BIOSYNTHESIS AND ENGINEERING OF LANTHIPEPTIDES

BY

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DISSESSATION

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ABSTRACT

The emergence of antibiotic-resistant bacterial strains is a growing concern as antimicrobial drug discovery is not proceeding at the same pace as the growth of resistance. Genome sequencing techniques have advanced in the last 20 years and have revealed the hidden capacity of microbes to generate natural products including antibiotics. Most of these compounds were not detected with the traditional techniques due to conditional expression and low production. One group of natural products that is much larger than anticipated based on the genomes identified is the ribosomally-synthesized and post-translational modified peptides (RiPPs). Lanthipeptides are a class of RiPPs with pharmaceutically valuable properties including antimicrobial activity against clinically-relevant bacterial pathogens, even including drug-resistant strains. These peptides are characterized by thioether crosslinks making them more resistant to degradation under physiological conditions. A detailed understanding of the biosynthesis and mode of action could allow engineering of these peptides for better pharmacological properties. In this thesis, biosynthesis of several lanthipeptides belonging to the cinnamycin group of peptides is described, and the biosynthetic enzymes of cinnamycin were studied in detail. In Chapter II, the biosynthesis of cinnamycin was investigated by reconstitution of the cinnamycin biosynthetic enzyme activities in vitro, and through heterologous expression of the cinnamycin biosynthetic genes in E. coli. These two approaches employed to
produce cinnamycin have their own advantages. For instance, *in vitro* reconstitution of the lantibiotic synthetase CinM and the tailoring enzyme CinX allowed for detailed studies of these enzymes as described in Chapter III. On the other hand, through production of cinnamycin in *E. coli*, the activity of a new enzyme called cinorf7 was discovered, which could not be achieved in the *in vitro* experiments. Therefore, Chapter III also contains our attempts to identify the catalytically important residues of cinorf7 by mutational studies. These experiments on cinnamycin biosynthesis set the stage for the biosynthesis of other lanthipeptides in the cinnamycin-group of peptides, which is described in Chapter IV. Lanthipeptide synthetases have been shown to have relaxed substrate specificity, creating new possibilities in the engineering of these molecules for desired functions. Peptide libraries have been commonly used for engineering and selection of pharmaceutically valuable molecules. Lanthipeptides have great potential for drug development compared to linear peptides as they are chemically and metabolically more stable because of the thioether crosslinks. Therefore, large molecular libraries of lanthipeptides with designed ring structure would be useful. In Chapter V, phage display of the well known lanthipeptide nisin is described, which will form a platform for the production and selection of lanthipeptide libraries.
To my family
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It all started with me hearing about DNA in detail and genomic studies performed by different laboratories all over the world towards the end of 90’s. The people who know me would remember that I really wanted to be close to science and had decided to continue my education abroad by an early age. And now that it has actually happened, it has been tremendously dense with a lot of unexpected events. My earlier dream was that I was going to travel, meet cool scientists, learn more about research, experience different cultures, and taste different food. Now looking back to my PhD years, I had experienced everything I dreamed of, which doesn’t leave many words to say, but that I am very very lucky.

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TABLE OF CONTENTS

LIST OF FIGURES ................................................................................................................... xiv
LIST OF TABLES ................................................................................................................... xvii
CHAPTER I: INTRODUCTION TO LANTHIPEPTIDES ...................................................... 1
  1.1 General view on natural products .................................................................................... 1
  1.2 Lanthipeptides and their biosynthesis .......................................................................... 2
    1.2.1 Classes of lanthipeptides ...................................................................................... 6
    1.2.2 Role of leader peptides ....................................................................................... 8
  1.3 Mode of action of lanthipeptides .................................................................................... 9
  1.4 Engineering of lanthipeptides and their biosynthetic enzymes .................................... 12
    1.4.1 In vitro techniques .............................................................................................. 12
    1.4.2 Production of lanthipeptides in E. coli and their native producing strains ................. 14
    1.4.3 Engineering of lanthipeptides via employing leader peptides ............................... 14
    1.4.4 Engineering of the enzymes involved in biosynthesis of lanthipeptides ..................... 16
    1.4.5 Chemical synthesis .............................................................................................. 17
    1.4.6 Summary and outlook ......................................................................................... 17
  1.5 REFERENCES ................................................................................................................. 20

CHAPTER II: NINE POSTTRANSLATIONAL MODIFICATIONS DURING THE BIOSYNTHESIS OF CINNAMYCIN ................................................................. 30
  2.1 INTRODUCTION ........................................................................................................... 30
  2.2 RESULTS ....................................................................................................................... 35
    2.2.1 In vitro reconstitution of the enzymatic activity of the lanthionine synthetase, CinM ................................................................. 35
    2.2.2 In vitro reconstitution of CinX ............................................................................. 38
    2.2.3 Lysinoalanine formation and bioactivity assays .................................................... 42
    2.2.4 Coexpression of CinA, CinM, CinX in E. coli (NB10, p28, p35) ......................... 44
    2.2.5 Coexpression of CinA, CinM, CinX and Cinorf7 in E. coli ................................. 49
2.3 DISCUSSION ........................................................................................................... 51
2.4 MATERIALS ........................................................................................................... 55
2.5 GENERAL METHODS ............................................................................................. 56
2.6 EXPERIMENTAL METHODS .................................................................................. 57
  2.6.1 Procedure for LysC cleavage........................................................................... 57
  2.6.2 CinM cyclization activity assay (NB2, p54) .................................................... 57
  2.6.3 Hydroxylation activity assay with CinX (NB5, p61) ........................................ 58
  2.6.4 Tandem MS analysis to determine the hydroxylation site (NB4, p66)........... 58
  2.6.5 Coexpression of CinA(A–1K), CinM, and CinX with and without Cinorf7 in E. coli (NB8, p50)......................................................................................... 59
  2.6.6 Bioactivity assays with B. subtilis LH45 (NB8, p54)...................................... 60
  2.6.7 Cloning of cinA, cinM, cinX and cinorf7 and construction of expression plasmids (NB7, NB9, p6) .......................................................................................... 61
  2.6.8 Construction of the CinA (A–1K) (NB5)......................................................... 63
  2.6.9 Generation of a coexpression system in E. coli (NB9, p8)............................. 65
  2.6.10 Overexpression and purification of His6-CinA, His6-CinA A–1K, and His6-CinA A–1K D15A (NB7, p18, p36).......................................................... 66
  2.6.11 Overexpression of his10-cinM and his6-cinX (NB5, 34, 73; NB1, p17-19, p41) .................................................................................................................. 67
  2.6.12 Purification of His10-CinM and His6-CinX (NB7, p26; NB5, p34)............. 67
  2.6.13 Detection of lysinoalanine (Lal) on CinA by gas chromatography-mass spectrometry analysis (GC-MSMS) (NB5, 94-95) ........................................ 69
2.7 REFERENCES .......................................................................................................... 72

CHAPTER III: BIOCHEMICAL CHARACTERIZATION OF THE TAILORING ENZYMES CINX AND CINORF7 INVOLVED IN CINNAMYCIN BIOSYNTHESIS ................................................................................................................................. 76

3.1 INTRODUCTION ....................................................................................................... 76
3.2 RESULTS .................................................................................................................. 79
  3.2.1 Order of modification by CinX and Cinorf7.................................................... 79
  3.2.2 Purification and Characterization of Cinorf7.................................................. 83
  3.2.3 Crystallization Trials...................................................................................... 86
4.2.1.1 Duramycin gene cluster from *Streptovercitillium cinnamoneum forma azacoluta* (ATCC 12686) .......................................................................................... 123
4.2.1.2 Coexpression of *durA*, *durM*, *cinX*, and *cinorf7* in *E. coli* .......... 126
4.2.1.3 One step removal of the leader peptide of DurA with commercially available proteases.................................................................................................................. 131
4.2.1.4 Isothermal calorimetry (ITC) studies of duramycin and cinnamycin for binding to PE .......................................................................................................................... 133
4.2.2 Frankisin Biosynthesis ............................................................................. 135
4.2.2.1 Frankisin biosynthetic gene cluster from *Frankia Sp. EUN1f* ........ 135
4.2.2.2 Coexpression of *franA*, *franM*, *franX*, and *franorf7* in *E. coli* .... 139
4.2.3 Engineering of cinnamycin analogs ....................................................... 141
4.2.3.1 Biosynthesis of ancovenin .................................................................. 141
4.2.3.2 Preparation of cinnamycin and duramycin analysis containing Phe7Val or Phe7Leu mutations .......................................................... 143
4.3 DISCUSSION .............................................................................................. 148
4.4 MATERIALS ............................................................................................. 151
4.5 GENERAL METHODS ................................................................................ 152
4.6 EXPERIMENTAL METHODS .................................................................... 153
4.6.1 Isolation of genomic DNA from *Streptovercitillium cinnamoneum forma azacoluta* and genomic sequencing (NB 13, p14) ....................................................... 153
4.6.2 Cloning ................................................................................................... 153
4.6.2.1 Cloning of *durA*, *durM*, *durX*, and *durorf7* into pDuet vectors (NB14, p7-17) ............................................................................................................... 153
4.6.2.2 Cloning of *franA*, *franM*, *franX*, and *franorf7* into pDuet vectors (NB10, p66; NB11, p24) ............................................................ 155
4.6.2.4 Generation of CinA-K R2V, F10L, F12W, V13S mutations in pRSFDuet-1 MCS-I for ancovenin production ........................................ 157
4.6.2.5 Generation of *cinA-K Phe7Val* and *Phe7Leu*, and *durA Phe7Val* and *Phe7Leu* (NB10, p70; NB11, p45) .............................................. 159
4.6.3 Coexpression studies ........................................................................... 160
4.6.3.1 Coexpression and purification of $duR$, $duM$, and $cinX$ with and without $cinorf7$ in $E. coli$ (NB14, p58-60, 63, 65; NB15, p26)..........................160
4.6.3.2 Coexpression and purification of $franA$, $franM$, $franX$, and $franorf7$ in $E. coli$ (NB14, p59).........................................................161
4.6.4 Bioactivity assays with $B. subtilis$ 6633 (NB11, p98; NB14, p77)......161
4.6.5 Protease cleavage of DurA, FranA, NisA, and CinA-K (NB11, p94) .162
4.6.6 Aminopeptidase cleavage of GluC-cleaved DurA and ArgC-cleaved FranA and analysis by MALDI-ToF MS (NB14, p 56)..........................163
4.6.7 Isothermal Calorimetry (ITC) experiments (NB11, p16, 19, 20, 25, 99) .................................................................................................163

4.7 REFERENCES.............................................................................................166

CHAPTER V: PHAGE DISPLAY OF LANTHYPEPTIDES ..................................171

5.1 INTRODUCTION........................................................................................171
5.2 RESULTS....................................................................................................176
  5.2.1 Experimental design .............................................................................176
  5.2.2 C-terminal display of nisin via pJF3H phagemid.....................................181
  5.2.3 Phage display of other lanthipeptides....................................................188
  5.2.4 Construction of cyclic peptide libraries using ProcM............................192
  5.2.5 ProcM Promiscuity Test .......................................................................195
5.3 DISCUSSION...............................................................................................198
5.4 MATERIALS...............................................................................................202
5.5 GENERAL METHODS ...............................................................................203
5.6 EXPERIMENTAL METHODS.....................................................................204
  5.6.1 Cloning..................................................................................................204
  5.6.1.1 Cloning of the nisin coexpression system (NB6, 34-35) .................204
  5.6.1.2 Cloning of $cinA(A–1K)$ into pSEX81 and pJF3H, cloning of $nisA$ into pSEX81, and factor Xa cleavage site insertion (NB6, p48-65) ........205
  5.6.1.3 Cloning of $pelB$-$cinA(A–1K)$-$Xa$, and $pelB$-$cinA(A–1K)$ into pRSFDuet-1.................................................................207
  5.6.1.4 Preparation of the constructs for peptides attached to the ProcA leader 2.8.................................................................209
5.6.2 Preparation of phagemids for display of NisA, CinA, and LctA (NB10, p27, 29) .......................................................................................................................... 211
5.6.3 Phage preparation for nisin display (NB11, p84) ................................. 212
5.6.4 Phage preparation for cinnamycin display (NB11, p59-60)................. 213
5.6.5 Phage preparation for lacticin 481 display (NB12, p45) ...................... 214
5.6.6 Determination of phage titer (NB9 p 30, 36, 84; NB10 p73) ............. 215
5.6.7 Western blot analysis (NB9, p85) .............................................................. 215
5.6.8 Enzyme-linked immunosorbent assay (ELISA) analysis (NB12, p24) 216
5.6.9 Mass spectrometry analysis of phage displayed peptides (NB15, p80) ................................................................................................................................. 217
5.6.10 Purification of modified CinA from the periplasmic space (NB1, p61, p63, p69) ......................................................................................................................... 217
5.6.11 Coexpression studies for nisin, lacticin 481, haloduracin, and prochlorosins ......................................................................................................................... 219
5.7 REFERENCES ..................................................................................................................... 222
LIST OF FIGURES

Figure 1.1 Formation of lanthipeptides ................................................................. 3
Figure 1.2 Representation of several different lanthipeptides ............................. 5
Figure 1.3 Representation of the lanthionine forming enzymes that define the classes of lanthipeptides ................................................................. 7
Figure 1.4 Representation of peptidoglycan formation on the cell membrane.... 12

Figure 2.1 The structure of cinnamycin ................................................................. 33
Figure 2.2 The gene cluster for cinnamycin production, regulation, and immunity from Streptomyces cinnamoneus DSM 40005 ........................................ 34
Figure 2.3 MALDI-ToF MS analysis of IAA modification assay of CinA modified in vitro by CinM ................................................................. 37
Figure 2.4 Tris-HCl 4-20% Gradient SDS-PAGE for (A) His_{10}-CinM (121,312 Da) and (B) His_{6}-CinX (MW 38,859 Da) after purification by IMAC .............. 39
Figure 2.5 MALDI-ToF MS analysis of hydroxylation by CinX ......................... 39
Figure 2.6 Order of CinM and CinX post-translational modifications for CinA .... 40
Figure 2.7 Importance of the leader sequence for enzymatic activity of CinM and CinX ................................................................. 41
Figure 2.8 Tandem MS/MS analysis of CinA1-19 treated with His_{6}-CinX in vitro ................................................................. 41
Figure 2.9 Detection of lysinoalanine ................................................................. 44
Figure 2.10 MALDI-ToF MS analysis of His_{6}-CinA(A–1K) modified by CinM and CinX in E. coli ................................................................. 46
Figure 2.11 Bioassay with the indicator strain B. subtilis LH45 ......................... 47
Figure 2.12 Use of o-phthalaldehyde (OPA) to monitor lysinoalanine formation 48
Figure 2.13 Use of o-phthalaldehyde (OPA) to monitor lysinoalanine formation and bioassay for the peptide obtained from coexpression studies .......... 50
Figure 2.14 Two other genes with high sequence similarity to cinorf7 were identified in the protein databases ................................................................. 54

Figure 3.1 Sequence alignment of Cinorf7-like genes ......................................... 78
Figure 3.2 Use of o-phthalaldehyde (OPA) to monitor lysinoalanine formation 82
Figure 3.3 Order of CinX and Cinorf7 modification ........................................... 83
LIST OF TABLES

Table 2.1 List of primers.................................................................................................................. 71
Table 3.1 List of primers.................................................................................................................. 112
Table 4.1 List of primers.................................................................................................................. 165
Table 5.1 List of the randomized peptides possessing the ProcA 2.8 wild type leader peptide attached to a core region with the sequence C(XXX)₅S MPP S(XXX)₅C.......................................................................................................................... 220
Table 5.2 List of primers.................................................................................................................. 221
CHAPTER I: INTRODUCTION TO LANTHIPEPTIDES

1.1 General view on natural products

Natural products produced by living organisms such as animals, plants, and microbes often, have valuable biological activities and thus, they have been employed in a variety of areas including medicine, cosmetics, and the food industry (1). The isolation and discovery of pharmaceutically valuable compounds for the use of humans peaked in the period of 1970-1980 (2-3); however, the lack of new approaches to isolate new natural products in addition to a long and expensive development process has caused pharmaceutical companies to retract from this field in recent years (2). However, a new urgency for the discovery of new antibiotics that operate with different mechanisms of action compared to current antibiotics emerged as antibiotic-resistant infectious bacterial strains, such as methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant Enterococci (VRE), have started to appear in clinical settings (3). Many studies have demonstrated a clear relationship between exposure time to an antibiotic and the appearance of resistant strains (4). Therefore, higher usage of an antibiotic leads to development of resistant bacterial strains, requiring the continual discovery of new pharmaceutically available antibiotics.

The advancements in genome sequencing in the last two decades has allowed for re-investigation of the strains studied previously, yielding discovery of new natural products, which could not be obtained by the older techniques for
several reasons such as low amount of production leading to detection problems (5). One class of natural products that is much more abundant in the genomes than anticipated is the ribosomally-synthesized and post-translationally modified peptides (RiPPs) (6). These peptides contain an immense structural diversity compared to linear peptides, and the post-translational modifications provide tight target binding surfaces and increased metabolic and chemical stability of the molecules.

1.2 Lanthipeptides and their biosynthesis

Lanthipeptides are ribosomally-synthesized polycyclic peptides characterized by post-translationally introduced thioether rings called lanthionines (Lan) and methyllanthionines (Melan) (Figure 1.1A). These rings form by activation of specific serine (Ser) or threonine (Thr) residues followed by β-elimination to form dehydroalanine (Dha) and dehydrobutyrine (Dhb), respectively. Subsequent stereoselective intramolecular Michael-type addition of cysteine thiols to the dehydroamino acids completes the ring formation (Figure 1.2A). In addition to providing bioactivity to the peptide, these cross-links provide chemical and thermal stability to the peptide, important features in pharmaceutical applications.
Figure 1.1 Formation of lanthipeptides. (A) Representation of the mechanism of (Me)Lanthionine formation, (B) Representation of processing of lanthipeptides by enzymes to yield mature RiPP.
When the lanthipeptide precursors are synthesized by the ribosomes, they have an N-terminal sequence called the leader peptide that is recognized by various enzymes that process the C-terminal core peptide (Figure 1.1B). In addition to the enzymes that introduce (Me)Lan modifications, there are proteins that catalyze the formation of more than 15 different structural motifs such as lysinoalanine, β-hydroxyl-aspartate, D-alanine, 2-oxobutyrate, pyruvate, lactate, S-aminovinyl-D-cysteine and S-aminovinyl D-methylcysteine. Moreover recently, the first glycosylated labionin-containing lanthipeptide called NAI-112 was discovered (7). After the core peptide is processed, the leader peptide needs to be removed to afford the mature, biologically active compound. More than sixty lanthipeptides have been discovered to date and hundreds of them have been identified in genomic databases (Figure 1.2).
Figure 1.2 Representation of several different lanthipeptides. Lipid II binding motif of class I lantibiotics is circled in green, whereas class II-lipid II binding domain is circled in red. Lan are shown in red, MeLan are shown in blue. Several other post-translational modifications are shown in different colors.
1.2.1 Classes of lanthipeptides

Lanthipeptides can be classified into four classes based on the type of lanthionine synthetase that forms the lanthionine rings (Figure 1.3). In the first class, the dehydration and cyclization activities are carried out by individual enzymes called LanB and LanC whereas, in classes II, III and IV, both reactions are catalyzed by bifunctional lanthionine synthetases. The lanthionine synthetase LanM in class II contains a cyclization domain at the C-terminus that shows homology with the LanC cyclases of class I. However, the N-terminal dehydration domains of class II enzymes do not display sequence homology with other enzymes in the databases (Figure 1.3). Both class III and class IV enzymes contain a bifunctional enzyme with three domains. The central kinase domain and N-terminal phosphoSer/phosphoThr lyase domain of these two classes show homology to each other. However, the C-terminal cyclization domain of class III enzymes differ from class IV and the other LanC proteins as they lack the zinc ion that is used to activate the cysteines in the substrate for nucleophilic attack (5). A subset of class III enzymes generates an additional carbon–carbon crosslink, putatively by attack of the initially generated enolate onto a second dehydroalanine (Figure 1.1A) (8-9). The structure thus formed is called a labionin (Lab), which was first detected in the labyrinthopeptins (7, 10). The recently discovered Class IV enzymes (LanL) have three different domains, including N-terminal lyase and kinase domains as in class III, but its C-terminal cyclase domain is homologous to LanC (Figure 1.3) (11).
Some lantibiotics are composed of two components encoded by two different structural genes, *lanA1* and *lanA2*, that are subsequently modified by two different LanM protein, LanM1 and LanM2. Then, both peptides are exported and their leader sequences are removed by a multifunctional protein possessing an N-terminal protease domain called LanT as observed in class II lantipeptides. Generally, the α- and β-peptides of two-component lantibiotics formed from LanA1 and LanA2, respectively act synergistically to exert their full antibacterial activity, and currently this group includes eight lantibiotics including haloduracin and lacticin 3147 (12).
1.2.2 Role of leader peptides

The majority of the leader peptides of lantipeptides do not show homology to secretion signals commonly used to direct proteins to the sec and twin-arginine translocation (tat) secretion pathways (13). Their lengths range between approximately 20 to 60 amino acids and their sequences vary largely (13). Leader peptides have been proposed to have several different functions. Firstly, the leader peptide is required for recognition of the precursor peptide by several of the processing enzymes (14-18). It is proposed that the leader peptide might induce a conformational change in the processing enzymes, causing the enzymes to shift from an inactive form to an active form (13, 19). For example, studies on nisin biosynthesis demonstrated that the highly conserved residues in the leader peptide of NisA between –18 and –15 are crucial for lanthionine incorporation into the precursor peptide (20). Secondly, the leader peptide acts as a secretion signal, directing the peptide to the extracellular space of the producing organism (20-24). Finally, in most of the lantibiotics, the leader peptide provides immunity to the producing organism as biological activity is not gained until the removal of the leader peptide from the core (19-21), which is accompanied by export of the mature peptide outside the cell (13, 22, 25-27).

The removal of the leader peptide is performed by different sets of enzymes in different classes of the lantipeptides. In class I lantipeptides, a proteolytic enzyme called LanP, which has similarity to subtilisin-like serine proteases, removes the core region from the leader (25, 28-31) and a separate
enzyme called LanT is responsible for export of the molecule before or after function of LanP, depending on where LanP is located. In class II lanthipeptides, both proteolysis and secretion are typically performed by the dedicated ATP-binding cassette (ABC) transporter family proteins, called LanTs, which have a protease domain that utilizes Cys, His, and/or Asp residues for catalysis (32). The protease domains of LanTs share sequence homology with papain-like cysteine proteases. Site-directed mutagenesis of the protease domain of the lacticin 481 transporter showed that Cys12 is important for the cleavage reaction, whereas His90 affects regio-specificity (27). The prepeptide substrates of LanT share a conserved double-glycine type cleavage site (27).

1.3 Mode of action of lanthipeptides

Lantibiotics, lanthipeptides with antimicrobial activity, form the majority of known lanthipeptides. Among these peptides, a derivative of actagardine is in clinical trial for the treatment of Clostridium difficile infections (33-34), whereas mutacin 1140 is currently in preclinical development for the treatment of gram-positive bacterial infections (35). Lantibiotics also possess potent activity against many clinically relevant gram-positive bacteria (5), in addition to having potent activity against some gram-negative pathogens, such as Neisseria and Helicobacter (36). Lanthipeptides with low or no antimicrobial activity can also have pharmaceutically important properties. For example, despite possessing weak antibacterial activity, NAI-112 and the labyrinthopeptins are effective against nociceptive pain (7), and labyrinthopeptin A1 demonstrates broad anti-
human immunodeficiency virus (HIV) and anti-herpes simplex virus (HSV) activity (37). Moreover, the class II lantibiotic duramycin is in clinical trials for the treatment of cystic fibrosis (38-42).

Lanthipeptides gained interest due to the use of a lantibiotic called nisin as a food preservative for more than 50 years without significant development of resistant bacteria (43). The reason bacteria could not develop resistance to nisin may be due to its unique dual mode of action (44). Nisin kills mainly gram-positive bacteria through first binding to the pyrophosphate motif of lipid II by its A and B thioether rings. This binding lipid II inhibits cell wall biosynthesis (Figure 1.3, Figure 1.4) (45). Lipid II, which is composed of a polyprenyl pyrophosphate, N-acetyl-D-muramic acid pentapeptide (MurNAc-pp), and N-acetyl-D-glucosamine (GlcNAc), is a membrane-anchored cell-wall precursor that is essential for bacterial cell-wall biosynthesis (46). Through binding to lipid II, nisin uses its flexible hinge region located between residues 20-22 to orient itself (47) and form pores by insertion of its C-terminal tail into the cell membrane (48). Studies of nisin on the cell membrane suggests that eight nisin molecules form a complex together with four lipid II molecules to create pores in the membrane, which results in efflux of molecules from the cytoplasm of the gram-positive bacteria (49).

Since the discovery of the mode of action of nisin, many other lantibiotics have been shown to target lipid II to prevent the synthesis of peptidoglycan (46). For example, the A and B rings of nisin are conserved in other class I
lanthipeptides. Therefore, the lanthipeptides microbiosporicin (50) and mutacin 1140 (51) also exert their antimicrobial activity through binding to lipid II.

The first lanthipeptide known to bind to lipid II among class II lanthipeptides was mersacidin. Unlike nisin, mersacidin has a different binding motif, conserved in some of the other class II lanthipeptides (Figure 1.3), and it inhibits transglycosylation in a Ca^{2+}- dependent manner without forming pores (Figure 1.4) (52-53). Recently, lacticin 481 was shown to inhibit PBP1b-catalyzed peptidoglycan formation by binding to lipid II and not form pores in the membranes of susceptible bacteria (54). For the two-component lantibiotics lacticin 3147 and haloduracin, the α-peptide shares the mersacidin-binding motif and interacts with lipid II, which then recruits the β-peptide to form pores (55-56). Fluorescent labeling of lacticin 481 and haloduracin α showed that they localized predominantly at sites of new and old cell division as well as in punctate patterns along the long axis of rod-shaped bacilli, similar to the localization of lipid II. On the other hand, fluorophore labeled haloduracin β was localized nonspecifically in the absence of haloduracin α, but formed specific patterns when coadministered with haloduracin α. These data with living cells supports a model in which the α component recognizes lipid II and then recruits the β-component (57).

Molecular targets of a few more lanthipeptides are known, however little is known about the mode of action of other lanthipeptides. For example, Pep5 and epilancin K7 do not bind to lipid II but form pores on bacteria membrane suggesting other targets. In addition, cinnamycin and duramycins were shown to
bind to phosphatidylethanolamine (PE) with micromolar affinities, destabilizing the membrane without forming pores (38, 58-61).

Figure 1.4 Representation of peptidoglycan formation on the cell membrane. Nisin and well-known antibiotic vancomycin binding sites are labeled with red on lipid II (blue). Peptidoglycan has a dynamic structure provided by transglycosylation (green) and transpeptidation reactions (green).

1.4 Engineering of lanthipeptides and their biosynthetic enzymes

Lanthipeptide engineering methods are divided into two approaches, preparation in vitro or in vivo in the producing strain or other bacteria such as E. coli. These two techniques have their own advantages and limitations.

1.4.1 in vitro techniques

In vitro reconstitution of lantibiotic synthetases and other modifying enzymes involved in lanthipeptide biosynthesis has been accomplished for many
lanthipeptides. Activity has been reconstituted for the class I LanB and LanC enzymes involved in nisin biosynthesis: NisB (62) and NisC (63), and for many class II LanM enzymes such as CinM, LctM, HalM1 and HalM2, and ProcM which are involved in the formation of cinnamycin (16), lacticin 481 (64), haloduracin (15), and prochlorosins (18), respectively. In addition, activity has been reconstituted for class III enzymes LabKC, CurKC, and AciKC involved in the biosynthesis of labyrinthopeptin A2 (9), curvopeptin (8), and catenulipeptin (65) respectively, and for the class IV enzyme VenL involved in the biosynthesis of a lanthipeptide from *Streptomyces venezuelae* (11, 66).

*In vitro* reconstitution of these enzymes allows substrate preparation by chemical techniques such as solid phase peptide synthesis (SPPS), native chemical ligation, and click chemistry (67-75). The use of these chemical synthesis techniques drastically increased the range of chemical modifications which have been used to make chemically and biologically more stable and/or more effective lanthipeptides. For example, mutants of lacticin 481 containing the unnatural amino acids 3-(4-pyridyl)alanine (Pal) and 3-(2-naphthyl)alanine (Nal) were prepared via SPPS and were demonstrated to have more potent growth inhibition activity compared to authentic lacticin 481 (54, 76).

Many lanthipeptide producing enzymes have relaxed substrate specificity for substrates prepared by site-directed mutagenesis. To illustrate, a series of mutants of lacticin 481 was prepared *in vitro* by incubating mutant precursor
peptides prepared via site-directed mutagenesis of LctA and the processing enzyme LctM to yield peptides with altered antimicrobial potencies (19, 76).

1.4.2 Production of lanthipeptides in *E. coli* and their native producing strains

Production of lanthipeptides inside the cells has many other advantages compared to the *in vitro* techniques. Firstly, reconstitution of enzyme activity *in vitro* is a laborious process as it can be difficult to identify the small molecules needed for activity. In addition, the proteins that are active *in vivo* might not be stable *in vitro*, and in the cases where heterologous strains are used for expression, problems with codon usage can arise. Finally, *in vivo* techniques can result in a higher yield of pure lanthipeptides as many steps, for instance purification of enzymes is avoided.

Many lanthipeptides from the first three classes have been expressed and isolated from the producing strains (77-80). In addition, *E. coli* production of several lanthipeptides including nisin (56), lacticin 481 (56), haloduracin (56), prochlorosins (81), cinnamycin (16), two lantibiotics from *Geobacillus thermodenitrificans* (82), lichenicidin (83), bovicin HJ50 (84), and ala(0)actagardine (85) has been described.

1.4.3 Engineering of lanthipeptides via employing leader peptides

Lanthionine processing enzymes modify the C-terminal core region of the precursor peptides via guidance of the leader peptide as described above. The presence of the leader peptide is crucial for enzyme activity, however as it needs
to be removed for bioactivity of the product it presents drawbacks for *in vitro* studies with synthetic substrates, which require ligation strategies. A study performed on the lacticin 481 lantibiotic synthetase, LctM, showed that the leader region of LctA, can be attached to the N-terminus of the enzyme to make it *act in vitro* on the core peptides without leader peptides (19). Also, this engineered enzyme allowed the processing of lacticin 481 analogues containing non-proteinogenic amino acids (19).

The fact that different core regions can be processed by different lantibiotic synthetases as long as the matching leader peptide is attached to the core region (13) opens up new possibilities to engineer new lanthipeptides. Discovery of 29 natural lanthipeptides called prochlorosins, which are formed by the action of a single enzyme called ProcM, shows that nature already exploits the substrate substrate tolerant property of these enzymes (86). These findings suggest that lantibiotic synthetases can be used to engineer natural products, perhaps with desired properties and defined ring structures (86-87). This approach can also be used to make lanthipeptide libraries. Recently, a study showed that bacterial display of lanthipeptide libraries with defined structures can be achieved by post-translational modification of the peptides by lanthionine-synthetase enzymes in *Lactococcus lactis*, followed by export and sortase-mediated covalent coupling to the lactococcal cell wall (88). In addition, a library with $10^{11}$ different lanthionine containing peptides was formed using *in vitro*
translation coupled with an enzyme-free protocol for post-translational modification (89).

Lanthionine synthetases have also uses for preparation of a unique functional group for chemoselective modification to label lanthipeptides and thus visualize their cellular localization. For example, the cellular locations of lacticin 481, haloduracin α, and haloduracin β were identified by aminooxy-derivatized alkyne or fluorophore labeling an α-ketoamide, which was spontaneously generated by hydrolysis of the N-terminal enamine as a result of introduction of an N-terminal dehydro amino acid by the cognate biosynthetic enzymes (57).

1.4.4 Engineering of the enzymes involved in biosynthesis of lanthipeptides

Engineering of the enzymes involved in biosynthesis of lanthipeptides is another way to utilize the lanthipeptide biosynthesis machinery. Lanthionine synthetases in classes II, III, and IV have been shown to activate serine and threonine residues in the core region by phosphorylation, which is followed by elimination. This property of lanthionine synthetases was exploited in a study where the class II lanthipeptide synthetase ProcM was engineered to generate a variety of peptides containing O-phosphoserine (pSer) and O-phosphothreonine (pThr) residues, either in vitro or in vivo (90). Phosphorylation is an abundant post-translational modification involved in a myriad of cell signaling pathways thus through the help of this study one can prepare pharmaceutically valuable phosphorylated peptides to study cell signaling enzymes and processes (91-92).
1.4.5 Chemical synthesis

Chemical synthesis of lanthipeptides is a challenging task, since these molecules are composed of several thioether crosslinks at different locations. In addition, these molecules contain other post-translational modifications in addition to lanthionines and introduction of these modifications on a peptide with a variety of reactive groups of the amino acid side chains is tough chemically (67). However, although challenging, in recent years scientists developed creative ways to synthesize these large molecules. The first successful total chemical synthesis of a lanthipeptide was the remarkable solution-phase synthesis of nisin (93). After this successful representation and with the employment of orthogonally protected Lan amino acid, chemical synthesis of the α-peptide of lacticin 3147 containing overlapping rings (94), lactocin S (74), analogs of the β-peptide of the lacticin 3147 (94-95), epilancin 15X (96), and lacticin 481 (97) have also been achieved.

