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ANTIMICROBIAL PROPERTIES OF SYRINGOPEPTIN 25A AND RHAMNOLIPIDS

by

Prerak T. Desai

A thesis submitted in partial fulfillment
of the requirements for the degree

of

MASTER OF SCIENCE

in

Nutrition and Food Sciences

UTAH STATE UNIVERSITY
Logan, Utah

2006

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ABSTRACT

Antimicrobial Properties of Syringopeptin 25A and Rhamnolipids

by

Prerak T. Desai, Master of Science

Utah State University, 2005

Major Professor: Dr. Bart C. Weimer
Department: Nutrition and Food Sciences

The increasing bacterial resistance to available antibiotics requires the search for new antibacterial compounds to be broadened. This study investigated the antimicrobial properties of two secondary metabolites from fluorescent pseudomonads -- syringopeptin 25A, a lipodepsipeptide produced by *Pseudomonas syringae* pv. *syringae,* and a rhamnolipid mixture produced by *Pseudomonas aeruginosa.* The rate of antimicrobial action was determined by monitoring the rate of uptake of propidium iodide during exposure to the compounds. Inhibition was also confirmed by the microbroth dilution method to determine the MICs. Both the compounds inhibited growth of Gram-positive organisms, including *Mycobacterium smegmatis,* staphylococci, and listeria. Inhibition of spore germination was also notable. SP 25A inhibited two multiple antibiotic strains of *Staphylococcus aureus* subsp. *aureus* and *Enterococcus faecalis,* while RLs failed to do

so, even at 60 μg/ml. Addition of the compounds together showed a synergistic activity against *Listeria monocytogenes*. Neither compound was toxic to human cells in vitro at 8 μg/ml.

It is postulated that both compounds exert their antimicrobial effect by forming pores in the bacterial cell membrane, but we did not observe a relation between membrane permeabilization and inhibition of growth in each case. At sub-MIC concentrations RLs did cause pores in the membrane of *L. monocytogenes,* while SP 25A did not. However, RLs did not inhibit cell growth, while SP 25A completely inhibited cell growth.

To investigate these effects gene expression was monitored just before treating the cells with the antimicrobials, 30 min after treatment and 120 min after treatment. The gene expression profile was distinct when cells were treated with both the antimicrobials. SP 25A repressed genes related to cell division, intermediary metabolism, transcription, translation, and virulence genes. These effects were not produced when cells were treated with RLs, hence giving indications that even though both the antimicrobials may act on the same site (i.e. the cell membrane), the cellular response was different, which led to different phenotypes for growth.

This work indicates that SP 25A holds promise for further development as a therapeutic agent and provides evidence that the proposed pore-forming model alone does not suffice to explain the mode of action of SP 25A.

(105 pages)

ACKNOWLEDGMENTS

I would like to express my deepest gratitude to my professor and mentor, Dr. Bart C. Weimer, for giving me the opportunity to learn and discover in his group. In the same spirit, I also thank my committee members Dr. Jon Takemoto and Dr. Marie Walsh for their guidance and encouragement.

I would also like to thank all the members of Weimer lab: Dr. Dong Chen and Dr. Balasubramanian Ganesan in particular for guiding me, challenging my ideas, and motivating me to improve. A special acknowledgment needs to be mentioned to all of the CIB staff, Patricia and Mark in particular for all their help. I also express appreciation for the members of Takemoto lab, Mekki F. Bensaci in particular for helping me purify syringopeptins.

I also thank all my friends here in Logan: Harshil, Bhavik, Shaunak, and Vishal for sharing with me the best and worst of the times. Many others are not listed here for the lack of space but I truly acknowledge their support.

Above all I dedicate the realization of this endeavor to my parents, Sh.Tushar Desai and Smt. Priti Desai, without whose support it would not have been possible for me to pursue higher education.

Prerak T. Desai

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CHAPTER 1

INTRODUCTION

Very few developments in the history of health science have had such a profound impact upon human life as the advances in controlling pathogenic microorganisms since the discovery of penicillin. Though the application of antimicrobial agents preceded the understanding of their action, it was not until the late 19th and early 20th century that the work of Pasteur and Koch firmly established that microorganisms as the cause of infectious diseases and provided strategies that led to rational prevention and control strategies. The first group of compounds discovered to suppress bacterial infections were sulphonamides (2). The success of sulphonamides along with the world wars stimulated a massive hunt for more effective antimicrobials. Florey and Chain (3) succeeded in isolating an impure but highly active preparation of penicillin and published their results in 1940. The enormous success of penicillin quickly diverted a great deal of scientific effort towards search of other antibiotics that culminated in the discovery or approximately 3,000 named antibiotics. Of these, 50 have met with clinical use and many fewer are regularly employed in therapy of infectious diseases (2).

The initial success of those antibiotics is now marred with emergence of resistant organisms. Antibiotic resistance is a complex problem accelerated by the versatility of the microbes, overuse of antibiotics, and the lack of patients completing the prescribed dosage (1). Curable diseases such as gonorrhoeae and typhoid are becoming difficult to

treat (4) due to resistance issues. However, the most troubling issue is the establishment of vancomycin resistant organisms (4). The solution to the problem of antimicrobial resistance is very complex and a tough scientific issue. The primary path forward for keeping infectious agents at bay is to continuously discover new antimicrobial compounds. This path becomes easier if we better understand the mode of resistance and susceptibility. With the advances in genomics, this task become should become easier and faster. The knowledge obtained from system-wide genome analysis will help us in designing better molecules to inhibit microbes in multiple modes.

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CHAPTER 2

LITERATURE REVIEW

Molecular mechanisms of antibiotic action

The word antibiotic brings together a varied group of compounds with little in common except for their antimicrobial activity. Hence, it is not surprising that they prevent the growth of susceptible bacteria by different molecular mechanisms. The five major mechanisms by which the antibiotics attack bacterial cells are (25, 35, 40):

1. *Blocking steps in cell wall synthesis*. This group is exemplified by penicillin, cephalosporin, cycloserine, and vancomycin. These antibiotics interfere with the biosynthesis of peptidoglycan and damage its cross linked macromolecular structure which can arrest growth and kill the bacteria.
2. *Permeabilizing cell membrane*. Polymyxin, tyrocidin, and valinomycin are examples of this mode of action. When cells are treated with lethal doses of these antibiotics they interact with the components of cell membrane, probably proteins, and induce a lesion in the cell membrane hence impairing its ability to act as a semi permeable barrier between the cell and its environment causing the cell components to leak out.
3. *Inhibition of nucleic acid function*. This group has examples including rifampicin, actinomycin D, and acridines. These compounds interfere at various stages (nucleotide biosynthesis, polymerization of nucleotides) of DNA replication and ultimately cause loss of gene expression, which is fatal to bacterial cells.

4. *Inhibition of protein synthesis*. For example streptomycin, tetracyclines, and chloramphenicol are used that act in this mode. These compounds bind the subunits of ribosome and distort the ribosome enough to prevent normal codon anticodon interaction leading to either inhibition of protein synthesis or faulty protein synthesis.
5. *Inhibition of cellular metabolism*. This is exemplified by sulphonamides. These compounds inhibit the synthesis of folic acid by competing with *p*-amino benzoic acid as a substrate for enzyme tetrahydropteroic acid synthetases that incorporates *p*-amino benzoic acid into the folic acid molecule.

Antibiotic resistance in microorganisms

The development of microbial strains that are resistant to antibiotics is unfortunately an ever increasingly common phenomenon. Antibiotic resistance may be divided into two categories:

Intrinsic resistance: Generally Gram-negative bacteria are more resistant to antibiotics than Gram-positive bacteria (4, 17, 35). This greater intrinsic resistance of Gram-negative bacteria to antimicrobial agents may depend upon the nonspecific permeability barrier presented by the outer membrane, preventing access of the antibiotic molecules to their active site (4, 17, 35). Some bacteria may be inherently resistant due to presence of various defense mechanisms like presence of antibiotic degrading enzymes, presence of antibiotic efflux mechanisms or the organism may altogether lack the drug target (4, 17, 35).

Acquired resistance: Overuse of antibiotics and consequent antibiotic selective pressure is thought to be the most important factor contributing to the organisms gaining antibiotic resistance (4, 17, 25, 35, 39). Mechanisms by which the microbes gain resistance may be due to spontaneous mutations, transduction, transposons or conjugation (25). Bernard Davis (1952) first outlined the possible biochemical mechanisms of drug resistance. They may be summarized as follows (4, 17, 35):

1. Conversion of an active drug to an inactive derivative (e.g. inactivation of β-lactam antibiotics by β-lactamases).
2. Enhancement of alternative metabolic pathways (e.g. the pathways responsible for the salvage of purine and pyrimidine bases from nucleic acid catabolism); thereby allowing their reutilization into new nucleic acids. This may be used to circumvent the antibacterial activity of certain nucleic acid analogues.
3. Reduction in physiological importance of the target site, for e.g. bacteria may overcome the antimicrobial action of sulphonamides by accumulating large amounts of PABA (Para amino benzoic acid).
4. Loss of cell permeability to a drug due to synthesis of an additional permeability barrier (if the antibiotic gains access through passive transport) or due to loss of a specific transport mechanism (i.e. if the antibiotic gains access through a particular transport mechanism).
5. Modification of the antibiotic sensitive site (e.g. resistance to erythromycin in several bacterial species depends on an alteration in a protein of the 50S ribosome subunit that leads to a reduced affinity of ribosomes for erythromycin).

6. Active efflux of the antibiotic from the cytoplasm (e.g. resistance to tetracycline in several Gram-positive as well as Gram-negative bacteria depends upon an ATP dependent efflux system present in the cytoplasmic membrane).

Antibiotic resistance is a global health problem and is increasing. With every new antimicrobial compound discovered or synthesized we are only a step ahead of microbes before they become resistant. Recent increases in bacterial and fungal infections, the few available antibiotics and the increasing resistance to the available antibiotics have resulted in a broadening of the search for new inhibitory compounds.

Secondary metabolites from pseudomonads as potential antimicrobials

Secondary metabolites from microbes are compounds produced and typically secreted into the environment during the stationary phase of growth, and so are not produced or required for growth (12, 30). The physiology of stationary phase cells is adapted to restricted nutrients or another nutritional change, which allows the cell to shift metabolism leading to the production of these compounds. One can think of this phase as an environmental stress that result in changes in cellular metabolism that induces the production of new compounds that are known as secondary metabolites. Often these secondary metabolites are directed at inhibiting competing organisms that occupy the same niche to give the producing organism a selective advantage. There are two ways to increase ones competitive fitness in a 'tight' environment: self-improvement (increasing ones own ability to gather nutrients), or by decreasing the fitness of ones competitors.

Siderophores and high-affinity nutrient uptake mechanisms are examples of how a cell can increase its supply of nutrients (12, 30). Antibiotics and bacteriocins are examples of how an organism can increase its supply of nutrients by crippling its competition (6, 12, 30).

Pseudomonads produce a variety of secondary metabolites that act as phytotoxins or antimicrobial/antifungal agents (6, 12, 30) (Table 2.1).Pseudomonads produce a wide spectrum of chemically distinct and biologically active compounds that inhibit other microbes. Among the most well characterized compounds are those produced by plant pathogen *Pseudomonas syringae* and opportunistic pathogen *Pseudomonas aeruginosa.* This study would be focusing on two compounds; syringopeptins produced by *P. syringae* pv. *syringae* and rhamnolipids produced by *P. aeruginosa* owing to very promising results obtained in the preliminary studies.

Syringopeptins

Syringopeptins (SPs) are a class of cyclic peptides substituted with fatty acids known as cyclic lipodepsipeptides that are produced by several strains of *P. syringae* pv. *syringae*. Currently, five different syringopeptins have been identified that vary in their fatty acid chain length and number and composition of amino acids in the peptide moiety (2, 3, 6, 13, 15, 16, 19, 23, 43). Syringopeptins contain either 22 or 25 amino acids depending upon the specific bacterial strain from which it was purified. Often the N-terminal (2, 3-dehydro-2-aminobutyric acid) is acylated with either 3-hydroxydecanoic or

3-hydroxydodecanoic acid (5, 6, 24). An eight-member lactone ring is formed due to the ester bond between allothreonine and C-terminal tyrosine (5, 6, 24). A high percentage of hydrophobic amino acids are found in syringopeptins and it has been determined that the peptide moiety is primarily composed of D-amino acids (5, 6, 24). The peptide sequence of the syringopeptins may vary from strain to strain (5, 6, 24).

The conformation is characterized by three structural regions: 1) a loop including the residues from Pro2 to Val6, 2) a helicoidal zone including the residues from Ala8 to Ala15, and 3) the lactone ring including Thr18 to Tyr25. This three-dimensional conformation likely leads to changes in the membrane environment as discussed later in this review (31) (Fig. 2.2). Primarily, it is a phytotoxin and functions as a virulence factor for *P. syringae* by playing a major role in inducing necrosis in plant cells (6). Studies with knock out mutants have shown that organisms deficient in SP production are less virulent, though some diseases may also occur in its absence (18). It has the ability to cause electrolyte leakage by forming pores in plant plasma membranes, thereby promoting transmembrane ion flux that leads to necrotic symptoms (23). It also displays biosurfactant properties with a critical micelle concentration of 0.9 mM for SP 25A and 0.4 mM for SP 22A, which may aid in the spread of organisms on the plant surface (6, 21).

