# THE ISOLATION AND PARTIAL CHARACTERIZATION OF NOVEL BACTERIOCINS FROM THE ORAL BACTERIUM *Streptococcus salivarius*

A Thesis

by

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## ABSTRACT

Antimicrobial resistance is a wide-reaching concern, having garnished increased saliency amongst all corners of society. These fears are well founded and attempts to moderate and effectively respond to this potential crisis is with the identification of new life saving compounds. We have presently reported the identification and partial characterization of one novel bacteriocin produced by *Streptococcus salivarius* U3. Through the use of a specialized growth medium, we have been able to induce the production of antibacterial metabolites and have successfully isolated one of these compounds. The compound in question possesses a highly limited spectrum of activity. The compound is also evidently amphipathic (as evidenced by its point of elution during High Performance Liquid Chromatography) and proline rich, based on the amino acid analysis that has been performed. The presence of contaminants in the isolated product has made pinpointing an exact mass somewhat difficult. However, cross-referencing mass spectrometry data with current NMR assignments leads us to suspect that the compound is between 2.0kDa and 2.6kDa in size. The NMR data also reveals a compound that is highly structured based upon the observed spectral width and amide to amide proton interactions.

# DEDICATION

I first and foremost dedicate the following work to my mother and father, Donna and Melvin Williams, whose undying commitment to my wellbeing and success has enabled me to be where I am today. I also dedicate this work to all members of my graduate committee including Dr. Joseph Sorg and Dr. Ravi Kumar. I would like to give particular thanks to Dr. James Smith, whose guidance, patience, and professional insights as a mentor have shaped this project into something promising and worthy of investigation far beyond the scope of this thesis. This appreciation also extends to all of my friends/colleagues that are/have been in his employ. I offer additional thanks to Mr. Ali Kayani, a man whom I greatly respect and who has expressed his support for my endeavors many times. Lastly, I dedicate this work to the families of Williams and Rigsby, who, through their various gifts, have made substantive contributions in the areas of industry, academia, mathematics, science, and various other social and intellectual pursuits. This present investigation should not serve only to reflect my own intellectual contribution but as a part of the greater continuum of work performed by members of these families in commensurate areas of study. I am proud to be a member of the Smith lab and a member of the Williams/Rigsby family and I only hope that my initial efforts displayed herein are greeted charitably by all aforementioned.

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## Contributors

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# CHAPTER 1

# GENERAL INTRODUCTION TO SALIVARICINS

## *General Overview*

The widespread implementation of antibiotics has been a major facet of managing disease in the western hemisphere since their introduction at the end of the Second World War. The public at large has considered the existence of these compounds as a panacea and, indeed, their application and availability has been responsible for the survival of millions (Ventola, 2015). However, even during the earliest period of their use, many scientists had begun to observe the development of antibiotic resistance, beginning with penicillin resistance in *Staphylococcus aureus* (Chambers, 2001). Indeed, this matter has loomed over the medical world in perpetuity, with concerns about drug efficacy oscillating in intensity for over half a century (Spellberg & Gilbert, 2014). Recently, a new wave of agitation over this question has surfaced in light of a new set of antibiotic resistant organisms, including highly virulent strains of gonorrhea and highly lethal strains of *Candida auris* (Chowdhary et. al, 2017; Saga & Yamaguchi, 2009). This resurgence constitutes a severe problem which can be redressed with the identification of new antibiotics. Fortunately, an expansive depository for these antibiotics can be found in the oral cavity of humans along with their major progenitors, *Streptococcus salivarius*.

*S. salivarius* is a non-pathogenic Gram-positive bacterium that is localized predominately in the mouth and, to a lesser extent, in the upper respiratory system of humans (Wescombe, 2009). As a niche, the oral cavity is a highly competitive environment which *S. salivarius* efficiently occupies. This fact can be attributed to its ability to produce several effective antimicrobial compounds broadly defined as salivaricins. Their presence in this environment is not inconsequential, with research suggesting that they play a major role in controlling the floral

composition of the mouth and, by extension, maintaining the health of the carrier (Wescombe et. al, 2006). The potential value of these salivaricins as alternative antibiotics is typified by their effectiveness against pathogenic species of *Streptococcus*, including *S. pyogenes* and *S. agalactiae* (Wescombe et. al, 2006; Patras et. al, 2015). Additionally, their usefulness has proven to possess market viability in the form of a commercially available strain, K12, used as both an oral probiotic and as a treatment for halitosis (Wescombe et. al, 2011).

### *Classifications*

Salivaricin is a term that describes all varieties of bacteriostatic/bactericidal compounds that are produced by both *S. salivarius* and the distantly related *Lactobacillus salivarius*. These compounds are ribosomally synthesized/proteinaceous and can exhibit either acute target specificity or have broad spectrum toxicity (Hols et. al, 2019). These compounds can be divided and subdivided in accordance with their size, chemistry, biosynthesis, and mode of action. Specifically, bacteriocins are subdivided into 4 main groups: (I) lantibiotics, (II) small peptide, (III) large peptide, (IV) cyclized (Wescombe et. al, 2009). Groups (II) and (III) both lack post-translational modifications (PTMs) in their structures and are easily distinguished from one another by whether they fall above or below a 10kD threshold. Group IV bacteriocins are the least understood of all bacteriocin classes. They are characterized by the presence of PTMs and the ring formations they achieve through covalent interactions between their terminal ends. Group I bacteriocins are some of the best studied compounds of this kind and will be the primary focus of this introduction.

The lantibiotics are characterized by a number of post-translational modifications, the most notable of these being the lanthionine rings. These ring formations are synthesized via dehydration reactions which convert serine and threonine residues into dehydroalanine and dehydrobutyrine

residues, respectively, which can then form thioether linkages with cysteines (Repka et. al 2017). The efficient formation/positioning of these linkages is achieved enzymatically through cyclases that forge covalent bonds between the cysteine and dehydro-amino acids (Li. et. al. 2006). It is worth noting that non-catalyzed cyclizations are possible, being spontaneously generated as a result of stereoselective Michael additions occurring between the substrates (Toogood, 1993). Be that as it may, the spontaneous synthesis of lanthionine is not a conventional way by which such structures are produced in functional lantibiotics.

Similar to the broader classification of bacteriocins, the lantibiotics can be divided and subdivided into various groups based upon different criteria, including their chemistry and structural geometries (Dicks et. al, 2018). For this review, the biosynthetic classification system implemented in Willey & van der Donk (2007) will be used and discussion will be restricted to the first two classes. Class I and Class II lantibiotics are distinguished by the biosynthetic mechanisms involved in the generation of their ring motifs. For class I, the dehydration of the serine/threonine amino acids is facilitated through a dehydratase that deprotonates the amino acids via glutamylation (Garg et. al, 2013). For class IIs, the dehydration of the serine/threonine amino acids is achieved through a dual phosphorylation/elimination reaction, whereby the introduction and subsequent removal of phosphates is catalyzed by specific sub-domains within the effector enzyme (Chatterjee et. al, 2005a; You et. al, 2007; You et. al, 2009). Additionally, the dehydration and cyclization steps in class IIs are performed by a single bifunctional enzyme, in contrast to class I lantibiotics which possess distinct enzymes for both reactions. Class II lantibiotics can be further subdivided between single component and two-component varieties.

Two-component lantibiotics have two distinct precursor peptides that undergo extensive processing before acquiring synergistic activity (Garneau et. al, 2002). Along with the addition of

the standard lanthionine rings, two-component lantibiotics, such as lacticin 3147, also include the addition of D-alanine amino acids, a distinct post-translational modification that is generated after the synthesis of the prepeptide (Skaugen et. al, 1994; Ryan et.al, 1999). These modifications are catalyzed by LtnJ, a reductase/dehydrogenase that converts dehydroalanines present within the mature lantibiotic into D-alanine amino acids (Cotter et. al, 2005). The addition of these D-alanine amino acids appears to play a critical role in bioactivity, evidenced by the fact that when these elements are absent, bioactivity is reduced substantially (Cotter et. al, 2005). At the moment, these moieties appear to be exclusive to two-component lantibiotics including lacticin 3147, carnolysin, and bicereucin (Repka et. al, 2017). The biosynthesis of these two-component lantibiotics is identical to other class II lantibiotics except that two LanM proteins are involved in the synthesis of each of the components (Dougherty et. al, 1998).

### *General Biosynthesis*

The synthesis of lantibiotics is regulated through operons and organized into gene clusters which encode several key elements, including those directly involved in protein modification, immunity, secretion, and those that possess regulatory features (de Vos & Kuipers, 1995; Siezen et. al, 1996). If we are to consider class I lantibiotics, such as the one featured in Figure 1-1(A), the first gene in the operon encodes LanA. LanA is the unmodified precursor peptide that, after synthesis, is subjected to various chemical modifications. This precursor protein is comprised of two segments: the leader peptide and the core peptide. The leader peptide consists of N-terminal elements that will be removed after the fully modified peptide is transported out of the cell (Repka et. al, 2017). Despite not being a site for modification, it is still relevant for the maturation of the peptide. More specifically, the leader peptide serves as a binding region for LanB (dehydratase) and possibly LanC (cyclase), enabling the efficient incorporation of PTMs (Yang & van der Donk,

2013). The region whereupon these modification enzymes interact is referred to as the FNLD box (EV and/or EL box in class II lantibiotics), a highly conserved region of the leader domain characterized by its importance to lantibiotic synthesis (Mavarro et. al, 2011; Abts et. al, 2013). Furthermore, the FNLD boxes have an almost ubiquitous distribution across class I leader domains, however, a novel binding region, the EDLF box, has been identified in mutacin 1140 (Escano et. al, 2014).

There are other roles that leader peptides are believed to play in addition to chemical maturation. It is thought that leader peptides are essential for keeping functional lantibiotics in an inactive conformation prior to their secretion, ensuring that the host cell is not poisoned by its products (Patton et. al., 2006). Additionally, evidence exists suggesting that sections of the leader domain, in particular the FNLD-box motif, may be important for the efficient secretion of the compound via LanT transporters (Plat et. al 2013; Plat et. al 2011). It should be noted that research pertaining to this LanT/leader peptide interaction only measured transport of modified product out of the cell and as such, a conclusion cannot be drawn regarding this matter. One alternative explanation to these findings may be that core peptide modifications are a general prerequisite for protein secretion, with said secretions not being dependent upon direct binding between the propeptide and the transporter. With all of this in mind, it is clear that leader peptides are vital to lantibiotics production albeit in a largely supplemental fashion. The important role of the leader peptide as enzymatic scaffolding stands in contrast to the core peptide which is the beneficiary of these modifications. The true effect of these modifications only manifest once the peptide is fully matured, a state that is achieved after the core peptide is cleaved from the leader peptide domain.

The excision of the leader peptide from the core peptide is facilitated through LanP (protease) which will cleave at a site, specific to each lantibiotic (Oman & Van der Donk, 2010).

The *lanB* of the operon encodes the dehydratase that dehydrates serines and threonines present within the core peptide. The dehydroalanine/dehydrobutyrine residues generated from this reaction will participate in thioether linkages with cysteine residues. This formation is achieved via LanC, which is needed for the site-specific coupling of thiol-bearing cysteines to dehydro-amino acids (Meyer et. al, 1995). Biosynthesis for class II lantibiotics is quite similar in many respects, however, they differ in that a major component of the compound's maturation is facilitated by a single enzyme, LanM (Gilmore et. al, 1994). LanM works as a bifunctional synthase responsible for the installation of both the dehydro-amino acids and the thioether bridges (Xie et.al, 2004). This dual capacity is the result of the enzyme possessing two key domains: a dehydration domain and a cyclization domain (Repka et. al, 2017). Dehydration in class II lantibiotics occurs via phosphorylation and elimination reactions, the former being dependent upon the availability of ATP and $Mg^{2+}$ ions and the latter being dependent upon ADP (Chatterjee et. al, 2005a; You & van der Donk, 2007). Cyclase domains operate in a fashion identical to the cyclase enzyme of class I lantibiotics.

