C. difficile, C. perfringens, E. faecalis, E. faecium, L. ivanovii, S. aureus*.

3.3 RESULTS

3.3.1 Generation of nisin derivatives

3.3.1.1 *Cloning the original nisA gene in pUK200*

The aim was to create *nisA* mutants to incorporate features of the ClosA1 peptide. The original nisin structure was used as a template. The original *nisA* gene from *L. lactis* FI5876 was subcloned on a pUK200 expression vector in *L. lactis* FI7847 (Δ*nisA*).

The best cloning strategy is illustrated in Figure 21 and it involved the use of a two cohesive ends insert and a dephosphorylated vector.

To confirm the formation of the desired mutation and the integrity of the wild type *nisA* gene, selected colonies were sequenced to confirm 100 % match to the original nisin gene sequence (data not shown).

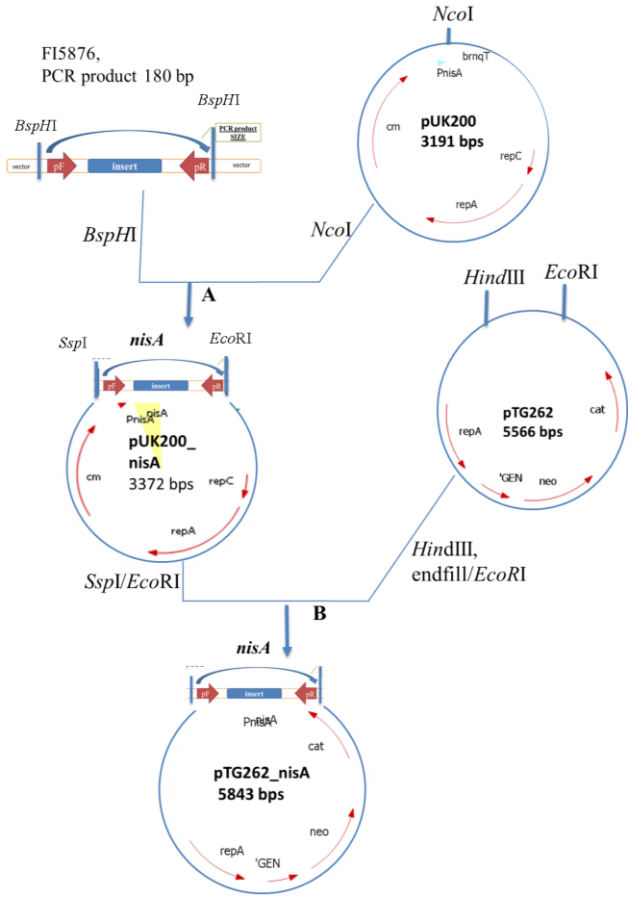


Figure 21 - Cloning *nisA* gene from genomic DNA of FI5876 to make A. pUK200_*nisA* and B. pTG262_*nisA* expression vectors.

3.3.1.2 *Design of clos-like nisin mutants*

The bioengineered nisin template pUK200_*nisA* was used to incorporate single and multiple mutations to generate a range of *clos*-like nisin mutant peptides (Figure 22).

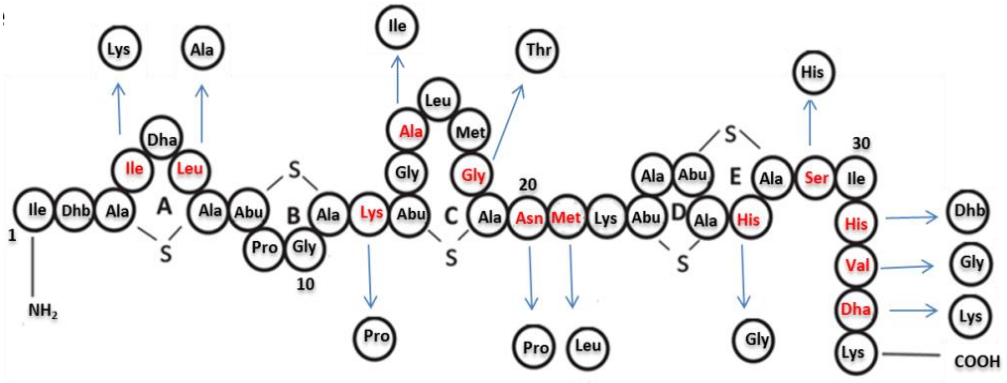


Figure 22 - Generation of *clos*-like nisin mutants.

A range of nisin variants that have features of the ClosA1 peptide were generated by subsequent amino acid substitution in the original nisin structure as highlighted in red.

The original *nisA* gene was subcloned in two expression vectors: pUK200 and pTG262 to make pUK200_*nisA* and pTG262_*nisA* constructs, which were expressed in *L. lactis* containing the nisin gene cluster (Δ*nisA*). Induction of the nisin promoter (P_{nisA}) by nisin and the expression of *nisA* by the nisin biosynthetic machinery in *L. lactis* strain were achieved with subinhibitory amounts of exogenous mature nisin and the antimicrobial activity was assessed (Figure 23).

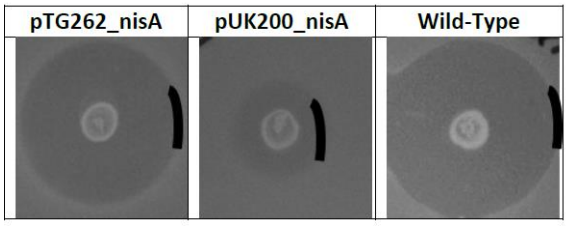


Figure 23 - Antimicrobial activity of *nisA L. lactis* producing strains overlaid with *L. lactis* MG1614 indicator strain. This picture is a representative image of three independent agar plate diffusion assays.

The pTG262_nisA construct was more effective compared to pUK200_nisA and it allowed the production of antimicrobial activity which proved to be nearly as high as the wild type nisin, FI5876; this plasmid was then used as a template to introduce subsequent mutations, followed by a short *nisA* structural gene.

The difference in expression levels could be attributed to the plasmid copy number, as the halo size was different when *nisA* was expressed from either pUK200 or pTG262.

These results demonstrate that the *nisA* gene was successfully expressed by the nisin biosynthetic machinery of *L. lactis* strain. The pTG262_*nisA* construct was therefore selected for subsequent generation of the nisin mutants.

3.3.1.3 *Construction of novel clos-like nisin variants by site directed mutagenesis*

One of the aims of the project was to make successive changes in the *nisA* gene of the nisin cluster using a nisin construct to alter the sequence and to generate a range of active *clos*-like nisin mutants. The aim of the work was to understand how flexible the nisin machinery is at tolerating amino acid substitution before it fails and can no longer modify and /or express any active peptide. Therefore, the effect of introducing single or multiple mutations in the rings was first assessed (Figure 22). The initial efforts to make nisin variants focussed on introducing modifications in the Rings A, C, E and the hinge region of nisin where they were previously found to have the largest effects on activity [40, 91]. Standard genetic engineering and manipulations were performed using established procedures. The splice overlap extension PCR strategy was employed to mutate the *nisA* gene which replaced the wild type *nisA* gene to make three nisin variants (I4K, L6A, A15I). Conventional PCR reactions with designed oligonucleotides allowed the successful construction of 9 additional nisin mutants. Forward and reverse single stranded oligomers were annealed to make a 73 bp insert (*Sac*I/*BamH*I) or a 123 bp insert with a *Sac*I site inside (*BspH*I/*BamH*I). The following mutants where made using the 73 bp insert: G18T, N20P, M21L, H27G, H27G_S29H_ H31T_V32G_ S33K, H31T_V32G_ S33K. Using the 123 bp insert the following mutant was made: I4K_L6A. The cloning process to make mutants A15I_G18T, N20P_M21L and H31T_V32G_ S33K will be described below.

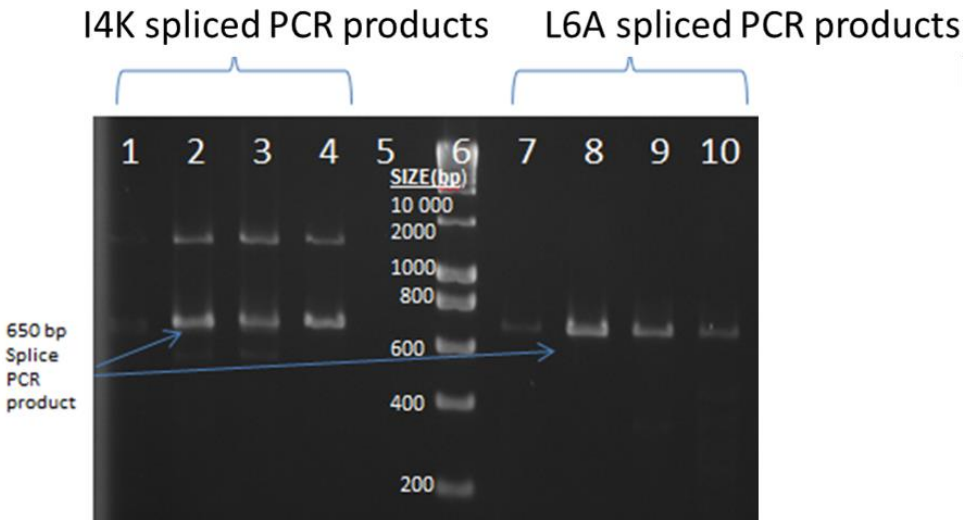


Figure 24 - Splice overlap extension PCR products to introduce either I4K or L6A mutation in the pUK200_*nisA* construct.

Two PCR products were made using one vector primer (pUK_R or pUK_F) and one primer that incorporated the changed nucleotides: *nisA*_I4K_F or *nisA*_I4K_R. The resulting 2 PCR products were then mixed and amplified with the vector primers (pUK_F/pUK_R) to give a splice overlap extension PCR product of 650 bp. Lane 1: reaction with 2 ng of the 2 PCR products; Lane 2: reaction with 8 ng of the 2 PCR products, lane 3: control with one PCR product; lane 4: control with the second PCR product; lane 5: control with UPH_2O, lane 6: marker, lane 7: reaction with used 2 ng of 2 PCR products, lane 8: reaction with used 8 ng of 2 PCR products, lane 9: control with one PCR product; lane 10: control with the second PCR product.

The pUK200_*nisA*_I4K construct was made using splice overlap extension PCR and pairs of primers: *nisA*_I4K_F/pUK_R and pUK_F/*nisA*_I4K_R. The engineered splice overlap extension PCR fragment with the I4K mutation in was ligated in *L. lactis* MG1614 host strain (Figure 25). Sequencing of pUK200_*nisA*_I4K or L6A or A15I constructs confirmed the nisin mutant peptides had the correct mutation in. These pUK200 constructs were then successfully electroporated into *L. lactis* FI7847 and confirmed by PCR from colonies obtained with antibiotic selection.

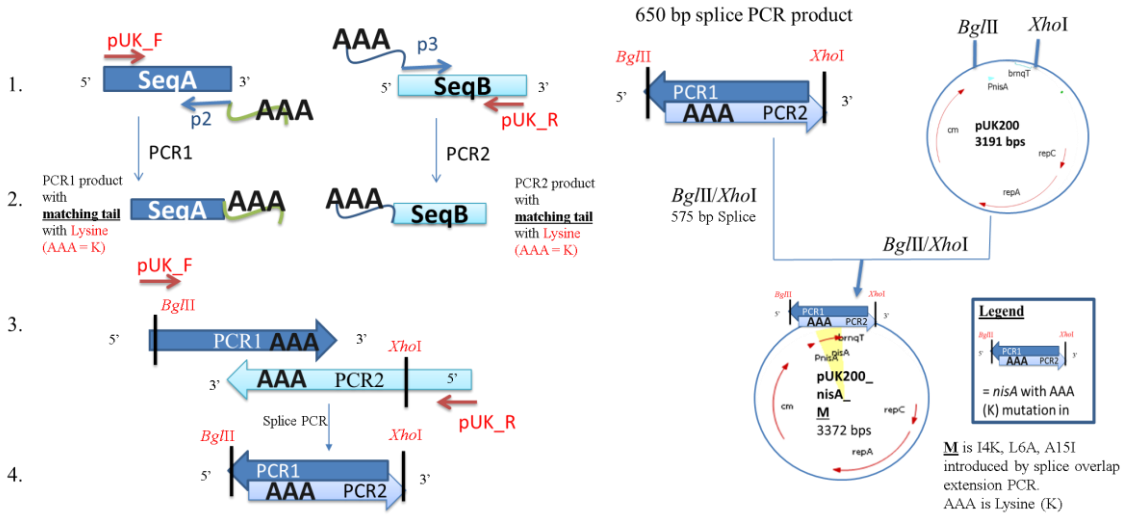


Figure 25 - Diagram to illustrate site directed mutagenesis of *nisA* gene to make I4K nisin single mutant.

A. Splice overlap extension PCR to introduce Lysine mutation (K = AAA) at position 4 instead of Isoleucine (I = ATT) in nisin, B. cloning to make I4K mutant in pUK200 expression vector. Primer p3 is the *nisA*_I4K_F primer and p2 is the *nisA*_I4K_R primer.

Since the cloning in the pTG262 plasmid improved expression of *nisA*, the three individual *nisA* mutated inserts (I4K, L6A and A15I) were excised and cloned as described in section 3.2.1.2. The pTG262_*nisA* plasmids with the incorporated mutations were finally cloned in *L. lactis* FI7847 (Δ*nisA*) host strain.

To make the A15I_G18T double mutant in the pTG262_*nisA* expression vector, a two-step cloning strategy was used: 1. pUK200_*nisA*_A15I construct was used as a template to introduce the G18T mutation by splice overlap extension PCR. 2. The A15I_G18T insert was excised and cloned to make pTG262_*nisA*_ A15I_G18T construct.

Splice overlap extension PCR was performed as before using G18T_F/pUK_R and pUK_F/A15I_R sets of primers. The two PCR products were overlapped with the outer primers pUK_F and pUK_R, then the 650 bp insert was cut with *Bgl*II/*Xho*I. The 575 bp insert was cloned in pUK200_*nisA*_A15I vector previously digested with *Bgl*II and *Xho*I. Ligation, transformation and sequencing were performed as described in chapter 2. The new construct pUK200_*nisA*_A15I_G18T was digested with *Hind*III, end filled, and digested with *Xba*I and cloned into the pTG262_*nisA* vector digested with *Ssp*I and *Xba*I to make the pTG262_A15I_G18T.

After numerous attempts to make mutants I4K_L6A, G18T, N20P using the same splice overlap extension PCR strategy, an alternative method was used for subsequent cloning which was routinely giving multiple bands. Initially, bands of interest were excised and purified from gel, however any subsequent attempts to create the correct constructs were

not successful according to DNA sequencing. Instead, pairs of single stranded DNA sequences, known as oligomers, were designed as detailed in Table 17. A *Sac*I site identified at position 15 in the nisin sequence was used to plan a cloning strategy with the aim to introduce mutations in the *nisA* structural gene.

Mutations in Ile1 to Ala15 of *nisA* (N-terminal to the *Sac*I). This involved a two-step cloning strategy.

1. A pUK200_I4K_L6A construct was made with splice overlap extension PCR.
2. The entire nisin mutated region was excised and cloned in pTG262.

Firstly, a 123 bp insert that comprises mutations before the *Sac*I site (e.g. I4K_L6A nisin mutant) was made using a designed pair of forward and reverse oligonucleotides which were annealed to make a *BspH*I and a *BamH*I restriction site at each end (Figure 27, C). The 123 bp double stranded insert had a *Sac*I site within the PCR overlap sequence, which was used for subsequent cloning. The insert was cloned in a pUK200 vector previously digested with *Nco*I and *BamH*I enzymes. Secondly, the resulting construct was digested with *Sac*I and *BamH*I and ligated to a 73 bp PCR product (amplified with pTG262_S_F/ pTG262_B_R from pTG262_*nisA*), which was cut with *Sac*I/*BamH*I. This step created the second part of nisin (Figure 26, Figure 27).

The pTG262_*nisA*_I4K_L6A construct was made to allow for an enhanced expression of the mutant peptide. The pTG262 vector was digested with *Hind*III, end filled and digested with *EcoR*I. A 220 bp fragment was prepared as follows: the pUK200_*nisA*_I4K_L6A construct was digested with *Ssp*I/*EcoR*I and ligated to the prepared vector.

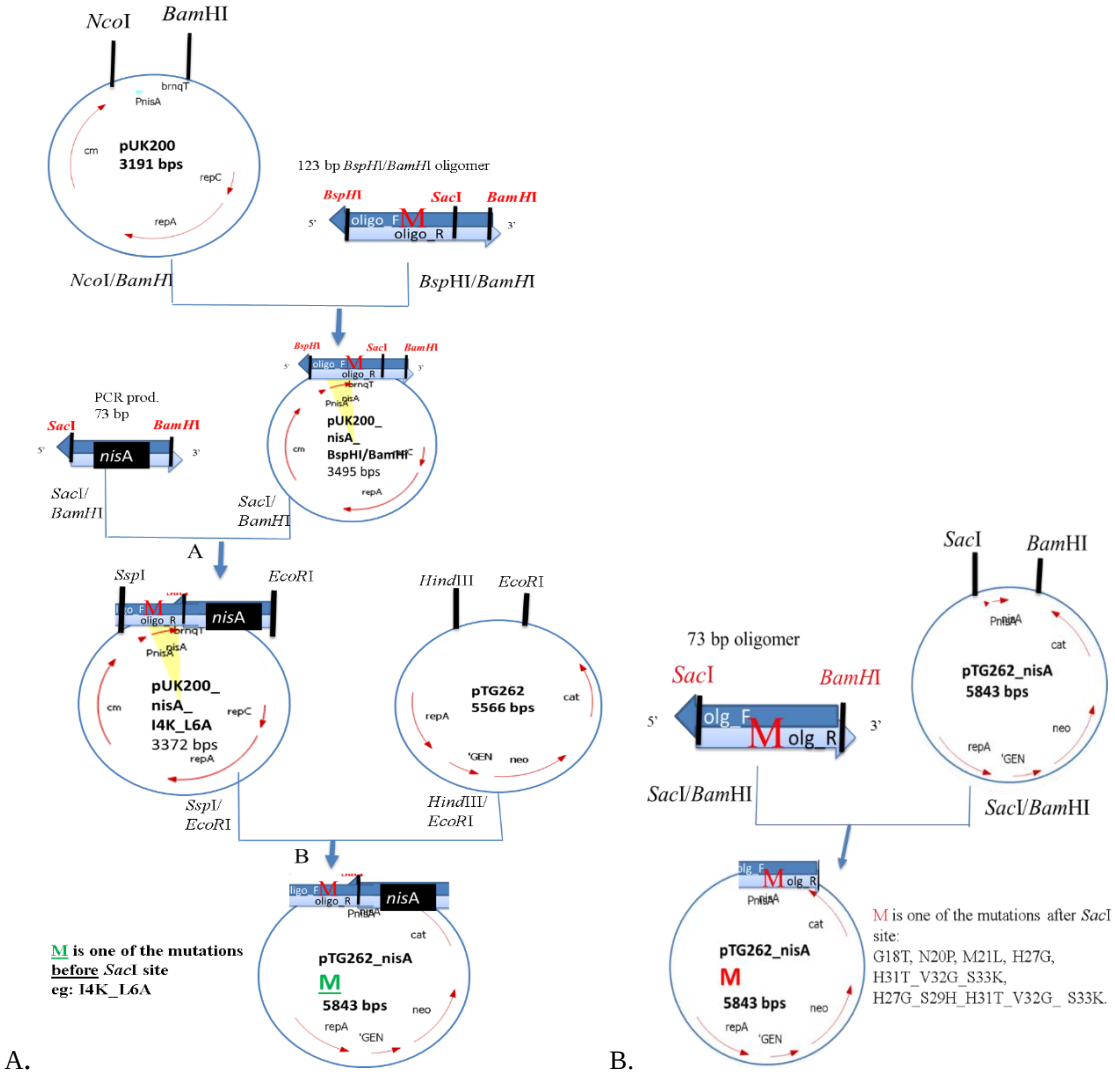


Figure 26 - Diagram to illustrate cloning in Ile1 to Ala15 of *nisA* (N-terminal to the *Sac*I) to create the I4K_L6A mutant (A) and cloning in Leu16 to Lys34 (C-terminal to the *Sac*I) (B).

I4K_L6A mutant was created using A. pUK200 *Nco*I/*BamH*I cut vector, 123 bp oligomer to introduce the I4K_L6A mutation and 73 bp oligomer to create the second half of nisin; B. pTG262 *Hind*III/*Eco*RI cut vector and *Ssp*I/*Eco*RI excised *nisA*_I4K_L6A fragment. M is I4K_L6A.

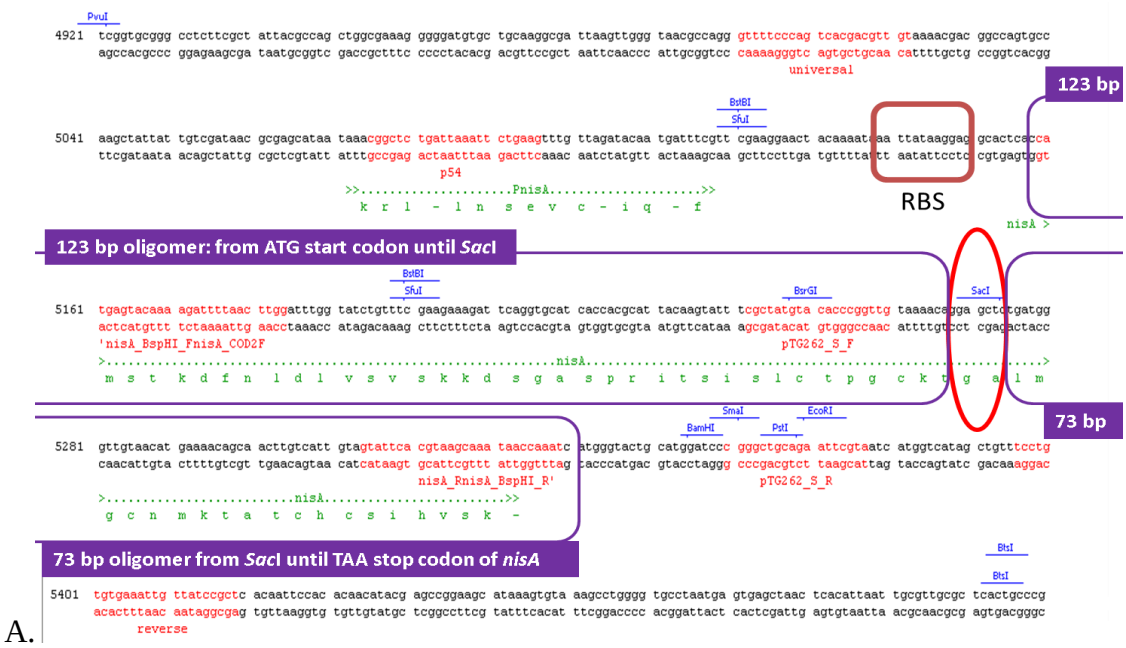
Cloning after Leu16 to Lys34 (C-terminal to the *Sac*I) was done in pTG262_*nisA* construct using a 73 bp oligomer. M is one of the mutants: G18T, N20P, M21L, H27G, H31T_V32G_S33K, H27G_S29H_ H31T_V32G_ S33K.

Mutations in Leu16 to Lys34 of *nisA* (C-terminal to the *Sac*I). The *Sac*I restriction site at position 15 in nisin was also used to generate the following mutants: G18T, N20P, M21L, H27G, H27G_S29H_ H31T_V32G_ S33K, H31T_V32G_ S33K.

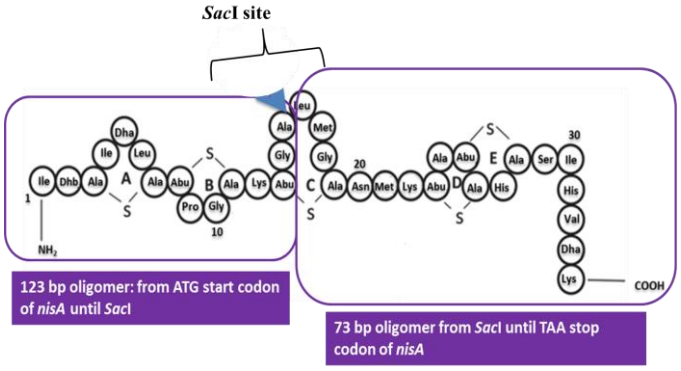
pTG262_*nisA* vector was restricted with *Sac*I and *BamH*I. A 73 bp insert that comprised mutations after the *Sac*I site was made using designed pairs of forward and reverse oligonucleotides which were annealed (Table 17). The 73 bp double stranded insert had a *Sac*I/*BamH*I site already created by annealing as described above and in chapter 2.

The new cloning strategy involved the use of the unique *Sac*I restriction site that was located at position 15 in the Ring C of nisin (Figure 27). Pairs of forward and reverse single stranded oligonucleotides were annealed to make a 73 bp double stranded DNA insert bearing mutations and cloning was performed as in section 3.2.1.2. After the pair of oligonucleotides was annealed the resulting insert created the restriction sites for *Sac*I and *BamH*I on each end. The *Sac*I/*BamH*I fragments, incorporating mutations in *nisA*, were subsequently cloned into the multicloning site of the *Sac*I/*BamH*I cut pTG262_*nisA* shuttle vector. The ligated construct was transformed into *L. lactis* MG1614.

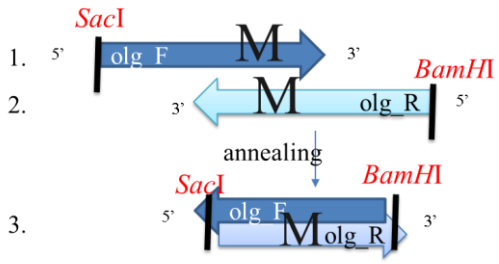
PCR and sequence analysis of *L. lactis* MG1614 transformants confirmed successful ligations and verified the position of amplified DNA within the *nisA* gene, creating an uninterrupted *nisA* structural coding region with the desired substitutions in the nisin template.



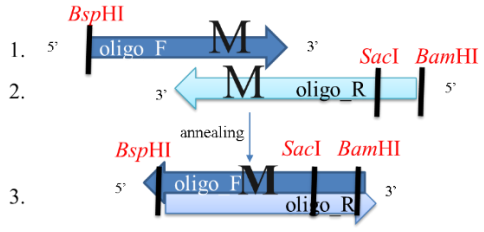
A.



B.



C.



D.

Figure 27 - Illustration to introduce mutations in *nisA* sequence using oligomers.
M = mutation
A. *nisA* sequence with the *Sac*I restriction site.
Primer pair, universal and reverse, were used in PCR to confirm successful incorporation of mutations.
B. Nisin structure in which *Sac*I site is highlighted by a blue arrow.
C. 73 bp (*Sac*I/*BamH*I) fragment to make the second part of native nisin where 1. Single stranded DNA, forward oligomer (olg_F); 2. Single stranded DNA, forward oligomer (olg_R); 3. Double stranded DNA, with *Sac*I restriction site at 5' and *BamH*I site at 3', there is no need to cut the insert as sites are already created by oligomer annealing.
D. 123 bp (*BspH*I/ *BamH*I) fragment was used to make the first part of native nisin where 1. Single stranded DNA, forward oligomer (oligo_F); 2. Single stranded DNA, reverse oligomer (oligo_R); 3. 123 bp double stranded DNA, with *BspH*I restriction site at 5', *Sac*I restriction site and *BamH*I site at 3' end.

An uninterrupted nisin sequence was created by using a combination of primers in PCR which included a 73 bp and a 123 bp sequence of amplified DNA fragments as illustrated in Figure 27. Splice overlap extension PCR and designed oligonucleotides were used to successfully introduce mutations in the nisin template. The successful generation of different cloning products during different steps of the cloning process were assessed by agarose gel electrophoresis (Figure 24).

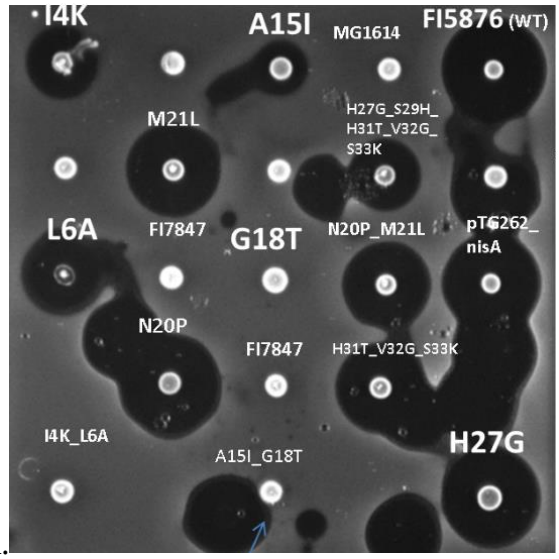
3.3.2 Production and characterisation of nisin variants

Several derivatives of nisin have so far been generated in which single or multiple amino acids were simultaneously changed (Figure 22). All nisin mutants were subject to bioassays such as the overlay assay, drop test or plate diffusion assay against indicator strains, most typically *L. lactis* MG1614, to identify the effect of amino acid substitutions.

3.3.2.1 *Antimicrobial spectrum of nisin variants*

The overlay assay was used as a sensitive method for the initial screening of bioactivity of nisin mutant producing strains against nisin-sensitive indicator strains. The bioactivity of the mutants was compared to the nisin positive control strain: pTG262_*nisA* in FI7847 (Δ*nisA*). The overlay assay was supported by other bioassays to determine if this enhanced bioactivity is attributable to enhanced specific activity [13, 16], production, solubility or to diffusion [17].

A.



B.

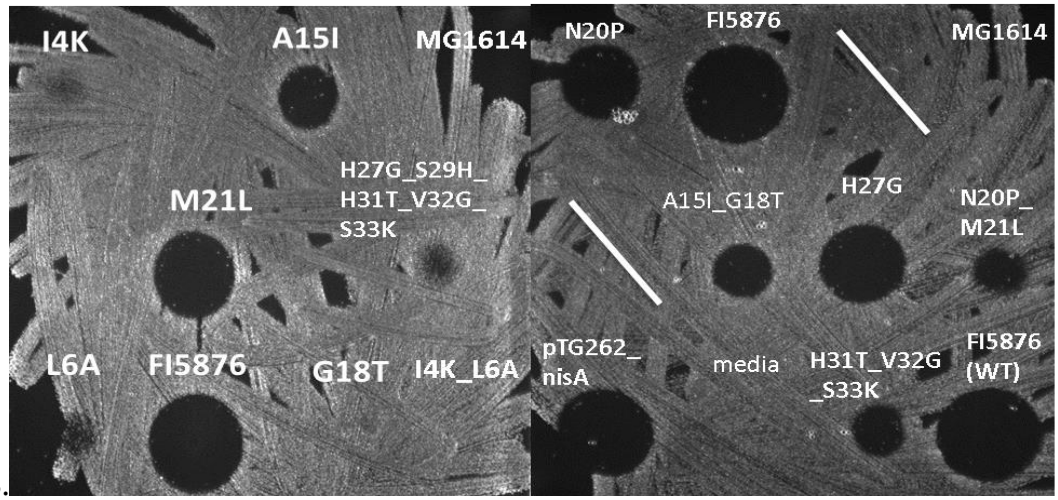


Figure 28 - Antimicrobial activity of nisin-variant strains with induction by native nisin as monitored by (A) overlay assay and by (B) drop test against *M. luteus.*
Antimicrobial activity shown as zones of clearing/bacterial inhibition against *M. luteus*. The pictures are a representative image of two independent agar plate diffusion assays (A) and drop tests (B).

Colony overlay assay (A). Indicator strain: *M. luteus*. Positive controls: colonies of FI5876 wild type strain, nisin producing strain pTG262_*nisA* in *L. lactis* FI7847 (Δ*nisA*) and the negative control strain: colonies of plasmid free *L. lactis* MG1614 and acidified GM17 media. Nisin mutants and Clos hybrids were expressed from pTG262 in *L. lactis* strain (Δ*nisA*) (see section 3.2.5 and 3.3.5).

Drop test (B). The level of bioactivity of acidified nisin mutant supernatant was determined from diffusion of sample spotted on a lawn of nisin sensitive *M. luteus* indicator strain.

Similar bioactivity assays as in Figure 28 were performed using different indicator strains.

The results were summarised in Table 18 and Table 19.

		Colony	Supernatant	
Nisin mutant	**Location of mutation**	**Activity in overlay assay**	**Activity in drop test**	**Activity in supernatant* (plate diffusion assay)**
I4K	Ring A	++	+ (v. low)	++
L6A		+++	+ (v. low)	+++
I4K_L6A		-	-	+
A15I	Ring C	+	++	+
G18T		-	-	-
A15I_G18T		-	+/++	+/-
N20P	Hinge region	+++ (higher)	+++	++
M21L		+++	+++	+++
N20P_M21L		+++	++	++
H27G	C-terminal end of nisin	+++	+++	+++
H27G_S29H_ H31T_V32G_ S33K		++	+	+
H31T_V32G_ S33K		++	++	+

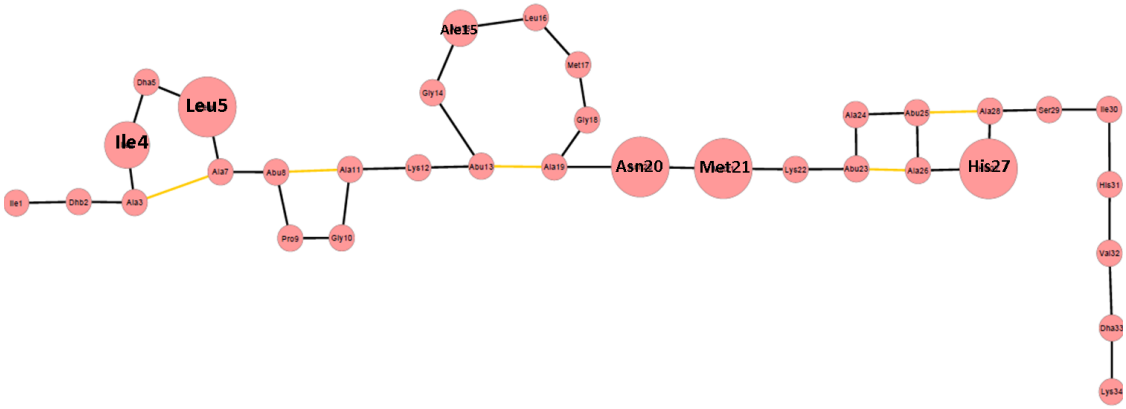


Table 18 - The antimicrobial activity of Ring A, Ring C, Hinge region, Ring E and the C-terminal nisin mutants tested against *M. luteus* or *L. lactis* MG1614 indicator strains.
The table highlights the bioactivity of each nisin mutant compared to nisin.
Illustration of the nisin structure and the effect of the mutations on individual amino acids. The size of each amino acid corresponds to the antimicrobial activity of each nisin mutants as represented by the cytoscape software.
*Bioactivity of nisin and nisin mutants' from acidified culture supernatants tested against *L. lactis* MG1614; liquid cultures initially induced with 100 ng/ml of nisin as described in section 3.3.2.3 (Figure 30).
FI7369 *L. lactis* strain is a nisin producer positive control with pTG262_*nisA* construct.
Legend for overlay assay to assess bioactivity:
'+++' = 80-100 %; '++' = 50 - 80 %; '+' = 20 - 50 %; '-' = < 20 %.
Legend for drop test to assess bioactivity/diffusion:
'+++' = 50-100 %; '++' = 50 %, '+' = 20 %; '-' = < 20 %.
80 – 100 %, is wild-type nisin and it was used as a reference.

Table 18 summarises the relative antimicrobial activity of the nisin mutants compared to positive control: pTG262_*nisA* in FI7847 (Δ*nisA*) depicted from assays against *M. luteus* and *L. lactis* MG1614. It should be considered that for accurate comparison of bioactivity the peptides need to be purified, otherwise the amount of peptide on plate or exported in the supernatant remains unknown. The results confirm that the production, secretion and export of active peptides differ depending on the growth of mutants on liquid or solid media.

Indicator strain/ nisin mutants	Ring A mutants			Ring C mutants			Hinge mutants			Ring E & C-terminal mutants		
	I4K	L6A	I4K_ L6A	A15I	G18T	A15I_ G18T	N20P	M21L	N20P_ M21L	H27G	H27G_S29H_H31T _V32G_S33K	H31T_V32G _S33K
L. lactis MG1614	+++	+++	-	+	-	-	+++	+++	+++	+++	+	++
B. cereus	-	-	-	-	-	-	+++	+	+	-	-	-
M. luteus	++	+++	-	+	-	-	+++/ ++	+++/ ++	+++	+++/ ++	++	+++
S. aureus	+	+	-	-	-	-	+++	++	+	+	-	-
L. mesenteroides sub mes.22θ	++	-	-	+	-	-	+++	+++	++	+++	+	+
C. perfringens	++	++	-	+	-	-	+++/ ++	+++/++	++	+++	++	+++/ ++
C. difficile	+	+	-	-	-	-	+++	++	++	+	++	+++
E. faecalis	IC	IC	IC	-	IC	-	IC	IC	IC	+	+	+
L. ivanovii/ L innocua	+	+	-	+ / -	-	-	+	+	+	+	+	+

Table 19 - Summary of antimicrobial activity of different nisin mutants from overlay assay using *L. lactis* MG1614 and nine other bacterial strains.

All tested mutants and hybrids were expressed from pTG262 in the *L. lactis* FI7847 (Δ*nisA*) strain induced with 10 ng/ml of nisin. The pTG262_*nisA* in *L. lactis* FI7847 (Δ*nisA*) was used as a reference and it was active against all the tested indicators. The activity of this positive control was set as 100 %. Legend: +++ = 80-100 % activity; ++ = 50 - 80 % activity; + = 20 - 50 % activity; - = no activity. IC means inconclusive at it was difficult to distinguish the bioactivity from background. '+++/++' means that the antimicrobial activity was very close to 80 %. '+/-' means that the antimicrobial activity was very close or below 20 %.

When *L. ivanovii* and *L. innocua* indicator strains were tested separately, the antimicrobial activity was very similar.

Nisin was used as a reference and the antimicrobial activity of nisin mutants was compared to nisin. The results from the assays with 10 target indicator strains including pathogens are shown in Table 19. The best results were obtained from the producer of the H27G and N20P which exhibited very similar (80 – 100 % activity) of bioactivity compared to the nisin positive control. The nisin producer strain for the Ring A single mutant, I4K, generally showed as low levels of bioactivity compared to nisin when tested against *L. mesenteroides.* I4K was inactive against *B. cereus* whereas the double mutant was inactive against all tested strains. All three hinge mutants had high activity against all strains, with N20P having an equal level of activity against *S. aureus*, which is in line

with the results in literature [87] and very similar activity against *M. luteus*, *C. perfringens* and *C. difficile*. The Ring E and the C-terminal mutants were also active against different strains. H27G had very high activity against most of the strains; however, it was active against *B. cereus* in the same way as H27G_S29H_H31T_V32G_S33K and H31T_V32G_S33K peptides.

Ring A mutants:

In plate diffusion assays, the I4K mutant is less active than the positive control whereas the L6A mutant is more active both when induced with 10 or 100 ng/ml of nisin compared to nisin (Figure 30) as also summarised in Table 18. The I4K_L6A double mutant showed no activity in the bioassays against *M. luteus*. Interestingly, in the plate diffusion assay the I4K_L6A mutant (only the 100 ng/ml induced mutant was tested) there is an indication that antimicrobial production is present even if at low levels, when this is compared to the *nisA* construct or the vector only control (induced with 100 ng/ml). Overall I4K is more active than L6A in overlay assays, however L6A seems to give a bigger zone of inhibition than I4K when tested against *C. perfringens.* I4K, L6A and *nisA* have the same bioactivity when tested against *C. difficile.* However, L6A is not active against *S. aureus* and *L. mesenteroides*. Both nisin mutants lost their antimicrobial activity against *B. cereus*, but L6A was highly active against *L. lactis* MG1614 and *M. luteus*. Both single mutants were active against *L. innocua*, *L. ivanovii*, whereas the I4K_L6A double mutant was inactive.

Ring C mutants:

The A15I nisin mutant retained its bioactivity in the assays (Table 18, Table 19) even if it was at the lower limit of detection. The G18T single mutant had no bioactivity in either of the tests against *M. luteus* or *L. lactis* MG1614. In the plate diffusion assay, the zone of inhibition in the presence of 100 ng/ml is the same as the vector only control with nisin. Interestingly, A15I_G18T double mutant is active in the drop test, which is different from the lack of bioactivity seen with A15I_G18T and G18T in overlay bioassays. The most obvious bioactivity is indicated in the plate diffusion assay when A15I_G18T was grown with 10 ng/ml, which confirms antimicrobial production hence the zone of inhibition. With 100 ng/ml, the zone was similar to the vector control with added nisin.

Screening of mutants in overlay assays indicated that mutations in Ring C had a drastic effect on the activity of the peptide. Mutation of both Ile4 and Leu6 residues in the I4K_L6A double mutant, or in Ala15, Gly18 residues in the A15I, G18T or A15I_G18T

double mutants may result in disruption of cyclization for other rings. This may explain the decreased/lack of antimicrobial activity from these mutant peptides (Figure 28, Figure 30). The bioactivity of A15I was detected against *L. mesenteroides*, *L. ivanovii*, *C. perfringens*, *M. luteus* and *L. lactis* MG1614.

Hinge mutants:

For the pore formation step, the flexible hinge region between Rings A, B, C and Rings D, E is important as it allows for the translocation of the C-terminus of nisin across the membrane. The N20P nisin mutant had been previously synthesised and results with antimicrobial activity were reported [87], however for the purpose of having a direct comparison especially against the selected tested strains, N20P was also included in this study. Overall, mutations but not truncations in the hinge region seem to have a beneficial effect: mutants like N20P, M21V and K22S have been shown to have an improved antimicrobial activity against *L. monocytogenes* and/or *S. aureus* as well as other Gram-positive pathogens [13, 15, 87]

The N20P mutant produced a zone of inhibition comparable to the *nisA* construct tested against MG1614 as indicator organism (Figure 30). Compared to the nisin positive control, the bioactivity of N20P was enhanced in the overlay assay against *M. luteus*, whereas M12L was as good as the *nisA* construct in inhibiting the growth of the same organism. Interestingly, M21L proved to be self-inducing as it was active with no added nisin as illustrated in Figure 30. The zone of inhibition of M21L was comparable to the bioactivity of N20P. The N20P_M21L double hinge mutant was very active in all the tests.

Ring E and C-terminal mutants:

H27G seems to be the best of mutants in this region since H27G was highly active in all the tests (Table 18) and it produced zones of inhibition against *M. luteus* and *L. lactis* MG1614, which were comparable to nisin. In the plate diffusion assay, the two multiple mutants (H31T_V32G_ S33K and H27G_S29H_ H31T_V32G_ S33K) produced a good zone of inhibition with both 10 and 100 ng/ml of added nisin, which confirms that these mutants are active even if their bioactivity seems to be lowest compared to H27G or the *nisA* construct. H31T_V32G_ S33K was less active compared to the positive control but much more active than the multiple mutant H27G_S29H_ H31T_V32G_ S33K.

Interestingly, H31T_V32G_ S33K seemed to be more active than H27G (Table 19) and as good as *nisA* when tested in overlay assay against *C. perfringens*. The activity of

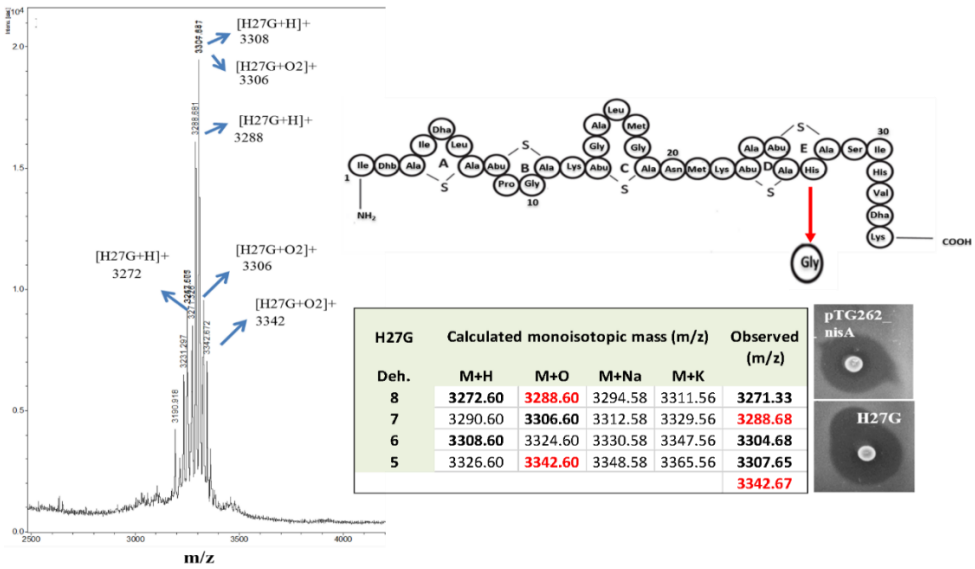
H27G_S29H_ H31T_V32G_ S33K was similar to M21L and N20P_M21L but less against the positive control tested against *C. perfringens*. The multiple mutants totally lost their bioactivity when tested against *S. aureus* and *B. cereus*, activity was apparently present against *L. mesenteroides*, *L. ivanovii*, *L. innocua* and *E. faecalis*.

3.3.2.2 ***Confirmation of the engineered nisin variants by MS***

All the nisin mutants were grown and analysed to investigate the production of the corresponding peptides. Prior to the MS analysis, samples were prepared using a modified version as described in section 2.4.5.3 [87]. This was used to analyse the dehydration pattern of the nisin variants as described in chapter 2.

Results obtained by using different bioassays are summarised in Figure 28, Table 18 and Table 19 and confirm the presence or the absence of an active antimicrobial nisin mutant peptide. The Maldi-ToF analysis described in here was used as a rapid test to confirm that the peptide was produced since some nisin variants may be stable, but they may lack antimicrobial activity.

Figure 29 and Appendix 1 indicates that unique peptide masses, which could correspond to modified peptides were detected by MS. Peptides from the wild type FI5876 and the positive control, *nisA* construct, also gave matching observed masses. Figure 29 is an example of the Maldi-ToF spectrum, obtained after analysis of H27G nisin mutant peptide.



H27G	Calculated monoisotopic mass (m/z)				Observed (m/z)
Deh.	M+H	M+O	M+Na	M+K	
8	**3272.60**	**3288.60**	3294.58	3311.56	**3271.33**
7	3290.60	**3306.60**	3312.58	3329.56	**3288.68**
6	**3308.60**	3324.60	3330.58	3347.56	**3304.68**
5	3326.60	**3342.60**	3348.58	3365.56	**3307.65**
					3342.67

Figure 29 - Maldi-ToF analysis of the Ring E nisin mutant, H27G, a *clos*-like nisin mutant peptide.

The observed masses correspond to the calculated masses of different monoisotopes. In red, observed and calculated masses are 100 % match; in black and bold, there is 1-unit difference between the observed and calculated mass of the peptide. H27G, is highlighted in red. A mixture of dehydrated forms of the nisin mutant ranging from 8- to 5- dehydrations was detected. The picture on the right is the overlay assay of H27G and nisin positive control also shown in Figure 28.

([M+H]+ = 3272.580, [M+H]+ = 3326.610, [M+Na]+ = 3294.57, [M+Na]+ = 3330.610, [M+K]+ = 3311.581).

Results in Figure 29 and Appendix 1 suggested that the peptide masses corresponded to several dehydrated forms of the mutants; besides the expected fully dehydrated residues, additional products with partially dehydrated residues were also found. For instance, the observed mass of [M+H]+ = 3272.580 Da correlated to fully dehydrated peptide (8 dehydrations). Since H27G was a nisin mutant and it is known that there is no mass change resulting from ring formation [129], it was assumed that the modification enzymes from FI7847 (nisin gene cluster, Δ*nisA*) would introduce both dehydrations and rings. As in the case of nisin, the most common forms range from 8- to 5- dehydrations. Fully modified nisin has 8 dehydrations. pTG262_*nisA* showed masses for nisin ranging from 8- to 5- dehydrations and 2- to 1- dehydration (Appendix 1). Similarly, the single and multiple mutants in Ring E and the rest of the C-terminus part of nisin showed a number of different peptides, which included the predicted peaks of which the masses corresponded to 8- to 5- dehydrated residues. However, mutations in Ring A and in the hinge region showed a slightly different pattern: L6A had 8- to 4- dehydrations. Interestingly, the N20P_M21L double mutant in the hinge region is dehydrated almost fully (8- and 7 - dehydrations) and as a result it remained active in the overlay assay

(Table 18, Table 19, Figure 28). I4K, N20P and M21L single mutants have 8- to 6-dehydrations (for I4K), 8- to 6-, 4- to 2- (for N20P) and 8- to 4- dehydrations (for M21L). Mutations of the residues in Ring A and Ring C (I4K_L6A and G18T, respectively) resulted in an alternative dehydration pattern, which may explain the lack of antimicrobial activity. The I4K_L6A is the only mutant that had a spectrum with peaks, which corresponded to a mixture of dehydrations with either cleaved or uncleaved forms of the mutant peptide (Appendix 1); this may explain the lack of antimicrobial activity seen with this Ring A double mutant. Therefore, the newly installed amino acids at positions 4 and 6, which are the closest to the protease restriction site, may have an effect on their structure which may prevent effective proteolytic cleavage and full dehydration by NisB. MS analysis of the G18T peptide clearly indicated the presence of mass peaks that correspond to unmodified peptide (0 dehydrations) as well as a range of dehydrated peptides that carry 8- to 5-, 3- and 1- dehydrations. Surprisingly, there was no indication of antimicrobial activity in G18T. Thus, despite the complete dehydration of the peptides, the rings may not have formed which could explain the lack of antimicrobial activity.

In conclusion, the nisin mutants were characterised by Maldi-ToF to assess production, to identify and to confirm the modifications made by the nisin machinery especially looking at their dehydration pattern.

3.3.2.3 ***Self-induction capacity of nisin mutants***

In order to test the autoinduction capacity of the mutant peptides, the induced strains were subcultured in different inducible levels of native nisin during liquid growth. The activation of the nisin operon is positively regulated by nisin [74], therefore it was interesting to investigate if the nisin mutants could act as signal molecules that initiate the transcription of the structural gene using the nisin gene cluster [74].

Initial bioassays showed that there was a difference in the expression levels of the mutated *nisA* genes, as observed by assessment of antimicrobial activity (Figure 28). It was hypothesised that the variation may be due to the difference in induction capacity of the nisin mutants.

Nisin mutants were grown with a subinhibitory amount of nisin (10 ng/ml of nisin) to allow for the nisin operon to be switched on. Then, the bioactivity present in the cell-free supernatants of the 12 engineered nisin mutant strains was determined using a plate diffusion assay (Figure 30).

All except for the M21L mutant peptide required continuous induction with native nisin in order to allow for antimicrobial production (Figure 30) which proved that M21L is a self-inducing mutant peptide. Baseline level of activity was set for the vector only control pTG262 in FI7847, induced with 100 ng/ml of native nisin, which produced a small hazy zone of growth inhibition. Although, there was a small zone of inhibition that was outside the range of the nisin standard curve, the inhibition zones were still taken into consideration. All of the nisin variants tested here were novel except for the M21L and the N20P hinge mutants which were constructed and described previously [16, 87, 123]. The 12 engineered nisin mutant strains, the pTG262_*nisA* positive control and the nisin wild type strain FI5876, gave an increased zone of inhibition when liquid cultures were subcultured in media that contained 100 ng/ml of nisin A (Figure 30). The G18T remained inactive when cultured with external nisin. Interestingly, A15I_G18T mutant was active. A zone of inhibition was only detected when the mutant was grown with 10 ng/ml of nisin. The inhibition zones of H27G, M21L, N21L and L6A supernatants were slightly lower than that of the positive control. The diffusion zones of I4K and N20P_M21L were at approximately 75 % of the size generated by the nisin positive control. Since there was no difference between the zone of inhibition of nisin variant I4K_L6A, H27G_S29H_ H31T_V32G_ S33K and H31T_V32G_ S33K compared to the pTG262 vector control induced with 100 ng/ml of nisin, it was concluded that the small halo was given by the external added nisin. The antimicrobial activity in the supernatant from the A15I_G18T and G18T nisin mutants was below the level of detection using this bioassay method. Therefore, no zone of inhibition from the mutant itself was observed for these two Ring C mutants.

It was unclear if the diffusion zones from supernatants of nisin mutant strains were due to an improved or a deficient induction, or whether they were a reflection of the external nisin. The problem seems to be that the lowest end of the scale lacks sufficient resolution to measure small differences therefore, a different and perhaps more sensitive assay is required. Nevertheless, the biosynthesis of most of the nisin mutants was stimulated in here by using increased amounts of nisin. The levels of antimicrobial production were potentially detectable by plate diffusion bioassay as illustrated in Figure 30.

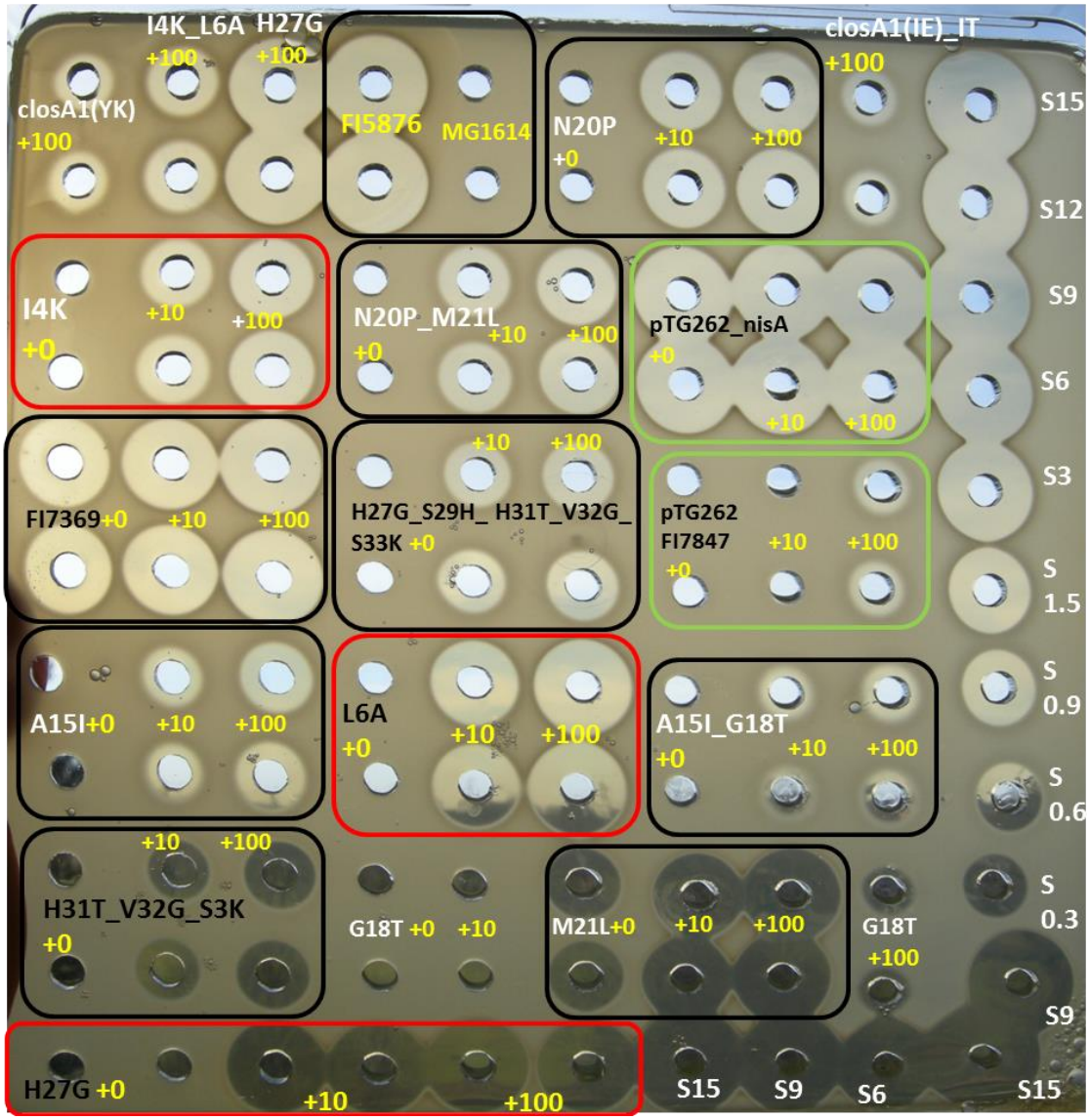


Figure 30 - Plate-diffusion assay to assess the bioactivity of the supernatant of 12 nisin-variant strains when non-induced (no nisin) and induced with exogenous native nisin (10 or 100 ng/ml) against *L. lactis* MG1614 indicator strain. Control strains: pTG262 empty vector in FI7847, FI5876 wild type strain. The FI7369 positive control is the *L. lactis* FI7847 with the pTG262_*nisA* plasmid. S is standard nisin (µg/ml). pTG262_nisA is positive control. This picture is a representative image of two independent agar plate diffusion assays.

In summary, all the engineered strains were capable of peptide biosynthesis in the presence of exogenous nisin. The assays confirmed that these nisin mutant peptides, except for M21L, have lost their capacity to act as signalling molecules as a result of introducing single or multiple mutations, which is in line with other studies on the signalling capacity on nisin mutant peptides [8, 130]. In the plate assay, the absence of external nisin (no nisin) did not allow for the expression of mutated *nisA* (Figure 30).

3.3.2.4 ***Phenotypic analysis of nisin variants***

During the growth of the FI7847 (Δ*nisA*) strain containing pTG262_*nisA*_L6A or pTG262_*nisA*_H27G_S29H_ H31T_V32G_ S33K, it was noted that these strains grew better in liquid media or broth than on solid media. Some nisin-variant strains displayed a highly flocculent phenotype when grown in broth at high OD value compared to other liquid cultures that aggregated on the bottom of the vial. There seemed to be a distinctive phenotype with the L6A mutant when grown on solid media for the overlay assays (Figure 28), thus transmission electron microscopy (TEM) was employed to examine and compare cell morphology. TEM of the nisin variant producer strains, which offered valuable information on the complex phenotype that may be arising from changes in the bacterial cell wall and/or cell membrane.