Though the antimicrobial properties of this compound have yet to be fully assessed, preliminary studies show promising results to inhibit many different bacteria. Initial studies found these compounds inhibit Gram-positive bacteria at micromolar

concentrations, with the MIC values ranging from about 1.5 to 37 μg/ml for various organisms (Table 2.2).

Little information about the mechanism is published describing the mode of action of the SPs against bacteria, but the initial studies indicate that SPs form pores in model membranes (3, 21, 23, 31, 43). It has been predicted that the SP molecule first adsorbs onto the cell membrane with the hydrophobic acyl chain inserted between those of the phospholipids of cell membrane, while the hydrophobic linear peptide portion and the hydrophilic cyclic moiety remain folded as they are in solution. Presumably, the adsorbed monomers form aggregates that eventually form the pore, since more than one monomer is required to form a pore (14, 21). After forming aggregates the hydrophobic portion unfolds and aligns with the lipid tails spanning the membrane that causes the formation of a pore. This pore formation is voltage dependent as observed in planar lipid membranes (21). Once the pore is formed, the cell would loose its permeability barrier and ultimately lead to cell death.

Rhamnolipids

Rhamnolipids (RLs) are biosurfactants produced by several strains of *Pseudomonas aeruginosa* (1, 7, 8, 12, 20, 30, 32, 33, 38). What makes these compounds interesting is that it also shows antimicrobial activity the potential applications of which could be enormous if they inhibit many types of bacteria (1, 7, 20). RLs are often a mixture of various homologues, depending upon the strain and carbon source provided

during growth (33). Eleven different homologues have been identified in cultures of *P. aeruginosa* (20) (Fig. 2.3). The physiological role of a specific RL is not well understood, but since they have very good surface active properties they may:

1. Emulsify hydrocarbons or hydrophobic substrates making them available for cell metabolism (28, 36).
2. Help the cells in swarming motility under nutrient limitations (28).
3. Cripple the competing organisms for nutrients by the virtue of it antimicrobial activity (1, 7, 20).
4. They may play a role as a virulence factor (34). *In vivo* RLs prevent phagocytosis of the organism by macrophages by bringing about structural changes in the macrophages so they cannot associate with the bacteria (34).

RLs have an array of applications due their versatility in bioactivity. A few of them are as follows:

1. They are useful in bioremediation and biodegradation of organic compounds, both aliphatic and aromatic. Addition of RLs to pure cultures of bacteria increase biodegradation of hexadecane, octadecane, n-paraffin, phenanthrene, tetradecane, pristine and creosote (32). This phenomenon could be due to their surface-active properties since they increase the solubility of the hydrocarbons and hence make them readily available to the degrading cells, or they may increase the surface hydrophobicity of the cells by removing lipopolysaccharides from the cell wall improving the association of more hydrophobic substrates with the cells (32).

2. They have high affinity for a variety of toxic metals including cadmium, copper, lead, zinc, and lanthanum. This property makes them a potential flushing agent in sites where bioremediation may be too slow or infeasible (32).
3. They are effective against zoosporic plant pathogens, such as *Pythuim aphonidermatum, Phytophthara capsici, and Plasmopara lactucae-radicis.* In their pure forms RLs render the zoospores nonmotile and bring about their lysis in less than a minute at a concentration of 5-30 μg/ml (32). Hence, they show potential for biological control, too.
4. Due to their low toxicity ready biodegradability and excellent surface-active properties they could be of great value in the cosmetic industry (42).

The antibacterial activity of RLs has not been fully explored nor exploited for commercial value. These compounds are active against both Gram-positive as well Gram-negative bacteria (1, 7, 20). The target for the action of rhamnolipids is the cell envelope (1), presumably the physicochemical properties of the compound are responsible for the permeabilization effect on the cell surface.

Use of high density oligonucleotide microarrays to determine cellular response to inhibitors

The entire genome of several bacteria are fully sequenced, annotated, and available in the public domain that can be used to assess the global impact of bacterial inhibitors on gene expression. This along with the latest developments in high density oligonucleotide microarrays represents a very powerful resource to understand the

differential gene expression patterns in bacteria when subjected to different antimicrobial compound treatments (22, 41, 44). By analyzing differential gene expression patterns of bacteria when subjected to sub-MIC doses of antimicrobials, we have an opportunity to uncover adaptive mechanisms employed by bacteria at the genomic level to overcome the antimicrobial stress and also look at various secondary cellular responses. Stress responses thus induced can be used to detect and characterize the mode of action of these antimicrobials (9). In contrast to using single copy gene fusions (9) use of high density oligonucleotide arrays are better tools to understand the stress responses and when combined to various pathway mapping tools like KEGG (26, 37) or Metacyc (27), they confer an opportunity to uncover the precise metabolic shifts leading to the expression of a particular phenotype.

In context to the antimicrobials under investigation here, evidence is mounting that for poreforming peptides, membrane permealization may not be the only mechanism by which they inhibit bacteria (10). They may flocculate intracellular contents, alter cytoplasmic membrane septum formation, inhibit cell wall synthesis, bind nucleic acids, inhibit protein synthesis or inhibit enzymatic activity (10). Also, it has been observed that the antimicrobial activity may or may not correlate with the pore forming activity of the peptide because evidence is available that bacteria can repair their cytoplasmic membrane even when treated with lethal concentrations of the antimicrobial (45) giving conclusive evidence that alternate mechanisms of cell death may be involved. Hence, in the light of recent discoveries it becomes necessary to investigate the alternate mechanisms of the

bactericidal effects of these compounds. Use of whole genome expression studies currently seems to be the best alternative to achieve that.

In light of the need for new antibiotics and the broad industrial applications of SPs and RLs, we have selected them for further study. We will use these compounds to determine their potential to inhibit foodborne illness organisms as well as other pathogens important to the agricultural sector.

Hypothesis

Syringopeptin 25A (SP 25A) and rhamnolipids (RLs) inhibit many species of bacteria and their mode of inhibitions are distinct from each other.

The above hypothesis would be verified by realizing the following objectives.

Objectives

1. Screen candidate compounds for microbial inhibition and determine the minimum inhibitory concentration for these compounds against selected bacteria
2. Based on results from the first objective, one organism will be used to determine the cellular response to sub-MIC levels of SP 25A and RLs using gene expression arrays.

Completion of these objectives will demonstrate the utility of a two new classes of natural antibiotics to inhibit bacteria found in food and the environment. Specific commercial applications of these compounds may be possible, but demonstration of those

applications is beyond the scope of this initial project. Use of gene expression arrays will offer insight into the mechanism of action and potential targets that are being inhibited by the compounds in the cell. Most importantly, this study will determine the differences in the cellular response between two different classes of antibacterial compounds that are produced by pseudomonads to inhibit other bacteria.

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Table 2.1. Antimicrobial/antifungal secondary metabolites from pseudomonads.

Toxin	*Pseudomonas* species	Chemical Class	Reference
Pyo Compounds	*P. aeruginosa*	Quinoline derivatives	8, 10
Pseudomonic acid	*P. fluorescens*	Organic acid	8
Pyrrolnitrin	*P. cepacia,* *P. aureofaciens*	Pyrrol derivatives	8, 10
Pyoluteorin	*P. aeruginosa*	Pyrrol derivatives	10
Azomycin	*P. fluorescens*	Pyrrol derivatives	10
Rhamnolipids	*P. aeruginosa*	Glycolipids	8, 10, 11
Tabtoxin	*P. syringae*	Monocyclic β-lactam	8, 9
Phaseolotoxin	*P. syringae*	Sulfodiaminophosphinyl peptide	8, 9, 10
Sperabillins	*P. fluorescens*	Substituted peptides	10
2-n-hexyl-5-n-pentyl resorcinol & 5-n-heptyl resorcinol	*Pseudomonas* spp.	Substituted resorcinols	8, 10
2,4 diacetyl phloroglucinol	*P. fluorescens*	Substituted Phenols	8, 10
Obafluorin	*P. fluorescens*	β-lactones	10
Tropolone	*Pseudomonas* spp.	Tropolone	10
7-hydroxy tropolone	*P. cepacia*	Tropolone derivative	10
Thiotropocin	*P. seudomonas spp.*	Tropolone derivative	10
Phosphonomycin	*P. syringae*	Unknown	10
Syringomycin	*P. syringae,* *P. fuscovaginae*	Lipodepsinonapeptide	9, 10

Pseudomycin	*P. syringae,* *P. fuscovaginae*	Lipodepsinonapeptide	9, 10
Syringotoxin	*P. syringae,* *P. fuscovaginae*	Lipodepsinonapeptide	9, 10
Syringostatin	*P. syringae,* *P. fuscovaginae*	Lipodepsinonapeptide	9, 10
Syringopeptin	*P syringae*	Lipodepsipeptide	9,10
Tolaasin	*P tolaasii*	Lipodepsipeptide	9
Viscosin	*P fluorescens*	Lipodepsipeptide	9

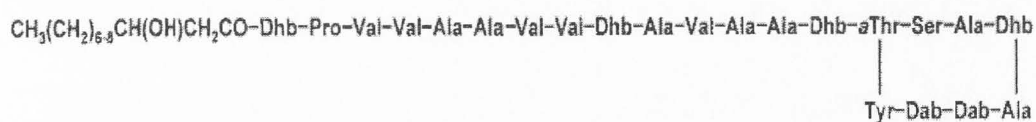
Table 2.2. Reported MIC values for SP 22A and SP 25A against selected organisms.

Organism	MIC (μM)		Reference
	SP 22A	SP 25A	
Bacillus megaterium	1.56	3.12	(29)
Bacillus globigii spores	2.08	2.08	Weimer, unpublished data
Rhodococcus fascians	6.25	12.50	(29)
Micrococcus luteus	12.50	37.50	(29)
Listeria innocua	6.25	4.17	Weimer, unpublished data
Salmonella enteridis	6.25	8.33	Weimer, unpublished data
Mycobacterium smegmatis	1.63	NA	(11)

Table 2.3. MIC values of rhamnolipids against selected organisms.

Organism	MIC (μg/ml)	RL Mixture	References
Bacillus cereus var *mycoides* ATCC 11778	64	RL47T2	(7, 20)
	4	RLLB1	
Enterobacter aerogenes CECT 689	4	RLLB1	(1, 7)
	>256	M7	
Staphylococcus aureus ATCC 6538	8	RLLB1	(1, 7)
	128	M7	
Alternaria alternata	4	RLLB1	(7)
Proteus mirabilis CECT 170	8	RLLB1	(7)
Alcaligenes faecalis	32	M7	(1)
Serratia marcescens	16	M7	(1, 20)
	8	RL47T2	
Klebsiella pneumoniae CECT 17832	0.5	RL47T2	(20)
Clostridium perfringens	256	M7	(1, 20)
	128	RL47T2	
Escherichia coli	32	M7	(1, 20)
	64	RL47T2	

SP22



SP25

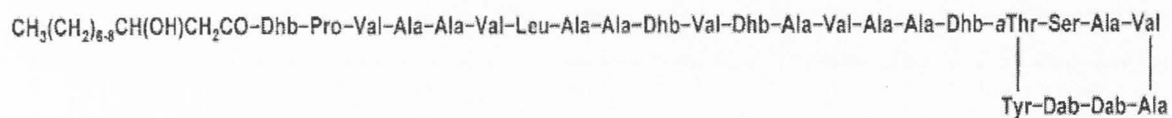


Fig. 2.1. Structures of two syringopeptins with an amino acid backbone of 22 and 25 residues (6). The fatty acids can either be 3-hydroxydecanoic or 3-hydroxydodecanoic acid. Abbreviations for non-standard amino acids are: Dhb is 2, 3-dehydroaminobutyric acid; aThr is allothreonine.

