EXPLORING COMBINATORIAL LEADER PEPTIDES AND THEIR ROLE IN POST-RIBOSOMAL MODIFICATIONS

MASTER'S RESEARCH PROJECT

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ABSTRACT

The resistance of microbial pathogens to classic antibiotics is ever-rising. Ribosomally synthesized and post-translationally modified/processed peptides (RiPPs) are a promising group of antimicrobial peptides to counter these pathogens. RiPPs generally are translated as prepeptides, consisting of a leader peptide fused to a core peptide. The leader peptide plays an important role in the modification, export and activation of the core. In this project, the core peptides of the RiPPs Microcin B17 and Nisin have been fused to combinatorial leaders, to investigate the influence of these leaders on the activity, export and post-translational modifications of the core peptide. Besides setting the optimal expression conditions and asserting the activity of these peptides, structure analysis via software prediction has also been undertaken. Three out of four variants tested showed activity, indicating the potential for hybrid leaders to be used in further experiments. Further analysis required purification, which was performed by his-tagging the peptides. One Microcin B17 variant carrying a hybrid leader was successfully modified while remaining its activity. Nisin his-tagging deemed unsuccessful as the peptides carrying hybrid leaders lost their activity. Furthermore, one variant of Microcin B17 was not tested, while his-tagging wildtype Nisin caused the loss of the C-terminal lysin in the core peptide. Structure analysis by predictive software was considered not confident enough to subtract conclusions. Conclusively, hybrid leaders are not disabling the antimicrobial activity of Microcin B17 and Nisin, opening up the potential for peptides to carry post-translational modifications from multiple RiPPs at the same time. The B17-Nis leader is of most interest, as it was suitable with both Microcin B17 and Nisin.

1 INTRODUCTION

1.1 ANTIMICROBIAL PEPTIDES AND RIPPS

Antimicrobial peptides (AMPs) are relatively small (usually <100 amino acids), gene coded and ribosomally synthesized peptides that are bioactive against a large scale of micro-organisms including, but not limited to, Gram-positive and Gram-negative bacteria, protozoa and fungi[1,2]. Other common characteristics are that they mostly are strongly cationic and have a mode of action that should restrict the selection of resistant strains[3]; their antimicrobial activity is applied in several ways thanks to their multifunctional properties which makes it more difficult to develop resistance by microorganisms. AMP's appear throughout nature and have been found in organisms ranging from prokaryotes to higher eukaryotes such as humans[4,5].

Since the introduction of antibiotics in the early 20th century, an increased number of pathogenic organisms have acquired resistance against the traditional antibiotics. Due to their antimicrobial activity, which differs from that of the traditional antibiotics, AMPs are an exciting prospect as new pharmaceuticals. Next to the antimicrobial properties of AMPs, recent discoveries also describe them to be able to neutralize endotoxins, to have chemokine-like activities, immunomodulating activity and induction of both angiogenesis and wound repair[6,7].

A possible disadvantage of AMPs is their potential toxicity in humans. The issue of toxicity to humans has not been discussed much for all AMPs and although many peptides are prokaryotic membrane-active, they seem to also possess the ability to disrupt eukaryotic cell membranes[8]. In order to apply AMPs as pharmaceuticals, studies are needed to evaluate the toxicity of the peptides.

AMPs have been characterized in almost all living organisms and are difficult to classify because of their great diversity. Different classifications are based on amino acid composition, size and secondary structures[3,5]. Despite this ongoing debate on the classification of AMPs, an interesting class has been characterized as the ribosomally-synthesized and post-translationally-modified peptides, or RiPPs. These peptides undergo extensive post-translational and/or co-translational (altering one or more amino acids in a protein after translation has begun but before the protein has been released from the ribosome) modifications to obtain structures that are unusual for natural ribosomal peptides. Typically to allow better target recognition, to increase metabolic and chemical stability and to augment chemical functionality[9]. In combination with their relative simplicity these peptides make excellent candidates for bio-engineering in order to adjust their functionality to conform better to e.g. industrial or pharmaceutical usage.

Among the RiPPs there are several common features, most importantly that they are initially synthesized as longer precursor peptides. These precursor peptides usually contain a N-terminal, or alternatively a C-terminal, leader peptide next to the core peptide, which is important for recognition by many of the post-translational modification and exportation enzymes[10]. Modification of the leader peptide offers potential for a wide diversity of structures and functions applicable to the natural core peptide that are not found in nature.

There are over thousands of RiPPs, sorted in over 20 classes[9], but in this research the focus will be placed on only two peptides: Microcin B17 and Nisin.

1.2 MICROCIN B17 AND NISIN BIOSYNTHESIS

Microcin B17 belongs to the class of microcins, RiPPs with low molecular masses (<10 kDa) and a resistance to proteases, extreme pH and temperatures. Through post-translational modifications Microcin B17 carries four thiazole and four oxazole heterocycles[11]. Nisin is a part of the class of lanthipeptides, peptides containing (methyl-)lanthionines, which are a serine or threonine residue crosslinked with a cysteine via a thioether linkage that connects their β-carbons. The lanthipeptides are further divided into four sub-classes (Classes I, II, III and IV) depending on the biosynthetic enzymes that install the (methyl-)lanthonine motifs[9].

Microcin B17 is translated as a precursor peptide containing an N-terminal leader peptide. The core peptide undergoes post-translational modifications prior to becoming the mature active microcin[13].

Mature Microcin B17 is formed from the precursor McbA in the presence of the modification enzymes McbB, McbC, and McbD that form the Microcin B17 synthetase complex to accommodate the formation of the

heterocycles[14], as illustrated in figure 1A. During the modification process, the McbBCD complex recognizes the leader peptide that has a helical structure[15]. Upon recognition of the precursor peptide select cysteine and serine undergo a backbone cyclodehydration reaction. Catalyzed by the C/D proteins, this reaction yields an azoline heterocycle; thiazole from cysteine or oxazole from serine. Upon removal of the N-terminal 26-amino acid leader peptide by a yet unknown protease, mature Microcin B17, as illustrated in figure 1B, is exported from the cell by the dedicated ABC transporter McbEF. McbEF also contributes to self-immunity towards Microcin B17 acting together with McbG[16].

