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Engineering the synthesis of lantibiotics in *E. coli* by combining the cinnamycin and nisin modification systems

Tesi in

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CONTENTS

INTRODUCTION

The following project was carried out at MolGen research group of the Rijksuniversiteit Groningen, NL.

The aim of this work is to provide new tools to engineer lantibiotic peptides.

Lantibiotics are post-translationally modified antimicrobial peptides that show antimicrobial activity against a wide group of Gram-positive bacteria.

To obtain antimicrobial activity lantibiotics are processed by a post-translational modification machiney formed by a set of enzymes.

Lantibiotics represent a potential solution to figth multi-drugs resistant pathogens.

Apromising way in the use of lantibiotics as new antibiotics involves a Synthetic Biology approach.

Synthetic Biology aims to apply the principles of modularity and orthogonality of engineering to Biology.

On lantibiotics, this means standardization of parts of the peptide or the biosynthetic machinery in such a way that they can be predictably combined to produce a new antimicrobial molecule.

Lantibiotics structure involves two regions, a leader-peptide and a core-peptide.

Core-peptide is the region that harbour the modifications and that possess antimicrobial activity.

A leader peptide is necessary for recognition of the molecule by modification enzymes.

The cleavage of the leader peptide is requested to have antimicrobial activity.

Structure, classification and mode of action of lantibiotics are described in the first chapter.

This project involved two lantibiotics and their modification machinery: nisin and cinnamycin.

Nisin is a lantibiotic with a rod-like structure that after modification possesses lanthionine and metylanthionine rings. These rings are fundamentals for its antimicrobial activity.

The modification machinery of nisin involves the enzymes NisB and NisC, responsible for the lanthionine and methylantionine formation; NisP, necessary for the cleavage of the leader peptide; and NisT that transports the modified nisin out of the cell.

Unlike nisin, cinnamycin has a globular structure and in addition to lanthionine and methylanthione ring possess others modifications, a hydroxylated aspartic acid and a lysinoalanine bridge.

The modification enzymes are: CinM, the single enzyme responsible for lanthionine and methylantionine formation; CinX the hydroxylase; Orf7 that form the lysinoalanine bridge.

The second chapter of this thesis focuses on nisin and cinnamycin structure and describes their modification machinery.

The main goal of this work is to prove that cinnamycin enzyme CinM can modify nisin to obtain a fully modified molecule that show antimicrobial activity.

To do that, a cinnamycin heterologous expression system was implemented in *E. coli*, confirming that cinnamycin can be produced and modified in a heterologous producer.

The activity of the peptide was confirmed by the modifications on core peptide demonstrated by mass spectrometry analysis, and by activity tests.

These result confirmed that CinM can be produced and work in *E. coli*.

To allow modification of nisin by CinM was necessary to design a specific molecule formed by a chimeric leader peptide and nisin core peptide.

The chimeric leader peptide was formed by fusion of cinnamycin leader peptide and nisin leader peptide, in this way CinM could recognize the cinnamycin leader and modify nisin.

The production of this chimeric nisin and its modification by nisin system was tested beforehead.

The results proved that the presence of cinnamycin leader does not affect the nisin production and modification, as well its activity.

Finally, the modification of nisin by CinM was tested on the same chimeric molecule.

The result showed that CinM can modify nisin, this means that a single enzyme as CinM can act in the same way as the combination of NisC and NisB.

This result proves that CinM can be used to modify lantibiotics using a chimeric leader system, even if optimal modification conditions are to be found.

In addition, the use of CinM open the way to the introduction of lysinoalanine bridge formation by Orf7 in nisin or designed substrates, because this last enzyme require the presence of CinM to work.

The experiments carried out and the results are described and discussed in chapters three and four.

CHAPTER 1: Lantibiotic definition and mode of action

1.1 Definition of lantibiotic

Lantipeptides are polycyclic peptides characterized by the presence of the thioether-cross-linked amino acids *meso*-lanthionine (Lan) and (2S, 3S, 6R)-3-methyllanthionine (Melan) [1].

Lantipeptides that shows antimicrobial activity are called lantibiotics.

Small antimicrobial peptides produced by Gram-positive bacteria are named bacteriocins.

Bacteriocins that do not require post-traslational modifications for their antimicrobial activity are classified as non-lantibiotics, whereas members of the lantibiotic group need modifications to show antimicrobial activity.

Lantibiotics are produced by ribosomes as inactive pre-peptides, consisting in an N-terminal leader-peptide and a C-terminal core-peptide part [2].

The modifications are carried out by lantibiotic-specific modification enzymes, that requires the presence of a leader peptide to recognize the target molecule.

The modifications involves only the core-peptide part, in which most of the serine (Ser) and threonine (Thr) residues are dehydrated to dehydroalanine (Dha) and dehydrobutyrine (Dhb) respectively.

Subsequently this dehydroresidues are coupled to cysteines (Cys) residues, this leads to the formation of (methyl)lanthionine rings. This step is generally called cyclization.

In the end, the cleavage of the leader peptide by dedicated protease is necessary to obtain the active compound.

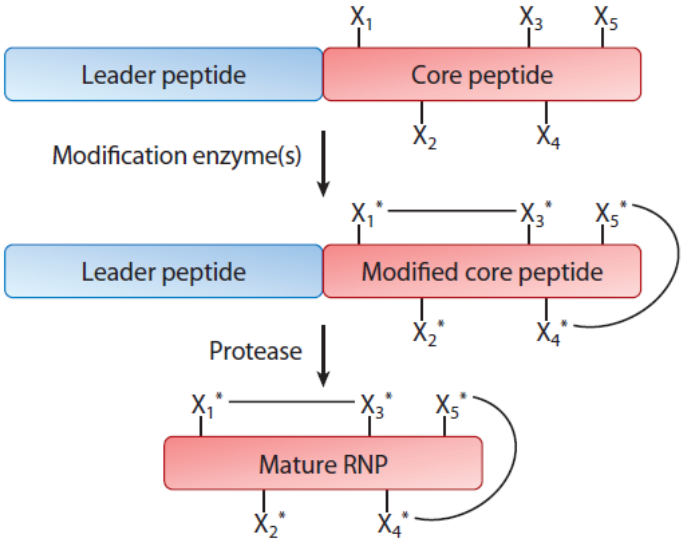


Fig 1: General representation of ribosomally synthetsized natural product (RNP) biosynthesis.Xn represent modified amino acidic residues*

On the basis of the biosynthetic machinery involved in the formation of (methyl)lanthionine rings, is possible to define four classes of lantipeptides.

For class I, two distinct enzymes, the dehydratase LanB and the cyclase LanC, perform Ser/Thr dehydration and cyclization respectively.

The term “Lan” is a general abbreviation for proteins involved in lantibiotic biosynthesis.

A member of Class I lantibiotic is nisin.

For class II, these reactions are carried out by a single bifunctional enzyme, LanM, containing both the dehydratase (N-terminal part) and cyclase (C-terminal part) domains.

This is the case of cinnamycin.

Class III lantibiotics are formed by two subunits called α-peptide and β-peptide that are linked together.

Lantipeptides that are members of Class III, are modified by a trifunctional synthetase, containing an N-terminal lyase domain, a central kinase domain, and a putative cyclase domain in the C-terminal part.

For this last domain is used the term putative because it lacks many of the conserved active-site motif found in LanM and LanC ezymes.

In the end, recently identified Class IV lantipeptides, are modified by a single enzyme similar as Class III synthetase, LanL. This enzyme contains the lyase domain and kinase domain as the Class III enzyme, but its C-terminal cyclase domain is analogous to LanC

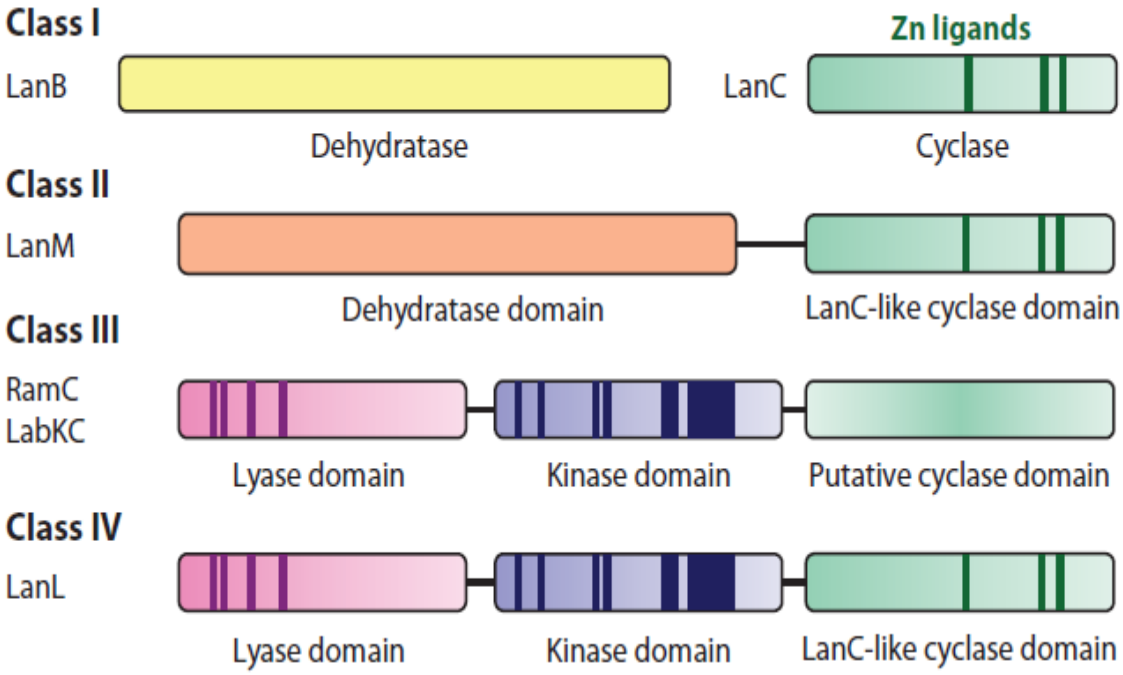


Fig. 2: Schematic representation of the four classes of lantipeptides modification enzymes. Highlighted part represent conserved motifs.

1.2 Clinical use of lantibiotics

Due to an increasing resistance of bacteria to available antibiotics, there is an urgent need to search for substances active against multidrugs resistant pathogens [2].

In the last years, the increase of multidrug resistant bacteria, especially in hospital environments, has caused an increasing concern in medicine.

Some of this multidrug resistant bacteria are: methicillin-resistant *Staphilococcus aureus* (MRSA), vancomycin-resistant *Enterococcus faecalis* (VRE), third generation cephalosporin-resistant *E. coli.*

This topic has become of major interest in antibacterial therapy, highlighting the need of new ways to treat infections.

In literature is possible to find several approaches to tackle down this problem, such as isolation of infected patients in hospitals, reduction of non-human antibiotics, rational use of antimicrobials, or phage therapy [6-8].

Another way to fight this growing threat is to find new antibiotics with a new mechanism of action.

Under this respect a new approach in the research of antibacterial agents is needed, because the research in traditional antibiotics did not provide good results.

According to a recent report of European Medicines Agency, only 15 compounds of 167, investigated in clinical trial, shows a new mechanism of action or involve a new target.

The situation is even worse for Gram-negative bacteria, where only 2 of the 167 compounds had a novel target or mechanism of action.

This results lead to a reduced interest from pharmaceutical sector (1.6% of the total molecules under development in 2004), and should be compensated by investments from public funds [9].

It has already been shown that some lantibiotics exhibit activity against antibiotic-resistant pathogens at nanomolar concentrations, so at concentration similar to commercial antibiotics, targeting a different compound of bacterial membrane compared with traditional antibodies.

Clinical applications of lantibiotics are currently of great interest.

Lantibiotics possess potent activity against several clinically relevant Gram-positive bacteria, such as *Staphylococcus*, *Streptococcus*, *Enterococcus*, but also against Gram-negative bacteria as *Neisseria* and *Helicobacter.*

Currently there are several lantibiotics in clinical and preclinical development such as duramicycin for the treatment of cystic fibrosis-associated pneumonias, mersacidin, that shows activity against methicillin-resistant *Streptococcus aureus* strains, and lacticin 3147 proved to be a successful antimicrobial agent against vancomycin-resistant *Enterococcus faecalis* andpenicillin-resistant *Streptococcus pneumonia*.

1.3 Mode of action of lantibiotics

The mechanism of action by which lantibiotics exert bactericidal activity has been studied only in few cases, but most of this bactericidal peptides are believed to inhibit cell wall biosynthesis and/or form pores through the membrane, leading to cell disruption.

Target of lantibiotics seems to be Lipid II, an amphipathic peptidoglycan precursor molecule involved in the synthesis of the cell wall of bacteria.

Lipid II is responsible to deliver the complete peptidoglycan,formed at cytoplasmic side of the membrane, to the extracellular side (Fig 3) [4].

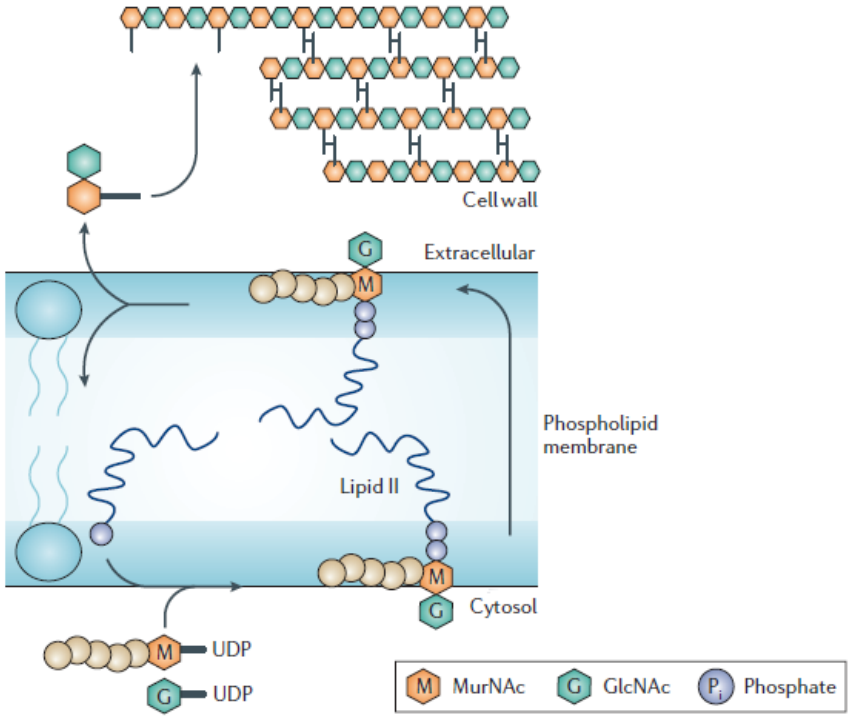


Fig 3: Schematic representation of cell wall biosynthesis steps. GLCNAc (N-acetylglucosamine) and MurNAc (N-acetylmuramic acid)are compounds of peptidoglycan involved in cell wall synthesis

Binding of Lipid II leads to inhibition of transglycosylation reaction, necessary in the synthesis of peptidioglycan and therefore in the synthesis of the cell wall.

Bound Lipid II are sequestered from the cell wall division site (septum) and translocate in a non-functional locations, in this way the cell wall biosynthesis is stopped.[3]

As mentioned above, the other mechanism of action for lantibiotics involves the formation of pores through the membrane of bacteria.

This mechanism has been extensively studied for the Class I lantibiotic nisin.

The first two rings in nisin, called A and B, and formed by a Lan and a MeLan respectively, can bind the pyrophosphate portion of Lipid II (Fig 4).

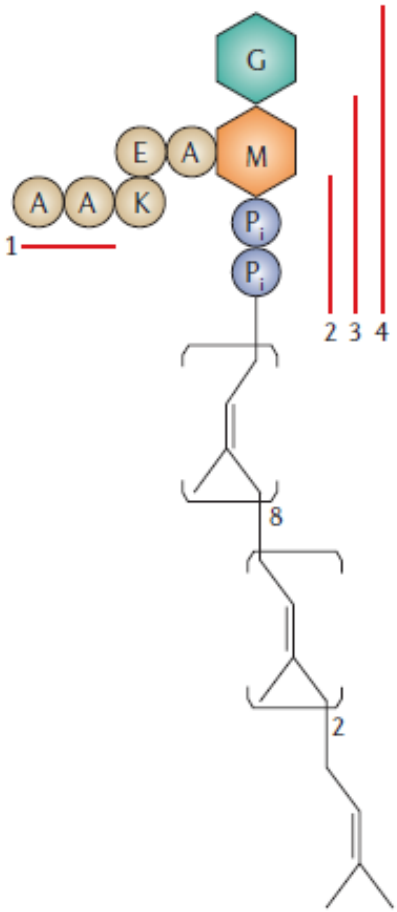


Fig 4: Structure of Lipid II. The red bars indicate the minimal binding sites in Lipid II of glycopeptide antibiotics (1), nisin (2), ramoplanin (3) and mersacidin (4).
GlcNAc (N-acetylglucosamine); MurNAc (N-acetylmuramic acid).

As the A and B rings are conserved in most of Class I lantibiotics, this mode of action is credited to other class I lantipeptides.

Once the two rings bind Lipid II, nisin is able to insert its tail into the membrane.

A group of eight nisin, and four Lipid II, form a stable pore that leads to the loss of intracellular compounds in the extracellular environment and consequently to the death of the bacteria (Fig 6) [3].

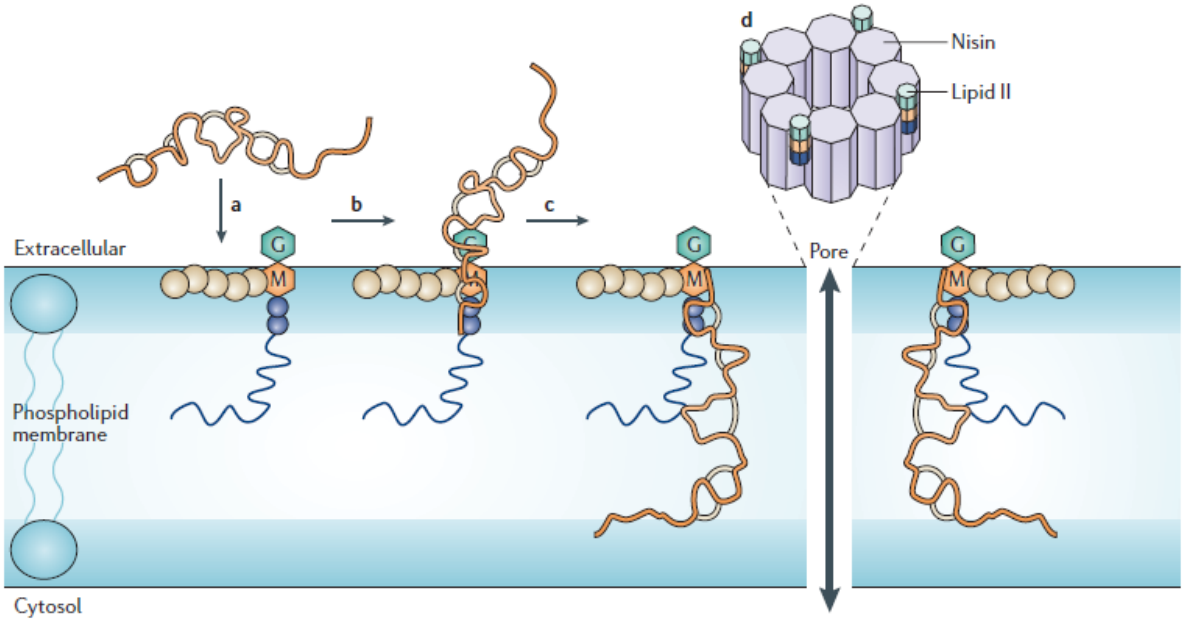


Fig 6: Schematic representation of pore-formation nisin mechanism. (a): nisin reach the extracellular side of the membrane; (b): nisin rings A and B bind the pyrophosphates of LipidII; (c): transmembrane insertion of nisin; (d): four nisin bind four Lipid II, and four additional nisin are recruited for pore formation.

As mentioned above, and shown in Fig. 5, nisin can bind the Lipid II at pyrophosphate level. General glycopeptide antibiotics, such as vancomycin, can bind Lipid II at level of the pentapeptide. This kind of binding is related to the specific sequence of this oligopeptide.

This means that changes in the amino acidic sequence can compromise the binding. Bacteria can change this sequence and thus develop resistance to general glycopeptide antibiotics. On the other hand, the pyrophosphates are a fundamental mojeties of Lipid II and difficult to replace. This could represent a potential advantage for using lantibiotics, as bacteria cannot easily develop resistance. This is proved from the fact that in 50 years of use of nisin as food preservative no significant resistance has been observed.

Some kind of resistance mechanism has been detected, as an increising in the cell wall thickness in an attempt to reduce the access to the Lipid II, but this does not lead to a significant decreasing of nisin activity.

As nisin, classII lantibiotics (e.s. mersacidin) kill bacteria with a mechanism that inhibits cell wall replication by binding Lipid II, but this mechanismdoes not involve pore formation.

The binding site of mersacidin involves the GLcNAc sugar, but cannot bind it in LipidII.

The mechanism of Lipid II binding seems to be related with positive charge, in particular seems that this mechanism is dependent to the bivalent ion Ca^{2+} that is responsible for the formation of a bridge between the lantibiotic and the sugar of Lipid II [4].

Not all the class II lantibiotics act in this way, cinnamycin e.g. does not target Lipid II.

Mode of action of Class III lantibiotics involves both the subunits.

The α-peptide behave like mersacidin, binding the LipidII then, the β-peptide is recruited to form pores.

An example of this lantibiotics is haloduracin.

In this case, a complex formed by a Lipid II, two α-peptide and two β-peptide is necessary for the pore formation.

This mode of action explains why a single subunit of a two component lantibiotic shows low or no activity. [1].

The literaturereports of other lantibiotics, such as Pep5 and epilacin K7, that form pores without the binding of Lipid II, suggesting a specific but alternative molecular target [10].

Another advantage in the use of antibiotics targeting Lipid II is the low toxicity in human, due to the fact that Lipid II production is restricted to bacteria.

However, there are still obstacles that must be overcome before lantibiotics can be introduced in clinical applications.

For example, nisin shows poor pharmacokinetic that represents the main impediment to its clinical development.

Anyway, the development of nisin as therapeutic molecule can benefit of the large amount of information collected from more 50 years of usage of nisin as food preservative, for example, this information can be used on issues relating stability and conditions of use [4].

In conclusion, the available data support the great capabilities of lantibiotics as potent antimicrobials with a novel mechanism of action [5].

1.3.1 Effect of the presence of thioether bridges in lantibiotics

As mentioned before, lantibiotics have still to overcome some obstacles before their clinical use is confirmed.

On the other hand, lantibiotics have a characteristic that is very important for a therapeutic peptide: the presence of thioether-bridges.

The main limitation in the application of therapeutic peptides is their rapid degradation by proteases.

A strategy to prolong the clearance of these peptides is to bind them to a protein as albumin.

This problem does not affect lantibiotics, because of the presence of thioether-bridges that confer strong resistance against proteolitic degradation [6].

In lantibiotics, thioether-bridges are present in the amino acids lanthionine and methylanthionine, introduced as post-traslational modifications.

Therefore, Lan and MeLan rings are useful both for the activity and for the resistance against proteolitic degradation.

In lantibiotics the Lan and MeLan formation follow this steps: 1) dehydratation of serines (Ser) and threonines (Thr); 2) resulting dehydroalanines and dehydrobutyrines are coupled to cysteines. This process is catalized by a cyclase enzyme (Fig 7) [6].