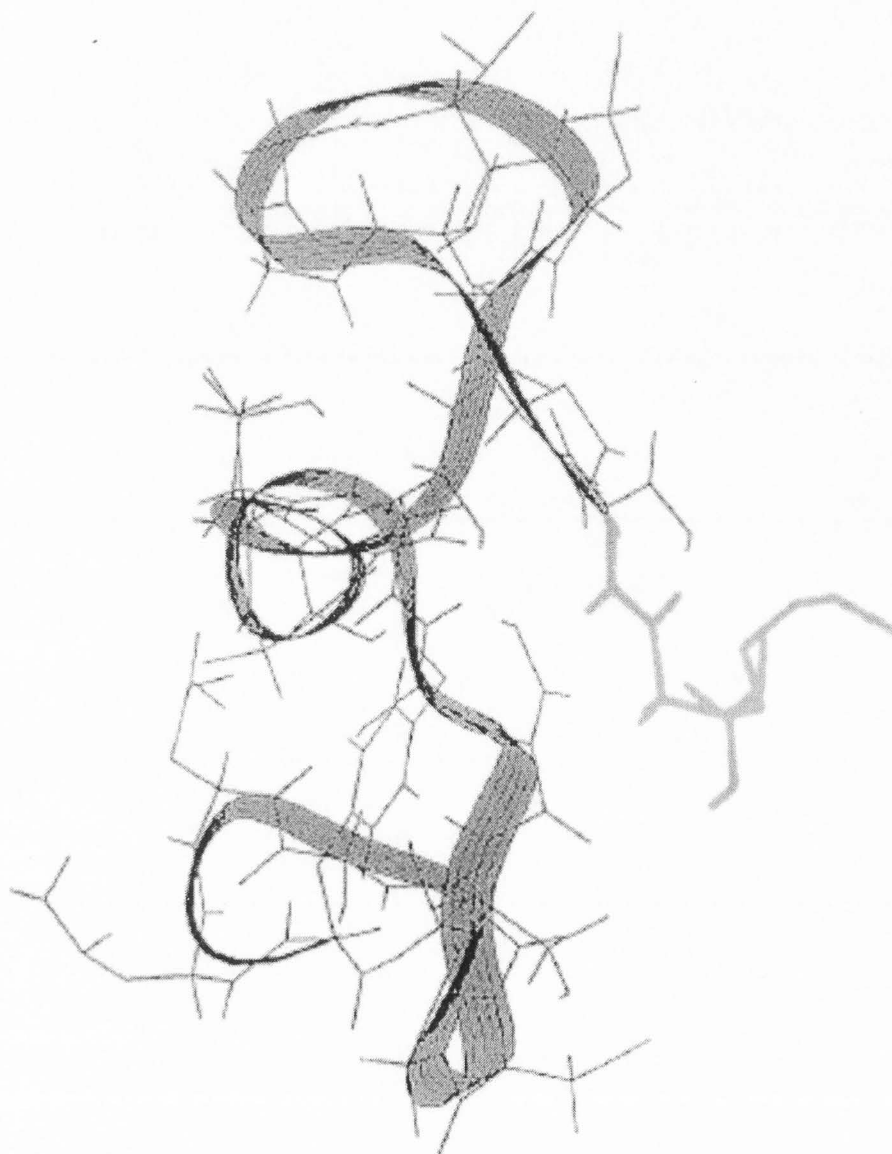


Fig. 2.2 Three-dimensional structure of SP 25A obtained by distance geometry calculations from NMR analysis of the syringopeptin in D_2O at pH 3.6 (31).

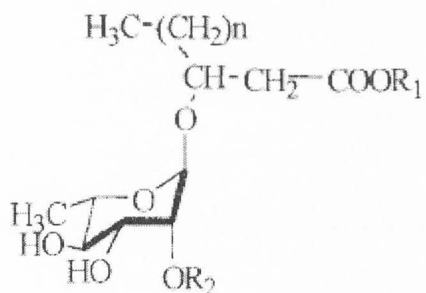


Fig. 2.3. Generalized structure of a rhamnolipid. The carbon chain length may be n = 4, 6, 8 of C_8, C_{10}, C_{12}. In dirhamnolipids, R_1 is H or 3-hydroxydecanoate and R_2 is L rhamnosyl (1).

CHAPTER 3

ANTIMICROBIAL SPECTRUM OF SP 25A AND RL'S AGAINST BACTERIA[1]

Abstract

Secondary metabolites from microorganisms are common sources of antibiotics. However, recent increases in bacterial infections, the decreasing availability of potent antibiotics and the increasing bacterial resistance to available antibiotics requires the search for new antibacterial compounds to be broadened. In this study we investigated the antimicrobial properties of two secondary metabolites from fluorescent pseudomonads - syringopeptin 25A (SP 25A), a lipodepsipeptide produced by *Pseudomonas syringae* pv. syringae, and a rhamnolipid mixture, which contained two primary types of glycolipids produced by *Pseudomonas aeruginosa*. Human pathogens, food spoilage organisms, and fermentative bacteria from both Gram-positive and Gram-negative classes were tested to determine the inhibitory potential of these compounds. The rate of antimicrobial action was determined by monitoring the rate of uptake of propidium iodide during exposure to the compounds. Both compounds compromised the membrane of all the Gram-positive bacteria with the rhamnolipids acting significantly faster (3-433 times depending upon the organism tested) ($p<0.05$) than SP 25A. Inhibition was also confirmed by the microbroth dilution method to determine the minimum inhibitory concentration (MIC). Both compounds inhibited all the Gram-positive organisms tested, as well as

[1] Coauthored by Prerak T. Desai, Patricia J. Champine, and Bart C. Weimer.

Flavobacterium devorans with MICs ranging from 3 μg/ml to 32 μg/ml. Both compounds inhibited *Mycobacterium smegmatis*, *Bacillus subtilis* spores, and *Clostridium sporogenes* spores with an MIC of 4 μg/ml. Interestingly, these compounds acted synergistically to inhibit *Listeria monocytogenes*, thereby lowering the MIC for *L. monocytogenes*. No toxicity was observed during exposure of these compounds to mouse enteroendocrine, human embryonic kidney, and human lung fibroblasts. Taken together these data support the conclusion that both of these compounds have potential for use as antibacterial compounds which needs to be confirmed with in vivo studies.

Introduction

The ability to control infections due to microorganisms has been one of the single most profound developments in the history of health science. The initial success of antibiotics is now marred by the emergence of resistant organisms (4). Antibiotic resistance is a complex problem exacerbated owing to the versatility of the microbes, overuse of antibiotics, and the lack of patients completing the prescribed dosage (4). Among many issues of antibiotic resistance, one of the most troubling issue is the establishment of vancomycin resistant organisms (36). The solution to this complex problem remains to be identified, but discovery of new compounds is essential in solving this issue.

One source of new antibiotics is the secondary metabolites of bacteria with different modes of action. The antimicrobial potential of secondary metabolites of fluorescent pseudomonads have been studied extensively (6, 11, 27). Among the most

well characterized compounds are those produced by *Pseudomonas syringae* and *Pseudomonas aeruginosa.* The ability of these organisms to inhibit competing microorganisms via a myriad of mechanisms has inspired the search for new compounds from these and other pseudomonads (6, 11, 27). Many pathovars of *P. syringae* produce non-specific toxins (e.g. syringomycin, syringopeptin, coronatine, phaseolotoxin, syringotoxin, and tabtoxin) that increase the virulence of the organism to the host plant (6). Some of these toxins have antimicrobial and antifungal properties (6) which fuels their use as biocontrol agents (22). The most promising antibacterial activity is shown by syringopeptins (SP) (6, 10, 26, 42). In nature, SPs cause electrolyte leakage by forming pores in the plasma membrane of plant cells, thereby promoting transmembrane ion flux that leads to plant necrosis (20). SPs also have biosurfactant properties with a low critical micelle concentration (0.4-0.9 mM) (12) that may aid in the spread of the organisms on the plant surface (6, 19). Additionally SPs, along with syringomycin induce stomatal closure in plants, thereby preventing entry of other pathogens, which effectively reduces competition by other organisms on the plant surface (14).

SPs are cyclic lipodepsipeptides and are produced by many strains of *P. syringae* pv. syringae (2, 16, 38, 42). Currently, five different SPs have been identified. They vary in the fatty acid chain length and number and composition of amino acids in the peptide moiety (2, 5, 6, 16, 21) . SPs contain either 22 or 25 amino acids with the N-terminal being acylated with either 3-hydroxydecanoic or 3-hydroxydodecanoic acid to 2, 3-dehydro-2-aminobutyric acid (5, 6, 21). An eight-member lactone ring is formed due to the ester bond between allothreonine and C-terminal tyrosine (5, 6, 21). A high

percentage of hydrophobic amino acids are found in SPs with the peptide being composed of D-amino acids primarily (5, 6, 21); however, the peptide sequence of the SP varies from strain to strain (5, 6, 21).

The mechanism of bacterial inhibition by SPs is unknown, however, initial studies indicate that SPs form pores in the cell membrane (3, 19, 20, 28, 40). It is hypothesized that SP molecules adsorb onto the cell membrane via the hydrophobic acyl chain inserted between the phospholipids in the membrane. Once the adsorbed monomers form aggregates of sufficient concentration, a pore is formed (12, 19). However, this mode of action remains to be proven, as does the minimum number of SP molecules required for pore formation.

P. aeruginosa is the epitome of opportunistic pathogens in humans, but the wide interest in this organism not only stems from this but also from its wide catabolic potential and the array of compounds with antibiotic activity that it produces (11). Cell free culture supernatants from *P. aeruginosa* were extensively used in therapy of diphtheria, influenza and meningitis in the first quarter of the previous century, and since it exhibited enzymatic properties it was called pyocyanase (27). Subsequently, nearly 50 antimicrobial substances have been characterized from fluorescent pseudomonads (11).

This study focused on the antimicrobial properties of rhamnolipids (RL), which are glycolipids produced by some strains of *P. aeruginosa* (7, 11, 29, 35). RLs are usually a mixture of various homologues, depending upon the strain and carbon source provided during growth (30). Eleven different RL homologues have been identified in cultures of *P. aeruginosa* (17) and consist of one or two moieties of rhamnose covalently

linked to a 3-β hydroxy acid, where the chain length of the acid is 8, 10, or 12 carbon atoms. In some cases they may also have 3-hydroxy decanoate linked to the former fatty acid via an ester bond. The physiological role of a specific RL is not well understood, but they are thought to aid in emulsifying hydrophobic substrates for cellular metabolism (24, 34); aid the cells in swarming motility under nutrient limitations (24); cripple the competing organisms for nutrients by the virtue of the surfactant activity (1, 7, 17); and may act as a virulence factor (31). *In vivo* RLs prevent macrophage phagocytosis of the organism by bringing about structural changes in the macrophages so they cannot associate with the bacteria (31). The target for the action of RLs against other bacteria is the cell membrane (1), presumably the surfactant properties of the compound are responsible for the permeabilization effect on the cell surface. In this study we hypothesized that SPs and RLs inhibit a wide spectrum of bacteria without causing toxicity to mammalian cell lines. If true, these compounds may represent new options for antibacterial therapy.

Materials and Methods

Purification of antimicrobials. Syringopeptin 25A (SP 25A) was produced and purified from *Pseudomonas syringae* pv. *syringae* M1 as described by Bidwai et al. (9). In brief, the culture was grown to stationary phase for 10 d standing culture at room temperature (~25°C). After collection of the supernatant SP 25A was extracted with acidified acetone, concentrated with a rotary evaporator, purified to homogeneity by

reverse phase HPLC, and lyophilized for storage at 4°C until further use. Purity and the molecular weight of the compound were verified by MALDI-TOF analysis at the Center for Integrated BioSystems (Logan, UT).

Commercial RL samples were obtained as 25.1% aqueous solution (product JBR-425; Lot#021004) from Jeneil Biotech, Inc. (Saukville, WI). The purity and molecular weight of the RLs were determined using MALDI-TOF at the Center for Integrated BioSystems. The relative concentrations via molar ratios between the two different rhamnolipid moieties (Decanoic acid, 3-[(6-deoxy-L-mannopyranosyl) oxy]-1-(carboxymethyl) octyl ester, and Decanoic acid, 3-[(6-deoxy-2-O (6-deoxy-L-mannopyranosyl)-L-mannopyranosyl] oxy]-1-(carboxymethyl)octyl ester) in the commercial rhamnolipid mixture were determined by ^{13}C NMR as described by Sim et al. (39) .

Determination of rate of antimicrobial action and the MICs against bacteria. The antimicrobial action for each compound was initially determined by the rate of uptake of propidium iodide (PI) (Fluropure grade, Molecular Probes, Inc.; Eugene, OR) as previously described (18). In brief, all cultures were grown overnight in their respective optimal growth medium and temperature from freezer vials (Table 3.1). Each culture was sub-cultured twice, harvested in mid log phase, washed with saline and adjusted to an OD_{600} of 0.25 in saline. PI, with an excitation wavelength of 535 and an emission wavelength of 617, was added to the culture suspension at a final concentration of 10 μM. Each organism was treated with 50 μg/ml SP 25A and 60 μg/ml of the RL mixture in a final volume of 2.2 ml. The increase in fluorescence was measured with a Shimadzu

RF 1501 spectrophotoflurometer (Columbia, MD) at 15 s intervals for a maximum period of 120 min. Saline was added in place of SP 25A or RLs as a negative control. All inhibition experiments were done in two biological replicates.

The rate of antimicrobial action was expressed as the inhibition rate (IR) (Eq. 1). The curve fitting was done using OriginPro Ver 7.0 (Natick, MA).

$$IR = ((\text{Log RFU}/ (\text{Time})) - C) / \text{Time} \quad (\text{when } d \text{ Log RFU}/ dT > 0) \qquad (\text{Eq. 1})$$

Where RFU = relative fluorescent units; and C = Y intercept

The minimum inhibitory concentration (MIC) for the organisms were determined by microbroth dilution method as prescribed by the National Committee for Clinical Laboratory Standards (NCCLS) (44). The microorganisms were prepared as described above and resuspended in their optimal growth media (Table 3.1) to ~10^5 CFU/ml containing SP 25A at 2, 3, 4, 5, 6, 7, 8, 16, 32 and 50 μg/ml in a total volume of 550 μl. RL concentrations of 2, 3, 4, 5, 6, 7, 8, 16, 32 and 60 μg/ml in a total volume of 550 μl were tested in a 48-well plate (Corning, NY), unless otherwise noted. The plates were incubated in optimal growth conditions for the respective organism and monitored for an increase in OD_{600} after 48 h by a Perkin-Elmer (HTS 7000) plate reader (Downers Grove, IL). A positive control (inhibition of growth) using Polymyxin B (Sigma-Aldrich Cat# P0972) at 1000 μg/ml for all Gram-negative organisms, Penicillin G (Sigma-Aldrich Cat# P3032) at 1000 μg/ml for the Gram-positive organisms, Rifampicin (Sigma-Aldrich Cat# 3501) at 1000 μg/ml was used for *M. smegmatis, Enterococcus faecalis* and *Staphylococcus aureus.* Negative controls (no inhibition of growth) were included using saline in the assay for each compound. The least concentration at which there was no

increase in OD over 48 h was reported as the MIC. Each MIC was determined in two biological replicates with triplicate tests per replication. The triplicates were averaged for each replicate reported.