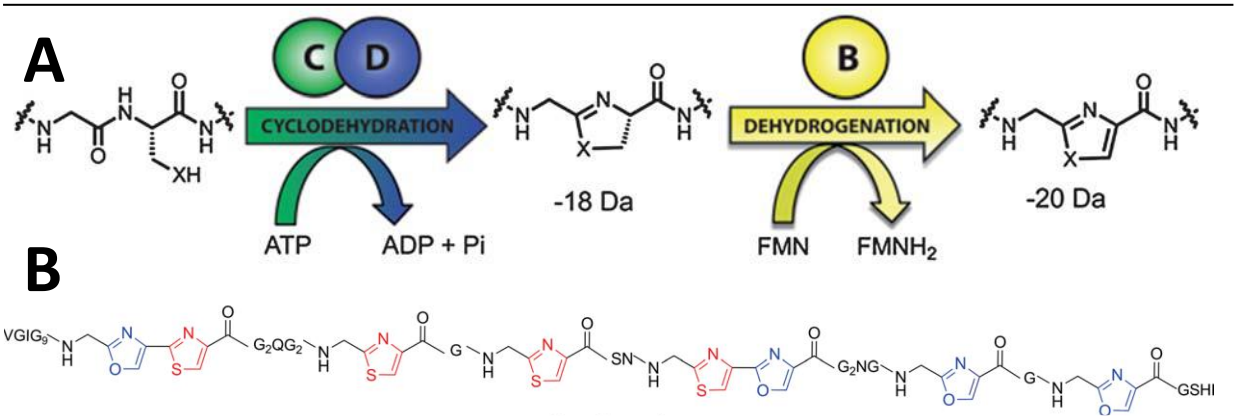

Figure 1 Biosynthetic scheme (A) and mature structure (B) of Microcin B17. The post-translational modifications are color-coded: thiazoles are red and oxazoles blue. Illustrations obtained from Arnison et al. (2013).

Nisin is a bacteriocin that is naturally produced in Gram-positive bacteria, such as *Lactococcus lactis.* Nisin is synthesized from the precursor NisA, whereafter specific serine and threonine residues are converted by the dehydratase NisB into dehydroalanines and dehydrobutyrines, respectively. The dehydrated residues are coupled to cysteine residues by the cyclase NisC yielding five thioether rings comprising one lanthionine (a) and four methyllanthionines (b-e). The fully modified prenisin is subsequently exported by NisT and processed by the extracellular protease NisP that cleaves off the leader peptide to liberate active Nisin. This maturation is illustrated in figure 2. After being matured and exported Nisin acts as a pheromone which is sensed by NisK and leads NisR to promote further Nisin synthesis. To prevent the cell from killing itself, NisI recognizes Nisin and activates NisE, NisF and NisG which provide self-immunity[17].

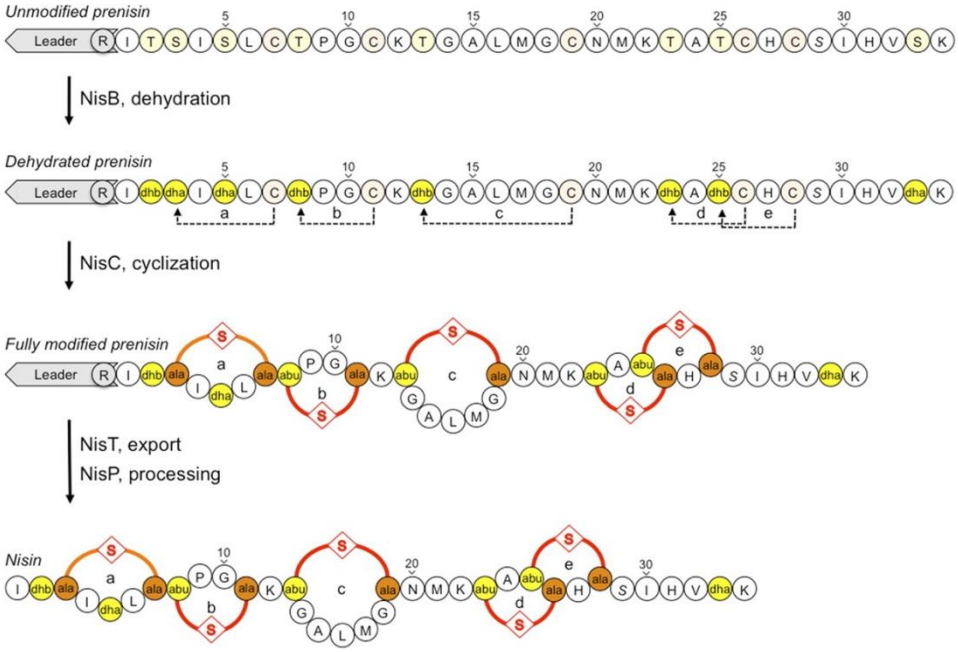

Figure 2 Posttranslational modification of nisin. Serine and threonine residues (highlighted in yellow) are converted by NisB into dehydroalanines (dha) and dehydrobutyrines (dhb).. These residues are coupled to cysteine residues (highlighted in orange) by NisC yielding

five thioether rings comprising one lanthionine (a) and four methyllanthionines (b-e). Prenisin is subsequently exported by NisT and processed NisP. Illustration obtained from Mavaro et al. (2011)[18].

As established, the leader peptides of the RiPPs play an important role in the post-translational modifications of the RiPPs. The leaders however are found to not be absolutely necessary for biosynthesis of the peptide, as shown in Nisin[20]. It is therefore possible to combine leaders to introduce new post-translational modifications in RiPPs. By fusing these hybrid leaders to existing core peptides and expressing them with the necessary machinery, entirely new RiPPs could be synthesized.

1.3 Project aims

This project is mainly aimed at the hypothesis that combinatorial leaders could be recognized by the modification machinery of the core peptide, and thus can allow the post-translational modifications to occur. Sequences have been designed that combine the core peptide of Microcin B17 and Nisin with a hybrid leader. The first aim of this project is to set optimal expression conditions for Microcin B17 with a hybrid leader consisting of part Nisin leader and part Microcin B17 leader and a variant with these parts switched. After establishing these conditions, C-terminal His-Tags need to be introduced to these peptides, and Nisin with combinatorial leaders in order to improve purification efficiency. Ultimately, the purified peptides could be used in other experiments to establish their activity and verify structural modifications.