1.4.6 Summary and outlook

Natural product discovery is an important way to combat the growing bacterial resistance problem observed in clinical settings (98). The current pool of antibiotics is unable to handle the challenges arising in this field. One way to fight so-called superbugs is to discover new antimicrobials with different modes of action. Lanthipeptides have proved to be pharmaceutically valuable, which includes having antimicrobial activity against clinically-relevant bacterial pathogens including drug-resistant strains. Investigations on the biosynthesis and
mode of action of lanthipeptides would be useful for engineering of these peptides to obtain better pharmacological properties. In this thesis, biosynthesis of several lanthipeptides belonging to the cinnamycin group of peptides is described, and the biosynthetic enzymes of cinnamycin were studied in detail. In Chapter II, the biosynthesis of cinnamycin was investigated by reconstitution of the cinnamycin biosynthetic enzyme activities in vitro, and through heterologous expression of the cinnamycin biosynthetic genes in E. coli. These two approaches employed to produce cinnamycin have their own advantages. For instance, in vitro reconstitution of the lantibiotic synthetase CinM and the tailoring enzyme CinX allowed for detailed studies of these enzymes as described in Chapter III. On the other hand, through production of cinnamycin in E. coli, the activity of a new enzyme called Cinorf7 was discovered, which could not be achieved in the in vitro experiments. Therefore, Chapter III also contains our attempts to identify the catalytically important residues of Cinorf7 by mutational studies. These experiments on cinnamycin biosynthesis set the stage for the biosynthesis of other lanthipeptides in the cinnamycin-group of peptides, which is described in Chapter IV. Lanthipeptide synthetases have been shown to have relaxed substrate specificity, creating new possibilities in the engineering of these molecules for desired functions. Peptide libraries have been commonly used for engineering and selection of pharmaceutically valuable molecules. Lanthipeptides have great potential for drug development compared to linear peptides as they are chemically and metabolically more stable because of the
thioether crosslinks. Therefore, large molecular libraries of lanthipeptides with
designed ring structure would be useful. In Chapter V, phage display of the well-
known lanthipeptide nisin is described, which will form a platform for the
production and selection of lanthipeptide libraries.
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CHAPTER II: NINE POSTTRANSLATIONAL MODIFICATIONS DURING THE BIOSYNTHESIS OF CINNAMYCIN

2.1 INTRODUCTION

Cinnamycin is a 19 amino acid lantibiotic with antimicrobial activity against gram-positive rods such as bacilli, *Clostridium botulinum*, and mycobacteria (1). The compound also causes transbilayer phospholipid movement in the cell membrane of mammalian cells to access phosphatidylethanolamine (PE) residing in the inner leaflet of the membrane (2). The interaction of cinnamycin with PE renders the compound pharmacologically valuable. For example, duramycin, a close structural analog of cinnamycin, promotes chloride secretion in lung epithelial cells by binding to PE; this activity in turn promotes mucus clearance from the lungs. As a result, the compound is in phase II clinical trials for the treatment of cystic fibrosis, a common genetic disorder characterized by abnormal chloride ion transport (2-3). Cinnamycin has also been suggested as an alternative treatment for atherosclerosis through its ability to inhibit phospholipase A2 by binding to its substrate PE (4-5). Cinnamycin has a compact globular and relatively rigid structure with one lanthionine (Lan) and two methyllanthionine (MeLan) rings in addition to an unusual lysinoalanine (Lal) crosslink formed from lysine 19 and serine 6 (Figure 2.1).

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Unlike most lantibiotics, the rings are generated in a bidirectional manner such that the MeLan bridges are formed by cysteines that are located N-terminally to the dehydrobutyrine residues with which they react, and the Lan bridge is formed from a cysteine located C-terminal to the dehydroalanine with which it reacts (Figure 2.1). Similarly, the Lal crosslink is generated from a Lys located C-terminal to the serine with which it forms a bridge. Unlike the Lan and MeLan structures, which have D-stereochemistry at the α-carbon originating from dehydroalanine/dehydrobutyrine (Dha/Dhb), the Lal crosslink has the L-configuration at the α-carbon originating from Ser, raising the question whether it is formed with the intermediacy of a Dha or not (6-8). In addition to the macrocyclic structures, cinnamycin also contains an erythro-3-hydroxy-L-aspartic acid resulting from the hydroxylation of L-aspartate at position 15 (Figure 2.1) (6-8). The binding of cinnamycin to its target PE is primarily mediated by interaction of this posttranslational modification with the ammonium group of PE (4).

The cinnamycin biosynthetic gene cluster (cin) from *Streptomyces cinnamoneus* DSM 40005 was reported providing opportunities to investigate the biochemistry of its biosynthetic pathway (9). The cinnamycin gene cluster contains 21 open reading frames (17,083 bp), which include the genes for cinnamycin production, regulation, and self-resistance (Figure 2.2) (9). The cinnamycin precursor peptide, CinA, consists of a C-terminal core region that is 19 residues long and that will be transformed into cinnamycin through posttranslational modifications. Appended to the N-terminus of the core peptide
is an unusually long 59 amino acid leader sequence that is not modified. Instead of a GG or GA protease cleavage motif seen in most class II lantibiotics, an AXA motif is present between the leader sequence and the core region of CinA (Figure 2.1). This sequence is recognized by type I signal peptidases of the general secretory (sec) pathway, which explains the absence of a cinnamycin-specific protease in the gene cluster (10). The CinM sequence shows high homology with other class II LanM enzymes which are bifunctional enzymes that catalyze both dehydration and cyclization (9, 11). However, the enzymes responsible for Asp hydroxylation and Lal bridge formation have not been explored. After the core peptide is processed, the leader peptide is removed proteolytically to afford the mature, biologically active compound (12).

Understanding the biosynthetic pathway of cinnamycin may be used to generate analogs with potentially improved properties for treatment of cystic fibrosis or clostridium infections. Herein, we investigated the biosynthetic machinery of cinnamycin in vitro and characterized the enzymes involved in the formation of the thioether crosslinks and the β-hydroxylation of aspartate 15. In addition, we developed a coexpression system in E. coli that resulted in fully modified CinA and identified cinorf7, an open reading frame of unknown function, as a critical determinant for lysinoalanine formation.
Figure 2.1 The structure of cinnamycin. Lan and MeLan are shown in red and blue respectively, Lal is depicted in purple, and the hydroxylated Asp is shown in green. The sequence of the CinA core and leader peptides is depicted as well as the residues that form the crosslinks. The sec-cleavage sequence at the end of the leader peptide is shown in bold underlined font.
Figure 2.2 The gene cluster for cinnamycin production, regulation, and immunity from *Streptomyces cinnamoneus cinnamoneus* DSM 40005 (9).
2.2 RESULTS

2.2.1 *In vitro* reconstitution of the enzymatic activity of the lanthionine synthetase, CinM

The gene for the precursor peptide CinA was cloned into a pET15b vector and the gene encoding CinM was cloned into a pET16b vector to generate N-terminal histidine tagged fusion proteins upon heterologous expression. Site-directed mutagenesis was used to mutate the alanine residue at the last position of the leader peptide of CinA (position –1) to lysine (Dr. Lisa E. Cooper, Thesis 2009). The resulting His$_6$-CinA(A–1K) mutant enables removal of the leader peptide using the commercial endoproteinase LysC for bioactivity assays. This substrate analog was used as a precursor peptide throughout this study. Both His$_6$-CinA(A–1K) and His$_{10}$-CinM were heterologously expressed in *E. coli* BL21 DE3 cells and purified by immobilized metal ion affinity chromatography (IMAC). Approximately 6-10 mg of His$_{10}$-CinM was obtained per liter of cell culture. The His$_6$-CinA(A–1K) peptide was further purified after IMAC by reversed-phase high-performance liquid chromatography (RP-HPLC) resulting in 40 mg of purified peptide per liter of cell culture that was detected as the Na$^+$ adduct after LysC protease cleavage (Figure 2.3A). The enzyme CinM migrated as a protein of approximately 120 kDa by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and gel filtration analysis, close to the predicted monomeric molecular weight of His$_{10}$-CinM (118.8 kDa) (Figure 2.4A).
In order to evaluate dehydration activity, His$_6$-CinA(A–1K) (20 μM) was incubated with His$_{10}$-CinM (5 μM) at 25 ºC for 6 h in the presence of adenosine triphosphate (ATP), MgCl$_2$, and immobilized tris(2-carboxyethyl)phosphine (TCEP). Use of immobilized reducing agent was required to prevent unwanted additions of reducing agents (TCEP/ dithiothreitol) to the dehydrated precursor peptide. Analysis of the core peptide by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-ToF MS) after LysC endoproteinase treatment revealed a mass corresponding to loss of four water molecules (Figure 2.3B). This finding demonstrates that CinM is responsible for the dehydration of the two threonine (Thr11 and Thr18) and two serine residues (Ser4 and Ser6) in the core region of CinA. Addition of cysteine thiols to the dehydroamino acids does not cause a change in mass, which prevents observation of cyclization by MALDI-ToF MS. To investigate the cyclization activity of CinM, iodoacetamide (IAA), a thiol alkylating agent that induces a mass increase upon reaction with cysteine thiols, was used. His$_6$-CinA(A–1K) and His$_6$-CinA(A–1K) modified by His$_{10}$-CinM were treated with IAA and LysC protease and then subjected to MALDI-ToF MS analysis. As expected, His$_6$-CinA(A–1K) that had not been treated with His$_{10}$-CinM showed a mass increase corresponding to alkylation of the three cysteines in the core peptide (Figure 2.3D). In contrast, only minor alkylation products were observed in the His$_{10}$-CinM treated sample (Figure 2.3C). The data strongly support conversion of the majority of the free thiols to thioether rings by His$_{10}$-CinM, although one
Cys appears to be less efficiently cyclized resulting in some peptide with one IAA adduct (Figure 2.3C). Taken together, these results demonstrate that CinM functions as a bifunctional LanM enzyme that catalyzes both dehydration and cyclization reactions to form the thioether crosslinks. CinM did not, however, form the lysinoalanine crosslink (*vide infra*).

Figure 2.3 MALDI-ToF MS analysis of IAA modification assay of CinA modified *in vitro* by CinM. (A) MALDI-ToF MS of His$_6$-CinA(A–1K) treated with LysC, (B) MALDI-ToF MS of His$_6$-CinA(A–1K) after incubation with His$_{10}$-CinM followed by LysC treatment, (C) MALDI-ToF MS of His$_6$-CinA(A–1K) after incubation with His$_{10}$-CinM followed by IAA and LysC treatment, and (D) MALDI mass spectrum of His$_6$-CinA(A–1K) after IAA and LysC treatment. CP = CinA core peptide.

*Calculated mass for CP + Na$^+$ + H = 2097.3 + 23 + 1 = 2121 Da, Obsvd: 2120.27 Da; CP – 4 H$_2$O + H; 2097.3 – 4 * 18 + 1 = 2026.3, Obsvd: 2026.2 Da; CP – 4 H$_2$O + IAA + H (M$^+$); 2097.3 – 4 * 18 + 57 + 1 = 2083.3, Obsvd: 2084.6 Da; CP + 3 IAA + H = 2097.3 + 3 * 57 + 1 = 2269 Da, Obsvd: 2271.3 Da.
2.2.2 In vitro reconstitution of CinX

The *cinX* gene was cloned into pET28b and was heterologously expressed in *E. coli* with an N-terminal His<sub>6</sub>-tag, and purified by immobilized metal ion affinity chromatography (IMAC). His<sub>6</sub>-CinX migrated as a monomer protein of approximately 38 kDa in gel filtration and SDS-PAGE analysis (Figure 2.4B). Sequence analysis suggested that CinX may be a member of the non-heme Fe(II)-dependent family of enzymes that utilizes β-ketoglutarate (α-KG) as cofactor.

His<sub>6</sub>-CinA(A–1K) was modified with His<sub>10</sub>-CinM followed by His<sub>6</sub>-CinX treatment in the presence of Fe(II) and α-KG for 2 h. After subsequent digestion with LysC, four dehydrations and a hydroxylation were observed in the core region of CinA by MALDI-ToF MS (Figure 2.5). In the absence of either Fe(II) or α-KG, no mass change corresponding to hydroxylation was observed, which supports the hypothesis from its sequence that CinX is an α-KG/Fe(II)-dependent hydroxylase.

The order in which CinM and CinX carry out their posttranslational modifications was also investigated. *In vitro* these two enzymes did not have a compulsory order of action as His<sub>10</sub>-CinM was able to dehydrate and cyclize His<sub>6</sub>-CinA that had been hydroxylated by His<sub>6</sub>-CinX (Figure 2.6), and His<sub>6</sub>-CinX was able to hydroxylate His<sub>6</sub>-CinA that had been dehydrated and cyclized (Figure 2.5). To validate the site of hydroxylation, aspartate 15 was mutated to alanine
and the mutant CinA peptide was incubated with His$_6$-CinX. MALDI-ToF MS analysis showed no change in the mass of the mutant peptide (data not shown).

Figure 2.4 Tris-HCl 4-20% Gradient SDS-PAGE for (A) His$_{10}$-CinM (121,312 Da) and (B) His$_6$-CinX (MW 38,859 Da) after purification by IMAC.

Figure 2.5 MALDI-ToF MS analysis of hydroxylation by CinX. His$_6$-CinA(A–1K) modified by His$_{10}$-CinM and subsequently digested with LysC is shown in black. His$_6$-CinA(A–1K) modified by His$_{10}$-CinM and then His$_6$-CinX and subsequent digestion with LysC is shown in red. CP = CinA core peptide.

*Calculated mass for black; CP – 4 H$_2$O + H (SM); 2097.3 – 4 * 18 + 1 = 2026.3, Obsrvd: 2026.2 Da, red; CP – 4 H$_2$O + OH + H (SM); 2097.3 – 4 * 18 + 16 + 1 = 2042.3, Obsrvd: 2043.7 Da.
In lantibiotics, the leader peptide is required for activity of most of the biosynthetic enzymes (12). To evaluate if this was also the case for His_{10}-CinM and His_{6}-CinX, the core peptide of CinA (CinA1-19) was synthesized by solid phase peptide synthesis. As expected, His_{10}-CinM showed no activity in the absence of the leader peptide (Figure 2.7A). In contrast, His_{6}-CinX was capable of hydroxylating CinA(1-19) (Figure 2.7B). LC-MS/MS analysis of the product further verified that the hydroxylation occurs on Asp15 (Figure 2.8).

![Figure 2.6 Order of CinM and CinX post-translational modifications for CinA.](image)

**Figure 2.6 Order of CinM and CinX post-translational modifications for CinA.** MALDI-TOF mass spectra of CinA peptide modified by CinX before (black line) and after incubation with CinM (red line). Samples were cleaved with LysC prior to MS analysis. A peptide fragment arising from LysC cleavage of CinX (CinA A25-51) causes suppression of the other peaks. *Calculated mass for M - 4 H_2O + OH + H (M') = 2097.3 – 4 * 18 + 16 + 1 = 2042.3, Obsrvd: 2043.4 Da.*
Figure 2.7 Importance of the leader sequence for enzymatic activity of CinM and CinX. (A) MALDI mass spectrum of CinA1-19 treated with His$_{10}$-CinM, (B) MALDI mass spectrum of CinA1-19 treated with His$_{6}$-CinX.


Figure 2.8 Tandem MS/MS analysis of CinA1-19 treated with His$_{6}$-CinX in vitro. The y''4 and y''5 ions define Asp15 as the site of hydroxylation, which is also consistent with all other observed fragment ions.
2.2.3 Lysinoalanine formation and bioactivity assays

Genetic studies in the producing organism have shown that \textit{cinA}, \textit{cinM}, \textit{cinX} and \textit{cinorf7} are required for formation of cinnamycin (13). Having assigned the function of the first two enzymes, we investigated whether Cinorf7 was responsible for Lal formation. Cinorf7 was expressed and purified as an N-terminal hexa histidine fusion protein and added to various assays containing His\textsubscript{6}-CinA and His\textsubscript{10}-CinM and/or His\textsubscript{6}-CinX, with various orders of addition and supplementation with cofactors. Unfortunately, Lal was not formed under any defined conditions as determined using a variety of assays (see Chapter III for detailed description). Non-enzymatic formation of Lal, which results in both L and D configurations, has been observed in food products under alkaline conditions by chemical dehydration of Ser and non-selective conjugate addition of lysine to the resulting Dha (14). Based on these observations, we investigated the non-enzymatic \textit{in vitro} formation of the Lal ring under mild alkaline conditions (pH 9.5) after the precursor peptide His\textsubscript{6}-CinA(A–1K) had been processed with His\textsubscript{10}-CinM and His\textsubscript{6}-CinX. The leader peptide was removed with LysC endoproteinase, and the resulting product was analyzed for Lal formation. The product was first hydrolyzed in strongly acidic conditions and the resulting amino acids were derivatized to the corresponding methyl esters and pentafluoropropionyl amides (15-16). Authentic cinnamycin was subjected to the same treatment to provide a standard. Subsequent analysis by gas chromatography-tandem mass spectrometry (GC-MS/MS) demonstrated that derivatized Lal from authentic
cinnamycin eluted as two peaks, probably due to epimerization during the acidic hydrolysis. The same retention times for these two peaks were observed for Lal derived from both authentic cinnamycin and the in vitro assay sample (Figure 2.9A & B). The fragmentation pattern of the peaks in the GC-MS were also identical and consistent with those in the literature for lysinoalanine derivatives (Figure 2.9C & D) (17). Thus, these experiments verify formation of the lysinoalanine ring at increased pH, but because the Lal residue originating from authentic cinnamycin was epimerized, we were unable to establish whether non-enzymatic Lal formation was stereoselective.

To investigate if the non-enzymatic Lal cyclization product has the correct stereochemistry, an antimicrobial assay was employed, under the assumption that cyclization with the incorrect stereochemistry would give an inactive product. A well diffusion assay against the indicator strain B. subtilis LH45 demonstrated clear antimicrobial activity of cinnamycin synthesized in vitro. A sample that was not treated under alkaline conditions did not produce a zone of growth inhibition, which demonstrates the requirement of Lal for antimicrobial activity of cinnamycin. Unfortunately, this in vitro process to produce cinnamycin did not allow quantitative comparison of its bioactivity with authentic cinnamycin because the sample contained unknown amounts of material derived from the biosynthetic enzymes, the leader peptide, and LysC. Therefore, the concentration of cinnamycin within the sample was not known. Attempts to purify the cyclized core
peptide by RP-HPLC produced very low quantities of cinnamycin, preventing comparison with authentic cinnamycin.

Figure 2.9 Detection of lysinoalanine. (A) GC-MS analysis of His$_6$-CinA(A–1K) processed with His$_{10}$-CinM and His$_6$-CinX in vitro followed by LysC protease treatment, acid hydrolysis, and derivatization (B) GC-MS analysis of cinnamycin standard (Novacta) after acidic hydrolysis and derivatization (C) Mass spectrum of the lysinoalanine (Lal) peak of the sample in A, (D) Mass spectrum of the lysinoalanine (Lal) peak of cinnamycin standard (Novacta). All samples were treated with 6 M HCl and derivatized as described in the Methods section. The major peaks observed in panels C and D are 190 Da (CF$_3$CF$_2$CONHC$_2$H$_4$), 230 Da (CF$_3$CF$_2$CONHC$_2$H$_6$), 465 Da (M – CF$_3$CF$_2$CONHCHCO- OCH$_3$), 405 Da (465 Da-CH$_3$OCOH), 433 Da (465 Da-CH$_3$OH), 640 (M – CH$_3$OCO), M is Lal: 699 Da.

2.2.4 Coexpression of CinA, CinM, CinX in E. coli (NB10, p28, p35)

Quantification of Lal formation after alkaline treatment by GC-MS has been challenging because of the large background resulting from the leader peptide and the biosynthetic enzymes. In an effort to improve both the amounts
of material and their purity, we turned to coexpression of the CinA peptide and the CinM and CinX modification enzymes in *E. coli*. Recent studies in several laboratories have demonstrated the feasibility of conducting the post-translational modifications that generate lantibiotics in this heterologous host (18-21). In our laboratory, pRSFDuet-1 and pACYCDuet-1 plasmids were used for these studies, which offer a convenient purification approach of the post-translationally modified peptide using IMAC because of a His$_6$-tag at the N-terminus of the leader peptide of the substrate peptide (20). A similar approach was employed here in an attempt to obtain higher quantities of peptide processed by CinM and CinX.

The gene encoding CinA(A–1K) with an N-terminal flag tag was inserted into multiple cloning site I (MCSI) of the pRSFDuet-1 vector to generate His$_6$-Flag-CinA(A–1K) (22). The gene encoding CinM was inserted into MCSII of the same plasmid resulting in a protein without a His-tag, thus preventing co-purification of the CinM enzyme, which complicated the *in vitro* experiments. Similarly, *cinX* was cloned into MCSII of the pACYCDuet-1 vector, which also produces CinX without a His-tag.
Figure 2.10 MALDI-ToF MS analysis of His$_6$-CinA(A–1K) modified by CinM and CinX in E. coli. (A) MALDI mass spectrum of His$_6$-CinA(A–1K) modified by CinM and CinX in E. coli and treated with LysC; (B) MALDI mass spectrum of iodoacetamide (IAA) treatment of the coexpression product followed by LysC cleavage. The ion marked with an asterisk is M – 4 H$_2$O.

*Calculated mass for M – 4 H$_2$O + OH + H (M'); 2097.3 – 4 * 18 + 16 +1 = 2042.3, Obsrvd: 2043.1 Da, M' + IAA = 2042.3 + 57 = 2099.3 Da, Obsrvd: 2099.5 Da.

Coexpression of CinM and CinX together with His$_6$-Flag-CinA(A–1K) yielded 2 mg of His$_6$-Flag-CinA(A–1K) peptide per liter of cell culture after IMAC purification and subsequent RP-HPLC. MALDI-ToF MS analysis of the LysC-treated peptide showed a mass difference with respect to unmodified core peptide corresponding to four dehydrations and one hydroxylation (Figure 2.10A). In vivo thioether cyclization activity was investigated by IAA treatment and analysis by MALDI-ToF MS as previously described. Similar to the in vitro results, CinM formed predominantly three thioether rings in the CinA core region (Figure 2.10B).
The peptide obtained from coexpression did not have antimicrobial activity towards the indicator strain *B. subtilis* LH45 similar to the observations for the product of CinM and CinX treatment *in vitro*. However, when Lal cyclization was induced by mild alkaline treatment, the peptide showed strong antimicrobial activity comparable to authentic cinnamycin (Figure 2.11).

![Figure 2.11 Bioassay with the indicator strain *B. subtilis* LH45. Sector 1, authentic cinnamycin; sector 2, His₆-CinA(A–1K) modified by CinM and CinX in *E. coli* and treated with LysC followed by incubation at pH 9.5; sector 3, His₆-CinA(A–1K) modified by CinM and CinX in *E. coli* treated with LysC (no alkaline treatment); sector 4 was not used.](image)

With larger quantities of material in hand, the peptide produced in *E. coli* was used to investigate non-enzymatic Lal formation in more detail. o-Phthalaldehyde (OPA) is a reagent that reacts selectively with primary amines in the presence of a thiol such as β-mercaptoethanol but does not react with secondary amines (23). Thus, OPA was used to monitor conversion of the primary ε-amine of Lys to the secondary amine in Lal. CinA(A–1K) that had been
modified by CinM and CinX in *E. coli* and then incubated with LysC was treated with OPA, causing a mass increase corresponding to two OPA additions in the core region. These two OPA adducts originate from the N-terminal amino group and the ε-amino group of Lys. Furthermore, one β-mercaptoethanol addition was observed as a consequence of Michael-type addition to dehydroalanine 6 (Figure 2.12A).

**Figure 2.12 Use of o-phthalaldehyde (OPA) to monitor lysinoalanine formation.** (A) CinA(A–1K) modified by CinM and CinX, and treated with LysC was incubated with OPA and β-mercaptoethanol. (B) CinA(A–1K) modified by CinM and CinX followed by alkaline treatment and removal of the leader peptide with LysC was incubated with OPA and β-mercaptoethanol. (C) Authentic cinnamycin was incubated with OPA and β-mercaptoethanol. SM = mass of core peptide after 3 dehydrations and one hydroxylation. Asterisk indicates fourfold dehydrated core peptide that did not react with OPA.

*Calculated mass for CP – 4 H₂O + OH + H (SM): 2097.3 – 4 * 18 + 16 + 1 = 2042.3, SM + OPA = 2042.3 + 180.1 = 2222.4, Obsrvd: 2221.84 Da, Cinnamycin + OPA: SM + OPA, Obsrvd: 2220.2 Da, SM + 2 OPA + BME + H = 2042.3 + 2 *180.1 + 78.1 = 2481.6, Obsrvd: 2481.4 Da.*
Collectively, these data clearly show the absence of a lysinoalanine crosslink. In contrast, the OPA assay of the same sample after alkaline treatment resulted in only one OPA addition and no β-mercaptoethanol adducts, which was also observed when authentic cinnamycin was used as a positive control (Figure 2.12B, C). The absence of the peak corresponding to the formation of two OPA and one β-mercaptoethanol adducts in this sample suggests that the cyclization occurred with near quantitative efficiency.

2.2.5 Coexpression of CinA, CinM, CinX and Cinorf7 in E. coli

The in vitro studies did not provide any insights into the possible role of Cinorf7 in cinnamycin biosynthesis. In an alternative approach, the coexpression system in E. coli was used to investigate whether Cinorf7 is involved in formation of the Lal crosslink in vivo. For this purpose, the cinorf7 gene was cloned into MCSI of pACYCDuet-1 that already contained the gene encoding CinX in MCSII. The resulting plasmid was used with the pRSFDuet-1 vector containing the genes for CinA(A–1K) and CinM for coexpression in E. coli. The resulting peptide was purified by IMAC, treated with LysC and analyzed with the OPA assay. The resulting mass spectrum showed that the Lal crosslink was formed without the need of raising the pH (Figure 2.13A). Also, an antimicrobial assay towards the indicator strain B. subtilis LH45 verified that the activity of cinnamycin synthesized heterologously in E. coli was similar to the antimicrobial activity of authentic cinnamycin (Figure 2.13B). Because in the absence of Cinorf7 both the OPA assay and the antimicrobial assay were negative for the peptide produced
in *E. coli*, these results reveal that Cinorf7 is required for the formation of the Lal crosslink.

Figure 2.13 Use of o-phthalaldehyde (OPA) to monitor lysinoalanine formation and bioassay for the peptide obtained from coexpression studies. (A, top) CinA(A–1K) modified in *E. coli* by CinM, CinX, and Cinorf7 followed by *in vitro* LysC cleavage; (A, middle) The peptide form the top spectrum analyzed by OPA assay as described in the text; (A, bottom) Authentic cinnamycin analyzed by OPA assay as a positive control. (B) The outer ring of spots shows various concentrations of authentic cinnamycin, the inner ring of spots shows LysC-cleaved coexpression product (CinA(A–1K), CinM, CinX, Cinorf7). Concentrations: spot 1: 60 μg/mL, 2: 30 μg/mL, 3: 15 μg/mL, 4: 10 μg/mL, 5: 7.5 μg/mL, 6: 5 μg/mL, 7: 2.5 μg/mL, 8: 1 μg/mL.

*Calculated mass for CP – 4 H₂O + OH + H (M'); 2097.3 – 4 * 18 + 16 = 2042.3, M' + OPA = 2042.3 + 180.13 + 1 = 2222.4 Observed mass (B) 2221.8 Da, (C) 2218.36 Da.*
2.3 DISCUSSION

The *in vitro* biosynthesis of cinnamycin was achieved in this work by reconstitution of the activities of two enzymes in the biosynthetic pathway, CinM and CinX. His_{10}^-CinM dehydrated two threonine and two serine residues in the core region of His_{6}^-CinA and catalyzed the subsequent cyclization by cysteine thiols to form three thioether rings. The observation that CinM dehydrated Ser6 confirms that the Lal crosslink is formed with the intermediacy of a dehydroalanine despite the L-stereochemistry at the α-carbon of the former Ser residue. Although His_{10}^-CinM dehydrated Ser6, it did not catalyze the formation of the Lal ring. Considering that LanC/LanM enzymes typically stereoselectively protonate the enolate formed during the Michael-type addition to provide D-stereochemistry at the α-carbon, it is not surprising that Lal formation, which results in the L-configuration at the newly formed stereogenic center, is not catalyzed by CinM. In addition, CinM is much better suited to activate a thiol nucleophile with the Zn^{2+} in its active site than to activate a primary amine (24-25).

CinX was shown to be an Fe(II)/α-ketoglutarate-dependent hydroxylase that catalyzes the β-hydroxylation of Asp15 in the CinA precursor peptide. Interestingly, His_{6}^-CinX accepted the core region of CinA as a substrate without the need of the leader sequence. Similar observations were made for the oxidative decarboxylases MrsD and EpiD (26-27), suggesting that tailoring enzymes that introduce post-translational modifications beyond dehydration and
cyclization may not require the leader peptide. However, as observed for other lanthionine synthetases (28-30), His\textsubscript{10}-CinM required the leader peptide for efficient processing. Changing the order of incubation of the precursor peptide with His\textsubscript{10}-CinM or His\textsubscript{6}-CinX resulted in the same product.

*In vitro* biosynthesis of cinnamycin was accomplished by inducing non-enzymatic formation of the Lal by raising the pH after treatment of CinA with His\textsubscript{10}-CinM and His\textsubscript{6}-CinX *in vitro* or after heterologous coexpression of CinM and CinX with the precursor peptide in *E. coli*. The high bioactivity of the resulting peptide suggests that the non-enzymatic cyclization occurs with high stereoselectivity, assuming that cinnamycin with a Lal residue with incorrect stereochemistry has no or low antimicrobial activity. High stereoselectivity for non-enzymatic formation of Lan and MeLan has been demonstrated previously (31-36), but no such investigations have been conducted for Lal formation. The Lan and MeLan residues generated by CinM may pre-organize the conformation of the peptide to favor stereoselective formation of Lal, similar to a recent proposal for a lanthionine synthetase that generates a large number of thioether crosslinks of high structural diversity (16).

Genetic studies have identified the cinorf7 gene to be essential in the biosynthesis pathway of cinnamycin (13). This gene, composed of just 360 nucleotides, is located immediately upstream of cinA and the two genes are cotranscribed (9). We show here that Cinorf7 is required for lysinoalanine formation. Unfortunately, *in vitro* reconstitution of its activity has been
unsuccessful to date. Sequence analysis of Cinorf7 shows that it does not have significant homology to other functionally-characterized proteins. Two other genes encoding proteins with high sequence similarity to Cinorf7 were identified in the protein databases (Figure 2.14A). One of these, found in the genome of *Lyngbya majuscule*, has two nearby likely precursor peptides whereas the second, found in the genome of *Frankia Sp.*, has one gene encoding a possible precursor peptide nearby (Figure 2.14B). Both cinorf7 orthologs also are near LanM-like genes. Although the sequences of the predicted leader peptides of these putative precursor peptides vary, their core regions showed very high sequence identity to the cinnamycin group (Figure 2.14B), suggesting these gene clusters have the genetic capacity to generate cinnamycin-like peptides. Experimental investigations of cinnamycin-like gene clusters are presented in Chapter IV.

In summary, I demonstrated in the studies described in this chapter that CinM is responsible for dehydration of two Ser and two Thr residues and also generates the three thioether crosslinks in cinnamycin. Furthermore, CinX hydroxylates a single Asp in a 78-residue substrate and also accepts just the core peptide as substrate. Finally, the data indicate that Cinorf7 is critical for the formation of the unique lysinoalanine crosslink.
Figure 2.14 Two other genes with high sequence similarity to cinorf7 were identified in the protein databases. (A) The ortholog from Lyngbya majuscula (ZP_08425231) has two possible precursor peptides close by in the genome. These two peptides are shown in panel B (ZP_08425235_02840 and ZP_08425237_02860). The second ortholog was found in Frankia Sp. It has one possible precursor peptide nearby (ZP_06416733_6431 in panel B) in addition to having one LanM-like gene. (B) The sequences of the predicted leader peptide of the three putative precursor peptides vary, but the core region showed very high sequence similarity to cinnamycin group of peptides. The predicted start of the core peptide is indicated with a vertical black line.
2.4 MATERIALS

Oligonucleotide primers for mutagenesis were synthesized by Operon Technologies. *Taq* and *Platinum Pfx* DNA polymerases, DpnI, restriction endonucleases, and bacteriophage T4 DNA ligase were purchased from Invitrogen. *Fusion* DNA polymerase was purchased from New England Biolabs. Cloning vectors (pET and pDuet) were obtained from Novagen. Gel extraction, plasmid mini-prep, and PCR purification kits were purchased from Qiagen. All strains were grown in media acquired from Difco laboratories. Other items procured include isopropyl-1-thio-D-galactopyranoside (IPTG, CalBiochem), iodoacetamide (IAA, Acros Organics), tris(2-carboxyethyl) phosphine hydrochloride (TCEP, Aldrich), and dithiothreitol (DTT, Sigma). Endoproteinase LysC was purchased from Roche Applied Science.
2.5 GENERAL METHODS

Molecular biological manipulations were carried out using standard techniques (37). PCR was performed using an automatic thermocycler (PTC 150, MJ Research) and DNA sequencing was performed at the Biotechnology Center of the University of Illinois at Urbana-Champaign (UIUC). *Escherichia coli* DH5α cells (UIUC Cell Media Facility) and *E. coli* BL21 (DE3) cells (Stratagene) were used for plasmid DNA preparation and protein expression, respectively. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-ToF MS) was carried out on a Voyager-DE-STR (Applied Biosystems). Liquid chromatography electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS) was carried out and processed using a Synapt ESI quadrupole ToF MS Mass Spectrometry System (Waters) equipped with an Acquity Ultra Performance Liquid Chromatography (UPLC) system (Waters). GC/MS-MS analysis (Agilent HP 5973 mass spectrometer and a Supelco SPB-1701 30 m x 0.25 mm fused silica capillary column) was performed at the Metabolomics Center of the Roy J. Carver Biotechnology Center, University of Illinois at Urbana-Champaign.
2.6 EXPERIMENTAL METHODS

2.6.1 Procedure for LysC cleavage

Enzymatic assay products were separated from the immobilized TCEP gel by centrifugation and dried on a centrivap concentrator. The products were resuspended in 19 μL of 100 mM Tris, pH 8.3 and 2 μL of LysC (0.4 μg/mL in 100 mM Tris, pH 8.3) was added. The cleavage reaction was incubated at 37 ºC for 3 h and then desalted using by ZipTip (Millipore). The product was eluted in 5 μL of sinapinic acid and analyzed by MALDI-ToF MS.

2.6.2 CinM cyclization activity assay (NB2, p54)

CinM dehydration and cyclization activities in vitro CinM assay components consisted of 50 mM MOPS, pH 7.5, 10 mM MgCl₂, 20 μM CinA(A–1K) peptide, 2.5 mM ATP, 5 μM enzyme and 1 mM tris(2-carboxyethyl) phosphine hydrochloride (TCEP) or dithiothreitol (DTT). MALDI-ToF analysis after LysC treatment showed that CinM is responsible for four dehydrations in the core region. However, assays using TCEP and DTT formed side products of +180 Da or +250 Da for TCEP and +154 Da for DTT (Dr. Lisa E. Cooper, Thesis 2009). In order to prevent the formation of these adducts, enzyme assays were conducted in the presence of TCEP-immobilized gel (20 μL, Thermo Scientific) in 50 mM MOPS, pH 7.5, 10 mM MgCl₂, and 2.5 mM ATP (60 μL final volume) for 6 h while gently rocking the suspension.

After incubation of His₅-CinA(A–1K) with His₁₀-CinM, the assay components were separated from the immobilized TCEP resin by centrifugation.
and products were dried in an eppendorf tube on a centrivap concentrator. The assay products were resuspended in 100 mM Tris, pH 8.3, 1 mM TCEP, and 5 mM iodoacetamide and then incubated at 25 °C for 90 min in the dark, desalted using ZipTipC18, and subjected to MALDI-ToF MS.