Synergistic activity between SP 25A and RLs was measured by exposing *L. monocytogenes* to RLs at a concentration of 0, 0.5, 1, 1.5, 3, and 6 μg/ml alone and in combination with 3 μg/ml SP 25A and monitoring PI uptake as previously described. The experiment was done in two biological replicates.

Determination of cytotoxicity in cell culture. Toxicity of the two compounds to mammalian cells was assayed in cell culture using mouse enteroendocrine cells (STC-1) (37), human embryonic kidney cells (HEK 293; ATCC CRL-1573), and human lung fibroblasts (LL47; ATCC CCL-135). Each cell line was subjected to SP 25A and RLs at the observed MIC (e.g. 4 μg/ml and 8 μg/ml). The human embryonic kidney cells and human lung fibroblasts were grown as per the ATCC recommendation, while the mouse enteroendocrine cells were grown as described by Vincent et al. (37). Media and sera were purchased from HyClone Laboratories (Logan, UT). All cells were grown in 10% fetal bovine serum (FBS).

The number of total cells and dead cells were counted after 6, 24, and 48 h using a Nucleocounter Automated cell counting system (New Brunswick; Edison, NJ). In brief, cells (STC - 200,000 cells/well, HEK 293 - 200,000 cells/well and LL47 - 100,000 cells/well) were incubated with the appropriate medium for 24 h prior to addition of fresh media containing the antimicrobial compounds. After addition of the antimicrobial compound the cell cultures were incubated at 37°C with 5% CO_2 for 6, 24, and 48 h.

Cells were harvested by trypsinization using 0.25% trypsin-EDTA for 2 min. The trypsin was neutralized by addition of 200 μl of serum containing fresh medium. The cells were harvested and transferred to 1.5 ml tubes, centrifuged (3-5 min at <100 x g), and resuspended in 200 μl of fresh medium. Subsequently, for total cell count 100 μl of the cell suspension was added to the Lysis buffer (Reagent A100 in the starting kit (Cat No. M1293-0020, New Brunswick Scientific) for 30 s, which was stabilized using 100 μl of Reagent B. A positive control of completely lysed cells by lysing all the cells with triton was used along with a negative control using sterile PBS (pH 7.4). For dead cell counts 100 μl of cell lysate was counted without use of lysis buffer or stabilizing buffer. All cell counts were obtained using the Nucleocounter automated cell counting system. Data were reported as the percent of cell death. The toxicity testing was done in two biological replicates with triplicate wells per replication. The triplicates wells per replication were averaged before reporting the replicate reading.

Results and Discsussion

Compound purity. After purification SP 25A was subjected to MALDI-TOF and HPLC analysis to confirm the purity of the fractionated compound. HPLC analysis revealed a single peak, as did MALDI TOF (Appendix A, Fig A1). This single major peak had a molecular weight of 2,400.37 Da, which was in agreement with the reported size (33).

The commercial RL preparation was subjected to MALDI-TOF analysis and ^{13}C NMR to determine the relative concentration and isoform content, respectively. MS analysis revealed Rhamnose-C10-C10 (MW = 503.31) and Rhamnose-Rhamnose-C10-C10 (MW = 649.33) (Appendix A, Fig A2). These observations are in agreement with the product data sheet. NMR analysis demonstrated that the isoforms were present in an equimolar ratio.

Bacterial inhibition by SP and RLs. It is thought that these compounds target the cell membrane, inducing lysis (6, 12, 25). This study used PI, a membrane impermeant nucleic acid stain, as a probe to monitor cell membrane integrity during cellular exposure to both compound types (18). PI accumulation directly correlated to increasing exposure time for each compound, indicating that the compounds compromised the cell membrane. As such, the rate of PI accumulation (Eq. 1) was used to compare the inhibitory rate for each organism tested (Fig. 3.1).

SP 25A was not inhibitory to any of Gram-negative organisms tested except *F. devorans*, while it inhibited all Gram-positive organisms tested. Also, SP 25A did not inhibit the growth of any yeast tested (*Brettonomyces bruxellensis, Candida vini, Pichia fermentans, Saccharomyces luduigi, Metschinikowia pulcherrima, Kloeckera apiculata)* (data not shown). The greatest rate of inhibition was found for *Brevibacterium linens*, while *E. faecalis* had the slowest rate of inhibition (Fig. 3.1).

As observed with SP, RLs inhibited only Gram-positive bacteria (except *F. devorans)*, with activity being the fastest against *B. subtilis* (Fig. 3.1) and slowest against both the two *Listeria* species tested. The rate of action was distributed differently relative

to SP 25A, but the same Gram-reacting organisms had the same inhibition for each compound.

There was a significant difference ($p<0.01$) in the rate of PI accumulation (IR) between SP 25A and RLs. Depending upon the species RLs were 3 to 433 times faster in the compromising the cell membrane as compared to SP 25A, the difference being highest for enterococci and lowest being for the *Listeria* species

While the rate of action indicates the speed to compromise the membrane, an indication of the MIC is required to demonstrate inhibition of growth. Therefore, the MIC for each compound was determined (Table 3.2). Measuring inhibition with MICs confirmed the observations using PI.

The SP 25A MIC ranged from 3 to 16µg/ml, while the MICs for RL ranged from 4 to 32µg/ml for the organisms tested. Interestingly, a difference in the SP 25A and RL MIC with *E. faecalis* and *S. aureus* was observed (Table 3.2). While the MIC for RLs for both these organisms was >60 µg/ml, SP 25A completely inhibited both the organisms at 8 µg/ml. For all the other organisms SP 25A had a similar or lower MIC as compared to RLs. Interestingly, both compounds inhibited spore germination from *Bacillus* and *Clostridium* at 4µg/ml. This work is the first report of anti-spore activity by these compounds. Both the organisms inhibited growth of *M. smegmatis* at 4µg/ml.

Lavermicocca et al. (26) measured the antibacterial activity of SP 25A using six organisms. No inhibition was observed for the three Gram-negative organisms, even at 120 µg/ml. However, all three Gram-positive bacteria were inhibited (*Micrococcus luteus*, *Bacillus megaterium*, and *Rhodococcus facians*). While the overall observations

are in agreement between this study and Lavermicocca et al. (26), the exact concentrations are not comparable, unfortunately, due to differences in methodology.

Antimycobacterial activity was found by Buber et al. (10), but due to the method of isolation they were unable to conclusively assign this activity to SP 25A. In this study we conclusively found that SP 25A inhibited *M. smegmatis*, a surrogate organism for *Mycobacterium tuberculosis*, at 4 μg/ml.

The literature contains conflicting reports on the spectrum of activity for RLs. This may be attributed to the fact that different groups have used RL mixtures with different compositions of RL homologues. For example, Abalos et al. (1), Benincasa et al. (7), and Haba et al. (17) reported that RL mixtures are active against both Gram-positive and Gram-negative bacteria. In this study, we found RLs that were active against Gram-positive bacteria and only one Gram-negative bacterium (*F. devorans*) at <60 μg/ml. Kim et al. (8) reported the ability of RLs to lyse zoospores from *Phytophthora capsici* within 1 min at a concentrations <50 μg/ml. Conversely, we observed that RLs inhibited bacterial spore germination in *B. subtilis* and *C. sporogenes* at 4 μg/ml.

Synergistic activity. Since both compounds demonstrated a similar range of activity and MICs, we determined the synergistic activity in an effort to reduce the MIC for each compound. This was done by exposing *L. monocytogens* to mixtures of SP 25A at 3 μg/ml with various RLs concentrations. The IR for the mixture of both the compounds was significantly different ($p<0.05$) than the IR of the compounds used alone across all concentrations tested, hence satisfying the classic definition of synergism. We achieved a higher rate of antimicrobial action when both the compounds were used in

combination as compared to individual use. Using the compounds together we were able to achieve the same level of inhibition with up to 6-fold less RLs. The increase in synergism followed a sigmoidal curve (Fig 3.2).

This is the first study to define the synergistic activity of SP 25A and RLs. Woo et al. (43) reported synergistic activity of SPs with fungal cell wall degrading enzymes to inhibit fungal pathogens. Also, there have been reports of synergism between cationic pore forming peptides (23), but there has been no report of synergism between a lipodepsipeptide and a glycolipid. The importance of this finding is best refined for use in specific applications, and is therefore beyond the scope of this work.

Cellular toxicity. Three mammalian cell lines were used to assess cytotoxic effects for each compound at 4μg/ml and 8μg/ml. No significant ($p>0.05$) cytotoxicity was observed at 6, 24, and 48 h after treatment for each compound at either concentrations (Fig. 3.3). While a small amount of lysis was observed, it was not above background. Cells treated with triton (positive control) showed 100% lysis. These observations indicate that neither compound compromised the host membrane. Various groups (13, 15, 41) reported haemolytic activity of SP 25A. Menestrina et al. (15) reported a C_{50} value of 8.88 μg/ml of SP 25A for RBC hemolysis. In contrast, at similar concentrations, we did not observe membrane permeabilization of any of the three cell lines tested. A possible explanation of this observation is that RBC's lack an endomembrane, which is thought to play a central role in the rapid resealing response in event of plasma membrane disruption (32).

Although structurally different both the compounds have a similar spectrum of activity for the strains tested. One aim of this study was to determine the use of these compounds against multiple drug resistant strains. This was done using *E. faecalis* and *S. aureus* that are gentamicin/vancomycin/teicoplanin resistant and MRSA/VISA, respectively. We observed that RLs had a higher IR than SP 25A, yet RLs were unable to inhibit growth at 60 μg/ml. This is similar to the concept described by Wu et al. (45), who found that cationic peptides were not correlated with the ability to permeabilize the cell membrane and the antimicrobial activity.

Several inferences can be made from this lack of correlation. The biochemical changes brought about by RLs were overcome by a stress response that repaired the compromised membrane, but they could not repair the changes brought about by SP 25A. This reveals that either the compounds have a differing mode of action on the cell membrane or SP 25A has multiple modes of action (i.e. it may act on multiple cellular targets). Membrane repair in response to cationic peptides was reported by Wu et al. (45). They observed that some peptides did not depolarize the cell membrane at MIC concentrations, suggesting that at these MICs bacteria repaired their cell membrane and that a mechanism other than membrane disruption leads to cell death. In this study, we observed RLs to disrupt the membrane enough to up take PI, yet the organisms retained the ability to replicate.

This study demonstrated the ability of SP 25A and RLs to compromise the membrane of Gram-positive bacteria with MICs of ≤8 μg/ml. The compounds acted synergistically to inhibit *L. monocytogenes* resulting in lower MICs for each compound

when used in combination. Considering the inhibition of multiple drug resistant enterococci and staphylococci, *Mycobacterium, Bacillus* spores, and the lack of toxicity towards mammalian cells makes SP 25A a very promising therapeutic agent, which needs to be confirmed with in vivo studies.

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Table 3.1. List of bacteria used for antimicrobial screening and their growth conditions.

Organism	Strain	Temperature (°C)	Oxygen Demand	Medium
Aeromonas caviae	13137	30	Aerobic	Nutrient agar
Bacillus cereus	10987	30	Aerobic	Nutrient agar
Bacillus subtilis	23857	26	Aerobic	Nutrient agar
Bacillus megaterium	14581	30	Aerobic	NB
Brevibacterium linens	BL1 MGE	37	Aerobic	TSB
Citrobacter fruendii	11811	37	Aerobic	Nutrient agar
Clostridium sporogenes	10000	37	Anaerobic	Reinforced clostridial medium
Enterobacter aerogenes	13048	30	Aerobic	Nutrient agar
Enterococcus faecalis	700802	37	Aerobic	BHI
Erwinia herbicola	33243	37	Aerobic	Nutrient agar
Eschereschia coli	K12	37	Aerobic	Nutrient agar
Eschereschia coli H7:0157	35150	37	Aerobic	Nutrient agar
Flavobacterium devorans	10829	30	Aerobic	Nutrient agar
Klebsiella pneumoniae sub sp pneumoniae	700721	37	Aerobic	NB
Lactobacillus plantarum	8014	37	Microaerophilic	MRS
Lactobacillus acidophilus	4355	37	Microaerophilic	MRS
Lactococcus lactis subsp lactis	IL1403	30	Microaerophilic	Ellikers Broth
Listeria innocua	33090	37	Aerobic	BHI
Listeria monocytogenes	43251	37	Aerobic	BHI
Micrococcus luteus	21102	30	Aerobic	BHI
Mycobacterium smegmatis	14468	37	Aerobic	Luria Broth
Salmonella typhimurium	13076	37	Aerobic	Nutrient

				agar
Salmonella enteridis	700931	37	Aerobic	TSB
Staphylococcus aureus subsp aures	700699	37	Aerobic	BHI
Streptococcus mutans	89/1591	37	Aerobic	BHI
Streptococcus suis	700610	37	Aerobic	BHI
Streptococcus agalacticae	12403	37	Aerobic	BHI
Bacillus subtilis (spores)	6633	26	Aerobic	TSB
Clostridium sporogenes(spores)	11437	37	Anaerobic	Reinforced clostridial medium

Table 3.2. MICs (Two biological replicates done in duplicate) and mean IR's (2 biological replicates) of SP 25A and Rhamnolipid mixture against screened organisms. (ND represents Not Determined).