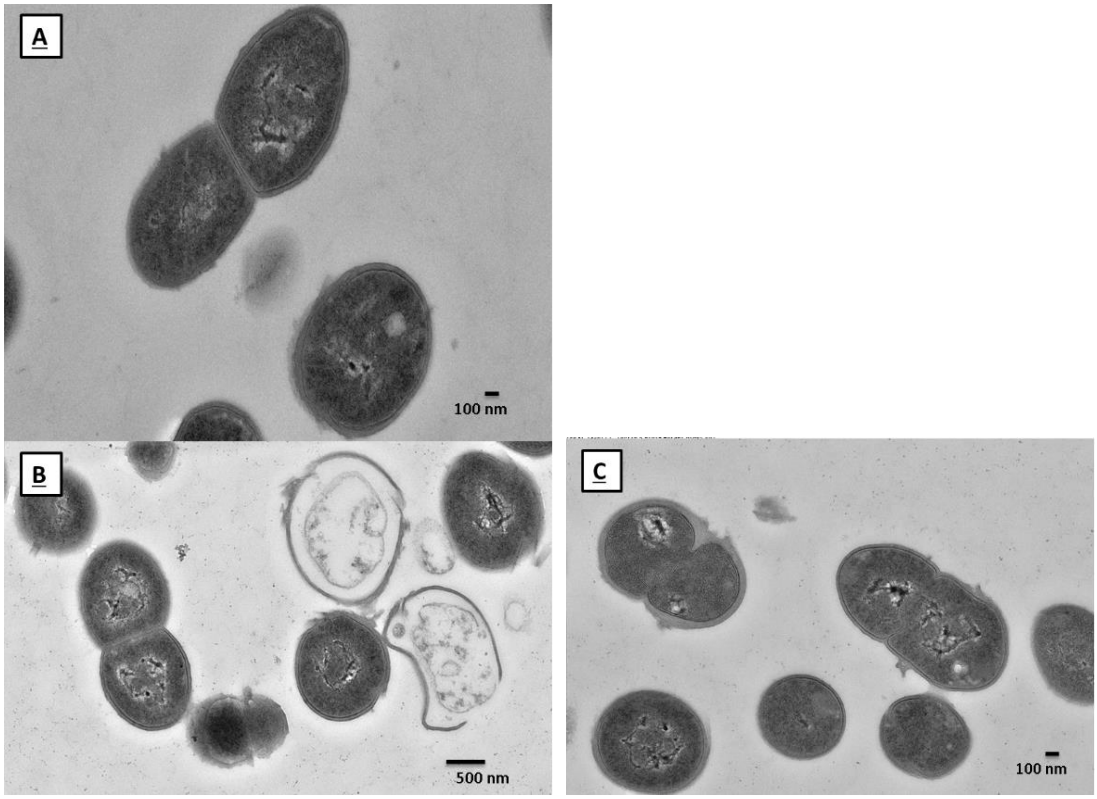


Figure 31 - TEM images of *L. lactis* FI7847 (Δ*nisA*) cells expressing different constructs.

A. pTG262_*nisA*

B. pTG262_*nisA*_H27G_S29H_ H31T_V32G_ S33K

C. pTG262_*nisA*_L6A.

The TEM images for L6A mutant (Figure 31, C) showed a strong similarity in phenotype to the nisin positive control (Figure 31, A). Cells for the nisin positive control (pTG262_*nisA*) had a normal elongated shape, half of the cells were dividing and had characteristic scars on the surface for division. There was a mixed population of viable and very few nonviable cells. In contrast, the H27G_S29H_ H31T_V32G_ S33K nisin mutant cells were damaged since many cells appeared to have lysed suggesting a greater proportion of nonviable cells (Figure 31, B).

The FI7847 strain contains the nisin A biosynthetic machinery. It may be that the nisin transport machinery could be clogged by the conformation of the peptides and pores may not be formed properly which would explain the observed phenotypes. The amino acid substitution in the nisin original strain to create different constructs in the FI7847 cells, is likely to have an impact on the antimicrobial activity of the nisin mutants. Another explanation for the phenotype could be the presence of a mixture of forms of the peptide: the precursor peptide with the leader peptide still attached (inactive form) or of the partially or fully modified mutant peptide.

3.3.3 **Purification of nisin variants**

I4K and L6A are Ring A nisin mutants, whereas H27G is a Ring E mutant. Previous results with these mutants demonstrated that they display an increased antimicrobial activity, therefore I4K, L6A and H27G were selected for further characterisation. The first aim was to induce the mutants with different amounts of nisin to maximise the production of the peptides, then these nisin variants were produced in large scale, purified and their specific activity was assessed against nisin. The specific activity of purified nisin mutants peptides would provide a more accurate comparison of the inhibitory effects of nisin variants. The variation in the observed bioactivity due to the induction capacity of the mutant peptides or variation in the level of peptide biosynthesis could therefore be eliminated. Liquid cultures were induced with a range of nisin concentrations to maximise the expression of the mutant peptide (Figure 32).

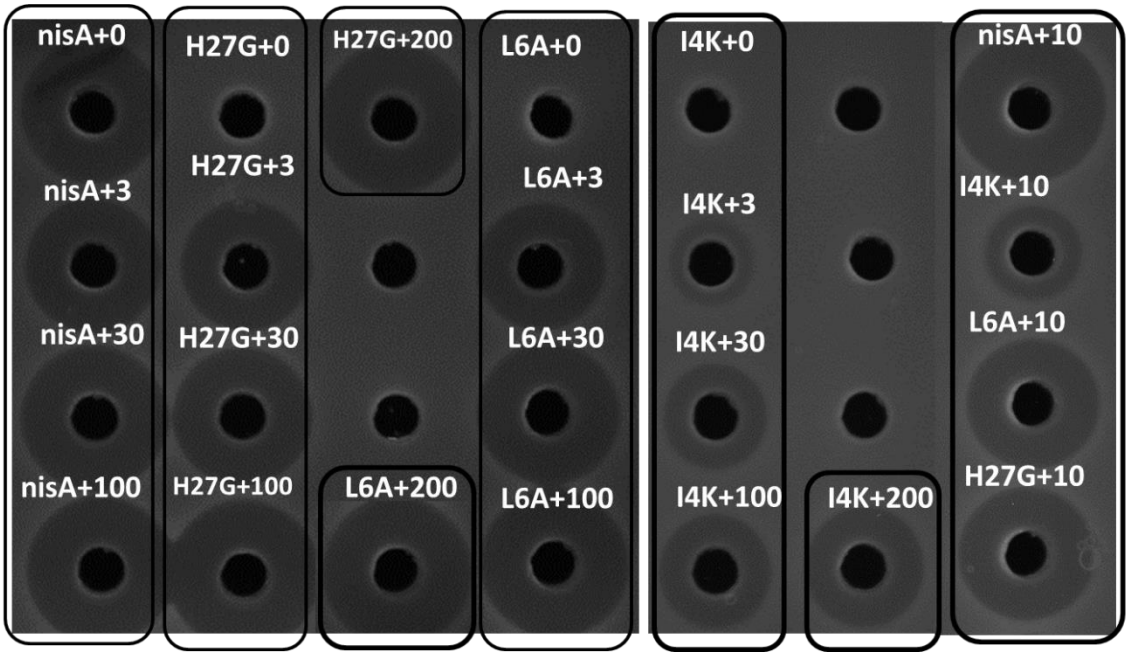


Figure 32 - Supernatant antimicrobial activity in the engineered mutant producer strains (I4K, L6A, H27G) with induction by 0, 3, 30, 100 and 200 ng/ml nisin and monitored by plate-diffusion assay using *L. lactis* MG1614 as indicator strain. This picture is a representative image of three independent agar plate diffusion assays.

The levels of supernatant activity were determined from the inhibition zones of *L. lactis* MG1614 using a standard curve of the positive control *nisA* which was also induced with nisin (data not shown).

It was observed that induction of nisin mutants with 100 ng/ml of nisin resulted in maximal levels of antimicrobial activity, so this nisin concentration was selected for scaled up production and for future purification of L6A and H27G. For the I4K producing strain, 200 ng/ml of nisin was used. It is expected that the inducible amount of nisin used

in the large scale fermenters would elute at different elution time points compared to the elution peak of nisin. In order to maximize production, purify and further characterise the nisin mutants, the liquid cultures were grown in 11-15 L fermenters ensuring the pH was kept constant to pH 6.0 (Figure 33).

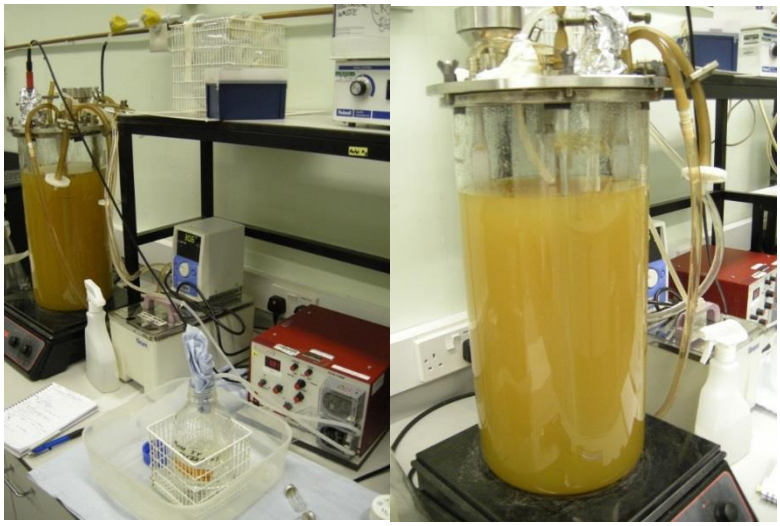


Figure 33 - Scaled up production of nisin mutant peptides.

Nisin mutant producing strains were incubated in a fermenter at 30°C with stirring and the pH was maintained at 6.0 by a pH controller, using 5 M NaOH solution.

Nisin is a low molecular weight peptide (active nisin is approx. 3.5 kDa) and it has a high number of hydrophobic residues which makes it amenable to be separated from other media components by the process of hydrophobic interaction chromatography.

I4K and H27G were successfully purified using an optimised protocol and buffers as described in section 2.3. The yield obtained from the first step of purification on a Fractogel Toyopearl column was different depending on the nisin variant that was purified. 11 L of liquid cultures were grown in fermenters allowing for the production of 150 mg and 50 mg for H27G and I4K, respectively. The large volume in the fermenters and an increase in cell density due to using optimal fermentation culture conditions and growth medium should have allowed for the protein yield to be improved. The yield of the I4K was 4.55 mg/l, and for H27G was 13.5 mg/l. Pure nisin was not purified in this work in order to have a direct comparison to the yield of the three peptides. However, even if the yield for I4K and H27G seemed to be low, the amount of peptide that was purified was sufficient for further characterisation.

Active peptide was eluted with HCl, which was an essential step to ensure the release of mutant peptide from its hydrophobic attachment to the Fractogel Toyopearl column. It was previously shown that the presence of HCl in subsequent freeze-drying concentration

steps inactivates nisin [97]. To overcome that, the pH was changed to neutral with 5 M of NaOH. The pH was then decreased with glacial acetic acid, which allowed the formation of a salt that was removed in further purification steps. In this first step of purification, degradation products as well as media components were removed by the elution chromatogram and by SDS-PAGE gels (data not shown).

The second step of purification involved to use the lyophilised powder from Fractogel and load onto a C18 RP-HPLC column to separate modified peptides. The H27G, L6A and I4K mutations introduced a change in the hydrophobicity of the mutants which means that the mutants had different retention time.

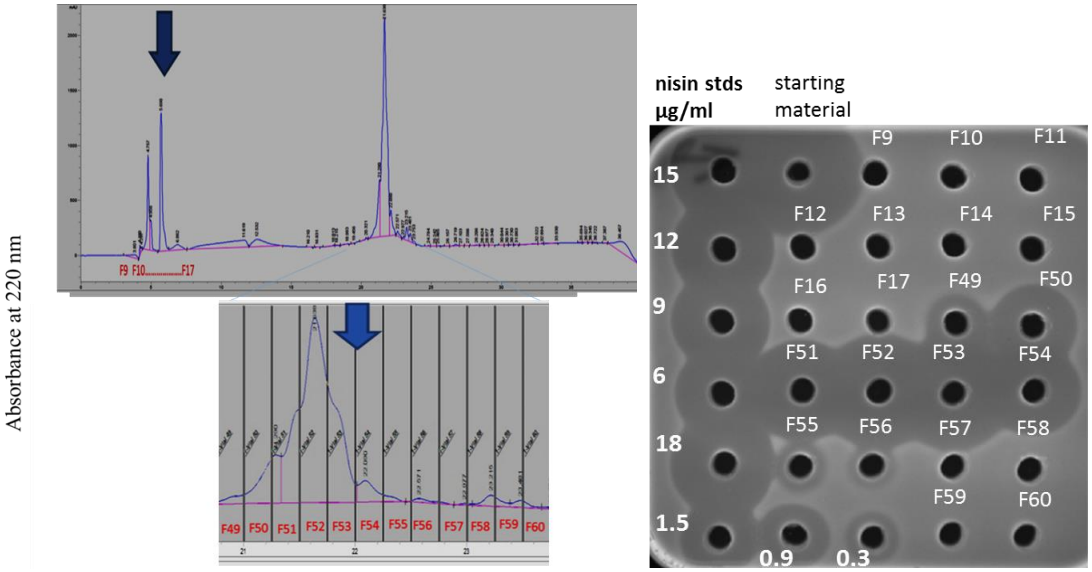


Figure 34 - C18 RP-HPLC chromatogram of H27G (left), H27G supernatant antimicrobial activity of fractions collected from C18 semi-preparative column (right).
Top chromatogram is the elution peak of the H27G from the C18 column during RP-HPLC purification step. Bottom chromatogram is a zoom in the main elution peaks.
The first fractions, called F9 to F17, eluted from the C18 column after 5 min retention time. The second main peak and the set of fractions eluted after 20.8 min retention time correspond to fractions F49 to F60.
The engineered mutant was purified from a liquid culture previously induced with 100 ng/ml of Sigma nisin and fractions were screened for their antimicrobial activity in plate diffusion assays.
The levels of specific activity were determined from inhibition zones of *L. lactis* MG1614 using native nisin standard curve (in µg/ml) (stds).

The Fractogel purification step resulted in the elimination of most of the contaminating material absorbing at 220 nm as illustrated for the H27G nisin mutant (Figure 34). A lyophilised pool of active fractions that eluted from the Fractogel column was taken on the C18 column for further purification. The main fraction was eluted from the C18 after a 27 min retention time.

Initial screening of the nisin mutants against different indicator strains suggested that L6A would be an interesting candidate to purify (Table 18 and Table 19).

Despite exhaustive attempts to purify the L6A mutant peptide, it was not possible to produce any quantifiable amounts of this mutant peptide. The activity was not affected by low pH (HCl), since the pre-purified fractions of L6A from Fractogel were fully active for up to 48-hours at pH 3.5 (data not shown). The unsuccessful purification can be explained by a low level of expression even with optimal amount of inducer nisin peptide. Figure 35 shows that the L6A, I4K and H27G nisin variants binded to the C18 RP HPLC column. Since the interaction with the C18 column was moderate, it was therefore possible to elute the largest and most hydrophobic nisin mutant peptides using a gradient of a weak solvent such as acetonitrile. The more hydrophobic the mutation was, the stronger it bound to the column and the later it was eluted from the column (as seen in the elution gradient profiles, Figure 35). I4K and H27G mutants had different retention times of approx. 17 to 21 min and 21 to 22 min, respectively. The elution profile from RP-HPLC C18 column was therefore monitored on a UV detector and the elution peaks for the purified peptide were collected at 220 nm (Figure 35).

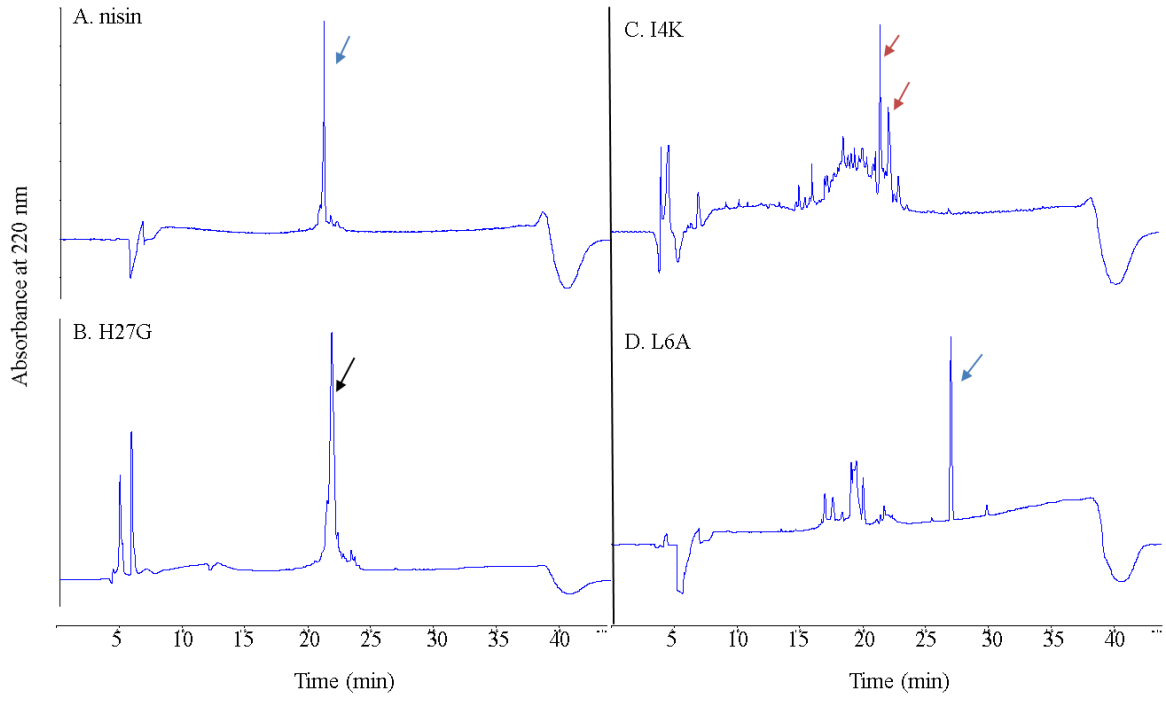


Figure 35 - RP-HPLC analysis of purified A. nisin, B. H27G, C. I4K, D. L6A.

The concentration and the purity of nisin mutants was confirmed by analytical HPLC on an Agilent C18 RP-HPLC column (data not shown). The area under the peak corresponds to the amount of injected peptide onto the C18 semi-preparative column used for purification. For accurate quantification of the peptides, the concentration of pure peptide was determined in plate diffusion assay against *L. lactis* MG1614 as indicator strain to test pure peptide solutions.

For the purification of H27G, the chromatogram indicated the presence of clear peaks at 20 min retention time, which were collected without contamination; this was confirmed by further analytical RP-HPLC and Maldi-ToF (Figure 35). The elution profile of I4K nisin mutant revealed the presence of two very close main peaks on the separation gradient at 17 - 21 min retention time (red arrows). The fractions for I4K and H27G were assessed for their inhibitory effect in a plate diffusion assay against MG1614 (Figure 34, results for H27G) and the active fractions were pooled and lyophilised.

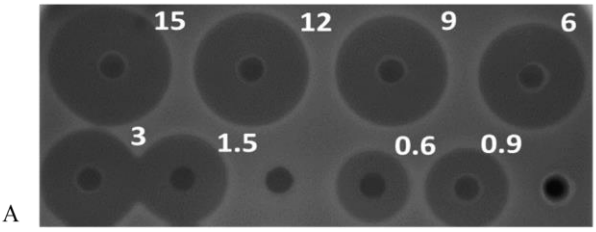
In the case of I4K mutant, eluted peaks contained low levels of pure peptide. The activity was mainly lost during the C18 purification step. As in the case of L6A, the yield could perhaps be improved by loading more starting material to counteract the possibility that the L6A and I4K are poor producer strains. The relative molecular mass of I4K and H27G nisin variants was confirmed by Maldi-ToF. The observed masses are in agreement with the theoretical values as shown in Appendix 1.

In conclusion, some of the nisin species were purified by chromatography on Fractogel TSK butyl 650-S followed by RP-HPLC. Analytical C18 HPLC was used to test the purity of the eluted fractions and the peptides were further purified on semi-preparative RP-HPLC. The bioactivity of purified fractions was assessed using plate diffusion assay against the *L. lactis* MG1614 strain as before.

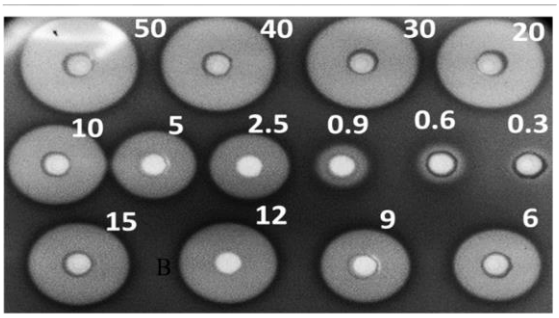
3.3.4 Characterisation of pure nisin mutants

3.3.4.1 *Determining the specific activity of I4K and H27G nisin variants*

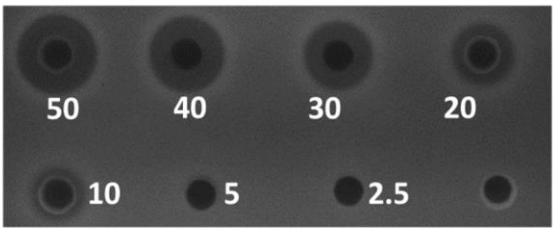
The specific activities of nisin, I4K and H27G were determined in plate diffusion assays by loading different standard concentrations and are shown in Figure 36. The standard curves for nisin, I4K and H27G were compared.



A



B



C

D.

µg/ml	50	40	30	20	15	12	9	5	2.5	0.9	0.6	0.3
log_{10} [peptide]	1.7	1.6	1.5	1.3	1.2	1.1	1.0	0.7	0.4	0.0	-0.2	
y Nisin					186.3	162.6	150.4	130.2	109.0	0.0	-0.1	
y H27G	162.3	149.4	144.2	125.5			91.6	59.8	47.4	11.4	3.3	0.2
y I4K	28.6	24.5	15.7	9.8			3.9	0.1				

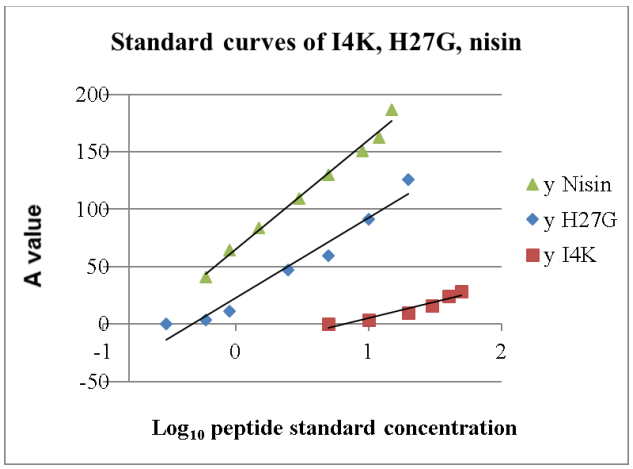


Figure 36 - The purified nisin mutants were loaded at different concentrations in plate diffusion assay to make standard curves. Plot and calculations (D) of standard curves for (A) nisin, (B) H27G and (C) I4K. Concentrations of pure peptides are written in white and expressed as µg/ml.
These pictures are a representative image of three independent agar plate diffusion assays.
The levels of activity of the pure peptide were determined from inhibition zones of *L. lactis* MG1614 strain. All standards are in µg/ml. The bioactivity given by 9 µg/ml of H27G and 40 µg/ml of I4K should fit on

the nisin standard curve to allow for quantification of peptide concentration relative to nisin (as expressed in nisin units). Figure 9 has details on how plot the standard curves to create the graph in D.

The zone of inhibition produced by each peptide was measured and the level of antimicrobial activity was determined by comparison to their own standard curve (Figure 36). Pure nisin was used as a positive control. The inhibition zone of pure H27G at 9 µg/ml does fit to the nisin standard curve and it is a suitable concentration to be used for further testing. The activity of I4K at 9 µg/ml was too close to the lower level of detection to accurately determine its bioactivity at this concentration.

Interestingly, the diffusion zone/halo for I4K did not have a sharp defined edge compared to H27G and nisin. The I4K mutant seemed to exhibit two zones of inhibition that were related to the activity of native nisin. For instance, at 40 µg/ml of I4K, the inner clear zone displayed an inhibition level equal to the 0.6 µg/ml native nisin standard, whereas the outer hazy zone gave a value that was between 0.6 and 0.9 µg/ml on the nisin standard curve. I4K had completely lost activity at 5 µg/ml (no halos were observed).

We wanted to determine how much variant protein was needed in nisin units to achieve the same activity as nisin. For this purpose, high equimolar concentrations of I4K nisin mutant were required. In this study, there was an insufficient amount of I4K available to be fully tested. When H27G was used at a concentration of 50 µg/ml, it was observed that the indicator strain was still growing. Compared to H27G and nisin, I4K is far less active. Therefore, I4K peptide amounts beyond those available in these experiments would need to be added in order to obtain the same antimicrobial activity as for nisin at a level that is within the nisin standard curve.

In order to test for the specific activity and stability of H27G, a concentration of 9 µg/ml was selected, since this concentration would give an equivalent level of activity compared to the nisin positive control standard curve. A starting concentration of 40 µg/ml was decided to be used for I4K. This is because any drop in the bioactivity of I4K due to degradation should still give a measurable zone of inhibition that would fit the nisin standard curve.

In conclusion, the results from the plate diffusion assay shown in Figure 36 suggested that the H27G and nisin at 9 µg/ml, I4K at 40 µg/ml gave a good zone of inhibition that fitting on the nisin standard curve, therefore these concentrations were selected for further testing.

3.3.4.2 *Characterising the activity of nisin mutants under GI tract temperature and pH conditions*

In a previous study [47], the effect of pH from 2.0 to 8.0 and temperature (20˚C and 37˚C) on nisin activity was measured. At 37˚C there was a distinct pH dependence: bioactivity and stability of nisin A decreased as determined by RP-HPLC and in bioassay tests.

Based on these observations, the pure nisin mutant peptides and nisin positive controls were prepared in different buffers and different pH and incubated at 37˚C over time.

Aliquots of the I4K, H27G and nisin samples were collected at different time points and the effect of temperature and pH on the peptides was monitored by plate diffusion assays. Different concentration to create standard curves for each nisin mutant peptide were also loaded on the plates.

The aim was to compare and assess the stability and specific activity of the mutants compared to the nisin positive control. Figure 36 shows that at T0, I4K was significantly less active than both nisin and H27G. Even if 40 µg/ml of I4K and 9 µg/ml of nisin and of H27G were used, the amount of peptides was normalised to 9 µg/ml of nisin equivalents in order to compare the bioactivity of equivalent amounts of peptide. There was a major decrease in the level of antimicrobial activity: I4K was approximately 1 % active compared to nisin whereas H27G had 30 to 40 % antimicrobial activity in comparison to nisin. The biological activity of the peptides against *L. lactis* was monitored over time in three independent agar plate diffusion assays (three biological replicates and three technical replicates).

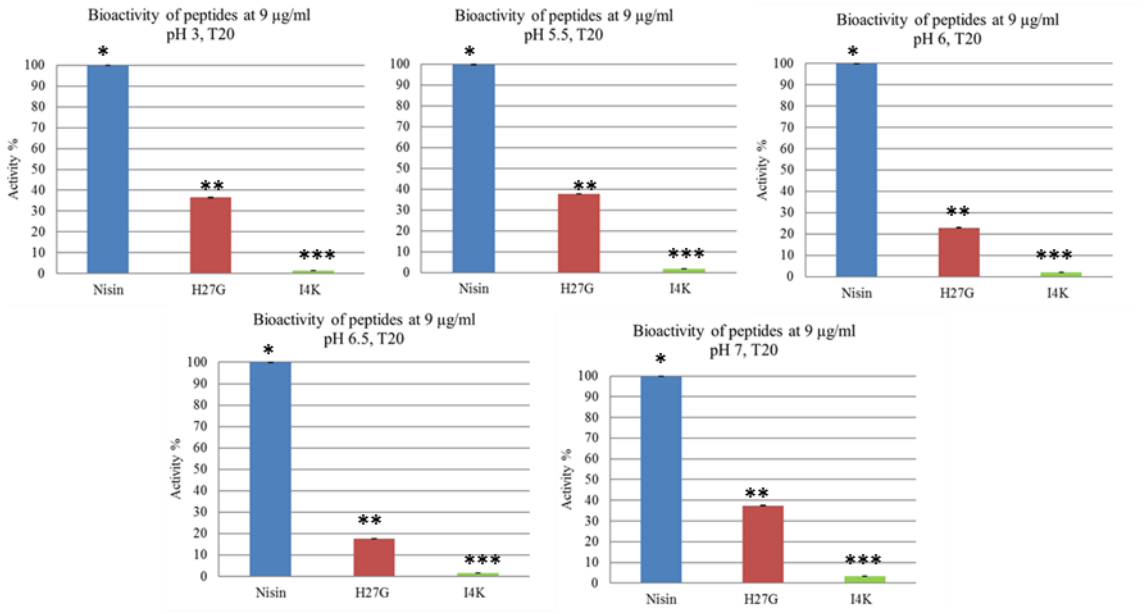


Figure 37 - Comparison of the antimicrobial activity of equivalent amounts of peptides at different pH conditions after 20 min incubation at 37˚C.
The amount of I4K, H27G was normalised to 9 µg/ml to compare the antimicrobial activity of equivalent amounts of peptides. The activity of nisin was set as 100 %. Shown are means ± standard error of the means (SEM) calculated as ± SD of mean, SD is standard deviation (3). The means and standard deviations plotted were based on three independent agar plate diffusion assays (three biological replicates and three technical replicates).
'*' means 100% initial activity (T0) of nisin, H27G and I4K at pH 3.0, 5.5, 6.0, 6.5 and 7.0 respectively.
'**' means that there is significant difference in the bioactivity of H27G compared to nisin at the tested pH.
'***' means that there is significant difference in the bioactivity of I4K compared to nisin at the tested pH.

The activity of I4K looked promising from plate diffusion assay with the supernatants from non-purified peptide (Figure 30), however it became clear that using the purified peptide the activity was a lot less (Figure 38, C). It was concluded that at any given time point and pH, the activity of I4K is 99 % less compared to nisin (Appendix 2).

It was therefore concluded that due to the very low antimicrobial activity displayed by the two engineered purified nisin mutants compared to the wild type nisin, in the future, a higher amount of the pure peptides would be required to achieve a similar level of antimicrobial activity as the activity of the wild type nisin. The decreased activity of the pure peptides must be a consequence of the incorporation of mutations either at position 4 or position 27. This means that these amino acid substitutions did not have a beneficial effect on activity, despite the fact that the stability seems to have improved slightly for the two mutant peptides.

3.3.4.3 ***Stability of the nisin mutants***

We wanted to monitor the stability of the peptides under GI tract conditions. The same samples as in the previous section were used. The zones of inhibition from plate diffusion assays were compared to investigate the antimicrobial activity when the peptide was challenged at different pH, temperature and incubation time. In previous sections, it was established that the I4K and H27G nisin variants are less active compared, both in the supernatant as well as when testing their specific activity. In here, the inherent stability to low pH is considered. One of the controls was the buffer only control and the nisin variants are being compared against it; all the samples were treated the same to mimic the GI tract conditions.

The specific activity at equivalent levels of protein was measured by normalising the values of the zones of inhibition (Figure 37). The bioactivity was reported for 9 µg/ml of nisin, 9 µg/ml of I4K and 9 µg/ml of H27G. The initial starting point (T0) was set as 100% activity for each peptide. Note that 100 % activity of I4K is not the same as 100% activity as H27G and is different from 100 % activity of nisin. One needs to take into account how low the level of activity is for I4K and H27G at 9 µg/ml compared to the equivalent level of activity from 9 µg/ml of nisin (Figure 37).

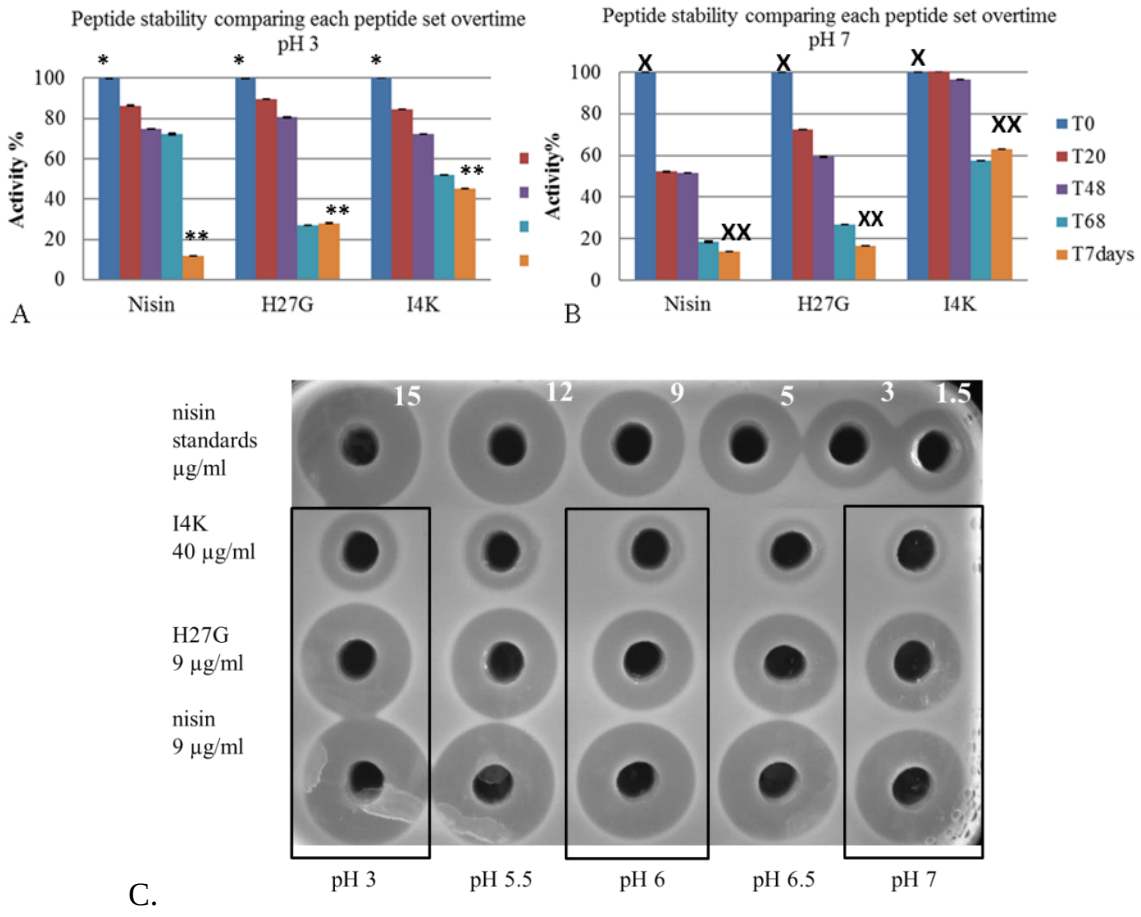


Figure 38 - Testing the stability of nisin mutants under GI tract conditions.
A. Testing the stability at pH 3.0 for nisin over time, H27G over time and I4K over time.
B. Testing the stability at pH 7.0 for nisin over time, H27G over time and I4K over time.
C. Plate diffusion assay to test bioactivity of solutions for I4K at 40 µg/ml, H27G and nisin at 9 µg/ml at pH 3.0, pH 5.5, pH 6.0, pH 6.5 and pH 7.0 after incubation at 37°C for 24 h. *L. lactis* MG1614 was used as an indicator strain. This picture is a representative image of three independent agar plate diffusion assays.
'*' means 100% initial activity (T0) of nisin, H27G and I4K at pH 3.0.
'**' means that at T7 days, there is significant difference in the bioactivity of nisin, H27G and I4K compared to the bioactivity at T0 for the same peptide when tested for pH 3.0.
'x' means 100% initial activity (T0) of nisin, H27G and I4K at pH 7.0.
'xx' means that at T7 days, there is significant difference in the bioactivity of nisin, H27G and I4K compared to the bioactivity at T0 for the same peptide when tested for pH 7.0. The means and standard deviations plotted were based on three independent agar plate diffusion assays (three biological replicates and three technical replicates).

The amount of I4K and H27G was normalised to 9 µg/ml to compare the antimicrobial activity of equivalent amounts of peptides (Figure 38). T0 value of each individual peptide was set as 100 % level of activity and the activity levels of each peptide determined over time are expressed as a percentage of these individual T0 values.

The stability of I4K over 7 days was compared against its initial 100 % activity at a given pH. I4K has a longer term stability compared to H27G and nisin. The inhibitory activity of active I4K was reduced only to 40-50 %. At pH 6.0, the activity drop was to 30 % (Figure 39).

A similar pattern in the drop of activity was observed for I4K at pH range 3.0 to 7.0, particularly in the first 48 h. However, Figure 37 clearly shows that the initial starting activity of I4K is already low (1/900th of that of nisin), so this needs to be taken into consideration when comparing the level of stability of I4K to the level of stability of nisin. Apparently, the low level of activity that the I4K mutant has retained suggests I4K may be more stable than nisin at higher pH (see orange bar). However, the zone of inhibition of I4K is outside the range of nisin standards curves, therefore the error of measurement for these concentrations is higher as shown in Figure 38, C and these results cannot be conclusive.

The effect of pH 3.0 to 6.5 on the stability of nisin A was moderate for 48 hours whereas at pH 7.0 the activity dropped rapidly to 50 % compared to a value of 15 % reduction for any of the other tested pH. After 68 h incubation at 37°C, the activity of nisin at pH 3.0 to 5.5 was of 70 %. At pH 6.0-7.0, there was a 50 % reduction in its bioactivity. After 7 days, the level of nisin activity dropped dramatically to approx. 10 % in any tested pH. Therefore, nisin is indeed more stable at lower pH for the first 48 h, however it becomes very unstable and it degrades rapidly over time.

In comparison to I4K and H27G, nisin retains a higher activity for 48 h at pH 3.0-6.5, however at pH 7.0 nisin is only 50 % active, whereas H27G is 65 % as active and I4K is 95 % active. This would imply that at this pH, the 2 nisin mutants are more stable. However, the zones of inhibition measured with 9 μg/ml of nisin and H27G were more reliable to be measured as they all fit on the nisin standard curve; this is not the case with I4K. Also, one needs to consider that I4K was degrading when stored at – 20°C and during handling, an effect that was enhanced during freeze thawing. To enable statistical comparison between the nisin variants with a fresh and hopefully non-degrading I4K peptides, using different treatments (pH, temperature), further replicates of these tests are necessary.

H27G followed a similar trend in activity and stability as for nisin at pH 3.0-6.0. The mutant had a lower stability at both pH 6.5-7.0 of 60 % after 48 h and at pH 7.0 activity dropped down to 50 %. Unlike nisin, the activity of H27G decreased even further after 68 h and it remained the same after 7 days. At pH 7.0, H27G seemed to be more stable (15 % to 30 %) compared to nisin (10 % on average) based on the 7-day time point. The activity of nisin drops by 90 % whereas for H27G the activity drops by 75 %. Based on Figure 37, the level of H27G is in theory 35 % of nisin units compared to 100 % of nisin

therefore one would need to add three times more pure H27G to get to 100 % activity of nisin. The 75 % drop in activity of H27G would be commercially significant/interesting if H27G had the same level of 100 % activity at equivalent amounts of peptide in nisin units.

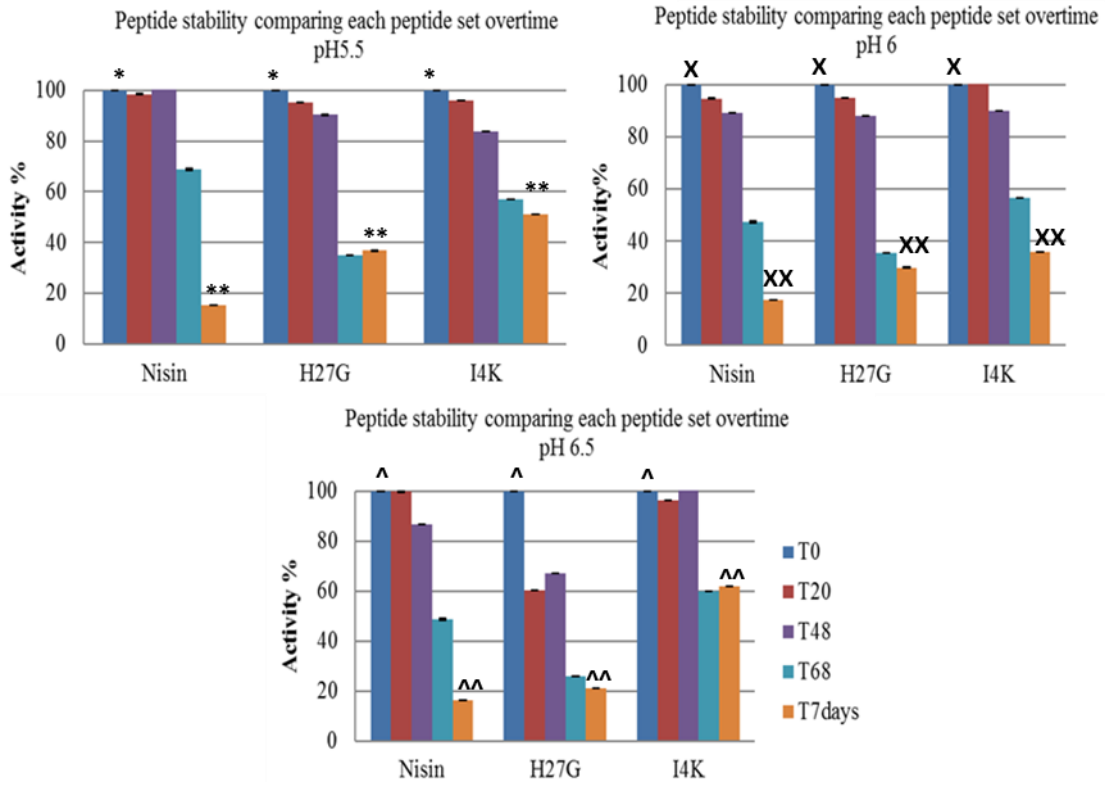


Figure 39 - Testing stability of nisin mutants under GI tract conditions: pH 5.5, 6.0 and 6.5 from T0 to T7 days. The means and standard deviations plotted were based on three independent agar plate diffusion assays (three biological replicates and three technical replicates).

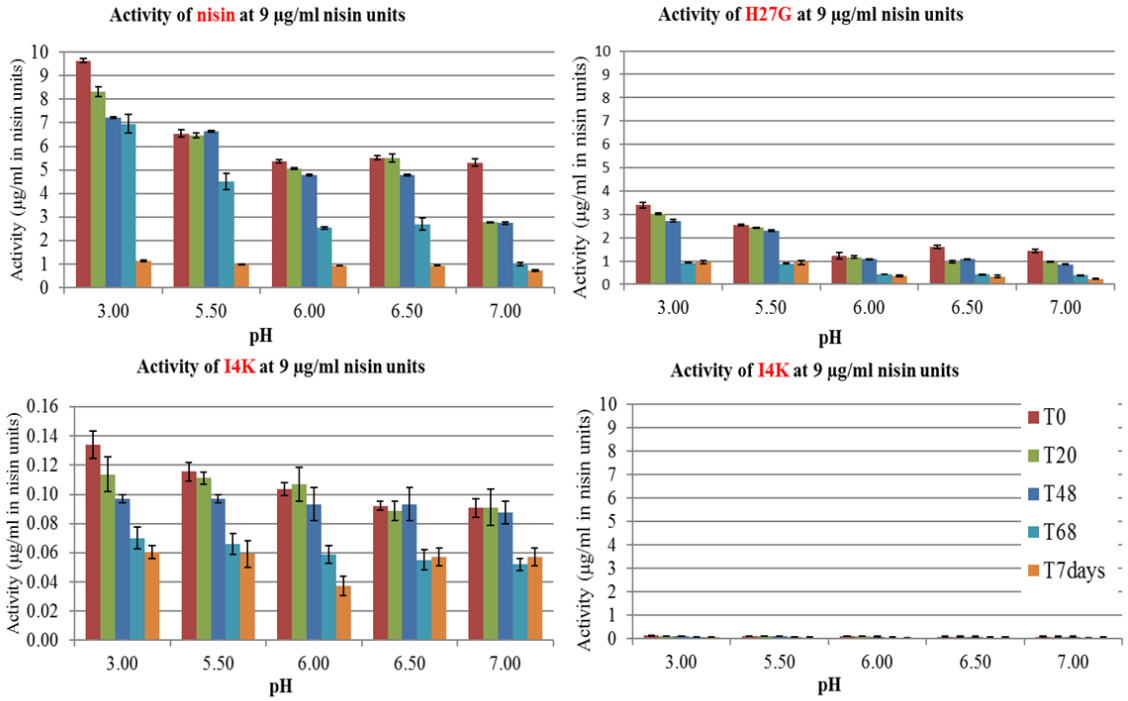
'*, x, ^' means 100% initial activity (T0) of nisin, H27G and I4K at pH 5.5, 6.0 and 6.5 respectively.
'**' means that at T7 days, there is significant difference in the bioactivity of nisin, H27G and I4K compared to the bioactivity at T0 for the same peptide when tested for pH 5.5.
'xx' means that at T7 days, there is significant difference in the bioactivity of nisin, H27G and I4K compared to the bioactivity at T0 for the same peptide when tested for pH 6.0.
'^^' means that at T7 days, there is significant difference in the bioactivity of nisin, H27G and I4K compared to the bioactivity at T0 for the same peptide when tested for pH 6.5.

In all experiments, a reasonable correlation between the increase in pH (same peptide content/equivalent amount) and the decrease in biological activity against *L. lactis* strain was observed. A typical example is shown in Figure 39. There is a consistently higher biological activity relative to the amount of active nisin (at pH 3.0 to 6.0: 80-90 % active for 48 h for nisin, I4K and H27G). The peptides are most stable at pH 3.0 to 5.5 for the first 48 h of incubation at 37°C.

In the case of I4K nisin mutant, it is unclear if the observed stability is due to a significant improvement from protein engineering, or a reflection of the errors introduced by measuring the zone of inhibition in this pH range (3.0 to 7.0). Further repetition of

experiments using a higher amount of I4K would be required for clarification. Due to the time constrains, it was not possible to repeat the purification of I4K to produce higher yields to be used in further testing.

Nisin, I4K and H27G were still stable and had antimicrobial activity until pH 7.0. The trend for their antimicrobial activities is shown in Figure 40. In conclusion, the results indicated that I4K and H27G did not have considerable enhanced activity, they maintained their stability compared to wild type nisin for the first 48 h when tested at 37˚C (Figure 40).



pH	3	5.5	6	6.5	7
nisin	89	85	81	81	85
H27G	71	60	80	78	85
I4K	57	45	40	33	33

Figure 40 - Stability and activity of peptides at 9 µg/ml tested at 37˚C, pH 3.0, 5.5, 6.0, 6.5 and 7.0 at different time points: T0, T20, T48, T68 and T7days.

The means and standard deviations plotted were based on three independent agar plate diffusion assays (three biological replicates and three technical replicates).

Stability of nisin, H27G and I4K over time. Note that the Y scale was set at 10 µg/ml for the 3 mutants in one graph and in another graph, the y scale was modified to 0.5 µg/ml for the 3 mutants. There is also a graph on I4K where the Y scale was modified and zoomed to a maximum of 0.16 µg/ml.

The table represents the decrease in percentage in antimicrobial activity calculated between T0 and T7 days for each pH. The data was collected from the same assays (3 independent agar plate diffusion assays) as described in section 3.3.4.2.

3.3.4.3.1 **Stability of I4K and H27G at storage conditions**

The observation that the I4K mutant is unstable under storage condition suggests that the mutation has no beneficial effect on stability (Figure 41). After purification, the yield of I4K was much lower compared to H27G (50 mg compared to 150 mg, respectively). In order to understand the drop in stability under storage conditions, standard curves of I4K were compared using: 1. freshly purified I4K, 2. I4K after incubation at -20°C for approx. 8 weeks (data not shown). The I4K was unstable at storage conditions of -20°C. Although degradation was noticed during the handling of the I4K mutant, it was decided to continue with the characterisation of I4K since the peptide was still active.

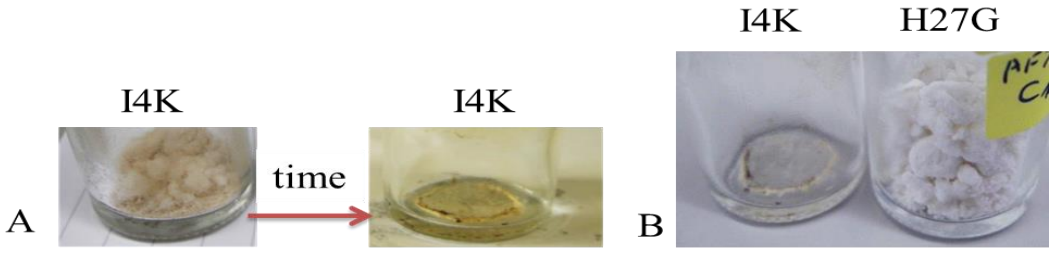


Figure 41 - Stability of I4K and H27G mutants at storage conditions.

A. Freshly purified I4K mutant and after 6 weeks of incubation at -20°C. The initial colour of I4K was never white, unlike for H27G, B. H27G powder after incubation for 6 weeks at -20°C. During this time, both mutants were removed from – 20°C for several times to prepare stocks and to perform plate diffusion assays as described above.

3.3.4.3.2 **Spectrum of activity of pure *clos*-like nisin mutants**

Here, we aimed to verify the stability of nisin, I4K and H27G, and to evaluate their antimicrobial activity at pH 6.0 against GI tract pathogens: *B. cereus*, *E. faecalis*, *E. faecium*, *L. ivanovii*, *C. perfringens*, *C. difficile* and *L. lactis* MG1614 (for control). It was not possible to test the I4K nisin mutant as it proved to be unstable at storing conditions, it was degrading and the amount of the material was insufficient.

Solutions of H27G and nisin prepared at pH 6.0 were loaded onto the agar plates seeded with indicator strains. The agar was adjusted to pH 6.0 and the plates were incubated at 37°C in order to mimic the conditions relevant to the GI tract.

It was not possible to measure the antimicrobial activity against any of the other tested strains since the tested amounts of pure nisin mutants did not give any zone of inhibition at pH 6.0 and 37°C. H27G did not show any activity compared to the nisin control because the tested concentration was too low or because it was unstable in these GI tract conditions. However, nisin did display bioactivity against all tested strains.

3.4 DISCUSSION

In this chapter the genetic engineering of *clos*-like nisin mutants, induction and characterisation of their antimicrobial activity as well as optimisation of large-scale production and peptide purification were addressed.

This work stemmed from the discovery of a novel *clos* gene cluster from a GI tract bacterium, *Blautia obeum* A2-162, and the identification of unique amino acids in the predicted structure of the three identical ClosA1 peptides. Compared to the well characterised lantibiotic prototype, nisin, ClosA1 has 12 different amino acids. It was noted before that nine natural nisin variants which differ by a few amino acids have been identified in *L. lactis* species, *Streptococcus uberis* [16], a *Streptococcus hyointestinalis* strain isolated from the humn gut, *Streptococcus gallolyticus* subsp. *pasteurianus* and *Streptococcus sui* [48]. Some of these natural nisin variants are known to have antimicrobial activity against a range of Gram-positive bacteria like staphylococci, streptococci, *Listeria* spp., bacilli, and enterococci.

The hypothesis was therefore: the *clos* cluster was identified in a gut bacterium, any putative antimicrobial peptides should be stable and active under GI tract conditions and have antimicrobial activity against GI tract pathogens. It was speculated that *Blautia obeum* A2-162 could have bacteriocin like properties similar to nisin from *L. lactis* since their gene clusters and their peptides proved to have structure similarities. The well-prototype of lantibiotics, nisin, has antimicrobial activity against pathogenic

Gram-positive bacteria such as *C. difficile* and *C. perfringens.* The creation of a library of *clos*-like nisin mutants and their successful characterisation using known methods [16, 131] was therefore the focus of this chapter. The aim was to identify mutants that could be used as treatment for certain GI diseases potentially caused by GI pathogens [132].

The construction of a range of *clos*-like nisin mutants was particularly interesting as they would incorporate one or more amino acid substitutions to make them look more like the putative ClosA1 peptide as illustrated in section 1.9, chapter 1 (Figure 8). These novel mutants would aid in the identification of novel bacteriocins that would have improved antimicrobial activity, enhanced stability at physiological pH and wider spectrum of activity particularly against gut pathogens.

Site directed and random mutagenesis approaches have already been applied to create a vast library of nisin mutant peptides as reported in different studies [16]. Genetic engineering of the nisin A structural gene has recently been applied and nisin mutants

with improved antimicrobial activity have been identified [87]. The predicted putative antimicrobial peptides from the *clos* cluster bear structural similarities to nisin, which suggests that new peptides with improved properties that are active against GI tract pathogens may be generated.

The pTG262_*nisA* construct had an antimicrobial activity that is similar to the wild type strain, FI5876. This plasmid was then used as a template to introduce subsequent mutations followed by a short *nisA* structural gene which includes a transcriptional terminator. The plasmid copy number influenced the expression levels of *nisA* when cloned in either pUK200 or pTG262. The zones of inhibition given by the pTG262_*nisA* construct were bigger. Both plasmids are known to be high copy number plasmids: pUK200 is a 3191 bp vector, pTG262 is a bigger size vector (5566 bp) and it is derived from the lactococcal plasmid pCK17 replicon [133]. One would expect that with low copy number the expression would be lower, however this is not the case in here. Results show that the expression of nisin in pTG262 was enhanced possibly due to the higher copy number vector. However, this is counter intuitive as it is expected that the bigger the plasmid the lower the copy number. The poor expression of nisin using pUK200 may be due to instability in this host strain and to the presence of a strong terminator. Therefore, low levels of expression for nisin or nisin mutant peptides were detected when pUK200 was used.

Examination of the literature revealed that previous experiments using site-directed mutagenesis and amino acid randomisation in the nisin template [16, 87] gave useful insight onto the importance of the conserved amino acids and the effects of altering and introducing alternative charges in the nisin molecule. The ability to produce nisin mutants by site-directed mutagenesis has proven an extremely powerful asset in the study of their modes of action. Studies which involved the deletion or the substitution of residues in the C-terminal part of nisin have shown that the C-terminal may not be important for biological activity [79, 130, 134]. On the other hand, other studies strongly suggested that the N-terminal of nisin is essential for binding to lipid II and hence it will have a greater effect on the overall activity of the mutant peptide [60].

Previous studies using engineered nisin mutants and truncated nisin peptides (fragments) suggested that the N-terminal residues of a fully modified nisin peptide are also essential for the induction capacity of a mutant peptide [74, 95]. In a study with engineered truncated nisin, it was demonstrated that a nisin fragment that comprises intact Ring A to

Ring C is very important for the growth-inhibition activity and a truncated nisin 1-20 is sufficient in rendering an active peptide [134].

Other studies suggested that in order for nisin to act as an antimicrobial peptide, the pore formation step is not essential, since a nisin fragment 1-28 that lacked any pore formation capacity was still active, while other C-terminally truncated mutants had only a slightly decreased activity. However, the ability of nisin to form pores is actually an advantage as nisin can indeed kill bacteria more efficiently and is more resistant to the development of resistance. Mutations in the hinge region are generally regarded as beneficial as attempts to engineer new nisin mutants with altered antimicrobial activity against a variety of organism and the enhancement of physicochemical properties by using site-directed mutagenesis have been successful. As a result, nisin mutants with enhanced antimicrobial activity against *S. aureus* and *L. monocytogenes* have been generated [87, 123, 135].

3.4.1 **Genetic engineering of *clos*-like nisin mutants.**

In this study, site-directed mutagenesis of nisin with controlled and targeted specific amino acids substitutions was based on the structure comparison between nisin and the predicted ClosA1 peptide. In this PhD project, the aim was to use the nisin machinery and the genes identified in the novel *clos* cluster to make a variety of engineered variants to investigate the production and activity of the novel lantibiotics by expression in *L. lactis* host strain. Genetic engineering of *clos*-like nisin mutants was achieved by subcloning the *nisA* gene routinely on pTG262. The plasmid was designed by fusing a single or multiple mutated *nisA* gene to the nisin promoter (P_{nisA}) with the aim of substituting either individual or multiple amino acids in the nisin structure. The resulting constructs were confirmed by sequencing and the novel *clos*-like nisin mutants were subcloned in *L. lactis* strain (Δ*nisA*). Positive clones were characterised and tested for their antimicrobial activity. A survey of the literature indicated that most of the resulting 12 *clos*-like nisin variants were novel except for M21L and N20P hinge mutants, which had been synthesised previously and were highly active against pathogens as confirmed in this study [13, 16, 87].

All the nisin mutant peptides described in this project were secreted both in the culture supernatant or on the agar plate as the nisin biosynthetic machinery worked well to introduce unusual amino acid and modifications, to transport and to process the leaderless peptides. However, limitations of the *in vivo* engineering may render the peptides inactive.

All the nisin variants biosynthesised and tested in this work maintained their antimicrobial activity except for one mutant in Ring C. Altering both the charge and the size of the new residues had an important effect on one or both mechanisms of action known for nisin: binding to lipid II and pore formation. In comparison to nisin, mutants with decreased activity were detected when they were screened against pathogens. Examination of literature revealed that the results obtained this work can be correlated with similar observations on amino acid substitution in nisin variants.

All the mutants except for M21L lost their autoinduction capacity which was expected based on the observation that the first three lanthionine Rings A, B, C are essential for induction [74, 95, 96]. The importance and the integrity of Ring A and the modified amino acid Dha5 as a hydrophobic moiety for the biological activity had been previously shown [47]. Therefore, the I4K and L6A mutants may have disturbed the Dha5 rending them less active. The importance of Ring C for the biological activity of nisin was also demonstrated by replacing this Lan ring with a disulphide bond [130]. In this work, A15I and G18T, Ring C mutants, had very low antimicrobial activity and some were inactive such as the double nisin mutant, A15I_G18T.

Another study, examined the interaction of lipid II with a number of nisin variant producers to identify structural elements of the nisin molecule. It was found that amino acid substitutions that affected the conformation of Rings A, B, C led to a reduction in binding of lipid II and the concentration of the peptide needed for formation of pores was increased [79].

The N-terminal rings in nisin play an important role in lipid II binding [80, 134] and genetic engineering resulting in minor variations within this region, greatly reduced the specific activity of the peptides. For instance, Ser3Thr (changing the first Lan residue to MetLan) led to a dramatic loss in activity [80]. Mutation in Ser3Thr sterically hinders the formation of the H-bond, thereby it decreased the affinity of the mutant for lipid II by 50-fold [79]. Therefore, this may explain why cleavage or alteration of Ring A may ultimately lead to the complete elimination of bioactivity [134] which was the case with the Ring A double mutant: I4K_L6A.