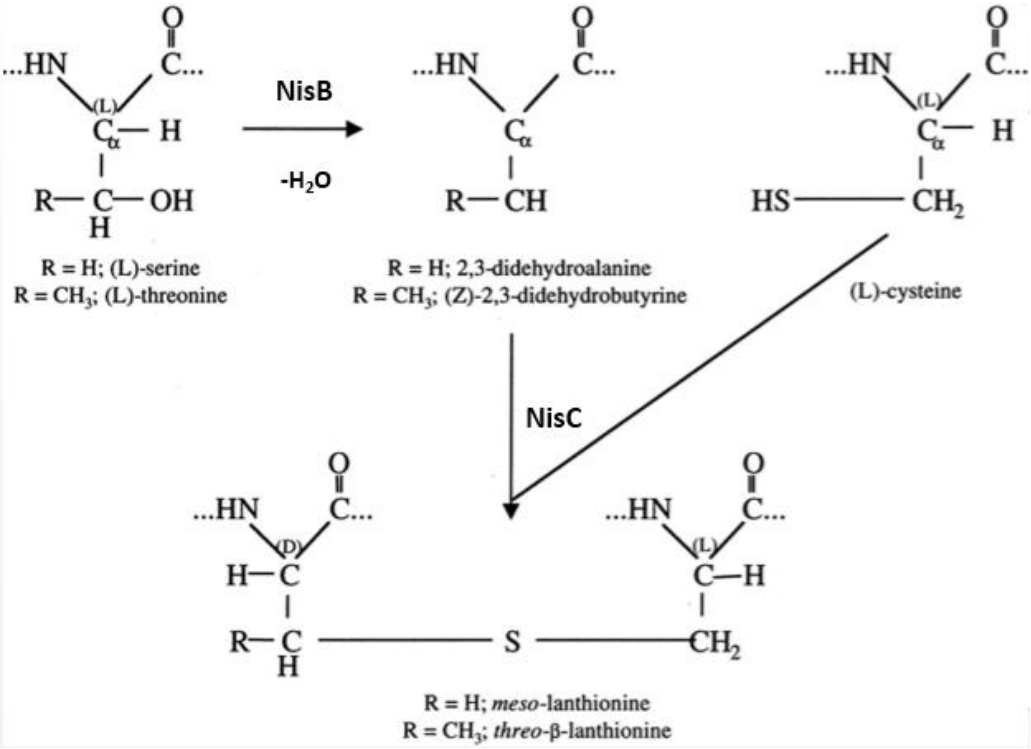


Fig 7: Schematic representation of thioether-bridge formation.

Resistance against proteases gives the advantage that a higher concentration of therapeutic peptide can be reached in the body and can be kept for longer time.

More important, proteolitic stability prevent the formation of toxic compounds from lysate peptides.

The presence of thioether-bridges seems to open the way to the production of oral delivery peptide drugs.

This kind of delivery is very difficult to reach in peptide drugs that do not contain thioethers links, but seems to be possible in those that have these bridges, as shown

for thioether-bridged angiotensin, that could be successfully delivered both orally and airborne [5].

1.4 Strategies to develop new lantibiotics.

As stated in the previous paragraph, the use of lantibiotics represents a valid and novel strategy to fight the growing problem of multi-resistant pathogens.

To increase the success of lantibiotics as new drugs several strategies can be used.

For example exploiting the possibility to realize fusion leader-peptide and the substrate tolerance for modification enzymes, the design of novel lantibiotics or an improved activity of bioactive compounds is possible.

In this paragraph possible strategies in the discover of new lantibiotics are described, including both a natural and a synthetic biology approach. Furthermore, methods to improve activity of these lantibiotics, as additional modifications or mutagenesis, are illustrated.

1.4.1 Traditional approach: discover new lantibiotics using wild-type producers

The traditional approach to search new potential antibiotic compounds is based on the isolation of producer organisms from diverse habitats.

After isolation of the producer, the next step is to check the antimicrobial activity.

The most common way to do that is an agar diffusion assay (or activity test).

With this assay is possible to check if the producer actually can produce antimicrobial molecules (lantipeptides in our case), and this is confirmed by an inhibition of growth of the indicator strain on the plate (Fig 7).

For this kind of assay two variable are to be taken into account: the indicator strain and the conditions for the growth of the producer.

The number and diversity of indicator strains that can be tested is a limitation, but is also true that we can limit this number selecting only strain suitable for certain application.

Also, finding the growth condition in which the producer can express the lantibiotic is not an easy task. [5]

If areas of growth inhibitions are detected is necessary to check if the inhibition actually depends from the antimicrobial peptide or if it is related to others substances, for example catabolities as organic acids.

Therefore, additional steps are necessary to check the antimicrobial molecule, but anyway this method is still a good strategy to screen potential producers.

The main disadvantage of this kind of approach is the wide screening area that has to be covered, which lead to a high demand of resources. This point can be partially overcome by automatic high throughput screening systems [5].

On the other hand, the advantage is that identified antimicrobial compound are already been selected in the appropriate growth conditions.

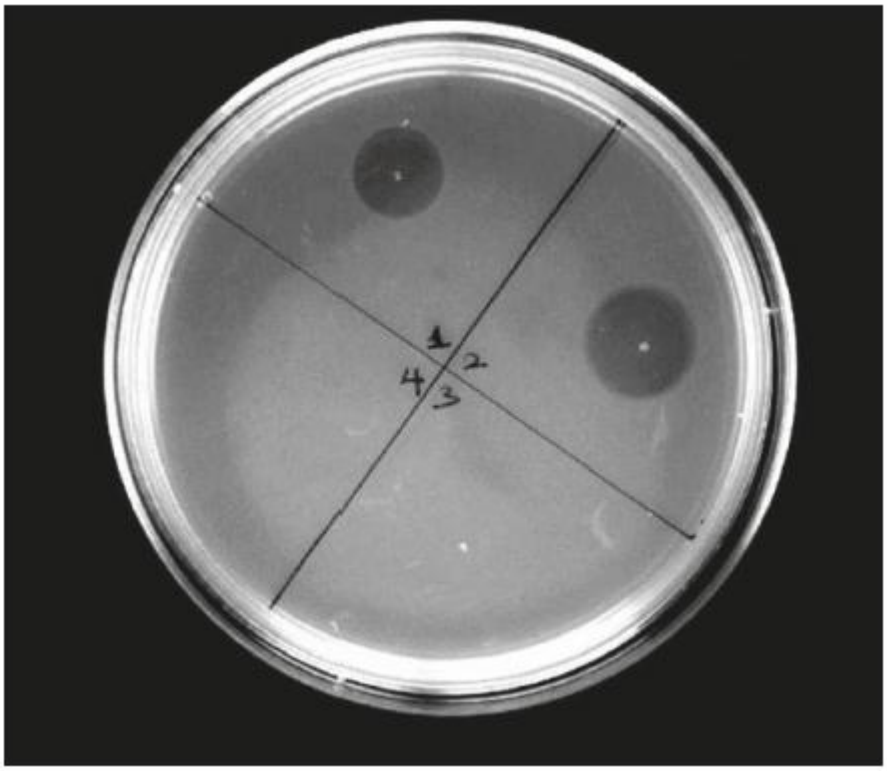


Fig 7: Example of activity test. In this case the activity of cinnamycin samples against Bacillus subtilis LH45 (indicator strain) is shown on LB-agar plate.

1.4.2*In silico* genomic mining-tools to discover new lantibiotics

An alternative way to discover new lantibiotics involve the analysis of the genome of potential producers.

Anyway, lantibiotic structural genes (genes that codify LanA) are too small and show low homology to use a similarity oriented search.

Instead of searching for homology of structural genes, a better strategy is search for homology for modification enzymes, because of their larger size and conserved domains.

Literature report that structural genes for lantibiotics are surrounded by "Open Reading Frames" (ORFs) for modification enzymes.

That means that searching in the genome of a potential producer for a modification enzyme gene can lead to the entire gene cluster for the expression of the lantibiotic.
The use of*in silico* mining tools, such as “Bagel2” and “Bagel3” softwares developed by “Molgen” group of the Groningen University, is necessary to this aim.
This searching engine allow the identification in the genome of a gene cluster related to the production of lantibiotics , considering the structure of the putative structural gene, the surrounding ORFs and their homology to modification enzymes.
This kind of strategy allow to check exotic and non-culturable bacteria, and if a positive result is found is possible to try heterologous expression.
Other application of *in silico*analysis, oriented to microbial ecology, permit to restrict the environments to screen to find new potential producers.

1.4.3 Improve the bioactivity through mutagenesis

Once a novel lantibiotic has been defined it’s possible to improve its properties as stability, potency or spectrum using mutagenesis.
That means the replacement one or more amino acids in the peptide sequence.
Mutagenesis can be also use to better understand the lantibiotic’s mechanism of action, its maturation process and the bacterial immunity system.
Experiments carried out on the leader peptide highlight the role of this portion on the maturation step, because after mutation the modification enzymes could not more recognize the leader and modify the core peptide.
However, point mutation can improve molecules, this is the case of nisin Z, in which after a substitution was observed an improved stability and solubility without decreasing the activity. This was possible by an insertion of a positively charge amino acids in the first ring (Fig 8).
Nowadays, it is possible obtain libraries of mutants lantibiotics, with all the possible point mutation, or even with several mutation at the same time, and to test

thesecompounds with high throughput screening systems to identify variants with higher expression level, better stability or specific bioactivity.

Of course this strategy can be used on wild type lantibiotics as well as on new synthetized ones.

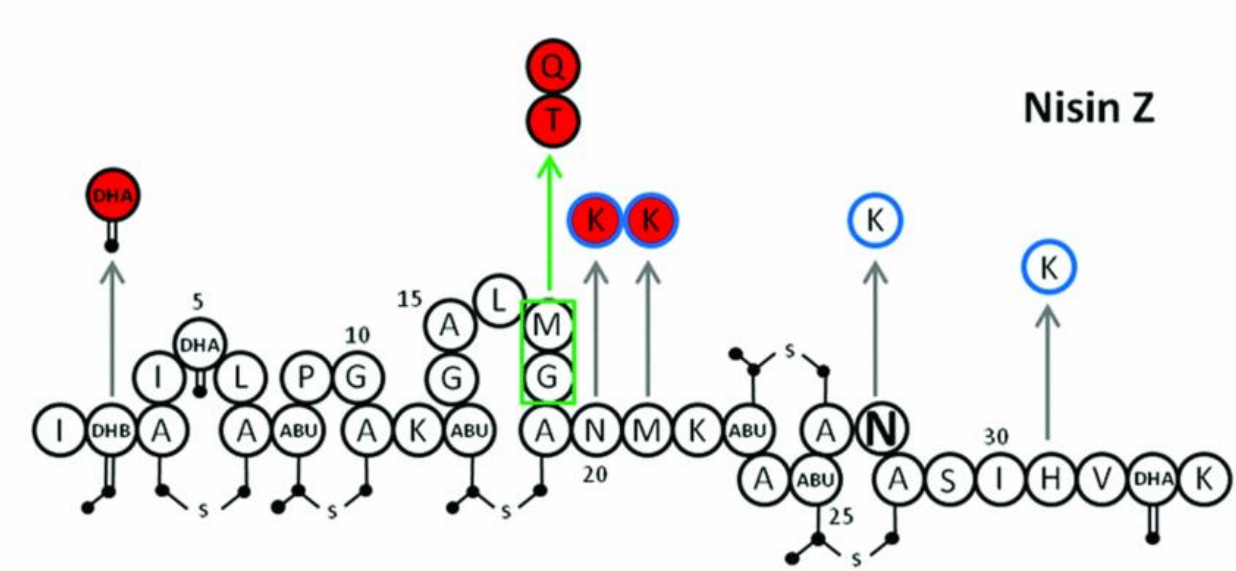


Fig 8: Nisin Z with possible mutations. Red circles indicates improved activity, blue circle indicates improved physiochemical proprieties.

1.4.4 Synthetic Biology approach

Synthetic Biology aims to apply the principles of modularity and orthogonallity of engineering to Biology.

Thus, the isolation of parts of the peptide or the biosynthetic machinery should be standardized in such a way that they can be predictably combined to produce a new antimicrobial molecule [5].

As mentioned above, modification enzymes of lantibiotics can modify a wide range of substrate peptides as long as they are linked to the leader peptide that is necessary for the recognition from the enzymes.

This point has been reported for nisin modification enzymes nisB, nisC and nisT.

In the chapter 3 of this thesis the modification of nisin with a chimeric leader peptide is reported. The synthetic leader is formed by the fusion of cinnamycin and nisin leaders by the class LanM modification enzyme CinM, from cinnamycin modification machinery.

Indeed, it has been proved that "silent" lantibiotics from *S. pneumoniae,*can be produced and modified by nisBCT in *L.lactis,* thusin a heterologous host, if the core-peptide is linked to nisin leader.

This means that the enzymes are able not only to dehydrate and cyclize the peptide, but also to match dehydroamino acids and cysteines in the correct way.

This is a clear example of modularity applied to lantibiotics production.

Heterologous expression allow to produce lantibiotics of non-culturable bacteria without engineering the original producer.

In addition, heterologous expression of lantibiotics modified by nisin system in *E.coli* has been reported.

In addition to the characteristic lanthionine and methilantionine, lantibiotics carry additional post-translational modification important for stability and modification, such as lysinoalanine bridge in cinnamycin.

Exploiting the concept of modularity it is possible to introduce theseadditional modifications of some lantibiotics to hypermodify existing molecules, and confer to them improved bioactivity or new proprieties.

However,it is necessary to characterize this modification enzymes in order to use them in a rational and predictable way.

Modularity in lantibiotics is not only referred to leader peptide, but also to the core peptide sequence.

Three different regions can be described in nisin: 1)a leader peptide that leads the modification enzymes; 2) a Lipid II binding domain, formed by the first two rings of nisin; 3) a membrane insertion tail.

That means that is theoretically possible to design a lantibiotic with the Lipid II binding domain from nisin and a C-terminal tail to introduce additional post-traslational modification to create a novel molecule.

Modularity is present also in Class III lantibiotics, in which one subunit is necessary to bind Lipid II and the other is necessary for pore formation.

Moreover, combination of lantibiotics with traditional antibiotic could represent a valid strategy.

*In vitro*combination of nisin with vancomycin via chemical semi-synthesis has been reported, and improved activity was detected for vancomycin[11].

The possibilities in design of novel lantibiotics with a synthetic biology approach are enormous because the number of combinations is very high, and can be raised with the discover of new lantibiotics and the modification by mutagenesis method.

This is particularly true if we consider the possibility to introduce non-canonical amino acids in the sequence of lantibiotics.

These non-canonical amino acids can allow introduction of new properties for the peptides.

In conclusion, all the strategies described above should be combined to obtain a considerable improvement in the research of new lantibiotics (Fig8).

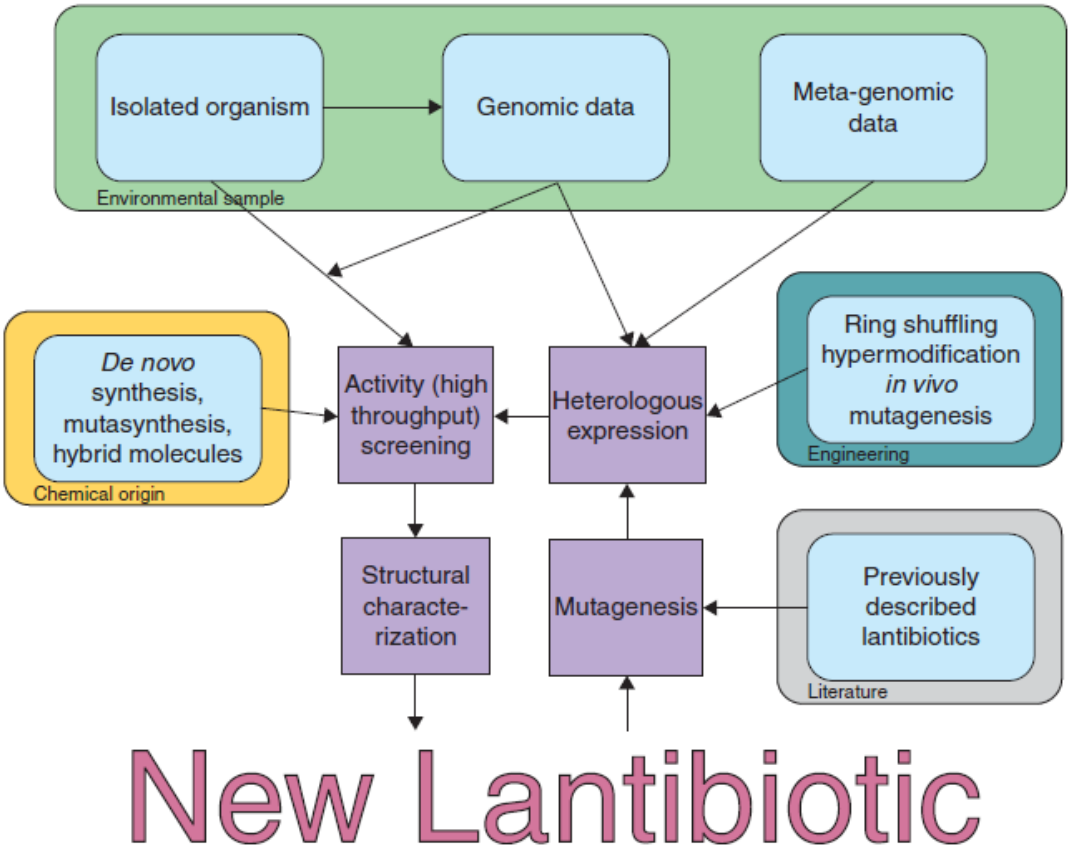


Fig 8: Representation of the possible strategy in the develop of new lantibiotics

CHAPTER 2:Analysis of the lantibiotics Nisin and Cinnamycin

The project reported in this work involves two lantipeptides, the class I lantibiotic nisin, and the class II lantibiotic cinnamycin.

In this chapter, structure, modification machinery and mode of action of these two lantibiotics are described.

Furthermore, some examples of application of synthetic biology approach on nisin are reported.

2.1 Structure, biosynthesis, modification machinery of nisin. Design of new lantibiotics through a Synthetic Biology approach

Nisin is a class I lantibiotic produced by *Lactococcus lactis*, is one of the oldest know antibiotic compounds, and shows antimicrobial activity against Gram-positive bacteria.

The genes necessary for nisin biosynthesis process - that involves production, maturation, immunity and regulation - are located in a conjugative transposon [20].

Nisin was used as food preservative for 50 years, and its high efficiency makes it one of only few commercial applied lantibiotics.

Target for nisin antimicrobial activity are Gram-positive bacteria such as *Bacillus cerus*, *Listeria monocytogenes*, *Enterococci*, *Staphylococci* and *Streptococci*.

For this very high antimicrobial activity, nisin has been proposed to be used as model to develop new antibiotics.

Belonging to class I lantibiotics, nisin harbours thioether bridges in the Lan and MeLan resiudues, introduced after the action of the modification enzymes NisB and NisC.

In the following paragraphs biosynthesis, modification machinery and nisin role in developing of new lantibiotics through a Synthetic Biology approach are described.

2.2.1 Biosynthesis of nisin.

Nisin biosynthetic genes are organized in four operons, *nisABTCPIRK*, *nisI*, *nisRK* and *nisFEG*.

Nisin precursor peptide is encoded in the gene *nisA*, the result of the translation of this gene is a peptide of 57 aminoacidic(a.a.) residues.

To obtain the mature lantibiotic is necessary that the modification machinery processes the precursor peptide.

The modification system is encoded in the four genes *nisB,nisC,nisT,nisP*.

The first one encodes the dehydratase NisB, responsible for the dehydration of three serine and five threonine.

Some of this dehydrated residues are coupled with cysteins by the cyclase NisC.

This result in the formation of one lanthionine and four methyllantionine, and also one dehydrobutyrine and two dehydroalanines that are not coupled with cysteins.

The next step in the biosynthesis of nisin is the exportation of the modified molecule through the membrane.

This process is carred out by the transporter NisT, an ABC-type transporter [21].

Both for the modification and for transportation the presence of the leader peptide is necessary for the enzymes to recognize the substrate molecule.

The leader peptide is a peptide of 23 a.a., and until it is binding the modified nisin the molecule does not show any activity.

Thus, the last step involves the cleavage of the leader peptide.

This process is carried out by the membrane protein NisP.

The mature nisin is also capable to induce the complex NisRK, the system that regulates the transcription of nisin gene cluster [20].

The analyis of nisin transposon (Fig. 9) highlights the presence of a regulation operon, formed by *nisRK*, and an immunity operon formed by *nisI* and *nisFEG*.

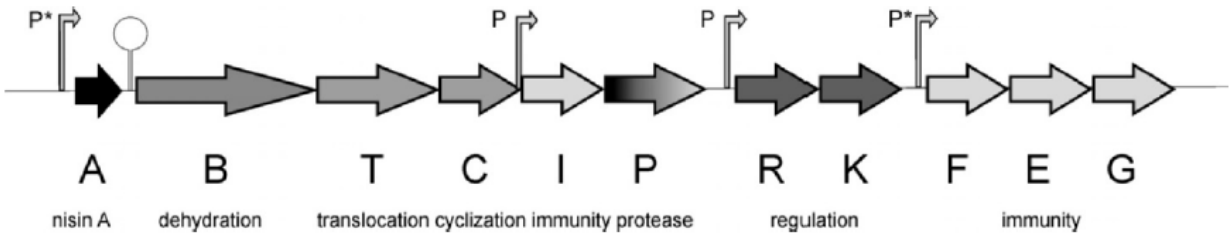


Fig 9: Transcriptional organization of nisin biosynthetic gene cluster.

The regulatory system for nisin biosynthesis is a two-component system, formed by NisR and NisK.

NisK is a histidine sensor kinase that is located in the extracellular side of the membrane and act as receptor for fully matured nisin.

The binding of NisK with nisin initiate a signal cascade, that starts with the auto-phosphorylation of NisK.

Subsequently, the phosphate is transferred to NisR, which is a transcriptional activator that binds the promoter regions of *nisABCPRK* and *nisFEG*. This induces the transcription of genes required for nisin biosynthesis and immunity.

The promoter of *nisRK* was shown to be independent from nisin regulation and the two genes to be constitutively expressed.

This system provide a tight regulation of gene expression, for this reason nisin-controlled expression system (NICE) [22] were developed and used to control overexpression of protein in heterologous expression systems.

*Lactococcus lactis*has developed an immunity system against the bactericidal activity of nisin itself.

The immunity system consist in a two-component complex made by the lipoprotein NisI and the ABC-type transporter NisFEG [23].

The two components strongly interact to confer immunity as proved by the fact that each component alone provide 5-20% of the total immunity level.

NisI is a lipoprotein anchored on the extracellular side of the membrane, but also free NisI was detected.

The function of NisI is to interact with nisin by binding it and avoid that nisin itself can target the Lipid II of the membrane.

NisFEG form a transporter system that has the role to export nisin from the cytoplasmatic side of the membrane.

The two systems interact because NisFEG can provide high local concentration region for nisin, so that NisI can easily intercept nisin and avoid its movement in the extracellular environment [20].

In the figure below the biosynthetic system of nisin is shown.

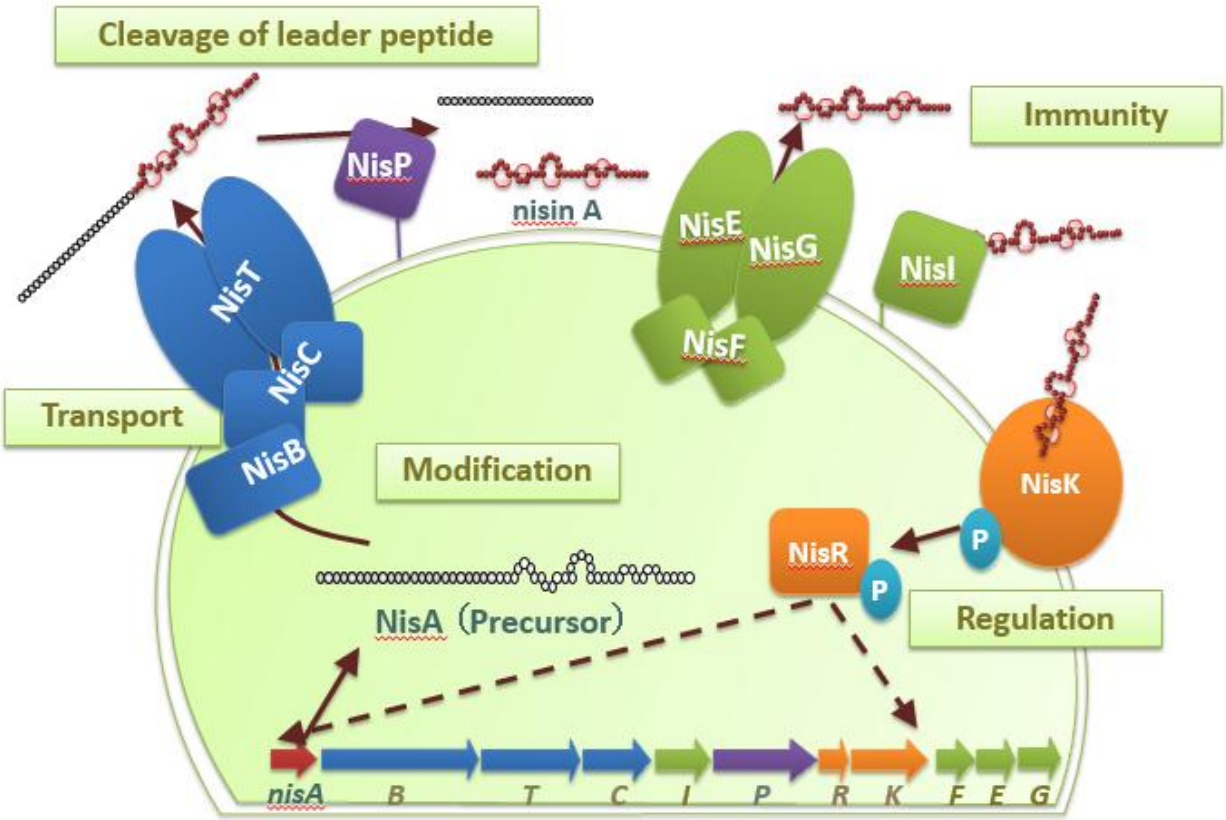


Fig 10: Representation of nisin biosynthetic system. P in the blue circle indicates the phosphorylation of NisK and NisR.