Genus	IR Rhamnolipids (60μg/ml)	MIC(μg/ml)	IR SP 25A (50μg/ml)	MIC(μg/ml)
Bacillus megaterium	1.043	4	0.005	3
Listeria innocua	0.014	5	0.005	3
Listeria monocytogenes	0.032	6	0.005	3
Bacillus cereus	0.834	4	0.004	4
Bacillus subtilis	1.807	4	0.006	4
Clostridium sporogenes	0.698	4	0.008	4
Flavobacterium devorans	0.518	16	0.002	4
Lactococcus lactis subsp. *Lactis*	1.219	4	0.008	4
Micrococcus luteus	0.183	8	0.006	4
Mycobacterium smegmatis	ND	4	ND	4
Streptococcus mutans	0.164	4	0.003	4
Streptococcus suis	1.018	4	0.006	4
Bacillus subtilis (spores)	ND	4	ND	4
Clostridium sporogenes(spores)	ND	4	ND	4
Enterococcus faecalis	0.482	>60	0.001	8
Lactobacillus acidophilus	0.196	16	0.003	8
Staphylococcus aureus subsp. *aureus*	0.894	>60	0.003	8
Streptococcus agalacticae	1.073	4	0.004	8
Lactobacillus plantarum	0.287	32	0.003	16
Aeromonas caviae	0.000	>60	0.000	>50
Citrobacter fruendii	0.000	>60	0.000	>50
Enterobacter aerogenes	0.000	>60	0.000	>50
Erwinia herbicola	0.000	>60	0.000	>50
Eschereschia coli K12	0.000	>60	0.000	>50
Klebsiella pneumoniae subsp. *Pneumoniae*	0.000	>60	0.000	>50
Salmonella typhimurium	0.000	>60	0.000	>50
Salmonella enteridis	0.000	>60	0.000	>50
Brevibacterium linens	0.512	ND	0.009	ND
Eschereschia coli H7:0157	0.000	ND	0.000	ND

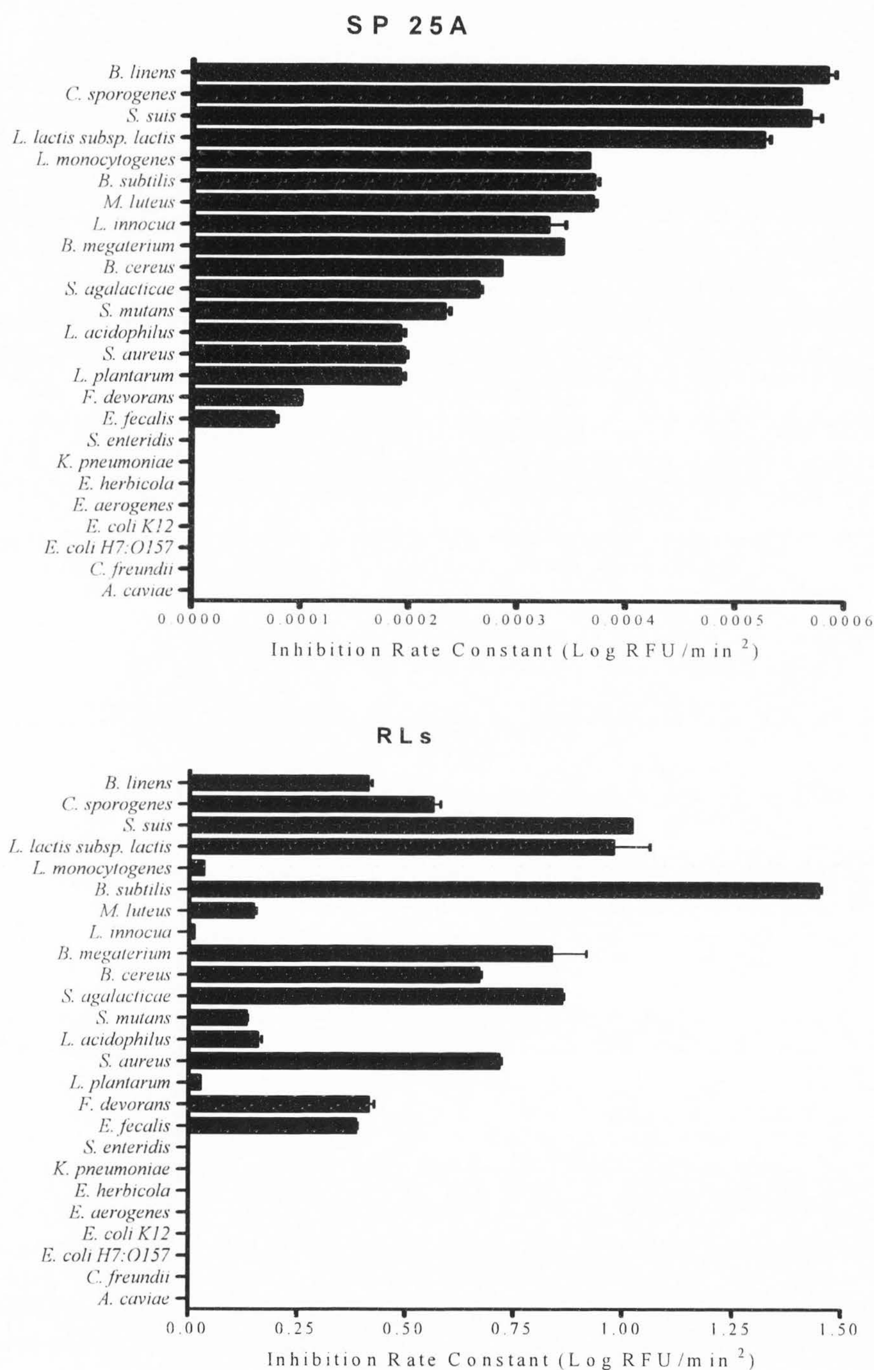

Fig 3.1. Inhibition rate for SP 25A at 50 μg/ml; Inhibition rate for RLs at 60 μg/ml. The error bars represent SEM from two biological replicates.

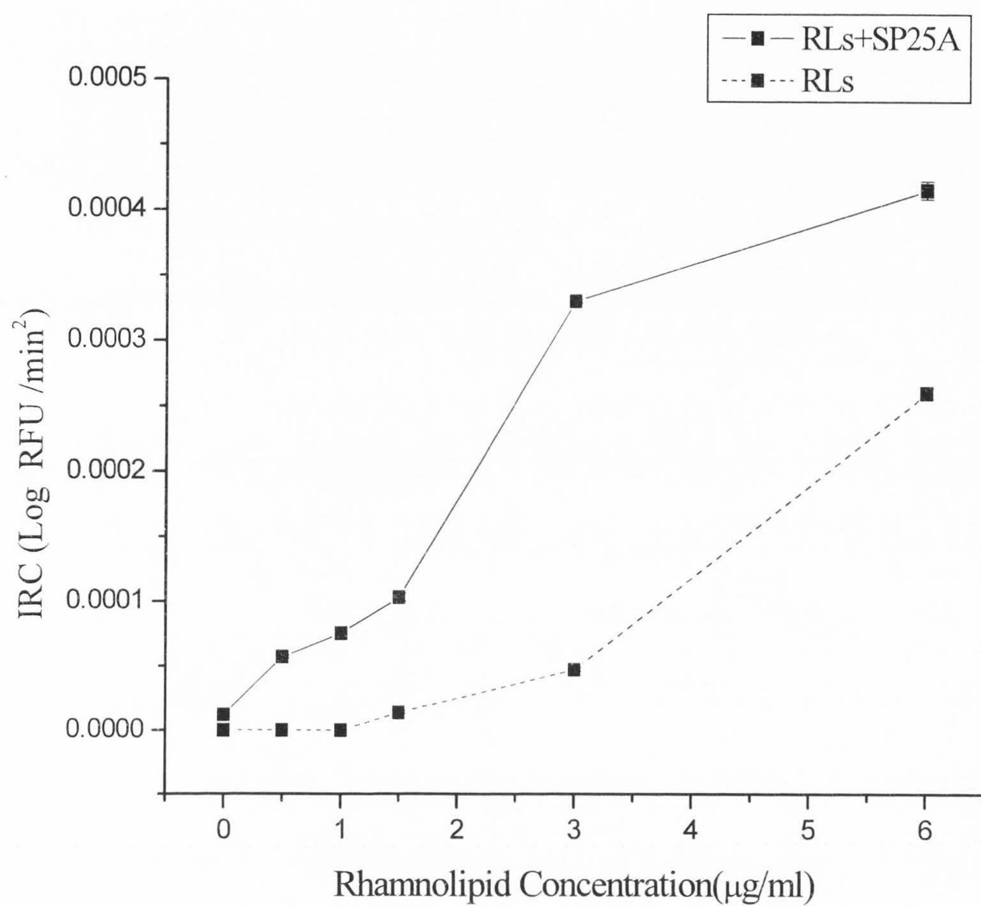

Fig 3.2. Inhibition rate for *L. monocytogenes* with RLs alone (0, 0.5, 1, 1.5, 3 and 6 µg/ml) and with rhamnolipids and SP 25A (3 µg/ml). The error bars (smaller than the symbols) represents SEM from two biological replicates.

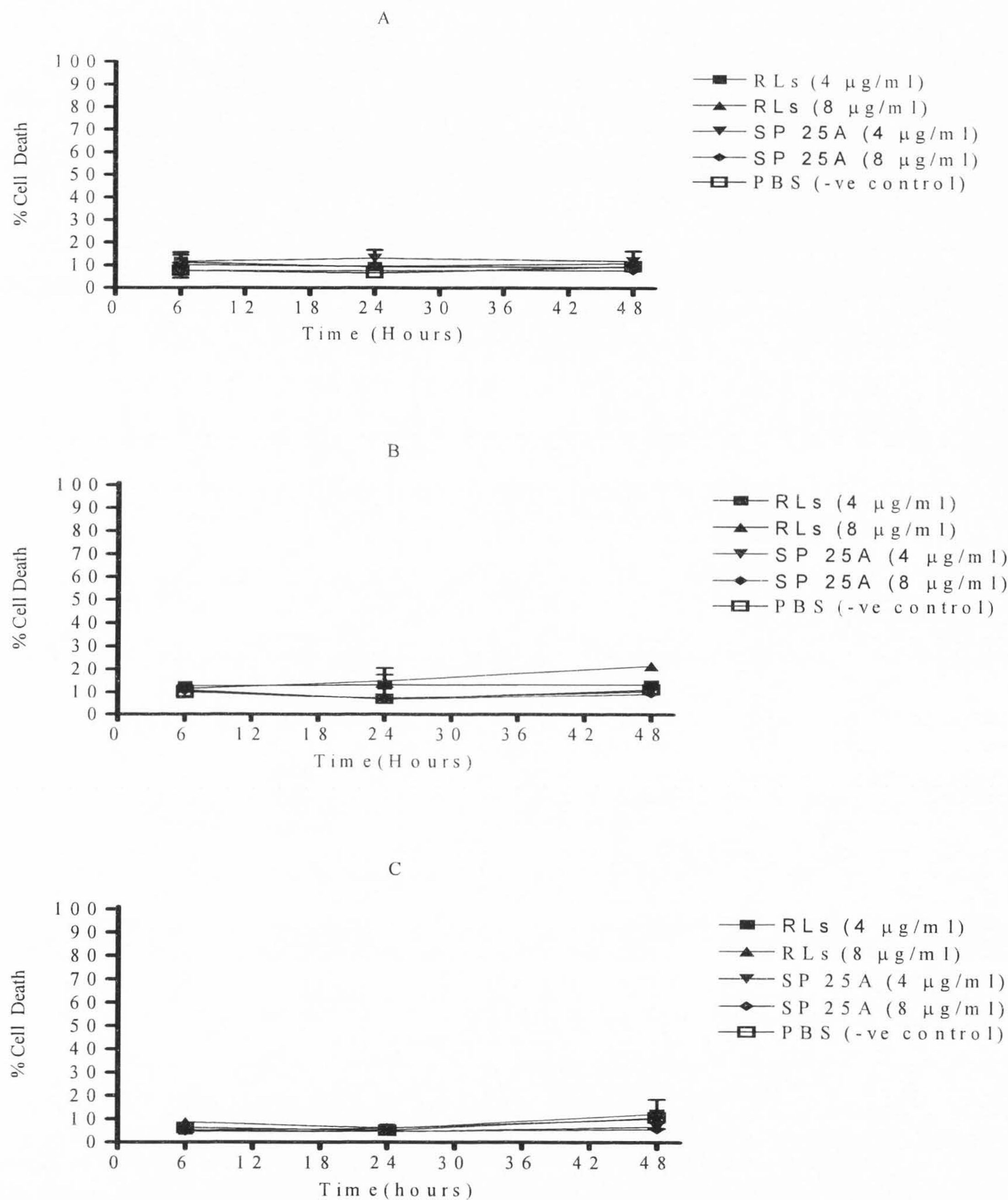

Fig 3.3. Toxicity of RLs and SP 25A to STC (panel A), HEK 293 (panel B) and LL-47 (panel C) in culture. The number in the parenthesis represents the concentration of each compound in µg/ml. The percent cell death was benchmarked to 100% lysis with the positive control. The error bars represents SEM from two biological replicates.