Mutation in the hinge region which reduced the flexibility of nisin had a negative effect on monolayer interactions and bioactivity, whereas mutations in the C-terminal part will impact the ionic interactions of nisin in a monomeric or oligomeric form with anionic lipids [82, 136, 137]. The hinge mutants generated in this work had similar or only slightly

decreased bioactivity compared to nisin which means that the introduction of Leu or Pro nonpolar residues had a neutral effect on bioactivity.

It was previously shown that C-terminally truncated nisin A mutants that lack Rings D and E were capable of retaining significant bioactivity but they are unable to permeabilise the target membrane [88]. Most of the positive charges carried by nisin are held by C-terminal residues: Lys-22, Lys-34, His-27, His-31 and it was shown that they are crucial for the initial interaction of nisin with the target membrane. Introduction of a negatively charged glutamate (Val32Glu) drastically reduced the interaction of nisin Z with the anionic lipids, whereas the addition of an extra positive charge (Val32Lys) led to improved binding ability [82]. In this work, H27G had an antimicrobial activity similar to nisin, whereas all the other C-terminal mutants retained their bioactivity but it decreased compared to nisin. Figure 42 illustrates the charges of the substituted residues.

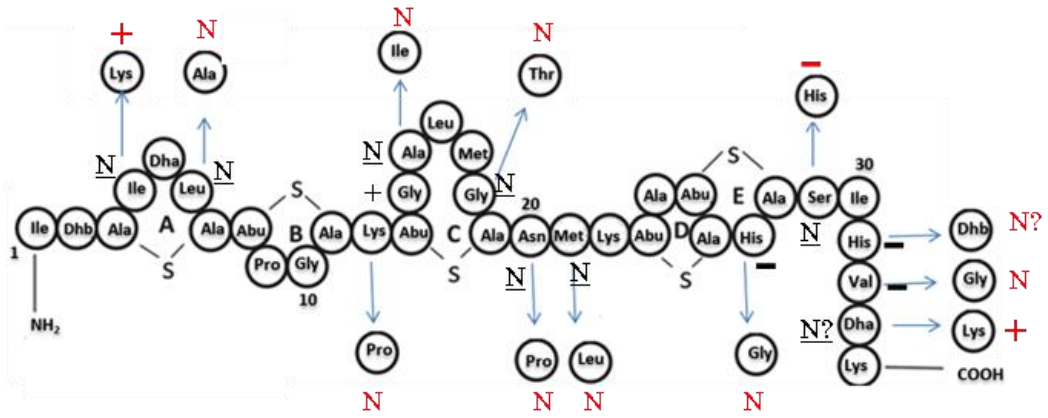


Figure 42 - Illustration of the structure and the charges of nisin and *clos*-like nisin mutants.

The charges of residues in the original structure of nisin and in the new peptides are highlighted. N represents charge of the original nisin, plus (+) is positive charge, minus (-) is negative charge.

3.4.2 Characterisation of *clos*-like nisin mutants

Preliminary characterisation of non-purified peptides did allow for comparison of the relative antimicrobial activity. To account for the specific activity, purified peptides were compared to commercial purified nisin. Even if dehydration and cleavage of the peptides were confirmed by Maldi-ToF, in the case of the double mutants in Rings A and Ring C or for the G18T mutant, their lack of antimicrobial activity against indicator strains can be explained by different contributing factors. A possible explanation that could account for this is the improper modification by NisB and NisC; this may lead to degradation of the intermediate form of the peptides or to abolished production. Even though fully processed novel mutants can be isolated, it has been observed that degraded products or incompletely modified products are often present [60].

Previous studies [138] have shown that an inactive nisin that was expressed in a strain where the nisin transport protein, NisT, had been mutated rendered a form of nisin that was incapable of inducing the nisin Z promoter, P_{nisZ}, hence no nisin was secreted or exported outside the cell; instead it accumulated inside. This work has also shown that these bacteriocins will predominately accumulate as immature mutant peptides that are partially modified or that remain unmodified. Initial attempts to induce nisin mutant peptides using subinhibitory amounts of nisin indicated that there is a deficiency in the process of biosynthesis, either in their expression, processing, transport or secretion.

Preliminary induction and characterisation of the peptides suggested that all nisin mutants, except for M21L, would need to be continuously induced with exogenous nisin. Since most of the mutants were active in the bioassays, it was speculated that the nisin leader must have been cleaved either by a dedicated nisin protease (NisP) or by intracellular proteolytic activity [138] to allow for the leaderless mutants to be exported. It was assumed that the lack of self-induction capacity was most likely due to a problem with the nisin modification machinery resulting from the mutations, which in turn it did not allow for secretion of predominantly fully modified mutant peptide. As a result, the high proportion of partially or unmodified peptide that accumulated in the cell was not capable of interacting with the signal recognition domain of the NisK for signal transduction, therefore none of the nisin mutants, except for M21L, could act as signal molecules.

M21L was an interesting mutant as it regulated its own induction by accumulating in sufficient amount in the supernatant and by being successfully modified and processed

by the nisin machinery. These observations are in line with the Maldi-ToF results, which confirm that M21L was mainly 8- to 6- dehydrated, even though there were also indications that 5- to 4- dehydrations were also present. Since no measurements were made to investigate ring formation, it can only be assumed that the rings were indeed made since M21L has a very high activity similar to nisin. A fully modified form of nisin peptide is one of the pre-requisites for antimicrobial activity [139].

Biological production by means of *in vivo* biosynthesis is very commercially attractive and it is targeted at fostering lanthipeptides manufacturing and their ultimate use in a clinical setting. Therefore, *in vitro* protein engineering permits the exploration of the structural and functional tolerance of the biosynthetic enzymes: the non-proteinogenic amino acids can be added to natural amino acids [60]. In the future, it is speculated that any problems with the inactive nisin mutants (i.e.: lack of modifications) could be overcome. Synthetic biology could be used to co-express ClosB and ClosC enzymes to make strain with new functions.

Based on the example of Venter's recent work [140], it would be possible to apply a whole-genome rational design and synthesis so as to create organisms that would synthesise the desired lantibiotics. Their aim is to create such a simple cell with essential 'core sets of conserved genetic functions'; the cell would allow to identify both the molecular and biological function of genes. This approach could be used in the future to explore the potential *clos* genes. However, the success of using the co-expression with ClosBC approach could depend for instance on whether the nisin leader is suitable for the substrate specificity of the *clos* modification machinery.

The genetic engineering approach taken in here allowed for the production of some potent nisin-variant producing strains. However, none of the engineered strains produced a lantibiotic peptide with improved properties compared to nisin possibly because of degradation as well as their altered regulation. Therefore, the inability to induce *in vivo* synthesis will ultimately result in reduced or absence of antimicrobial production. This is the case with the inactive mutants generated in this work: Ring A and Ring C double mutants (I4K_L6A, A15I_G18T) and the Ring C single mutant (G18T) [130].

In general, the replacement of hydrophobic amino acids, which are found naturally in nisin (nisin Q, U or U2) with another hydrophobic amino acid to give M21L is known to result in the retention of relatively high levels of bioactivity as seen in overlay assays against *L. lactis*, *C. perfringens* or *M. luteus*. However, it was reported that the bioactivity

of the producer of M21L is somewhat decreased to 58–76 % when expressed in a slightly different system [87]. In this project, asparagine substitution also had very interesting consequence in that the N20P strain had increased or similar relative bioactivity levels against all tested species. This effect was expected since it was previously established that the N20P mutant generally displays enhanced bioactivity against a number of *S. aureus* strains. With respect to the N20P_M21L double mutant, the bioactivity was only slightly decreased in the overlay assay against different indicator strains.

Another interesting mutant created in this project is H27G located in Ring E. The consequence of incorporating the smaller glycine residue, to make H27G, resulted in a strain exhibiting similar relative bioactivity against *L. lactis*, *C. perfringens* and *M. luteus*. This is in good agreement with observations made in other studies where other small amino acids were used [141, 142]. The results from the bioassays confirmed these finding. Following site saturation mutagenesis of the C-terminal region of nisin, it was also now possible to assess the impact on bioactivity of two nisin mutant peptides that have three and five mutations, respectively. The two C-terminal nisin variants with multiple substitutions had slightly decreased levels of activity against *C. perfringens* and *M. luteus*. Their bioactivity had dramatically decreased when tested against *L. lactis*. This variation could be a consequence of the different sensitivity of the indicator strains or could be a consequence of measuring on the relative bioactivity rather than on specific activity.

In contrast, the alanine- and lysine- containing Ring A mutants, I4K and L6A, had varying degrees of bioactivity (70-90 %) against all the tested strains, therefore establishing that at positions 4-6, replacement by small aminoacids is well tolerated. Interestingly, a peptide corresponding to I4K could not be detected by CMS, indicating a negative impact of the substitution on peptide production even though the mutant was indeed active in bioassays. With respect to the double mutant I4K_L6A, the effect of mutations was dramatic, as peptides were inactive against all indicator strains. This is perhaps because of the opening of Ring A or as a consequence of preventing the dehydration of the threonine residue in Ring A at position 5. In addition, the presence of masses corresponding to the uncleaved peptides suggested that the NisP proteolytic activity may be hampered in this mutant. Even if the introduction or the exchange of positively charged residues in nisin is generally tolerated, there are also structural considerations, with the bulkier lysine residue having the most negative influence, which seems to be the case with I4K_L6A.

Screening of nisin mutants against indicator strains indicated that some mutants have different levels of bioactivity. A possible increase in the bioactivity of nisin mutants compared to the positive control could be explained by a general increase in production or by an enhanced diffusion rate in the agar plate. The hinge mutants had an increased bioactivity which it could possibly be because of a greater specific activity against the target strain.

The self-induction capacity of the nisin mutant peptides was also assessed. Their relative antimicrobial level of activity was compared against the vector control. The plate diffusion bioassay was not used for the purpose of quantifying the amount of bioactivity in nisin units as the amount of produced peptide in the supernatant was unknown. Despite generating a standard curve from positive control samples on the plate, the zones of inhibition produced by mutants were too low to accurately create their standard curves. The amount of peptide produced and then exported in the liquid culture could vary for every mutant peptide as a consequence of amino acid substitution. In future, for more accurate assessment of the amount of antimicrobial peptide, all the mutant peptides should be purified and their level of antimicrobial activity should be assessed relative to the amount of pure nisin mutant.

To summarise, the work in this chapter involved the substitution of amino acids first in the rings of the nisin structure, then in the hinge region and in the C-terminal part of nisin. The number of *clos*-like nisin mutants was narrowed down in order to select and purify the ones that are potentially interesting candidates of bacteriocins with improved properties. It was hypothesized that the antimicrobial activity, stability, spectrum of activity and specific activity could be enhanced if mutations were first introduced in the rings. It was possible to assess the effect of mutations on the antimicrobial activity. Furthermore, preliminary results on the bioactivity of nisin mutants in culture supernatant and in assays with colonies from liquid cultures against ten indicator strains were also revealed. N20P and M21L hinge mutants had been previously synthesised, and as in other studies they had high antimicrobial activity. Therefore, they were not considered for purification. Assessment of the bioactivity of N20P had enhanced antimicrobial activity against *S. aureus* and *L. monocytogenes* which is in line with the finding from literature. Nisin mutants with amino acid substitutions in the hinge region that are not capable of pore formation but are still potent antibiotics also exist [125]. H27G is a Ring D nisin mutant that proved to be highly active. I4K and L6A are Ring A single mutant that

produced a zone of inhibition which was less than nisin. Therefore, I4K, L6A and H27G mutants were targeted for purification and subsequent bioactivity studies.

3.4.3 Purification and characterisation of pure I4K and H27G nisin mutants

Fractogel TSK butyl 650-S (Merk) column chromatography, ultrafiltration, preparative RP-HPLC on C18 column, and lyophilisation allowed the successful removal of any non-specific products from biosynthesis or any low molecular weight contaminants from the media. The purified I4K and H27G were successfully challenged to GI tract conditions and a more accurate and reproducible assessment of high specific biological activity was therefore possible.

Initial attempts to produce and purify the I4K, L6A and H27G mutants from a 11 L fermenter using in-house optimised methods to purify nisin-like lantibiotic methods were only successful for I4K and H27G but not for the L6A nisin mutant; the unsuccessful purification of L6A could be explained by its poor production levels despite using an optimised scale-up purification method.

Nisin shows a slow loss of activity during storage with respect to practical applications [123] and its solubility and stability is strongly dependent on pH. Nisin contains unusual unsaturated amino acids: dehydroalanine (Dha5, Dha33) and dehydrobutyrine (Dhb2). Due to these structural features and potential intermolecular nucleophilic addition reactions, the solubility and bioactivity of nisin drops in alkaline pH [47], therefore it was interesting to assess the effect of applying treatment to mimic the GI tract conditions: different pH and high temperature of 37°C.

Although full characterisation of the purified I4K was not completed to investigate the host range, the Ring A nisin mutant was successfully purified from culture supernatant by hydrophobic interaction chromatography using Fractogel Butyl-Toyopearl resins. The activity from I4K, L6A and H27G nisin mutants that were taken on Fractogel and on a C18 column (RP-HPLC purification step) was detected throughout the process using two steps of purification. Unique peaks for I4K and H27G were detected in Maldi-ToF, but not for L6A, and the successful purification of I4K and H27G was confirmed. Further Maldi-ToF analysis using fractions from the first purification step indicated that L6A was isolated and it was still active. However, no active antimicrobial peptide was detected from fractions collected from the C18 column even if they were further concentrated by freeze-drying.

Preliminary characterisation of the I4K antimicrobial showed that it was unstable over time. Using the plate diffusion assay however, it was possible to look for the specific activity of pure I4K and pure H27G in comparison to wild type nisin. All the mutants were incubated at pH relevant to the GI tract and incubated at 37°C for 7 days during which time aliquots were removed and tested for their antimicrobial activity at different time points. Although, I4K was a promising good candidate to be purified and its specific activity was investigated, I4K is only 1 % as active as nisin in its pure form, and it degraded under storage conditions. To investigate this degradation phenomenon, I4K should be re-purified at a higher yield. On the other hand, pure H27G has a starting concentration of 35 to 40 % of nisin units compared to nisin when tested at any given pH. As with nisin, H27G was more stable at acidic pH over time and the biological activity dropped over time and as a result of increased in pH which is in line with previous observations from literature [47].

In this study, preliminary bioassays with H27G, I4K and L6A against vegetative spores of *C. perfringens* and *C. difficile* indicated that these mutants had inhibitory activity. In previous studies [143], it was suggested that besides having two killing mechanism, the inhibition of the cell wall biosynthesis and pore formation, nisin can stop the outgrowth of spores by the release of two hydrolysing enzymes that promote autolysis of the cell wall in spores. A Dha residue at position 5 is essential for this activity [139]. It was speculated that since mutations in I4K and L6A are in the vicinity of the Dha5, the mutations may have interesting effects on bioactivity. Both I4K and L6A seemed to be active, however when I4K was finally successfully purified, it proved to be unstable. Also, a very high amount of the I4K nisin mutant peptide would need to be used to attain an equivalent level of activity as nisin. In general, I4K, H27G and nisin appeared to degrade in a similar fashion when exposed to GI tract conditions.

Measurement of the specific activity of nisin variants indicated that the activity of nisin at any pH has exhibited a sharp decrease in the biological activity but particularly at higher pH, whereas for H27G and I4K the decrease was moderate. The activity declined to 10-15 % in the case of nisin, for H27G it was 30 % active on average (15-40 % active) whereas I4K maintained its activity to 40-67 % at any given pH. H27G was almost fully degraded after 68 h, with nearly complete loss of its antimicrobial activity particularly at pH 6.0 to 7.0. Nisin was still active at all pH values tested after 7 days which makes it the most stable peptide tested in this study.

The biggest challenges in this work involved identification of antimicrobial activity from C18 fractions with L6A due to low yield from culture supernatant or loss of antimicrobial activity during the purification steps, which is something that has been reported before for bacteriocin-like peptides [144].

In this work, a limited number of *clos*-like nisin mutants was generated. Site-directed mutagenesis targeted the rings in the N-terminal part of nisin, the hinge region and the rings in the entire C-terminal part of nisin and the effect of the amino acids substitutions was tested. Their antimicrobial activity and spectrum of activity was assessed in order to confirm that certain mutations do not render the nisin variant inactive. Based on the results, it was concluded that a nisin variant that would comprise all the 12 possible mutations present in the ClosA1 peptide would not be a good candidate for the development of a lantibiotic peptide with enhanced activity and spectrum. In the future, the specific activity of the novel mutants made in this work could be assessed and the knowledge could inform on the rational design of potent lantibiotic peptides that would be *clos*-like nisin mutants.

In conclusion, we have successfully produced and tested a range of nisin mutants with characteristics of the ClosA1 putative peptides and demonstrated the production of modified peptides by MS. Increased activity and variation in host range have been demonstrated as seen with results with N20P and M21L against *C. difficile* and *C. perfringens* (Table 18, data not shown in this work). Purification of H27G and I4K allowed for comparison of specific activity against nisin, none of the peptides had enhanced activity, stability of spectrum of activity against indicator strains. Future work would involve further attempts to purify and characterise L6A and I4K as well as introduce mutations in the nisin peptide to make the fully modified Clos peptide or nisin mutants with modifications in all the rings.

CHAPTER FOUR

4. Identification and functional characterisation of promoters in the *clos* operon

4.1 INTRODUCTION

During the genome mining of the GI tract, a novel gene cluster, known as *clos*, was identified and sequenced [97]. This nisin-like *clos* gene cluster is considered to encode four peptides whose sequences have been predicted based on structure comparison to the nisin gene sequence (Figure 8, chapter 1). However, the real structures for the Clos peptides remain unknown and they have yet to be characterised. In order to understand if the *clos* cluster is active or if it is a silent operon, the functionality of the putative *clos* promoters was investigated. If the *clos* promoters are activated by induction with nisin, this will provide evidence that the associated genes are possibly expressed in the *clos* gene cluster. The *L. lactis* UKLc10 host strain chromosome contains the *nisRK* genes which regulate the activation of the P_{nisA} promoter, one of the nisin-inducible promoters found in the nisin gene cluster [100, 145]. The *L. lactis* UKLc10 strain was therefore selected as a suitable host strain where the *clos* promoters were cloned to create a reporter gene expression (P_{ClosA_pepI}) system that could potentially be controlled by induction with nisin.

The *clos* cluster of the *Blautia obeum* A2-162 strain is comprised of four copies of the *closA* genes: three are identical copies of *closA* (*closA1*, *closA2*, *closA3*) and they are predicted to encode for nisin-like lantibiotics, whereas the forth *closA* gene (*closA4*) may encode for a protein whose structure differs to any known lantibiotic [97] (chapter 3).

Lactic acid bacteria often produce antimicrobial peptides (AMP) via quorum sensing and the autoregulation of nisin biosynthesis by the nisin lantibiotic peptide itself is a special example of this process [146]. The nisin response regulator, NisR, and the nisin sensor histidine kinase, NisK, proteins are part of a two-component regulatory system which is switched on by addition of subinhibitory amounts of extracellular nisin [74, 92]. Extracellular nisin acts as a signal which is transduced by the NisK auto-phosphorylation and this is followed by phosphate transfer to NisR. The transcription of any genes under control of the *nisA* promoter (P_{nisA} and P_{nisF}) is then activated (Figure 43). In nisin, transcripts that are comprised of *nisA*, *nisABTCIP*, *nisABTCIPR*K and *nisFEG* are formed as a result of transcriptional activation since both P_{nisA} and P_{nisF} are inducible promoters as they require nisin to be activated [147]. In contrast, the *nisRK* genes are expressed independent of induction, since the NisR promoter, (P_{nisR}), is a constitutive non-inducible promoter [8].

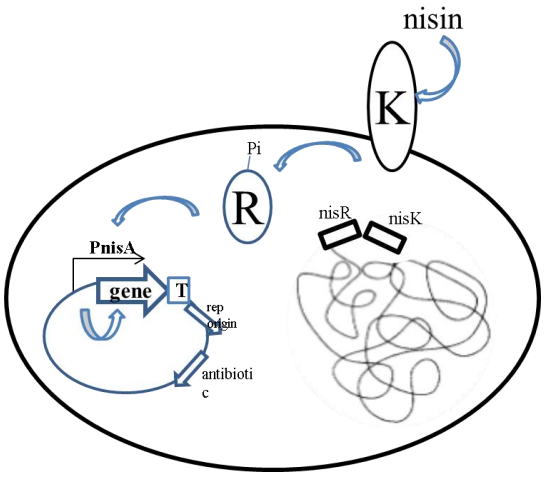


Figure 43 - Model for nisin signal transduction pathway by the two-component regulatory system that consists of the nisin sensor histidine kinase, the NisK protein, and the nisin response regulator, NisR.

NisR is phosphorylated, annotated as R-Pi. The *nisRK* genes are integrated on a chromosome. Adapted from Kuipers *et al.* [148]. T is terminator. 'Rep origin' is origin or replication.

Figure 43 illustrates an UKLc10 *L. lactis* host strain with the *nisRK* genes chromosomally integrated where the target gene from the plasmid is expressed as a result of nisin induction and *nisA* promoter, P_{nisA}, subsequent activation. UKLc10 was used as a host strain in which the nisin promoter is fused to a *pepI* reporter gene whose expression gives rise to a colour development and the *pepI* specific activity can be measured accurately [100]. The well-established concept behind the nisin signal transduction in the positive control strain, known as pUK200_ P_{nisA}_*pepI* (pP_{nisA}_I), has confirmed the need to develop similar expression systems in order to investigate the activation of the promoters *in the clos* cluster.

A reporter gene, *pepI*, from *Lactobacillus delbrueckii* subsp. *lactis* [100] was used to investigate the activation of the four putative *clos* promoters identified in the *clos* cluster of the original gut bacterium, *Blautia obeum* A2-162. The reporter gene promoter systems were used to investigate the possible regulation of the *clos* promoters by either *closRK* or *nisRK* genes, the two-component regulatory systems as described in section 4.3.3.

L. lactis UKLc10 is one of the few most suitable expression systems which relies on the use of nisin to induce fusions of *clos* or nisin promoters with a target *pepI* gene via the two-component nisin regulatory system, *nisRK* [100, 149]. The positive control has the advantage that expression of a target gene in *L. lactis* UKLc10 is inducible by subinhibitory amounts of nisin (10 ng/ml), in a series of up to several hundred fold (specific activity is approx. 35,000 nmol/mg/min) [147].

We aimed to use this reporter system to investigate the activation of *clos* promoters.

4.2 MATERIALS AND METHODS

4.2.1 Designing of a *pepI* reporter gene promoter system

4.2.1.1 *Bioinformatics analysis to identify the putative promoters*

The *clos* cluster was analysed using soft berry software to search for functional motifs/promoters. Putative regions which may be part of the RNA binding site in a promoter, which means it is the DNA sequences located at the -10 position, -35 position, and the transcription start site (TSS) as well as the ribosome binding site (RBS) were identified. The nisin and subtilin gene clusters were also used for comparison between the characteristic elements of a promoter sequence.
http://www.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb.

4.2.1.1.1 Cloning of the *pepI* reporter gene

The P_{ClosA}, $P_{ClosR2K2}$, $P_{ClosBTC}$ and $P_{ClosFEG}$ promoters from the *clos* cluster were cloned individually using the primer pairs as listed in chapter 2 (section 2.2.1). For instance, PClosA_F/ PCA_R and PCA_F/ *pepI*-R or *pepI*_R primer pairs were used. The two PCR products where then spliced with PClosA_F/ *pepI*-R or *pepI*_R to generate approx. 510 bp, 150 bp, 372 bp and 774 bp PCR products, for P_{ClosA}, $P_{ClosR2K2}$, $P_{ClosBTC}$ and $P_{ClosFEG}$ promoters, respectively.
In each case, splice overlap extension PCR involved the use of two PCR products, one from each reaction (A and B) and the amplified fragments from each reaction were used as templates for the final splice reaction which involved the forward primer from reaction A and reverse primer from reaction B (see chapter 2). The splice products were then digested with *Bgl*II and *Xho*I, unique cloning sites to be used for translational fusions, and ligated into pIL253 plasmid cut with *BamH*I and *Sal*I to create pP_{ClosA}_I, $pP_{ClosR2K2}$_I, $pP_{ClosBTC}$_I and $pP_{ClosFEG}$ _I constructs.

4.2.1.1.2 Cloning of *closRK* in pUK200_ P_{nisA}

The sequence was aligned to the original expected sequences designed in Clonemanager and after sequence confirmation, the plasmid was transformed in ultra competent *L. lactis* UKLc10 cells for further testing as described in section 4.2.3.
The forward primers, ClosR1K1_F and ClosR2K2_F, were designed to create *Nco*I restriction enzyme sites, whereas the reverse primers, ClosR1K1_R_BamHI and

ClosR2K2_R_BamHI or ClosR1K1_R_SalI and ClosR2K2_R_SalI, were designed to create either *BamH*I or *Sal*I restriction enzyme sites, respectively. The *closR1K1* and *closR2K2* were fused to P_{nisA} promoter individually using primer pair as listed in chapter 2. For instance, primer pairs ClosR1K1_F/ClosR1K1_R_SalI were used to make a PCR product that was restricted with *Nco*I/*Sal*I and cloned into pUK200_ P_{nisA} restricted with *Nco*I and *Xho*I.

4.2.1.1.3 Generation of frameshift in *closR1*

The pP_{nisA}_cRK construct encodes for one of the two sets of *clos* two-component regulatory system. The UKLc10 *L. lactis* strain harbouring plasmid pP_{nisA}_cR1K1 was grown overnight then harvested and the plasmid DNA was extracted using the Qiagen plasmid mini prep kit. The plasmid DNA was restricted for 2 h with *Afl*II at 37°C, the enzymes were heat inactivated at 80°C for 20 min. The plasmid was blunt ended with T4 DNA polymerase, purified by Sure Clean and the success of the digestion was assessed on an agarose DNA gel. For ligation, Fast link DNA ligase and the blunt end protocol were used. Restriction digests and all the cloning steps were performed using the optimal conditions described by the manufacturer (NEB, Herts, UK) (chapter 2). Each pP_{nisA}_cRK plasmid was transformed into electrocompetent *L. lactis* MG1614. Clones were identified by colony PCR using primers p54/p181 and plasmid DNA from positive clones were sequenced with primer p54. The sequences were aligned to the original expected sequences designed in Clonemanager and the absence of one nucleotide introduced by *Afl*II digestion was confirmed. The plasmid with the frameshift in the *closR1* gene was transformed in ultra competent UKLc10 cells.

4.2.2 Transformation to make reporter gene and *clos* regulatory system

A two plasmid system was created in *L. lactis* UKLc10: pP_{Clos}_I which encodes for the *pepI* reporter gene and pP_{nisA}_cRK which encodes for one of the two sets of *closRK* two-component regulatory system. One of the two plasmid systems incorporated pP_{ClosA}_I construct and pP_{nisA}_cfsR1_cK1 that encodes for a mutated *closR1* (frameshift). The positive control, pP_{nisA}_I in *L. lactis* UKLc10, was kindly donated by Nikki Horn from the IFR collection. The *pepI* expression was achieved by induction with 10 ng/ml of nisin. Controls without nisin induction were also included. Strains that contain the pUK200 and/or pTG262 expression vectors are chloramphenicol and/or erythromycin resistant, respectively.

4.2.3 Measurement of *pepI* specific activity

4.2.3.1 *Preparation of cell extracts*

Two 10 ml cultures of constructs in *L. lactis* UKLc10, MG1614 and FI7847 strains were grown from glycerol stock overnight, then two 100 ml fresh cultures were inoculated and grown to an optical density at 600 nm (OD_{600}) of 0.5, then they were typically induced with nisin at 10 ng/ml for 2 h to activate the *nisA* and *closA* promoters, unless otherwise specified. Cell volumes equivalent to 37 OD_{600} units from the two separate cultures were harvested by centrifugation at 10,000 × g for 15 min at 4°C and the cell extracts were prepared following the method of Wegmann *et al.* [100, 149]. The resulting cell pellets were washed once in 0.2 M Tris HCl (pH 7.4) before freezing at -20°C. Individual thawed cell pellets was resuspended in 250 µl of 0.2 M Tris HCl (pH 7.4). Sonication to disrupt the cells was performed with four 10 s pulses, 6 µm amplitude, with 5 to 10 min pauses on ice between each pulse. The resulting supernatants containing the cell extracts were obtained after a 30 min centrifugation at 14,000 × g at 4°C.

To maximize Clos peptide production, strains were grown for 12 h induced with 10 ng/ml of nisin and aliquots were collected at time zero, 2 h, 4 h, 6 h, 12 h and cell extracts were prepared and loaded on protein gels and analysed by western blotting.

4.2.3.2 *PepI enzyme assays*

The specific activity of the peptidase I was assayed by using 50 µl of undiluted or diluted cell-free samples in 0.2 M Tris HCl (pH 7.4), with 0.7 mM L-proline *p*-nitroanilide trifluoroacetate salt (Sigma) as the substrate. The *p*-nitrophenol release was measured at A_{405} at 37°C for 15 minutes. Sample protein determination was performed at A_{600} using the Bio-Rad protein assay (Bio-Rad, United Kingdom) with standard BSA (New England BioLabs UK Ltd.) and typically 1/200 dilution of cell extracts, 1/1000 and 1/5000 dilutions for the cell extract positive control. The specific peptidase activity was expressed as nanomoles of *p*-nitrophenol released from the chromogenic substrate per milligram of protein per minute (nmol/mg/min). The means and standard deviations plotted were based on two biological replicates each with two technical replicates.

4.3 RESULTS

4.3.1 Investigation of the clos promoters - bioinformatics analysis

Computational analysis of the *clos* cluster with the soft berry software identified four putative *clos* promoter sites as illustrated in Figure 44. The characteristic elements of the *clos* promoters, -35 box, -10 box and RBS, are listed in Table 20, and they were compared to the promoters of nisin and subtilin.

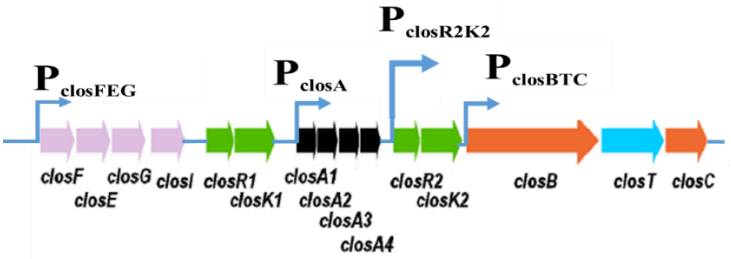


Figure 44 - Diagram of the *clos* cluster to highlight the four *clos* promoters detected by computational analysis in order to understand which one of the two sets of *closRK* genes regulates the biosynthesis of *clos*.

Promoter (nucleotide start site)	-10 box			-35 box (lan –box)				
	Promoter position		score	Promoter position		score	RBS*	
P_{ClosA} 633	6320	TTTTATATT	66	6304	TTGATA	58	AGGAGG	6 bp to ATG
$P_{ClosFEG}$ 717	702	TTTTAATCT	55	684	TTTACA	47	AAGGAGA	11 bp to ATG
$P_{ClosR2K2}$ 7236	7221	TGTTATTAT	79	7202	GTGATT	20	AAGGAGC	5 bp to ATG
$P_{ClosBTC}$ 9605	9590	TTTGATAAT	50	9569	TTCAAA	40	AGGAGG	5 bp to ATG
P_{nisA}	TACAAT		54	TCTGAN6TCTGA		81		
P_{nisF}	TATACT		39	TCTGAN6TCTGA		66		
P_{spaB}	TATAGT		122	TTGATN6TTGAT		148		
P_{spaS}	TACTAT		31	ATGATN6TTGAT		57		
P_{spaI}	TAGAAT		109	TTGATN6TTGAT		135		

Table 20 - Characteristic regions in the *clos* promoters and comparison to nisin and subtilin.
*Represents the number of nucleotides (in base pairs) relative to the ATG start codon.
-35 region is the lantibiotic box: subtilin box or nisin box. Details for nisin and subtilin promoter sites were adapted from Kleerebezem *et al.* [83, 146]. The scores for the search of *clos* promoters are given in the table and the positions of the predicted *clos* promoter sites where 1 is the first amino acid in the *closF* gene in the *clos* gene cluster [146]. TTGAT in -35 box represents half a SPA box. N_6 represents 6 nucleotides in any combination.

Previous transcription analysis of the P_{LanA} promoter in nisin and subtilin suggested the presence of a nisin box [146], however further experiments need to be performed to identify an equivalent *clos* box.

Target genes can be expressed in *L. lactis* host strains provided that the process is regulated by strong constitutive or inducible promoters via a two-component regulatory system. For nisin, it was demonstrated that constitutive expression of *nisRK* in an

uninduced state is possible since *nisRK* genes are expressed continuously to ensure a rapid build-up of immunity. In an induced state, the *nisRK* gene expression in the nisin gene cluster is enhanced [146].

After the *clos* and nisin gene clusters were compared as they share a high level of structural similarity, it was predicted that their genes encode for highly similar proteins. Interestingly, the -35 box in P_{ClosA} has a half subtilin box. The spa-box is known to be involved in the subtilin-mediated regulation of *spa* gene expression as it represents the binding site of the SpaR regulator [146]. Therefore, it is believed that perhaps the half subtilin box may act as the binding site for ClosR since it was previously shown that the ClosR1K1 protein seems to be more similar to SpaR1K1 than its equivalent in nisin operon [97]. However, additional sigma factors may contribute to the transcription initiation of *clos* which is something that could be investigated in the future.

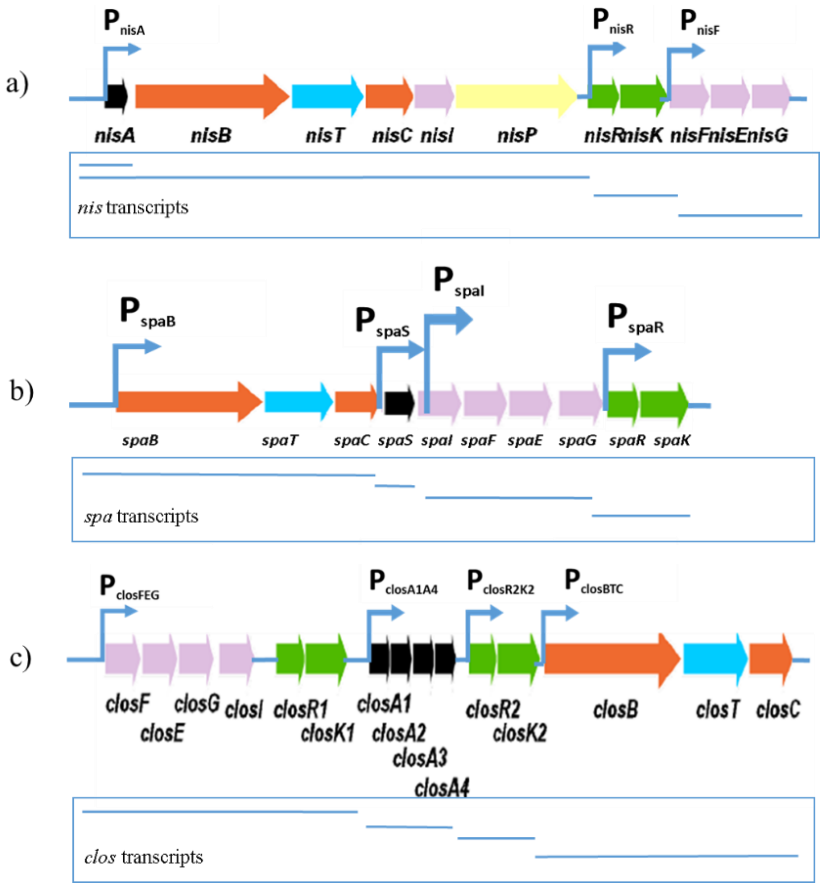


Figure 45 - Comparison between the organisations of a) nisin, b) subtilin and c) *clos* biosynthetic gene clusters (predicted *clos* transcripts).

The structural genes (in black), genes that encode proteins involved in maturation (in orange) and in transport (in blue), in leader processing (for nisin in yellow), in immunity of the producer (in purple), and in regulation (in green) are indicated. Adapted from Kleerebezem *et al.* [146]. The predicted transcripts would be formed due to activation of promoters.

Figure 45 illustrates the known location for both promoters and transcripts involved in the nisin and subtilin biosynthesis. Based on the *in silico* search, the *clos* promoters were also highlighted for the production of Clos.

4.3.2 Construction of plasmids to study P_{Clos} promoters

A number of constructs were successfully made using the methods described before (section 4.2).

Fusions of P_{Clos} promoters with *pepI* reporter genes were first created in *L. lactis* MG1614 host strain, known as P_{ClosA}*_pepI*, $P_{ClosBTC}$*_pepI*, $P_{ClosFEG}$*_pepI* and $P_{ClosR2K2}$*_pepI*. Splice overlap extension PCR was used to amplify the sequence before the *closA1-4*, *closBTC*, *closFEG* and *closR2K2* genes in the *clos* cluster sequence. These sequences include the essential elements present in any *clos* promoter.

The primers for splice overlap extension PCR were designed to be complementary to the ATG start site of the *pepI* gene (reverse primer) and to the 3' end of the promoter sequence. The outer primers were designed to create *Bgl*II and *Xho*I restriction enzyme sites at each end. After subsequent cloning steps and sequence confirmation these constructs were transformed into the *L. lactis* UKLc10 or FI7847 host strains. In order to determine if the P_{Clos} promoters are activated by nisin and regulated by *nisRK* genes, the specific activity of *pepI* was monitored in an enzyme assay.

To identify if either of the two *closRK* systems can switch on any of the P_{Clos} promoters, the *closRK* genes were cloned under the control of the nisin promoter, P_{nisA}, on a pTG262 expression vector to make pTG262_P_{nisA}_ClosRK constructs (Figure 46). The outer primers were designed to create *Nco*I and *Sal*I or *BamH*I restriction enzyme sites at each end.

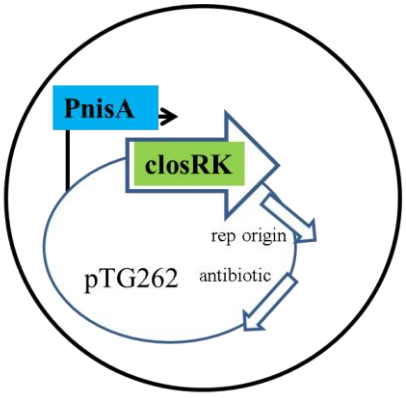


Figure 46 - Expression of the *closRK* genes under the control of P_{nisA} in a pTG262 expression vector. *L. lactis* MG1614 host strain with no *lanRK* genes.

Similarly, to test if a non-functional ClosR1 would allow for activation of P_{Clos} promoters, a frameshift was introduced in ClosR1. A mutated *closR1* (*fscR1*) and *closK1* genes were expressed under the control of P_{nisA} in a pTG262 expression vector to investigate the effect of a frameshift on the expression of a non-functional ClosR1K1 protein and results are presented in section 4.3.4.

4.3.3 Generation of reporter gene and *clos* promoter regulatory dual systems

The first hypothesis was to assess if one of *closRK* systems is able to activate any of the four predicted promoters in the *clos* gene cluster. To test this idea, the first aim was to create a two plasmid system composed of one promoter reporter gene plasmid, known as pIL253_P_{Clos}_*pepI* or pP_{Clos}_I, and one nisin induced regulatory system, named pTG262_ P_{nisA}_*closRK* or p P_{nisA}_cRK. To further investigate if the regulatory genes from the nisin cluster alone would switch on *clos* promoters, the second aim was to create a set of strains to test if the *nisRK* system alone in the UKLc10 strain is able to regulate any of the P_{Clos} promoters (Figure 47). The expression of *pepI* reporter gene in UKLc10 host strain would give rise to a colour development and the *pepI* specific activity can be measured accurately.

The second hypothesis was that the NisR and ClosR proteins in the dual system are competing for the same binding site in the P_{Clos} promoters. To investigate this, the aim was to mutate the *closR* gene to prevent binding of a non-functional ClosR protein to the P_{Clos} promoter (Table 21).

Proline iminopeptidase I, *pepI*, reporter gene and expression vectors for *L. lactis* (pIL253 and pTG262) were used to create a series of different constructs; using fusions to the *pepI* marker gene, high or low level protein expression vectors were created. The predicted four *clos* promoters were each fused to the *pepI* reporter gene, whereas each of the two *closRK* sets of two-component *clos* regulatory system (*closR1K1* or *closR2K2*) were expressed under the control of the nisin induced P_{nisA} via *nisRK* genes (in the chromosome of the *L. lactis* UKLc10 strain). To achieve optimal expression and to measure the full activity of the *pepI* reporter gene, alternative expression hosts were used. Different constructs were transformed in *L. lactis* MG1614 or UKLc10 host strains as listed in Table 21.

Full strain name - Hypothesis 1 - Aim 1		**Shorter strain name**	Regulatory system
pUK200_ P_{nisA}_*pepI*	In UKLc10 Positive control	p P_{nisA}_*pepI* in UKLc10	*nisRK* genes on the chromosome
pIL253_ P_{ClosA}_*pepI* + pTG262_ P_{nisA}_*closR1K1*	In UKLc10[a]	p P_{ClosA}_I+ p P_{nisA}_*cR1K1*	*nisRK* genes on the chromosome
pIL253_$P_{ClosBTC}$_*pepI*+ pTG262_ P_{nisA}_*closR1K1*	Test strains	p$P_{ClosBTC}$_I+ pP_{nisA} _*cR1K1*	
pIL253_ $P_{ClosFEG}$ _*pepI*+ pTG262_ P_{nisA} _*closR1K1*		p $P_{ClosFEG}$ _I+ p P_{nisA} _*cR1K1*	
pIL253_ $P_{ClosR2K2}$_*pepI*+ pTG262_ P_{nisA} _*closR1K1*		p $P_{ClosR2K2}$_I+ p P_{nisA} _*cR1K1*	
pIL253_ P_{ClosA}_*pepI*+ pTG262_ P_{nisA} _*closR2K2*		p P_{ClosA}_I+ p P_{nisA} _*cR2K2*	
pIL253_$P_{ClosBTC}$_*pepI*+ pTG262_ P_{nisA} _*closR2K2*		p$P_{ClosBTC}$_I+ p P_{nisA} _*cR2K2*	
pIL253_ $P_{ClosFEG}$ _*pepI*+ pTG262_ P_{nisA} _closR2K2		p $P_{ClosFEG}$ _I+ p P_{nisA} _*cR2K2*	
pIL253_$P_{ClosR2K2}$_*pepI*+ pTG262_ P_{nisA} _*closR2K2*		p $P_{ClosR2K2}$_I+ p P_{nisA} _*cR2K2*	
pIL253_ P_{ClosA}_*pepI* + pTG262_ P_{nisA}	empty vector control	p P_{ClosA}_I+ pP_{nisA}	*nisRK* genes on the chromosome
pIL253_ P_{ClosA}_*pepI* + pTG262_ P_{nisA} _*closR1K1*	in MG1614[b] control strains	p P_{ClosA}_I+ p P_{nisA}_*cR1K1*	no *lanRK* genes for MG1614 control strains
pIL253_ $P_{ClosR2K2}$_*pepI*+ pTG262_ P_{nisA} _*closR1K1*		p $P_{ClosR2K2}$_I+ p P_{nisA}_*cR1K1*	
pIL253_ P_{ClosA}_*pepI*+ pTG262_ P_{nisA} _*closR2K2*		p P_{ClosA}_I+ p P_{nisA} _*cR2K2*	
pIL253_$P_{ClosR2K2}$_*pepI*+ pTG262_ P_{nisA} _*closR2K2*		p $P_{ClosR2K2}$_I+ p P_{nisA} _*cR2K2*	
Full strain name – Hypothesis 1 - Aim 2[c]		**Shorter strain name**	Regulatory system
pIL253_P_{ClosA}_*pepI* pIL253_$P_{ClosBTC}$_*pepI* pIL253_ $P_{ClosFEG}$ _*pepI* pIL253_ $P_{ClosR2K2}$_*pepI*	In UKLc10 Test strains	p P_{ClosA} _I p$P_{ClosBTC}$_I p $P_{ClosFEG}$ _I p $P_{ClosR2K2}$_I in UKLc10	*nisRK* genes on the chromosome
pIL253_ P_{ClosA} _*pepI* pIL253_$P_{ClosBTC}$_*pepI* pIL253_ $P_{ClosFEG}$ _*pepI* pIL253_ $P_{ClosR2K2}$_*pepI*	In MG1614 Control strains	p P_{ClosA} _I p$P_{ClosBTC}$_I p $P_{ClosFEG}$ _I p $P_{ClosR2K2}$_I in MG1614	no *lanRK* genes for MG1614
Full strain name – Hypothesis 2 - Aim 1[d]		**Shorter strain name**	Regulatory system
pIL253_ P_{ClosA} _*pepI* + pTG262_ P_{nisA} _ _fsclosR1_*closK1*	In UKLc10 control strain	p P_{ClosA} _I+ p P_{nisA} _fscR1_*cK1*	frameshift in *closR1* *nisRK* genes on the chromosome

Table 21 - List of constructs expressed in *L. lactis* UKLc10 (test strain) or MG1614 (control strain).
To investigate if P_{Clos} promoters are active by measuring the *pepI* specific activity, two systems were created: a one plasmid system (hypothesis 1, aim 2) or two plasmid system (hypothesis 1, aim 1).
The activation of individual P_{Clos} promoters fused to the *pepI* is mediated by one of the *closRK* systems. The *closRK* genes (*closR1K1* or *closR2K2*) were each cloned and expressed under the control of P_{nisA} and regulated by the *nisRK* genes.
[a] pP_{nisA}_I in UKLc10 strain is the positive control. The *L. lactis* UKLc10 strain has the nisin two-component regulatory system (*nisRK* genes) on the chromosome.
[b] The *L. lactis* MG1614 strain is plasmid free.
[c] The *clos* promoter reporter gene system (pP_{Clos}_I) was cloned in a pIL253 plasmid.
[d] Control strains for hypothesis 2 contained a frameshift in the *closR1* regulatory gene (*closfsR1*).
In hypothesis 1, the *pepI* expression is expected to take place as a result of expression of *closRK* and the subsequent activation of P_{Clos}; in hypothesis 2, activation of P_{Clos} is controlled by *nisRK*.

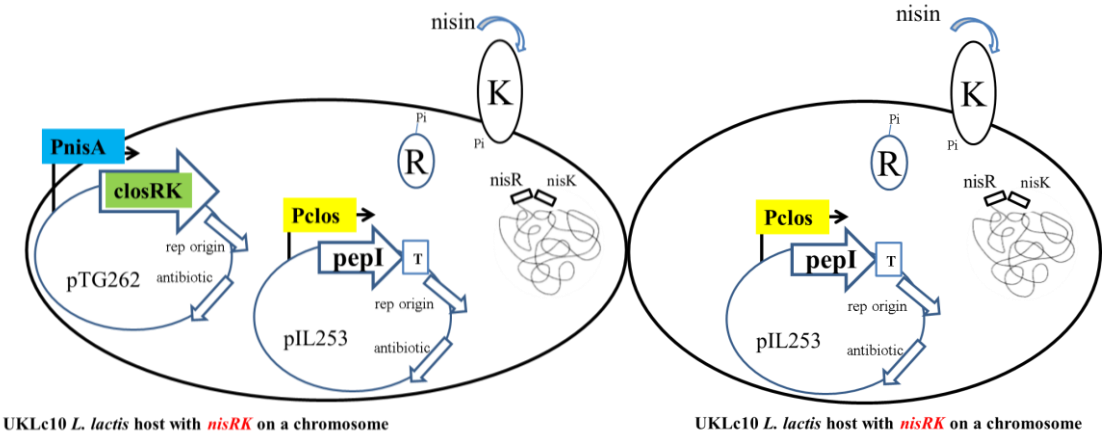


Figure 47 - Diagram to illustrate hypothesis 1 (left): dual plasmid system, or hypothesis 2 (right): 1 plasmid system, to investigate the activation of *clos* promoters, P_{Clos}.

In hypothesis 1, the *pepI* expression is expected to take place as a result of expression of *closRK* and the subsequent activation of P_{Clos}; in hypothesis 2, activation of P_{Clos} is controlled by *nisRK*. T is a terminator.

Based on the diagrams in Figure 47, several constructs were made to investigate the putative activation of *clos* promoters via *nisRK* or *closRK* signal transduction event. The aim was to create a set of expression vectors that will allow the detection of different levels of protein expression: high, low or constitutive, using the *pepI* reporter system.

4.3.4 Studying the functionality of P_{ClosA} promoter

4.3.4.1 *PepI expression under the control of ClosA promoter (P_{ClosA})*

Wegmann *et al.* [149] have shown that the pP_{nisA}_I in *L. lactis* UKLc10 positive control has a very high *pepI* activity of approx. 35,000 nmol/mg/min. This is based on the fact that nisin binds to P_{nisA} promoter with high affinity and it induces the promoter through the signal transduction initiated by the *nisRK* genes. Therefore, the specific *pepI* activity of the constructs in Table 21 was measured to determine which set of *closRK* system activates which P_{Clos} promoter.

The *L. lactis* UKLc10 strain has the *nisRK* genes on its chromosome, therefore it is expected that the P_{nisA} promoter would be activated; this binding should allow for the subsequent expression of the target gene in constructs known as pP_{Clos} _I + pP_{nisA}_cRK. The pP_{nisA}_cRK construct encodes for one of the two *closRK* sets of regulatory genes: *closR1K1* or *closR2K2*. Cell extracts were prepared and the *pepI* content was quantified by measuring the *pepI* specific activity by quantification of PepI.

The control strains known as pP_{Clos}_I in UKLc10 were designed to express the *pepI* reporter gene; this would take place if the activation of *clos* promoters is regulated by the

NisRK system alone. Therefore, cell extracts and the *pepI* activity assays were also performed with these constructs as illustrated in Figure 48.

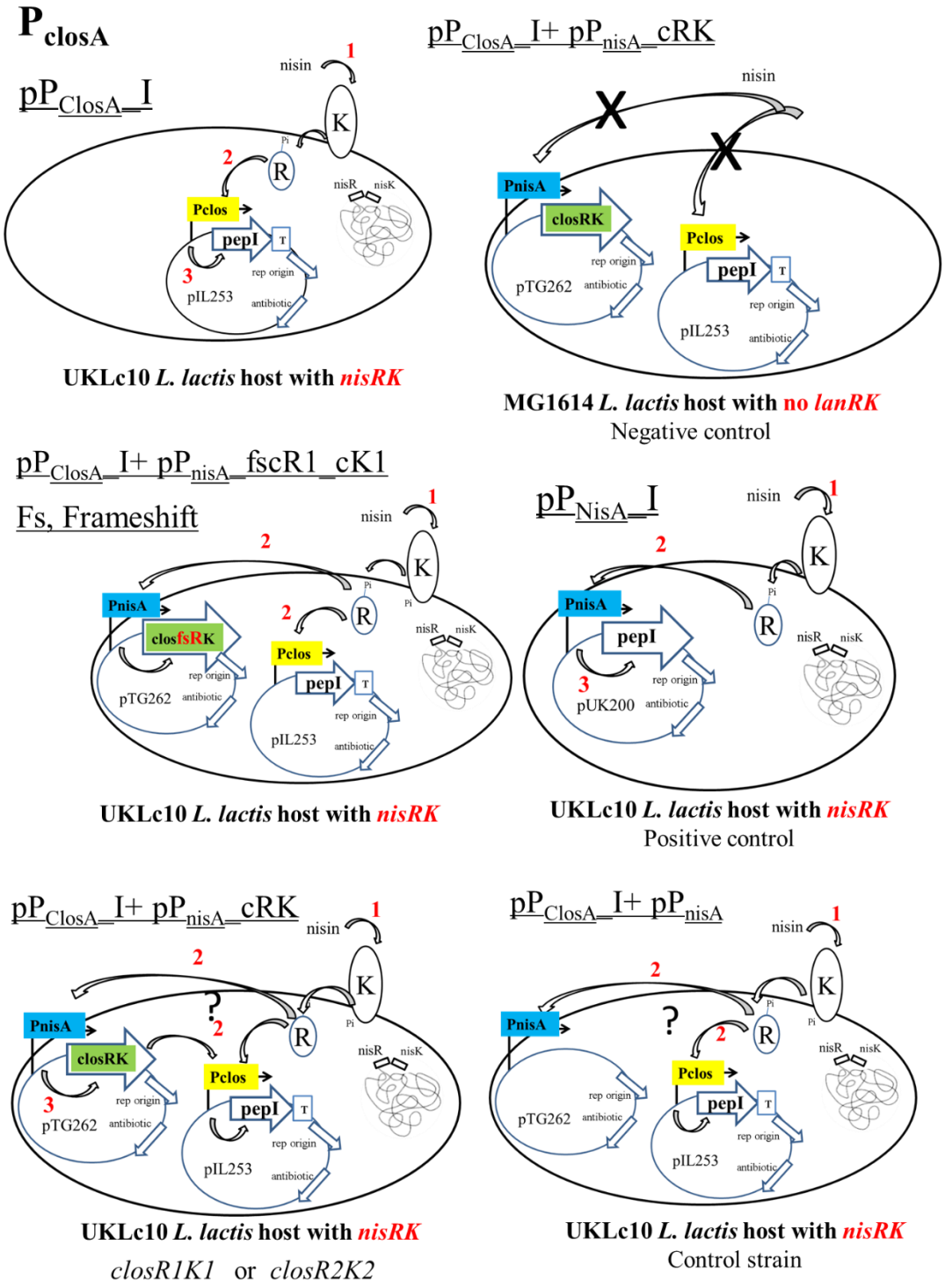


Figure 48 - Diagrams of different constructs to investigate the activation of P_{ClosA} via *nisRK* or *closRK* genes in *L. lactis* MG1614 negative control strain or in *L. lactis* UKLc10 test strains.

Extracellular nisin (1) was added in order to activate the nisin or *clos* promoters (2). For details on the plasmids see Table 21. I is *pepI*, cRK is *closR1K1* or *closR2K2*. The proposed steps for signal transductions were numbered and indicated by arrows.

The specific *pepI* enzyme activity of the cell extracts of constructs described in Table 21 was measured to determine if any of the *clos* promoters is activated: P_{ClosA}, $P_{ClosR2K2}$, $P_{ClosFEG}$, $P_{ClosBTC}$. The results are plotted in Figure 49 to Figure 53.

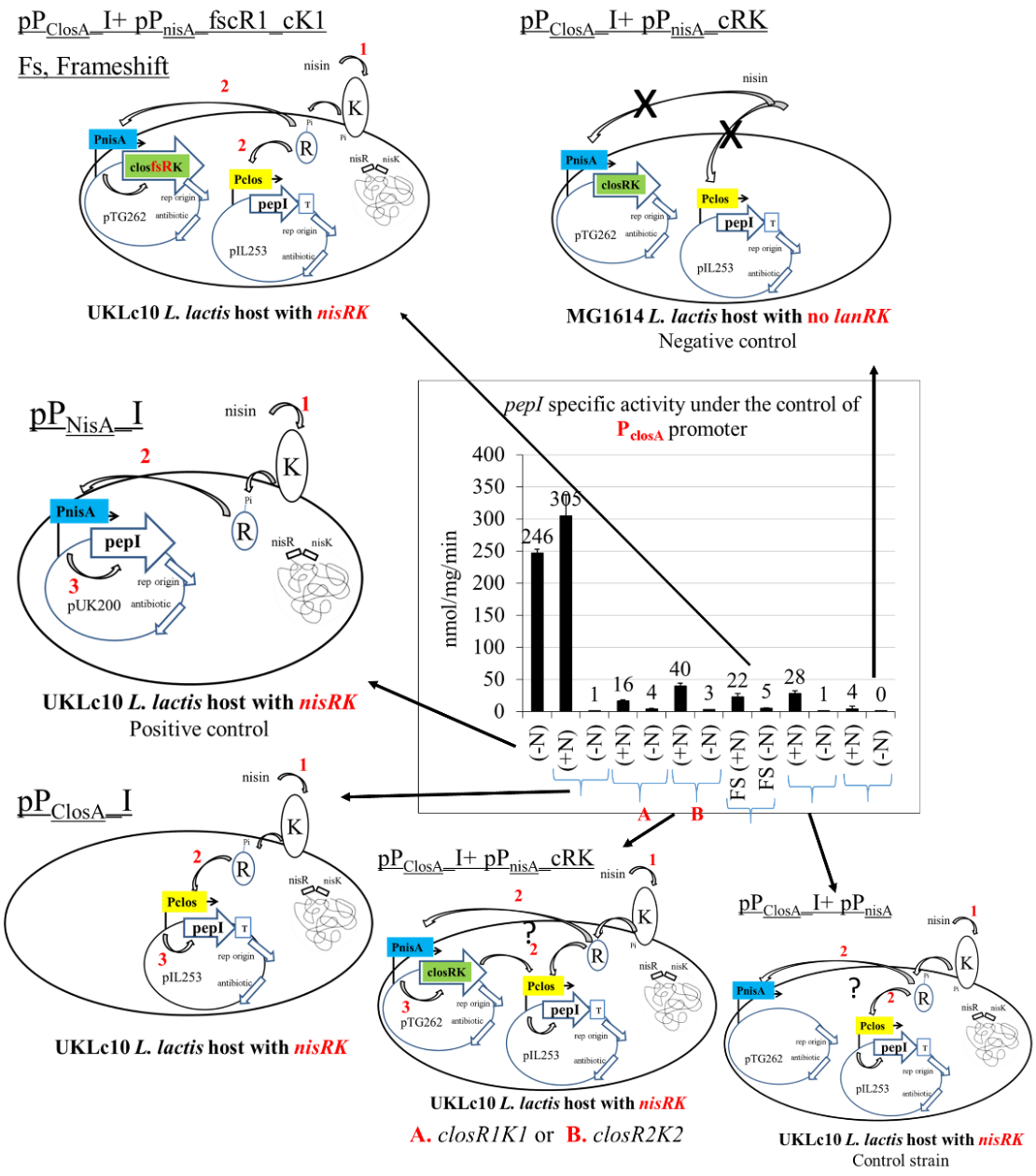


Figure 49 - Expression of *pepI* specific activity under the control of P_{ClosA} in *L. lactis* UKLc10 and MG1614 host strains.

Induction with nisin (+N), uninduced state (-N), N is nisin. Illustrations of signal transduction in a P_{ClosA} reporter gene construct, p P_{ClosA}_I, which is regulated only by *nisRK* system; in the dual plasmid systems, pP_{ClosA}_I + pP_{nisA}_cRK, the activation may be regulated by *nisRK* and/or *closRK*. The control strain in MG1614, pP_{ClosA}_I + pP_{nisA}_cRK, is devoid of the *nisRK* regulatory genes.

