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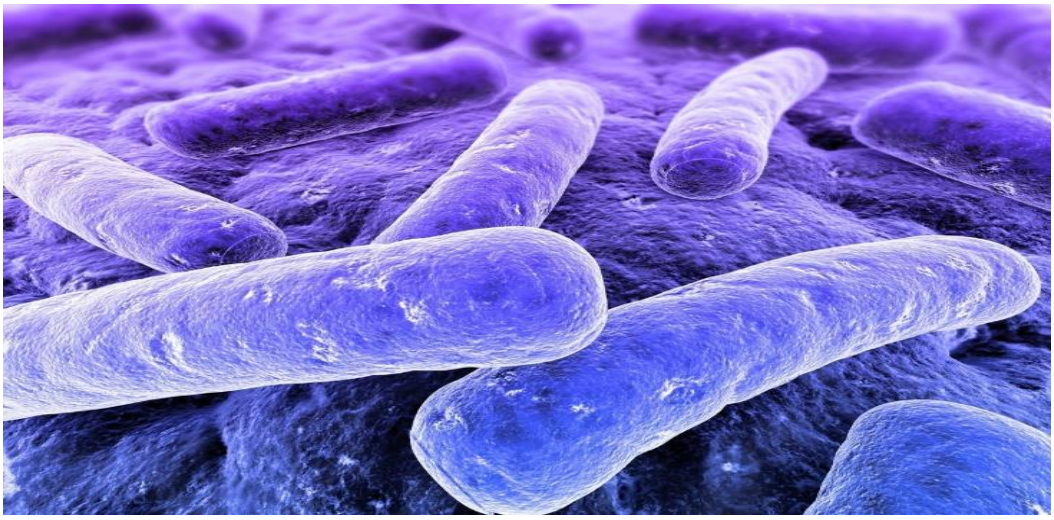
Comparison of different prenisin expression systems in *E. coli*



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Master research project

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Contents

Abstract

The huge void on novel antibiotic production during the past two decades due to rapidly increasing numbers of resistant pathogenic bacterial strains, has raised worldwide concern regarding public health. Therefore, attention has been drawn to novel ways of developing new antimicrobial substances.

Lantipeptides represent a whole new spectrum of potent bioactive drugs. They are characterized by the presence of cross-link thioether bonds in the core peptide, which are formed after the action of modification enzymes, which recognize designated sequences within the preceding leader peptide. Nisin is a class I lantipeptide which has been thoroughly examined for its antimicrobial activity and used in food industry for decades.

From another perspective, in the endeavor to enhance substrate diversity, further promoting antimicrobials, modification enzymes are being used to peptides that are unrelated to their native counterparts. Recently, rSAM homologous enzymes belonging to the *poy* gene cluster have shown to produce biologically intricate structures via numerous modifications like hydroxylations, N-methylations and epimerizations, capacity which endorses their use in antibiotic production.

In this project, at first nisin was fused with a hybrid leader containing recognition sites for modification enzymes belonging to the *poy* gene cluster (PoyF, PoyE, PoyD and co-expressed with NisBTC machinery in *Lactococcus lactis*, as we tried to observe if any modifications to the NisA core peptide would be present.

Further on, there was an effort to compare different prenisin expression systems in *E. coli* under various conditions, in order to observe whether modification and production were possible. After evaluating and comparing these systems, the best was selected for insertion of hybrid leader(s) containing recognition sites for OspD epimerase, a PoyD homologue, along with part of the native nisin leader. Production of modified prenisin was observed of the hybrid leader(s) constructs, in different temperatures (18^{o}C, 37^{o}C) and induction points (4h, 20h).

Introduction

Since the dawn of the 21st century, there has been a substantial gap concerning the production of new antibiotics, despite the fact that the endeavors have been perpetual both from science labs and pharmaceutical companies of every scale and status. There have been many challenges to these efforts, mainly deriving from the bacteria's ability to progressively adapt to changes in their environment, thus leading to evolutionary advanced organisms, resistant to more and more types of antibiotics, via different mechanisms, such as enzymatic degradation, changes in cell membrane permeability to antibiotics, or of sequence alteration of the proteins that are targeted by specific antibiotics. Hence, scientific world has turned to other routes on its relentless effort to generate improved antimicrobial drugs that will contribute in coping with resistance.

During the past decade, wide genome-mining strategies have assisted in discovering the rapidly increasing diversity of ribosomally synthesized natural products (RNPs). Specifically, a variety of different posttranslational modifications (PTMs), such as dehydration, cyclization, epimerization etc., result in amplifying the diversity of these compounds, rendering them a great option to experiment on and develop new antimicrobial drugs.

Lantipeptides

A certain class of RNPs that has been thoroughly studied and is also the one that will be examined in this report, are lantipeptides.

Lantipeptides stand for polycyclic peptides which are defined by the presence of the thioether cross-linked amino acids meso-lanthionine (Lan) and (2S,3S,6R)-3-methyllanthionine (MeLan) (Donk and Knerr, 2012). These non-canonical amino acids are formed after PTMs and then introduce intramolecular cyclic structures (Sahl and Bierbaum, 2009). Initially, the term ''lantibiotic'' was used to describe that class, although it shifted to ''lantipeptide'', so as to incorporate compounds that share the same structure but do not exert microbial activity (Donk and Knerr, 2012). Thioether bonds in peptides with antimicrobial properties, such as most lantipeptides, have been shown to offer stability and to protect from protein degradation (Mavaro et al., 2011). Thus far, over 60 different compounds have been isolated and described (Sahl and Bierbaum, 2009).

Lantipeptides are also characterized by the presence of a leader peptide prior to the core peptide sequence. Leader peptide has pleiotropic functions, as its sequence serves as recognition site for the modification enzymes so that they are able to make the designated modifications in the core peptide. Moreover, it serves as a translocation signal, and also keeps the peptide inactive (Plat et al., 2011), until it is exported to the cell membrane and cleaved by a specific protease.

The mechanism of action of lantipeptides has been studied meticulously in a small number of cases, but in general it is believed that the majority of them act by inhibiting cell wall synthesis via interaction with lipid II, a docking molecule which is critical for cell wall formation, and/or distorting cell membrane structure through pore formation (Donk and Knerr, 2012).

Lantipeptides of different classes have already been successfully expressed in *E. coli* expression systems (Shi et al., 2010), thus widely opening the route for large amounts of production.

Nisin

First lantibiotic to ever be described was nisin (Mattick and Hirsch, 1947). Nisin is 3354 Da peptide in its mature, active form and is consisted of 57 amino acids prior to leader peptide cleavage. It was isolated by Mattick and Hirsch in 1947 from the Gram-positive bacteria *Lactococcus lactis* (Mattick and Hirsch, 1947), and has been used successfully as food preservative ever since, to prevent growth of spoilage and pathogenic bacteria (Plat et al., 2011), without showing any significant signs of antimicrobial resistance (Donk and Knerr, 2012). In the case of nisin, the N-terminus of the mature molecule binds to the pyrophosphate moiety of lipid II by direct hydrogen bonding with the polypeptide backbone (de Kruijff et al., 2008). This results not only in inhibiting transglycosylation reaction, which is essential for peptidoglycan synthesis, but also in isolating lipid II in nonfunctional locations (Donk and Knerr, 2012).

Nisin belongs to the 1st class of lantipeptides. In that class, two separate modification enzymes act subsequently to eventually form the characteristic thioether rings. These enzymes, are dehydratase NisB and cyclase NisC, and are encoded by lanB and lanC genes, respectively. The enzyme NisB dehydrates the serine and threonine residues present in the core peptide, forming dehydroalanines (Dha) and dehydrobutyrines (Dhb), respectively, and cyclase NisC later comes to catalyze the coupling of the dehydrated amino acids to the thiol groups of cysteines (Plat et al., 2011), via bridge formation, resulting in the assembly of five thioether bonds (**Figure 1c**). After its modification, the core peptide with the leader still attached to it, is transferred across the lipid bilayer via the ATP-consuming transporter NisT, where the leader is cleaved by protease NisP processing.

The NisBTC modification machinery has been shown to work better when all members are present (Lubenski et al., 2009), as absence of either of the three components of the complex greatly compromised nisin production (van de Berg et al., 2008). However, after a plethora of *in vitro* and *in vivo* studies, it has been shown that each separate enzyme can act independently (Lubelski et al., 2009).

Lubelski et al., contributed a lot in research on nisin biosynthesis when they conducted experiments to investigate the sequence of processing of NisB and NisC to the core peptide, where the concluded that NisB and NisC act in an alternating fashion, where the processing of the core peptide follows a N- to C- terminus pattern, meaning the enzymes NisB and NisC catalyze the formation of ring A and then proceed to ring B and so on. (Lubelski et al., 2009)

Leader peptide

The leader peptide of nisin consists of 23 amino acids (**Figure 1**) and, as already mentioned, is critical for the production of nisin and also its antimicrobial activity. Arguably one of the most important parts within the leader sequence is the highly conserved F(N/D)LD box, which is located near the N-terminus. Complete removal of this box eliminated modification of the core peptide, illustrating that the FNLD box is utterly important as a recognition site for the NisBC enzymes (Plat et al., 2011) Replacing the box residues with alanines had similar results, while on the contrary, single or double substitutions demonstrated different results, as they allowed modification. However, production was compromised with most mutants, while surprisingly, a single mutant (L-16A) led to higher production (Plat et al., 2011). More recent data states that STKD (-22-19) and PR (-2-1) residues also play a critical role in interacting with NisB and NisC, as alanine substitutions of these residues led to decreased interaction with NisB, while it completely negated the capability of NisC to interact with the leader (Khusainov et al., 2013).

Furthermore, the distance between the recognition sites and the serine/threonine residues is considered imperative for interaction with NisB, as deletion of residues -14 to -1 led to 4 maximum dehydration, instead of the 8 designated ones (Plat et al., 2011). Therefore, these residues might serve as a spatial bridge that facilitates NisB to interact and fully modify precursor nisin (Plat et al., 2011).