2.6.3 Hydroxylation activity assay with CinX (NB5, p61)

A solution with the final concentrations of 100 mM MOPS pH 7.5, 1 mM α-ketoglutaric acid sodium salt (Sigma), 2 mM of L-(+)-ascorbic acid (Acros), 0.1 mM ferrous sulfate 7-hydrate (Mallinckrodt Chemicals), 20 μM peptide, and 25 μM of CinX was prepared in a final volume of 25 μL and incubated at room temperature for 1 h. The assay was quenched by the addition of TFA to a final concentration of 0.1% and the assay mixture was desalted using a C18 ZipTip and eluted with 5 μL of sinapinic acid. Then, the samples were analyzed by MALDI-ToF MS analysis.

2.6.4 Tandem MS analysis to determine the hydroxylation site (NB4, p66)

CinA was modified by CinX in vitro and the leader peptide removed by LysC. The resulting products were first treated with 5 mM TCEP to reduce the disulfides that might have formed and then analyzed by LC-ESI-MSMS using a Synapt ESI quadrupole ToF Mass Spectrometry System (Waters) equipped with an Acquity Ultra Performance Liquid Chromatography (UPLC) system (Waters).
2.6.5 Coexpression of CinA(A–1K), CinM, and CinX with and without Cinorf7 in *E. coli* (NB8. p50)

In order to coexpress CinA(A–1K), CinM and CinX, *E. coli* BL21 (DE3) cells were transformed with a pRSFDuet-1 vector containing cinA(A–1K) and cinM and a pACYCDuet-1 vector containing cinX. Similarly, for coexpression of CinA(A–1K), CinM and CinX together with Cinorf7, *E. coli* BL21 (DE3) cells were transformed with a pRSFDuet-1 vector containing cinA(A–1K) and cinM and a pACYCDuet-1 vector containing cinX and cinorf7. Single colony transformants were grown in a 37 °C shaker for 12-15 h in 50 mL of Luria-Bertani (LB) medium supplemented with 25 μg/mL kanamycin/12.5 μg/mL chloramphenicol. A 20 mL aliquot was centrifuged at 5000×g for 10 min, the spent LB medium was discarded, and the cell pellet was resuspended in fresh LB medium. The resuspended cells (20 mL) were added to 2 L of LB supplemented with the appropriate antibiotics, and the culture was grown aerobically at 37 °C until the A$_{600}$ was ~ 0.6 to 0.8. IPTG was added to a final concentration of 0.5 mM and the culture was transferred to 18 °C for aerobic growth for an additional 20 h. Cells were harvested by centrifugation at 5000×g for 20 min at 4 °C. The cell paste (~ 3-4 g) was stored at –80 °C until use.

Peptides were purified from *E. coli* BL21 (DE3) cultures as described for other His$_6$-LanA peptides (38) by use of a 5 mL HiTrap chelating HP nickel affinity column (GE Healthcare) followed by HPLC.
2.6.6 Bioactivity assays with B. subtilis LH45 (NB8, p54)

CinM (5 μM) was incubated with 50 mM MOPS, pH 7.5, 10 mM MgCl\textsubscript{2}, 20 μM CinA(A–1K) peptide, 2.5 mM ATP, and 150 μL immobilized TCEP (600 μL total volume) at 25 °C for 6 h. Enzymatic assay products were separated from the TCEP immobilized resin by centrifugation and dried via on a centrivap concentrator. The CinM-modified peptide was resuspended in CinX assay components and hydroxylation was carried out for 2 h as described for the CinX assay. Then the leader peptide was cleaved with 15 μL of LysC (0.4 μg/mL in 100 mM Tris, pH 8.3) at 37 °C for 12 h. The pH of the solution was carefully increased to pH 9.5 by addition of 5 M NaOH and the solution was incubated at room temperature for 12 h. A solid phase extraction column (SPE, 1 mL, Discovery DSC-18) was used to partially purify the peptide from the assay components. The C18 column was first washed with 2 mL of 80% acetonitrile (ACN)/water and equilibrated with 2 mL of water and then the sample was applied. The column was washed with 4 mL of water and the sample was eluted with 1 mL 80% ACN/water solution. The sample was dried on a centrivap concentrator and the peptide was dissolved in 10 μL of 100 mM Tris pH 7.5, analyzed by MALDI-ToF MS, and used for the bioassay.

Similarly, 0.5 mg modified peptide obtained from the coexpression of CinM and CinX (sample A) and from coexpression of CinM, CinX and, Cinorf7 (sample B) were separately dissolved in 1 mL of 100 mM Tris-HCl pH 8.3 buffer and digested with 50 μL of LysC (0.4 μg/mL in 100 mM Tris, pH 8.3) at 37 °C for 12 h.
Then, the pH of sample A was carefully increased to pH 9.5 by addition of 5 M NaOH and the solution was incubated at room temperature for 12 h for non-enzymatic formation of Lal. Both samples were then further purified by SPE as described above.

*B. subtilis* LH45 was grown at 37 °C for 15 h in 5 mL of LB supplemented with 0.1% D- (+)-glucose (Calbiochem). LB medium containing 1.5 % agar was autoclaved and cooled to 42 °C in a water bath, and 15 mL of this solution was mixed with 375 μL of *B. subtilis* LH45 culture and with a D- (+)-glucose solution to give a 0.1% final glucose concentration. The agar was poured into sterile plates and shallow wells were generated after the agar solidified. The peptide samples (10 μL in 50 mM Tris Buffer) were spotted and the plates were incubated for 10 h at 25 °C.

### 2.6.7 Cloning of *cinA, cinM, cinX* and *cinorf7* and construction of expression plasmids (NB7, NB9, p6)

The genes *cinA, cinM, cinX* and *cinorf7* were cloned from *S. cinnamomeous* genomic DNA. The amplification of *cinA* was conducted via 30 cycles of denaturing (98 °C for 10 s), annealing (55 °C for 30 s), and extending (72 °C for 135 s) using CinA_wt_FP_NdeI_pET15b and CinA_wt_RP_BamHI_pET15b as forward and reverse primers (Table 2.1). The PCRs (50 μL) included 1x HF buffer (Finnzymes), DMSO (4%), Fusion DNA Polymerase (Finnzymes) (0.02 unit/μL), dNTPs (1 mM), *S. cinnamomeous* genomic DNA, and primers (1 μM each). Amplification of the final PCR product
was confirmed by 2% agarose gel electrophoresis, and the products were purified using QIAquick PCR purification kits (QIAGEN). The resulting DNA fragment and the pET15b vector were digested in 1 x NEBuffer 4 (New England Biolabs) with NdeI (for 15 h) and BamHI at 37 °C (for 3 h). The digested products were purified by agarose gel electrophoresis followed by use of a QIAquick gel extraction kit (QIAGEN). The resulting DNA insert was ligated with the digested pET15b vector at 16 °C for 15 h using T4 DNA ligase.

The ligation reaction mixture was diluted 5 times with water prior to transformation. E. coli DH5α cells were transformed with the ligation product via heat shock, plated on LB-ampicillin agar plates, and grown at 37 °C for 15 h. Colonies were picked and incubated in 5 mL of LB-ampicillin medium at 37 °C for 15 h, followed by isolation of the plasmids using a QIAprep Spin Miniprep Kit (QIAGEN). The sequences of the resulting plasmids were confirmed by DNA sequencing.

Similarly, cinM, cinX and cinorf7 were amplified using appropriate primers (Table 2.1) by PCR. The reactions contained 1xFailSafe PreMix G (PICENTRE Biotechnologies), DMSO (4%), Platinum Pfx DNA polymerase (0.025 unit/μL), Taq DNA polymerase (0.05 unit/μL), and primers (1 μM each). Amplification of the final PCR products were confirmed by 1% agarose gel electrophoresis and the products were purified using QIAquick PCR purification kits. The resulting DNA fragments and the pET16b and pET28b vectors were digested in 1 x NEBuffer 4 with NdeI and XhoI at 37 °C (for 15 h). The digested products were
purified by agarose gel electrophoresis followed using a QIAGen gel extraction kit. The resulting DNA inserts were ligated with the digested corresponding vectors at 16 °C for 15 h using T4 DNA ligase to generate $\text{cinM-pET16b}$, $\text{cinX-pET28b}$ and $\text{cinorf7-pET28b}$. The ligation reaction mixture was diluted 5 times with water prior to transformation. $\text{E. coli}$ DH5α cells were transformed with the ligation product via heat shock, plated on LB-ampicilin (for pET16b) and LB-kanamycin (for pET28b) agar plates, and grown at 37 °C for 15 h. Colonies were picked and incubated in 5 mL of LB-ampicilin/kanamycin medium at 37 °C for 15 h, followed by isolation of the plasmids using a QIAprep Spin Miniprep Kit. The sequences of the resulting plasmids were confirmed by DNA sequencing.

2.6.8 Construction of the CinA (A−1K) (NB5)

Site-directed mutagenesis of $\text{cinA}$ was performed by multistep overlap extension PCR. First, the amplification of $\text{cinA}$ was conducted via 30 cycles of denaturing (94 °C for 20 s), annealing (55 °C for 30 s), and extending (72 °C for 120 s) using the $\text{cinA-NdeI-FP}$ primer and $\text{CinA-K_RP_(A−1K)}$ pET15b reverse primer (see Table 2.1) to yield a 5’ fragment of the mutated CinA(A−1K) gene (FP reaction). The PCR mixtures included 1x FailSafe PreMix G, DMSO (4%), Platinum Pfx DNA polymerase (0.025 unit/μL), Taq DNA polymerase (0.05 unit/μL), and primers (1 μM each). In parallel, a PCR using $\text{CinA-K_FP_(A−1K)}$ pET15b forward primer and the $\text{CinA_wt_RP_BamHI}_pET15b$ primer was also conducted to produce the 3’ fragment of the mutated CinA(A−1K) gene using the same PCR conditions that were used for the FP reaction (RP
reaction). The overlapping products from the FP reaction and RP reaction were combined in equal amounts and extended by five cycles of denaturing, annealing, and extending in a solution containing 1x FailSafe PreMix G, DMSO (4%), and Platinum Pfx DNA polymerase (0.025 unit/μL). Following the extension, the CinA_Ndel_FP and CinA_wt_RP_BamHI_pET15b primers were added (final concentration of 2 μM), and the reaction mixture was incubated for 25 additional cycles of denaturing, annealing, and extending.

Amplification of the final PCR product was confirmed by 2% agarose gel electrophoresis, and the products were purified using QIAquick PCR purification kits. The resulting DNA fragment and the pET28 vector were digested in 1x NEBuffer 4 with Ndel (15 h) and BamHI (3 h) at 37 °C. The digested products were purified by agarose gel electrophoresis followed using a QIAquick gel extraction kit. The resulting DNA insert was ligated with the digested pET15b vector at 16 °C for 15 h using T4 DNA ligase. The ligation reaction mixture was diluted 5 times with water prior to transformation. E. coli DH5α cells were transformed with the ligation product via heat shock, plated on LB-ampicilin agar plates, and grown at 37 °C for 15 h. Three colonies were picked and incubated in 5 mL of LB-ampicilin medium at 37 °C for 15 h, followed by isolation of the plasmids using a QIAprep Spin Miniprep Kit. The sequences of the resulting plasmids were confirmed by DNA sequencing.
2.6.9 Generation of a coexpression system in *E. coli* (NB9, p8)

The genes encoding CinA(A–1K), CinM, CinX and Cinorf7 were amplified using appropriate primers (Table 2.1) using CinA(A–1K)-pET15b, CinM-pET16b, CinX-pET28b and Cinorf 7-pET28b as templates following the protocols described above. The gene encoding CinA(A–1K) with an N-terminal FLAG-tag (DYKDDDDK) was placed into multiple cloning site I (MCSI) of pRSFDuet-1 by using AscI and HindIII restriction sites, whereas *cinM* was placed into MCSII of the same vector by using EcoRV and KpnI restriction sites. Double digestion for the corresponding gene and the corresponding vector was carried out with AscI and HindIII-HF enzymes by use of 1x NEBuffer 4 for 10h at 37 °C. Similarly, double digestion for the corresponding gene and the corresponding vector was carried out with EcoRV-HF and KpnI-HF enzymes by use of 1x NEBuffer 4 for 10h at 37 °C. In addition, two pACYCDuet-1 vectors were constructed, one contains only *cinX* in MCSII, and one contains *cinorf7* in MCSI and *cinX* in MSCII. NcoI and HindIII restriction sites were used to introduce *cinorf7* whereas EcoRV and XhoI cut sites were used to place *cinX* into pACYCDuet-1. For NcoI and HindIII, a double digest reaction was carried out in 1x NEBuffer 2 at 37 °C for 10 h, whereas for EcoRV and XhoI, a double digest reaction was carried out in the presence of 1x NEBuffer 3 together with 1x BSA (NEB) for 10 h at 37 °C. The digested products were purified by agarose gel electrophoresis followed using a QIAquick gel extraction kit. The resulting DNA inserts were ligated with the digested corresponding vectors at 16 °C for 15 h using T4 DNA ligase. The
ligation reaction mixture was diluted 5 times with water prior to transformation. *E. coli* DH5α cells were transformed with the 10 μL of the ligation product via heat shock, plated on LB-kanamycin plates for pRSFDuet-1 vector, LB-chloramphenicol agar plates for pACYCDuet-1 vector, and grown at 37 ºC for 15 h. Three colonies were picked and incubated in 5 mL of LB-kanamycin/chloramphenicol medium at 37 ºC for 15 h, followed by isolation of the plasmids using a QIAprep Spin Miniprep Kit. The sequences of the resulting plasmids were confirmed by DNA sequencing.

2.6.10 Overexpression and purification of His₆-CinA, His₆-CinA A−1K, and His₆-CinA A−1K D15A (NB7, p18, p36)

Peptides were overexpressed and purified from *E. coli* BL21 (DE3) cultures as described for other His₆-LanA peptides (38). Briefly, purification was accomplished by solubilizing the inclusion bodies from the overexpression experiments using 4 M guanidine hydrochloride and loading this solution onto a 5 mL HiTrap chelating HP nickel affinity column (GE Healthcare). The desired peptides were eluted in 1-2 column volumes of elution buffer containing 4 M guanidine hydrochloride, 20 mM sodium phosphate, pH 7.5, 100 mM NaCl, and 1 M imidazole. Desalting was achieved by preparative RP-HPLC on a Waters system using a C4 PrepLC column. Solvents for preparative RP-HPLC were solvent A (0.1% TFA in water) and solvent B (0.086% TFA in 80% ACN/20% water). A gradient of 2 – 100 % of solvent B over 45 min was executed with a flow rate of 8 mL/min. Peptides were detected by absorbance at 220 nm.
Lyophilized powder was analyzed by matrix-assisted laser desorption/ionization-time of flight mass spectrometry MALDI-ToF MS) and desired products were stored at – 20 ºC. MALDI-ToF MS was carried out on a Voyager-DE-STR mass spectrometer (Applied Biosystems).

2.6.11 Overexpression of his₁₀-cinM and his₆-cinX (NB5, 34, 73; NB1, p17-19, p41)

*E. coli* BL21 (DE3) cells were transformed with pET16b-cinM and pET28b-cinX. Single colony transformants were grown in a 37 ºC shaker for 12-15 h in 50 mL of Luria-Bertani (LB) medium supplemented with 100 μg/mL ampicillin (for pET16b-cinM) or 50 μg/mL kanamycin (for pET28b-cinX). A 20 mL aliquot was centrifuged at 5000×g for 10 min, the spent LB medium was discarded, and the cell pellet was resuspended in fresh LB medium. The resuspended cells (20 mL) were added to 2 L of LB supplemented containing corresponding antibiotic, and the culture was grown aerobically at 37 ºC until the A600 was ~ 0.6 to 0.8. IPTG was added to a final concentration of 1 mM and the culture was transferred to 18 ºC for aerobic growth for an additional 20 h. Cells were harvested by centrifugation at 5000×g for 20 min at 4 ºC. The cell paste (~ 4 g) was stored at – 80 ºC until use.

2.6.12 Purification of His₁₀-CinM and His₆-CinX (NB7, p26; NB5, p34)

Both His₁₀-CinM and His₆-CinX were kept at 4 ºC for the duration of the purification process. For each enzyme, the cell paste was resuspended in ~ 20 mL of LanM Start Buffer (20 mM Tris, pH 7.6, 500 mM NaCl, 10 % glycerol) and
lysed by sonication (35% amplitude, 4.0 s pulse, 9.9 s pause, 15 min). The sample was centrifuged at 23,700×g for 45 min (Beckman JA-20 rotor) and the supernatant was clarified through 0.45 μm syringe filters (Corning). The sample was then loaded onto a 5 mL HisTrap HP column (GE Healthcare Life Sciences). The column was washed with 20 mL each of start buffer containing 25 and 50 mM imidazole and then 10 mL each of start buffer containing 100, 200, and 500 mM imidazole. The eluent was collected in several fractions, which were analyzed by Tris-SDS-PAGE (4-20% acrylamide gradient). The fractions containing the desired protein (200-500 mM imidazole) were combined and concentrated using an Amicon Ultra-15 centrifugal filter unit (30 kDa molecular mass cut off for His₆-CinM, 10 kDa molecular mass cutoff for His₆-CinX, Millipore) to less than 2 mL. Buffer exchange of the concentrated proteins with LanM Final Buffer (20 mM Tris, pH 7.6, 500 mM KCl, and 10% glycerol) was conducted twice via size exclusion chromatography (GE Healthcare Life Sciences). The resulting protein samples were aliquoted, frozen with liquid nitrogen, stored at −80 °C. Protein concentrations were determined using A280 measurements and were calculated with theoretical extinction coefficient obtained from the ProtParam function on the ExPASy Proteomics Server (His₁₀-CinM, ε₂₈₀ = 158,180 M⁻¹ cm⁻¹, His₆-CinX, ε₂₈₀ = 62,005 M⁻¹ cm⁻¹). For CinM, typical protein yields ranged from 6 -10 mg per liter of overexpressed cells, whereas for CinX protein yields ranged from 1.7 mg to 2 mg per liter of overexpressed cells.
2.6.13 Detection of lysinoalanine (Lal) on CinA by gas chromatography-mass spectrometry analysis (GC-MSMS) (NB5, 94-95)

The presence of Lal in CinA modified with CinM and CinX followed by LysC protease cleavage and alkaline treatment was confirmed via hydrolysis and derivatization of the peptide, followed by analysis by gas chromatography mass spectrometry (GC/MS) with comparison to Lal in authentic cinnamycin (Novacta) treated in the same way. Modified CinA (~ 0.9 mg) prepared in vitro as described above was dissolved in 6 M HCl (3 mL) and heated with stirring at 110 °C in a high-pressure, sealed vessel for 22 h. The reaction was cooled and concentrated under reduced pressure. Methanol (3 mL) was chilled in an ice-water bath, and acetyl chloride (1 mL) was added dropwise. This solution was added to the hydrolysate, and the mixture was heated at 110 °C for 45 min. The reaction was again allowed to cool and concentrated under reduced pressure to dryness, then chilled in an ice-water bath. Dichloromethane (2.5 mL) and pentafluoropropionic anhydride (1 mL) were added, and the mixture was heated at 110 °C for 15 min. The reaction was allowed to cool and was dried under a stream of nitrogen. The residue was taken up in 100 μL of methanol, transferred to a clean vial and dried under a stream of nitrogen. Similarly, cinnamycin (Novacta; 0.9 mg) was hydrolyzed and derivatized in the same way. The sample and the standard were analyzed individually by GC/MS using an Agilent HP 5973 mass spectrometer and a Supelco SPB-1701 30 m x 0.25 mm fused silica capillary column. Samples were dissolved in methanol and introduced to the instrument via splitless
injection and a flow rate of 1.5 mL/min. The temperature gradient used was 100 °C for 5 min, then 100 °C to 280 °C over 18 min, then held at 280 °C for 1 min.
List of Primers * From 5’ to 3’

CinA wt FP Ndel pET15b: GGGAAATCCATATGACCGCTTCGATTCTTCAGCAG
CinA wt RP BamHI pET15b: TATAAACGCGGATCTTACTTGGTGTGTCGACGCA
CinM FP Ndel pET15b: GGGAAATCCATATGATGGGTATGGAATACGCATATCCG
CinM RP XhoI pET16b: TATAAACGCGCTCGAGTTACTGTCGCTTGTCGACGCG
CinX Ndel FP pET28b: GGA ATT CCA TAT GGC CCT GAA GAC CTG CGA GG
CinX XhoI RP pET28b: CCG CTC GAG TCA GCC GTG GTG G

Cinor7 FP Ndel pET28b:
GCTCTGTCGCGCGCGCGACGCTATGAAAGTGCTCAAGGAACGCGATCTACC
Cinor7 RP XhoI pET28b:
GGGGTTTTTGCAGGCGATCTTCGAGCAGCTCTGTGCTACGACCCAGCC
CinA-K FP (A−1K) pET15b: CACGGAAGCTTTCAAGTGGCGCCGAGAGCT
CinA-K RP (A−1K) pET15b: CAGCTCTGCGCGACACTTGAAGGCTTTCCGT

pDuet System Cloning

CinA-K Ascl pRSFDuet-1 MCSI FP:
TTCGAGCTCGGCGCCCTGATGACCGCT TCGATTCTTCAGCAG
CinA-K HindIII pRSFDuet-1 MCSI RP:
GCG GCC GCAAGCTTTACTTGGTGTGTCGACGAC
CinX EcoRV pACYCDuet-1 MSCI FP:
TCTCAATTGAGTCAGTGGCCCTAGACCTGCAGAGGAATTC
CinX XhoI pACYCDuet-1 MCSI RP:
TTAACCAGAATCGAGTCAGCCGTTGTGGCAGGGAAGGCGTCC
Flag-CinA-K BamHI pACYCDuet-1 FP:
GTCGAGACACGATTATGATAAGGATACGCAGACGATAAATTC
CinA-K HindIII pRSFDuet-1 and pACYCDuet-1 RP:
GCGGCCGCAAGCTTTTACTTGGTGTGTCGACGAC
Cinor7 Neol pACYCDuet-1 MCSI FP:
AGGGTCAGACCCATGAGCAGGAAAGTGCTCAAGGAACGCGAC
Cinor7 HindIII pACYCDuet-1 MCSI RP:
TCGATGCGACCTTTCGAGCTACGCTGTGCGAC
CinM EcoRV pRSFDuet-1 FP:
TCTCAATTGAGTCAGTGGCAGGAAATTCGCGATATGGAATGGTACGCG
CinM KpnI pRSFDuet-1 RP:
AGACTCGAGGAACTTTACTGCGTGTCGACGCG

*the restriction sites are underlined.

Table 2.1 List of primers.
2.7 REFERENCES


22. The flag tag was incorporated to provide an additional purification handle if needed. For the experiments in this study, the flag tag was not used.


CHAPTER III: BIOCHEMICAL CHARACTERIZATION OF THE TAILORING ENZYMES CINX AND CINORF7 INVOLVED IN CINNAMYCIN BIOSYNTHESIS

3.1 INTRODUCTION

Enzymes that share the same mechanism usually possess similar amino acid sequences (1). Conserved residues in the amino acid sequences are commonly used to explore enzyme mechanism as they may be necessary for catalysis. Mutational studies have been useful for understanding the mechanism of the enzymes in lantibiotic biosynthesis as described for several lantibiotics (2-6). Understanding the mechanism of lantibiotic enzymes may allow for preparation of non-natural analogs of a lantibiotic, thus potentially contributing to drug design.

The work presented in the previous chapter showed that four genes (cinA, cinM, cinX, and cinorf7) are essential for cinnamycin biosynthesis (7). Sequence alignment of CinX with other enzymes indicated that it is a member of the Fe(II)/α-ketoglutarate (αKG)-dependent hydroxylases, a group of enzymes that catalyzes hydroxylation through oxidative decomposition of αKG (11). Hydroxylases dependent on αKG have a characteristic iron/αKG binding motif of H-X-D/E-Xₙ-H-Xₘ-R, where n=43-153 and m=8-19 (8-9). The H-X-D/E-Xₙ-H motif is responsible for binding to the Fe(II) while the arginine has been shown to be important in binding the distal carboxylate of αKG. Analysis of the CinX sequence identified four possible binding motifs; H₁₅₈-R-D₁₆₀-X₇₃-H₂₃₃-X₉-R₂₄₄ n=73, m=9, H₁₈₈-V-D₁₉₀-X₄₃-H₂₃₃-X₈-R₂₄₂ n=43, m=8, H₁₈₈-V-D₁₉₀-X₇₅-H₂₆₅-X₁₃-
In vitro reconstitution of the CinX enzyme by former post-doctoral researcher Dr. Emily J. Fogle demonstrated that this enzyme indeed catalyzes the \( \beta \)-hydroxylation of Asp15 in the CinA precursor peptide. Further studies performed together with post-doctoral researcher Dr. Remco Merkx on the CinX enzyme showed that it does not require the leader peptide and is capable of hydroxylating substrates CinA-(1-19) and CinA-(10-19).

A small protein encoded by \textit{cinorf7} was shown in Chapter II be critical for the formation of the lysinoalanine (Lal) crosslink, which is important for the bioactivity of cinnamycin (7). The Cinorf7 protein has no significant sequence homology to other functionally-characterized proteins. Six other genes encoding proteins with high sequence similarity to Cinorf7 were identified in the protein databases and in the genome of the duramycin producing organism, \textit{Streptoverticillium cinnamoneum forma azacoluta} (ATCC 12686), that was sequenced by our laboratory as discussed in Chapter IV. These \textit{cinorf7} orthologs also are near \textit{lanM}-like genes. Although the sequences of the predicted leader peptides of the putative precursor peptides located near these Cinorf7 orthologs vary, their core regions have very high sequence similarity to those of the cinnamycin group, suggesting that these gene clusters have the genetic capacity to generate cinnamycin-like peptides.

Herein, we investigated the tailoring proteins CinX and Cinorf7 through a series of \textit{in vitro} and in \textit{E. coli} experiments. The order of CinX and Cinorf7
modifications were investigated by coexpression of CinA, CinM and Cinorf7 excluding CinX in *E. coli*. Furthermore, Cinorf7 activity was investigated in *E. coli* by mutating the residues that are conserved in Cinorf7 and six Cinorf7-like protein sequences, found in various genomes (Figure 3.1). Additionally, trials to crystallize and reconstitute the *in vitro* activity of Cinorf7 were undertaken. The substrate tolerance of CinX was also explored by testing its activity using several short synthetic peptides, and N-terminal truncations of CinX were prepared for crystallization studies.

![Alignment of Cinorf7-like genes](alignment.png)

**Figure 3.1 Sequence alignment of Cinorf7-like genes.** Durorf7 was found in the genome of the duramycin producing organism *Streptovorticillium cinnamoneum forma azacoluta* (ATCC 12686), Noc7 was found in the genome of *Nocardiosis potens*, Osc7 was found in the genome of *Oscillatoria sp. PCC 10802*, Lyn7 was found in the genome of *Lyngbya majuscule*, Fran7 was found in the genome of *Frankia Sp.*, and Act7 was found in the genome of *Actinomadura atramentaria.*
3.2 RESULTS

3.2.1 Order of modification by CinX and Cinorf7

The ability of Cinorf7 to form Lal in the absence of CinX was tested by the coexpression of \textit{cinA(A−1K)}, \textit{cinM} and \textit{cinorf7} in \textit{E. coli}. For this purpose, the \textit{cinorf7} gene was cloned into the first multiple cloning site of pACYCDuet-1 and used for the coexpression with a pRSFDuet-1 plasmid containing \textit{his}_6-\textit{cinA(A−1K)} and \textit{cinM} genes. The \textit{His}_6-\textit{CinA(A−1K)} peptide from CinM and Cinorf7 coexpression in \textit{E. coli} was purified by immobilized metal ion affinity chromatography (IMAC) followed by reversed-phase high-performance liquid chromatography (RP-HPLC) resulting in 2-3 mg of purified peptide per liter of cell culture. Analyses of the core peptide by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-ToF MS) after LysC endoproteinase treatment revealed a mass difference corresponding to loss of four water molecules as expected. This finding demonstrates that CinM was able to dehydrate the two threonine (Thr11 and Thr18) and two serine residues (Ser4 and Ser6) in the core region of CinA in the presence of Cinorf7. Addition of cysteine thiols to the dehydroamino acids does not cause a change in mass, which prevents observation of cyclization by MALDI-ToF MS. To investigate the cyclization activity of CinM in the absence of CinX, iodoacetamide (IAA) was used. The peptide obtained from the coexpression system was treated with IAA followed by LysC protease incubation. The analysis of the peptide by MALDI-ToF MS showed only minor alkylation products, whereas the positive
control, unprocessed peptide His$_6$-CinA(A–1K), showed a mass increase corresponding to alkylation of the three cysteines in the core peptide. This result showed that the (Me)Lan residues obtained from coexpression of CinM and Cinorf7 were formed successfully. The presence of the lysinoalanine (Lal) crosslink in this peptide was tested by o-phthalaldehyde (OPA), which as described in Chapter II, reacts selectively with primary amines in the presence of a thiol such as β-mercaptoethanol but does not react with secondary amines (Figure 3.2D) (10). CinA that had been modified by CinM and CinX in E. coli did not possess Lal, because a mass increase corresponding to two OPA additions and one β-mercaptoethanol addition in the core peptide was observed (Figure 3.2A). These two OPA adducts originated from the N-terminal amino group and the ε-amino group of Lys, and one β-mercaptoethanol addition as a consequence of Michael-type addition to dehydroalanine 6. The OPA assay of CinM- and Cinorf7-modified peptide resulted in only one OPA addition and no β-mercaptoethanol adducts, which was also observed when authentic cinnamycin was used as a positive control (Figure 3.2B, 3.2C). This observation clearly shows that in the presence of CinM and Cinorf7 Lal was formed and that CinX was neither involved nor important for the formation of this crosslink. In the bioassay of this peptide towards indicator strain B. subtilis after cleavage of the leader peptide with LysC, no zone of inhibition was observed (Figure 3.3A), suggesting the (Me)Lan and Lal crosslinks are not sufficient for bioactivity. Next, the CinM and Cinorf7 modified peptide was treated with CinX in vitro.
Surprisingly, CinX was not able to hydroxylate this peptide as no mass change was observed in mass spectrometry studies after LysC cleavage (Figure 3.3B). Thus, no zone of inhibition was observed for this CinX treated peptide in the bioactivity assay towards the indicator strain (Figure 3.3A). In comparison, CinX was able to hydroxylate CinM-modified precursor peptide as expected based on the data in Chapter II (Figure 3.3C). These results strongly suggest that hydroxylation is required for antimicrobial activity of cinnamycin, and that CinX only acts on the substrate before Lal crosslink formation.
Figure 3.2 Use of o-phthalaldehyde (OPA) to monitor lysinoalanine formation. MALDI-TOF analysis of (A) CinA(A–1K) modified by CinM and CinX in E. coli, and treated with LysC and with OPA and β-mercaptoethanol. (B) CinA(A–1K) modified by CinM and Cinorf7 in E. coli and treated with LysC was incubated with OPA and β-mercaptopethanol. (C) Authentic cinnamycin was incubated with OPA and β-mercaptopethanol. Asterisk indicates four-fold dehydrated core peptide that did not react with OPA. (D) Chemical reaction of o-phthalaldehyde (OPA) reagent with primary amines in the presence of a thiol. AMX = CinA(A–1K) modified by CinM and CinX in E. coli followed by LysC treatment, AM7 = CinA(A–1K) modified by CinM and Cinorf7 in E. coli followed by LysC treatment.

*Calculated mass for (A) AMX + 2 OPA + BME = 2042.3 + 2 *180.1 + 78.1 = 2480.6 Da, (B) AM7 + OPA; 2027 + 180.1 = 2207.1 Da, (C) M + OPA; 2042.3 + 180.1 = 2222.4 Da. Obsrvd mass for (A) AMX + 2 OPA + BME is 2474.7 Da, (B) AM7 + OPA is 2219.9 Da, (C) M + OPA is 2220.19 Da.
Figure 3.3 Order of CinX and Cinorf7 modification. (A) Inhibition assay of \textit{in vitro} CinX treated His$_6$-CinA(A–1K) which was modified by CinM and Cinorf7 in \textit{E. coli} followed by LysC treatment. The activity is towards \textit{B. subtilis} 6633. Spot 1: Authentic cinnamycin, Spot 2: His$_6$-CinA(A–1K) modified by CinM and Cinorf7 in \textit{E. coli} and then treated with LysC and CinX, (B) MALDI-ToF MS analysis of the peptide used for spot 2, (C) MALDI-ToF MS analysis of \textit{in vitro} CinX assay performed with His$_6$-CinA(A–1K) modified with CinM in \textit{E. coli}, followed by LysC treatment, CP = CinA core peptide.

*Calculated mass for (B) CP – 4 H$_2$O + H; 2097.3 – 4 \times 18 + 1 = 2026.3, (C) CP – 4 H$_2$O + OH +H; 2097.3 – 4 \times 18 + 1 = 2042.3. Observed mass (B) 2026.3 Da, (C) 2043.1 Da.

3.2.2 Purification and Characterization of Cinorf7

Although, Cinorf7 purification was demonstrated by Dr. Lisa Cooper, a previous PhD student in our laboratory, by use of a pET28b vector, a method for the purification of this protein from the coexpression system of CinA(A–1K), CinM, CinX and Cinorf7 was developed. A His$_6$-tag was introduced at the N-terminus of the \textit{cinorf7} gene through insertion of \textit{cinorf7} into MCS I of pACYCDuet-1 containing the \textit{cinX} gene in MSC II. Use of this plasmid together with a pRSFDuet-1 vector containing \textit{cinA}(A–1K) and \textit{cinM} genes resulted in His$_6$-tagged Cinorf7 that was purified by IMAC and desalted by gel filtration.
Purified Cinorf7 samples were treated with 5 mM DTT and boiled for 20 minutes, and analyzed by SDS-PAGE. Interestingly, after protein staining, two bands were observed on the gel; one corresponding to the size of monomeric Cinorf7, and a second corresponding to the size of dimerized Cinorf7 (Figure 3.4). Increasing the DTT concentration from 5 mM DTT to 100 mM resulted in decrease of the thickness of the band corresponding to the dimer. Indeed, in gel digestion of the two bands with trypsin endoproteinase followed by MALDI-ToF-MS analysis confirmed that the second band was dimerized Cinorf7. Close analysis of the peaks from the monomeric and dimeric form also showed that Cinorf7 is not post-translationally modified during the biosynthesis of cinnamycin. Gel filtration studies showed that the majority of IMAC-purified His$_6$-Cinorf7 migrated as if it was a trimer, although dimer and monomer were also observed. Circular dichroism (CD) analysis of the purified His$_6$-Cinorf7 showed that the protein was predominantly $\alpha$-helical (Figure 3.5). To confirm that the addition of the His tag did not interfere with Cinorf7 function, CinA(A$\rightarrow$1K) peptide was purified from the coexpression of His$_6$-CinA(A$\rightarrow$1K), CinM, CinX and His$_6$-Cinorf7 in *E. coli* by IMAC followed by RP-HPLC. His$_6$-Cinorf7 was highly soluble, whereas modified His$_6$-CinA(A$\rightarrow$1K) was found in the pellet. Therefore, IMAC purification of His$_6$-CinA(A$\rightarrow$1K) was performed by applying the insoluble fraction onto the Ni-NTA column after solubilization with guanidine hydrochloride. Mass spectrometric analysis of the LysC-cleaved peptide showed that four dehydrations and a hydroxylation were introduced. The bioassay of LysC-cleaved peptide towards
the indicator strain *B. subtilis* resulted in a zone of growth inhibition, which suggests that the peptide contained Lal, which is crucial for antimicrobial activity. To conclude, the His tag does not interfere with Cinorf7 function.