The error bars represent standard deviations based on two biological replicates with two technical replicates each. Induction with 10 ng/ml of nisin was represented by (+N) and the uninduced form with (-N), where N stands for nisin.

The *pepI* specific activity in fusions to the P_{ClosA} promoter was determined using the constructs in Figure 49 and the non-normalised reported values were highlighted on the graph to account for differences in *pepI* expression levels. Hypothesis 1, aim 1 comprises the two-component plasmid system, whereas aim 2 is represented by the one plasmid system as detailed in Figure 50, Table 21.

The results from Figure 49 indicated that the P_{ClosA} promoter in p P_{ClosA}_I did not display nisin-independent constitutive characteristics since without nisin no activity was detected. As it was shown previously [149], in an uninduced state. *nisRK* genes are constitutively expressed; this was also confirmed in here with the positive control, p P_{nisA}_I, (-N), without nisin, where the *pepI* specific activity was approx. 250 nmol/mg/min (Figure 49). In contrast, no activity was detected for p P_{ClosA}_I without nisin which confirms that P_{ClosA} is a DNA binding site for the activated NisR only when nisin is present.

In the positive control, pP_{ClosA}_I, where *pepI* expression is under the control of P_{nisA}, NisR has a higher affinity for the nisin promoter (value of approx. 35 k nmol/mg/min, +N, section 4.3.4.4) and approx. 250 ng/mol/min without nisin (Figure 49, positive control, -N). In contrast, NisR also binds to the P_{ClosA} promoter in pP_{ClosA}_I, (+N) construct, however the specific activity is lower (305 nmol/mg/min) when induced with nisin. The *pepI* activity seen with pP_{ClosA}_I (+N) is a very interesting result which means that the transcription of the *pepI* reporter gene is driven by the activated P_{ClosA} promoter which is triggered by nisin-mediated, NisRK-dependant signal transduction.

To understand if the *closRK* system activates P_{nisA}, several constructs known as pP_{ClosA}_I+ p P_{nisA}_cRK were created and they were comprised of both P_{ClosA} and P_{nisA} promoters (Figure 49). The first thing to note was the extremely low *pepI* activity. The pP_{ClosA}_I + pP_{nisA}_cRK constructs induced with nisin (+N) gave 16 and 40 nmol/mg/min, for *closR1K1* and *closR2K2*, respectively, whereas the empty vector control strain, pP_{ClosA}_I + pP_{nisA}, had 28 nmol/mg/min. Therefore, there was no significant *pepI* activity using both promoters.

Results using all the strains in Figure 49, including the negative controls in MG1614, have indicated that there was no obvious difference between functional and non-functional ClosR1 (*fsclosR1*) proteins using the frameshift constructs. The assumption is that the presence of two promoters, which are both competing for the same NisR, are responsible for the very poor *pepI* signal. Since NisR is binding with a higher affinity to the nisin box of P_{nisA}, P_{nisA} is favoured hence the lack of activation of P_{ClosA} and the lack

of *pepI* expression in the dual plasmid system. In contrast, the pP_{ClosA}_I control strain that contains only the P_{ClosA} promoter is clearly activated by nisin via the *nisRK* system present on the chromosome of UKLc10 strain.

The clearly distinguishable *pepI* activity of 305 nmol/mg/min in pP_{ClosA}_I in UKLc10 host strain with nisin (Figure 49), confirmed that since both *nisR* and *nisK* genes are integrated on the chromosome of UKLc10 strain, the expression of *pepI* was inducible by the wild type nisin. These results suggest that *nisRK* genes are necessary and perhaps sufficient for the activation of P_{ClosA} in this UKLc10 heterologous expression system which is not a perfect system. Therefore, in the one plasmid system, pP_{ClosA}_I, the *closA* promoter is definitely activated by nisin in this scenario (Figure 50).

In contrast, in the dual plasmid system pP_{ClosA}_I + p P_{nisA}_cRK, the *pepI* values were very close to the background, therefore activation of the P_{ClosA} promoter can not be confirmed in this system. Consequently, the aim 2 of hypothesis 1 was validated (Figure 50). It was interesting to observe that the presence of P_{nisA} prevented notable activation of P_{ClosA}.

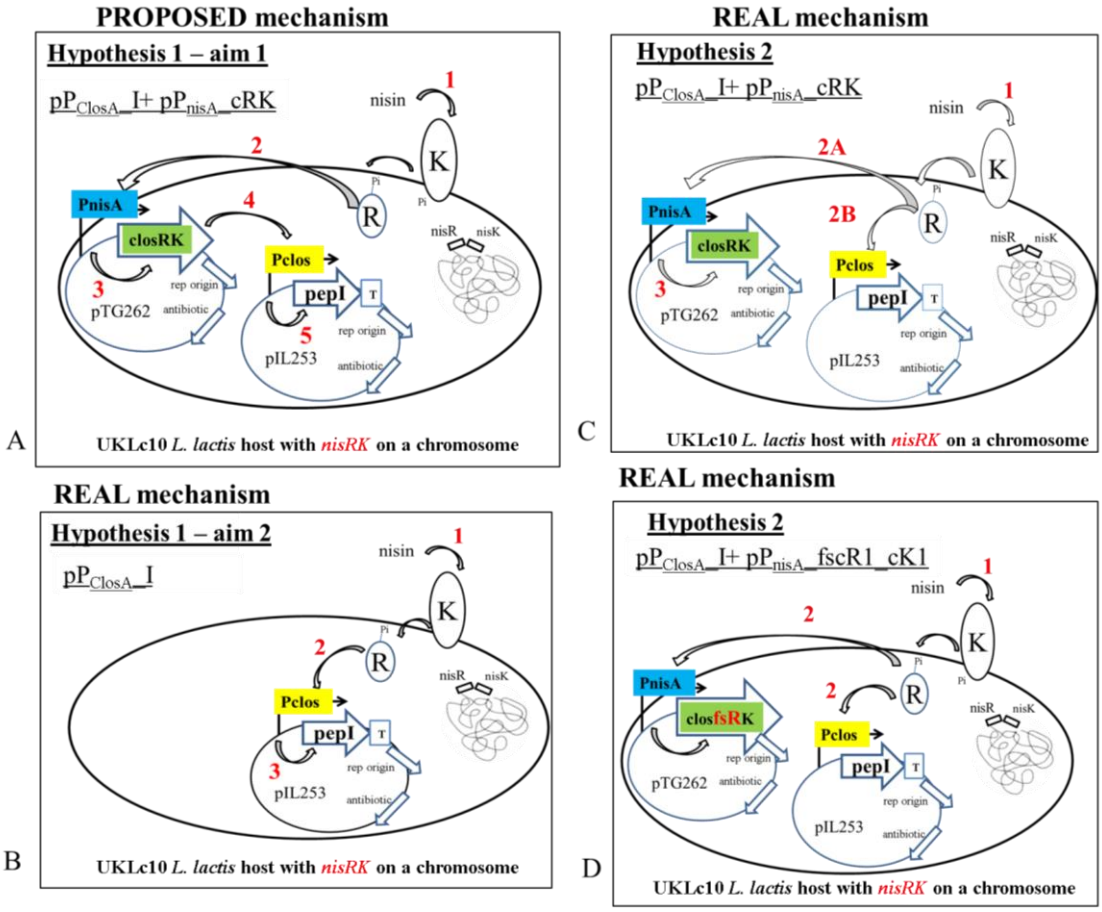


Figure 50 - Illustration of activation of P_{ClosA} promoter in different constructs mediated by the *nisRK* system in the UKLc10 strain.

Based on the lack of specific activity seen from the dual plasmid system, a new hypothesis was considered by which the NisR and ClosR are competing against each other for the same binding site in the P_{ClosA} promoter. To test this, frameshift in the potential N-terminal of ClosR was introduced in order to change the expressed protein and to disallow its binding to the P_{ClosA}. The frameshift removed 1 nucleotide to generate a new random sequence that has a stop codon created. The assumption was that the frameshift will favour the binding of NisR to the available target site in P_{ClosA}. Constructs known as pP_{ClosA}_I+p P_{nisA}_fscR1_ cK1 were created that encode for non-functional ClosR proteins. Therefore, it was interesting to investigate the effect on the *pepI* activity in the new scenario (hypothesis 2, Figure 50, D). For proof of principle, it was decided that similar strains carrying a plasmid with a mutated *closR2* was not necessary. Since pP_{ClosA}_I in UKLc10 was the only strain that gave a good *pepI* signal, no extra strains that had a frameshift in *closR* were tested.

Figure 50 illustrates the proposed (A) and real mechanism (B) for hypothesis 1 including the real mechanism for hypothesis 2 (C, D). The proposed mechanism for hypothesis 1

(A) suggests that P_{ClosA} promoter would be activated by one set of the *closRK* genes; P_{ClosA} was fused to *pepI* and *closRK* were expressed under the control of P_{nisA}. Based on the measurements for the *pepI* specific activity, the real mechanism for hypothesis 1 is that both *clos* and nisin promoters appear to be competing for the available NisR and that, based on the positive control, NisR has a higher affinity for P_{nisA}, therefore the binding between the two is favoured. Since NisR is no longer available, NisR does not bind P_{ClosA} so it does not get activated. The assumption that NisR and ClosR are competing for the same DNA target site in P_{ClosA} is not valid if the empty vector control and the mechanism in hypothesis 2, are considered. The mechanism illustrated for hypothesis 2 (Figure 50, C, D) suggests that in the presence of two promoters, the NisR will still favour the P_{nisA} binding site (Figure 50, D).

4.3.4.2 *PepI expression under the control of ClosR2K2 promoter ($P_{ClosR2K2}$)*

Based on previous results (Figure 50), the P_{ClosA} is definitely an inducible promoter as it is activated by the *nisRK* regulatory system as seen with pP_{ClosA}_I in UKLc10 strain. However, the *pepI* specific activity observed with the p $P_{ClosR2K2}$_I control in Figure 51 is very low and there is not a significant difference between the specific activity in an induced (74 nmol/mg/min) compared to an uninduced state (51 nmol/mg/min). Interestingly, the *L. lactis* MG1614 control strain with p $P_{ClosR2K2}$_I + p P_{nisA}_cRK, which lacks the essential *nisRK* genes for signal transduction, displayed a background level for the *pepI* specific activity of around 40 nmol/mg/min (for *closR1K1*) and 30 nmol/mg/min (for *closR2K2*) respectively in the presence and absence of an inducible amount of extracellular nisin. The background level with the $P_{ClosR2K2}$ constructs is very different from the equivalent control strain with P_{ClosA} constructs. The P_{ClosA} in *L. lactis* MG1614 controls displayed a value of 4 nmol/mg/min (+N) and 0 nmol/mg/min (-N), respectively.

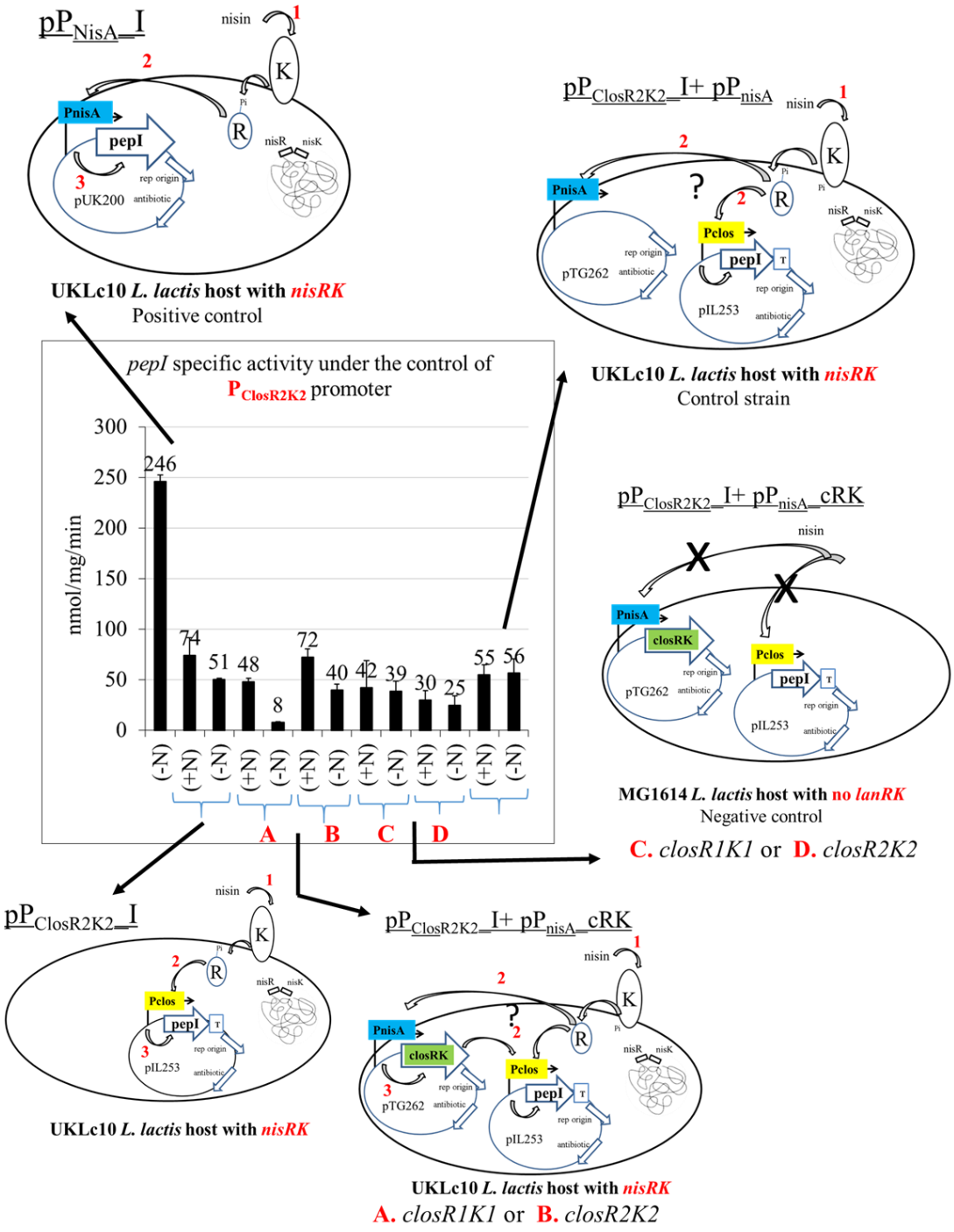


Figure 51 - Expression of the *pepI* under the control of $P_{ClosR2K2}$ in *L. lactis* UKLc10 and MG1614 host strains.

N represents nisin, induction with nisin (+N), uninduced state (-N). Illustrations of signal transduction in a $P_{ClosR2K2}$ reporter gene construct, $pP_{ClosR2K2}$_I, which is regulated only by *nisRK* system; in the dual plasmid system, $pP_{ClosR2K2}$_I + pP_{nisA}_cRK, the activation may be regulated by *nisRK* and/or *closRK*. An *L. lactis* MG1614, $pP_{ClosR2K2}$_I+ pP_{nisA}_cRK, control strain was also included where no *nisRK* regulatory genes were present.

If the results for the PepI assay with the $P_{ClosR2K2}$_I and pP_{ClosA}_I in UKLc10 strains were compared, it would be difficult to conclude that $P_{ClosR2K2}$ is switched on by *nisRK* genes using this heterologous expression system due to the fact that the observed values are close to the background level seen with the MG1614 control strains (Figure 51).

The *pepI* specific activity observed in the two plasmid system, indicate that perhaps the two promoters, $P_{ClosR2K2}$ and P_{nisA}, are not competing for the activated NisR regulatory protein in this heterologous system.

The lack of competition is confirmed by the PepI assay results. The PepI values for p $P_{ClosR2K2}$_I + p P_{nisA}_cRK when induced with nisin was 48 and 72 nmol/mg/min whereas for p $P_{ClosR2K2}$_I, it was 74 nmol/mg/min.

In conclusion, unlike the PepI assay with the P_{ClosA} constructs, the P_{nisA} and $P_{ClosR2K2}$ do not appear to be competing for the same translational regulatory protein, NisR, in this expression system.

4.3.4.3 *PepI specific activity assay under the control of ClosFEG and ClosBTC promoter ($P_{ClosFEG}$ and $P_{ClosBTC}$)*

Figure 52 and Figure 53 illustrate the *pepI* specific activity obtained with p $P_{ClosFEG}$ _I or p$P_{ClosBTC}$_I in UKLc10 strain. Results with the dual plasmid systems with p $P_{ClosFEG}$ _I+ pP_{nisA}_cRK and p $P_{ClosBTC}$_I+ p P_{nisA}_cRK were also included. The first thing to note was the very low *pepI* activity particularly in the constructs with $P_{ClosFEG}$, demonstrating almost the complete absence of the *pepI* enzyme activity in the cell extracts. The constructs displayed a negligible amount of activity ranging from 1 to 4 nmol/mg/min for $P_{ClosFEG}$ and values between 5 to 6 nmol/mg/min for $P_{ClosBTC}$, respectively.

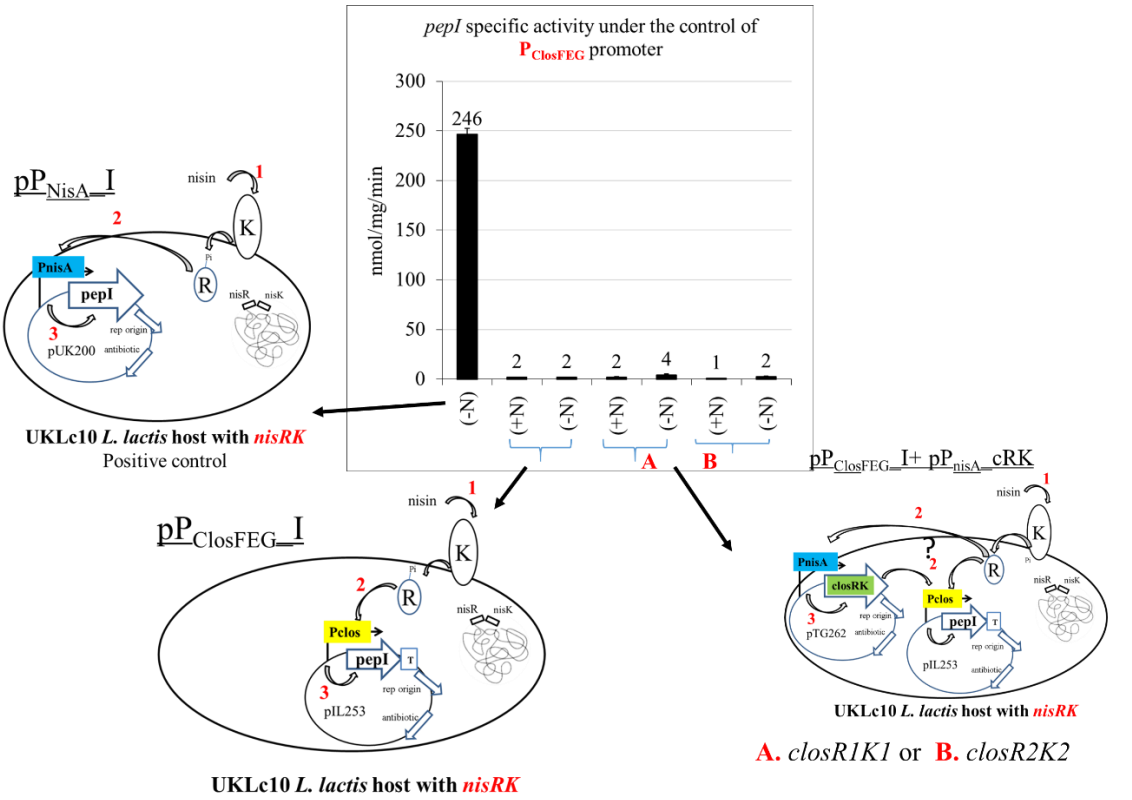


Figure 52 - Expression of *pepI* specific activity under the control of $P_{ClosFEG}$ in *L. lactis* UKLc10 host strain. Induction with nisin (+N), uninduced state (-N), N is nisin. Illustrations of signal transduction in a $P_{ClosFEG}$ reporter gene construct, $pP_{ClosFEG}$ _I, which is regulated only by *nisRK* system; in the dual plasmid system, $pP_{ClosFEG}$ _I + pP_{nisA}_cRK, the activation may be regulated by *nisRK* and/or *closRK*.

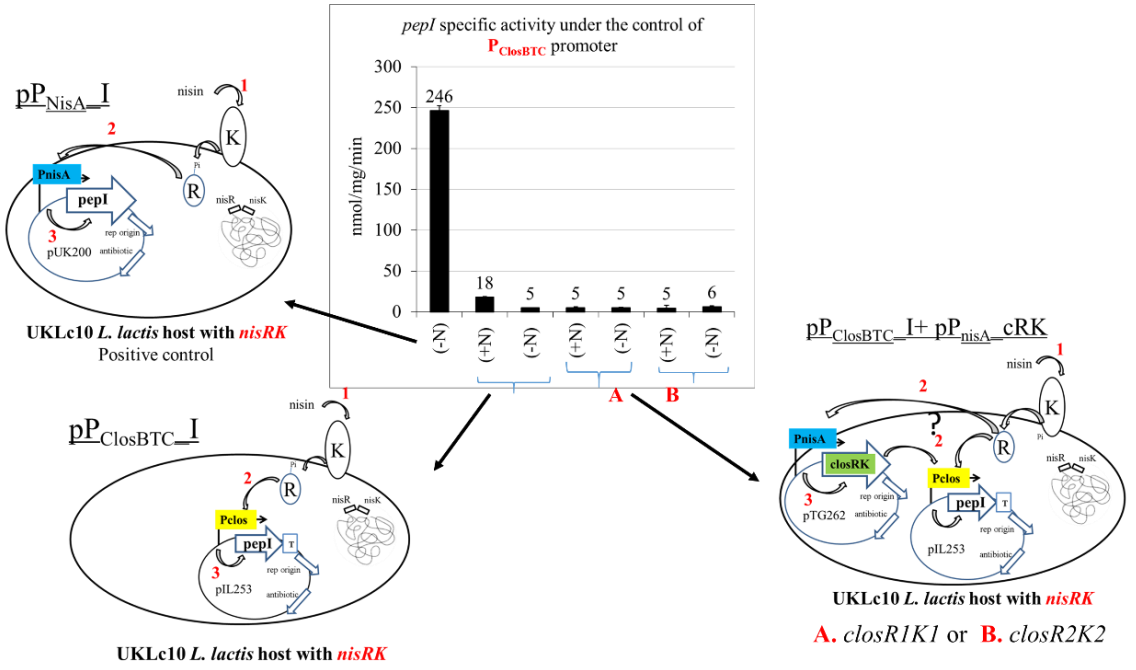


Figure 53 - Expression of *pepI* specific activity of cell extract under the control of $P_{ClosBTC}$ in *L. lactis* UKLc10 host strain.
Induction with nisin (+N), uninduced state (-N), N is nisin. Illustrations of signal transduction in a $P_{ClosBTC}$ reporter gene construct, p $P_{ClosBTC}$_I, which is regulated only by *nisRK* system; in the dual plasmid, p $P_{ClosFEG}$ _I + p P_{nisA}_cRK, the activation may be regulated by *nisRK* and/or *closRK*.

Interestingly, in a nisin induced state (+N) the *pepI* activity of pP$_{ClosBTC}$_I strain is 18 nmol/mg/min which is higher than all the other readings but still a low value. In conclusion, the *closRK* or the *nisRK* two-component regulatory systems are not capable of switching on the putative P$_{ClosBTC}$ and P$_{ClosFEG}$ *clos* promoters when they are expressed in the *L. lactis* UKLc10 heterologous system.

4.3.4.4 *Comparison of the pepI specific activity assay under the control of different clos promoters*

To summarise, the sequences upstream of *pepI* in pP$_{ClosBTC}$_I, p P$_{ClosFEG}$ _I and p P$_{ClosR2K2}$_I which were predicted to be *clos* promoters, did not act as DNA binding sites for NisR regulatory protein. This is confirmed by comparing the *pepI* activity against the positive control when induced with nisin (Figure 54).

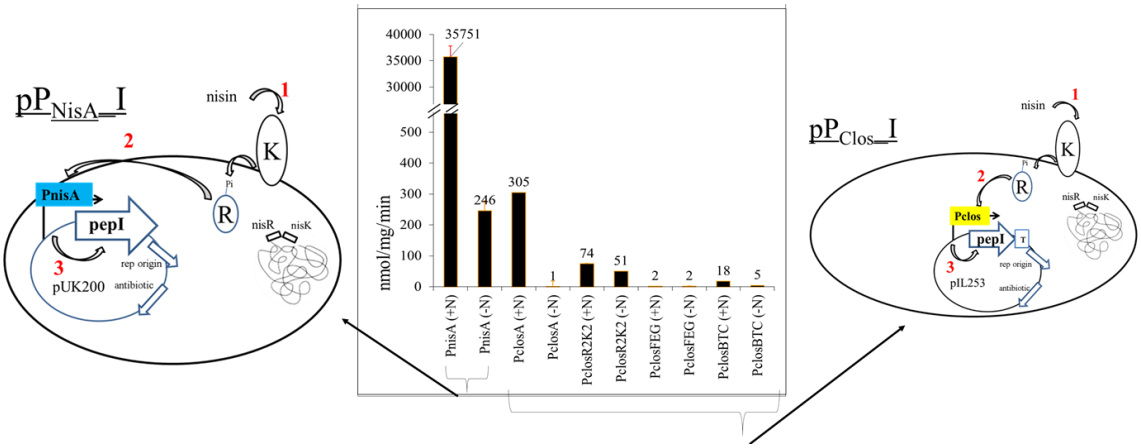


Figure 54 - Specific *pepI* enzyme activity of cell extract of the P$_{nisA}$_*pepI* positive control was compared to pP$_{Clos}$ fused to *pepI* type of constructs in a nisin induced (+N) and an uninduced state (-N). The UKLc10 with a *nisRK* genes on the chromosome was used as a host strain.

The specific activity of *pepI* was measured from constructs (pP$_{ClosA}$_I) where *pepI* was expressed under the control of one of the four predicted *clos* promoters to determine if they are activated by the *nisRK* system in the UKLc10 host strain. N stands for nisin.

The first striking observation in Figure 54 was the total lack of any *pepI* activity in pP$_{ClosFEG}$_I , as well as the very low activity close to the background levels for pP$_{ClosBTC}$_I. The same applies for pP$_{ClosR2K2}$_I compared to the *L. lactis* MG1614 background (Figure 51 and Figure 54). Compared to the positive control, the observed activation of the *closA* promoter by the *nisRK* system is demonstrated by the *pepI* specific activity of 305 nmol/mg/min with nisin induction.

In conclusion, the *pepI* specific activity measured with the one plasmid reported gene constructs, pP$_{Clos}$_I, demostrated that none of the P$_{Clos}$ promoters are constitutive promoters and that they are poor promoters in this *L. lactis* UKLc10 expression system.

P_{ClosA} is clearly switched on by induction with nisin when it is expressed and regulated by the *nisRK* genes in the *L. lactis* UKLc10 strain. The P_{Clos}_I fusion plasmids were similar except for the P_{ClosA} promoter which definitely has half a spa box. Therefore, the differences in the specific activity must probably be due to the fact that a promoter may not be active or activated by the *nisRK* system, or there may be a post-transcriptional effect like the stability of mRNA, the efficiency and the level of translation as well as protein stability that may explain the current results.

4.4 DISCUSSION

In this chapter, the activation of putative *clos* promoters in the *clos* gene cluster was explored. The *clos* promoters were expressed in a *pepI* reporter gene expression system which is under the control of the *nisRK* genes.

A survey of the literature suggests that a lantibiotic biosynthetic machinery is encoded by a gene cluster that is comprised of genes and promoters [150]. It was hypothesised that if *clos* promoters are activated by an inducer molecule, the *clos* cluster in the wild type strain could potentially express active lantibiotic peptides. *In silico* analysis of the 17 kb *clos* operon has led to the identification of four putative *clos* promoters, where the P_{ClosA} has half a spa box; this suggested that P_{ClosA} is a potential target recognition site for NisR or even for the ClosR regulatory proteins. The spa-box is the DNA target site for the regulatory gene in another class I lantibiotic gene cluster of subtilin [83, 126]. The sequences for the putative promoters were fused to the *pepI* reporter gene following the example of Wegmann *et al.* [100, 149].

Based on previous published results [151] on the characterisation of the subtilin-regulated gene expression (SURE), a more robust *pepI* dual plasmid reporter gene promoter system was engineered.

In this chapter, it was demonstrated that the P_{ClosA} putative *clos* promoter is activated by induction with nisin in a similar fashion as P_{nisA} promoter. Results from the *pepI* liquid assay (data not shown) indicated that $P_{ClosR2K2}$ and $P_{ClosBTC}$ putative promoters may respond to the presence of nisin; this activation is dependent on the presence of the NisRK system, however these results were not validated by more accurate measurements in the *pepI* assay.

Since the expression of the *nisRK* two-component regulatory system is constitutive, the hypothesis was that perhaps the *closRK* genes are also expressed nisin-independent, therefore *closR1K1* and *closR2K2* may follow a similar pattern as *nisRK*. The results from the *pepI* assay suggested that the *clos* promoters are not constitutive and that neither of the two *closRK* set of regulatory genes possess any role in the activation of *clos* promoters using this heterologous system. The presence of both nisin and *clos* promoters in the same system had a negative impact since they may have interfered with downstream reactions. Future work may involve to incorporate the *closRK* genes on a chromosome to create an alternative host strain with the pP_{nisA}_I plasmid in by using the example of the UKLc10 strain.

The same strong positive control, p P_{nisA}_I in the UKLc10 strain, was used in this study as before [149]. The nisin promoter, P_{nisA}, represents the DNA binding/target site for the phosphorylated NisR regulatory protein [152]. The P_{Clos}_*pepI* fusions were expressed on the high copy number pIL253 plasmid [153], whereas the positive control was under the control of P_{nisA} and it was expressed in pUK200. Therefore, a direct comparison to the positive control is not strictly conclusive. The current results could only give a relative level of the promoter activity considering that the values could not be normalised and qPCR data was not available in here to account for the differences for individual transcript levels.

A dual plasmid reporter gene system was used and results suggested that the NisR and ClosR may be competing for the same binding site in the P_{ClosA} promoter. It was shown that a frameshift in ClosR1 did not restore the signal. Apparently, competitive binding of NisR would favour P_{nisA} rather than P_{ClosA}. Competition between P_{nisA} and P_{ClosA} was confirmed by measurements of the *pepI* activity using strains pP_{ClosA}_I+p P_{nisA}_cRK, pP_{ClosA}_I+p P_{nisA} and pP_{ClosA}_I+p P_{nisA}_fscR1_cK1; the values with the dual plasmid system were lower than the ones for pP_{ClosA}_I constructs, the one reporter gene plasmid strains, using nisin induction.

Measurements of the specific *pepI* activity using the *closRK* or *nisRK* two-component regulatory systems demonstrated that the *lanRK* genes are not capable of switching on the putative $P_{ClosBTC}$ and $P_{ClosFEG}$ *clos* promoters when they are expressed in the *L. lactis* UKLc10 heterologous system. The *clos* promoters may be functional when they are present on the *clos* gene cluster, however UKLc10 may lack the correct transcriptional repressors and/or activators. Even if sigma factors in *L. lactis* host strain are present, the lactococcus sigma levels in this strain may not be suitable enough to bind to a *Blautia obeum* A2-162 type of promoters.

The *clos* gene cluster has two sets of regulatory genes, one is under the direct control of the *clos* promoter, $P_{ClosR2K2}$. Previous work has shown that expression of P_{nisR} promoter is constitutive [154]. Therefore, it was assumed that if the *clos* cluster was actively producing lantibiotic peptides, the ClosRK system should also be constitutive in order to allow for a fast accumulation of immunity. The results obtained in this study did not indicate that $P_{ClosR2K2}$ is either constitutive or that it can be activated by nisin induction, therefore the original hypothesis could not be validated.

In this chapter, the role of the *closRK* regulatory system has not been defined in this work. Even if it seems that the *closRK* genes may not interact with the *clos* promoters when nisin is present, preClosA production and activation/maturation of *closA* may be necessary for the ClosRK systems to become regulatory. Based on the knowledge about the location of the NisRK proteins in the nisin operon, it can be assumed that the ClosK sensor kinase of the two-component regulatory ClosRK system should also be exposed to the outside of the cell. This is because ClosK should respond to an extracellular inducer signal when the inducing peptide is added. The subsequent phosphorylation of the internal ClosR should stimulate the *closA* transcription provided that appropriate levels of the inducer peptide are added to the broth or to the agar media. It may be possible that the signal molecule such as the Clos peptide itself is synthesised in a higher concentration when cultured on agar plates rather than in liquid. In contrast, Clos may be too diluted when grown in liquid broth cultures thus any small amounts of Clos can not initiate transcription of the *closA* structural genes.

In conclusion, the transcription of *pepI* genes is activated by nisin-mediated autoinduction and involves signal transduction by the nisin two-component regulatory system composed of NisR and NisK. The NisK sensor kinase will detect the nisin signal molecule, and after phosphotransfer to the NisR, the response regulator will be activated and it will bind to P_{ClosA}. This step will finally lead to increased *pepI* expression levels. Induction with 10 ng/ml of nisin did not allow for a similar *pepI* specific activity to be detected when either $P_{ClosFEG}$, $P_{ClosBTC}$ or $P_{ClosR2K2}$ promoters were present. In nisin, the activity levels of P_{nisA} and P_{nisF} promoters directly depend on the added nisin concentration; perhaps, there is also a linear dose response relationship between nisin and the resulting transcriptional level of *pepI* that could contribute to the activation of the three *clos* promoters. In the future, an increased amount of nisin should be added to test the possible activation of the *clos* promoters.

Therefore, current results confirmed that P_{ClosA} is activated by NisRK proteins and perhaps additional activators or different sigma factors are required for the switching on of $P_{ClosFEG}$, $P_{ClosBTC}$ and even of $P_{ClosR2K2}$. DNA fingerprinting could help to further understand the activation of the *clos* promoters [155]. This study is based on *in silico* predictions for the location of *clos* promoters. Similar to other studies that involved to test the regulation of lantibiotic gene clusters, RNA analysis may allow for a more accurate evaluation of the levels of *clos* gene transcription [119, 149].

There is a great diversity in the regulation of the lantibiotic immunity and biosynthesis. The regulatory systems in lantibiotic gene clusters are responsible for both the biosynthesis of bacteriocins and their autoinduction, and it was shown that some *lanRK* regulatory genes target both pathogenic bacteria and the survival events inside the host organism [7]. Some examples of the diversity of the regulatory systems are given below. Several two-component lantibiotics are known to autoregulate their biosynthesis such as cytolysin, staphylococcin C55 and lacticin 3147 [7, 156]. A two-component regulatory systems (LanR, LanK) is responsible for controlling the regulation of lantibiotics except in the case of epidermin where regulation is done by a single protein (EpiQ) [157].

Nisin and subtilin, two-component signal transduction systems regulate their biosynthesis and LanR acts on the promoter of the *lanA*, lantibiotic structural gene [7]. Nisin acts as a peptide pheromone in the process of quorum sensing in order to autoregulate its own biosynthesis through NisR and NisK proteins [74].

The gene cluster for mersacidin encodes for three regulatory genes out of which MrsR2/K2 encode for a two-component regulatory system which controls the immunity gene transcription; the third gene encodes for a response regulator-like protein [158-160].

The gene cluster for epidermin encodes for a response regulator type of protein, LanQ, which binds to the epiABCD promoter region to activate the *lanA* expression. However, it was proposed that the signal transduction process is completed by a histidine kinase from the host [157, 161].

Similar to the *pepI* assay work performed in this chapter, a β-glucuronidase assay was used to investigate the activation of the four psp promoters cloned to *gusA*, the β-glucuronidase reporter gene. The promoters were identified in the gene cluster of planosporicin. Three regulatory proteins were determined such as 'an extracytoplasmic function σ factor (PspX), its cognate anti-σ factor (PspW), and a transcriptional activator (PspR) with a C-terminal helix-turn-helix DNA-binding domain' [23].

It is known that ruminococcin C (RumC) is a bacteriocin which is a trypsin-dependent peptide that was isolated from human feces [50, 162]. The impact of the environment on the regulation of lantibiotic biosynthesis had been previously reported [71, 163]. The transcription of the putative regulatory *rumRK* was shown to increase when trypsin was added and it was constitutive under non-inducing conditions [164].

It was previously suggested that *Blautia obeum* A2-162 should respond to trypsin [97]. Since the *clos* cluster was isolated from the *Blautia obeum* A2-162 gut bacteria, it should

be noted that additional factors from the GI tract are necessary to investigate the activation of *clos* promoters which are yet to be identified.

In order to understand if the *clos* gene cluster is actively producing, modifying and secreting an antimicrobial peptide, we needed to determine if the *clos* promoters are active to ensure that the required genes are expressed. Common difficulties with gene clusters are that the antimicrobial peptide remains inside the cell and it is not secreted which makes the lantibiotic peptide a less interesting candidate to explore further. Knowledge on the efficiency of the promoters to ensure that each lantibiotic biosynthesis step does takes place will ultimately allow us to develop an expression system to produce high yield (active) Clos peptide.

Based on the nisin induced activation of the P_{ClosA} promoter using the NisRK regulatory system demonstrated in this chapter, the *clos* gene cluster will be expressed in the same UKLc10 heterologous expression system to investigate the production, detection and activation of the preClosA peptides as described in chapter 5.

CHAPTER FIVE

5. Heterologous expression of Clos peptides

5.1 INTRODUCTION

In a previous study by Hatziioanou [97], the entire novel *clos* gene cluster was subcloned and expressed in *L. lactis.* Despite complementation with the *closA* gene on an inducible plasmid to try to improve expression of the Clos peptide, the expression of biologically active mature ClosA peptides was unsuccessful. However, a peptide of the size of the preClosA1 was detected at very low levels using an antibody designed to the ClosA1 leader. The *closA* structural sequences were also expressed fused to the nisin A leader in a nisin-producing *L. lactis* strain of NisL_closA. The hybrid genes which included the N-terminal nisin leader sequence and the nisin promoter (P_{nisA}) fused to the C-terminal of ClosA1(IE), ClosA1(YK) or ClosA4 protein were created in pUK200 expression vector and were expressed in Δ*nisA* and Δ*nisP L. lactis* host strains (Table 22).

Since the exact end of the Clos leader and the start of the nisin-like *closA* structural sequence were uncertain, two constructs were previously designed with the N-terminal amino acids as IE or YK fused to the nisin leader [97]. Based on the comparison of known lantibiotic LanP cleavage sequences and with the sequences of other active lantibiotics, the cleavage site for ClosA leader peptide was proposed to be before either IE or YK start site.

The first aim of this chapter was to investigate whether the nisin biosynthetic machinery would be able to recognise the nisin leader in a 'nisin leader_closA' hybrid, to modify, to secrete and to cleave the leader in order to produce active forms of the ClosA peptides.

The role of a leader peptide in the precursor molecule is to maintain the peptide in an inactive form and to induce PTM such as the LanB and the LanC catalysed dehydration and ring formation of the prepeptide. For nisin, the leader peptide also plays a role in mediating the interaction with the NisT in the export of nisin [165].

In the nisin leader_closA hybrids, it was expected that the NisP from the nisin biosynthetic system would remove the nisin leader peptide [150] even if the constructs do not have a typical recognition NisP site. If the NisP would not recognise the PR↓IE or PR↓YK cleavage site, a 6 kDa prepeptide is expected to be formed. To address this and based on the knowledge of the predicted amino acid content in the nisin leader peptide, a new cleavage site was designed as another aim of this chapter.

Previous work [97] indicated that attempts to express the Clos peptide in *L. lactis* FI7847 (*L. lactis* FI5876 Δ*nisA*) and FI8438 (*L. lactis* FI5876 Δ*nisP*) indicated that the strains may be capable of synthesizing a truncated nisin A homologue and possibly a full length unprocessed nisin precursor of 5961 Da, respectively. Previous results with the strain containing the nisin leader_*closA1(YK)* hybrid indicated that a prepeptide was visible in extracts from the culture supernatant and additional protein bands around 3.5 kDa in FI7847 may have been detected in western blot. A consistent band appeared at 6 kDa in this strain which was absent in any of the controls. No antimicrobial activity or specific MS peaks that would confirm the presence of a Clos peptide was detected. However, unique proteins (Table 22) that hybridised to the antinisin leader antibody in a *closA1(YK)* containing strain, confirms that the Clos peptide was made in the *L. lactis* MG1614 strain. Despite the western blot band was observed, the 6 kDa protein was not extracted due to poor yield, therefore processing of the peptide including PTM were not established.

	Nisin leader _ClosA1(IE)	nisin leader _ClosA1(YK)	nisin leader_ClosA4	nisin
Prepeptide with nisin leader	6304	5836	6064	5961
Leaderless peptide	3840	3372	3599	3497
Prepeptide with clos leader	5837	5837	6417	NA

Table 22 - Calculated masses of nisin leader_clos, clos leader_clos hybrids and leaderless precursor Clos peptides.

The *L. lactis* FI7847 and FI8468 strains produce a 5962 Da nisin homologue; the mass of the Clos peptides should allow to distinguish between nisin and Clos. Based on Hatziioanou [97].

Previous attempts to purify and characterise active Clos peptides were unsuccessful due to a number of possible reasons: poor yield, the absence of a protease and presence of unpredicted peptides or the use of the incorrect indicator strains. Understanding if and how active lantibiotics from the novel *clos* gene cluster are made could enable the development of variants with improved clinical activity since results from this thesis (chapter 3) showed that nisin expression was more effective from pTG262 expression vector.

The first task was to excise the nisin leader_clos hybrids and to express them on a better pTG262 expression vector. In this chapter, the nisin leader_clos hybrids were also cloned with a more efficient designed putative recognition site for the nisin protease, NisP. The new nisin leader_closA hybrids that had the IT start site instead of IE or YK, were created and tested for the possible production and secretion of a leaderless ClosA peptide.

The nisin protease is a highly specific enzyme that removes the leader peptide from the fully modified and exported prenisin. Interestingly, it was shown that both *L. lactis* and *B. subtilis* lantibiotic producing strains, contain intracellular and cytoplasmic proteolytic activity. Secretion of leaderless lantibiotics was demonstrated in a deletion mutant strain or in the absence of *lanP* in subtilin and nisin [166-168]. It was hypothesised that the Clos leader peptide would play a similar role as the nisin leader peptide and that perhaps *Blautia obeum* A2-162 may have, still undetectable, internal proteolytic activity like other lantibiotics from the same family [83].

The second task of this chapter was to express the entire *clos* cluster in the same nisin-inducible controlled expression system, *L. lactis* UKLc10 strain which was used in chapter 4.

In a comparison between nisin and *clos* biosystems, *nisA* and *closA1-4* encode the precursor Clos and nisin peptides (4 copies of *closA* and one copy of *nisA*, respectively). The clusters are also comprised of genes that encode for proteins involved in the maturation of the precursor preClosA or prenisin peptides: for PTM, ClosBC and NisBC, respectively, for export of the modified precursor peptide ClosT and NisT, respectively, and for immunity and for the protection of *Blautia obeum* A2-162 and *L. lactis* organisms: ClosFEGI and NisIFEG. Notably, a gene encoding an extracellular protease (NisP) responsible for cleavage of the nisin leader sequence is present in the nisin cluster to generate a mature nisin molecule. However, both subtilin and *clos* gene clusters do not contain a gene that encodes for a similar function, however unspecific serine proteases secreted by *B. subtilis* that are involved in the maturation of subtilin have been detected [146]. Finally, the nisin gene clusters contain genes that encode a *nisRK* two-component regulation systems, whereas two sets of *closRK* genes composed of a sensor kinase (ClosK1 or ClosK2) and a response regulator (ClosR1 or ClosR2) have been identified in the *clos* gene cluster.

We aim to test the expression of the clos peptides from nisin leader_clos using the nisin biosynthetic machinery or clos leader_clos hybrids using the *nisRK* regulatory system of nisin. The aim is to also engineer an expression system to allow for improved Clos peptide production and to assess the biological activity of Clos. Another aim was to investigate if the pClosCluster in *L. lactis* in UKLc10 producing strain is able to synthesise *clos* when the producer is grown on plate and if trypsin is able to cleave the predicted Clos leader from the synthesised preClosA peptide to generate a series of Clos tryptic peptides. Finally, we

wanted to understand if the mature leaderless Clos peptide or the tryptic peptides display antimicrobial activity against a range of indicator strains.

5.2 MATERIALS AND METHODS

5.2.1 Generation of nisin leader_clos hybrids and the design of NisP cleavage site

Constructs in the pUK200 vector (Table 23) were used as templates to excise the nisin leader+closA insert using a set of primers as detailed in chapter 2. The inserts were cut with *Ssp*I and *Xba*I and cloned in a pTG262 vector digested with *Hind*III, endfilled and cut with *Xba*I to generate the constructs in Table 23.

Plasmids in pUK200	Plasmids in pTG262
pUK200_NisL_closA1(IE)	pTG262_NisL_closA1(IE)
pUK200_NisL_closA1(YK)	pTG262_NisL_closA1(YK)
pUK200_NisL_closA4	pTG262_NisL_closA4

Table 23 - List of nisin leader_clos hybrids expressed in two expression vectors.

The nisin leader_clos hybrids were cloned in pTG262 expression vector in *L. lactis* MG1614 first to confirm the identity of the plasmid by sequencing, then they were transfered to FI7847 to allow for *clos* expression including cleavage of the nisin leader by NisP protease and export of leaderless Clos peptide across the membrane. Constructs were also transformed in FI8438 (Δ*nisP*).

To create a new potential cleavage site for NisP, the IE and the YK predicted start sites were substituted by IT. Cloning was done with primer pairs as detailed in Table 24.

Also, pTG262 vector was digested with *Sal*I and *Sac*I restriction enzymes and cloned with the 401 bp or 380 bp PCR product previously restricted with *Sal*I and *Sac*I restriction enzymes. After sequence confirmation, the new plasmids were transformed in *L. lactis* FI7847 (Δ*nisA*) and FI8438 (Δ*nisP*) strains.

Mutation	Primer pair	Insert size
ClosA1(IE)_IT	ClosA1(IE)_IT_F & pUK200_Reverse SalI_F & ClosA1(IE)_IT_R	401 bp
ClosA1(YK)_IT	ClosA1(YK)_IT_F & pUK200_Reverse SalI_F & ClosA1(YK)_IT_R	380 bp

Table 24 - Details to introduce mutations in the nisin leader to create the putative NisP cleavage site by designing two oligonucleotide sequences (forward and reverse).

Annealing was done with equimolar concentrations at 90-100°C for 5 min, with slow cooling to room temperature for 60 min. A double stranded insert with restriction sites at each end was made and finally cloned in the pTG262 vector. The mutations were introduced by splice overlap extension PCR in a pUK200 vector. The nisin fragment with the IT mutations in were excised and cloned in pTG262 vector.

5.2.2 Biological activity and characterisation of Clos hybrids

The nisin leader_clos hybrids obtained in the previous section were screened for their antimicrobial activity and for their self-induction capacity by inducing the strains with 0, 10 or 100 ng/ml of nisin. Indicator strains used in drop tests, overlay assays and plate diffusion assays included *B. subtilis*, *C. difficile*, *C. perfringens*, *E. faecalis*, *E. faecium*, *L. ivanovii* and *S. aureus* using methods described in chapter 2. Maldi-ToF was performed as described in section 2.5.1.

5.2.3 Design of a model expression system for production of Clos

5.2.3.1 *Cloning of the clos cluster in L. lactis UKLc10 to improve expression of clos*

The nisin biosynthetic machinery and/or the *clos* biosynthetic machinery were used to investigate if fully modified and biologically active Clos can be generated in *L. lactis* UKLc10 (*nisRK*) or FI7847 (Δ*nisA*) host strains. The plasmid pP_{nisA}_ closLeader_*closA34*, which is comprised of one copy of the three identical copies (*closA3*) and the fourth unique *closA* structural gene, was used to investigate if expression of *clos* is enhanced with additional copies of the structural gene.

Each pClosCluster plasmid and pClosCluster(Δ*cA*) plasmid was transformed into electrocompetent *L. lactis* UKLc10 strain. Clones were identified by colony PCR using primers pIL253F:/pIL253R: and plasmid DNA from positive clones was checked by restriction profiling with *Hind*III using methods as described in chapter 2.

5.2.3.2 *Detection of preClosA peptide*

Cell extract and TCA precipitated supernatant of pClosCluster and pClosCluster+ pTG262_P_{nisA}_ closLeader_*closA34* in UKLc10 were loaded on 4-12 % Sodium Dodecyl sulphate PolyAcrylamide Gel Electrophoresis (SDS-PAGE), then the samples were blotted with 1/100 dilution of antiClos leader antibody on western blot following the same method as described in chapter 2.

5.2.4 **Testing biological activity of preClosA peptides**

The pClosCluster in UKLc10 strain was used as the test strain, positive control pTG262_*nisA* in FI7847, negative controls: pClosCluster (Δ*cA*) and pIL253 in UKLc10, UKLc10 strain alone.

Strains were grown from glycerol in 10 ml of GM17, then subcultured for a number of days in fresh media and 10 ng/ml of nisin for induction. Colonies from 5 µl of liquid culture were grown on solid agar with $NaHCO_3$, to prevent non-specific antimicrobial peptide activity, and induced on plate with 10 ng/ml nisin. The colonies were overlaid with agar medium containing the indicator strain and trypsin. Two different kinds of trypsin were used: Sequencing Grade Modified Trypsin (Promega, Southampton, UK), and Trypsin TPCK Treated from Bovine Pancreas (Sigma-Aldrich, Dorset, UK). The trypsin concentration was 1 or 5 ng/µl (Promega) or 1, 5, 10 or 15 ng/µl (Sigma). A plate with no trypsin was also included as negative control. The same method was followed as described in section 2.1.3 in chapter 2. For screening the antimicrobial activity several indicator strains were used as listed in chapter 2.

5.3 RESULTS

5.3.1 Generation of nisin leader_clos hybrids and the design of NisP cleavage site

Previous results with expression of *nisA* (chapter 3) indicated that pTG262 is a better expression vector. Here, the nisin leader *closA1(IE)*, *closA1(YK)* or *closA4* hybrids were excised from pUK200 clones and subcloned into pTG262 as illustrated in Figure 55. Table 25 has details of the sequences of the new constructs: pTG262_NisL_ClosA1(IE), pTG262_NisL_ClosA1(YK) and pTG262_NisL_ClosA4 (Figure 55, A).

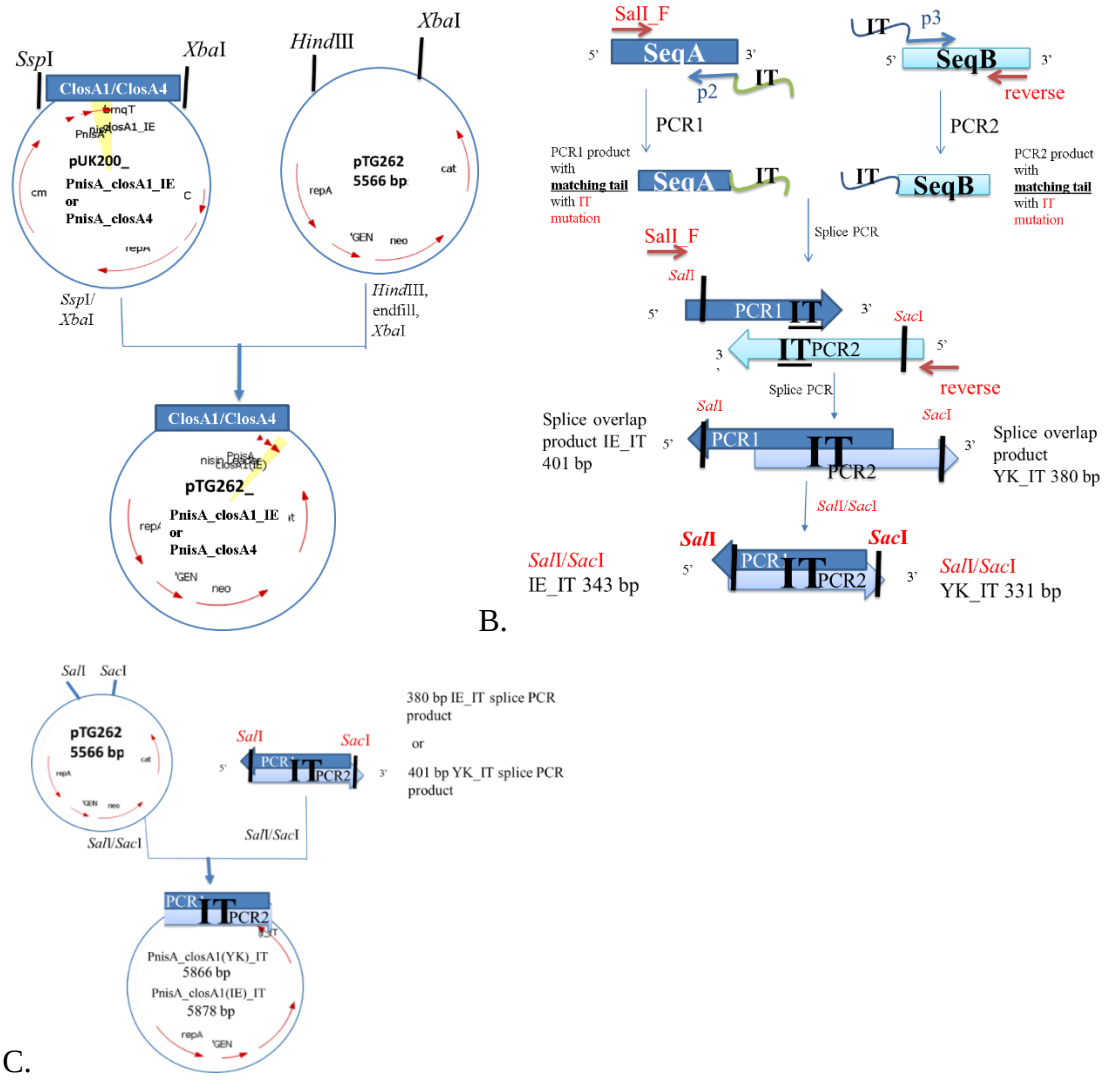


Figure 55 - Cloning to express nisin leader_ClosA1 and nisin leader_ClosA4 hybrids and to engineer a new NisP putative cleavage site by splice overlap extension PCR
A. Expression of hybrids in pTG262 expression vector.
B. Splice overlap extension PCR to introduce IT mutation in nisin leader_ClosA1(IE) or nisin leader_ ClosA1(YK) fusions.
C. Cloning of mutated fusions into templates (pTG262_NisL_ClosA1(IE), pTG262_NisL_ClosA1(YK)).
N is the nisin leader, IE and YK are the predicted start sites for ClosA1.

In order to create a more efficient recognition site for the nisin protease (NisP), the nisin leader peptide was fused to a peptide that has isoleucine and threonine (IT) as its start site. Splice overlap extension PCR was used to change the IE or the YK start sites in ClosA1 into IT to generate the following constructs: pTG262_NisL_ClosA1(IE)_IT, pTG262_NisL_ClosA1(YK)_IT constructs as described in Table 26 and Figure 55.

Four PCR products were made by using pTG262_NisL_ClosA1(IE), pTG262_NisL_ClosA1(YK) constructs as templates and primer pairs: 1. ClosA1(IE)_IT_F & pUK_Reverse, SalI_F & ClosA1(IE)_IT_R and 2. ClosA1(YK)_IT_F & pUK_Reverse and SalI_F & ClosA1(YK)_IT_R, respectively. Each set of PCR products were then overlapped using outer primers SalI_F & pUK_Reverse primer pair. A similar splice overlap extension PCR cloning strategy was used as described in section 2.2.7 and in Figure 55. The spliced PCR products were then digested with *Sal*I and *Sac*I enzymes to give a 401 bp splice product (IE_IT) and a 380 bp product (YK_IT) and cloned in pTG262 vector restricted with *Sal*I and *Sac*I. Ligation, transformation and sequence confirmation were performed as described in chapter 2.

The *clos* cluster lacks a protease that would cleave the leader peptide. Therefore, the constructs in Table 25 may express either a leaderless peptide or a peptide with the leader still attached.

The nisin leader_clos peptide constructs do not have a typical recognisable NisP cleavage site. The new PR↓IT site should act as a cleavage site for NisP. Table 26 lists the mutations introduced in the YK or IE nisin leader_clos hybrids to introduce IT at the N-terminal of the core peptide.

Clos hybrids	Strain & vector	Expected products
ClosA1 (IE)	pTG262 (Δ*nisP*)	MSTKDFNLDLVSVSKKDSGASPRIEPKYKSKSACTPGCPTGILMTCPLKTATCGCHITGK
ClosA1 (IE)	pTG262 (Δ*nisA*)	IEPKYKSKSACTPGCPTGILMTCPLKTATCGCHITGK MSTKDFNLDLVSVSKKDSGASPR↓IEPKYKSKSACTPGCPTGILMTCPLKTATCGCHITGK
ClosA1 (YK)	pTG262 (Δ*nisA*)	MSTKDFNLDLVSVSKKDSGASPRYKSKSACTPGCPTGILMTCPLKTATCGCHITGK
ClosA1 (YK)	pTG262 (Δ*nisA*)	YKSKSACTPGCPTGILMTCPLKTATCGCHITGK MSTKDFNLDLVSVSKKDSGASPR↓YKSKSACTPGCPTGILMTCPLKTATCGCHITGK
ClosA4	pTG262 (Δ*nisA*)	MSTKDFNLDLVSVSKKDSGASPRITSQHSFCTPNCLTGFLCPPKTQLTCTCKLKGQ
ClosA4	pTG262 (Δ*nisA*)	ITSQHSFCTPNCLTGFLCPPKTQLTCTCKLKGQ MSTKDFNLDLVSVSKKDSGASPR↓ITSQHSFCTPNCLTGFLCPPKTQLTCTCKLKGQ

Table 25 - Expression of nisin leader_clos hybrids in pTG262 expression vector.
Nisin leader_ClosA hybrids were previously designed [97] to contain the full nisin leader peptide sequence 'MSTKDFNLDLVSVSKKDSGASPR' followed by one of the predicted *closA* structural sequences to make:
Nisin leader_closA1 (with IE possible start site to give a longer lantibiotic than nisin): IEPKYKSKSACTPGCPTGILMTCPLKTATCGCHITGK, or
Nisin leader_ClosA1 (with YK possible start site to give a similar size peptide but with different start site than nisin):
YKSKSACTPGCPTGILMTCPLKTATCGCHITGK
Nisin leader_ClosA4 (unique structure, similar length to nisin when cut before IT): ITSQHSFCTPNCLTGFLCPPKTQLTCTCKLKGQ.