The second aim is to clone the modification machinery of Microcin B17 and Nisin into low-copy expression plasmid pACYC in order to establish better yields when expressing the genes.

The third aim is to compare the predicted secondary and 3D structure of the RiPPs for any structural similarity between the peptides with hybrid leader and their wildtype. When similar, it would be more probable that these hybrid leaders could be recognized by the modification machineries of the RiPPs.

2 EXPERIMENTAL PROCEDURES

Bacterial strains and plasmids

Escherichia coli strain BL21(DE3) was used in all protein expression experiments and as indicator strain for activity against Gram-negative bacteria. The strain TOP10 was used for genetic modifications. *Lactococcus lactis* NZ9000 was used as an indicator strain for activity against Gramm positive bacteria. An overview of the strains and plasmids they contain is summarized in table 2.

Table 2 Strains and plasmids used in experiments with additional information.

Strain	**Info**
E. coli BL21(DE3)	Expression and indicator strain
E. coli TOP10	Cloning strain
L. lactis NZ9000	Indicator strain
Plasmid	**Info**
pRSF-Duet-1	Kanamycin resistance, double T7 promoter
pBAD24	Ampicillin resistance, AraC promoter
pNZ-nisP	NisP production
pIL253	Erythromycin resistance
pACYC-Duet-1	Chlorampenicol resistance, double T7 promoter

pRSF-Duet-1 plasmids containing the gene coding for core peptide McbA or NisA fused to various leader peptides under the control of T7 promoter were used together with pBAD24 vectors containing *mcbB-G* genes under the control of an arabinose inducible promoter.

Growth conditions

All *E. coli* strains were grown in LB medium supplemented with 100 µg/ml ampicillin and 20 µg/ml kanamycin at 37^{O}C, in a 220 rpm shaking incubator. *L. lactis* strains were grown in GM17 medium at 30^{O}C.

Protein overexpression

E. coli BL21(DE3) containing plasmid pBAD24_mcbB-G and pRSF-Duet1_T2 with McbA fused to different hybrid leader were grown to various OD_{600} of interest and induced with varying concentrations of IPTG and arabinose. Subsequently, samples were taken after 4 and 20 hours after the induction. Samples were spun down with table centrifuge for 1 min at 13800 rpm. Both supernatant and cell pellet were collected. Cell pellets were resuspended in 50mM Tris HCl 5mM $CaCl_2$ buffer (pH 6.9) and disrupted with glassbeader by 2 cycles of 1 minute. Disrupted cell pellets were centrifuged for 1 min at max speed with table centrifuge and obtained supernatants were optionally further analyzed by cleaving the leader peptides and applying the samples for antimicrobial assays. Cleavage of the leader was done by mixing 40 µl of sample with 1 µl of 1 µg/ml trypsin for at least 3 hours in 37^{o}c.

Antimicrobial activity assay

Plates were made by pouring of 4 ml LB agar (1.5%) as a matrix and then, after the matrix solidifies, 7 ml of soft agar (0.7%) mixed with either 28 µl of *E. coli* or 150 µl of *L. lactis* indicator strain on top. After solidifying up to eight wells were punctured in the plates wherein 40 µl of trypsin treated samples were applied. The plates were left O/N at 37^{O}C and then checked for halo's where the indicator strain had not grown.

Plasmid isolation

Plasmid isolation was performed using the Macherey-Nagel plasmid purification kit. Concentrations were checked by Nanodrop.

Round-PCR

His-tags were added to McbA by means of Round-PCR. Primers were designed to introduce 6 histidine residues at the C-terminus. The reaction was performed with 10 µl 5x HF buffer, 1 µl template DNA, 0.5 µl 10 mM dNTP's, 0.25 µl 7x Phusion polymerase, 1 µl 10 mM primer mcbA_6H_FWD (5'-CATCACCATCATC ACCACTAATGACGTCGGTACCCTCGAGTCTG-3'), 1 µl 10 mM primer mcbA_6H_REV (5'-GATATGTGAACCACTTCCG CCGCTGCC-3') and 36.25 µl MQ H_2O to a total volume of 50 µl. Initial denaturation at 96^{O}C for 1 minute, then 35 cycles of denaturation at 96^{O}C for 30 seconds, annealing at 52^{O}C for 30 seconds and extension at 72^{O}C for 4

minutes and 5 seconds. After these cycles final extension at 72^{O}C for 10 minutes. For the addition of His-tags to NisA, the primers nisA_6H_FWD (5'-CACCATCACCATCACCATTAAGCTTTCTTTGAACCAAAATTAG-3') and pBAD24_REV (5'- GGCGGATTTGTCCTACTCAG-3') were used.

Subsequently, the quality of the PCR products were checked by DNA Electrophoresis on a 1% agarose gel. The samples were purified by means of the Machery-Nagel PCR Clean-up kit and its concentrations checked by Nanodrop.

Digestion
Digestion was performed with Thermo Scientific FastDigest restriction enzymes according to the Thermo Scientific FastDigest protocol.

Ligation
Ligation was performed with T4 DNA ligase according to the Thermo Scientific T4 DNA ligase protocol.

Electrotransformation
Plasmids were cloned into competent cells by electrotransformation. 0.5-1 µl template DNA was added to 40 µl competent cells and transferred to a cold 0.2 cm cuvette (BioRad). These cuvettes were placed into a BioRad Gene Pulser, with the capacitor set at 25 µF and the Pulse Controller at 200 Ohm and had one pulse of 2.25V applied. After transformation cells were suspended in 1 ml LB medium and shaken for 1 hour at 37^{O}C and 220 rpm. Ultimately cells were plated in varying concentrations on selective LB agar.