2.2.2 Analysis of nisin structure

After modification nisin is a peptide of 31 amino acids containing one lanthionine ring and four methyllanthionine rings, plus two dehydroalanine and one dehydrobutyrine. The five rings on nisin molecule are named as A, B, C, D, and E starting from the N-terminal side to the C-terminal side of the molecule.

The main part of nisin consist of the lanthionine ring A, and the two methyllanthionine rings B, C. This part of the molecule is linked by a “hinge” region to two intertwined double methyllanthione rings D and E.

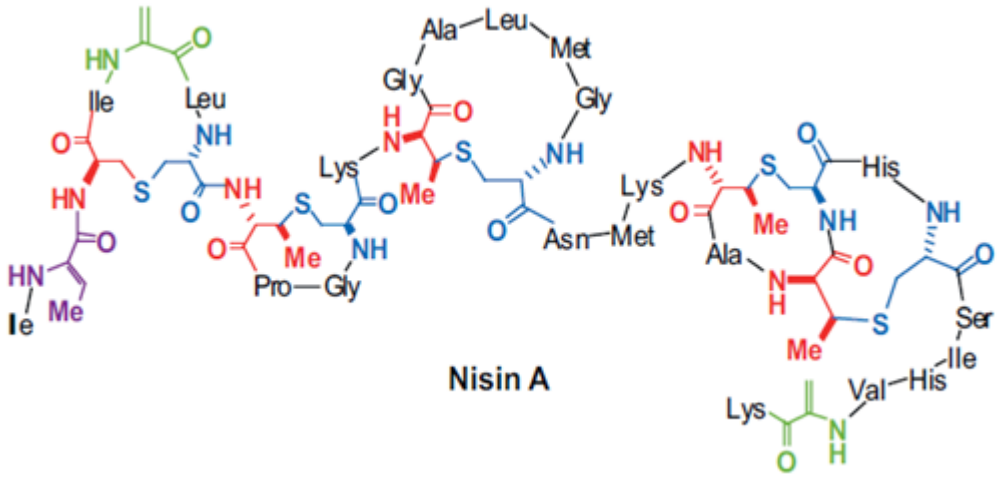
Thus, nisin shows as a rod-like molecule with a hinge region that confer flexibility to the total structure, and also the two parts are both quite flexible.

It is remarkable that rings A, B and C share the feature that hydrophobic part is situated opposite to the thioether bonds. The hydrophobic part is composed of the residues Ile4, Leu6, Pro9, Leu16 and Mey17 whereas the hydrophilic Lys12 is located at the opposite face [20].

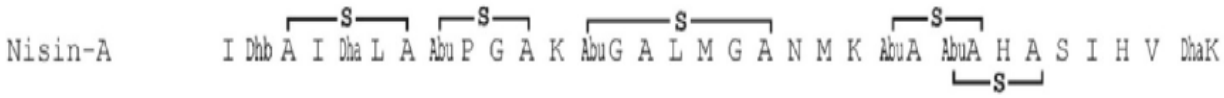
Taken together, the molecule is amphipathic in two ways: first, most of the residues in the N-terminal part are hydrophobic and only a single charged residue is present (Lys12), whereas the charged and hydrophilic amino acids are mainly located in the C-terminal half of the molecule.

Secondly, both the N-sided domain, with rings A, B and C as well the C-sided domain, containing rings D and E, have a hydrophobic and hydrophilic side [20].

Nisin amino acidic sequence and structure is shown in the figure below.



(a)



(b)

Figure 11: (a) Structure of fully modified nisin. Red regions represent dehydrobutyrines and dehydroalanines involved in Lan and MeLan formation. Blue regions represent cysteines involved in Lan and MeLan formation. Green regions are dehydroalanines. Purple region is dehydrobutyrine.

(b) Amino acidic sequence of fully modified nisin.

2.2.3 Nisin modification machinery

Nisin modification machinery is composed by four enzymes: the dehydratase NisB, the cyclase NisC, the ABC-transporter type NisT and the protease NisP.

All the modification steps seems to be carried out in proximity of the membrane.

This was dimostrated by immunoprecipitation assay [24], furthermore it was demonstrated that physical interaction between the modification enzymes is requested for the modification of nisin pre-peptide.

However, *in vitro*studies show that the modification enzymes can work separately, this means that the lanthionine sintetase complex, formed by all four modification enzymes, is not a prerequisite for functioning of any of the modification and transport enzymes, and it is likely highly unstable and transient in nature [20].

This means that the single modification enzymes canbe use separately to modify lantibiotics using a synthetic biology approach, exploiting the not strict specificity of these enzymes for their substrate.

It was demonstrated that the overexpression of *nisBCT* gene leads to insertion of Lan and MeLan rings in non-lantibiotic peptides if they are linked to nisin leader.

This proves that nisin modification enzymes do not have highly specificity and that the main prerequisite for modification is the presence of the leader peptide upstream the substrate.

Figure 12 show nisin modification steps and lanthionine sintetase complex located in the cytoplasmic side of the membrane.

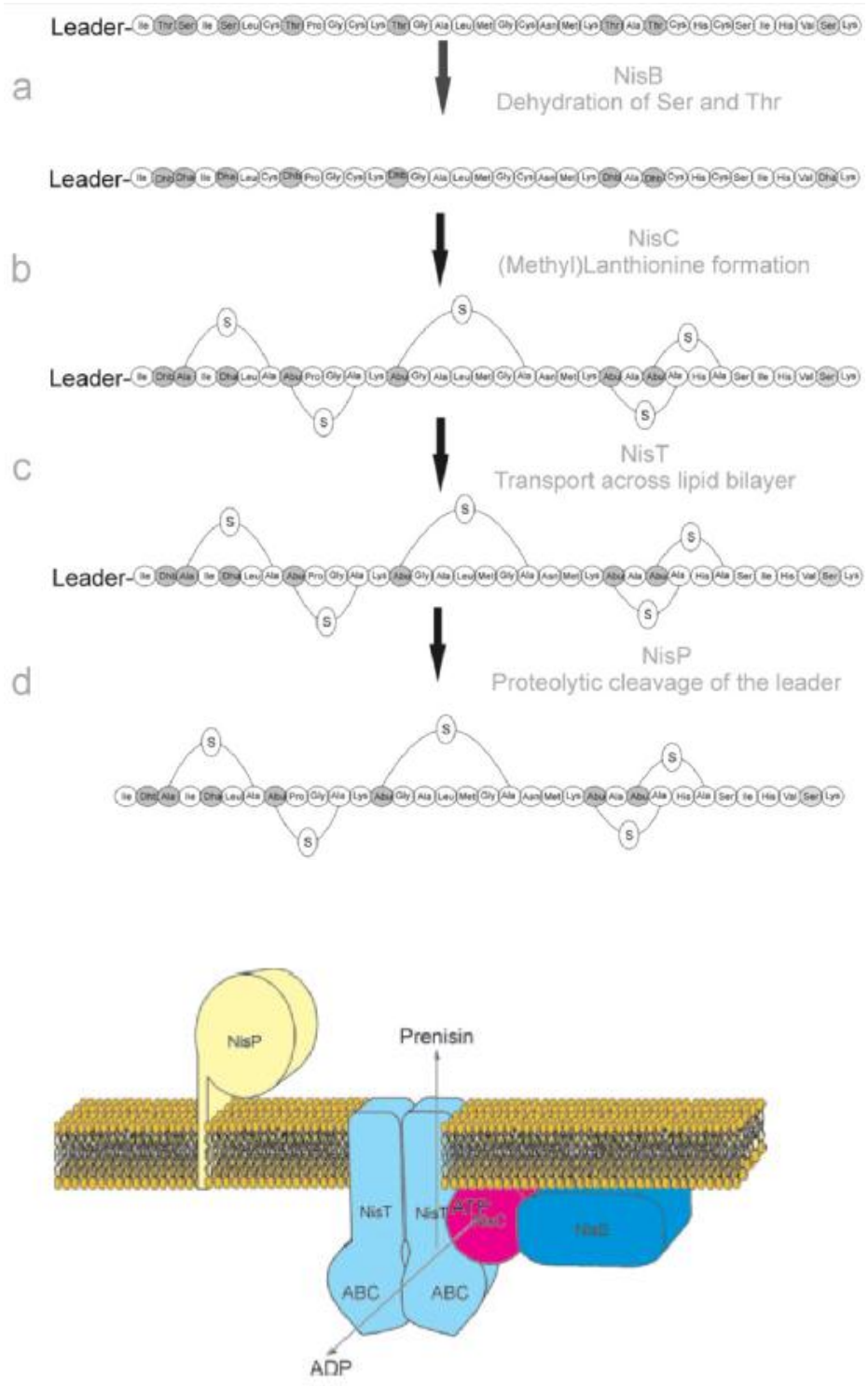


Figure 12: Nisin modification steps and lanthionine sintetase complex.

NisB, a protein of 117.5 kDa, is the dehydratase responsible for the dehydratation of Ser and Thr residues in the nisin core-peptide.

This was proved by the inhibition of *nisB*gene. This operation leads to a complete loss of nisin production. Furthermore, overexpression of this enzyme rises the efficiency of dehydratation in nisin.

To carry out its function NisB requires the presence of the leader peptide.

Furthermore, it was proved that NisB could dehydrate Ser and Thr also in non-lantibiotic peptides, showing a relaxed substrate specificity.

Apparently the substrate specificity is related to the amino acidic residues near Ser and Thr residues.

Studies demonstrates that the nature of residues close to dehydrated serine and dehydrated threonine may influence the dehydratation step [20].

These residues should be surrounded by hydrophobic residues to obtain the dehydratation of Ser residues.This is the main limitation for NisB substrates.

NisC is the cyclase that carry out the coupling of dehydrate residues with free cysteine, it is a 47.9 kDa protein.

Thus, the ring formation is lead by NisC and all the rings are oriented in the same manner, from N-terminal side, where the dehydro residue is located, to C-terminal side where the cysteine is positioned.

The role of NisC was confirmed by knoking out this gene in nisin gene cluster. This operation leads to the production of nisin with dehydro- residues without lanthionine and menthyllanthionine rings.

This proves that NisC is fundamental in cyclization.

It was also proved that NisC can work separately from the other modification enzymes.

It was demonstrated that NisC need the presence of the leader peptide to carry out its function.

So, as for NisB the leader peptide is fundamental, and seems that NisC can recognize the leader by a specific region formed by hydrophobic and negatively charges residues that form a channel in which the leader peptide is supposed to be trapped.

NisT is an ABC half-transporter that consist of putative α-helices that crosses the cytoplasmic membrane five times and a hydrophilic nucleotide-binding domain that binds ATP needed to energize the transport.

A typical ABC transporter includes two modules, i.e., two trans-membrane segments and two nucleotide-binding domains.

Therefore, NisT is an half ABC transporter and most likely requires another half-transporter to form an active unit[20].

This putative partner is likely another NisT.

The function of NisT was confirmed bythe knock out of the gene *nisT.*As a result the secretion of nisin was inhibit.

It was also proved that NisT can transport unmodified or partially modified nisin, but also non-lantibiotic peptides if this are fused with the nisin leader sequence.

NisP is the protease that carry out the cleavage of nisin leader peptide.

This was proved by knocking out the gene encoding NisP.

As a result, no active compound was detected because the cleavage of the leader is necessaryto obtain active nisin.

The activity was restored by incubation with cells expressing NisP.

NisP contain a Sec-signal in the N-terminal side, that is likely responsible for targeting and transporting NisP out of the cell via *Sec* pathway.

NisP contains also a C-terminally located LPXTG sequence, that suggests its anchoring to the cell surface [20].

NisP can cleave only the nisin leader peptide, furthermore neither unmodified nor dehydrated pre-nisin could be cleaved by NisP, indicating that one or more thioether rings are required for its activity [25].

2.2.3 Use of nisin and nisin modification enzymes in synthetic biology

As mentioned in the previous chapter, Synthetic Biology represent a valid strategy in the design of new lantibiotics.

The Synthetic Biology approach is exploiting the relaxed substrate specificity of enzymes involved in lantibiotic modification, and also the modular structure of lantibiotics themselves.

At least two separate modules are detectable in latibiotic, a leader peptide and a core peptide. The first one is involved in the recognition of the molecule from modification enzymes.

Using this peculiarity it is possible design non-lantibiotic peptides fused with a leader peptide that can be modified by modification enzymes.

It is also possible to introduce in a given lantibiotic post-translational modifications that belong to other lantipeptides.

The following are example of use of Synthetic Biology in the modification of lantibiotics.

The first one describe the use of nisin modification machinery in the production of a two-component lantibiotic of *S.pneumoniae,* whereas the second one shows the possibility of using modification enzymes from different lantibiotics to design novel molecules.

The first example is reported in [2], and reports the expression of a class II two-component lantibiotic from *S.pneumoniae*using the nisin modification machinery.

To do that it was necessary toisolate the gene encoding for the lantibiotic peptide and to modifyit to obtain a gene that encodes the fusion of nisin leader peptide with the two-component lantibiotic peptide.

Then the overexpression of this novel gene and of the *nisBCT*genes was performed.

The peptide was secreted out and found in the supernatant of the culture, meaning that the transporter nisT can export the peptide.

Mass spectrometry analysis dimostrated the presence and partial localization of multiple dehydratated serines and/or threonines and (methyl)lanthionines in both peptides.

Furthermore, after the cleavage of the leader peptide from both the components antimicrobial activity against *Micrococcus flavus*was detected.

These results proved that the nisin modification machinery can be succefully used to modify and produce other antimicrobially active lantibiotics, and confirm the validity of the synthetic biology approach.

The second example is reported in [26] and is related with the design and production of novel lantibiotics using a combination of different lantibiotic modification enzymes.

The specific results highlight:i)the modification of the lantibiotic gallidermin by nisin modification enzymes NisBTC and gallidermin modification enzyme GdmD ; ii) the design and production of a novel lantibiotic formed by the nisin region containing rings ABC and a gallidermin tail. This hybrid molecule was modified by the combination of NisBTC and GdmD; iii) the design and production of modified nisin by introduction of a modification carried out by lacticin modification enzyme LntJ.

GdmD is responsible for C-terminal decarboxylation, and LntJ is a reductase that stereospecifically converts dehydroalanine in D-alanine.

The modification and transport of this molecule was proved by heterologous expression in *L.lactis*, this results provide a plug and play system that can be used to

select different sets of modification enzymes to work on diverse, specifically designed substrates, with the aim to obtain new to nature molecules with antimicrobial activity and/or new physicochemical proprieties.

The representation of the system is shown in figure 13.

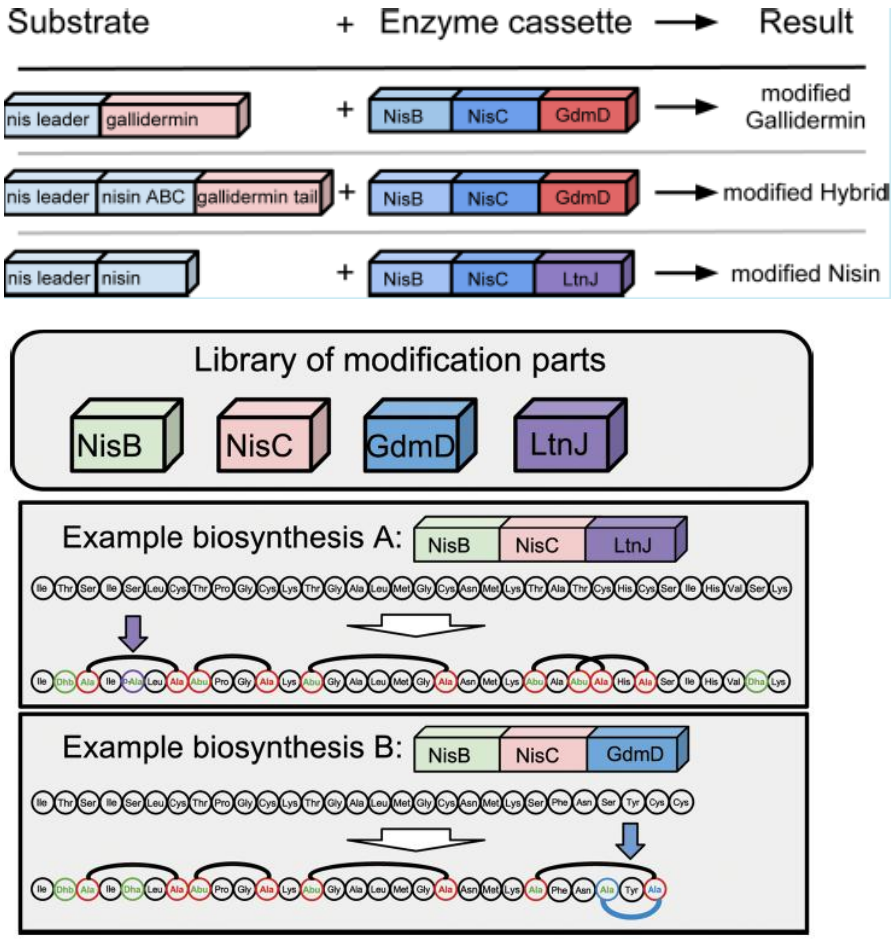


Figure 13: Schematic representation of the synthetic biology plug and play system for the design and production of new to nature lantibiotic.

2.2 Structure, mode of action and modification machinery of the class II lantibiotic cinnamycin

Cinnamycin is an antibiotic peptide produced by several *Streptomyces* strains, including *Streptomyces cinnamoneus* DSM 40005 [13].

Cinnamycin belongs to lantibiotic group as it carries lanthionine and methyllanthioninebridges.

As in other lantibiotics, these bridges are formed from dehydro serine and threonine residues, coupled with cysteines.

These modifications are carried out by a LanM modification enzyme, called CinM, given that cinnamycin belongs to class II lantibiotics.

Unlike nisin or others class I lantibiotics, cinnamycin possesses other post-translational modification.

Cinnamycin contains a β-hydroxy-aspartate residue and a lysinoalanine bridge (Lal).

The hydroxylation of the aspartic acid residue (Asp) is carried out by the modification enzyme CinX and the formation of the Lal bridge is made by the small enzyme Orf7.

Class II lantibiotics are less studied and usually have globular and inflexible tertiary structure (Fig 14), unlike the class I lantibiotics that are more flexible and have linear structure [14].

Another difference between class I molecules as nisin and class II lantibiotics as cinnamycin is in the mode of action.

As mentioned in the previous chapter, class I lantibiotics exercise their antimicrobial activity interacting with Lipid II of the cell wall, this generally lead to pore formation.

Some class II lantibiotics as cinnamycin and duramycin seems to exercise their activity binding enzymes involved in the cell wall biosynthesis.

Similar to other lantibiotics the non-mature peptide is formed by the fusion of a leader peptide and a core peptide.

The core peptide is a 19a.a.-long peptide, and it is the portion that harbour the lanthionine and methyllantiones residues, and the other post-translational modification.

The leader peptide is a 59a.a.-sequence, a very long sequence if compared to leader peptide of others lantibiotics, especially in class I.

The leader peptide is important for the modification enzymes to recognize their target.

To obtain the active compound the cleavage of the leader is necessary.

Unlike nisin, it seems that there is not a dedicated protease for this taskin the cinnamycin gene cluster.

Therefore, it seems that the last three amino acids, "AFA", of the leader are involved in the cleavage, fitting with the "AXA" motif recognized by type I signal peptidases of the general secretory pathway (Sec) [13].

This "AFA" sequence will be taken into account in the heterologous expression of cinnamycin in *E.coli* (described in chapter 3), to engineer the cleavage of the leader peptide.

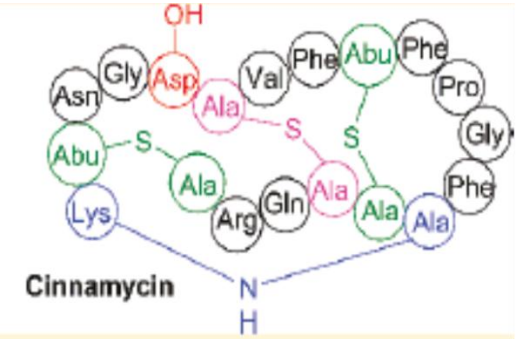


Fig 14: Schematic structure of cinnamycin. MeLan bridges are shown in green. Lan bridge is shown in pink. Blue is used to label the Lal bridge. Red is used to label the hydroxylation of Asp.

2.2.1 Analysis of the cinnamycin gene cluster

The study of the gene cluster involved in the expression of cinnamycin in *S. cinnamoneum*was possiblethanks to genomic analysis tools[13].

The schematic representation of this cluster is illustrated in Fig 15.

The genome contains 21 genes, but it is not yet clear which is the functionfor some of them.

The function of each gene was studied through a homology approach, thus searching proteins similar to those encoded in the *cin* gene cluster.

Most lantibiotic gene clusters contain the following genes: i) *lanA*, the lantibiotic prepeptide structural gene; ii) *lanBC* or *lanM*, the genes that encodes the enzyme for dehydratation an cyclization of dehydrated serine and threonine residues with cysteines; iii) *lanT*, which encodes a transporter enzyme for export the lantibiotic; iv) *lanP*, the gene encoding the protease for the cleavage of the leader peptide; v) *lanRK*, which encodes a two-component regulatory system; vi) *lanFEG*, encoding a transporter enzyme responsible for the immunity of the producer bacteria to mature lantibiotic [13].

This are the most common genes in a general lantibiotic gene cluster.

Proceeding from left to right on the cluster represented in Fig 15, the genes in cinnamycin gene cluster are the following.

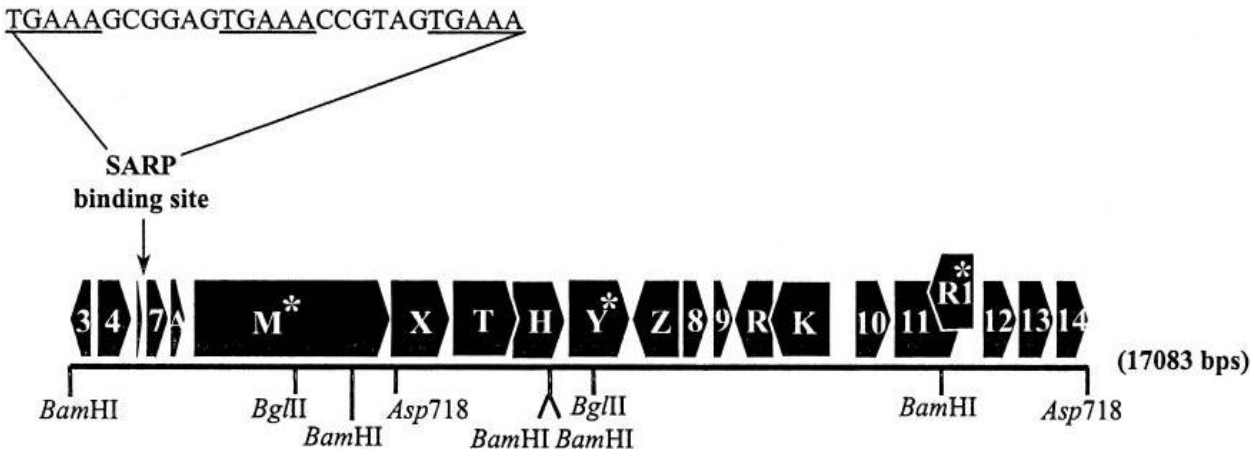


Fig15: Representation of the cinnamycin gene cluster

cinorf3: encodes an N-terminal signal peptide. It shares 78% homology with a membrane protein of *Streptomyces coelicolor* [15]

cinorf4: seems to encode a regulatory protein with a helix-turn-helix DNA-binding motif similar for the 67% to an *S.coelicolor* protein [15].

cinorf7: is a 360 bp long gene. It seems to encode the enzyme responsible to synthesize the lysinoalanine bridge, as reported in [12].

cinA: is the gene encoding the cinnamycin prepeptide. It has a length of 234 bp.

cinM: encodes the modification enzyme cinM, member of LanM family, thus responsible both for the dehydratation and cyclization of Ser and Thr residues. This gene contains the rare leucine codon TTA, for this reason in this project the cinM gene was modified to replace this rare codon. The gene has a length of 3276 bp [16].

cinX: this gene has a length of 968 bp. It encodes the protein for the hydroxylation of Asp [12].

cinT and *cinH*: cinT share homology with a transporter membrane protein of ABC family transporter. CinH seems to be similar to a protein involved in drug efflux and resistance, suggesting that it could have a role in the immunologic system [13].

cinY: encodes a product that may possess an N-terminal signal peptide, suggesting that it may be exported.

cinZ: has a length of 627 bp. The function in unknown.

cinorf8: has a length of 420 bp and seems to be a member of the CoA-binding protein. [13]

cinorf9: has a length of 321 bp. The encoded protein may have a possible N-terminal signal peptide suggesting that could be exported [13].

cinR and *cinK*: Encodes a two-component regulatory system and shares homology with two protein in the regulatory system of *Bacillus subtilis* [13].

cinorf10 and *cinorf11*: does not show significant similarity with other proteins.

cinR1: encodes a protein involved in *Streptomyces* regulatory system. [13].

cinorf12, *cinorf13* and *cinorf14*: does not show similarity with other proteins [13].