The arrow (↓) in Table 25 highlights the predicted site for NisP protease cleavage. The sequence of the nisin leader peptide is before the arrow and it is expected to be removed by NisP in the *L. lactis* FI7847(Δ*nisA*) strain.

ClosA hybrid mutants	Strain & vector	Prenisin with engineered NisP cleavage site*
ClosA1 (IE)_IT	pTG262 (Δ*nisA*)	MSTKDFNLDLVSVSKKDSGASPR↓ITPKYKSKSACTPGCPTGILMTCPLKTATCGCHITGK
ClosA1 (YK)_IT	pTG262 (Δ*nisA*)	MSTKDFNLDLVSVSKKDSGASPR↓ITSKSACTPGCPTGILMTCPLKTATCGCHITGK

Table 26 - The bioengineering of a putative NisP cleavage site by splice overlap extension PCR in two templates: ClosA1(IE) and ClosA1(YK) nisin leader_clos hybrids (see section 2.2.7).
*Substituted amino acids are indicated in boldface type. The arrow in black represents the expected NisP cleavage site.

In conclusion, three existing Clos hybrids, that include the nisin leader sequence fused to the *closA* structural sequences, were subcloned in the pTG262 expression vector. None of the hybrids were detected by colony MS in their cleaved or uncleaved form by Maldi-ToF. The nisin biosynthetic machinery in the *L. lactis* FI7847 host strain was not able to express any of the predicted 6 kDa peptide. The potential cleavage site for NisP was re-designed (Table 26) as described above, however the 6 kDa peptides or their cleaved forms were not detected in MS.

The Clos hybrids were all screened in drop tests and antimicrobial activity assays against selected indicator strains: *B. subtilis*, *C. difficile*, *C. perfringens*, *E. faecalis*, *E. faecium*, *L. ivanovii*, *S. aureus*.

The self-induction capacity of nisin leader_clos hybrids was tested by inducing the peptides with exogenous nisin. No antimicrobial activity was detected in the supernatants of any of the hybrids in Table 25 or Table 26. Results (Figure 56) indicated that *closA1(YK)* and *closA1(IE)_IT* displayed zones of inhibition which are similar to pTG262 empty vector control induced with 100 ng/ml of nisin. It was concluded that the observed bioactivity was due to the extracellularly added nisin and not from the modified active Clos peptide. Their small zone of inhibition made it difficult to realistically distinguish between any real antimicrobial activity and the control strain.

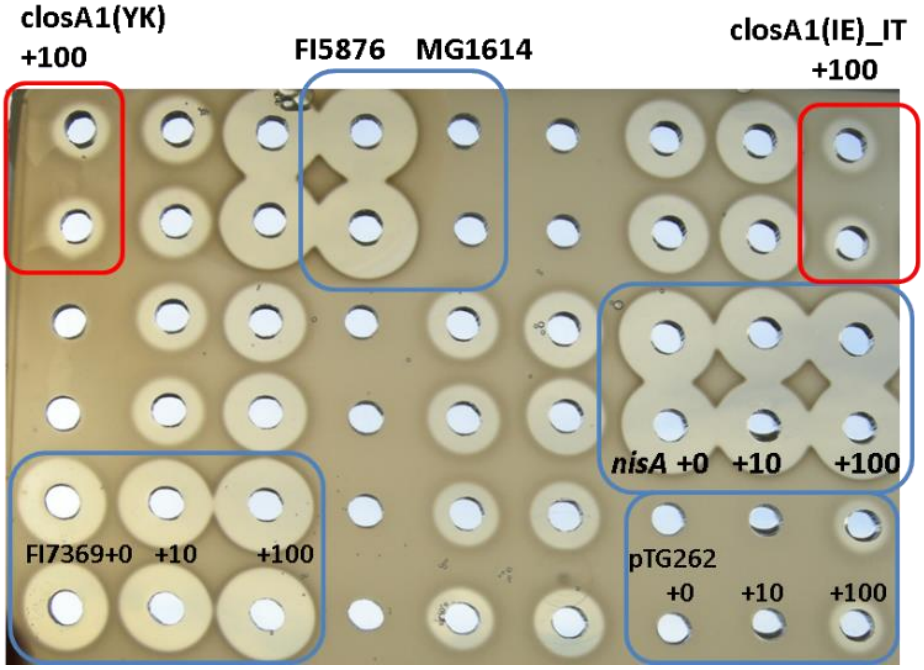


Figure 56 - Plate diffusion assay of nisin leader_clos hybrids induced with 100 ng/ml of nisin.

The activity of the peptide in the supernatant was determined from inhibition zones of *L. lactis* MG1614. The biosynthesis of the Clos hybrids and of the control strains was induced by adding 10 ng/ml of native nisin (Day 2). The cultures were then subcultured and induced (Day 3) with 0, 10 or 100 ng/ml native nisin overnight (as marked in black, '+ 0, 10, 100').

5.3.2 **Designing a model expression system for production of preClosA**

Previous work on the investigation of *clos* biosynthesis involved the cloning of the gene cluster on the pIL253 vector in *L. lactis* MG1614 host strain [97]. A 6 kDa band was detected in a western blot with the antiClos leader antibody when copies of the *closA3*, *closA4* were overexpressed on a vector. However, the presence of the peptide was not demonstrated by Maldi-ToF. An explanation would be that the methods used at that time were not suitable enough for the detection of the nisin-like lantibiotic. The antiClos leader antibody was designed to bind to the Clos leader of ClosA1-3 peptides. It was expected that only a ClosA1 precursor peptide would be detected by hybridisation in a western blot whereas the ClosA4 precursor should not, since their leader peptides are different. The yield of this 6 kDa peptide was very low which prevented any further investigation into the structure, activity or function characterisation of this 6 kDa peptide.

One hypothesis was that the Clos production may improve if the entire *clos* biosynthetic machinery would be switched on by the NisRK system. This could therefore facilitate the modification, secretion and processing of the Clos precursor peptides. To address this, the first aim was to switch on the P_{Clos} promoters via nisin induction, by expression of the *clos* gene cluster in the UKLc10 strain where the *nisRK* regulatory system is on the chromosome. In the proposed system, the two ClosRK regulatory systems are also present in the *clos* gene cluster and they could potentially be activated by induction with nisin. This assumption that nisin induces the activation of the P_{ClosA} promoter through the *nisRK* but not via the *closRK* regulatory system is based on the observations with the *pepI* assay (chapter 4).

The second aim in this chapter was to express the *clos* cluster in the *L. lactis* FI7847 host strain which has the *nisRK* genes in the nisin biosynthetic cluster. As the *clos* cluster lacks a gene that encodes for a protease, we hypothesised that NisP may recognize and remove the Clos leader peptide from pClosCluster in *L. lactis* FI7847 host strain. Control strains with pClosCluster and with pClosCluster(Δ*cA*) expressed in *L. lactis* MG1614 and in FI7847 were also included. The *closA34* genes were overexpressed on a pTG262 vector in a strain where the *clos* cluster was already present.

The third aim in this work was to test if once the cluster is switched on, the structural genes can be expressed and the Clos production can be amplified in the UKLc10 heterologous expression system. Therefore, the pClosCluster(Δ*cA*) in UKLc10 host strain was selected since the *clos* gene cluster was present with the structural genes knocked out to make the pClosCluster(Δ*cA*) + p P_{nisA} _ *cA34* in UKLc10 strain. This strain is based on a dual plasmid

system where the strong interaction between the activated NisR with the P_{nisA} promoter should allow the expression, modification and secretion of the ClosA34 peptides in the presence of the *clos* biosynthetic machinery. The dual plasmid system was also cloned in FI7847 to facilitate the potential removal of the Clos leader peptide by NisP.

The fourth aim in this work was addressed by expressing the entire *clos* cluster in the presence of additional copies of both *closA3* and *closA4* genes in either *L. lactis* UKLc10 or FI7847 host strains. It was assumed that if ClosA34 peptides are co-expressed, they may act as inducers for Clos peptide production and further processing in strains with two plasmids: pClosCluster+p P_{nisA}_cA34.

A number of strains were made (Table 27) in order to achieve the four aims.

Full strain name		Shorter strain name	Regulatory genes	Reference
pIL253_ClosCluster	MG1614[b]	pClosCluster in MG1614	*closR1K1 closR2K2*	[97]
pIL253_ClosCluster	FI7847[c]	pClosCluster in FI7847	*closR1K1 closR2K2 nisRK*	This work
pIL253_ClosCluster	UKLc10[a]	pClosCluster in UKLc10	*closR1K1 closR2K2 nisRK*	This work
pIL253_ClosCluster(Δ*closA*)	MG1614	PClosCluster (Δ*cA*) in MG1614	*closR1K1 closR2K2*	[97]
pIL253_ClosCluster(Δ*closA*)	FI7847	pClosCluster (Δ*cA*) in FI7847	*closR1K1 closR2K2 nisRK*	This work
pIL253_ClosCluster(Δ*closA*)	UKLc10	pClosCluster (Δ*cA*) in UKLc10	*closR1K1 closR2K2 nisRK*	This work
pTG262_P_{nisA} _closA3_closA4	MG1614	p P_{nisA} _cA34 in MG1614	*none*	This work
pTG262_P_{nisA} _closA3_closA4	FI7847	p P_{nisA} _cA34 in FI7847	*nisRK*	This work
pTG262_P_{nisA} _closA3_closA4	UKLc10	p P_{nisA} _cA34 in UKLc10	*nisRK*	This work
pIL253 control strain	UKLc10	pIL253 in UKLc10	*nisRK*	This work
pIL253_ClosCluster (Δ*closA*) + pTG262_ P_{nisA} control strain	UKLc10	pClosCluster (Δ*cA*)	*closR1K1 closR2K2 nisRK*	This work
pIL253_ClosCluster + pTG262_P_{nisA} _closA3_closA4	MG1614	pClosCluster + p P_{nisA} _cA34	*closR1K1 closR2K2*	[97]
pIL253_ClosCluster + pTG262_P_{nisA} _closA3_closA4	UKLc10	pClosCluster + p P_{nisA} _cA34	*closR1K1 closR2K2 nisRK*	This work
pIL253_ClosCluster (Δ*closA*) +pTG262_P_{nisA} _closA3_closA4 Test strain and control strain	UKLc10	pClosCluster (Δ*cA*) + p P_{nisA} _cA34	*closR1K1 closR2K2 nisRK*	This work

pIL253_ClosCluster + pTG262_P_{nisA} _closA3_closA4	FI7847	pClosCluster + p P_{nisA} _cA34	*closR1K1 closR2K2 nisRK*	This work
pIL253_ClosCluster (Δ*closA*) +pTG262_P_{nisA} _closA3_closA4 Test strain and control strain	FI7847	pClosCluster (Δ*cA*) + p P_{nisA} _cA34	*closR1K1 closR2K2 nisRK*	This work
pIL253_ClosCluster (Δ*closA*) + pTG262_ P_{nisA} control strain	FI7847	pClosCluster (Δ*cA*) + p P_{nisA}	*closR1K1 closR2K2 nisRK*	This work
pIL253+ pTG262_ P_{nisA} empty vector control strain	FI7847	pIL253+ pP_{nisA}	*nisRK*	This work

Table 27 - A number of expression systems were designed in order to improve the *clos* cluster production in *L. lactis* UKLc10 or FI7847 host strains. pClos_pepI constructs were cloned in pIL253.
[a] Strain in UKLc10 has the nisin two-component regulatory system, *nisRK*, on the chromosome.
[b] The *L. lactis* MG1614 strain is plasmid free.
[c] The *L. lactis* FI7847 strain is the FI5876 with the nisin biosynthetic machinery (nisin gene cluster) with the structural genes knocked out (Δ*nisA*). It has one set of *nisRK* genes in the nisin cluster.
[d] The pP_{nisA} _ *cA34* construct has two different copies of *clos* structural genes (*closA3* and *closA4).*

Besides examining if the *nisRK* genes switch on the P_{ClosA} promoter when the *clos* cluster is incorporated on a plasmid in UKLc10 host strain, we want to understand if the expected 6 kDa Clos prepeptide is modified by the *clos* biosynthetic machinery and finally if the modified prepeptide is released or it becomes trapped in the *clos* transporter machinery. The time for Clos prepeptide maximum production, after incubation with 10 ng/ml of nisin, was optimised. In order to confirm that the 6 kDa band is the result of the *closA1-4* expression, a pClosCluster (Δ*cA*) in UKLc10 control strain with the *clos* structural genes knocked out was included.

To achieve optimal expression and purification of the Clos (pre)peptide from Clos leader_Clos peptide, alternative expression hosts were used.

Table 27 also highlights the presence of any *lanRK* regulatory genes.

In pP_{nisA}_*cA34* construct, there are two copies of the *clos* structural genes that encodes for two structurally different peptides. Both *closA3* and *closA4* structural genes are expressed under the control of the nisin promoter, P_{nisA}. It is expected that either a preClosA precursor (Clos leader_Clos peptide) or a leaderless Clos peptide would be expressed from this pP_{nisA}_*cA34* construct.

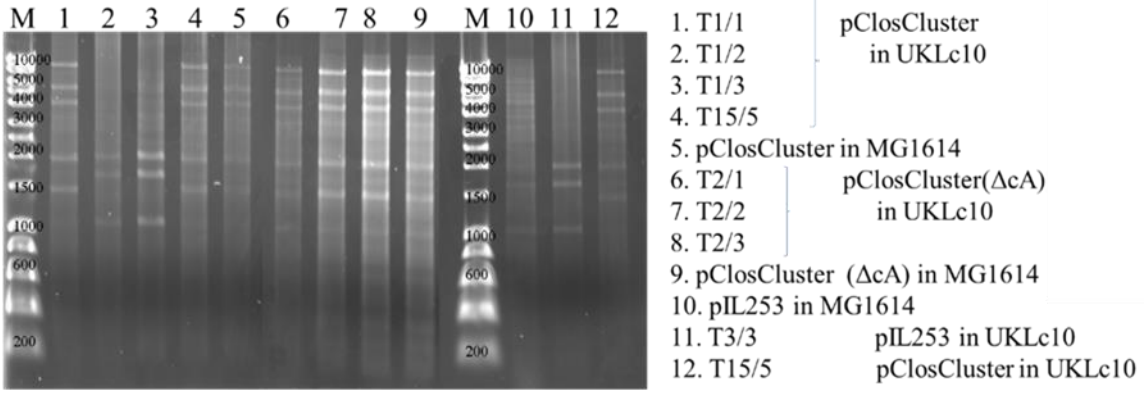


Figure 57 - Confirmation of the plasmids present in different *L. lactis* host strains.

Agarose gel with to confirm the correct constructs in *L. lactis* MG1614 and UKLc10 strains. The gel bands are fragments obtained from plasmid digestion with *Hind*III.

Colonies for pClosCluster strains are notated as T15/5 and T1/1, T1/2, T1/3 (lanes 1-4).

Colonies for control pClosCluster (Δ*cA*) in UKLc10 strains are notated as T2/1, T2/2, T2/3 (lanes 6-8).

Colonies for control pIL253 in UKLc10 strains are notated as T3/3 (lane 11).

Reference strains used in here (Table 27) to verify the integrity of the clos cluster are as follows:

pIL253 empty vector in MG1614, pIL253_ClosCluster in MG1614, pIL253_ClosCluster (Δ*cA*) in MG1614.

M is a DNA gel marker (Hyperladder I).

Expected masses for pClosCluster: 419bp+1066 bp = 1500 bp, 1430 bp, 5209 bp, 9162 bp and for pIL253 is 1066 bp, 3897 bp.

Agarose gel with the digested plasmids revealed that some transformants, like T1/2 and T1/3, were expected to have the 17 kDA insert. However, when they were restricted with *Hind*III, a different restriction profile which is specific to an empty vector control was obtained (pIL253 in MG1614, lane 10 as reference, Figure 57). The results indicate that the *clos* cluster is unstable, therefore the strain with the correct pattern carring with the pIL253_ClosCluster plasmid, was selected for further investigation.

5.3.3 Detection of 6 kDa peptide from heterologous systems

To investigate if secretion or intracellular accumulation of Clos precursor peptides from these strains (Table 27) can be detected after expression in *L. lactis* UKLc10 or FI7847, a number of tests were used.

Different methods were used for the detection of a 6 kDa or a 3.5 kDa peptide:

1) CMS, LC-MS and Maldi-ToF with colonies from plates or liquid culture,
2) western blotting with two sets of samples against antiClos (*closA1*) leader antibody (cell extracts and TCA precipitated supernatant),
3) excised protein gel bands (two sets of samples) were analysed by LC-MS. The cysteines were labelled with S-alkylators. The samples were trypsin digested (chapter 6) and we purified 6 kDa Clos peptide (3). The results are described in chapter 6.

Previous work involving the expression in FI7847 should have allowed for the detection of a 6 kDa preClosA peptide or of a leaderless Clos peptide of 3.5 kDa provided that NisP cleaved the Clos leader peptide.

In this work, the expression in UKLc10 should allow for production of a 6 kDa prepeptide and possibly of a 3.5 kDa proved that if intracellular or cytoplasmic unspecific protease activity in *L. lactis* does take place to remove the Clos leader peptide. Unspecific protease activity is known to occur in other lantibiotics such as in nisin and in subtilin [166].

Further work involving the labelling of cysteine residues, trypsin digestion to obtain tryptic fragments, fragmentation and analysis on LC-MS and Maldi-ToF described in chapter 6, would allow us to investigate the efficiency of the *clos* modification machinery to introduce PTM. Similar to other lantibiotic leader peptides such as nisin, the Clos leader peptide is expected to facilitate dehydration of amino acids by ClosB and to guide the cyclization reactions as performed by ClosC, that is formation of (Me)Lan rings.

To optimise the maximum production of Clos, pClosCluster in MG1614 and pClosCluster in UKLc10 strains were induced with 10 ng/ml of nisin and incubated overnight. Aliquots of liquid cultures were collected at time zero, after 2 h, 4 h, 6 h, 12 h after nisin induction of UKLc10 strains. For *L. lactis* MG1614 and FI7847, the cultures were harvested at time zero, 2 h and 12 h. In a previous study [97], a 6 kDa band was detected by western blot from the MG1614 strain induced for 12 h, however despite attempts to replicate the test, no Clos peptide was detected in this work. Also, the identity of the 6 kDa peptide in the MG1614 strain was not revealed by MS analysis which would be required to validate and to confirm

the presence of preClosA in the western blot. Both cell extracts and TCA precipitated supernatant were prepared for several strains listed in

Table 27. It was previously shown that induction with nisin is sufficient for 2 h in a UKLc10 strain [149], therefore it was expected that if Clos was produced in any of the strains, it would have hybridised to the antiClos leader antibody in the aliquots collected at the 2 h time point.

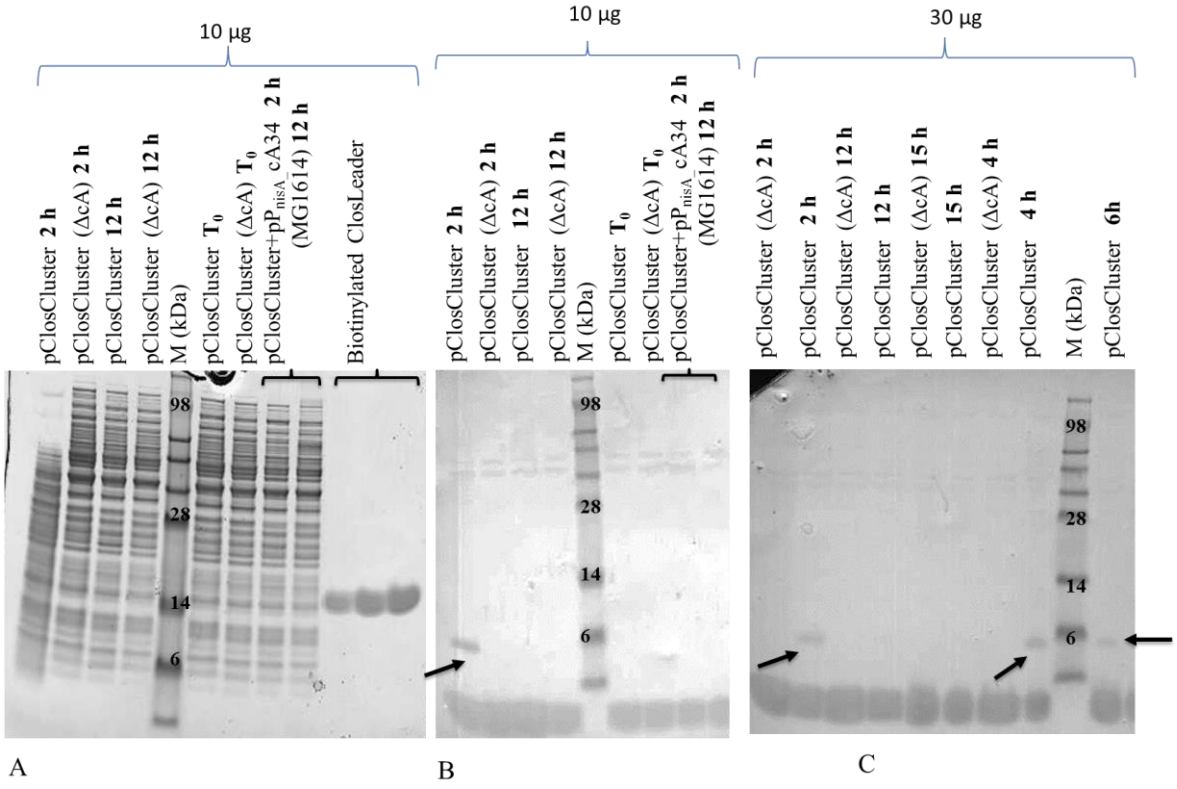


Figure 58 - Detection of Clos in a protein gel and in western blot.

A. 10 µg of cell extracts were prepared from pClosCluster in *L. lactis* MG1614 and UKLc10 strain previously incubated with nisin for T0, 2 h, 12 h before loading on SDS-PAGE.

B. Western blot with antiClos leader antibody of cell extracts bead beaten from *L. lactis* strains. Same samples were loaded (10 µg of cell extracts) as in A.

C. Western blot with 30 µg of cell extracts from pClosCluster in UKLc10 harvested from nisin induced liquid cultures for T0, 2 h, 4 h, 6 h and 12 h.

The antibody was used at 1/100 dilution. All the cloned constructs were expressed in UKLc10 host strain unless otherwise stated. N means nisin; T0 negative control means that the liquid culture was grown for 12 h without nisin induction. The protein standards were M: SeeBluePlus2, Pre-stained Protein Standard.

The protein gel in Figure 58, A, shows that a band for a 6 kDa peptide could not be easily visualised and that the cell extract contains a mixture of proteins. The western blots in Figure 58, B-C, clearly shows that there is no equivalent 6 kDa peptide produced from pClosCluster + pP_{nisA}_ *cA34* in MG1614 strain negative control after induction with nisin for either 2 h or

12 h. In here, the 6 kDa peptide was not identified in a western blot from the same MG1614 strain after 12 h incubation with nisin.

The pClosCluster in *L. lactis* UKLc10 strain successfully produced a 6 kDa peptide as detected by hybridisation with the antiClos leader antibody (Figure 58, B-C) that should have hybridised to the Clos leader_ClosA1 peptide. This band was later confirmed by MS as being the precursor preClosA1 peptide (chapter 6). The bands in the western blot confirmed that the size of the secreted Clos peptide was approx. 6 kDa, which matches the size of the expected preClosA precursor peptide of 57 amino acids (excluding the N-terminal methionine residue). Detection was from cell extracts prepared from liquid cultures that were previously induced with nisin for 2 h, 4 h, 6 h but not for 12 h. The absence of a detectable preClosA peptide may suggest that the preClosA is only stable up to 6 h and maybe it is degraded after 6 h of being induced with nisin.

Results in Figure 59 shows that a 6 kDa band of the expected size for preClosA can be detected from both samples pClosCluster and pClosCluster + pP_{nisA}_ *cA34* in UKLc10 strains. This band was detected both inside the cell from the cell extracts as well as in the media in the TCA precipitated supernatant. This means that a proportion of the 6 kDa peptide is trapped within the cell as well as another proportion is secreted in the medium.

The pClosCluster (ΔcA) + pP_{nisA}_ *cA34* in MG1614 strain has the *closA* structural genes knocked out; a 6 kDa peptide would be detected if ClosA34 proteins was biosynthesised. Since the NisRK proteins are absent, it was suggested that ClosR binds to P_{nisA} to allow for expression of ClosA34 proteins. Consequently, only the mature ClosA34 proteins may act as an inducer which will activate the *clos* promoters to allow for the *clos* modification and transport machinery to become functional.

These preliminary western blot results (Figure 58, Figure 59) confirmed that nisin-mediated activation of transcription of the *clos* genes involve signal transduction by the two-component regulatory system (NisRK). A control with pClosCluster in UKLc10 without nisin was also included (Figure 58, sample at T0). Current results would indicate that there is no confirmation of the extent to which either or both ClosR1 and ClosK1 and/or ClosR2 and ClosK2 proteins are involved in the activation of the *closA* transcription. Therefore, further testing would be necessary.

The strain known as pClosCluster in FI7847 was designed to incorporate both the *clos* and the *nis* gene biosynthetic machinery. The aim was to have a system in which the *nisRK* genes and the *nisP* genes are present on the nisin cluster. This strain would facilitate the activation

of *clos* promoters and the possible cleavage of the Clos leader by the nisin protease. The *clos* cluster was expressed in the FI7847 host strain, however no form of a Clos peptide was detected after induction with 10 ng/ml of nisin; the cells were harvested after induction with nisin for 2 h and for 12 h.

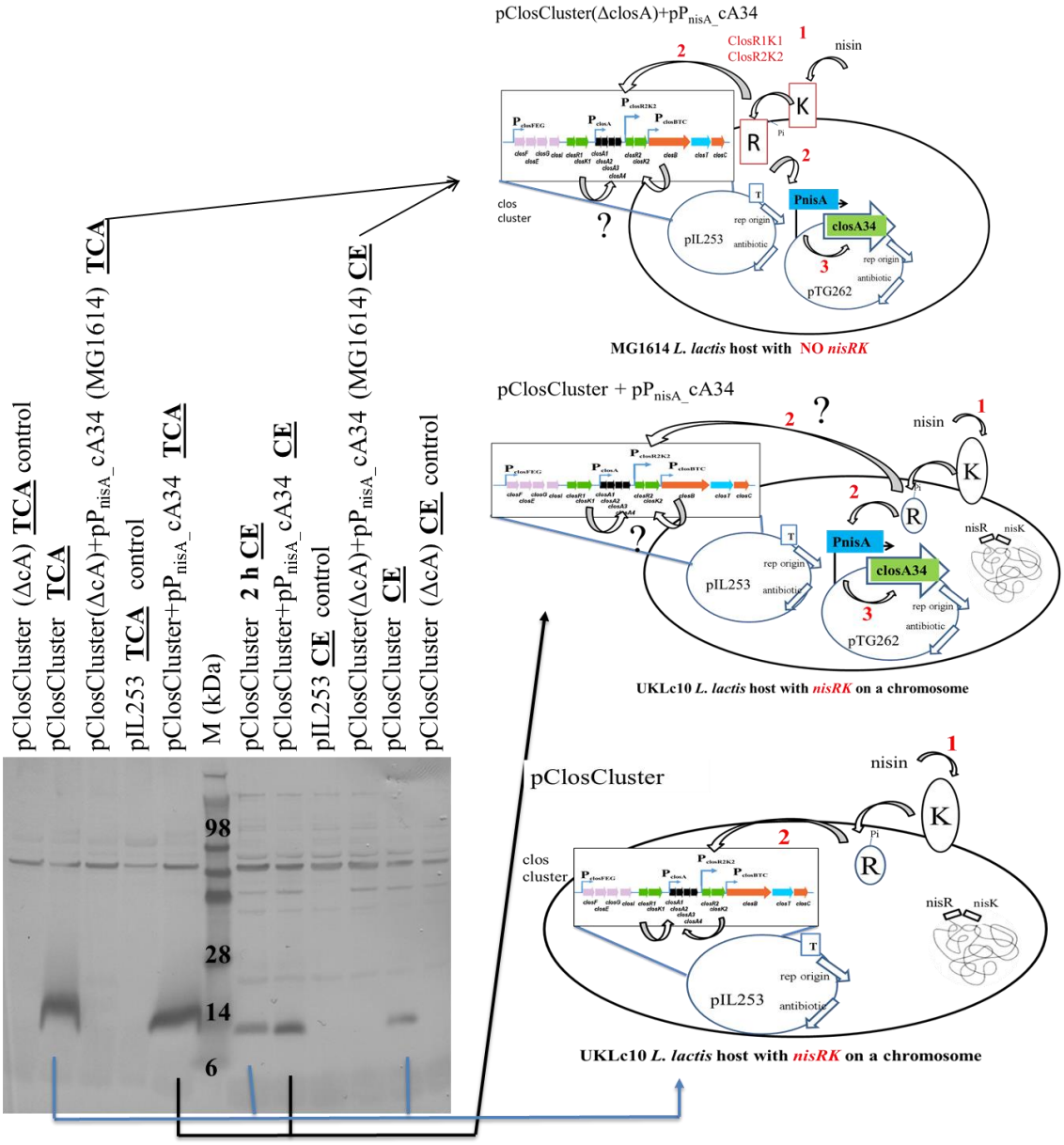


Figure 59 - TCA precipitated supernatant and cell extracts (CE) from *clos* cluster containing strains.
The strains were induced with nisin for 3 h before harvesting unless specified otherwise. The legend of strains (all strain are in UKLc10 unless specified otherwise):
pClosCluster (Δ*cA*) + pP$_{nisA_}$ *cA34* in MG1614,
pClosCluster + pP$_{nisA_}$ *cA34* in UKLc10, .
pClosCluster in UKLc10, *closR1K1* and *closR2K2* genes in the *clos* gene cluster. The *nisRK* genes are on the chromosome of the UKLc10.
M is SeeBluePlus2 marker in kDa. 30 µl of cell extract (CE) of total protein was loaded.
To be noted that an inactive ClosA34 protein would not work an inducer in pClosCluster + pP$_{nisA_}$ *cA34* in UKLc10 strain.

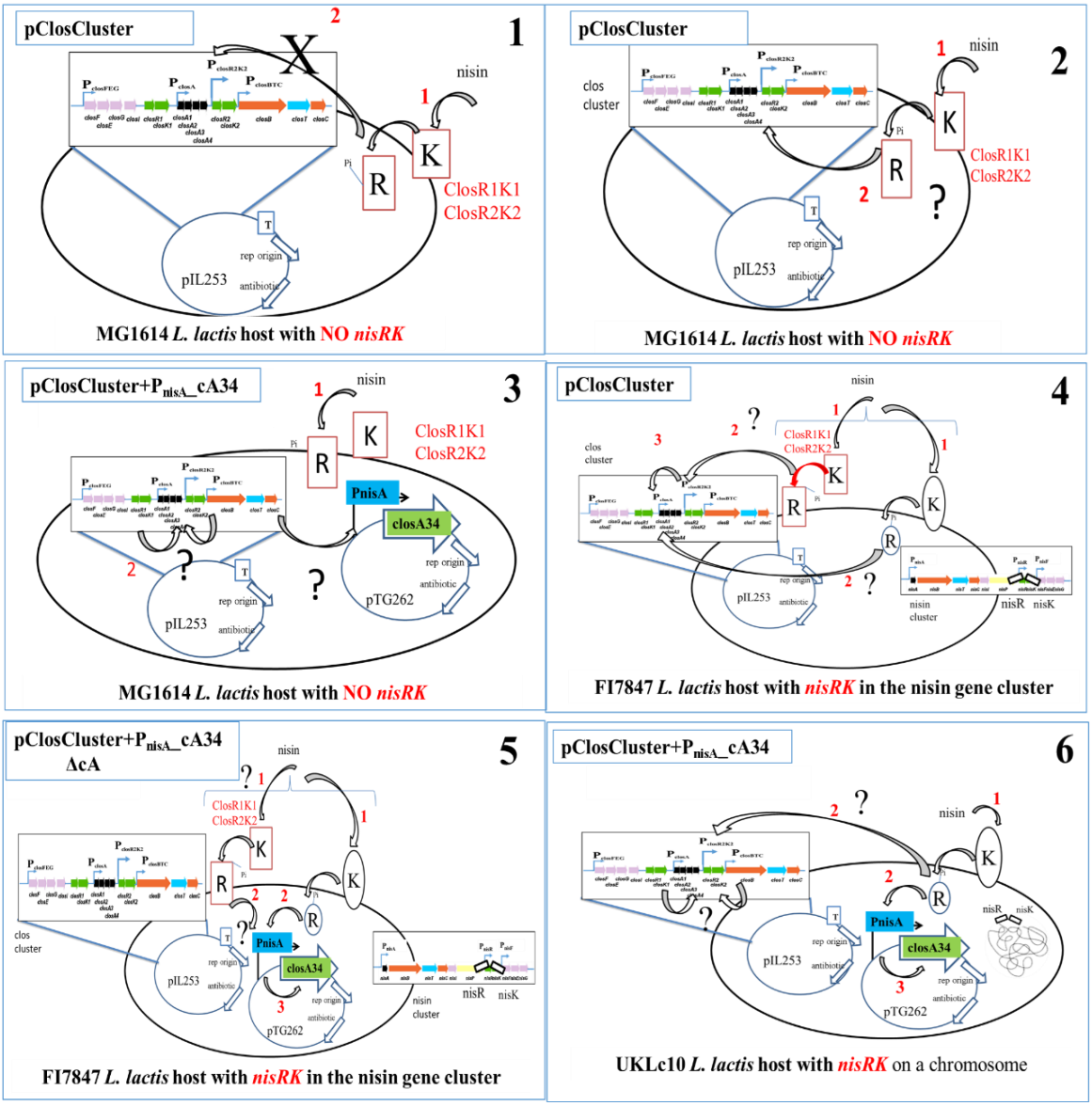


Figure 60 - Proposed mechanisms for signal transduction in different strains where the *clos* cluster is expressed on a plasmid.
The legend is:
1. pClosCluster in MG1614, with no *nisRK* genes; mechanism based on western blot (Table 25).
2. pClosCluster in MG1614, with no *nisRK* genes; mechanism is based on previous western blot results [97].
3. pClosCluster + $pP_{nisA\_}$ *cA34* in MG1614, with no *nisRK* genes; mechanism is based on the assumption that *closRK* genes are working as suggested by previous work [97]. In this work, no band for a 6kDa peptide was detected in western blot [97].
4. pClosCluster in FI7847, with *nisRK* genes and two sets of the *closRK* genes.
Bands detected for 6 kDa preClosA peptide (Figure 62).
5. pClosCluster + $pP_{nisA\_}$ *cA34* in FI7847, with *nisRK* genes, two sets of *closRK* genes.
Proposed mechanism if all the regulatory genes mediate *clos* transcription.
No band was detected in western blot in this work.
6. pClosCluster + $pP_{nisA\_}$ *cA34* in UKLc10, with *nisRK* genes, two sets of *closRK* genes. Proposed mechanism only if *nisRK* genes regulate transcription.
Bands detected for 6 kDa preClosA peptide.

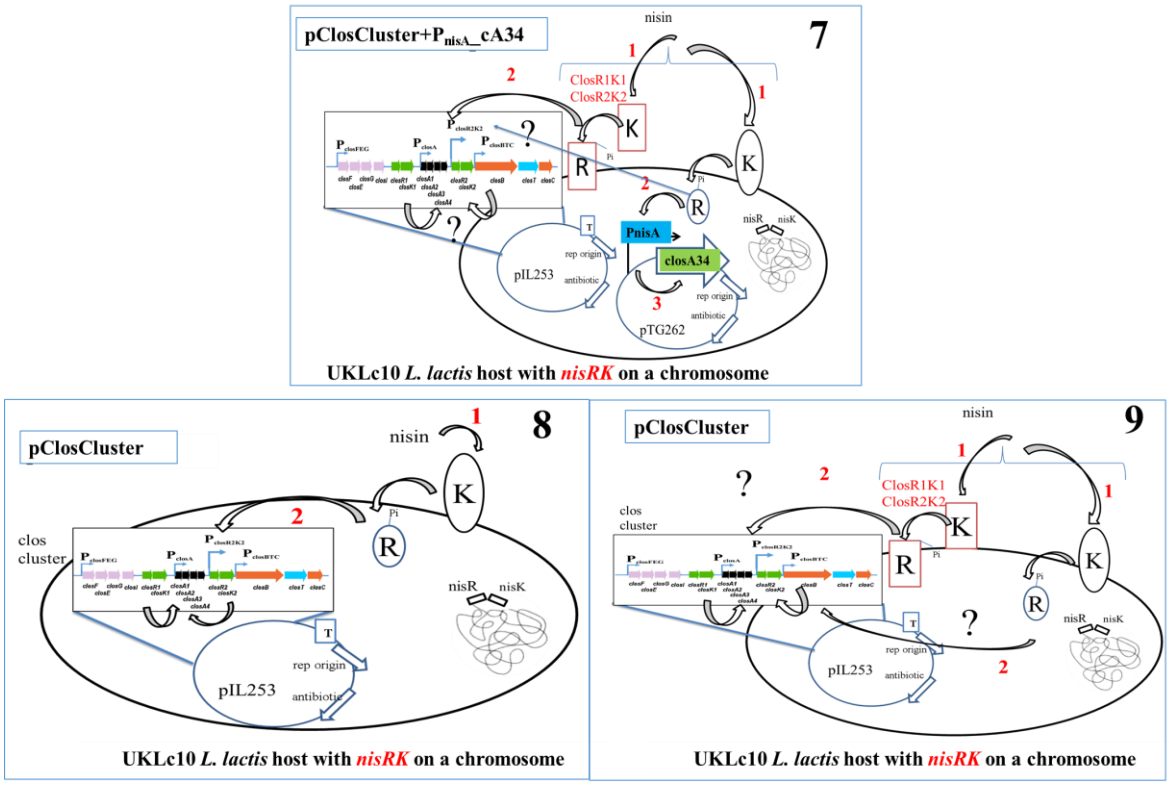
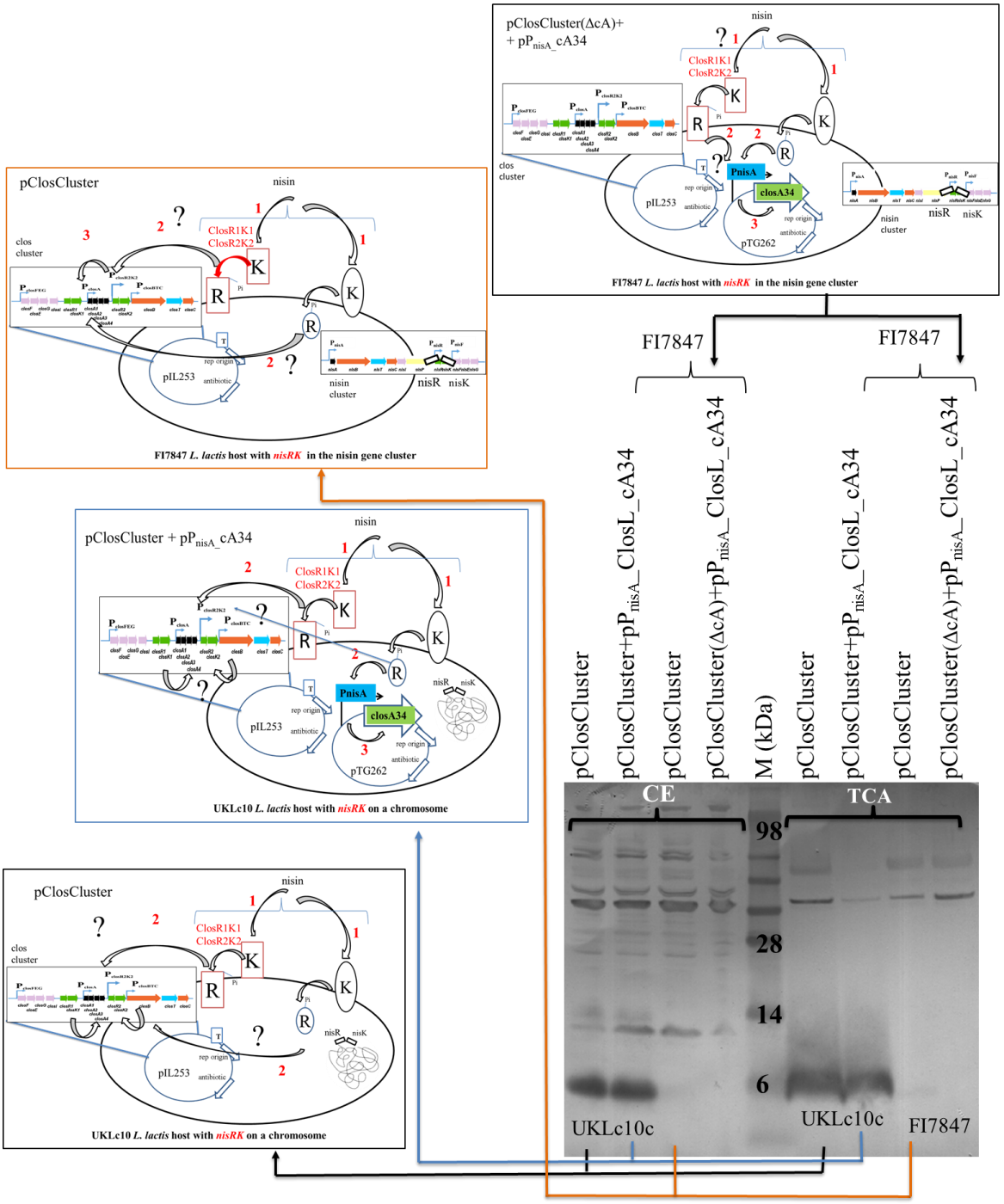


Figure 61 - Proposed mechanisms for signal transduction in different strains where the *clos* cluster is expressed on a plasmid.
The legend is:
7. pClosCluster + pP_{nisA}_ *cA34* in UKLc10, with *nisRK* genes and two sets of *closRK* genes.
Proposed mechanism if all the regulatory genes mediate *clos* transcription. Bands detected for 6 kDa preClosA peptide.
8. pClosCluster, in UKLc10, with *nisRK* genes, two sets of *closRK* genes.
Proposed mechanism only if *nisRK* genes regulate transcription. Bands detected for 6 kDa preClosA peptide.
9. pClosCluster, in UKLc10, with *nisRK* genes, two sets of *closRK* genes.
Proposed mechanism if all the regulatory genes mediate *clos* transcription. Bands detected for 6 kDa preClosA peptide.

Figure 60 and Figure 61 are illustrations of the proposed mechanisms for signal transduction which are based on the results from the western blot obtained in current and previous work (for MG1614 strains).

The results from the western blot in Figure 62 indicated that a very faint band for a 6 kDa peptide expressed by pClosCluster in the FI7847 was detected when probed with the antiClos leader antibody. However, as for the other strains, the specificity of the antibody was unknown and further MS analysis would be required to confirm the identity of the preClosA peptide. A 6 kDa band was detected only in the TCA precipitated supernatant (Figure 62).



UKLc10 strains	FI7847 strains
pClosCluster (CE) in UKLc10 pClosCluster + pP$_{nisA}$_ClosL_A34 (CE)	pClosCluster (CE) in FI7847 pClosCluster(Δ*cA*) + pP$_{nisA}$_ClosL_A34 (CE)
pClosCluster (TCA) in UKLc10 pClosCluster + pP$_{nisA}$_ClosL_A34 (TCA)	pClosCluster (TCA) in FI7847 pClosCluster(Δ*cA*) + pP$_{nisA}$_ClosL_A34

Figure 62 - Western blot to test for the presence of the 6 kDa peptide, potentially the preClosA peptide, in cell extracts and TCA precipitated supernatant of constructs expressed in *L. lactis* UKLc10 or FI7847 host strain.

Tested strains were listed in the table. The strains were grown and the samples were prepared as described in chapter 2; expression of the preClosA peptide was induced with 10 ng/ml of nisin for 3 h at 30˚C unless otherwise specified. CE is cell extract of total protein and 30 µg/ml were loaded. Western blotted with 1/100 dilution with the antiClos leader antibody. M is SeeBluePlus2 marker in kDa.

Results in Figure 62 indicate the successful expression of a number of peptides in the UKLc10 strains. Based on the very high intensity of the bands, the peptide was produced in very good yield in both inside and outside the UKLc10 strains. However, from the current results it is not possible to suggest if this 6 kDa peptide is preferentially located either inside or outside the cell.

In comparison to the samples in *L. lactis* UKLc10, FI7847 only showed a faint band and the western blot was prepared to compare the two strains. The potential expression of a 6 kDa peptide from pClosCluster in FI7847 strain is interesting. Only a 6 kDa protein band from the TCA precipitated supernatant could be detected from this strain which means that this peptide did not exclusively accumulate inside the cell or the peptide was secreted outside the cell. The pClosCluster construct in FI7847 would produce a preClosA peptide if the *closA1-4* structural genes were expressed. The observed poor yield may be due to the instability of the 17 kb *clos* cluster itself since it is expressed in a strain that already has the nisin gene cluster in. In contrast, no 6 kDa peptide was expressed by pClosCluster(Δ*cA*) + pP_{nisA}_ closLeader_*closA34* in UKLc10 strain. A 6 kDa peptide would be synthesised from this strain provided that the activated P_{nisA} promoter would allow the expression of the *closA34* genes in the presence of the *nisRK* regulatory system.

In conclusion, a 6 kDa peptide was identified in both TCA precipitated supernatant and CE from UKLc10 strains that hybridised to the antiClos leader antibody.

5.3.4 Biological activity of trypsin cleaved preClosA peptides

Earlier results in this chapter suggested that the preClosA peptide was expressed in broth culture and it was detected both in the cell extract and in the supernatant of a strain where the *clos* cluster functionality is under the control of *nisRK* regulatory system.

The lack of proteases in the *clos* operon would suggest that a modified precursor peptide with the leader still attached could be produced in the UKLc10 heterologous system. The hypothesis is that external proteases are necessary for the cleavage of the leader peptide in order to obtain functional active Clos peptide.

To investigate this hypothesis, the pClosCluster in *L. lactis* UKLc10 strain was grown on an agar plate and induced with nisin to allow for the biosynthesis of the preClosA peptide. The fact that colonies did grow on the solid media in the presence of inducible nisin did suggest that the *clos* immunity genes must also be expressed in agar cultures along with the other *clos* genes, therefore the Clos prepeptide was expected to be present on the plate.

After colonies were grown on plates, they were treated with UV light, then they were overlaid with soft agar in which different indicator strains as well as various trypsin concentrations were added. Two different sources of trypsin were used: trypsin in a mix and pure trypsin from a kit at concentrations from 0 to 15 ng/µl. Consequently, a mixture of Clos tryptic peptides is predicted to be formed on the plates with trypsin. Colonies grown on plates overlaid with trypsin displayed antimicrobial activity against *C. perfringens*, *C. difficile* and *L. lactis* MG1614 indicator strains (Figure 63). Control plates overlaid with indicator strains but without trypsin were included and the results confirmed that antimicrobial activity was only possible if preClosA was digested with trypsin. This suggested that a 6 kDa preClosA does not display antimicrobial activity since the Clos leader is still attached. However, after trypsin digestion the resulting full length leaderless Clos or the tryptic peptides displayed antimicrobial activity against the tested sensitive strains.

A number of other indicator strains were also tested, however the tryptic fragments were obtained by using a trypsin concentration between 1 to 15 ng/µl of trypsin. The nisin positive control strain was expected to produce a leaderless nisin peptide, therefore it displayed antimicrobial activity in plates with and without trypsin. Interestingly, even if nisin must have also been digested by trypsin, the resulting nisin tryptic peptides accumulated in high enough concentration to display biological activity against all the tested indicator strains. It may be that trypsin has been used at a concentration that has only allowed for the partial cleavage and the release of specific nisin fragments.

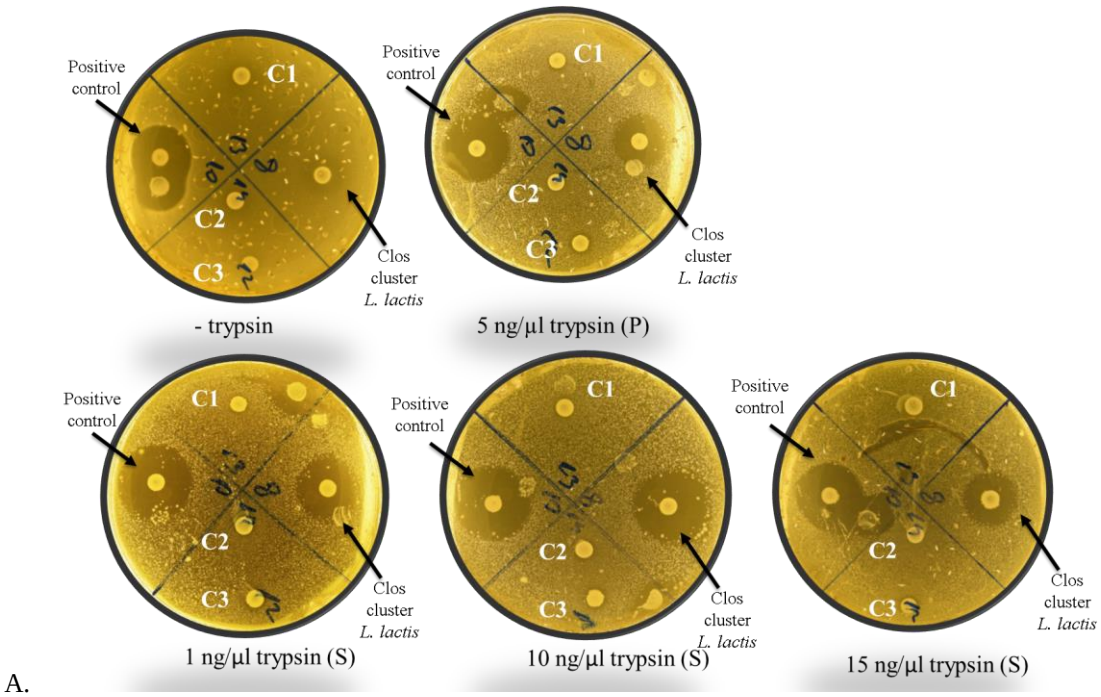
At higher trypsin concentrations, trypsin may have facilitated the accumulation of specific peptide that have biological activity. However, in the case of the indicator strains where antimicrobial activity from Clos fragments was not detected, a higher amount of Clos may facilitate the release of the active fragments if tested in the future.

Interestingly, the *closA* gene expression was confirmed and the bioactivity that resulted after trypsin digestion of preClosA on the plate was significantly increased in a dose-dependent fashion which can be especially seen in the plates with *L. lactis* MG1614 (Figure 63). This observation was made by comparing the increase in the zone of inhibition given by raising amounts of trypsin. It was therefore concluded that increasing the trypsin concentration in the overlaying soft agar may facilitate the complete digestion of preClosA and the full accumulation of active Clos tryptic peptides.

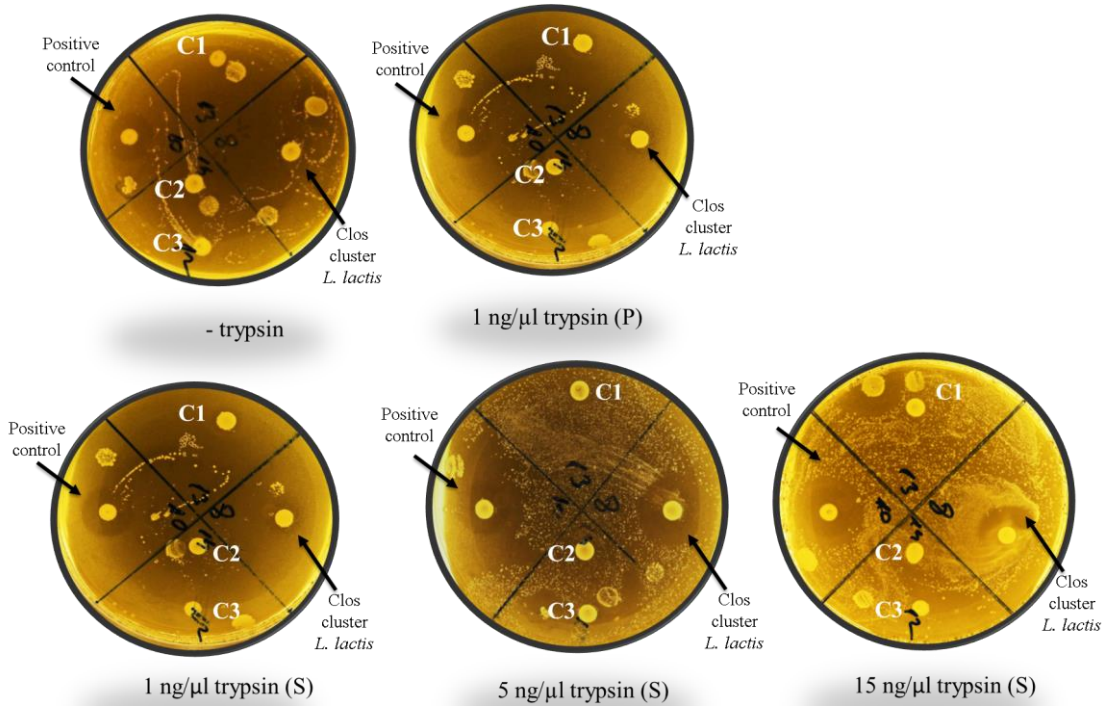
The biological activity obtained from trypsin treated preClosA follows the same trend when tested against *L. lactis* MG1614 as with the *Clostridiales* species. The biological activity increased until 10 ng/µl of trypsin, then it decreased at 15 ng/µl against *C. perfringens*. For *C. difficile*, the bioacitivity is high until 5 ng/µl of trypsin then it decreased with 15 ng/µl.

The highest antimicrobial activity displayed by tryptic peptides was seen in plates tested against *C. perfringes*. Antimicrobial activity was compared between plates where the nisin positive control (nisin in FI7827) and the negative controls (no trypsin plates) were included. Results indicated that both Clos and nisin can be digested with trypsin to give a series of tryptic peptides that display biological activity when screened in overlay assay against *C. perfringens* and *C. difficile* as well as *L. lactis* MG1614.

Trypsin was not killing the indicator strains since activity can only be observed around the growing cells and nisin and/or Clos production and not on the whole plate. Future work may involve to design of a good system to follow the time course cleavage of nisin and Clos peptides in solution or in on the plate.


Positive control
C1
C2
C3
Clos cluster L. lactis
- trypsin
5 ng/µl trypsin (P)
1 ng/µl trypsin (S)
10 ng/µl trypsin (S)
15 ng/µl trypsin (S)

A.


Positive control
C1
C2
C3
Clos cluster L. lactis
- trypsin
1 ng/µl trypsin (P)
1 ng/µl trypsin (S)
5 ng/µl trypsin (S)
15 ng/µl trypsin (S)

B.

C.

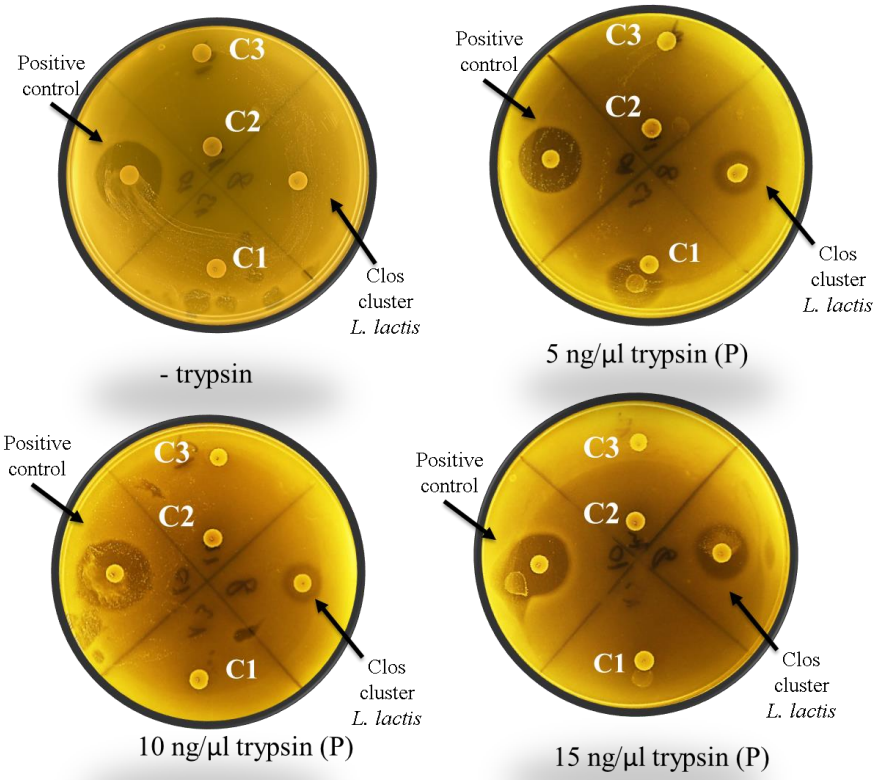


Figure 63 - Overlay assay of *L. lactis* strains against A. *C. perfringens*, B. *C. difficile*, C. *L. lactis* MG1614 indicator strains.
These pictures are a representative image of three independent overlay assays.
The samples were: pTG262_*nisA* in FI7847 (positive control), pClosCluster in UKLc10 strain.
Negative control strains (C1, C2, C3): pClosCluster (Δ*cA*) and pIL253 in UKLc10, UKLc10 strain alone, respectively. Colonies were grown on solid agar with $NaHCO_3$, and 10 ng/ml of nisin in order to induce *clos* and *nis* expression. The assay was overlaid with indicator strains and with different amounts of trypsin from two sources: 1 or 5 ng/µl of pure trypsin or 1, 5, 10 or 15 ng/µl of trypsin from a Promega, Mass Spec Grade kit. Negative control plates with no trypsin was also included. P means pure trypsin stock from kit. S means trypsin stock from Sigma. $NaHCO_3$ was used to prevent non-specific antimicrobial peptide activity.
Both the nisin and the clos tryptic peptides were active against the tested indicator strains.

5.4 DISCUSSION

In this chapter, fusions of the *closA* structural genes to the nisin A leader peptide were expressed and a putative cleavage site for the NisP protease was created. All the constructs were cloned in *L. lactis* and they were tested to investigate the possible expression and modification of active Clos peptide by suing the the nisin biosynthetic machinery.