Colony PCR
Colony PCR reactions were performed with 2 µl 10x DTaq buffer, 0.25 µl 10 mM dNTP's, 0.5 µl 10 mM pRSFDuet1_T7_2_FW (5'-GGCCGCATAATGCTTAAGTC-3'), 0.5 µl 10 mM pRSFDuet1_T7_2_Rev (5'-AGCAGCGGTT TCTTTACCAG-3'), 0.15 µl 7x Phy polymerase and 16.6 µl MQ H_2O to a total volume of 20 µl and inoculated by toothpick in case of his-tagging. For the cloning of *mcbB-G* and *nisBC* into pACYC the primers pRSF-Duet_1_T7_1_FW (5'-TCACCACCCTGAATTGACTC-3') and B17_OP_Seq_rev_7 (5'-AGGTTAGTGCTGAAATAATAC-3') were used.
Initial denaturation at 95^{O}C for 5 minutes, then 33 cycles of denaturation at 96^{O}C for 30 seconds, annealing at 52^{O}C for 30 seconds and extension at 72^{O}C for 30 seconds. After these cycles final extension at 72^{O}C for 1 minute. Products were checked by DNA Electrophoresis on a 1% agarose gel.

Sequencing
Isolated plasmids were prepared as prescribed by MacroGen and sent for sequencing by MacroGen.

Peptide purification by Ni-NTA column
Overnight culture was diluted 1:50 to 700 ml of fresh LB medium containing appropriate antibiotics and grown until OD_{600} reached 0.6-0.8. Induction was performed by adding with IPTG and arabinose to the concentrations of 1mM. Cells were harvested 20 hours post-induction by centrifugation (30 minutes, 6000 rpm). Cell pellet was sonicated (30 min, pulse 30s on/30s off, 75% amplitude) and lysed cells spun down for 40 minutes at 9500 rpm. Both supernatant and lysed cells were filtered through a .45 µm filter and loaded onto Ni-NTA column.

Silver staining
Purified peptides were loaded on SDS-Page gels and run at 80V through the stacking gel and 130V through the running gel. After electrophoresis, all following steps were performed on a horizontal shaker with gentle shaking.

At least one hour or overnight	Fixing solution (50% v/v ethanol, 12% v/v acetic acid, 0.1% v/v formaldehyde 37%, ad Millipore)
3 × 20 min	50 % (v/v) ethanol
1 min	Sensitizer (0.03% w/v $Na_2S_2O_3 \cdot 5\ H_2O$)
3 × 20 sec	Millipore water
15-25 min	Silver nitrate solution (0.2% w/v $AgNO_3$, 0.1% v/v formaldehyde 37%, ad Millipore)
3 × 20 sec	Millipore water

1-30 min (until staining is sufficient)	Developer (6% w/v Na_2CO_3, 2% sensitizer, 0.05% v/v formaldehyde 37%, ad Millipore)
2 × 20 sec	Millipore water
10 min	Stop solution (50% methanol, 12% acetic acid, ad Millipore)
2 × 20 sec	Millipore water

Secondary structure prediction

Secondary structures of peptides of interest (sequences included in appendix I, names in table I) were predicted with the software Jpred 4, available at http://www.compbio.dundee.ac.uk/jpred/. Alignment of the sequences on similarity was performed manually with Adobe Photoshop CS6.

Table 1 Names of peptides of interest with description of their constitution.

Peptide name	Description of leader	Description of core peptide
Nisin WT	Wildtype Nisin	Wildtype Nisin
Microcin B17 WT	Wildtype Microcin B17	Wildtype Microcin B17
Microcin J25 WT	Wildtype Microcin J25	Wildtype Microcin J25
LanJ25	Part Nisin and part Microcin J25.	Part Microcin J25 and part Nisin
Hybrid Nisin	Hybrid Microcin B17 and Nisin .	Hybrid Nisin and Microcin B17
Hybrid Nisin 6xH	Hybrid Microcin B17 and Nisin .	Hybrid Nisin and Microcin B17 with a C-terminal His-tag
Hybrid Nisin II	Hybrid Nisin and Microcin B17 and Nisin .	Hybrid Nisin and Microcin B17.
Lasso Hybrid Nisin	Part Nisin and part Microcin J25	Wildtype Nisin
Lasso Hybrid J25	Part Nisin and part Microcin J25	Wildtype Microcin J25
HCM Hybrid Nisin	Hybrid Microcin B17 and Nisin	Wildtype Nisin
HCM Hybrid B17	Hybrid Microcin B17 and Nisin	Wildtype Microcin B17
HCM Hybrid Nisin II	Hybrid Nisin and Microcin B17	Wildtype Nisin
HCM Hybrid B17 II	Hybrid Nisin and Microcin B17	Wildtype Microcin B17

3D structure prediction

3D structures of the peptides of interested were predicted with the software I-TASSER, available at http://zhanglab.ccmb.med.umich.edu/I-TASSER/. Comparison of models was performed with molecular visualization system PyMOL.

3 RESULTS

3.1 OPTIMAL GROWTH CONDITIONS OF E. COLI BL21(DE3) FOR OVERPRODUCTION OF HCM HYBRID B17 AND HCM HYBRID B17 II

Expression strains containing the structural Microcin B17 peptide fused to one of the hybrid leaders were tested on overproduction of the peptide by measuring its activity. These strains were grown under varying conditions to determine the following five aspects for the optimization of overproduction:

- the optimal OD_{600} to induce;
- the optimal concentration of the inducers;
- the optimal times of induction;
- the necessity of leader cleavage by trypsin
- the optimal time of harvest and cellular location of the peptide.

Samples of all possible combinations were tested in varying dilutions on activity assays against Gramm negative bacteria. The highest dilution with activity prevalent was determined to be the best condition to grow the strain for overproduction of Microcin B17. For both strains it was determined that the optimal OD_{600} to induce is 0.6, with activity present in both supernatant and cell extract, as shown in figure 3. In this experiment samples were induced with 1 mM IPTG and 2 mM arabinose, harvested after 4 hours and digested with trypsin.

Figure 3 Activity of supernatant (left) and cell extract (right) samples of HCM Hybrid B17 II on *E.coli* sensitive strain. Samples were induced at the following OD's: 6) OD_{600} 0.3; 7) OD_{600} 0.6; 8) OD_{600} 1.0; 9) OD_{600} 1.5 and 10) not induced.