**Figure 3.4** Cinorf7 purification and dimer formation analyzed by SDS-PAGE. (A) SDS-PAGE analysis of His<sub>6</sub>-Cinorf7.

**Figure 3.5** Circular dichroism (CD) analysis of the purified His<sub>6</sub>-Cinorf7. Results suggests that Cinorf7 has alpha-helical secondary structure.
3.2.3 Crystallization Trials

Initial attempts to crystallize Cinorf7 have been unsuccessful, possibly due to the presence of multimeric forms of the protein after purification. Cinorf7 has only one cysteine residue at position 26, which might be responsible for the dimer formation by forming disulfide bonds. In an attempt to increase the solubility and stability of this small protein for crystallization studies, Cys26 was mutated to a serine residue. To determine if this mutation impacted the activity of Cinorf7, the bioactivity of modified CinA(A-1K), purified after coexpression with His\textsubscript{6}-CinA(A-1K), CinM, CinX, and Cinorf7-Cys26Ser, was tested towards the indicator strain after LysC endoproteinase cleavage of the leader peptide. A zone of growth inhibition was observed suggesting that the mutation Cys26Ser in Cinorf7 does not hamper activity and thus can be used for crystallization studies. His-tagged Cinorf7-Cys26Ser co-expressed from a pACYCDuet-1 vector that also encoded CinX was purified by IMAC. Interestingly, the mutation did not prevent the formation of the dimer in SDS-PAGE analysis. It is possible that polymerization is due to hydrophobic and/or hydrophilic interactions between Cinorf7 proteins.

3.2.4 Mutational studies of Cinorf7

At the time of this study, two other genes encoding proteins with high sequence similarity to Cinorf7 were identified in the protein databases. One of these is present in the genome of *Lyngbya majuscule*, which encodes two nearby likely precursor peptides, whereas the second, found in the genome of *Frankia*
Sp., has one gene encoding a possible precursor peptide nearby. Both cinorf7 orthologs also are near lanM-like and lanX-like genes, suggesting these gene clusters may be responsible for the biosynthesis of cinnamycin-like peptides.

Comparison of Cinorf7 with these orthologs allowed the identification of conserved residues, which may be important in the catalysis of Lal formation. Thus, the conserved residues in Cinorf7 (Figure 3.6A), with the exception of Cys26, were individually mutated to alanine. The plasmid pACYCDuet-1 containing cinorf7 mutants and cinX genes together with pRSFDuet-1 containing His<sub>6</sub>-CinA(A−1K) and cinM were used to co-express these genes in E. coli. His<sub>6</sub>-CinA(A−1K) peptide was purified by IMAC followed by RP-HPLC. After LysC cleavage, the bioactivity of the peptide modified by each mutant towards the indicator strain was tested (Table 3.6B). Several modifications of Cinorf7 did not prevent Lal formation. Interestingly, removal of 20 amino acids from either the N-terminus or C-terminus of this protein also did not abolish Lal formation. Mutation of Arg52 and Trp87 to alanine individually reduced the efficiency, whereas mutation of Trp68 to alanine resulted in loss of Cinorf7 activity in E. coli. Reduction of the efficiency of Lal formation was evaluated based on weaker bioactivity when the same amount of peptide was applied in the bioassay. However, due to solubility problems of the peptides, quantitative analysis of Lal formation could not be performed, which will be needed for understanding the loss of activity better.
Figure 3.6. Mutational studies on Cinorf7. (A) Two proteins with high sequence similarity to cinorf7 were identified in the databases. The orthologs originate from *Lyngbya majuscula* (ZP_08425231) and *Frankia Sp.* (ZP06416732). The mutations introduced are highlighted in red, (B) List of the mutations introduced to the cinorf7 gene, and results for bioactivity tests conducted towards the indicator strain *B. subtilis* 6633.
3.2.5 Interaction of Cinorf7 with CinM

It has been unclear whether Cinorf7 is an enzyme catalyzing Lal formation or if it is acting as a chaperone for CinM, allowing CinM to catalyze the reaction. To test this latter hypothesis, a pull down experiment with Cinorf7 was performed. A DNA sequence encoding an His\_6-tag was placed at the N-terminus of cinorf7 in pACYCDuet-1 containing cinX, and this vector was used to co-express His\_6-CinA(A−1K) and CinM from pRSFDuet-1 together with CinX and His\_6-Cinorf7. The protein His\_6-Cinorf7 was purified by IMAC as explained in the purification procedure above. SDS-PAGE analysis of different imidazole fractions of purified Cinorf7 showed that CinM co-eluted, suggesting an interaction between these two proteins (Figure 3.7).

![Figure 3.7 Pull-down experiment with His\_6-Cinorf7. Lane 1: Broad range marker, Lane 2: His\_6-Cinorf7 from coexpression purification pulls down CinM.]

Figure 3.7 Pull-down experiment with His\_6-Cinorf7. Lane 1: Broad range marker, Lane 2: His\_6-Cinorf7 from coexpression purification pulls down CinM.
### 3.2.6 Substrate specificity for CinX hydroxylation in *E. coli*

In the coexpression studies, CinX successfully hydroxylated CinM-modified CinA. A system where CinX hydroxylation occurs without CinM would be beneficial for substrate specificity analysis of this enzyme in *E. coli*. Therefore, *his*<sub>6</sub>*-cinA(A−1K)*, inserted in MCS I of pRSFDuet-1, was co-expressed with *cinX*, inserted in pACYCDuet-1 MCS II. The *his*<sub>6</sub>-CinA(A−1K) was purified by IMAC followed by RP-HPLC, and after LysC cleavage, the peptide was analyzed by MALDI-ToF MS. However, the hydroxylated product could not be identified. In order to simplify the construct and possible problems arising from a two plasmid system, *cinX* was cloned into the MCS II of pRSFDuet-1 containing *cinA(A−1K)* in MCS I, and these two genes were co-expressed in *E. coli*. Although the mass of the hydroxylated peptide was identified using this construct, the peak intensity was very low (Figure 3.8). Both CinA core peptide and its hydroxylated form do not ionize well in MALDI-ToF MS analysis. In addition, the presence of a sodium adduct further complicates detection of this peptide. Hence, we were unable to use coexpression in *E. coli* to investigate substrate selectivity.
Figure 3.8 Hydroxylation of CinA(A−1K) by CinX in *E. coli*. His$_6$-CinA(A−1K) modified by CinX in *E. coli* and treated with LysC to give M (core region) and M+OH. *Calculated mass for M + OH; 2097.3 + 16 = 2113.3. Obsrvd: 2113.1 Da.*

3.2.7 CinX substrate specificity studies

Tandem MS studies described in Chapter II showed that CinX can hydroxylate both CinA (1-19) and CinA (10-19) in the absence of the leader sequence, allowing analysis of the substrate specificity of CinX by using peptides synthesized by solid phase peptide synthesis. In this study, twelve different peptides were tested for CinX activity (Figure 3.9). The site of hydroxylation in the peptides that served as a substrate was investigated by tandem mass spectrometry. Peptides 1-6 were designed to identify the importance of the amino acids located directly N-terminal and C-terminal to Asp15, whereas peptides 9-11 were used to investigate the importance of the position of the aspartate. The
possibility of double hydroxylation in one sequence was also investigated with peptide 12, which has two potential Asp hydroxylation sites in the sequence FTFVCDGRFTFVCDG.

<table>
<thead>
<tr>
<th>Flanking residues</th>
<th>Substrates</th>
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</thead>
<tbody>
<tr>
<td>CinA (1-19) CRQSCSFGPFTFVCDGNTK</td>
<td><del>VCDG</del></td>
</tr>
<tr>
<td>CinA (10-19) FTFVCDGNTK</td>
<td><del>VCDG</del></td>
</tr>
<tr>
<td>1 TFCVCDGNTK</td>
<td>+</td>
</tr>
<tr>
<td>2 FVCDGNTK</td>
<td>+</td>
</tr>
<tr>
<td>3 VCDGNTK</td>
<td>-</td>
</tr>
<tr>
<td>4 CDGNTK</td>
<td>-</td>
</tr>
<tr>
<td>5 DNGNTK</td>
<td>-</td>
</tr>
<tr>
<td>6 FGPFTFVCDG</td>
<td>+</td>
</tr>
<tr>
<td>7 FTFCVGNTK <del>CDGV</del></td>
<td>-</td>
</tr>
<tr>
<td>8 FTFCVDCGNTK <del>VDCG</del></td>
<td>+</td>
</tr>
<tr>
<td>9 FTFCVCDGNTK <del>VGDN</del></td>
<td>-</td>
</tr>
<tr>
<td>10 DFTFVCNNTK DF~</td>
<td>-</td>
</tr>
<tr>
<td>11 FTFCVCDNGKD ~KD</td>
<td>-</td>
</tr>
<tr>
<td>12 FTFVCDGRFTFVCDG</td>
<td>+/-</td>
</tr>
</tbody>
</table>

**Figure 3.9 Peptides tested for CinX activity.** Peptides 1-6 (in red) are N-terminal or C-terminal truncations of CinA (10-19), whereas in peptides 7-11 the location of the aspartic acid is varied. Peptide 12 has two possible hydroxylation sites.

The peptides were synthesized and purified by Dr. Remco Merkx. The CinX assays with peptides 1-6 showed that peptides 1, 2, and 6 were hydroxylated (confirmed via MALDI-ToF MS analysis for peptides 1 and 2, and MS/MS analysis for peptide 6). This observation demonstrates that phenylalanine
10 and threonine 11 N-terminal to Asp15 at the N-terminus and asparagine, threonine and lysine (NTK) at the C-terminus of Asp15 are not necessary for the execution of hydroxylation. Because CinX can accept both 2 and 6 as a substrate, the FVCDG sequence appears sufficient for CinX substrate recognition. Prolyl-4-hydroxylase and lysyl 5-hydroxylase, other members of the Fe(II)/αKG dependent enzyme family, can hydroxylate their targets with a minimum chain length of six amino acids containing a Xaa-Pro/Lys-Gly sequence (11). Our results suggest that the recognition sequence for hydroxylation by CinX might be Xaa-Asp-Gly. However, the presence of additional amino acid residues around this consensus sequence might be needed for substrate-enzyme complex formation, which could explain the inability of CinX to process peptides 3, 4 and 5.

Among peptides 6-11, CinX only accepted peptide 8 as a substrate showing the importance of flanking residues at the modification site, which explains the inability of CinX to process 6, 7, 9, 10, and 11. The difference between wild-type substrate and peptide 8 is that this peptide has a VDCG amino acid sequence at the site of modification instead of VCDG. Mass spectrometry analysis of the processed peptide showed that the modification on the aspartate residue resulted in a mass increase of 12 instead of 16 normally observed upon hydroxylation (Figure 3.9A, B). CinX might further oxidize the hydroxylated Asp to form a ketone and a desaturated C-N bond in peptide 8, accounting for this mass difference (Figure 3.10C). In the hydroxylation reaction of Fe(II)/αKG dependent
hydroxylases, the primary substrate is bound near the metal in the active site (9).

The positioning of the substrate towards the active site might be the determining factor for the type of reaction that this group of enzymes carries out, as they can catalyze a variety of reactions such as desaturation, oxidation, and ring expansion/closure using the same active site and co-substrates that are used for hydroxylation (9).
The assay with peptide 12 showed that CinX can introduce one or two hydroxyl groups to the FTFVCDGRFTFVCDG sequence; however, double hydroxylation occurs with low efficiency (Figure 3.11). The placement of the two possible hydroxylation sites close to each other might account for the low activity.

![Figure 3.11 CinX assay with peptide 12](image)

**Figure 3.11 CinX assay with peptide 12.** Two aspartic acids in this peptide were hydroxylated with low efficiency. M is the mass of unmodified peptide 12.

*Calculated M + OH + H: 1713.9 + 16 +1 = 1730.9 Da, M + 2 OH + H = 1746.9 Da. Observed M: 1712.13 Da, M + OH + H: 1729.5, M + 2 OH + H: 1744.8 Da.

### 3.2.8 Attempts to characterize the kinetics of CinX catalysis

An enzyme coupled assay was developed to perform kinetic studies on CinX with the twelve substrates tested previously *in vitro*. The assay was designed such that succinate produced by CinX activity is used by another enzyme causing a signal to be detected. In this assay, succinic acid (succinate)
produced by CinX is converted to succinyl-CoA in the presence of adenosine-5'-triphosphate (ATP) by the enzyme succinyl-CoA synthetase (SCS), with the concurrent formation of adenosine-5'-diphosphate (ADP) and inorganic phosphate (Pi). Then, pyruvate kinase uses the ADP produced to react with phosphoenolpyruvate (PEP) to form pyruvate and ATP. The pyruvate produced is reduced to L-lactate by L-lactate dehydrogenase (L-LDH) in the presence of reduced nicotinamide adenine dinucleotide (NADH), with the production of NAD$^+$. The amount of NAD$^+$ formed in this coupled reaction pathway is stoichiometric with the amount of succinic acid produced. Thus, the rate of succinic acid production can be obtained by assessing NADH consumption, which is measured by the decrease in absorbance at 340 nm. However, in the experiments using this system, low solubility of the peptides under the reaction conditions prevented accurate kinetic characterization.

3.2.9 Crystallization studies of CinX

Although CinX was obtained in high purity, attempts to obtain crystals by Vinayak Agarwal in the Nair laboratory, including by use of Cd$^{2+}$ and α-ketoglutarate, have not been successful to date. Interestingly, 49 amino acids at the N-terminal region of CinX are not present in the newly identified CinX-analogs in Lyngbya majuscule and Frankia Sp. genomes (Figure 3.12). This region shows homology to a Nif11 domain, which is found in a nitrogen fixation protein of unknown function. Nif11 was also shown to have high sequence homology to the leader region of ProcA which is recognized by ProcM to yield 29
different lanthipeptides named prochlorosins (Figure 3.13) (12). Genome mining experiments have shown that several cyanobacterial genomes contain Nif11 domains on peptides surrounded by lanthionine biosynthetic machinery (13). For one such system in *Prochlorococcus* lantibiotic biosynthesis has been established (12). In this system, the leader peptide shows homology with the Nif11 domain. Hypothesizing that the N-terminal region of CinX may cause instability in crystallization studies, four N-terminal truncated forms of CinX; −20 (amino acid), −35, −50 and −75 were prepared, and their activities were tested in the coexpression studies. For this purpose, N-terminal truncated forms of CinX were cloned into MCS II of pACYCDuet-1 containing Cinorf7 in MCS I, and this vector was used for coexpression experiments with His$_6$-CinA(A−1K), CinM, Cinorf7 and CinX analogs in *E. coli*. The peptide His$_6$-CinA(A−1K) produced was purified by IMAC followed by RP-HPLC. Bioassay and MALDI-ToF MS studies performed after LysC cleavage of the peptide were used to evaluate the activity of CinX. The results showed that only the −20 CinX was able to efficiently hydroxylate CinA and a very small extent of hydroxylation was observed with the −35 CinX truncated protein. Thus, −20 *cinX* and −35 *cinX* were cloned into pET28b with a His-tag to facilitate purification. The protein produced from these vectors will be used in future crystallization studies.
Figure 3.12 Nif11 sequence in CinX. The Nif11-like sequence of CinX is shown in red. Sequence alignment of CinX with its analogs from Lyngbya majuscule (LYNGBM3L_02810) and Frankia Sp. EUN1f (WP_006544296.1).

Figure 3.13 Sequence alignment of Nif11 sequence with CinX and ProcA 2.8 leader.
3.3 DISCUSSION

This chapter described further analysis of Cinorf7 and CinX showing that CinX must hydroxylate the precursor peptide CinA before the action of Cinorf7. The knowledge of the order of the modifications in cinnamycin biosynthesis is valuable for making cinnamycin derivatives. Previously, coexpression studies in *E. coli* showed that CinM dehydrates Ser6 in CinA core region, and that Cinorf7 is required for lysinoalane (Lal) formation with Dha6. Coexpression of CinA-K with CinM and Cinorf7 was sufficient to introduce (Me)Lan and Lal modifications. This finding clearly shows that it is possible to make a cinnamycin-like peptide that has all post-translational modifications except for hydroxylation.

The efforts towards the *in vitro* reconstitution of Cinorf7 activity and crystallization of this enzyme are still ongoing. Gel filtration analysis suggested that the protein exists in several different oligomerization states. The hypothesis that Cinorf7 may act as a chaperone for CinM was investigated by purification of Cinorf7 from *E. coli* expressing all of the cinnamycin biosynthetic genes. Affinity purification of His6-Cinorf7 pulled-down CinM, which supports an interaction between these proteins. However, considering that LanM enzymes typically stereoselectively protonate the enolate formed during the Michael-type addition to provide d-stereochemistry at the α-carbon, it may be that Lal formation, which results in the L-configuration at the newly formed stereogenic center, (14-15) is not carried out by CinM. Conversely, recent studies have shown that several LanM proteins can protonate the enolate with either stereochemistry (16).
Mutational studies on Cinorf7 were carried out as well. Several modifications to Cinorf7 did not affect its activity drastically. Mutation of Arg52 and Trp87 to alanine individually reduced the efficiency of Lal formation, whereas mutation of Trp68 to alanine resulted in loss of Cinorf7 activity. Currently, it is unknown whether these mutations directly affected Lal formation or folding/solubility of the protein, which would also result in loss of activity. It is possible that these mutations are important for the interaction of Cinorf7 with CinM and/or the precursor peptide CinA. Further studies to assess the loss of activity are needed for the mutants that caused partial loss of function. In future studies, the exact Lal amount on a peptide can in principle be determined by use of the o-phthalaldehyde reagent, which was employed for identification of Lal in this study as a sensitive fluorescent detection reagent for amines.

CinX showed promiscuity towards various substrate peptides. It accepted substrates as small as 7 amino acids without the need of the leader sequence. However, it was unable to process the core region of CinA containing (Me)Lan and Lal modifications. The NMR structure of cinnamycin bound to PE clearly shows that Asp15 is facing the interior of the cylinder formed by this molecule around its target (17-18). Thus, formation of the Lal crosslink might lock this peptide in a state where Asp15 is not available for CinX hydroxylation. The inability of CinX to hydroxylate CinM- and Cinorf7-modified peptide also indicates that Cinorf7 acts after CinX modification. Therefore, if hydroxylation needs to be
incorporated on the cinnamycin or its derivatives, the precursor peptide should be treated with CinX before the action of Cinorf7 to form Lal.

The peptide that lacked the hydroxylation on Asp15 compared to cinnamycin was not bioactive towards indicator strain *B. subtilis* 6633. This observation is not surprising as cinnamycin binds to its substrate phosphotidylethanolamine (PE) with a binding constant of $10^7$-$10^8$ M$^{-1}$, primarily through the interaction between the β-hydroxyl group of the hydroxylated aspartic acid and the ammonium ion of PE, and inhibits the growth of certain Gram-positive bacteria by disrupting membrane integrity through this binding (19-20). Hence, the absence of the hydroxyl group on Asp likely results in a much lower affinity for PE.
Oligonucleotide primers for mutagenesis were synthesized by Operon Technologies. *Taq* and *Platinum Pfx* DNA polymerases, *DpnI*, restriction endonucleases, and bacteriophage T4 DNA ligase were purchased from Invitrogen. *Fusion* DNA polymerase was purchased from New England Biolabs. Cloning vectors (pET and pDuet) were obtained from Novagen. Gel extraction, plasmid mini-prep, and PCR purification kits were purchased from Qiagen. All strains were grown in media acquired from Difco laboratories. Other items procured include isopropyl-1-thio-D-galactopyranoside (IPTG, CalBiochem), iodoacetamide (IAA, Acros Organics), tris(2-carboxyethyl) phosphine hydrochloride (TCEP, Aldrich), and dithiothreitol (DTT, Sigma). Endoproteinase LysC was purchased from Roche Applied Science.

3.4 MATERIALS
3.5 GENERAL METHODS

Molecular biological manipulations were carried out using standard techniques \( (21) \). PCR was performed using an automatic thermocycler (PTC 150, MJ Research) and DNA sequencing was performed at the Biotechnology Center of the University of Illinois at Urbana-Champaign (UIUC). *Escherichia coli* (*E. coli*) DH5α cells (UIUC Cell Media Facility) and *E. coli* BL21 (DE3) cells (Stratagene) were used for plasmid preparation and protein expression, respectively. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-ToF MS) was carried out on a Voyager-DE-STR (Applied Biosystems). Liquid chromatography electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS) was carried out and processed using a Synapt ESI quadrupole ToF Mass Spectrometry System (Waters) equipped with an Acquity Ultra Performance Liquid Chromatography (UPLC) system (Waters).
3.6 EXPERIMENTAL METHODS

3.6.1 Cloning

3.6.1.1 Cloning of cinA, cinM, cinX and cinorf7 and generation of a coexpression system in E. coli (NB6)

Cloning of the genes cinA, cinM, cinX and cinorf7 from S. cinnamomeus genomic DNA into pRSFDuet-1 and pACYCDuet-1 was described previously (7). In this study, a histidine tag was inserted at the N-terminus of CinM in MCSII of pRSFDuet-1, and Cinorf7 in MCSI of pACYCDuet-1 respectively. For His\textsubscript{10}-CinM cloning, two primers His\textsubscript{10}-CinM\textsubscript{pRSFDuet-1}\textsubscript{MCSII_EcoRV_FP} and CinM\textsubscript{pRSFDuet-1}\textsubscript{MCSII_KpnI_RP} (Table 3.1) were used for the amplification of the cinM gene from a template pET16b plasmid containing a gene encoding His\textsubscript{10}-CinM (obtained from previous PhD student Dr. Lisa Cooper) whereas for His\textsubscript{6}-Cinorf7 cloning of the gene encoding for His\textsubscript{6}-Cinorf7, Cinorf7\textsubscript{pACYCDuet-1}\textsubscript{NcoI_His\textsubscript{6}-Tag_FP} and Cinorf7\textsubscript{pACYCDuet-1}\textsubscript{HindIII_RP} (Table 3.1) were used for the amplification of the cinorf7 gene from a template pET28b containing His\textsubscript{6}-Cinorf7 (obtained from Dr. Lisa Cooper). The reactions contained 1xFailSafe PreMix G (PICENTRE Biotechnologies), DMSO (4%), Platinum Pfx DNA polymerase (0.025 unit/μL), Taq DNA polymerase (0.05 unit/μL), and primers (1 μM each). Amplification of the final PCR products were confirmed by 1% agarose gel electrophoresis and the products were purified using QIAquick PCR purification kits. The resulting DNA fragments and the corresponding vectors were digested in 1 x NEBuffer 4 with EcoRV-HF and KpnI-HF at 37 °C (for 15 h)
for insertion of CinM, and NcoI-HF and HindIII-HF for insertion of Cinorf7. The digested products were purified by agarose gel electrophoresis followed using a QIAquick gel extraction kit. The resulting DNA inserts were ligated with the digested corresponding vectors at 16 °C for 15 h using T4 DNA ligase to generate His$_6$-CinA/His$_{10}$-CinM/pRSFDuet-1, His$_6$-Cinorf7/CinX/pACYCDuet-1. The ligation reaction mixture was diluted 5 times with water prior to transformation. *E. coli* DH5α cells were transformed with the ligation product via heat shock, plated on 50 mg/mL LB-kanamycin (for pRSFDuet-1) and 25 mg/mL LB-chloramphenicol (for pACYCDuet-1) agar plates, and grown at 37 °C for 15 h. Colonies were picked and incubated in 5 mL of LB-kanamycin or LB-chloramphenicol medium at 37 °C for 15 h, followed by isolation of the plasmids using a QIAprep Spin Miniprep Kit. The sequences of the resulting plasmids were confirmed by DNA sequencing.

Similarly, *cinX* was cloned into pRSFDuet-1 containing CinA(A−1K) by use of CinX pRSFDuet-1 MCSII EcoRV FP and CinX pRSFDuet-1 MCSII PvuI RP as forward and reverse primers (Table 3.1). Cloning of DNA encoding N-terminal truncations of CinX into MCSII of pACYCDuet-1 containing *cinorf7* in MCSI was performed using the primers listed in Table 3.1. The reactions contained 1xFailSafe PreMix G (PICENTRE Biotechnologies), DMSO (4%), Platinum Pfx DNA polymerase (0.025 unit/μL), Taq DNA polymerase (0.05 unit/μL), and primers (1 μM each). Amplification of the final PCR products were confirmed by 1% agarose gel electrophoresis and the products were purified using QIAquick
PCR purification kits. The resulting DNA fragments and the corresponding vectors were digested in 1 x NEBuffer 4 with EcoRV-HF and PvuI-HF at 37 °C (for 15 h) for insertion of CinX into pRSFDuet-1, and EcoRV-HF and XhoI-HF for insertion of short CinX forms into pACYCDuet-1. The digested products were purified by agarose gel electrophoresis followed using a QIAquick gel extraction kit. The resulting DNA inserts were ligated with the digested corresponding vectors, use to transform E. coli cells, purified, and sequenced as described above. A similar process was followed for cloning the genes for −20 CinX and −35 CinX cloning into pET28b by use of the primers indicated in Table 3.1.

3.6.1.2 Cloning of Cinorf7 mutants (NB 9-10, p25-28)

The amplification of Cinorf7/CinX/pACYCDuet-1 was conducted via 30 cycles of denaturing (98 °C for 10 s), annealing (60 °C for 30 s), and extending (72 °C for 135 s) using appropriate mutant primers (see Table 3.1 for oligonucleotide sequences). The PCRs (50 μL) included 1_ HF buffer (Finnzymes), DMSO (4%), Fusion Hot Start High-Fidelity DNA Polymerase (Finnzymes) (0.04 unit/μL), dNTPs (2 mM), Cinorf7/CinX/pACYCDuet-1 (20 ng), and primers (1 μM each). The PCR product was checked on a 1% agarose gel and purified using a QIAquick PCR purification kit (QIAGEN). The resulting DNA was treated with DpnI at 37 °C for 15 h to digest the methylated template, and E. coli DH5R cells were transformed. The desired mutations were confirmed by DNA sequencing.
3.6.2 *In vitro* Cinorf7 assays (NB9, p. 13-16, 49, 53)

His$_6$-CinA(A−1K) modified by CinM and CinX in *E. coli* (0.5 mg) was treated with 10% reaction buffer from RTS 100 *E. coli* HY kit, 20% cell extract prepared according to the literature (7), 2 mM ATP, 10 mM MgCl$_2$, 2.5 mM DTT, 5 μM Cinorf7, 5 μM CinM, and 20 μM CinX followed by incubation with LysC.

3.6.3 Circular dichroism spectrometry measurements of Cinorf7 (NB8, p75)

A solution of 100 mM Na$_2$HPO$_4$ was used as a blank in a 1 cm cuvette. The CD spectrometer (Prof. Martin Gruebele Lab) was adjusted to 80 mV, 1 nm step resolution and 100 mdeg sensitivity. The CD spectrum of 3.6 μM His$_6$-Cinorf7 in 50 mM sodium phosphate buffer pH 7.5 was measured between 200-250 nm after 100 mM Na$_2$HPO$_4$ was used as a blank in a 1 cm cuvette. The webpage Expasy APSSP2 was used for the estimation of the secondary structure.

3.6.4 OPA Assay (NB7, p.70)

CinA(A−1K) (~0.5 mg), which had been modified by CinM and CinX, and CinA(A−1K) (~0.5 mg), which had been modified by CinM and Cinorf7 in *E. coli*, were incubated with LysC endoproteinase (50 μL, 1 mg/mL) followed by SPE purification as described in Chapter I. Then the samples were purified and concentrated by solid phase extraction column (SPE, 1 mL, Discovery DSC-18). The C18 column was first washed with 2 mL of 80% ACN/water and equilibrated with 2 mL of water, and then the sample was applied. The column was washed with 4 mL of water and the sample was eluted with 1 mL of 80% ACN/water solution. The sample was dried on a centrivap concentrator and the peptide was
dissolved in 25 μL of autoclaved water, and analyzed by MALDI-ToF MS. The samples and 25 μL of authentic cinnamycin (0.1 mg/mL) were separately incubated with 75 μL of a solution containing 35 mg of OPA, 0.5 mL of methanol, and 42.5 mL of buffer (25 g/L boric acid, 0.3 % Brij 35, 0.2 % β-mercaptoethanol) for 1 h. Then, the samples were analyzed by MALDI-ToF MS.

3.6.5 Tandem mass spectrometry analysis to determine the hydroxylation site in CinA (NB4, p65-66; NB8, p59)

The peptides were modified by CinX in vitro and analyzed by LC-ESI-MSMS using a Synapt ESI quadrupole ToF Mass Spectrometry System (Waters) equipped with an Acquity Ultra Performance Liquid Chromatography (UPLC) system (Waters). A collision energy ramp of 15-50 V was used over 20 min in the LC MS program with a C18 column using a mass range of 50-2000 Da. In most of the peptides analyzed, the +2 charged ions were the dominant peaks, which were used as parent ion for fragmentation.

3.6.6 Overexpression and purification of His6-CinA(A−1K) from coexpression studies (NB9, p37)

Peptides were co-expressed with different enzymes and purified from E. coli BL21 (DE3) cultures as described in previous coexpression studies (7). Briefly, purification was accomplished by solubilizing the inclusion bodies from the coexpression experiments using 4 M guanidine hydrochloride as even in the modified form, CinA stays in the insoluble fraction during purification. Then this solution was loaded onto a 5 mL HiTrap chelating HP nickel affinity column (GE
Healthcare). The desired peptides were eluted in 1-2 column volumes of elution buffer containing 4 M guanidine hydrochloride, 20 mM sodium phosphate, pH 7.5, 100 mM NaCl, and 1 M imidazole. Desalting was achieved by preparative reversed phase high performance liquid chromatography (RP-HPLC) on a Waters system using a C4 PrepLC column. Solvents for preparative RP-HPLC were solvent A (0.1% TFA in water) and solvent B (0.086% TFA in 80% ACN/20% water). A gradient of 2 – 100 % of solvent B over 45 min was executed with a flow rate of 8 mL/min. Peptides were detected by absorbance at 220 nm. Lyophilized powder was analyzed by MALDI-ToF MS and the desired products were stored at –20 ºC.

3.6.7 Purification of His$_6$-Cinorf7 and His$_{10}$-CinM from the coexpression system in E. coli (NB 8, p97-98)

*E. coli* BL21 (DE3) cells were co-transformed with His$_6$-CinA(A−1K)/CinM/pRSFDuet-1 and CinX/ His$_6$-Cinorf7/pACYCDuet-1 for His$_6$-Cinorf7 purification, whereas the cells were co-transformed with His$_6$-CinA(A−1K)/His$_{10}$-CinM/pRSFDuet-1 and CinX/Cinorf7/pACYCDuet-1 for His$_{10}$-CinM purification. Single colony transformants were grown in a 37 ºC shaker for 12-15 h in 50 mL of Luria-Bertani (LB) medium supplemented with 25 μg/mL kanamycin (for pRSFDuet-1) and 12.5 μg/mL chloramphenicol (for pACYCDuet-1). A 20 mL aliquot was centrifuged at 5000×g for 10 min, the spent LB medium was discarded, and the cell pellet was resuspended in fresh LB medium. The resuspended cells (20 mL) were added to 2 L of LB containing the two
antibiotics, and the culture was grown aerobically at 37 °C until the A600 was ~ 0.6 to 0.8. IPTG was added to a final concentration of 0.5 mM and the culture was transferred to 18 °C for aerobic growth for an additional 20 h. Cells were harvested by centrifugation at 5000×g for 20 min at 4 °C. The cell paste (~ 4 g) was stored at –80 °C until use.