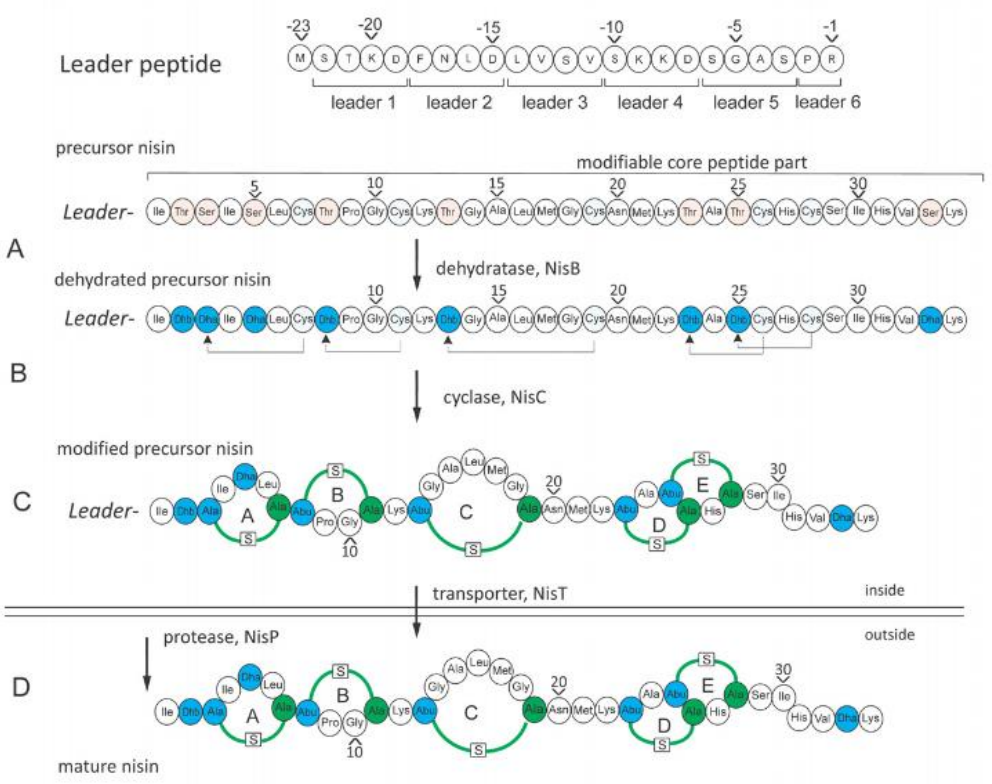


Figure 1. Schematic representation of nisin posttranslational modification. [A] Unmodified precurson nisin, consisting of leader and core peptide. Residues to be dehydrated are depicted in pink. [B] Dehydrated precusor nisin, after action of NisB. Dehydrated residues, dehydroalanines (Dha) and dehydroburytines (Dhb), are portrayed in blue. [C] Fully modified precursor nisin, after cyclase NisC

catalysis. Letters A-E correspond to the formed thioether bonds. [D] Mature nisin, after transportation and cleavage by NisT and NisP, respectively. (Khusainov et al., 2013).

Nisin-controlled expression (NICE) system

All the genes encoding the proteins and enzymes responsible for modification, transportation, regulation and immunity are embedded to a single gene cluster (**Figure 2**). That cluster contains 3 promoters, nisA, nisF and nisR. Among these three, nisR mediates the expression of *nisRK* genes, while nisA and nisF are responsible for driving expression by nisin-induced autoregulation (Zhou et al., 2006).

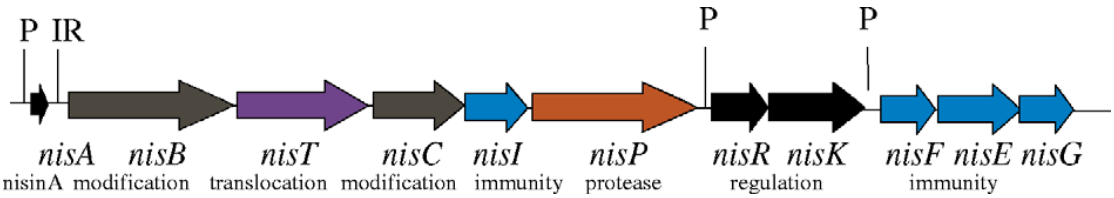


Figure 2. Outline of the nisin gene cluster. P denotes promoter; IR denotes interval region and PnisA and PnisF can be induced by nisin (Zhou et al., 2006).

Specifically, nisin initiates auto-regulation via a two-component regulatory system, consisting of the histidine kinase NisK and the response regulator, NisR. At first, nisin binds extra-cellularly to the N-terminus of NisK, prompting NisK to auto-phosphorylate and transfer a phospate group to NisR, which subsequently, acts as a transcription factor of nisA and nisF, and induces gene expression (Zhou et al.,2006). NICE system is well summarized in **Figure 3.**

As already mentioned, nisin gene cluster also contains genes that encode two proteins systems which are expressed and act cooperatively, granting immunity to the host producer. *NisI* encodes for a cell membrane related protein, while *nisFEG* encodes for a membrane localized ABC transporter (AlKhatib et al., 2014).

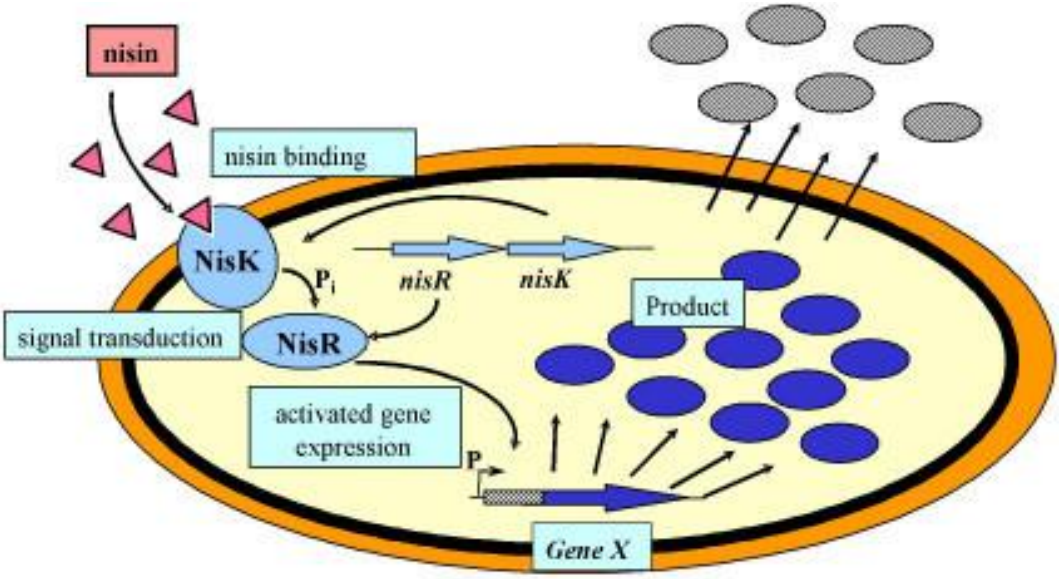


Figure 3. Schematic overview of the NICE system, its components and its function. NisK and NisR are the sensor protein and the response regulator, respectively. The product of the expressed gene can either accumulate in the cell or be secreted into the extracellular medium depending on the presence of a signal sequence in the construct (Mierau et al., 2005).

In order to increase diversity which will further assist in the attempts of making new antibiotics, different modification machineries could be tested to peptide leaders which are different to their native ones. In fact, there have already been successful research, where NisB had the capacity to modify therapeutical peptides unrelated to nisin, providing that nisin leader recognition sites were present (Kluskens et al., 2005). Such results maintain that as long as the designated sequences that are important for recognition by modification enzymes are present, all modifications can take place regardless of the nature of the substrate. Therefore, attention should be focused in the construction of combinatorial leaders, meaning, peptide sequences that contain multiple recognition sites for many different modification enzymes. That will generate even more different combinations of interacting peptides that were initially isolated from completely unrelated strains.

Proteusins

Recently, there has been interest about an intriguing modification machinery, that has been shown to create a unique structural complexity in specific compounds called polytheonamides A and B (PoyA), highly cytotoxic substances which were isolated from sponge *Theonella swinhoei* and are the first members of a new forming family, called proteusins (Freeman et al., 2012). These ribosomally synthesized metabolites share identical chemical composition, comprising of 48 amino-acids, 13 of them being nonproteogenic. To generate polytheonamides A and B, a total number of 48 post-translational modifications needs to be introduced, including 18 epimerizations, at least 21 carbon methylations and 4 hydroxylations (Freeman et al., 2012). These large number of modifications is credited to a specific gene operon in the sponge *Theonella swinhoei* (Figure 4). In this cluster, genes called *poyB, poyC and poyD* encode proteins

which are homologous to members of the radical S-adenosylmethionine (rSAM) superfamily, with PoyD assumed to be responsible for the altering from L- to D-configuration amino acids (with exception of achiral glycine) (Freeman et al., 2016). PoyE is homologous to SAM-dependent methyltransferases and responsible for the N-methylations of the asparagine residues inside the PoyA precursor peptide. PoyI is homologous to Fe(II)/a-ketoglutarate oxidoreductases and is speculated to catalyze hydroxylation of valines in PoyA. Finally, PoyF is homologous to the dehydratase domain of LanM-type lantibiotic synthetases. Besides these six enzymes, the cluster encodes other proteins which are probably involved in regulation, transport, and proteolytic removal of the leader region. No homology was found for PoyK, and it is unclear if it belongs to the specific pathway (Freeman et al., 2012).

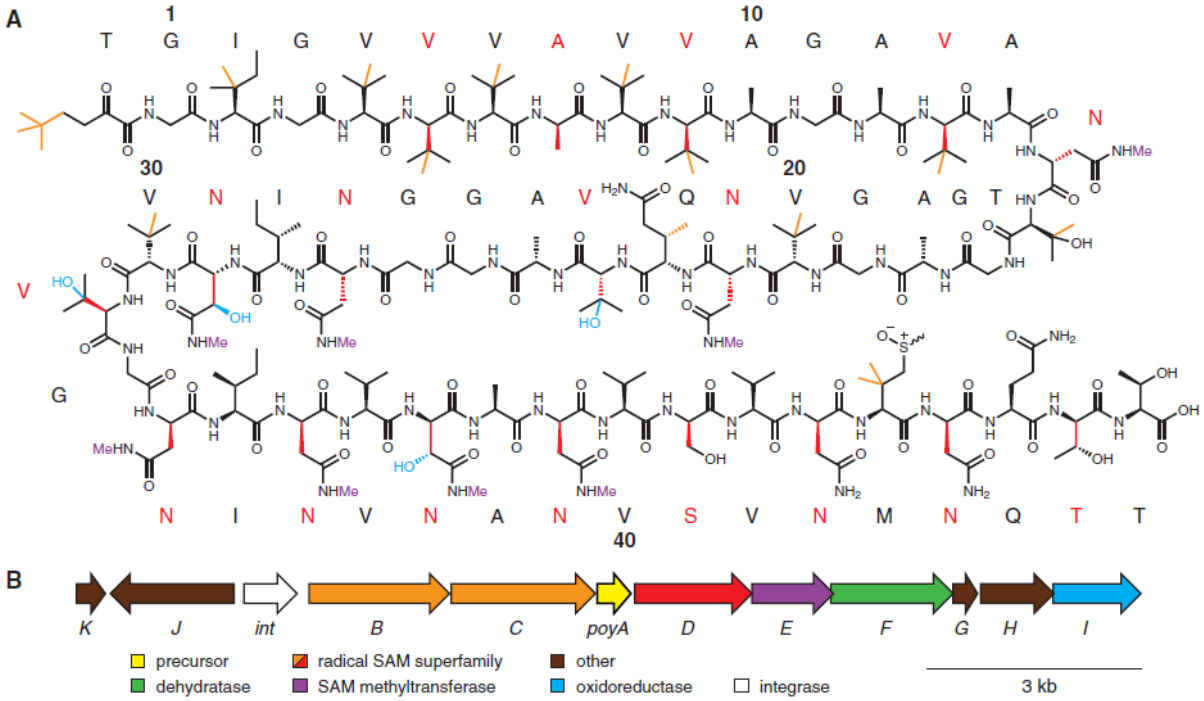


Figure 4. Polytheonamides: structures, genes, and biosynthetic model. (A) Polytheonamides A and B (shown) differ in the configuration of the sulfoxide moiety in residue 44. The sulfoxide arises from spontaneous oxidation during polytheonamide isolation. Residues are numbered on the basis of the typical notation for polytheonamides (2). The core peptide sequence is indicated by bold letters, with the color red denoting posttranslational epimerization. All other biosynthetic transformations during maturation of the core peptide are colored as follows: orange, C-methylation; purple, N-methylation; blue, hydroxylation; and green, dehydration (C). (B) Map of the polytheonamide (poy) biosynthetic gene cluster (Freeman et al., 2012).