Besides lanthionine rings, the core peptide can also acquire class-specific/compound-specific augmentations known as tailoring modifications. These modifications can include amino vinyl cysteines, which are generated by lantibiotic-specific LanD enzymes, as well as D-alanine amino acids (Chatterjee et. al, 2005b; Ryan et. al, 1999). Other examples include 2-oxobutrates/2-oxopropionyls, moieties which are not enzymatically generated but rather the result of unstable N-terminal dehydrobutyrine/dehydroalanine residues that have undergone deamination (Kellner et. al, 1989; Skaugen et. al, 1994). It should be stated that while these specific N-terminal modifications are produced spontaneously, the +1 site of the peptide can still be an area for enzymatically configured tailoring motifs. For example, committed oxidoreductases have been

identified in some lantibiotic gene clusters which are responsible for catalyzing the conversion of oxopropionyl residues into 2-hydroxyproprionyls (Heidrich et. al, 1998; Velazquez et. al 2011). One final tailoring modification of note is the 2S,8R-lysinoalanine, which is generated by the duramycin exclusive enzyme, DurN (Huo et. al, 2017; Repka et. al 2017). Once the lanthionines have been introduced to the nascent peptide and subsequently hydroxylated via Dur X (another tailoring enzyme unique to duramycin), DurN can then catalyze the formation of novel lysinoalanine bridges (Huo et. al, 2017). The development of these bridges also entails the formation of cyclic peptides. There are several more of these modifications that could be discussed in detail. The expanse of literature would indeed warrant elaboration far beyond the scope of this chapter but suffice it to say that further investigation is required to better understand all of these structural elements.

### ***Regulation***

Typically, lantibiotic biosynthesis is a function of cellular density, requiring a very large number of cells in proximity to one another to engender production. Interestingly, salivaricin production can be induced at a low cellular density if the organisms are grown in the presence of a lantibiotic for which they are able to synthesize (Barbour et.al, 2013). In this manner, the bacteriocins serve as signaling elements in quorum sensing, promoting an autoinductive synthesis of the lantibiotic between cellular neighbors (Jimenez & Federle, 2014). The use of these bacteriocins as communication signals is not restricted only between conspecifics. The production of SalA in *S. salivarius* can undergo transcriptional upregulation when in the presence of *S. pyogenes*, which also produces a variant of SalA (Upton et. al, 2001). In some alternative systems, such as the novel TprA/PhrA system in *Streptococcus pneumoniae* D39, lantibiotic synthesis via quorum sensing can be tied to the environmental availability of galactose, a carbohydrate

commonly found in the nasopharynx (Hoover et. al, 2015). Similarly, lantibiotic regulation for subtilin is heavily influenced by the availability of glucose in the extracellular space (Bochman et. al, 2015). It may be argued that these functional correlations between bacteriocin production and nutrient rich environments is an evolved genetic interaction which enables organisms to proactively hegemonize a favorable environment. Taken together, the interspecies communication and nutrient dependent synthesis of certain lantibiotics confers a substantive advantage to producer organisms occupying critical niches.

Mechanistically, the signaling for and regulation of salivaricin biosynthesis is achieved by a two-component regulatory system, with the bacteriocins operating as a sort of pheromone (Kleerebezem, et. al, 1997). The operons of lantibiotic-producing species encode *lanR* and *lanK* whose products regulate expression via phosphate mediated signal transduction (Klein et. al, 1993; Engelke et al, 1994). The lantibiotic-associated histidine kinase (LanK) possesses extracellular/transmembrane ligand receptors along its N-terminus which specifically interact with lanthionine rings (Teng et. al, 2014; Ge et. al, 2016; Ge et. al, 2017). The ligand binding-domain/input-domain of these kinases engage in direct binding between themselves and the complementary bacteriocin to induce phosphorylation (Kleerebezem, 2004). LanK can transfer this phosphate to the lantibiotic-associated response regulator (LanR) through the transmitter domain localized on its C-terminus (Kuipers et. al, 1995; Draper et. al., 2008). LanR has a DNA binding domain which, when phosphorylated, activate the *lan* operon through direct binding at the promoter (Ni et al. 2011). For some lantibiotic-producing species, additional environmental constraints exist to control the biosynthesis of these bacteriocins. Within the context of natural subtilin production among *B. subtilis*, biosynthesis is growth-phase-dependent (Gutowski-Eckel et. al, 1994). This observation would suggest that subtilin production is controlled by the transition

state regulator AbrB and, by extension, Sigma H (Weir et. al, 1991; Dubnau et. al, 1988). Indeed, it has been shown experimentally that Sigma H, along with LanR and LanK, work as part of a dual control system, with lantibiotic expression of each mechanism being tied to, if not constrained by, specific metabolic and environmental requirements (Stein et. al, 2002).

### *Immunity*

The synthesis of these lantibiotics is invaluable to the organisms that produce them, however, without a mechanism to confer immunity; the act would be entirely moot as the producing-organism would become poisoned. For lantibiotic producing species, this immunity can exist in the form of LanI proteins and LanFEG complexes, which help to manage issues of self-toxicity within the extra/intracellular space (Repka et. al. 2017). The exact structure and functionality of these immunity proteins is by no means uniform, varying across several different operons and subclasses (Okuda & Sonomoto, 2011; Deng et. al, 2014). The compositional modularity of these proteins has at times been an encumbrance into their investigation, particularly in the study and identification of LanI proteins (Draper et. al, 2009; Alkhatib et. al, 2012).

Broadly speaking, LanI proteins are thought to work by a sequestration mechanism that shields the producing organism from bacteriocins that have accumulated in the extracellular space. This is achieved via direct binding of LanI with its cognate lantibiotic, ensuring that these compounds are prevented from contacting the cell membrane and inducing inhibition or autolysis (Hacker et. al 2015; Geiger et. al 2019). Interestingly, while this sequestration mechanism is effective, it may not be the only way that LanI can manage toxic concentrations of lantibiotics. In nisin-producing species, when the extracellular concentration of nisin reaches a certain threshold, the organisms will arrest growth, begin to take on a cocci-like morphology, and organize themselves into long chains (Alkhatib et. al, 2014). This behavior was only observed in those

strains that encoded *lanI* and was accompanied with higher overall survivability in the presence of the lantibiotic. This suggests that this strategy may help manage toxicity at extremely high concentrations (Alkhatib et. al, 2014).

The binding domains associated with LanI proteins appear to be compound-specific in many cases, such that organisms that encode SpaI (immunity protein in subtilin) will not interact with nisin in spite of the high structural similarity between the bacteriocins (Stein et. al, 2005). In general, cross-immunity between LanI proteins is rare, which may be attributed to evolutionary divergence between the different *lanI* genes (Draper et. al, 2009; Khosa et. al, 2016). Indeed, there is structural diversity among LanI, so much so that there exists very little structural homology between any of the studied proteins (Twomey et. al, 2002; Draper et. al, 2008). As a consequence of such sizable variation in the structure and localization of LanI, these proteins have been divided into 3 groups: Lipoproteins, Membranous/Extracellular, and Trans-membranous (Okuda & Sonomoto, 2011). Even when high structural homology is identified between two or more LanI proteins, this metric fails to be a reliable or consistent means to determine cross-immunity as evidenced by the previous example of the failed cross-immunity between SpaI and NisI.

While LanI is important in conferring immunity, *lanI* mutations are not lethal. Indeed, when *lanI* mutants of *L. lactis* were measured for immunity against extracellular nisin, only a 60-90% reduction in viability was observed, when compared to the control group (Ra et. al, 1999). When a similar experiment was performed using *E. coli* expressing *nisI*, *in trans*, the observed cells presented less than 5% of the control group's viability (Qiao et. al, 1995). These observations suggested that other genes likely work in consort with the LanI to confer full immunity. There is some uncertainty as to the full role other genes play in immunity, however, some explanations may lie with expression of other lantibiotic genes. The expression of LanI in some lantibiotic operons,

such as those that encode nisin, is tied to the phosphorylation of LanR while the same expression in subtilin encoding operons is associated with expression of the unmodified prepeptide, LanA (Saris et. al, 1996). The interaction between LanI and other gene products may also extend to bioactivity, such as in the case of NisI. As a lipoprotein, NisI undergoes several post-translational modifications before acquiring full functionality, including cysteine modification, cleavage of signal motifs, and acylation (Zuckert, 2014; Qiao et. al, 1995; Nakayama et. al, 2012). Some research has suggested that NisI lacking these modifications (denoted as lipid-free) may enhance the bactericidal properties of nisin (Koponen et. al, 2004). However, this particular attribution is suspect since the study reporting this observation lacked sufficient controls to determine whether this increase in activity was only specific to the indicator strains used or if the activity was directly related to the use of lipid-free NisI. This more dubious finding aside, LanI proteins are still crucial elements. The role that they play in immunity cannot be understated, however, it is a role that is often supplemented by other genes. For many lantibiotic producing species, LanI is not the only committed immunity protein available. A significant amount of the work done to protect the cell can be performed by LanFEG.

Alternatively, or in addition to LanI, many lantibiotic operons encode LanFEG complexes that help prevent self-poisoning. The LanFEG complexes have features that are structurally homologous to ABC transporters and are responsible for preventing the activated lantibiotic from accumulating in the plasma membrane (Bolhuis et. al, 1996). There are several important structural elements that characterize a functioning ABC transporter, however, for the purpose of both precision and brevity, this section will only briefly discuss 3: the transmembrane domain (TMD), the nucleotide binding domain (NBD), and the E loops.

The architecture of LanFEG complexes consist of 2 TMDs that are positioned within the phospholipid bilayer and 2 nucleotide binding NBDs which are localized in the cytoplasmic space (Rees et. al, 2009). The LanE and LanG subunits form a heterodimeric complex which serves as the TMD whilst two LanF subunits form a homodimeric complex acting as the NBD (Okuda et. al, 2010). All subunits are important for conferring immunity, although the LanF component is the most vital of these (Peschel & Gotz, 1996). The importance of LanF has been attributed to the presence of the E-loop motif common to all LanF orthologs, although the exact role of this motif is still not entirely understood (Okuda et. al, 2010). These LanFEG transporters, like LanI, have compound-specific affinity for the proteins that they secrete (Otto et. al, 1998). The nature of this specificity has yet to be fully elucidated.

Wild-type immunity to lantibiotics is conferred through the co-expression of both LanI and LanFEG proteins (Stein et. al, 2003). Coordinated expression has a synergistic effect on the producer's immunity (Takala et. al, 2004; Draper et. al, 2009). Additional immunity proteins play a role under limited circumstances. In the case of Nukacin ISK-1, LanFEG complexes are aided by the accessory protein LanH. LanH may serve the ABC transporter by providing a more efficient method to capture intracellular lantibiotic (Okuda et. al, 2008). Similar to LanI and LanFEG, the interaction of LanFEG and LanH appears to be multiplicative in its ability to confer immunity to the host (Aso et. al, 2005). Based upon phylogenetic comparisons of various LanFEG complexes, only 1/3 could be identified as being associated with bacteriocin synthesis or regulation, implying that additional roles may exist for these structures (Gebhard, 2012).

### *Modes of Action*

The bacteriostatic/bactericidal nature of lantibiotics is not uniform and can have modes of action including, but not limited to, pore-formation, lipid II sequestration, and growth inhibition

(Smith & Hillman, 2008; Chatterjee et. al, 2005b; Hols et. al 2019). These differences are attributed to the differences in structural elements by which the compounds interact with their targets (Yeaman & Yount, 2003). In the case of class I bacteriocins (lantibiotics), the lanthionine rings, as illustrated in Figure 1-2, serve as the primary source of bioactivity and chemical stability. Broadly, lantibiotics work by binding to the cell wall precursor molecule lipid II, although some exceptions do exist (Repka et. al 2017). When the lantibiotic complexes with lipid II, it undergoes a conformational change and interacts with the pyrophosphate group of the target, forming a cage-like heterodimer in the process (Breukink et. al, 2003; Hsu et. al, 2004). At best (for the cell) this interaction can be purely inhibitory, only minimizing growth but not killing the targets outright. Conversely, these interactions can induce multiple disruptions including perturbations of membrane potential, efflux of vital salts, influx of cytotoxic compounds, loss of ATP, and cell death (Moll et. al, 1999).

