

**Genetic and Structural Characterization of Pneumolancidin, a
Broad Spectrum Inhibitory Lantibiotic, Produced by
*Streptococcus pneumoniae***

by

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DEDICATION

In loving memory of my mother, Estera Maricic

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ABSTRACT

Streptococcus pneumoniae, a member of the diverse microbial community of the human nasopharynx, is subjected to competitive interactions. Since colonization is a prerequisite for pneumococcal pathogenesis, understanding the dynamics of bacterial competition is important for identifying factors that aid in colonization and carriage. To eliminate competitors, pneumococcus is known to secrete antimicrobial peptides or bacteriocins. Pneumococcal bacteriocins and their role in competition have been well characterized. However, a class of modified bacteriocins named lantibiotics, and their role in promoting pneumococcal competition is less well understood. Though several lantibiotic loci have been identified in pneumococcus, none have been found with antimicrobial activity. Recently, our laboratory identified a clinical isolate of pneumococcus, P174, with a broad spectrum of inhibitory activity attributed to a novel lantibiotic locus, termed pneumolancidin (*pld*). In addition to encoding genes required for the modification, processing, regulation and immunity to lantibiotic peptides, four open reading frames predicted to encode four highly homologous lantibiotic peptides were also found. This posed the question of whether the Pld peptides were redundant in function or had specialized roles. Lantibiotic peptides are known to function as antimicrobials and autoinducers. To determine the role of each peptide as it relates to inhibition and induction, individual in-frame peptide

deletions were constructed. Strains carrying the mutation were assayed for their ability to inhibit the growth of other strains and their ability to upregulate the *p/d* locus. The first three peptides, PldA1-3, were found to be required for signaling while PldA4 was found to be dispensable. Because upregulation of the locus is needed to determine whether specific peptides were involved in inhibition, the ability of the peptide deletion strains to inhibit could not be evaluated. However, a serendipitous mutant, P174act, was discovered that allowed for distinct phenotypes to be observed for each of the Pld peptide deletion strains. Through structural elucidation, it was found that PldA1 and PldA3 are structurally similar yet have specialized roles in signaling and inhibition, respectively. The Pld peptides represent a novel strategy for bacterial competition and provide insight into structure-function relationships of lantibiotics.

CHAPTER I

INTRODUCTION

Abstract

Lantibiotics are a class of bacteriocins produced by many Gram-positive bacteria with activity primarily against other Gram-positive strains. Lantibiotics undergo extensive posttranslational modifications that are required for activity as antimicrobials and as autoinducers that upregulate their own expression. They are promising for use as potential therapeutics for many reasons. First, lantibiotics are quite potent; nanomolar concentrations are typically sufficient to inhibit bacterial growth. Second, many lantibiotics demonstrate a dual mechanism of action, which make it more difficult for target bacteria to develop resistance. While these key features could certainly prove useful in the treatment of antibiotic resistant bacteria, the reliance of lantibiotics on intracellular machinery for their functionality make lantibiotics notoriously difficult to synthesize and current methods of purification from host cells are laborious. Understanding how the production of lantibiotic peptides is regulated within the cell will be useful to improve output and purification. Likewise, characterization of the protein structures and how those structures relate to overall function will be fundamental to our understanding of these entities as therapeutics and will provide new insight into the design of innovative, potentially breakthrough

antimicrobials. This review will focus on the biosynthesis, regulation, and structure of lantibiotic peptides produced by *S. pneumoniae* and closely related Gram-positive bacteria.

1.1 Bacterial Competition

Bacteria often exist in polymicrobial communities found either in the environment or in the host. To survive, bacteria have evolved multiple mechanisms to promote either cooperative or competitive relationships for access to limited resources. Cooperative interactions result in beneficial outcomes to all groups involved. Competitive interactions, on the other hand, result in a negative outcome for one group and can occur in two ways, indirect (exploitative) or direct (interference). Indirect competition refers to a bacterial cell's ability to utilize nutrients more efficiently than neighboring bacteria, preventing it from being able to utilize the required nutrients essential for survival. This mechanism has been noted to play a role in shaping the gut microbiota. As an example, commensal *Escherichia coli* strains were able to outcompete pathogenic enterohemorrhagic *E. coli* (EHEC). This was shown using a mouse model in which mice were treated with streptomycin to eliminate facultative anaerobic bacteria so that specific *E.coli* strains can be introduced into the mouse and competitive experiments can be performed. Commensal *E. coli* was shown to confer a protective effect by utilizing sugars more efficiently than EHEC [1, 2]. Direct competition refers to bacteria producing specific factors or altering abiotic factors that can directly inhibit their competitors' growth. An example of direct

competition through manipulation of abiotic factors can be seen with a recently discovered *Streptococcus* species found in the oral cavity that has the capacity to alter the pH of the environment through increased arginine catabolism [3]. Higher pH values inhibit the growth of *Streptococcus mutans*, a caries causing pathogen, effectively eliminating the competition by creating an inhospitable environment [3]. In addition to altering abiotic variables, bacteria can also produce factors that are directly (or actively) inhibitory to bacteria. Production of antimicrobial peptides is one common strategy used by bacteria.

1.2 Bacteriocins

Bacteriocins are antibiotics that are ribosomally synthesized small peptides. Bacteriocins exert an antimicrobial effect on either a wide or narrow range of competing bacteria [4]. Nearly all bacteria that have been studied encode at least one bacteriocin in their genome, however, the Gram-positive lactic acid bacteria (LAB) are the best-known producers of bacteriocins [5].

The LAB, which comprise the *Lactobacillus*, *Streptococcus*, *Leuconostoc*, *Pediococcus*, and *Lactococcus* genera primarily, are named for their ability to convert glucose into lactic acid. This property makes them useful in the food industry, not only for their ability to ferment certain foods, but also for their ability to prevent spoilage and colonization by pathogenic bacteria by either production of bacteriocins and/or lowering the pH through lactic acid production. Some of the LAB or their bacteriocin products can be added to food because they fall into the category of generally recognized as safe by the food industry. Bacteriocins

that are produced by LAB can be divided into two classes: Class I are post-translationally modified lanthionine-containing peptides (otherwise known as the lantibiotics), and Class II are unmodified, non-lanthionine containing peptides [4]. Class II bacteriocins can be further divided into four subclasses: Class IIa are known as pediocin-like and are characterized by their listericidal activity, class IIb are two peptide bacteriocins, class IIc are circular bacteriocins, and class IId are other linear, non-pediocin single peptide bacteriocins.

The class I lantibiotics will be the main focus of this introduction because of their potent antimicrobial activity. With the rise of antibiotic resistant bacteria, there is a demand for new antimicrobials. One option that is being pursued is the use of lantibiotics to treat bacterial infections. Lantibiotics undergo posttranslational modifications that are required for their antimicrobial activity. These modifications impart additional benefits including resistance to proteolytic degradation and stability at high temperatures. Most importantly, resistance to lantibiotics is more difficult to acquire because of their dual mechanism of action in killing bacteria, a combination of binding to lipid II and pore formation in the bacterial membrane. Further understanding of how these lantibiotic peptides function and their structures will be useful in the development of new antimicrobials.

1.3 Lantibiotics

Approximately 100 lantibiotics have been discovered and it is expected that this number will continue to grow as more microbial genomes are sequenced

and mined for lantibiotic associated genes. Lantibiotics undergo extensive posttranslational modifications that are required for their function as both an antimicrobial and auto-inducer. The following sections will explore the biosynthesis, regulation, structures, and mechanisms of self-immunity of some of the best-characterized lantibiotics.

1.3.1 Biosynthesis of lantibiotics

Lantibiotic loci can be found encoded on the chromosome, often as part of an integrative conjugative element (ICE) or on an extra chromosomal plasmid. This suggests that lantibiotic loci can be transmitted through horizontal gene transfer between strains and species. Lantibiotics are subject to extensive posttranslational modifications that result in the creation of lanthionine or methyllanthionine residues. The name lantibiotic arises from the presence of the unusual residue lanthionine combined with the antimicrobial properties of the lanthionine containing peptides, eg, **lanthionine-containing antibiotics** [6]. The nomenclature for generic lantibiotic genes uses *lan*, or a specific abbreviation of the lantibiotic, i.e. *nis* for nisin, followed by a letter to designate the function of the gene product. The lantibiotic is first translated into a prepeptide that is encoded by *lanA*. The prepeptide lantibiotic has an N-terminal leader sequence that is required for recognition by the modification machinery and for transport outside of the cell and a C-terminal propeptide that will be modified. Lantibiotics can be divided into three different classes depending on their modification machinery and function [7, 8].

Class I lantibiotics are modified by two separate modification enzymes encoded by *lanBC*. In the case of nisin, the prototypical lantibiotic produced by *Lactococcus lactis* and discovered in 1928, modification is catalyzed by NisB and NisC [9, 10]. Figure 1.1 is included as an overview of nisin biosynthesis. Nisin is encoded by *nisA* and is first translated into a prepeptide form of 57 amino acids. NisB is responsible for dehydration of serines and threonines that are found in the C terminal propeptide creating 2,3-didehydroalanine (Dha) or (Z)-2,3-didehydrobutyrine (Dhb), respectively. NisC catalyzes formation of thioether linkages of cysteines to either Dha or Dhb creating lanthionine (Lan) or methyllanthionine (MeLan) residues, respectively [11-14]. NisBC has been shown to form a complex at the membrane and interacts with dedicated ABC transporter, NisT [15]. Once transported outside of the cell, the leader sequence of the prenisin is cleaved by a membrane bound subtilisin-like serine protease, NisP, removing 23 amino acids from the N-terminus [16]. Not all *lan* systems encode a dedicated serine protease. In the case of subtilin, three different serine proteases, not encoded by the locus, were identified that could process subtilin [17]. After modification and cleavage of the leader sequence, nisin is able to function as an antimicrobial and autoinducer for the upregulation of the *nis* locus by interaction with its cognate two component system, NisKR [18]. It was determined that the N-terminus of fully modified nisin was required for interaction with NisK to initiate a signaling cascade involving the phosphorylation of NisR [16, 18, 19]. Phosphorylated NisR is able to bind to the promoter regions of *nisABTCIP*, *nisRK*, and *nisFEG* [18, 20, 21]. There is a transcriptional attenuator

located between *nisA* and *nisBTCIP* which allows for higher expression of *nisA* but allows some read through of the downstream genes encoding the biosynthetic machinery under basal conditions [21]. This arrangement likely ensures the correct ratio of prenisin peptides to biosynthetic machinery enzymes. In addition to being upregulated in response to exogenous nisin, *nisRK* is also expressed constitutively so that the cell can immediately respond to nisin allowing for the rapid upregulation of the genes encoding immunity proteins, *nisFEG* and *nisl* [22]. An internal promoter upstream of *nisl* within the operon *nisABTCIP* was recently discovered which contributes to a basal level of immunity, further protecting nisin producing cells from nisin mediated inhibition [23].

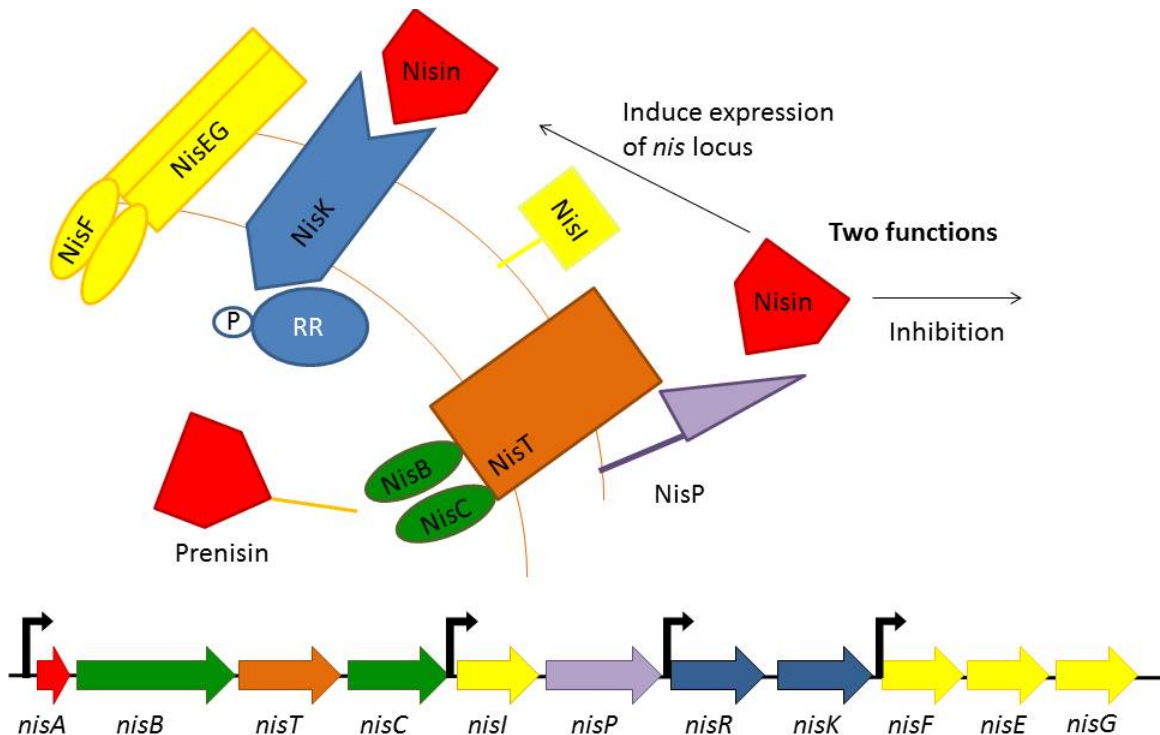


Figure 1.1 Biosynthesis of Nisin. A schematic of the nisin locus is shown above. An arrow above an open reading frame (ORF) depicts a promoter region. The promoters of *nisl* and *nisRK* are constitutively expressed. The other promoters are activated upon nisin exposure through NisRK signaling, creating a positive feedback loop as NisA is expressed. There is read-through allowing *nisABTCIP* to be transcribed on a single transcript. A transcriptional attenuator found in between *nisA* and *nisBTCIP* is present to allow for the correct stoichiometry of nisin to biosynthetic machinery. Nisin is first translated along with an N-terminal leader peptide that is needed for recognition by NisBC. After modification of NisBC, prenisin is secreted out of the cell by NisT. The leader peptide is cleaved by NisP resulting in the release of functional nisin which can either activate the *nis* locus in other *nis* positive strains or kill sensitive cells through lipid II binding and subsequent pore formation.

Class II lantibiotics combine the action of dehydration and cyclization into a single modification enzyme called LanM [24]. Another difference between class I and class II lantibiotics is the N-terminal cleavage event. Instead of the cleavage occurring through an outer membrane protease, in class II lantibiotics the dedicated ABC transporter, LanT carries out the cleavage. LanT contains an N-terminal peptidase domain that recognizes a conserved cleavage motif ending in double glycine, GA, or GS sequence. This sequence is cleaved concomitantly with secretion releasing the mature peptide [25, 26]. In the case of mersacidin, a class II lantibiotic produced by *Bacillus* sp., it was found that the regulation of mersacidin locus was under the control of a single response regulator, MrsR1[27]. Immunity to mersacidin was found to be under the control of the two component system MrsR2/K2 [27]. Although functional mersacidin is needed to induce expression of *mrsA*, it is unclear how MrsR1 is activated because it does not rely on MrsK2-dependent phosphorylation. This suggests that there might another histidine kinase not encoded by the locus that interacts with mersacidin

[27]. Two-peptide lantibiotics are members of the class II lantibiotics but their loci encode two separate LanM proteins that each modifies its own specific peptide [28, 29]. Two-peptide lantibiotics work synergistically to achieve an antimicrobial effect [30].

Class III lantibiotics, unlike class I and II, do not have antibacterial activity and will not be discussed further.

While the four different classes of lantibiotic synthetases create either Lan, MeLan, or other Lab structures, many lantibiotics contain alternative modifications that are catalyzed by other enzymes. Some of the structures include *S*-aminovinyl-*D*-cysteine (AviCys) or *S*-aminovinyl-3-methyl-*D*-cysteine (AviMeCys) which can be found on the lantibiotics epidermin and mersacidin, respectively, and are a result of either EpiD or MrsD catalysis, respectively [31-33]. Lantibiotics containing AviCys or AviMeCys have been found to be required for antimicrobial activity [34, 35]. Other modifications include the creation of a lysinoalanine bridge and a hydroxy-aspartate that are both found in the lantibiotic cinnamycin and duramycin [36, 37]. Formation of lysinoalanine and hydroxyl-aspartate was attributed to the action of Cinorf7 and CinX in cinnamycin [36]. Interestingly, the hydroxyl-aspartate plays a role in cinnamycin's ability to bind phosphatidylethanolamine (PE), which is different from the typical target, lipid II [38, 39]. Binding of PE prevents activation of phospholipase A2 that can lead to immunomodulatory effects on eukaryotic cells [40]. Duramycin, a lantibiotic that is structurally similar to cinnamycin, also has the same mechanism of action and is currently in phase II clinical trials for treatment of cystic fibrosis (CF) because it

was able to improve mucus clearance in CF patients. The mechanism of action likely affects membrane permeability of epithelial cells lining the airways that increases efflux of chloride ions, and reverses the defect in CF. [41-43].

1.3.2 Structure-Function Relationships

Lantibiotics are capable of inhibiting sensitive strains at nanomolar concentrations and this has been attributed to the lantibiotic's mechanism of action. Nisin, which is part of the type-A(I) classification class of lantibiotics based on structure, is cationic and linear [44]. It has a total of five rings, three located on the N-terminus named A-C, and two more rings on the C-terminus named D-E (Fig.1.2). The two domains are separated by a flexible hinge region. Rings A and B of nisin are able to bind to the pyrophosphate of lipid II, which differs from the binding site of vancomycin [45, 46]. Deletion of the N-terminal rings or even changing the Lan to MeLan in ring A is sufficient to abrogate lipid II binding. Lipid II serves an important role in transporting cell wall precursors from the cytoplasm to the peptidoglycan layer. Lipid II is made in limited amounts and therefore, binding of nisin sequesters lipid II and cell wall synthesis is arrested. In addition to sequestering lipid II, nisin through its flexible hinge is able to insert its C-terminus into the membrane forming pores, which depolarizes the membrane potential leading to rapid cell lysis [47]. Decreasing the length of the hinge region in nisin from three amino acids to one was sufficient to abrogate its ability to form pores but lipid II binding was unaffected [48]. However, random mutagenesis yielding smaller sized amino acids such as alanine in the hinge region could enhance antimicrobial effect of either nisin A or nisin Z, presumably by increasing

accessibility of the C-terminus to the membrane and decreasing steric hindrance [49, 50]. Because of nisin's dual mechanism of action, resistance to nisin is more difficult to acquire and has been used by the food industry for over 50 years.

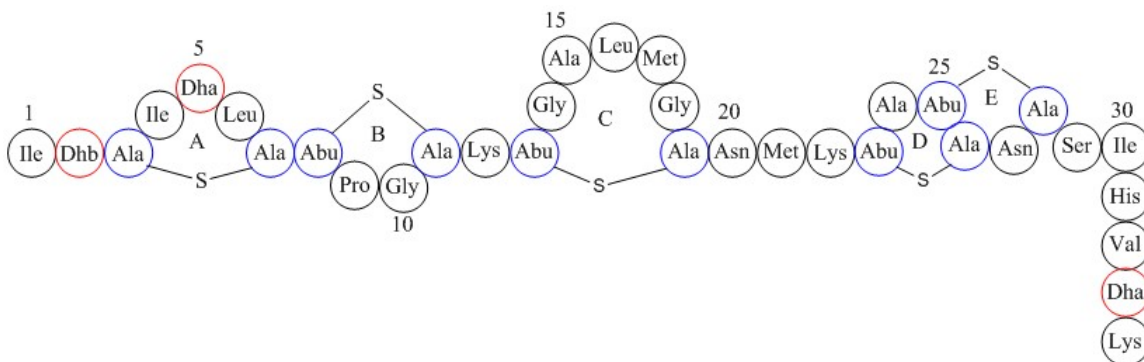


Figure 1.2. Structure of Nisin. Nisin is a linear, cationic lantibiotic peptide. Functional nisin is 34 amino acids in length. Rings A-E are labeled in the above figure. Rings A and B are involved in lipid II binding and rings D and E are involved in pore formation. The hinge region is located from amino acid 20-22. Dehydroalanine and dehydrobutyrine are represented by the symbol Dha and Dhb, respectively, and are depicted in blue circles. Lanthionine (Ala-S-Ala) and methylanthionine (Abu-S-Ala) rings are represented in blue.

Mersacidin, a type-B lantibiotic, is globular in shape consisting of four rings (A-D) and does not have a net charge [35, 51]. Mersacidin is able to bind lipid II via its ring C which is conserved in other mersacidin like lantibiotics but does not form pores like nisin and other flexible, cationic lantibiotics, most likely because it does not have a hinge region connecting a positively charged C-terminus [52-54]. Through site directed mutagenesis, it was found that replacement of threonine residues involved in ring formation with serines in mersacidin abrogated lantibiotic production either because it cannot be properly modified or processed or because the resulting structure could not function as an autoinducer [55]. It was also found that the structure of ring B in mersacidin was most important for activity as insertions and deletions were not tolerated yet

amino acid substitutions could be made [55]. Overall, Appleyard et al. concluded that predictions of the activity of lantibiotics based on amino acid changes are hard to generalize [55].

Whereas the lantibiotics of structure class A and B have been single peptide lantibiotics, there are cases of multi-peptide lantibiotics. Two peptide lantibiotics work synergistically to bring about antimicrobial activity, which includes binding to lipid II and formation of pores in most cases [56]. The two peptides (Lan α and Lan β) tend to have very limited sequence identity and each peptide undergoes modification by a specific LanM protein encoded by *lanM1* and *lanM2*. After modification, the structures of Lan α and Lan β are different [28, 57]. Lan α tends to have a globular structure similar to mersacidin while lan β is flexible and linear [30, 58]. It was shown in the case of lactacin 3147 that Ltn α binds to lipid II, which then recruits Ltn β causing pore formation [30].

Enterococcal cytolysins represent a unique approach to lantibiotic-mediated inhibition that includes the ability to sense the presence of sensitive cells. A unique property of cytolysin is that it is able to inhibit both eukaryotic and prokaryotic cells. Unlike other examples of two peptide lantibiotics, cytolysin, has only one modification enzyme that modifies both CylL_L and CylL_S [59, 60]. Interestingly, after a secondary cleavage event by CylA, the two peptides, CylL_L" and CylL_S" , play different roles [60, 61] . In the absence of target cells, CylL_L" and CylL_S" form an inactive complex and cytolysin expression is not upregulated in *Enterococcus faecalis* [62]. However, in the presence of eukaryotic cells,

Cyl_L" has a higher affinity for binding to eukaryotic membranes, freeing up Cyl_S" which then is able to relieve derepression of the cytolysin locus [62].

1.3.3 Mechanism of Self-Immunity

In addition to encoding the genes for the structural peptide, a lantibiotic producing strain must also express genes required for protection against the effect of its own lantibiotic. Two mechanisms of immunity exist in most lan systems encoded by the *lanFEG* and *lanI* genes. The systems that protect nisin producing strains from their own peptide have been best studied and some version of these strategies are used by all previously reported lantibiotic systems.

NisFEG is an immunity ABC transporter that confers protection against nisin [63]. NisF is the nucleotide binding domain (NBD) of the ABC transporter that provides energy to the transporter by binding and hydrolyzing ATP as it contains the characteristic motifs found in other ATPases such as Walker A, Walker B, E-loop, and the H-loop as well as the C- and D-loops that are specifically found in some ABC transporters [64-67]. This E-loop was found to be important for immunity as mutating this glutamic acid residue resulted in little to no immunity [67]. This E-loop may be important in communication between NisF and the transmembrane domains (TMDs), NisE and NisG, to activate transport; the Q-loop was found to have this role in non-immunity transporters [67, 68]. NisE and NisG make up the heterodimer that forms the permease component of the transporter. Deletion of any single component of the ABC transporter disrupts immunity [63]. NisFEG is able to export nisin from the membrane into the environment without altering or destroying the peptide.

NisI is a two domain protein consisting of 245 amino acid residues that is lipid anchored at its N-terminus to the outside of the cell membrane [69]. Some NisI escapes membrane attachment and is secreted into the environment [70]. Secreted NisI is able to protect the cell from nisin mediated killing by binding to nisin without modifying or degrading the molecule [22, 70-73]. The C-terminus of NisI seems to play a role in the protective effect of NisI as it was shown that deleting up to 22 amino acids from the C-terminus decreased immunity and could not prevent nisin mediated pore formation [73, 74]. To verify that this phenotype was not attributed to an improper folding of NisI, a chimera immunity protein was created fusing the C-terminus of NisI to the N-terminus of a different lantibiotic immunity protein, Spal, which confers protection against the lantibiotic subtilin. Although the structures of subtilin and nisin are very similar, the immunity proteins are very specific and Spal does not protect against nisin. The chimera immunity protein with the C-terminus of NisI was found to protect against nisin [74]. This suggests that the C-terminus of NisI is able to inhibit pore formation mediated by nisin although the exact mechanism is unclear.

Nisin binding directly to NisI is unlikely to represent the only mechanism for protection. It was recently found that when *L. lactis* was exposed to high concentrations of nisin that would saturate NisI binding, *L. lactis* was still protected from nisin-, mediated inhibition in a NisI dependent manner. AlKhatib et al. propose that the C-terminus of NisI might be able to bind to lipid II, preventing nisin from binding and inhibiting subsequent pore formation. Additionally, *L. lactis* expressing either NisI or NisI with the 22 amino acids deleted from the C-

terminus important for binding to nisin, were shown to induce long chains of bacteria at high concentrations of nisin [73]. As nisin concentrations decrease, the phenotype was restored to wildtype (WT). Although the exact mechanism of how long chain formation impacts immunity or how NisI induces this morphological change is unknown.

NisI and NisFEG are believed to function cooperatively to provide full immunity. A strain containing a deletion of either immunity protein was shown to have only 10-30% of full immunity. It appears that NisI contributes quantitatively more to immunity than NisFEG [72, 75]. Although some lantibiotics like the mersacidin locus only encode MrsFGE, it is believed that lantibiotics capable of binding to lipid II and forming pores require the actions of both immunity proteins [27, 76].

1.4 *Streptococcus pneumoniae*

S. pneumoniae, a Gram-positive bacteria, is a common colonizer of the human nasopharynx [77]. It has been shown that colonization by pneumococcus happens early in life and often with more than one capsule type or serotype [78]. By adulthood, carriage decreases, therefore, young children represent the major pneumococcal reservoir [79]. Although, pneumococcus is a commensal, it has the ability to transition to a pathogenic form. Once it gains access to other sites in the body, pneumococcus is able to cause a variety of diseases ranging from otitis media to more severe, invasive diseases such as pneumonia, meningitis, and bacteremia [80]. Worldwide it has been estimated that about 1.6 million people

die from pneumococcal infections [81]. In the United States, about 4 million people have a pneumococcal related illness each year and 22,000 of those infections result in death [82]. Of the 4 million, 1.2 million of those cases are caused by drug resistant pneumococcus [82].

Currently, there are vaccines to protect against specific serotypes of pneumococcus that are known to cause severe disease. Although these vaccines have proven effective at preventing pneumococcal disease in susceptible populations, the use of these vaccines has altered the composition of colonizing pneumococcal populations to include less common serotypes. Many of these replacement serotypes are capable of causing severe infections [83, 84]. The reason for this is attributed to the ability of pneumococcus to evade the immune system by undergoing capsule switching along with the high pre-existing diversity in capsule type [85].

Antibiotics can be used to treat pneumococcal infections. However, the continued use of penicillin, which was historically the first drug of choice, resulted in the rapid emergence of penicillin resistant strains [86]. Although the introduction of vaccines has eliminated certain antibiotic resistant serotypes, antibiotic resistance continues to be a problem and further complicates treatment [87-89].

Studying pneumococcal competition is important not only for the identification of bacteriocins which may be used as a future therapeutic, but also for identifying factors that are important for successful colonization. By knowing what factors are important for colonization, specifically through competitive

interactions mediated by the secretion of bacteriocins, we can predict which pneumococcal strain will be gain prominence in a population and target those specific strains. The following sections will examine what is known about pneumococcal bacteriocins, especially lantibiotics.