There has already been efforts in integrating these newly characterized modification enzymes and/or homologues in *E. coli* expression systems (Morinaka et al., 2014, Freeman et al., 2016). *In vitro* studies have revealed a dependence of PoyA solubility and PoyD expression, as co-expressing PoyD and PoyA generated a soluble product, while PoyA, when expressed alone, generated an insoluble form of the protein (Freeman et al., 2016). Moreover, data illustrated that these enzymes seem to be highly regioselective and act on a great range of different substrates (Morinaka et al., 2014),

giving access to extensive peptide modifications of taxonomically irrelevant bacteria, in order to increase biological activity and bio-stability.

Maltose Binding Protein (MBP)

During this project there was also an attempt to fuse maltose binding protein to nisin precursor. Reasons were mainly to facilitate purification of the passenger protein, and also to enhance solubility, which have been observed to be the primary advantages of MBP compared to other fusing proteins (David S, PhD).

Aims of the project

The current project targets mainly on evaluating and comparing different expression systems of prenisin in *E. coli*, so as to observe whether it retains the ability to produce while still illustrating antimicrobial activity. Evaluation and comparison of these expression systems will help us to opt for the best one and integrate hybrid leader(s) containing both native nisin leader and OspA leader sequences, which are recognized by OspD epimerase, to investigate whether prenisin production is still possible.

Furthermore, introduction of *poy* genes and expression in *Lactococcus lactis* will take place, during the effort to observe whether these genes are capable of making modifications to nisin core peptide.

Materials and methods

Bacterial strains, plasmids and growth conditions

Lactococcus lactis NZ9000 was used as sensitive strain for activity assays of the different constructs that were made in *E. coli* expression systems. *L. lactis* was grown in M17 broth, supplemented with 0.5% glucose (GM17) with or without chloramphenicol (Cm, 5µg/ml) and/or erythromycin (Ery, 5µg/ml). Same strain was also as a host for incorporating *poy* genes and co-expressing them with NisBTC machinery.

Escherichia coli TOP10 electro-competent cells were used to introduce the vectors which contained the target genes in each different construct, after digestion and ligation, following transformation via electroporation. After confirmation of successful gene instertion via colony PCR, restriction analysis and plasmid sequencing, plasmids where transformed into expression *E. coli* BL21 (DE3) cells. List of bacterial strains and *E. coli* plasmids is presented in table 1**.**

Table 1. Strains and plasmids used in this study.

Strains	Characteristic(s)
***Lactococcus lactis* NZ9000**	***nisP***
***Escherichia coli* TOP10**	**Competent cells**
***Escherichia coli* BL21(DE3)**	**Competent cells used for expression**
Plasmids (*E. coli*)	**Characteristic(s)**
pET28b*nisA*	***nisA* gene, Km^R**
pET28b-*MBP-nisA*	***MBP* gene fused to nisA gene, Km^R**
pCDFDuet-1*nisBC*	***nisBC* cloned in 1st T7 promoter, $Spec^R$**
pRSF_T2_*nisA*	***nisA* gene cloned in 2nd T7 promoter, Km^R**
pBAD*nisB(T)C*	***nisB(T)C*, Amp^R**
pRSF*nisOsp1*$^{not\ mod}$	***nisOsp1*$^{not\ mod}$ with hybrid leader, Km^R**
pRSF*nisOsp1*^{mod}	***nisOsp1*^{mod} with modified hybrid leader, Km^R**
Plasmids (*L. lactis*)	**Characteristic(s)**
pNZ*nisP*	***nisP***
pIL253	
pTK41	***poyF* , Ery^R**
pTK44	***poyE* , Ery^R**
pTK45	***poyE* , Cm^R**
pTK50	***poyD*, Ery^R**
pTK51	***poyD* , Cm^R**
pIL3nisBTC	***nisBTC*, Cm^R**
pIL3ErynisBTC	***nisBTC* , Ery^R**

Molecular cloning

All plasmid isolations, PCR product and gel clean-ups were performed with Macherey-Nagel® respective DNA-RNA purification and PCR clean-up kits, according to the manufacturer's instruction manuals. However, during plasmid isolation, two extra incubation steps at 55°C were added before DNA elution, to improve product yield.

Genetic manipulations were performed by using standard and/or alternative procedures (Geu-Flores et al., 2007). Hereby are presented the different constructs that were made along with their own elaborate cloning plan that was followed:

- *L. lactis constructs**

pTK44, containing *poyE* (Ery^R)	pTK44, containing *poyE* (*Ery^R*) + pIL3*nisBTC* (Cm^R)
pTK45, containing *poyE* (Cm^R)	pTK45, containing *poyE* (*Cm^R*) + pIL3Ery*nisBTC* (Ery^R)
pTK50, containing *poyD* (Ery^R)	pTK50, containing *poyD* (*Ery^R*) + pIL3*nisBTC* (Cm^R)
pTK51, containing *poyD* (Cm^R)	pTK51, containing *poyD* (*Cm^R*) + pIL3Ery*nisBTC* (Ery^R)

*All the above listed plasmids were provided intact by the lab stock. However, before proceeding in any experiments, sequence of pTK plasmids was checked via restriction analysis.

Specifically, for each plasmid one particular restriction enzyme was selected, with the criteria that it cuts it in two different positions, with one of them located inside the insert gene. For the restriction analysis, 800 ng of DNA were used, following concentration calculation of each sample with NanoDrop. Plasmid restrictions were made in 20µl final volume, containing 2µl of 10X FastDigest Enzyme, 1µl of desired enzyme and the DNA template. Final volume was obtained by adjusting with miliQ H_2O. Enzymes used for each different plasmid are shown in table **2.**

Table 2. Restriction enzymes used for restriction analysis for each different plasmid

Plasmid	Restriction enzyme
pTK41	**SacI**
pTK44	**KpnI**
pTK45	**KpnI**
pTK50	**AseI**
pTK51	**BsaI**

.

E. coli constructs

Main cloning strategy that was followed during this project was based on using restriction sites containing primers, with these sites also being present in the target vector, so as to facilitate cloning process.

pET28b*nisA* + pCDFDuet-1*nisBC*, pET28b-*MBP-nisA*[1] + pCDFDuet-1*nisBC*[2]

Structural *nisA* gene was obtained from pNZnisA vector by PCR.

Primers used:

NisA_BspHI (forward) $^{5'}$ATCATCATGAAATTATAAGGAGGCACTCAC$_{3'}$ (for [1])

NisA_Fw_NdeI (forward) $^{5'}$ GGGAGCATATGAGTACAAAAGATTTTAACTTGGATTTGGTATCTG$_{3'}$ (for [2])

FISH_Lan_Rv_HindIII_EcoRV_KpnI (reverse) $^{5'}$CGAGGATATCGGTACCACGTTTCAAGCCTTGGTTTTC$_{3'}$

Reaction mixtures (50µl) contained: 5X HF buffer (10µl), dNTPs (0.8µl from 10mM stock), 0.5µl of each primer (10µM stock), Pfu X7 DNA polymerase (0.2µl of 1U/µl), DNA template (1µl from 5ng/µl pNZnisA) and miliQ H_2O (37µl).

PCR reaction program was: 1. Denaturation: 96^{o}C, 5min

2. Denaturation: 96^{o}C, 30s
   Annealing: 52^{o}C, 30s
   Extension: 72^{o}C, 30s
   } 35 cycles
3. Final extension: 72^{o}C, 1min

Colony PCR reactions were performed for clone screening. Reaction mixtures consisted of: 10X DreamTaq buffer (2 µl), dNTPs (0.5 µl of 10 mM stock), 0.5µl of each primer (10 µM stock),), Pfu X7 DNA polymerase (0.15 µl of 1U/µl) and miliQ H_2O (16,35µl). Selected colonies were picked with a sterile toothpick and gently grinded into empty PCR tubes. Subsequently, PCR reaction mixture was added to these tubes. Empty vector (0.5µl from 5ng/µl pNZnisA) was used as a positive control (**Figure 5**).