The number of lanthionine rings and the proper modality of key binding motifs are vital for conferring target specificity (Ghergisan-Filip et. al, 2018; Ross et. al, 1993). Additionally, these ring motifs are vital in conferring both substantial thermostatic and proteolytic stability to the compounds (Bierbaum et.al, 1996; Suda et.al, 2010). Depending upon the nature of these structures, certain bactericidal responses can be seen. In the case of gallidermin, both bacteriostatic and bactericidal mechanism are present. When this compound binds to lipid II in the target, it will block cell wall synthesis while simultaneously inducing pore formation (Bonelli et. al, 2006). The effectiveness of this dual attack is modulated by the thickness of the target's membrane (Bonelli et. al, 2006). In contrast, salivaricin B induces cell death without damaging the membrane. Similar to vancomycin, salivaricin B blocks transglycosylation of the lipid II subunits, resulting in cell wall malformation and death (Barbour et. al, 2016). In addition to pore formation, nisin can

prevent cell wall synthesis via the abduction of lipid II from the septum (Hasper et. al, 2006; Islam et. al, 2012). Salivaricin D, which shares high structural identity with nisin, may use a similar mode of action, however, this has yet to be determined experimentally (Birri et. al, 2012).

In the case of two-component lantibiotics, the modes of action are generally identical to one-component varieties – through lipid II binding and introducing membrane perforation and/or blocking transglycosylation (Mcauliffe et. al, 1998; Morgan et. al, 2005). Like nisin for class I lantibiotics, lacticin 3147 is one of the best characterized of the two-component lantibiotics in regards to its structure, homology, and its mechanism for cell-membrane interaction (Mcauliffe et. al, 1998; Ryan et. al 1999; O'Sullivan et. al, 2003; Martin et. al, 2004; O'Connor et. al, 2007; Iancu et. al, 2012). From this research, a robust model for these compounds' mode of action has been established.

For two-component lantibiotics, the alpha and beta units work together to permeabilize the cell membrane of the target (Garneau et. al, 2002; Bindman & van der Donk, 2013). The alpha unit of these compounds will initiate this process by strongly binding to lipid II, after which the beta unit will complex with the α-lipid II heterodimer allowing for pore-formation to occur (Bindman & van der Donk, 2013; Baktiary et. al, 2017). This high-affinity binding is achieved by the alpha unit possessing a binding region for lipid II (which is structurally similar to the class II lantibiotic, mersacidin) (Knerr & van der Donk, 2012). Optimum activity of these compounds is generally (although not always) achieved when both components are present in equimolar concentrations (Navaratna et. al, 1998; Morgan et. al, 2005; Oman & van der Donk, 2009). The synergy between the subunits appears to be dependent upon the presence of lipid II and its accessibility to the alpha unit. This is evidenced by the fact that when beta units are added to a target before alpha units, the holopeptides do not form and no activity is observed (Morgan et. al,

2005). Based upon these observations, it was proposed that these compounds synergize via a three-step process: (I) association of the alpha unit with lipid II, (II) subsequent conformational change of complex enabling docking of the beta unit, (III) docking of beta unit and formation of a trimer capable of introducing pores into the target membrane (Morgan et. al, 2005; Wiedemann, et. al, 2006). This model seems to hold true for several two-component lantibiotics including lacticin 3147, haloduracin, and thusin (Wiedemann et. al, 2006; McClerren et. al, 2006; Oman & van der Donk, 2009; Xin et. al, 2016)

### *Current Methods for Characterization of Salivaricin Structures & Bioactivity*

Acquiring an understanding of the varied properties of these bacteriocins is the first and most obvious step in the development of novel antibiotics and food preservatives. Many techniques developed to aid in the isolation and characterization of these compounds including High-Performance Liquid Chromatography (HPLC), deferred antagonism/minimum inhibitory assays (MIC), Nuclear Mass Resonance (NMR), Flow Cytometry, and Mass Spectrometry (Yoshikawa et. al, 1980; Andrews et. al 2001; Wheat et. al, 2001; Moran et. al, 2016; Sahl et. al, 1995; Balouri et. al, 2016; Makloufi et. al, 2013). Several of these tools have proven invaluable in the identification of lifesaving compounds or in evaluating the pharmacokinetics of established drugs (Fleming, 1929; Yoshikawa et. al, 1980). Moreover, the understanding of PTMs has been greatly enhanced through size/mass determination methods such as mass spectrometry (Hindre et. al, 2002; Guyonnet et. al, 2000).

The deferred-antagonism assay and minimum inhibitory assay (MIC) are flagship procedures for investigating antimicrobial activity (Andrews et. al, 2001; Wheat et. al, 2001). The deferred antagonism assay involves exposing an indicator strain to an active compound produced by some other organism and using the size of the inhibition zones as a proxy for bioactivity

(Fleming, 1929; Moran et. al, 2016). The producer strain is generally incubated over night before being killed or removed, and the indictor strain is subsequently added to the plate. This should not be confused with the overlay assay, whereby a sample (purified protein band, HPLC fraction, or any other product of either known or unknown concentration) is applied directly to the surface of a plate containing an indicator strain to determine its antimicrobial effect (Maricic & Dawid, 2014). The MIC assay is a technique that allows researchers to quantitatively show the potency of a bacteriocin against an indicator strain (Fleming, 1929; Balouiri et. al, 2016). Serial dilutions of antibiotic are used to acquire antibiotic potency using a standard inoculum of an indicator strain (Fleming, 1929; Syal et. al, 2017). The MIC is identified by the first well/tube (in order of dilution) that contains no noticeable growth or turbidity (Syal et. al, 2017).

Determining a substance's bioactivity is a critical part of investigations into antimicrobials. However, understanding the manner in which a compound facilitates the eradication/inhibition of its target is also important. Kinetic studies offer clues as to how an antibiotic works. One method to help determine this is flow cytometry (Balouiri et. al, 2016). "SYTOX Green" and propidium iodine (PI) are membrane impermeable dyes that allow the discrimination between viable and dead cells (Lebaron et. al, 1998; Boulos et. al, 1999). More specifically, "SYTOX Green" is a fluorescent DNA intercalating agent that concentrates inside cells with damaged membranes and is measured based upon the amount of observed fluorescence (Thakur et. al, 2015; Roth et. al, 1997). PI is an older compound and is a precursor to "SYTOX Green". Today, PI is commonly used in membrane integrity assays where cellular damage can be reliably correlated to the measured fluorescence (Ko et. al, 2019; Jenkins et. al, 1997). The functional interchangeability of these two agents should make the use of either one sufficient (at least in theory) for determining whether the compound's mode of action is related to membrane disruption. In the case of *Barbour*

*et. al, 2013*, both compounds were used to establish the mechanism underlying the bioactivity of the sal9 lantibiotic (Barbour et. al, 2013). They found that sal9 functioned by a pore formation mechanism.

One of the most important, albeit difficult, areas of lantibiotic investigation is in the structural characterization. Determination of a compounds primary structure (amino acid sequence) can be critical for predicting a molecule's steric configurations in scenarios where experimental methods are unavailable (Deng et. al, 2018; Chakraborty et. al, 2019; Hyink et. al, 2007). That being said, the dynamic modifications that define the lantibiotics have presented severe problems in deducing these compounds architectural nuances. Classical methods for determining protein sequence, such as Edman degradation, have, at times, been made ineffective due to the notable post-translational modifications that make the process unusable (Kraiij et. al, 1999; Garneau et. al, 2002). More precisely, when certain lantibiotics are subjected to Edman degradation, the removal of N-terminal dehydrobutyrines facilitates their conversion into 2-oxobutryls (Kellner et. al, 1988). This moiety cannot be resolved or bypassed in the procedure thus obscuring analysis of all amino acids downstream of the blockage (Kellner et. al, 1988). To overcome this limitation, N-terminally blocked lantibiotics can undergo a derivatization reaction when exposed to ethanethiol and trifluoroperacetic acid (Meyer et. al, 1994). These reactions result in the oxidation and subsequent removal of the 2-oxobutrate from the compound, allowing for complete Edman sequencing of the lantibiotic (Meyer et. al, 1994). Continued refinement of the derivatization reaction led to the development of the "double-labeling technique" for Edman sequencing. This method expanded upon the older technique by including an additional chemical reaction by first using sodium borohydride to reduce the dehydrated amino acids before the addition of ethanethiol to label the lanthionine rings. The method enables researchers to distinguish

between dehydrated and thioether residues (Smith et. al, 2000). While these technical improvements have enhanced the ability to investigate post-translational modifications, these methods should not be seen as a panacea. Even when a lantibiotic undergoes derivatization, more perspicuous methods are still required to determine the positioning and topology of structural elements, such as lanthionine rings (Lohans & Vederas, 2014)

Methods for direct structural characterization, such as X-ray crystallography have proven to be far less fruitful in their returns. Early into the investigation of nisin, researchers attempted to acquire a crystal structure of the bacteriocin, however, all attempts resulted in failure (Sahl et. al, 1995). Much of the difficulty associated with using crystallographic methods for these compounds is tied to them possessing highly unstructured regions not readily amenable to crystallization (Antanaskovic & Kleanthous, 2019). To date, mersacidin is the only known lantibiotic that has had a crystal structure produced (Schneider et. al, 2000). Due to these limitations, most investigators altered course and decided to utilize proton-proton interactions in NMR and sophisticated computer modeling to determine the structure of lantibiotics (Sahl et. al, 1995; Palmer et. al, 1989). Furthermore, NMR has proven indispensable in this field since, to date, it is the only method known to provide definitive evidence for the correct pairing of lanthionine rings (van de Ven & Jung, 1996).

### *Salivaricins Identified*

Several salivaricin-based bacteriocins have been isolated over the course of the last two decades and all are either class I (lantibiotic) or class II bacteriocins. All novel lantibiotic salivaricins to date have derived from *S. salivarius* and include SalA (and its variants), SalB, SalD, SalE, Sal9, and SalG32 (Ross et. al, 1993; Barbour et. al, 2013, 2016; Birri et. al, 2012; Walker et. al, 2016; Wescombe et. al, 2006, 2011, 2012). The oldest documented salivaricin, SalA, was

characterized as a class I bacteriocins by a thiol derivatization with ortho-phthalaldehyde, a reaction which confirmed the presence of a lanthionine (Ross et. al, 1993). With advancements in both sequencing and bioinformatic approaches, it became common to deduce the identity of compounds based upon the conserved synthesis genes observed within a given gene cluster (*e.g.*, salivaricin A2-A5, G32, B, D, and 9) (Wescombe et. al, 2006, 2012; Hyink et. al, 2007; Birri et. al, 2012; Barbour et. al, 2013, 2016). From these studies, we know that most lantibiotic salivaricins belong to the class II subdivision, with salivaricin D being the only currently documented example of a class I lantibiotic.

The class II salivaricins include SalT, SalL, SalP, CRL1328, and mmaye1 (O'Shea et. al, 2011; Barrett et. al, 2007; Pingitore et. al, 2009; Wayah & Philip, 2018). These compounds are produced by *Lactobacillus salivarius*. Characteristically, these compounds appear not to have post-translational modifications and are small, with an average molecular weight of 4,133 Da. Most of our understanding on the structural properties of these compounds has been deduced through genomic studies, similar to many of the class I salivaricins. Interestingly, the array of currently identified class II salivaricins represent many of the major subdivisions currently defined for this category of molecules. Both mmaye1 and CRL 1328 fall into the class IIa category (one-component). By contrast, SalT and SalP belong to the class IIb subdivision (two-component). SalL falls into class IId, having unique molecular features, not commonly observed amongst other class II bacteriocins (O'Shea et. al, 2011; Iwatani et. al, 2011).

Strikingly, there is the lack of structural data available for many of these compounds with the structures for the class I salivaricins (SalB, SalD, and Sal9) being hypothetical in nature. SalA2 is the only lantibiotic salivaricin that has structural data acquired through direct experimental

means, *i.e.* NMR and mass spectrometry (Geng et. al, 2018). Other key features of these salivaricins are showcased in Figure 1-3 and Table 1-1.