In conclusion, the genes involved in the modification and expression of cinnamycin are *cinA*, *cinM*, *cinX* and cinorf7. Thus these genes were the ones selected for the heterologous expression in *E.coli* described in chapter 3.

2.2.2 Modification machinery of cinnamycin

Only the genes *cinM*, *cinX* and *cinorf7* encode a modification enzyme in the entire gene cluster of the lantibiotic cinnamycin.
These modification enzymes act on the pre-peptide encoded by the gene *cinA*.
As in others lantibiotics the modification enzymes recognize the target molecule thanks to the presence of a leader peptide.
The cinnamycin leader peptide has a length of 59 a.a., whereas the core peptide is long 19 a.a.
The cleavage of the leader peptide is necessary to obtain the active molecule after the modification of the core peptide.

Mature cinnamycin has a globular structure, unlike the rod-like structure of nisin, and carries several kind of post-traslational modification.

Cinnamycin belongs to class II lantibiotics: this means that the introduction of Lan and MeLan rings depends on a single LanM enzyme, CinM.

Unlike nisin, the introduction of the thioether-bridge containing a.a is not the only modification.

Cinnamycin harbour an *erythro*-3-hydroxy-L.aspartic acid resulting from the hydroxylation of L-aspartate by CinX [12].

The last modification in cinnamycin core peptide is the lysinoalanine bridge, carried out by enzyme Orf7.

This modification is fundamental to confer antimicrobial activityto cinnamycin, because this bridge can bind the target of cinnamycin in the membrane of bacteria.

The two modifications described above are illustrated in figure 16.

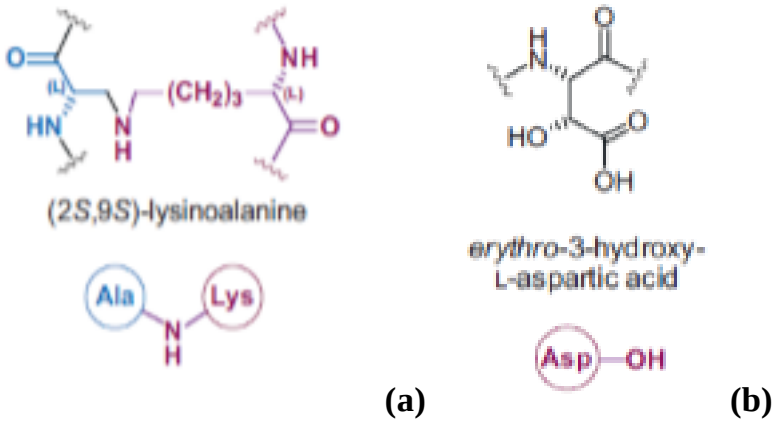


Fig 16: Representation of lisynoalanine bridge (a) and of hydroxylated aspartic acid (b)

The modification enzyme CinM requires the presence of a leader peptide to modify the substrate.

This class LanM enzyme is responsible for the dehydratation of Ser and Thr residues in cinnamycin core peptide.

In total four residues are modified, two serine at positions 4 and 6 and two threonine at positions 11 and 18.

This process leads to the formation of two dehydroalanine resulting from dehydration of Ser residues, and two dehydrobutyrines, resulting from dehydration of Thr residues.

LanM enzymes as CinM carry out also the cyclization of these dehydro-residues matching them with cysteine.

Unlike the case of nisin, where the cyclization is driven by the single enzyme nisC, in cinnamycin the Lan and MeLan rings are generated in a bidirectional way, such that the MeLan rings are formed using cysteines that are located N-terminal to the dehydrobutyrines, and the Lan bridge is formed using a cysteine located C-terminal to dehydroalanine with which it reacts [12].

In total the action of CinM introduces one lanthionine ring and two methillanthionine rings, and also an additional dehydroalanine involved in lysinoalanine bridge formation.

The action of CinM is summarized in the figure below.

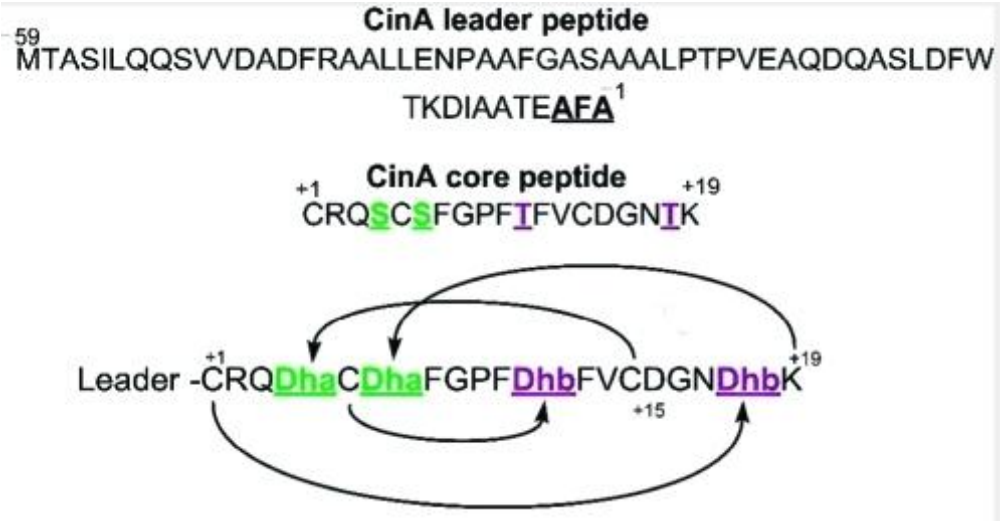


Fig 17: Schematic representation of the action of CinM. It is possible to note the different orientation of Lan and MeLan bridges. The link between the second Dha and the last lysine is the lysinoalanine bridge.

Analysis on cyclase enzymes (ex. Subtilin SpaC and nisin nisC) dimostrated that this protein contains zinc.

These results were confirmed from the anlaysis of the crystal structure of NisC.

The zinc seems to be involved in the formation of thioether rings [17]].

LanM enzyme share sequence homology at the C-terminus with LanC cyclases, including two Cys and one His that coordinate the Zn^{2+} in LanC proteins.

Thus, it was hypothesized that LanM cyclase domains might act through a similar zinc-involved mechanism.

This hypothesis was confirmed with a spectrophotometric assay using metallochromic indicator [12].

The peculiarity of CinM to drive the formation of Lan and MeLan bridge in a bidirectional manner makes this enzyme interesting from the Synthetic Biology point of view, because it could be used to introduce Lan and MeLan ring in a different manner.

CinX is the enzyme responsible for the hydroxylation of Asp in position 15 of core peptide.

It was demonstrated [12] that the action of CinX can happen both after or before the formation of Lan and Melan rings.

This means that there is not a specific order in the modification steps for cinnamycin.

Furthermore, it was demonstrated that CinX does not need the presence of a leader peptide to carry out its action, unlike most lantibiotics modification enzymes.

Orf7 is the protein encoded in the gene *cinorf7*. This small protein (13 kDa) carries out the formation of the lysinoalanine bridge, matching the last lysine of the core peptide with the dehydroalanine in position 4.

Activity test for cinnamycin missing lysinoalanine bridge gives negative result, showing that this modification is fundamental in the antimicrobial activity.

Therefore, *in vitro* modification of partially modified cinnamycin (modified by CinM and CinX) with Orf7 shows again negative result.

Thus, this could mean that Orf7 requires the presence of the other modification enzymes to carry out its own function, and not only a dehydratated serines and a lysine.

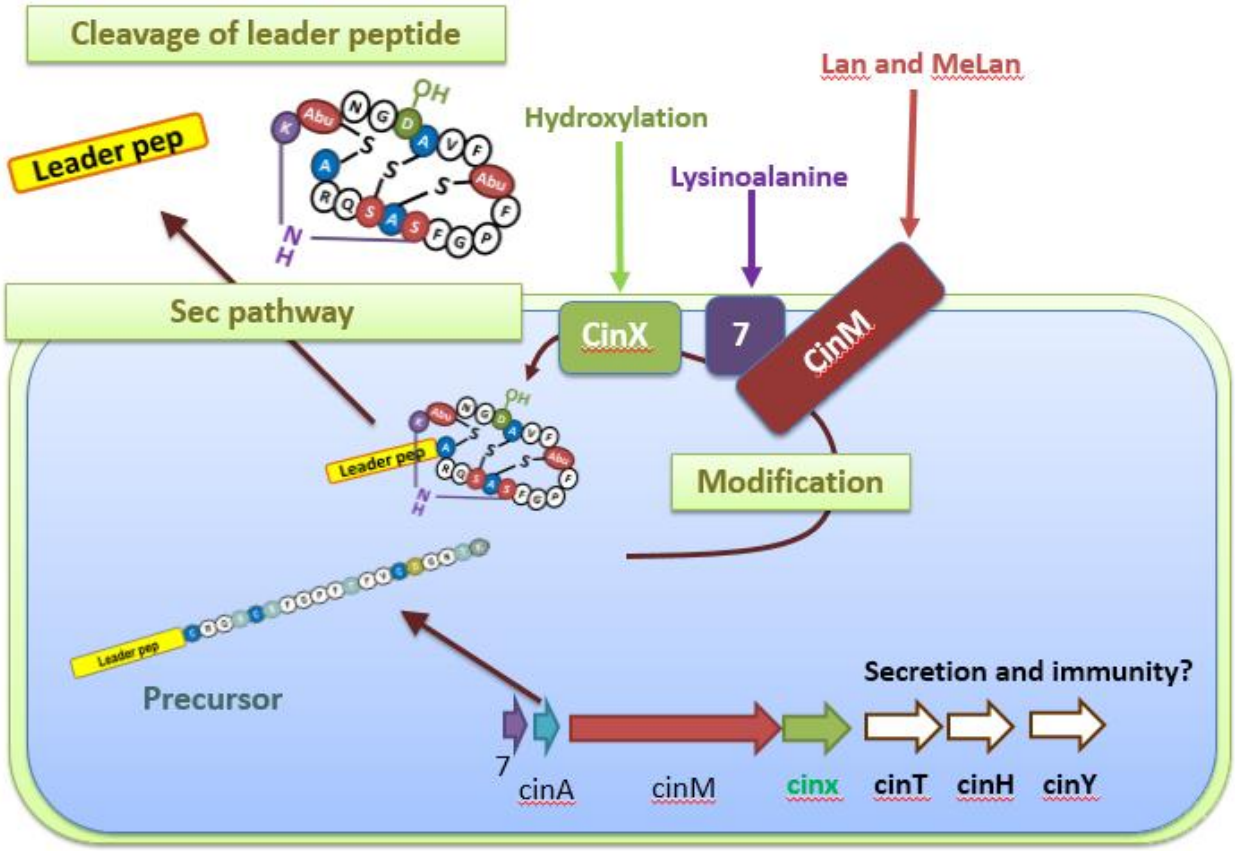


Fig 18: Schematic representation of the biosynthesis of cinnamycin by S.cinnamoneus.

2.2.3 Mode of action of cinnamycin

Unlike nisin and others class I lantibiotics, or class II lantibiotic mersacidin, cinnamycin does not target Lipid II, but interacts with membrane proteins such as phospholipase A2.

Phospholipase A2 has a major role in the release ofarachidonic acid from phospholipids in the cell membranes. Further oxidative metabolism of free

arachidonic acid leads to prostaglandins and leukotrienes. Several of these eicosanoids are potent mediators of diseases, such as inflammation and allergy [18].
In addition, A2 phospholipase is involved in atherosclerosis.
Inhibition of the enzymatic activity of phospholipase A2 may therefore be therapeutically beneficial.
Cinnamycin shows activity against Gram-positive bacteria as *Clostridium botilinum* and *Mycobacterium* [14].
This lantibiotic also causes the trans-bilayer phospholipid movement in the membrane of mammalian cells to access phosphotidylethanolamine (PE) residing predominantly in the inner side of the membrane [19].
PE is also a substrate for the phospholipase A2.
The lantibiotic duramycin, a close structural analogus of cinnamycin, promotes chloride secretion in lung ephitelial cells by binding to PE.
This secretion lead to a mucus clearance from the lung, as result this compound I is in phase II clinical trials for the treatment of cystic fibrosis [12].
For this reason cinnamycin has been suggested as an alternative treatment for atherosclerosis, because can inhibit phospholipase A2 by binding its substrate PE.

CHAPTER 3: Material & Methods

This research project can be divided in three modules.
The first one involves the heterologous expression of cinnamycin in *E. coli.*
Ti do that was necessary to introduce in the bacteria the gene encoding for the LanA peptide and the genes encoding for the modification enzymes.
The peptide was expressed by this heterologous system, purified and its activity

tested.

The second module involves the modification of nisin fused with a chimeric leader peptide formed by the fusion of cinnamycin leader peptide and nisin leader peptide. Nisin was modified by nisin system (NisBC) and the leader peptide was cleaved by NisP.

The aim of this module was to check if the presence of the cinnamycin leader peptide upstream nisin leader could affect nisin modification and activity.

The last part of the project aimsto prove the modification of nisin by a single class LanM enzyme, CinM.

Therefore, the modification of CinM on nisin was tested with the previous chimeric leader peptide.

Figure 19 illustrates a summary of the project.

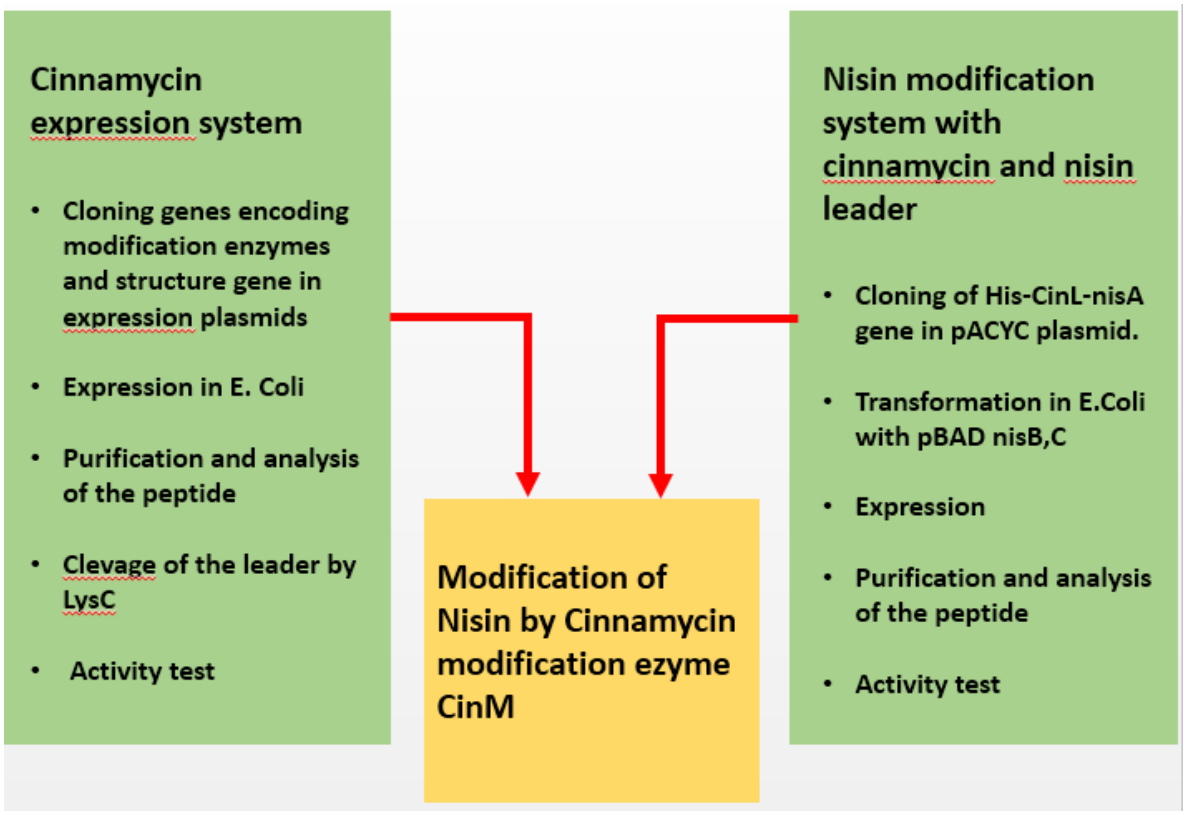


Figure 19: Outline of the research project.

3.1 Heterologous expression of cinnamycin in *E. coli*

The heterologous expression of cinnamycin in *E.coli*involves several steps.
Firstit was necessary to select the correct genes for the expression of the cinnamycin pre-peptide and for the expression of the modification enzymes.
In particular it was necessary to use a mutated variant of the structure gene *cinA* to bypass the absence of a dedicated protease for the cleavage of the leader peptide in cinnamycin gene cluster.
Once the genes were selected, the next step was the cloning of these genes in plasmid vectors.
Then the plasmid carrying the genes were introduced in the bacteria by electroporation.
After that, fresh-transformed cells were used to start a culture and obtain the expression of the cinnamycin.
After that, the cells werelysated and the peptide molecules purified.
A digestion step with protease LysC was necessary for the cleavage of the leader peptide.
Finally, the activity of fully modified cinnamycin was tested against Gram-positive bacteria *B.subtilis* and *M.luteus.*

3.1.1 Cloning of genes involved in expression and modification

To obtain cinnamycin expression are necessary the structure gene *cinA*, encoding the cinnamycin pre-petide, and the genes encoding the modification enzymes CinM, CinX and Orf7.
The aim of these cloning steps is to obtain a pACYC plasmid that harbors the two modification enzymes *orf7* and *cinX*, and a pRSF plasmid with *cinA* and *cinM.*

The genes used for this project are not the wild type genes, but are modified to increase the expression level for *E.coli.*

This genes were provided by Lanthiopharma, a company which collaborate with Molgen research group of the Groningen University.

In particular,*cinM*was modified to replace the rare codon "TTA" that encodes for leucine with a more common codon. In this way the expression of CinM is optimized for an heterologous producer.

The ribosome binding site (RBS) or *orf7*was removedbecause an RBS was already present in the first cloning site of pACYC plasmid, in this way a double RBS situation was prevented.

The modified Orf7 is called Orf7b.

The most important modification involves the structure gene *cinA.* This gene encodes for the cinnamycin pre-peptide, formed by cinnamycin leader peptide and core peptide.

As reported in chapter 2 there is not a specific protease for the cleavage of the leader peptide in cinnamycin gene cluster. It seems that in the natural producer strain this is obtained by peptidase of the general secretory pathway *Sec.*

In *E.coli*the cleavage cannot be obtained in this way, so itis necessary to use a specific protease.

For this reason,*cinA* was mutated to replace the alanine at the last position of the leader peptide (position -1) with a lysine. After this mutation the gene is named *cinA(A-1K).*

In this way is possible to obtain the cleavage by protease LysC that can break the peptide bond at the C-terminal side of lysine.

Only two lysine are present In cinnamycin sequence after this mutation, one at the last position of the leader peptide and the second at the last position of the core peptide.

Therefore, using LysC lead to a single cut that allow the separation of the leader peptide from the core peptide.

The genes were cloned into a pRSF and a pACYC plasmid.

Both these plasmids possess two separate cloning site, each one controlled by an IPTG inducible promoter.

For this reason a gene encoding lactose inhibitor is present in both plasmids under the regulation of a constitutive promoter.

Kanamycin resistance gene is present in pRSF plasmid, whereas a gene encoding for chloramphenicol resistance is present in pACYC. A constitutive promoter regulates these two genes.

These two plasmids possess the same origin of replication (ori), this mean the will be present in the same number inside the host.

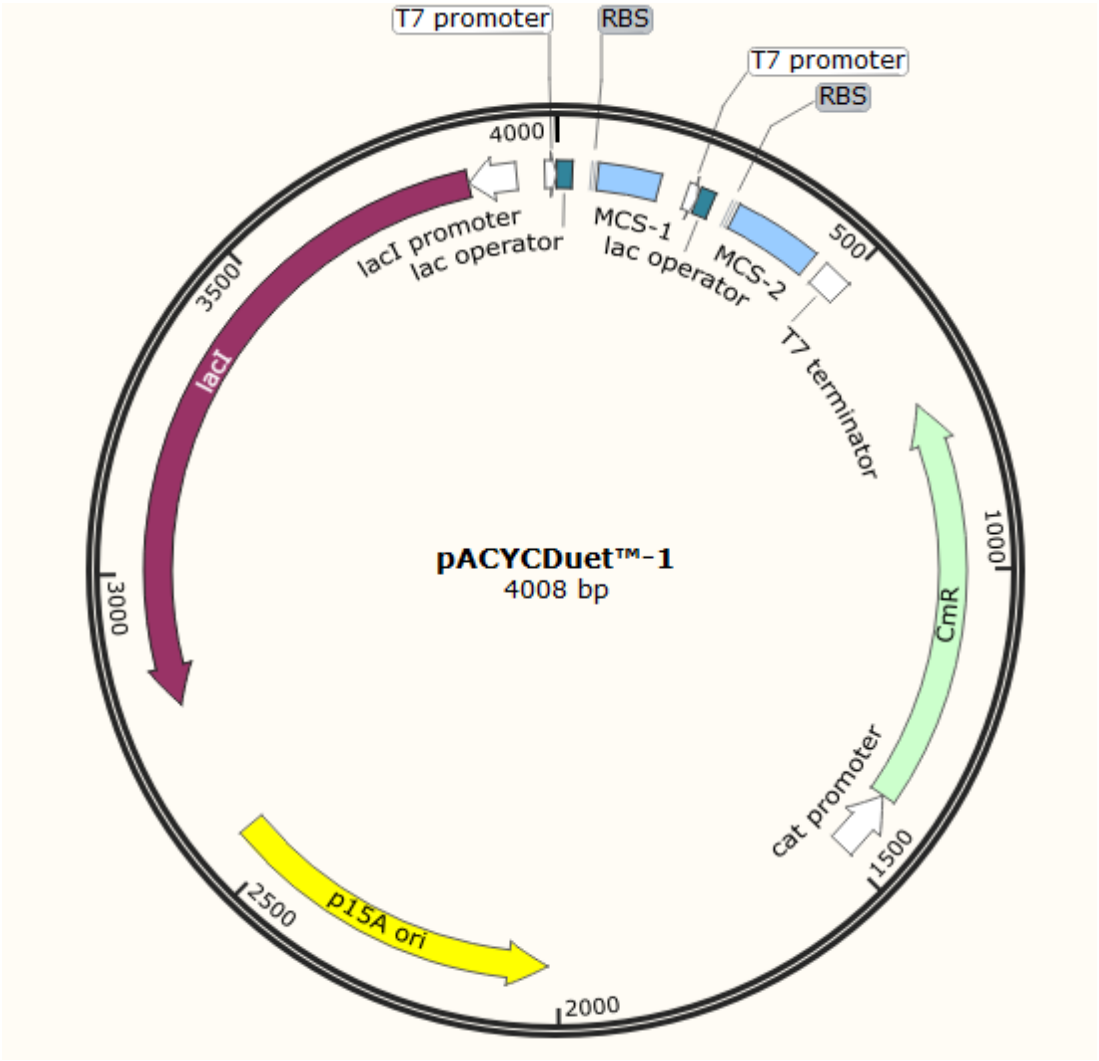
The first cloning site of the pRF plasmid possess a his-tag sequence.

Therefore, cloning of a gene downstream this sequence to obtain the protein expressed in the gene with a sequence of histidine at N-teeminal side is possible.

In this case the gene in the first cloning site of the pRSF plasmid was *cinA(A-1K)*.

The his-tag (formed by six histidine residues) is necessary to detect the expression of the pre-peptide and for its purification by affinity chromatography.

The structure of the two plasmids used in this project are reported tIn the figure below.