Primers used for this colony PCR:

NisA_Fw_NdeI (forward) $^{5'}$ GGGAGCATATGAGTACAAAAGATTTTAACTTGGATTTGGTATCTG$_{3'}$

pET_rev_GOOD (reverse) $^{5'}$CCTCAAGACCCGTTTAGAGG$_{3'}$

PCR reaction program was: 1. Denaturation: 96^{o}C, 5min

2. Denaturation: 96^{o}C, 30s
   Annealing: 55^{o}C, 30s
   Extension: 72^{o}C, 30s
   } 35 cycles
3. Final extension: 72^{o}C, 1min

A B

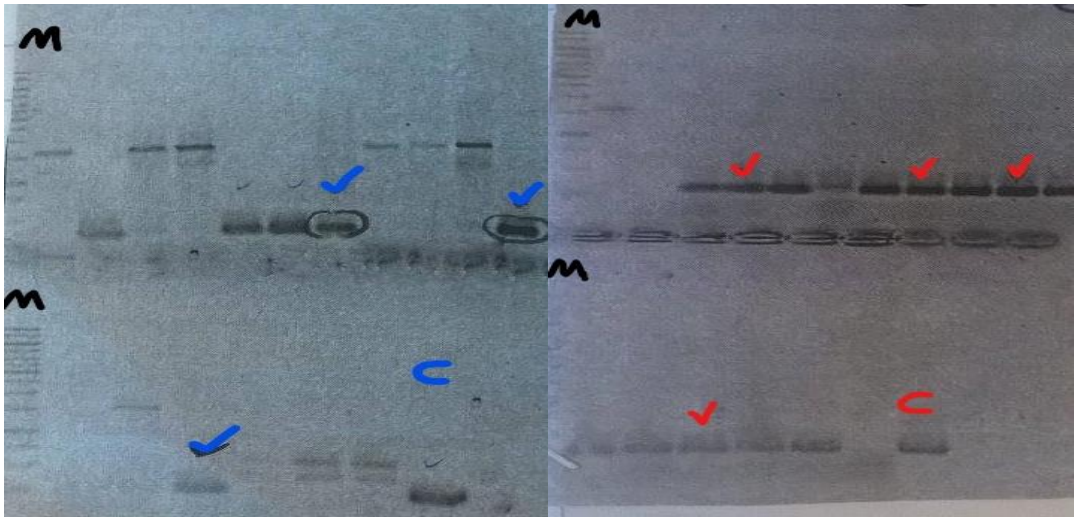


Figure 5. Gels of colony PCRs for **(A) pET28b*nisA*** ***and*** ***(B)*** **pET28b-*MBP-nisA*. (A)** M; marker. Blue color represents selected colonies who seem to have the expected size (~270bp) and control (C). **(B)** M; marker. Red color represents selected colonies who seem to have the expected size (~270bp) and control (C)

The primary strategy regarding that construct was to obtain the structural *nisBC* gene from pBADnisBC vector and clone in under the 2nd pCDFDuet-1 T7 promoter, via USER cloning method. This procedure requires specific primers that were designed according to relevant literature (Geu-Flores et al., 2007).

USER primers used:

pCDF_USER _FW (forward): 5'ATGGCAGA**U**CTCAATTGGATATCG3'
pCDF_USER_REV (reverse): 5'ATGTATA**U**CTCCTTCTTATACTTAAC3' } For vector (pCDF)

pCDF_nBC_FW_USER (forward): 5'ATATACA**U**ATGATAAAAAGTTCATTTAAAG3'
pCDF_nBC_Rev_USER (reverse): 5'ATCTGCCA**U**GTACAAGAAAGCTGGGTCTC3' } For instert (*nisBC* from pBAD)

Gradient PCR was performed for the amplification of the DNA fragments. Reaction mixtures (50µl, one for each fragment) contained 5X HF buffer (10µl), $MgCl_2$ (1µl from 50mM stock), dNTPs (0.5µl from 10mM stock), 0.5µl of each primer, Pfu X7 DNA polymerase (0.15 µl of 1U/µl), DNA template (1µl from pBADnisBC and pCDFDuet-1, for each respective mixture), and miliQ H_2O (37,4µl).

PCR was run as follows:

1. Denaturation: 96°C, 1min
2. Denaturation: 96°C, 1min
   Annealing: 60°C-50°C, 30s
   Extension: 68°C, 10min
   } 35 cycles
3. Final extension: 20°C, ∞

USER protocol was performed as described below:

USER mixture (11µl) consisted of approximately 100ng DNA from the target vector (pCDFDuet-1) and DNA from the desired insert (pBAD*nisBC*), and USER enzyme

(1µl, 5U/µl). Mixture was then incubated at 37ºC from 15min, following a 15min incubation at room temperature (25ºC).

Despite multiple efforts, a correct sequence for pCDFDuet-1*nisBC* could not be obtained. Reason may be attributed to DNA template overload in gradient PCR reaction. In any case, it was decided to opt for the regular cloning strategy, where *nisBC* would be obtained from pIL3nis*BC* vector and would be cloned into the 1st T7 promoter of pCDFDuet-1, via standard amplification PCR.

Primers used:

*nisB*_Fw_BspHI (forward):	5'AGGCTTCATGATAAAAAGTTCATTTAAAGCTCAACCGTTTTTAG3'
*nisC*_Rev_SalI (reverse):	5'AAGCTGGGTCTCATTTCCTCTTCC3'

Reaction mixture (50µl) contained: 5X HF buffer (10µl), dNTPs (0.8µl from 10mM stock), 0.5µl of each primer (10µM stock), Pfu X7 DNA polymerase (0.2µl of 1U/µl), DNA template (1µl from 5ng/µl pIL3BC) and miliQ H_2O (37µl).

PCR reaction program was: 1. Denaturation: 96ºC, 10min

2. Denaturation: 96ºC, 30s
   Annealing: 52ºC, 30s
   Extension: 72ºC, 5min
   } 35 cycles
3. Final extension: 72ºC, 10min

Colony PCR was performed as described previously (**Figure 6, (A)**).

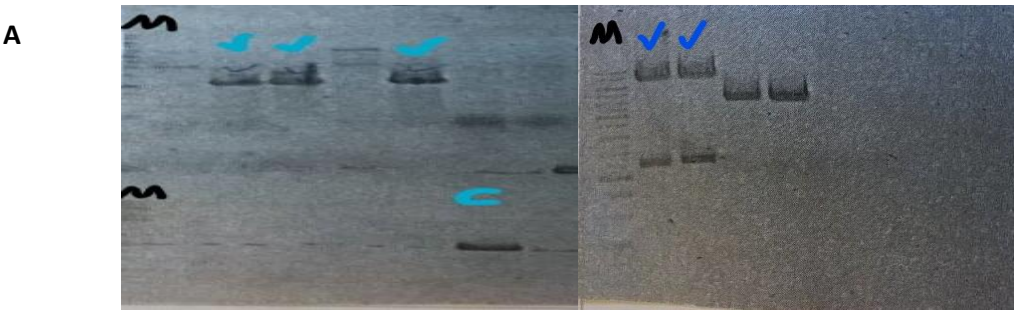


Figure 6. Gels of colony PCR **(A)** and restriction analysis **(B)** for **pCDFDuet-1*nisBC***. **(A).** M; marker**.** Blue color represents selected colonies who seem to have the expected size (~1100bp) and control (C). **(B)** M; marker. Blue color represents selected colonies with expected sizes (~ 6900bp and 1100bp) that were sent for sequencing

pRSF_T2_*nisA* + pCDFDuet-1*nisBC*

pRSF_T2_*nisA* plasmid was obtained from lab stock. Along with pCDFDuet-1*nisBC* (obtained as described), they were both transformed into BL21 (DE3) cells.

pRSF_T2_*nisA* + pBAD*nisB(T)C*

This construct was provided intact by the lab.

pRSF*nisOsp1*$^{not\ mod}$ + pBAD*nisBC* *(1)*, pRSF*nisOsp1*^{mod} + pBAD*nisBC* *(2)*

pRSF_T2_*nisA* plasmid was obtained from lab stock. Structural *nisOsp1* gene was obtained after amplification PCR, from pET28b*nisOsp1* vector, which was provided by the lab. This synthetic gene contains recognition sites for NisBC machinery and potential OspD recognition sites (**Figure 8**). In construct (1), *NisOsp* $^{not\ mod}$ was cloned under the 1st T7 promoter of pRSF vector, while in construct (2), **pRSF*nisOsp1*^{mod}** was cloned under the 2nd T7 promoter. Vector pBAD*nisBC* was provided by the lab.

Primers used:

pRSF_T1_Fw: $^{5'}$TCACCACCCTGAATTGACTC$_{3'}$ (for **(1)**)

pRSF_*nisOsp1*_NdeI_Fw:
$^{5'}$AAGGCATATGGGCCATCATCATCATCATCACTCCACCCGTAAAGAAGC$_{3'}$ (for **(2)**)

pET_rev_GOOD (reverse) $^{5'}$CCTCAAGACCCGTTTAGAGG$_{3'}$

Reaction mixtures (50µl) contained: 5X HF buffer (10µl), dNTPs (0.8µl from 10mM stock), 0.5µl of each primer (10µM stock), DNA template (1µl from 5ng/µl pET28b-*nisOsp1* in both cases) and miliQ H_2O (37,25µl).Phusion DNA polymerase (0,25µl of 2U/µl) was used for (1), while Pfu X7 DNA polymerase (0,25µl of 1U/µl), was used for (2).

PCR programs:

pRSF*nisOsp1*$^{not\ mod}$		**pRSF*nisOsp1*^{mod}**	
1. Denaturation: 96°C, 1min		1. Denaturation: 96°C, 1min	
2. Denaturation: 96°C, 30s		2. Denaturation: 96°C, 30s	
Annealing: 52°C, 30s	35 cycles	Annealing: 54°C, 30s	35 cycles
Extension: 72°C, 1min		Extension: 72°C, 30s	
3. Final extension: 72°C, 5min		3. Final extension: 72°C, 2min	

Colony PCR reactions were performed as described previously. PCR result is depicted in figure 7.

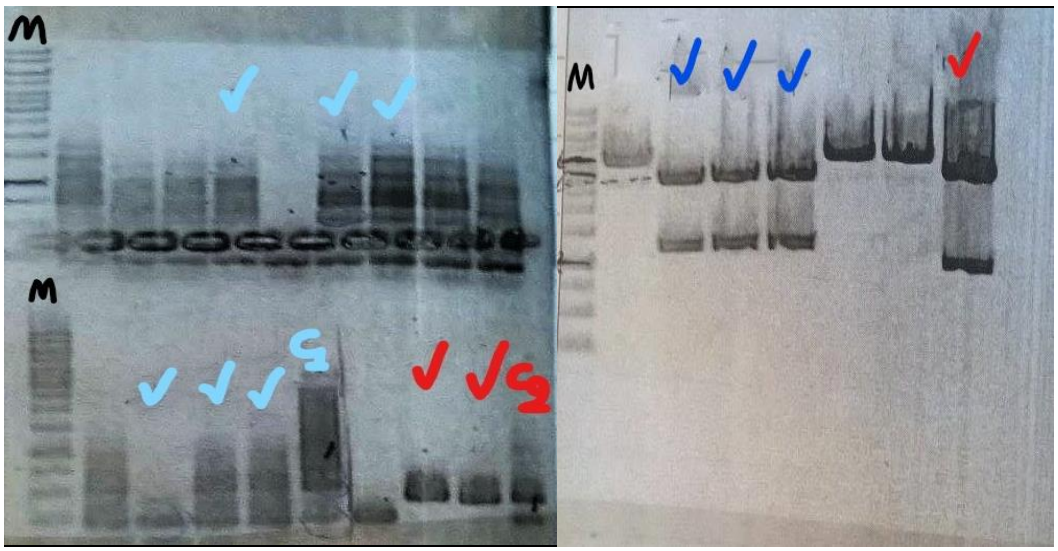
Reaction mixture was identical for both constructs and consisted of: 10X DreamTaq buffer (2 µl), dNTPs (0.5 µl of 10 mM stock), 0.5µl of each primer (10 µM stock),), Dream Tag DNA polymerase (0.3 µl of 2 U/µl) and miliQ H_2O (16,2µl)