### *The Value & Goals of Salivaricin Research*

The increasing prevalence of antibiotic resistant organisms requires a substantial response by the scientific community and must be a highly aggressive effort to isolate and characterize novel antibiotics. With this in mind, the lantibiotics produced by *S. salivarius* represent excellent candidates, in so far as their binding to an evolutionarily constrained target (lipid II) insures functional longevity. The low cytotoxicity of several of these compounds would also make them a preferable alternative to certain chemotherapeutic antibiotics that, while effective against resistant organisms, can be extremely toxic and have deleterious metabolic effects.

The K12 strain of *S. salivarius* is known to synthesize SalA and SalB. One study found that many of the lantibiotic genes encoded by K12 (and several others) were encoded on large >100 kB megaplasmids suggesting that could spread through horizontal gene transfer mechanisms (Wescombe et.al, 2006). Combined with our knowledge of how competitive these lantibiotic producing organisms are, it seems reasonable to conclude that the study and isolation of these salivaricins is a rich endeavor. The investigation herein is a continuation of this greater body of research. Our research intends to provide greater understanding of the biological and chemical subtleties of the bacteriocins produced by this species. More precisely, the aims of my investigation are as followed: determining the spectrum of activity and the production of a quantitative/qualitative appraisal of the physical attributes including structural characteristics of the compound. We hope to make a meaningful contribution to the area of antibiotic research by providing compounds that can be augmented, enhanced, and eventually put to good use by a public which is at an increased risk of falling prey to an ever-widening array of incurable maladies.

Table 1-1: Table displaying all LanA sequences for lantibiotic salivaricins with leader and core peptides color coded as red and green respectively. The FNLD, EV, and EL binding motifs are displayed in bold face and underlined.

| Lantibiotic Salivaricins | LanA Sequences |
|---|---|
| SalA | MKNSKDILNNAIE**EV**SEK**EL**MEVAGG-<br>KRGTGWFATITDDCPNSVFVCC |
| SalB | MAKQQMNLVEIEAMNSLQ**EL**TLE**EL**DNVLGA-<br>GGGVIQTISHECRMNSWQFLFTCCS |
| SalD | MSTKD**FNLD**LVEVSKSNTGASAR-<br>FTSHSLCTPGCITGVLMGCHIQSIGCNVHIHISK |
| SalE | MRKNNNRKEIDTLDF**EV**KNQ**EL**SGKSGS-<br>GWFTAVQLTLAGRCGRWFTGSYECTTNNVKCG |
| SalG32 | MKKDAIIESIK**EV**SLE**EL**DQIIA-GNGVFKTISHECHLNTWAFLATCCS |
| Sal9 | MKSTNNQSIAEIAAVNSLQ**EV**SME**EL**DQIIGA-<br>GNGVVLTLTHECNLATWTKKLKCC |

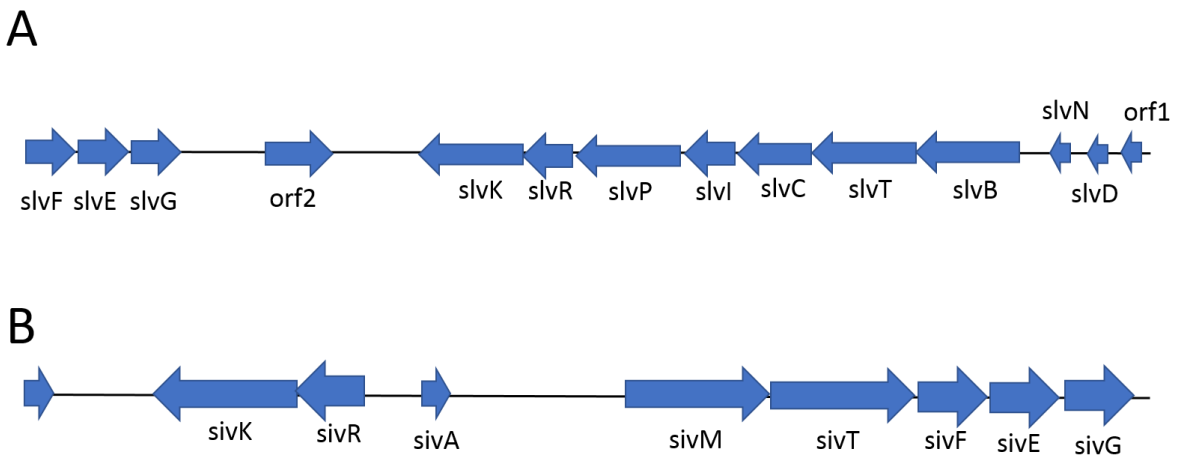



Figure 1-1. Representative schematic of salivaricin operons. (A) Class I lantibiotic operon, sal D; (B) Class II lantibiotic operon, sal 9. Class I operons are characterized by the presence of individuated cyclase and dehydratase (LanB and LanC respectively). Class II possess the bifunctional synthase enzyme, LanM

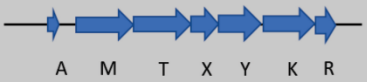
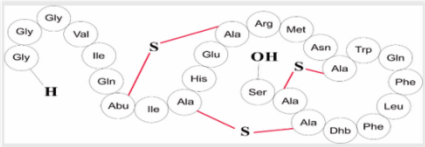
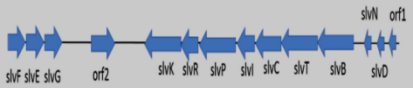
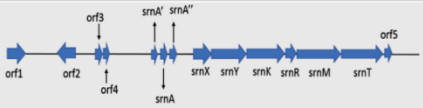
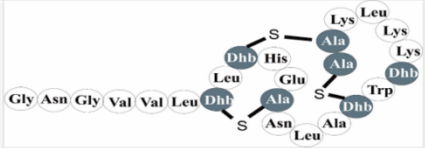
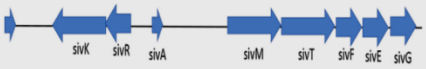
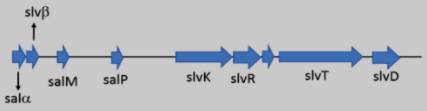
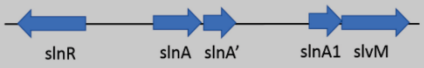
| Name | Covalent Structures | Molecular Weight | Gene Clusters/Operons |
|---|---|---|---|
| Salivaricin A(2)$^{\perp}$ |  | 2367.19$^{+}$ |   |
| Salivaricin B$^{\perp}$ |  | 2732.39 |   |
| Salivaricin D$^{\perp}$ |  | 3467.55 |   |
| Salivaricin E$^{\perp}$ | NA | 3565.90 |   |
| Salivaricin P | NA | sln1=4096<br>Sln2=4284 |   |
| Salivaricin 9$^{\perp}$ |  | 2560.60 |   |
| CRL 1328 | NA | α=4096.14<br>β=4333.12 |   |
| mmaye1 | NA | 1221.07 | NA |
| G32$^{\perp}$* | NA | 2667.00 |   |
| Salivaricin T | NA | α=5656.25<br>β=5270.51 |   |
| Salivarcin L* | NA | 4117.00 | NA |

Figure 1-2. List of Salivaricins. Figure shows all the identified salivaricins with their corresponding structures and gene clusters. Molecular weights for masses (Da) are rounded to the nearest 100$^{th}$. $^{+}$ Only the mass of salivaricin A2 is displayed. $^{\perp}$ Denotes confirmed lantibiotic salivaricins. $^{*}$ Denotes incomplete or unavailable gene clusters.

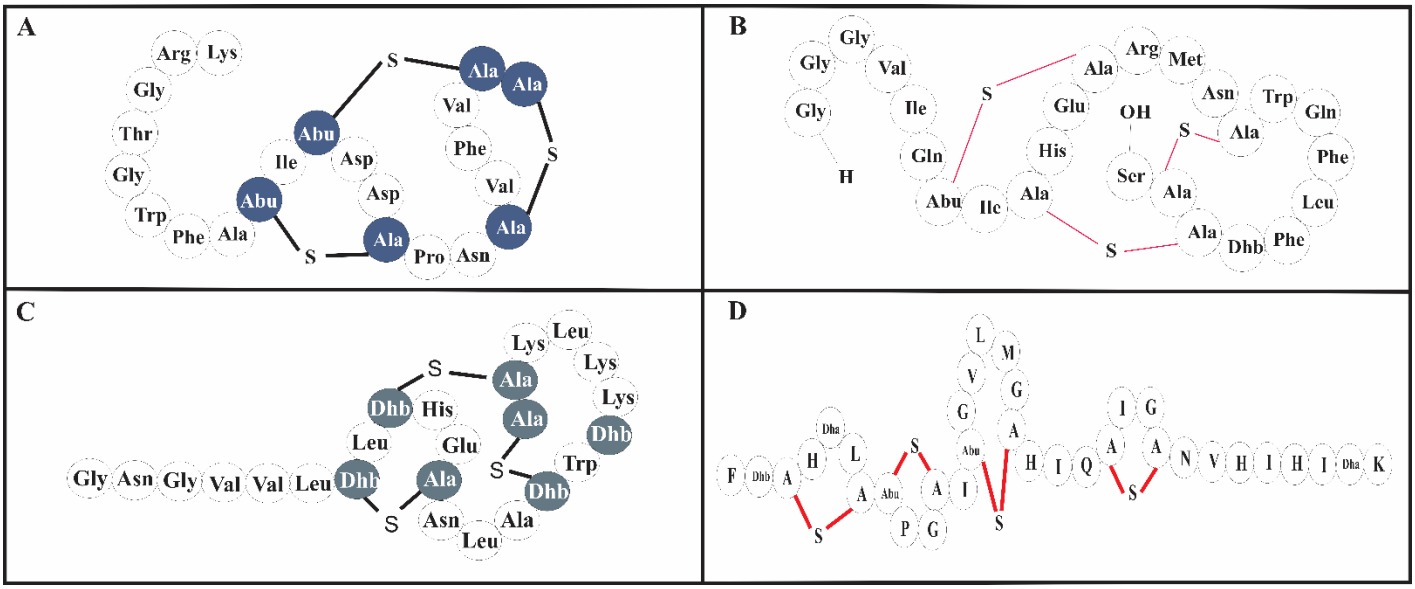



Figure 1-3: Covalent Structures of 4 lantibiotic salivaricins with Post-translational modifications, including dehydro-amino acids and lanthionine rings. (A) Salivaricin A2, (B) Salivaricin B, (C) Salivaricin 9, (D) Salivaricin D

# CHAPTER 2

# BIOACTIVITY AND STRUCTURAL CHARACTERIZATION OF A NOVEL SALIVARICIN

## *General Introduction*

The resurgence of antibiotic-resistant species in recent times has been of great concern to many over the previous decade. The increased length of hospital stays and mortality due to antibiotic resistance is viewed in the medical profession as an indication of worse things to come. It is for this reason that our research is focused on the isolation and characterization of novel antibiotic compounds. We are particularly interested in the isolation of the lantibiotics produced by certain members of the lactic acid-producing, Gram-positive bacteria (LAB), specifically those of *S. salivarius* U3.

Ecologically, the human oral cavity is an intricate environment, characterized by a web of complex symbiotic interactions that have formed between the various microbial inhabitants including bacteria, phage, and fungi (Guo et. al, 2014; Baker et. al, 2017). The full extent of this interaction is not fully appreciated. What is recognized, though, is the generalized stability of the oral niche. The microflora that colonize the mouth are highly resistant to fluctuations in organismal composition and appear to be resistant to outside colonization (Belstrom et. al, 2016; He et. al, 2014). Much of the research pertaining to ecological stability and oral health appear to implicate several different species of Streptococci bacteria, demonstrating their importance in either suppressing or managing invasive and/or pathogenic elements (Lopez-Lopez et. al, 2017; He et. al, 2014; Dierksen & Tagg, 2000). *S. salivarius* is one such species that has an interesting role to play in oral health given its probiotic qualities and antimicrobial efficiency against several infectious agents (Dierksen & Tagg, 2000; Wescombe et. al, 2006; Patras et. al, 2015). As an oral

commensal and as a producer of several bacteriocins, with both broad and narrow bioactivity, it is of definite interest to isolate new compounds.