T7 promoter
RBS
T7 promoter
RBS
4000
lacI promoter
lac operator
MCS-1
lac operator
MCS-2
500
T7 terminator
3500
lacI
1000
pACYCDuet™-1
4008 bp
3000
CmR
cat promoter
1500
p15A ori
2500
2000

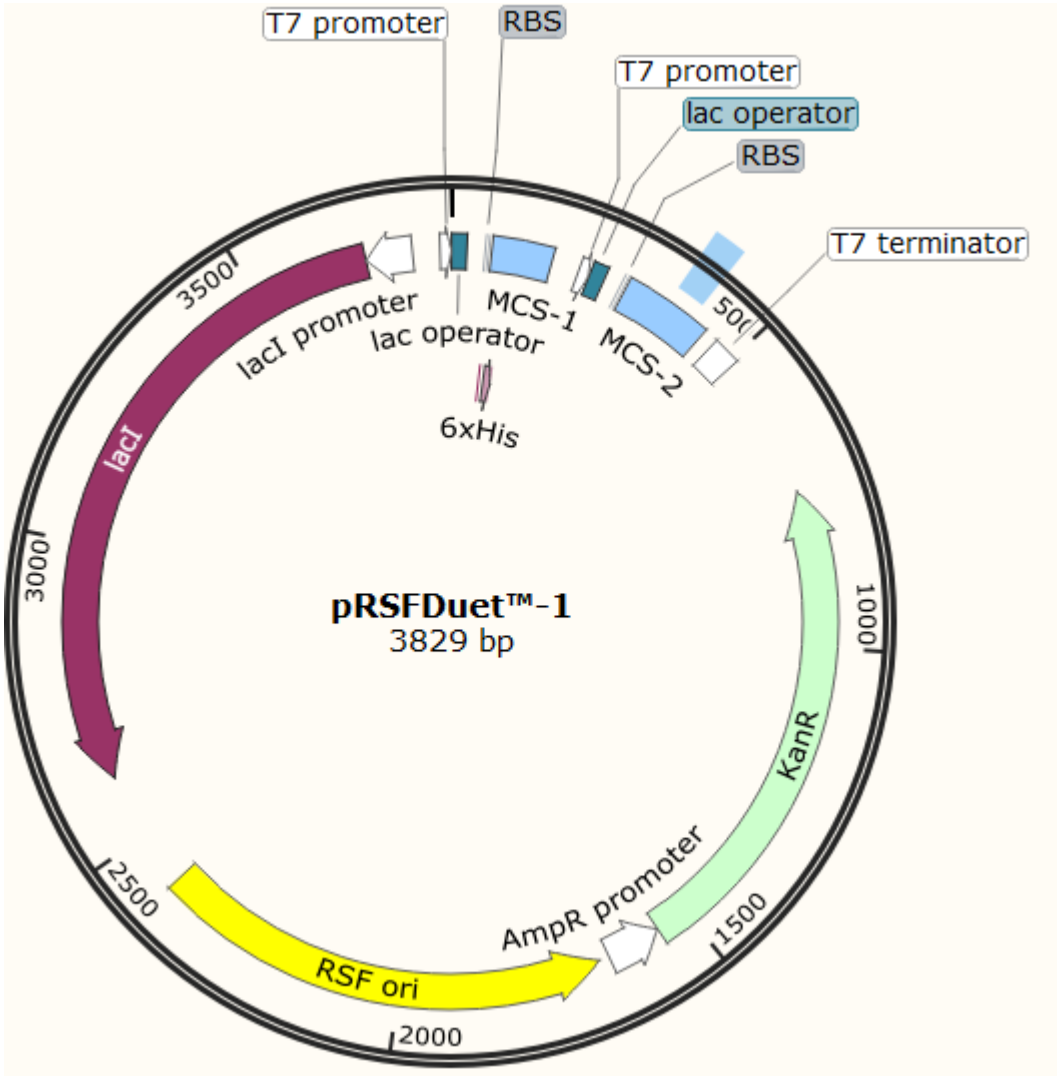


Fig 20: Structure of pACYC and pRSF plasmids

The final expression system consist in a pACYC plasmid carrying in the first cloning site *orf7b* and in the second cloning site *cinX* (pACYC *orf7b-cinX*), and a pRSF plasmid carrying *cinA(A-1K)* in the first cloning site and *cinM* in the second (pRSF *cinA(A-1K)-cinM*).

The project started from a pACYC *orf7b* and a pRSF *cinA(A-1K)* already built by Lanthiopharma.

To obtain the final expression system the following steps were performed:

- PCR to amplify the genes *cinX* and *cinM*

- Digestion
- Ligation
- Transformation
- Colony PCR
- Sequencing

The first step was to amplify the gene by PCR.

To do that it was necessary to design primers to amplify *cinX*, whereas for *cinM* case was possible to use the general primers for pACYC plasmid.

In fact, a *cinM* gene was contained in the second cloning site of a pACYC plasmid,thus was possible to amplify it using the couple of primers pACYC 2nd-Fw and pACYC 2nd-RV, that allow to amplify the second cloning site of the plasmid when used together in a PCR step. (Primers sequences are reported in supporting information section) [SI 1].

CinX was contained in a pET plasmid, so it was necessary to design the correct primers to amplify the gene.

The primers were designed using the software Clone Manager.

PCR reaction was carried out using phusion polymerase enzyme by Thermo scientific, and result analyzed by electrophoresis.

According to Clone Manager, PCR product for *cinX* case is 1030 bp, whereas for *cinM* case is 3456 bp.

These results are reported below.

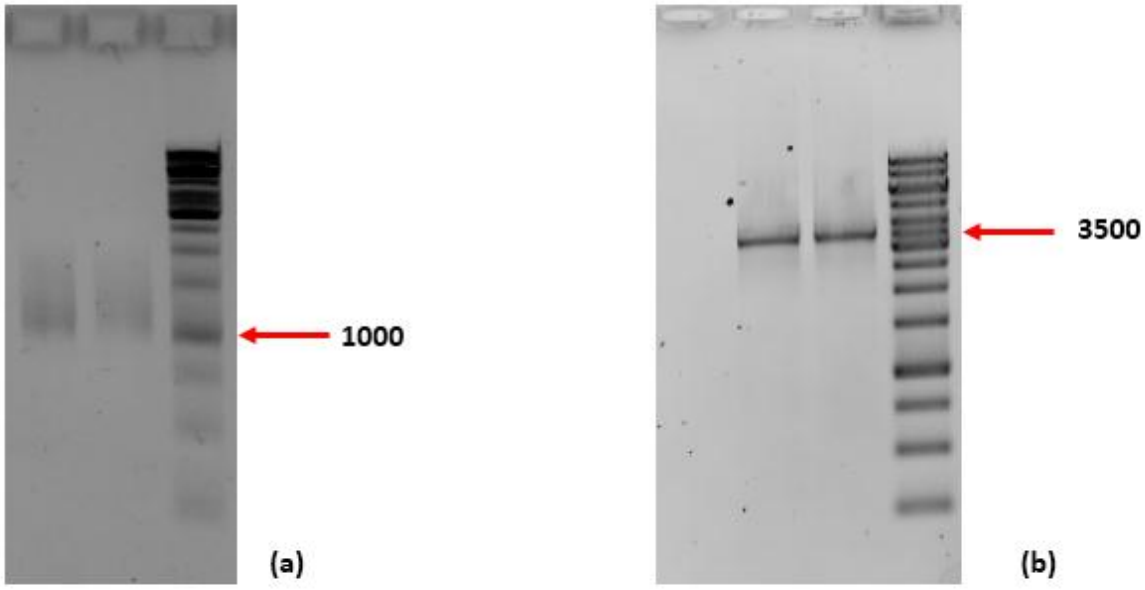


Fig 21: Analysis by electrophoresis of (a) PCR products for cinX case, (b) PCR products for cinM case.

The PCR product contains the gene sequence plus additional DNA, whichharbors restriction sites for digestion enzymes.

Using the correct combination of digestions enzyme it is possible to introduce the gene sequence into the plasmid.

For *cinM* PCR product was possible to cut the DNA sequence by enzymes NdeI and KpnI (the same enzyme cut the pRSF plasmid).

This leaded to an open plasmid that can harbor the gene in its second cloning site.

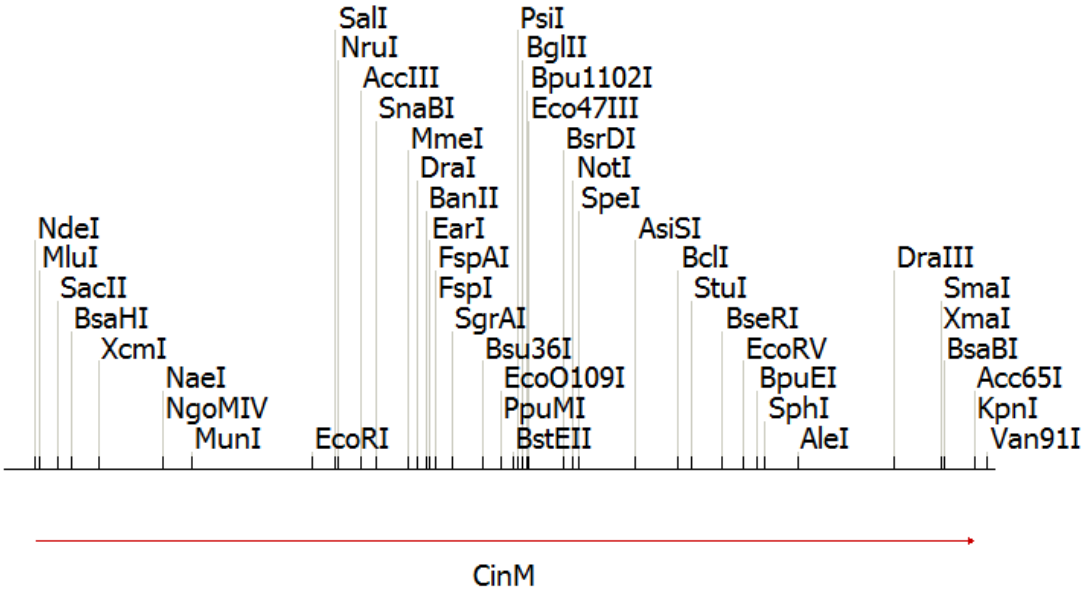


Fig 22: cinM PCR product. The restriction sites are shown. The upstream and downstream enzymes NdeI and KpnI were used to cut this sequence.

For the *cinX* case the restriction enzymes NdeI and XhoI were used.

The same enzymes were used to cut the pACYC plasmid.

For digestion steps were used FastDigest enzymes by ThermoScientific.

The results were analyzed by electrophoresis, and the digestion reactions were then purified to separate the DNA sequences and perform the ligation step.

In the ligation step opened plasmids and gene sequences were mixed making the correct couples (pACYC *orf7b* + *cinX*, pRSF *cinA(A-1K)* + *cinM*), an then ligation reaction was performed overnight at 4°C using T4 DNA ligase enzyme.

Ligation products were then used for transformation.

The plasmids were separately introduced in *E.coli*TOP10 strain by electroporation, and plated in Petri dishes with LB (Luria-Bertani) medium with the correct antibiotic.

The plates were left in incubation overnight at 37°C and the day after colonies were used for colony PCR (CPCR) step.

Colony PCR (CPCR) technique allow to check if cells of a certain colony possess the plasmid with the insert in it. In this way is possible to exclude cells with the plasmid that do not carry the insert.

CPCR is similar to classic PCR with the difference that instead of DNA template is used a colony.

Therefore is necessary a longer step at 98 °C to break the cell wall and make available the DNA to primers and polymerase.

Using the correct couple of primers is possible amplify a region of the plasmid contained in the cells.

CPCR allow to amplify only fragment shorter than 1000 bp.

This is not a problem for the *cinX* case, because cinX is 980 bp, so it was possible used the previous primers.

For *cinM* case was necessary design two specific primers called cinM-Fw and cinM-Rv.

Coupling this two primers with pRSF-2nd-Rv and pRSF 2nd-Fw was possible amplify the 3' and 5' part of cinM.

The products using these primers couples are around 300 bp.

CPCR results are available in supporting information [SI 2].

Using the colonies that gave good result in the CPCR step a culture was started and cells left growth overnight to amplify the plasmids.

Then the cells was treated to obtain plasmids that were sent to sequencing.

Sequencing operation was necessary to confirm the correct nucleotidic sequence of the genes carried on the plasmids.

Once the sequencing confirmed the correctness of the gene sequences, plasmids were used together in a transformation step, using *E.coli* strain BL21.

This strain is optimized for the expression of the genes carried in the plasmids.

After this transformation the cells contains the entire heterologous expression system.

The expression system is illustrated in the figure below

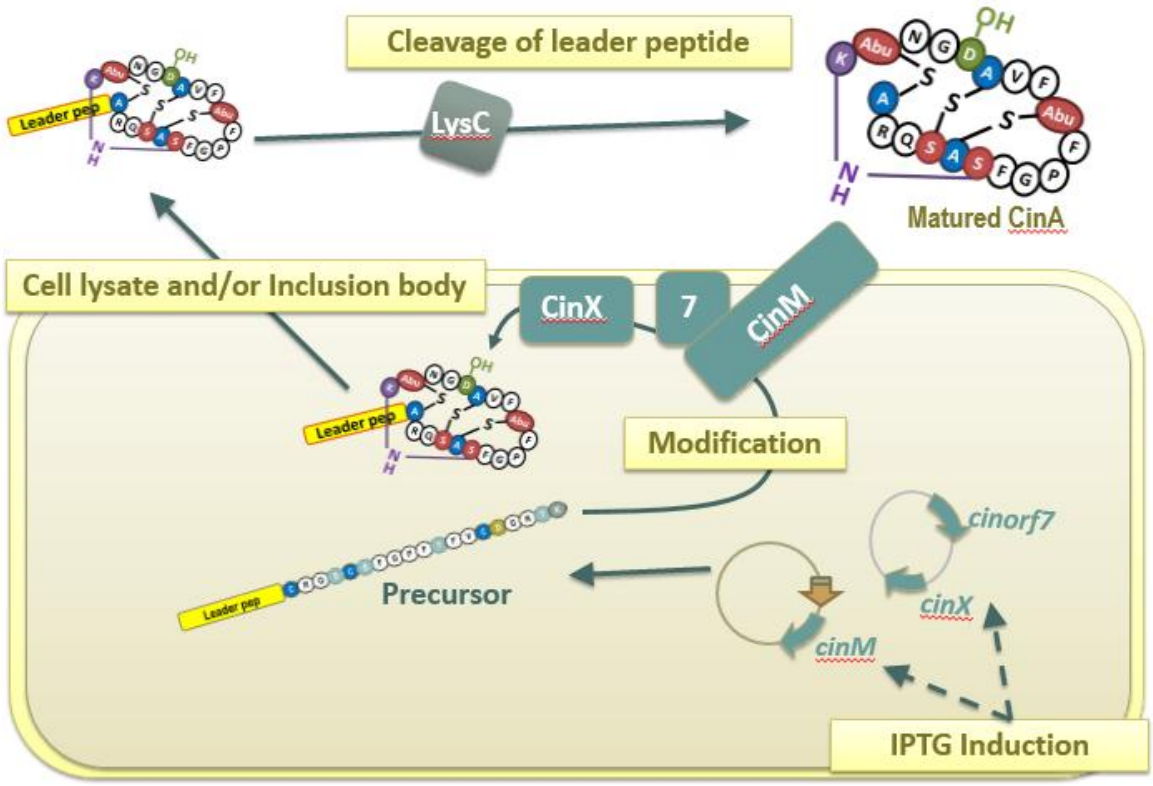


Fig 23: Heterologous expression system for cinnamycin.

3.1.2 Expression and detection of the cinnamycin peptide.

Cells containing the two-plasmid system were inoculated in 2 l of LB medium with 5 µg/ml of chloramphenicol and 25 µg/ml of kanamycin.

Cells were grown at 37°C until an OD value between 0.6-0.8 at 600nm was reached.

At this point 1mM IPTG was added to induce the transcription of the genes carried on the two plasmids, this leaded to the expression of cinnamycin pre-peptide and modification enzymes.

Culture was left in incubation overnight at room temperature with shacking.

As shown in the figure above, *E.coli* heterologous expression system for cinnamycin lacks of a transportation system to export the peptide in the extracellular environment.

Therefore, to break the cells and analyze the cell lysate was necessary to detect the presence of the molecule.

In addition, from literature is known that overexpressed protein could be stored in inclusion bodies, protein aggregate made by the cells.

First, the cellular pellet was harvested and resuspended in a phosphate buffer called Start Buffer [SI 3].

Then the cells were broken by sonication and centrifuged [27].

The supernatant, that is the cell lysate, was kept and named "1st Start Buffer".

The pellet was then resuspended again in Start Buffer and centrifuged.

This step was necessary to remove all the residues of medium from the pellet.

Then the pellet, that contains inclusion bodies, was treated with an8M Urea Denaturing Buffer [SI 3].

Urea is a strong denaturing agent that allow to solubilize the inclusion bodies.

The sample was centrifuged again and the supernatant kept and named "Inclusion Bodies".

Samples were then analyzed by SDS-PAGE and Western blot to check the expression of modification enzymes and cinnamycin.

SDS-PAGE confirmed the expression of all the modification enzymes, whereas Western Blot with anti-his-tag antibodies confirmed the presence of cinnamycin pre-peptide [SI 4].

After these steps was necessary purify the peptide by Affinity Chromatography, before check the activity of cinnamycin.

Affinity Chromatogpahy allow the isolation of the peptide exploiting the presence of the histidine contained in his-tag sequence.

These residues can bind the nickel contained in a column in which the sample is load, leading to the separation of the target molecule from the others compounds.

The target molecule is then eluted using an imidazole containing buffer that can bind hisitidine and therefore drag the molecule out of the column.

Affinity Chromatography was performed by NGC machine by BioRad using a GE HealthCare nickel coloumn.

Buffers used for this step are described in [SI 5] .

NGC machine has a spectrophotometer at the exit of the column that allow checkingof the elution of compounds.

The profile for the inculsion bodies sample is shown below.

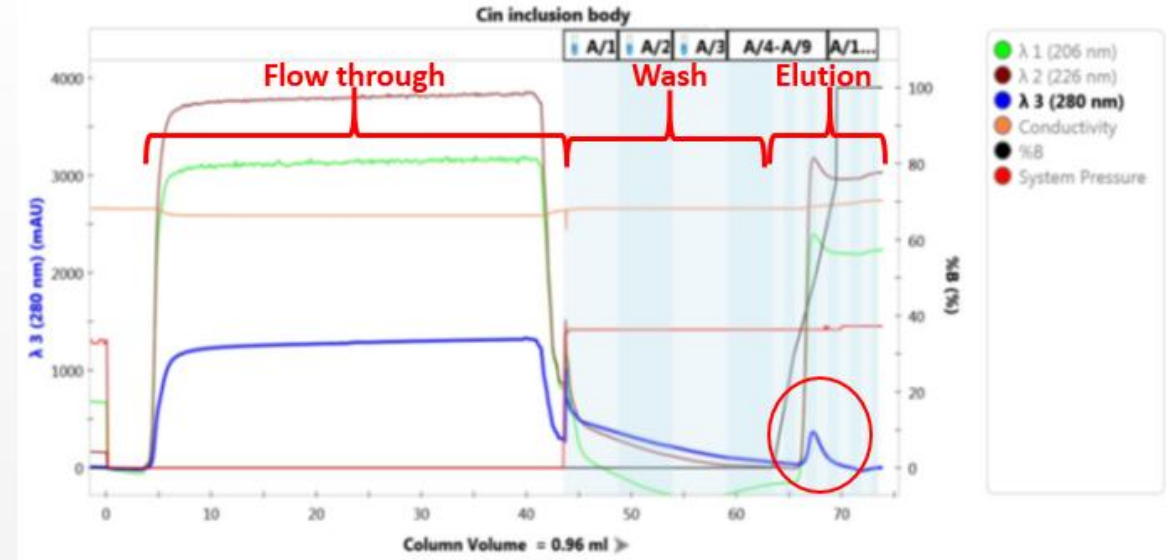


Fig 24: NGC Affinithy Chromatography profile for inclusion bodies sample. The peak labeled with a red circle represent the elution of the target molecule.

The blue line represent the absorbance level at 280 nm.

At this wavelength tryptophan, show a high absorbance.

Checking the leader peptide sequence a tryptophan residue was found.

This mean that the peak labeled with the red circle represent the elution of the target molecule.
The result was further confirmed by western blot performed for the elution fractions on which the peak was detected.
As negative control, Affinity Chromatography was performed also for sample after inclusion bodies treatment obtained from a culture that was not induced by IPTG.
The profile for this sample [SI 6] did not show any peak, meaning that there is no production of cinnamycin.
Western blot confirm this result.
This mean that IPTG inducible promoters tightly regulate this heterologous expression system.

3.1.3 Purification and mass spectrometry analysis

After affinity chromatography, the sample was analyzed by mass spectrometry.
Mass spectrometry allow detection of the molecular weights of the compounds of a solution.
In this case was used to detect if the peptide is actually present by checking the know molecular weight.
Modified/unmodified cinnamycin with the leader peptide and his-tag still attached has a molecular weight of approx. 9800 Da.
Unfortunately, for this sample the MALDI-ToF MS (Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry) analysis did not show clear result.
A non-clear peak was detected around 9600-10100 Da, highlighting the need of further purification.

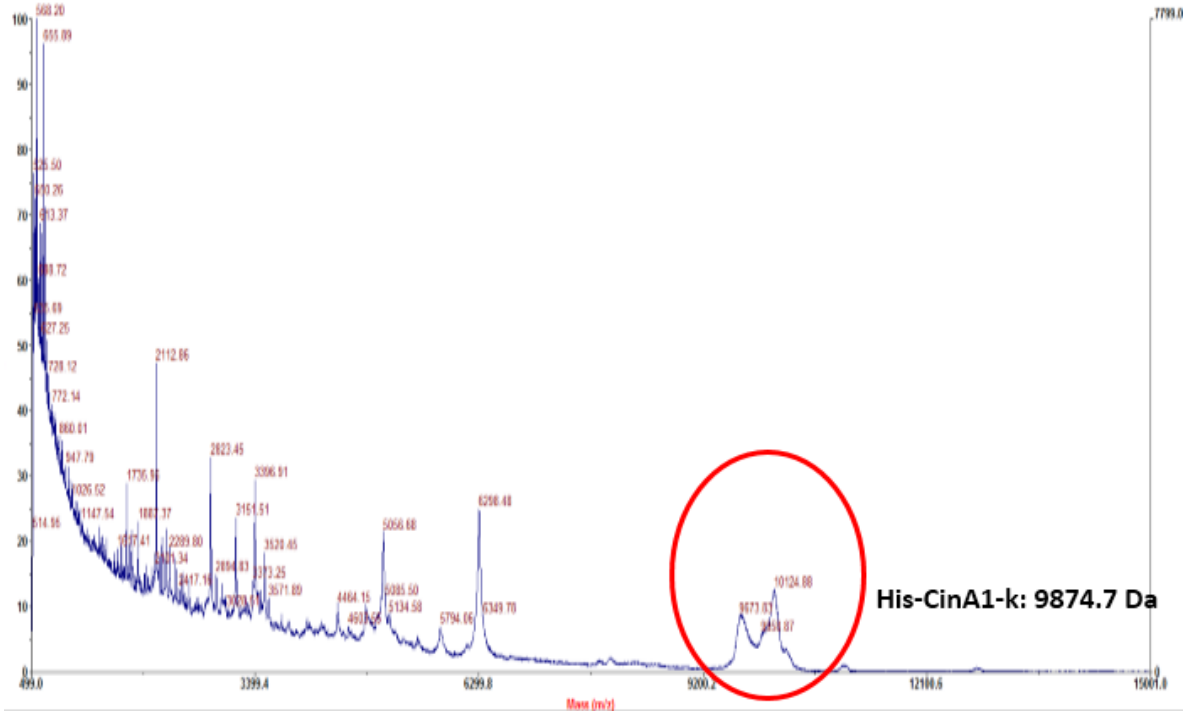


Figure 25: MALDI-Tof profile of inclusion bodies sample after NGC. The red circle represent the area of interest in which the peak representing the cinamycin peptide should be present. The indication of the theoretical molecular weight of the target molecule is reported.

Therefore, to obtain a pure peptide a HPLC was performed.

HPLC (High Performance Liquid Chromatography)allows to separate the several compounds of a solution exploiting the different affinity of each compounds between a “stationary solution” and a “mobile solution”.

It relies on pumps to pass a pressurized liquid solution containing the sample mixture through a column.Each component in the sample interacts slightly differently with an elution solution that flows through the column, causing different flow rates for the different components and leading to the separation of the components as they flow out the column.

A spectrophotometer positioned outside the column consents to detect the eluted compounds.

After this step several samples, each representing the elution of a different compound, were obtained

The HPLC output profile for the previous sample is reported.