Both CinM and Cinorf7 were kept at 4 °C for the duration of the purification process. For each enzyme, the cell paste was resuspended in ~ 20 mL of LanM Start Buffer (20 mM Tris, pH 7.6, 500 mM NaCl, 10 % glycerol) and lysed by sonication (35% amplitude, 4.0 s pulse, 9.9 s pause, 15 min). The sample was centrifuged at 23,700×g for 45 min (Beckman JA-20 rotor) and the supernatant was clarified through 0.45 μm syringe filters (Corning). The sample was then loaded onto a 5 mL HisTrap HP column (GE Healthcare Life Sciences). The column was washed with 20 mL each of start buffer containing 25 and 50 mM imidazole and then 10 mL each of start buffer containing 100, 200, and 500 mM imidazole. The eluent was collected in several fractions, which were analyzed by Tris-SDS-PAGE (4-20% acrylamide gradient). The fractions containing the desired protein (200-500 mM imidazole) were combined and concentrated using an Amicon Ultra-15 centrifugal filter unit (30 kDa molecular mass cut off for His10-CinM, 3 kDa molecular mass cut off for His6-Cinorf7, Millipore) to less than 2 mL. Buffer exchange of the concentrated proteins with Final Buffer (20 mM Tris, pH 7.6, 500 mM KCl, 10% glycerol) was conducted twice via size exclusion chromatography (GE Healthcare Life Sciences). The resulting protein samples
were aliquoted, frozen with liquid nitrogen, and stored at −80 °C. Protein concentrations were determined using absorbance at 280 nm and were calculated with a theoretical extinction coefficient obtained from the ProtParam function on the ExPASy Proteomics Server (His$_{10}$-CinM, $\varepsilon_{280} = 158,180$ M$^{-1}$cm$^{-1}$, His$_{6}$-Cinorf7, $\varepsilon_{280} = 17,990$ M$^{-1}$cm$^{-1}$). For CinM, typical protein yields ranged from 6 -10 mg per liter of overexpressed cells, whereas for Cinorf7 protein yields ranged from 1.5 mg to 2 mg per liter of overexpressed cells.
List of primers * From 5' to 3'

His-tag Insertion to Cinorf7 and CinM

Cinorf7 pACYCDuet-1 Ncol His-Tag FP: AATTCGATCCCATGGGCAGCAGCCATCATCATCATC
Cinorf7 pACYCDuet-1 HindIII RP: TCGATGCAAGCTTTCGAGACGTGCTGCTGAC
His-CinM pRSFDuet-1 MCSII EcoRV FP:
TCTCAATTTGGATATCGATGCGCCATCATCATCATCAT
CinM pRSFDuet-1 MCSII KpnI RP: AGACTCGAGGGTACCTTACTGTTCTGTTGCTGACGAGG

Cinorf7 Mutational Studies

Cinorf7 - 20 pACYCDuet-1 Ncol FP: AGGTGAACCCATGGGC GAG CTC ATG GTT CTG TGC TCC
Cinorf7 - C-tr. trunc. RP HindIII: TCGATGCG AGCTTTTCACAGAATGGGCGGCTGCTGTTG
Cinorf7 R52A QC FP: GAGGAAGCCGCTACTGGCGCAATCGGCCTTCACTACCAACT
Cinorf7 R52A QC RP: GCCGAGTACCCGGTCCTCGAGCGAGAGG
Cinorf7 W689-69AA QC FP: CTTGCGAGAAGAAGGCGCAGCAGGCTGGTCGCTG
Cinorf7 W689-69AA QC RP: GCTTTTCGTCGCAAGGGGATGAGAGTT
Cinorf7 E81A QC FP: GAGCGGTACCTTCAGCGAGGCAAGAGGCTGCGGCTG
Cinorf7 E82A QC RP: TCTGCTGGAACGCGTCCACGAGCCACAG
Cinorf7 W87A FP: GAGAAGCCGCTGCTGCGAGGACAAACACAGGACAC
Cinorf7 W87A RP: GGCGACGAGTCTCTTTCTCTGCTCGGAAAC
Cinorf7 Q88A FP: GAGCTGCTGCGCTCATTCAACAAGCAGACATG
Cinorf7 Q88A RP: CCAGGCGACCAGCTCCTCTCCTCGCTGGAAC
Cinorf7 E100A FP: GAGACGCACATCGCTGCGGTCACAGAATGCCGAAC
Cinorf7 E100A RP: AGAATGGCGGCTCAGGTATTGCTGCTG

CinX Primers

CinX pRSFDuet-1 MCSII EcoRV FP: TCTCAATTTGGATATCGATGCGCCCTGGAAGACTCTGCGAGGATT
CinX pRSFDuet-1 MCSII PvuII RP: GAAGCGATTACGCGATCGCTACGCCTCGTGCTGGCGAGGGCGGTCA
CinX FP EcoRV -20: TCTCAATTTG GATATCGCGAGAGATGAAGCGGTCA
CinX FP EcoRV -35: TCTCAATTTGGATATCGCGAGAGATGAAGCGGTCA
CinX FP EcoRV -50: TCTCAATTTGGATATCG AGAAGGCATGACCTTCCAGGGACAC
CinX FP EcoRV -70: TCTCAATTTGGATATCGCGGCGGCAACAGGGCC
CinX pACYCDuet-1 MCSII XhoI RP: TTACAGGAGCTCGAGTCGAGCGGTGGTGGCGAGGGCGGTCC

For pET28b

-20 CinX Ndel: GGA ATT CCATATGCGCGAGATGAAGGCGGTCAC
-35 CinX Ndel: GGA ATTCATATGCGCGCAGCGACGGCTAC
CinX pET28b XhoI RP: CCCTCGAGTCGAGCGGTGGTG

LctC Studies

LctCE pRSFDuet-1 BglII MCSII FP: ATGCATGCG AGATTCATGAAAGAAAACACTCTTTTAAT
LctCE pRSFDuet-1 PvuII MCSII RP: CGCGATGCGATCCGTTAATCAACATATGGCATAAAGAC

*Restriction sites and mutation sites are underlined

Table 3.1. List of primers.
3.7 REFERENCES


CHAPTER IV: BIOSYNTHESIS OF CINNAMYCIN-LIKE NATURAL PRODUCTS

4.1 INTRODUCTION

The currently known members of the family of cinnamycin-like peptides share the same post-translational modifications with cinnamycin, which are one lanthionine, two methyllanthionines, a lysinoalanine linkage and, with the exception of ancovenin, an erythro-3-hydroxyl-L-aspartate residue (Figure 2.1). These peptides, including cinnamycin, duramycin, duramycins B, duramycin C, and ancovenin have been isolated and structurally characterized (1-3). However, many putative cinnamycin-like peptides have yet to be isolated and studied; their biosynthetic gene clusters have only been predicted bioinformatically.

A BLAST search on the NCBI genome database identified fourteen cinnamycin-like peptides including the previously isolated peptides (Figure 4.1). Interestingly, three sets of two cinnamycin-like peptides, which could be two-component lantibiotics, were also identified (Figure 4.1). From these, the gene clusters found in Moorea producens, and Oscillatoria sp. PCC 10802 contain cinX-like and cinorf7-like genes like the other members of the cinnamycin group of peptides; however the cluster identified in Cylindrospermum stagnale PCC 7417 does not contain these genes. Among the precursor peptides, OscA1, OscA2, and CylinA2 have interesting amino acid sequences where double cinnamycin-like core sequences are attached to each other after putative leader peptide sequences (Figure 4.2A). Also, MooA2, OscA2, and CylinA2 lack the Ser to make the Lal
crosslink (Figure 4.2A). OscA2 and CylinA2 show high sequence identity, both having two cinnamycin-like core regions connected by the amino acids PN. However, OscA1 and CylinA1 differ from each other; CylinA1 has a second cinnamycin-like core sequence, whereas OscA1 does not (Figure 4.2B).

Duramycin and duramycin B are closely related to cinnamycin with the exception of a substitution in each peptide, arginine 2 to lysine and phenylalanine 10 to leucine, respectively. Duramycin C differs from cinnamycin by five amino acids, which are arginine 2 to lysine, glutamine 3 to asparagine, phenylalanine 7 to tyrosine, phenylalanine 12 to tryptophan, and valine 13 to serine (4). Cinnamycin, duramycin, duramycin B, and duramycin C strictly bind to the phosphatidylethanolamine (PE) within the membrane and form a 1:1 complex (4-7). These peptides cannot bind phospholipids closely related to PE, such as phosphatidylcholine (8). The NMR structures of cinnamycin and the duramycins showed that the four crosslinks form a cylindrical shape offering a binding scaffold for the recognition of the glycerol moiety of PE (4, 9-11). Specific interactions between a region spanning the 7th and 15th amino acids of cinnamycin and the head group of the phospholipid ensures that only PE can bind to cinnamycin (9). Specifically, 3-hydroxyaspartic acid at position 15 is involved in the binding of the free amino group of PE and the hydrophobic residues, and Gly-8, Pro-9 and Val-13 are involved in the binding to the glycerol moiety (4, 9). The amino acids involved in the binding of the amino group and the glycerol moiety are conserved in several of the putative group members.
suggesting that these cinnamycin-like peptides may also be involved in binding to PE or other phospholipids.

Cinnamycin, duramycin, and duramycins B and C inhibit phospholipase A2 (PLA2) by sequestering its substrate PE with micromolar affinity (8, 12). PLA2 utilizes PE as a substrate to form arachidonic acid, which leads to the biosynthesis of prostaglandins and other mediators of inflammation (13). Inflammation is an important contributing factor to atherosclerosis and increases the risk of high blood pressure, heart disease and stroke (14-15). Duramycin has also been shown to mitigate pulmonary secretions related to cystic fibrosis (CF) and is currently in clinical trials for the treatment of CF (16). Duramycin binds to PE and induces pore formation, which leads to an increase of the net chloride ion secretion in airway epithelium. Therefore, duramycin contributes to increased fluidity of the mucus and alleviation of blocked tubes (16). This mode of action of duramycin suggests that cinnamycin, also a PE binder, may play a similar role for the treatment of CF (17). The duramycin biosynthetic gene cluster had not been described prior to the studies described in this chapter, which allowed production of duramycin in E. coli.
Figure 4.1 Alignment of cinnamycin-like peptide precursors. The bacteria for which the sequences for precursor peptides have been identified are as follows: NocA in *Nocardiopsis potens*, ActA in *Actinomadura atramentaria*, DurA in ATCC 12686 *Streptoverticillium cinnamoneum* forma *azacoluta*, OscA in *Oscillatoria* sp. PCC 10802, MooA1 and MooA2 in *Moorea producens*, OscA1 and OscA2 in *Oscillatoria* sp. PCC 10802, and CylinA1 and CylinA2 in *Cylindrospermum stagnale* PCC 7417. However the latter gene cluster does not have a cinorf7-like gene while others depicted here except ancovenin do. Duramycins B and C have been isolated from *Streptoverticillium* strain R2075 and *Streptomyces griseoluteus* R2107, ancovenin was isolated from the culture broth of a *Streptomyces* species.
**A.**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>OscA2</td>
<td>MSNSFEYSVILEKPSVEDLELLQRSVIDQFBSELVDNEAPFGIA-ADREKIELPEKVEQ</td>
</tr>
<tr>
<td>CylinA2</td>
<td>MSN-----KTLPRIIPSYLDQLLHRAIVDSEFRIELLSRPEEFGITKADVVLALLTSVEQ</td>
</tr>
<tr>
<td>OscA2</td>
<td>QDMSFVELV TEDNIF- AE</td>
</tr>
<tr>
<td>CylinA2</td>
<td>CGSTCVSGETILCDGNTQ PN CRRTCVS GWTIRCDGATV</td>
</tr>
</tbody>
</table>

**B.**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>OscA1</td>
<td>MSIT--LEKPSVIYLEQIFQKAIDAFDRDEILANPGAELALPAAVEKQDSV</td>
</tr>
<tr>
<td>CylinA1</td>
<td>MPTQNLTKSPVKYHELQRAIVDAEFRHELCLRPEEFGITKEDLERLPDAVEKQDNMT</td>
</tr>
<tr>
<td>OscA1</td>
<td>FELLDDALGELNIAVA--------------------------------------------------------</td>
</tr>
<tr>
<td>CylinA1</td>
<td>VELVVESGNSDIKAAGTCKSGLYTCDGDRDNPPGD T</td>
</tr>
</tbody>
</table>

---

Figure 4.2 OscA1, OscA2, and CylinA2 are cinnamycin-like peptides with interesting amino acid sequences, where double cinnamycin-like sequences are attached to each other after each putative leader peptide. (A) OscA2 and CylinA2 have high sequence identity and have two cinnamycin-like core regions attached to each other separated by the amino acids PN (in red). However OscA1 and CylinA1 differ from each other such that CylinA1 has a second cinnamycin-like core sequence whereas OscA1 does not (B).

Ancovenin is a potent inhibitor of angiotensin-converting enzyme (ACE), a well-studied enzyme known to regulate inflammation. ACE inhibitors decrease systemic vascular resistance without increasing heart rate and are effective in the treatment of hypertension, (3, 18-22). The biosynthetic gene cluster of ancovenin is not known, therefore, it is not possible to produce ancovenin in vitro currently. Furthermore, the ancovenin producing strain is claimed to be lost at Fujirebio Diagnostics, Inc (23). This chapter describes an alternative route to ancovenin.

Cinnamycin biosynthesis was previously described by our group (24) and is used as a representative system for the study of the other members and
unnatural analogs described in this chapter. The mode of action and/or binding partner of cinnamycin-like lanthipeptides other than cinnamycin, duramycins and ancovenin are currently unknown. A biotinylated derivative of cinnamycin that retains high specificity to PE has been used as a probe for the investigation of the transbilayer movement of PE (7, 25). It is valuable to find small peptide ligands for different phospholipids as they can be used as a marker for cancer, viral infections and related diseases (26). For example, 99mTc or biotin labeled duramycin has been used for the detection of cancer, apoptosis, acute cardiac cell death in mice, vascular PE as a critical anticoagulant, and rat cerebral ischemia-reperfusion injury in rat brain after middle cerebral artery (MCA) occlusion (25, 27-29).

The cinnamycin-like group of peptides has the potential to bind to phospholipids with diverse head groups. Amino acids recognizing the glycerol moiety and aspartic acid 15 that is hydroxylated are conserved within the members of the group. Hydroxylation within the peptide is crucial for the interaction with the amino group in the phospholipid PE (24) and CinX-like proteins are responsible for this hydroxylation and were found in most, but not all, of the biosynthetic gene clusters of uncharacterized peptides. Lanthionine and lysinoalanine crosslinks on cinnamycin can be formed successfully in the absence of CinX as described in Chapter II. Therefore, it is possible to make cinnamycin analogs without the hydroxylation on Asp15 and investigate their binding properties. Moreover, as cinnamycin-like peptides exhibit different
properties towards the pulmonary and cardiovascular systems it would be valuable to study their biosynthesis. Biosynthesis of duramycin, ancovenin, and a peptide from \textit{Frankia Sp.} EUNf1 (frankisin), whose biosynthetic gene cluster contains a biosynthetic cluster that is very similar to the cinnamycin biosynthetic genes, was studied in this chapter. Computer modeling studies performed by Prof. Ming Zhao`s group at Northwestern University suggested analogs of cinnamycin and duramycin could bind to different phospholipids. Thus, several analogs of cinnamycin and duramycin were prepared by site directed mutagenesis and tested for binding to phosphotidylcholine (PC), phosphatidylinositol (PI), and phosphatidylserine (PS) by isothermal calorimetry (ITC).
4.2 RESULTS

4.2.1 Duramycin Biosynthesis

4.2.1.1 Duramycin gene cluster from *Streptovercillium cinnamoneum forma azacoluta* (ATCC 12686)

I extracted the genomic DNA from *Streptovercillium cinnamoneum forma azacoluta*, which was sequenced by Dr. Yun-juan Chang, by Illumina shot gun sequencing. According to the sequencing results, the duramycin gene cluster contains four biosynthetic genes as in the case of the cinnamycin biosynthetic gene cluster (Figure 4.3). Similar to the findings for the cinnamycin precursor peptide, DurA consists of a C-terminal core region that is 19 residues long and that is transformed into duramycin through post-translational modifications. Appended to the N-terminus of the core peptide is a 59 amino acid leader sequence that is not modified. Instead of a GG or GA protease cleavage motif seen in most class II lantibiotics, an AXA motif is present between the leader sequence and the core region of DurA (Figure 4.3). This sequence is recognized by type I signal peptidases of the general secretory (*sec*) pathway (30), which explains the absence of a duramycin-specific protease in the gene cluster. DurA (78 aa), the precursor peptide for duramycin, showed 92% identity to CinA with 100% sequence coverage. The DurM sequence shows high homolgy with other class II LanM enzymes, which are bifunctional enzymes that catalyze both dehydration and cyclization (31-32). In particular, DurM (1083 aa) and CinM (1088 aa) showed 85% identity with 100% coverage. Furthermore, DurX (327 aa)
and CinX (325 aa) showed 80% identity with 100% coverage, suggesting that DurX is a member of the non-heme Fe(II)-dependent family of enzymes that utilizes β-ketoglutarate (α-KG) as cofactor for catalyzing the hydroxylation of the β-carbon on Asp15. Durorf7 (125 aa) and Cinorf7 (119 aa) showed 89% identity with 97% coverage, which suggests that this protein is involved in Lal formation in duramycin biosynthesis. After the core peptide is processed by these enzymes, the leader peptide is removed proteolytically to afford the mature, biologically active compound (33).

Figure 4.3 Duramycin biosynthesis. (A) Genomic DNA from duramycin producing strain, *Streptoverticillium cinnamoneum forma azacoluta* ATCC 12686 producing the duramycin biosynthetic gene cluster was sequenced by Illumina short gun sequencing and compared to the cinnamycin biosynthetic gene cluster from *S. cinnamoneus*. (B) Representation of biosynthesis of duramycin from the precursor peptide DurA. The directionality of the formation of the thioether crosslinks (black) and Lal (blue) is shown. Hydroxylation of Asp15 is represented in red. The sequence of the DurA core and leader peptides is depicted as well as the residues that form the crosslinks. The sec-cleavage sequence at the end of the leader peptide is shown in underlined font.

In addition to the genes required for duramycin biosynthesis, the gene sequences showing high sequence identity and coverage to cinY, cinZ, cinZ,
cinH, cinT, and cinorf4 were also identified in the gene cluster in the same locations compared to the corresponding genes in the cinnamycin gene cluster (Figure 4.4). From these genes durorf4 (570 bp) encodes a helix–turn–helix DNA-binding motif similar to the pfam TETR family. DurT and durH (864 and 870 bp, respectively) encode for subunits of an ABC transporter. DurT appears to be the ATP-binding subunit and is a member of the pfam family of ABC transporters; it shows 94% identity to CinT. The protein DurH appears to be an integral membrane subunit of the pfam family of ABC-2 type transporters commonly involved in drug efflux and resistance, or carbohydrate export. The durY gene (945 bp) encodes a protein that may possess an N-terminal signal peptide, suggesting that it may be exported, but displays no other significant homologies to known proteins. The durZ gene has a length of 627 bp and the encoded protein appears to be a member of a family of proteins (pfam UPF0029) of unknown function.

Figure 4.4 The gene cluster for duramycin production, regulation, and immunity from *Streptoverticillium cinnamoneum forma azacoluta* ATCC 12686.
4.2.1.2 Coexpression of *durA*, *durM*, *cinX*, and *cinorf7* in *E. coli*

Recent studies in several laboratories have demonstrated the feasibility of conducting the post-translational modifications that generate lantibiotics in *E. coli* (34-38). In our laboratory, pRSFDuet-1 and pACYCDuet-1 plasmids were used for these studies, which offer a convenient purification approach of the post-translationally modified peptide using IMAC because of a His$_6$-tag appended to the N-terminus of the leader peptide of the substrate peptide (36). A similar approach was employed here in an attempt to obtain duramycin.

The gene encoding for DurA was inserted into multiple cloning site I (MCSI) of the pRSFDuet-1 vector to generate His$_6$-DurA. The gene encoding for DurM was inserted into MCSII of the same plasmid resulting in a protein without a His-tag, thus preventing co-purification of the DurM enzyme. Similarly, *durX* was cloned into MCSII and *durorf7* was cloned into MCSI of the pACYCDuet-1 vector, which also produces DurX and Durorf7 without a His-tag (24).

Coexpression of *durM*, *durX*, and *durorf7* together with *his$_6$-durA* caused the cells to die and this experiment has not been repeated. However, when *his$_6$-durA* and *durM* were coexpressed with *cinX* and *cinorf7*, ~2 mg of modified His$_6$-DurA peptide per liter of cell culture after IMAC purification and subsequent RP-HPLC was obtained. Removal of the leader peptide was accomplished by GluC protease cleavage, which cleaves at the C-terminus of Glu located at the −4 position, followed by aminopeptidase treatment of the peptide to remove the AFA amino acid sequence at the N-terminus. Since duramycin starts with a cysteine
that is involved in lanthionine crosslink formation, aminopeptidase does not recognize this amino acid, and thus aminopeptidase treatment results in removal of the AFA sequence to yield duramycin. MALDI-ToF MS analysis of the GluC and aminopeptidase treated peptide showed a mass difference with respect to unmodified core peptide corresponding to four dehydrations and one hydroxylation (Figure 4.5A). *In vivo* thioether cyclization activity was investigated by iodoacetemide (IAA) treatment and analysis by MALDI-ToF MS as previously described. Only minor amounts of IAA adducts were observed indicating that DurM formed predominantly three thioether rings in the DurA core region (Figure 4.5B). An agar diffusion assay of the modified core region of DurA towards *B. subtilis* 6633 confirmed that the peptide was bioactive, and thus duramycin was successfully prepared by heterologous expression of the genes encoding for DurA, DurM, CinX, and Cinorf7 in *E. coli* (Figure 4.6B).
Figure 4.5 MALDI-ToF MS analysis of His$_6$-DurA modified by DurM, CinX, and Cinorf7 in E. coli. (A) MALDI-ToF MS analysis of His$_6$-DurA modified by DurM, CinX, and Cinorf7 in E. coli and treated with GluC followed by aminopeptidase treatment; (B) MALDI-ToF MS analysis of iodoacetamide (IAA) treatment of the coexpression product followed by GluC cleavage and aminopeptidase treatment. *DurA core region with different numbers of dehydrations. **Duramycin with an alanine at $-1$ position that remains from the leader peptide due to less than 100% efficiency of leader peptide removal by aminopeptidase.

*Calculated mass for $M - 4$ H$_2$O + OH + H ($M^*$); 2069.3 – 4 * 18.0 + 16 +1 = 2014.3 Da, Obsrvd: 2013.3 Da (B) $M^* +$ IAA = 2013.3 + 57 = 2071.3 Da; Obsrvd $M^*:2013.4$ Da. $M^* +$ IAA = 2071.7 Da.

In Chapter II, the Lal crosslink was also formed in vitro by treating the dehydrated, cyclized, and hydroxylated CinA in alkaline solution overnight. Similar experiments were performed for the formation of the Lal crosslink in duramycin. The formation of the crosslink in this peptide was investigated by o-phthalaldehyde (OPA) (39). IMAC purified His$_6$-DurA that had been modified by DurM and DurX in E. coli were incubated with GluC followed by aminopeptidase. Subsequently, the proteolyzed peptides were treated with OPA resulting in three OPA adducts originating from the N-terminal amino group and the ε-amino groups of Lys2 and Lys19. However, when His$_6$-DurA that had been modified by
DurM and CinX in *E. coli* and authentic duramycin were treated with OPA a mass increase corresponding to two OPA additions in the core region was observed (Figure 4.7A, B). These two OPA adducts originate from the N-terminal amino group and the ε-amino groups of Lys2 with Lys19 not involved in a Lal crosslink. In addition, one β-mercaptoethanol addition was observed, presumably as a consequence of Michael-type addition to dehydroalanine 6 (Figure 4.7C).

![Figure 4.6 Bioassay with the indicator strain *B. subtilis* 6633.](image)

(A) Sector 1, authentic duramycin; sector 2, His\(^6\)-DurA modified by DurM and DurX in *E. coli* and treated with GluC and aminopeptidase followed by incubation at pH 9.5; sector 3, His\(^6\)-DurA modified by DurM and DurX in *E. coli* treated with GluC and aminopeptidase (no alkaline treatment); sector 4 was not used. (B) Sector 1, authentic duramycin; sector 2, His\(^6\)-DurA modified by DurM, CinX, and Cinorf7 in *E. coli* and treated with GluC and aminopeptidase.

The bioactivity analysis of the DurA core peptide containing (Me)Lanthionine crosslinks and hydroxylated Asp before and after the alkaline treatment also suggested that Lal formed at pH 9.5 as alkaline treated peptide
formed a zone of inhibition, whereas the untreated peptide did not (Figure 4.6A). As observed with cinnamycin, these results show that Lal is crucial for the bioactivity of these peptides and that this crosslink can be formed \textit{in vitro} by increasing the pH.

\textbf{Figure 4.7 Use of o-phthalaldehyd (OPA) to monitor lysinoalanine formation.} (A) MALDI-ToF MS of authentic duramycin incubated with OPA and β-mercaptoethanol. M = mass of core peptide after 4 dehydrations and one hydroxylation. (B) DurA was modified by DurM and CinX followed by alkaline treatment and removal of the leader peptide with GluC and aminopeptidase. The resulting peptide was then incubated with OPA and β-mercaptoethanol and analyzed by MALDI-ToF MS. (C) MALDI-ToF MS of DurA modified by DurM and DurX, and treated with GluC and aminopeptidase incubated with OPA and β-mercaptoethanol.

*Calculated mass for authentic duramycin + 2 * OPA + H = 2013 + 2 * 180 + 1 = 2374 Da, for GluC digested fully-modified is 2302.6 Da (M); M + OPA + βME +H; 2302.6 + 180 + 78 +1 = 2561.6 Da, M+ 2 * OPA + H; 2302.6 + 2 * 180 + 1= 2663.6 Da, M + 2 OPA + BME + H; 2013.3 + 2 * 180 + 78 +1 = 2741 Da, M + 3 OPA + BME + H; 2302.6 + 3 * 180 + 78 +1= 2921.6 Da, Obsrvd (A) authentic duramycin + 2 OPA + H: 2365.0 Da, (B) M + 2 OPA + H: 2656 Da, (C) M + 3 OPA + BME + H = 2924 Da.
4.2.1.3 One step removal of the leader peptide of DurA with commercially available proteases

As aminopeptidases are expensive, we aimed to remove the leader peptide of DurA in one step by testing two constructs; DurA A−1E and DurA A−1M whose encoding genes were prepared by site directed mutagenesis. His$_6$-DurA A−1E and A−1M were purified by IMAC after coexpression with the genes encoding for DurM, CinX, and Cinorf7. Then, the peptides were analyzed by mass spectrometry after GluC digestion. The mass spectrometry results showed that both of these two mutant peptides had undergone four dehydrations and a hydroxylation (Figure 4.8A, B). Bioactivity analysis of the DurA core region obtained from digestion of these peptides isolated from the coexpression studies showed that fully-modified, and bioactive duramycin can be obtained for both of these mutants (Figure 4.9). However, large peaks corresponding to the unmodified core peptide and/or hydroxylated core peptide were also observed for both DurA A−1M and DurA A−1E mutant peptides (Figure 4.8). In addition, GluC activity was partially inhibited by the thioether crosslink at the Cys1 position and thus partially cleaved precursor peptide was observed when the DurA A−1E peptide was digested. To summarize, these results suggest that although it is more expensive, the best way to remove the leader sequence of modified DurA is via cleavage of the wild-type precursor peptide by GluC followed by aminopeptidase treatment.
Figure 4.8 Removal of leader peptide with commercially available proteases in one step. MALDI-ToF MS analysis of (A) His₆-DurA A−1E purified by IMAC from coexpression of this peptide with the genes encoding for DurM, CinX, and Cinorf7 followed by GluC digestion; (B) His₆-DurA A−1M purified by IMAC from coexpression of this peptide with the genes encoding for DurM, CinX, and Cinorf7 followed by GluC digestion. M: core peptide and M* is AFM attached to the N-terminus of the core peptide.

Calculated mass for $M - 4\ H_2O + OH + H$ ($M'$); $2069.3 - 4 \times 18.0 + 16 + H = 2014.3$ Da, $M^* + OH + H$; $2416.7 + 16 + 1 = 2433.7$ Da, $M^* - 4\ H_2O + OH + H$; $2416.7 - 4 \times 18 + 16 + 1 = 2361.7$ Da, Obsrvd $M'$ (A) 2012.2 Da (B) $M^* - 4\ H_2O + OH + H$; 2362.5 Da.

Figure 4.9 Bioassay with His₆-DurA A−1E purified by IMAC from coexpression of this peptide with the genes encoding for DurM, CinX, and Cinorf7 followed by GluC digestion. Sector 1, authentic duramycin; sector 2, Soluble fraction of His₆-DurA modified by DurM, CinX, and Cinorf7 in $E.\ coli$ and treated with GluC; sector 3, Insoluble fraction of His₆-DurA modified by DurM, CinX, and Cinorf7 in $E.\ coli$ and treated with GluC.
4.2.1.4 Isothermal calorimetry (ITC) studies of duramycin and cinnamycin for binding to PE

Both cinnamycin and duramycin have been shown to bind specifically to PE by ITC experiments (5, 11). In order to test the binding activity of the peptides that are synthesized in our laboratory, binding experiments with both duramycin and cinnamycin were carried out. The association constant for binding of cinnamycin to PE in octyl glucoside (OG) micelles was $(1.0 \pm 0.4) \times 10^7 \text{M}^{-1}$ for 1:1 binding (Figure 4.10B, D), whereas it was found to be $(3 \pm 1) \times 10^6 \text{M}^{-1}$ in the paper published by Prof. Seelig (5). This slight difference in the results might be arising from the low solubility of cinnamycin. For duramycin, 200 μm diameter liposomes consisting of a 1:1 ratio of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphotidylcholine (POPC) to 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphotidyl ethanolamine (POPE) (0.925 mM, 1 μL additions in the ITC titration) were used to titrate 200 μM duramycin at 37 ºC and the $K_a$ was found to be $6.1 \times 10^6 \text{M}^{-1}$, whereas $K_d$ is 164 nM (N= 0.97, ΔH = 22.9 kJ /mol) (Figure 4.10A, C). In the previous studies, the binding constants of the 1:1 complexes of cinnamycin and duramycin to PE were determined with isothermal titration calorimetry to be $10^7$-$10^8 \text{M}^{-1}$ depending on the size and composition of the liposomes for the 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine matrix (5).

ITC studies with cinnamycin and its mutants have been challenging due to the low solubility of the peptide (~ 50-100 μM), which can also be observed from the ITC data of wild-type cinnamycin titrated with PE (Figure 4.10B, D). In
In comparison, duramycin has higher solubility (~ 500 μM) as a solvent exposed Arg2 is replaced with Lys (4, 10).

Figure 4.10 ITC data for the titration of (A) duramycin, and (B) cinnamycin with phosphatidylethanolamine-containing POPC liposomes and OG micelles, respectively. (C) duramycin and PE, (D) cinnamycin and PE binding curve. (A & D) Authentic duramycin (200 μM, 350 μL) was titrated with 200 μm diameter liposomes consisting of a 1:1 ratio of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphotidylcholine (POPC) to 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphotidylethanolamine (POPE) (0.925 mM) at 37 °C. (B & D) Authentic cinnamycin (0.025 μM, 350 μL) was titrated with phosphatidylethanolamine (0.267 mM) in 36 mM OG solution. 1 μL was used for each titration.
4.2.2 Frankisin Biosynthesis

4.2.2.1 Frankisin biosynthetic gene cluster from Frankia Sp. EUN1f

Frankia sp. EUNf1 is a nitrogen-fixing bacterium that lives in the soil and has a symbiotic relationship with many plants (40). It supplies most or all of the host plant’s nitrogen needs, and thus can establish a nitrogen-fixing symbiosis with host plants where nitrogen is the limiting factor in the growth of the host. Phospholipid analysis of the cell membranes of this organism shows that they contain phosphatidylinositol, phosphatidylinositol mannosides, and diphosphatidylglycerol; however, nitrogen-containing phospholipids such as PE and PC are absent (41).

The genomic sequence of the bacterial strain Frankia Sp. EUN1f was determined and genomic DNA was kindly provided by Prof. Tisa S. Louis (University of New Hampshire) (42). The BLAST analysis of the genome identified a gene cluster that contains four putative lanthipeptide biosynthesis genes as in the case of the cinnamycin and duramycin biosynthetic gene clusters and in this study the peptide that could be produced by this gene cluster is named frankisin (Figure 4.11A). The putative precursor peptide FranA consists of a C-terminal core region that is 19 residues long. Appended to the N-terminus of the core peptide is a 65 amino acid leader sequence. Instead of a GG or GA protease cleavage motif seen in most class II lantibiotics or the sec-cleavage site found in CinA and DurA, no known motif is present between the leader sequence and the core region of FranA (Figure 4.11B). The FranA core region contains four
threonines and two serines, which could be processed by a lantibiotic synthetase in the biosynthetic cluster (Figure 4.11B). FranA drastically differs from CinA and DurA in the leader region. However, the core region varies from cinnamycin at only 6 positions; R2K, Q3S, T4S, F7S, F10I, and F12I. Interestingly, these variations introduce two more dehydratable amino acids. The FranM sequence shows high identity with other class II LanM enzymes, which are bifunctional enzymes that catalyze both dehydration and cyclization (31-32). In particular, FranM (1116 aa) and CinM (1088 aa) show 52% identity with 95% coverage. Furthermore, FranX (253 aa) and CinX (325 aa) show 51% identity with 91% coverage, suggesting that FranX is a member of the non-heme Fe(II)-dependent family of enzymes that utilized β-ketoglutarate (α-KG) as cofactor and that it catalyzes the hydroxylation of Asp15. Franorf7 (110 aa), when compared to Cinorf7 (119 aa) showed 40% identity with 80% coverage, which suggests that this protein is involved in Lal formation in frankisin biosynthesis. After the core peptide is processed by these enzymes, the leader peptide is likely removed proteolytically to afford the mature, biologically active compound as observed in other lantibiotics (33).

The putative genes involved in frankisin biosynthesis *franA, franM, franX* and *franorf7* are not similar to those in the cinnamycin biosynthetic gene cluster (Figure 4.12). Upstream of the biosynthetic genes, two putative aliphatic sulfonate ABC transporter ATP-binding proteins T1 (280 aa) and T2 (111 aa) are found, which might be responsible for the transport of the mature natural product.
outside the producing strain. In addition, FranR (952 aa), located upstream of the transporter, is a transcriptional regulator from the LuxR family containing the pfam:PF00196 motif. The genes labeled as 1 and 2 encode for small proteins (84 aa and 43 aa, respectively) with unknown functions, and the gene labeled as 3 encodes for a protein (197 aa), which shows similarity to Het-C heterokaryon incompatibility gene suggesting that this protein might be a cell wall protein (43).

Figure 4.11 Biosynthesis of frankisin. (A) Biosynthetic gene cluster and phosphatidylinositol-specific phospholipase C region identified downstream of the biosynthesis genes, (B) Biosynthesis of frankisin. The crosslinks on the modified FranA are drawn based on the structure of the known cinnamycin group of peptides, (C) Structure of phosphatidylinositol. The cleavage point of phospholipase C enzymes, which is between phosphorus and oxygen, is shown in red on the structure.

The gene labeled as PIC encodes for phosphotidylinositol specific phospholipase C (731 aa), which catalyzes the production of inositol phosphate (IP) and diacylglycerol (DAG) (Figure 4.11C). This enzyme is considered to be
involved in phosphotidylinositol (PI) breakdown in cells, which occurs in response to several external stimuli. Hydrolysis of membrane PI of a number of eukaryotic cells and tissues by the bacterial PIC has been shown to cause substantial release of acetylcholinesterase, alkaline phosphatase and 5′-nucleotidase in free, soluble form (44). As stated above, removal of the leader sequence is required for lanthipeptides to be biologically active and several lanthipeptides have genes encoding for unique protease proteins for the removal of their leader sequences (45-46). No protease sequence was found within the frankisin biosynthetic cluster. This lack of protease gene might be due to the short contig that ends right after the PIC gene. It is possible that there are other genes downstream of PIC that are important for other functions such as leader peptide removal and immunity. These genes may not have been assembled in the draft genome.