The compounds that have been identified as salivaricins have so far come from either *S. salivarius* or from the more distantly related *L. salivarius* (Ross et. al, 1993; Barbour et. al, 2013, 2016; Birri et. al, 2012; Walker et. al, 2016; Wescombe et. al, 2012; O'Shea et. al, 2011; Barrett et. al, 2007; Pingitore et. al, 2009; Wayah & Philip, 2018). Additionally, the salivaricins so far documented have either fallen into the category of either class I or class II bacteriocins. Between the two species, class I bacteriocins are exclusively synthesized by *S. salivarius* and are characterized by their large array of post-translational modifications, the most notable of these being the lanthionine rings (Ross et. al, 1993; Barbour et. al, 2013, 2016; Birri et. al, 2012; Walker et. al, 2016; Wescombe et. al, 2012; Repka et. al, 2017). Novel salivaricins produced from *L. salivarius* are all class II bacteriocins, identified by their apparent lack of observable post-translational modification and their small molecular weights (O'Shea et. al, 2011; Barrett et. al, 2007; Pingitore et. al, 2009; Wayah & Philip, 2018). Both classes disrupt the membrane potential of effected species by binding to specialized target moieties such as lipid II, in the case of many lantibiotics, or the IIC/IID subunits of the mannose phosphotransferase, in the case of class II bacteriocins (Repka et. al, 2017; Kjos et. al, 2010).

While an abundance of genomic data exists confirming the identity of these compounds through their association with class specific synthesis elements, very little direct structural characterization of these products has been done. Characterization of class I/II bacteriocins has proven to be an extraordinarily difficult task, primarily due to the inability to purify these compounds in sufficient quantities for structural characterization. One very common method for structural characterization, X-ray crystallography, has proven to have dismal output, particularly

in light of the total number of bacteriocins that have been documented (Sahl et. al, 1995). Exceptions to this are the class I bacteriocin, mersacidin, (the only documented example for a lantibiotic) and the colicins (Schneider et. al, 2000; Hilsenbeck et. al, 2004; Grinter et. al, 2012). This limitation in crystalizing bacteriocins is thought to be the result of these molecules having flexible regions which interfere with directional nucleation, thus serving as an impediment to proper crystal formation (Antanaskovic & Kleanthous, 2019; Holcomb et. al, 2017). To date, NMR is the preferred method of structural characterization of these compounds, especially since this method is able to reveal the disulfide bonds/thioether bridges characteristic of lantibiotics (Rosengren et. al, 2009; van de Ven & Jung, 1996). With this in mind, it is surprising that only one salivaricin, SalA2, has been documented through direct means (Geng et. al, 2018). To be clear, this dearth of structurally characterized bacteriocins is significant because the structural chemistry underlying the lantibiotics bioactivity is diverse among this class of molecules. By thoroughly investigating and documenting all of the chemical and topological properties of these molecules, we can in time hope to understand the full efficaciousness of these compounds. This understanding would allow for the development of manufactured drugs which possess enhanced effectiveness. In this study, the partial characterization of the structure and bioactivity of a novel bacteriocin isolated from *S. salivarius* U3 is presented.

## *Materials and Methods*

**Bacterial strains and growth conditions.** The organisms used for this study are listed in Table 2-1. *S. salivarius* U3, *Micrococcus luteus* ATCC 10240, *Bacillus subtilis* PY79, wks1555, 34A, *Streptococcus pneumoniae* ATCC 27336, *Bacillus megaterium* ATCC 14581, and MRSA TCH-1516 were cultured on Todd-Hewitt yeast extract (THyex) agar plates (containing 30 g/liter Todd-Hewitt broth, 3 g/liter yeast extract, and 15 g/liter agar) or in THyex broth (containing 30 g/liter Todd-Hewitt broth and 3 g/liter yeast extract). All bacterial strains were cultured at 37 °C.

**Determination of Bioactivity for U3 metabolites.** Colonies of a newly plated bacteriocin-producer are taken from a fresh THyex agar plate and are suspended in THyex broth between $OD_{600}$ = 0.2-0.3. A 2µl sample of this bacterial suspension is seeded onto the plate in triplicate. The plate is then incubated for 24 hours at 37 ℃ after which time it is then heat killed via placement into an oven set to 65 ℃ for 1 hour. Fresh colonies of an indicator organism are then added to THyex broth to an $OD_{600}$ of 0.2. Then, 800 µl of this suspension isadded to 20 ml of molten top agar (30g/liter Todd-Hewitt broth and 7.5g/liter agar heated to 42 ℃), and 5 ml of this mixture is poured onto the surface of the plate. The plate is allowed to cool and solidify before being inverted and incubated for 24 hours at 37℃. The radius of each inhibition zones that appears is measured in millimeters and the average area is then calculated.

**Purification of U3 bacteriocin.** *Streptococcus salivarius* U3 was grown on a modified THyex soft agar containing 30 g/liter Todd-Hewitt medium, 3 g/liter yeast extract, 1 g/liter $KH_2PO_4$, 0.1 g/liter $K_2HPO_4$, 0.3 g/liter $MgSO_4$, 0.005 g/liter $FeSO_4$, 0.005 g/liter $MnSO_4$, and 0.3% agar. The medium was stab-inoculated with *S. salivarius* U3 and incubated at 37°C for 2 days. After that, the media was immediately frozen at −80°C overnight and was thawed the next day in a 65°C incubator. The thawed media was centrifuged at 20,000 × *g* for 30 min at 4°C in 250-ml centrifuge bottles to remove insoluble elements such as agar (Beckman J2-21 centrifuge). The supernatant of these bottles was decanted into a clean beaker and was subjected to an ammonium sulfate extraction. The amount of $NH_4SO_2$ salt dissolved into the supernatant was equivalent to 50% of the supernatants volume (50g/100ml). The extract was refrigerated at 4-5°C for two days or until proteinaceous layer was visible. The mixture was then separated into 250ml centrifugation tubes and centrifuged at 20,000 × *g* for 30 min at 4°C. The supernatant would be decanted and disposed of and the brown proteinaceous pellet would be dissolved in 3-5ml of 50%

(vol/vol) acetonitrile (ACN)-water with 0.1% trifluoroacetic acid (TFA). This extract would first be run on a semipreparative $C_{18}$ column (SinoChrom [ODS-BP; 5 μm × 10 mm × 250 mm]). After this, the cleaned product would then be run using an analytical column (SinoChrom [ODS-BP; 5 μm× 4.6 mm × 250 mm]). All solvents for HPLC contained 0.1% TFA. 1:4 extract to ½ ACN mixture produced optimal resolution. The sample was separated through a water-ACN gradient, starting with 90% to 70% water over 18 min, followed by an isocratic flow at 70% water for 20-25 min, followed by a linear gradient from 70% to 55% water for 8 minutes, followed by an isocratic flow at 55% for 3 minutes, followed by a concluding linear gradient from 55% to 20%. The fractions eluted at 60 to 55% water and were analyzed by mass spectrometry. The desired fractions were dried by lyophilization and resuspended in 1 ml of 10% ACN with 0.1% TFA. The samples were rerun on an analytical column with the same gradient from 90% to 20% water for 40 min.

**Confirmation of activity from purified fraction.** Verifying that collected fractions contained antimicrobial properties was performed using an overlay assay. *M. luteus* ATCC 10240 colonies grown on Todd-Hewitt yeast extract (THyex) agar plates (containing 30 g/liter Todd-Hewitt broth, 3 g/liter yeast extract, and 15 g/liter agar) would be added to THyex broth (containing 30 g/liter Todd-Hewitt broth and 3 g/liter yeast extract) to 0.2-0.3 $OD_{600}$. One ml of this suspension would be added to molten top agar (30g/liter Todd-Hewitt broth and 7.5g/liter agar heated to 42℃). Five ml of this mixture would be poured over the surface of a non-inoculated THyex plate and allowed to cool. 5μl of crude extract would be spotted onto the surface of the plate. A dried HPLC fraction of unknown concentration would be resuspended in 300μl of 50% can and 5ul of this material would be spotted onto the plate at designated positions. The spots would then be allowed

to dry at which time the plate was inverted and incubated at 37℃ for 24 hours. The plate would then be checked for inhibitory zones.

**MIC Determination.** The purified salivaricin fraction was dried and weighed on an analytical balance (Adventurer Pro AV114C; Ohaus Corporation, USA) and suspended in 100% DMSO. The minimal inhibitory concentration (MIC) is the lowest concentration of compound that inhibits the visible growth of the bacteria after 24 hours of incubation at 37°C, and was performed following a modified version of the broth microdilution method described in M07-A8 by the Clinical Laboratory Standards Institute. Commercially available antibiotic kanamycin was used as a comparison. The MICs were determined against *M. luteus* ATCC 10240.

**Stability assays / Mass disambiguation.** The protease stability of salivaricin U3 fraction was tested via trypsin digest. The trypsin treatment, a stock solution of sodium phosphate buffer (0.2M, pH 7.3) and a 10× trypsin stock solution (5.19 mg of trypsin in 1 ml of 1 mM HCl solution) were used. Each reaction mixture consisted of 200 µl 10× protease stock solution, 1 ml 2× stock solution, 780 µl dd$H_2O$, and 20 µl DMSO. Then, 100 µl of the reaction mixture was aliquoted into 1.8-ml centrifuge tubes. Using a prepared 1µg/µl stock solution of the purified fraction in 50% acetonitrile, 12 µg of salivaricin U3 was added to each tube and incubated for 0.5hr at 37°C. After incubation, the samples would be run on the HPLC and the masses of each collected fraction was determined via mass spectrometry.

**Mass spectrometry.** A Shimadzu/Kratos matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometer or an Electro-spray ionization mass spectrometer was used to determine the mass of the reversed-phase HPLC (RP-HPLC) fractions. Briefly, for the MALDI-TOF mass spectrometer, 1 µl of each fraction was directly mixed with 1 µl of α-cyano-4-

hydroxycinnamic acid matrix (30 mg/ml in methanol [MeOH]-0.1% TFA and then diluted with 50% ACN-water with 0.1% TFA to 6 mg/ml) and dried on the MALDI-TOF target plate in a 37°C incubator. MALDI-TOF MS was performed in the positive linear mode or reflective mode. For the Electrospray ionization mass spectrometer, 100µl of an HPLC fraction were lyophilized and resuspended in 300 µl of 50% (vol/vol) ESI-grade methanol-water. The ESI-MS was performed in the positive mode.

**Nuclear Magnetic Resonance (NMR).** NMR analysis of salivaricin U3 was performed as described previously (Ravichandran et.al, 2013). Salivaricin U3 was dissolved in dimethyl sulfoxide-$d_6$ and the NMR data were collected on a Bruker Advance III-HD spectrometer operating at a proton frequency of 850 MHz, using a TCI Cryoprobe. The $^{1}H$ resonances were assigned according to standard methods (Wüthrich, 1986) using COSY, TOCSY (Braunschweiler & Ernst, 1983), NOESY (Kumar et.al, 1980) and 13C-HSQC (Bodenhausen & Ruben, 1980) experiments. NMR experiments were collected at 25°C. The TOCSY experiment was acquired with a 60 ms mixing time using the Bruker DIPSI-2 spinlock sequence. The NOESY experiment was acquired with 400 and 500 ms mixing times. Phase sensitive indirect detection for NOESY, ROESY, TOCSY, and COSY experiments was achieved using the standard Bruker pulse sequences. Peaks were assigned using NMRView (Johnson & Blevin, 1994).

## *Results*

**Purification and Bioactivity Characterization.** For S. salivarius U3 that was used, it was essential to first understand whether or not the organism was capable of generating metabolites with antimicrobial properties. To do this, we used a deferred antagonism assay. By first seeding a fresh plate of medium with *S. salivarius* U3 inoculum and then allowing it to grow at 37°C for 24 hours, the plate was assayed for secondary metabolites that inhibit the growth either *M. luteus*

ATCC 10240, *B. megaterium* ATCC 14581, *S. pneumoniae* ATCC 27336, 3 strains of *B. subtilis* (PY79, 34A, wks1555), and MRSA TCH-1516. A zone of inhibitions was only observed against *M. luteus* ATCC 10240 (Table 2-2; Figure 2-1). These results indicated that antimicrobial metabolites produced by *S. salivarius* U3 are narrow spectrum in activity.