After establishing the optimal OD_{600}, cells were induced with varying concentrations of IPTG and arabinose, as summarized in table 2; IPTG was added in concentrations of 0.1, 0.2, 0.5 or 1 mM and arabinose in concentrations of 1 or 2 mM. Samples were harvested after 4 hours and 20 hours after induction and tested on activity.

Table 2 Combinations of concentrations of IPTG and arabinose used in the study.

Sample #	Concentration IPTG (mM)	Concentration arabinose (mM)
1	0.1	2
2	0.2	2
3	0.5	2
4	1	2
5	0.1	1
6	0.2	1
7	0.5	1
8	1	1
9	0	0

As visible in figure 4, out of all possible combinations four showed activity in all cell fractions: samples 2, 3, 6 and 8. Further dilution of samples revealed that condition 8 was the most active overall, meaning the optimal concentration of the inducers are 1 mM IPTG and 1 mM arabinose.

The influence of the time of induction was also tested. Samples were either induced with IPTG and arabinose simultaneously or first with IPTG and one hour later with arabinose. Figure 5 shows an overview in which it is clear that activity is most prevalent in samples that were induced simultaneously. Furthermore they showed that the peptides are most active when harvested 20 hours after induction from cell extract.

Ultimately, both HCM Hybrid B17 and HCM Hybrid B17 II were tested on activity when not treated with trypsin. HCM Hybrid B17 showed no activity, but HCM Hybrid B17 II did show activity in its supernatant fractions, as shown in figure 6.

All in all these results show the optimal growing conditions for expression of both peptides to be simultaneous induction of 1 mM IPTG and 1 mM arabinose at OD_{600} 0.6 and harvesting 20 hours later. For HCM Hybrid B17 trypsin digestion is necessary and most peptide is found in the cell extract, while HCM Hybrid B17 II does not need to be digested and is found most in the supernatant.

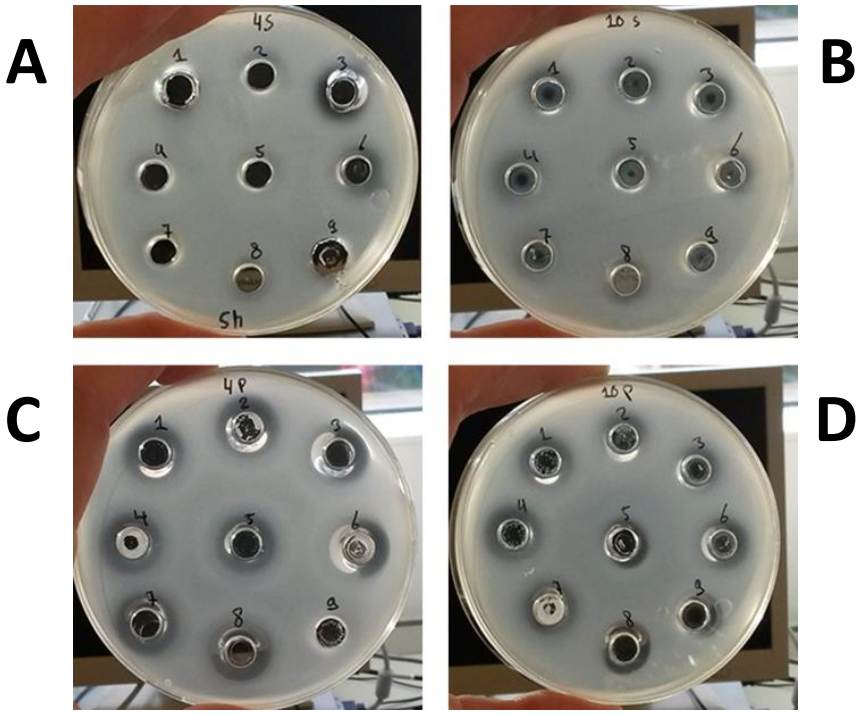

Figure 4 Activity of HCM Hybrid B17 II samples on *E.coli* sensitive strain when induced with different combinations of IPTG and arabinose. Explanation of sample numbers in table 3. Pictures correspond to samples as follows: Supernatant harvested 4 hours (A) and 20 hours (B) after induction, and cell extract harvested after 4 hours (C) and 20 hours (D) after induction.

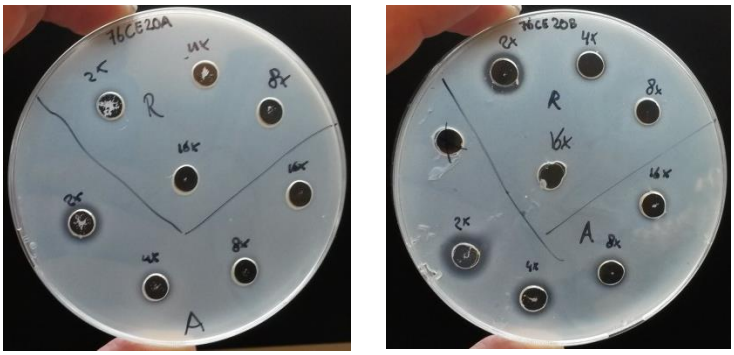

Figure 5 HCM Hybrid B17 II samples that were induced with IPTG and arabinose simultaneously (right) show activity at higher dilutions than those that were induced with one hour in between (left) on *E.coli* sensitive strain. Two, four, eight and sixteen times diluted samples were used.

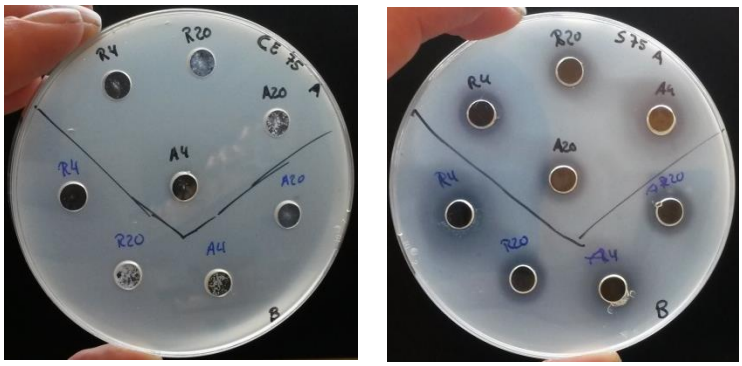

Figure 6 HCM Hybrid B17 II samples that have not been treated with trypsin show activity in supernatant (right), but not in cell extract fractions (left) on *E.coli* sensitive strain.