**Figure 4.12** Biosynthetic gene cluster of frankisin.
4.2.2.2 Coexpression of \textit{franA}, \textit{franM}, \textit{franX}, and \textit{franorf7} in \textit{E. coli}

Similar to the approach taken to produce duramycin, pRSFDuet-1 and pACYCDuet-1 plasmids were employed for coexpression of the four \textit{Frankia} sp. genes. The gene encoding for FranA was inserted into multiple cloning site I (MCSI) of the pRSFDuet-1 vector to generate His\textsubscript{6}-FranA. The gene encoding FranM was inserted into MCSII of the same plasmid resulting in a protein without a His-tag, thus preventing co-purification of the FranM enzyme. Similarly, \textit{franX} was cloned into MCSII and \textit{franorf7} was cloned into MCSI of the pACYCDuet-1 vector, which also produces FranX and Franorf7 without a His-tag (24). Coexpression of \textit{franM}, \textit{franX}, and \textit{franorf7} together with his\textsubscript{6}-\textit{franA} yielded \(\sim0.5\) mg of His\textsubscript{6}-FranA peptide per liter of cell culture after HiTrap IMAC column purification and subsequent reversed-phase high performance liquid chromatography (RP-HPLC). Removal of the leader peptide was achieved by treatment with the ArgC protease, which cleaves at the C-terminus of Arg at the \(-2\) position (Figure 4.11B), followed by aminopeptidase treatment of the peptide to remove aspartic acid at position \(-1\). MALDI-ToF MS analysis of the peptide treated with ArgC followed by aminopeptidase showed a mass difference with respect to the unmodified core peptide corresponding to six dehydrations and one hydroxylation (Figures 4.13). This result suggests that all four threonines and two serines in the core region are dehydrated (Figure 4.11B). Thioether cyclization activity of FranM was investigated by IAA treatment and analysis by MALDI-ToF MS as previously described. However, after IAA assay no mass
corresponding to the core region or its IAA adducts was observed, which may be
due to low ionization of the peptide in MALDI-ToF analysis. Thus, in future
studies cyclization and formation of the Lal crosslink on FranA should be
confirmed and the residues forming the thioether crosslinks should be identified
as there are multiple possibilities due to the presence of two additional
dehydrated residues with compared cinnamycin and duramycin.

The peptide obtained from coexpression was tested for antimicrobial
activity towards several indicator strains including B. subtilis 6633, B. subtilis 168,
B. simulance, Micrococcus luteus, Bacillus thuringiensis 4 AWL, Streptococcus
agalactiae, and Lactococcus lactis HP. However, no zone of inhibition was
observed indicating that the peptide obtained is not bioactive towards these
strains. This result might arise due to several reasons, which are discussed in the
discussion section.
Figure 4.13 MALDI-ToF mass spectrum of His$_6$-FranA modified by FranM, FranX, and Franorf7 in *E. coli*. His$_6$-FranA modified by FranM, FranX, and Franorf7 in *E. coli* and treated with ArgC protease followed by aminopeptidase.

*Calculated mass for M – 6 H$_2$O + OH (M$^-$): 1928.2 – 6 * 18.0 + 16 = 1836.2 Da, Obsrvd; 1835.1 Da.

4.2.3 Engineering of cinnamycin analogs

4.2.3.1 Biosynthesis of ancovenin

The biosynthetic gene cluster for ancovenin is not known because the producing strain appears to have been lost at Fujirebio Diagnostics, Inc. (23). Ancovenin differs at only four positions (Val2, Leu10, Trp12, and Ser13) from cinnamycin (Arg2, Phe10, Phe12, and Val13) allowing ancovenin production as a cinnamycin analog. Using site-directed mutagenesis, four mutations were introduced in CinA(A–1K) to yield a peptide consisting of the cinnamycin leader.
peptide attached to the ancovenin core region. Then, \( \text{his}_6 \)-cinA(A–1K) R2V/F10L/F12W/V14S and cinM and cinorf7 were coexpressed and, modified His\(_6\)-CinA(A–1K) R2V/F10L/F12W/V14S peptide was purified by IMAC followed by RP-HPLC. Ancovenin does not contain the hydroxylation on the Asp15 (1), therefore cinX was not used in the coexpression. Analysis of the peptide by MALDI-ToF MS after the removal of the leader peptide by LysC endoproteinase showed a mass corresponding to the core region with 4 dehydrations in addition to a peak with 3 dehydrations (Figure 4.14). Cyclization of the cysteines was investigated with the IAA assay of LysC-cleaved peptide by MALDI-ToF MS analysis. The results showed no IAA adducts suggesting that all three cysteines were crosslinked to the dehydrated amino acids (Figure 4.14).
**Figure 4.14 Biosynthesis of ancovenin.** (A) MALDI-ToF MS analysis of the IAA assay for the sample obtained as follows. His<sub>6</sub>-cinA(A–1K) R2V/F10L/F12W/V14S, two copies of cinM, and cinX were coexpressed, and modified His<sub>6</sub>-CinA(A–1K) peptide was purified by IMAC column purification followed by RP-HPLC. Analysis of the peptide by MALDI-ToF MS after the removal of the leader peptide by LysC endoproteinase showed that the major peak corresponds to the core region with 4 dehydrations in addition to a peak with 3 dehydrations. No IAA adducts were observed.

*Calculated mass for M – 4 H<sub>2</sub>O + H (M'); 2033.3 – 4 * 18 + 1 = 1962.3 Da, Obsrvd; 1962.2 Da.

4.2.3.2 Preparation of cinnamycin and duramycin analysis containing Phe7Val or Phe7Leu mutations

Molecular dynamics (MD) studies performed by Prof. Ming Zhao at Northwestern University suggested that cinnamycin and/or duramycin with the mutations Phe7Val and Phe7Leu might bind phosphatidylserine (PS), which differs from PE by a carboxyl group at the head group (Figure 4.15A, D). NMR studies also clearly show that the amino group of PE points towards Phe7 and thus its mutation to smaller amino acids such as Leu and Val might create more
space in the binding scaffold and accommodate the carboxylate of PS (Figure 4.15D) or possibly the three methylations on the amino group of PC (Figure 4.15B). Therefore, the genes encoding CinA Phe7Leu and Phe7Val, and DurA Phe7Leu and Phe7Val were prepared via site-directed mutagenesis, followed by coexpression with the appropriate LanM, LanX and Lanorf7 enzymes, and purification by IMAC. Then, the leader peptides were removed using the appropriate proteases, as specifically described for cinnamycin in Chapter II and for duramycin in section 4.2.1.2. The peptides were analyzed by MALDI-ToF MS and determined to contain the three (Me)Lan and one Lal crosslinks as well as the hydroxylation of Asp15 (Figure 4.16A, B). Interestingly, when the bioactivity of these cinnamycin and duramycin mutants were tested, formation of a zone of growth inhibition in agar diffusion assays was lost towards *B. subtilis* 6633, suggesting that these mutants no longer bind to PE. To test this hypothesis, the four mutant peptides were titrated with PE (1,2-diacyl-sn-glycero-3-phosphoethanolamine from egg yolk) in OG micelles and the heat exchange was monitored by ITC.
Figure 4.15 Preparation of cinnamycin analogs for binding to phosphatidylcholine (PC). Representation of (A) phosphatidylethanolamine (PE), (B) phosphatidylcholine (PC), (C) phosphatidylinositol (PI), and (D) phosphatidylserine (PS). The differences of PE, PC, and PS are highlighted in red, (E) NMR structure of duramycin complexed with PE; Phe7 and hydroxylated Asp15 are shown in yellow.
Figure 4.16 Mass spectrometry analysis of iodoacetamide treated (A) duramycin Phe7Leu and (B) duramycin Phe7Val. The peptides were obtained from the coexpression studies in E. coli, and subsequent IMAC and RP-HPLC purification followed by GluC and aminopeptidase treatment.

*Calculated mass for (A) M – 4 H₂O + OH; 2035.3 – 4 * 18.0 + 16 = 1979.3 Da, Obsrvd: 1979.6 Da; (B) Calculated mass for M – 4 H₂O + OH; 2021.3 – 4 * 18.0 + 16 = 1965.3 Da, Obsrvd mass (A) 1979.6 Da, (B) 1965 Da.

Figure 4.17 ITC studies for cinnamycin Phe7Val with PC containing OG micelles.
Although ITC experiments of cinnamycin mutants for binding to PE, PC, and PS were performed, the results were not considered convincing due to low solubility issues mentioned in the section for cinnamycin ITC studies. Therefore, the same experiments were repeated for duramycin Phe7Leu and duramycin Phe7Val, which have much higher solubility due to the solvent exposed Lys at the second position. The ITC experiments for duramycin mutants showed that they no longer bind to PE but started to weakly recognize PC. These results resemble the ITC results obtained for the binding of cinnamycin mutants to these phospholipids (Figure 4.17). Interestingly, the ITC curves for PC binding do not suggest 1:1 binding but suggest that duramycin Phe7Leu and Phe7Val are aggregating on the micelles in a PC dependent manner. Therefore, more experiments should be performed to confirm the binding interaction between PC and these duramycin mutants such as fluorescence resonance energy transfer studies. None of the mutants recognized phosphatidylserine (PS), in ITC studies. Also, negative control experiments where duramycin was titrated with PC-containing micelles, peptide buffer was titrated with PC or PE micelles or POPE:POPC liposomes, and mutant peptide solution was titrated with micelles without any phospholipids showed no interaction as expected.
4.3 DISCUSSION

In this chapter, the biosyntheses of duramycin, ancovenin, and a peptide from *Frankia Sp. EUNf1* (frankisin), whose genome contains a biosynthetic cluster that is very similar to the cinnamycin biosynthetic genes, are described. To do so, the genomic DNA from ATCC *Streptoverticillium cinnamoneum forma azacoluta*, which produces duramycin, was sequenced by Dr. Yun-juan Chang at the University of Illinois at Urbana-Champaign. The genomic DNA of *Frankia Sp. EUNf1* was obtained from Prof. Tisa S. Louis (University of New Hampshire). Duramycin and frankisin were biosynthesized by heterologous expression of their corresponding biosynthetic genes in *E. coli*. Mass spectrometry analysis confirmed that fully-modified duramycin can be formed with this approach; however more experiments are needed to confirm the formation of fully-modified frankisin.

The peptide obtained from the coexpression of the putative frankisin biosynthetic genes did not show bioactivity towards many strains tested, which might be due to several reasons. Firstly, although dehydration of the six Ser/Thr residues in the core region of FranA was confirmed, the formation of Me(Lan) and Lal crosslinks could not be verified due to difficulties encountered in mass spectrometry analysis. Therefore, it is possible that the peptide obtained is not fully cyclized. Secondly, it is possible that this peptide is binding to PE as other members of the group do, but might not be able to induce bacterial cell death. Therefore, ITC experiments should be performed for binding to PE. Thirdly, it is
possible that frankisin is not binding to PE but has a different target. The cinnamycin biosynthetic cluster contains a SAM-dependent enzyme, which methylates the amino group of all the PE in the producing strain, providing immunity to the organism (47). Frankia Sp. does not contain this gene, but is also does not have PE in its membranes. Therefore, the PIC enzyme found downstream of the biosynthetic genes is interesting, as it might be providing immunity to Frankia Sp if frankisin was a different target than PE. Similar to the other members in the cinnamycin group of peptides, frankisin contains the critical amino acids that are involved in direct interaction to the glycerol moiety and the phosphate on a phospholipid as identified by NMR studies with cinnamycin and duramycins (9). In addition, the FranA core region contains serine and two isoleucines in the positions of the bulky amino acid phenylalanine at positions 7, 10, and 12 in cinnamycin and the duramycins. These substitutions might generate space in the binding cavity for a phospholipid like PI with a larger head group compared to PE to bind to this peptide. More experiments should be performed to test these hypotheses.

Ancovenin, the third cinnamycin-like peptide studied herein, was obtained via site-directed mutagenesis of four amino acids of CinA (Arg2, Phe10, Phe12, and Val13) to Val2, Leu10, Trp12, and Ser13 and heterologous expression of the resulting CinA mutant together with CinM and Cinorf7 in E. coli. Mass spectrometry analysis of the peptide obtained from the coexpression showed that CinM was able to process this peptide and form four dehydrations with three
(Me)Lan crosslinks. The previous studies where sequence analysis of ancovenin was investigated showed that this peptide does not contain the hydroxylation on the Asp15, unlike the other members of the group (1). The preparation of this using the cinnamycin biosynthetic enzymes provides new opportunities to study ancovenin.

The possibility that cinnamycin and duramycin analogs might bind to different phospholipids than their natural binding partner PE was also investigated. The cinnamycin and duramycin analogs Phe7Val and Phe7Leu were by site-directed mutagenesis and tested for binding to phosphatidylcholine (PC), phosphatidylinositol (PI), and phosphatidylserine (PS) by ITC. All of the mutants prepared lost their bioactivity towards B. subtilis 6633, suggesting that they lost the ability to recognize PE. This hypothesis was tested by ITC studies, which showed that the cinnamycin Phe7Val mutant still recognized PE, whereas cinnamycin Phe7Leu lost its ability to bind to PE. On the other hand, both cinnamycin Phe7Val and Phe7Leu and duramycin Phe7Leu interacted weakly with PC, generating exothermic heat signals, whereas no heat exchange was observed in the ITC experiment of cinnamycin or duramycin with PC. However, high-quality data could not be obtained from the titrations therefore further experiments are needed for definitive conclusions. The experiments to test binding of the duramycin mutants to PE have not been performed yet.
4.4 MATERIALS

Oligonucleotide primers for mutagenesis were synthesized by Operon Technologies. Taq and Platinum Pfx DNA polymerases, DpnI, restriction endonucleases, and bacteriophage T4 DNA ligase were purchased from Invitrogen. Fusion DNA polymerase was purchased from New England Biolabs. Cloning vectors (pET and pDuet) were obtained from Novagen. Gel extraction, plasmid mini-prep, and PCR purification kits were purchased from Qiagen. All strains were grown in media acquired from Difco laboratories. Other items procured include isopropyl-1-thio-D-galactopyranoside (IPTG, CalBiochem), iodoacetamide (IAA, Acros Organics), tris(2-carboxyethyl) phosphine hydrochloride (TCEP, Aldrich), and dithiothreitol (DTT, Sigma). Endoproteinase LysC was purchased from Roche Applied Science. 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE), and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) were purchased from Avanti Lipids (Alabaster, AL). Octyl β-D-glucopyranoside was purchased from Sigma Aldrich.
4.5 GENERAL METHODS

Molecular biological manipulations were carried out using standard techniques (48). PCR was performed using an automatic thermocycler (PTC 150, MJ Research) and DNA sequencing was performed at the Biotechnology Center of the University of Illinois at Urbana-Champaign (UIUC). *Escherichia coli* DH5α cells (UIUC Cell Media Facility) and *E. coli* BL21 (DE3) cells (Stratagene) were used for plasmid preparation and protein expression, respectively. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-ToF MS) was carried out on a Voyager-DE-STR (Applied Biosystems). Liquid chromatography electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS) was carried out and processed using a Synapt ESI quadrupole ToF Mass Spectrometry System (Waters) equipped with an Acquity Ultra Performance Liquid Chromatography (UPLC) system (Waters).
4.6 EXPERIMENTAL METHODS

4.6.1 Isolation of genomic DNA from *Streptoverticillium cinnamoneum forma azacoluta* and genomic sequencing (NB 13, p14)

*Streptoverticillium cinnamoneum forma azacoluta* (ATCC 12686) was grown in BBL™ Trypticase Soy Broth with shaking at 37 °C for 1-2 days. Then, the bacteria were harvested by centrifugation and washed twice with 0.05 M Tris, pH 8. The pellet was resuspended in 25% sucrose-Tris buffer (0.05 M, pH 8) at a concentration of 1 g wet weight per 10 mL of buffer. This suspension was homogenized in a glass tissue grinder to break up the clumps of mycelium. Lysozyme at 10 mg/mL was mixed with the cell suspension and incubated at 37 °C for 30 min. The solution of 0.25 M EDTA, pH 8 (5 mL) was added to 20 mL of cell suspension and incubated at 37 °C for another 15 min, which completed the cell lysis. Then, the genomic DNA was extracted with Promega Wizard Plus SV miniprep kit, which resulted in ~150 ng/μL of DNA. Then 200 μL of this DNA solution was submitted for shot-gun sequencing performed by Dr. Yun-juan Chang at the Biotechnology Center at UIUC.

4.6.2 Cloning

4.6.2.1 Cloning of *durA, durM, durX, and durorf7* into pDuet vectors (NB14, p7-17)

The genes *durA, durM, durX* and *durorf7* were cloned from *Streptoverticillium cinnamoneum forma azacoluta* genomic DNA. The amplification of *durA* was conducted via 30 cycles of denaturing (98 °C for 10 s),
annealing (55 °C for 30 s), and extending (72 °C for 30 s) using DurA_BamHI_FP and DurA_HindIII_RP as primers and using the genomic DNA as a template (Table 4.1). Similarly durM was amplified with DurM_EcorV_FP and DurM_KpnI_RP primers by denaturing (98 °C for 10 s) for 30 cycles, annealing (55 °C for 30 s), and extending (72 °C for 100 s). The PCRs (50 μL) included 1x HF buffer (Finnzymes), DMSO (4%), Fusion DNA Polymerase (Finnzymes) (0.02 unit/μL), dNTPs (1 mM), S. cinnamoneum forma azacoluta genomic DNA, and primers (1 μM each). Amplification of the final PCR product was confirmed by 2% and 1% agarose gel electrophoresis for durA and durM, respectively, and the products were purified using QIAquick PCR purification kits (QIAGEN). The resulting DNA fragments and the pRSFDuet-1 vector were digested in 1 x NEBuffer 4 (New England Biolabs) with BamHI-HF and HindIII-HF for durA, and EcorV-HF and KpnI-HF for durM at 37 °C (for 15 h). The digested products were purified by agarose gel electrophoresis followed by use of a QIAquick gel extraction kit (QIAGEN).

Similarly, durX and durorf7 were amplified using primers CinX_EcoRV_pACYCDuet-1_MCSI_FP and CinX_XhoI_pACYCDuet-1_MCSI_RP for durX, Cinorf7_Ncol_pACYCDuet-1_MCSI_FP and Cinorf7_HindIII_pACYCDuet-1_MCSI_RP for durorf7 (Table 4.1) by PCR. The reactions contained 1xFailSafe PreMix G (PICENTRE Biotechnologies), DMSO (4%), Platinum Pfx DNA polymerase (0.025 unit/μL), Taq DNA polymerase (0.05 unit/μL), and primers (1 μM each). Amplification of the final PCR products was
confirmed by 1% agarose gel electrophoresis and the products were purified using QIAquick PCR purification kits. The resulting DNA fragments and the pACYCDuet-1 vector was digested in 1 x NEBuffer 4 with EcoRV-HF and XhoI for insertion of durX and NcoI-HF and HindIII-HF for durorf7 at 37 ºC (for 15 h). The digested products were purified by agarose gel electrophoresis followed by use of a QIAquick gel extraction kit.

The resulting DNA inserts were ligated with the digested corresponding vectors at 16 ºC for 15 h using T4 DNA ligase to generate durA- and durM-containing pRSFDuet-1, and durX- and durorf7-containing pACYCDuet-1. The ligation reaction mixture was diluted 5 times with water prior to transformation. E. coli DH5α cells were transformed with the ligation product via heat shock, plated on LB-chloramphenicol (for pACYCDuet-1b) and LB-kanamycin (for pRSFDuet-1) agar plates, and grown at 37 ºC for 15 h. Colonies were picked and incubated in 5 mL of LB-chloramphenicol/kanamycin medium at 37 ºC for 15 h, followed by isolation of the plasmids using a QIAprep Spin Miniprep Kit. The desired sequences of the resulting plasmids were confirmed by DNA sequencing.

4.6.2.2 Cloning of franA, franM, franX, and franorf7 into pDuet vectors (NB10, p66; NB11, p24)

The genes franA, franM, franX and franorf7 were cloned from Frankia Sp. EUNf1 genomic DNA provided by Prof. Tisa Louis at the University of Hampshire. Similar to the duramycin biosynthetic genes, franA, franM, franX and franorf7 were amplified via 30 cycles of denaturing (98 ºC for 10 s), annealing
(55-65 °C for 30 s), and extending (72 °C for 30-180 s) using appropriate primers (Table 4.1). The PCRs (50 μL) included 1x HF buffer (Finnzymes), DMSO (4%), Fusion DNA Polymerase (Finnzymes) (0.02 unit/μL), dNTPs (1 mM), Frankia Sp. EUNf1 genomic DNA, and primers (1 μM each). Amplification of the final PCR product was confirmed by 1- or 2% agarose gel electrophoresis depending on the size of the gene, and the products were purified using QIAquick PCR purification kits (QIAGEN). The resulting DNA fragments and the pRSFDuet-1 or pACYCDuet-1 vector were digested in 1 x NEBuffer 4 (New England Biolabs) with EcoRI-HF and HindIII-HF for franA, EcoRV-HF and Xhol for franM, Ndel and EcoRV-HF for franX, and Ncol-HF and NotI-HF for franorf7 at 37 °C (for 15 h). The digested products were purified by agarose gel electrophoresis followed by use of a QIAquick gel extraction kit (QIAGEN). The resulting DNA inserts were ligated with the digested corresponding vectors at 16 °C for 15 h using T4 DNA ligase to generate franA- and franM-containing pRSFDuet-1, and franX- and franorf7-containing pACYCDuet-1. The ligation reaction mixture was diluted 5 times with water prior to transformation. E. coli DH5α cells were transformed with the ligation product via heat shock, plated on LB-chloramphenicol (for pACYCDuet-1b) and LB-kanamycin (for pRSFDuet-1) agar plates, and grown at 37 °C for 15 h. Colonies were picked and incubated in 5 mL of LB-chloramphenicol/kanamycin medium at 37 °C for 15 h, followed by isolation of the plasmids using a QIAprep Spin Miniprep Kit. The desired sequences of the resulting plasmids were confirmed by DNA sequencing.

The mutations A-1E and A-1M were generated in durA, and D-1E and D-1M were made in franA by Quikchange PCR. Briefly, the amplification of these genes from pRSFDuet-1 containing durA and durM and pRSFDuet-1 containing franA and franM was conducted via 30 cycles of denaturing (98 °C for 10 s), annealing (60 °C for 30 s), and extending (72 °C for 240 s) using the primers listed in Table 4.1. The PCRs (50 μL) included 1x HF buffer (Finnzymes), DMSO (4%), Fusion Hot Start High-Fidelity DNA Polymerase (Finnzymes) (0.04 unit/μL), dNTPs (2 mM), corresponding template (20 ng), and primers (1 μMeach). The PCR product was checked on a 1% agarose gel and purified using a QIAquick PCR purification kit (QIAGEN). The resulting DNA was treated with DpnI at 37 °C for 15 h to digest the methylated template, and E. coli DH5α cells were transformed. The desired mutations were confirmed by DNA sequencing.

4.6.2.4 Generation of CinA-K R2V, F10L, F12W, V13S mutations in pRSFDuet-1 MCS-1 for ancovenin production

Generation of the gene encoding the ancovenin precursor peptide was performed with multistep PCR reactions to fuse the core region of ancovenin to the CinA leader peptide. In the first reaction, CinAK_AscI_pRSFDuet-1_MCSI_FP and CinA-K_Ancovenin_pRSFDuet-1_MSCI_RP-1 were used as forward and reverse primers. The PCRs (50 μL) included 1x HF buffer (Finnzymes), DMSO (4%), Fusion DNA Polymerase (Finnzymes) (0.02 unit/μL),
dNTPs (1 mM), CinA(A–1K)/pET15b as template DNA (20 ng), and primers (1 μM each). The amplification was conducted via 30 cycles of denaturing (98 °C for 10 s), annealing (60 °C for 30 s), and extending (72 °C for 15 s). The final PCR product was confirmed by 2% agarose gel electrophoresis, and it was purified using a QIAquick gel purification kit (QIAGEN). The second PCR was performed by using the first PCR product as a template and CinAK_AscI_pRSFDuet1_MCSI_FP and CinA-K_Ancovenin_pRSFDuet1_MCSI_RP-2 as forward and reverse primers. The amplification was conducted via 30 cycles of denaturing (98 °C for 10 s), annealing (60 °C for 30 s), and extending (72 °C for 15 s). The successful generation of the final PCR product was confirmed by 2% agarose gel electrophoresis, and it was purified using a QIAquick gel purification kit (QIAGEN). The third and final PCR was performed by using the second PCR product as a template and, CinA-K_AscI_pRSFDuet-1_MCSI_FP and CinA-K_Ancovenin_pRSFDuet-1_MCSI_RP-3_HindIII as forward and reverse primers. The PCR was performed under the same conditions followed by purification of the PCR product by gel electrophoresis. The resulting DNA fragment and the pRSFDuet-1 containing cinM were digested in 1x NEBuffer 4 with Ascl (15 h) and HindIII-HF (3 h) at 37 °C and the product purified by agarose gel electrophoresis followed by use of a QIAquick gel extraction kit (QIAGEN). The resulting DNA insert was ligated with the digested vector at 16 °C for 15 h using T4 DNA ligase. The ligation reaction mixture was diluted 5 times with water prior to transformation. E. coli DH5α cells were transformed with the
ligation product via heat shock, plated on LB-ampicilin agar plates, and grown at 37 °C for 15 h. Colonies were picked and incubated in 5 mL of LB-ampicilin medium at 37 °C for 15 h, followed by isolation of the plasmids using a QIAprep Spin Miniprep Kit (QIAGEN). The desired sequences of the resulting plasmids were confirmed by DNA sequencing.

4.6.2.5 Generation of cinA-K Phe7Val and Phe7Leu, and durA Phe7Val and Phe7Leu (NB10, p70; NB11, p45)

Both Phe7Val and Phe7Leu mutations were made in the genes encoding CinA (A-1K) and DurA by Quikchange PCR. Briefly, the amplification of cinA-K- and cinM-containing pRSFDuet-1 and durA- and durM-containing pRSFDuet-1 was conducted via 30 cycles of denaturing (98 °C for 10 s), annealing (60 °C for 30 s), and extending (72 °C for 180 s) using primers CINA_PHE7VAL_FP or CINA_PHE7LEU_FP together with CINA_QC_PHE7VAL_7LEU_RP for both of the templates (See Table 4.1 for primer sequences). The PCRs (50 μL) included 1x HF buffer (Finnzymes), DMSO (4%), Fusion Hot Start High-Fidelity DNA Polymerase (Finnzymes) (0.04 unit/μL), dNTPs (2 mM), corresponding template (20 ng), and primers (1 μM each). The PCR product was checked on a 1% agarose gel and purified using a QIAquick PCR purification kit (QIAGEN). The resulting DNA was treated with DpnI at 37 °C for 15 h to digest the methylated template, and *E. coli* DH5α cells were transformed. The desired mutations were confirmed by DNA sequencing.
4.6.3 Coexpression studies

4.6.3.1 Coexpression and purification of \textit{durA}, \textit{durM}, and \textit{cinX} with and without \textit{cinorf7} in \textit{E. coli} (NB14, p58-60, 63, 65; NB15, p26)

In order to coexpress \textit{durA}, \textit{durM} and \textit{cinX}, \textit{E. coli} BL21 (DE3) cells were transformed with a pRSFduet-1 vector containing \textit{durA} and \textit{durM} and a pACYCDuet-1 vector containing \textit{cinX}. Similarly, for coexpression of \textit{durA}, \textit{durM} and \textit{cinX} together with \textit{cinorf7}, \textit{E. coli} BL21 (DE3) cells were transformed with a pRSFduet-1 vector containing \textit{durA} and \textit{durM} and a pACYCDuet-1 vector containing \textit{cinX} and \textit{cinorf7}. The \textit{durA} gene encoded for His$_6$-tagged DurA peptide. Single colony transformants were grown in a 37 °C shaker for 12-15 h in 50 mL of Luria-Bertani (LB) medium supplemented with 25 μg/mL kanamycin and 12.5 μg/mL chloramphenicol. A 20 mL aliquot was centrifuged at 5000×g for 10 min, the spent LB medium was discarded, and the cell pellet was resuspended in fresh LB medium. The resuspended cells (20 mL) were added to 2 L of LB supplemented with the appropriate antibiotics, and the culture was grown aerobically at 37 °C until the $A_{600}$ was ~ 0.6 to 0.8. IPTG was added to a final concentration of 0.5 mM and the culture was transferred to 18 °C for aerobic growth for an additional 20 h. Cells were harvested by centrifugation at 5000×g for 20 min at 4 °C. The cell paste (~ 3-4 g) was stored at –80 °C until use. Peptides were purified from \textit{E. coli} BL21 (DE3) cultures as described for other His$_6$-LanA peptides (49) by use of a 5 mL HiTrap chelating HP nickel affinity column (GE Healthcare) followed by HPLC.
4.6.3.2 Coexpression and purification of \(\text{franA}, \text{franM}, \text{franX},\) and \(\text{franorf7}\) in \(E. \text{coli}\) (NB14, p59)

In order to coexpress \(\text{franA}, \text{franM}\) and \(\text{franX}\) together with \(\text{franorf7}\), \(E. \text{coli}\) BL21 (DE3) cells were transformed with a pRSFDuet-1 vector containing \(\text{franA}\) and \(\text{franM}\) and a pACYCDuet-1 vector containing \(\text{franX}\) and \(\text{franorf7}\). The \(\text{franA}\) gene encoded for His\(_6\)-tagged Fra\(\text{nA}\) peptide. Single colony transformants were grown in a 37 °C shaker for 12-15 h in 50 mL of Luria-Bertani (LB) medium supplemented with 25 μg/mL kanamycin and 12.5 μg/mL chloramphenicol. A 20 mL aliquot was centrifuged at 5000×g for 10 min, the spent LB medium was discarded, and the cell pellet was resuspended in fresh LB medium. The resuspended cells (20 mL) were added to 2 L of LB supplemented with the appropriate antibiotics, and the culture was grown aerobically at 37 °C until the \(A_{600}\) was ~ 0.6 to 0.8. IPTG was added to a final concentration of 0.5 mM and the culture was transferred to 18 °C for aerobic growth for an additional 20 h. Cells were harvested by centrifugation at 5000×g for 20 min at 4 °C. The cell paste (~ 3-4 g) was stored at −80 °C until use. Peptides were purified from \(E. \text{coli}\) BL21 (DE3) cultures as described for other His\(_6\)-LanA peptides (49) by use of a 5 mL HiTrap chelating HP nickel affinity column (GE Healthcare) followed by HPLC.

4.6.4 Bioactivity assays with \(B. \text{subtilis}\) 6633 (NB11, p98; NB14, p77)

Modified peptide obtained from the coexpressions for duramycin and frankisin production (0.5 mg) were separately dissolved in 1 mL of 100 mM Tris-HCl pH 8.3 buffer and digested with 50 μL of LysC (0.4 μg/mL in 100 mM Tris,
pH 8.3) at 37 °C for 12 h. In the case where non-enzymatic Lal formation was induced *in vitro*, the pH of the solution was carefully increased to pH 9.5 by addition of 5 M NaOH and the solution was incubated at room temperature for 12 h. Both samples were then further purified by SPE as described above. *B. subtilis* 6633 was grown at 37 °C for 15 h in 5 mL of LB supplemented with 0.1% D-(-)-glucose (Calbiochem). LB medium containing 1.5 % agar was autoclaved and cooled to 42 °C in a water bath, and 15 mL of this solution was mixed with 375 μL of *B. subtilis* 6633 culture and with a D-(-)-glucose solution to give a 0.1% final glucose concentration. The agar was poured into sterile plates and shallow wells were generated after the agar solidified. The peptide samples (10 μL in 50 mM Tris Buffer) were spotted and the plates were incubated for 10 h at 25 °C.

4.6.5 Protease cleavage of DurA, FranA, NisA, and CinA-K (NB11, p94)

To lyophilized peptide (1mg), 50 mM HEPES buffer (1 mL per 5 mg of peptide, 1 mM TCEP, pH 7.5) and the necessary protease (5 μL/L overexpression culture; stock solutions used: LysC = 3 U/100 μL, GluC = 2 mg/mL, trypsin = 40 μM) were added. ArgC cleavage was performed according to the manufacturers protocol (Roche). The reaction was incubated overnight at room temperature (GluC, trypsin, and ArgC) or at 37 °C (LysC). After 12 h the progress of the reaction was analyzed by MALDI-TOF MS and additional enzyme was added in 5 μL aliquots if the reaction was not complete.
4.6.6 Aminopeptidase cleavage of GluC-cleaved DurA and ArgC-cleaved FranA and analysis by MALDI-ToF MS (NB14, p 56)

The DurA and FranA peptides (1mg/mL) digested with GluC and ArgC, respectively, in 50 mM HEPES buffer were treated with aminopeptidase (Sigma A8200, 6 μL/1 mg peptide, 100 U/mL stock solution). The reaction was incubated at 37 °C for 24 h and analyzed by mass spectrometry. After digestion by ArgC and aminopeptidase, the FranA peptide was analyzed in negative mode MALDI-ToF MS with 2,5-dihydroxybenzoic acid (DHB) matrix (1 mg/mL, in 60% acetonitrile(ACN):water). DurA digested with GluC and aminopeptidase was analyzed in positive mode with sinapinic acid matrix (1 mg/mL, in 60% acetonitrile(ACN):water).

4.6.7 Isothermal Calorimetry (ITC) experiments (NB11, p16, 19, 20, 25, 99)

The ITC measurements for cinnamycin were performed using micelles composed of octyl β-D-glucopyranoside (OG) as described (5). For this experiment, L-α-phosphatidylethanolamine (1,2-diacyl-sn-glycero-3-phosphoethanolamine) from egg yolk (Sigma, 0.25 mg) and cinnamycin (Novacta) (25 or 50 μM) were dissolved in 36 mM OG solution, 10 mM Tris-HCl and 0.1 M NaCl, pH 7.4. Then, cinnamycin/OG solution (350 μL) was titrated with 1 μL of the PE/OG micelles (50 μL in total) and measurements were collected. Solutions were degassed under vacuum before use.

The ITC measurement for duramycin was performed using POPC/POPE liposomes (17). Vesicles were prepared as POPC/POPE mixtures (10:1, w/w) at
a final lipid concentration of 10 mg/mL. For this purpose, stock solutions of POPC and POPE in methanol and chloroform were prepared. The appropriate amount of POPC was added to a 5 mL round-bottomed flask and was dried down under a stream of nitrogen. Residual solvent was removed by applying vacuum. The amount of POPC was controlled by weighing. Next, POPE dissolved in chloroform and methanol was added, and the procedure was repeated. A total of 2 mL of buffer (10 mM Tris-HCl and 0.1 M NaCl, pH 7.4) was added to the dry film, and the suspension was extensively vortexed. Then, 1 mL of liposome solution was extruded through a filter with 200 μm pore size 20 times and purified by a Sephadex column. Liposomes elute from the Sephadex column as an opaque dispersion. Then, the concentration of the liposome solution was calculated with phosphate quantification using ascorbic acid (50).