To isolate the compounds from extracts of the *S. salivarius* U3 strain, High-Performance Liquid Chromatography was used. During this analysis, a doublet peak is observed between 54-55% (Figure 2-2) in the chromatogram. These peaks were collected, separately, and their activity was tested using an overlay assay using the *M. luteus* indicator strain. The overlay assay clearly showed that both fractions had antimicrobial activity (Figure 2-3). Both HPLC fractions were mixed and spotted in the overlay assay to determine whether the fractions were part of a two-component antibiotic system. The combination of the fractions did not increase the inhibitory activity (data not shown). To quantify the potency of this compound, an MIC was performed using *M. luteus* ATCC 10240 as the susceptible strain. The MIC of the fraction 2 HPLC material was 128µg/µl (Table 2-3). The high concentration of material needed to inhibit growth is surprising given the size of the inhibitory zone on the overlay assay.

**Structural Characterization.** With bioactivity of the compound observed and quantified it became necessary to evaluate its chemical and structural attributes. Given the greater bioactivity of HPLC fraction 2, this sample was given priority for all chemical assays and collection. We began this facet of the investigation by first performing mass spectrometry and amino acid analyses. When subjected to electrospray ionization mass spectrometry (ESI-MS), the results were somewhat confounding. Although the compound was collected from a resolved HPLC peak, multiple masses were observed (Figure 2-4). From the amino acid analysis, we found that the compound was rich in proline (Table 2-4). Furthermore, it was apparent from the data the

compound contained no arginine or lysine residues. NMR was used to both independently verify the observations of the analysis as well as disambiguate/determine the mass of compound. Both the presence and molar concentration of glutamic acid/glutamine, valine, and leucine were supported by TOCSY (Table 2-5 and Figure 2-5). Additional confirmation for the presence of phenylalanine, glycine, and aspartic acid were also achieved through the TOCSY assignments. The presence of alanine was suggested by the amino acid analysis, however, this has yet to be confirmed by the data and may have been introduced through contamination.

### *Discussion*

In this study, we began the initial characterization of the bioactivity of a novel strain, U3, of *Streptococcus salivarius*. Purification efforts enabled the isolation of salivaricin U3 for additional bioactivity studies and structural characterization. Amino acid analysis and NMR analyses show that the bacterium produces a proline rich peptide with a narrow spectrum of activity. While there is some ambiguity regarding the true mass of this compound, it is reasonable to conclude from the TOCSY data that the compound is likely between 2 and 3 kDa, similar to other identified salivaricins. As predicted from the amino acid analysis, trypsin digestion did not help resolve the ambiguity in the mass spectrometry data due to the lack of lysine and arginine residues. The retention time and peak intensity of the resolved HPLC for the *S. salivarius* U3 product remained unchanged following digestion attempt with trypsin (Figure 2-6). Despite this, the result served as further experimental verification of amino acid analysis and its observation of nil arginine/lysine composition. While the presence of confounding contaminants is not ideal, the application of robust NOESY data should provide clarity.

Several novel compounds have been identified in *S. salivarius* and *L. salivarius* over the past two decades. Using *S. salivarius* U3, we further expanded the library of documented salivaricins

currently known. These results indicated that antimicrobial metabolites were being generated albeit having a very narrow spectrum of activity. Two things are to be kept in mind in light of these observations: (1) niche-specific activity and (2) cryptic bioactivity. To the first point, all organism that were tested, with exception to *S. pneumoniae,* are not conventional inhabitants/colonizers of the oral cavity. If the compound in question is anything other than a class I bacteriocin, it would stand to reason that the compound would have evolved to target a specific cadre of oral bacteria. Identification of bioactivity amongst species who are oral commensals is unquestionably worthwhile. It is possible that bacteria can produce compounds which, while not strong enough to decimate an antagonist, are potent enough to abate growth. This retardation in growth may be achieved to such an extent that the antagonistic species' ability to occupy the niche is sufficiently compromised through competitive exclusion. These sorts of cryptic responses could be tested for in the future through a kill kinetic assay.

The amino-acid and NMR analyses reveals two important things about the peptide in question. First, the spectral width of the spin systems is quite wide for peptide, being indicative of a highly structured molecule. Secondly, that the compound is proline rich, which also supports the first observation given the significant role prolines have in introducing sophisticated architecture such as beta turns. Also, the number of spin systems identified in the data suggest a peptide with a mass of no less than 2,300 Da. Further work and purification are needed to better resolve some of the ambiguities but the observations so far appear to be promising. It seems likely that with additional scrutiny with the aid of the TOCSY and NOESY data will eventually enable us to ascertain the class and function of this novel molecule.

Table 2-1. Table 2-1. Bacterial strains used in this study.

| Organism | Description | Reference or Source |
|---|---|---|
| *Streptococcus salivarius* U3 | Bacteriocin Producer | Texas A&M |
| *Micrococcus luteus* | Indicator Strain | ATCC 10240 |
| *Streptococcus pneumoniae* | Indicator Strain | ATCC 27336 |
| *Bacillus Megaterium* | Indicator Strain | ATCC 14581 |
| *Bacillus Subtilis* PY79 | Indicator Strain | Schroeder & Simmons, 2013 |
| *Bacillus Subtilis* wks1555 | Indicator Strain | Leiden University Medical Center |
| *Bacillus Subtilis* 34A | Indicator Strain | Browne et. al, 2015 |
| MRSA TCH-1516 | Indicator Strain | Hensler et. al, 2014 |

Table 2-2: List of all organisms used in deferred antagonism against U3 metabolites. "+" indicates an antibacterial growth response was observed and "-" indicates no observed response

| **Organism** | **Response** |
|---|---|
| *Micrococcus luteus* ATCC 10240 | + |
| *Bacillus megaterium* ATCC 14581 | - |
| *Streptococcus pneumonia* ATCC 27336 | - |
| *Bacillus subtilis* PY79 | - |
| *Bacillus subtilis* 34A | - |
| *Bacillus subtilis* wks1555 | - |
| MRSA TCH-1516 | - |

Table 2-3. MIC value for the 2nd fraction

| | Peak 2 | Kanamycin |
|---|---|---|
| **Strain** | **MIC (ug/ml)** | **MIC (ug/ml)** |
| M. luteus ATCC 10240 | 128 | 8 |

Table 2-4. The results of the amino acid analysis performed on the 2nd Fraction and ordered based upon molar % composition.

| | | **average** | | | | | **RAW DATA** | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | µgrams | | Molar | | | nanomoles | | | |
| AA | M.W. | from raw data | | % Composition | | | replicate 1 | replicate 2 | replicate 3 | average |
| PRO | 97.1 | 0.268 | | 28.2% | | PRO | 2.85 | 2.82 | 2.62 | 2.76 |
| GLX | 129.1 | 0.205 | | 16.1% | | GLX | 1.58 | 1.63 | 1.54 | 1.58 |
| VAL | 99.1 | 0.126 | | 13.0% | | VAL | 1.29 | 1.30 | 1.22 | 1.27 |
| LEU | 113.2 | 0.127 | | 11.4% | | LEU | 1.13 | 1.14 | 1.10 | 1.12 |
| THR | 101.1 | 0.076 | | 7.6% | | THR | 0.77 | 0.78 | 0.69 | 0.75 |
| ASX | 115.1 | 0.058 | | 5.1% | | ASX | 0.51 | 0.52 | 0.48 | 0.50 |
| ILE | 113.2 | 0.057 | | 5.1% | | ILE | 0.50 | 0.51 | 0.49 | 0.50 |
| PHE | 147.2 | 0.073 | | 5.0% | | PHE | 0.50 | 0.50 | 0.48 | 0.49 |
| SER | 87.1 | 0.030 | | 3.5% | | SER | 0.32 | 0.34 | 0.37 | 0.34 |
| GLY | 57.1 | 0.014 | | 2.5% | | GLY | 0.21 | 0.23 | 0.30 | 0.25 |
| ALA | 71.1 | 0.009 | | 1.3% | | ALA | 0.11 | 0.11 | 0.17 | 0.13 |
| TYR | 163.2 | 0.010 | | 0.6% | | TYR | 0.06 | 0.05 | 0.07 | 0.06 |
| ARG | 156.2 | 0.007 | | 0.4% | | ARG | 0.04 | 0.04 | 0.05 | 0.04 |
| HIS | 137.2 | 0.000 | | 0.0% | | HIS | 0.00 | 0.00 | 0.00 | 0.00 |
| MET | 131.2 | 0.000 | | 0.0% | | MET | 0.00 | 0.00 | 0.00 | 0.00 |
| LYS | 128.2 | 0.000 | | 0.0% | | LYS | 0.00 | 0.00 | 0.00 | 0.00 |
| IS(1) | | | | | | IS(1) | 5.00 | 5.00 | 5.00 | |
| IS(2) | | | | | | IS(2) | 5.00 | 5.00 | 5.00 | |
| | | | | | | | | | | |
| | Sum: | 1.06 | | 100% | | | 9.87 | 9.99 | 9.58 | 5.47 |

Table 2-5. Chemical shift values and corresponding amino acid assignments from NMR data of 2nd fraction. NMR data displaying spin systems with corresponding amino acid assignments.

| Assignment | Amide (6-12ppm) | Alpha (4-5ppm) | Side Chain (0-3ppm) |
|---|---|---|---|
| Proline | NA | 4.27 | 1.91/1.80 |
| Proline | NA | 4.32 | 2.28/2.18/1.49/0.85 |
| Proline | NA | 4.37 | 3.87/1.82/0.985 |
| Proline | NA | 4.53 | 2.13/1.95/1.81 |
| Valine | 7.56 | 4.34 | 1.71/0.86 |
| Cysteine | 7.58 | 4.19 | 3.99/1.00 |
| Glutamine | 7.72 | 4.42 | 1.92/0.78 |
| Phenylalanine | 7.7 | 4.44 | 3.01/2.84 |
| Isoleucine | 7.83 | 4.33 | 2.07/1.87/1.69/0.88 |
| Valine | 7.88 | 4.31 | 1.71/0.86 |
| Threonine / Dhb | 7.95 | 4.39 | 3.82/1.09 |
| Isoleucine | 8.018 | 4.26 | 1.50/1.65/0.82/0.87 |
| Glutamic Acid | 8.0419 | 4.44 | 2.14/1.88/1.72 |
| ??? | 8.0438 | 7.71 | 2.34/2.27 |
| Glycine | 8.06 | 3.84 | 3.59 |
| Aspartic Acid | 8.11 | 4.53 | 2.51/2.41 |
| Leucine | 8.12 | 4.18 | 2.11/1.72 |
| Threonine / Valine | 8.57 | 4.34 | 0.94/0.90 |
| Leucine | 8.6 | 4.59 | 1.65/1.48/0.91 |
| Threonine / Dhb | 7.95 | 4.39 | 3.82/1.09 |
| Isoleucine | 8.018 | 4.26 | 1.50/1.65/0.82/0.87 |