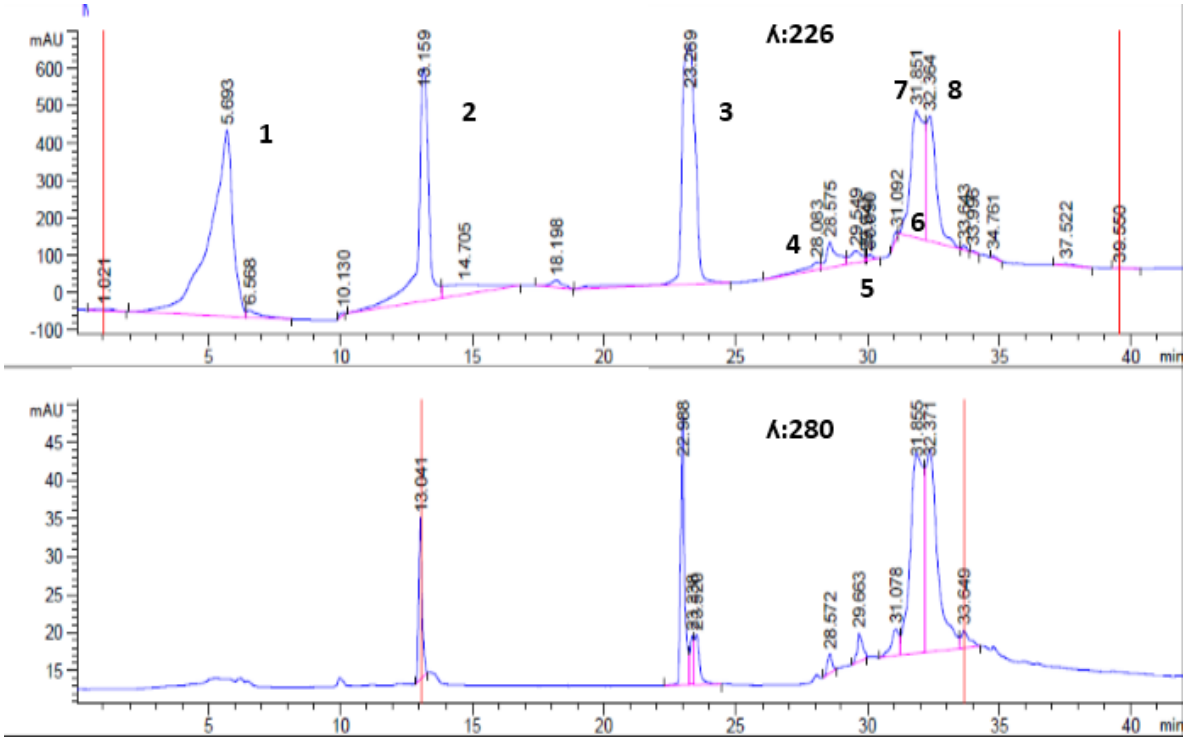


Fig 26: HPLC profile. Each peak represent the elution of a compound of the sample solution. The wave length used for the detection are reported. Each peak was labeled with a number.

In total eight samples were obtained after this purification step.

To check which of these samples contains the target molecule an additional MALDI-ToF analysis was performed.

Analysis of the samples number eight shown a very clear peak, even if the detected molecular weight did not fit with the theoretical molecular weight of modified cinnamycin with leader and his-tag, but it is smaller.

This could mean that also the leader peptide is dehydrated.

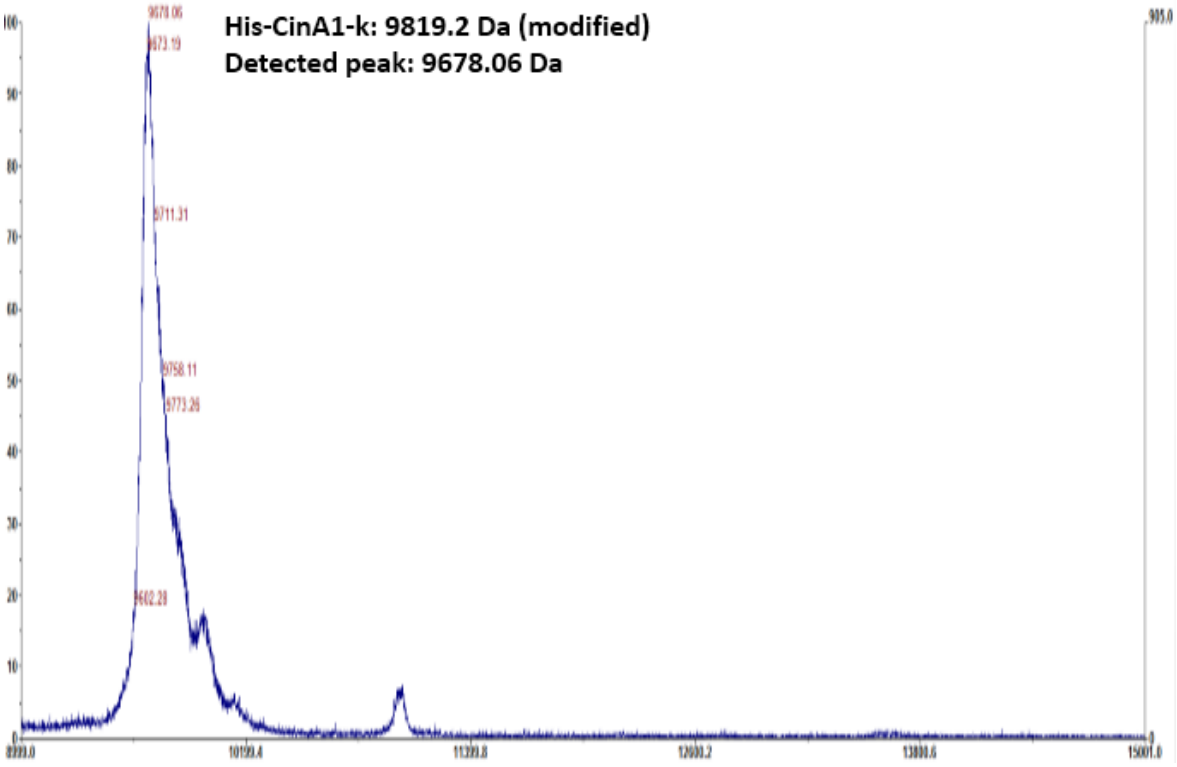


Figure 27: MALDI-ToF profile for sample number 8 obtained from HPLC.
The detected molecular weight and the theoretical molecular weight of the target molecule without modification are indicated.

The same sample was also digested by protease LysC, overnight at 37°C.

Before doing that it was necessary to lyophilize the sample and resuspend the powder in Tris-HCl Buffer.

After digestion by LysC the sample was again analyzed by MALDI-ToF.

This time the peak, representing only the modified core peptide fitted with the theoretical molecular weight.

The modification necessary to obtain cinnamycin that modify the molecular weight are four dehydration and ahydroxylation.

To consider a dehydration is necessary subtract from the molecular weight of the core peptide (2097,3 Da) the molecular weight of an oxygen (15.99 Da) and two hydrogen (1 Da).

The hydroxylation is taken into account by adding the molecular weight of an oxygen and of and hydrogen.

The molecular weight of fully modified cinnamycin is 2041,3 Da.

This result show that the molecule contained in the sample is probably fully modified cinnamycin.

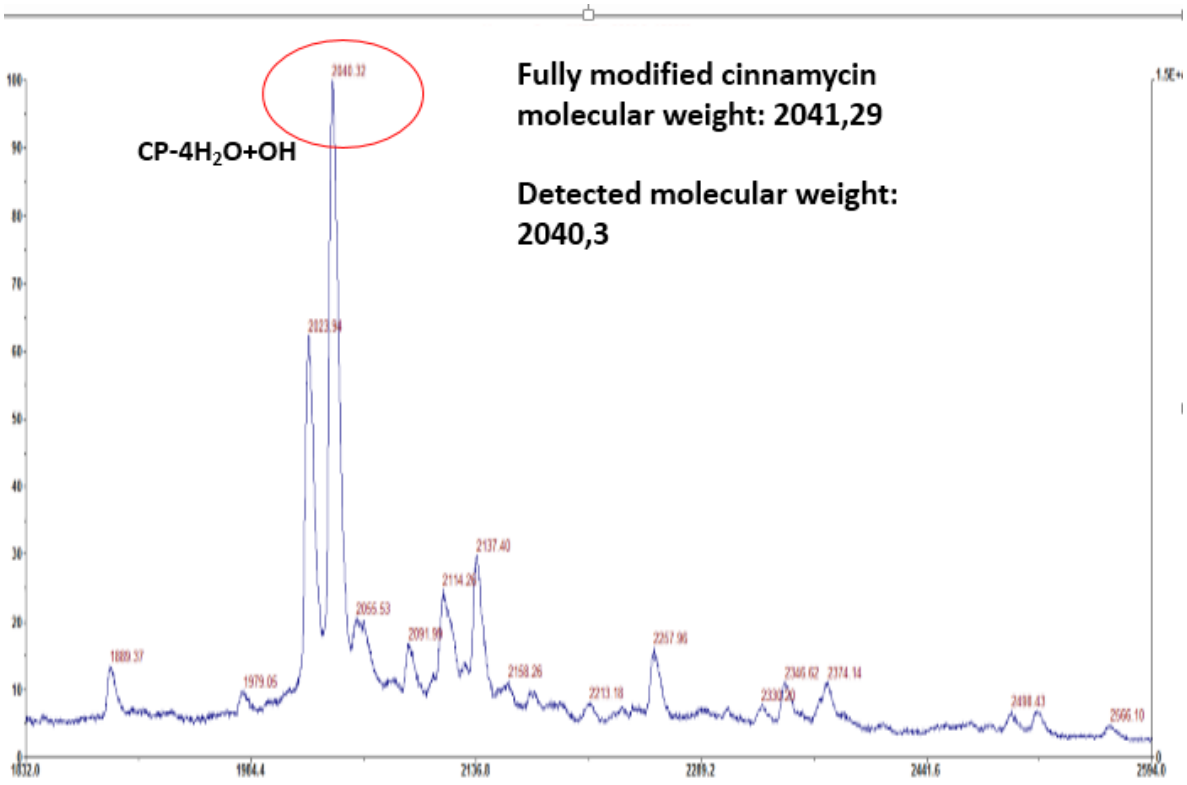


Figure 28: MALDI-ToF analysis after digestion by LysC. The theoretical molecular weight of fully modified cinnamycin and the detected molecular weight are reported.
Also the modification on the copre peptide are reported. CP: core peptide

To test this, an activity test was performed.

In the activity test an indicator strain (the strain that represent the target for the molecule) is add to a mixture of LB and agar.

The concentration of agar shouldn't be too high to allow the diffusion of the antimicrobial molecule.

The sample was spotted on the plate, with higher and lower concentration.

In addition, pure nisin was used as positive control.

The antimicrobial activity lead to area in which the growth of the indicator strain is inhibit, meaning that the molecule possess activity against the indicator strain.
This is the case of cinnamycin, which shown activity against *Bacillus subtilis* and *Micrococcus luteus*as reported in the figure below.
In addition, an activity test with samples before the HPLC treatment was performed [SI 7].
The result was negative and this confirms that the molecule do not have activity before the cleavage of the leader peptide, as expected.

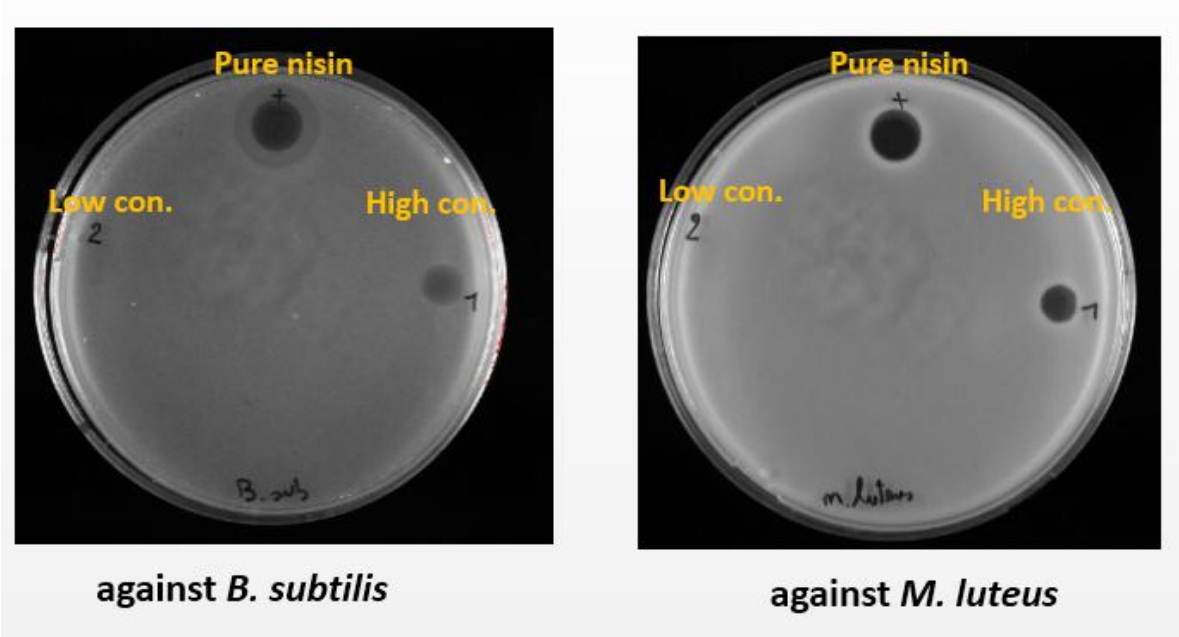


Figure 29: Activity test for heterologous expressed cinnamycin.

All this results show that cinnamycin and its modification enzymes can be expressed in *E. coli*and also that production of active cinnamycin by heterologous producer is possible.
In particular it was confirmed that the modification enzyme CinM can work in *E.coli*, and for that reason was tested for modification of nisin.

3.2 Test of nisin modification system in *E.coli* on nisin with chimeric leader peptide

The second part of the project involves the modification of nisin, modified to obtain a chimeric leader peptide formed by the fusion of cinnamycin leader and nisin leader, by the nisin modification enzymes NisB and NisC.
The aim of this experiment is to check if the presence of the cinnamycin leader peptide could affect the nisin modification, activity or expression in *E.coli*.
Once confirmed that the presence of the cinnamycin leader does not affect nisin production system the next step was tested the modification of nisin by CinM, as described in paragraph 3.3.

3.2.1 Design and cloning of the synthetic design encoding nisin with chimeric leader peptide.

The first step was the design of the synthetic gene encoding nisin with a chimeric leader peptide made by the fusion of cinnamycin leader and nisin leader peptide.
The synthetic gene was designed by Clone Manager, and includes the following modules: i) a his-tag region necessary for detection and purification of the peptidic molecule; ii) the DNA sequence encoding the leader peptide of cinnamycin; iii) the DNA sequence encoding for nisin leader peptide; iv) sequence encoding nisin core peptide.
In addition, upstream and downstream of this gene were positioned the restriction site for NcoI and HndIII restriction ezymes.
In total, the sequence is 419 bp long.
A representation of the modules of the synthetic gene is reported below

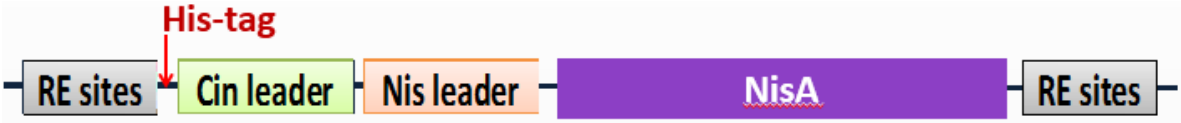


Fig 30: schematic representation of the synthetic gene

This gene was cloned in the first cloning site of a pACYC plasmid, therefore its expression was regulated by an IPTG inducible promoter.

The cloning of this plasmid involved the same steps of the plasmids construction in the previous paragraph:

- PCR to amplify the synthetic gene using two designed primers [SI 1]
- Digestion of the synthetic gene and of the empty pACYC plasmid by NcoI and HindIII
- Ligation
- Transformation in *E.coliTOP*10
- CPCR and Sequencing

The other part of the expression system is constituted by a pBAD plasmid carryng the two genes *nisB* and *nisC*, in two separate cloning sites.

This plasmid possess the resistance gene against ampicillin (Amp), and two arabinose inducible promoters.

The two enzymes encoded in this plasmid have the task to modify the peptide encoded in the synthetic gene to obtain active nisin.

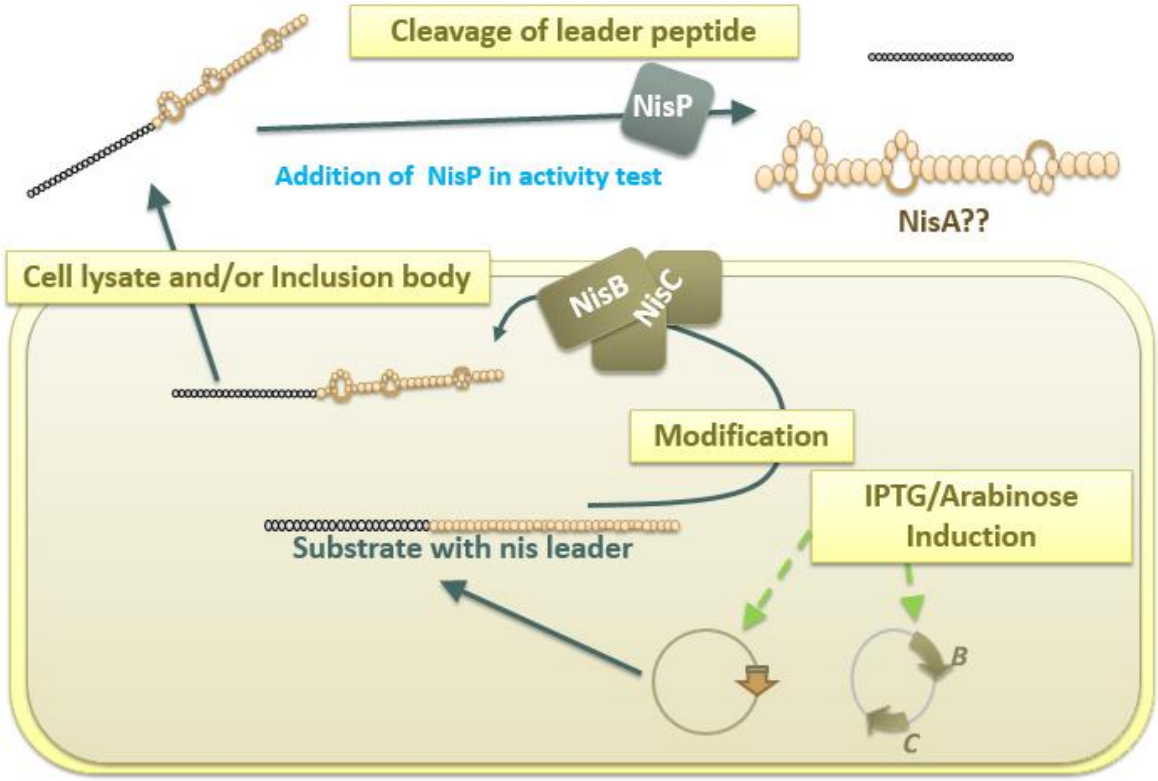


Fig 31: Representation of the two-plasmid system for the modification of nisin with chimeric peptide.

3.2.2 Expression and activity test

The two plasmids were introduced inside the cells (*E.coli* BL21) by electroporation. Transformed cells were used to inoculate a culture of 200 ml of LB with addition of chloramphenicol 5 µg/ml and ampicillin 100 µg/ml.

Cells were left grown until the OD measured at 600 nm reached a value of 0.6-0.8. Then transcription was induced by addition of 1mM IPTG and 0.05% arabinose. Culture was left in incubation overnight at room temperature with shacking.

As for the case of cinnamycin expression system, in this expression system there is not a transportator for the peptide, so it was necessary to break the cells and analyze cells lysate and inclusion bodies.

Cells were treated as described before and samples from cell lysate and inclusion bodies were analyze by SDS-PAGE and Western blot.

The peptide produced by this system has a molecular weight of approx. 13 kDa.

An intense band was detected in the SDS-PAGE for the inclusion bodies sample.

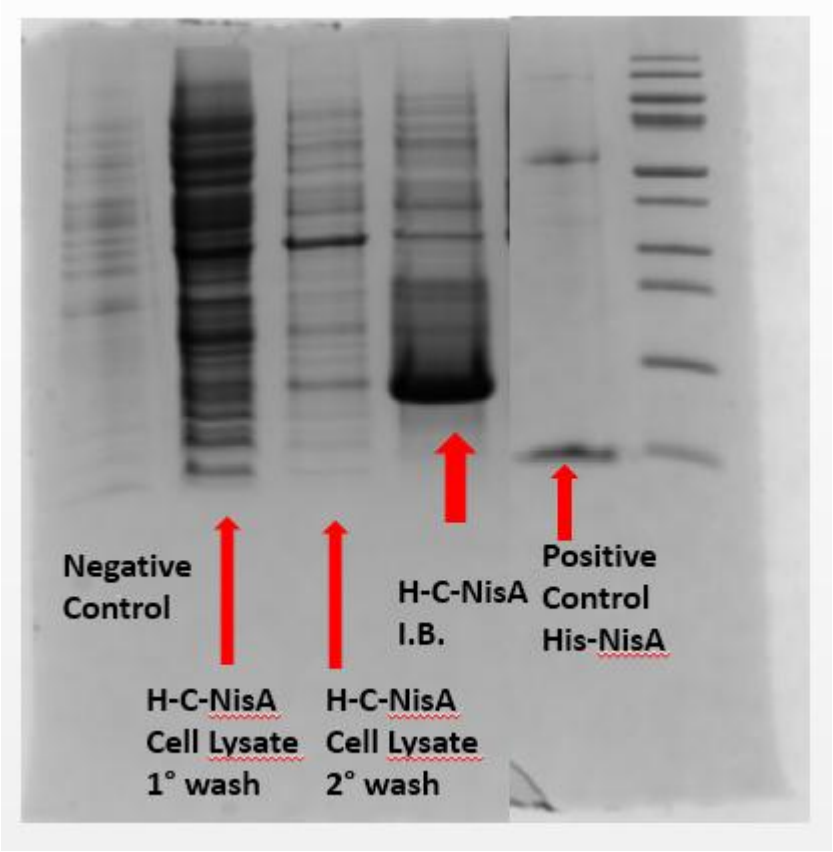


Fig 32: SDS-PAGE for cell lysate and inclusion bodies samples.

It is possible to obtain the cleavage of the leader peptide by NisP enzyme for this peptide, named H-CinL-nisA.

So the activity can be checked without the previous purification steps.

To do that it was necessary to perform an activity test with a particular indicator strain.

The indicator strain is *L.lactis* nz9000 modified with the introduction of a plasmid encoding NisP and a plasmid encoding the regulatory nisin enzymes NisK and NisR.

A constitutive promoter regulates the transcription of these two genes, whereas a nisin inducible promoter regulates the transcription of NisP.

This promoter is regulated by the action of NisK and NisR.

So in the preparation of the mixture for the activity test in addition to M17 (medium necessary for *L.lactis* growth) agar and strain, also a small amount of nisin was added.

This small amount of nisin cannot kill the indicator strain because is too low, but is enough to start the signal cascade that lead to the activation of *nisP* transcription by NisK and NisR.

Therefore, the indicator strain on the plate is the producer of NisP necessary for the cleavage of the chimeric leader peptide.

If nisin is correctly modified, area in which the growth is inhibit appears.

The samples obtained by treatment of cell lysate and incusion bodies were spotted on the plate and activity zone were detected especially for inclusion bodies sample.