1.4.1. *Pneumococcal Bacteriocins*

S. pneumoniae is known to secrete bacteriocins that mediate competition in the human nasopharynx [90]. The **bacteriocin- like peptide** (*blp*) locus is found in all sequenced pneumococcal strains and contains not only the genes encoding the bacteriocins but also genes required for transport, processing, regulation, and immunity [91, 92]. The Blp bacteriocins are small, unmodified peptides that contain an N-terminal leader peptide that directs the Blp bacteriocins to BlpAB. BlpAB is an ABC transporter complex that contains a peptidase domain that cleaves off the leader peptide upon secretion [93]. After cleavage and secretion, the Blp bacteriocins are functional and can inhibit strains that lack the corresponding immunity proteins [94]. Expression of the *blp*-associated genes is upregulated when the signaling pheromone, encoded by *blpC* interacts with the two component system, BlpRH [92]. Expression of *blpC* itself is also upregulated in response to BlpC signaling, which creates a positive feedback loop [92].

Another pneumococcal bacteriocin is the **competence induced bacteriocin** (Cib). Competence is the ability of bacteria to uptake exogenous DNA and integrate into the genome. Pneumococcal competence is controlled by a signaling pheromone named **competence stimulating peptide** (CSP) which interacts with its cognate histidine kinase, ComD, to upregulate genes necessary

for competence development [95] The genes upregulated through CSP-mediated signaling include, the two peptide bacteriocin, CibAB. CibAB has been shown to play a role in competence-mediated fratricide, resulting in autolysis of noncompetent cells [96].

Several putative lantibiotic loci have been identified in pneumococcal genomes although no antimicrobial activity has been attributed to these clusters [97-100]. Some loci have been shown to have antimicrobial function when expressed under heterologous conditions, for example, heterologous expression of a two-peptide lantibiotic, PneA1 and PneA2, derived from a sequence found in the pneumococcal strain, D39, was able to undergo modification using the nisin biosynthetic enzymes, NisBC [101]. Modification by NisBC was successful upon replacement of PneA's leader peptide sequence with that of nisin [101]. However, the two peptides did not act synergistically when combined and the resultant peptide was only found to have antimicrobial activity against *Micrococcus flavus* [101]. The modifications generated by NisBC may not reflect the true structure of PneA. This is an interesting method to study the function of lantibiotics that lack antimicrobial activity in their native backgrounds [101]. Recently, the same locus was found to be under the control of a quorum sensing system identified as TprA/PhrA and was upregulated in the presence of galactose but repressed under high glucose growth conditions [100]. Interestingly, the nasopharynx is high in galactose but low in glucose suggesting that the locus may be active on this surface. It is not clear whether the peptides that may be produced by this locus under high galactose conditions have

inhibitory activity as the authors in this study did not demonstrate any antimicrobial function [100]

Some lantibiotic loci that have been identified in pneumococcus have been located on integrative and conjugative elements (ICE). ICE are acquired by pneumococcal strains because they harbor genes with beneficial properties such as antibiotic resistance genes [102]. Pneumococcal strain ATCC700669 was found to contain a lantibiotic locus on an ICE termed ICES_{p23FST81} [97]. In addition to the lantibiotic locus, other cargo genes that code for antibiotic resistance and DNA repair enzymes were found which might explain the prevalence of this ICE in pneumococcal genomes [97]. Notably, pneumococcal strain ATCC700669 is a member of the serotype 23F sequence type 81 lineage, a pandemic strain, suggesting that having an ICES_{p23FST81} may have contributed to its fitness and transmission, although antimicrobial activity was not observed from its lantibiotic locus [97].

To summarize, lantibiotic loci in pneumococcus have been identified although antimicrobial activity has not been described for any of them. Two lantibiotic loci located on ICE have been disseminated in the pneumococcal population indicating these lantibiotic loci may be under positive selective pressures. The contribution of these lantibiotic loci to competitive dynamics and colonization is unknown but may represent an additional strategy to compete with other bacteria in the nasopharynx. My dissertation research examines the first functional lantibiotic locus expressed in a clinical isolate of *Streptococcus pneumoniae*, P174. This locus encodes the named lantibiotic, pneumolancidin

(pld) that demonstrates broad-spectrum antimicrobial activity. Understanding lantibiotic structure, function and regulation and will provide further insight into how bacterial metabolites can shape a host-associated microbial community.

1.6 Outline of the Thesis

The central goal of this thesis is to characterize the pneumolancidin (*pld*) locus in *Streptococcus pneumoniae*. The *pld* locus is noteworthy not only because of the broad spectrum of inhibition associated with it, but also the presence of four highly homologous lantibiotic peptides. Lantibiotic peptides are known to have two roles, acting as both a growth inhibitor for some cells and an auto-inducer. In Chapter I, I dissect the role of each Pld peptide as it relates to growth inhibition, locus upregulation, and immunity using a genetic approach. I also characterized the role of each gene in the *pld* locus. Additionally, I uncovered the reason why some strains that possess the *pld* locus are nonfunctional. Finally, I examined the biological significance of having a functional *pld* locus using a staggered colonization mouse model. In Chapter II, a biochemical approach was employed to assess the role of purified Pld peptides. PldA1 and PldA3 were successfully purified and their bioactivities were determined. The complete structural elucidation of PldA1 and PldA3 was also achieved. In Chapter IV, I discuss the significance of my thesis research, and the future directions needed to address near-term and longer-term questions.

CHAPTER II

CHARACTERIZATION OF A MULTI-PEPTIDE LANTIBIOTIC LOCUS IN *STREPTOCOCCUS PNEUMONIAE*

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Abstract

Bacterial communities are established through a combination of cooperative and antagonistic interactions between the inhabitants. Competitive interactions often involve the production of antimicrobial substances including bacteriocins, which are small antimicrobial peptides that target other community members. Despite the nearly ubiquitous presence of bacteriocin encoding loci, antimicrobial activity has only been attributed to a small fraction of gene clusters. In this study, we characterize a novel locus in the pathogen *Streptococcus pneumoniae* that drives the production of a bacteriocin called pneumolancidin (pld) with broad antimicrobial activity. The locus encodes an unusual tandem array of four inhibitory peptides, three of which are absolutely required for antibacterial activity. The three peptide sequences are similar, but appear to play distinct roles in regulation and inhibition. A modification enzyme typically found in loci encoding a class of highly modified bacteriocins called lantibiotics was required for inhibitory activity. The production of pneumolancidin is controlled by a two

component regulatory system that is activated by the accumulation of modified peptides. The locus is located on a mobile element that has been found in many pneumococcal lineages although not all elements carry the *pld* genes. Intriguingly, a minimal region encoding only the genes required for pneumolancidin immunity was found in several *Streptococcus mitis* strains. The pneumolancidin producing strain can inhibit nearly all pneumococci tested to date and provided a competitive advantage *in vivo*. These peptides not only represent a unique strategy for bacterial competition but are also an important resource to guide the development of new antimicrobials.

2.1 Introduction

Streptococcus pneumoniae is a common colonizer of the human nasopharynx, a highly diverse polymicrobial environment [103-105]. Bacterial competition between members of the microbiome is often mediated by antimicrobial peptides called bacteriocins. In pneumococci, competition is enhanced in strains with a functional bacteriocin locus during nasal colonization of the mouse [90]. Bacteriocin production in pneumococcus is controlled by the *blp* locus, which has been identified in all sequenced strains [90, 93, 106]. Most pneumococcal genomes also contain the *cib* locus which encodes a highly conserved two peptide bacteriocin, CibAB, that has been shown to play a partial role in competence mediated fratricide resulting in autolysis of noncompetent cells [96]. Both the *blp* and *cib* loci are predicted to encode Class II, or unmodified peptide bacteriocins.

Lantibiotics are a specific class of bacteriocins that are characterized by their extensive posttranslational modifications. Secretion of functional lantibiotics has not been described in pneumococcus. Precursor lantibiotic peptides are modified intracellularly by one or more enzymes encoded by genes contained within the specific *lan* locus. The most common modification involves the dehydration of serines and threonines and through a thioether linkage, cyclization of the dehydrated amino acids to cysteines creating either lanthionine or methyllanthionine, respectively [11-14, 24]. Following modification, the pre-lantibiotic is transported out of the cell by a dedicated ABC transporter that recognizes a conserved signal sequence [14]. The signal peptide is either cleaved concomitantly with secretion by the transporter, or by an alternative protease [16]. Cleavage of the signal sequence renders the lantibiotic active and able to exert an antimicrobial effect on susceptible cells by either binding to lipid II and blocking cell wall synthesis and/or forming pores [107]. This dual mechanism of action can be combined in a single lantibiotic peptide or split among two peptides. Modification of a two-peptide lantibiotic typically requires two LanM, each one specific for one peptide [28, 57, 108-110]. Self-immunity to lantibiotics can occur through expression of a lipoprotein that is thought to competitively bind to the lantibiotic or through production of an efflux ABC transporter. Some loci encode for both immunity strategies, in these cases partial immunity defects are noted when one gene is deleted. In most cases, the lantibiotics also function as signaling peptides and interact with a cognate histidine kinase, LanK. Binding of the lantibiotic to the histidine kinase results in

upregulation of the locus via a two component regulatory cascade [18, 111, 112]. Some potential lantibiotic encoding clusters have been described in pneumococcus, however, no activity has been attributed to these loci. Some members of the pandemic pneumococcal strain, Spain23F sequence type 81 lineage, that at one point was found to be responsible for 40% of pneumococcal disease in America, carry a locus that appears to have all required elements for lantibiotic expression, but no inhibitory activity has been detected in strains carrying this locus [97, 98]. In this study, we report the identification of the first functional lantibiotic locus in pneumococcus. The activity derived from this locus inhibits a significant number of pneumococci and other Gram-positive pathogens. It requires the concerted action of three similar but distinct structural peptides, all of which appear to be modified by the same LanM enzyme. To our knowledge, the requirement for three homologous peptides for full inhibitory activity of a lantibiotic has not been described previously and represents a unique approach to bacterial antagonism.

2.2 Results

2.2.1 Identification of a functional lantibiotic locus in P174

During previous work on the *blp* locus, we noted that an invasive serotype 23F isolate derived from a patient in South Africa had significant antibacterial activity against all but one pneumococcal isolate despite containing an inactivating mutation in the *blp* bacteriocin locus [93]. Deletion of the upstream regulator of the *cib* or *blp* bacteriocins (*comDE* or *blpC*, respectively) did not abrogate inhibitory activity suggesting that the inhibitory activity was derived from

a previously undescribed locus (Fig. 2.1A). Transposon mutagenesis localized the activity to a locus predicted to encode a series of lantibiotic peptides.

Fig.2.1. Inhibitory activity and genetic structure of the *pld* lantibiotic locus.

A) Overlay assays were performed using either P174 wildtype or deletion mutants of either upstream regulators of the *blp* or *cib* locus or a deletion of the *pldM* gene that was identified in the transposon mutagenesis screen. A TIGR4 strain was used as the overlay strain. B) The *pld* locus of P174 and the corresponding locus of *S. mitis*, and *S. pneumoniae* ATCC700669 are shown above. The percentage of amino acid identity between the predicted proteins found in *S. mitis* B6 and P174 homologues is noted above the B6 ORFs. Presumed functional designations are indicated by the color of the ORF. Regions of DNA homology between sequences are shown as grey background. C) Amino acid alignment demonstrating the homology between predicted structural proteins PldA1-4. The proposed signal peptide sequence cleavage point is shown with a vertical arrow. Shared amino acid residues in the functional peptide are highlighted in yellow. Amino acid residues in red indicate sites of possible modification catalyzed by PldM. D) Deletions of various genes in the *pld* locus of P174 were constructed and assayed for inhibitory and signaling activity as well as immunity to WT lantibiotic. Inhibition and signal secretion were tested by stabbing the strain of interest and overlaying with the sensitive indicator strain, TIGR4 or the reporter strain P174 *pldM-lacZ*, respectively. The chromogenic substrate, X-gal was included in the overlay mixture for signaling assay. Immunity was determined by stabbing P174 and overlaying with each of the deletion mutants.

A schematic of the locus is shown in Fig. 2.1B. We chose to designate this lantibiotic locus as **pneumolancidin** and the corresponding genes abbreviated to *pld* per the standards of nomenclature for lantibiotics. A nucleotide BLASTn of the *pld* locus found in P174 demonstrated that a homologous gene cluster is found in a pneumococcal strain designated PN1, isolated from Papua New Guinea in the 1970's [99]. The locus encodes two ABC transporters, the 5'-most genes consist of two ORFs, *pldFE* that encode an ATP binding protein and a permease, respectively. These genes share 99% amino acid identity with an ABC transporter identified in PN1 and 97% similarity with a transporter encoded in the *Streptococcus mitis* B6 genome. Based on conserved domains PldF

serves as the nucleotide binding domain of an immunity ABC transporter. Although it does not contain a typical E-loop which is conserved in all LanF proteins of immunity ABC transporters, we chose to name the genes *pldFE* based on the location of these genes in the locus, homology, and likely immunity function [67]. The proteins also share homology to bacitracin ABC transporters found in a number of streptococcal species that provide resistance to bacitracin and can provide cross resistance to heterologous lantibiotics such as nisin and gallidermin [113-115]. A second ABC transporter, *pldT*, was identified in the 3' region of the locus. The second ABC transporter shared 31% identity at the amino acid level to the mersacidin ABC transporter, MrsT, in *Bacillus sp. and contains conserved domains involved in lantibiotic export and cleavage*. The only modification enzyme found in the locus is encoded by the *pldM* gene. PldM is a member of the LanM family of modification enzymes. The *pld* locus contains coding sequences for a two component regulatory system, *pldKR*, which is homologous to genes found in the *S. mitis* B6 strain. Homologs of *S. mitis* B6 *pldKR* are adjacent to the immunity *pldFE* homologs, but the surrounding region lacks the remainder of the pneumococcal *pld* locus (Fig. 2.1B). Flanking either end of the *pld* locus in P174 are regions of significant homology to the ICE element found in the *S. pneumoniae* strain ATCC 700669. This strain has a lantibiotic locus in the precise location of the *pld* locus although the genes in the two loci share no homology (Fig 2.1B).

A region encoding the putative structural proteins was identified upstream of *pldFE*. A tandem array of four possible ORFs were identified (*pldA1-4*), each

encoding a peptide with a signal sequence followed by a sequence with a large number of serines, threonines and cysteines typically found in lantibiotic peptides. The predicted Pld peptides do not share homology to known lantibiotic peptides. The four ORFs are homologous to each other and the N-terminal leader sequence of each peptide is followed by a double glycine, GA, GS, or double alanine motif suggesting the point of peptidase cleavage. The active peptides of PldA1 and PldA2 would be predicted to differ by only two amino acids (Fig. 2.1C).

2.2.2 Identification of the genes required for lantibiotic activity and immunity.

To determine the role of each gene in the *pld* locus of P174, individual deletions were constructed and assayed for inhibition and immunity to the P174 secreted lantibiotic. Deletions in *pldM*, *pldT* and *pldK*, encoding the modification enzyme, lantibiotic transporter, and histidine kinase, respectively all resulted in loss of inhibition (Fig. 2.1D). When tested for immunity, the *pldM* and *pldT* deletion strains retained immunity to P174 while the *pldK* mutant lost immunity confirming the requirement for the regulatory proteins for activation of lantibiotic immunity. To determine the role of *pldFE*, an inframe, unmarked mutation of both genes was introduced into P174. This mutant, P174 Δ *pldFE*, lacked inhibition in overlay assays and was sensitive to WT secreted lantibiotic peptide, suggesting that this ABC transporter is required to initiate immunity or is directly involved in protection from its own lantibiotic (Fig. 2.1D). As expected, Pld peptides are required for inhibition but not for immunity since a strain carrying a deletion of all 4 peptides had loss of inhibition but retained immunity (Fig. 2.1D). P174 displays an

interesting self-inhibitory phenotype in which the formation of a halo of decreased density of growth can be seen when P174 is plated against itself, suggesting that there is a lapse in the development of immunity in this assay which allows for some degree of self-inhibition in the presence of functional lantibiotic peptides. This phenomenon is also seen when P174 is grown as a single strain lawn where it is noted to form occasional plaque-like structures that are characterized by areas of partial clearing. Although the mechanism for the development of plaques is unknown, we hypothesize that these are areas in which the *pld* locus is spontaneously activated in a portion of the population, resulting in the inhibition of any neighboring cells that are delayed in their production of immunity.

2.2.3 Modified lantibiotic peptides are required for activation of the locus.

In addition to their inhibitory activity, lantibiotic peptides also often serve as inducers by interaction with the cognate histidine kinase leading to upregulation of the entire *lan* locus. Upregulation of the locus is dependent upon the concentration of the lantibiotic peptides. Similar to other lantibiotic loci, we found that the Pld peptides are required for activation of transcription of the *pld* locus. We compared the activation of a *pld* reporter construct to the signal secreted by P174 in the P174 and P174 $\Delta pldA1-4$ background (Fig. A1). The reporter strain in the wildtype background produced a large zone of signaling when plated over a peptide secreting strain, while only a small zone was seen when the reporter in the P174 $\Delta pldA1-4$ background was tested. Because the stabbed strain secretes the same amount of peptide in each overlay, this result suggests that propagation of the signal within the overlay results in a larger activation zone and

further suggests that this propagation requires the lantibiotic peptides. Similarly, P174 $\Delta pldA1-4$ does not secrete a signal that can stimulate the reporter strain P174 $pldM-lacZ$, further supporting the role of Pld peptides as inducers of the *pld* locus (Fig. 2.1D). To determine if the Pld peptides need to be modified and secreted to act as inducers, P174 $\Delta pldM$ or P174 $\Delta pldT$ were stabbed and overlaid with the WT reporter (Fig. 2.1D). Stimulation of the *pld* locus was not seen in either overlay confirming that the induction signal of the locus is an extracellular, modified peptide. As expected, the P174 $\Delta pldK$ strain, which lacks the histidine kinase regulator was not able to secrete a signal, consistent with a loss of both inhibition and immunity in this strain (Fig. 2.1D). Surprisingly, the strain carrying the unmarked *pldFE* mutation, missing the genes that are proposed to play a role in immunity, also did not secrete a signal despite retaining the genes encoding the peptides and the regulatory, modification and secretion proteins (Fig. 2.1D). This may be due to development of a compensatory mutation that prevents the activation of the locus in the setting of reduced immunity, or because the immunity transporter plays an undefined role in regulation. We attempted to evaluate the kinetics of activation of the locus in broth culture using the *pldM-lacZ* reporter strain, but this strain failed to show any induction of the locus during growth in broth, presumably because the concentration of peptides never reaches the level required to support activation of the locus under these conditions (data not shown).

Given the unusual presence of four homologous but not identical putative functional peptides, we wanted to determine the function of each peptide

individually in inhibition and signaling. Individual, in frame, unmarked peptide deletions were tested for inhibition, immunity and evidence of signal secretion (Fig. 2.2). P174 lacking *pldA1*, *pldA2*, or *pldA3* had identical phenotypes in that all three deletion strains had a loss of inhibitory activity and signal secretion (Fig. 2.2A), suggesting that all three peptides are required for activation of the locus. These strains had nearly wildtype levels of immunity to P174, with only a small zone of clearing that most likely represents some degree of delayed production of immunity. P174 Δ *pldA4* was fully inhibitory and secreted a signal that was indistinguishable from P174 suggesting that this gene is dispensable for inhibition (Fig. 2.2A).

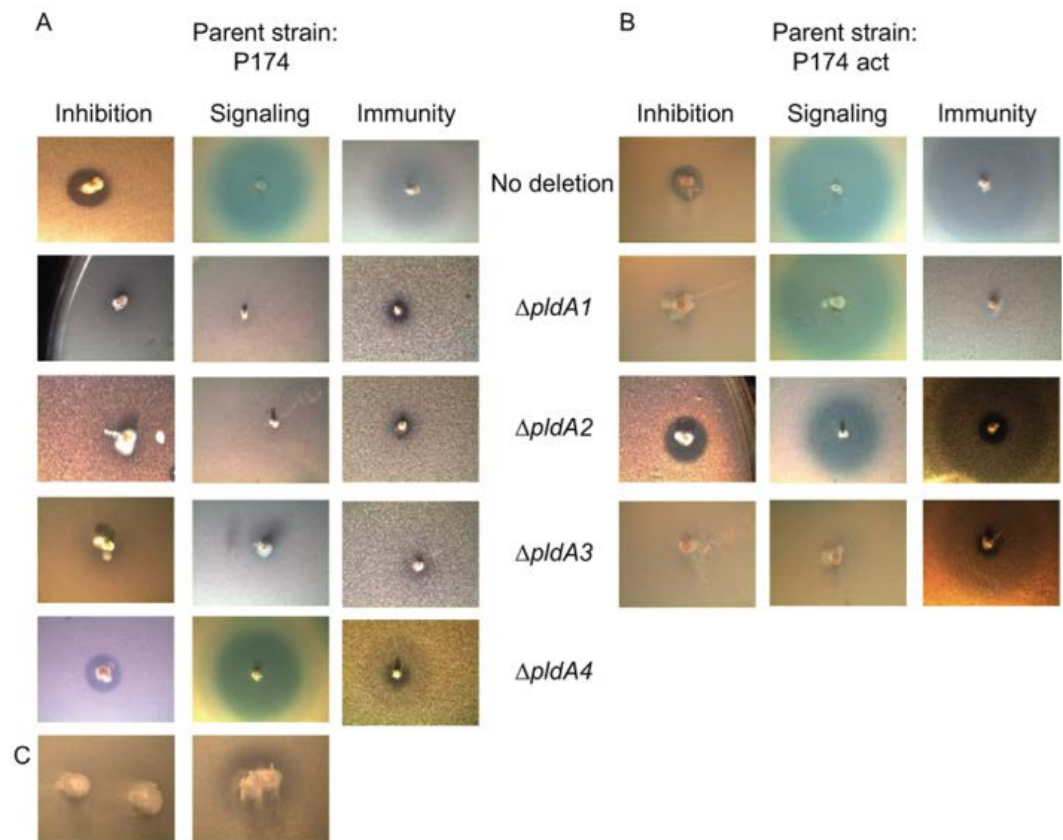


Figure 2.2. Deletion of the lantibiotic peptide in either P174 or the hyperinducible P174act background. In frame, unmarked peptide deletion

mutants were constructed and assayed for inhibitory and signaling activity as well as immunity to WT lantibiotic. Evidence of inhibition and signal secretion were tested by stabbing the strain of interest and overlaying with the sensitive indicator strain, TIGR4, or the reporter strain P174 *pldM-lacZ*, respectively. Immunity was determined by stabbing P174 and overlaying with each of the deletion mutants. Peptide deletion mutants that were made in either the P174 background (A) or the P174act background (B). C) Phenotypic complementation was assayed using P174act Δ *pldA3* and P174act Δ *pldA1*. Both strains were stabbed progressively more closely to each other and the plate subsequently overlaid with TIGR4. Pictures were taken at a higher magnification (2x) than other overlays to better appreciate the inhibitory effect.

2.2.4 Hyperinducible strain of P174 has a decreased threshold for locus activation.

Surprisingly, we found that certain isolates derived from allelic exchange of the counter selectable Janus cassette with a cloned copy of the peptide region that was being used for constructing peptide deletions, had an altered lantibiotic production phenotype which we have designated as P174act. When the *pldM* reporter plasmid was integrated into this strain, it exhibited evidence of greater *pldM* transcription in overlay assays when stimulated with P174 and produced a large number of plaque-like formations in the overlay lawn when compared with the reporter in the wildtype background (Fig.2.3A). When P174act was stabbed into plates, there was no significant difference in inhibition or signaling compared with the wildtype strain (Fig. 2.2A & B). To determine the mutation responsible for the hyperinducible phenotype of P174act, we sequenced the entire fragment that was used for allelic exchange. Sequencing revealed a single base pair mutation presumably attributable to a PCR error that was located in the intergenic region between *pldA4* and *pldFE* (Fig. 2.3B). The mutation was found in the cloned fragment used for transformation and, using linkage analysis on transformants

that resulted from allelic exchange with the plasmid carrying the mutation, was absolutely linked to the hyperinducible phenotype (data not shown). The mutation resulted in a change of a thymidine to a cytosine at the first T of the proposed TATA box preceding *pldFE* (Fig. 2.3B). Because this mutation was in an intergenic region downstream of the peptide ORFs, we reasoned that this single base pair change was affecting transcription of key genes in the locus by either disrupting a transcriptional attenuator (allowing for increased read-through) or affecting a promoter element (in particular by altering RNA polymerase binding at the -10 region) either of which might result in changes in downstream gene expression. DNA analysis failed to demonstrate any sequences likely to form a stem-loop typical of a transcriptional attenuator. To further examine this, a 4bp deletion that included the site of the mutation was constructed in the P174 and both the inhibition and immunity phenotype of the resulting strain was assessed (Fig.2.3C). The strain carrying this deletion lost both inhibition and immunity making the presence of a transcriptional terminator at this site unlikely and suggesting that the mutation that results in the act phenotype was affecting the activity of a promoter element preceding *pldEF*.

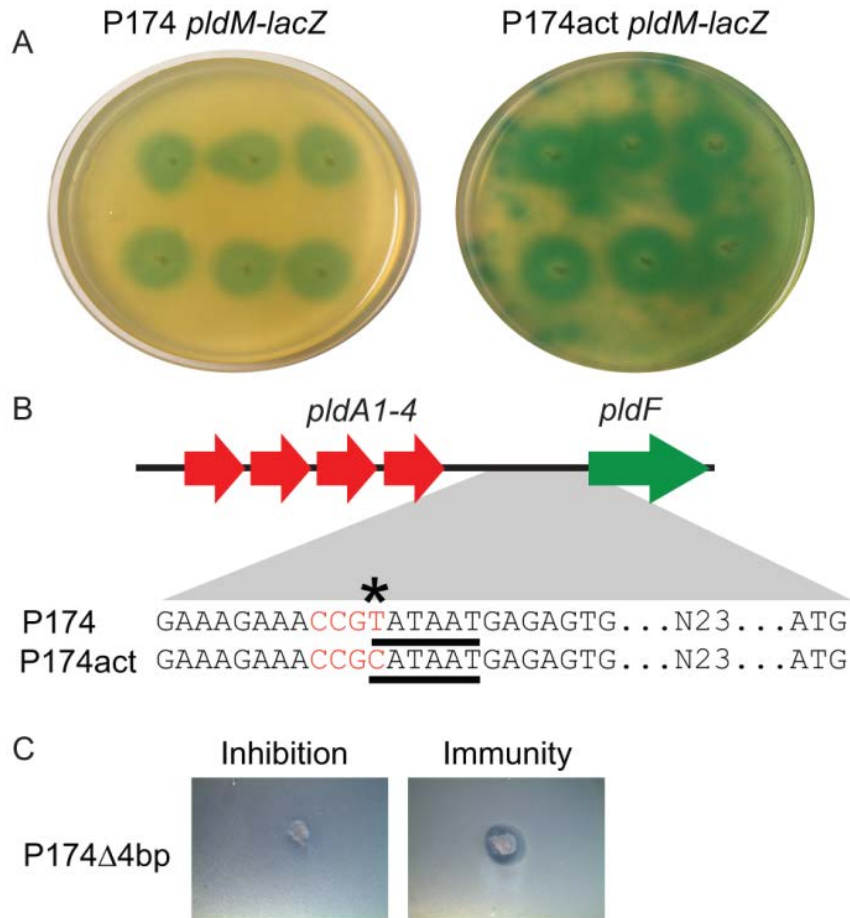


Figure 2.3. A hyperinducible strain contains a mutation that is affecting the promoter upstream of *pldFE*. A) Response to exogenous peptides was tested in either P174 *pldM-lacZ* or P174act *pldM-lacZ*. P174 was stabbed multiple times into TS plates and overlaid with either reporter. B) Location of the single base pair mutation resulting in the hyperinducible phenotype in the intergenic region between *pldA4* and *pldFE*. The site of the mutation is marked by an asterisk. The 4 bp region shown in red was deleted in strain P174Δ4bp. The proposed TATA box preceding the *pldF* ORF is underlined. The distance to the start codon of *pldF* as denoted by N. C) Overlay assays assessing inhibition and immunity phenotype of the 4bp deletion that included the site of the activating mutation. For inhibition, the P174Δ4bp strain was stabbed and overlaid with TIGR4. For immunity, P174 was stabbed onto a TS plate and overlaid with the P174Δ4bp strain.