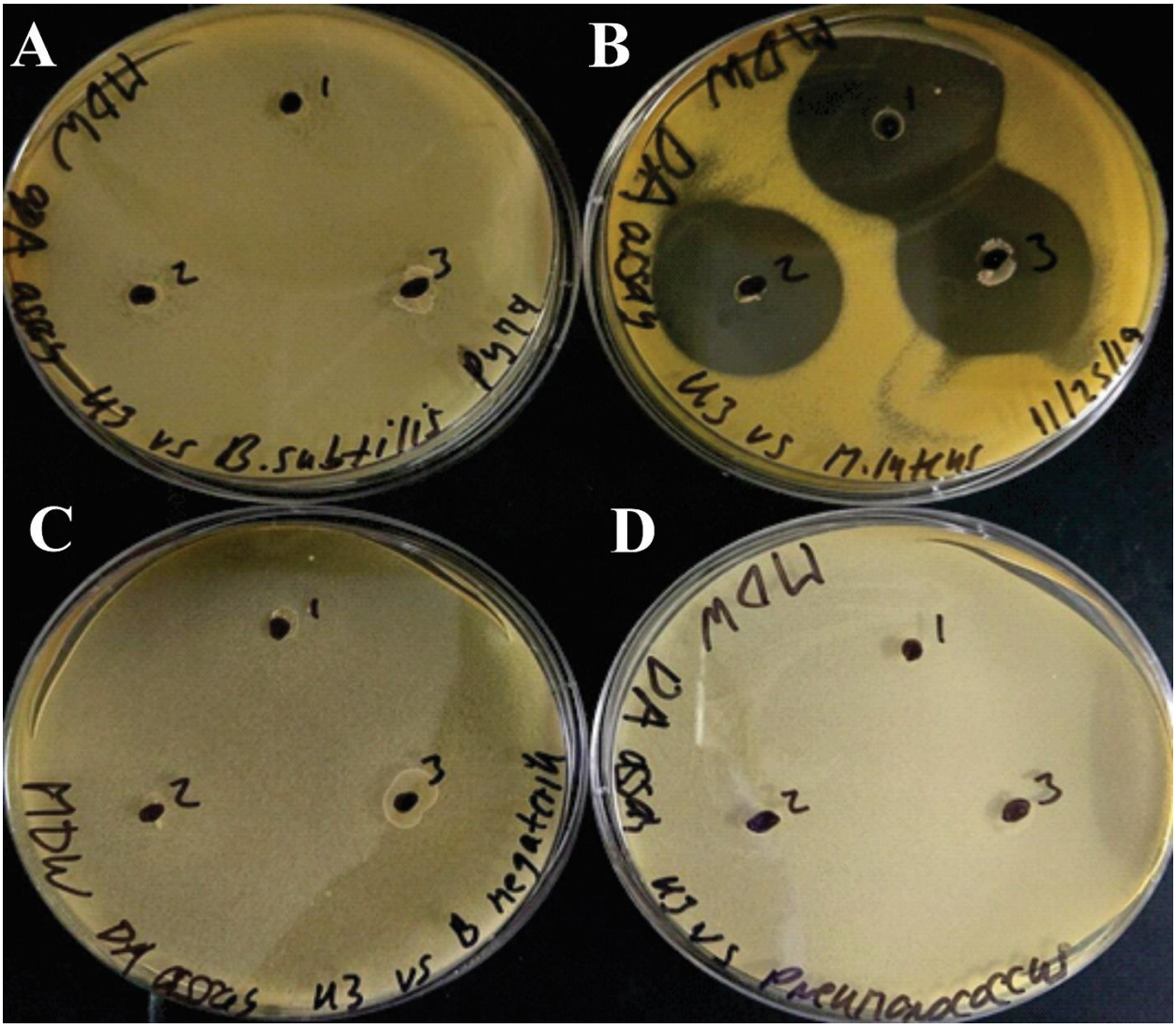



Figure 2-1: Deferred antagonism assay performed against four Gram-positive species. (A) *B. subtilis* PY79, (B) *M. luteus* ATCC 10240, (C) *B. megaterium* ATCC 14581, and (D) *S. pneumonia* ATCC 27336

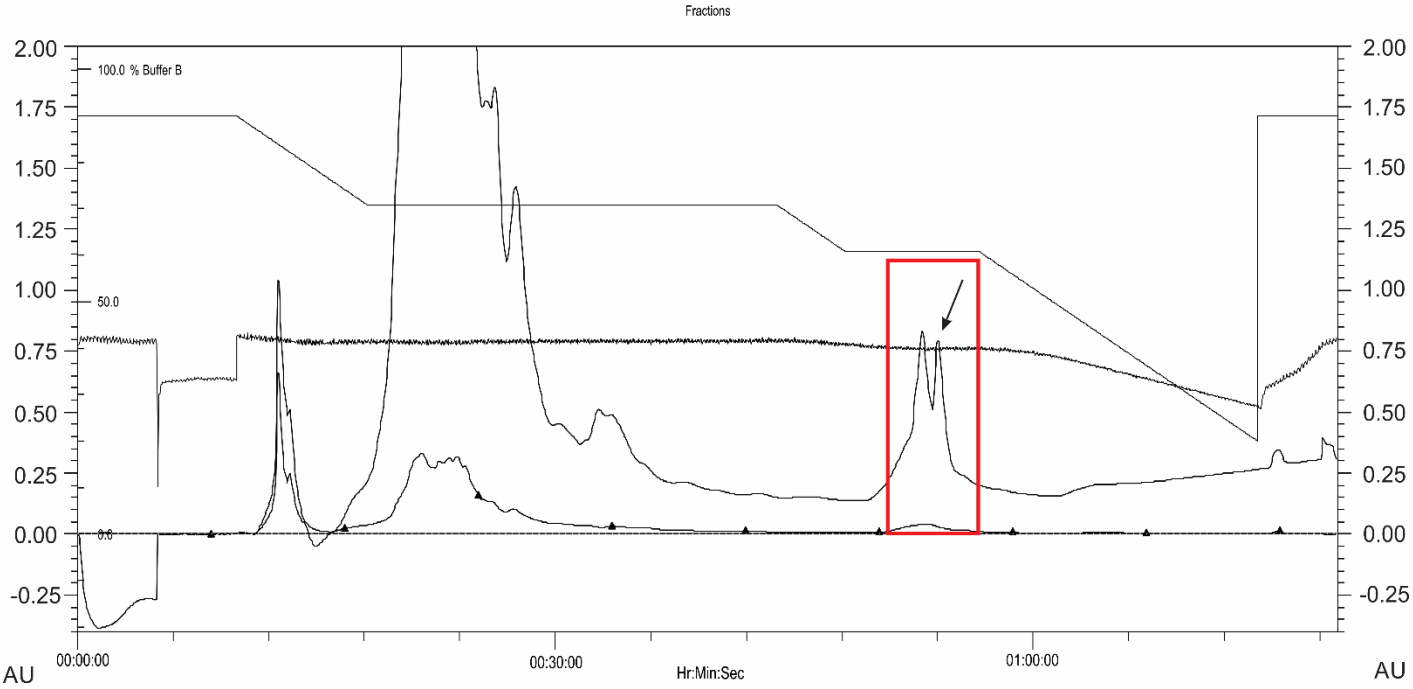



Figure 2-2: Chromatogram of $NH_4SO_2$ extract eluting off a semi-prep column. Red box encapsulates area of the chromatogram where fractions exhibits antibacterial properties. Black arrow indicates the fraction that has been subjected to chemical/structural analysis.

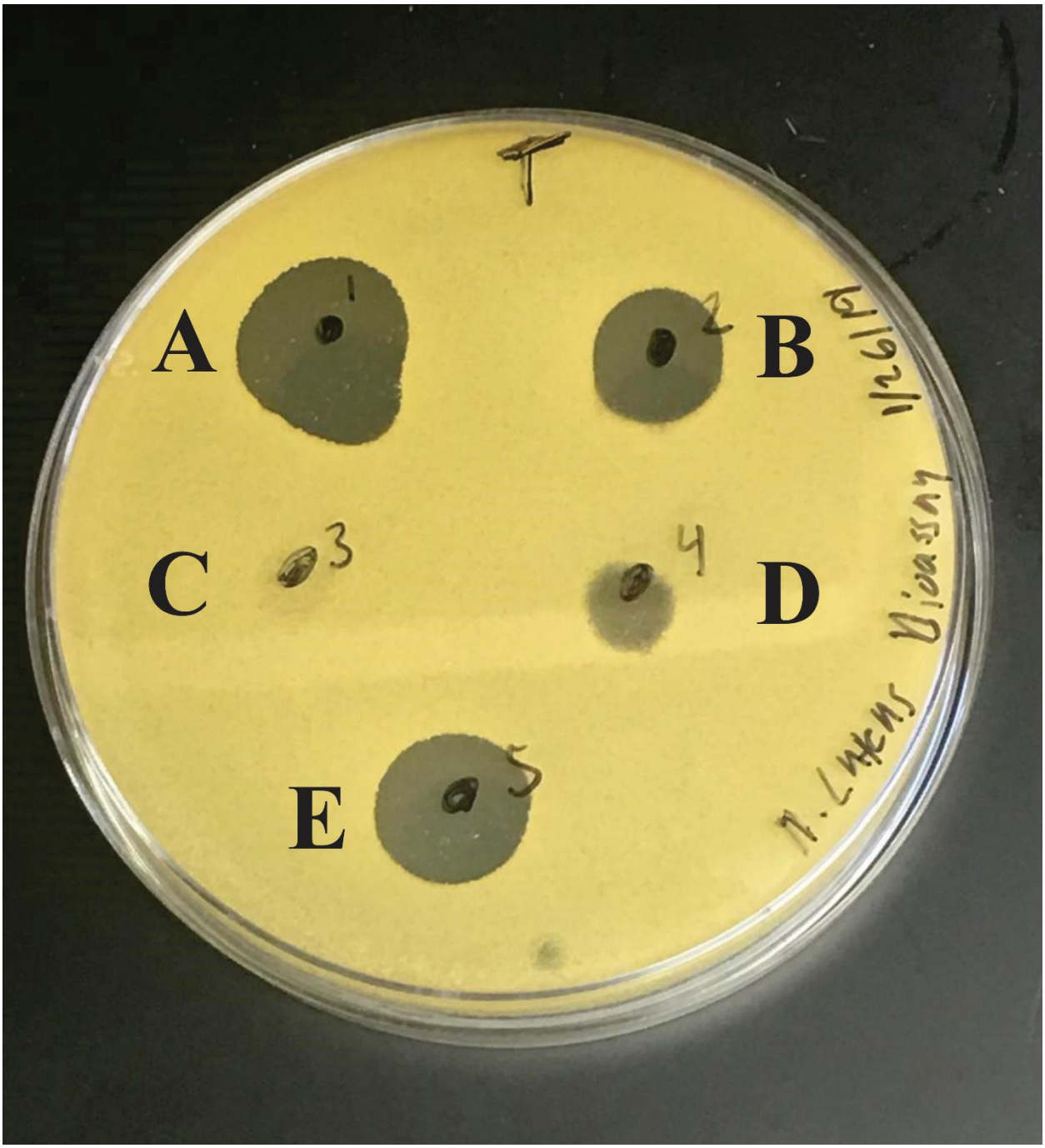



Figure 2-3. Overlay Assay involving different HPLC fractions against *M. luteus* indicator strain. Positions A and B had crude $NH_4SO_2$ added to them and served as positive controls. Position C had a non-active shoulder peak added. Position D had fraction 1 added to it, producing an inhibition zone of 7mm. Position E had fraction 2 spotted to it, producing an inhibition zone of 12mm.