Isothermal titration calorimetry (ITC) was performed with a Nano ITC titration calorimeter (TA Instruments). Solutions were degassed under vacuum before use. Injection volumes varied between 1 and 3 μL. Peptide concentrations varied between 50 and 200 μM. The calorimeter cell had a reaction volume of 350 μL. The titration pattern was analyzed in terms of a 1:1 peptide-lipid complex.
List of Primers *From 5’ to 3’*

duet System Cloning

Franamycin Coexpression System
FranA EcoRI pRSFDuet-1 MCS1 FP:  
ATCGCTACGAAATTCTGATGATCACATCTGCATCGAAAGATTTCCG
FranA HindIII pRSFDuet-1 MCS1 RP:  
GGCCGCGCAAGCTTTTACTTGCTTTTCCGTACAGAC
FranM EcoRV pRSFDuet-1 MCS1 FP:  
TCTCAATTGGATATCCGATGCGCCGAACGGCCTGGATAC
FranM XhoI pRSFDuet-1 MCS1 RP:  
TAGCATGACCTGAGTTATCGCTGTAAGGTTCTGCTTAG
Fran7 NcoI pACYCDuet-1 MCS1 FP:  
AGGTCCAGCCATGGCGATGCAAAAGGAAGTACCTGACG
Fran7 NolI pACYCDuet-1 MCS1 RP:  
TAGCATGCGCGCCGCTCACTGGTTTCAGGTTCCGAGTG
FranX NdeI pACYCDuet-1 MCS1 FP:  
AGGTAGATATACATATGATGGCCGCGCACTGTC
FranX EcoRV pACYCDuet-1 MCS1 RP:  
AGCGTATAGATACCTAGGCCTAGCCGCTCGG

Duramycin Coexpression System
DurA BamHI pRSFDuet-1 MCS1 FP:  
TCTCAATTGGATATCCGATGCGCCGAACGGGCTTGCTAGTC
DurA HindIII pRSFDuet-1 MCS1 RP:  
GGCCGCGCAAGCTTTTACTTGCTTTTCCGTACAC
DurM EcoRV pRSFDuet-1 MCS1 FP:  
TCTCAATTGGATATCCGATGCGCCGAACGGGCTTGCTAGTC
DurM KpnI pRSFDuet-1 MCS1 RP:  
AGCGTATAGATACCTAGGCCTAGCCGCTCGG

Site-Directed Mutagenesis
DurA F7V FP:  
GAGCAGAGCTGCAGCGTGCGGGCCGCTTCACCTTC
DurA F7V RP:  
GAGGCGACGAGCCGACGTCGCTTCACCTTC
DurA F7L FP:  
GAGGCGACGAGCCGACGTCGCTTCACCTTC
DurA F7L RP:  
GAGGCGACGAGCCGACGTCGCTTCACCTTC
CinA F7V FP:  
GAGGCGACGAGCCGACGTCGCTTCACCTTC
CinA F7L FP:  
GAGGCGACGAGCCGACGTCGCTTCACCTTC
CinA QC F7V F7L RP:  
GAGGCGACGAGCCGACGTCGCTTCACCTTC

DurA A -1 M FP:  
GGCCGCTACGAAATTCTGACGATGCAAGCAGGCTGCAGC
DurA A -1 M RP:  
GCTGAGCTTCTGTGCTTGACATGAAAGCCTTCGATCAGGCG
DurA A -1 E FP:  
GGCCGCTACGAAATTCTGACGATGCAAGCAGGCTGCAGC
DurA A -1 E RP:  
GCTGAGCTTCTGTGCTTGACATGAAAGCCTTCGATCAGGCG
FranA D-1M FP:  
GACGATCAGGGAAAGCAGCAGTGCAAGCAGGCTGCAGC
FranA D-1E FP:  
GACGATCAGGGAAAGCAGCAGTGCAAGCAGGCTGCAGC
FranA D QC RP:  
GCTGAGCTTCTGTGCTTGACATGAAAGCCTTCGATCAGGCG

*the restriction and mutation sites are underlined.

Table 4.1 List of primers.
4.7 REFERENCES


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47. Bibb, M. J. *personal communication*.


CHAPTER V: PHAGE DISPLAY OF LANTHIPEPTIDES

5.1 INTRODUCTION

Cyclic peptides are promising candidates to use as molecular scaffolds for peptide libraries, which in turn may provide powerful tools for drug design and investigation of protein-protein interactions (1). The characteristic thioether crosslinks of lanthipeptides provide stability to different physical conditions (pH, temperature), resistance to proteases, and form scaffolds to selectively and tightly bind to small molecules such as lipid II and phosphotidylethanolamine (PE) (2-5). These crosslinks are necessary for the activity of antimicrobial lanthipeptides, called lantibiotics, as in the absence of these modifications, binding to their target is not observed. Previous studies showed that the solubility, stability, and therapeutic effect of lanthipeptides can be improved by replacement of amino acids at different positions in the sequence with natural and/or unnatural residues (6-7). However, only a few systematic methods for discovery of new, pharmaceutically valuable lanthipeptides are available such as bacterial display, and in vitro non-ribosomal translation of lanthipeptides with designed ring structures (8-9). Therefore, a method that enables selection of a desired function of a lanthipeptide would be highly valuable. The molecular details of the biosynthesis of several lantibiotics such as nisin, cinnamycin, lacticin 481, and haloduracin have been revealed by our laboratory and provide a strong basis for engineering of these molecules (Figure 5.1) (10-13).
Nisin is a polycyclic antibacterial peptide with 34 amino acid residues and five cyclic thioether cross-links of varying sizes. After the precursor peptide NisA is synthesized by the ribosome, the cross-links are installed by sequential actions of the enzymes NisB and NisC on NisA (Figure 5.2). Then, the mature lantibiotic is formed through the cleavage of the leader peptide located at the N-terminus of NisA by NisP, a serine protease (Figure 5.2) (14). NisB dehydrates threonines and serines in the core region of NisA whereas NisC forms the five thioether cross-links by Michael-type addition of cysteine thiols to the dehydrated Thr/Ser. Nisin specifically binds to the pyrophosphate group of lipid II and forms pores in the cell membrane to kill gram positive bacteria (15), therefore it can potentially be used as a scaffold to target other components of the cell wall.

**Figure 5.1** Structural representation of nisin, lacticin 481, cinnamycin and Halα, one of the two components of haloduracin. Dha, dehydrolalanine; Dhb, dehydrobutyrine; Abu, 2-aminobutyric acid.
Phage display is widely used for identification of peptide/protein-binders from large libraries of peptides (16-17). However, introduction of post-translational modifications onto the phage-displayed peptides is a fairly new concept. Three approaches can be used to introduce modifications on a phage-displayed peptide. One way to alter the structure of a phage-displayed peptide is chemical modification by reagents that selectively react with the peptide (18-20). For example, in a recent study peptides containing three cysteine residues separated by several random amino acid residues were fused to the phage pIII protein and reacted with tris-(bromomethyl)benzene to form bicyclic peptide libraries on phage (19). Another way to modify phage-displayed peptides is attachment of both the substrate peptide and the modifying enzyme onto the phage surface so that the enzyme catalyzes the formation of the product on phage. This method has been used to engineer enzymes catalyzing desired reactions through directed mutagenesis (21-22). Finally, the third method that has been successful to modify the peptide displayed on phage is in vitro modification of the peptide by an enzyme (23). The drawbacks of these applications include the necessity of reconstitution of the enzyme activity in vitro, and efficient substrate recognition and/or appropriate folding of the enzyme attached to the phage surface. Therefore, a more efficient method to prepare post-translationally modified phage display peptide libraries in vivo would be valuable.
Previous studies showed that a naturally occurring enzyme in phage infected bacteria can modify peptides fused to a phage protein *in vivo*, which are then displayed on the newly formed phage (24). For example, phage designed to display a specific 15 amino acid long substrate on their pIII protein was biotinylated by the *Escherichia coli* (*E. coli*) enzyme called biotin holoenzyme synthetase (BHS; product of the *birA* gene) *in vivo* (25). Herein, we describe the display of the lantibiotic nisin on M13 phage by adapting a previously developed nisin production system, in which the precursor peptide NisA is modified by the enzymes NisB and NisC in *E. coli* (26). In this case, phage display of nisin was envisioned by heterologous expression of the precursor peptide NisA fused to a phage display protein, the modifying enzymes NisB and NisC, and the genes required for forming other phage components in *E. coli*. In this strategy, the NisA peptide fused to the phage display protein was expected to be modified in *E. coli* and be incorporated with the phage components into phage displaying the corresponding lantibiotic.
Figure 5.2 Posttranslational modifications in nisin biosynthesis. NisB dehydrates seven threonines/serines in NisA, whereas NisC forms five thioether crosslinks by Michael-type addition of cysteine thiols onto the dehydrated amino acids.
5.2 RESULTS

5.2.1 Experimental design

M13 phage contains five pIII proteins on M13 phage that are responsible for the attachment of phage on to the pilus of E. coli during the infection process with which the phage propagates. This protein is the most common protein used for display of peptides, however fusing a peptide onto pIII impairs its function. In conventional phage display, phagemid encodes for pIII proteins which are fused to the displaying peptide, whereas helper phage M13K07 or VCSM13 encode for wild type pIII sustaining the infection function.

The phage display technique generally utilizes two main components. One is a phagemid or phasmid, which is a plasmid that contains an f1 origin of replication enabling single stranded DNA replication observed in f1 phage. In other words, a phagemid can replicate as a plasmid, and also can provide single stranded DNA that can be packaged into viral particles (Figure 5.3A). The second component is a helper phage M13KO7, which is an M13 phage derivative with the mutation Met40Ile in pII. This helper phage carries an origin of replication from the p15A plasmid and the kanamycin (Kan) resistance gene from Tn903 both inserted within the M13 origin of replication, thus disrupting the M13 origin (Figure 5.3B) (27). M13KO7 is able to replicate in the absence of phagemid DNA. In the presence of a phagemid bearing a wild-type M13 or f1 origin, single-stranded phagemid is packaged preferentially over helper phage.
DNA and secreted into the culture medium. This strategy allows easy production of single-stranded phagemid DNA for mutagenesis or sequencing.

Figure 5.3 Components of phage display. A) Phagemid is engineered to encode for a fusion protein to pIII (encoded by gIII). It consists of an origin of replication in E. coli (dsDNA ori; double stranded DNA origin) and the f1 origin that is used for propagation of phage. (B) Helper phage consists of a single stranded DNA encoding for phage proteins including pIII, which was used for display of the lantibiotic nisin in this study. M13 K07 helper phage genome is shown in red. Other genes on the genome are not shown. In this study, phagemid pSEX81 was used for N-terminal display.


In phage display, a desired peptide is presented on a phage surface protein by infection of the bacteria (for example E. coli cells), which are harboring a phagemid containing a fusion gene encoding of the display peptide fused to a phage surface protein, by the helper phage (Figure 5.4). This approach allows expression of phage proteins and peptide-phage protein fusion in E. coli, which results in the assembly of phage displaying peptide-phage protein on their surfaces. In addition, preferential packaging of the phagemid over helper phage DNA is favored as packaging of wild-type M13 or f1 origin on the phagemid is
more efficient than packaging using the origin of replication from P15A on the helper phage genome (Figure 5.4).

Figure 5.4 Phage propagation in M13 phage display via helper phage.

Peptides can be displayed either at the N-terminus or C-terminus of a phage coat protein (16). In this project, C-terminal display was chosen by using pJF3H phagemid, because C-terminal display exposes the modified-core region for direct interaction with a ligand of interest without interference of the leader peptide. The pJF3H phagemid enables peptide display at the C-terminus of a small peptide called Fos (Fos peptide: ~6.2 kDa), which dimerizes with the Jun peptide fused to the N-terminus of truncated plll on the phage surface (22, 28). Therefore, this method allows for fusion of the peptide of interest with a small
protein rather than fusion with a big protein such as pIII (~42.5 Da), which can be important in substrate recognition of the modifying enzymes. Previous studies showed that addition of N-terminal tags such as histidine tags to the lanthipeptide precursor peptides did not prevent the activity of lanthipeptide synthetases (29). Therefore, the modifying enzymes were expected to accept their substrates fused to the Fos peptide.

Phage propagates at 30 °C instead of 18 °C, which is the temperature used for the E. coli coexpression system described for the lantibiotics employed in this study (12, 26). Therefore, one potential concern was incomplete modification of the peptides displayed on phage due to non-functioning enzymes at 30 °C. In order to test whether the enzymes can work at 30 °C, nisA was coexpressed with nisB and nisC at 30 °C. Analysis of the isolated NisA peptide by MALDI-ToF MS showed that the NisA was completely modified demonstrating that this temperature can be successfully employed for nisin production.
Figure 5.5 Phage display using pJF3H phagemid. The phagemid pJF3H allows for display through the dimerization of Jun and Fos peptides. Jun peptide displayed on pIII protein dimerizes with fos-peptide fusion through two disulfide bonds.
5.2.2 C-terminal display of nisin via pJF3H phagemid

The gene encoding the precursor peptide NisA was successfully cloned into phagemid pJF3H. Also, a flag-tag was added to the N-terminus of the peptide to observe the peptide display with western blotting or enzyme-linked immunosorbent assay (ELISA). Nisin was displayed on phage by first coexpression of *nisB* and *nisC* from pACYCDuet-1, and the *fos-nisA* fusion from the pJF3H phagemid by induction with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 3 hours, followed by infection of cells with helper phage M13KO7. Then, the cells were collected by centrifugation and transferred to new media lacking IPTG but containing the appropriate antibiotic, and cells were grown overnight at 30 ºC for phage to be produced. Then, phage was isolated from the culture medium by PEG precipitation and analyzed with different techniques.

Western blot analysis with anti-flag antibody successfully detected the Fos-Flag-NisA (~13.5 kDa) fusion peptide that was detached from Jun-pIII protein by disrupting the disulfide bond between Jun and Fos by addition of the reducing agent DTT (Figure 5.6A). In addition, western blot analysis with anti-pIII antibody identified two bands, Jun-pIII and wild type pIII, which are required for display and infection respectively (Figure 5.6B). These results show that phage can proliferate under coexpression conditions, and that it can display the NisA precursor peptide effectively.
In order to test whether post-translational modifications were incorporated on the NisA precursor peptide, a custom-made commercial antibody was raised in a rabbit towards nisin, followed by purification with immobilized nisin and removal of antibodies recognizing immobilized unmodified NisA precursor peptide. Therefore, the antibody was expected to only bind to mature nisin but not unmodified NisA.

The core region of NisA can be cleaved by trypsin at positions 12 and 22 as shown in red in Figure 5.7A. However, when post-translational modifications are introduced, trypsin does not readily cleave the core peptide (30). Should NisB and NisC modified peptide be incorporated into the phage, trypsin protease digestion of the phage obtained by the coexpression studies was expected to release the modified nisin into the solution where it could be recognized by the
anti-nisin antibody. On the other hand, if the precursor peptide NisA is not processed by NisB and NisC, treatment with trypsin would cause the core region to be digested into three pieces not recognized by the antibody. In addition, anti-nisin antibody was able to recognize NisA modified by NisB and NisC, even though leader peptide was attached, providing another avenue to test for successful modification by NisB and NisC.

![Image](image.png)

**Figure 5.7 Western blot analysis with anti-nisin antibody.** (A) Structure of nisin. Trypsin cleavage sites within nisin are shown in red. When modifications are introduced trypsin does not readily cleave after these two lysines. (B) Western blot analysis, lane 1: NisA peptide digested with trypsin, lane 2: Peptide obtained from coexpression of nisA with nisB digested with trypsin; lane 3: Peptide obtained from coexpression of nisA with nisB and nisC and digested with trypsin; lane 4: Authentic nisin. A 12%, tris-glycine gel blotted onto a PVDF membrane was used.

The selective binding of the anti-nisin antibody to post-translationally modified NisA and nisin was tested by western blot analysis with trypsin digested peptides, including unmodified NisA, NisB-modified NisA, NisA modified by NisB and NisC, and authentic nisin (Figure 5.7B). The results showed that anti-nisin antibody successfully detects NisA modified by NisB and NisC and treated with
trypsin, as well as authentic nisin but neither unmodified NisA nor NisB-modified NisA. This observation clearly shows that the anti-nisin antibody can be used for detection of nisin displayed on phage. Thus, phage displaying unmodified NisA and phage obtained from coexpression studies were prepared in parallel, digested by trypsin, and analyzed by anti-nisin antibody. Western blot analysis detected nisin from the phage obtained by coexpression but not in NisA-displaying phage (Figure 5.8).

![Figure 5.8 Analysis of phage](image)

**Figure 5.8 Analysis of phage.** Western blot analysis of lane 1: NisA-displaying phage, lane 2: NisA-displaying phage treated with trypsin, lane 3: Phage obtained from the coexpression of *nisA* with *nisB* and *nisC*, lane 4: Phage obtained from the coexpression of *nisA* with *nisB* and *nisC* and subsequent digestion with trypsin.

Characterization of post-translational modifications on phage is also possible by mass spectrometry analysis as recently described (31). Purification of phage from PEG, which is used to precipitate phage from the growth medium, is needed for identification of the peptides by mass spectrometry. Here, the purification process of phage was simplified to one step, which involved C18 solid phase extraction (SPE) purification after trypsin digest of the phage, rather than using a size exclusion column to separate the phage from PEG, which was
followed by filtration to separate the protease digested peptide (31). MALDI-ToF mass spectrometry was used to analyze trypsin-digested phage peptides eluted from the SPE column. The MS data are consistent with the mass of nisin (1-32) (3154 Da), which does not have the two C-terminal amino acids, in the samples obtained from the coexpression system but not from NisA-displaying phage (Figure 5.9 A, B). This degradation product of nisin is commonly observed as a consequence of hydrolysis of dehydrated serine at position 33 (32).

Figure 5.9 Mass spectrometry analysis of trypsin-digested phage followed by C18 SPE purification. (A) Phage obtained from coexpression studies with nisA, nisB, and nisC is shown in red, whereas phage displaying unmodified NisA is shown in black; (B) Zoom in of the sample shown in red in (A). (C) Iodoacetamide (IAA) assay of trypsin-digested phage obtained from coexpression studies with nisA, nisB, and nisC.

*Calculation of the mass for NisA (1-32) + 2 * IAA; 3154 + 2 * 57 = 3268 Da, Obsrvd: 3268 Da.

To investigate whether the displayed NisA peptide was fully-cyclized by NisC, iodoacetamide (IAA), a thiol alkylating agent that induces a mass increase
upon reaction with cysteine thiols, was reacted with this peptide in the presence of a reducing agent. The mass spectrometry analysis demonstrated two IAA additions to the peptide suggesting that two cysteines were not cyclized in nisin(1-32) (Figure 5.9C). Evaluation of antimicrobial activity of this product resulted in a faint zone of inhibition in an agar diffusion assay with *B. subtilis* 6633, whereas no zone of inhibition was observed for peptide obtained from trypsin digestion of NisA-displaying phage, which was purified and concentrated with a SPE column.

The pJF3H phagemid uses the sec-pathway to direct both Fos-NisA and Jun-pIII fusions into the periplasmic space. The sec-pathway transports unfolded proteins either post- or co-translationally through use of different mediators SecB and SRP, respectively (33). SecA accepts the unfolded proteins and threads them through the transmembrane channel at SecY that is known to not accept molecules larger than 2.8 nm in diameter (34). On the other hand the twin-arginine transport (tat) pathway, another major export pathway in *E. coli*, is known to transport large molecules such as proteins, which are fully-folded in the cytoplasm (35) allowing more time in the cytoplasm before the export (36). Therefore, in order to display fully-modified nisin on phage, I aimed to transport nisin via the twin arginine transport (tat)-pathway. This strategy would increase the contact time between NisA and the enzymes NisB and NisC before the export into the periplasm, and help the formation of fully-modified nisin before the export.
For this purpose, a previously described system using the tat-pathway for display of peptides on phage was employed (37). In this experimental design, the pJF3H plasmid was modified to export the Fos-NisA fusion through the tat-pathway by placing a TorA signal sequence in the place of the OmpA signal sequence, while the gene encoding for the Jun-pIII fusion was kept the same such that it was transported via the sec-pathway by employing the PelB signal sequence as in the case of the original pJF3H.

Nisin was displayed on phage through the tat-pathway by first coexpression of nisB and nisC from pACYCDuet-1 and nisC in pCDFDuet-1, and the torA-fos-nisA fusion from the pJF3H phagemid by induction with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 3-4 hours, followed by infection of cells with helper phage M13KO7. Then, the cells were collected by centrifugation and transferred to new media lacking IPTG but containing the appropriate antibiotics, and cells were grown overnight at 30 ºC for phage to be produced. Then, phage was isolated from the culture medium by PEG precipitation and analyzed with different techniques. Mass spectrometry analysis, which was performed after trypsin digestion followed by C18 SPE column purification as described above, showed the mass corresponding to nisin(1-32), suggesting that modified NisA can be displayed on phage through export by the tat-pathway. Furthermore, an IAA assay confirmed that the thioether crosslinks on nisin(1-32) were successfully introduced on the peptide displayed on the phage (Figure 5.10A). Finally, bioassay analysis of the peptide towards B. subtilis
6633 revealed a zone of growth inhibition, further verifying the formation of the lanthionine rings on nisin(1-32) (Figure 5.10B). Further experiments will be performed where Ser33 is mutated into an alanine to display the full length sequence of nisin on the M13 phage.

![Figure 5.10. Analysis of trypsin-digested phage produced using tat-pathway.](image)

(A) Mass spectrometry analysis of iodoacetamide-treated, trypsin-digested and SPE-purified (C18) phage obtained from coexpression studies with tat-fos-nisA, nisB, and nisC; (B) Growth inhibition zone formation assay with peptide obtained by trypsin-digested and SPE-purified (C18) phage obtained from coexpression studies with tat-fos-nisA, nisB, and nisC at 30 ºC (spot 2). Spot 3 is trypsin-digested and SPE-purified (C18) phage obtained from coexpression studies with tat-fos-nisA, nisB, and nisC at 18 ºC (negative control). Spot 1 is authentic nisin.

*Calculation of the mass for NisA (1-32) + Na^+ + H^+; 3154 + 23 + 1 = 3178 Da, Obsrvd: 3177 Da.

### 5.2.3 Phage display of other lanthipeptides

As mentioned above, phage propagates at 30 ºC instead of 18 ºC which is the temperature used for the *E. coli* coexpression systems described for cinnamycin, lacticin 481, haloduracin, and prochlorosins (12, 26). Therefore, one
possible concern was the incomplete modification of the peptides displayed on phage due to non-functioning enzymes at this temperature (e.g. because of solubility problems). In order to test whether the biosynthetic enzymes of these lanthipeptides could work at 30 ºC, several previously reported coexpression systems were evaluated: lctA and lctM; cinA(A–1K) with cinM, cinX and cinorf7; halα with halM; and procA2.8 with procM. Analysis of the purified peptides by MALDI-ToF MS showed that the LctA, CinA, Halα and ProcA2.8 peptides were all completely modified at 30 ºC demonstrating that this temperature can be successfully employed for these enzymes.

In phage display the peptide/protein to be displayed is directed to the periplasmic space of E. coli by a signal sequence such as PelB or OmpA at the N-terminus. Once the peptide/protein is translocated into the periplasmic space, this signal sequence is removed by periplasmic proteases and phage assembly takes place. Therefore, another issue to address for the display of lantibiotics on phage was the possible incomplete modification of the precursor peptide, which might arise if the transport of the precursor peptide to the periplasmic space does not allow enough time for enzyme substrate interaction in the cytoplasm. To test whether CinM, CinX and Cinorf7 function is impaired by periplasmic translocation, pelB-his6-cinA(A–1K) was cloned into the first multiple cloning site (MCSI) of pRSFDuet-1 containing the cinM gene in the MCSII. Furthermore, cinX and cinorf7 were inserted into pACYCDuet-1 as described in chapter II. Then, pelB-cinA(A–1K) together with cinM, and cinX, and cinorf7 in pACYCDuet-1 were
heterologously expressed in *E. coli*. The periplasmic fraction was separated from the cytosolic fraction by disrupting only cell membrane by osmotic pressure, and the CinA peptide was purified by IMAC followed by C18 SPE purification. The MALDI-ToF MS analysis of the peptide from the periplasmic fraction after LysC treatment showed four dehydrations and a hydroxylation of the core region. Cyclization by the IAA assay could not be performed due to low amount of peptide. In comparison, no peptide was identified in the cytoplasmic fraction of the cells.

After eliminating our concerns about this approach with at least one system, phage designed to display lacticin 481 and cinnamycin were prepared as follows. The genes encoding for the precursor peptide CinA (A–1K) of cinnamycin or LctA of lacticin 481 were cloned into pJF3H with a flag tag fused to the N-terminus of the peptides to observe the displayed peptide with western blotting analysis or ELISA using anti-flag tag antibody. *E. coli* SS320 cells were transformed with pCDFDuet-1 containing the *cinM* gene, pACYCDuet-1 containing the *cinX* and *cinorf7* genes, and the pJF3H phagemid containing the *fos-cinA* fusion gene for cinnamycin phage preparation. Similarly, *E. coli* SS320 was transformed with *lctM*-containing pACYCDuet-1, and *fos-lctA* containing pJF3H phagemid for lacticin 481 phage preparation. After phage production was induced with 0.1 mM IPTG, western blot analysis of the phage produced did not display the corresponding peptides as no bands were detected by anti-flag antibody. Surprisingly, western blot analysis with anti-pIII antibody also did not
detect the display of Jun-pIII on the phage. To investigate whether selected IPTG levels were causing problem or not, the cells were induced at different IPTG levels after infection with M13K07 helper phage and cultures were grown overnight at 30 °C. Then, the phage were analyzed by sandwich ELISA where anti-flag antibody was immobilized on the surface of a 97-well plate and binding of phage onto the plate was analyzed by a secondary antibody recognizing M13 phage. Unfortunately, the ELISA analysis of the cinnamycin and lacticin 481 phage prepared as described at different IPTG concentrations yielded no or low display levels, compared to the high signal per phage arising from the nisin phage coexpression system as described above (Figure 5.11).
Figure 5.1. ELISA to determine display levels of CinA, LctA, and NisA on phage produced by coexpression. Anti-flag antibody-coated ELISA plate was treated with phage produced by cinnamycin, lacticin 481, and nisin coexpression systems. Phage employed in this study was prepared by 0 mM (samples 1 and 5), 0.1 mM (samples 2 and 6), 0.5 mM (samples 3 and 7), and 1 mM (samples 4 and 8) IPTG induction of the helper phage infected E. coli SS320 cells, which were transformed with pJF3H phagemid containing fos-CinA(A–1K), pCDFDuet-1 containing the cinM gene, and pACYCDuet-1 containing the cinX and cinorf7 genes (samples 1-5). IPTG was added to helper phage infected E. coli SS320 cells, which were transformed with pJF3H phagemid containing fos-LctA, and pACYCDuet-1 containing LctM gene (samples 5-8). Sample 9 is E. coli SS320 cells, which were transformed with pJF3H phagemid containing fos-nisA, and pACYCDuet-1 containing nisB and nisC genes. Sample 10 is a negative control where phage was prepared by 0.1 mM IPTG induction of helper phage infected or E. coli SS320 cells which were transformed with pJF3H phagemid containing fos-HalA with no flag tag.

5.2.4 Construction of cyclic peptide libraries using ProcM

Initially, we aimed to design phage display libraries by use of the highly promiscuous enzyme ProcM. This enzyme has 29 natural substrates composed of a highly conserved leader domain attached to highly diverse core regions containing serines/threonines and cysteines at different positions (38). Therefore,
ProcM was considered a good candidate for constructing a library of peptides containing two lanthionine crosslinks (Figure 5.12).

![Image](ProcA 2.8 AACXXXXSMPPSXX)

**Figure 5.12 Peptide design for cyclic peptides containing a double ring structure.** ProcA2.8 leader peptide was attached to a core region which could lead to the formation of two rings.

The peptides in the library consisted of the ProcA2.8 wild type leader sequence attached to a core region coding for C(X)₅S MPP S(X)₅C, where X is an amino acid encoded by the NWY degenerate codon. The peptides were encoded in a pRSFDuet-1 vector containing the gene encoding for ProcM (prepared by PhD student Xiao Yang). The NWY codon was selected for the library because it does not encode for Cys or Ser/Thr, and therefore would not complicate lanthionine formation at the designed positions. Twelve clones were investigated as shown in Table 5.1, and peptides 2.8-5, 2.8-9, and 2.8-10 were coexpressed with ProcM and purified by IMAC followed by RP-HPLC. The analysis of the peptides by MALDI-ToF tandem mass spectrometry after GluC endoproteinase cleavage showed that ProcM dehydrated and cyclized all three peptides (Figure 5.13A, B, and C).
Figure 5.13 Analysis of peptide (A) 2.8-5, (B) 2.8-9, and (C) 2.8-10 modified by ProcM in vivo. Left: Mass spectrometry analysis of the peptides modified by ProcM in *E. coli* and treated with GluC, Right: Tandem mass spectrometry analysis of the peptides.

*Calculated mass for M – 2 H₂O + H for (A) 2460.6 – 2 * 18 + 1 = 2425.6 Da, (B) 2438 – 2 * 18 + 1 = 2403 Da, (C) 2468.8 – 2 * 18 + 1 = 2432.8 Da, Obsrvd (A) 2424.1 Da, (B) 2402 Da, (C) 2432 Da.*
5.2.5 ProcM Promiscuity Test

We initially started our phage display studies by use of the pSEX81 phagemid. In phage display using this phagemid, the peptides are displayed on the phage surface while they are attached to the N-terminus of the pIII protein. This fusion protein contains a signal sequence PelB at the N-terminus to direct it to the periplasmic space, where the phage assembly takes place. One concern in this system is that the pIII protein (~45 kDa) at the C-terminal of the precursor peptide might affect the dehydration and cyclization activities of lanthionine synthetase used.

Previously, I was able to show that ProcM could successfully modify peptide 2.8-1 and introduce two lanthionine rings. To test the ability of ProcM to process a fusion protein of 2.8-1 and pIII, a gene encoding for the fusion of 2.8-1 peptide-pIII, with an N-terminal flag tag and a factor Xa cleavage site separating the precursor peptide and the pIII protein, was cloned into the first cloning site of a pRFSDuet-1 vector containing procM in MSC II (Figure 5.1). After heterologous coexpression of the genes encoding the fusion of 2.8-1 peptide-pIII and ProcM using this vector in E. coli, the peptide-pIII fusion was purified by IMAC column followed by PD10 desalting. MALDI-ToF MS analysis of the peptide after Factor Xa and GluC endoproteinase cleavage showed that two dehydrations were introduced into the peptide (Figure 5.1B). Tandem mass spectrometry analysis (MALDI-ToF) of the peptide also confirmed that the rings were successfully formed (Figure 5.1D). However, IAA treatment of the
peptide-protein fusion followed by Factor Xa and GluC cleavage also showed 1 and 2 IAA additions to the core region showing that cyclization was not complete (Figure 5.14C). To conclude, ProcM was able to process precursor peptide 2.8-1 when it was attached to the N-terminus of the pIII protein but with low efficiency in cyclization.
Figure 5.14 Analysis of 2.8-1-pIII modified by ProcM in vivo. (A) Schematic representation of peptide 2.8-1 attached to pIII, (B) Mass spectrometry analysis of the 2.8-1-pIII fusion after GluC and Factor Xa cleavage; (C) Iodoacetamide analysis of the peptide; (D) Tandem mass spectrometry analysis of GluC- and Factor Xa-cleaved peptide.

*Calculated mass for M – 2 H₂O + H: 2861 – 2 * 18 + 1 = 2825 Da, Obsrvd; 2826 Da; M – 2 H₂O + IAA + H; 2825 + 57 = 2882 Da, Obsrvd; 2882 Da; M – 2 H₂O + 2 * IAA + H; 2882 + 57 = 2939 Da, Obsrvd; 2939 Da.
5.3 DISCUSSION

In this chapter, the lantibiotic biosynthesis machinery of nisin, cinnamycin, and lacticin 481 were employed to attempt display of these peptides on the M13 phage. It was shown that nisin(1-32) can be displayed on phage by the export of a Fos-NisA fusion through the tat-pathway while the Jun-pIII fusion was transported via the sec-pathway. In addition, it was demonstrated that fully-dehydrated NisA(1-32) peptide containing three thioether rings instead of the five observed in wild-type nisin structure can be displayed on phage using the sec-pathway.

This observation can be the result of several reasons. Firstly, it is possible that the sec-pathway is incapable of transporting fully modified peptide with large rings to the periplasmic space. Previous studies, which attempted nisin production using export via the sec-pathway in its producing strain Lactococcus lactis, and in B. subtilis, failed to isolate any bioactive material although fully-dehydrated NisA peptide was obtained from this system (39). In another study however, the sec-pathway in E. coli was shown to export post-translationally biotinylated and phosphopantetheinylated recombinant proteins successfully, suggesting that sec-transport mechanism can carry post-translationally modified molecules across the membrane (40). In an experiment performed by Kenton Hetrick, a PhD student in our laboratory, a large amount of modified NisA was encountered inside cells coexpressing the genes fos-nisA and jun-pIII in pJF3H together with nisB and nisC in pACYCDuet-1. This experimental set up
resembles that of nisin displaying phage, and thus indicates that modified NisA accumulates in the cell because it is not able to efficiently pass through the pores provided by the sec-pathway components. The dimensions of the solvent-accessible surface of nisin are about 2.2 nm by 2.7 nm by 4.7 nm (41). Furthermore, the A and B rings of nisin form a horseshoe-like structure at the N-terminus causing an arrangement of a large segment that is 2.2 nm by 2.7 nm wide. On the other hand, the sizes of individual lanthionines on nisin range between 0.5-1.0 nm as observed from the NMR structure of nisin bound to lipid II (15). The SecY pore involved in the sec-pathway can accommodate structures of at least 2.2-2.4 nm (34). These findings suggest that the sec-pathway is able to incorporate and export each lanthionine crosslink on NisA when linear peptide is considered. However, the three-dimensional folding of A and B rings would prevent the passage of the modified peptide. Secondly, the fast translocation of the peptide to the periplasmic space by the sec-pathway might not allow sufficient time for full modification by NisC.