2.2.5 Genes involved in immunity, regulation and peptide modification are not part of an operon.

We attempted to determine the transcriptional units of the locus and to compare RNA levels in wildtype and hyperinducible backgrounds using both RT-PCR and northern blotting, however, transcripts could not be detected in broth grown organisms and RNA isolated from plate grown organisms was too degraded for use in RT experiments (data not shown). As an alternative approach to determining minimal transcriptional units and relative activity of various genes in the *pld* locus, we constructed three additional reporters in either the P174 or P174act background through plasmid integration in which the reporter gene *lacZ* was fused to the region upstream of either *pldK*, *pldFE*, or *pldA1*. All constructed reporter strains were tested for inhibition and signal secretion. All of the fusion constructs retained the wildtype inhibitory phenotype (Fig.A2a) demonstrating that insertion of the reporter plasmid at either of these locations did not disrupt the function of the locus. Like the *pldM* reporter, the *pldFE* reporter demonstrated signal detection when overlaid over P174. The *pldA1-lacZ* fusion in either background was upregulated in response to exogenous peptides indicating that the peptides are autoregulated (Fig.A2a). Although the *pldK* reporter can secrete wildtype levels of peptide by signal secretion assay and inhibits the TIGR4 strain in overlay assays, this reporter in either P174 or P174act background was not activated by P174 when used in an overlay assays (Fig.A2b). This indicates that the genes involved in regulation are not inducible with exogenous peptides and that the *pldFE*, *pldKR* and *pldM* genes are all controlled by separate promoters since the peptide inducible promoters of *pldFE* and *pldM* flank either side of the non-induced *pldFE* genes (Fig. 2.1B). The lack of appreciable *lacZ* expression in

the *pldKR* reporter suggests that the regulatory genes are produced at very low levels and are not part of the positive feedback regulation in response to secreted peptides. In addition, the lack of appreciable activity of this reporter in the P174act background suggests that the alteration of the promoter in front of *pldFE* that is responsible for the hyperinducible phenotype does not appreciably alter expression of the downstream regulatory genes.

*2.2.6 The hyperinducible mutation functions only when directly upstream of *pldFE*.*

To better determine the role of the mutation in the hyperinducible phenotype, we created two additional reporter plasmid integrations into the promoter of *pldF* in which the P174act mutation was placed upstream of *lacZ* only or upstream of *pldF* only (Fig. 2.4A). The activity of these strains in reporter overlay assays was compared with the wildtype reporter strain, P174 *pldF-lacZ* by using cell free supernatant preparations of cultures of P174act to activate the locus (Fig. 2.4A). Only the reporter that had the mutation directly upstream of the *pldF* gene had the hyperinducible (multiple plaque forming) phenotype as seen with the multiple spots of *lacZ* expression (Fig.2.4A). When comparing the relative activity of the wildtype reporter strain with the strain containing the mutation only in front of *lacZ*, it appeared that the reporter with the mutation had relatively less induction suggesting that the mutation may decrease the activity of the *pldF* promoter (Fig.2.4A).

To better understand the hyperinducible phenotype, we used cell free supernatant preparations of cultures of P174act as an inducer to measure β -

galactosidase activity (Fig. 2.4B). Two fold dilutions of the cell free supernatant were used to induce the wildtype P174 *pldF-lacZ* reporter strain and the same reporter with the act mutation in front of *lacZ* (P174 P_{act}*pldF-lacZ*) or in front of *pldF* (P174act P₁₇₄*pldF-lacZ*). The P174act P₁₇₄*pldF-lacZ* strain showed a clear dose response to increasing concentrations of supernatant (Fig 2.4B). P174 *pldF-lacZ* or P174 P_{174act}*pldF-lacZ* strains showed no appreciable activation of the locus (Fig 2.4B). These findings suggest that the act mutation results in a decreased threshold for signaling resulting in enhanced transcription of the locus even at low peptide concentrations and confirms our observations on plates that the mutation must lie directly 5' to the start codon of *pldF* to demonstrate the hyperinducible phenotype.

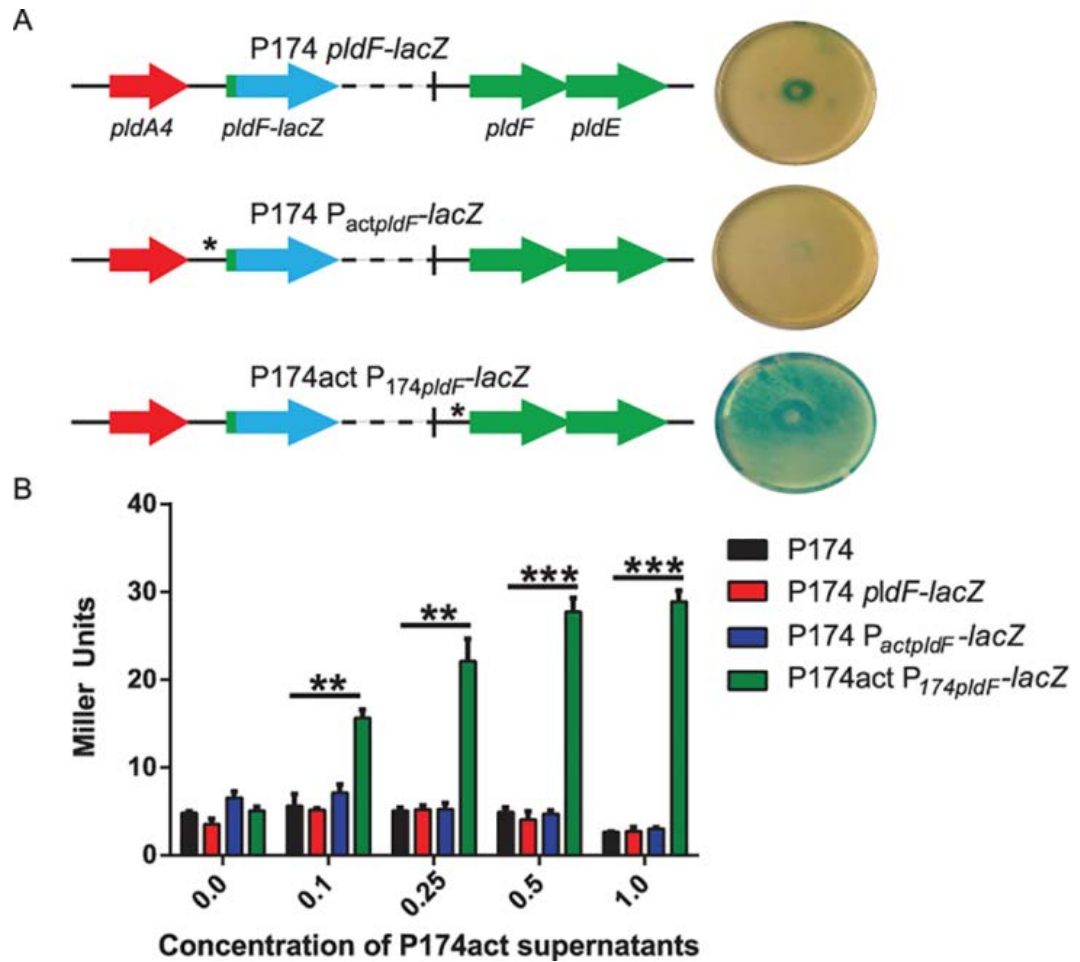


Figure 2.4. Transcriptional Activity of *pldF-lacZ* fusion strains in either P174 or P174act background. A) Schematics of the *pldF-lacZ* reporter strains are shown after plasmid integration. Dotted lines denote the plasmid-derived sequence; the *lacZ* gene is shown as a light blue arrow. An asterisk depicts the site of the act mutation. To the right of the corresponding schematic of the locus is the phenotype of each construct grown on TS plates containing X-gal in which 5 μ l of crude P174act derived supernatant was added to the center of a lawn for induction. Response to supernatants was evidenced by the blue halos. B) Transcriptional activity of the promoter driving *lacZ* was assessed in in broth grown organisms using strains P174, P174 *pldF-lacZ*, P174 $P_{actpldF-lacZ}$, and P174act $P_{174pldF-lacZ}$. Two fold dilutions of crude P174act derived supernatant was added to the strains at an OD₆₂₀ of 0.2 and induced for 1.5 hours. Activity was determined by calculating Miller Units. To account for endogenous β -galactosidase activity, wildtype P174 was included.

2.2.7 Three distinct phenotypes for peptide found in the P174act background.

Given the decreased threshold for signaling in the hyperinducible background, the three unmarked peptide mutations that lacked inhibition in overlay assays in the wildtype background were moved into this background and assessed for secretion of signaling and inhibitory peptides (Fig. 2.2B). In this background, the three deletion strains had three distinct phenotypes in overlay assays. The *pldA1* deletion in the P174act background retained signal secretion but lost inhibition. The strain carrying the *pldA2* deletion was indistinguishable from the P174 wildtype strain. The P174act Δ *pldA3* deletion strain had the most dramatic phenotype and lost all signal secretion and inhibition. The inhibitory defect in the P174act Δ *pldA1* strain could be phenotypically complemented by placing an adjacent stab of the P174act Δ *pldA3* strain confirming the overall integrity of the locus in each deletion mutant (Fig. 2.2C). It is important to note here that the active peptide sequences of PldA1 and PldA2 only differ by two conserved amino acids. The difference in the phenotypes of the *pldA1* and *pldA2* knockout in the P174 and P174act backgrounds suggest that both are required for signaling when thresholds are at wildtype levels, but PldA2 plays less of a role in inhibition than PldA1 when the signaling threshold is low.

2.2.8 Broad spectrum of inhibitory activity derived from pld locus.

P174 lantibiotic derived inhibitory activity was examined using a previously described collection of 50 pneumococcal isolates from South Africa and the alternative lantibiotic expressing ATCC 700669 [93]. Additionally, a selection of non-pneumococcal strains was tested for sensitivity to P174. Inhibitory activity was found against all but one of the pneumococcal strains as well as isolates of

Streptococcus oralis, *Listeria monocytogenes*, *Streptococcus pyogenes*, *Streptococcus agalactiae*, and *Lactococcus lactis* (Table 2.1). Further information on the pneumococcal and streptococcal strains tested can be seen in Table A1. Strains that were sensitive to P174 were also tested against P174 $\Delta pldK$ to confirm that activity was attributed to the *pld* locus. A single strain, P130 was the only pneumococcal isolate that had immunity when tested against P174 in the overlay assay.

Table 2.1. Spectrum of Inhibitory Activity for P174.

Strain	Inhibition by P174
<i>pld</i> negative <i>Streptococcus pneumoniae</i>	+ (50/50)
<i>pld</i> positive <i>Streptococcus pneumoniae</i>	+ (4/5)
<i>Lactococcus lactis</i> ATCC 14365	+
<i>Streptococcus pyogenes</i> Clinical Isolates and Lab strains	+ (12/12) [¥]
<i>Listeria monocytogenes</i> 10403S	+
<i>Streptococcus agalactiae</i> Clinical and Lab Strains	+ (6/15) [¥]
<i>Streptococcus mitis</i> ATCC 49456	-
<i>Staphylococcus aureus</i> Clinical Isolate	-
<i>Enterococcus faecalis</i> ATCC 29212	-
Vancomycin resistant <i>Enterococcus faecalis</i> ATCC 51299	-

*=includes all but one member of the South African strain collection, ATCC 700669, and the four *lanM* positive strains from the University of Michigan clinical isolate collection.

+ = zone of clearance was detected.

- = no inhibitory activity was detected.

¥ = the number of strains killed over the total number of strains tested

2.2.9 *Pneumolancidin* producing strains have an advantage in invasion of colonization *in vivo*.

To determine if pneumolancidin production provides a competitive advantage during colonization we compared the ability of a producing strain to invade a colonized mucosal surface to an otherwise isogenic non-producer. Using a

staggered inoculation model, colonization was first established with the sensitive P174 $\Delta pldK$ strain for three days followed by introduction of either producer P174st^R or non-producer P174 $\Delta pldA1-4$. P174st^R was able to invade the established community of P174 $\Delta pldK$ in the nasopharynx better than P174 $\Delta pldA1-4$ (Fig. 2.5). Levels of P174 $\Delta pldK$ were lower in the mice inoculated with the wildtype strain suggesting that colonization results in the eviction of some resident organisms, however this difference was not statistically significant. To account for difference in overall colonization density, we assessed the ratio of invading to resident (I/R) CFU per mouse in each condition. While, only 1 of 20 of the P174 $\Delta pldA1-4$ inoculated mice had an I/R greater than 1, 7/19 mice inoculated with the wildtype strain had a predominance of the invading strain (Fig. A3).

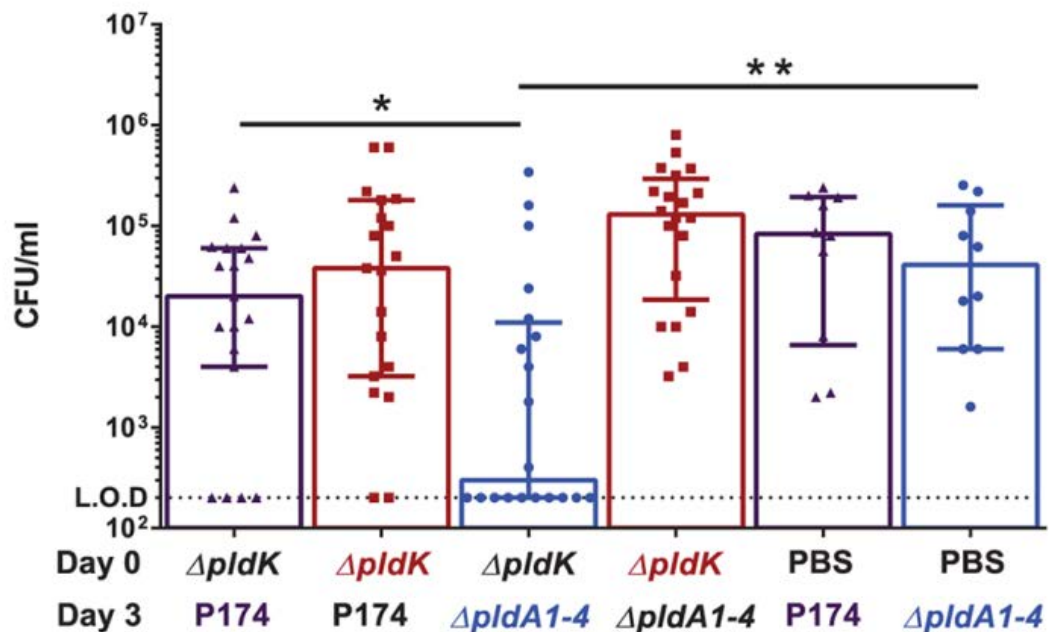


Figure 2.5. Competitive advantage of the *pld* locus *in vivo*. Mice were either colonized with P174 $\Delta pldK$ or sterile PBS at day zero. At day 3, mice were challenged intranasally with either P174 or P174 $\Delta pldA1-4$. Nasal washes were

obtained 3 days post inoculation and CFU calculated by differential plating. Data for P174 are shown in purple, P174 Δ *pldK* in red and P174 Δ *pldA1-4* in blue. Median and interquartile range are shown. Dotted line indicates the limit of detection (L.O.D). Statistical analysis was performed using an unpaired Mann-Whitney test. * = $p < 0.05$. ** = $p < 0.01$.

2.2.10 Identification and characterization of additional strains with a *pld*-like locus.

P130, which has immunity to pneumolancidin was shown to encode a *pld* locus by PCR and sequencing. The locus in P130 has the same gene content as P174, but overlay assays failed to demonstrate any evidence of lantibiotic-mediated inhibition. The *pld* locus of P130 is nearly identical to the locus in the previously identified PN1 strain and the fully sequenced BHN418 strain and does not contain any large deletions or frameshift mutations that might account for the lack of activity. MLST analysis of the *pld* positive strains P174, P130, BHN418, and PN1 was determined to assess their genetic similarity (Table A2). P130 and BHN418 differed by only a single MLST allele while P174 did not share any common alleles with these strains. It appears that acquisition of the *pld* locus is a relatively unique property of P174 because the locus is not found in three publicly available, fully sequenced strains (GA05245, GA17227 and GA41301) that share the same founder ST as P174 (ST242). In fact, these strains lack the associated ICE element all together suggesting that P174 may have recently acquired the element.

To determine if more pneumococcal strains could be identified that contain the locus, we screened a collection of over 400 clinical isolates for the presence of the *pldM* gene using PCR. Four strains were identified and were categorized

by serotyping and MLST analysis (Table A2). One of the newly identified clinical isolates was found to be from the same clonal complex as BHN418 and P130, but this isolate was neither active nor immune to P174. Three NT isolates with the locus were identified as belonging to the 448 sequence type. A genome sequenced isolate from this same ST, MNZ14 was found to have a *pld* locus that encodes all of the elements found in P174 with the exception of an apparent disruption of the gene encoding the peptide transporter, *pldT* [116]. The three *pldM* positive NT isolates from our collection lacked both inhibition and immunity to P174 mediated inhibition (not shown).

2.2.11 P130 contains an inactive locus due to a mutation in PldM.

Using allelic replacement, lysates from P130 were used to transform P174 containing an exchangeable cassette replacing the entire *pld* locus. All of the resulting transformants lacked inhibitory activity suggesting that a mutation in the *pld* locus was responsible for the lack of P130 derived inhibitory activity. Amino acid sequence alignment of the PldM gene products from both strains demonstrated that the P130 strain contained a C867Y mutation in one of the critical residues required for zinc coordination (Fig.A4). It was shown previously that mutating any one of the conserved cysteine residues resulted in a loss of cyclization activity in the NisC enzyme [117]. To confirm that the mutation alone would result in a strain with P130 phenotype, the region of the *pldM* gene in P174 containing the site of the mutation was exchanged with the P130 allele. The resulting strain, P174 PldM C869Y, was unable to inhibit (Fig.A4). Replacement of the P174 locus with the P130 *pld* versions of *pldA1-4*, *pldF*, and *pldKR*

resulted in a fully inhibitory strain (not shown) suggesting that the *pldM* mutation alone was responsible for the P130 phenotype. Reciprocal gain of function experiments in P130 could not be performed because P130 was not transformable.

2.3 Discussion

This work describes the first functional lantibiotic locus found in pneumococcus. The locus encodes the genes required for inhibitory activity against other pneumococci and closely related Gram positive organisms. It is found as cargo of an ICE element and has been identified in unrelated isolates suggesting that the locus is moving through the pneumococcal population via either conjugation or horizontal gene transfer. Not only has this locus disseminated in distinct pneumococcal lineages, but the two component system and *pldFE* genes of the locus share significant homology to a region in the *S. mitis* B6 genome. Genetic exchange between *S. pneumoniae* and *S. mitis* is common and contributes to the genomic diversity of the species [118]. The region of homology in the *S. mitis* B6 genome is sufficient for immunity to the *pld* peptides via the PldKR mediated upregulation of the PldFE ABC transporter. *S. mitis* ATCC 49456, was found to contain the *pldK* gene by PCR (data not shown). This isolate was immune to inhibition by P174 which may be due to the presence of the four *pld* genes, although without deletion analysis, the requirement for the *pld* homologues for immunity cannot be verified.

All pneumococcal strains that we have identified apart from P174 that contain the *pld* locus lack inhibitory activity. P130 has *pld* mediated immunity

that seems to derive from the *pld* locus. The mutation responsible for lack of inhibition was localized to a SNP within the *pldM* gene that results in the mutation of a critical residue involved in zinc coordination which is required for enzymatic activity of the modification enzyme. This same mutation is found in the sequenced strains PN1 and BHN418, perhaps explaining why no inhibitory activity has been attributed to these strains [99]. Disruption of the modification enzyme has been seen in the lantibiotic locus encoded by *S. suis* as way to prevent production of active lantibiotics but retain lantibiotic immunity [119]. Four other pneumococcal isolates were identified that were *pld* positive but these strains were not immune to P174, unlike P130. Loss of lantibiotic production may occur because of the energetic cost of production leading to the selection of a mutation rendering the locus nonfunctional. The energetic cost of the *pld* locus may be particularly high in pneumococcal strains due to in-vitro evidence of imperfect self-immunity demonstrated by plaque-like structure formation when strains are grown at high density. Staggered colonization experiments demonstrate that pneumolancidin production does provide a competitive advantage in vivo, even in the relatively difficult task of invading an existing community. Only 9 of 20 pre-colonized mice that were challenged with the non-producing strain had evidence of any appreciable invasion, while 15 of 19 mice challenged with the producing strain were colonized with the invading strain. P174 lantibiotic peptides do not share homology to any other known lantibiotic peptides. Interestingly, the peptides are homologous to each other which may indicate a remote gene duplication event. The tandem array of similar genes

encoding peptide antibiotics is reminiscent of some loci that encode the highly modified thiocillins. Several of these loci are characterized by a tandem array of identical structural peptides that are modified by a series of enzymes to create the antibacterial molecule. The whole cluster of four structural peptides has been shown to be required for the antimicrobial activity of the *tcl* locus in *Bacillus subtilis*, however, to our knowledge, the requirement for multiple copies has not been explored but may be related to optimal gene dosage because a strain carrying a deletion of the four structural genes can be complemented with a single copy expressed on a multi-copy plasmid [120].

Other, more typical *lan* systems that express two structural peptides usually encode two separate modification enzymes where each is uniquely dedicated to the modification of one peptide [28, 57]. A seven lantibiotic peptide, cerecidin, in *Bacillus cereus* has been described that is associated with a single modification enzyme, although it was not shown to be functional *in vivo* [121]. We are currently working on purifying the active peptides to determine the specific role of each peptide in inhibition and stability.

P174 is immune to its own lantibiotic although in overlay assays there is some degree of self-inhibition that is characterized by a halo of decreased growth around the stab. This phenotype is only seen when strains that can produce inhibition themselves are grown in the overlay. This may be indicative of a lapse in developing immunity in response to exogenous peptides when broth grown organisms are applied over stabs in which the locus has already been upregulated. When the overlay strain is placed over an actively secreting stab,

lantibiotic-mediated signaling derived from the stabbed strain activates some cells in the overlay and kills others. The activated overlay strains in turn kill or activate surrounding strains resulting in a wave of combined signaling and inhibition. The fact that the wave of signaling and inhibition requires signal propagation within the overlay strain is supported by the activity of the three reporter strains (*pldA1-4* knockout, P174, and P174 act) with increasing ability to amplify the response to secreted signals and correlated increased zones of signaling when placed in overlay over P174.

Although our inability to examine *pld* specific transcripts directly has hampered characterization of transcriptional control of the locus, we have described the activity of a series of reporter constructs in plate assays in an attempt to determine the likely transcriptional units. Unlike many other lantibiotic loci in which the entire cluster of *lan* genes is encoded on a single transcript, we have shown that the genes clusters downstream of *pldA1-4* are controlled by separate promoters. The separate control of each of the gene clusters is further supported by the fact that plasmid integrations into the presumed promoters of *pldFE*, *pldKR* and *pldM* did not disrupt inhibitory activity. In fact, unlike the *pldFE* and *pldM* genes, the intervening *pldKR* genes are not upregulated in response to P174 peptide at all. The lack of peptide responsiveness of these genes may serve to dampen the positive feedback loop that occurs during peptide stimulation, by limiting the amount of regulatory proteins on the cell surface. We describe the identification of a serendipitously isolated hyperinducible strain. Based on the location of mutation in the presumed TATA box of the promoter

preceding *pldFE* and our results with the *pldFE* reporter strains, the most likely explanation of the phenotype is that the mutation affects expression of only *pldFE*, the proposed immunity transporter. Based on preliminary data, the variant phenotype of P174act seems to be attributable to decreased levels of PldFE, leading to increased sensitivity of the cell to exogenous peptides. The *pldFEKR* cluster falls in to the family of Bce regulatory/transporter proteins typified by the bacitracin resistance gene cluster BceRS-BceAB. In these cases, the transporter appears have dual role, functioning as a resistance protein by pumping out antibiotics and as a regulator interacting with the two component system to upregulate gene expression [122-124]. One possible interpretation of our findings is that altering the ratio of immunity transporter to regulatory gene products results in increased sensitivity to signaling peptide. Alternatively, it is possible that PldFE binds the Pld peptides with different affinity. If this is true, then decreased production of the immunity transporter complex may change effective concentrations of the peptides.

We have not yet tested whether the *pld* locus in P174 provides resistance against other cell wall targeting antimicrobials. Since bacitracin transporters often mediate resistance to other antibiotics, this could explain the maintenance of the *pld* locus in strains that are not producing functional lantibiotics [113-115]. Wildtype P174 only produces appreciable *pld* mediated inhibition in plate grown organisms. This property has hampered our attempts at large-scale purification. We have noted, however, that the P174act strain can produce appreciable quantities of peptides during growth in broth, presumably due to the decreased

threshold for signaling. This mutant may serve as a useful tool for large-scale purification of this potent lantibiotic that inhibits nearly all pneumococcal strains tested. In addition the manipulation of immunity transporter quantities for increased yield of inhibitory peptides may be translatable for use in other *lan* systems with a bacitracin-like immunity transporter.

We have shown that the P174 *pld* locus has the interesting property of requiring the presence of three very similar peptides for self-signaling to occur in a wildtype background. This may be a result of a gene dosage effect, such that three copies of nearly identical genes are required for to reach the threshold for activation; this assumes that all three peptides have the same function. Alternatively, the three peptides may form a complex and each peptide of the complex is uniquely required for signaling. The potential for separate roles of the individual peptides could not be assessed in the P174 background because the block in signaling does not allow us to assess differences in inhibition. In contrast to the individual peptide knockouts in the wildtype background, the individual knockouts in the P174^{act} background each had a distinct phenotype, most likely due to the lower threshold for activation noted in this strain. The phenotypes of individual peptide knockouts noted in this background confirm the absolute requirement for PldA3 for signaling. Of note, PldA3 is the most divergent of the three peptides, with two more cysteine amino acids in the active domain compared with PldA1 and 2 and would be predicted to have a very different structure. In addition, the single peptide deletions in the P174^{act} background demonstrated that either PldA1 or PldA2 are sufficient to promote signal

secretion in combination with PldA3, but that PldA1 is more important for inhibition than PldA2. This may be either because more PldA1 is made than PldA2 or because the two peptides differ in their inhibitory activity on the target cell surface. We are currently working to isolate each of the three peptides to better understand their contribution to the remarkable anti-pneumococcal activity of pneumolancidin.

2.4 Materials and Methods

Bacterial strains, plasmids, and growth conditions

All pneumococcal strains used are described in Table A2 and all primers used are described in Table A3. All pneumococcal strains were plated on either 5% sheep's blood (SBA) or tryptic soy agar plates (TSA) with 0.5% catalase (Worthington, Lakewood, NJ) (4,741 U) and incubated at 37 °C with 5% CO₂. For growth in liquid culture, all pneumococcal strains were grown in Todd-Hewitt broth supplemented with 0.5% yeast extract (THY). *Escherichia coli* strains were grown in Luria-Bertani (LB) broth or LB agar.

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neomycin, 2 µg/ml chloramphenicol, 1 µg/ml erythromycin, and 200 µg/ml spectinomycin; and for *E. coli*, 50 µg/ml kanamycin, 20 µg/ml chloramphenicol, 100 µg/ml erythromycin, and 100 µg/ml spectinomycin. The South African collection of pneumococcal isolates has been previously described [93]. The clinical isolate collection consists of 457 disease causing pneumococcal isolates that were collected by the microbiology lab from existing patient samples at the University of Michigan between 2004 and 2006.

Disruption of blp and com loci in P174

To disrupt the *blp* or *cib* locus, P537 was made that contained the Janus cassette in place of the *blp* locus or P1535 that contained a Janus insertion at the *comD* gene [93, 125]. Genomic DNA was extracted from either P537 or P72 and used to transform competent P174. Colonies were selected for the presence of the Janus cassette by plating on kanamycin plates. Allelic exchange of the appropriate locus for the Janus was confirmed by PCR [125].

Transposon mutagenesis of P174

Mutagenesis of P174 genomic DNA was performed as previously described by van Opijnen *et al.*, 2007[126]. The transposon mutagenized genomic DNA was then used to transform P174 and transposon insertion mutants selected on spectinomycin plates. Transformants were screened for loss of inhibitory activity by overlay assay using a 96 well replicator to stab the strains into agar plates. The overlay strain used was the universally sensitive pneumococcal strain, P537. Lysates of transformants unable to inhibit strain

P537 were used to back transform strain P174 and the original phenotype confirmed on overlay assay. Confirmed mutants were sequenced using universal transposon specific and universal primers as previously described to determine the location of the transposon insertion [126]. The entire *pld* locus from P174 and P130 was sequenced by using a combination of chromosome walking and plasmid rescue.

Construction of individual pldA deletions, whole peptide deletions, and complementation.

In frame, unmarked deletions of the genes encoding individual peptides were constructed using the counter-selectable Janus cassette. First, the *pldA1-4* region plus 500bp up- and downstream was amplified using primers 1 and 2. This product was cloned into pCR2.1 generating plasmid pE93. A blunt-ended janus cassette amplified using primers 5 and 6 was ligated into an inverse PCR product generated using primers 3 and 4. A streptomycin resistant version of P174 was transformed with the ligation product creating P174 *pldA1-4::Janus* [127]. This strain, containing the Janus cassette in place of the four putative peptide ORFs was used to make individual peptide and whole peptide deletions. Four separate inverse PCR reactions followed by plasmid religation were performed on plasmid E93 to create individual in frame unmarked deletions of the individual peptides using primer pairs 7 and 8, 9 and 10, 11 and 12 and 13 and 14. Primers 3 and 4 were used to create an unmarked whole deletion of the peptide region. Resultant plasmids were transformed into P174 *pldA1-4::Janus*.