Primers used for this colony PCR:

pRSFDuet-1_T7_1_seq_FW $^{5'}$TCACCACCCTGAATTGACTC$_{3'}$
pRSFDuet1_T7_1_Rev $^{5'}$GGCCGTGTACAATACGATTACTTTC$_{3'}$ — For **pRSF*nisOsp1*$^{not\ mod}$**

pRSFDuet1_T7_2_FW $^{5'}$GGCCGCATAATGCTTAAGTC$_{3'}$
pRSFduet-1_T7_2_Rev $^{5'}$AGCAGCGGTTTCTTTACCAG$_{3'}$ — For **pRSF*nisOsp1*^{mod}**

A



B

Figure 7. Gels of colony PCRs **(A)** and restriction analysis (**B)** for **pRSF*nisOsp1*$^{not\ mod}$ and pRSF*nisOsp1* ^{mod}**. **(A)** M; marker. Light blue color represents selected colonies who seem to have the expected size (~ 514bp), and control (C_1) for **pRSF*nisOsp1*$^{not\ mod}$**. Red color represents selected colonies who seem to have the expected size (~ 435bp), and control (C_2) for **pRSF*nisOsp1* ^{mod}. (B)** M; marker. Blue color represents selected colonies with expected sizes of **pRSF*nisOsp1*$^{not\ mod}$** (~ 1185bp and 2845bp) that were sent for sequencing. Red color represents selected colonies with expected sizes of **pRSF*nisOsp1* ^{mod}** (~ 989bp and 3035bp) that were sent for sequencing. Both restriction analysis were done using SmaI as restriction enzyme.

MGSSHHHHHHSSGLVPRGSHMSTRKEAEEQLAIKALKDFNLDLVSVSKKDSFASPRITSISLCTPGCKTGALMGCNMKTATCHCSIHVSK

MGHHHHHHSTRKEAEEQLAIKALKDFNLDLVSVSKKDSFASPRITSISLCTPGCKTGALMGCNMKTATCHCSIHVSK

Figure 8. Peptide sequences of the hybrid leaders of the *NisOsp1* gene used in this study. (Top) Initial peptide sequence of hybrid leader. (Bottom) Modified sequence of the initial hybrid leader. 6xHis-tag was introduced to facilitate prenisin purification. Orange; part of OspA native leader; Yellow; part of native nisin leader; Green; nisin core peptide; Colorless part contains His-Tag to facilitate purification (Bottom), as well as Thrombin cleavage recognition site (LVPRGS) (Top).

Digestions and ligations of vectors

For digestions of donor and recipient vectors, 800ng of DNA was used for plasmids and ~200ng for PCR clean-up products Amount (µl) in reaction mixture was determined after sample concentration measurement with NanoDrop. Total volume of reaction mixture (20µl for plasmids, 30µl for PCR products), was also supplemented with 10X FastDigest enzyme (2µl), the two selected restriction enzymes (1µl each), and miliQ H_2O. Enzymes that were used for each different construct are presented in table 3.

Following digestion, cut products were purified and subjected into ligation. Ligations were performed using 100ng of target vector and opting for a 3:1 to 5:1 molar ratio of insert. Reaction mix (20µl) contained T4 ligase (1µl of 5U/µl, Fermentas LIFE SCIENCES), 10X T4 ligase buffer (2µl). Final volume was reached with miliQ H_2O. Ligation

reaction was incubated for at least 1h at room temperature (25°C). Ultimately, ligation reaction ends with ligase heat-inactivation at 70°C for 5 minutes.

Table 3. List of restriction enzymes used for the digestion reaction of each different construct

Constructs	Vector	Insert
pET28b*nisA*	NcoI, HindIII	BspHI, HindIII
pET28b-*MBP-nisA*	NdeI, HindIII	NdeI, HindIII
pRSF*nisOsp1*$^{not\ mod}$	NcoI, HindIII	NcoI, HindIII
pRSF*nisOsp1*^{mod}	NdeI, XhoI	NdeI, XhoI
pCDFDuet-1*nisBC*	NcoI, SalI	BspHI,SalI

.

Preparation of electro-competent cells

L. lactis. ~1ml of ON culture (10ml) grown in M17 medium, supplemented with 0.5M sucrose, 0.5% glucose and 1% sterile glycine (SMGG) was regrown in 100ml of SMGG medium at 30°C without shaking, until OD_{600} reaches 0.2-0.7. Subsequently, cells were transferred in sterile falcons (50ml) and centrifuged at 6000g, for 15min at 4°C. Afterwards, cells were harvested and washed three times with ice cold buffer (50ml), containing: 0.5M sucrose and 10% glycerol (sterile), diluted in miliQ H_2O. Finally, cells were resuspended in 1ml of ice cold buffer and split into 40µl aliquots, to be stored in -80°C freezer.

E. coli. ~1ml of ON culture (6ml) grown in LB medium, were regrown in 100ml LB in 500ml flask at 37°C shaker, until OD_{600} reaches 0.6-0.8. Later on, cells were transferred in sterile falcons (50ml), and were left on ice for approximately 30min, to be finally centrifuged at 5000g for 10min, 4°C. Cells were harvested and resuspended in ice cold 100, 50 and 50ml of miliQ H_2O to be centrifuged at 6000g for 20min, 4°C, at 6000g for 25min, 4°C and at 7000g for 15min, 4°C, respectively Subsequently, cells were transferred in eppendorf tubes (2ml) and resuspended in 10% sterile glycerol (2ml), to be centrifuged at 13800rpm for 1min. Finally, cells were resuspended in 10% glycerol (0.2ml) and distributed in 40µl aliquots, to be stored at -80°C freezer.

Transformations

40 µl electro competent cells (TOP10, BL21(DE3)) were thawed on ice and mixed with 0.5-1µl of the ligation reaction, Afterwards, the mixture was transferred to an ice cold, 0.2 cm electroporation cuvette and pulsed (on a BioRad Gene Pulser) with the following settings: capacitor 25µF, Pulse Controller 200 Ω, voltage 250 kV. 1 mL of LB was added and suspension was transferred to a 1.5ml eppendorf tube and incubated at 37°C, 240 rpm for 1h. Different dilutions of concentrated cells were plated out on plates containing the appropriate antibiotics.

In case of *L. lactis* electro competent cells, cells were suspended in 1ml SM17 medium containing 0.5% Glucose, 20mM MgCl2 and 2mM CaCl2 (SGM17MC) placed in 1.5ml eppendorf tube and incubated at 30°C for 2h.

Activity assays

Activity assays were tested on sensitive strain plates. Sensitive strain plates comprised of a basic GM17 agar layer (4ml) on the bottom and the sensitive strain layer (7ml), which had a 3:1 GM17 agar:GM17 broth ratio, and contained sensitive strain ON culture (120µl) and nisin (1µl from 10µg/µl stock) to induce nisin production.

L. lactis

For each one of the *L. lactis* constructs, 3 ml of fresh ON culture (GM17 6ml, proper antibiotics) were spun down at 13800rpm for 1min. Cells were, then, resuspended in 200µl of MEM medium, that was supplemented with 0,4% glucose, 90Mµ $CaCl_2$, 2mM $MgCl_2$, 1000X vitamins stock, and proper antibiotics (Ery, Cm, 5µg/ml). Subsequently, cells were transferred to sterile bottles and grown in GM17 medium (15ml). Nisin (5ng/ml from 10µg/ml stock) was also added to induce the cells. Cells were incubated at 30ºC until they reach an OD_{600} of 0.6, to be induced again with nisin (15ng/ml). After 4h and 20h, 1.8ml of each construct was spun down (13800rpm, 1min), and supernatant was collected, to be stored at 4ºC.

Trichloroacetic acid (TCA) precipitation

Cells were then subjected to TCA precipitation. In each tube, TCA was added (200µl) and cells were left on ice for 2h. Every 30min tubes were inverted a few times and placed again on ice. Later on, cells were centrifuged at 13800rpm for 1h, 4ºC. Afterwards, the precipitate was resuspended in 1ml ice cold acetone and centrifuged at 13800rpm for 45min, 4ºC. Supernatant was discarded and subsequently, pellet was left at room temperature to be air-dried (open tubes). Finally, miliQ H_2O was added (25µl) for the peptide to be dissolved. Samples could then be used also for further analysis (SDS-PAGE, MS).

E. coli

800µl of fresh ON culture (LB 6ml, proper antibiotics) was re-inoculated in 250ml flask containing 40ml of LB with proper antibiotics, and was incubated at 37ºC shaker (initial OD_{600} ~ 0.1). Short list of antibiotics used for each construct is presented in table 3.

For constructs also containing the NisT transporter, 800µl of ON culture (LB 6ml, proper antibiotics) was spun down at 7000rpm for 1min and then resuspended in 800µl fresh LB, prior to being re-inoculated in 250ml flask, which did not contain antibiotics.

At first, in order for the optimal conditions that would grant decent nisin production and activity to be determined, several OD_{600} points and induction concentrations (only for IPTG) were used. Testing constructs, in which several conditions were tested, were our initial genetic devices, namely: **pET28b*nisA* + pCDFDuet-1*nisBC***, **pET28b-*MBP-nisA* + pCDFDuet-1*nisBC*** and **pRSF_T2_*nisA* + pCDFDuet-1*nisBC*.** OD_{600} points ranged from 0.6 to 1.5, and when the optimal point was determined, different IPTG concentrations, ranging from 0.5mM to 2mM, were also used.

When cells reached the desired OD_{600}, amount of flask content (30ml) was split into two sterile bottles. One would be induced, so as to activate *nisA* gene and modification

enzyme genes expression, while the other would serve as control. Above mentioned constructs were induced only with IPTG, as their vectors contain only T7 promoters, while **pRSF*nisOsp1*$^{not\ mod}$ + pBAD*nisBC*** and **pRSF*nisOsp1*^{mod} + pBAD*nisBC*,** were induced both with IPTG (1mM) and Arabinose (1mM), as pBAD vector contains arabinose promoter. Samples were, then, incubated either at 18°C or at 37°C. After 4h and 20h of incubation, 4ml of culture was spun down at 13800rpm for 1min. Supernatant was discarded and were, finally, resuspended in 50mM TrisHCl and 5mM CaCl buffer (pH=6.9). For constructs containing the NisT transporter, supernatant was also loaded.

Further on, cells were glass beaded 2 times for 1min with a 1min interval in between, and spun down at 138000rpm for 5min. Finally, part of the supernatant (40µl) was subjected into Trypsin treatment (1µl from 1mg/µl stock), and was incubated at 37°C for 4h or ON.