CHAPTER 4

GENE EXPRESSION PROFILE OF *LISTERIA MONOCYTOGENES EGDE* IN RESPONSE TO SUB-MIC DOSES OF SYRINGOPEPTIN 25A AND RHAMNOLIPIDS

Abstract

Two antimicrobial compounds that target the cell membrane, syringopeptin 25A and an equimolar mixture of two rhamnolipids, were used to determine the cellular response during sub-MIC treatment with *Listeria monocytogenes* EGDe. Cell growth, membrane permeabilization and the gene expression profile was determined immediately prior to treating the cells with either antimicrobial, after 30 min of exposure, and after 120 min of exposure. Cell membrane permeabilization was highest after treating the cells with rhamnolipids, which also resulted in a 47 % reduction in growth. However, growth inhibition was 100% after treatment with SP 25A, despite having only ~2% increase in dye uptake. Treatment of *L. monocytogenes* with SP 25A significantly changed the expression of 139 genes, while treatment with rhamnolipids resulted in significantly different expression of 39 genes. SP 25A repressed 97% of the differentially regulated genes, while RLs induced 70% of the differentially regulated genes after 120 min of treatment. The effective reduction in cell density after treatment with SP 25A was associated with the repression of key genes involved in cell division and genome replication; hence, inhibiting cell division. SP 25A also repressed key genes in central

metabolism, generation of precursor metabolites, transcription, and translation resulting in repression of RNA production, protein biosynthesis, cellular energy, and virulence. Rhamnolipids, conversely, affected only a few genes within any single functional category that were not associated with any single phenotypic observation. Hence, SP 25A caused repression in the cell's metabolism, which was independent of the observed pore forming activity. Taken together these data led us to conclude that both the compounds, even-though acting on the cell membrane, produced distinctly different gene expression profiles with SP 25A being more effective to inhibit cell growth in *L. monocytogenes*.

Introduction

The increase in multiple antibiotic resistant strains, which leads to more nosocomial infections and community-acquired infections, has fueled the search for new antibacterial compounds, including peptides. Antimicrobial peptides are pervasive components of prokaryotic and eukaryotic defense mechanisms against invading organisms (9). Though structurally diverse, the amphipathicity and cationic nature of these peptides allows them to interact and disrupt the bacterial cell membrane leading to cell death (35). Subsequent effects on intracellular molecules have also been observed, leaving some investigators to conclude that membrane disruption is only a portion of the antimicrobial activity (16, 36).

The mode of action to disrupt the membrane with peptide antibiotics is thought to follow the "barrel stave" model that form pores across the membrane or the "carpet"

model, where the molecules align orient parallel to the membrane and disrupt the lipid structure (28). While it seems alluring to endow the antibacterial effect of these compounds solely to these two models, these mechanisms alone do not account for the physiological effects that these peptides produce in bacterial cultures (8). Wu et al. (35) and Friedrich et al. (16) observed that some cationic peptides failed to depolarize the bacterial membrane, yet effectively inhibited growth. At the MIC concentration these peptides did not disrupt the bacterial membrane, giving conclusive evidence that a general membrane disruption mechanism alone cannot account for antimicrobial properties of all peptides (15, 35). Further, Tomasinsig et al. (32) and Hong et al. (17) found that cationic peptides resulted in changes in gene expression profiles in *E. coli* in addition to permeabilizing the membrane. Taken together, these data provide evidence that it is unlikely that the diverse groups of peptides act via only membrane disruption alone. Once inside the cytoplasm these peptides may bind proteins, DNA, RNA, or other macromolecules to alter replication and metabolism (8).

Cyclic lipopeptides are potent antimicrobial and two compounds of this class polymyxin B (14) and daptomycin (26) are approved by the Food and Drug Administration for clinical use. Syringopeptin 25A (SP 25A), a cyclic lipodepsipeptide produced by *Pseudomonas syringae* pv. syringae is structurally similar to daptomycin. SP 25A consists of 25 amino acid residues with the N-terminal being acylated with 3-hydroxydecanoic acid. An eight-member lactone ring is formed via an ester bond between allothreonine and C-terminal tyrosine (3, 5, 19). Hydrophobic amino acids

account for 68% in syringopeptins with the peptide being composed of primarily D-amino acids (3, 5, 19).

In a previous study, rhamnolipids (RLs) showed a similar spectrum of activity to inhibit bacteria, as compared to SP 25A (13). RLs are surfactants with 11 homologues produced by some strains *Pseudomonas aeruginosa* and consist of one or two moieties of rhamnose covalently linked to a 3-β hydroxy acid, where the chain length of the acid is 8, 10, or 12 carbon atoms (6). In some cases they may also have 3-hydroxy decanoate linked to the fatty acid moiety via an ester bond. RLs are usually a mixture of various homologues, depending upon the strain and carbon source provided during growth (23). In a previous study Desai and Weimer (13) found no correlation between the bactericidal activity and membrane permeabilization of SPs and RLs. In a multidrug resistant strain of *Staphylococcus aureus,* RLs induced higher propidium iodide (PI) uptake, indicating an increase in membrane permeabilization, as compared to SP 25A, but RLs did not inhibit the cell growth. This confirms the observations of others for the disconnection of membrane disruption and growth inhibition (8, 13, 15, 16, 18, 33, 35).

A number of groups have used transcription profiling during challenges with sub-MIC doses of antimicrobial compounds to find that some antimicrobial peptides invoke cellular changes that are not lytic and not lethal (1, 10, 17, 32, 34). These experiments are leading to the discovery of the multiple targets for antimicrobial peptides beyond the membrane. Such observations imply that the peptides directly or indirectly interact with membrane proteins leading to regulatory components, which affects the susceptibility of the organism to the compound, which has been observed in *Salmonella enterca* and *P.*

aeruginosa (32). In this study, we hypothesized that sub-MIC doses of SP 25A or RLs will evoke distinct gene expression responses that will provide genetic targets that are additional mechanisms of action to inhibit bacterial growth that are not membrane associated.

Materials and Methods

Purification of antimicrobials. P. syringae pv. syringae M1 was obtained from the Utah State University culture collection. SP 25A was produced and purified as described by Bidwai et al. (7). In brief, the culture was grown to stationary phase for 10 d standing culture at room temperature (~25°C). After collecting the supernatant, SP 25A was extracted with acidified acetone, concentrated with a rotary evaporator, purified to homogeneity by reverse phase HPLC, and lyophilized for storage at 4°C for further use. The purity and molecular weight were verified by MALDI-TOF analysis at the Center for Integrated BioSystems (Utah State University, Logan, UT).

Commercial RL samples were obtained as a 25.1% aqueous solution (product JBR-425; Lot#021004) from Jeneil Biotech, Inc. (Saukville, WI). The purity and molecular weight of the RLs were determined using MALDI-TOF at the Center for Integrated BioSystems. The relative concentrations via molar ratios between the two different rhamnolipid moieties (Decanoic acid, 3-[(6-deoxy-L-mannopyranosyl) oxy]-1-(carboxymethyl) octyl ester, and Decanoic acid, 3-[(6-deoxy-2-O (6-deoxy-L-mannopyranosyl)-L-mannopyranosyl] oxy]-1-(carboxymethyl)octyl ester) in the

commercial rhamnolipid mixture were determined by ^{13}C NMR as described by Sim et al. (29).

Bacterial strain, growth conditions and gene expression profiling. Listeria monocytogenes EGDe was thawed and subcultured twice at 37°C in brain heart infusion broth (BHI) (DIFCO, Franklin Lanes, NJ). An overnight culture was diluted 10 fold in sterile medium and grown for 4 h to an OD_{600} of 1.7, which corresponded to ~10^9 cfu/ml. The cell preparation was exposed to either 3μg/ml SP 25A or 6 μg/ml RLs. Previously, Desai and Weimer (13) determined the MIC for *L. monocytogenes* with SP 25A to be 3 μg/ml and 6 μg/ml with RLs. This study used these concentrations, but increased the cell population to ~10^9 cfu/ml, rather than 10^5 cfu/ml, the cell density used to determine the MIC. Thereby, making SP 25A and RLs sub-MIC at 3 μg/ml and 6 μg/ml, respectively.

Total RNA was extracted from 1.8 ml culture immediately before treating the cells with SP 25A and RLs (T_0), after 30 min (T_{30}), and at 120 min (T_{120}) of exposure at 37°C. Simultaneously, the cell density was measured at OD_{600}. Membrane permeabilization was determined by measuring the PI uptake as described by Desai and Weimer (13). Total RNA extraction and reverse transcription (from 10 μg total RNA) was done as described by Yi et al. (36) to produce biotinylated cDNA, which was sheared with DNase1 as described by the protocol of NimbleGen Systems (Madison, WI). The custom, optimized NimbleScreen chip contained 12 wells, enabling the entire experiment to be done on a single chip. Each well contained five probes for each open reading frame in the entire genome.

Hybridization of the fluorescently-labeled (Cy3 –streptavidin; Amersham Biosciences, Piscataway, NJ) cDNA (500 ng) was done using a custom NimbleScreen chip optimized for *L. monocytogenes* EGDe, as described by the NimbleGen Systems protocol. Hybridization was detected with a Genepix 4200A array scanner (Axon Instruments, Union City, CA) at the Center for Integrated BioSystems. Data extraction from the scanned images was completed at NimbleGen Systems. The raw expression data from the entire experiment were normalized together using R with the robust multichip average (RMA) method (18). Appendix C lists the R code used for RMA normalization. Annotations for *Listeria monocytogens* EGDe were obtained from the ERGO database (Integrated Genomics, Chicago, IL).

Statistical analysis and data visualization. RMA normalized data were analyzed using SAM Version 2.01 (33) with a one class time course experimental design using the xCluster R module (Center for Integrated BioSystems). Any gene with at least a $\log_2$ ratio of ±0.58, which is equivalent to a 1.5 fold change, and a $Q<0.3$ was considered significant (30). The entire biological experiment was replicated twice.

Heat maps were drawn with $\log_2$ values of the RMA normalized data after calculating the average of the biological replicates using Hierarchical Clustering Explorer version 3.0 (27). The $\log_2$ ratios were calculated by taking a difference in $\log_2$ intensity of a single time point with the preceding time point.

Results

Compound purity and growth inhibition. The commercial RL preparation was tested using MS and ^{13}C NMR to determine the isoform content and relative concentration, respectively. ^{13}C NMR analysis revealed Rhamnose-C10-C10 (MW = 503.31) and Rhamnose-Rhamnose-C10-C10 (MW = 649.33) to be present in an equimolar ratio. These observations are in agreement with the product data sheet.

The compound purity was tested to ensure the compound identity before use in the study of growth inhibition. SP 25A isolated in this study was a single compound (Appendix A, Fig A1), but the commercial RL preparation was a mixture of two isoforms (Appendix A, Fig A2). The pure SP 25A had a molecular weight of 2,400.37 Da, which was in agreement with the reported molecular weight for SP 25A (24).

Both antibacterial treatments caused membrane permeabilization (Fig. 4.1A), but reduced cell growth (Fig. 4.1B) of *L. monocytogenes* by different amounts. Addition of RLs resulted in more membrane permeabilization than did the addition of SP 25A. After 30 min of incubation with RLs the permeabilization increased by 53%; while permeabilization due to SP 25A increased 2.6% during the same time. Interestingly, the permeabilization declined after 120 min of treatment with RLs.

The cell density was highest in the control, as expected, and lowest with addition of SP 25A. The control culture increased growth by 15.3% during 120 min, while the culture treated with RLs increased by 8%, but those treated with SP 25A did not increase. The membrane permeabilization and the cell density changes did not correlate (Fig. 4.1), suggesting that the mechanism of action for both compounds was not just membrane

permeabilization. To determine the underlying mechanisms responsible for these differences, gene expression profiles were determined.

Gene expression profiling with RLs. The expression data were examined for genes that were constitutively expressed above the mean expression level, but none were found, indicating that gene expression changed over the exposure time. At T_{30} RLs induced eight genes and repressed two genes. Treating the cells for 120 min with RLs significantly altered the expression of 39 genes. Regulation of 21 common genes was observed between SP 25A and RLs (Table 4.1). Despite regulating these common genes, the patterns of expression between the two classes of the antimicrobial compounds were different. At T_{120}, 27 genes were induced and eight were repressed.