All transformants were verified by PCR using primers 7 and 14 and sequenced to ensure the absence of PCR generated mutations. To complement P174*pldA1-4::Janus*, E93 was used to transform back all four of the peptides and the resulting strain tested for inhibition and immunity.

Disruption or deletions of other genes in lan locus.

To disrupt *pldK* and *pldT*, a small internal fragment of both genes was amplified using either primer pair 15 and 16, or 17 and 18, respectively. Both PCR products were cloned into pCR2.1 and then moved into E68, an integrative plasmid with the spectinomycin resistance gene. The resulting plasmids were used for transformation into P174 and plated on spectinomycin plates. Transformants were PCR verified using forward primer 15 or 17 for either the *pldK* or the *pldT*, respectively, and either primer 19 or 20 to determine orientation.

Disruption of *pldM* was achieved by using the transposon mutant identified in the transposon mutagenesis screen. To verify inhibitory activity was attributable to disruption of *lanM*, lysates were prepared from the original transposon mutant and re-transformed into P174. To delete *pldEF*, an in-frame, unmarked deletion of this region was made by allelic exchange. The Janus cassette was amplified from P174*pldA1-4::Janus* using primer pair 1 and 23 creating a NsiI site at the 3' end of the Janus. This PCR product was digested with NsiI and ligated to the NsiI digested PCR product of 22 and 16. The ligated product was transformed into P174strep^R kanamycin resistant transformants

were verified by size by PCR with primers 1 and 16. To generate a *pldFE* deletion, PCR products using primer pair 1 and 21 and primer pair 22 and 16 were ligated and transformed into P174*pldA1-4EF::Janus* with selection on streptomycin. The appropriate deletion was confirmed by PCR and sequencing.

To create a 4 bp deletion in the intergenic region between *pldA4* and *pldFE* which included the site of the mutation, the Janus cassette was extended from P174*pldA1-4::Janus* to cover the site of the mutation. The Janus from P174*pldA1-4::Janus* was amplified using primer pair 1 and 23 and ligated to the product of primer pair 33 and 34 after digestion with NsiI. The ligated product was transformed into P174strep^R, resulting in P174*pldA1-4mut::Janus*. Next, site directed mutagenesis was used on plasmid E93 using primer pair 35 and 36 the resulting product used to transform P174*pldA1-4mut::Janus*. The mutation was verified by sequencing.

Construction of Reporter Plasmid and Strains

The reporter plasmid was constructed in a derivative of the *lacZ* containing integrative plasmid, pEVP3. This plasmid was first made kanamycin-resistant due to the inherent chloramphenicol resistance of P174. The kanamycin resistance cassette was added to pEVP3 at a unique EcoRI site creating plasmid E65. A PCR product containing 500bp upstream of the *pldM* gene and the first 207 nt of the *pldM* coding sequence was amplified using primer pair 24 and 25. This product was cloned into E63 creating a transcriptional fusion of the *pldM* promoter and *lacZ*. This reporter plasmid was used to transform P174, P174

$\Delta pldA3$, or in P174act and transformants selected on kanamycin. Colonies were screened using primer pair 24 and 26.

To generate the *pldK* reporter, a 500bp fragment containing 451 bp upstream of *pldK* through the first 15 codons was amplified with primer pair 37 and 38. This PCR product was cloned into pE65 into unique XbaI and NsiI sites. The resulting plasmid E180 was transformed into either P174 or P174act. To generate the *pldF-lacZ* fusion strain, a 500 bp fragment containing the first 36 bp of *pldF* from either P174 or P174act was cloned into E65 using XbaI and NsiI sites with primer pair 39 and 40. The resulting plasmid E181 or E182, containing P174 or P174act DNA, respectively, was then transformed into either P174 or P174act. Confirmation of correct integration of the plasmid was done using primer pair 39 and 26. To generate a *pldA1-lacZ* fusion, a PCR product containing approximately 500 bp upstream of *pldA1* and through the first 13 codons of *pldA1* was generated and cloned into E65 using XbaI and NsiI sites with primer pair 47 and 48. The resulting plasmid E188 was transformed into either P174 or P174act. Transformants were verified with primer pair 47 and 26.

Linkage analysis

To demonstrate that the lack of inhibitory activity in P130 was linked to the *pld* locus, an exchangeable Janus cassette was used to replace the entire *pld* locus of P174. The deletion was created by ligating the product of primer pair 1 and 23 on P174 *pldA1-4::Janus* to the product of primer pair 39 and 40 from P174 and transforming into P174 strep^R. The correct insertion was verified using

primer pairs 1 and 40. P174 *pldA1-4EFKRMT::Janus* was transformed with genomic DNA of P130 and selected on streptomycin. Transformants were verified by PCR.

To make the specific mutation in *pldM* in the P174 background, a Janus cassette was introduced into a unique BsrGI site in a cloned version of *pldM* made using primer pair 41 and 42. The resulting ligation was then transformed into P174 strep^R, creating P174 *pldM::Janus*. Verification of transformants was done using primer pair 41 and 42. To exchange just the area of difference between the two alleles, P174 *pldM::Janus* was transformed with a PCR product from P130 produced using primer pair 45 and 46.

Overlay assays for inhibition, signaling, and immunity

Overlay assays were used to screen inhibition, immunity and signaling were performed as described previously [128]. All overlays were repeated at least three times and performed on three separate occasions to evaluate for consistency to the methods. To test activity of supernatants on plates obtained from P174act grown to an OD₆₂₀ of 0.5, a lawn of a reporter strain was spread onto a TS plate containing X-gal and catalase. Spots of the filter sterilized supernatants were added on top of the dried lawn and then incubated at 37°C overnight.

Screening of the South African and Clinical Isolate Collection for lan genes

To identify other strains that might possess the *pld* locus, the collection was screened by PCR using primers 27 and 28 that amplified an internal conserved region of *pldM*. Reactions were run on an agarose gel and the presence of a 300 bp band indicated *pldM* positive strain. To determine if these strains had the same peptide region as P174 any positive strains were also screened with primers 31 and 32.

MLST of clinical isolates

MLST analysis was performed by sequencing the seven housekeeping genes using previously described methods [129]. Sequences obtained from using universal primers for each allele were entered into the website <http://spneumoniae.mlst.net/> to generate an allele type contributing to a sequence type.

Miller Assays

Reporter strains containing *pldF-lacZ* in either P174 or P174 act background were grown to an OD of 0.2, 40 μ l was added to a microtiter plate containing catalase and either 40 μ l of supernatants from Δ *pldK* or from P174 act that was diluted in two fold serial dilutions in THY. Plates were incubated for 1.5 hours and Miller Units determined as described [130, 131].

Staggered mouse colonization assays

All mice were purchased from Jackson laboratories and were housed in accordance with Institutional Animal Care and Use Committee protocols. This

protocol was described previously with some modifications [90]. Ten 6 to 7-week-old BALB/c mice were inoculated intranasally with 10 μ l containing 4×10^7 CFU of animal passaged strain of P174 $\Delta pldK$ resuspended in PBS. At 3 days post inoculation, approximately 10^7 CFU of either animal passaged P174 strep^R or P174 $\Delta pldA1-4$ strep^R were intranasally inoculated into mice either previously colonized with P174 $\Delta pldK$ or PBS. Five control mice received PBS followed by the two invading strains. After three days, mice were sacrificed and nasal washes were collected by tracheal lavage using 200 μ l of PBS. The lavage fluid was diluted in PBS and plated on TSA with selection. All plates contained neomycin to prevent growth of the natural flora. Strains were differentiated on streptomycin or spectinomycin containing media. The experiment was repeated once and the cumulative data from both experiments is shown. One mouse in the producer arm died during the course of the experiment, colonization for this mouse was not determined.

CHAPTER III

LANTIBIOTIC PEPTIDES, PNEUMOLANCIDIN A1 AND A3, OF *STREPTOCOCCUS PNEUMONIAE* HAVE DIFFERENT BIOACTIVITIES DESPITE STRUCTURAL SIMILARITIES

Abstract

Lantibiotics undergo posttranslational modifications that result in the creation of lanthionine or methyllanthione rings that are required for their function both as antimicrobials and autoinducers. Two-peptide lantibiotics require the actions of two modification enzymes and are structurally different. This difference in structures contributes to their specialized role in inhibition. Recently, a lantibiotic locus, encoding pneumolancidin (*pld*), was found in *Streptococcus pneumoniae*. This locus contains four open reading frames arranged in a tandem array that are predicted to encode four highly homologous lantibiotic peptides. A gene encoding a single modification enzyme, *pldM*, was found in the locus suggesting that the four Pld peptides might be modified by the same enzyme and thus adopt similar structures. Our previous data demonstrate that these peptides, despite significant homology, have specialized functions related to the antimicrobial and autoinduction function of the lantibiotic locus. Using genetic analysis, it was shown that the Pld peptides (1-3) play specialized and unique roles in inhibition and the ability to upregulate the locus. In the WT setting,

each peptide was required for inhibition. This could be explained by a requirement for a specific gene dosage rather than a requirement for the individual properties of each peptide. To determine if gene dosage rather than unique activity attributable to each peptide might explain the requirement for all three Pld peptides, we created a *pld* locus that contained a *pldA1* duplication that replaced the ORF of *pldA2*. If gene dosage alone explained the requirement for *pldA2*, then we predicted that this strain would retain inhibitory activity. However, this strain was not able to kill or signal arguing in favor of a specific role for PldA2. Additionally, expression of PldA3 alone was not sufficient to kill or signal in the WT (P174) or lab generated hyperinducible strain (P174act) background despite being required for signaling. This data combined suggests that a complex may be required for activity consisting of PldA3 and at least one other Pld peptide. The structures of pneumolancidin A1 and A3 from *Streptococcus pneumoniae* have been solved using nuclear magnetic resonance spectroscopy (NMR) and tandem mass spectrometry (MS/MS). Purified PldA1 and PldA3 were found to be structurally homologous but with distinct functions that were not consistent with genetic analysis. Despite having similar structures, PldA1 was only able to upregulate the *pld* locus in a reporter strain but did not have any inhibitory activity while PldA3 was able to inhibit a sensitive strain but lacked evidence of signaling activity. The Pld peptide structures will provide further insight into structure-function relationships.

3.1 Introduction

Lantibiotics are a class of bacteriocins that undergo posttranslational modifications resulting in creation of lanthionine or methylanthione residues [6]. Creation of these residues involves the dehydration of serines and threonines which are then linked to cysteines via a thioether bridge. In type II lantibiotics, the dehydration and thioether formation is catalyzed by a single enzyme, LanM [24]. In type I lantibiotics, two modification enzymes are required for both dehydration and cyclization, LanB and LanC, respectively [11-14]. After modification, the prelantibiotic peptide is secreted by a dedicated ABC transporter encoded by LanT [14]. The signal peptide which is needed both for recognition by the modification enzyme is then either cleaved by LanT or by an outer membrane protease, LanP [16, 25, 26]. After cleavage of the signal peptide, the lantibiotic is functional. Lantibiotics exert an antimicrobial effect by targeting lipid II, an important cell wall precursor, and some lantibiotics are capable of subsequent pore formation [45-47, 56]. Lantibiotics act as signaling pheromones and are able to interact with a cognate histidine kinase, LanK, initiating transcription of the *lan* locus [18, 111, 112]. Immunity is conferred by either an ABC transporter and/or an immunity protein, LanI [63, 71, 72, 132].

Streptococcus pneumoniae is known to secrete antimicrobial peptides called bacteriocins, an important mediator of competition [90, 93, 96, 106]. Although putative lantibiotic loci are encoded in many pneumococcal strains it is unknown if expression of these lantibiotics occur [97-101]. We recently described the first functional lantibiotic locus in a clinical isolate of

pneumococcus, P174 [133]. The pneumolanicidin (*pld*) locus of P174 encodes four highly homologous Pld peptides. This locus only encodes a single modification enzyme, PldM, unlike other examples of multi-peptide lantibiotic loci, suggesting that the structures of the peptides may be similar. Using genetic analysis, we found three of the four peptides, PldA1-3, were required for both inhibition and signaling of the reporter strain. A hyperinducible mutant strain (P174act) was discovered in which a lowered threshold of exogenous Pld peptides was needed for activation. When individual PldA deletions were tested in this background, the Pld peptides were found to have specialized roles. A PldA1 deletion in P174act was found to secrete a signal but inhibition was lost, a PldA2 deletion strain was able to signal and inhibit, and a PldA3 deletion strain did not inhibit or signal. This suggested that PldA1 was important for inhibition, PldA2 was dispensable for inhibition and signaling, and PldA3 was required for signaling with an undetermined role in inhibition. To better dissect the role of these Pld peptides, we tested the bioactivity of purified Pld peptides against pneumococcus.

The *pld* locus has been found in a number of pneumococcal strains and in *Streptococcus salivarius*, however the structure of the Pld peptides has not been solved [99, 134]. From a structure perspective, lantibiotics can be separated into two classes that predict their activity. Type-A lantibiotics have a linear structure that is important for forming pores in the membranes of sensitive strains [47]. Type-B lantibiotics are globular in shape and do not form pores but bind to lipid II [53]. In the case of known two-peptide lantibiotics, one is typically structurally

similar to type-A and the other is similar to type-B [135]. The Pld peptides do not share homology with any known lantibiotics so structure predictions could not be made based on primary sequence alone. To better understand the function of these unique peptides, we purified two of the three functional peptides and determined their structure using a combination of MS/MS and NMR. Here we demonstrate that despite the similarity in structures of the Pld peptides, they are able to play specialized roles in antimicrobial activity and auto-induction which may require complex formation.

3.2 Results

3.2.1 Gene dosage is not a contributing factor for functioning of Pld peptides and PldA3 alone does not activate the pld locus

We previously showed that individual in-frame unmarked deletions of the genes encoding the first three peptides, PldA1, 2 or 3 resulted in a strain that could neither inhibit nor upregulate the locus in the reporter strain. A deletion of the gene encoding the last putative peptide, PldA4, did not result in loss of signaling or inhibition. This suggested that in the WT setting, PldA1-3 were required for signaling to occur, most likely forming a complex that is recognized by PldK. Since the peptides are homologous to one another, a deletion of the genes encoding any of the three functional peptides, PldA1-3, could perturb the stoichiometry that is needed for optimal activity. To test this hypothesis, a duplication of *pldA1* was made in place of *pldA2*, essentially creating a *pldA2* deletion with a *pldA1* duplication. Although PldA2 was deleted, the relative ratio

of Pld peptides to PldFE should be unchanged as compared to the WT containing PldA1-4. The resulting strain, P174*pldA1*+/ Δ *pldA2*::*pldA1* was unable to inhibit or signal suggesting that altered gene dosage does not explain the phenotype of how the Pld peptides might be functioning (Fig 3.1).

Peptide deletions created in the hyperinducible strain, P174act, had distinct phenotypes. P174act Δ *pldA1* could not inhibit but could still secrete a signal indicating that PldA1 may be important for inhibition but was dispensable for signaling, at least in this background. P174act Δ *pldA2* was able to inhibit and signal suggesting that the role of PldA2 was dispensable in this background. P174act Δ *pldA3* was unable to inhibit or signal suggesting that PldA3 is required for signaling at a minimum, its role in inhibition could not be assessed using a genetic approach. We had shown previously that the mutant, P174act, required less of the Pld peptides for activation of the locus. Since the mutation in P174act results in the downregulation of the genes encoding the immunity transporter, PldFE, it is possible that excess of PldFE might play a role in sequestering free peptides.

Since PldA3 was required in both wildtype and P174 act backgrounds for both signaling and inhibition, strains were constructed that only expressed PldA3 and PldA4 in either P174 or P174act backgrounds. Consistent with previous data, the *pldA1-2* deletion containing strain, P174 Δ *pldA1* Δ *pldA2*, did not exhibit any inhibitory or signaling activity. Because we had previously shown that in P174act, expression of PldA3 in combination with either PldA1 or PldA2 was sufficient for signaling, we wanted to determine if PldA3 alone was sufficient for

signaling when expressed in this background. P174act Δ pIdA1 Δ pIdA2 was unable to inhibit or signal the reporter strain suggesting that at least one other PId peptide, either PIdA1 or PIdA2, is needed for signaling to occur (Fig. 3.1).

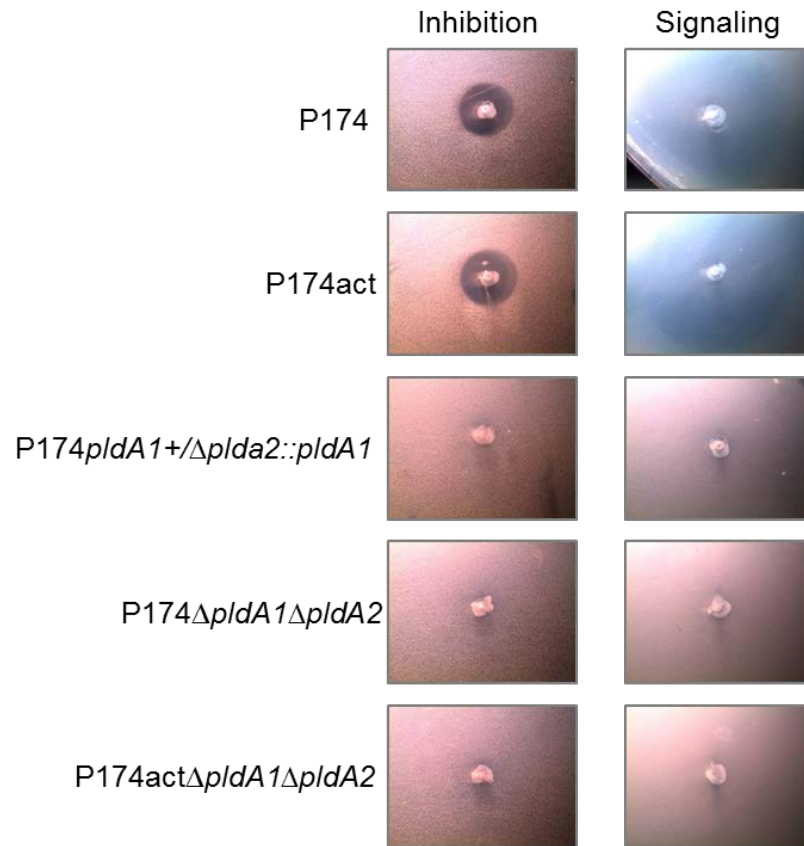


Figure 3.1. Evaluation of signaling and inhibition in either PIdA1 duplication or PIdA3 only expressing strains. Inhibition and signal secretion was assessed using the overlay assay. For the overlays, each strain was stabbed onto an agar plate and then overlaid with either a PId sensitive strain, TGR4, or a reporter strain P174pIdM-lacZ to detect either inhibition or signal secretion, respectively. As controls, P174 and P174act were included for comparison.

3.2.2 Purified PIdA1 and PIdA3 have different bioactivities and undergo a secondary cleavage event

To gain a better understanding of the role of the PId peptides, we separated the PId peptides by purification to test their bioactivity directly. Since expression of

PId peptides is only seen with growth on solid media, multiple stabs of P174act were made onto BSA plates. Broth media, THY, was overlaid on top of the plates. The 5.5L of media, containing PId peptides, was collected and subjected to an organic resin extraction. Three different solvents, methanol, ethanol, and propanol were used to elute the PId peptides from the resin. Of the three, the ethanol with 0.1%TFA contained the most PId peptides based on activity of fractions upon dilution (Fig. 3.2). The specific activity of the ethanol fraction was higher as compared to either methanol or propanol fractions (Fig.3.2).

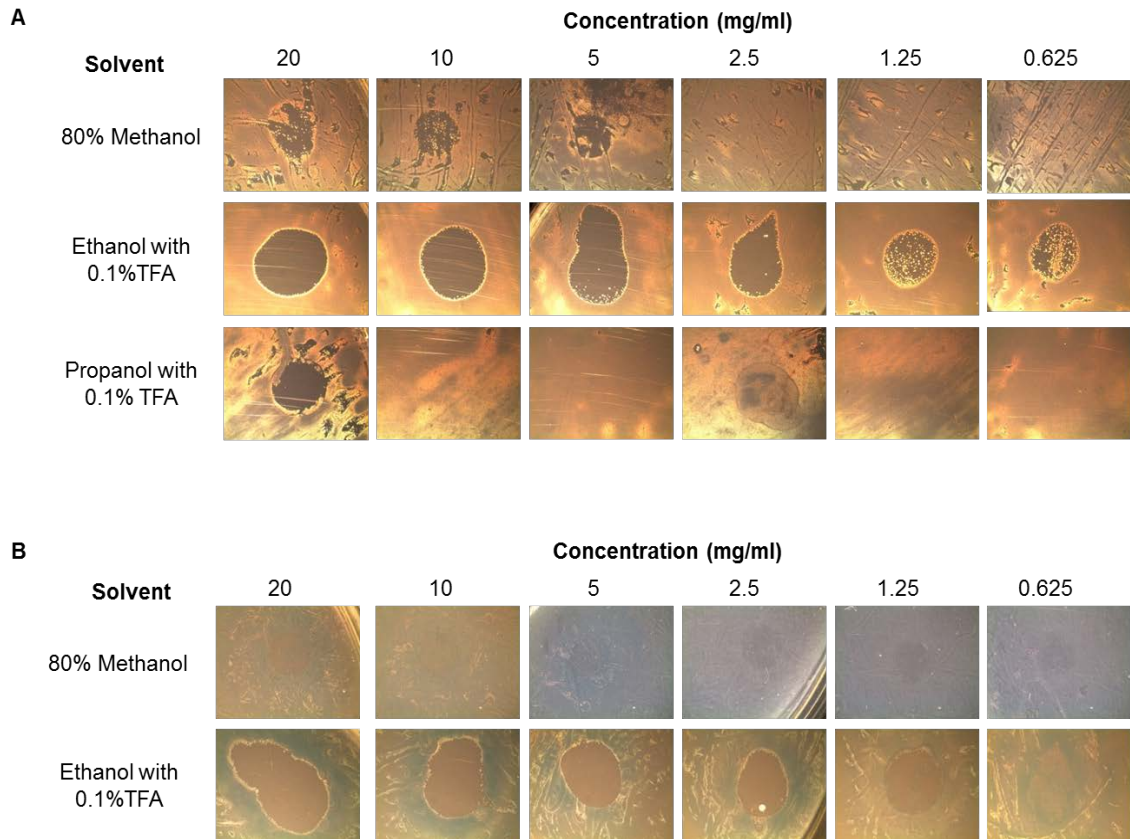


Figure 3.2. Inhibitory and signaling activity of different solvents used for elution of Pld peptides from resin. The following solvents, 80% methanol, ethanol with 0.1%TFA, and propanol with 0.1% TFA were used sequentially to wash the resin beads. To quantify the amount of Pld peptides that were eluted, serial dilutions starting from 20mg/ml of total protein were made and 5 μ l were spotted on either a plate containing a lawn of a Pld sensitive strain, TGR4, to assess inhibition (A) or a plate containing a lawn of reporter strain, P174*pIdM-lacZ*, to detect exogenous Pld peptides (B). Ethanol with 0.1% TFA exhibited inhibition and signaling at higher dilutions as compared to either 80% methanol or propanol. Signaling activity of propanol eluted resin fraction was not seen and not included in the above figure (B). DMSO was also included as a control and did not exhibit inhibition or signaling activity (not shown).

The ethanol fraction collected from P174act was subjected to HPLC analysis. To determine which peaks may contain Pld peptides. P174 Δ *pIdK* was used as a control to determine relevant peaks to collect for purification since Pld peptide production is abrogated in this mutant. Peaks absent in the chromatogram of

P174 Δ *pldK* but present in P174*act* represented potential peaks containing Pld peptides. Using this comparison, peaks 1-6 were collected and tested for inhibition and signaling. Two peaks, 3 and 6, were identified that contained PldA1 and PldA3 based on MS/MS analysis (Fig 3.3,B1,B9). The fraction containing PldA1 was able to activate the reporter strain but could not inhibit the sensitive strain. The fraction containing PldA3 was able to inhibit a sensitive strain, albeit at a higher concentration than expected, but this substance could not upregulate the reporter strain. The specialized roles determined for each purified Pld peptide was in direct contrast to what was previously deduced using information from the individual peptide deletions [136].

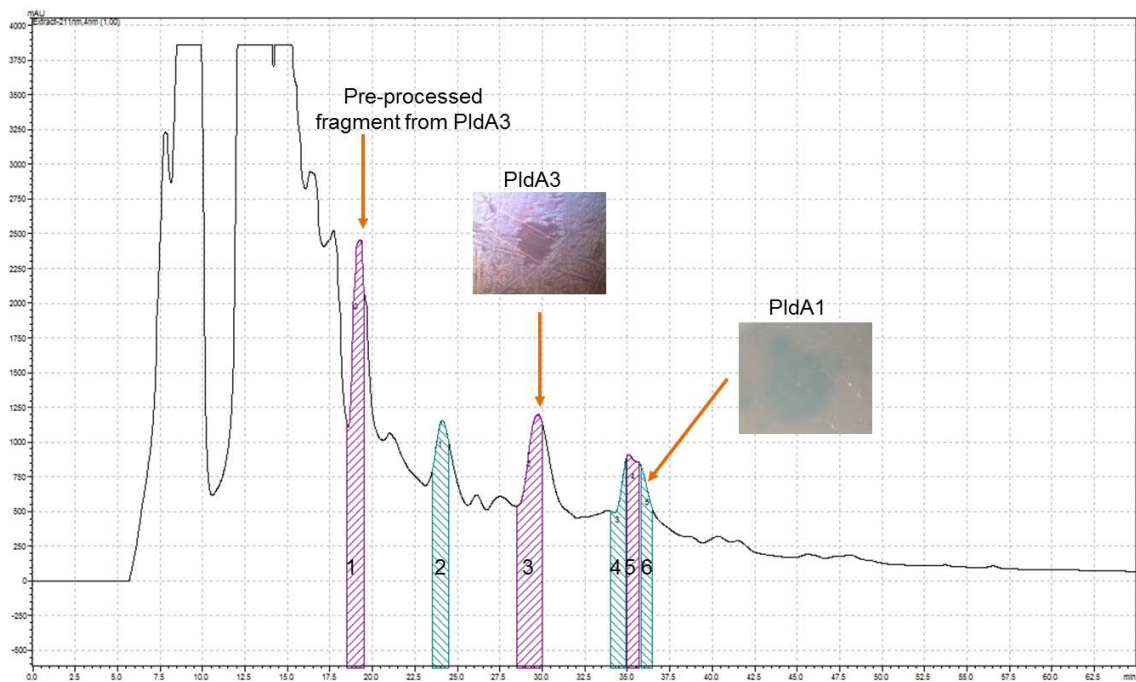


Figure 3.3 HPLC purification of Pld peptides and bioactivity. Reverse-phase HPLC separation of the peptides was done using a C-8 column. Each numbered peak represents a fraction that was collected and assayed for inhibition or signaling by pipetting 5 μ l spots of a 0.1mg/ μ l starting concentration dissolved in DMSO to either a lawn of a Pld sensitive strain, TGR4, or a reporter strain, P174*pldM-lacZ*, respectively. Peaks in which PldA3 and PldA1 were found are shown by arrows along with an inset depicting their bioactivity. Additionally, a

fragment containing the first eight N-terminal amino acids was found in peak 1 but did not contain activity.

Purified PldA1 was unstable as attempts to quantify the activity of PldA1 failed and activity could not be reproduced. Additionally, upon dilution of PldA3 to determine the MIC, inhibition was seen only at a two-fold dilution and activity was lost at higher dilutions. The instability and low activity prevented any synergistic activity experiments.

3.2.3 PldA1 and PldA3 are structurally similar and found to undergo a secondary processing event

Structures of PldA1 and PldA3 were elucidated using extensive 1D/2D NMR and HRMS/MS studies (Appendix B). The predicted amino acid sequence of PldA3 acted as preliminary template towards the primary structure of the peptide but the monoisotopic mass of $[M - 3H_2O + H]^+$ was observed to be 2511.3772 Da, suggesting further modifications. We proceeded with MS/MS mass spectrometry data for initial characterization, which suggested that PldA3 was truncated eight amino acids (between lysine and methionine) after the predicted leader sequence cleavage site. The truncated N-terminus of PldA3 was detected by MS/MS in peak 1 during HPLC purification, suggesting that a secondary processing event is occurring outside of the cell (Figs.B3 and 3.3).