Several studies reported the successful production and characterisation of novel lantibiotics using fusions of nisin leader and structural gene sequences and the biosynthetic machinery of nisin [93, 165]. Based on a recent example [169] and in order to overcome problems with the functional expression of NisP, the native nisin leader cleavage site was engineered in nisin leader_clos hybrids to resemble the one in nisin. Despite the introduction of the NisP cleavage site, the expression, modification, leader peptide cleavage and the transport of an active Clos peptide from nisin leader_clos fusions were not possible for a number of reasons. A survey of literature suggested that the nisin leader peptide is capable of guiding and of inducing PTM via NisB and NisC. Dehydrated amino acids and the ring formation (cyclization) reactions were therefore expected to take place in Clos provided that the expression of the nisin leader_clos hybrids was efficient in the first instance. Despite the fact that the engineered nisin leader is known to have mutational freedom [169] and induction of preClosA biosynthesis should be possible, accumulation or secretion of the Clos peptides was not detected by Maldi-ToF.

The nisin leader plays a role in mediating the interaction with the NisT in the export of nisin. An intact nisin leader peptide is essential for NisB and NisC modification as it determines the substrate recognition and specificity of the two nisin modification enzymes [170]. The nisin leader is responsible for the functional binding of both enzymes; it also targets the substrate peptide to the NisT dedicated nisin transporter [165, 168]. NisT can transport different forms of nisin outside the cell such as fully modified, dehydrated as well as unmodified precursor nisin peptide or hybrids of nisin leader fused to lantibiotic or non-lantibiotic peptides [78]. Either of the three enzymes bind to their substrate (various nisin forms) with different affinities which explain the variation in the extent of modifications [165]. Unfortunately, in this project the nisin machinery was not able to biosynthesise any detectable form of the Clos peptide.

Van Heel *et al.* [165, 169, 171] have successfully produced modified active peptides by using a similar approach when testing the nisin leader hybrids. Therefore, the nisin expression and modification machinery have been shown to be functional when fusions of

the nisin leader peptide to the *clos* structural gene are expressed in a *L. lactis* host strain. The study suggested that when the original nisin leader cleavage site was used the expression of functional peptides was enhanced. In here, in order to overcome the challenge that the *clos* cluster may be a silent operon, the coding sequence of the lantibiotic core peptide was previously fused to the nisin leader peptide with two potential start sites: IE or YK [97]. Also, to address the issue with a non-compatible cleavage site, a novel NisP cleavage site was also created by introducing the IT mutation to substitute the IE and the YK start sites of the Clos core peptide.

Aminoacid substitution in the N-terminus of lantibiotics can be detrimental for their antimicrobial activity. Therefore, it is of utmost importance to correctly predict the end of the candidate leader peptide as well as the start of the candidate core peptide. Alternatively, additional modifications that take place in the original structure of the core peptide might be essential for rendering an active Clos peptide and may explain the lack of detection of Clos in this work. In the case of Clos, it may be possible that modifications can only be facilitated and guided by the original Clos leader peptide and not by the nisin leader.

The nisin modification machinery has a broad substrate tolerance [165, 172]. One explanation for the lack of preClosA could be explained by the partial incompatibility with the nisin modification system used in this work. Therefore, future work may involve to express both the nisin leader and *closA1* (IE or YK) as well as *closA4* hybrids into the same heterologous system. Structure comparison of the *clos* cluster with other lantibiotics would suggest that ClosA peptides are possibly part of a two-component system. Expression and antimicrobial activity would therefore require the presence of both ClosA1 and ClosA4 peptides.

In a study by Majchrzykiewicz *et al.* [173], a novel class II two-component lantibiotics which is not a homologue of nisin was biosynthesised using fusions to the nisin leader sequence. The peptides were produced by the nisin machinery, fully modified (dehydrate serine and threonine residues, lanthionine rings were introduced) and the biologically active peptides were secreted.

Another task of this chapter was to investigate if Clos production can be improved when the entire *clos* gene cluster is expressed in different host strains. Based on the *pepI* activity assays in chapter 4, the activation of P_{ClosA} is mediated by nisin induction and it involves the signal transduction by the nisin two-component regulatory system composed of NisR and NisK proteins.

In chapter 4, an increased specific *pepI* activity under the control of P_{ClosA} promoter was confirmed when induced with nisin which means that an external signal peptide like nisin could initiate transcription particularly of the *closA* structural genes. Therefore, in this chapter, the assumption was that if the entire *clos* gene cluster was expressed in the presence of *nisRK* genes, the P_{ClosA} would be activated by the phosphorylated NisR. If correct transcription activators were present, the other promoters may be switched on in order to allow for the biosynthesis of the preClosA peptide. Based on the current results, the *nisRK* genes may be essential for the good production of the intermediate Clos peptide, when the strain is induced with nisin.

In this chapter, in order to design an improved heterologous expression system that would allow for the production of Clos in higher yield and in order to achieve optimal expression and purification of preClosA, several host strains (*L. lactis* FI7847 and UKLc10) were used to clone the *clos* cluster. The *L. lactis* UKLc10 strain has the nisin two-component regulatory system, the *nisRK* genes, integrated on the chromosome. FI7847 is comprised of the nisin biosynthetic machinery (nisin gene cluster) with the structural gene knocked out (Δ*nisA*) and the *nisRK* genes present in the nisin gene cluster.

A western blot with the antiClos leader antibody of the strain pClosCluster(Δ*cA*) + pP_{nisA}_ ClosLeader_*closA34* in FI7847 did not indicate presence of any specific bands, however the antibody did successfully hybridise to a 6 kDa peptide in the UKLc10 strain that contained the same construct. The precursor Clos peptide was not detected in a western blot or in MS when it was expressed in FI7847 strains. This means that even if the P_{nisA} was switched on by nisin via the *nisRK* genes, the inducing signal was not strong enough to allow for the Clos leader_ *closA34* prepeptide to be synthesised or the product was not stable. Nevertheless, the UKLc10 strain allowed for the successful biosynthesis of the preClosA peptide in high yield as well as for the export and its detection after induction with external nisin.

The two pClosCluster and pClosCluster(Δ*closA*)+ pP_{nisA}_ ClosLeader_*closA34* constructs in FI7847, were expressed under the control of both *nisRK* and *closRK* sets of two-component regulatory system. However, the structural genes are located either on the *clos*

cluster or on a plasmid which could have an important impact on the expression efficiency. An extremely faint band for a 6 kDa band was detected in the western blot for the pClosCluster in FI7847 strain. This suggests that even though the background for the two constructs in FI7847 is the same (+/- *closA34*), the location and the copy number of the structural genes are important. Detection of the peptide was only possible in the TCA precipitated supernatant perhaps due to lower production or even degradation. It may be that the competition between the promoters prevented the accumulation of the preClosA peptide to reasonable detectable levels compared to other positive controls.

The absence of a 6 kDa band in the pClosCluster(Δ*cA*)+pP_{nisA}_ClosL_ *cA34* may be due to either the location of the structural genes or the poor Clos production in this host strain. Another possibility would be that the Clos leader may have been cleaved by NisP and a 3.5 kDa band for the leaderless Clos peptide could not hybrise to the antibody. To test this, gel slices were excised and they were subject to LC-MS, however no proteins were detected which confirmed that this scenario was not possible. The idea of expressing pClosCluster in the FI7847 host strain was based on the presence of the NisP protease on the nisin cluster. In contrast, the *clos* cluster in the UKLc10 host strain does not encode for a similar protease. It is known that the P_{nisR} promoter displays nisin-independent and constitutive traits to allow for a fast accumulation of immunity to protect the producer against the effect of its own AMP [8, 146]. In FI7847, the *nisRK* genes are expressed continuously which should mediate the activation of the P_{ClosA} promoter in the *clos* cluster by induction with nisin. Therefore, the extracellular nisin should have enhanced the *nisRK* expression as part of the nisin operon to amplify the response triggered by nisin even further in any *L. lactis* FI7847 strain. However, the presence of two gene clusters, *clos* and *nis*, may not have been tolerated well by the hosts. One possibility is that the *clos* cluster may be unstable if cloned in strains with other plasmids or gene clusters already in.

A survey of the literature suggests that the biosynthesis of some lantibiotic peptides is possible through a quorum sensing process [8, 83, 146]. Therefore, the *clos* cluster/Clos peptide may regulate similar phenotypic traits in the bacterial host strains and the Clos peptide may be a typical lantibiotic peptide that acts both as an inducing pheromone as well as an antimicrobial peptide. However, this is yet to be investigated in detail. The expression of a 6 kDa band was successfully confirmed in the UKLc10 strains from either the pClosCluster or pClosCluster+ pP_{nisA}_ ClosLeader_*closA34* constructs.

In this chapter, successful cleavage and detection of antimicrobial activity from active Clos tryptic peptides was demonstrated. The level of antimicrobial activity of the tryptic peptides against three tested indicator strains was similar when concentrations of 1 to 5 ng/µl of trypsin was used. Previous work on the *Blautia obeum* A2-162 indicated that antimicrobial activivity against *C. perfringens* was possible however it was not established if it was because of the Clos peptides [97].

In here, the trysin treated preClos peptides were responsible for the observed antimicrobial activity against *C. perfringens* and for the first time good biological activity against *C. difficile* and *L. lactis* MG1614 was also confirmed.

10 ng/µl of trypsin was a good concentration to give a big enough halo against *C. perfringens* whereas for *C. difficile* the biggest zone of inhibition was given by treatment with 5 ng/µl of trypsin. The antimicrobial activity from trypsin digested preClosA and nisin seemed greater and more reliable when tested against *C. perfringens* at 1-15 ng/µl of trypsin; at 1 ng/µl of trypsin there was a very big halo which means that at very low trypsin concentration, *C. perfringens* is more sensitive against the released Clos peptides compared to the other tested indicator strains. Further testing for antimicrobial activity should include the use of a pure stock of trypsin. These test would allow us to make more accurate comparison between plates with different indicator strains as well as test for the reproducibility of their biological activity. Compared to the nisin positive control, the antimicrobial activity from digested preClosA was slightly lower at any given trypsin concentration.

However, an increasing amount of trypsin allowed for the accumulation of completely digested tryptic peptides, therefore the size of the halo increased proportionally. Since we do not have the molecular tools to manipulate bacterial strains isolated from the GI tract, the well described and developed *L. lactis* UKLc10 system used in this project can be manipulated to further investigate the functionality of the *clos* cluster [24, 174, 175].

Similarly, heterologous expression, biosynthesis, and mutagenesis of type II lantibiotics from *Bacillus licheniformis* in *E. coli* was previously reported. A type II two-component lantibiotic, linchenicidin was heterologously expressed in a Gram-negative host, *E. coli* which also allowed for the further engineering and characterisation of the leaderless and active lantibiotic peptides [176].

The heterologous expression of a two-component, lanthionine-containing bacteriocins produced by *L. lactis* and *E. faecalis*, respectively, lacticin 3147 and cytolysin, was done in *E. faecalis* host strain. Only the lacticin 3147 biosynthetic machinery was successfully

heterologously expressed in the host strain to allow for the production of the lantibiotic with no haemolytic activity [175]. The use of a heterologous expression system allowed the mechanism of regulation for lacticin 3147 to be studied [177]. Similar to the work with lacticin 3147, in this study it was decided to investigate the expression system for *clos* by expressing the whole *clos* cluster in a *L. lactis* system which is more detectable and for which the molecular tools are known.

Bibb *et al.* [23] have reported the identification, heterologous expression and characterisation of several gene clusters that encode for novel lantibiotics that are regulated differently. Some of the heterologous expression systems were excellent molecular tools for the functional analysis of the genes in the cluster. In the absence of a dedicated LanP protease from the gene cluster that removes the leader peptide from microbisporicin, the *Nonomuraea* expression host strain allowed for the biosynthesis of a mature lantibiotic peptide since it also had the role for production of a general protease that successfully removed the leader peptide from the precursor [24]. The gene cluster for microbisporicin encodes for a number of putative regulatory proteins, such as 'an extra cytoplasmic function (ECF) σ factor, σMibX, a likely cognate anti-σ factor, MibW, and a potential helix-turn-helix DNA binding protein, MibR'. A model for the regulation of the biosynthesis of microbisporicin was proposed which was based on Maldi-ToF and qRT-PCR [23, 124].

In conclusion, in this chapter, a Clos peptide was successfully expressed in a *L. lactis* heterologous system and novel antimicrobial activity against two important pathogens was demonstrated. In the future, the aim would be to isolate the tryptic peptides obtained from cleaved of preClosA, characterise their biological activity and test the peptides for their potential use as inducers of the *Blautia obeum* A2-162 system. We have successfully showed the improved expression of biologically active Clos peptide and in the next chapter pure Clos peptide will be obtained and its structure will be confirmed.

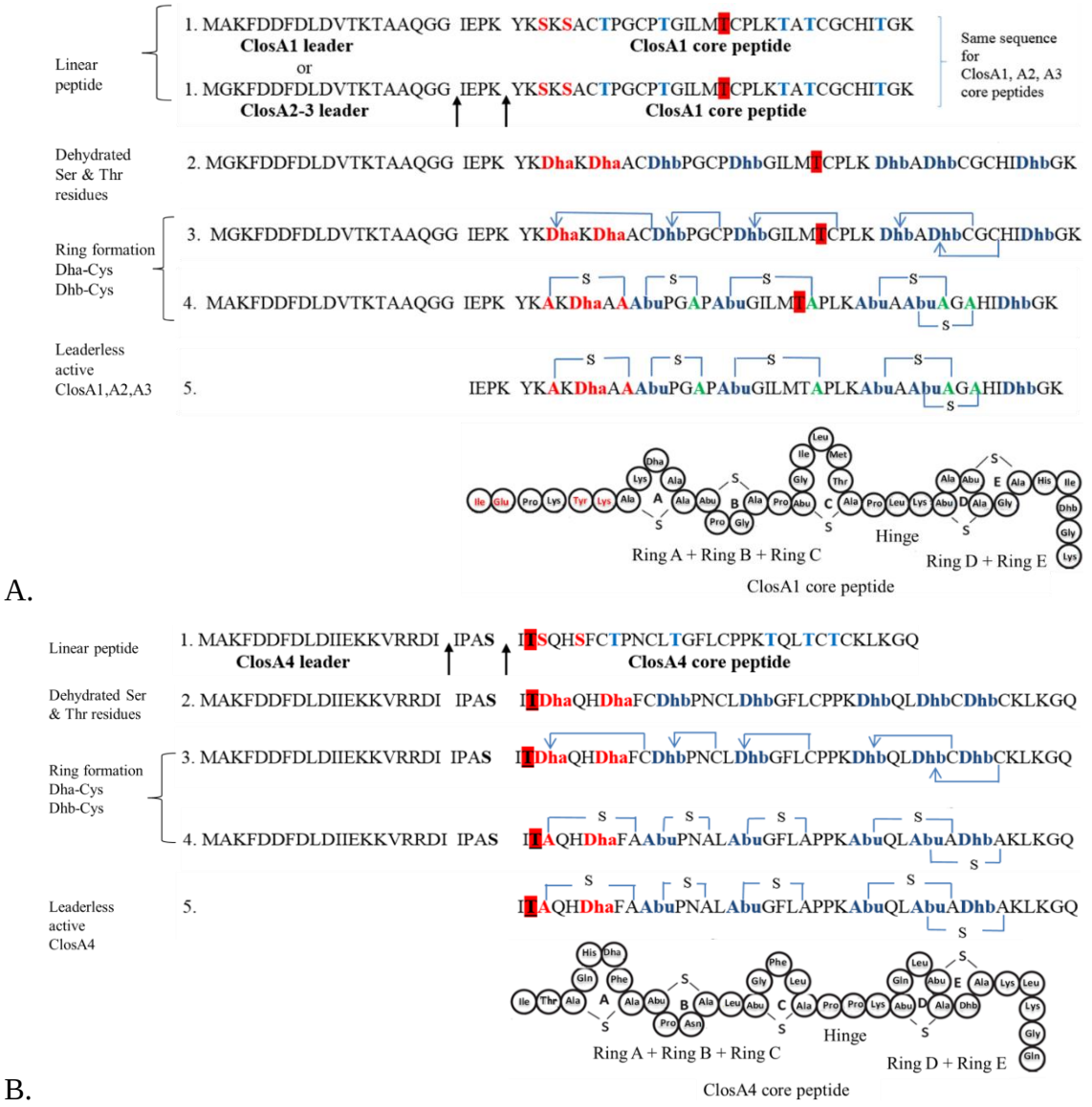
CHAPTER SIX

6. Structure confirmation of the preClosA peptide

6.1 INTRODUCTION

The *clos* cluster is comprised of four *clos* structural genes: three are identical except for their Clos leader peptide (ClosA1 leader + ClosA1 core peptide, ClosA23 leader + ClosA23 core peptide) and the fourth is a unique peptide (ClosA4 leader + ClosA4 core peptide) (Figure 64). In here, preClosA1-3 and ClosA1-3 were referred to as preClosA1 (there is 1 amino acid difference in the leader peptide) and ClosA1 respectively, unless otherwise stated. The biosynthesis of the active *clos* cluster is illustrated in Figure 64 and it was predicted to involve the expression of a linear preClosA peptide and the introduction of modifications such as dehydration of serine and threonine residues and the subsequent ring formation between the dehydrated amino acids and the cysteine residues.

Figure 64 illustrates the proposed steps for the biosynthesis of preClosA peptides which is based on comparison to other lantibiotic peptides [63, 69].



A.

B.

Figure 64 - Illustration of the predicted structure of preClosA and the steps for its biosynthesis: (A) preClosA1, (B) preClosA4.

ClosA1 leaderless peptides have the same sequence. However, only the sequences of the leader peptides for ClosA2 and ClosA3 are identical. The ClosA1 leader peptide has a G instead of an A after the first methionine amino acid.

Black arrows show two putative leader cleavage sites for ClosA1 and for ClosA4 as hypothesised by Hatziioanou [97].

The legend for the steps involved in the biosynthesis and the PTM of preClosA peptides is as follows:

1. Linear preClosA precursor peptide; the amino acids that will be dehydrated by ClosB are in red (serine) and in blue (threonine). Highlighted in red are also the threonine residues that are not being modified by ClosB as hypothesised by Hatziioanou [97].
2. Linear peptide with dehydrations in to make dehydroalanine (Dha) and dehydrobutyrine (Dhb).
3. The blue arrow highlights the amino acids that are involved in the (Me)Lan ring formation.
4. PreClosA precursor peptide with dehydrations and rings formed.
5. Leaderless and active Clos peptide with dehydrations and rings formed.

The predicted structure of the preClosA peptides is based on the comparison to the nisin lantibiotic peptide since *clos* has high sequence homology to nisin. The predicted start site for the ClosA1 peptide is either IE or YK (Figure 64, A), whereas the start site for ClosA4 could be IP or more likely IT (Figure 64, B) as highlighted by the black arrow. The sequence of ClosA1(IE), with IE as start site, would give a lantibiotic which is 3 - 4 amino acids longer than nisin. The ClosA1(YK) peptide, with YK start site, would have the same size as nisin but with a leader peptide that has a different C-terminal sequence. The unique ClosA4 peptide with an IT start site would be of a similar length to nisin; the PRIT amino acid sequence in ClosA4 is common to other lantibiotics such as nisin, epidermin and staphylococcin T [97, 161, 169, 178].

The hypothesis was that the *clos* machinery introduces PTM in the preClosA peptides. Therefore, the aims of this chapter are to purify and to identify the preClosA peptides, to determine the structure of different forms of the preClosA peptide and to investigate their PTM. In order to investigate this, mass spectrometry analyses were performed on gel slices and pure concentrated Clos peptides produced by immunoprecipitation (IP) from an *L. lactis* strain encoding for the *clos* cluster using the heterologous system described in chapter 5. LC-MS provides highly accurate mass, and after trypsin digestion, there is also information about Clos peptide identity and its sequence.

6.2 MATERIALS AND METHODS

6.2.1 Small scale IP purification of preClosA peptide using antibody antigen method

The pClosCluster in *L. lactis* UKLc10 strain was selected for the scaled up production and purification of Clos peptides. For the IP reaction, named WB2 (Table 28) the pClosCluster+pP_{nisA}_ *cA34* in UKLc10 strain was selected. The strain was grown to OD_{600} of 0.5, then it was induced with 10 ng/ml of nisin for 3 h at 30˚C before harvesting to prepare cell extracts. Cell extracts and cell-free medium samples were prepared and used as the starting material for preparation of the 6 kDa preClosA peptide. The Thermo Scientific Pierce IP Kit was used to obtain purified Clos peptide as described in sections 2.4.6 and 2.5.

At first, a few small scale immunoprecipitation (IP) reactions were performed to optimise the purification steps. The optimisation included the testing of different ratios of antigen/antibody (Ab/Ag) and the use of crosslinking (DSS) to immobilize the antiClos leader antibody to the Protein A/G agarose resin. Two types of samples were used as starting material for the IP reactions: 1. cell extracts in 0.025 M Tris HCl, pH 7.4 buffer or in IP Lysis/Wash buffer from the kit, both in the presence/absence of a protease inhibitor cocktail; 2. cell-free culture supernatant.

Aliquots of all flow through and elution fractions were collected at every stage of the purification process and the presence of the Clos peptides was visualised by western blotting as described in section 2.4.5. In addition, Maldi-ToF MS and LC-MS were used to monitor the purification and the elution of Clos from the resins.

After the ratio of antibody to antigen was adjusted (based on the rough estimation that 3 mg of antibody can bind approx. 200 µg of Clos), a large amount of Clos was purified in one scaled up IP reaction performed with cell extract (0.025 M Tris HCl, pH 7.4 buffer). Again, aliquots of the fresh starting material and any collected fractions were analysed by western blotting and MS to detect the preClosA peptide.

Some of the final eluted fractions still contained detergent from the buffers in the kit, and they were therefore re-purified on C8 cartridges (HyperSep). The preClosA peptide was eluted from the cartridge with steps of increasing concentrations of acetonitrile (in increments of 10 %) in 0.1 % formic acid (see section 2.5).

Name of experiment	**Details of buffers and protocol**	
WB1	CE in 0.025 M Tris HCl pH 7.4 as cell extraction buffer pClosCluster in UKLc10 (50 ml culture)	1 ml of CE (~15-20 mg) + 100 µl of Ab (¬3000 µg of total protein) incubated overnight at 4°C while vortexing. 50 µl of slurry (resins) incubated overnight in a falcon tube at 4°C while vortexing. Expect: antibody antigen complex to elute.
WB2	CE in 0.025 M Tris HCl pH 7.4 as cell extraction buffer pClosCluster+pP_{nisA}_ *cA34* in UKLc10 (50 ml culture)	
WB3	CE in 0.025 M Tris HCl pH 7.4 as cell extraction buffer (650 ml culture)	
WB4	CE in 0.025 M Tris HCl pH 7.4 + protease inhibitors as cell extraction buffer (650 ml culture)	
WB5	CE in IP Lysis + protease inhibitors as cell extraction buffer (650 ml culture)	
WB8	1 ml cell free medium	1 ml cell free (no cell extract/no TCA) adjusted to pH 7.4 mixed with 0.2 % NP-40 + 100 µl Ab Incubated overnight at 4°C, vortexing
WB9 a	CE in 0.025 M Tris HCl pH 7.4 as cell extraction buffer (650 ml culture) No crosslinker	Same protocol as for WB1 to WB5 Expect: pure Clos to elute.
WB9 b Big reaction Scale up	CE in 0.025 M Tris HCl pH 7.4 as cell extraction buffer (4 x 650 ml culture) With crosslinker	1 ml of slurry (500 µl of resin) + 0.5 ml 20 x coupling buffer + 2 ml antibody (¬64 mg total protein) + 6.5 ml UPH_2O, incubating for 2 h vortexing; Put on column and wash a few times: 1 ml of 1 x coupling buffer and 2 x 3 ml of 1x coupling buffer; Use 1 ml of 2.5 mM DSS and add 3.75 ml water and 250 µl of 20 x coupling buffer to 5 ml total volume (for crosslinking); Mix in the column and incubate; Spin and wash the column with elution buffer and IP Lysis/Wash buffer; Add 20 ml of Clos cell extract (approx. 300 mg total protein) in 0.025 M Tris HCl pH 7.4, incubate overnight in falcon tube, vortexing; Recover the mix and place back in the column, wash with IP Lysis and 1 x 0.025 M Tris HCl pH 7.4 and conditioning buffer. Elute with 5 x 0.5 ml elution buffer. Expect: ~200 µg of Clos to elute.

Table 28 - Details for small scale IP reactions with/without crosslinker, different extraction buffers were used; one large scale IP reaction with crosslinking. CE is cell extract. Ab is antibody, DSS crosslinker is from the IP kit.

For the small scale IP reactions, the same protocol as in the manufacturer's instructions was used unless otherwise specified. For experiments WB1 to WB5 and WB8 no crosslinker was used so an Ab-Ag complex is expected to elute from the small scale IP reactions. WB9a is a small scale reaction with crosslinker and WB9b is a scaled up IP reaction; only purified preClosA, not in a complex, is expected to elute because of using the crosslinker.

6.2.2 Large scale IP purification of preClosA peptide

Large scale IP purification of preClosA was performed in order to isolate the peptide in high yield, and to identify preClosA, for activity measurements of preClosA in solution and to understand PTM introduced by the *clos* machinery in the preClosA peptide.

Based on previous estimations on the optimal antibody antigen ratios, a large scale IP reaction experiment was designed (Table 28). The cell extract was prepared in 0.025 M Tris HCl, pH 7.4 using the DSS crosslinker as follows: 1 ml of slurry (500 µl of resin) was first incubated with coupling buffer and 2 ml of antibody (¬64 mg total protein) and water for 2 h, vortexing in order to allow for the binding to occur. The mix was loaded on a column, washed and the crosslinking reaction was set up by incubating 1 ml of 2.5 mM DSS crosslinker with coupling buffer and water up to 5 ml total volume. The mix was incubated for several hours on a column, then washed. Next, the crosslinked antibody coated resins were vortexed with 20 ml of Clos cell extract (¬ 300 mg total protein) in 0.025 M Tris HCl pH 7.4 overnight. This step was performed to allow for the specific binding of preClosA to the antiClos leader antibody. The subsequent steps were performed as described in chapter 2 and the fractions were assessed in western blot to confirm the elution of preClosA.

6.2.3 Structure elucidation of the 6 kDa preClosA peptide

The method was provided by JIC Proteomics Facility and performed by Dr. Gerhard Saalbach.

Purified intact preClosA peptide was characterised by LC-MS on a Synapt G2-Si mass spectrometer coupled to an Acquity UPLC system (Waters, Manchester, UK). Aliquots of the samples were injected onto an Aeris WIDEPORE 3.6µ C4 column (Phenomenex, Macclesfield, UK) and eluted with a gradient of 1-95 % (Acquity curve 7) in 14 min with a flow rate of 0.2 ml min^{-1}. The mass spectrometer was controlled by the Masslynx 4.1

software (Waters) and operated in positive MS-ToF and resolution mode with a capillary voltage of 2.5 kV and a cone voltage of 40 V in the m/z range of 50-1600. Leu-enkephalin peptide (1 ng ml^{-1}, Waters) was infused at 10 µl min-1 as a lock mass and measured every 30 s. Spectra were generated in Masslynx 4.1 by combining a number of scans, and the spectra were deconvoluted using the MaxEnt3 tool in Masslynx.

Gel slices containing a 6 kDa band reacting with the antiClos leader antibody (chapter 2) were washed, treated with trypsin, and extracted according to standard procedures adapted from Shevchenko *et al.* [121]. Aliquots were analysed by nanoLC-MSMS on an Orbitrap Fusion™ Tribrid™ Mass Spectrometer coupled to an UltiMate® 3000 RSLCnano LC system (Thermo Scientific, Hemel Hempstead, UK). The sample was separated on a PepMap™ 100 C18 LC Column (C18, 2 µm, 500x0.75 mm, Thermo) using a gradient of 0.75 % min^{-1} acetonitrile from 6 % to 40 % in water/0.1 % formic acid at a flow rate of 0.3 µl min^{-1} and infused directly into the mass spectrometer. The mass spectrometer was run in positive ion mode with quad isolation at 120K resolution over the mass range 400-1600 (*m/z*) for the precursor scans (orbitrap). One microscan of 50 ms with an AGC target of 2e5 was used. MS2 threshold was set to 2e4 and precursors fragmented by both CID and HCD with CE=30 and an isolation window of 1.6 Da (quadrupole) using the automatic maximum speed option with ion injection for all available parallelizable time. Dynamic exclusion was set to 1 count and 40 s. Recalibrated peaklists were generated using MaxQuant 1.5.3.30 [122] and the database search was performed with the merged HCD and CID peaklists using Mascot 2.4.1 (Matrixscience, London, UK). The search was performed on a *Lactobacillus lactis* protein sequence database (uniprot) to which the *clos* peptide sequences had been added with a precursor tolerance of 6 ppm and a fragment tolerance of 0.6 Da. The enzyme was set to trypsin/P with a maximum of 2 allowed missed cleavages. All used modifications (C(-33) and C(-1) of cysteine, dehydration (-18) of serine and threonine, S(-17) of serine, S(+15) of serine, oxidation of methionine and carbamidomethylation (CAM) of cysteine) were set as variable modifications. The Mascot search results were imported into Scaffold 4.4.1.1 (www.proteomsoftware.com) using identification probabilities of 99 % and 95 % for proteins and peptides.

6.3 RESULTS AND DISCUSSION

6.3.1 Small scale IP purification and investigation of the 6 kDa peptide on western blot and MS

In order to perform structure characterisation and to test the activity of pure peptide in solutions, a method was developed to specifically extract preClosA from cell cultures. Previous work (chapter 5) involved the loading of cell extracts or TCA precipitated proteins on a gel and to excise the 6 kDa gel bands with the aim to have MS confirmation that the peptide was indeed preClosA.

MS analysis of 8 individual protein gel slices with constructs in UKLc10 confirmed the presence of several forms of preClosA peptides. MS results (Appendix 3) using preClosA from the excised protein bands, suggested that they still contained a large number of proteins, and other methods would be needed for the selective purification of preClosA from a mixture of proteins.

After developing a pClosCluster construct in UKLc10 that allowed for an improved expression of the preClosA target protein, detection of the preClosA peptide was addressed. Western blot analysis only allowed for the detection of a 6 kDa peptide and further analysis is needed to confirm the protein identity.

A small scale IP reaction was performed using the manufacturer's instruction with optimised buffers and concentrations. The initial signal for the detection of preClosA was very weak in the western blot (elution 2, 3, Figure 65). A 6 kDa peptide was detected by the antiClos leader antibody in different fractions: in the starting material (cell extract in IP Lysis buffer), in elution 2 and elution 3, in different flow though wash steps. Eluate 2 gave a positive signal in the western blot (Figure 65) and it was selected and analysed by LC-MS and by Maldi-ToF (section 6.3.2-6.3.3). The amount of purified preClosA was enough for its detection on Maldi-ToF which confirmed the presence of the preClosA peptide (Figure 66).

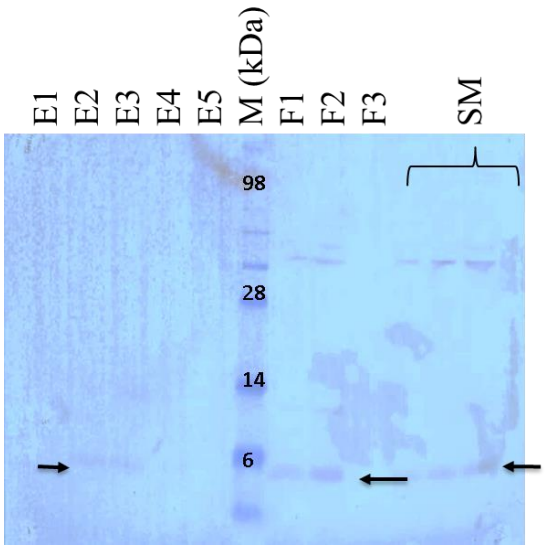


Figure 65 - Detection of a 6 kDa peptide on western blot with antiClos leader antibody.

Samples were eluates (E) from antibody antigen purification reaction using crosslinking and IP lysis buffer.

SM is cell extract total protein as the starting material. F1 is flow through after 4 h incubation, F2 is flow after the resin was washed, F3 is wash step after elution. The antiClos leader antibody was used at 1/100 dilution. The black arrow highlights the 6 kDa peptide. M is the SeeBluePlus 2 marker in kDa.

Results in Figure 66 indicated that one of the washing buffers used for the purification of preClosA contains a detergent; this was carried over and the eluted preClosA was contaminated with the detergent which interfered with the Maldi-ToF signal (Figure 66). The IP Lysis/wash buffer contains the 1 % NP-10 detergent so in order to remove contamination, a different extraction and wash buffer were considered. Alternatively, C8 cartridges were used to re-purify some of the eluates.

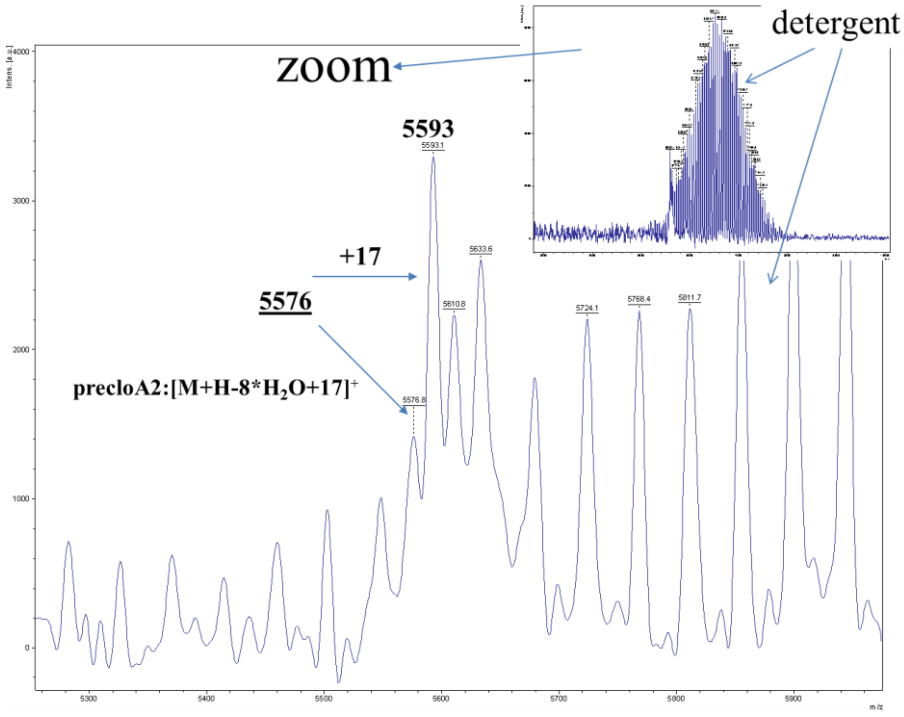


Figure 66 - Maldi-ToF analysis of purified preClosA (elution 2) from an IP reaction using crosslinker.

The values for the Maldi-ToF measurement are not perfectly accurate due to the heavy contamination with detergent. The peak at 5576 most likely corresponds to preClosA2 (expected *m/z* = 5574.7 Da).

Detection of nisin from isopropanol extraction using Maldi-ToF is one of the most common methods for the characterisation of the peptide. In general, this method did not work for the detection of preClosA. Only in the presented attempt (Figure 66) peaks which would fit the expected mass of preClosA were detected. The masses of the main observed peaks most likely correspond to preClosA2 with 8 dehydrations (expected m/z = 5574.7 Da) and the same compound but with an addition of 17 Da was detected (peak at 5593 Da). The additional mass of 17 Da has also been observed in many spectra acquired for preClosA by LC-MS and seems to be a common pattern encountered in this work during the MS analysis of preClosA. The shift in mass is due to the fact that the instrument was not perfectly calibrated for this mass range however the most abundant peak is most likely preClosA2 at 5576 Da. LC-MS was also used for the detection of preClosA, however it will be discussed along with the other spectra in a later section.

Several factors were varied: buffers for cell extraction and for the actual purification (incubation time with the beads, the use of a crosslinker, and the main source for the preClosA peptide). Some of the cell extractions and the subsequent steps were performed in IP Lysis buffer or in 0.025 M Tris HCl, pH 7.4 buffer. It was considered that the crosslinker could affect the binding of the antiClos leader peptide antibody, therefore two

different preClosA immobilization methods were tested: a one step IP method (with crosslinker) or a sequential IP method (no crosslinker) as described in chapter 2.

It was assumed that the elution buffer from the kit could be too weak and preClosA might still be bound on the column which may explain the low yield in the eluates. To test if any amount of preClosA is still bound on the column after the final elution step, 10 to 20 µl of the beads were boiled with SDS loading buffer (using the protocol in section 2.5), and analysed by western blotting (Figure 67).

Six small scale IP reactions were prepared using no crosslinker to investigate if preClosA (in complex with the antibody) was eluted in a much higher yield. The ratio between the antibody and the antigen that were needed were estimated and it was decided that 1 ml of CE (¬15-20 mg of total protein) and 100 µl of Ab (¬3 mg of total protein) would allow for the purification of the preClosA peptide as detailed in Table 28.

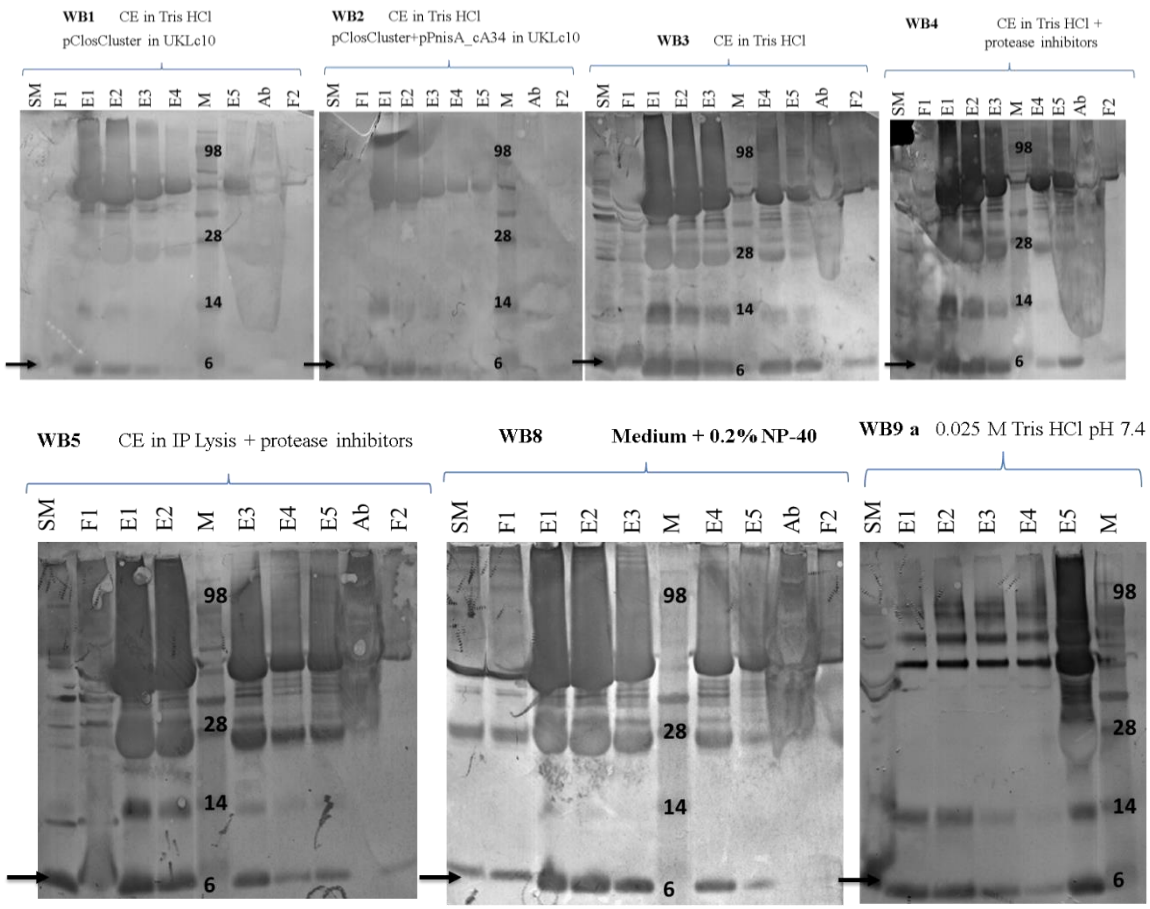


Figure 67 - Western blots to compare different extraction buffers in order to maximise the yields of extracted preClosA peptide. Efficient extraction of 6 kDa using different buffers.

Several small scale IP reactions were performed and analysed here using the no crosslinking method. The same amount of sample and eluates was loaded in each well to compare the amount of extracted preClosA. The antiClos leader antibody was used to visualise the bands at 1/100 dilution. The IP reactions and their western blots were called WB1 to WB5, WB8, WB9 a.

Samples: eluates 1-4 (E), E5 is a complex mix of resin, antibody and the uneluted preClosA peptide. F1 is flow after overnight incubation with the sample, F2 is the flow through after wash step, M is SeeBluePlus2 marker in kDa, SM is the starting material, CE is cell extract total protein as the starting material. The black arrow highlights the presence of the 6 kDa peptide.

The IP lysis buffer contains 1 % of NP-10 detergent and if used it is expected that more total protein will be extracted compared to using Tris HCl only buffer; it is unclear if increased total protein mix would mean an increased amount of the preClosA peptide. Therefore, in the WB8 experiment, the detergent containing buffer as well as the cell free supernatant/medium were used to facilitate the binding of the preClosA peptide to the antibody coated resins.

Here, the pClosCluster in *L. lactis* UKLc10 strain has the *clos* cluster coding sequence expressed on a plasmid, therefore the preClosA peptide is expected to be accumulated and/or secreted outside the cell. Results in Figure 67 indicated that a 6 kDa peptide reacting with the preClosA antibody in western blots was efficiently extracted from the

complex total protein using different buffers. A 6 kDa band was mainly detected in elution 2 and 3 (Figure 67) and MS analysis of these eluates confirmed that the 6 kDa preClosA was purified both from the total protein in the cell extracts as well as from the cell free medium. Very high intensity bands were detected in elutions 1 to 3 but also in elutions 4 and 5 in some IP reactions (Figure 67).

Therefore, successful purification of preClosA peptide was possible using two different samples (cell extract and TCA precipitated supernatant) and three buffers: 1) 0.025 M Tris HCl, pH 7.4, 2) 0.025 M Tris HCl, pH 7.4 plus protease inhibitors or 3) IP Lysis/wash buffer plus protease inhibitors buffer. Elution 5 contains unbound preClosA protein recovered from the resins after elution 4. Elution 5 in the western blots (Figure 67) confirmed that a small amount of the preClosA was still bound on the column.

Some western blots for samples where the crosslinker IP method was used are illustrated in Figure 67, WB4 and results confirmed that the eluates contained an antibody-preClosA complex because high intensity bands were detected for the antibody in the upper area of the blot. The LC-MS analysis of the eluates confirmed that preClosA can be isolated in the absence of a detergent containing buffer (section 6.3.3).

Based on the results in WB9a, where the 6 kDa peptide was purified in 0.025 M Tris HCl, pH 7.4 buffer, it was decided that this buffer system as well as the crosslinking method should be used for the large scale purification and high yield elution of the preClosA peptide described in section 6.3.3.

The advantage of using the one step IP method is that it allows the elution of preClosA without contamination from antiClos leader peptide antibody, whereas in the traditional sequential IP method preClosA is eluted in a complex with the antibody (Figure 67). TCA precipitated supernatant was not used as a source for the starting material since TCA can denature proteins/peptides irreversibly which would affect the binding of preClosA peptide to the antibody.

Even though a large amount of preClosA peptide was successfully isolated, the activity of the pure peptide in solution was not tested in this work. Future work should involve to take the preClosA peptide and place it on the agar plate which will then be overlaid with trypsin to allow for the release of active peptides tested against different indicator strains.

6.3.2 Protein extraction and analysis of preClosA peptide from the excised gel slices

6.3.2.1 *Mascot database search for identity of (pre)ClosA tryptic peptides*

In order to identify the tryptic peptides according to their specific masses and sequences, the acquired spectra were used for database searches. Spectra were from a few protein gels with cell extracts from the two strains and a total of 8 gel slices corresponding to 6 kDa were cut from the protein gels and analysed by trypsin digestion and nano-LC-MS/MS. Both pClosCluster and pClosCluster + pP_{nisA}_ *cA34* in UKLc10 strains were used.

The results shown in Appendix 3, clearly demonstrate the presence of preClosA in all analysed gel slices. All possible detectable tryptic peptides of preClosA1 and preClosA23 have been detected with at least 95 % probability, so that the complete sequence of those preClosA forms has been covered. A peptide from the leader sequence of preClosA4 has also been detected indicating the presence of a certain amount of this form as well. Altogether, 368 spectra were matched to the three identical preClosA1 peptides (Table 29). This is the first piece of evidence that the preClosA peptide with the correct amino acid sequence is present in the analysed samples.

Number of PSM's	**Type of preClosA23 tryptic peptide**	**Location**
368 total number of tryptic peptides		
109	AKFDDFDLDVTK	Leader peptide
1	AKFDDFDLDVTKTAAQGGIEPK	
30	FDDFDLDVTK	
127	SACTPGCPTGILMTCPLK	Rings A-C area (Core peptide)
5	SKSACTPGCPTGILMTCPLK	
22	TAAQGGIEPK	Leader peptide
4	TAAQGGIEPKIEPKYKSK	Leader peptide & start of Core
66	TATCGCHITGK	Rings D-E area (Core peptide)
4	YKSKSACTPGCPTGILMTCPLK	

Table 29 - Typical tryptic peptides identified in the mascot search. PSM's: peptide spectra matches.

Using the variable modification settings described above and listed in Appendix 3, a number of peptides with dehydrated Ser and Thr residues (Dha and Dhb) were detected (Table 29). Altogether, the results from the 8 samples show that every Ser and Thr residue (8 residues) in the core *clos* sequence were dehydrated, while no dehydration was detected in the leader sequence as expected.

Cysteine residues were alkylated with iodoacetamide (IAA) during sample preparation, and this modification was also detected by the database search. Overall it was found that all 5 Cys residues of the *clos* sequence can be modified. However, by checking individual spectrum/peptide matches a number of spectra were observed with unmodified (unlabbeled) Cys residues.

For instance, a mixture of peptides for the C-terminal part of preClosA1 (Ring D-E, TATCGCHITGK) was identified, each with at least 95 % probability and the possible ring formation for this peptide is discussed in section 6.3.2.3. Altogether, 66 spectra were matched to this peptide. Some of the peptides (11) were dehydrated (all 3 Thr residues or the 2 Thr residues essential for rings). Only those dehydrated peptides had free Cys residues (no IAA modification), all other peptides had at least 1 Cys residue modified, 26 had both Cys residues modified (Table 30).

At the same time, in the spectra for the dehydrated peptides no MS2 fragments (neither b- nor y-ions) were formed from the part of the sequence potentially forming the Rings D and E as described in section 6.3.2.3 (Figure 68).

Number of PSM's		Modifications seen in the **TATCGCHITGK** peptide in Mascot in	Number of possible rings
66 total number of tryptic peptides			
26		Both C + CAM	No rings
8		Only C4 + CAM	1 possible ring
21		Only C6 + CAM	1 possible ring
11		Both C free	2 possible rings
11	7	3 dehydro T	
	4	2 dehydro T (T1, T3)	
1		with C(-1) only	No rings

Table 30 - Detection of alkylated cysteine residues and dehydrated amino acids in TATCGCHITGK peptide. PSM's: peptide spectra matches.

T1AT3C4GC6HIT9GK. T is threonine and the number of the residue is associated with this peptide. Plus CAM (+CAM = carbamidomethyl) means that cysteine (C) residue is alkylated since it is not forming a ring and it is free in the peptide.

6.3.2.2 ***Cleavage of the preClosA peptide with trypsin***

Cleavage of the preClosA peptides with trypsin, known as trypsinolysis, typically involves the digestion of a peptide after the lysine (K) or arginine (R) residue when they are not followed by proline. Some lysine sites in the Clos peptides may be more prone to be digested than others such as the Lys sites around the ring structures. It was assumed that trypsin can cleave after a lysine residue which is incorporated inside a ring structure, however fragments will not be formed as they are held together by the ring structure (section 6.3.2.5). The ClosA1 peptide does not have any arginine residues whereas the leader peptide of ClosA4 contains Arg. ClosA1 has the advantage that trypsin can cleave at the predicted start site (the YK), therefore one of the tryptic peptides that is formed could be the active form of the Clos peptide. Based on the predicted cleavage sites for trypsin (blue arrow), preClosA can be fragmented into several tryptic peptides that are listed in Table 31 and in more detail in Appendix 3.

MAK FDDFDLDVTK TAAQGGIEPK YK SK SACTPGCPTGILMTCPLK TATCGCHITGK
FDDFDLDVTK [a] TAAQGGIEPK SACTPGCPTGILMTCPLK TATCGCHITGK
AKFDDFDLDVTK [b] SKSACTPGCPTGILMTCPLK YKSKSACTPGCPTGILMTCPLK

Table 31 - Sequence of preClosA1 with cleavages sites for trypsin (blue arrow) and tryptic peptides detected in the Mascot search.

[a] The results in Appendix 3 indicate that there are no missed cleavages, therefore 4 peptides were more frequently observed. However, the small peptides YK and KS are not observable.

[b] Missed cleavages have been observed for K3, K25, K27 resulting in three observed peptides.

According to the fragmentation pattern given by digestion with trypsin (Table 31, Appendix 3), it was observed that the preferential cleavage site for trypsin is at the K↓SAC site (within the region for Ring A which proves no Ring A) as well as around the rings leaving the fragmentation within the (Me)Lan rings for some peptides of the C-terminal part of preClosA untouched [134].

Table 31 illustrates the location of the trypsin cleavage sites in the ClosA1 core peptides: Lys4-Tyr5, Lys8-Dha9, Lys6-Ala7, Lys26-Abu27; for the ClosA4 core peptide, the cleavage sites are: Lys 21-Abu 22, Lys 29-Leu 30, Lys 31-Gly 32 (if IT is the start site). It is possible that unidentified trypsin sites could be discovered in the future.

6.3.2.3 ***Detection of PTM in the C-terminal part of the preClosA peptide***

A mixture of different forms of tryptic peptide for TATCGCHITGK were seen where some had no rings, others had one ring or even the two expected rings. Results in Figure 68 indicated that there is a strong indication that cysteine was non-alkylated/unmodified by IAA. These cysteine residues and the dehydrated amino acids must have formed two (Me)Lan rings. The first observation on the spectra in Figure 68 is that all the serines and threonine residues were dehydrated, labelled as T-18 (threonine minus 18 Da for loss of water). This means that that they could form ring structures with the neighbour cysteine residues. Next, there are no peaks assigned for TATCGC peptide (in black, forward and reverse) as no fragmentation and no ions could be assigned. A lack of fragmentation indicates that both Rings D and Ring E are likely to be present.

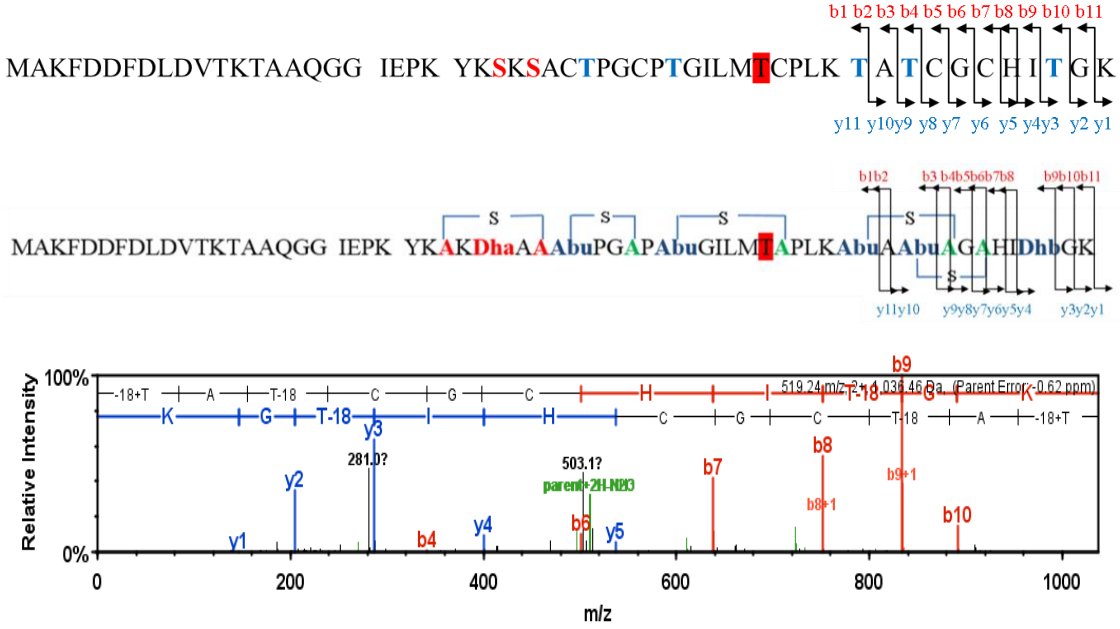


Figure 68 - Mascot search to identify modifications in the C-terminal part of preClosA peptide.

Analysed tryptic peptide: TATCGCHITGK from ClosA1 core peptide detected with 95 % probability. B4 is at the noise level.

The modifications used to identify the presence of rings and of dehydrated residues were: Ser->Dha (T-18), Thr->Dhb (T-18), Cys -> Cys+57. Modifications within the rings were not investigated as they are not expected to take place and the total mass remains the same. The colour coded b and y ions were detected by LC-MS fragmentation. The assigned b4 ion is most likely a false assignment; the intensity of this ion is at the noise level.

Mascot search for the C-terminal peptide, TATCGCHITGK, identified a mixture of fragments with different modifications with 95 % probability. The b and y ions (1-11)

were assigned on the structure of preClosA1 (in red and blue) as well as a spectrum with assigned ions is included (Figure 68). The spectrum shows that there are no ions assigned for T1 to C4 (b1 to b4, the software assigned b4 but it is at the noise level) and for T3 to C6 (y6 to y11) which means that fragmentation was not possible; this is an indication that rings were present since rings are generally known to prevent the MS fragmentation. None of the Cys residues were labelled with IAA since Cys residues did not have a 57 Da corresponding to added IAA. Therefore, the absence of labelling with IAA of TATCGCHITGK was not possible due to the presence of rings. Also, all the Ser and Thr residues are dehydrated as illustrated by the loss of 18 Da (T-18).

The b4 ion appears on the spectrum however, a closer inspection of the spectra reveals that the software assigned b4 ion is at the noise level. Therefore, these ions do not really exist whereas all the other ions in the table and on the spectrum confirm a fragmentation around Rings D-E in TATCGCHITGK but not within the ring region. This peptide was determined with 95 % probability and MS analysis (Figure 70) confirms it contains all the dehydrations and the two possible rings.

6.3.2.4 *Detection of PTM in the N-terminal part of the preClosA peptide*

The *clos* modification machinery is expected to introduce PTM such as dehydrations and rings and so far only a few peptides seem to have all the PTM in the C-terminal part of Clos.

From the N- and C-terminal sequences of the detected tryptic peptides in Appendix 3, it can be concluded that all the potential tryptic cleavage sites (which means all Lys residues) can experimentally be cleaved by complete digestion with trypsin. This includes the Lys27 residue, which is located in the predicted Ring A region of preClosA. The cleavage at this site together with cleavage at the next downstream site (Lys45) leads to the formation of the peptide SACTPGCPTGILMTCPL (Figure 69). This peptide has been identified by 132 spectra (out of a total of 368 spectra for ClosA1 core peptide) from the gel samples. In the vast majority of those peptides all three Cys residues are modified by IAA (e.g. 122x C3, 110x C7, 127x C15, and 107x all three C, were found). Dehydration of Thr residues was observed in 65 spectra.

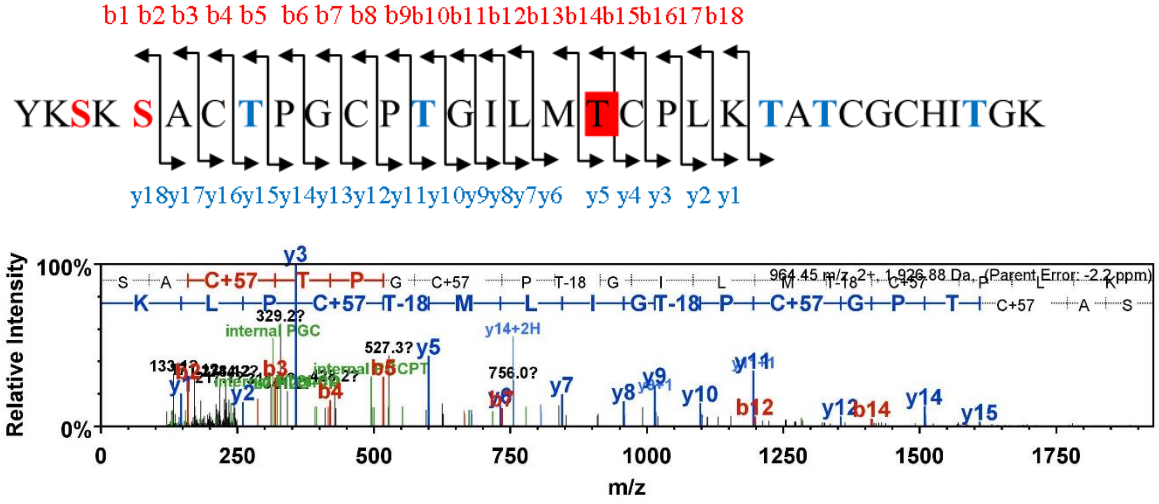


Figure 69 - Mascot search to identify modifications in the N-terminal part of preClosA.

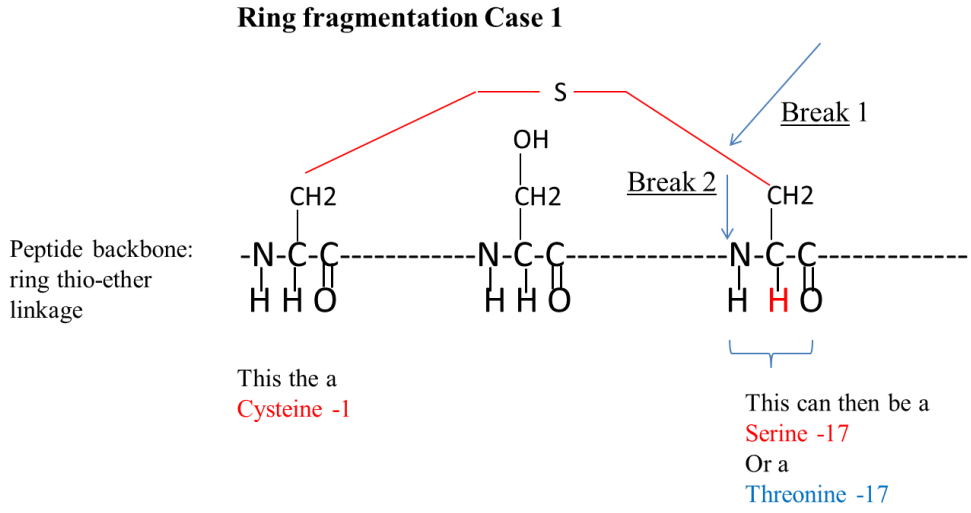
The b and y ions were detected in MS and they were colour coded as highlighted in the table after MS fragmentation. Analysed tryptic peptide: SAC3TPGC7PTGILMTC15PLK from preClosA1 core peptide detected with 99 % probability.