3.2 Activity test of HCM Hybrid Nisin and HCM Hybrid Nisin II

Under the established optimal growth conditions, *E. coli* BL21(DE3) containing pRSF Duet-1 with either HCM Hybrid Nisin or HCM Hybrid Nisin II coding genes and pBAD24 containing *nisBC* were expressed and tested for antimicrobial activity against Gramm positive bacteria. HCM Hybrid Nisin showed activity, but HCM Hybrid Nisin II did not, as visible in figure 7.

Figure 7 HCM Hybrid Nisin (marked as 80) shows activity on sensitive *L. lactis* when harvested after 4 hours (left) and 20 hours (right), when either digested (marked with D (left) or T (right)) or undigested with trypsin. HCM Hybrid Nisin II (78) shows no activity at all. Prenisin and buffer were added as positive and negative control, respectively.

3.3 Addition of C-terminal His-tag and subsequent activity tests

In order to simplify the purification process of the peptides, C-terminal His-tags were introduced by performing round PCR (rPCR) to pRSF Duet-1 containing the genes that encode HCM Hybrid Nisin, HCM Hybrid Nisin II and Nisin WT respectively. As shown in figure 8, PCR products were validated by DNA electrophoresis. In addition, pRSF Duet-1 containing genes that encode peptides HCM Hybrid B17, HCM Hybrid B17 II and Lasso Hybrid Nisin with a C-terminal His-tag were obtained.

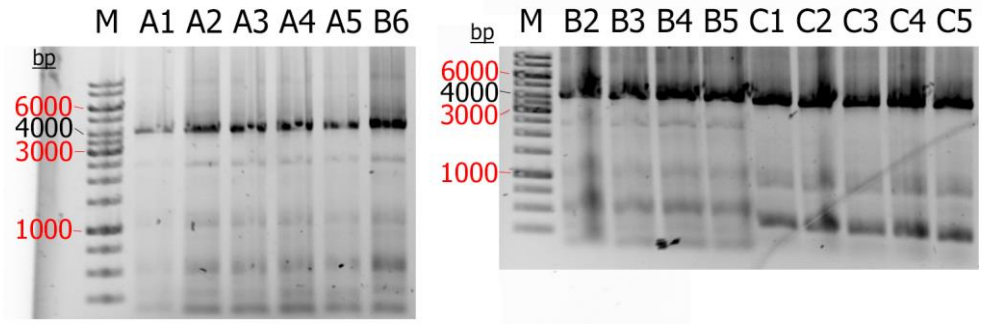

Figure 8 rPCR products of his-tagged HCM Hybrid B17 II (A), HCM Hybrid B17 (B) and Nisin WT (C).Gene sizes are approximately 4 kb.

Each plasmid was transformed into *E. coli* TOP 10 by electroporation and plated on selective medium. Single colonies were picked and checked on incorporation of the gene by colony PCR as shown in figure 9. For every mutant, one to four successfully transformed colonies were sequenced by MacroGen to ensure the introduction of the His-tag in the gene. Sequence analysis confirmed successful incorporation for all mutants, except for Nisin WT. Nisin WT colonies that had the his-tag incorporated missed the C-terminal Lysin.

Consequently, the pRSF duet-1 plasmids coding for the His-tagged peptides were transformed with pBAD24 containing the modification machinery genes (*mcbB-G* for B17 mutants and *nisBC* for Nisin mutants) into *E. coli* BL21(DE3) and plated on selective medium. Single colonies were picked for expression and checked for activity in supernatant and cell extract, 4 and 20 hours after induction and either digested by trypsin or not. None of the his-tagged NisA mutants (figure 10) showed any activity. His-tagged wildtype McbA showed activity in supernatant fractions, either digested or undigested by trypsin, and his-tagged HCM Hybrid B17 showed activity in cell extract when digested by trypsin (figure 11). His-tagged HCM Hybrid B17 II has not been tested due to contamination of the strain.

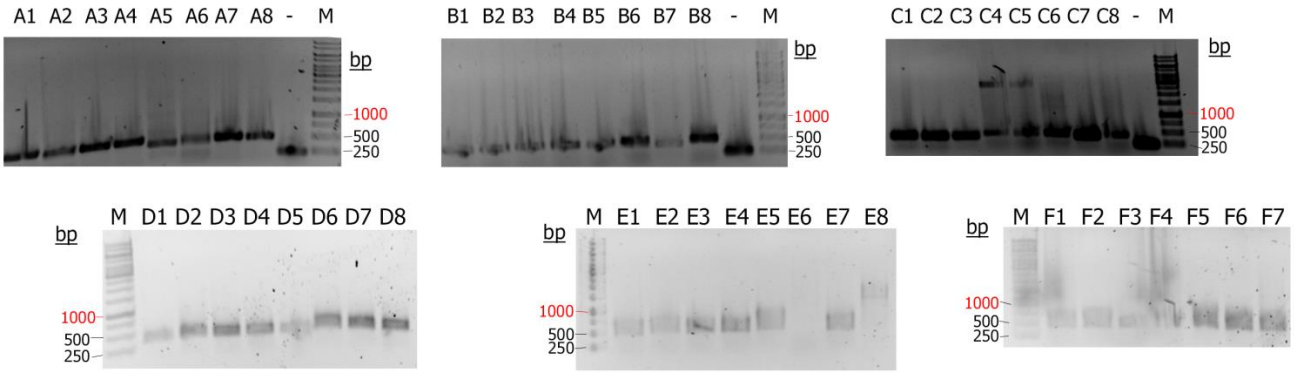

Figure 9 cPCR products of his-tagged HCM Hybrid II (A), HCM Hybrid I (B), Nisin WT (C), Lasso Hybrid Nisin (D), HCM Hybrid Nisin (E) and HCM Hybrid Nisin II (F).Bands were expected at approximately 400 bp.