To determine structures, NMR spectroscopic data were obtained for PldA3 (Fig.B4-8). In addition, HSQC spectra unambiguously established no aliphatic unsaturation by absence of NMR signal in pre-requisite spectral region of 5.0 – 6.5 ppm, confirming the absence of Dha and Dhb residues in PldA3(Fig. B6).

The Lan/MeLan rings in PldA3 were established with short range NOESY, (Fig.B7). The C-terminal MeLan spin system S--Ala24-His23-Val22-Asn21-Asn20-Abu19—S was established based on NOE observed between assigned downfield Ala24-H α (4.17 ppm) and Thr18- β (3.38 ppm) along with correlation between NH doublet at 7.25 ppm, CH singlet at 6.71 ppm in His23 ring residues and Thr18-H β proton at 3.79 ppm. Moreover, the largest MeLan ring in PldA3 between S-Ala17 and Abu2 was assigned through an unequivocal NOE observed between Thr18- β (3.38 ppm) and CH₃ singlet at 1.73 ppm in Met1; the spin system also showed distinct connectivity between Ala17-H α (4.18 ppm), NH17 (7.74 ppm) and CH₃ doublets in Leu3 (1.10 and 0.99 ppm).

A similar experimental approach using HR MS/MS and extensive 1D/ 2D NMR was followed to determine the structure of PldA1(Fig. B9-13). Pld A1 showed a high resolution time of flight electrospray ionization mass spectrometry (ESIMS) [M-2H₂O+H]⁺ ion peak at m/z 2610.4430. Again, the observed m/z for PldA1 was less than calculated m/z of mature peptide, indicating truncation in PldA1 apart from predicted leader peptide cleavage side, which corresponded to removal of the first 7 amino acids at the N-terminus. As in the case of PldA3, fragmentation between amino acids situated within the Lan and MeLan rings is typically not observed by MS/MS, enabling us to map out topological ring systems in PldA1. Interestingly, unlike PldA3, here all the Cys residues were observed to be involved in forming Lan/ MeLan rings. All Lan/MeLan rings were confirmed with NOESY (Fig.B13).

To affirm the MS/MS fragmentation and structural obscurities, 2D gHSQCAD, gCOSY and NOESY spectrums were recorded in CD3OD -d6 (Fig. B11-13). Interestingly, PldA1 contained an isopeptide linkage between Glu16 and Lys23 which was confirmed by NOESY and MS/MS (Fig. B9 & B13). Although the presence of an isopeptide in a lantibiotic peptide has not been described before, it may be important in maintaining the correct shape and protein stability which is needed for PldA1's role in signaling. The isopeptide bond has been shown to be important for providing thermal stability and protection against proteolysis in other proteins such as pili [137]. Furthermore, there were no chemical shifts observed within the expected spectral region of 5.0-6.5 ppm in gHSQCAD, ruling out the possibility of any dehydration of Thr14 and Thr18 for completing the structure of PldA1 (Fig. B11).

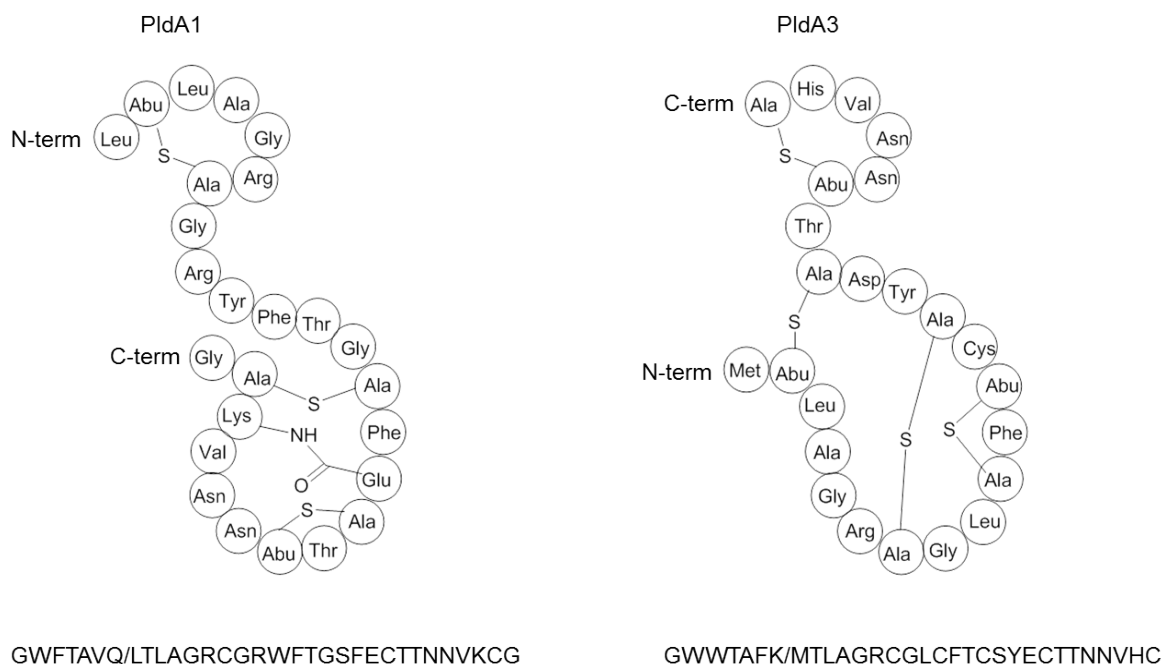


Figure 3.4 Deduced structures of PldA1 and PldA3. The structures of both PldA1 and PldA3 are shown above and resemble type-B lantibiotics. Both peptides contain a single lanthionine (Ala-S-Ala) and either two methyllanthionine

rings (Abu-S-Ala) in PldA1 or three methyllanthionines in PldA3. PldA3 contains a single unreactive Cys.

3.3 Discussion

This work describes the structural characterization of two pneumolancidin peptides, PldA1 and PldA3, which are secreted by a clinical isolate of pneumococcus, P174. We had previously shown using genetic analysis that PldA3 is required for upregulation of the *pld* locus because strains with a PldA3 deletion in either the wildtype P174 or P174act background lacked evidence of signaling or inhibition. Genetic analysis also suggested that PldA1 was required for inhibition as a *pldA1* knockout in the P174act background lacked evidence of inhibition, but retained the ability to upregulate the locus of the reporter strain [133]. We hypothesized that gene dosage might be playing a role in how these peptides function because PldA1 and PldA2 are nearly identical in sequence. The intergenic region upstream of *pldA2* was retained in the construction of the PldA1 duplication to eliminate any possibility that the intergenic region might be important in the difference of function. There are examples of lantibiotics in which the ratios of peptides is controlled by gene dosage, and is important for their biological activity. For example, *Bacillus cereus* SJI encodes three precursor lantibiotic peptides named bicereucin [138]. Two of the peptides, BsjA1 and BsjA3, are identical in sequence, and were found to exhibit synergistic antimicrobial activity with BsjA2 in a 2:1 ratio [138]. Hence, two copies of BsjA1 are needed to form the optimal ratio needed for antimicrobial effect. Given this

example from a similar system, we thought gene dosage may explain the requirement for all three Pld peptides in the WT background. PldA1 and PldA2 differ by only two amino acids, F15Y and T19S, in the active peptide sequence with F15Y representing a conserved change in overall shape of the two amino acids. A deletion of the gene encoding either PldA1 or PldA2 in a P174 background resulted in a strain that did not signaling activity. We reasoned that if gene dosage was the explanation for the requirement for both PldA1 and PldA2 rather than any unique function for the peptides, increasing the amount of PldA1 in a strain deficient in PldA2 would restore the strain to wildtype function. However, signaling was not restored in the $\Delta pldA2$ strain expressing the PldA1 duplication indicating that gene dosage is not the explanation for the requirement for both peptides. We are currently creating a *pldA1* duplication in the P174act background. We hypothesize that this strain should look identical to the *pldA2* deletion strain in this background with wildtype inhibition and signaling.

Compared with the single *pldA1* or *pldA2* mutations in the P174act background, a deletion of both *pldA1* and *pldA2* resulted in a complete loss of activity supporting the hypothesis that complex formation may needed for recognition by PldK [133]. Specifically, PldA3 might require either PldA1 or PldA2 to form a signaling complex. When examining the function of the purified peptides, PldA3 alone inhibits a Pld sensitive strain but does not signal our reporter strain (Fig.3.3). Conversely, purified PldA1 upregulates the *pld* locus in the reporter strain but lacks evidence of inhibition (Fig. 3.3). Based on the instability of purified PldA1, this would suggest the role of PldA3 as a stabilizing

factor for PldA1 and possibly PldA2. Without PldA3, PldA1 is unstable and cannot signal the reporter strain nor inhibit which is the case when *pldA3* is deleted in both P174 and P174act [133]. Since we were not able to recover purified PldA2, it was not possible to determine its role in either signaling or inhibition. PldA2 seems to play a less important role in inhibition as compared to PldA1 based on the loss of inhibition that is observed when PldA2 and PldA3 are expressed but not when PldA1 and PldA3 are expressed. Although, the PldA1 duplication replacing PldA2 was not sufficient to recover inhibitory activity it may indicate that relative ratios of each peptide may be important in forming a functional complex. Currently, we do not know the expression levels of these Pld peptides or the stoichiometric requirement of these complexes. Additionally, it is unknown if PldA3 can inhibit in complex with PldA1, or if the formation of this complex is just needed for signaling. Another possibility is that PldA3 may be required to stabilize either PldA1 or PldA2 or both. In the absence of PldA3 in either P174 or P174act, inhibition and signaling is abrogated which may be explained by the fact that without PldA3, PldA1 and/or PldA2 are unstable and therefore, nonfunctional (Fig. 3.1). This may also be the reason why purified PldA1 is unstable. In the future, we plan to study the stability of PldA1 with and without PldA3.

Since lantibiotics are known to exert an antimicrobial effect at even nanomolar concentrations, it was puzzling that PldA3 was not able to inhibit at more than two fold dilution. One hypothesis might be that the secondary cleavage event was an artifact of the purification strategy and might not

represent the functional peptides *in vivo*. To address this issue, Laser Ablation Electrospray Ionization (LAESI) -MS will be used to analyze the lantibiotic peptides that are found in the zone of clearance of overlay plates. A secondary cleavage event was not predicted because the *pld* locus does not encode a second protease that would be predicted to cleave beyond the typical GG motif recognized by PldT during export. Some *lan* loci do not encode either a LanT- or LanP-like protease. Instead, proteolytic cleavage is achieved by co-opting a protease not encoded by the *lan* locus. In the case of subtilin, three different serine proteases not encoded in the subtilin locus, were able to cleave the leader peptide [17]. Other *lan* loci encoding type II lantibiotics, such as cytolysin and bicereucin, encode a dedicated protease in addition to the ABC transporter containing a peptidase domain [60, 138]. To identify a protease, a PCR of P174 was performed to detect the presence of a surface exposed serine protease named **Subtilase Family Protein (SFP)**. SFP was identified in D39 and was found to play a minimal role in virulence [139]. However, PCR did not detect the presence of the gene encoding SPF in P174 suggesting that there might be another protease present. Genomic sequencing of P174 is currently being performed which may help to identify other proteases that might contribute to PldA processing.

The structures of PldA1 and PldA3 resemble the globular lantibiotics of the type-B classification similar to the well characterized lantibiotic, mersacidin. Mersacidin binds to lipid II but does not form pores in membranes. PldA3 might also be able to bind lipid II but further experiments are needed to confirm

mechanism of action. Interestingly, the Pld peptides play specialized roles despite sharing similar structures. Site directed mutagenesis will need to be done to identify residues that are required for signaling and those required for inhibition. PldA3 contains an extra methyllanthionine that may be important for inhibition. Interestingly, PldA1 contains an isopeptide bond that has been found in other proteins to play an important role in maintaining protein shape and rigidity [137]. It is possible that the isopeptide bond is needed for the correct structure to interact with PldK and would be a likely candidate for mutagenesis. It is also possible that the methyllanthionine ring in PldA1 which is replaced by a lanthionine ring in PldA2 might play an important role in antimicrobial activity. Despite similar structures, the Pld peptides are specialized and able to perform diverse functions, reminiscent of the Enterococcal lantibiotic, cytolysin. Cytolysin consists of two peptides, Cyl_L" and Cyl_S", that form an inactive complex when target cells are absent. When bacteria encounter target cells, Cyl_L" has a higher affinity for membranes than Cyl_S". Cyl_L" binding frees up Cyl_S" which is then able to activate the cytolysin complex.

We believe that the Pld peptides are also able to form complexes with each other, which contributes to their specialized activity despite similarity in structures. We present a model in which the Pld peptides are forming complexes for bioactivity and increased stability of PldA1 and PldA3. When PldA1 and PldA3 are in complex together, inhibition of sensitive cells and upregulation of the locus is able to occur. Although PldA2 was not recovered and bioactivity of PldA2 is

unknown, it is possible that PldA2 also forms a complex with PldA3 that is needed for signaling but not for inhibition.

This represents a novel strategy for producing specialized, highly homologous lantibiotic peptides that may prove useful in the development of new antimicrobials.

3.4 Materials and Methods

Bacterial strains, plasmids, and growth conditions.

All pneumococcal strains and plasmids used are described in Table 3.1. All primers used are described in Table 3.2. All pneumococcal strains were plated on either 5% sheep's blood agar (SBA) or tryptic soy agar (TSA) plates with 0.5% catalase (Worthington, Lakewood, NJ) (4,741 U) and incubated at 37°C with 5% CO₂. For growth in liquid culture, all pneumococcal strains were grown in Todd-Hewitt broth supplemented with 0.5% yeast extract (THY). Antibiotics were added when indicated in the following amounts: 500 µg/ml kanamycin and 100 µg/ml streptomycin. *Escherichia coli* strains were grown in Luria-Bertani (LB) broth or LB agar and supplemented with 50 µg/ml of kanamycin when indicated.

Table 3.1. List of strains and plasmids used.

Strain	Description	Source
P174	Clinical isolate from South Africa, contains <i>pld</i> locus; Erm ^R , Chl ^R , Neo ^R	[93]
P174act	P174 containing a single base pair mutation T->C in the intergenic region upstream of <i>pldF</i>	[136]
P174strep ^R	P174 backtransformed with lysates of a strep ^R strain to confer strep resistance	
P174 <i>pldM-lacZ</i>	P174 with <i>lacZ</i> fused to <i>pldM</i> and used	

	as reporter to detect the presence of exogenous peptides	
TGR4	Pld sensitive strain	
P174 <i>pldA1-4::Janus</i>	P174 with Janus replacing peptide region of the <i>pld</i> locus; Kan ^R , Strep ^S	
P174 <i>pldA1pldA2::Janus</i>	P174 with Janus replacing <i>pldA1pldA2</i>	In this study
P174act <i>pldA1pldA2::Janus</i>	P174act with Janus replacing <i>pldA1pldA2</i>	In this study
P174 <i>pldA1+ΔpldA2::pldA1</i>	P174 containing a <i>pldA1</i> duplication in place of <i>pldA2</i>	In this study
P174Δ <i>pldA1ΔpldA2</i>	P174 containing a deletion of <i>pldA1pldA2</i> along with the intergenic region between <i>pldA1</i> and <i>pldA2</i>	In this study
P174actΔ <i>pldA1ΔpldA2</i>	P174act containing a deletion of <i>pldA1pldA2</i> along with the intergenic region between <i>pldA1</i> and <i>pldA2</i>	In this study
Plasmids	Description	Source
E115	pCR2.1 TOPO vector containing the <i>pldA1-4</i> region with <i>pldA1</i> deleted using inverse PCR	[136]
E123	pCR2.1 TOPO vector containing the <i>pldA1-4</i> region with <i>pldA2</i> deleted using inverse PCR	
E186	pCR2.1 TOPO vector containing 500bp up and downstream of <i>pldA1-4</i> region but with <i>pldA1pldA2</i> deleted	In this study
E187	pUC57 containing <i>pldA1</i> duplication in place of <i>pldA2</i> along with 500bp upstream and downstream	In this study

Table 3.2 List of primers used.

Primer #	Sequence	Description
1	5'- GGCCGCTCCCGGGATCCGTTTGATTTTAAATGGAT AAT-3'	Forward primer for amplification of Janus cassette and adds a SmaI site
2	5'-ACCTCCCGGGCCCCTTTCCTTATGCTTTTGGAC- 3'	Reverse primer for amplification

		of Janus cassette and adds a SmaI site
3	5'-GGTGTGGACTTTGAAGAACA-3'	Forward primer 500 bp upstream of Pld peptide region
4	5'-GAGAAGTGTTGTTTTCCAC-3	Reverse primer 500 bp upstream of Pld peptide region

*Construction of *pldA1* duplication and *pldA1pldA2* deletion strains*

To create a duplication of *pldA1*, a strain containing a Janus cassette replacing *pldA1* and *pldA2* was made first. The Janus cassette contains a kanamycin resistance marker (*kan*) and a counterselectable *rpsL+* marker and is used as genetic tool to construct allelic replacements. P174 *pldA1pldA2::Janus* was made by digesting plasmid E115 and E123 with SmaI. E115 contains the *pldA* region along with 500bp up and downstream DNA and has a *pldA1* deletion that was created through inverse PCR and contains a SmaI site in between the start and stop codon of *pldA1*. E123 is like E115 but *pldA2* has a SmaI site in between start and stop codon. Both plasmids were digested with BamHI and SmaI site. The digest released product from E123 was used to ligate into the digested plasmid E115 so that the upstream region of *pldA1* was included and the intergenic region between *pldA2* and *pldA3* creating plasmid E186. The Janus cassette from P174 *pldA1-4::Janus* was amplified using primer pair 1 and 2 adding a SmaI site on either end of the Janus. The SmaI digested Janus and

E186 were ligated together and transformed into P174strep^R creating P174 *pldA1pldA2::Janus*.

A DNA fragment of 1500bp was synthesized by GenScript containing 500bp upstream of P174's *pldA1* and 500bp downstream of *pldA2*. The ORF encoding *pldA2* was replaced with *pldA1* and the intergenic region between *pldA1* and *pldA2* was unchanged. This DNA fragment contained two copies of *pldA1* and was ligated into pUC57 creating E187. The resulting plasmid E187 was transformed into P174 containing a Janus cassette replacing both the *pldA1* and *pldA2* genes. Allelic replacement of the Janus was confirmed with primer pair 3 and 4.

For construction of the *pldA1pldA2* deletion, plasmid E186 was transformed into either P174*pldA1pldA2::Janus* or P174act*pldA1pldA2::Janus*. Confirmation that the Janus cassette had been replaced was determined with primer pair 3 and 4 as well as sequencing.

Inhibition and signal secretion overlay assays

Overlays assays used to detect inhibition and signaling were performed as described previously [133].

Organic resin extraction

Since Pld peptides are only expressed when grown as stabs on agar plates, a biphasic growth system was used for purification. P174act was stabbed into plates and 30ml of growth media, THY, was overlaid on top of the plates. Following incubation overnight at 37 °C and 5% CO₂, media was collected and centrifuged. Supernatants were filtered sterilized using a 0.22 µm size filter. XAD-

16 resin was added to supernatants to extract organic material from the media and incubated with shaking overnight. Three sequential washes ranging in polarity, starting with 80% methanol, ethanol with 0.1% TFA, and propanol were used to elute lantibiotic peptides from the resin. Washes were collected and dried using a rotary evaporator and pH was maintained between 4 and 5. Dried extracts were then resuspended in DMSO at a concentration of 20 mg/ml. Two fold serial dilutions were made in DMSO and 5µl spots were made on plates containing a lawn of a sensitive strain, P250, or a reporter strain, P174*pIdM-lacZ*, that responds to exogenous PId peptides along with X-gal.

HPLC analysis of Ethanol fraction

After determining that ethanol with 0.1% TFA contained the highest specific activity, fractions were dried down using a rotary evaporator. The dried fraction was dissolved in HPLC grade methanol at a concentration of 50 mg/µl and 50 µl was injected into the HPLC using a reverse phase Luna 5 µm C8 (2) 100 Å 50 × 1.0 mm column. The solvent used was H₂O with 0.1% TFA (A) and acetonitrile with 0.1% TFA (B). A linear gradient of 30% to 40% of solvent B in 53 minutes at a flow rate of 6 ml/min was used for separation. To identify peaks that contained possible PId peptides, fractions collected from a isogenic nonproducer strain, P174Δ*pIdK*, was also analyzed. Peaks present in the chromatogram of P174act but absent chromatogram of P174Δ*pIdK* were chosen for bioactivity analysis. Peaks collected were dried down and dissolved in DMSO at a final concentration of 0.1mg/µl. Samples were tested by pipetting 5µl on plates

containing a lawn of a sensitive strain, P250, or a reporter strain, P174*pIdM-lacZ*, that responds to exogenous PId peptides along with X-gal.

MS/MS and NMR

Mass spectrometric analysis of extracts was performed using an Agilent 6520 Q-TOF mass spectrometer equipped with an Agilent 1290 HPLC system, maintained at the University of Michigan core facility in the Department of Chemistry. LC for mass spectrometry was conducted using Luna 5 μm C8 (2) 100 Å 50 × 1.0 mm column and a solvent system of methanol (w/ 0.1% FA) and H₂O (w/ 0.1% FA) at flow rate of 0.3 mL/ min with linear gradient from 30% B to 90% B in 11 min. Preliminary LCMS analysis of HPLC fractions was performed on a Shimadzu 2010 EV ESI spectrometer.

All NMR spectra were acquired on a Varian INOVA 600 MHz at the NMR core facility in Life Sciences Institute University of Michigan using DMSO-d₆ solvent.

3.5 Notes

This work was done in collaboration with Drs. David Sherman and Ashootosh Tripathi at the University of Michigan.

CHAPTER IV

DISCUSSION

Introduction

Streptococcus pneumoniae is subject to competitive interactions in the nasopharynx. Since colonization is a prerequisite for pathogenesis, understanding the competitive interactions of pneumococcus among other co-colonizing bacteria is important in identifying factors that enhance the likelihood of a successful colonization. One factor important in pneumococcal competition is the production of antimicrobial peptides. The Blp and Cib peptides are two well-described examples of unmodified bacteriocins that pneumococcus secretes to eliminate competitors [90, 93, 96, 106]. Additionally, production of the Blp peptides is upregulated when competence is initiated, indicating that, in addition to removing competitors, DNA released during bacterial lysis can be used for recombination [140, 141]. Together these activities, the elimination of competitors and subsequent uptake of DNA, has made pneumococcus a successful, adaptable pathogen. Although vaccines and antibiotics are used to remove the more pathogenic pneumococcal strains from the population, this has only provided an opportunity for other pneumococcal strains to colonize and cause disease [84].

Possible alternatives that are being investigated for eradicating pathogenic bacteria include the use of a modified bacteriocin or lantibiotic. Unlike unmodified bacteriocins, lantibiotics undergo posttranslation modifications that make them resistant to proteolytic degradation, thermal inactivation, and provide stability at low pH [142]. These biochemical properties make them useful to pursue as a therapeutic. Therefore, we became interested in finding a lantibiotic that would demonstrate potent anti-pneumococcal activity. Although several lantibiotic loci have been described in pneumococcus, none have been associated with antimicrobial activity until recently.

My thesis work has been centered around characterizing the pneumococcal lantibiotic, pneumolancidin (*pld*), secreted by P174 using a comprehensive approach that involved careful genetic and biochemical dissection of the function of genes in the locus, described in chapter II and III, respectively. The *pld* locus had broad spectrum inhibitory activity, including the ability to kill 54 out of 55 pneumococcal strains, making it an attractive candidate for future development as a therapeutic.

Notably, four homologous *pld* peptides were found in tandem. A single modification enzyme encoded by the gene *pldM* was also found on the locus. The presence of a single modification enzyme with multiple lantibiotic peptides is different from the previously described two peptide lantibiotics. For most two peptide lantibiotics, two different modification enzymes are encoded that separately modify each peptide [28]. The sequence homology between the LanA1 and LanA2 is typically low, and therefore, the requirement for two different

LanM enzymes, LanM1 and LanM2, is necessary to have both peptides properly modified [28, 57]. After processing and cleavage, the peptides are functional yielding Lan α and Lan β . As expected, the structures of Lan α and Lan β are different from each other and are required for their specific role in inhibition of sensitive strains. Lan α typically resembles type-B lantibiotics like mersacidin, and binds to lipid II, an important bacterial cell wall precursor. Lan β resembles type-A lantibiotics like nisin, and forms pores upon recognition of the complex between Lan α and lipid II [30]. In other multipeptide lan systems in which a single *lanM* is encoded in the locus, the lan peptides are identical in sequence suggesting similar structures with redundancy in the peptide's function. The *pld* locus did not express the same characteristics as of other multipeptide lantibiotic systems that have been described. Rather than expressing several identical peptides, the amino acid sequences of PldA1-4 were found to be similar but not identical in the context of a single *pldM* gene, indicating that there might be discrete functions of these Pld peptides.

Since lantibiotic peptides are known to function as both antimicrobials and as autoinducers, the aim of my dissertation was to dissect the individual the roles of the putative Pld peptides A1-4 using both a genetic approach (chapter II) and a biochemical approach (chapter III).

The role of the pld peptides

In the WT setting, it was found that PldA1-3 were each required for signaling with the exception of PldA4 (Fig. 2.2).. This suggested that Pld peptide

A1-3 were each required for upregulation of the locus, perhaps forming a complex that is recognized by the histidine kinase, PldK. The requirement for complex formation for signaling is unprecedented in other lantibiotic systems.

Luckily, a serendipitous mutant of P174 was discovered which was characterized by a lowered threshold for activation of the *pld* locus (Fig.2.4). A single base pair mutation was found in the intergenic region between *pldA4* and *pldF* that resulted in downregulation of the immunity transporter presumably due to the disruption of the -10 RNA polymerase binding site, leading to activation of the *pld* locus at lower peptide concentrations (Fig. 2.3). Peptide deletions were assayed in this background and found to have distinct phenotypes (Fig 2.2). PldA1 was implicated in inhibition, PldA2 was found to be dispensable for inhibition and signaling, and PldA3 was required for signaling and possibly for inhibition (Fig 2.2).

While the peptide deletions in the P174act background helped to provide clues as to the function of each of the Pld peptides in signaling, we sought to purify the individual peptides to clarify the specific role of each peptide in both signaling and inhibition. We also wanted to assess the bioactivity of purified Pld peptides and elucidate their structures. Using HPLC, we were only able to purify PldA1 and PldA3. Based on our genetic studies, we predicted that PldA1 would kill sensitive strains and PldA3 would be the autoinducer although data from the wildtype strains suggested complex formation was likely to be vital. The activities of purified PldA1 and PldA3 were somewhat surprising. A preparation of PldA1 only had evidence of signaling but not inhibition, while a preparation of

PldA3 only had evidence of inhibitory activity but not signaling. Both preparations were active only at very high concentrations that are unlikely to be physiologic.

One explanation for difference in activity between purified Pld peptides and the phenotypes of the peptide deletion strains, might be that PldA3 helps to stabilize PldA1 and PldA2 through formation of a complex. In the absence of PldA3 in P174act, signaling is abrogated even in the presence of PldA1 which was found to signal in its purified form (Fig 2.2 & 3.3). If PldA1 is unstable without PldA3, it would make sense that P174act Δ pldA3 is unable to signal or inhibit. PldA1 is unstable and cannot upregulate the locus including expression of PldA3, which is needed for inhibition. In summary, PldA3 is necessary for signaling and requires the presence of either PldA1 or PldA2 presumably through the formation of a complex which stabilizes PldA2 and PldA1. When PldA3 and PldA1 are expressed by P174act, inhibition occurs (Fig 2.2). In contrast, expression of PldA3 and PldA2 does not result in inhibitory activity (Fig. 2.2). This suggests that PldA2 is not as important for inhibition compared to PldA1. This is surprising because of the high sequence similarity between PldA1 and PldA2, differing by only two amino acids. Specifically, T19S, is involved in the formation of a methyllanthionine ring in PldA1 which would be replaced by a lanthionine ring in PldA2. This may account for their difference in activity. However, since PldA2 was not purified, we cannot state this with certainty nor determine the function of PldA2 directly. Supernatants that were collected from biphasically grown P174act were able to signal and inhibit months after being collected suggesting that either

having all the Pld peptides together is important for stability or that the crude preparation has other stabilizing factors. Additionally, when concentrating supernatants it was noted that activity was retained above a 50kDa cut off column. This suggests that the Pld peptides are forming complexes of very high molecular weights. To support the theory of a complex being formed between PldA1 and PldA3 that is needed for stability, purified PldA1 and PldA3 will be combined and tested for signaling and inhibition over time as compared to Pld peptides alone. If complex formation is needed, then we should expect to see bioactivity over longer periods of time and more potent inhibition than PldA1 or PldA3 alone.