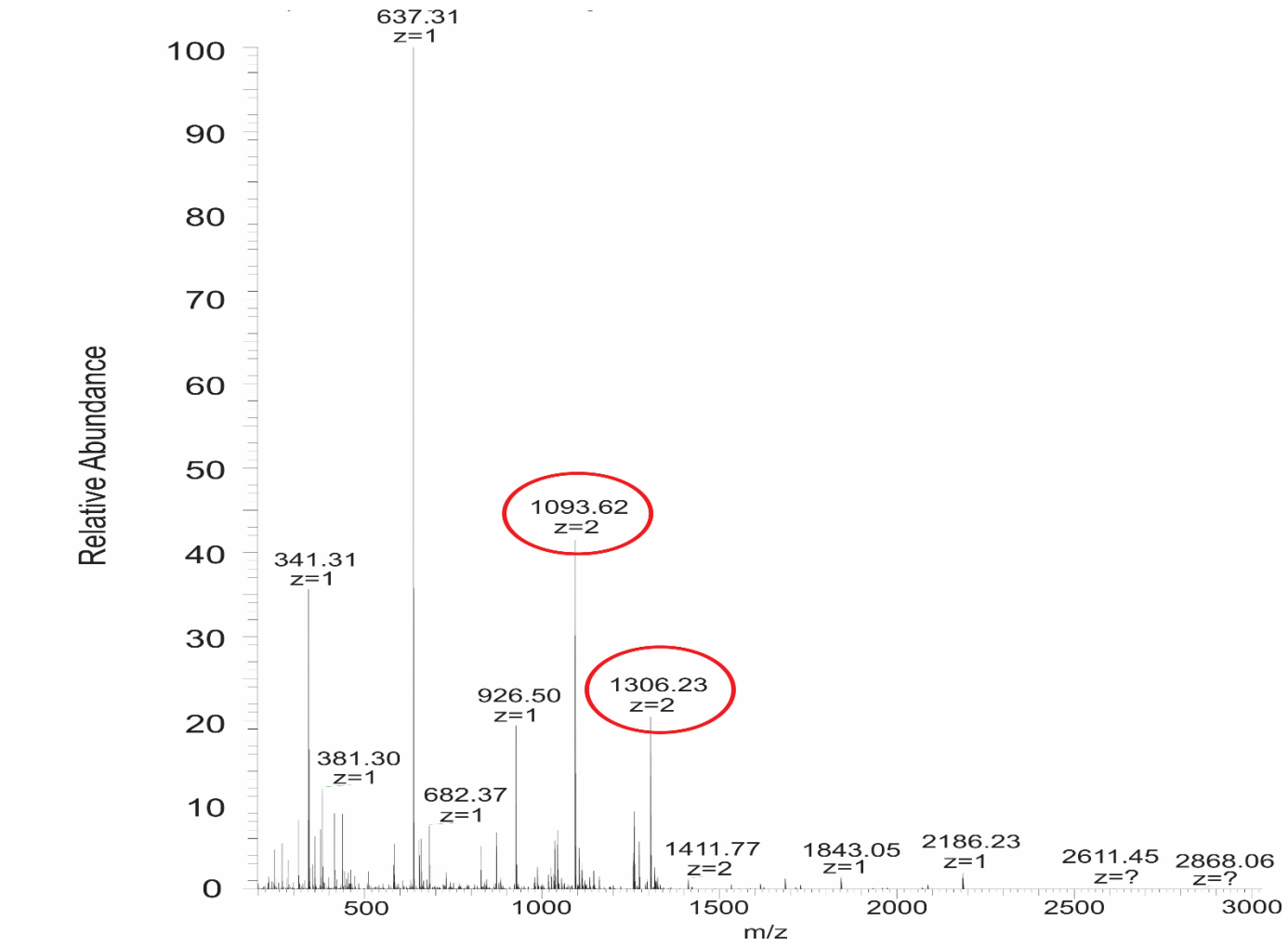



Figure 2-4. Electrospray Ionization mass spectrometry data for the 2nd HPLC fraction. The circled masses indicate those most likely to be the candidate for the compound of interest

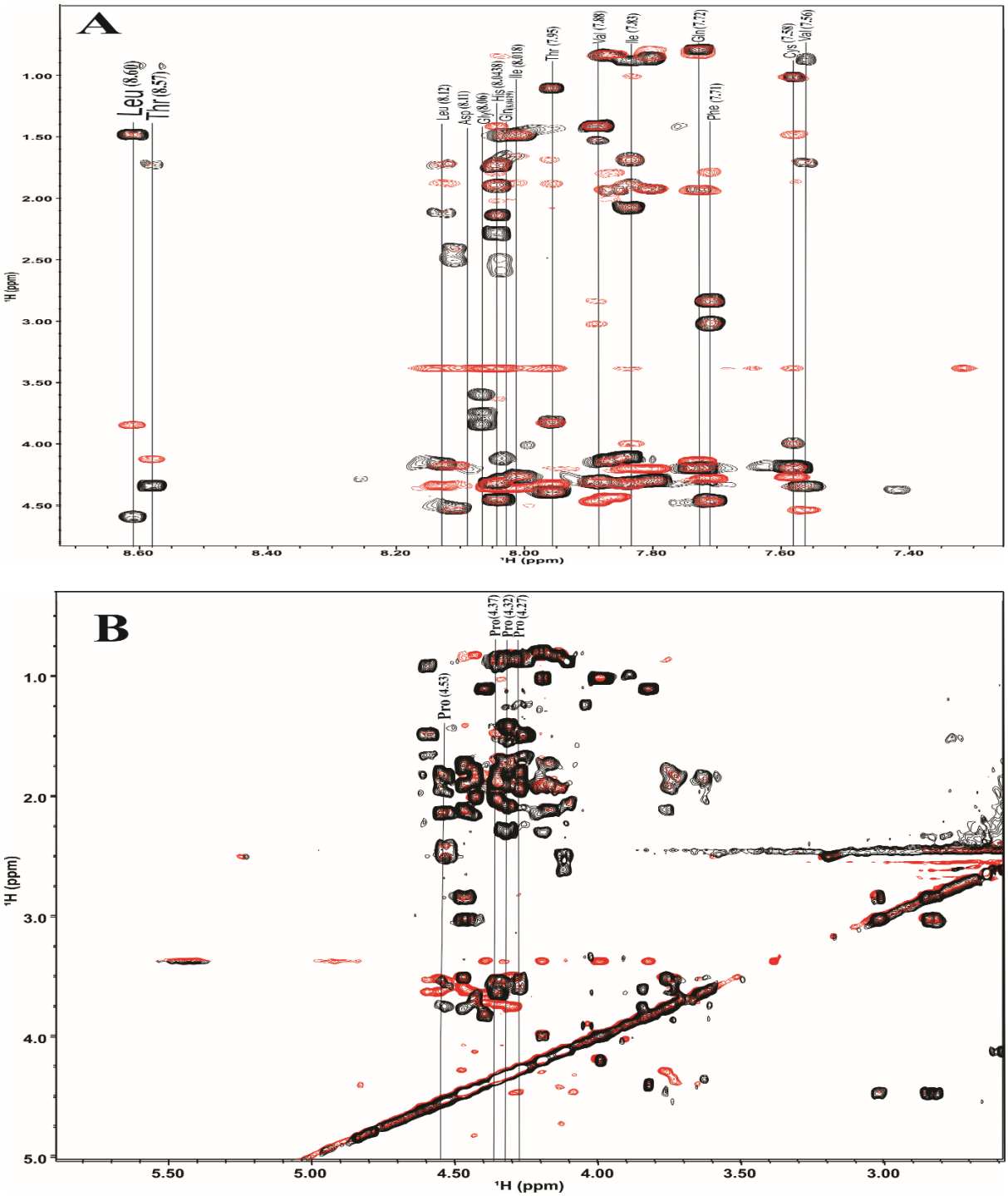



Figure 2-5. NMR data displaying spin systems with corresponding amino acid assignments. A.) Peptide fingerprint region, showing the NH correlations to alpha and side chain protons. B.) Region showing alpha proton and side chain proton correlations showing the presence of multiple prolines in the peptide structure.

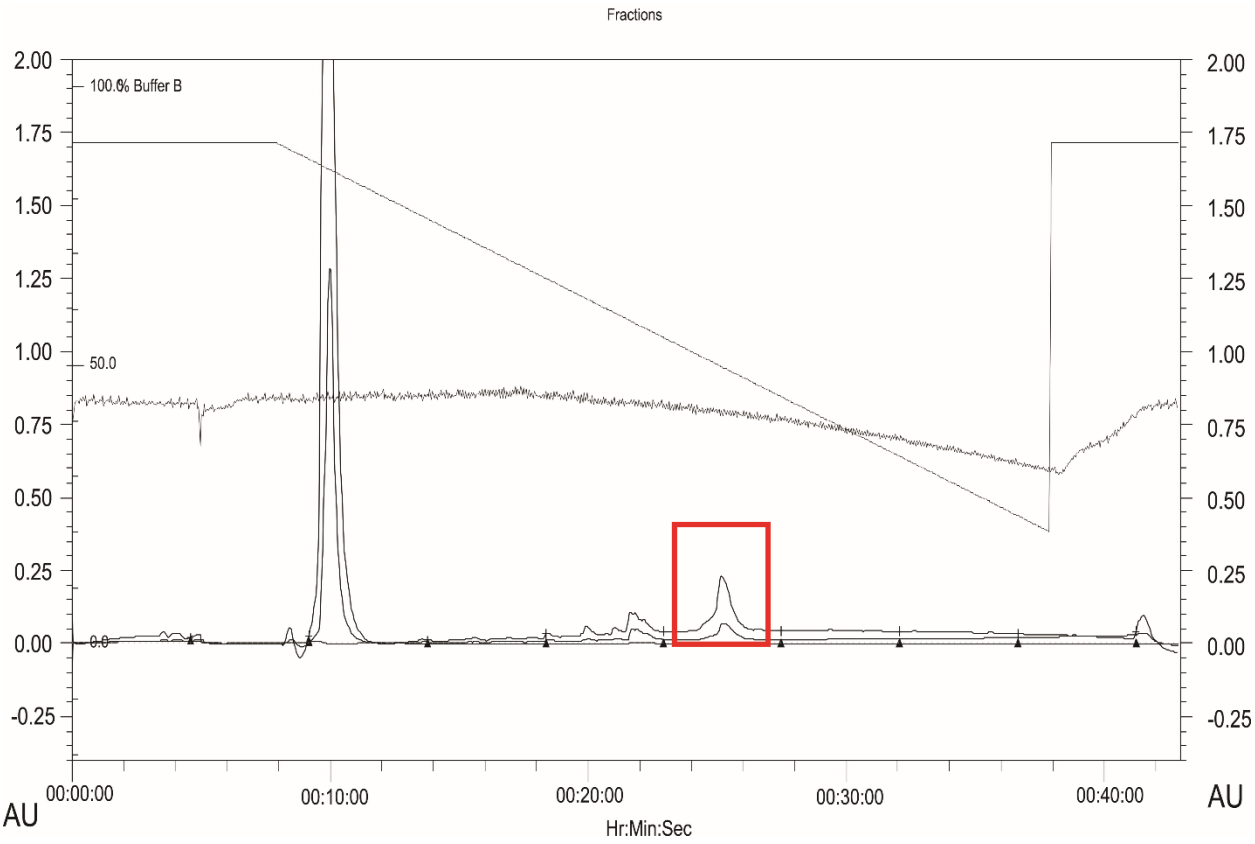



Figure 2-6. Chromatogram of peak 2 fraction after trypsin digest was performed. Red box indicates Peak 2 fraction. Identical masses were observed in the peak before and after digest indicating no reaction.

# CHAPTER 3

## CONCLUSION AND FUTURE DIRECTIONS

The idea that antibiotics may one day fail us is a concern not beyond interminable recapitulation. The frequency at which bacteria acquire immunity/resistance may be an omen for dire things to come. It therefore goes without saying that identification and potential synthesis of novel drugs is of the utmost importance if we are to prevent the resurfacing of past torments and plagues. Surmising the chemistry of these antimicrobials will undoubtedly open new venues for drug development. It has already been conveyed herein that the LAB are a viable source of many novel compounds which are specifically made to target both essential and conserved features of bacterial morphology. We know that *S. salivarius* is aggressive in its subjugation of preferred environments and that this predominance is aided by the production of several novel antibacterials that have yet to be discovered or elucidated. Indeed, the literature has shown that many have made a substantive effort in expanding upon the available number of compounds available to us however it may not be enough. As discussed in previous chapters, several of the compounds for which genetic and some physical data exist, have no supporting structural data. Experimentally determining the orientation of specific post-translational modification is quite important.

My research is reflective of only a partial characterization of one salivaricin. Many ambiguities persist regarding this compounds mass and chemistry. Future goals will involve achieving a comprehensive appraisal of this compound's qualities. More broadly, we will seek to apply a more directly empirical approach to the characterization of more compounds derived from novel strains of *S. salivarius*. We must attempt to push forward in this area of research, tenaciously exploiting these reservoirs. From there we will want to use the methods available to us to isolate, purify, and characterize this diverse array of compounds. The deficiencies that currently persist

in NMR data for salivaricins, let alone most bacteriocins, is in some way particularly problematic. Only when we can evaluate the structural nuances of these compounds do we have the ability to eventually emulate their chemistry in drug development. Our research will attempt to aggressively account for this oversight in the investigations to come. Characterization will also include scrutiny into the potency, stability, and range of these identified compounds. In achieving these goals, we intend to expand upon the current foray of knowledge and contribute to the laudable goal of providing new medicines and opportunities for future generations.

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