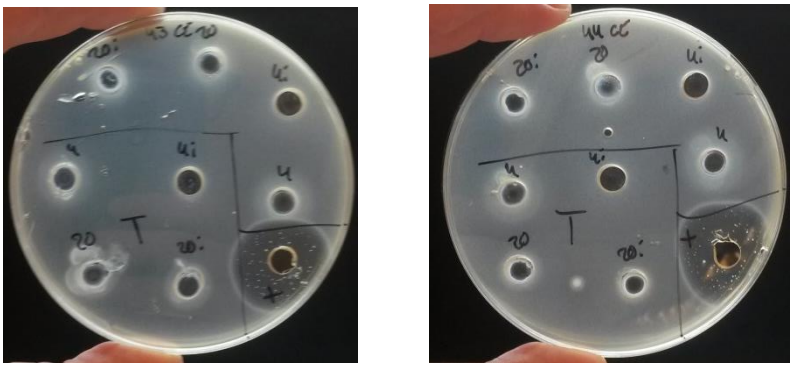

Figure 10 Neither his-tagged HCM Hybrid Nisin (left) or his-tagged HCM Hybrid Nisin II (right) samples show any antimicrobial activity on sensitive *E. coli*..

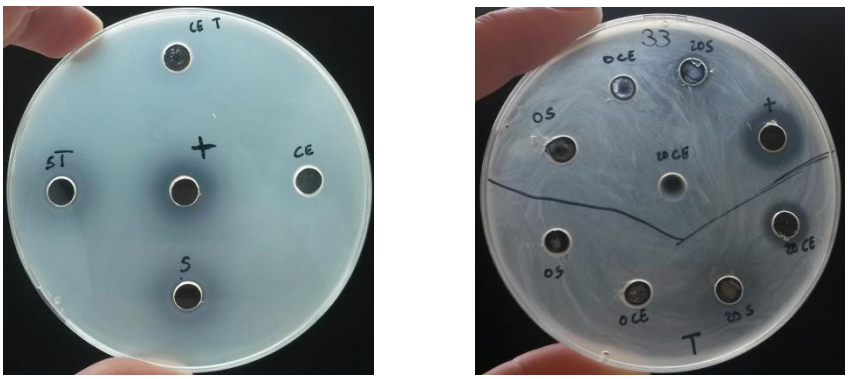

Figure 11 His-tagged wildtype mcbA (left) shows antimicrobial activity on sensitive *E. coli* in supernatant fractions that were digested (marked sT) or undigested (s) by trypsin. His-tagged HCM Hybrid B17 (right) shows activity on *E. coli* in trypsin digested cell extract.

3.4 Analysis of purified peptides

All his-tagged variants of McbA and NisA, including the wildtype NisA missing the C-terminal lysin, were purified by means of a Ni-NTA column. Wash and elution fragments were loaded on SDS-Page gels and dyed by silver staining. These gels did not provide insight into the presence of the peptide as the staining failed to visualize.

3.5 Secondary and 3D structure prediction of various peptides of interest

Peptides of interest, which names and sequences are specified in table 1 and appendix 1 respectively, were tested for structural similarity. After sequences were processed with Jpred 4, sequences were aligned manually on similarity of the predicted secondary structure in the leaders, as depicted in figure 12. The confidence of the software on the prediction of the secondary structures in the leaders varies greatly. On a scale from 0-10, the secondary structure of the Nisin leader averages a 7, whereas those of the Microcin B17 and Microcin J25 lay around 2. In general, the peptides with hybrid leaders show no significant structural differences to the wildtype peptides.

Figure 12 Overview of the various peptides that had their secondary structure predicted by Jpred 4. Every peptide has three rows: QUERY displays the peptide sequence; jnet displays the secondary structures that are predicted, with yellow arrows symbolising beta-strands and pink barrels symbolising alpha-helices, and conf corresponds to the confidence of the prediction on a scale of 1 to 10.

The sequences were also processed through I-TASSER software to predict their 3D structure. The models predicted by this software did not provide useful however for several reasons. First of all, the software are not able to predict the post-translational modifications in the peptides, as demonstrated in the model for Microcin B17, displayed in figure 13, where it clearly lacks the helical structures present in the leader and the heterocycles in the core peptide. Secondly, the quality estimates of the models by the software lay around 50% and lastly comparison between all different peptides proved to be too difficult to obtain useful results.

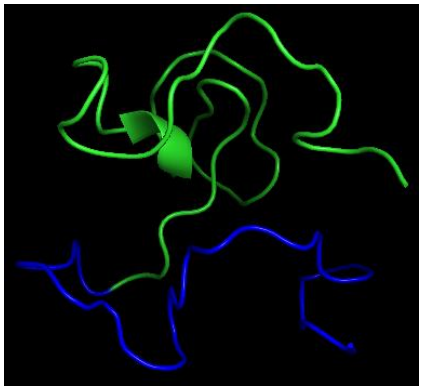

Figure 13 I-TASSER model of Microcin B17 viewed in PyMOL. The leader (blue) lacks the helical structure and the core peptide (blue) the heterocyclic modifications.

3.6 Cloning *mcbB-G* and *nisBC* into pACYC-Duet-1

The genes *mcbB-G* and *nisBC* have been amplified from isolated plasmids pBAD24 and were digested by restriction enzymes NcoI & BamHI and NcoI and SalI, respectively. These genes were then mixed with pACYC-Duet-1 digested by the same restriction enzymes and ligated. The ligated plasmids were transformed into *E. coli* TOP10 cells and plated on selective medium, but colonies that were subsequently picked for colony PCR did not show insertion of the gene.

4 Discussion

The optimal growing conditions for expression of Microcin B17 fused to a hybrid leader are similar in both variants tested. In essence both peptides are best grown to an OD_{600} of 0.6 to be simultaneously induced with 1 mM IPTG and 1 mM arabinose. Then, 20 hours after induction these cells are best harvested. HCM Hybrid B17 needs to be digested by trypsin and is most abundant in the cell extract. HCM Hybrid B17 II can be found in its active form in the supernatant, without further processing, but is also active when processed with trypsin. In the case of HCM Hybrid Nisin and HCM Hybrid Nisin II, the first mutant shows activity when processed and unprocessed, while the second remains inactive at all times. The presence of active peptide in supernatant when undigested by trypsin indicates that these variants get recognized by the modification machinery, leader protease and transporter.