The spectra in Figure 69 shows that some cysteine residues did not form a ring in the original sequence as they were free. Therefore, these Cys residues were labelled by IAA, 57 Da mass addition, (C3+57 and C15+57). Appendix 3 indicates that the cysteine residues were all modified by IAA (C+57), which reconfirms that there is strong evidence that this peptide does not contain rings. All the other tryptic peptides for this region of preClosA were the same. The software also assigned dehydration of the terminal Ser residue in 10 spectra (out of the 132), but visual inspection showed that there is no direct evidence for dehydration of this Ser28 residue. Inspection of the spectra also reveals that MS2 fragmentation occurs throughout the complete predicted ring regions for Rings A to C without the occurrence of the predicted modified amino acid residues for ring breakage as discussed below (section 6.3.2.5).

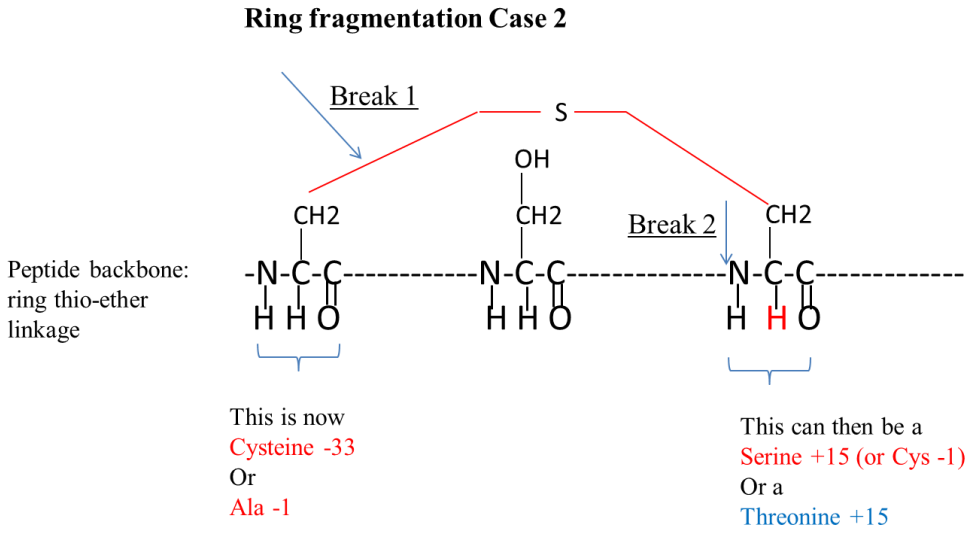
This kind of modification has only been assigned for 6 out of the 132 spectra in the SACTPGCPTGILMTCPL peptide. Visual inspection revealed that 5 of those spectra are of very low quality, and in the remaining spectrum there is no direct evidence for the modified residues (no peaks directly covering the modified residues).

6.3.2.5 *Investigation of ring fragmentation in lantibiotics*

Two possible scenarios for ring fragmentation are illustrated in Figure 70, where the linkages in the rings could be broken by MS. This phenomenon had previously been demonstrated in experiments where different lantibiotics were fragmented based on their structure such as for nisin, lacticin 481, haluduracin β [179-181].



A.



B.

Figure 70 - Illustration of fragmentation in lantibiotics of a ring thio-ether linkage within the backbone of lantibiotic peptides.

(A) Break 1 – sulfur goes to Cys to give Cys (-1), (B) Break 2 – Sulfur goes to Ser to give Ser (+15), Cys (-1) or Thr (+15). Using these proposed modification in database search, they were not detected as being present in the actual peptides.

Fragmentation gives b and y ions and their masses are used to identify the amino acid sequence of the fragmentation product [179].

In case 1, the break will give rise to cysteine (-1 Da) and serine (-17 Da) or threonine (-17 Da). In case 2, cysteine (-33 Da) or alanine (-1 Da) and serine (+15 Da) or cysteine (-1 Da) or possibly threonine (+15 Da) could be formed. These modifications were used in a database search with tandem mass spectra, and no peptides that had the special modifications highlighted in Figure 70 were detected.

In conclusion, it was demonstrated that specific modifications associated with the breakage of a (Me)Lan ring were not identified in the database search even if fragmentation of the ring may take place in preClosA. For the N-terminal part of preClosA, no tryptic peptides that have Rings A-C were detected, however some dehydrations did occur in this peptide. It was demonstrated that in the presence of a linear N-terminal peptide, the full fragmentation of this region of preClosA is possible. This is a strong indication that no rings are present. For the C-terminal part of preClosA, a small number of peptides that did have Rings D-E was detected. However, there were more peptides of this kind without any rings present. Even if the number of tryptic peptides with Rings D-E was small, the results demonstrated the presence of some ring formation. A survey of the literature suggests that there is one example where the Ring A of nisin was fragmented by MS and the b and y ions confirmed that the ring structure was completely cleaved and it split into two fragments [182]. The possible special modifications were discussed and searches were made in the database to confirm that no fragmentation inside the rings was possible in the work with preClosA since no tryptic peptides with these special modifications were found. This is not surprising since the same behaviour is observed with other lantibiotics in contrast to the unusual case of nisin.

6.3.3 Scaled-up production of preClosA and structure elucidation

6.3.3.1 *Large scale IP preparation of the preClosA peptide*

According to the results in the previous chapter, the overexpression of *closA3* and *closA4* genes did not improve the production of *clos* heterologous expression. The pClosCluster in UKLc10 strain was selected for scaled up production, as well as for further antibody antigen purification.

The aim was to increase the amount of the purified preClosA peptide, therefore the same ratio of antibody antigen as in the small IP reactions was maintained (Table 28).

The binding capacity of the resins is 50 mg/ml and the assumption was that the beads would really bind approx. 32 mg of antibody, which would correspond to 64 % of the binding capacity of the used resin. In here, the antibody did bind efficiently to the resins as it was measured by direct detect machine from Millipore (data not shown). A drop in the concentration of the antibody, when it was measured before incubation and after incubation with the resins, was detected. Therefore, the crosslinking reaction was successful as the antibodies were crosslinked to the resins. The crosslinker antibody resin complex was incubated overnight with the cell extract and the bound preClosA peptide was then eluted from the resins as confirmed both by the western blot (Figure 71) and the LC-MS analysis.

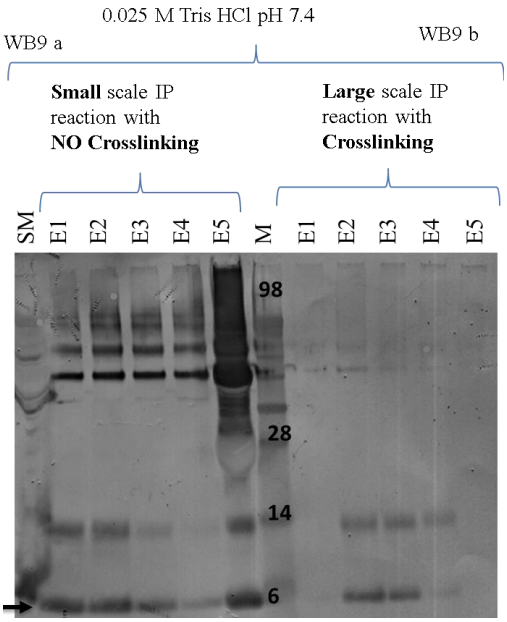


Figure 71 - Western blot to detect preClosA peptide.
Samples are eluates (E) and aliquots from the scaled up purification of Clos using 0.025 M Tris HCl, pH 7.4 buffer and crosslinker. WB9 a is a small scale reaction with no crosslinker, WB9 b is a large scale reaction performed with the crosslinking step. M is SeeBluePlus 2 marker in kDa. The black arrow highlights the peptide at 6 kDa.

Figure 71 is a comparison of a small scale and a large scale IP reaction using the same buffers but with and without crosslinker. Results in WB9,a confirm that preClosA was mainly eluted as a complex with the Ab in elutions 1-3 and a significant amount of Clos in complex with the Ab was still bound to the resins as shown by elution 5. Therefore, the elution of Ab-Clos complex from the beads (Wb9,a) was not entirely efficient since the beads still contained a high amount of non-eluted Clos. Instead, the results in Figure 71 (WB9 b) confirms that 0.025 M Tris HCl, pH 7.4 is a suitable buffer for the successful extraction of specific preClosA peptides. The advantage to use the crosslinking method (Figure 71, WB9 b), is to exclusively extract the preClosA peptide. The 'no crosslinking' method would allow for the extraction of an Ab-Ag complex which is not be suitable to be used for further structure characterisation in LC-MS. This is because preClosA is a much smaller peptide of only 6 kDa compared to the antiClos leader antibody which is typically 150 kDa.

6.3.3.2 *Structure characterisation of the preClosA peptide*

The post-translational modifications (PTM) in preClosA, dehydrations and ring formations, were investigated in this section. PTM are expected to be introduced by the *clos* biosynthetic machinery (Figure 72) [15, 63, 69, 183].

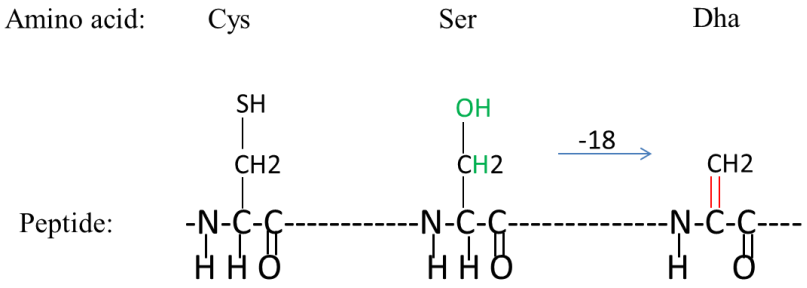


Figure 72 - Illustration of amino acid dehydration which involves the loss of a water molecule (-18 Da) and the formation of a double bond in the new unusual amino acid: dehydroalanine (Dha) or dehydrobutyrine (Dhb) [184]. Relationship between Cys, Ser, Dha residues.

In this process, serine and threonine residues are dehydrated by ClosB. Then, ClosC will mediate the formation of rings between cysteine and dehydrated amino acids residues. The ClosC cyclase couples the unusual amino acids formed in the first step, such as dehydroalanine and dehydrobutyrine, to cysteine residues to generate formation of (methyl)lanthionine ring structures. The dehydration step involves the loss of 18 Da (Figure 72) equivalent to a water molecule. The following cyclization reaction does not involve a mass change since hydrogen only moves across the structures (Figure 73) [184].

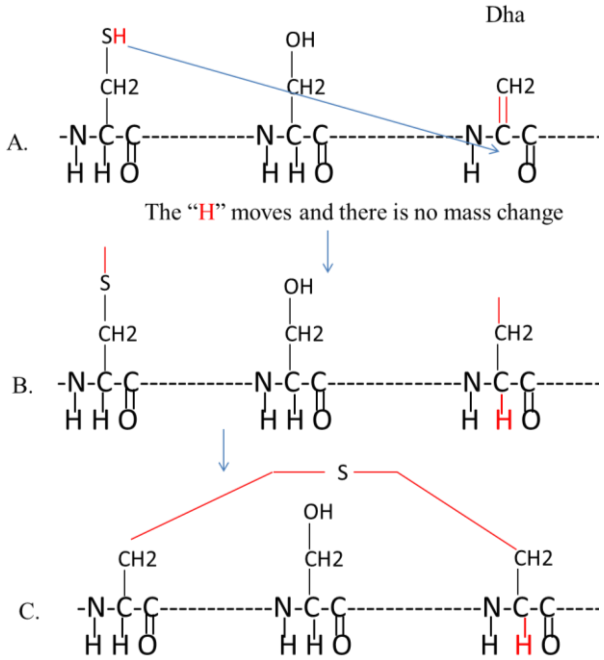


Figure 73 - Illustration of the cyclization between cysteine residues and dehydrated amino acids (A-B) [184, 185].

An essential observation about lantibiotic peptides is that the dehydrated residues (deydroalanine and dehydrobutyrine) have a double bond (Figure 73, A) which is a prerequisite for sulphur to attach to carbon (Figure 73, B) to form a new bond (Figure 73, C) [184]. Even though the PTM processes are drawn separately as a complete dehydration event before the initiation of cyclization, different studies proposed that the dehydration and cyclization steps are interchanging [185, 186].

Therefore, the work in this section involved taking two types of pepties: 1. pure IP eluted preClosA, 2. preClosA from excised protein gel slices, to alkylate the cysteine residues in preClos, to trypsin digest and to characterise the peptide on MS for PTM and structure confirmation.

To study PTM processes in preClosA, the tryptic peptides were generated and analysed by tandem MS. The tandem mass spectra allow for identification of the peptide sequence including the detection of modified (dehydrated) amino acid residues. The observed fragmentation patterns also indicated the presence of rings because these structures usually do not fragment in MS. Furthermore, the commonly used alkylation of the cysteine residues helps to identify free cysteine residues not involved in ring formation.

Since a rare case of ring fragmentation has been reported for nisin [182], the acquired Clos spectra were also checked for possible ring fragmentation.

The preClosA peptide can be synthesised as a linear peptide that is fully or partially dehydrated. The masses for the sequences of the tryptic peptides were determined using the software: http://web.expasy.org/peptide_mass/ (data not shown). The monoisotopic mass was calculated using this software.

To investigate the PTM introduced by the *clos* biosynthetic machinery, dehydrations and (Me)Lan rings, the cysteine residues were labelled with an S-alkylating agent such as iodoacetamide, IAA. Similar work was done to elucidate the structure of the two-component lantibiotic, haloduracin, lacticin 481 [187], subtilin and nisin [188-190].

Ring formation is otherwise difficult to detect in lantibiotics [69, 191, 192]. In here, the structure characterisation of preClosA was possible by using IAA labelling of cysteine residues as well as digestion of lysine residues with trypsin which are methods for common structure characterisation of lantibiotic peptides.

The eluates from the small scale IP reactions that were previously confirmed by western blot (Figure 67) were collected and analysed on LC-MS. The sample was named ‘clos pulldown, big elution 2’. A similar characterisation process of the fractions collected from

the scaled-up IP reaction was performed, however the LC-MS analysis reported in here was performed on the 'clos pulldown, big elution 2'.

Eluate 2 from the 'clos pull down' from several small scale reaction (Figure 67) was loaded on a C4 column for LC-MS analysis. Figure 74 shows the gradient and a base peak chromatogram where the m/z of the most abundant charge state of Clos (8+) was highlighted.

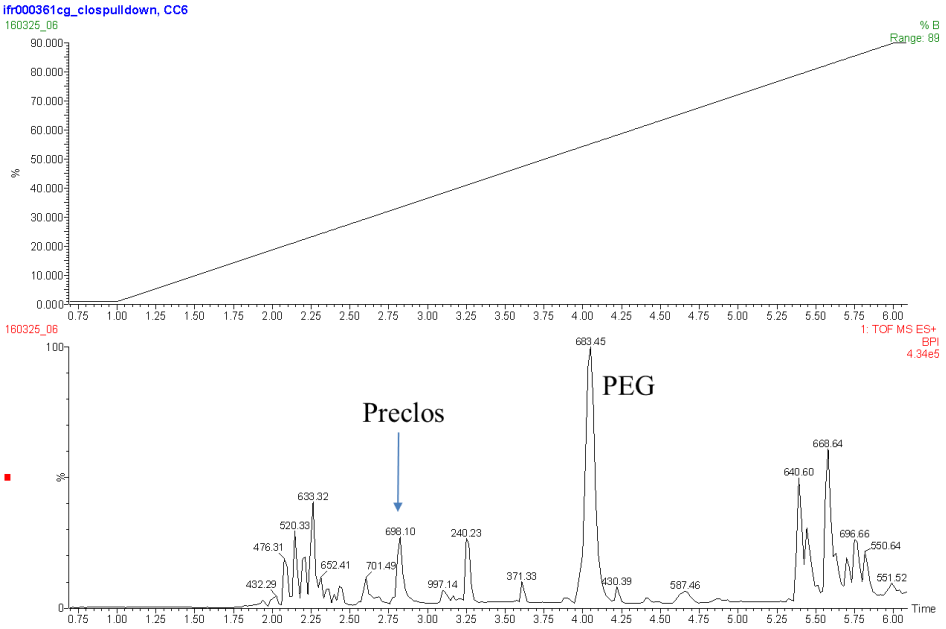


Figure 74 - Elution profile of preClosA (pulldown, big elution 2 sample) on C4 column on LC-MS.

ClosA eluted in 30 % acetonitrile at 2.75 min as highlighted by the arrow; a standard gradient from 10-90 % acetonitrile in 0.1 % formic acid was used.

The spectra under the LC-MS peak at 698.32 (Figure 74) were combined to reveal a series of charge states from 4+ to 9+ (Figure 75).

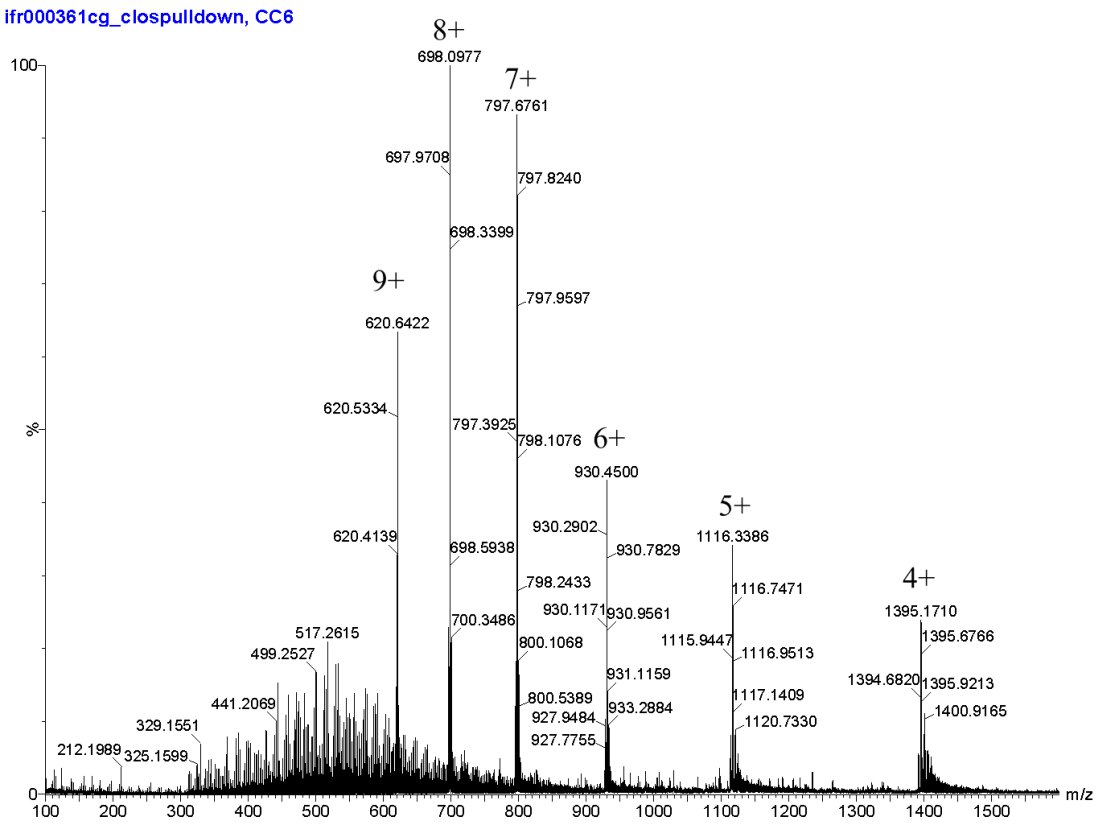


Figure 75 - Different charge states of preClosA obtained from the C4 column.

PreClosA is a 6 kDa peptide that can be multiply charged and it appears as a series of different charge states (Figure 75). The charge state series was deconvolunted to determine the actual mass of the protein. By simplifying the complicated appearance of the spectra, a series of peaks of the charge states is reduced to one single peak of the peptide (Figure 76). The deconvoluted mass was given by the MS software as the singly charged monoisotopic mass [M+H]+, the calculated mass and the observed masses were compared and the matches were highlighted in red (Figure 76,C).

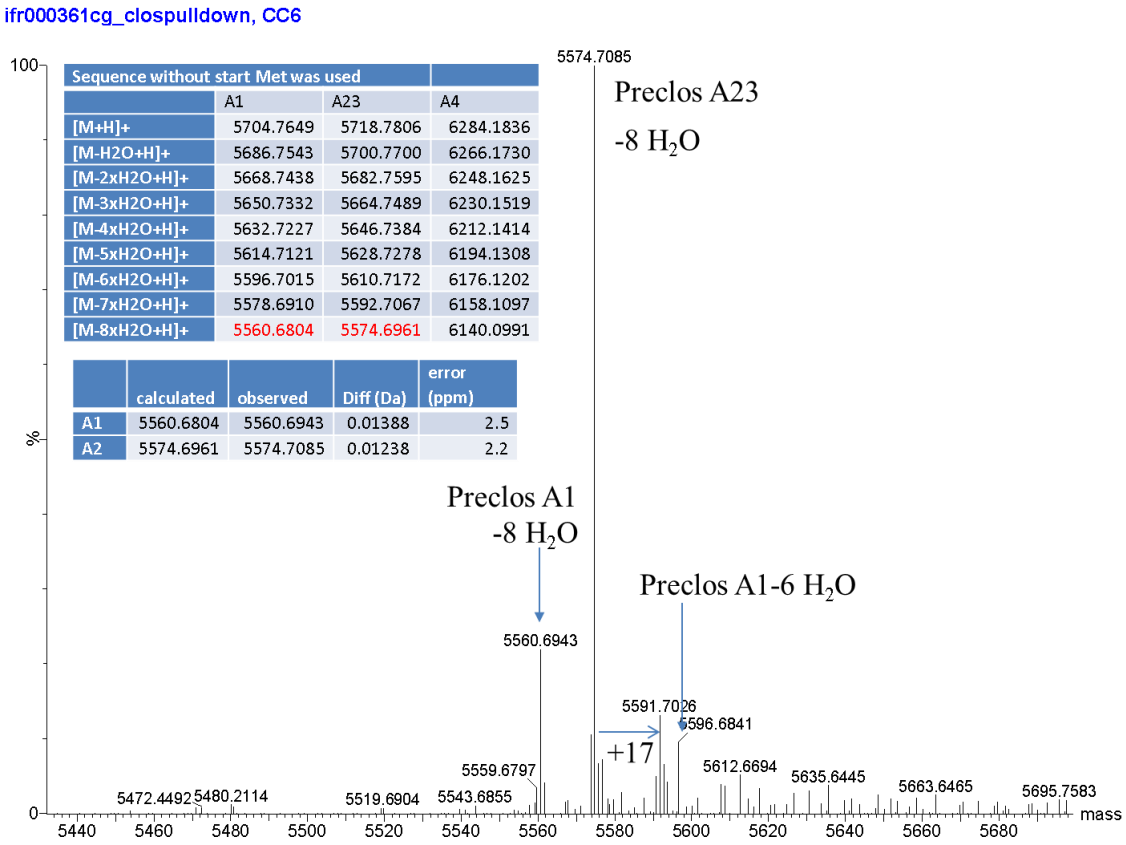
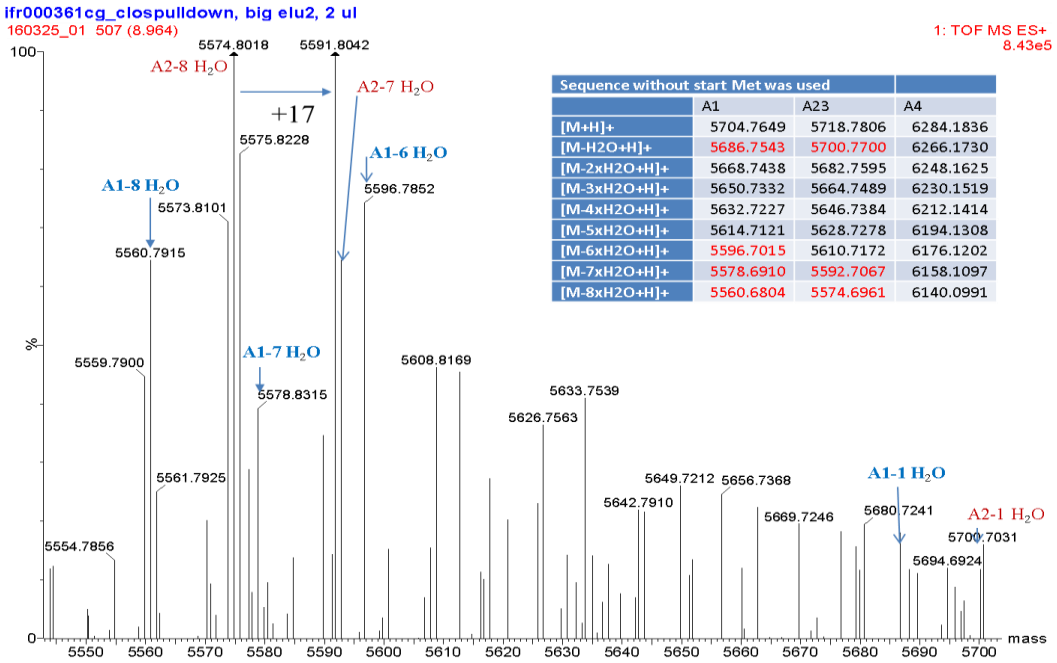


Figure 76 - LC-MS spectra to detect preClosA1 peptide at different dehydrations.

The calculated ppm error confirms that the masses of the peaks fit with the calculate masses in red0. Ppm is part per million.

Figure 76 illustrated the deconvoluted spectrum (Figure 75) showing the m/z for 1+ charge state. The two main observed masses match the predicted masses for preClosA1 and preClosA23 (-8H2O) as seen in the table (Figure 76) as well as one peak has an addional mass of 17 Da.

Figure 77 is a deconvoluted spectrum from another eluted sample and it shows stronger peaks fitting in different dehydration stages of preClosA1 and preClosA23. The dehydrations of specific amino acid residues to give dehydroalanine and dehydrobutyrine was investigated by analysing the tryptic peptides obtained in the Mascot search (Appendix 3) as well as by detecting them in LC-MS analysis (Figure 77). In here, a complex mixture of dehydrated preClosA peptides, mainly for the three preClosA1, with different states of dehydrations for preClosA1 and preclosA23 were observed.



Sequence without start Met was used			
	A1	A23	A4
[M+H]+	5704.7649	5718.7806	6284.1836
[M-H2O+H]+	5686.7543	5700.7700	6266.1730
[M-2xH2O+H]+	5668.7438	5682.7595	6248.1625
[M-3xH2O+H]+	5650.7332	5664.7489	6230.1519
[M-4xH2O+H]+	5632.7227	5646.7384	6212.1414
[M-5xH2O+H]+	5614.7121	5628.7278	6194.1308
[M-6xH2O+H]+	5596.7015	5610.7172	6176.1202
[M-7xH2O+H]+	5578.6910	5592.7067	6158.1097
[M-8xH2O+H]+	5560.6804	5574.6961	6140.0991

Figure 77 - Deconvoluted spectrum showing a complex mix of dehydrated preClosA1 and preClosA23 peptides in red.

Interestingly there was one isolated LC-MS case where the preClosA peptide was detected possibly with a loss of the C-terminal lysine (K) to give group 1; the work done on this particular form of preClos is presented below (Figure 78 to Figure 80). Figure 78 shows the charge state spectrum from another eluted sample of preClosA, however in here there is a second group of charge states shifted to lower m/z as highlighted by the blue arrows. Interestingly, a common feature in the spectra for preClosA is the presence of a peak with an addition of 17 Da (Figure 76, Figure 77).

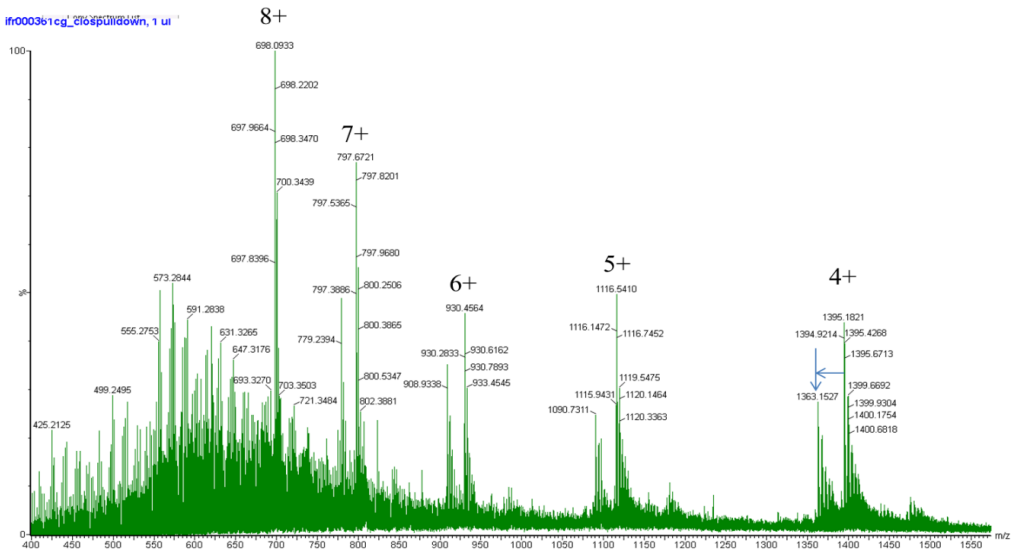


Figure 78 - Different charge states of preClosA1 (group 1, no terminal Lys) from the C4 column. Two peaks per charge state.

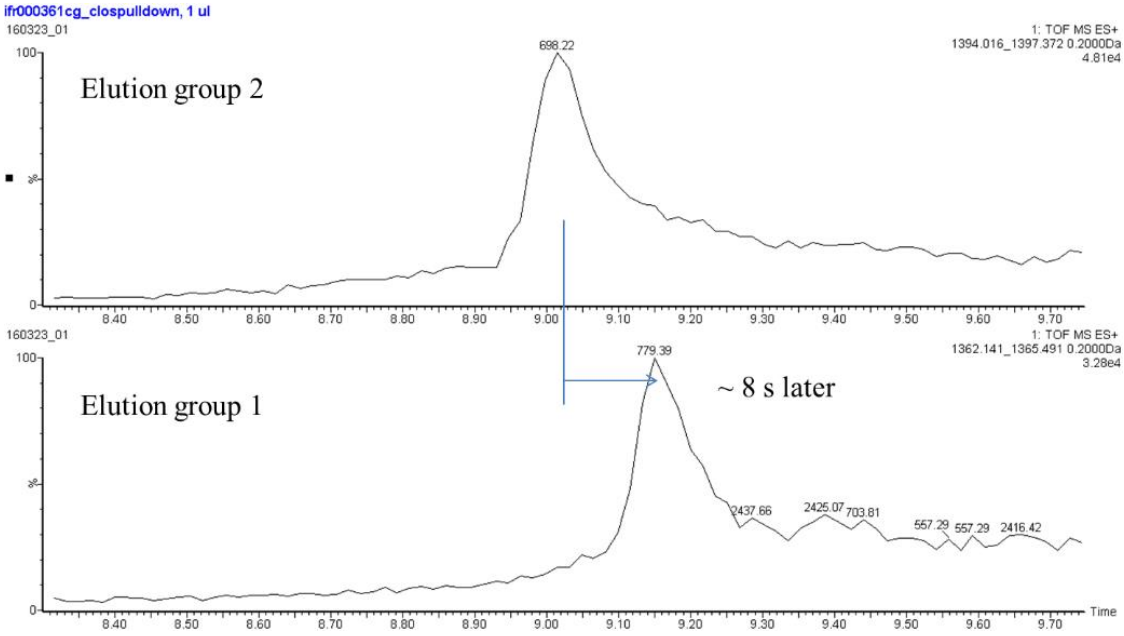


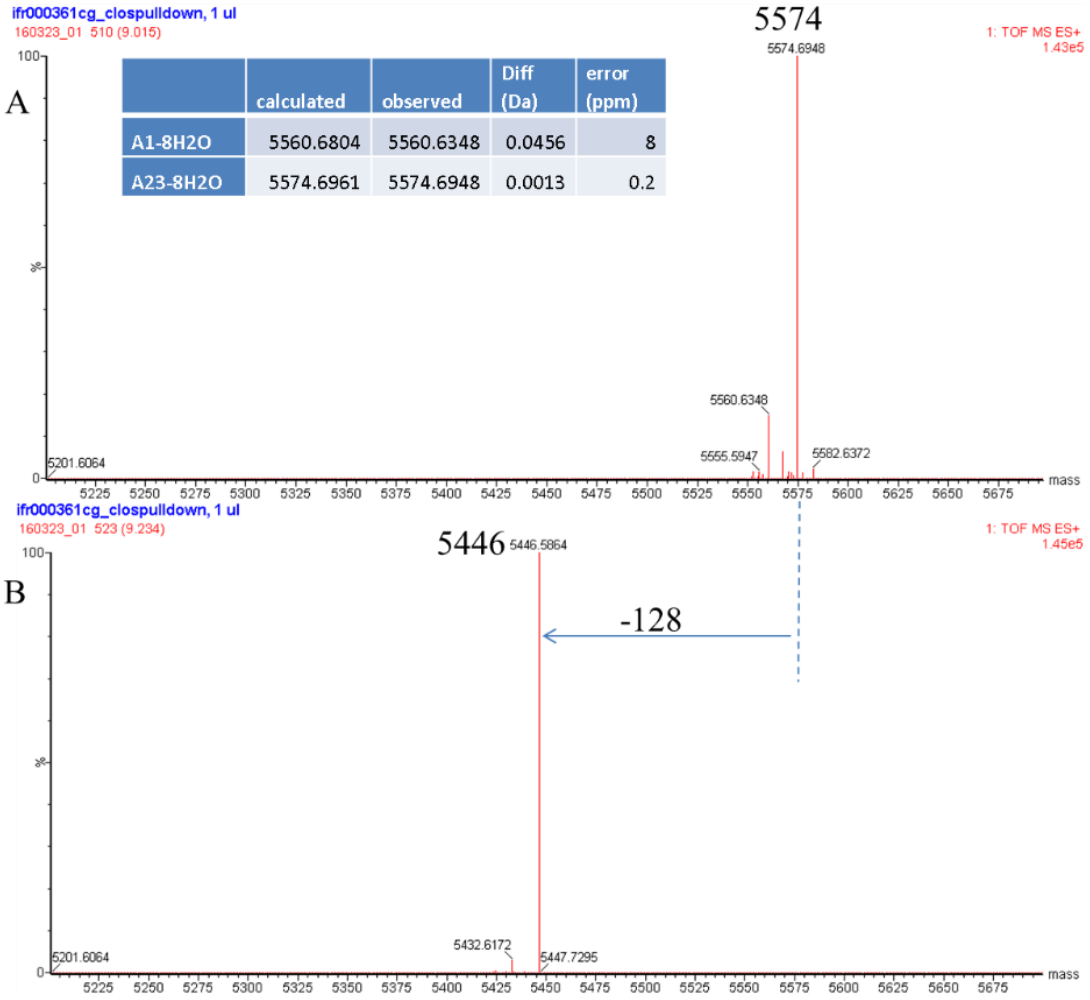
Figure 79 - Retention times of group 1 and group 2 of preClosA. Peak for group 1 elutes later.

Group 2 is the unmodified dehydrated preClosA1 and preClosA23. The two groups elute slightly differently and the deconvoluted spectrum is in Figure 79.

The charge state 4+ was zoomed in to reveal the presence of two forms of preClosA. The spectra for group 1 and group 2 were deconvoluted, and as observed in other experiments, the group 2 contained fully dehydrated preClosA1 (m/z = 5560) and preClosA23 (m/z = 5574) (Figure 80).

The calculated error (ppm) had a very low value which indicates that the observed masses were very accurate and they were identified compared to the calculated masses (see tables in Figure 80).

PreClosA23 is the strongest and most abundant peak since the *clos* gene cluster encodes for three copies of the *closA1* structural genes, but the group 1 and 2 peaks for preClosA1 are also present. In both cases, the mass difference between group 1 and 2 is -128 Da for group 2. This mass difference could be associated with a loss of a Lys(K) from the C-terminus of preClosA which may have been removed during biosynthesis or post-translational processing. Alternatively, preClos may have been degraded or the pClosCluster in the *L. lactis* strain is producing a new form of preClosA peptide.



	calculated	observed	Diff (Da)	error (ppm)
A1-8H2O	5560.6804	5560.6348	0.0456	8
A23-8H2O	5574.6961	5574.6948	0.0013	0.2

	[M+H]+	Lys residue Da	[M-128+H]+ calculated	observed	Diff Da	error (ppm)
A23-8H2O	5574.69612	128.09495	5446.60117	5446.5864	0.01477	2.7
A1-8H2O	5560.68042	128.09495	5432.58547	5432.6172	0.03173	5.8

Figure 80 - Deconvoluted spectra for charge state 4+ from two groups of preClosA1 and preClosA23 peptides: group 2 (A), group 1 (B). Special case, difference between 2 peaks is 128 Da for terminal Lys residue.

Group 1 shows a loss of 128 Da compared to group 2 which corresponds to the previously observed masses of preClosA1 and preClosA23 minus 8H2O, 8 water molecules (in red). 128 is the mass of a Lys residue, and it could mean that both preClosA1 and preClosA23 have lost the C-terminal Lys residue.

Some eluates of the preClosA peptide were treated with IAA with the aim to label the free cysteine residues and to measure the intact mass.

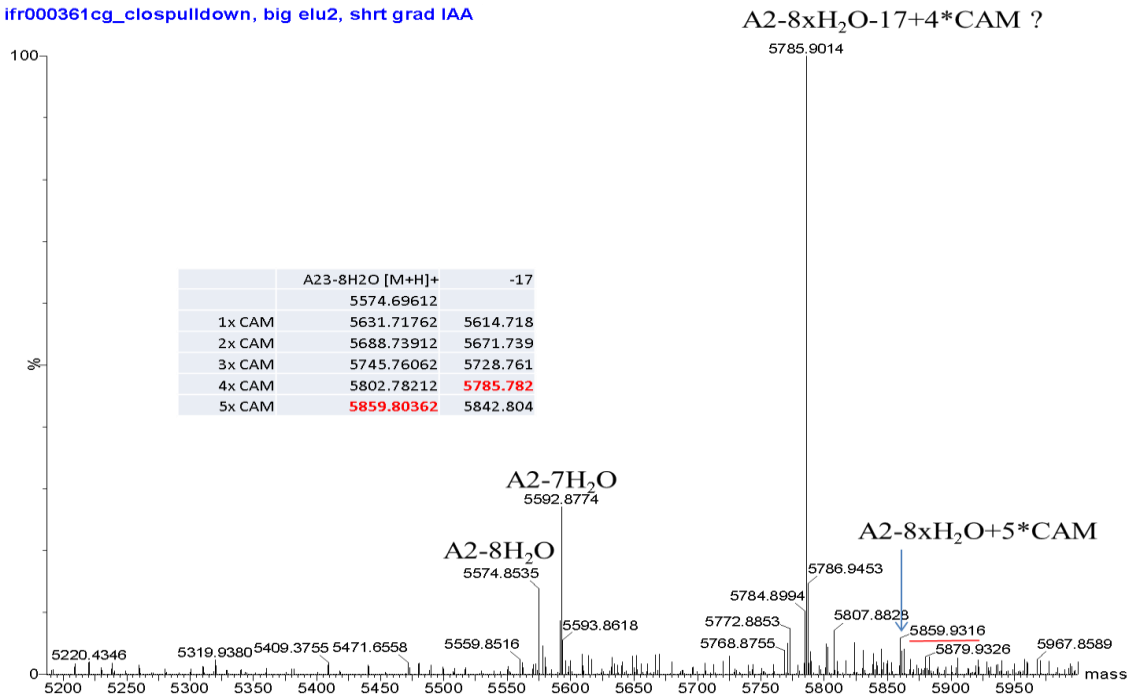


Figure 81 - Detection of preClosA23 in a sample that was treated with IAA.

The IAA labelled Cys residues, marked by 1 CAM per 1 Cys residue, indicated that the amino acids are free; free Cys residues do not form a ring with the neighbouring dehydrated amino acids. CAM adds a mass of 57 Da. In red is the calculated mass for preClosA that matched the observed mass for alkylated preClosA23. CAM is carbaimidomethyl. In the linear preclosA23, one, two, three, four or all the 5 Cys residues can be labelled with IAA.

The results in Figure 81 with IAA treated preClosA validate the observations previously made about the labelling of cysteine residues of the preClos peptide in the gel slices (Mascot search, Appendix 3). In here, a solution of pure preClosA peptide was treated with IAA and peaks for dehydrated preClosA23 were detected. The masses for preClosA23 that were identified were:

4) one peak is 5574 Da for preClosA23 with 8 dehydrations: [M+H-8x18] + = 5574
5) one peak is 5592 Da for preClosA23 with 7 dehydrations: [M+H-7x18] + = 5592.

The main new peak at 5785 matching to preClosA23 with 8 dehydrations and with 4 Cys residues labelled (IAA) but with a loss of a mass of 17. This is evidence of absence of rings, only one possible ring can be formed which is likely to be in the C-terminal part of the ClosA peptide. Another weaker peak was detected for a form of preClosA23 with all the 5 cysteine residues labelled by IAA at m/z 5859, which is evidence of absence of rings. The most abundant peak in here is for preClosA with no rings since 4 out of 5 cysteine residues were probably labelled with IAA which means that they were part of a linear preClosA23 peptide. The mass difference of 17 Da occurred in many spectra and

seems to be a common pattern encountered in the LC-MS analysis of preClosA. There may be indication that one cysteine residue was forming a ring since it was not alkylated. Peaks for completely unmodified (not alkylated) forms of preClosA1 and preClosA23 are also present in the spectrum which suggests that the peptides contain all the rings. Complete ring formation has however not been detected when analysing the tryptic digests from protein gel bands (Figure 68, Figure 69).

In conclusion, an optimal Ab-Ag ratio was determined and the crosslinking method was used for the purification of high yield pure preClosA peptide. The amino acid sequences and PTM for the novel antimicrobial peptides, preClosA1 and preClosA23 were confirmed and future work with the pure preClosA peptides should allow for the isolation of the active tryptic Clos peptides and their further structural and biological activity characterisation.

Apart from analysing preClosA from solution, Mascot analysis of 8 independent and excised protein gel slices from cell extracts and TCA precipitated supernatant samples revealed that they were all comprised of exactly the same tryptic peptides. This means that the preClosA peptide is present both inside the cell but it is also exported in the supernatant. There are two types of PTMs that can be considered: 1. PTM introduced by the *clos* modification machinery, 2. modifications of cysteine residues in preClosA introduced by labelling with IAA alkylator. Fragmentation of preClosA by LC-MS is also possible. The initial modifications involved dehydrations such as Ser -> Dha (S) and Thr -> Dhb with a change in mass of 18.010565 Da per dehydrated residue. According to literature [184, 187, 193], there is no mass change for ring formation when Cys is converted to Ala in ring formation (Cys -> Ala). This required IAA labelling of Cys residues. Therefore, it was interesting to investigate fragmentation of a thiol-ether crosslinkage by MS. The typical modification introduced during alkylation of preClosA and detected by LC-MS include a +57 Da mass change introduced because of IAA labelling (carbamidomethyl (C)) which made it easy to distinguish between free cysteines and cysteine in rings. The IAA S-alkylating agent is most commonly used for the structure-function characterisation of lantibiotics [181, 187, 194, 195]. IAA was selected for further target labelling of Cys residues. The small size of IAA is an advantage over other alkylators.

Only the ClosA4 leader peptide was detected and it was interesting to observe that even if the antiClos leader antibody was designed to bind to the sequence of the leader preClosA1 peptide, the preClosA4 leader also hybridised to the Ab.

The biological activity of the trypsin treated Clos peptide was assessed in chapter 5 using an overlay assay which proved to be a good method for the confirmation of antimicrobial activity. Interestingly, preClosA1-3 peptides have the advantage that trypsin can cleave at the predicted start site (the YK), therefore one of the tryptic peptides that is formed could be the full length leaderless form of the Clos peptide. Also, two major tryptic peptides were detected: one for the N-terminal part of preClos which proved to have no rings and another one for the C-terminal part of preClosA, where some peptides had the rings introduced. All the possible Ser and Thr residues were dehydrated efficiently by the *clos* modification machinery as confirmed by MS.

Therefore, there was no evidence for the presence of Rings A to C in the MS data from tryptic digests of IP Clos sample (Figure 81). These results are in contradiction with the argument that the presence of ring structure would be necessary for the antimicrobial activity in Clos. Based on previous work on structure elucidation of nisin and subtilin [89, 196-198], it is highly unlikely that in the absence of Rings A-C the full leaderless peptide would be active. Nisin and subtilin are lantibiotics to which preClosA peptide is predicted to have structural similarity. Different studies suggest that the modification machinery introduces the dehydrations in the precursor peptide, then the rings are formed possibly starting with Ring A [187, 193]. Consequently, more peptides with the Rings A-C were expected to be observed in the analysed samples which is not the case in the present study. Even if we assumed that fully modified preClosA with no leader present would be formed and would give the antimicrobial activity, the MS analysis indicates that two main tryptic fragments are mainly present: N-terminal peptide (linear peptide also comprising the hinge region) and C-terminal peptide (a mixture of linear, partial ring and full Rings D-E). This would suggest that these fragments are also most likely formed during the screening for biological activity using the overlay assays rather than the full leaderless sequence of Clos (see the evidence of a mixture of peptides as listed in Appendix 3).

6.3.4 **CONCLUDING REMARKS**

In this chapter, I was able to demonstrate the following:

1) Using the IP reactions, the ClosA1 prepeptide was successfully purified in high yield,

2) Using Mascot search, Maldi-ToF and LC-MS analysis of two types of samples:

2.1 excised 6 kDa gel slices and 2.2 eluted Clos in solution, confirmed the identity of preClosA according to the detected masses. Therefore, the structure of the preClosA peptide and the PTM were determined. A mixture of fully and partially dehydrated preClosA peptide were obtained, Rings D and E are more likely to be formed, however no evidence for Rings A-C was found.

3) PreClosA was completely digested with trypsin, all the cysteine residues were labelled with the IAA alkylator to obtain tryptic peptides with PTM (dehydrations and ring formations) as listed in Appendix 3. The following peptides were more currently found: FDDFDLDVTK, TAAQGGIEPK, SACTPGCPTGILMTCPLK and TATCGCHITGK, whereas if trypsin missed a cleavage site, some examples of these peptides can also be found but in a very low proportion: AKFDDFDLDVTK, SKSACTPGCPTGILMTCPLK and YKSKSACTPGCPTGILMTCPLK.

The observations on the MS data confirmed that the results were not consistent with the previously predicted structure for the N-terminal peptide, however they were consistent for hinge region and the C-terminal peptide. However, there was no MS data as evidence for the presence of a full peptide with the leader removed (IE or most likely YK as start site). This could be because trypsin normally removes the leader but it also further digests the leaderless core peptide at the cleavage sites along the peptide to form more tryptic fragments.

The heterologous expression system used in here has its limitations in that it produced peptides with the leader still attached at different stages of secretion, however the system is able to export precursor peptides that are partially modified.

In here there is no evidence of the machine exporting fully modified peptides with all dehydrations and all rings formed. It is known that during processing of the precursor peptide the leader is attached and it is necessary for the maturation of the lantibiotic peptide [168, 169]. However, if that were to be the case we would expect for the same to happen with Clos. Instead, it is likely that preClosA not fully converted into the mature form (with complete dehydrations and ring formation). More rings are expected to be formed in the early part of the preClosA in the N-terminal part and current results could

suggest that the system is different compared to nisin and subtilin. Successful and complete PTM of preClosA may require the presence of another component from the original bacteria that is not present in the heterologous system such as a protease.

Despite problems with preClosA peptide detection by Maldi-ToF due to detergent contamination in the eluted fractions, there was good indication that preClosA is present. The fragmentation pattern observed in the Mascot search indicated that preClosA is completely digested by trypsin. Searches for ring fragmentation using special modifications and identification of tryptic peptides that start from inside the region of Ring A (K↓SAC) confirmed that Ring A is not introduced by the *clos* biosynthetic machinery. The rest of the N-terminal peptide is likely to be devoid of rings as well since fragmentation characteristic to a linear peptide was observed. Some peptides for the C-terminal part of preClosA were linear, some were with one ring and a few others had both rings present.

CHAPTER SEVEN

7. Conclusions and future perspectives

The work in this PhD thesis demonstrated that novel and active lantibiotics can be obtained from the novel *clos* gene cluster isolated from a gut bacterium that have biological activity against gut pathogens. The activation of *clos* promoters and the structure of the preClosA peptides have been addressed. In addition, *clos*-like nisin mutants were generated and characterised, however peptides with improved properties compared to nisin were not detected in this work. Exploring the complex GI tract for novel antimicrobials still holds the potential for the discovery of novel lantibiotics active against gut pathogens such as in the case with the Clos peptides identified in this PhD thesis.

Chapter 3 - Generation and characterisation of clos-like nisin mutants

In chapter 3, it was shown that although it was possible to use a *nisA* construct as a template to incorporate features of the ClosA1 peptide to successfully substitute individual or multiple amino acids in 12 nisin variants, their biological activity was not better or significantly improved in any of the biological tests when compared to nisin and tested at pH 3.0 to 7.0.

A preliminary antimicrobial screening approach was used to assess the biological activity of the mutants and their induction/self-induction properties. Results indicated that only M21L was a self-inducer, whereas all the other mutants required continuous induction with nisin. All the nisin mutants were produced either in liquid or solid media. Testing the mutants against different indicator strains and colony MS allowed the successful detection of most of the peptides and the identification of mutants with similar or less antimicrobial activities to nisin A was possible. Purification and further characterisation of the specific activity of I4K and H27G at the temperature, pH and using indicator strains relevant to the GI tract did not demonstrate improved thermostability and/or spectrum of activity for the purified peptides. Future work could involve generating mutants to make them look more like ClosA1 or ClosA4. The observations made in this PhD project on the effect of particular amino acid substitutions can be used to rationally design future Clos mutant peptides by introducing particular amino acid substitutions in the rings and in the C-terminal part of nisin. However, generation of a nisin mutant with improved properties is limited by the lack of knowledge on the tolerance of the nisin biosynthetic machinery to the amino acid substitutions which is something that was explored in chapter 3.

Chapter 4 - Identification and functional characterisation of promoters in the *clos* operon

In chapter 4, bioinformatics led to the identification of four putative *clos* promoters; the expression system confirmed that P_{ClosA} and possibly $P_{ClosR2K2}$ are a potential target recognition site for NisR or potentially for the ClosR regulatory proteins. The other two *clos* promoters either showed no activation with no induction with nisin or they were not real *clos* promoters. Current results indicate that the *nisRK* genes may be essential for the production of the intermediate preClos peptide when the strain is induced with nisin.

Measurements of the specific *pepI* activity using the *closRK* or *nisRK* two-component regulatory systems demonstrated that the LanRK regulatory system is not capable of switching on the other putative *clos* promoters when expressed in the UKLc10 heterologous expression system. It was speculated that perhaps the ClosRK systems do play a role in the regulation of preClosA biosynthesis, however this is only possible when the preClosA peptide is already present in the medium. Inducer peptides regulating lantibiotic biosynthesis are generally encoded by genes from the same lantibiotic gene clusters [162]. Perhaps after the accumulated Clos inducer peptide reaches a threshold concentration during growth in a cell density-dependent manner, a positive feedback mechanism creates a burst of the signal hence the fast response. Future work may demonstrate that all *clos* promoters are functional when they are present on the *clos* gene cluster, and tests should include the correct transcriptional repressors and transcription activators from the *Blautia obeum* A2-162 strain.

Heterologous expression of active Clos peptides described in chapter 5 also involved creating and testing of fusions of the *closA* structural genes to the nisin A leader peptide; a putative cleavage site for the NisP was also created and expression in *L. lactis* indicated that the nisin biosynthetic machinery was incapable of producing, modifying or releasing active Clos peptides.

However, based on the knowledge of the activation and expression of the *pepI* gene in nisin inducible and *nisRK* controlled UKLc10 host strain, expression of the entire gene cluster with nisin induction allowed for the successful identification by MS and preClosA detection, using the antibody designed for the preClosA leader peptide. The presence of the specific preClosA peptides was confirmed in both TCA precipitated supernatant and in the cell extracts from nisin induced *clos* cluster containing strains.

It was hypothesised that the preClosA biosynthetic machinery is able to produce, modify and export the preClosA peptide and that external proteases would be capable to remove the leader peptide and generate active Clos peptide or Clos fragments.

Chapter 5 - Heterologous expression of Clos peptides

Heterologous expression was shown to be a beneficial system for the production of lantibiotic peptides from gene clusters isolated from different organisms [182]. Following the successful biosynthesis and post-translational modification of preClosA when it is expressed in the presence of the nisin regulatory genes, trypsin treatment of the preClosA peptide generated fragments with high biological activity compared to nisin. These trypsin-cleaved ClosA peptides are expressed from more than one structural *closA* gene. Antimicrobial activity was demonstrated against Gram-positive gut pathogens such as C. *difficile,* a major cause of hospital acquired infections and *C. perfringens* as well as against *L. lactis* MG1614. Therefore, Clos has the potential to be developed as a novel clinical antimicrobial peptide active against infections with MDR bacteria. Interestingly, the leaderless nisin was also active both in the absence and in the presence of trypsin. This suggests that the tryptic nisin fragments obtained by treatment with 1 to 15 ng/µl of trypsin were active despite not comprising the entire original nisin structure which is consistent with previous reports in literature [199, 200]. Possibly the same takes place with the Clos peptide. Also, it was demonstrated that an increasing amount of trypsin allowed for the accumulation of a higher number of completely digested tryptic peptides, therefore the size of the halo increased proportionally in the overlay assay.

In chapter 5, it was demonstrated that good preClosA production takes place between 2 and 6 h of nisin induction whereas the biosynthesis is switched off after 6 to 12 h. It is known that leaderless lantibiotic peptides, despite being active, can be degraded by internal proteases. This may imply that at lower trypsin concentrations there are different forms of tryptic peptides are produced due to the incomplete cleavage of preClosA at low trypsin concentration. Since antimicrobial activity against other tested indicator bacterial strains was not seen due to their different sensitivity to digested Clos peptide, in the future a higher amount of preClosA may allow for the accumulation of the correct active tryptic peptides. Future work on the screening for active Clos peptide should involve testing the purified trypsin digested preClosA and comparing it with current results obtained on plate assays where preClosA was produced by the *L. lactis* UKLc10 producing strain.

Since the GI tract is such a complex ecosystem, the effectiveness of nisin induction and trypsin-dependent active Clos production and the regulation of these processes *in vivo* is likely to depend on relations with numerous GI tract environmental factors. Also, these processes could be inhibited by digestive proteases, however the significance of these enzymes is yet to be determined in future *in vivo* studies.

Similar work was performed on another GI tract derived lantibiotic peptide, Ruminococcin A produced by the *Ruminococcus gnavus* strain from the human intestinal microbiota [162]. It would be interesting to study the impact of the gut environmental factors on the *clos* promoter activity in the gut-derived *Blautia obeum* A2-162, as it was previously investigated for another gut-derived bacteriocin produced by *Lactobacillus salivarius* [201]. Similarly, the GI survival of the Clos lantibiotic-producing strain, *L. lactis* UKLc10 could be investigated in *in vitro* and *in vivo* studies. The aim would be to further use the producing strain as a delivery system for biologically active Clos peptides to the gut in order to treat infections with *Clostridiales* gut pathogens. Similar work has already been done to study the fate and efficacy of the lacticin 3147 lantibiotic peptide [202].

Chapter 6 - Structure confirmation of the preClosA peptide

In chapter 6, the heterologously produced and nisin induced preClosA peptide was successfully purified in very high yield by using an optimised immunoprecipitation (IP) reaction with the antiClos leader antibody. Mascot database search, Maldi-ToF and LC-MS analysis of two types of samples including the 6 kDa protein gel slices and eluted preClosA in solution, confirmed the identity of the investigated peptides as preClosA. This was achieved by sequence identification of tryptic peptides and by comparing accurate experimental masses to the calculated masses and sequences. The Mascot database search also allowed for the detection of the post-translational modifications including the presence of possible ring structures. Results demonstrated that the *clos* biosynthetic machinery is either not efficient in introducing the PTM (e.g.: all the predicted ring structures) or the predicted structures of preClosA are incorrect.

Evidence of a mixture of fully and partially dehydrated preClosA peptides was found. A few spectra for some tryptic peptides indicated that one or both Rings D and E can be formed. However, no evidence for Rings A-C was found. This would suggest that the *clos* modification machinery can introduce the dehydrations, however the thio-ether linkages

are not efficiently introduced perhaps due to the lack of the correct Clos signal/inducing molecule.

In vitro work with immune-purified preClosA peptides could involve trypsin digestion and RP-HPLC separation of the individual tryptic peptides. C18 RP-HPLC was used in chapter 3 to purify *clos*-like nisin mutants, therefore the methods are in place for the isolation of the tryptic Clos peptides. Next, the purified peptides would need to be tested for their antimicrobial spectrum and activity, host range and their stability against a range of Gram-positive species. As a result, tryptic peptides with improved characteristics compared to nisin may be detected. The interaction and mode of action of tryptic peptides with the bacterial cell membrane can be assessed in comparison to nisin by solid state NMR [203, 204]. This future work will involve looking at the ability of the Clos lantibiotics to bind to membrane constituents and/or components involved in cell wall biosynthesis. The potential of the Clos leader peptide to form pores can be studied with the aim of investigating their mechanism of action against sensitive indicator strains including clinically important pathogens. Once the Clos tryptic peptides are isolated, it would be interesting to investigate if they can act as an inducer in the heterologous expression system or in the original *Blautia obeum* A2-162. The stability of the active Clos peptides *in vitro* can also be tested.