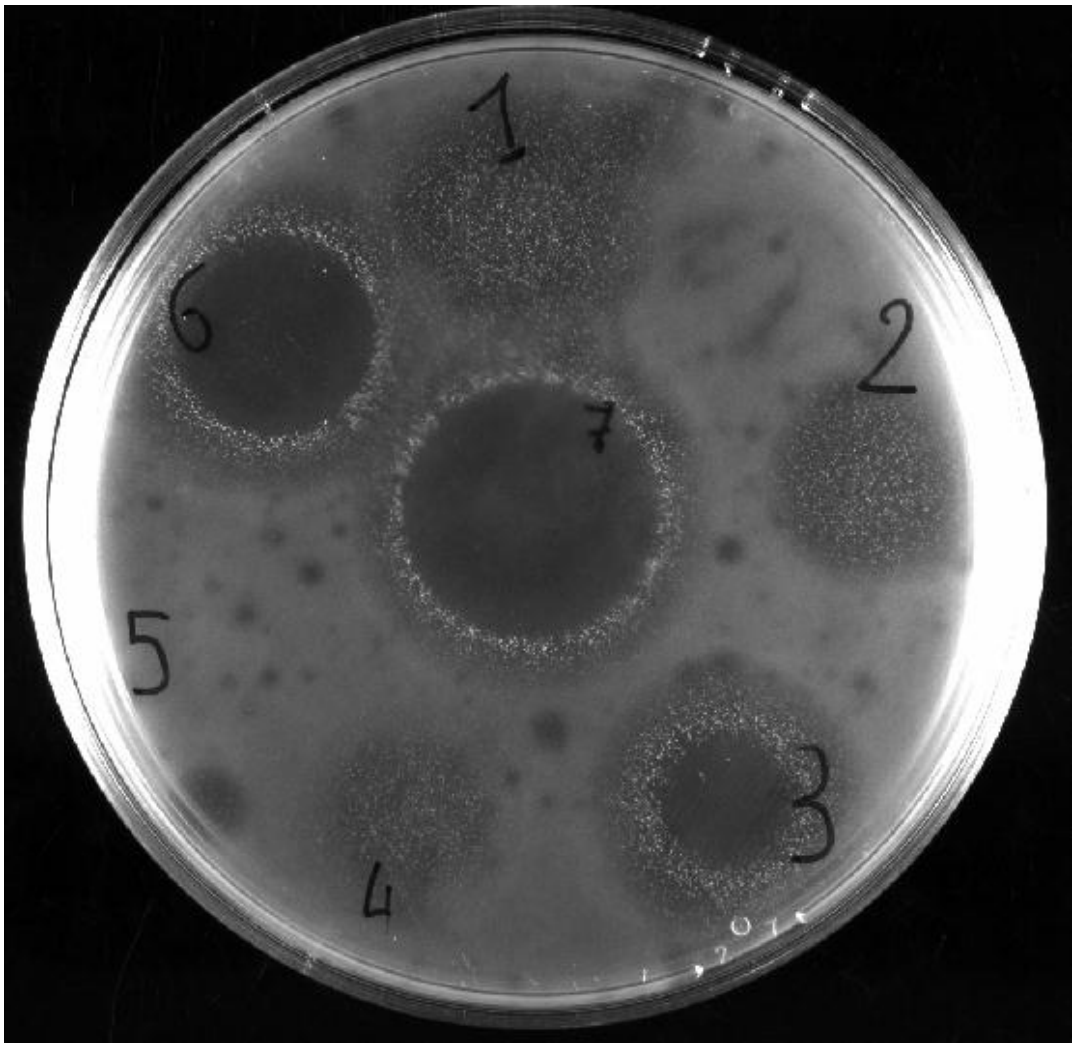


Fig 33: Activity test for H-CinL-nisA samples. 1 and 2 are samples from first and second wash of cell lysate with Start Buffer. 3 is the sample from incusion bodies.
4 and 5 are negative control (cells transformed with empty plasmid). 6 is the positive control necessary to check the correct cleavage of the leader (is modified nisin with original leader peptide). 7 is pure nisin.

3.2.3 Purification and mass spectrometry analysis

Inclusion bodies sample was then further purified by affinity chromatography and HPLC [SI 9-10].

After this two purification steps the activity test was performed again, and the sample obtained from elution peak number 5 of previous HPLC showed activity.

Also sample from peak number 6 showed weak activity.

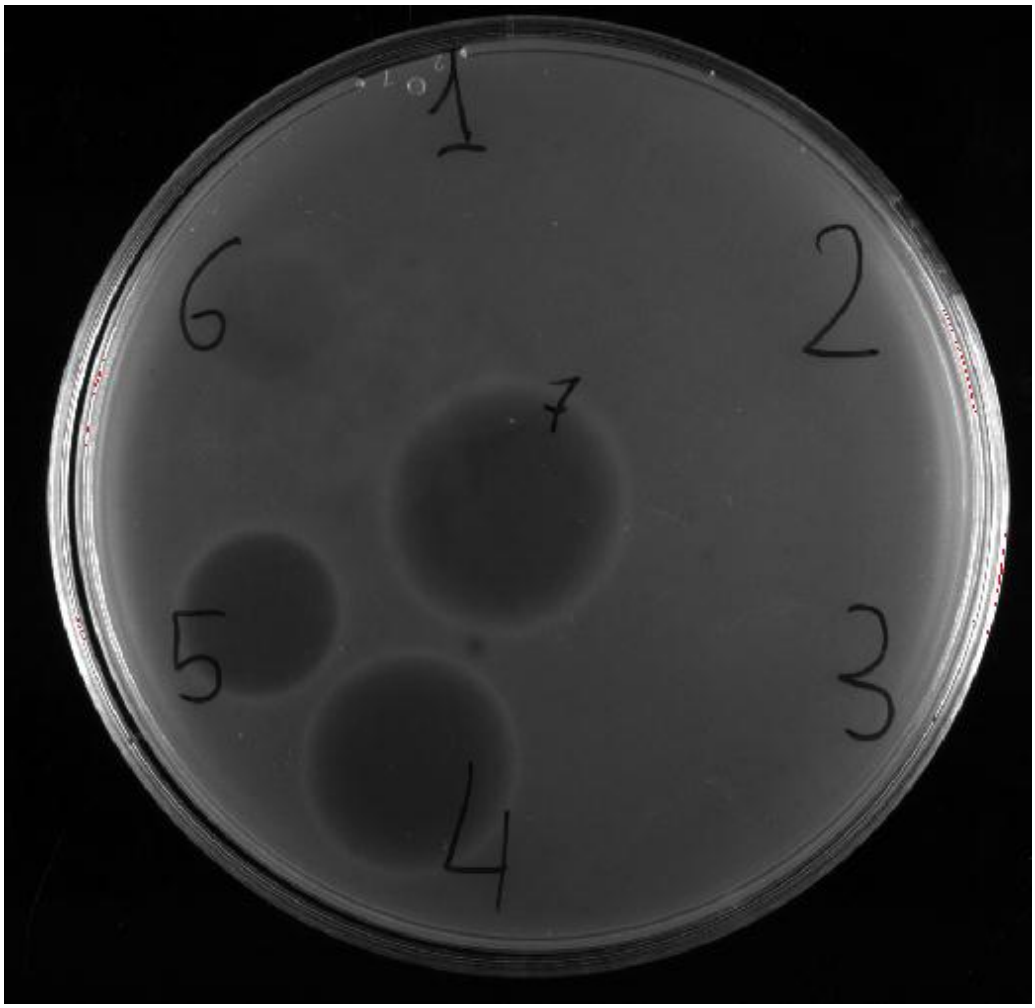


Fig 34: activity test after HPLC purification step. 1,2,5,6 are the samples obtained from HPLC. 3 represent the negative control. 4 is the positive control and 7 is pure nisin.

Sample number 5 was then analyzed by MALDI-ToF.

The result is a clear peak at 13585 Da.

According to Clone Manager, the theoretical molecular weight of H-CinL-nisA without modification is 13740 Da.

In nisin modification eight dehydration are carried out, this means that the theoretical molecular weight of the fully modified peptide is 13596,38 Da, close to the one detected.

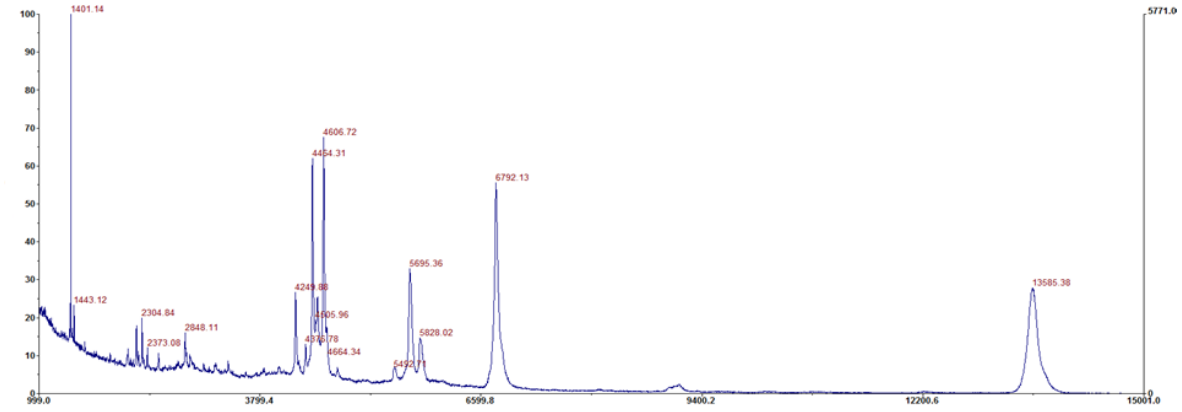


Fig 35: MALDI-ToF analysis of H-CinL-nisA.

The results proved that the expression of nisin with chimeric leader peptide is possible.

Therefore, the modification and activity of nisin modified by NisB and NisCwas also proved.

In addition, the results proved that the presence of the cinnamycin leader peptide does not affect the nisin production, modification and activity.

Exploiting the presence of the cinnamycin leader peptide is then possible to try to modify nisin by the LanM enzyme CinM of cinnamycin modification machinery.

3.3 Modification of nisin with chimeric leader peptide by CinM

The results reported in the previous paragraphs open the way to the modification of nisin by modification enzyme CinM, exploiting the presence of the cinnamycin leader peptide in the chimeric leader peptide upstream nisin core peptide.

Theoretically, CinM should recognize the cinnamycin leader peptide and then modify nisin core peptide.

Belonging to class LanM modification enzymes, CinM carry out both dehydration and cyclization on nisin, leading to the production of mature and active nisin.

To test this hypothesis the previous synthetic gene and cinM were cloned into a pACYC plasmid.

Unlike the previous case, this system is formed by only one plasmid as illustrated in the figure below.

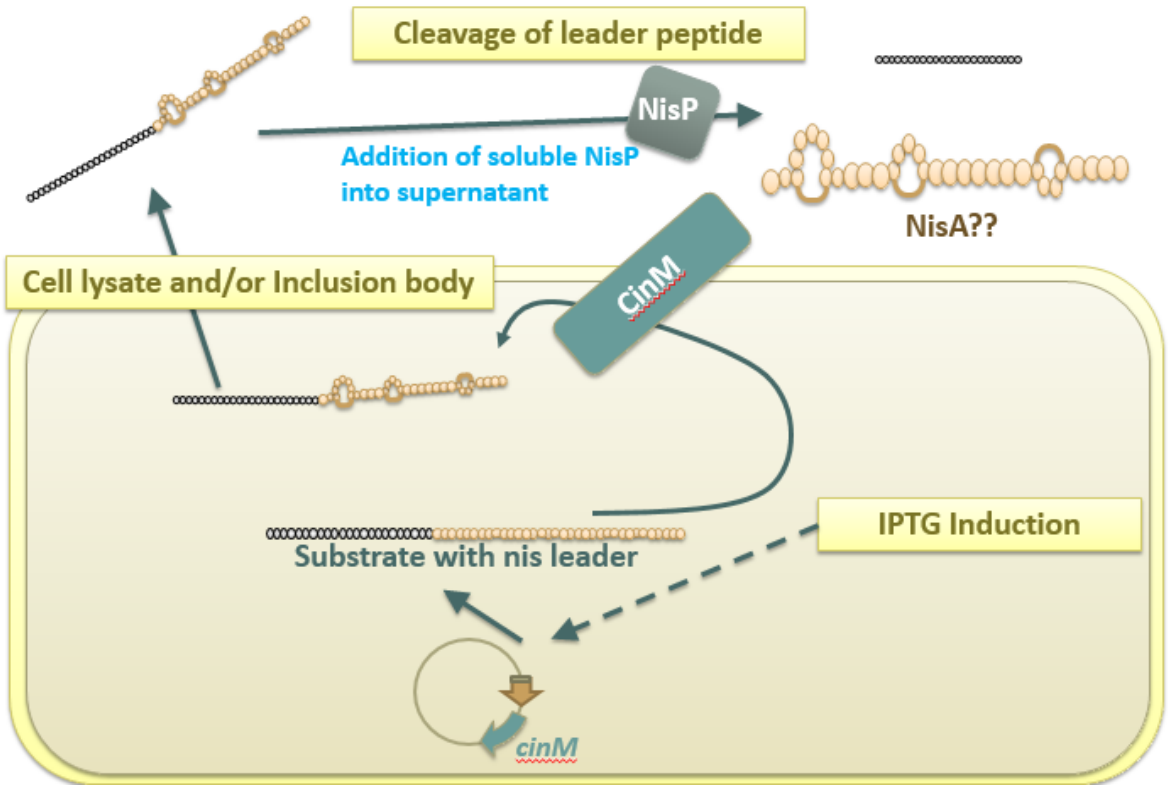


Fig 36: Chimeric leader-peptide nisin modification system.

Being a one-plasmid system the main differences with the previous nisin expression system are that is necessary add only chloramphenicol during the cells growth and IPTG for the induction.

Basically the same steps used for nisin modification by NisB and NisC were followed.

All the steps are summarized in the next figure.

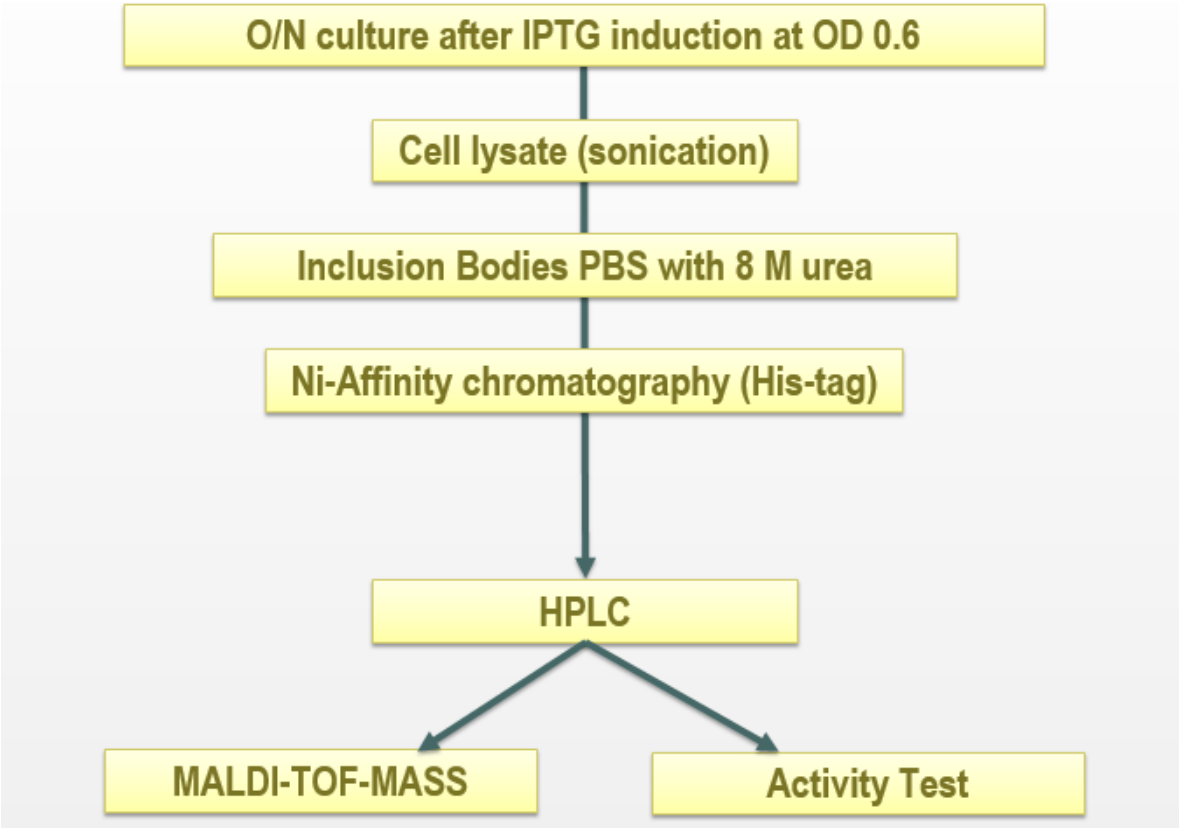


Figure 37: Steps involved to test nisin modification by CinM.

Weak activity was confirmed for inclusion bodies sample by activity test against *L.lactis* nz9000 expressing NisP.

This sample was then purified by affinity chromatography and HPLC.

HPLC result show the presence of a clear elution peak.

The elution fraction referred to this peak was then tested by activity test.

Both the HPLC profile and activity test are reported below.

For activity test was checked also if the sample has activity with the leader peptide.

To do that an activity test against *L.lactis* without NisP expression system was performed.

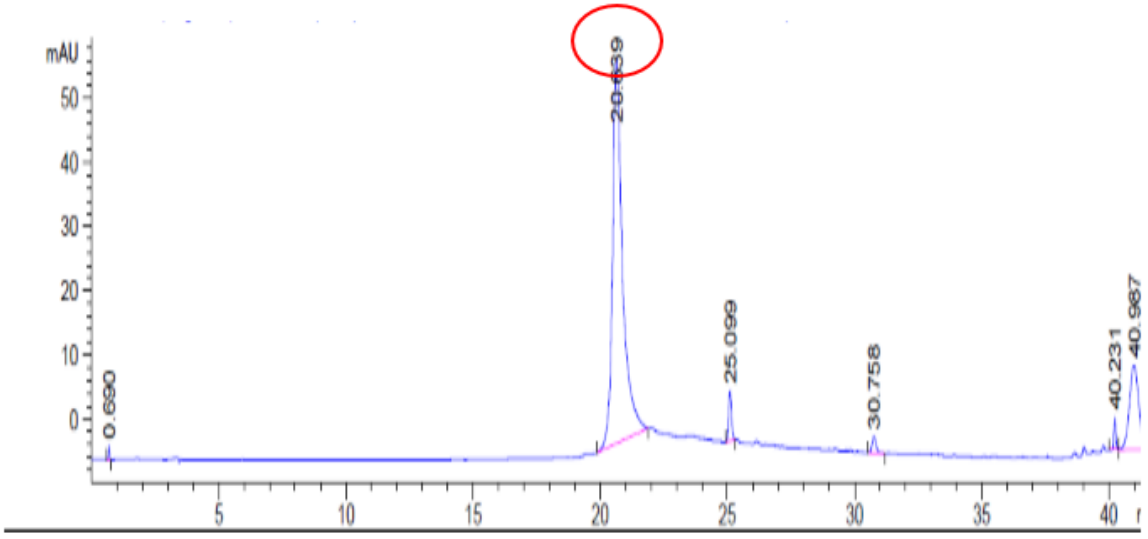


Fig 38: HPLC profile of inclusion bodies sample. Labeled peak represent the putative nisin modified by CinM with the leader peptide still attached.

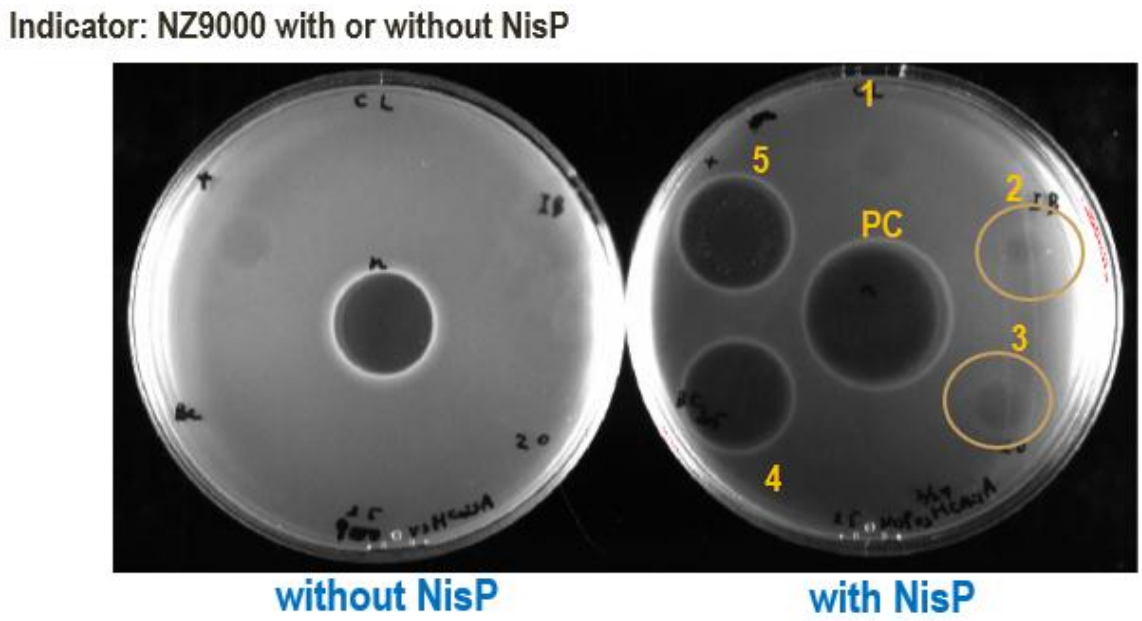


Figure 39: Activity test against L.lactis nz9000 with or without NisP expression of H-cinL-nisA +CinM

1: cell lysate sample. 2: inclusion bodies sample. 3: sample obtained after HPLC.

4: nisin with chimeric leader modified by nisin system. 5: nsin with natural leader

PC: positive control (pure nisin)

From activity test it is possible to see weak but clear activity in sample 2 and 3. This means that CinM can modify nisin if this one is fused with a chimeric leader peptide containing cinnamycin leader.

The result was confirmed also by MALDI-ToF analysis for sample 3 before cleavage of the leader pre-peptide.

The peak size fits with the peak size detected for modification of H-CinL-nisA by nisin system.

These result seems confirm that CinM and combination of NisB and NisC carry out the sames modification on nisin, as expected.

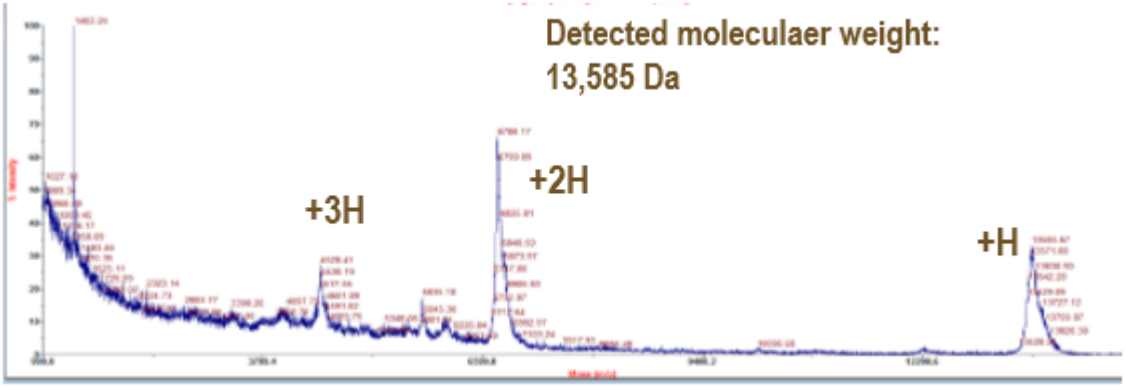


Figure 40: MALDI-ToF data for H-CinL-nisA modified by CinM. The detected molecular weight is reported. The others two peaks labeled by +2H and +3H seems to be the target molecule ionizied two and three times respectively

These results prove also that itis possible to use a modular schematization and conception for use of lantibiotics and modification enzymes, confirming that a Synthetic Biology approach seems to be a valid strategy in the design of novel lantibiotics.

CHAPTER 4: Results and Discussion

Heterologous expression system for cinnamycin was achieved by introduction of two plasmids, pACYC and pRSF, in *E.coli*.

The first one carries the two modification enzyems *orf7b*, responsible for lysinoalanine formation, and *cinX* necessary for hydroxylation.

The pRSF plasmid harbors the structure gene *cinA(A-1K)* that encodes cynnamycin pre-peptide, and *cinM* that encodes a class LanM enzyme responsible for both dehydration and cyclization.

The obtained results proved that the heterologous expression system allow the expression of cinnamycin pre-peptide and of all the modification enzymes.

This was proved by SDS-PAGE and Western Blot.

To check the activity of cinnamycin was necessary to cleave the leader peptide by protease LysC.

To do that affinity chromatography and HPLC were performed to purify the target molecule.

MALDI-ToF analysis confirms the cleavage of the leader peptide by LysC.

In addition, mass spectrometry analysis allowed the detection of a product with molecular weight very close to the theoretical molecular weight of fully modified cinnamycin.

Finally, the activity of heterologous expressed cinnamycin was confirmed by activity test against*Bacillus subtilis* and *Micrococcus luteus*.

To obtain enough active product it was necessary to start with a culture volume of 2 L.

Attempt to obtain active compound starting with lower volume gave negative results.

This means that the heterologous system has a low efficiency.

In addition, the concentration necessary to obtain a positive result on the activity test is unknown.