Intermediary metabolism and oxidative phosphorylation. RLs induced three PEP/PTS components, α-mannosidase (LMO0401), and five other genes involved in glycolysis and pentose phosphate pathway (Table 4.2). Conversely, all these genes were repressed when cells were treated with SP 25A during the same time period. The H+-transporting ATP synthase C (*atpE*) was induced at T_{30}, but subsequently repressed at T_{120}. Fig D1 and D2 from Appendix D gives the heatmaps of genes affected in these functional category

Protein biosynthesis and virulence factors. No genes related to transcription were differentially regulated after treatment with RLs. Only one gene related to protein biosynthesis, phenylalanyl-tRNA synthetase alpha chain (*pheS* (LMO1221)) was induced at T_{30}. An acetyltransferase (LMO0624) involved in post translation modification was

repressed at T_{30}, but induced at T_{120} (Table 4.2). Fig D3 and D4 from appendix D gives the heatmaps of genes affected in these functional category

Of the four virulence factors in the genome, only listeriolysin O (*hly*) was induced after 120 min of treatment. Expression of the remaining virulence factors was not changed by addition of RLs.

Stress genes. Four stress-related genes -- single strand binding protein (*ssb*), non-heme iron binding ferritin (*fri*), and heat shock protein *cspL*, and peroxide operon regulator *perR* (LMO1683) -- were induced after 120 min. However, none of the genes in the *perR* regulon were induced. No other genes were significantly regulated during treatment with RLs.

Gene expression profiling with exposure to SP 25A. Treating *L. monocytogenes* with SP 25A significantly altered the transcript profile of ~5% of the genes of the genome. Addition of SP 25A repressed 97% of the 139 differentially regulated genes (Table 4.3). The data set was also analyzed for genes that were constitutively expressed above the mean level during the treatment in an effort to find genes that may be essential for survival of bacteria under the antimicrobial stress. No genes were found that were constitutively expressed above the mean. Most functional categories contained genes that were repressed. No categories contained genes that were only induced. However, a few categories (ABC transporters, carbohydrate metabolism, transcription regulators, secretion, virulence factors, and unknown genes) contained genes that were induced and repressed.

Cell division. Four genes involved in cell division and chromosome replication were repressed. Genes required for cell division initiation protein, DivIVA (LMO1888), ATPase associated with chromosome architecture/replication (LMO2759), DNA gyrase subunit B (*gyrB*), and DNA gyrase subunit A (*gyrA*) were repressed with addition of SP 25A. The transcription factor, *lytR,* which is correlated to the decrease in activity of autolytic enzymes (11), was also repressed (Table 4.4).

Membrane proteins. PEP/PTS transporters specific for β-glucosides, fructose, and trehalose; α-mannosidase (a sugar hydrolase); and 22 other genes in carbohydrate metabolism were differentially expressed during the treatment time. From the entire set of genes in the intermediary metabolism category, only L-glutamine-fructose-6-phosphate transaminase (LMO0726) and 6-phospho-β-glucosidase (LMO0739) were induced at T_{30}. However, at T_{120} all of the 26 genes in sugar transport and intermediatery metabolism were repressed (Table 4.4). Each PEP/PTS components were repressed after 120 min (Table 4.4).

In addition to the sugar transporters and ATPases that were repressed, the large-conductance mechonosensitive ion channel (LMO2064) was induced at T_{30}, but repressed at T_{120}. This mechanoreceptor is involved in osmoregulation. Other studies using gene expression profiling did not observe an expression change in this ion channel, despite its importance in restoring the osmotic stability in a cell. This observation may be indicative of membrane perturbation early in the treatment time.

Intermediatery metabolism and respiration. The repressed genes in central intermediatery metabolism included genes involved in glycolysis - 6-phospho-beta-

glucosidase (LMO0739), the pyruvate dehydrogenase operon (*pdhA, pdhB, pdhC, pdhD),* lactate dehydrogenase *(ldh)*, pyruvate kinase (*pykA),* and phosphoglyceromutase (LMO2205). Repression of genes in the pentose phosphate pathway were also observed - ribose 5-phosphate isomerase (LMO0736), transaldolase (LMO2743), ribulose 5-phosphate 3-epimerase (LMO0735, LMO2659), and fructose-1,6-bisphosphate aldolase (*fbaA*). The dihydroxyacetone kinase enzyme complex (LMO2695, LMO2696 and LMO2697), which is responsible for phosphorylation of dihyroxyacetone and glycerol prior to entry into the glycolytic pathway, was also repressed. Four (out of six) genes involved in Fe-S cluster biosynthesis (*sufD, IscU, sufB,* cysteine desulfurase (LMO2413)) were repressed.

Repression of key genes for cellular respiration was observed. After 120 min, two genes (out of eight) of the H^+ transporting ATP synthase enzyme complex, which code for the alpha and c subunits (*atpA and atpE*), were repressed. Three of the four subunits for quinol oxidase (LMO0014, LMO0015, LMO0016) were also repressed.

Protein biosynthesis. At T_{30} two genes involved in protein biosynthesis, (LMO2511 and *rpsU*) were induced. The gene LMO2511 codes for the ribosome associated factor Y, which is a global translation inhibitor, while *rpsU* codes for the S21 protein in the 30s ribosomal complex. After 120 min of treatment with SP 25A, three of the four subunits of RNA polymerase (*rpoA, rpoB, rpoC)* were repressed. After 120 min 11 ribosomal proteins (out of 59) were repressed and two elongation factors (out of total four) were repressed. Hence, after 120 min genes needed for transcription and translation were repressed.

Virulence factors. At T_{30} two of the virulence genes *hly* (listeriolysin O precursor) and fibronectin binding protein (LMO0727) were induced, while *iap* (an invasion associated protein) was repressed. At T_{120}, phospholipase C (*plcA*) was also repressed. Hence, after 120 min SP 25A led to the repression of four genes directly required for host invasion by *L. monocytogenes.*

Stress response. At T_{30} five stress-related genes were repressed, while four genes were induced. Among the repressed genes two chaperone proteins (*groEL, grpE),* three oxidative stress genes (*sod, msrA, trxB*), and one gene related to toxic ion resistance (LMO1967). During the same period, the induced stress proteins were DNA binding protein (*fri*), organic hydroperoxide resistance protein (LMO2199), arsenate reductase (LMO2230), and a universal stress protein (LMO1580). After 120 min, an additional six stress related genes were repressed. These included *hrcA* (a negative regulator of class I heat shock genes), a general stress protein (LMO1601), a protein related to oxidative stress (*msrB),* and three genes involved in DNA recombination and repair (i.e. single strand binding protein (*ssb*), an endonuclease involved in recombination (LMO1502), and exinuclease ABC subunit A (*urvA*)). Three ATP-dependent endopeptidases needed for protein turnover (*clpE, clpB, clpX)*(22) were also repressed. At T_{30} one transcription regulator of the *marR* family (LMO2200), which is a negative regulator of antibiotic resistance proteins in *E. coli* (2) was induced, while at T_{120} another transcription of the same family (LMO0266) was repressed.

Discussion

This study explored the cellular response to treatment with two antibacterial compounds that presumably target the cell membrane for inhibition (5, 12, 21). The cell permeabilization, cell density, and the transcription profile between these compounds were markedly different. RLs caused an increase of ~53% in membrane permeabilization, and inhibited growth by ~47%, a reasonable correlation between membrane permeabilization and growth inhibition. Conversely, SP 25A permeabilized the membrane by ~2%, but led to complete inhibition of cell growth, demonstrating the disconnection between membrane permeabilization and cell growth inhibition. Other studies with *E. coli* and *S. aureus* have made similar observations (16, 35). Friedrich et al. (16) observed that treatment of *S. aureus* with CP26, an α-helical peptide, led to only partial membrane depolarization, even with 90% inhibition of the population. Wu et al. (35) found similar results using CP26 and bactenecin in *E. coli*. Tomasinsig et al. (32) found that a specific sequence of a proline-rich peptide interacted with the membrane leading to growth inhibition and that the same peptide fragment led to gene expression changes that were not related to membrane disruption. In contrast this study found RLs to be linked to membrane disruption to bacterial inhibition for lytic activities. Alternatively, use of SP 25A confirmed that additional mechanisms beyond membrane disruption for bacterial inhibition may be involved.

With this observation, gene expression profiles were used to determine the genetic targets of these bacterial inhibitors. SP 25A significantly changed expression of 139 genes, while RLs significantly changed expression of 39 genes. Both the compounds

affected twenty-one common genes, but the expression profile for all these genes (except *sufB*) was significantly different. While SP 25A caused repression of all these genes after 120 min of treatment, RLs caused an induction (Table 4.3), lending support to the observation of growth inhibition by SP 25A (Fig. 4.1). Tomasinsig et al. (32) found that genes from the same functional categories to be repressed with the Bac7 (1-35) peptide fragment in *E. coli*.

SP 25A repressed key genes involved in cell division. After 120 min four proteins involved in cell division were repressed; two DNA gyrase subunits, one was an ATPase associated with chromosome replication, and one was the cell division initiation protein, DivIVA, which is crucial for the initiation of cell division (15). Hence, repression of this protein alone would inhibit cell division (15). RLs, in contrast, did not affect any of the proteins involved in cell division and had only a small reduction in cell density (Fig. 1). In addition, repression of *lytR* was correlated to a decrease in autolytic enzyme activity, which resulted in inhibition of cell division in *S. mutans* (11). Use of SP 25A also led to repression of *lytR* in *L. monocytogenes* in this study. These observations explain the inhibition of growth, rather than membrane disruption, with the addition of SP 25A. It also points to the regulatory link between *lytR* and DivIVA to completely inhibit cell growth (Fig. 4.1A, Table 4.4).

SP 25A caused significant changes in intermediary metabolism, especially glycolysis and pathways needed for energy production (Table 4.4). Repression of central metabolism would lead to a lack of enzymes needed for generation of precursor metabolites and energy needed for growth. Repression of pyruvate dehydrogenase

complex (8- to 16-fold), pyruvate kinase (1.6 fold), and phosphoglyceromutase (1.5 fold) would virtually stop energy production from glycolysis.

Glycolytic intermediates are also important for generation of acetyl CoA, pyruvate and phosphoglycerate, which are precursor metabolites for production fatty acids and amino acids. RLs induced expression of the E3 subunit of pyruvate dehydrogenase, phosphoglyceromutase, and two more enzymes in the pentose phosphate pathway, indicating that glycolysis was induced and energy production improved. This may explain the reduction in membrane permeabilization after 120 min of exposure to RLs. In contrast, addition of SP 25A, five enzymes in the pentose phosphate pathway leading to generation of ribose-5-phosphate, which acts as a precursor metabolite in purine and pyrimidine metabolism, were repressed by 1.5-to 3- fold. The lack of induction of alternative pathways for formation of these metabolites left the cell with no method to produce energy or intermediates to use in cell division. The same repression of several key enzymes of the central intermediatery metabolism was also observed in *E. coli* when challenged with sub-MIC doses of $Bac7_{(1-35)}$, including sugar transporters and glycolytic intermediates, which was associated with inhibition of cell growth (32).

SP 25A led to a ~5-fold repression of four proteins needed for synthesis of Fe-S clusters. These are essential in diverse reactions, including electron transport, regulation of gene expression, and mediation of redox as well as non-redox catalysis (4). SP 25A also caused down regulation of two subunits of the proton pump by 1.5-fold and quinol oxidase by 2-fold, causing disruption of the oxidative phosphorylation machinery. RLs in contrast led to a 3-fold induction of one subunit of proton pump (*atpE*) at T_{30} and a 3.5-

fold repression at T_{120}. Hence, after 120 min SP 25A repressed the cells ability to generate precursor metabolites, as well as energy, while RLs did not have that effect at all. In *E. coli*, repression of iron metabolism was found only in transport (*fecA*), other genes were not affected (32).

Genes associated with transcription and translation were repressed between 1.5- and 12-fold with the addition of SP 25A. It repressed three out of four RNA polymerase subunits by 1.5- to 2-fold, which completely disrupted transcription. Coupled to this decline, translation activity was also repressed. This effect was widespread with repression (1.5- to 4-fold) of 11 ribosomal genes after 120 min, and two elongation factors (2.5- to 12-fold). Contrary to this study, Tomasinsig et al. (32) observed an induction of ribosomal genes after exposure to a proline rich antibacterial peptide.

Regulation of stress-related genes was widespread with the addition of SP 25A (Table 4.4). Multiple systems were regulated during the exposure time, which included osmotic regulation, DNA repair, chaparonenes, and peroxide resistance. For example, the large conductance mechonosensitive channel (*mscL*) was induced at T_{30}, but was repressed at T_{120}. This gene is associated with hypo-osmotic shock (31), likely caused by the interaction of SP 25A with the membrane. Induction of *mscL* demonstrates the cells effort to modulate the osmotic change with the addition of SP 25A. Repression of the membrane protein at T_{120} likely indicates that the cell is no longer under osmotic stress. This explanation seems likely considering the membrane permeabilization declined at T_{120}. No other group (17, 25, 32) has observed this phenomenon in response to an

antimicrobial peptide, despite observing changes in membrane and transport proteins associated with sugar and ion flux.