Future work with purified tryptic peptides could also involve assessing their antimicrobial activity in *in vitro* models. Lantibiotics with activity against colonic bacteria could be tested in pathogen challenged models using batch cultures as well as the full colon models that are designed to reproduce the microbiological and the physiological conditions of the human large intestine. These model systems mimic the GI tract conditions [205]. The effect of the lantibiotic on the composition and diversity of the commensal gut microbiota could be assessed for microbial profiling using 16S rDNA sequencing via the 454 platform. The data obtained would provide phylogenic information on any changes in the complex gut microbiota.

Furthermore, future studies could involve determining the possible induction conditions of the original *Blautia obeum* A2-162 strain where a number of Clos or tryptic peptides containing samples could be used to act as potent signal molecules. For instance, inactive Clos precursor peptides from supernatants or cell extracts from nisin induced pClosCluster in *L. lactis* UKLc10 strain could be incubated with various trypsin concentrations in the *Blautia obeum* A2-162 strain. Alternatively, active tryptic peptides

isolated from RP-HPLC or even IP pure preClosA in solution treated with various trypsin concentrations can act as inducing peptides.

More detailed analysis of the supernatant and cell extracts samples from the induced strains may confirm the presence of both ClosA3 and ClosA4 peptides. Future work could involve to understand if both ClosA3 and ClosA4 are necessary for the induction and antimicrobial activity since it was assumed that they are both part of a two-component system.

From the MS analysis of the IP enriched preClosA, it is apparent that preClosA23 is predominant and some preClosA1 is also present. PreClosA4 could not be detected which is most likely due to the difference in the sequence of the leader peptide which could not bind to the antiClos leader antibody originally designed for the ClosA1 leader peptide. The induction experiments would allow us to investigate if there is any Clos peptide production from the original strain as well as to test and establish the optimal conditions for antimicrobial biosynthesis under GI tract conditions. Tests could then include antimicrobial activity bioassays, RT-PCR, western blotting and mass spectrometry analysis.

Original Sequences[a]		
Hybrid name	Shorter name	
NisinA Leader+ Nisin	NisL+N	MSTKDFNLDLVSVSKKDSGASPR↓ITSISLCTPGC KTGALMGCNMKTATCHCSIHVSK
ClosLeader+ ClosA	ClosL+ClosA1 (GG_IE)	MGKFDDFDLDVTKTAAQGG↓IEPK↓YKSK SACTPGCPTGILMTCPLKTATCGCHITGK
	ClosL+ClosA2	MAKFDDFDLDVTKTAAQGG↓IEPK↓YKSK SACTPGCPTGILMTCPLKTATCGCHITGK
	ClosL+ClosA3	MAKFDDFDLDVTKTAAQGG↓IEPK↓YKSK SACTPGCPTGILMTCPLKTATCGCHITGK
	ClosL+ClosA4	MAKFDDFDLDIIEKKVRRDI↓IPAS↓ITSQHS FCTPNCLTGFLCPPKTQLTCTCKLKGQ
Mutagenesis – to cleave with ArgC (to introduce R)[*]		
ClosL + ClosA	ClosL+ClosA (GR_IE)	MGKFDDFDLDVTKTAAQGR↓IEPKYKSKSAC TPGCPTGILMTCPLKTATCGCHITGK
	ClosL+ClosA (GG_IEPR)	MGKFDDFDLDVTKTAAQGGIEPR↓YKSKSAC TPGCPTGILMTCPLKTATCGCHITGK
ClosL + ClosA	ClosL+ClosA4 (DR_IP)	MAKFDDFDLDIIEKKVRRDR↓IPASITSQHSFC TPNCLTGFLCPPKTQLTCTCKLKGQ
ClosL + ClosA	ClosL+ClosA4 (DI_IPAR)	MAKFDDFDLDIIEKKVRRDIIPAR↓ITSQHSFC TPNCLTGFLCPPKTQLTCTCKLKGQ

Table 32 - Cleavage of prenisin and preClosA with trypsin and with the engineered ArgC peptidase sites.
[*] Putative cleavage site in the preClosA peptides are indicated in green for trypsin and in red, boldface type for ArgC.
The peptides in the supernatant or in the cell extracts from the total protein can be identified by Maldi-ToF. The proposed hybrids that can be used to engineer the ArgC site are: nisinA Leader + nisin core peptide (as positive control), Clos leader (ClosL) + Clos core peptide. The conserved sequence of F(N/D)LD(L/V) in nisin and in the Clos leader peptide was highlighted in yellow [169].

Table 32 illustrates a strategy of introducing cleavage sites for ArgC protease in the sequences of ClosA1 and ClosA4 core peptides in order to facilitate the release of the full Clos peptide without the predicted leader attached. The ArgC sites would allow for the specific release of Clos peptides with either IE or YK start site since ArgC cleaves only after the Arg residue.

The IP reaction can be developed into a standard screening technique to identify and purify lantibiotics for which the leader peptide sequence is known. The IP reaction could allow for the purification of preClosA peptide in very high amounts. If the new Clos peptides are confirmed to be produced by the gut bacteria, antibodies could be used in western blotting for Clos leader detection, followed by the specific extractions and further MS characterisation to further confirm the identity of the Clos peptides.

From a clinical point of view, it would be interesting to explore if the *Blautia obeum* A2-162 has probiotic traits similar to other gut bacteria [29] and it could be used to develop a delivery system for the Clos peptide with a view to treat GI tract infections. One example would be the use of faecal microbiota transplantation [10, 206].

From a commercial, industrial and pharmaceutical perspective, Clos could be explored for its potential to inhibit food-poisoning pathogens such as *L. monocytogenes* or sporulation from different bacteria by using it as a food preservative. Investigations of the heat-stability and shelf life of pure preClos as well as the development of a scaled-up production system would also be interesting to explore.

In conclusion, the UKLc10 heterologous system with the *nisRK* genes on the chromosome allowed for the expression of the preClosA peptides in high yield, and the trypsin digested Clos displayed very high antimicrobial activity comparable to nisin when tested against *L. lactis* MG1614, *C. perfringens* and *C. difficile*. PreClosA was successfully purified by immunoprecipitation. This is the first demonstration of a bacteriocin from the *Blautia obeum* A2-162 gut species that could be digested by trypsin to give very significant antimicrobial activity against clinically important gut pathogens. This is also the first study on the post-translational modifications of the preClosA peptide as introduced by the *clos* biosynthetic machinery confirming the occurrence of dehydrations and some ring formations. The current study also showed that all the four forms of preClosA peptides were present, and that ClosA2 is the most abundant one. Future studies on the characterisation of the Clos structure using isolated tryptic peptides from the RP-HPLC column will improve the current knowledge on the role of the post-translational modification for the activity of the tryptic peptides.

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Appendix

Appendix 1 - Calculated and observed masses of clos-like nisin mutants that were detected in Maldi-ToF analysis

In red, observed and calculated are 100 % match; in black and bold, there is 1-unit difference between the observed and calculated mass of the peptide.

Nisin positive control

Nisin A	Calculated monoisotopic mass (m/z)				Observed (m/z)
Deh.	M+H	M+O	M+Na	M+K	
8	3352.64	3368.64	3374.62	3391.60	3352.64
7	3370.64	3386.64	3392.62	3409.60	3369.64
6	3388.64	3404.64	3410.62	3427.60	3373.17
5	3406.64	3422.64	3428.62	3445.60	3375.60
2	3460.64	3476.64	3482.62	3499.60	3386.63
1	3478.64	3494.64	3500.62	3517.60	3389.16
					3390.61
					3394.38
					3499.71

Ring A mutant peptides

I4K	Calculated monoisotopic mass (m/z)				Observed
Deh.	M+H	M+O	M+Na	M+K	(m/z)
8	3367.65	3383.65	3389.63	3406.61	3367.63
7	3385.65	3401.65	3407.63	3424.61	3383.66
6	3403.65	3419.65	3425.63	3442.61	3386.67
					3402.67

L6A	Calculated monoisotopic mass (m/z)				Observed
Deh.	M+H	M+O	M+Na	M+K	(m/z)
8	3310.59	3326.59	3332.57	3349.55	3327.34
7	3328.59	3344.59	3350.57	3367.55	3343.41
6	3346.59	3362.59	3368.57	3385.55	3383.54
5	3364.59	3380.59	3386.57	3403.55	3398.56
4	3382.59	3398.59	3404.57	3421.55	

	I4K_L6A	Calculated monoisotopic mass (m/z)				Observed (m/z)
	Deh.	M+H	M+O	M+Na	M+K	
active peptide	4	3397.60	3413.60	3419.58	3436.56	3413.47
	3	3415.60	3431.60	3437.58	3454.56	
propeptide (uncleaved)	6	5693.80	5709.80	5715.78	5732.77	5694.59
	5	5711.80	5727.80	5733.78	5750.77	5750.59

Ring C mutant peptides

A15I	Calculated monoisotopic mass (m/z)				Observed (m/z)
Deh.	M+H	M+O	M+Na	M+K	
8	3394.68	3410.68	3416.66	3433.65	3394.78
7	3412.68	3428.68	3434.66	3451.65	3412.35
6	3430.68	3446.68	3452.66	3469.65	3429.76
5	3448.68	3464.68	3470.66	3487.65	3467.77
4	3466.68	3482.68	3488.66	3505.65	3544.67
1	3520.68	3536.68	3542.66	3559.65	3450.35

G18T	Calculated monoisotopic mass (m/z)				Observed (m/z)
Deh.	M+H	M+O	M+Na	M+K	
8	3396.66	3412.66	3418.64	3435.63	3434.34
7	3414.66	3430.66	3436.64	3453.63	3473.10
6	3432.66	3448.66	3454.64	3471.63	3486.71
5	3450.66	3466.66	3472.64	3489.63	3560.27
3	3486.66	3502.66	3508.64	3525.63	
1	3522.66	3538.66	3544.64	3561.63	
0	3540.66	3556.66	3562.64	3579.63	

Hinge mutant peptides

N20P	Calculated monoisotopic mass (m/z)				Observed (m/z)
Deh.	M+H	M+O	M+Na	M+K	
8	3335.65	3351.65	3357.63	3374.61	3334.18
7	3353.65	3369.65	3375.63	3392.61	3353.36
6	3371.65	3387.65	3393.63	3410.61	3370.06
4	3407.65	3423.65	3429.63	3446.61	3386.19
3	3425.65	3441.65	3447.63	3464.61	3408.35
2	3443.65	3459.65	3465.63	3482.61	3425.47
					3442.01
					3460.02

M21L	Calculated monoisotopic mass (m/z)				Observed (m/z)
Deh.	M+H	M+O	M+Na	M+K	
8	3334.68	3350.68	3356.66	3373.64	3335.62
7	3352.68	3368.68	3374.66	3391.64	3350.64
6	3370.68	3386.68	3392.66	3409.64	3354.63
5	3388.68	3404.68	3410.66	3427.64	3369.68
4	3406.68	3422.68	3428.66	3445.64	3392.64
					3407.62

N20P_M21L	Calculated monoisotopic mass (m/z)				Observed (m/z)
Deh.	M+H	M+O	M+Na	M+K	
8	**3317.69**	**3333.69**	3339.67	3356.65	**3318.76**
7	**3335.69**	3351.69	3357.67	3374.65	**3333.80**
					3335.69

C-terminal mutant peptides

H27G	Calculated monoisotopic mass (m/z)				Observed (m/z)
Deh.	M+H	M+O	M+Na	M+K	
8	**3272.60**	**3288.60**	3294.58	3311.56	**3271.33**
7	3290.60	**3306.60**	3312.58	3329.56	**3288.68**
6	**3308.60**	3324.60	3330.58	3347.56	**3304.68**
5	3326.60	**3342.60**	3348.58	3365.56	**3307.65**
					3342.67

H27G_S29H_H31T_V32G_S33K	Calculated monoisotopic mass (m/z)				Observed (m/z)
Deh.	M+H	M+O	M+Na	M+K	
8	3285.63	**3301.63**	3307.61	3324.59	**3301.42**
7	**3303.63**	**3319.63**	3325.61	3342.59	**3317.70**
5	3339.63	**3355.63**	3361.61	3378.59	**3356.83**
4	**3357.63**	3373.63	3379.61	3396.59	

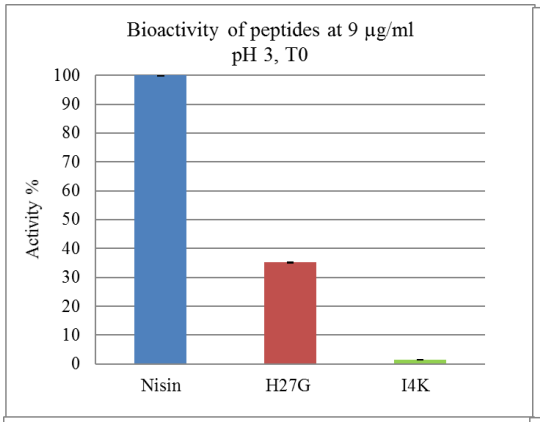
H31T_V32G_S33K	Calculated monoisotopic mass (m/z)				Observed
Deh.	M+H	M+O	M+Na	M+K	(m/z)
8	**3315.6417**	3331.64	3337.62	3354.61	**3312.56**
7	3333.64	3349.64	3355.62	3372.61	**3347.79**
6	3351.64	3367.64	3373.62	3390.61	**3369.83**
5	**3369.64**	**3385.64**	3391.62	3408.61	**3385.74**

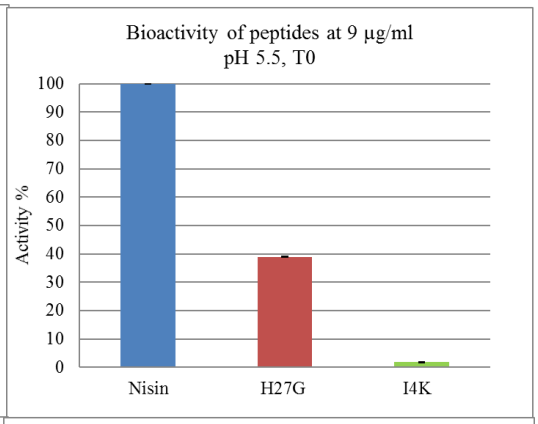
Appendix 2 - Activity and stability of I4K, H27G and nisin against L. lactis MG1614 under GI tract conditions (37°C, pH 3.0, 5.5, 6.0, 6.5, 7.0) measured for 7 days

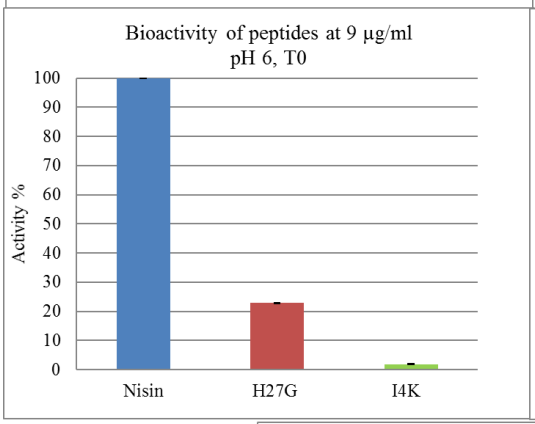
The specific activity of nisin, H27G and I4K given by 9 µg/ml of pure peptides was plotted for time points A. T0, B. T48, C. T68 hours.

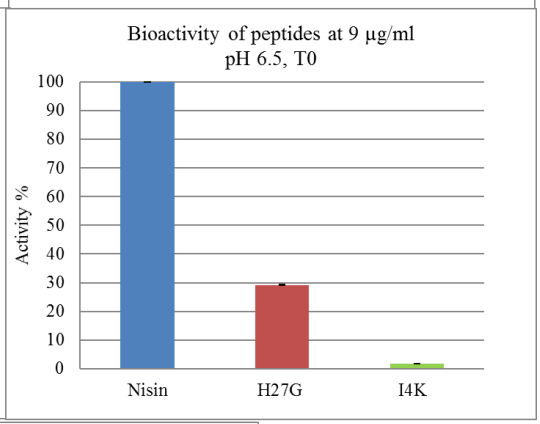
The bioactivity of the nisin variants and nisin follows the same pattern. At any given time point and pH, the activity of I4K is 99 % less and the activity of H27G is between 60-80 % less compared to nisin.

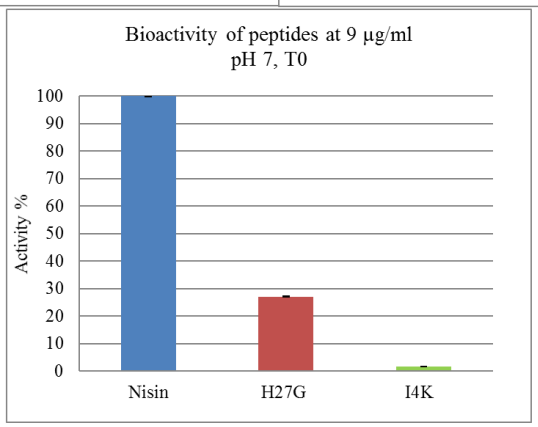
A.



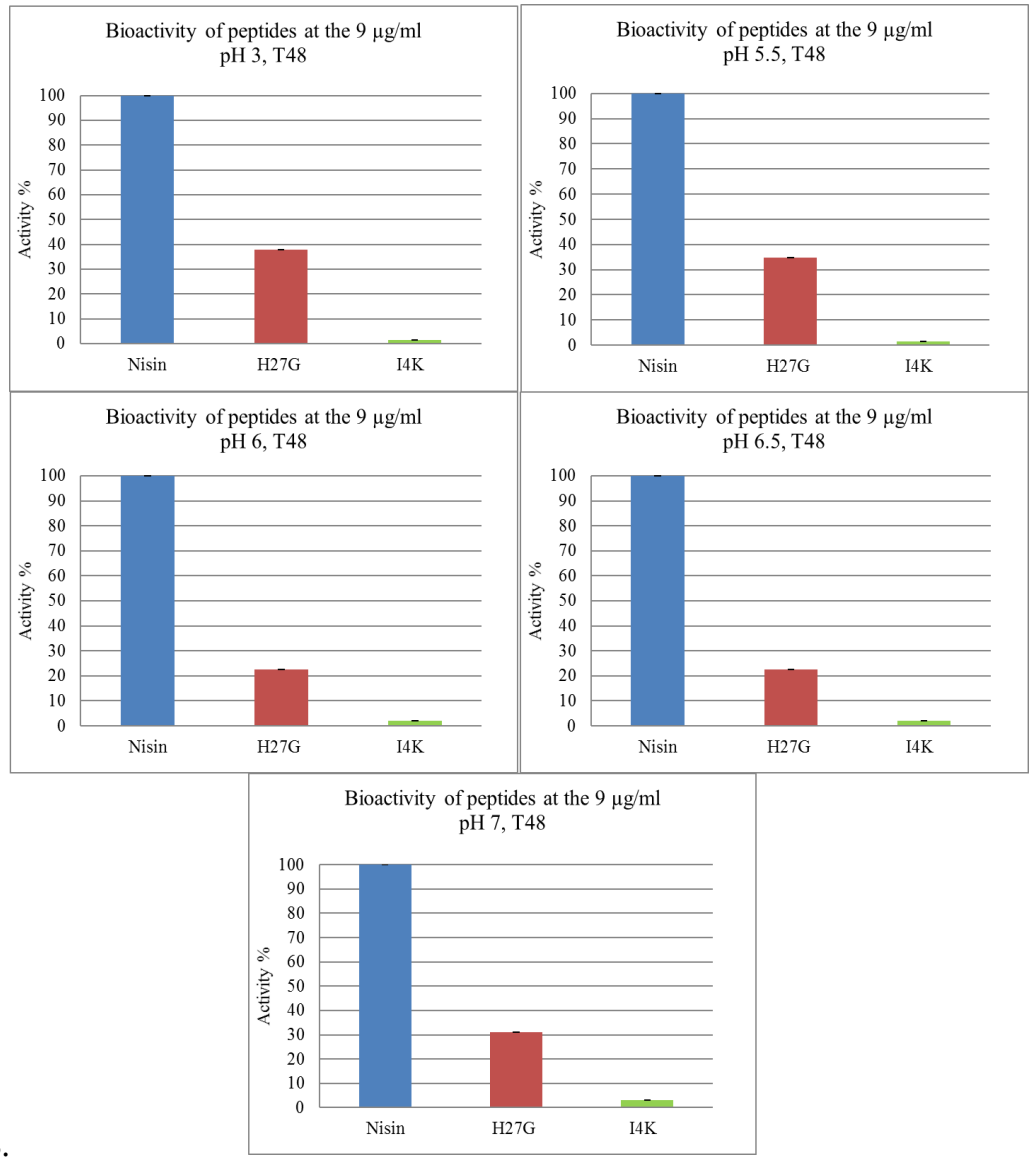




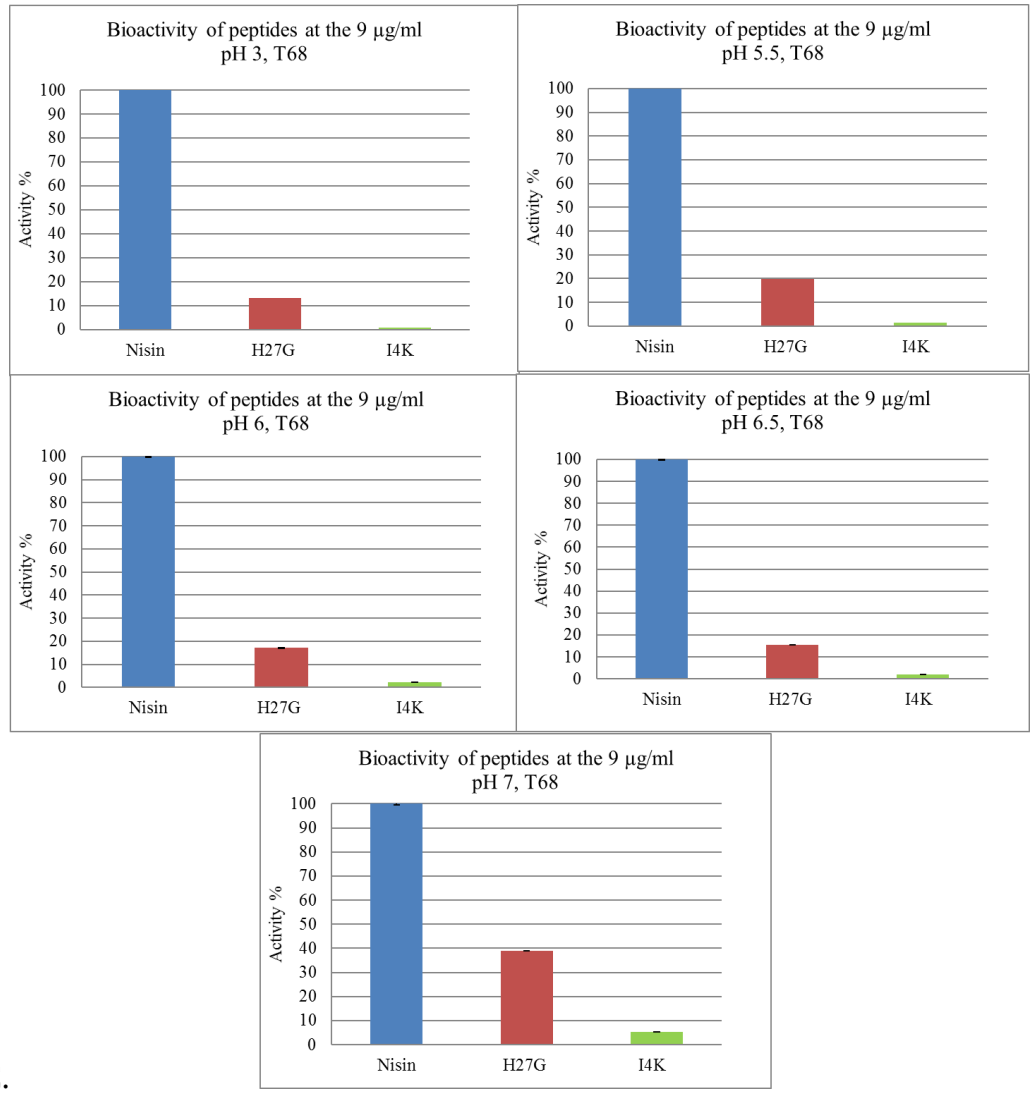




B.


Bioactivity of peptides at the 9 µg/ml
pH 3, T48
Activity %
100
90
80
70
60
50
40
30
20
10
0
Nisin
H27G
I4K
Bioactivity of peptides at the 9 µg/ml
pH 5.5, T48
Activity %
Nisin
H27G
I4K
Bioactivity of peptides at the 9 µg/ml
pH 6, T48
Activity %
Nisin
H27G
I4K
Bioactivity of peptides at the 9 µg/ml
pH 6.5, T48
Activity %
Nisin
H27G
I4K
Bioactivity of peptides at the 9 µg/ml
pH 7, T48
Activity %
Nisin
H27G
I4K

C.


Bioactivity of peptides at the 9 µg/ml
pH 3, T68
Activity %
100
90
80
70
60
50
40
30
20
10
0
Nisin
H27G
I4K
Bioactivity of peptides at the 9 µg/ml
pH 5.5, T68
Activity %
Nisin
H27G
I4K
Bioactivity of peptides at the 9 µg/ml
pH 6, T68
Activity %
Nisin
H27G
I4K
Bioactivity of peptides at the 9 µg/ml
pH 6.5, T68
Activity %
Nisin
H27G
I4K
Bioactivity of peptides at the 9 µg/ml
pH 7, T68
Activity %
Nisin
H27G
I4K

Appendix 3 - Mascot files for preClosA fragments labelled and digested with trypsin.

3.1. Mascot search for tryptic peptides from the preClosA1-4 leader peptides

List of peptide spectrum matches (PTM) for the leader peptide of the preClosA1-4 peptides extracted from a results file (Scaffold proteomics software) after a Mascot search with data from nano-LC-MS/MS of tryptic digested gel samples.

Table. A: Tryptic Peptide **AKFDDFDLDVTK** (1 missed cleavage) from **ClosA2-3** leader peptide

Total spectra observed: 109; No PTMs observed

Two PSMs of charge states 1 to 3 each are shown (Data extracted from Scaffold software)

Probability	Observed m/z	Charge	Actual mass	Calculated M+1H	Error (ppm)	Intensity (TIC)
0.997	471.9012	3	1412.6817	1413.6899	-0.652	18664300
0.997	471.9012	3	1412.6817	1413.6899	-0.652	13379400
0.997	707.3490	2	1412.6834	1413.6899	0.518	6238690
0.997	707.3490	2	1412.6834	1413.6899	0.518	5244830
0.997	1413.6915	1	1412.6843	1413.6899	1.123	332312
0.997	1413.6897	1	1412.6825	1413.6899	-0.144	268013

Table. B: Tryptic Peptide **FDDFDLDVTK** from **ClosA1-3** leader peptide

Total spectra observed: 30; No PTMs observed

Three PSMs of charge state 2 each are shown, no other charge states observed

Probability	Observed m/z	Charge	Actual mass	Calculated M+1H	Error (ppm)	Intensity (TIC)
0.997	607.7821	2	1213.5497	1214.5578	-0.706	1976410
0.997	607.7821	2	1213.5497	1214.5578	-0.706	1791220
0.997	607.7824	2	1213.5503	1214.5578	-0.188	358520

Table. C: Tryptic Peptide **TAAQGGIEPK** from **ClosA1-3** leader peptide

Total spectra observed: 22; No PTMs observed

Three PSMs of charge state 2 each are shown, no other charge states observed

Probability	Observed m/z	Charge	Actual mass	Calculated M+1H	Error (ppm)	Intensity (TIC)
0.997	486.2617	2	970.5089	971.5158	0.292	642814
0.997	486.2617	2	970.5089	971.5158	0.292	563809
0.997	486.2613	2	970.5081	971.5158	-0.461	168335

Table. D: Tryptic Peptide **TAAQGGIEPKYK** (1 missed cleavage) from **ClosA1-3** leader peptide

Total spectra observed: 4; No PTMs observed

Three PSMs of charge state 2 each are shown, no other charge states observed

Probability	Observed m/z	Charge	Actual mass	Calculated M+1H	Error (ppm)	Intensity (TIC)
0.997	631.8414	2	1261.6682	1262.6741	1.082	26473
0.997	631.8414	2	1261.6682	1262.6741	1.082	23005
0.997	631.8412	2	1261.6678	1262.6741	0.757	10428

Table. E: Tryptic Peptide **GKFDDFDLDVTK** from **ClosA1** leader peptide

Total spectra observed: 53; No PTMs observed

Two PSMs of charge states 1 to 3 each are shown (Data extracted from Scaffold software)

Probability	Observed m/z	Charge	Actual mass	Calculated M+1H	Error (ppm)	Intensity (TIC)
1.00	467.2299	3	1398.6678	1399.6739	0.492	1792850
1.00	467.2291	3	1398.6656	1399.6739	-1.062	1695910
1.00	700.3411	2	1398.6676	1399.6739	0.377	523015
1.00	700.3411	2	1398.6676	1399.6739	0.377	478187
1.00	1399.6755	1	1398.6682	1399.6739	0.799	78757
1.00	1399.6755	1	1398.6682	1399.6739	0.799	26022

3.2. Mascot search for tryptic peptides from the ClosA1-3 core peptides

List of peptide spectrum matches (PTM) for ClosA1-3 core tryptic peptides extracted from results file (Scaffold proteomics software) after a Mascot search with data from nano-LC-MS/MS of tryptic digested gel samples. Lower case residues mean that they were modified: dehydrated and/or with the Cys residues labelled by IAA. The modifications are located by the number associated with the residue.

Table 33 - List of all PSMs for tryptic peptide **<u>TATCGCHITGK</u>** covering the DE ring area of ClosA1-3 peptides.

Number of PSMs: 66, all modified, no missed cleavage,

Note that PSMs in bold have 2-3 dehydrations and are not (italic) or not fully modified by CAM (on Cysteines)

Visual inspection of the corresponding spectra showed missing fragmentation in ring area in some spectra (see chapter 6, Figure 68).

Abbreviations for all those tables: P=probability, CAM=Carbamidomethyl, Ox=Oxidation, dehydr=Dehydration, PSMs=Peptide Spectra Matches.

Peptide sequence	P	Variable modifications identified by spectrum
TATcGcHITGK	0.997	c4: Carbamidomethyl (+57.02), c6: Carbamidomethyl (+57.02)
TATcGcHITGK	0.997	c4: Carbamidomethyl (+57.02), c6: Carbamidomethyl (+57.02)
TATcGcHITGK	0.997	c4: Carbamidomethyl (+57.02), c6: Carbamidomethyl (+57.02)
TATcGcHITGK	0.984	c4: Carbamidomethyl (+57.02), c6: Carbamidomethyl (+57.02)
TATcGcHITGK	0.997	c4: Carbamidomethyl (+57.02), c6: Carbamidomethyl (+57.02)
TATcGcHITGK	0.997	c4: Carbamidomethyl (+57.02), c6: Carbamidomethyl (+57.02)
TATcGcHITGK	0.997	c4: Carbamidomethyl (+57.02), c6: Carbamidomethyl (+57.02)
TATcGcHITGK	0.997	c4: Carbamidomethyl (+57.02), c6: Carbamidomethyl (+57.02)
TATcGcHITGK	0.997	c4: Carbamidomethyl (+57.02), c6: Carbamidomethyl (+57.02)
TATcGcHITGK	0.997	c4: Carbamidomethyl (+57.02), c6: Carbamidomethyl (+57.02)
TATcGcHItGK	0.997	c4: Carbamidomethyl (+57.02), c6: Carbamidomethyl (+57.02), t9: Dehydrated (-18.01)
TATcGcHItGK	0.997	c4: Carbamidomethyl (+57.02), c6: Carbamidomethyl (+57.02), t9: Dehydrated (-18.01)
TATcGcHItGK	0.997	c4: Carbamidomethyl (+57.02), c6: Carbamidomethyl (+57.02), t9: Dehydrated (-18.01)
TATcGcHItGK	0.997	c4: Carbamidomethyl (+57.02), c6: Carbamidomethyl (+57.02), t9: Dehydrated (-18.01)
TATcGcHItGK	0.992	c4: Carbamidomethyl (+57.02), c6: Carbamidomethyl (+57.02), t9: Dehydrated (-18.01)
TATcGcHItGK	0.997	c4: Carbamidomethyl (+57.02), c6: Carbamidomethyl (+57.02), t9: Dehydrated (-18.01)
TATcGcHItGK	0.997	c4: Carbamidomethyl (+57.02), c6: Carbamidomethyl (+57.02), t9: Dehydrated (-18.01)
TATcGcHItGK	0.997	c4: Carbamidomethyl (+57.02), c6: Carbamidomethyl (+57.02), t9: Dehydrated (-18.01)
TATcGcHItGK	0.997	c4: Carbamidomethyl (+57.02), c6: Carbamidomethyl (+57.02), t9: Dehydrated (-18.01)

TATcGcHItGK	0.997	c4: Carbamidomethyl (+57.02), c6: Carbamidomethyl (+57.02), t9: Dehydrated (-18.01)
TATcGcHItGK	0.997	c4: Carbamidomethyl (+57.02), c6: Carbamidomethyl (+57.02), t9: Dehydrated (-18.01)
TATcGcHItGK	0.997	c4: Carbamidomethyl (+57.02), c6: Carbamidomethyl (+57.02), t9: Dehydrated (-18.01)
TATcGcHItGK	0.979	c4: Carbamidomethyl (+57.02), c6: Carbamidomethyl (+57.02), t9: Dehydrated (-18.01)
TATcGcHItGK	0.982	c4: Carbamidomethyl (+57.02), c6: Carbamidomethyl (+57.02), t9: Dehydrated (-18.01)
TATcGcHITGK	0.987	c4: Dehydro (-1.01), c6: Dehydro (-1.01)
tATCGcHItGK	0.952	t1: Dehydrated (-18.01), c6: Carbamidomethyl (+57.02), t9: Dehydrated (-18.01)
tAtCGCHITGK	***0.955***	***t1: Dehydrated (-18.01), t3: Dehydrated (-18.01)***
tAtCGCHITGK	***0.997***	***t1: Dehydrated (-18.01), t3: Dehydrated (-18.01)***
tAtCGCHITGK	***0.997***	***t1: Dehydrated (-18.01), t3: Dehydrated (-18.01)***
tAtCGCHITGK	***0.987***	***t1: Dehydrated (-18.01), t3: Dehydrated (-18.01)***
tAtCGCHItGK	***0.961***	***t1: Dehydrated (-18.01), t3: Dehydrated (-18.01), t9: Dehydrated (-18.01)***
tAtCGCHItGK	***0.997***	***t1: Dehydrated (-18.01), t3: Dehydrated (-18.01), t9: Dehydrated (-18.01)***
tAtCGCHItGK	***0.997***	***t1: Dehydrated (-18.01), t3: Dehydrated (-18.01), t9: Dehydrated (-18.01)***
tAtCGCHItGK	***0.997***	***t1: Dehydrated (-18.01), t3: Dehydrated (-18.01), t9: Dehydrated (-18.01)***
tAtCGCHItGK	***0.997***	***t1: Dehydrated (-18.01), t3: Dehydrated (-18.01), t9: Dehydrated (-18.01)***
tAtCGCHItGK	***0.997***	***t1: Dehydrated (-18.01), t3: Dehydrated (-18.01), t9: Dehydrated (-18.01)***
tAtCGCHItGK	***0.997***	***t1: Dehydrated (-18.01), t3: Dehydrated (-18.01), t9: Dehydrated (-18.01)***
TAtcGCHITGK	**0.955**	**t3: Dehydrated (-18.01), c4: Carbamidomethyl (+57.02)**
TAtcGCHITGK	**0.997**	**t3: Dehydrated (-18.01), c4: Carbamidomethyl (+57.02)**
TAtcGCHITGK	**0.997**	**t3: Dehydrated (-18.01), c4: Carbamidomethyl (+57.02)**
TAtcGCHITGK	**0.953**	**t3: Dehydrated (-18.01), c4: Carbamidomethyl (+57.02)**
TAtcGCHITGK	**0.965**	**t3: Dehydrated (-18.01), c4: Carbamidomethyl (+57.02)**
TAtcGcHItGK	0.997	t3: Dehydrated (-18.01), c4: Carbamidomethyl (+57.02), c6: Carbamidomethyl (+57.02), t9: Dehydrated (-18.01)
TAtcGCHItGK	**0.972**	**t3: Dehydrated (-18.01), c4: Carbamidomethyl (+57.02), t9: Dehydrated (-18.01)**
TAtcGCHItGK	**0.971**	**t3: Dehydrated (-18.01), c4: Carbamidomethyl (+57.02), t9: Dehydrated (-18.01)**
TAtcGCHItGK	**0.997**	**t3: Dehydrated (-18.01), c4: Carbamidomethyl (+57.02), t9: Dehydrated (-18.01)**

TAtCGcHITGK	0.997	t3: Dehydrated (-18.01), c6: Carbamidomethyl (+57.02)
TAtCGcHITGK	0.997	t3: Dehydrated (-18.01), c6: Carbamidomethyl (+57.02)
TAtCGcHITGK	0.997	t3: Dehydrated (-18.01), c6: Carbamidomethyl (+57.02)
TAtCGcHITGK	0.975	t3: Dehydrated (-18.01), c6: Carbamidomethyl (+57.02)
TAtCGcHITGK	0.975	t3: Dehydrated (-18.01), c6: Carbamidomethyl (+57.02)
TAtCGcHITGK	0.954	t3: Dehydrated (-18.01), c6: Carbamidomethyl (+57.02)
TAtCGcHITGK	0.959	t3: Dehydrated (-18.01), c6: Carbamidomethyl (+57.02)
TAtCGcHITGK	0.947	t3: Dehydrated (-18.01), c6: Carbamidomethyl (+57.02)
TAtCGcHITGK	0.997	t3: Dehydrated (-18.01), c6: Carbamidomethyl (+57.02)
TAtCGcHITGK	0.997	t3: Dehydrated (-18.01), c6: Carbamidomethyl (+57.02)
TAtCGcHItGK	**0.997**	**t3: Dehydrated (-18.01), c6: Carbamidomethyl (+57.02), t9: Dehydrated (-18.01)**
TAtCGcHItGK	**0.997**	**t3: Dehydrated (-18.01), c6: Carbamidomethyl (+57.02), t9: Dehydrated (-18.01)**
TAtCGcHItGK	**0.974**	**t3: Dehydrated (-18.01), c6: Carbamidomethyl (+57.02), t9: Dehydrated (-18.01)**
TAtCGcHItGK	**0.975**	**t3: Dehydrated (-18.01), c6: Carbamidomethyl (+57.02), t9: Dehydrated (-18.01)**
TAtCGcHItGK	**0.979**	**t3: Dehydrated (-18.01), c6: Carbamidomethyl (+57.02), t9: Dehydrated (-18.01)**
TAtCGcHItGK	**0.97**	**t3: Dehydrated (-18.01), c6: Carbamidomethyl (+57.02), t9: Dehydrated (-18.01)**
TAtCGcHItGK	**0.997**	**t3: Dehydrated (-18.01), c6: Carbamidomethyl (+57.02), t9: Dehydrated (-18.01)**
TAtCGcHItGK	**0.997**	**t3: Dehydrated (-18.01), c6: Carbamidomethyl (+57.02), t9: Dehydrated (-18.01)**
TAtCGcHItGK	**0.997**	**t3: Dehydrated (-18.01), c6: Carbamidomethyl (+57.02), t9: Dehydrated (-18.01)**
TAtCGcHItGK	**0.977**	**t3: Dehydrated (-18.01), c6: Carbamidomethyl (+57.02), t9: Dehydrated (-18.01)**

Table 34 - List of all PSMs for tryptic peptide **SACTPGCPTGILMTCPLK** covering the ABC ring area of ClosA1-3 peptides.

Number of PSMs: 127, all modified, no missed cleavage, note that all matches have at least 1 CAM modification.

Abbreviations for all those tables: P=probability, CAM=Carbamidomethyl, Ox=Oxidation, dehydr=Dehydration.

Visual inspection of the corresponding spectra showed missing fragmentation in ring area in some spectra (see chapter 6, Figure 69).

Peptide sequence	P	Variable modifications identified by spectrum
SAcTPGcPTGILmTcPLK	0.966	c3: CAM (+57.02), c7: CAM (+57.02), m13: Ox (+15.99), c15: CAM (+57.02)
SActPGCPTGILMtcPLK	0.972	c3: CAM (+57.02), t4: dehyr (-18.01), t14: dehyr (-18.01), c15: CAM (+57.02)
sAcTPGCPTGILMtcPLK	0.974	s1: dehyr (-18.01), c3: CAM (+57.02), t14: dehyr (-18.01), c15: CAM (+57.02)
SAcTPGcPtGILmtcPLK	0.98	c3: CAM (+57.02), c7: CAM (+57.02), t9: dehyr (-18.01), m13: Ox (+15.99), t14: dehyr (-18.01), c15: CAM (+57.02)
SAcTPGcPtGILMtcPLK	0.984	c3: CAM (+57.02), c7: CAM (+57.02), t9: dehyr (-18.01), t14: dehyr (-18.01), c15: CAM (+57.02)
SAcTPGcPTGILmtcPLK	0.989	c3: CAM (+57.02), c7: CAM (+57.02), m13: Ox (+15.99), t14: dehyr (-18.01), c15: CAM (+57.02)
SAcTPGcPtGILMtcPLK	0.99	c3: CAM (+57.02), c7: CAM (+57.02), t9: dehyr (-18.01), t14: dehyr (-18.01), c15: CAM (+57.02)
SAcTPGcPTGILMTcPLK	0.995	c3: CAM (+57.02), c7: CAM (+57.02), c15: CAM (+57.02)
SAcTPGcPTGILmtcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), m13: Ox (+15.99), t14: dehyr (-18.01), c15: CAM (+57.02)
SAcTPGcPTGILMTcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), c15: CAM (+57.02)
SAcTPGcPTGILMTcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), c15: CAM (+57.02)
SAcTPGcPTGILMTcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), c15: CAM (+57.02)
SAcTPGcPTGILmTcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), m13: Ox (+15.99), c15: CAM (+57.02)
SAcTPGcPTGILmTcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), m13: Ox (+15.99), c15: CAM (+57.02)
SAcTPGcPTGILmTcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), m13: Ox (+15.99), c15: CAM (+57.02)
SAcTPGcPtGILmtcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), t9: dehyr (-18.01), m13: Ox (+15.99), t14: dehyr (-18.01), c15: CAM (+57.02)
SAcTPGcPTGILmtcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), m13: Ox (+15.99), t14: dehyr (-18.01), c15: CAM (+57.02)
SAcTPGcPTGILmtcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), m13: Ox (+15.99), t14: dehyr (-18.01), c15: CAM (+57.02)
SActPGCPTGILMtcPLK	0.997	c3: CAM (+57.02), t4: dehyr (-18.01), t14: dehyr (-18.01), c15: CAM (+57.02)
SActPGcPTGILMtcPLK	0.997	c3: CAM (+57.02), t4: dehyr (-18.01), c7: CAM (+57.02), t14: dehyr (-18.01), c15: CAM (+57.02)

SAcTPGcPTGILmTcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), m13: Ox (+15.99), c15: CAM (+57.02)
SAcTPGcPTGILmTcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), m13: Ox (+15.99), c15: CAM (+57.02)
SAcTPGcPtGILmtcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), t9: dehyr (-18.01), m13: Ox (+15.99), t14: dehyr (-18.01), c15: CAM (+57.02)
SActPGCPTGILmTcPLK	0.997	c3: CAM (+57.02), t4: dehyr (-18.01), m13: Ox (+15.99), c15: CAM (+57.02)
SACTPGcPtGILMTcPLK	0.997	c7: CAM (+57.02), t9: dehyr (-18.01), c15: CAM (+57.02)
sAcTPGCPtGILMtcPLK	0.997	s1: dehyr (-18.01), c3: CAM (+57.02), t9: dehyr (-18.01), t14: dehyr (-18.01), c15: CAM (+57.02)
sAcTPGCPtGILMtcPLK	0.997	s1: dehyr (-18.01), c3: CAM (+57.02), t9: dehyr (-18.01), t14: dehyr (-18.01), c15: CAM (+57.02)
SAcTPGcPTGILmTcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), m13: Ox (+15.99), c15: CAM (+57.02)
SActPGCPTGILMTcPLK	0.997	c3: CAM (+57.02), t4: dehyr (-18.01), c15: CAM (+57.02)
SAcTPGcPTGILmTcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), m13: Ox (+15.99), c15: CAM (+57.02)
SActPGcPTGILmtcPLK	0.997	c3: CAM (+57.02), t4: dehyr (-18.01), c7: CAM (+57.02), m13: Ox (+15.99), t14: dehyr (-18.01), c15: CAM (+57.02)
sACTPGcPTGILMtcPLK	0.997	s1: dehyr (-18.01), c7: CAM (+57.02), t14: dehyr (-18.01), c15: CAM (+57.02)
SAcTPGcPTGILMtcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), t14: dehyr (-18.01), c15: CAM (+57.02)
SActPGCPTGILMTcPLK	0.997	c3: CAM (+57.02), t4: dehyr (-18.01), c15: CAM (+57.02)
SAcTPGcPTGILMtcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), t14: dehyr (-18.01), c15: CAM (+57.02)
SActPGCPTGILMtcPLK	0.997	c3: CAM (+57.02), t4: dehyr (-18.01), t14: dehyr (-18.01), c15: CAM (+57.02)
SAcTPGcPTGILMTcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), c15: CAM (+57.02)
SAcTPGcPtGILMtcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), t9: dehyr (-18.01), t14: dehyr (-18.01), c15: CAM (+57.02)
SAcTPGcPTGILmTcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), m13: Ox (+15.99), c15: CAM (+57.02)
SAcTPGcPTGILMtcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), t14: dehyr (-18.01), c15: CAM (+57.02)
sAcTPGCPtGILMtcPLK	0.997	s1: dehyr (-18.01), c3: CAM (+57.02), t9: dehyr (-18.01), t14: dehyr (-18.01), c15: CAM (+57.02)
SAcTPGcPtGILMtcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), t9: dehyr (-18.01), t14: dehyr (-18.01), c15: CAM (+57.02)
SAcTPGcPTGILMtcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), t14: dehyr (-18.01), c15: CAM (+57.02)
SAcTPGcPTGILmTcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), m13: Ox (+15.99), c15: CAM (+57.02)
SAcTPGcPTGILmtcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), m13: Ox (+15.99), t14: dehyr (-18.01), c15: CAM (+57.02)
SAcTPGcPTGILmTcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), m13: Ox (+15.99), c15: CAM (+57.02)
SAcTPGCPtGILmtcPLK	0.963	c3: CAM (+57.02), t9: dehyr (-18.01), m13: Ox (+15.99), t14: dehyr (-18.01), c15: CAM (+57.02)

SActPGCPTGILMTcPLK	0.963	c3: CAM (+57.02), t4: dehyr (-18.01), c15: CAM (+57.02)
SAcTPGcPTGILMTcPLK	0.972	c3: CAM (+57.02), c7: CAM (+57.02), c15: CAM (+57.02)
SActPGCPTGILMtcPLK	0.973	c3: CAM (+57.02), t4: dehyr (-18.01), t14: dehyr (-18.01), c15: CAM (+57.02)
SAcTPGcPTGILMTcPLK	0.974	c3: CAM (+57.02), c7: CAM (+57.02), c15: CAM (+57.02)
SAcTPGcPTGILMtcPLK	0.979	c3: CAM (+57.02), c7: CAM (+57.02), t14: dehyr (-18.01), c15: CAM (+57.02)
SAcTPGcPTGILmTcPLK	0.993	c3: CAM (+57.02), c7: CAM (+57.02), m13: Ox (+15.99), c15: CAM (+57.02)
SAcTPGcPTGILMTcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), c15: CAM (+57.02)
SAcTPGcPTGILmTcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), m13: Ox (+15.99), c15: CAM (+57.02)
SAcTPGcPTGILmTcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), m13: Ox (+15.99), c15: CAM (+57.02)
SAcTPGcPTGILmTcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), m13: Ox (+15.99), c15: CAM (+57.02)
SAcTPGcPTGILMTcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), c15: CAM (+57.02)
SAcTPGcPTGILMTcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), c15: CAM (+57.02)
SAcTPGcPTGILmTcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), m13: Ox (+15.99), c15: CAM (+57.02)
SAcTPGcPTGILMtcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), t14: dehyr (-18.01), c15: CAM (+57.02)
SAcTPGcPTGILMTcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), c15: CAM (+57.02)
SAcTPGcPtGILMtcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), t9: dehyr (-18.01), t14: dehyr (-18.01), c15: CAM (+57.02)
SAcTPGcPTGILMTcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), c15: CAM (+57.02)
SAcTPGcPtGILMtcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), t9: dehyr (-18.01), t14: dehyr (-18.01), c15: CAM (+57.02)
SAcTPGcPTGILMTcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), c15: CAM (+57.02)
SAcTPGcPTGILmtcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), m13: Ox (+15.99), t14: dehyr (-18.01), c15: CAM (+57.02)
SAcTPGcPTGILMTcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), c15: CAM (+57.02)
SAcTPGcPTGILmTcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), m13: Ox (+15.99), c15: CAM (+57.02)
SAcTPGcPTGILMTcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), c15: CAM (+57.02)
SAcTPGcPTGILmtcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), m13: Ox (+15.99), t14: dehyr (-18.01), c15: CAM (+57.02)
SAcTPGcPTGILmTcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), m13: Ox (+15.99), c15: CAM (+57.02)
SAcTPGcPTGILmTcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), m13: Ox (+15.99), c15: CAM (+57.02)
SAcTPGcPTGILMTcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), c15: CAM (+57.02)

SAcTPGcPtGILmtcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), t9: dehyr (-18.01), m13: Ox (+15.99), t14: dehyr (-18.01), c15: CAM (+57.02)
SAcTPGcPTGILmtcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), m13: Ox (+15.99), t14: dehyr (-18.01), c15: CAM (+57.02)
SAcTPGcPtGILMtcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), t9: dehyr (-18.01), t14: dehyr (-18.01), c15: CAM (+57.02)
SAcTPGcPtGILmtcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), t9: dehyr (-18.01), m13: Ox (+15.99), t14: dehyr (-18.01), c15: CAM (+57.02)
SAcTPGcPTGILMTcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), c15: CAM (+57.02)
SActPGCPTGILmTcPLK	0.997	c3: CAM (+57.02), t4: dehyr (-18.01), m13: Ox (+15.99), c15: CAM (+57.02)
SAcTPGcPTGILmTcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), m13: Ox (+15.99), c15: CAM (+57.02)
SAcTPGcPTGILmtcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), m13: Ox (+15.99), t14: dehyr (-18.01), c15: CAM (+57.02)
SAcTPGcPtGILMtcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), t9: dehyr (-18.01), t14: dehyr (-18.01), c15: CAM (+57.02)
SAcTPGcPTGILMTcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), c15: CAM (+57.02)
SAcTPGcPTGILmTcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), m13: Ox (+15.99), c15: CAM (+57.02)
SAcTPGcPTGILMTcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), c15: CAM (+57.02)
SAcTPGcPTGILMTcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), c15: CAM (+57.02)
SAcTPGcPTGILMtcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), t14: dehyr (-18.01), c15: CAM (+57.02)
SAcTPGcPTGILmtcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), m13: Ox (+15.99), t14: dehyr (-18.01), c15: CAM (+57.02)
SAcTPGcPtGILMtcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), t9: dehyr (-18.01), t14: dehyr (-18.01), c15: CAM (+57.02)
SAcTPGcPTGILMtcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), t14: dehyr (-18.01), c15: CAM (+57.02)
SAcTPGcPTGILMTcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), c15: CAM (+57.02)
SAcTPGcPTGILmTcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), m13: Ox (+15.99), c15: CAM (+57.02)
SAcTPGcPTGILmtcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), m13: Ox (+15.99), t14: dehyr (-18.01), c15: CAM (+57.02)
SAcTPGcPTGILmTcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), m13: Ox (+15.99), c15: CAM (+57.02)
SAcTPGcPTGILMTcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), c15: CAM (+57.02)
SAcTPGcPTGILMtcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), t14: dehyr (-18.01), c15: CAM (+57.02)
SAcTPGcPTGILMTcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), c15: CAM (+57.02)
SAcTPGcPTGILMTcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), c15: CAM (+57.02)
SAcTPGcPtGILmtcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), t9: dehyr (-18.01), m13: Ox (+15.99), t14: dehyr (-18.01), c15: CAM (+57.02)
SAcTPGcPTGILmtcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), m13: Ox (+15.99), t14: dehyr (-18.01), c15: CAM (+57.02)

SAcTPGcPtGILmtcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), t9: dehyr (-18.01), m13: Ox (+15.99), t14: dehyr (-18.01), c15: CAM (+57.02)
SActPGCPTGILMtcPLK	0.997	c3: CAM (+57.02), t4: dehyr (-18.01), t14: dehyr (-18.01), c15: CAM (+57.02)
SAcTPGcPtGILMtcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), t9: dehyr (-18.01), t14: dehyr (-18.01), c15: CAM (+57.02)
sACTPGcPTGILmTcPLK	0.997	s1: dehyr (-18.01), c7: CAM (+57.02), m13: Ox (+15.99), c15: CAM (+57.02)
SAcTPGcPTGILMtcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), t14: dehyr (-18.01), c15: CAM (+57.02)
SACTPGcPtGILMtcPLK	0.997	c7: CAM (+57.02), t9: dehyr (-18.01), t14: dehyr (-18.01), c15: CAM (+57.02)
SAcTPGcPtGILmtcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), t9: dehyr (-18.01), m13: Ox (+15.99), t14: dehyr (-18.01), c15: CAM (+57.02)
SAcTPGcPTGILmTcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), m13: Ox (+15.99), c15: CAM (+57.02)
SAcTPGcPTGILMtcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), t14: dehyr (-18.01), c15: CAM (+57.02)
SAcTPGcPTGILmTcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), m13: Ox (+15.99), c15: CAM (+57.02)
SAcTPGcPTGILmtcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), m13: Ox (+15.99), t14: dehyr (-18.01), c15: CAM (+57.02)
SAcTPGcPtGILMtcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), t9: dehyr (-18.01), t14: dehyr (-18.01), c15: CAM (+57.02)
SAcTPGcPTGILMTcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), c15: CAM (+57.02)
SActPGCPTGILMtcPLK	0.951	c3: CAM (+57.02), t4: dehyr (-18.01), t14: dehyr (-18.01), c15: CAM (+57.02)
sAcTPGcPtGILMtcPLK	0.951	s1: S(+15) (+14.97), c3: C(-33) (-32.98), c7: CAM (+57.02), t9: dehyr (-18.01), t14: dehyr (-18.01), c15: CAM (+57.02)
SAcTPGcPTGILmtcPLK	0.959	c3: CAM (+57.02), c7: CAM (+57.02), m13: Ox (+15.99), t14: dehyr (-18.01), c15: CAM (+57.02)
sAcTPGCPTGILMtcPLK	0.974	s1: dehyr (-18.01), c3: CAM (+57.02), t14: dehyr (-18.01), c15: CAM (+57.02)
SAcTPGcPtGILmtcPLK	0.975	c3: CAM (+57.02), c7: CAM (+57.02), t9: dehyr (-18.01), m13: Ox (+15.99), t14: dehyr (-18.01), c15: CAM (+57.02)
SAcTPGcPTGILmTcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), m13: Ox (+15.99), c15: CAM (+57.02)
SAcTPGcPTGILmTcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), m13: Ox (+15.99), c15: CAM (+57.02)
SAcTPGcPTGILmTcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), m13: Ox (+15.99), c15: CAM (+57.02)
SAcTPGcPTGILMTcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), c15: CAM (+57.02)
SAcTPGcPtGILmtcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), t9: dehyr (-18.01), m13: Ox (+15.99), t14: dehyr (-18.01), c15: CAM (+57.02)
SAcTPGcPtGILMTcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), t9: dehyr (-18.01), c15: CAM (+57.02)
SAcTPGcPtGILMTcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), t9: dehyr (-18.01), c15: CAM (+57.02)
SAcTPGcPTGILMTcPLK	0.997	c3: CAM (+57.02), c7: CAM (+57.02), c15: CAM (+57.02)

Table 35 - List of all PSMs for tryptic peptide **SKSACTPGCPTGILMTCPLK** covering the ABC ring area of ClosA1-3 peptides.

Number of PSMs: 5, all modified, 1 missed cleavage.

The last PSM in this list shows modifications which would be related to ring fragmentation, but visual inspection of the spectrum revealed that the spectrum is of poor quality.

Peptide sequence	P	Variable modifications identified by spectrum
sKSACTPGcPTGILMtcPLK	0.997	s1: dehydr (-18.01), c9: CAM (+57.02), t16: dehydr (-18.01), c17: CAM (+57.02)
SKsAcTPGcPTGILMtcPLK	0.997	s3: S(-17) (-17.00), c5: CAM (+57.02), c9: Dehydro (-1.01), t16: dehydr (-18.01), c17: CAM (+57.02)
SKSACTPGcPTGILMTcPLK	0.997	c9: CAM (+57.02), c17: CAM (+57.02)
SKSACTPGcPTGILMTcPLK	0.997	c9: CAM (+57.02), c17: CAM (+57.02)
sKsAcTPGcPtGILMtcPLK	0.997	s1: S(-17) (-17.00), s3: S(-17) (-17.00), c5: Dehydro (-1.01), c9: C(-33) (-32.98), t11: dehydr (-18.01), t16: dehydr (-18.01), c17: C(-33) (-32.98)

Table 36 - List of all PSMs for tryptic peptide **YKSKSACTPGCPTGILMTCPLK** covering the ABC ring area of ClosA1-3 peptides.

Number of PSMs: 4, all modified, 2 missed cleavages.

The last PSM in this list shows modifications which would be related to ring fragmentation, but visual inspection of the spectrum revealed that the spectrum is of poor quality.

Peptide sequence	P	Variable modifications identified by spectrum
YKSKsAcTPGcPtGILmTcPLK	0.997	s5: S(+15) (+14.97), c7: C(-33) (-32.98), c11: CAM (+57.02), t13: dehydr (-18.01), m17: Ox (+15.99), c19: CAM (+57.02)
YKSKsAcTPGcPTGILmtcPLK	0.997	s5: S(+15) (+14.97), c7: C(-33) (-32.98), c11: CAM (+57.02), m17: Ox (+15.99), t18: dehydr (-18.01), c19: CAM (+57.02)
YKsKSACTPGcPTGILmtcPLK	0.997	s3: dehydr (-18.01), c11: CAM (+57.02), m17: Ox (+15.99), t18: dehydr (-18.01), c19: CAM (+57.02)
YKsKsAcTPGcPtGILmTcPLK	0.997	s3: S(+15) (+14.97), s5: S(+15) (+14.97), c7: Dehydro (-1.01), c11: Dehydro (-1.01), t13: dehydr (-18.01), m17: Ox (+15.99), c19: Dehydro (-1.01)