Another explanation for the lack of inhibition seen in P174act Δ pldA1 could be that a complex is formed between PldA2 and PldA3, which is needed for signaling but not for inhibition. This would be reminiscent of the cytolysin system in which both peptides form a complex that is inactive. In the presence of target cells, the larger subunit is able to bind to eukaryotic membranes freeing up the smaller peptide that is able to activate the cytolysin locus [60-62]. In strains expressing only PldA3, inhibitory and signaling activity were abrogated which provides evidence in support of PldA3 most likely forming a complex under *in vivo* conditions that is needed for signaling or inhibition if in complex with PldA1 (Fig. 3.1).

All of this data seems to suggest that the role of the Pld peptides is more complex than originally expected and represents a unique strategy for specialization of function described for lantibiotic peptides. Interestingly, the

structures of PldA1 and PldA3 both contained globular domains on opposite ends of the peptides, and both had different bioactivity. It can be hypothesized that PldA2 most likely resembles PldA1 structurally and yet is not as important for inhibitory activity as compared to PldA1. This indicates that the two amino acids that are different between PldA1 and PldA2 are enough to confer specificity although site directed mutagenesis will have to be done to verify this hypothesis.

The sizes of the Pld peptides were found to be smaller than predicted and the cleaved N-terminal portion was identified in culture supernatants after HPLC separation (Fig. 3.3). These data suggest that a secondary cleavage event is occurring after removal of the N-terminal leader peptide by PldT. It is possible that the secondary cleavage event is an artifact of the purification process and is contributing to the instability of the peptides. The *pld* locus does not encode an additional protease in addition to PldT but it may be able susceptible to another protease for secondary cleavage. This has been described in other lantibiotic systems [17]. To answer this, genomic sequencing of P174 is underway to identify possible proteases. Additionally, to show that secondary processing is occurring *in vivo*, LAESI-MS will be used to detect the presence of doubly processed Pld peptides in the halos surrounding stabbed P174 in the inhibition overlay assay. This will also provide verification that the purification process is not responsible for the secondary cleavage of the lantibiotic peptides.

The role of the immunity transporter in regulation of pld locus

It is interesting and counterintuitive that decreased expression of an immunity ABC transporter needed for protection against the Pld peptides leads to an increase in the activation of the *pld* locus (Fig 2.4). In a WT background, it was shown that expression of *pldFE* is upregulated in response to exogenous Pld peptides which is absent in the P174act background following induction (Fig 2.4). One possible theory to explain how decreased expression of PldFE lowers the threshold for signaling of the *pld* locus is that PldFE may interact with PldK to initiate upregulation of the *pld* locus upon binding of exogenous *pld* peptides.

Some preliminary data that suggest that PldFE is involved in signaling is the fact that a deletion of *pldFE* resulted in a strain with a loss of inhibition and signaling activity. If PldFE was involved in immunity alone, then a deletion of *pldFE* should have resulted in a lethal mutation and no transformants should have been recovered. However, P174 Δ *pldFE* was easily constructed. The lack of transcriptional activity in this strain suggested that the *pld* locus is transcriptionally inactive either because of the essential role of PldFE in signaling or because a secondary mutation arose preventing the locus from being upregulated. If a secondary mutation is responsible for inactivity of the locus, sequencing of the entire *pld* locus in P174 Δ *pldFE* will reveal a secondary mutation and perhaps details regarding the role of the mutation on the regulation of the *pld* locus. Instead of sequencing, complementation of *pldFE* can also be utilized to determine if activity can be restored with an ectopic copy of the transporter gene.

In other lan systems, immunity is mediated by two proteins [63, 73]. One being the immunity transporter, LanFEG, which is thought to act as an efflux pump to remove lantibiotic peptides from the membrane [63]. The second immunity protein is a lipoprotein, LanI, that binds to the lantibiotic and prevents it from interacting with the membrane [70, 73]. In the *pld* locus, we found a small ORF predicted to encode PldI downstream of *pldA4* (data not shown). To verify the role of PldI, a clean deletion of *pldI* was constructed and assayed for immunity. An immunity defect was observed in Δ *pldI* suggesting that PldI is involved in conferring immunity. This provides evidence that a deletion of PldFE may not result in a lethal mutation because of the presence of a second immunity protein, PldI. There is still an immunity defect even with deletion of either PldFE or PldI and this suggests that full immunity is reached with expression of both immunity proteins. In chapter II, it was shown that *Streptococcus mitis* is immune to P174 most likely because it has a region with high homology to PldKR and PldFE (Fig. 2.1 & Table 2.1). After discovery of PldI, we reviewed the locus in *S.mitis* and found it also contains *pldI* and the putative promoter sequence driving expression of *pldI* which means that minimal requirement for immunity is expression of the two component system, PldKR, and both immunity proteins, PldFE and PldI. It is possible other strains encode the same region to ensure immunity against Pld.

Further evidence supporting the theory that PldFE may interact with PldK is the homology between PldFE and a family of ABC transporters that mediate

antibiotic resistance, BceAB-type. In a homologous system, *Bacillus subtilis* expresses a bacitracin immunity transporter, BceAB, that confers protection by binding to bacitracin, a cyclic antimicrobial peptide [124]. The binding of BceAB to bacitracin is recognized by the histidine kinase, BceS, which increases transcription of the bacitracin immunity transporter [124]. Given the homology, it is possible that PldFE might also be involved in signaling in a similar way by forming a sensory complex with PldK. We hypothesized that since expression of the two component system PldKR is not differentially upregulated in response to exogenous Pld peptides its levels likely remain constant. Altering the levels of PldFE may change signaling dynamics by either increasing or decreasing the likelihood of the two complexes finding each other. In the P174act background, less PldFE is expressed. When exposed to equal amounts of basal Pld peptides, the strain producing less PldFE will have a greater percentage of PldFE/Pld peptide complex to interact with and activate PldK. In the case of P174, since there is more PldFE than PldK, a greater proportion of PldFE does not have Pld peptides bound and fails to activate PldK at low peptide concentrations. This mechanism may also be a way to limit uncontrolled upregulation of the *pld* locus. At high exogenous Pld peptide levels, which have saturated PldFE binding resulting in PldK activation, upregulation of the *pldFE* operon occurs ensuring that the ratio of bound to unbound PldFE prevents further activation of PldK. Future experiments should be performed to verify that PldFE can form a sensory complex with PldK by performing pull-down assays. Since the expression level of PldFE has been deduced using reporter fusions, it would be useful to confirm at

the protein level that downregulation of PldFE is occurring in P174act. Also, altering the expression of PldFE using an inducible construct would be helpful in determining if changing ratios of PldFE to PldK levels is sufficient to recapitulate the lowered threshold requirement for signaling. Ultimately, manipulation of PldFE might help increase output of Pld peptides and facilitate purification.

An alternative explanation for how levels of PldFE might affect regulation of the *pld* locus is by sequestering of Pld peptides. Immunity transporters are known to bind to lantibiotic peptides [72]. It is possible that low levels of PldFE frees up Pld peptides which are then able to interact with PldK . Therefore, less Pld peptides are needed for activation of the *pld* locus. This explanation would fit if the reason for inactivity of the *pldFE* KO is attributable to a secondary mutation.

Advantages of having a functional pld locus

With the homology shared between PldFE and BceAB, it would be interesting to assay P174's resistance to cyclic antimicrobial peptides such as bacitracin and nisin. Cross protection to other antimicrobial peptides mediated by PldFE could also be a reason for the maintenance of the *pld* locus in pneumococcal strains. In chapter II, a pneumococcal strain, P130, was identified as the only strain immune to Pld peptides. Although it has immunity, inhibitory activity was not seen in this strain due to a mutation disrupting the function of PldM indicating that P130 is a cheater strain (Fig. A4). This also suggests that producing Pld peptides is energetically costly and instead of inactivating the entire locus, immunity is retained. However, other pneumococcal strains were

found that carry the *p/d* locus but are not immune suggesting that the locus is inactive. Why do pneumococcal strains retain an inactive *p/d* locus? It is possible that there are other advantageous cargo genes carried on the same ICE as the *p/d* locus that are transmitted between strains.

After screening over 400 pneumococcal strains, only P174 has been shown to have Pld-mediated antimicrobial activity. Using a mouse model of staggered colonization, we demonstrated that having a functional *p/d* locus confers a competitive advantage in invading a previously colonized surface. An interesting potential use of a strain that produces anti-pneumococcal antibiotic would be as a probiotic strain to prevent other pathogenic pneumococcal strains from colonizing. Inoculation of a Pld peptide expressing probiotic would be most beneficial in populations that are at high risk of acquiring pneumococcal disease. Currently, lantibiotic expressing strains are being developed for use as a probiotic in the oral cavity, preventing other *Streptococcus mutans* from colonizing. However, additional experiments would be needed to verify the safety, efficacy, and length of the protective effect afforded by the probiotic Pld expressing strain.

Concluding remarks

Pneumococcus, a member of the diverse microbial community in the nasopharynx, is able to compete with other strains by secreting bacteriocins. Understanding the dynamics of bacterial competition will help to predict which

pneumococcal strains will dominate in the population. Since colonization is a prerequisite for pathogenesis, those strains able to dominate are more likely to cause disease. One way to combat these pathogenic strains is the use of bacteriocins ,specifically lantibiotics.

There are many advantages to using lantibiotics in treating bacterial infections. Lantibiotics have attractive biochemical properties that make them desirable as therapeutics. Additionally, lantibiotic production is a common strategy used in competition and therefore, has evolved to be highly effective against bacteria. The food industry has benefitted from the use of lantibiotic producing strains by adding them directly to food to prevent spoilage and colonization by pathogenic bacteria. Nisin, a well characterized lantibiotic, has been used in the food industry for the past 50 years without generating any nisin resistant strains. Therefore, we were interested in finding a potent anti-pneumococcal lantibiotic that could ultimately be used as therapeutic.

We found that a clinical isolate of *S. pneumoniae* secretes a lantibiotic, pneumolancidin, that exhibits unprecedented broad spectrum antimicrobial activity. With the exception of the *pld* encoding strain, P130, we have not yet identified an isolate of *S. pneumoniae* that is immune or resistant to the pneumolancidin producing strain. The locus encoding pneumolancidin was of unique interest because it contained a tandem array of four genes encoding the structural peptides. My dissertation was focused on dissecting the role of each of the Pld peptides. In both chapters II and III, the interaction and function of these Pld peptides were shown to be more complex than originally predicted. The

discovery of the *pld* locus in several pneumococcal strains represents a unique strategy for competition. Our studies involving the Pld peptides will shed further light into how bacteria are able to shape the microbial community through production of these antimicrobial peptides. Additionally, a greater understanding of the function and structure of these Pld peptides will also aid in the development of future antimicrobials to combat pneumococcal infections.

APPENDICES

APPENDIX A

Supplemental figures for Chapter II.

Table A1. Description of pneumococcal and streptococcal strains used in inhibition overlay assays.

Bacteria	Strain	Description	Inhibition by P174	Reference
<i>Streptococcus pneumoniae</i>	ATCC700669	clinical isolate pandemic strain	Y	
	A74	University of Michigan clinical isolate collection	Y	This study
	B56	University of Michigan clinical isolate collection	Y	This study
	B59	University of Michigan clinical isolate collection	Y	This study
	D69	University of Michigan clinical isolate collection	Y	This study
	P124	South African collection-colonizing isolate	Y	[93]
	P125	South African collection-colonizing isolate	Y	
	P126	South African collection-colonizing isolate	Y	
	P127	South African collection-colonizing isolate	Y	
	P128	South African collection-colonizing isolate	Y	
	P129	South African collection-colonizing	Y	

	isolate	
P130	South African collection-colonizing isolate	N
P131	South African collection-colonizing isolate	Y
P132	South African collection-colonizing isolate	Y
P133	South African collection-colonizing isolate	Y
P134	South African collection-colonizing isolate	Y
P135	South African collection-colonizing isolate	Y
P136	South African collection-colonizing isolate	Y
P137	South African collection-colonizing isolate	Y
P138	South African collection-colonizing isolate	Y
P139	South African collection-colonizing isolate	Y
P140	South African collection-colonizing isolate	Y
P141	South African	Y

		collection-colonizing isolate	
P143		South African collection-colonizing isolate	Y
P144		South African collection-colonizing isolate	Y
P146		South African collection-colonizing isolate	Y
P147		South African collection-colonizing isolate	Y
P148		South African collection-colonizing isolate	Y
P149		South African collection-colonizing isolate	Y
P151		South African collection-colonizing isolate	Y
P152		South African collection-colonizing isolate	Y
P153		South African collection-colonizing isolate	Y
P154		South African collection-colonizing isolate	Y
P155		South African collection-colonizing	Y

		isolate	
	P156	South African collection-colonizing isolate	Y
	P157	South African collection-invasive isolate	Y
	P158	South African collection-invasive isolate	Y
	P159	South African collection-invasive isolate	Y
	P160	South African collection-invasive isolate	Y
	P161	South African collection-invasive isolate	Y
	P162	South African collection-invasive isolate	Y
	P163	South African collection-invasive isolate	Y
	P164	South African collection-invasive isolate	Y
	P165	South African collection-invasive isolate	Y
	P166	South African collection-invasive isolate	Y
	P167	South African collection-invasive isolate	Y
	P168	South African collection-invasive isolate	Y
	P169	South African collection-invasive isolate	Y
	P170	South African collection-	Y

		invasive isolate		
	P171	South African collection-invasive isolate	Y	
	P172	South African collection-invasive isolate	Y	
	P173	South African collection-invasive isolate	Y	
	P175	South African collection-invasive isolate	Y	
	P176	South African collection-invasive isolate	Y	
	P177	South African collection-invasive isolate	Y	
<i>Streptococcus pyogenes</i>	HSC5	Emm 14.0	Y	[143]
	SF370	Emm 1.0	Y	[144]
	MEW18	Emm 89.0	Y	MEW
	MEW19	Emm 28.0	Y	MEW
	MEW20	Emm 1.45	Y	MEW
	JRS4	Emm 6.0	Y	[145]
	MGAS166	Emm 1.0	Y	[146]
	MEW34	Emm 161.0	Y	MEW
	MEW62	Emm 4.0	Y	MEW
	MEW96	Emm 89.0	Y	MEW
	MEW97	Emm 118.0	Y	MEW
SLC826	Emm 28.0	Y	MEW	
<i>Streptococcus agalactiae</i>	ATCC12386		N	
	ATCC12403	III	Y	
	UMMC GBS 632	Clinical isolate	Y	BF
	A909	Ia	N	[147]
	COH1	III	N	[148]
	DK14	Ib	N	[149]
	DK23	II	Y	[149]
	TIGR 18RS21	II	Y	[147]
	CNCTC 1/82	IV	N	[150]
	JM9	VIII	N	[151]
	TIGR H36B	IB	Y	[147]
NT-6	VI	N	[152]	
H1A	Ia	N	BF	

	87-603	VII	Y	[151]
	ATCC BAA 611	V	N	[147]

MEW= Michael E. Watson's personal collection

BF= Betsy Foxman's personal collection

Table A2. MLST analysis of the strains positive for *pldM* and *pldA1-4*.

Collection	Strain	Serotype	ST	<i>aroE</i>	<i>gdh</i>	<i>gki</i>	<i>recP</i>	<i>spi</i>	<i>xpt</i>	<i>ddl</i>	Accession number
South African	174	23F	6279	15	29	4	21	30	1	5	KT630265
	130	6B	6389	7	25	8	5	10	6	14	KT630266
Clinical isolates	A74	6B	2452	7	2	8	5	10	6	14	
	B56	NT	448	8	5	2	27	2	11	71	
	B59	NT	448	8	5	2	27	2	11	71	
	D69	NT	448	8	5	2	27	2	11	71	
Genome sequenced	PN1	6A	7151	7	13	8	6	6	6	494	ERR163220
	BHN418	6B	138	7	5	8	5	10	6	14	ASHP01000000
	MNZ14	NT	448	8	5	2	27	2	11	71	ASJO01000000

Table A3. Strains and plasmids used in this study.

Strains	Description	Reference or source
P174	Clinical isolate from South Africa, contains <i>lan</i> locus; Erm ^R , Chl ^R , Neo ^R	[93]
P130	Clinical isolate from South Africa, contains nonfunctional <i>lan</i> locus	[93]
P537	Serotype 6A with Janus replacing blpT to upstream of blpY	[93]
P1535	D39 with Janus inserted in <i>comD</i>	[125]
P147	Clinical isolate from South Africa, belongs to the same MLST as P174 but does not contain the <i>lan</i> locus	[93]
P650	R6 strain with Strep ^R	
P174 strep ^R	P174 backtransformed with lysates of P650 to confer strep resistance	In this study
P174 <i>pldA1-4::Janus</i>	P174 with Janus replacing peptide region of the <i>pld</i> locus; Kan ^R , Strep ^S	In this study
P174 Δ <i>pldK</i>	P174 with Spec cassette inserted in <i>pldK</i>	In this study
P174 Δ <i>pldT</i>	P 174 with Spec cassette inserted in 3' ABC transporter	In this study
P174 Δ <i>pldM</i>	P174 with Spec cassette inserted in <i>lanM</i>	In this study
P174 Δ <i>pldA1</i>	P174 with deletion of <i>pldA1</i> ; Strep ^R	In this study
P174 Δ <i>pldA2</i>	P174 with deletion of <i>pldA2</i> ; Strep ^R	In this study
P174 Δ <i>pldA3</i>	P174 Δ peptide 2; Strep ^R	In this study
P174 <i>pldA1-4EF::Janus</i>	P174 strep ^R with Janus replacing <i>pldA1-4</i> and <i>pldFE</i> ; Strep ^S Kan ^R	In this study
P174act	P174 containing a single base pair mutation T->C in the intergenic region upstream of <i>pldF</i>	In this study

P174act $\Delta pldA1$	P174 act containing in frame deletion of <i>pldA1</i>	In this study
P174act $\Delta pldA2$	P174 act containing in frame deletion of <i>pldA2</i>	In this study
P174act $\Delta pldA3$	P174 act containing in frame deletion of <i>pldA3</i>	In this study
P174act <i>pldM-lacZ</i>	P174act plasmid integration of E110 used as a reporter to test for the presence of active exogenous peptides	In this study
P174 $\Delta pldFE$	P174 with <i>pldFE</i> deletion, Strep ^R	In this study
P174 $\Delta pldA1-4 pldM-lacZ$	P174 <i>pldA1-4::spect</i> with <i>pldM-lacZ</i> fusion	In this study
P174 <i>pldM-lacZ</i>	P174 plasmid integration of E110 used as a reporter to test for the presence of active exogenous peptides	In this study
P174 <i>pldF-lacZ</i>	P174 with <i>pldF</i> fused to <i>lacZ</i> as result of intergration of E181,; Kan ^R	In this study
P174 P _{actpldF} - <i>lacZ</i>	P174 with plasmid integration of E182 resulting in the <i>lacZ</i> being fused to the promoter of <i>pldF</i> including the mutation from P174 act; Kan ^R	In this study
P174 act P _{174pldF} - <i>lacZ</i>	P174 act with plasmid integration of E181 resulting in P174's promoter of <i>pldF</i> fused to <i>lacZ</i> ; Kan ^R	In this study
P174 P _{pldA1} - <i>lacZ</i>	P174 with plasmid integration of E188 resulting in promoter of <i>pldA1</i> fused to <i>lacZ</i> ; Kan ^R	In this study
P174act P _{pldA1} - <i>lacZ</i>	P174act with plasmid integration of E188 resulting in promoter of <i>pldA1</i> fused to <i>lacZ</i> ; Kan ^R	In this study
Plasmids	Description	Source
E65	pEVP3 with kan ^R	In this study
E68	pUC19 with	In this study

	spectinomycin cassette inserted in place of ampicillin	
E93	TOPO vector with <i>pldA1-4</i> + 500 bp upstream and downstream of P174 cloned into MCS ; KanR	In this study
E97	500 bp middle region of <i>pldT</i> cloned into TOPO vector using primers 17 and 18	In this study
E98	500 bp middle region of P174 <i>pldK</i> cloned into TOPO vector using primers 15 and 16	In this study
E102	E68 with P174 <i>pldK</i> middle region cloned in MCS using EcoRI	In this study
E103	E68 with P174 <i>pldT</i> cloned into MCS using EcoRI	In this study
E105	Inverse PCR on E93 with primers 3 and 4 creating full deletion of <i>pldA1-4</i> and unique SmaI site	In this study
E110	E65 with PCR fragment of primer pair 24 and 25 cloned upstream of <i>lacZ</i> , reporter plasmid; Kan ^R	In this study
E181	pEVP3 with P174's <i>pldF</i> fused to <i>lacZ</i> , Kan ^R	In this study
E182	pEVP3 with P174 act's <i>pldF</i> fused to <i>lacZ</i> , Kan ^R	In this study
E188	pEVP3 with <i>pldA1</i> fused to <i>lacZ</i> , kan ^R	In this study

Table A4. Primers used in this study.

Primer #	Primer Sequence	Description
1	5'-GGTGTGGACTTTGAAGAACA-3'	500 bp upstream of peptide region
2	5'-GAGAAGTGTTGTTTTTCCAC-3'	500 bp downstream of peptide region
3	5'-GAGGCGCCCGGGCTATTCTTTGACAGGAGAT-3'	Forward primer for full peptide deletion contains SmaI site
4	5'-GAGGCGCCCGGGTAGATGATCATCTTACAGT-3'	Reverse primer upstream of peptide 1 contains SmaI site
5	5'-GACTTGCCCGGGTCATTAGCTTTTTAGTGA-3'	Forward primer to amplify janus cassette from P271
6	5'-GAATCCCCGGGGAGCACTTTGTAAGTCTGTTG-3'	Reverse primer to amplify janus cassette from P271
7	5'-CCCGGGGGATAGCCTAGATTTTCGATA-3'	Forward primer used for inverse PCR to delete peptide 1
8	5'-CCCGGGCATAAGGTTGCCTCCTTC-3'	Reverse primer used for inverse PCR to delete peptide 1
9	5'-CCCGGGGGCTAGTTTTTCTCACCACT-3'	Forward primer used for inverse PCR to delete peptide 2
10	5'-CCCGGGCATTCTTCTACTCCTAC-3'	Reverse primer used for inverse PCR to delete peptide 2
11	5'-CCCGGGTGTTAGATAAAACAAAAGGA-3'	Forward primer used for inverse PCR to delete peptide 3
12	5'-CCCGGGCATAATTTTACTCCGTAATT-3'	Reverse primer used for inverse PCR to delete peptide 3
13	5'-CCCGGGGGATAAACTACCTCTATTC-3'	Forward primer used for inverse PCR to delete peptide 4
14	5'-CCCGGGCACTAAATCAAAGTGTTGTCA-3'	Reverse primer used for inverse PCR to delete peptide 4
15	5'-TGCCTTGTTTACGTCTTTC-3'	Forward primer to amplify middle of P174 lan histidine

		kinase
16	5'-TGAAATTTTTTATCGCCATC-3'	Reverse primer to amplify 500bp of P174 histidine kinase
17	5'-AAAGAATTTGGAACAAAAA-3'	Forward primer used to amplify 500 bp middle region of 3' ABC transporter
18	5'-CAATGCCAATTTGTTGACGA-3'	Reverse primer used to amplify 500 bp middle region of 3' ABC transporter
19	5'-CCGCTCTAGAACTAGTGGATCC- 3'	Forward primer to amplify spectinomycin
20	5'-CAATTTTTTTATAATTTTTTTAATCTG-3'	Reverse primer to amplify spectinomycin
21	5'-GGACGCATGCATCATCATTTTCATTCC TCCGTTTC-3'	Reverse primer upstream of first ORF of 5' ABC transporter with Nsil site
22	5'-GCTTGGATGCATTAAGTCAAATACCA GAGTTGC C-3'	Forward primer downstream of second ORF of 5' ABC transporter with Nsil site
23	5'-GATCATGCATGTTTGATTTTTAATGGA TAAT-3'	Forward primer that anneals to Janus contains Nsil site
24	5'-GAGTGATGCATATGGTTGTTCTAGTA AGGTT-3'	Forward primer anneals to response regulator 207 bp downstream of ATG adding a Nsil site
25	5'-CATCCTCTAGATAAACTTAACCATTTT TCAG-3'	Reverse primer used to amplify <i>lanM</i> 38 bp from ATG adding a XbaI site
26	5'-TCAAAAATAATTCGCGTCTG-3'	Reverse internal primer of <i>lacZ</i>
27	5'- ATGAATATGAGCTACAGAAC-3'	Forward internal primer of <i>pldM</i>
28	5'- CGGGCATTAAAATCAATACT-3'	Reverse internal primer of <i>pldM</i>
29	5'-GAAGCATCTTTAAAATCTGT-3'	Forward primer that anneals to peptide region found in ATCC 700669
30	5'-CGGATTTAAATATTTGCTAC-3'	Reverse primer that anneals to peptide region found in ATCC 700669
31	5'-GAAACTTGACGATTTACTC-3'	Forward primer that anneals to peptide region found in P174
32	5'-TGCCATGAGTATTTTGTGCA-3'	Reverse primer that anneals to peptide region found in P174
33	5'-GCGCGCATGCATGAAACGGAGGAAT G-3'	Forward primer that anneals 19 bp downstream of the site of the hyperinducible mutation

		and contains a Nsil restriction site
34	5'- TTGATAGTTTTCCCCTGAAT-3'	Reverse primer that anneals to 5'ABC transporter
35	5'- GGCAAACCTTGAAAGAAAATAATGAG AGTGTA AAAAAG-3	Forward primer of site directed mutagenesis deleting CCGT from intergenic region upstream of <i>pldFE</i>
36	5'- CTTTTTTACACTCTCATTATTTTCTTTC AAGGTTTGCC-3'	Reverse primer of site directed mutagenesis deleting CCGT from intergenic region upstream of <i>pldFE</i>
37	5'- GCGCATGCATGCGTCATCGTCTTTG - 3'	Forward primer used to construct <i>pldK-lacZ</i> reporter, anneals 451 bp upstream of first codon of <i>pldK</i> and adds Nsil site
38	5'- GCGCTCTAGACAATAAAAGTGAGTT- 3'	Reverse primer used to construct <i>pldK-lacZ</i> reporter, anneals 15 codons downstream of first ATG of <i>pldK</i> and adds Xbal site
39	5'- GCGCATGCATGAGAAATACAAATGA- 3'	Forward primer used to construct <i>pldF-lacZ</i> fusion, anneals 11bp upstream of <i>pldA4</i> and adds Nsil site
40	5'- GCGCTCTAGACCAAATGTTTTCGTT-3'	Reverse primer used to construct <i>pldF-lacZ</i> fusion, anneals 15 codons after start codon of <i>pldF</i> and adds Xbal site
41	5'-GTACAATTTCTTGAAACAAGCC-3'	Forward primer that anneals approximately 500bp upstream of second BsrGI site
42	5'-CGCTCTTTGTCAAGATATTC-3'	Reverse primer that anneals approximately 500bo downstream of second BsrGI site
43	5'- GCGCTGTACAGTTTGATTTTAAATGG ATAAT-3'	Forward primer that anneals to Janus cassette and adds a BsrGI site
44	5'- GCGCTGTACACTTTCCTTATGCTTTT GGAC-3'	Reverse primer used to amplify the Janus cassette adding a BsrGI site
45	5'-GGATTGTAGTCTATACAGTGG-3'	Forward primer that anneals 165 bp upstream of second

		BsrGI site in <i>lanM</i>
46	5'-AGAATAGCCTAAAATAGAAT-3'	Reverse primer that anneals 700 bp downstream of second BsrGI site in <i>lanM</i>
47	5'- GCGCATGCATGAAAGTCATGAATGAT -3'	Forward primer used to create <i>pldA1-lacZ</i> fusion that anneals 460 bp upstream of start codon of <i>pldA1</i> and adds a Nsil site
48	5'- GCGCTCTAGATTAGAGAATCAATCTC TGG-3'	Reverse primer used to create <i>pldA1-lacZ</i> fusion that anneals 37 bp after start codon of <i>pldA1</i> and adds a Xbal site

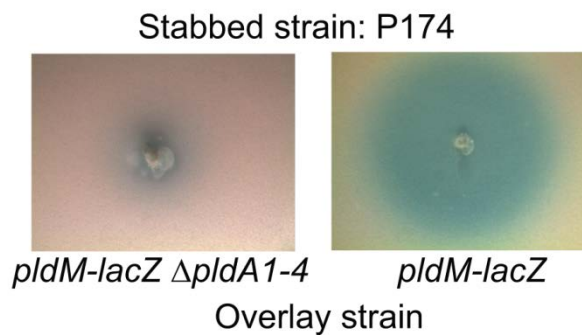


Figure A1. Lantibiotic Peptides Detected by Reporter Strain P174 *pldM-lacZ* and P174 Δ *pldA1-4* *pldM-lacZ*. P174 was stabbed into a TS plates and incubated for approximately 6 hours. Overlays were prepared with the chromogenic substrate, X-gal, included in the overlay mixture with either reporter strain both containing a *pldM-lacZ* fusion with intact peptides or the same reporter but lacking all four structural peptides.