The most probable explanation is that the end of the leader peptide, together with the start of the core peptide, needs to fit into the enzyme cleaving the leader. When this end is part of a different leader peptide, as is the case with HCM Hybrid B17, the Microcin B17 modification enzymes are not able to recognize and process this leader. When the parts are switched, so the natural leader sits next to the core peptide, recognition and cleavage is possible. In other words, the region at the end of the leader peptide is most important for recognition of modification enzymes and to transport the peptide. An illustration of the assembly of these peptides and their hybrid leaders is depicted in figure 45.

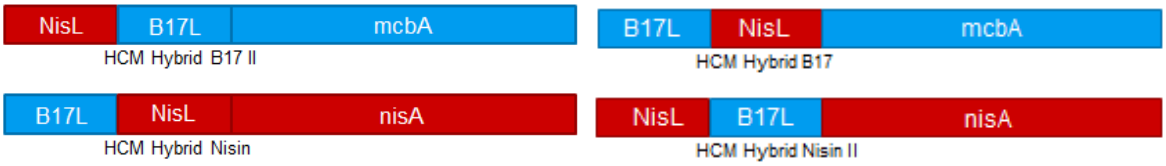

Figure 14 Illustration of the assembly of the HCM Hybrid mutants. Parts belonging to Nisin are in red, those of MccB17 in blue.

The loss of activity of the NisA mutants when his-tagged is uncommon, but not impossible. The loss of activity due to the his-tag could be explained by the impact of six additional, positively charged amino acids on such a short peptide. Whereas most proteins remain unaffected by the his-tag, Nisin is a small peptide, meaning six additional peptides is a relatively great number. The fact that his-tagged wildtype and mutant McbA remain active, however undermines this theory and indicates that it is more likely that either the strains have trouble expressing the mutant proteins or that the his-tags react with the core peptide or hybrid leader, potentially causing misfolding. Activity assays with correctly his-tagged wildtype Nisin should be able to further pin-point this.

The fact that HCM Hybrid B17 and HCM Hybrid Nisin are both active (albeit the first one with trypsin digestion) while carrying the same hybrid (B17-Nis)leader opens up potential for combined post-translational modifications. Prior to that, however, confirmation of the post-translational modifications within these peptides is necessary. This could be achieved by mass spectrometry, but to do that the peptide needs to be purified. Although the his-tagging method has proven unsuccessful for the NisA variant, other attempts could be made, such as a N-terminal his-tag. Still, a start could be made with HCM Hybrid B17.

The next step would be experimentation with hybrid core peptides to prove whether these cores are able to be modified by multiple modification machineries. If this works, the hybrid leaders and core peptides could be combined to create novel peptides carrying multiple post-translational modifications. These peptides would then hopefully be an answer to the ever-rising antibiotic resistance.

Insertion of *mcbB-G* and *nisBC* into pACYC-Duet-1 has failed as of yet, but seems achievable in the near future. Both the secondary and 3D structure predictions of the hybrid leaders that have been designed show neither enough confidence of prediction or such structural similarity that it provides a useful insight in the probability of these leaders to be recognized and/or processed by the various modification machineries of the peptides. It is improbable that the software for these predictions improve on a short term in such a manner that they can replace practical experimentation efficiently.

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6 APPENDICES

6.1 APPENDIX I – PEPTIDES OF INTEREST

Leaders are colored as follows:
Red: Nisin
Blue: Microcin B17
Green: Microcin J25

Nisin WT
MSTKDFNLDLVSVSKKDSGASPRITSISLCTPGCKTGALMGCNMKTATCHCSIHVSK
Microcin B17 WT
MELKASEFGVVLSVDALKLSRQSPLGVGIGGGGGGGGGGSCGGQGGGCGGCSNGCSGGNGGSGGSGSHI
Microcin J25 WT
MIKHFHFNKLSSGKKNNVPSPAKGVIQIKKSASQLTKGGAGHVPEYFVGIGTPISFYG

Lan J25
MSTKDFNLDLVRPAKGVIQIKKSASQLTKGGAGHVPEYFVGIGITSISLCTPG
Hybrid Nisin
MELKASEFGVVLSVDALKLSRQSPLGMSTKDFNLDLVSVSKKDSGIEGRITSISLCTPGVGIGGGGGGGGGGSCGGQGG
Hybrid Nisin6xH
MELKASEFGVVLSVDALKLSRQSPLGMSTKDFNLDLVSVSKKDSGIEGRITSISLCTPGVGIGGGGGGGGGGSCGGQGGHHHHHH
Hybrid Nisin II
MSTKDFNLDLVSVSKKDSGMELKASEFGVVLSVDALKLSRQSPLGIEGRITSISLCTPGVGIGGGGGGGGGGSCGGQGG

Lasso Hybrid Nisin
MSTKDFNLDLVRPAKGVIQIKKSASQLTKITSISLCTPGCKTGALMGCNMKTATCHCSIHVSK
Lasso Hybrid J25
MSTKDFNLDLVRPAKGVIQIKKSASQLTKGGAGHVPEYFVGIGTPISFYG

HCM Hybrid Nisin
MELKASEFGVVLSVDALKLSRQSPLGMSTKDFNLDLVSVSKKDSGIEGRITSISLCTPGCKTGALMGCNMKTATCHCSIHVSK
HCM Hybrid B17
MELKASEFGVVLSVDALKLSRQSPLGMSTKDFNLDLVSVSKKDSGIEGRVGIGGGGGGGGGSCGGQGGGCGGCSNGCSGGNGGSGGSGSHI
HCM Hybrid Nisin II
MSTKDFNLDLVSVSKKDSGMELKASEFGVVLSVDALKLSRQSPLGIEGRITSISLCTPGCKTGALMGCNMKTATCHCSIHVSK
HCM Hybrid B17 II
MSTKDFNLDLVSVSKKDSGMELKASEFGVVLSVDALKLSRQSPLGIEGRVGIGGGGGGGGGSCGGQGGGCGGCSNGCSGGNGGSGGSGSHI