Several dilutions of samples either with or without trypsin treatment were made and were subsequently loaded (40µl) in wells of the sensitive strain plates. If a sample produces active nisin, a halo around the well will be observed, and its radius depends on the range of the activity.

Table 4. List of antibiotics used for *E. coli constructs. Km; Kanamycin, Spec; Spectinomycin, Amp; Ampicillin.*

Constructs	Antibiotics
pET28b*nisA* + pCDFDuet1*nisBC*	Km(20µg/ml), Spec(100µg/ml)
pET28b-*MBP-nisA* + pCDFDuet-1*nisBC*	Km(20µg/ml), Spec(100µg/ml)
pRSF*nisA* + pCDFDuet-1*nisBC*	Km(20µg/ml), Spec(100µg/ml)
pRSF*nisOsp1*$^{not\ mod}$ + pBAD*nisBC*	Km(20µg/ml), Amp(100µg/ml)
pRSF*nisOsp1*^{mod} pBAD*nisBC*	Km(20µg/ml), Amp(100µg/ml)

Tricine SDS-PAGE

Stacking and running gels were prepared as described (Schägger 2006). Samples (18µl), containing 4X SDS solution (6µl), were heat-shocked for 5min at 100°C prior to being loaded in the wells, along with 5µl of proper marker (BIO-RAD pre-stained low-range). Gels were run at 80V until samples had cleared the stacking gel. At the point, voltage was shifted to 120V for the rest of the run (2hours).

Gel staining

Gels were stained using Silver staining method. At first, gels remained in fixing solution (50% ethanol, 12% acetic acid, 0.1% formaldeyde (37%), and miliQ H_2O) to get rid of interfering compounds, for at least an hour. Subsequently, gels were subjected into successive washes (20min) with 50% ethanol. Afterwards, sensitizer buffer (0.02% $Na_2S_2O_3$ dissolved in miliQ H_2O) was added for 1min, and then gels were washed three times (20sec) with miliQ H_2O.. Next, gels were incubated in silver nitrate solution (0.2% $AgNO_3$, 0.1% formaldeyde (37%) in miliQ H_2O) for 15-25min with gentle shaking. Gels were washed again three times (20sec) with miliQ H_2O and remained in developer solution (6% Na_2CO_3, 2% sensitizing solution, 0.1% formaldeyde (37%) and miliQ H_2O) to generate silver staining, until it is considered sufficient. Gels were finally transferred to stop solution (50% ethanol, 12% acetic acid), for 10min to halt staining.

His-tag protein purification (E. coli)

Prenisin purification was done using Ni-NTA agarose (0,5ml bed volume) columns.

1ml of fresh ON culture was added to LB (50ml, proper antibiotics) and was grown in 500ml flask (one for control, one for induction) at 37°C shaker to OD_{600}= 0.6-0.8. Cells were then induced (IPTG or IPTG and Arabinose) and left to grow for 20h, at 18°C or 37°C. Culture was subsequently transferred from flasks into sterile falcons (50ml), and then were centrifuged at 6000rpm for 25min, 4°C. Supernatant was discarded and pellet was resuspended in 3ml of binding buffer (20mM NaPi, 0.5M NaCl, pH=7.3) and remained in -20°C for 1h. Cells were, then, thawed and transferred to ice cold water bath, to be lysed via sonication (Device settings: big probe, amplitude 75%, Pulse on:30sec, Pulse off: 20sec, 15min). Following sonication, lysed cells were centrifuged at 9800rpm for 50min, 4°C. Supernatant was collected and loaded on Ni-NTA agarose columns for overnight incubation at 4°C with rotational mixing (20-25rpm).

Next day, flow-through was collected into sterile falcons (15ml). Columns were washed with 10 column volumes of washing buffer (20mM NaPi, 0.5M NaCl, 15mM imidazole), flow-through was collected. Finally, five column volumes elution buffer (0.5M imidazole) was added, and purified prenisin was finally collected in 1.5ml eppendorf tubes (0.5ml fractions). Flow-through, wash, and elution collections were then used for further analysis (activity assay, SDS-PAGE, MS).

Columns were then washed (10 volumes 2M NaCl ⟹ 10 volumes binding buffer ⟹ 10 volumes 20% ethanol) and stored at 4°C with 20% ethanol.

To be recharged, columns were firstly stripped using proper stripping buffer (20mM NaPi, 0.5M NaCl, 50mM EDTA, pH=7.4), and then 0.1M $NiSO_4$, dissolved in miliQ H_2O was added.

Desalination

After purification, samples were de-salted using gravity columns (~4ml bed volume). Columns were equilibrated using miliQ H_2O (~30ml). Sample (2.5ml) was, then, added to the column, and flow-through was discarded. Finally, miliQ H_2O (3.5ml) was used for elution of de-salted peptide. Samples were stored in -80°C before used for further analysis.

Columns, were subsequently washed (10 volumes miliQ H_2O) and stored at 4°C with 20% ethanol.

Mass spectrometry

Samples were applied by spotting 1 µl of the supernatant on the target. After the spots were dried, they were washed once with 4 µl miliQ H_2O to remove the salts. Subsequently, 1 µl of matrix (5 mg/ml _-cyano-4-hydroxycinnamic acid in 50% acetonitrile containing 0.1% (v/v) trifluoroacetic acid) was added to the target and allowed to dry. Mass spectra were recorded with a Voyager DE PRO matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometer (Applied Biosystems). External calibration was used so as to ensure higher sensitivity.

Results

Optimal conditions for production of active nisin

pET28b*nisA* + pCDFDuet-1*nisBC*

Brief summary of activity results for this construct are presented in table 5 and table 6**.** Comparing the halo radiuses that where observed in the different activity assays, three main conclusions were drawn. Firstly, it was illustrated that production of active nisin is higher at $0D_{600}$=0.6 (**Figure 9)**, prompting the use of that point in the next experiments. Moreover, active nisin was produced only after 20h of incubation after induction, while no activity was observed after 4h in any of the experiments performed. Also, it was noticed that when samples were subjected into ON trypsin treatment, they had less or no activity, compared to when subjected into 4h trypsin treatment, which even gave higher active nisin compared to untreated samples, while maintaining the same OD_{600} point (**Table 5**, columns 3-5). No activity was observed at control samples, as expected.

Acknowledging the above conclusions, the construct was subsequently induced in different IPTG concentrations, where it was observed decent activity in three different points, again better in trypsin treat samples, and the better point was selected for next experiments (**Table 6**).

On the other hand, activity assay at 18^{o}C repeatedly returned no results.

Table 5. Summarization of experiments for optimal OD_{600} for production of active nisin for construct **pET28b*nisA* + pCDFDuet-1*nisBC*.** (mm) correspond to halo radius observed in activity plates. Blue color; no activity. In the first column the incubation times after induction **(/4h, /20h)** and the manipulation of the peptide (**Tr^{-}; No trypsin treatment**, **Tr^{+}; Trypsin treatment)**, are described. First row describes the different OD_{600} points, and the duration of Trypsin treatment (4h; 4hours, ON; overnight), in the experiments. Incubation temperature after induction at all experiments was at 37^{o}C. Experiments for OD_{600} 0.6 and 0.8 were performed at least twice with same results.

OD_{600} Conditions	0.6	0.6 2x dillution	0.8(Tr^{4h})	0.8(Tr^{4h}) 2x dillution	0.8(Tr^{ON})	1.0(Tr^{ON})	1.5
Tr^{+}/4h							
Tr^{--}/4h							
Tr^{+}/20h	4-5mm	2-3mm	2-3mm	1-2mm			1-2mm
Tr^{--}/20h	2-3mm	1-2mm	1-2mm		1-2mm		

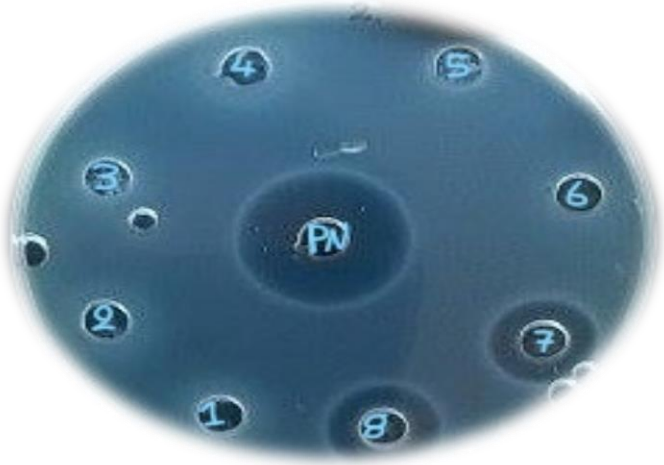


Figure 9. Activity plate for **pET28b*nisA* + pCDFDuet-1*nisBC***. C;control, I; induction, Tr^-; No trypsin treatment, Tr^+; Trypsin treatment. (/4h, /20h); hours of incubation after induction. **1**; C Tr^-/4h, **2**; C Tr^+/4h, **3**; C Tr^-/20h, **4**; C Tr^+/20h, **5**; I Tr^-/4h, **6**; I Tr^+/4h, **7**; I Tr^-/20h, **8**; I Tr^+/20h, PN; prenisin.

Table 6. Summarization of experiments for optimal IPTG concentration for production of active nisin for construct **pET28b*nisA* + pCDFDuet-1*nisBC*.** (mm) correspond to halo radius observed in activity plates. Blue color; no activity. In the first column the incubation times after induction **(/4h, /20h)** and the manipulation of the peptide (**Tr^-; No trypsin treatment**, **Tr^+; Trypsin treatment)**, are described. First row describes the different IPTG points used. Trypsin treatment for all samples was 4h, due to previous results. Incubation temperature after induction at all experiments was at 37°C.

IPTG (mM) OD_{600}=0.6	0.5mM	1mM	1mM 2x dillution	1.5mM	1.5mM 2x dillution	2mM
Tr^+/4h						
Tr^-/4h						
Tr^+/20h	1-2mm	4-5mm	2-3mm	4-5mm	1-2mm	2-3mm
Tr^-/20h		2-3mm	1-2mm	2-3mm	1-2mm	1-2mm

pET28b-*MBPnisA* + pCDFDuet-1*nisBC*

Results for this construct are depicted in tables 7 and 8. Similar to **pET28b-*MBPnisA* + pCDFDuet-1*nisBC*,** no activity was observed after 4h of incubation after induction. Also, once more trypsin treated samples gave higher active nisin, even when samples were treated ON (**Table 7,** columns 2, 3). However, control samples also displayed some activity in some experiments, both at 18°C and 37°C, thus rendering difficult to elicit a valid conclusion about the construct's credibility.