To overcome the low efficiency of the expression system it is necessary to check all the expression and purification steps, to find the better conditions.

For example, the desalting step necessary to remove imidazole used during affinity chromatography should be performed as soon as possible to prevent peptide precipitation that lead to a loss of product.

Also, to check the minimum amount of active product necessary to show activity in the activity test is necessary.

To do that it is possible to compare the effect of different aliquots of heterologous expressed protein with the effect of authentic cinnamycin at known concentrations.

The second part of the work involved the heterologous expression of nisin with a chimeric leader peptide formed by fusion of cinnamycin and nisin leader peptide, and the test of nisin modification enzymes on this molecule.

The results show that the expression of the chimeric molecule is possible and also its modification.

This means that the presence of cinnamycin leader peptide upstream nisin leader peptide do not affect the recognition by NisB and NisC of nisin leader peptide neither nisin modification.

The modifications was confirmed by activity test and partially by MALDI-ToF.

The activity detected was very clear, and comparable to normal nisin.

In the end, modification of the previous chimeric molecule by CinM enzyme was attempted.

The heterologous expression of cinnamycin confirmed that CinM can be expressed and can work in *E.coli*.

The idea was to exploiting the presence of cinnamycin leader peptide to obtain nisin modified by a single class LanM enzyme.

Activity was detected and confirmed by MALDI-ToF analysis, that gave a similar result to the mass spectorometry analysis of the same molecule modified by nisin modification enzymes.

This means that the two system provide the same modification on the molecule.

Therefore, CinM can modify nisin.

The detected activity was very weak if compared to normal nisin and nisin with chimeric leader peptide.

Expression levels for the chimeric molecule are very similar in both cases, meaning that the presence/absence of CinM expression do not affect the expression of the peptide.

Therefore seems that CinM action on this molecule is not so efficient.

Anyway, the expression system is formed by just one plasmid, unlike the two previous two-plasmid systems.

This leads to a lower metabolic load for the bacteria.

A way to increase the activity of CinM on nisin core peptide region is to optimize the chimeric leader peptide, for example with a shorter nisin leader.

This should provide an increase on the efficiency of CinM because there is less space between the cinnamycin leader and the core peptide region.

Anyway the cleavage of the leader peptide should be still possible using NisP.

This hypothesis has to be proved experimentally.

Even if this system have to be optimized to increase the production efficiency and activity of the product, the results are promising and lead to new challenges.

CinM, showed that in cinnamycincan lead to the formation of Lan and MeLan bridges opposite oriented with respect to the bridges present on nisin.

So, it would be interesting to check if nisin modified by CinM harbor the “inverted” bridges and what are the implications.

In addition, CinM activity should be tested on others lantibiotic using a similar chimeric leader method.

Finally, from cinnamycin modification system analysis seems that Orf7 can work only with the simultaneous presence of CinM.

Therefore, if CinM can work on nisin is also possible try to introduce the lysinoalanine bridge on nisin obtaining a new to nature molecule.

Another way is to design a fused core peptide made by the region that harbor the rings A, B and C of nisin and a designed tail.

The rings can be obtained using CinM whereas in the deigned tail can be introduced a lysinoalanine bridge by Orf7.

This kind of hypothetical new to nature molecule is shown below.

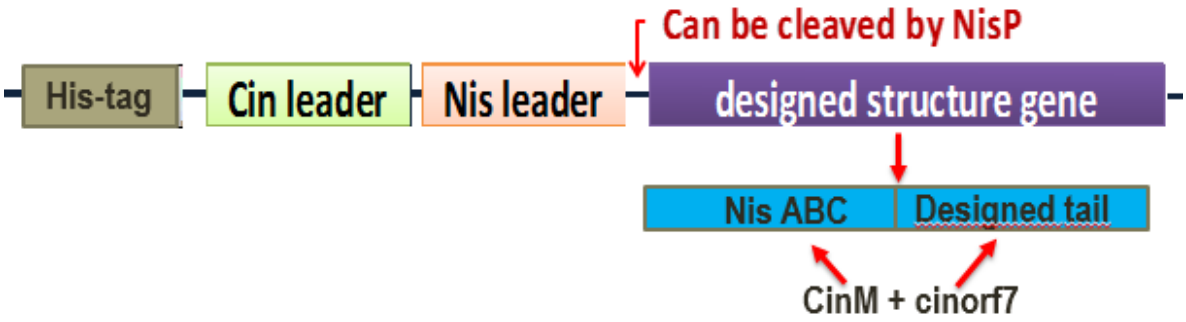


Figure 41: Hypothetical lantibiotic that can be produce by a synthetic biology approach using CinM and Orf7.

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CONCLUSIONS

Lantibiotic peptides possess strong activity against a wide range of Gram-positive and Gram-negative bacteria, and represent a potential solution to fight the growing problem of multi-drugs resistant pathogens.

Antimicrobial activity is due to post-translational modifications, that changes structure and proprieties of the peptide.

The modifications are carried out by specific modification enzymes that recognize the leader sequence and act on the core peptide.

Using a Synthetic Biology approach that involves the combination of different substrates and different modification enzymes it is possible to obtain new to nature lantibiotic.

Therefore, the final goal is to obtain a plug and play system that can be used to select different sets of modification enzymes to work on diverse, specifically designed substrates, with the aim to obtain new to nature molecules with antimicrobial activity and/or new physicochemical proprieties.

The intention was to enlarge the “library” of available modification enzymes that can be used in the plug and play system, by adding CinM, the enzyme responsible for lanthionine and methylanthionine formation in cinnamycin.

Therefore, the aim of this research was to prove the modification of nisin by the single class LanM enzyme, CinM belonging to cinnamycin modification machinery.

To do that, it was necessary to implement a heterologous expression system formed by two plasmids carrying the genes for modification enzymes and for cinnamycin pre-peptide.

The gene encoding for cinnamycin peptide was modified by substitution of alanine at the last position of leader peptide with a lysine.

This was necessary to obtain the cleavage of the leader peptide by protease LysC, bypassing the problem of absence of dedicated protease in the heterologous system for this aim.

MALDI-ToF and activity test confirmed the presence and activity of fully modified cinnamycin after the cleavage, proving that the heterologous system works well.

This result proved the possibility of expression and modification of cinnamycin by heterologous host, and also that cinM can be produced and works in *E.coli* as well as the others cinnamycin modification enzymes.

To obtain the modification of nisin by CinM, was necessary design a specific peptide, composed by a chimeric leader sequence obtained by fusion of cinnamycin leader and nisin leader upstream nisin core peptide.

The idea was that CinM could recognize the cinnamycin leader and then modify nisin.

Before proving that, it was necessary to check if the presence of cinnamycin leader affected the production and activity of nisin.

The synthetic gene encoding for the chimeric molecule was designed by Clone Manager and then introduced in *E. coli.*

Also a pBAD plasmid carrying the nisin modification enzymes NisB and NisC was introduced.

Expression of the chimeric molecule was proven by SDS-PAGE and Western blot.

MALDI-ToF and activity test confirm that active and fully modified nisin can be produced and modified by NisB and NisC even with the presence of cinnamycin leader peptide.

Once proven that cinnamycin leader does not affect nisin modification, the same molecule was modified by CinM.

For this aim, a single plasmid system was built and introduced in *E. coli*.

As before, SDS-PAGE and Western Blot proved peptide presence.

MALDI-ToF result was same as the previous case, meaning that CinM and combination of NisB and NisC carry out the same modification on nisin.

Finally, activity of nisin modified by CinM was detected by activity test.

The activity is weaker than natural nisin or chimeric nisin modified by nisin system, meaning that the efficiency of nisin modification by CinM is low.

For that reason, is necessary optimize the modification condition, for example reducing the nisn leader length.

In this way there is less space between cinnamycin leader and nisin core peptide.

Anyway, even if the activity is not strong, modification of nisin by CinM was proved.

This open the way to others possible experiments.

For example, test CinM on others lantibiotics, or check if the orientation of Lan and MeLan bridges introduced by cinM on nisin is same as natural nisin or are opposite oriented.

Furthermore, from literature is known that Orf7, the enzyme responsible for lysinoalanine formation, can work only with the presence of CinM.

So, is now possible try to introduce the lysinoalanine bridge on nisin, obtaining a hyper-modified molecule.

In conclusion, lantibiotics represent a potential solution to fight multi-drugs resistant pathogens.

Synthetic Biology seems a promising way to obtain designed and new-to nature lantibiotics, with desired proprieties and activity.

However, to obtain that is necessary standardized and master the behavior of modification enzymes and peptides.

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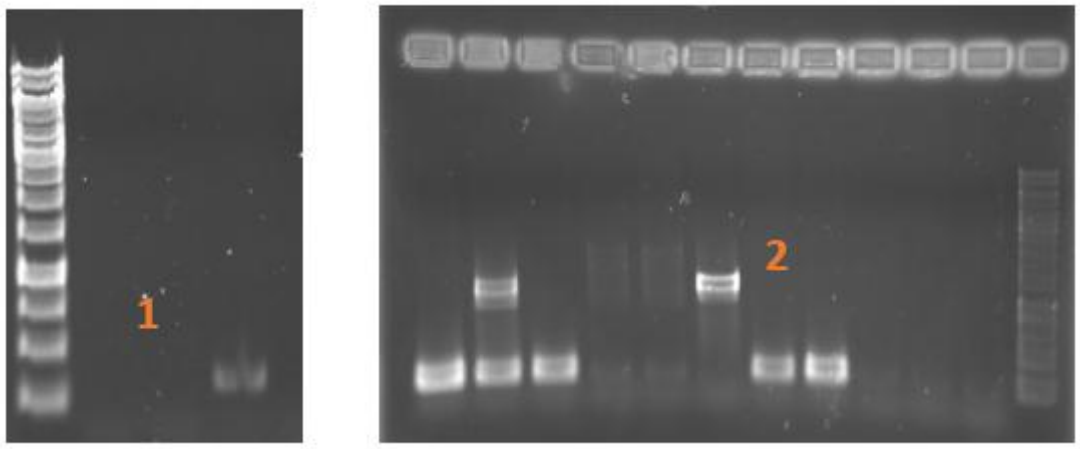
SUPPORTING INFORMATION

pRSF 1st-Fw: TCACCACCCTGAATTGACTC
pRSF 1st-Rv: GGCCGTGTACAATACGATTACTTTC
pRSF 2nd -Fw: GTACACGGCCGCATAATC
pRSF 2nd-Rw: TAAGCTGCGCTAGTAGAC

pACYC 1st-Fw: TCTCCCTTATGCGACTCCTG
pACYC 1st-Rv: CGGCCGTGTACAATACGATTACTTTC
pACYC 2nd-Fw: GTACACGGCCGCATAATC
pACYC 2nd-RV: TAAGCTGCGCTAGTAGAC
CinX-Fw: ATCCGGCTGCTAACAAAG
CinX-Rv: CTTTCGCTCGAGTTAACC
CinM-Fw: GACTGATGATCGGCATCTCC
CinM-Rv: GCGATTCCTGGTGGAACTTG
H-CinL-nisA Fw: ATCATACCATGGGCAGCAGCCATC
H-CinL-nisA Rv: ATCATTCGGTACCAAGCTTATTTGC

SI 1: Primers List



SI 2: 1)pRSF cinA(A-1K)-cinM CPCR; Primers pRSF 2nd –Fw CinM-Rv (342 bp)
2) pACYC orf7b-cinX CPCR; Primers pACYC 2nd-Fw pACYC 2nd-Rv (1161 bp)

Start Buffer:

20 mM NaH_2PO_4

500mM NaCl

0.5 mM imidazole

20% glycerol

pH 7.5

Denaturing Buffer:

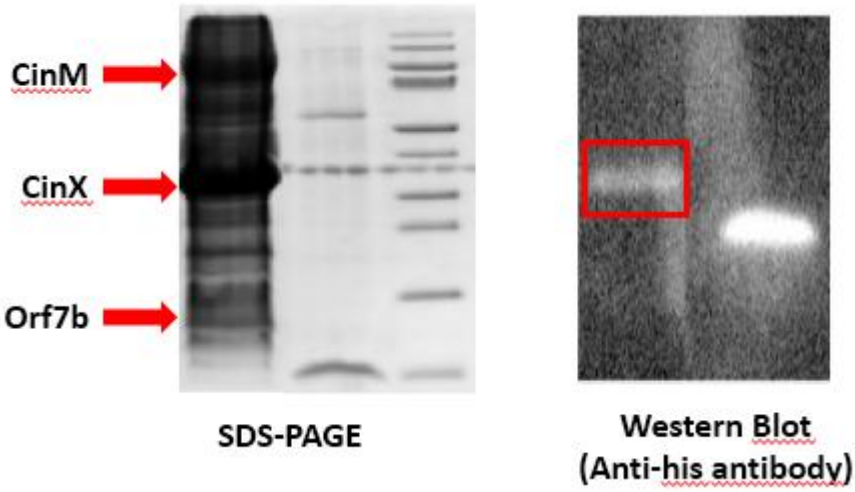
100mM NaH_2PO_4

10mM Tris

8M Urea

pH 8.0

SI 3: Buffers list for lysation of cells and denaturation of inclusion bodies



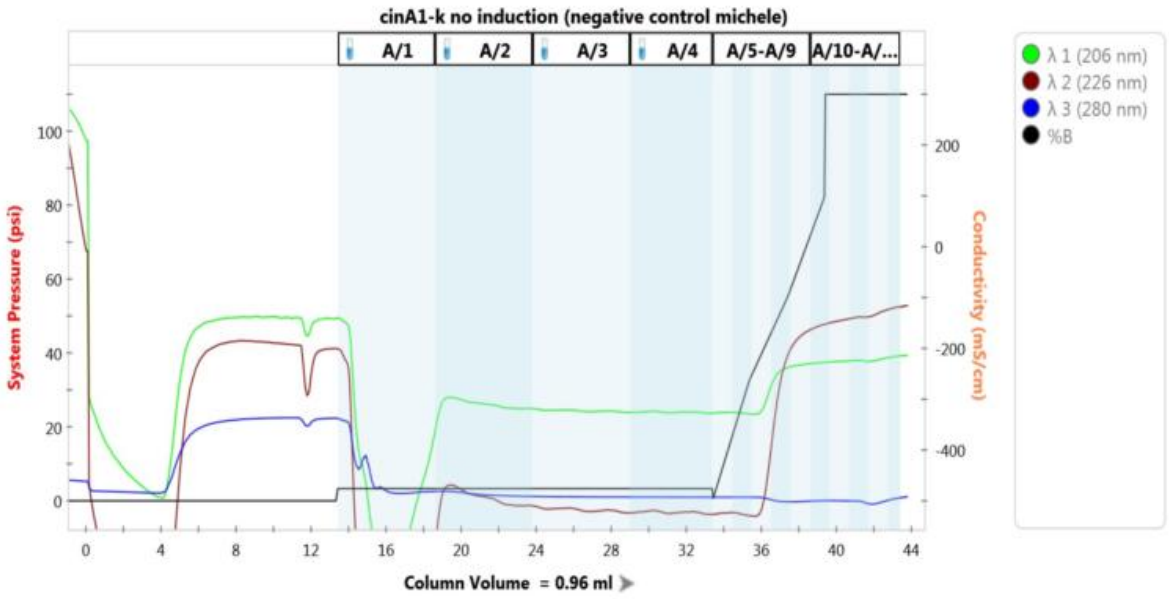
SI 4: SDS-PAGE and Western Blot. On the SDS-PAGE picture are indicated the modification enzymes. In western blot image band representing cinnamycin is highlighted

Equilibration buffer:
20 mM sodium phosphate,
0.5 M NaCl, pH 7.4

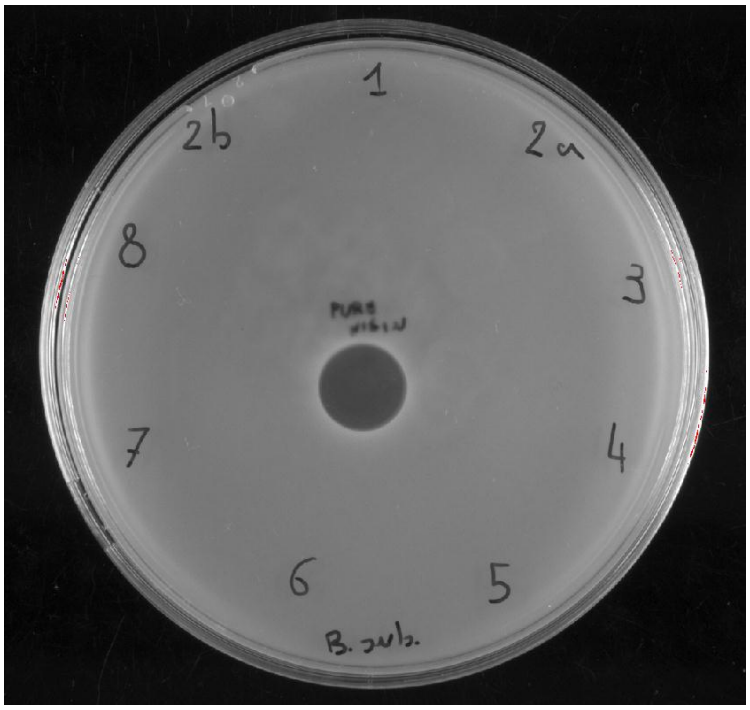
Wash buffer:
20 mM sodium phosphate
0.5 M NaCl
0 to 30 mM imidazole
pH 7.4

Elution buffer:
20 mM sodium phosphate
0.5 M NaCl
500 mM to 1 M imidazole
pH 7.4

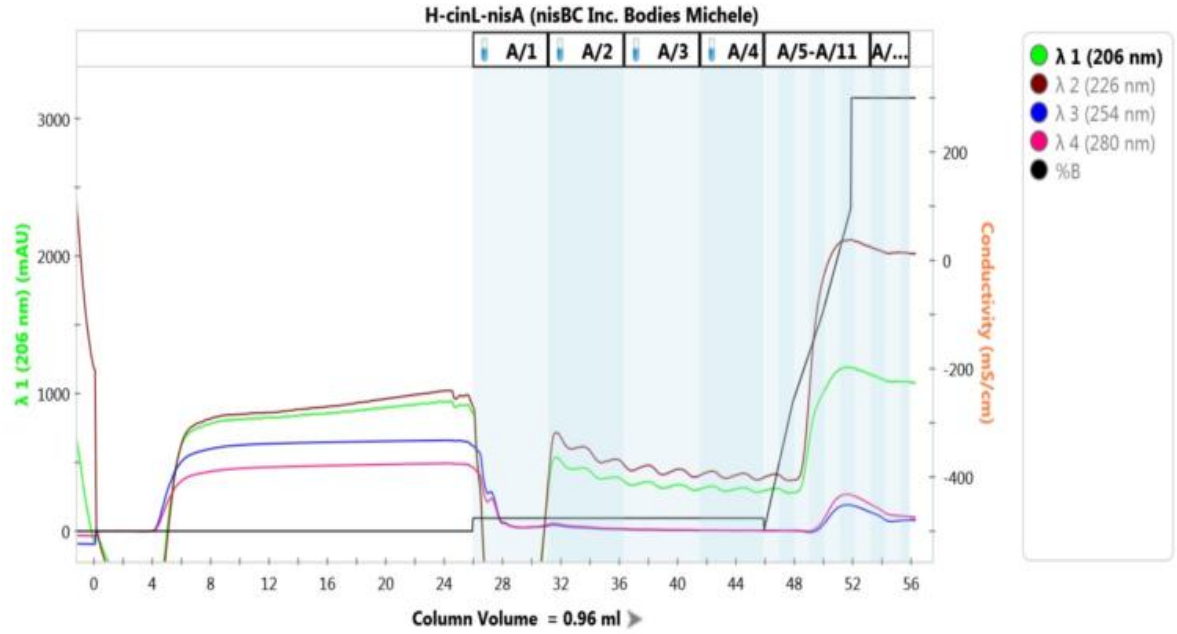
SI 5: Affinity Chromatography buffers



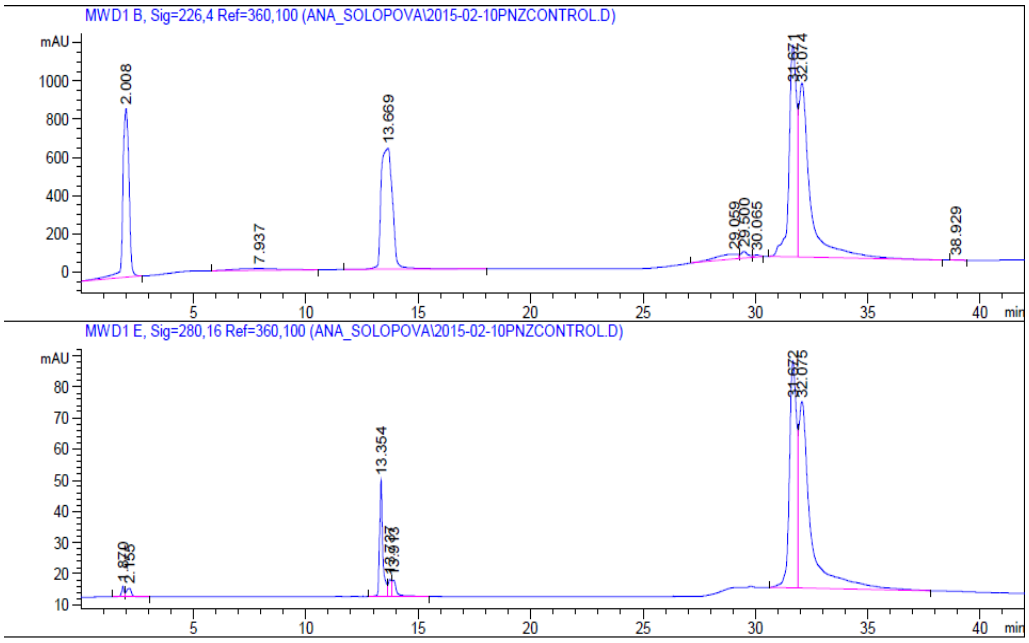
SI 6: Affinity Chromatography profile of a non-induced culture. No peaks were detected in elution steps, meaning that expression system is strictly regulated by IPTG induction



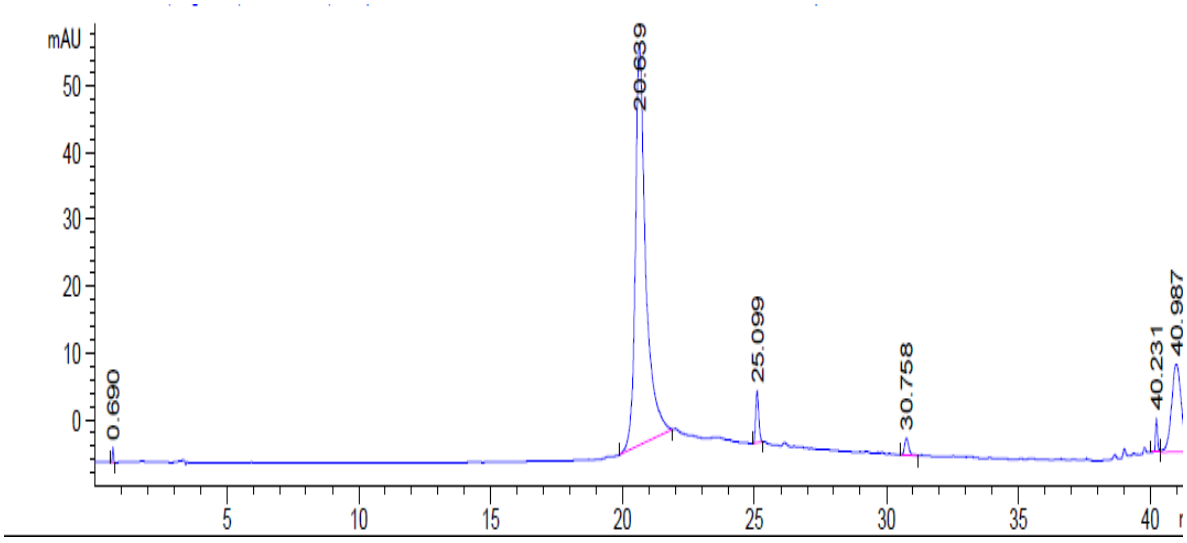
SI 8: Activity test for samples obtained after HPLC. Samples were not digested by LysC. No activity was detected meaning that peptides with leader peptide do not possess activity. Positive control: pure nisin.



SI 8: Affinity chromatography profile of H-CinL-nisA modified by nisin system. This sample came from inclusion bodies. A peak was detected during the elution phase



SI 9: HPLC profile of H-CinL-nisA of samples obtained after Affinity Chromatography.



SI 10: HPLC profile of H-CinL-nisA modified by CinM after Affinity Chromatography. The clear peak at 20 min was proven be the target peptide.