In order to increase the interaction time with NisA and NisC, a previous study which showed that post-translationally modified recombinant proteins can be exported via the sec-pathway in E. coli (40) was used. In this study, sodium azide was added to the culture medium that increases the modification efficiency, as sodium azide specifically inhibits the ATPase activity of the motor protein SecA which drives the translocation of preproteins across the cytoplasmic membrane (42). Therefore, it could be possible that the incomplete modification
observed in the nisin coexpression system can be overcome by use of sodium azide in the growth medium. However, in the experiments carried out with sodium azide, the cells died, thus this attempt also failed. Our previous experiments showed that phage can be formed at 18 °C even though the optimum temperature for multiplication is 30 °C. Thus, decreasing the temperature of coexpression to 18 °C might also be helpful to slow down the translocation of the peptide across the inner membrane, giving more time for substrate-enzyme interaction. However, no improvements in the formation of rings were observed in phage prepared at 18 °C. Alternatively, addition of multiple copies of the genes for lantibiotic biosynthesis was previously shown to increase the efficiency of modification simply due to a higher amount of enzyme being expressed in *E. coli* (43). Therefore, addition of more copies of *nisC* into the coexpression system was attempted; however this approach also did not result in the display of fully-cyclized nisin on phage. Overall, the fact that three of the five rings were formed on NisA suggests that the sec-pathway can be used to export peptides with smaller solvent exposed surfaces.

Both *tat*- and *sec*- transport approaches employed to display nisin on phage resulted in a degradation product of nisin at dehydrated Ser33, which is a common degradation pathway for nisin that is usually observed at higher pH (32). However, this breakdown product has not been observed in the coexpression studies described before (26). The pH of the periplasmic space directly depends on the pH of the exterior medium/solution (44). In our experiments we observed
that the growth media is becoming more alkaline after the coexpression, which might be the reason for the formation of the degradation product. However, our attempts to grow phage in phosphate buffered media failed to display nisin.

Phage display of lanthipeptides will enable the preparation of thioether-containing cyclic peptide libraries to advance or alter current properties of lantibiotics and/or to select for binders to new targets. Phage display of nisin may be useful for finding binders to molecules with structures similar to the pyrophosphate on lipid II and/or to solve nisin’s instability problem at physiological conditions by forming phage libraries displaying nisin-like peptides. To this end our laboratory has successfully produced prochlorosins, cinnamycin, haloduracin, and lacticin 481 and the processing enzymes have been shown to have high tolerance for different substrates (12, 26). Especially ProcM, the lanthionine synthetase of prochlorosins, may be very valuable to prepare lanthionine-containing peptide libraries as it can naturally process 29 substrates with rings that vary in size and topology (45-46). Therefore, this technique can be expanded for phage display of other lanthipeptides, which would enable preparation of cyclic peptide libraries with thioether crosslinks providing different scaffolds.
5.4 MATERIALS

Oligonucleotide primers for mutagenesis were synthesized by Operon Technologies. Taq and Platinum Pfx DNA polymerases, DpnI, restriction endonucleases, and bacteriophage T4 DNA ligase were purchased from Invitrogen. Fusion DNA polymerase was purchased from New England Biolabs (NEB). Cloning vectors (pET and pDuet) were obtained from Novagen. The phagemids pSEX81 and pJF3H were kindly provided by Prof. Jun Yin’s laboratory at the University of Chicago. Gel extraction, plasmid mini-prep, and PCR purification kits were purchased from Qiagen. M13K07 helper phage (NO315S), and anti-M13 pIII monoclonal antibody (E8033S) were obtained from NEB. Monoclonal anti-flag M2 antibody produced in mouse was purchased from Sigma (F3165). Anti-nisin antibody was ordered from Thermo Fisher Scientific by submitting 10 mg of purified nisin and purified fully modified NisA. ELISA plates (Nunc), and substrate for enzyme horseradish peroxidase (HRP) in ELISA studies, 1-step ultra TMB-ELISA (34028), was obtained from Thermo Fisher Scientific. Substrate for the HRP for western blot analysis was clarity western ECL substrate from Bio-rad (170-5060). All strains were grown in media acquired from Difco laboratories. Other items procured include isopropyl-1-thio-D-galactopyranoside (IPTG, CalBiochem), iodoacetamide (IAA, Acros Organics), tris(2-carboxyethyl) phosphine hydrochloride (TCEP, Aldrich), and dithiothreitol (DTT, Sigma). Endoproteinases LysC, GluC, and Factor Xa were purchased from Roche Applied Science.
5.5 GENERAL METHODS

Molecular biological manipulations were carried out using standard techniques (47). PCR was performed using an automatic thermocycler (PTC 150, MJ Research) and DNA sequencing was performed at the Biotechnology Center of the University of Illinois at Urbana-Champaign (UIUC). *E. coli* DH5α cells (UIUC Cell Media Facility) and *E. coli* BL21 (DE3) cells (Stratagene) were used for plasmid preparation and protein expression, respectively. MALDI-ToF MS analyses were conducted at the Mass Spectrometry Facility (UIUC) using an UltrafleXtreme TOF/TOF (Bruker Daltonics). Liquid chromatography electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS) was carried out and processed using a Synapt ESI quadrupole ToF Mass Spectrometry System (Waters) equipped with an Acquity Ultra Performance Liquid Chromatography (UPLC) system (Waters). ELISA readings were performed with a Synergy H4 reader.
5.6 EXPERIMENTAL METHODS

5.6.1 Cloning

5.6.1.1 Cloning of the nisin coexpression system (NB6, 34-35)

A gene encoding for NisA attached to a flag tag at its N-terminus was not available as template, therefore a two step PCR was performed to clone the gene into pJF3H. The primers Flag_NisA pJF3H_F1 and NisA_pJF3H_NotI_RP (see Table 5.2) were used to amplify nisA from nisA/pRSFDuet-1 (obtained from previous PhD student Dr. Neha Garg). The size of the final PCR product was confirmed by 2% agarose gel electrophoresis, and the product was purified using a QIAquick gel purification kit (QIAGEN). Then, a second PCR was conducted using Flag CinA-K/ NisAFP2 pJF3H_BssHII_F1 and NisA_pJF3H_NotI_RP as primers and the previous PCR product as a template (Table 5.2). The PCRs (50 μL) included 1x HF buffer (Finnzymes), DMSO (4%), Fusion DNA Polymerase (Finnzymes) (0.02 unit/μL), dNTPs (1 mM), template DNA, and primers (1 μM each). The amplification was conducted via 30 cycles of denaturing (98 °C for 10 s), annealing (60 °C for 30 s), and extending (72 °C for 15 s). The final PCR product was confirmed by 2% agarose gel electrophoresis, and the products were purified using QIAquick PCR purification kits (QIAGEN). The resulting DNA fragment and the pJF3H vector were digested in 1 x NEBuffer 4 (New England Biolabs) with BssHII and NotI-HF at 37 °C (for 15 h). The digested products were purified by agarose gel electrophoresis followed by use of a QIAquick gel extraction kit (QIAGEN). The
resulting DNA insert was ligated with the digested pJF3H vector at 16 °C for 15 h using T4 DNA ligase. The ligation reaction mixture was diluted 5 times with water prior to transformation. *E. coli* DH5α cells were transformed with the ligation product via heat shock, plated on LB-ampicillin/0.2 % glucose agar plates, and grown at 37 °C for 15 h. Colonies were picked and incubated in 5 mL of LB-ampicillin medium at 37 °C for 15 h, followed by isolation of the plasmids using a QIAPrep Spin Miniprep Kit (QIAGEN). The sequences of the resulting plasmids were confirmed by DNA sequencing. The pACYCDuet-1 vector containing *nisB* and *nisC* in MCSI and MCSII, respectively, was obtained from Noah Bindman in the van der Donk laboratory.

### 5.6.1.2 Cloning of cinA(A–1K) into pSEX81 and pJF3H, cloning of nisA into pSEX81, and factor Xa cleavage site insertion (NB6, p48-65)

Cloning of *cinA*(A–1K) into pSEX81 was performed by use of the primers Flag_CinA-K/NisA_FP2_NcoI pSEX81 FP and CinA-K_NotI pSEX81_RP (Table 5.1), whereas cloning into pJF3H was performed by use of Flag CinA-K/NisA_FP2_pJF3H_BssHII_FP and CinA-K_pJF3H_NotI_RP as forward and reverse primers. The PCRs (50 μL) included 1x HF buffer (Finnzymes), DMSO (4%), Fusion DNA Polymerase (Finnzymes) (0.02 unit/μL), dNTPs (1 mM), template DNA, and primers (1 μM each). As a template, *flag-tag-cinAK/pRSFDuet-1*, whose construction was described in chapter II, was used. The amplification was conducted via 30 cycles of denaturing (98 °C for 10 s), annealing (60 °C for 30 s), and extending (72 °C for 15 s). The final PCR product
was analyzed by 2% agarose gel electrophoresis, and the products were purified using QIAquick PCR purification kits (QIAGEN). The resulting DNA fragment and the corresponding vector were digested in NEBuffer 4 at 37 ºC for 15 h (New England Biolabs) with Ncol-HF and Notl-HF (for pSEX81) and, BssHI and Notl-HF (for pJF3H), respectively. The digested products were purified by agarose gel electrophoresis followed by use of a QIAquick gel extraction kit (QIAGEN). The resulting DNA insert was ligated with the digested vector at 16 ºC for 15 h using T4 DNA ligase. The ligation reaction mixture was diluted 5 times with water prior to transformation. E. coli DH5α cells were transformed with the ligation product via heat shock, plated on LB-ampicillin (for the plasmids pSEX81 and pJF3H)/0.2% glucose agar plates, and grown at 37 ºC for 15 h. Colonies were picked and incubated in 5 mL of LB-ampicillin medium at 37 ºC for 15 h, followed by isolation of the plasmids using a QIAprep Spin Miniprep Kit (QIAGEN). The sequences of the resulting plasmids were confirmed by DNA sequencing.

Since nisA encoding NisA with a flag tag at the N-terminus was not available for template, a two step PCR was performed to clone the gene encoding into pSEX81. For cloning into pSEX81, the primers Flag_NisA_pJF3H_FP-1 and NisA_Notl_pSEX81_RP were used to amplify nisA from nisA/prsFDuet-1 (provided by PhD student Noah Bindman). The size of the final PCR product was confirmed by 2% agarose gel electrophoresis, and the product was purified using a QIAquick gel purification kit (QIAGEN). Then, a second PCR was conducted using Flag_CinA-K/NisA_FP2/Ncol_pSEX81_FP
and NisA_NotI_pSEX81_RP as primers and the previous PCR product as a template. The digestion and ligation procedures were performed as explained above.

The factor Xa protease cleavage sequence was inserted between the leader and core regions of cinA(A–1K) and nisA to form cinA-K-Xa and nisA-Xa by Quikchange PCR. Briefly, the amplification from cinA(A–1K)-containing pSEX81 and nisA-containing pSEX81 was conducted via 30 cycles of denaturing (98 °C for 10 s), annealing (60 °C for 30 s), and extending (72 °C for 100 s) using appropriate mutant forward and reverse primers (see Table 5.2 for oligonucleotide sequences). The size of the PCRs (50 μL) included 1x HF buffer (Finnzymes), DMSO (4%), Fusion Hot Start High-Fidelity DNA Polymerase (Finnzymes) (0.04 unit/μL), dNTPs (2 mM), corresponding template (20 ng), and primers (1 μM each). The PCR product was checked on a 1% agarose gel and purified using a QIAquick PCR purification kit (QIAGEN). The resulting DNA was treated with DpnI at 37 °C for 15 h to digest the methylated template, and E. coli DH5α cells were transformed. The desired mutations were confirmed by DNA sequencing.

5.6.1.3 Cloning of pelB-cinA(A–1K)-Xa, and pelB-cinA(A–1K) into pRSFDuet-1

The genes encoding for PelB-CinA(A–1K)-Xa and PelB-CinA(A–1K) were cloned into the first MCS of pRSFDuet-1 containing a cinM in MCSII. For this purpose, PelB_CinAK/Xa_FP_Asc_I for_pRSFDuet_from_pSEX81 and CinA-
K_pRSFDuet-1_HindIII_RP were used as forward and reverse primers (Table 5.2). The PCRs (50 μL) included 1x HF buffer (Finnzymes), DMSO (4%), Fusion DNA Polymerase (Finnzymes) (0.02 unit/μL), dNTPs (1 mM), cinA(A–1K)-containing pSEX81 or cinA(A–1K)-Xa-containing pSEX81 as template DNA (20 ng), and primers (1 μM each). The amplification was conducted via 30 cycles of denaturing (98 °C for 10 s), annealing (60 °C for 30 s), and extending (72 °C for 15 s). The size of the final PCR product was confirmed by 2% agarose gel electrophoresis, and the products were purified using a QIAquick PCR purification kit (QIAGEN). The resulting DNA fragment and pRSFDuet-1 containing the gene encoding for CinM were digested in 1xNEBuffer 4 (New England Biolabs) with Ascl and HindIII-HF at 37 °C (for 15 h) respectively. The digested products were purified by agarose gel electrophoresis followed by use of a QIAquick gel extraction kit (QIAGEN). The resulting DNA insert was ligated with the digested vector at 16 °C for 15 h using T4 DNA ligase. The ligation reaction mixture was diluted 5 times with water prior to transformation. *E. coli* DH5α cells were transformed with the ligation product via heat shock, plated on LB-kanamycin (kan) agar plates, and grown at 37 °C for 15 h. Colonies were picked and incubated in 5 mL of LB-kan medium at 37 °C for 15 h, followed by isolation of the plasmids using a QIAprep Spin Miniprep Kit (QIAGEN). The sequences of the resulting plasmids were confirmed by DNA sequencing.
5.6.1.4 Preparation of the constructs for peptides attached to the ProcA leader 2.8

Cloning of the genes encoding the library of peptides attached to the ProcA 2.8 leader peptide (Table 5.1) into pSEX81 was performed by using Ncol and Notl cut sites using the primers in Table 5.2, Flag_2.8_ProcA_ldr_pSEX81_Ncol_FP and 2.8_ProcA_ldr_XiaoLibrary_pept_1_pSEX81_Notl_RP (or 2.8_ProcA_ldr_Xiao_library_pept_1/Factor Xa/pSEX81_Notl RP) primers were used as forward and reverse primers to amplify the ProcA 2.8 leader peptide library 1 sequence from template 2.8-peptide_1/prsfduet-1 provided by PhD student Xiao Yang in our group (see Table 5.2 for oligonucleotide sequences). The PCRs (50 μL) included 1x HF buffer (Finnzymes), DMSO (4%), Fusion DNA Polymerase (Finnzymes) (0.02 unit/μL), dNTPs (1 mM), template DNA, and primers (1 μM each). The amplification was conducted via 30 cycles of denaturing (98 °C for 10 s), annealing (60 °C for 30 s), and extending (72 °C for 15 s). The final PCR product was confirmed by 2% agarose gel electrophoresis, and the products were purified using QIAquick PCR purification kits (QIAGEN). The resulting DNA fragment and the pSEX81 vector were digested in 1x NEBuffer 4 (New England Biolabs) with Ncol-HF and Notl-HF at 37 °C (for 15 h). The digested products were purified by agarose gel electrophoresis followed by use of a QIAquick gel extraction kit (QIAGEN). The resulting DNA insert was ligated with the digested pSEX81 vector at 16 °C for 15 h using T4 DNA ligase. The ligation reaction
mixture was diluted 5 times with water prior to transformation. *E. coli* DH5α cells were transformed with the ligation product via heat shock, plated on LB-ampicillin/0.2 % glucose agar plates, and grown at 37 °C for 15 h. Colonies were picked and incubated in 5 mL of LB-ampicillin medium at 37 °C for 15 h, followed by isolation of the plasmids using a QIAprep Spin Miniprep Kit (QIAGEN). The correct sequences of the resulting plasmids were confirmed by DNA sequencing.

Similarly, the genes encoding for peptide library 1 attached to pIII were cloned into pRSFDuet-1 containing *procM* between the NdeI and Kpnl restriction sites. Flag 2.8_{ldr}_EcoRI_pRSFDuet-1_FP and pIII_pRSFDuet-1_Ascl_RP were used as forward and reverse primers to amplify the DNA with 1-FactorXa-pIII/pSEX81 as a template. The PCR conditions used were as indicated above. The resulting DNA fragment and the pRSFDuet-1 vector were digested in 1 x NEBuffer 4 (New England Biolabs) with EcoRI-HF and Ascl at 37 °C for 15 h. The digestion and ligation steps were performed as indicated above. *E. coli* DH5α cells were transformed with the ligation product via heat shock, plated on LB-kanamycin agar plates, and grown at 37 °C for 15 h. Colonies were picked and incubated in 5 mL of LB-kanamycin medium at 37 °C for 15 h, followed by isolation of the plasmids using a QIAprep Spin Miniprep Kit (QIAGEN). The sequences of the resulting plasmids were confirmed by DNA sequencing.
5.6.2 Preparation of phagemids for display of NisA, CinA, and LctA (NB10, p27, 29)

Electro-competent *E. coli* SS320 cells (50 μL) were transformed with 20 ng of phagemid (pJF3H) containing the desired gene, and plated on a Amp (100 mg/ml), Tet (5 mg/ml), 2% glucose LB plate. In this step, Amp is required for the phagemid, Tet is required for the F\(^+\) plasmid coding for the pili, which is needed for helper phage infection, and glucose is to prevent the expression of pIII since it slows down the cell growth overnight at 37 °C. After the plates were incubated overnight at 37 °C, a colony was picked and grown in 5 mL of 2xYT media (1.6% tryptone, 1.0% yeast extract, 0.5% NaCl, pH: 7, autoclaved) supplemented with Amp (100 mg/ml), Tet (5 mg/ml) and 2% glucose with shaking. Then, 20 mL 2xYT media was inoculated with 200 μL of the cells and the cells were grown until an O.D. of 0.5-0.6. Approximately 1x10\(^9\) bacteria per mL and 10\(^{10}\) helper phage M13K07 (NEB, 10 μL for 1 ml) were added at a phage/cell ratio of ~10-20. Then, the culture was incubated at 30 °C for 20 min without shaking, followed by 20 min with shaking at 30 °C. Infected cells were pelleted by centrifugation and resuspended in 2xYT medium (100 mL) supplemented with kanamycin (helper phage resistance) (50 mg/mL), ampicillin (100 mg/mL) and IPTG (0.1 mM) and grown for 15-18 h at 30 °C with shaking. The next day, cells were pelleted by centrifugation at 5000×g, and the phage particles in the supernatant were precipitated by adding poly(ethylene glycol) 8000 (PEG; 25 mL, 20%, w/v) and NaCl (2.5 M) followed by overnight incubation on ice. The next morning, the
precipitated phage was centrifuged for 30 min at 16,060 g. The supernatant was discarded, and the phage pellet at the bottom of the tubes was resuspended in phosphate buffered saline (PBS) buffer. Then, the phage was precipitated again with the PEG/NaCl addition and centrifuged for 30 min at 16,060 g (13,000 rpm). The supernatant was discarded and, the purified phage was resuspended in PBS (2 mL). The phage was stored at 4 °C. Phage concentration measurement was roughly estimated by using this conversion formula: O.D. 268 = 1 for a solution containing 5x 10^{12} phage/mL.

**5.6.3 Phage preparation for nisin display (NB11, p84)**

For coexpression studies to display nisin, electro-competent *E. coli* SS320 cells (50 μL) were transformed with *nisA*-containing pJF3H, and pACYCDuet-1 containing *nisB* and *nisC*, and plated on an Amp (50 mg/ml), Chl (25 mg/mL), Tet (5 mg/mL), and 2% glucose LB plate. After the plates were incubated overnight at 37 °C, a colony was picked and grown overnight at 37 °C in 5 mL of 2xYT media (1.6% Tryptone, 1.0% yeast extract, 0.5% NaCl, pH: 7, autoclaved) supplemented with Amp (50 mg/mL), Chl (25 mg/mL), Tet (5 mg/mL), and 2% glucose. Then, 5 mL of 2xYT media was inoculated with 50 μL of the overnight culture and the cells were grown until the O.D. was 0.7, and gene expression was induced by addition of 1 mM final concentration of IPTG to the 5 mL of cell culture, which was then incubated at 30 °C for 3 h with shaking. Afterwards, the cell culture was infected with 20 μL of helper phage M13K07 (10^{11} per mL, NEB) by incubation at 30 °C for 20 min followed by shaking at 30 °C for another 20
min. Infected cells were centrifuged at 5000×g, and the supernatant was removed. The cells were resuspended in 2xYT medium (200 mL) supplemented with Kan (50 mg/mL), Amp (50 mg/mL), and Chl (25 mg/mL) and grown for 15-18 h at 30 °C. After overnight shaking, cells were pelleted by centrifugation at 5000×g, and the phage particles in the supernatant were isolated by PEG purification as described above.

5.6.4 Phage preparation for cinnamycin display (NB11, p59-60)

For coexpression studies to display cinnamycin, electro-competent *E. coli* SS320 cells (50 μL) were transformed with *cinA-K*-containing pJF3H, *cinM*-containing pCDFDuet-1, and pACYCDuet-1 containing *cinX* and *cinorf7*, and plated on an Amp (50 mg/ml), Spec (15 mg/ml), Chl (25 mg/mL), Tet (5 mg/mL), and 2% glucose LB plate. After the plates were incubated overnight at 37 °C, a colony was picked and grown overnight at 37 °C in 5 mL of 2xYT media (1.6% Tryptone, 1.0% yeast extract, 0.5% NaCl, pH: 7, autoclaved) supplemented with Amp (50 mg/mL), Spec (15 mg/mL), Chl (25 mg/mL), Tet (5 mg/mL), and 2% glucose. After 12 h, 5 mL 2xYT media was inoculated with 50 μL of the overnight culture and the cells were grown until the O.D. was 0.7. The gene expression was induced by addition of 1 mM IPTG to the cell culture, which was then incubated at 30 °C for 3 h with shaking. Afterwards, the cell culture was infected with 20 μL of helper phage M13K07 (10^{11} per mL, NEB) by incubation at 30 °C for 20 min followed by shaking at 30 °C for another 20 min. Infected cells were centrifuged at 5000×g, and the supernatant was removed. The cells were
resuspended in 2xYT medium (200 mL) supplemented with Kan (helper phage resistance) (50 mg/mL), Amp (50 mg/mL), Spec (15 mg/ml), and Chl (25 mg/mL), and grown for 15-18 h at 30 °C. After overnight shaking, cells were pelleted by centrifugation at 5000×g, and the phage particles in the supernatant were isolated by PEG purification as described above.

5.6.5 Phage preparation for lacticin 481 display (NB12, p45)

For coexpression studies to display lacticin 481, electro-competent E. coli SS320 cells (50 µL) were transformed with lctA-containing pJF3H, lctM-containing pCDFDuet-1, and, lctM-containing pACYCDuet-1, plated on an Amp (50 mg/ml), Spec (15 mg/ml), Chl (25 mg/mL), Tet (5 mg/mL), 2% glucose LB plate. After the plates were incubated overnight at 37 °C, a colony was picked and grown overnight at 37 °C in 5 mL of 2xYT media (1.6% tryptone, 1.0% yeast extract, 0.5% NaCl, pH: 7, autoclaved) supplemented with Amp (50 mg/mL), Spec (15 mg/mL), Chl (25 mg/mL), Tet (5 mg/mL), and 2% glucose. Then, 5 mL of 2xYT media was inoculated with 50 µL of the overnight culture and the cells were grown until the O.D. was 0.7. The gene expression was induced by addition of 1 mM IPTG to the cell culture, which was then incubated at 30 °C for 3 h with shaking. Afterwards, the cell culture was infected with 20 µL of helper phage M13K07 (10^{11} per mL, NEB) by incubation at 30 °C for 20 min followed by shaking at 30 °C for another 20 min. Infected cells were centrifuged at 5000×g, and the supernatant was removed. The cells were resuspended in 2xYT medium (200 mL) supplemented with Kan (helper phage resistance) (50 mg/mL), Amp
(50 mg/mL), Spec (15 mg/ml), Chl (25 mg/mL), and grown for 15-18 h at 30 °C by shaking. After overnight shaking, cells were pelleted by centrifugation at 5000×g, and the phage particles in the supernatant were isolated by PEG purification as described above.

5.6.6 Determination of phage titer (NB9 p 30, 36, 84; NB10 p73)

Serial dilutions of phage particles (2 x10\(^{12}\)) were prepared by mixing 20 μL of the previous diluted phage stock with 180 μL of PBS. Then, 500 μL of \(E.\ coli\) SS320 or XL1blue (tet\(^R\)) at exponential phase was mixed with 200 μL of each phage mixture and 4 mL of 2YT top agar (microwaved and cooled to 42 °C before adding phage). Before the mixture solidified, it was poured onto LB/tet (5 mg/mL) plates resulted in double layers of agar. The phage particles were incubated for 8-15 h at 37 °C, and the plaques were counted. Then, phage particles per mL were calculated by dividing the number of plaques per plate per plated volume times the dilution factor. For example, if 347 plaques were counted when 0.1 mL of a 10\(^6\) diluted phage suspension was plated, the titer was 3.47 x 10\(^9\) phage particles per mL.

5.6.7 Western blot analysis (NB9, p85)

Phage (20 μL, 2x10\(^{12}\) phage/mL) was mixed with protein loading dye containing 25 mM DTT. After 30 min incubation, 15 μL of this solution was loaded on a 12% SDS-PAGE gel (Bio-Rad). After electrophoresis, the protein bands were electroblotted onto a piece of polyvinylidene fluoride (PVDF) membrane (Bio-Rad) at 250 mAmp for 50 min in 10 mM CAPS buffer pH 10.5. The
membrane was then blocked with 5% non-fat dry milk in PBS-T (137 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate dibasic, 2 mM potassium phosphate monobasic, 0.1% Tween-20, pH 7.4) for 1 h, followed by incubation for 1 h (RT) or overnight at 4 °C with an appropriate antibody (1 μg/mL; Sigma–Aldrich) in 5% milk/PBS-T solution. The membrane was then washed six times with PBS-T. An anti-mouse antibody–HRP conjugate (0.1 mg/mL antibody in 5% milk/PBS-T; Thermo Fisher Scientific) was incubated with the membrane for 1 h. Finally, the membrane was washed six times with PBS-T and twice with PBS followed by detection with the clarity ECL substrate (Bio-Rad, 170-5060).

5.6.8 Enzyme-linked immunosorbent assay (ELISA) analysis (NB12, p24)

The wells of a Nunc MaxiSorp® flat-bottom 96 well plate (Thermo Scientific) were coated with an anti-flag antibody at a concentration of 10 μg/mL in carbonate/bicarbonate buffer (pH 9.6, 100 μL) and the plate was incubated overnight at 4 °C. The next morning, the plate was washed twice with 20 μL of PBS buffer. The solutions or washes were removed. The remaining drops were removed by patting the plate on a paper towel. Then, the coated wells were blocked by addition of 300 μL of 5% non-fat dry milk/PBS per well and the plate was incubated for 2 h at room temperature. Then, 100 μL of 10x dilution of phage samples (~ 10^{12}) was added to each well and the plate was incubated for 90 min at 37 °C. The samples are removed and the plate was washed 6 times by filling the wells with 300 μL of PBS. Then, 100 μL of anti-M13 antibody-HRP in 5% milk solution was added to each well. The plate was incubated for another 1 h at room
temperature, followed by washing the plate for 6 times with PBS. The plate was developed with 1-Step Ultra TMB-ELISA (34028, Thermo scientific) and measurements were performed using a plate reader.

5.6.9 Mass spectrometry analysis of phage displayed peptides (NB15, p80)

Phage solutions in PBS buffer (1 mL, \(\sim 10^{12}\) phage/mL) were digested with 30 μL of appropriate proteases (stock solutions used: LysC = 3 U/100 μL, GluC = 2 mg/mL, trypsin = 40 μM) for 3 h at room temperature (GluC, trypsin) or 37 °C (LysC) followed by purification with a C18 solid phase extraction (SPE) column (1 mL, Discovery DSC-18). The C18 column was first washed with 2 mL of 80% acetonitrile (ACN)/water and equilibrated with 2 mL of water and then the sample was applied. The column was washed with 8 mL of water and the sample was eluted with 1 mL of 80% ACN/water solution. The eluent was dried on a centrivap concentrator, and the products were resuspended in 50 μL of miliQ water. The product (1 μL) was spotted on the MALDI plate together with sinapinic acid (1 μL) and analyzed by MALDI-ToF MS.

5.6.10 Purification of modified CinA from the periplasmic space (NB1, p61, p63, p69)

In order to coexpress PelB-CinA(A–1K), CinM and CinX, \(E.\ coli\) BL21 (DE3) cells were transformed with a pRSFDuet-1 vector containing \(pelb-cinA(A–1K)\) and \(cinM\) and a pACYCDuet-1 vector containing \(cinX\) and \(cinorf7\). Single colony transformants were grown in a 37 °C shaker for 12-15 h in 50 mL of Luria-Bertani (LB) medium supplemented with 25 μg/mL kanamycin/12.5 μg/mL
chloramphenicol. A 20 mL aliquot was centrifuged at 5000×g for 10 min, the spent LB medium was discarded, and the cell pellet was resuspended in fresh LB medium. The resuspended cells (20 mL) were added to 2 L of LB supplemented with the appropriate antibiotics for maintaining the vectors, and the culture was grown aerobically at 37 °C until the A$_{600}$ was ~ 0.6 to 0.8. IPTG was added to a final concentration of 0.5 mM and the culture was transferred to 18 °C for aerobic growth for an additional 20 h. Cells were harvested by centrifugation at 5000×g for 20 min at 4 °C. The cell paste (~ 3-4 g) was stored at −80 °C until use.

Bacterial cells (8 g) were harvested by centrifugation at 5,000 × g for 20 min at 4 °C. The supernatant was discarded and the pellet was gently resuspended in ice-cold 30 mM Tris-Cl, 20% sucrose (pH 8.0) at 80 mL per gram wet weight. Keeping the cells on ice, 500 mM EDTA was added dropwise to 1 mM and the cells were incubated for 5-10 min on ice with gentle agitation. The cells were centrifuged at 8000 x g for 20 minutes at 4 °C, the supernatant was discarded, and the pellet was resuspended in the same volume of ice-cold 5 mM MgSO$_4$. The sample was stirred for 10 min on an ice bath and centrifuged at 8000 x g for 20 min at 4 °C. The pellet has the intracellular components whereas the supernatant contains the periplasmic fraction. Peptides inside the cell and in the periplasmic region were purified as described for other His$_6$-LanA peptides (48) by use of a 5 mL HiTrap chelating HP nickel affinity column (GE Healthcare). A solid phase extraction column (SPE, 1 mL, Discovery DSC-18) was used to desalt the peptide. The C18 column was first washed with 2 mL of 80%
acetonitrile (ACN)/water and equilibrated with 2 mL of water and then the sample was applied. The column was washed with 4 mL of water and the sample was eluted with 1 mL of 80% ACN/water solution. The sample was dried on a centrivap concentrator and the peptide was dissolved in 100 μL of 100 mM Tris pH 7.5. The sample (20 μL) was mixed with 2 μL of LysC (0.4 μg/mL in 100 mM Tris, pH 8.3) and the protease cleavage reaction was incubated at 37 ºC for 3 h and then desalted using a ZipTip (Millipore). The product was eluted in 5 μL of sinapinic acid and analyzed by MALDI-ToF MS.

5.6.11 Coexpression studies for nisin, lacticin 481, haloduracin, and prochlorosins

Production of lanthipeptides nisin, lacticin 481, haloduracin, and prochlorosins and their derivatives such as ProcA2.8-pIII in E. coli was performed as described before (26); however, instead of using 18 ºC, the cells were grown at 30 ºC overnight.
Table 5.1. List of the randomized peptides possessing the ProcA 2.8 wild type leader peptide attached to a core region with the sequence C(XXX)_5 S MPP S(XXX)_5 C. The peptides shown in red were tested for dehydration and cyclization in this work.
List of primers

*Restriction sites and mutations are underlined*

**Flag 2.8 ProcA ldr pSEX81 Ncol FP:**
AGCGGGCACATGCCATGGATTATAAGGATGACGACGATAAAATTGTCACGAG AAG

**2.8 ProcA ldr Xia library pept. 1 pSEX81 NotI RP:**
TCCAAAAAGTCGCGCGCGCGCGCGCGCAATGGAAGCTTTATTGTGGGATGGG

**2.8 ProcA ldr Xia library pept. 1/ factor Xa/pSEX81 NotI RP:**
TCCAAAAAGTCGCGCGCGCGCGCGCGGCTCAATAGGAAGCTTTATTGTGGGATGGG

**ProcM in pRSFDUET-1 Ndel FP:**
GGTTGTTTATATGGAAAGTCCATCATCTTGG

**ProcM in pRSFDUET-1 KpnI RP:**
AATAGTTGTTACCTTATTCAGTACGCCAGAG

**Flag 2.8 ldr EcoRI pRSFduet-1 FP:**
GCCAGGATCCGAATTCGATTACAGGATGACGACG

**pll pRSFduet-1 Ascl RP:**
CATTATGCCGCCCGCGCTTTATCTACCATAGCCAGAGGACGACG

**pJF3H and pSEX81:**

**Flag CinA-K/NisA FP2 Ncol pSEX81 FP:**
GCTCGACGCGCGCGGCTGATTATAAGGATGACGAGATAAAATC

**CinA-K NotI pSEX81 RP:**
TTATGTCACGCGCGCGCTTTTGTTGCTCGGAGCACGAG

**NisA NotI pSEX81 RP:**
TTATGTCACGCGCGCGCTTTTGTTGCTCGGAGCACGAG

**Flag CinA-K/ NisAFP2 pJF3H BssHI RP:**
TTATGTCACGCGCGCGCTTTTGTTGCTCGGAGCACGAG

**CinA-K pJF3H NotI RP:**
ATGATGATGCGCGCTTTATCATTGTATTGCTCGGAGCACGAG

**Flag NisA pJF3H 1-1:**
GATGACGAGATAATTCATGACTACAAAAGATTATTTAATTGCTC

**NisA pJF3H NotI RP:**
ATGATGATGCGCGCTTTATCATTGTATTGCTCGGAGCACGAG

**FactorXa insertion:**

**CinA-K Factor Xa 1-1:**
CACCGGACCTCTAAACATTTGACGCGCGCGCTGCGCGCGAGAAGCTC

**CinA-K Factor Xa RP:**
GGCGCTCGGCGCAGCGCGGCTAAATCTCTGAGGCTTGGCTG

**NisA FactorXa FP:**
CGCGCTCGGCGCAGGCATTGACGCGCGGCGTAAATCTCTGAGGCTTGGCTG

**NisA FactorXa RP:**
CATAGGGAAATCATTGTGCTATCGGCGTACATGATGATGATGACGACGAG

**PelB CinA-K/Xa FP Ascl for pRSFduet from pSEX81:**
TTATGTCACGCGCGCGCTTTATCATTGTGCTACGCGCAGCGCG

Table 5.2 List of primers
5.7 REFERENCES