Table 7. Activity results for **pET28b-*MBPnisA* + pCDFDuet-1*nisBC***. (mm) correspond to halo radius observed in activity plates. Blue color; no activity. In the first column the incubation times after induction **(/4h, /20h)** and the manipulation of the peptide (**Tr^-; No trypsin treatment**, **Tr^+; Trypsin treatment)**, are described. First row describes the different OD_{600} points, and the duration of Trypsin treatment (4h; 4hours, ON; overnight), in the experiments. Incubation temperature after induction at all experiments was at 37ºC and IPTG=1mM.

OD_{600}	0.6 (Tr^{4h})	1.0(Tr^{ON})	1.0(Tr^{ON}) Control
Tr^+/4h			
Tr^{--}/4h			
Tr^+/20h	2mm	3mm	2mm
Tr^{--}/20h			

Comparing these two mentioned constructs, **pET28b*nisA* + pCDFDuet-1*nisBC*** seems to have superior activity, at least at 37ºC, since activity assay for **pET28b-*MBPnisA* + pCDFDuet-1*nisBC*** at 18ºC (**Table 8**), gave decently high active nisin levels, although controls again showed some activity as well.

Table 8. Activity results for **pET28b-*MBPnisA* + pCDFDuet-1*nisBC***. (mm) correspond to halo radius observed in activity plates. Blue color; no activity. In the first column the incubation times after induction **(/4h, /20h)** and the manipulation of the peptide (**Tr^-; No trypsin treatment**, **Tr^+; Trypsin treatment)**, are described. First row describes the different samples (control, induction) and dilutions in the experiments. Incubation temperature after induction at all experiments was at 18ºC, OD_{600}=0.6 and IPTG=1mM.

Conditions/Dillutions	Control	0	2x	4x
Tr^+/4h				
Tr^{--}/4h				
Tr^+/20h		2mm		
Tr^{--}/20h	4mm	6mm	3mm	1mm

pRSF*nisA* + pCDFDuet-1*nisBC*

Results of this construct are portrayed in table 9. Construct showed decent activity after 20h of incubation, again slightly better after being treated with trypsin (4h). Samples of 4h incubation time after induction again gave no results.

Table 9. Activity results for **pRSF*nisA* + pCDFDuet-1*nisBC*.** (mm) correspond to halo radius observed in activity plates. Blue color; no activity. In the first column the incubation times after induction **(/4h, /20h)** and the manipulation of the peptide (**Tr^-; No trypsin treatment**, **Tr^+; Trypsin treatment)**, are described. First row describes the dilutions in the experiments. Incubation temperature after induction at all experiments was at 37°C, OD_{600}=0.6 and IPTG=1mM. Experiment was repeated twice with same results.

Conditions/Dillutions	0	2x	4x
(Tr-/4h)			
(Tr+/4h)			
(Tr-20h)	4mm	3mm	
(Tr+/20h)	4mm	3mm	

Judging by the above mentioned results that were derived from all the activity assays and comparing the three initials constructs together, it was determined to choose **pET28b*nisA* + pCDFDuet-1*nisBC*** to further proceed and integrate the hybrid leader(s), as it seemed to have the best activity, at least at 37°C. Unfortunately, hybrid leader construct **pRSF*nisOsp1*$^{not\ mod}$ + pCDFDuet-1*nisBC*** repeatedly gave no activity, neither at 18°C nor at 37°C.

pRSF*nisA* + pBAD*nisB(T)C*

Since the above mentioned selected construct gave no active nisin, attention was drawn to previous constructs that had shown decent activity when tested in the beginning of this study (OD_{600}=0.8, Arabinose=1mM, IPTG=1mM, data not shown). Repetition of the experiment, both at 37°C and 18°C, returned similar encouraging results (**Figure 10, Table 10)**. Activity observed was way superior to any of the three initial constructs that were used, both after 4h and 20h after induction, with and without trypsin treatment. In addition, when NisBTC machinery was tested, supernatant activity was measured, where it was observed that highly active nisin was successfully exported out of the cell.

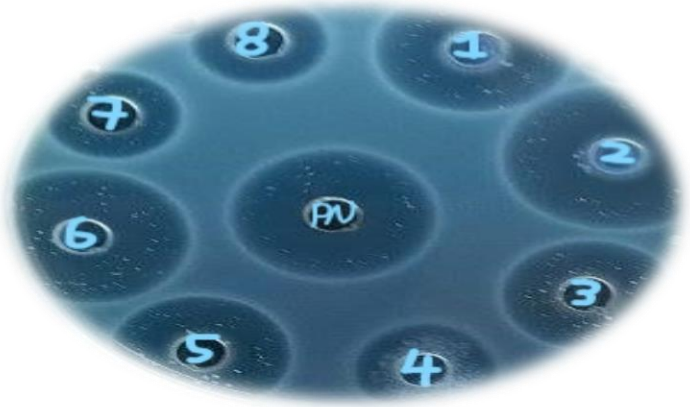


Figure 10. Activity plate of **pRSF*nisA* + pBAD*nisB(T)C***. BC; BC machinery, BTC; BTC machinery, Tr^-; No trypsin treatment, Tr^+; Trypsin treatment. (/4h, /20h); hours of incubation after induction. **1**; BTC Tr^-/4h, **2**; BTC Tr^-/20h, **3**; BC Tr^-/4h, **4**; BC Tr^-/20h, **5**; BTC Tr^+/4h, **6**; BTC Tr^+/20h, **7**; BC Tr^+/4h, **8**; BC Tr^+/20h, PN; prenisin.

Table 10. Activity results for **pRSF*nisA* + pBAD*nisB(T)C***. (mm) correspond to halo radius observed in activity plates. Blue color; no activity. In the first column the incubation times after induction **(/4h, /20h)** and the manipulation of the peptide (**Tr^-; No trypsin treatment**, **Tr^+; Trypsin treatment)**, are described. CE; cell extract, SN; supernatant. First row describes the dilutions in the experiments. Incubation temperature after induction at all experiments was at 37ºC for NisBC machinery and 18ºC for NisBTC machinery. OD_{600}=0.8, Arabinose=1mM and IPTG=1mM.

	Conditions/Dilutions	0	2x	4x	8x	16x
NisBTC machinery	*CE (Tr-/4h)*	8mm	7.5mm	7mm	7mm	6mm
	CE (Tr+/4h)	8mm	7mm	6mm	5mm	4mm
	CE (Tr-20h)	10mm	10mm	10mm	9mm	8.5mm
	CE (Tr+/20h)	10mm	10mm	9mm	6mm	5.5mm
	SN (Tr-/4h)	1,5mm				
	SN (Tr+/4h)					
	SN (Tr-20h)	8mm	7mm			
	SN (Tr+/20h)	5.5mm	5mm			
NisBC machinery	*CE (Tr-/4h)*	7mm	6mm	5mm	5mm	4mm
	CE (Tr+/4h)	5mm	4mm	3mm		
	CE (Tr-20h)	5mm	4mm	3mm		
	CE (Tr+/20h)	4.5mm	4mm	3mm		

These encouraging results prompted the introduction of the hybrid leaders firstly to the **pRSF*nisA* + pBAD*nisBC*** construct.

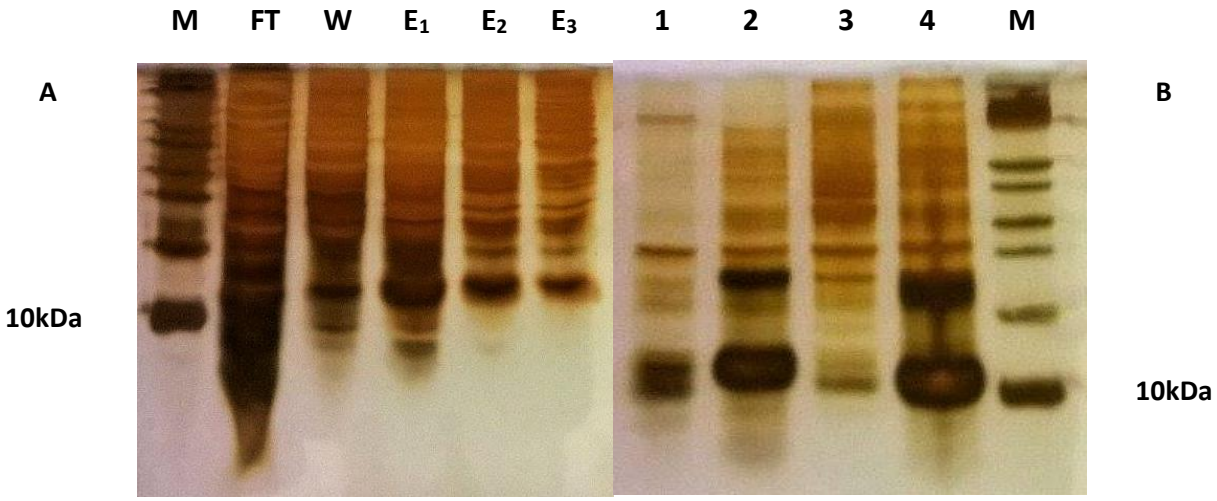


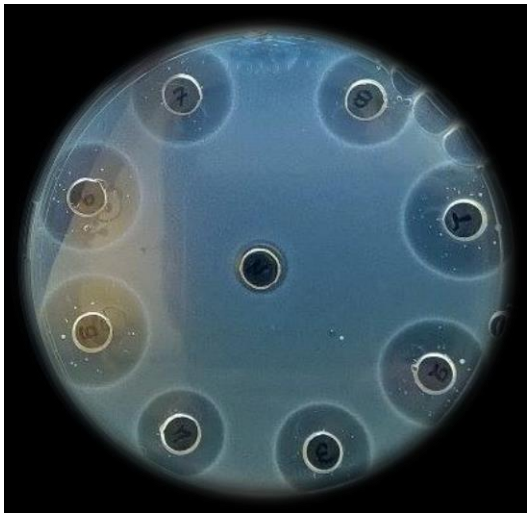
Figure 11. SDS-PAGE gels of **(A) pRSF*nisOsp1*$^{not\ mod}$ + pBAD*nisBC*** after purification and **(B) pRSF*nisOsp1*$^{not\ mod}$ + pBAD*nisBC*, pRSF*nisOsp1*^{mod} + pBAD*nisBC*** after purification and de-saltation. **(A)** Gel was obtained from purified sample after it had been induced (IPTG, Arabinose) and incubated for 20h at 37°C. M;marker, FT; flow-through, W; wash, E_{1-3}; elution fractions. E_1 and E_2 both contain a faint band (clearer in E_1) that corresponds to **pRSF*nisOsp1*$^{not\ mod}$** peptide with the leader still attached to it (~9600Da-9460Da, depending on whether it has been modified, either fully or partially, or not). **(B)** Gel was obtained from purified and de-salted samples after they had been induced (IPTG, Arabinose) and incubated for 20h at 18°C. All samples consist concentrated elution fractions. M; marker, **1-2**; Control-Induction of **pRSF*nisOsp1*$^{not\ mod}$ + pBAD*nisBC***, respectively. **3-4**; Control-Induction of **pRSF*nisOsp1*^{mod} + pBAD*nisBC***, respectively. Someone can easily observe a really intense and thick band near the 10kDa mark in both induction samples. Samples in both cases are overloaded resulting in protein smearing, however, it can be said that bands may correspond to the desired peptides, despite the fact that the bands are slightly above the 10kDa mark, as sample overloading causes proteins move downwards in a slower pace.

pRSF*nisOsp1*$^{not\ mod}$ + pBAD*nisBC*, pRSF*nisOsp1*^{mod} + pBAD*nisBC*

New hybrid leader(s) constructs were tested in the optimal conditions for this specific construct, as mentioned (OD_{600}=0.8, Arabinose=1mM, IPTG=1mM). Activity assay results were really encouraging, since highly active nisin was produced under certain conditions, mainly at 18°C (**Table 11** and **12, figures 12, 13, 14**). Also, modified leader construct had slightly better activity comparing to the initial hybrid leader. Surprisingly, trypsin treated samples gave no production of nisin.