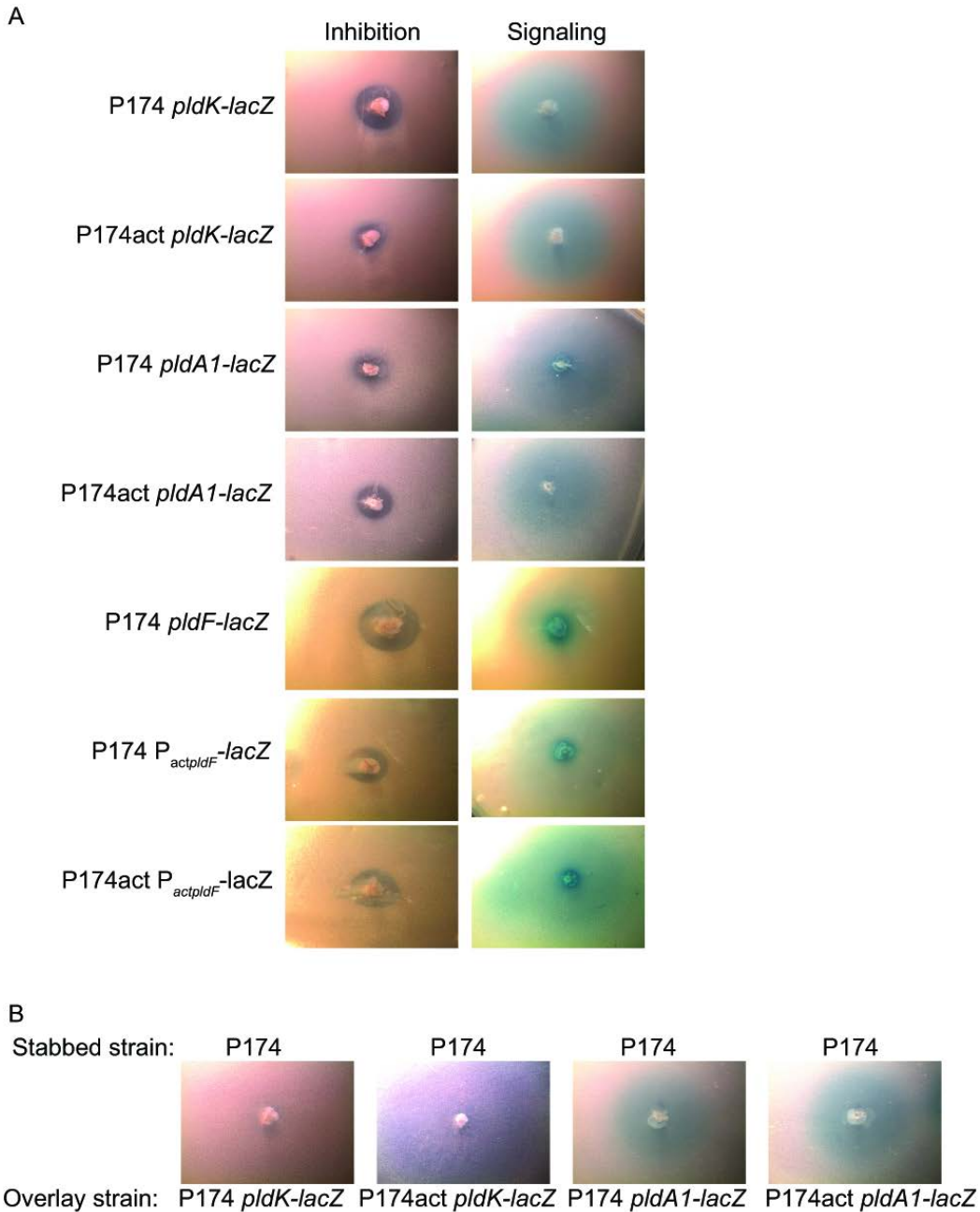


Figure A2. Inhibitory and signaling activity of *pldK* and *pldF* reporters.

A) To confirm that the reporter constructs of *pldK* and *pldF* in either P174 or P174act backgrounds retained a functional *pld* locus, reporters were stabbed into TSA plates and overlaid with either a sensitive strain for inhibition, or with the reporter, P174*pldM-lacZ* for signal secretion. B) The *pldK* reporter strains of either P174 or P174act was used as the overlay strain over stabbed P174 and assessed for their ability to respond to signal.

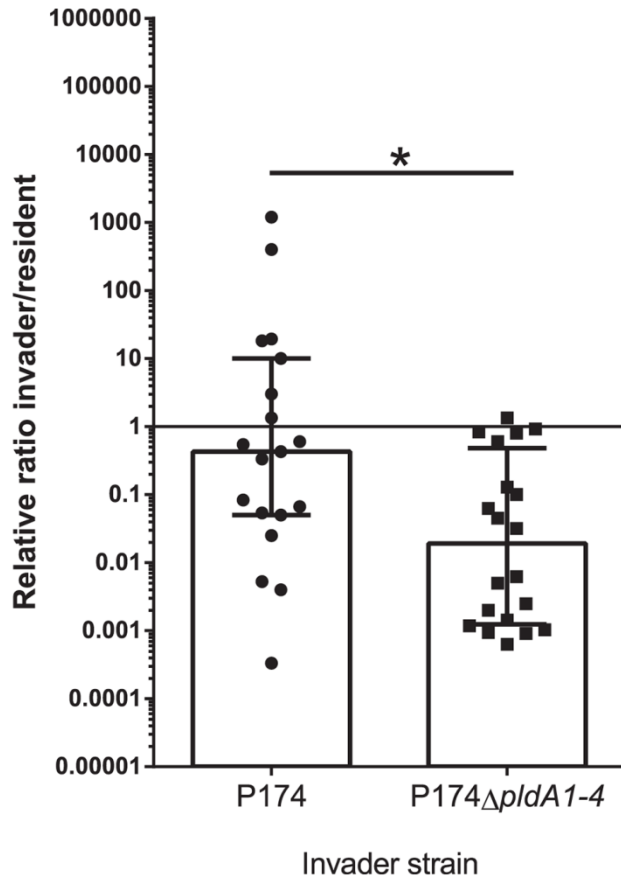


Figure A3. Paired relative ratios of invaders to resident. CFU/ml of the invader strain, P174 or P174 Δ pIdA1-4, was compared to the CFU/ml of the resident strain, P174 Δ pIdK, to calculate a relative ratio between strains in a single nasal wash. A relative ratio of 1 indicated by a line denotes equal colonization by the two strains. A relative ratio greater than 1 indicates more invader than resident strain. Statistical analysis was performed using unpaired Mann-Whitney test. *=p<0.05.

A

P174 KYYSW**C**NG..N38..DNSI**C**H 915
P130 KYYSW**Y**NG..N38..DNSI**C**H 913

B

Inhibition



Figure A4. Inactivity of *pld* locus in P130 linked to mutation in PldM. A) Amino acid alignment of PldM of P130 and P174 in the area of interest is shown. Amino acids that are conserved in a zinc binding domain of PldM are shown in red. P130 has a tyrosine in place of the conserved cysteine. B) Overlay assays demonstrating inhibition of TIGR4 were performed using strains P174, P130, and P174 PldM C869Y.

APPENDIX B

Supplemental figures for Chapter III.

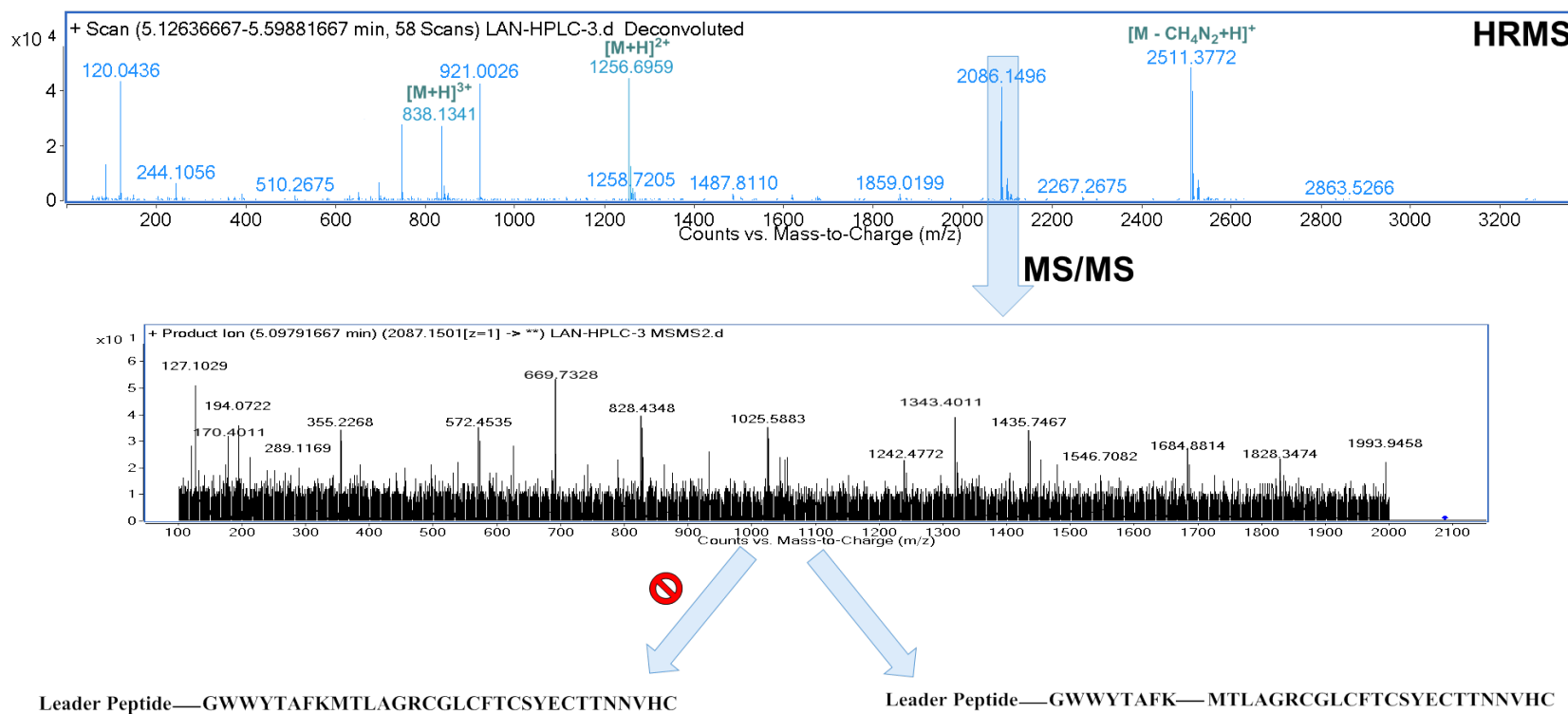


Figure B1. Q-TOF ESI MS deconvoluted data for PIdA3 and follow up MS/MS spectra on the most abundant TIC peak with m/z 2086.1496

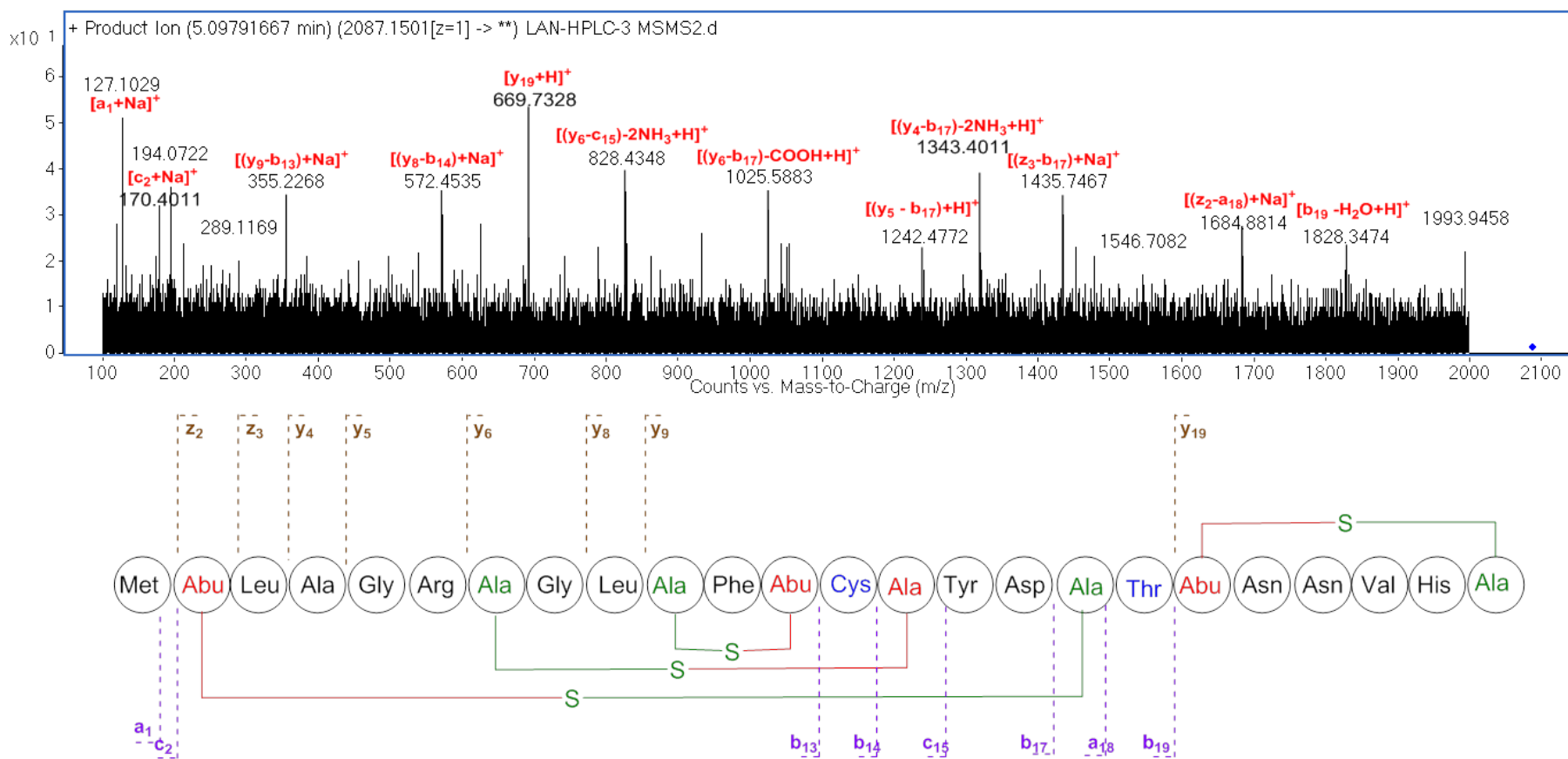


Figure B2. MS/MS fragmentation ions for PIdA3 and the schematic representing the tertiary structure of PIdA3.

Leader Peptide—**GWWYTAFK**—MTLAGRCGLCFTCSYECTTNNVHC

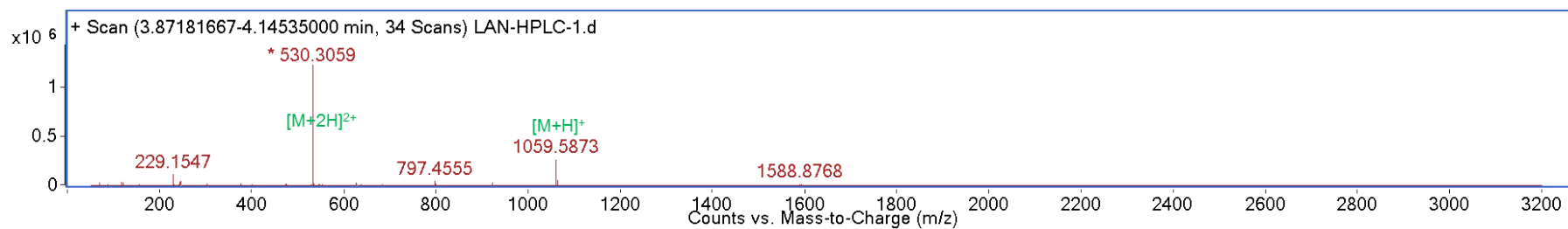
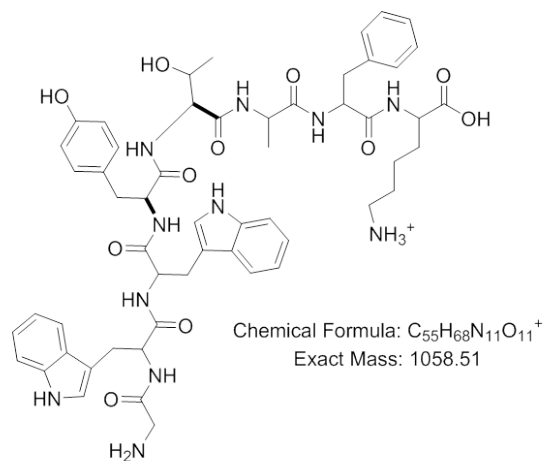


Figure B3. Q-TOF ESI MS chromatogram of N-terminus cleavage of PldA3.

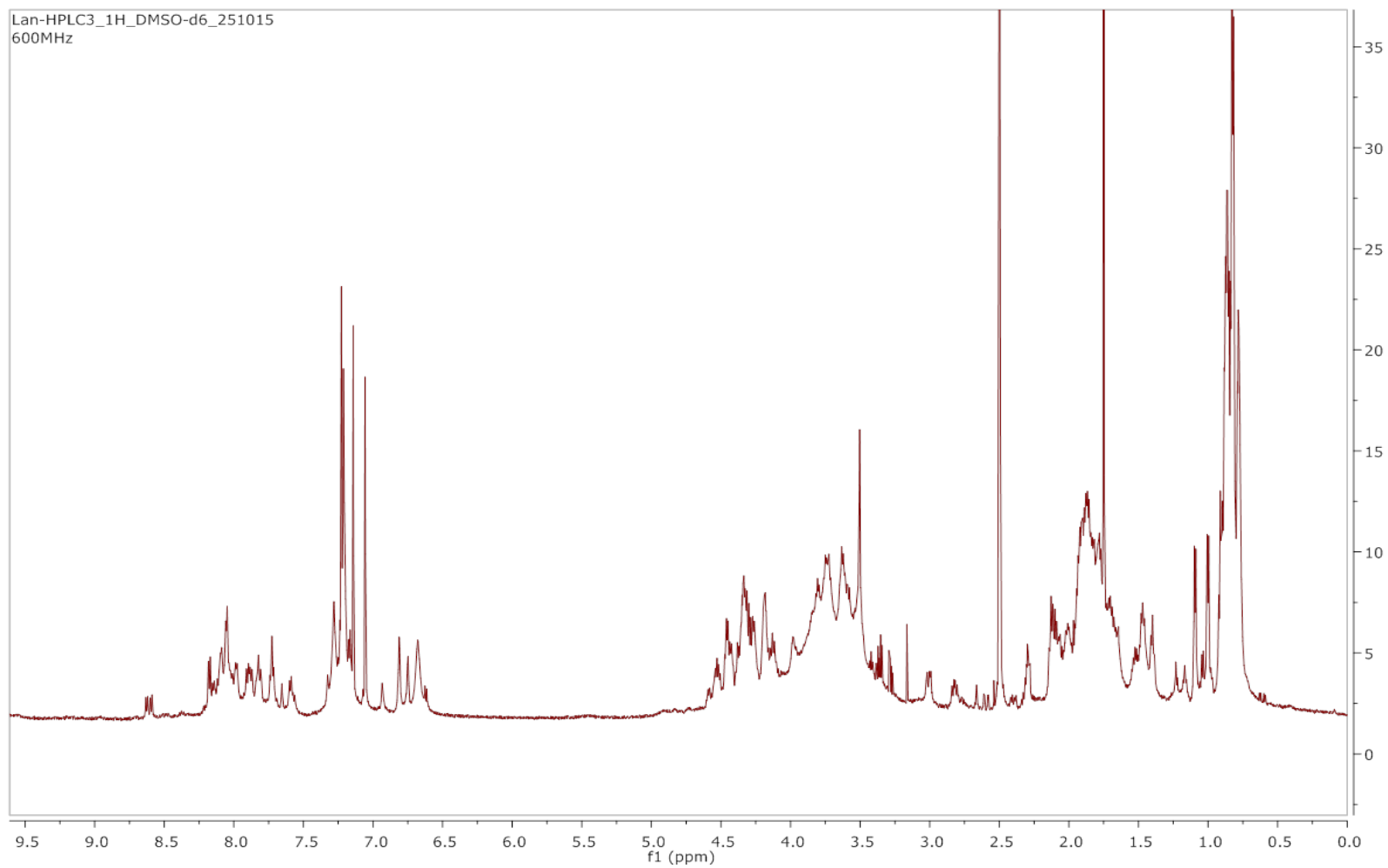


Figure B4. ^1H NMR (600 MHz, DMSO-d₆) spectrum of PlidA3

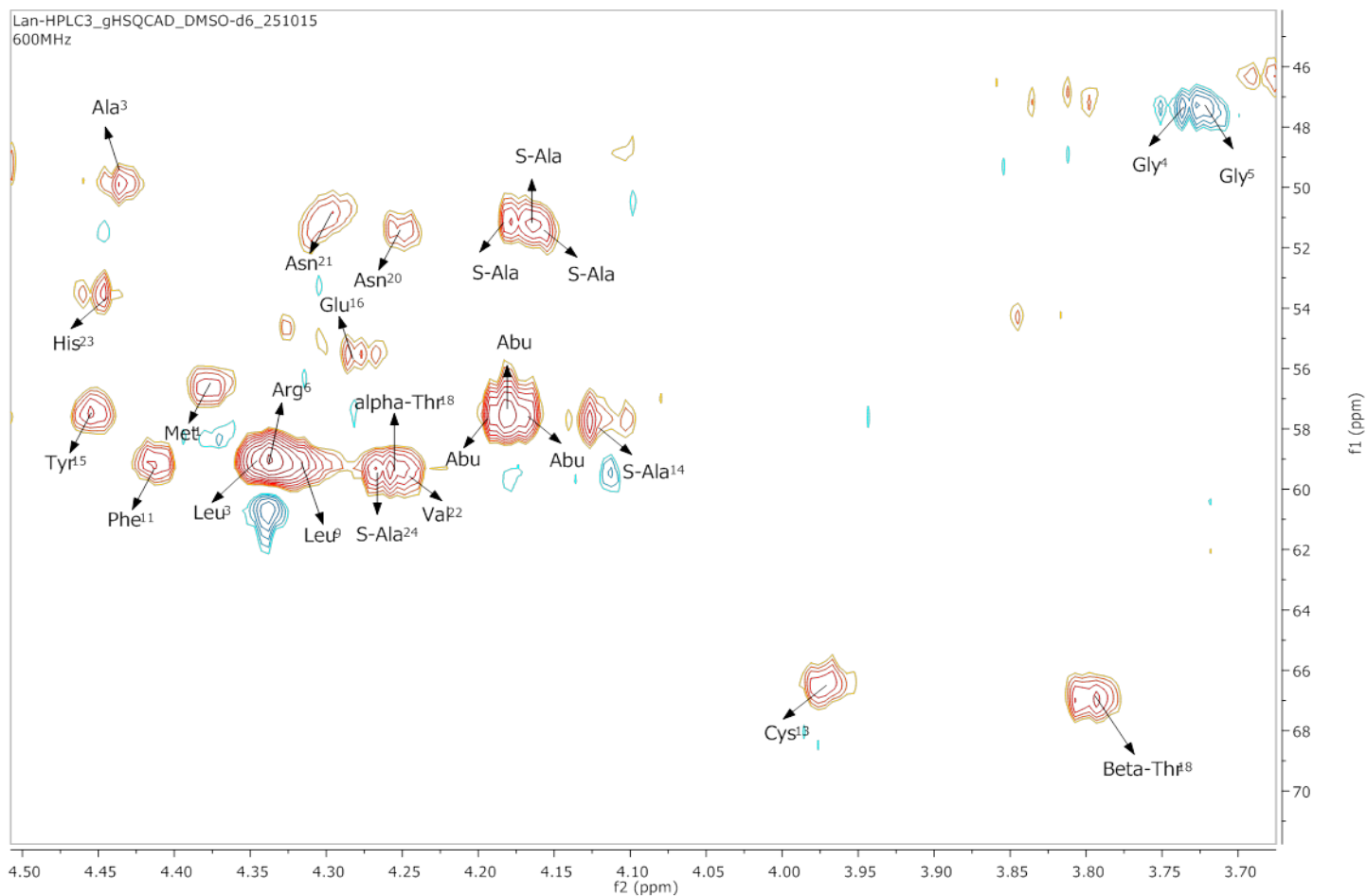


Figure B5. HSQCAD NMR (600 MHz, DMSO-d6) spectrum of PldA3 with resolved and annotated tertiary signals.

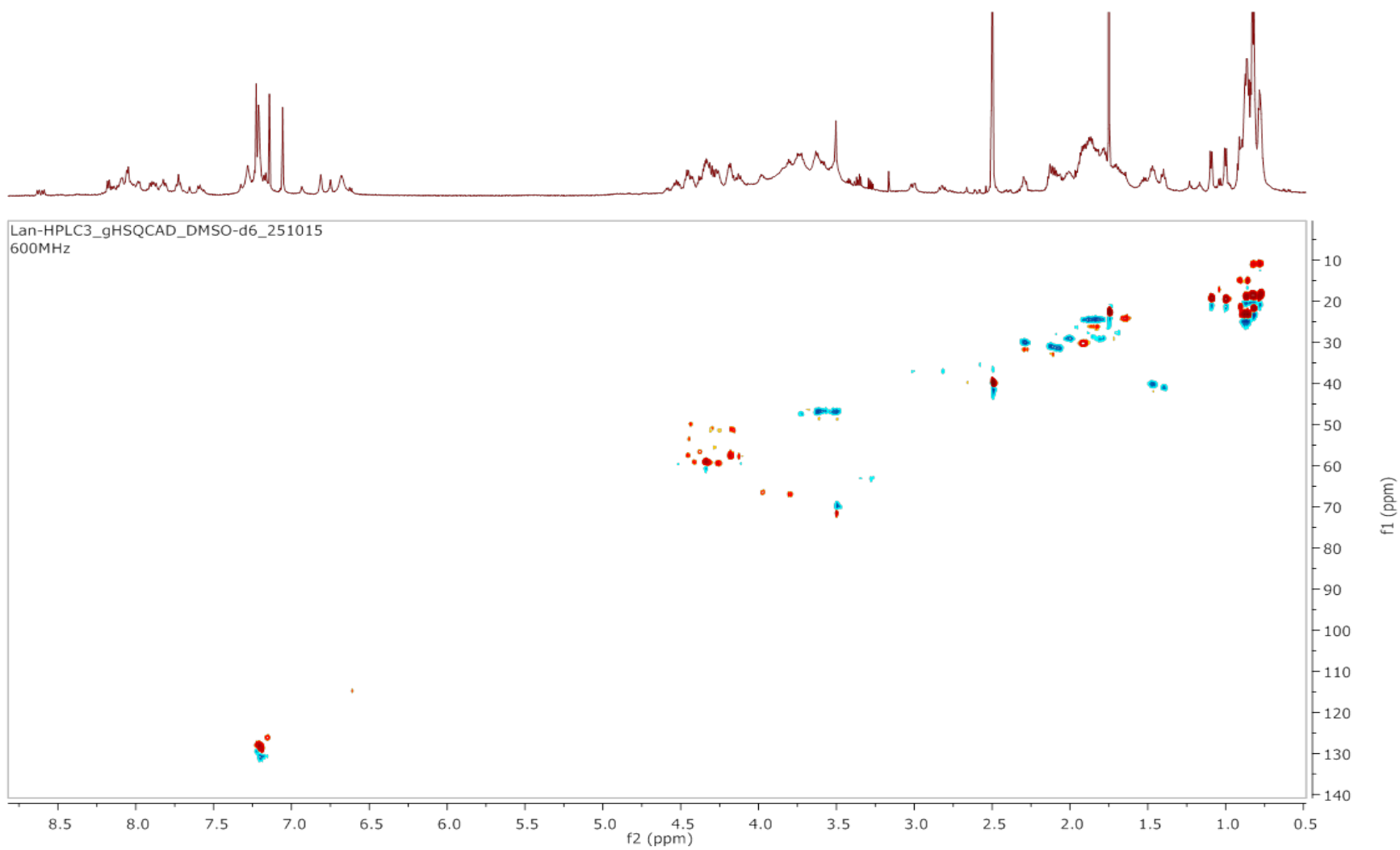


Figure B6. HSQCAD NMR (600 MHz, DMSO-d6) spectrum of PIdA3.

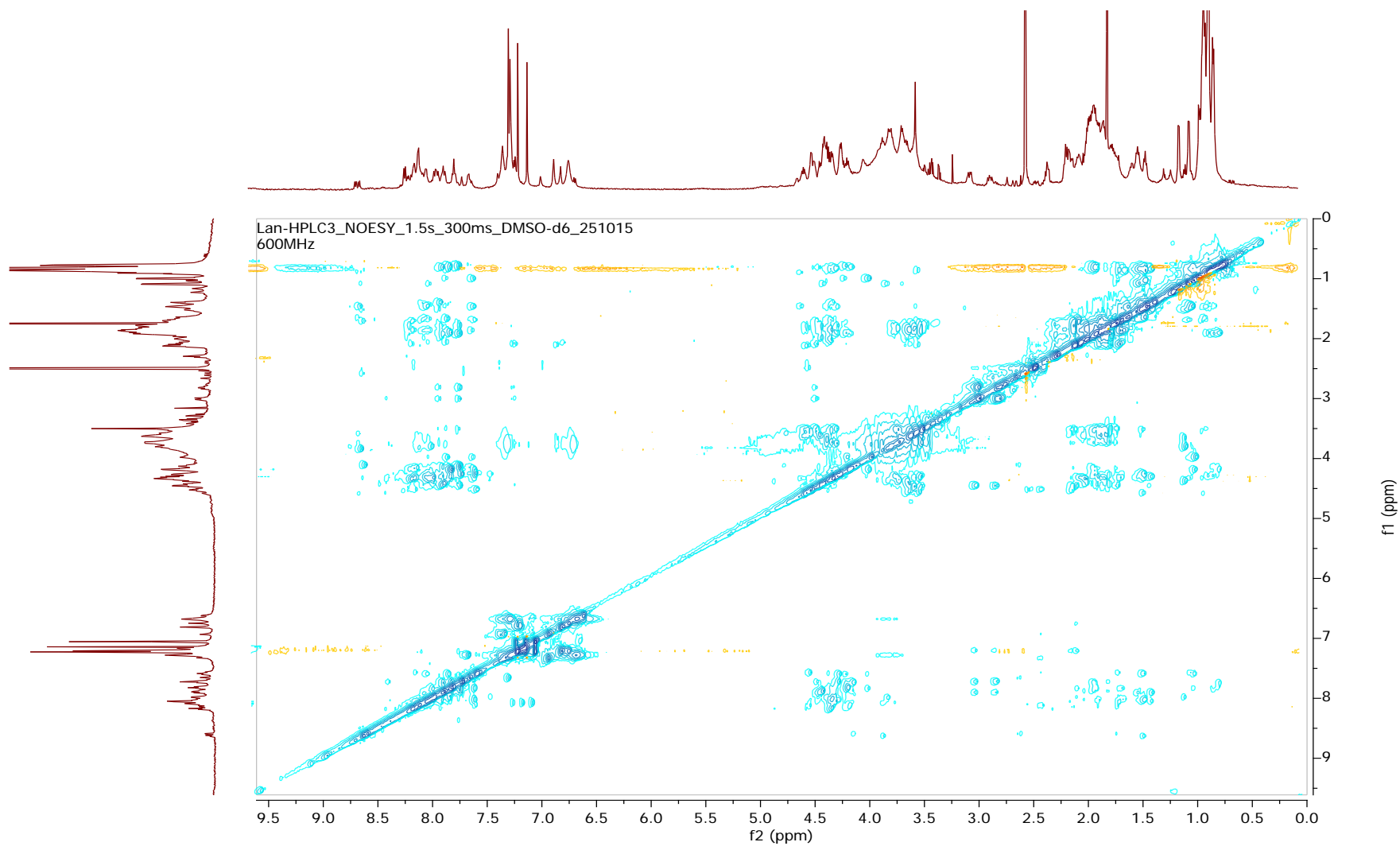


Figure B7. NOESYAD NMR (600 MHz, DMSO-d6) spectrum of PIdA3.

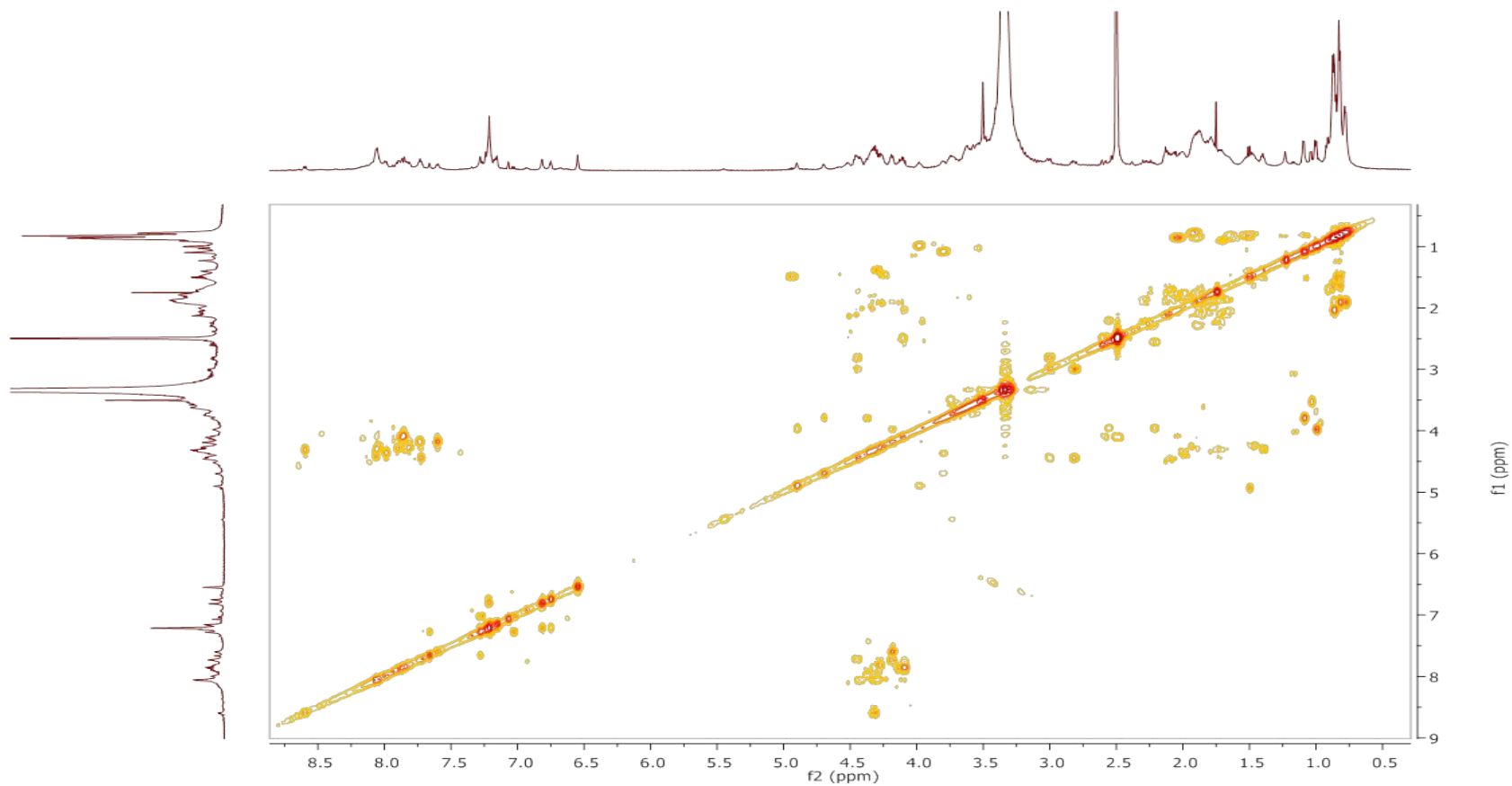


Figure B8. gCOSY NMR (600 MHz, DMSO-d₆) spectrum of PldA3.

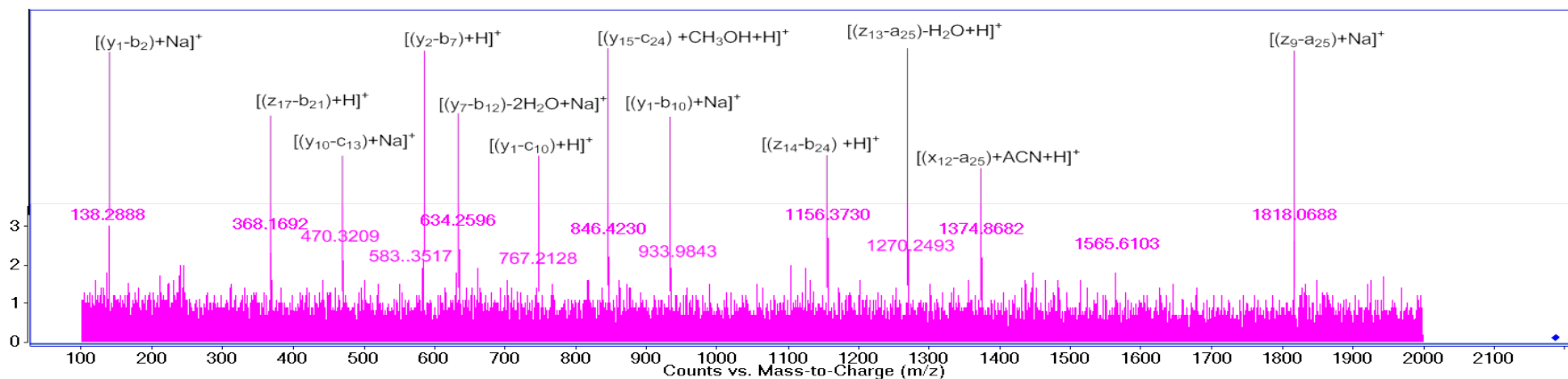
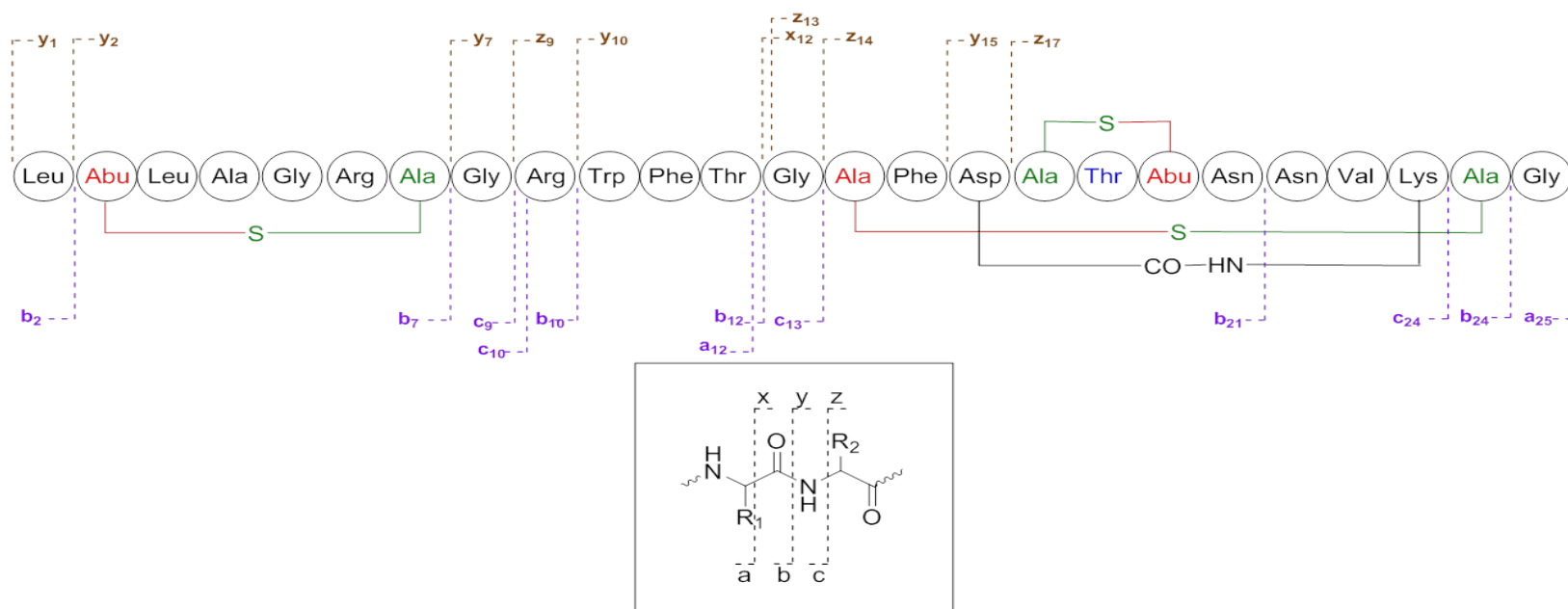


Figure B9. ESI MS for PIdA1 and follow up MS/MS spectra along with schematic representing the tertiary structure.

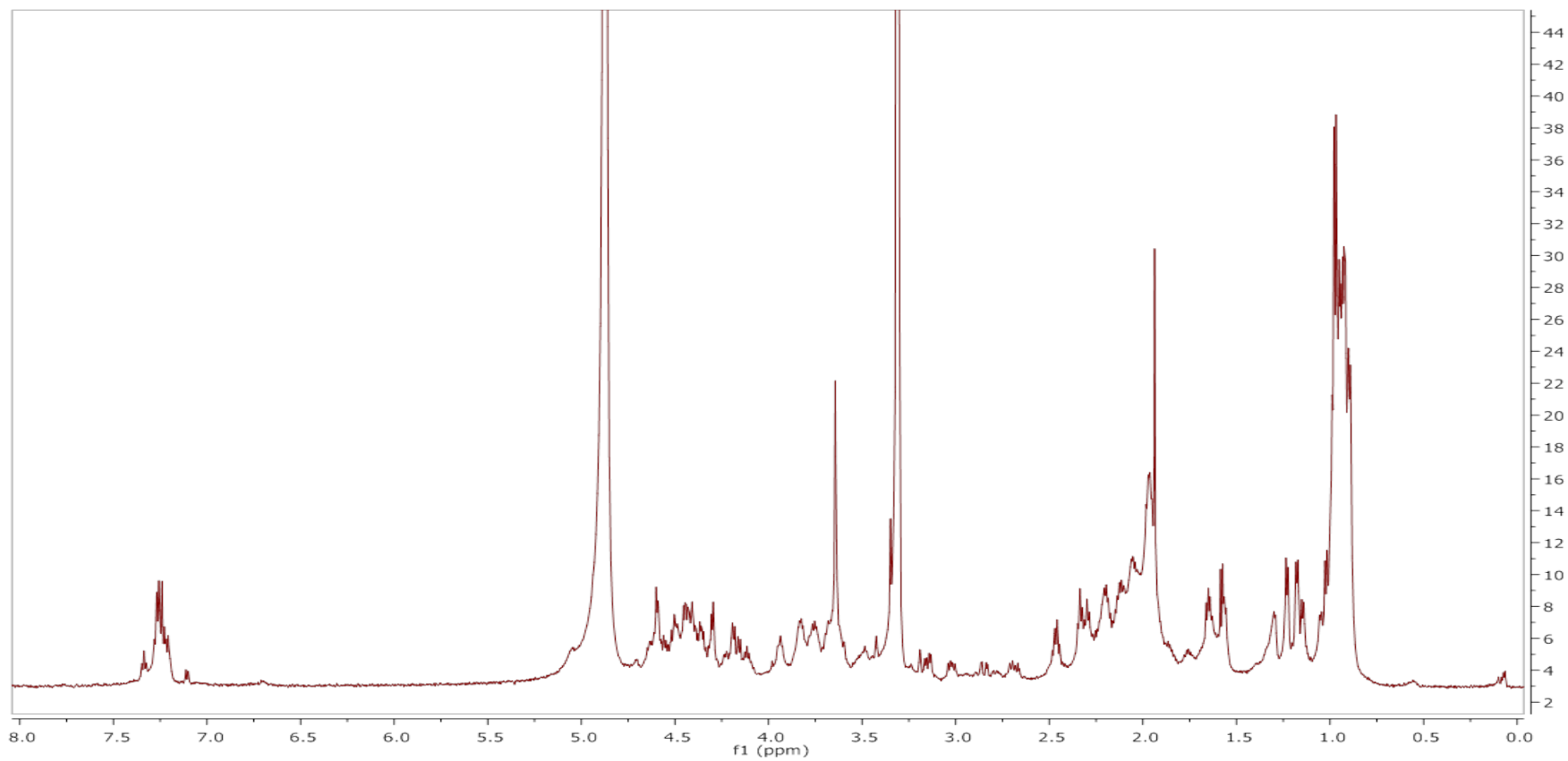


Figure B10. ^1H NMR (600 MHz, $\text{CD}_3\text{OD}-d_6$) spectrum of PldA1.

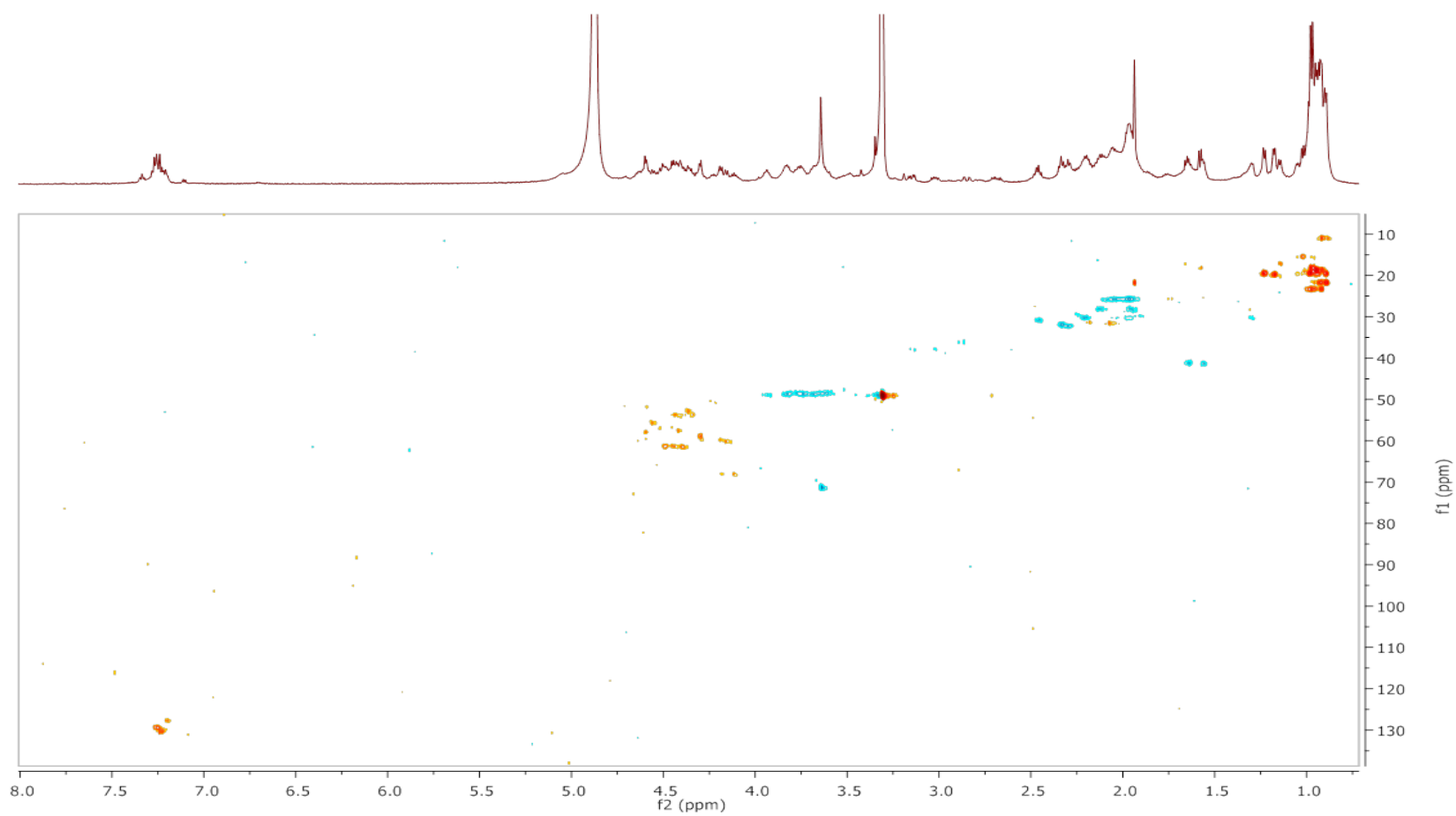


Figure B11. HSQCAD NMR (600 MHz, CD₃OD) spectrum of PldA1.

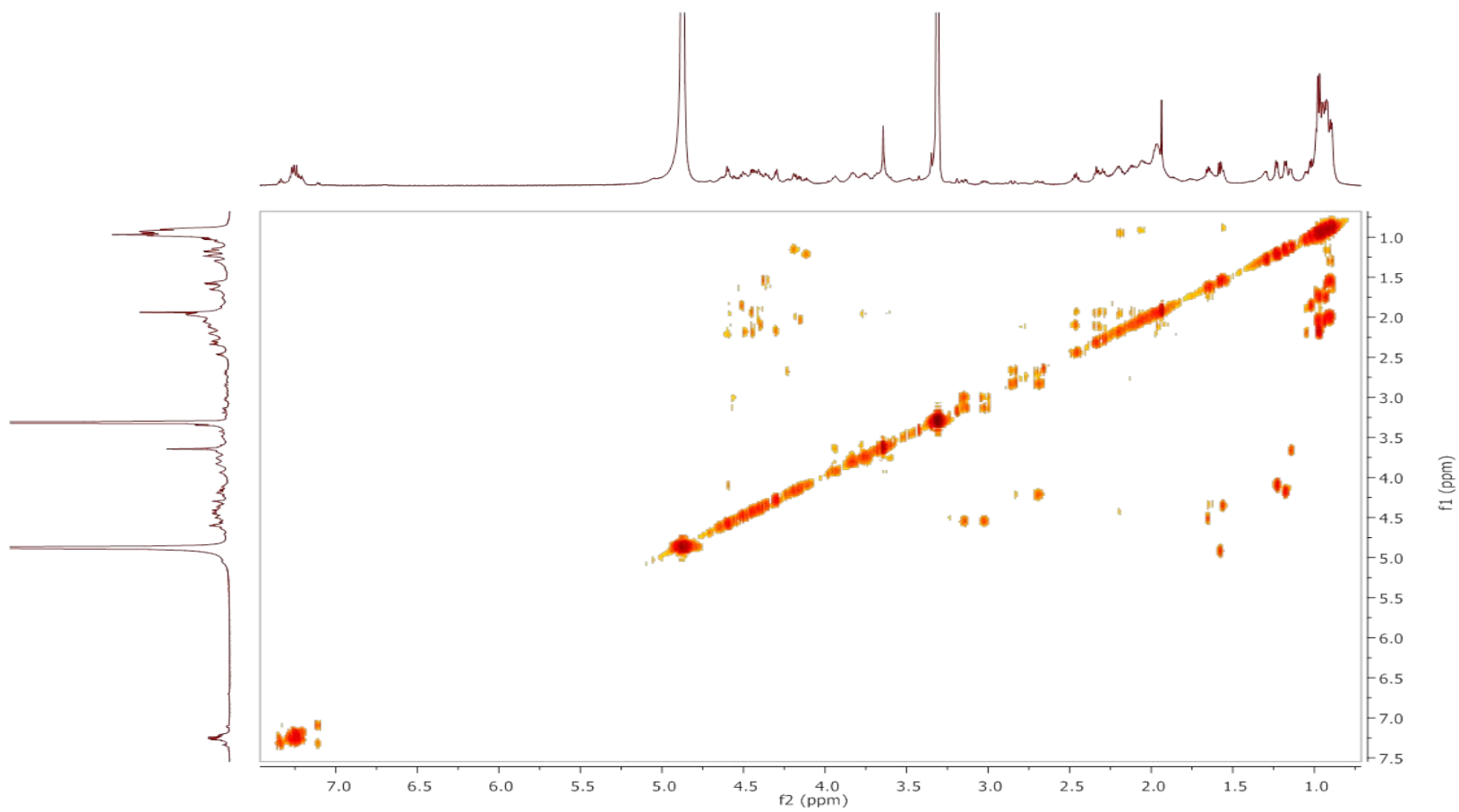


Figure B12. gCOSY NMR (600 MHz, CD₃OD) spectrum of PIdA1.

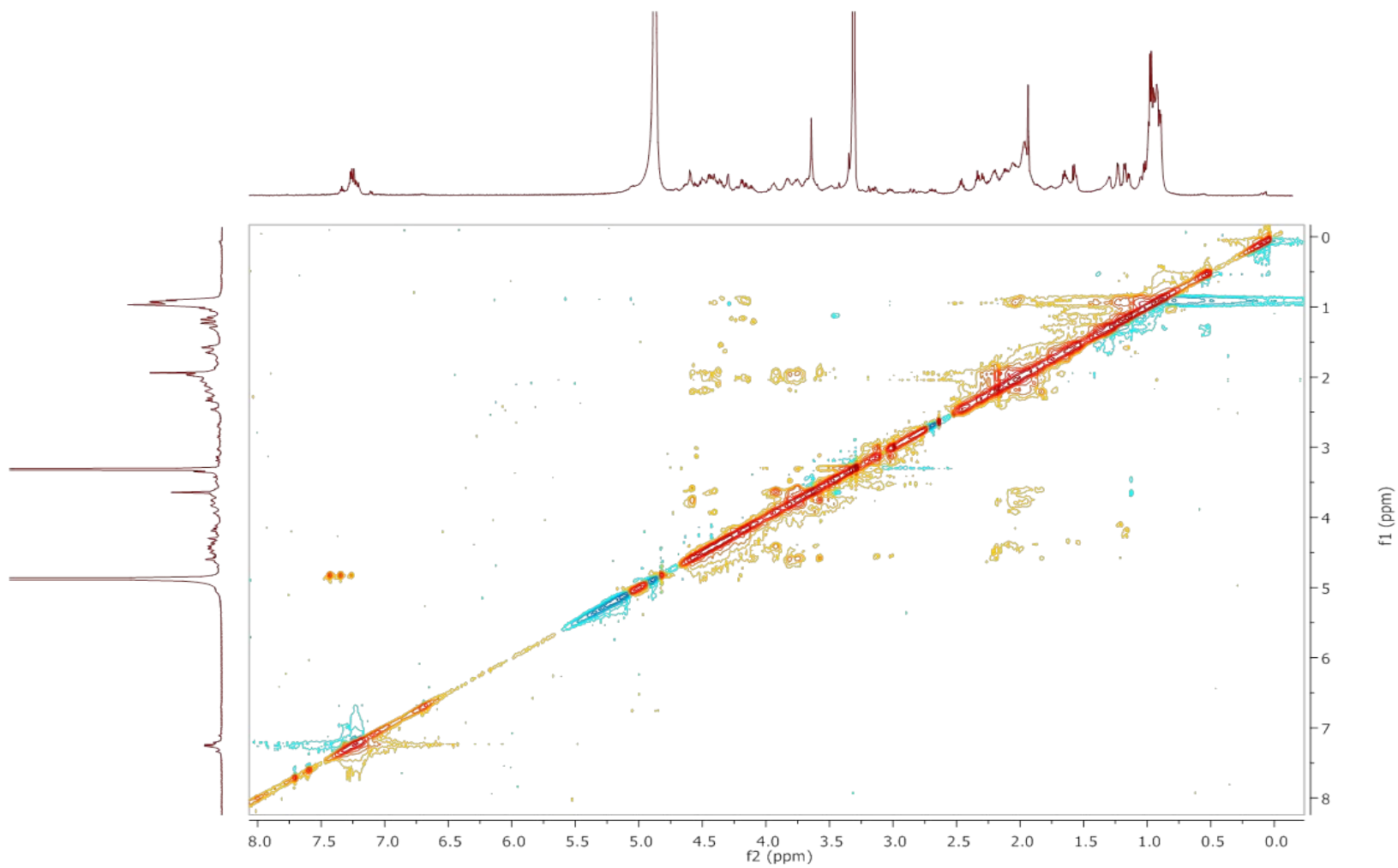


Figure B13. NOESYAD NMR (600 MHz, CD₃OD) spectrum of PIdA1.

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