On the other hand, control samples also gave some levels of activity in some cases, especially at 18°C, which raises questions concerning construct's reliability.

Constructs were further processed and subjected into purification and de-saltation both at 18°C and 37°C. Samples were then analyzed via SDS-PAGE (**Figure 11**), and a fraction was also used for activity assays in sensitive strain plates (**Tables 13, 14**).



Figures 12, 13. Activity plates of diluted samples of **pRSF*nisOsp1*^{mod} + pBAD*nisBC*** (left) and **pRSF*nisOsp1*$^{not\ mod}$ + pBAD*nisBC*** (right) at 37ºC. (/4h, /20h); hours of incubation after induction. All samples have not been trypsin treated. Both plates follow the same scheme. **1**; 2x/4h, **2**; 4x/4h, **3**; 8x/4h, **4**; 16x/4h, **5**; 2x/20h, **6**; 4x/20h, **7**; 8x/20h, **8**; 16x/20h.

Table 11. Activity results for **pRSF*nisOsp1*$^{not\ mod}$ + pBAD*nisBC*, pRSF*nisOsp1*^{mod} + pBAD*nisBC.*** (mm) correspond to halo radius observed in activity plates. Blue color; no activity. In the first column the incubation times after induction **(/4h, /20h)** and the manipulation of the peptide (**Tr$^-$; No trypsin treatment**, **Tr$^+$; Trypsin treatment)**, are described. First row describes the different samples (control, induction) and dilutions in the experiments. Incubation temperature after induction at all experiments was at 37ºC. OD_{600}=0.8, Arabinose=1mM and IPTG=1mM.

	Conditions/Dillutions	Control	0	2x	4x	8x	16x
Modified leader	*(Tr-/4h)*		8mm	6.5mm	5mm	5mm	4mm
	(Tr+/4h)		6mm	5mm	4mm	2mm	1mm
	(Tr-20h)	1mm	8mm	7mm	6mm	5mm	4mm
	(Tr+/20h)		5mm	4mm	2mm	1mm	
Unmodified leader	*(Tr-/4h)*	2.5mm	3.5mm	2mm	1mm		
	(Tr+/4h)						
	(Tr-20h)	2.5mm	7.5mm	5mm	5mm	4mm	4mm
	(Tr+/20h)						

Figure 14. Activity plate of **pRSF*nisOsp1*^{mod} + pBAD*nisBC*** and **pRSF*nisOsp1*$^{not\ mod}$ + pBAD*nisBC*** at 18°C. MC; Control modified leader, MI: Induction modified leader (when M is absent; unmodified leader), (/4h, /20h); hours of incubation after induction. All samples have not been trypsin treated. **1**; C/4h, **2**; C/20h, **3**; MC/4h, **4**; MC/20h, **5**; I/4h, **6**; I/20h, **7**; MI/4h, **8**; MI/20h.

Table 12. Activity results for **pRSF*nisOsp1*$^{not\ mod}$ + pBAD*nisBC*, pRSF*nisOsp1*^{mod} + pBAD*nisBC*. (**mm) correspond to halo radius observed in activity plates. Blue color; no activity, purple color; not measured. In the first column the incubation times after induction **(/4h, /20h)** and the manipulation of the peptide (**Tr$^-$; No trypsin treatment)**, are described. First row describes the different samples (control, induction) and dilutions in the experiments. Incubation temperature after induction at all experiments was at 18°C. OD_{600}=0.8, Arabinose=1mM and IPTG=1mM.

	Conditions/Dillutions	Control	0	2x	4x	8x	16x
Modified leader	*(Tr-/4h)*		8mm	7mm	7mm		
	(Tr-20h)	5mm	9mm	8mm	8mm		
Unmodified leader	*(Tr-/4h)*	2mm	8mm	7mm	6.5mm		
	(Tr-20h)	4mm	9mm	8mm	7.5mm		

Table 13. Activity results for **pRSF*nisOsp1*^{mod} + pBAD*nisBC*** after His-Tag purification **(A)** and de-saltation **(B)** at 37°C and after His-Tag purification and de-saltation at 18°C **(C)**. (mm) correspond to halo radius observed in activity plates. Blue color; no activity. FT; flow-through. Incubation temperature after induction at all experiments was at 37°C. OD_{600}=0.8, Arabinose=1mM and IPTG=1mM.

A

After HisTag Purification

Samples/Dillutions	0x
Elution 1	6mm
Elution 2	6mm
Elution 3	5mm
Elution 4	5mm
Elution 5	4mm
Elution 6	4mm

B

After de-saltation

Samples/Dillutions	0x
Control (FT)	
Control (Wash)	
Control (Elution)	
Induction (FT)	2mm
Induction (Wash)	1mm
Induction (Elution)	5mm

C

After HisTag Purification/ De-saltation (DS)

Samples/Dillutions	0x
Control (FT)	
Control (Wash)	
Control (Elution/DS)	4mm
Induction (FT)	10mm
Induction (W)	9mm
Induction (Elution/DS)	9mm

Discussion

Huge antibiotic production void over the recent years has emerged insurmountable problems concerning battle against pathogenic bacteria. Lantipeptides are a new family that seems to provide a viable option to counter the imminent crisis, consisting of RNPs which have shown great stability and antimicrobial activity, due to manipulation by specific modification enzymes.

In this project, there has been an initiative to try to integrate hybrid leader(s) into a previously tested optimal expression system for prenisin in *E. coli*. Besides the known native nisin leader sequence which is critical for NisB and NisC to recognize and modify the core peptide, these leaders also contain recognition sites that are believed to be recognized by OspD epimerase (Fuchs et al., 2016), a homologue of PoyD, which has been observed to alter chemical composition of a newly characterized family of peptides, called proteusins, in a very intricate way.

Production of active nisin was observed in all of the initial constructs (**pET28b*nisA* + pCDFDuet-1*nisBC*, pET28b-*MBPnisA* + pCDFDuet-1*nisBC*, pRSF*nisA* + pCDFDuet-1*nisBC***), only at 20h after induction, with or without trypsin treatment. However, ON trypsin treatment resulted in less or no production of active nisin. Thioether bonds protect nisin from internal cleavage of sensitive sites (arginine or lysine) from trypsin (Kluskens et al., 2005). Nevertheless, an assumption from such a result is that trypsin might be cutting inside the core peptide (e.g., position 12, where there is a lysine). This incident could be attributed to the fact that the produced nisin might not have been fully modified, thus the formed ring structures within the mature

molecule are incapable of offering stability and protecting the core peptide from undesirable cleavage.

In the case of **pET28b-*MBPnisA* + pCDFDuet-1*nisBC*,** inferior activity relative to the other constructs was observed. Maybe this could be attributed to the fact that MBP might be interacting with NisP and preventing it from cleaving the leader from the peptide. That may explain the fact that some activity was observed when samples were treated with trypsin.

Introducing the hybrid leader to the best of the initial constructs (**pET28b*nisA* + pCDFDuet-1*nisBC*, pET28b-*MBP-nisA* + pCDFDuet-1*nisBC*, pRSF_T2_*nisA* + pCDFDuet-1*nisBC*)** resulted in no activity, both at 37°C and at 18°C. Altering construct (**pRSF*nisA* + pBAD*nisBC***) and incorporating the hybrid leader, with and without modification, led to production of highly active nisin, way superior to our initial constructs. **pRSF*nisOsp1*^{mod} + pBAD*nisBC*** showed much better activity than **pRSF*nisOsp1*$^{not\ mod}$ + pBAD*nisBC*** at 37°C, thus indicating that removal of thrombin recognition site improves prenisin modification and production in a small extent. Both constructs had similar activity at 18°C. It would be an interesting proposition for future research and as an expansion to the current project, if the hybrid leader(s) were incorporated in the construct also containing the NisT transporter, as results so far have been quite hopeful, which has also been shown here.

Both **pRSF*nisOsp1*^{mod} + pBAD*nisBC*** and **pRSF*nisOsp1*$^{not\ mod}$ + pBAD*nisBC*** behaved better at 18°C than at 37°C. This might be credited to the fact that growing *E. coli* in such low temperatures compared to its optimal, slows down the growing process on one hand, but it provides time to the protein to be folded better and also maintain its solubility, thus resulting in protein production of better quality and status. Future research on these constructs might include time course experiments at this specific temperature, to optimize time points in different IPTG and Arabinose concentrations which elicit optimal protein yield.

On the other hand, the unexpected activity levels that were observed in control samples should be taken into consideration. Control activity could be attributed to leakiness of arabinose promoter, located into the pBAD vector, as otherwise, without modification, nisin cannot exert antimicrobial activity. However, the specific promoter has been long shown to be very tightly regulated, leading to extremely low background levels of expression (Siegele et al., 1997). Therefore, a valid reason that led to control activity production cannot yet be accounted for.

In any case, such encouraging evidence firmly advocates the fact that providing with the designated recognition sites, modification enzymes are able to work on foreign to them peptides, leading to increasing diversity, regarding the combinations of different recognition sites that can be incorporated in a single leader peptide. Both **pRSF*nisOsp1*^{mod} + pBAD*nisBC*** and **pRSF*nisOsp1*$^{not\ mod}$ + pBAD*nisBC*** have been successful in producing modified prenisin, therefore they might serve as the starting point of similar research attempts. Future research should probably include also the integration of OspD in the current expression system, so as to observe whether epimerization can also take place in synergy with the other modifications.

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