Interestingly, in all cases, stress-associated genes were repressed after 120 min of exposure to SP 25A. This may indicate that the cell has adapted to the effects of SP 25A, but it may also represent the inability to produce new RNA and proteins with the repression of the transcription and translation apparatus observed in this study. Three *Clp* ATPase implicated in regulation of the stresses by virtue of their protein reactivation, remodeling activities, and their capacity to target misfolded proteins for degradation (22) were repressed by SP 25A. There are no reports in literature of stress related genes being repressed in response to exposure with antimicrobial peptides (1, 10, 17, 32, 34).

Four virulence genes essential for intracellular survival of *L. monocytogenes* were repressed with treatment with SP 25A after 120 min of exposure. Fibronectin binding protein and *hly* were induced and *iap* was repressed at T_{30}, but after 120 min each of these genes and *plcA* were repressed. In contracts, addition of RLs induced *hlyA* expression after 120 min. Down-regulation of these genes would make *L. monocytogenes* EGDe less virulent with extended exposure by repressing the binding proteins and impairing the capacity for intracellular survival as well (20).

Conclusion

Treatment of *L monocytogenes* EGDe with sub-MIC doses of SP 25A and RLs led to complete inhibition of growth or a reduction of growth, respectively. However, this

inhibition was uncoupled from membrane permeabilization in the case of SP 25A. The gene expression profile of cells treated with SP 25A revealed that genes related to cell division, chromosome replication, intermediatery metabolism, transcription, translation, and virulence were repressed. These effects were not produced in cells treated with RLs. These observations indicate that SP 25A permeabilizes the membrane, but the mechanisms associated with cell death are likely related to other targets that inhibit cell division; while, the antibacterial activity of RLs is likely due to its interaction with the membrane.

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Table 4.1. Functional categories that contained the genes that were significantly differentially expressed in response to treatment with sub-MIC doses of RLs.

Functional category	Total genes in category	No. of differentially expressed genes			
		T_{30}		T_{120}	
		Induced	Repressed	Induced	Repressed
ABC transporters	134	0	0	2	1
Ion channels	4	0	0	0	0
PEP/PTS components	80	0	0	3	0
Polysaccharide degradation	18	0	0	1	0
Central intermediatery metabolism	272	0	1	4	0
Cofactor and coenzyme metabolism	116	0	0	0	0
Amino acid metabolism	179	0	0	2	0
Electron transport and oxidative phosphorylation	61	1	0	0	1
Cell wall metabolism	57	0	0	0	0
Transcription regulators	142	1	0	2	0
Transcription	36	0	0	0	0
Protein biosynthesis	158	1	0	1	1
Protein fate	92	0	1	1	0
Secretion	63	0	0	1	0
Virulence factors	131	0	0	1	0
Stress	50	0	0	2	0
Cell division	17	0	0	0	0
Phage proteins	18	1	0	0	2
Unknown/Hypothetical proteins	-	4	0	7	2
Total	1628	8	2	27	7

Table 4.2. Significantly differentially regulated genes during exposure to RLs.

Protein	ORF	Cellular Role	Log₂ Ratios		
			T_{30}	T_{120}	Q value
ABC transporter (Metal binding protein)	LMO1073	ABC transporters	-0.18	0.97	0.26
ABC transporter (ATP-binding protein)	LMO2193	ABC transporters	0.33	0.32	0.11
ABC transporter-associated protein, *sufB*	LMO2411	ABC transporters	-0.43	-0.17	0.23
Fructose-specific phosphotransferase enzyme IIB	LMO0399	PEP/PTS components	0.21	0.38	0.11
Fructose-specific phosphotransferase enzyme IIC	LMO0400	PEP/PTS components	0.05	2.54	0.11
Beta-glucoside-specific enzyme IIABC component	LMO0738	PEP/PTS components	0.46	1.36	0.11
Alpha mannosidase	LMO0401	Polysaccharide degradation	-0.15	2.70	0.11
Ribose 5-phosphate isomerase	LMO0736	Intermediatery metabolism	-0.03	1.72	0.11
6-phospho-beta-glucosidase	LMO0739	Intermediatery metabolism	0.04	2.86	0.11
Pyruvate dehydrogensae (dihydrolipoamide dehydrogenase, E3 subunit), *pdhD*	LMO1055	Intermediatery metabolism	0.24	0.96	0.11
Phosphoglyceromutase	LMO2205	Intermediatery metabolism	-0.73	0.86	0.15
Ribulose-phosphate 3-epimerase	LMO2659	Intermediatery metabolism	0.01	1.70	0.11
Glycine dehydrogenase (decarboxylating) subunit 2	LMO1350	Aminoacid metaboloism	-0.41	1.21	0.11
Threonine 3-dehydrogenase	LMO2663	Aminoacid metaboloism	-0.06	1.98	0.11
H+-transporting ATP synthase C chain, *atpE*	LMO2534	Respiration and oxidative phsphorylation	1.64	-1.81	0.11
Peroxide operon regulator, *perR*	LMO1683	Transcription regulator	-0.13	1.03	0.11
Transcriptional regulatory protein *degU*	LMO2515	Transcription regulator	0.12	1.39	0.16
Phenylalanyl-tRNA synthetase alpha chain, *pheS*	LMO1221	Protein biosynthesis	0.62	-0.45	0.29
Acetyltransferase	LMO0624	Post translational modification	-0.62	1.01	0.29
ATP-dependent endopeptidase clp ATP-binding subunit, *clpE*	LMO0997	Protein degradation	-0.03	1.28	0.11
Oxidoreducatse involved in TATpathway secreted proteins	LMO0737	Secretion	0.05	1.02	0.11

listeriolysin O precursor, *hly*	LMO0202	Virulence	0.24	1.90	0.11
Single-stranded DNA-binding protein, *ssb*	LMO0045	Stress	-0.01	1.33	0.11
Cold shock protein, *cspL*	LMO1364	Stress	0.14	0.45	0.29
Non-heme iron-binding ferritin, *fri*	LMO0943	Stress	0.09	0.81	0.11
Phage proteins	LMO2287	Phage proteins	0.66	-0.58	0.11
Phage proteins	LMO2327	Phage proteins	0.27	-0.61	0.11
Hypothetical Protein	LMO0743	Unknown	0.12	0.82	0.15
Hypothetical Protein	LMO1113	Unknown	0.77	-0.39	0.11
Hypothetical Protein	LMO2257	Unknown	0.00	0.94	0.11
Hypothetical Protein	LMO2432	Unknown	-0.20	3.24	0.11
Stage V sporulation protein G	LMO0197	Others	0.86	-0.61	0.11
Rhodanese-related sulfurtransferases	LMO1384	Others	0.60	-0.48	0.11
Glycerol uptake facilitator protein	LMO1539	Others	0.21	0.82	0.14
Creatinine amidohydrolase family protein	LMO1968	Others	0.77	-0.56	0.11
Protease I	LMO2256	Others	0.17	0.99	0.26
Putative transcriptional regulator, *MerR* family	LMO2728	Others	-0.05	0.63	0.15
Putative transcriptional regulator, *MerR* family	LMO2334	Others	0.57	-0.66	0.15

Table 4.3. Functional categories that contained the genes that were significantly differentially expressed in response to treatment with sub-MIC doses of SP 25A.

Functional category	Total genes in category	Number of differentially expressed genes			
		T_{30}		T_{120}	
		Induced	Repressed	Induced	Repressed
ABC transporters	134	1	3	2	8
Ion channels	4	1	0	0	1
PEP/PTS components	80	0	4	0	4
Polysaccharide degradation	18	0	1	0	1
Central intermediatery metabolism	272	2	13	0	24
Cofactor and coenzyme metabolism	116	0	4	0	5
Amino acid metabolism	179	0	1	0	4
Electron transport and oxidative phosphorylation	61	0	4	0	5
Cell wall metabolism	57	0	1	0	2
Transcription regulators	142	1	0	1	4
Transcription	36	0	0	0	3
Protein biosynthesis	158	2	3	0	12
Protein fate	92	0	3	0	4
Secretion	63	1	2	0	3
Virulence factors	131	2	1	0	4
Stress	50	4	5	0	13
Cell division	17	0	2	0	4
Phage proteins	18	0	0	0	0
Unknown/Hypothetical proteins	-	12	13	2	34
Total	1628	26	60	5	135

Table 4.4. Significantly differentially regulated genes during exposure to SP 25A.

Protein	ORF	Cellular Role	Log2 Ratios		
			T_{30}	T_{120}	Q value
Manganese uptake Mn ABC transporter	LMO1847	ABC transporters	0.66	-3.85	0.00
Metal cations ABC transporter, permease protein	LMO1848	ABC transporters	-3.43	-2.43	0.00
ABC transporter, ATP-binding protein	LMO2415	ABC transporters	-0.47	-0.65	0.00
Heavy metal-transporting ATPase	LMO0641	ABC transporters	-0.45	-0.42	0.01
Manganese transport proteins NRAMP	LMO1424	ABC transporters	-2.10	0.01	0.01
Metal cations ABC transporter, ATP-binding proteins	LMO1849	ABC transporters	-0.89	-0.78	0.04
ABC transporter-associated protein (sufB)	LMO2411	ABC transporters	-0.51	-0.20	0.04
Oligopeptide ABC transporter (ATP-binding protein)	LMO2193	ABC transporters	-0.24	-0.46	0.17
Acetoin uptake permease protein	LMO2239	ABC transporters	-0.24	0.68	0.17
ABC transporter (ATP-binding protein)	LMO2139	ABC transporters	0.07	0.60	0.23
Large conductance mechanosensitive channel	LMO2064	Ion Channel	1.96	-2.70	0.00
Fructose-specific phosphotransferase enzyme IIC	LMO0400	PEP/PTS components	-1.78	-0.34	0.00
Beta-glucoside-specific enzyme IIABC component	LMO2373	PEP/PTS components	0.39	-0.83	0.00
Beta-glucoside-specific enzyme IIABC component	LMO0738	PEP/PTS components	-1.04	-1.04	0.00
Trehalose specific enzyme IIBC	LMO1255	PEP/PTS components	-3.74	-0.40	0.00
Alpha mannosidase	LMO0401	Polysaccharide degradation	-2.61	-0.78	0.00
6-phospho-beta-glucosidase	LMO0739	Intermediatery metabolism	0.97	-2.39	0.00
6-phospho-beta-glucosidase	LMO0536	Intermediatery metabolism	-0.38	-0.24	0.23
Alpha,alpha-phosphotrehalase	LMO1254	Intermediatery metabolism	-2.10	-0.04	0.00
Pyruvate dehydrogenase (E1 alpha subunit), *pdhA*	LMO1052	Intermediatery metabolism	-2.74	-0.38	0.00
Pyruvate dehydrogenase (E1 beta subunit), *pdhB*	LMO1053	Intermediatery metabolism	-3.04	-1.38	0.00
Pyruvate dehydrogenase (dihydrolipoamide acetyltransferase E2	LMO1054	Intermediatery metabolism	-2.04	-2.62	0.00

subunit), *pdhC*

Pyruvate dehydrogensae (dihydrolipoamide dehydrogenase, E3 subunit), *pdhD*	LMO1055	Intermediatery metabolism	-2.58	-2.31	0.00
L-lactate dehydrogenase, *ldh*	LMO1057	Intermediatery metabolism	-1.11	-0.52	0.04
Pyruvate kinases, *pykA*	LMO1570	Intermediatery metabolism	-0.60	0.38	0.03
Ribose 5-phosphate isomerase	LMO0736	Intermediatery metabolism	-0.81	-0.32	0.00
Ribulose-5-Phosphate 3-Epimerase	LMO0735	Intermediatery metabolism	-0.43	-0.48	0.00
Transaldolase	LMO2743	Intermediatery metabolism	-0.62	-0.13	0.29
Fructose-1,6-bisphosphate aldolase, *fbaA*	LMO2556	Intermediatery metabolism	-0.22	-0.38	0.07
Dihydroxyacetone kinase	LMO2695	Intermediatery metabolism	-0.49	-0.71	0.03
Dihydroxyacetone kinase	LMO2696	Intermediatery metabolism	-0.51	-0.81	0.00
Dihydroxyacetone kinase phosphotransfer protein	LMO2697	Intermediatery metabolism	0.41	-1.51	0.01
Glucosamine-6-Phoasphate isomerase	LMO0957	Intermediatery metabolism	-0.52	-0.31	0.07
L-glutamine-D-fructose-6-phosphate amidotransferase	LMO0727	Intermediatery metabolism	0.76	-2.90	0.00
Phosphoglyceromutase	LMO2205	Intermediatery metabolism	-0.21	-0.42	0.03
Branched-chain alpha-keto acid dehydrogenase E2 subunit (lipoamide acyltransferase)	LMO1374	Amino acid metabolism	-0.56	-0.11	0.03
Glycerate dehydrogenases	LMO1684	Amino acid metabolism	-1.26	-1.22	0.00
Glycine dehydrogenase (decarboxylating) subunit 2	LMO1350	Amino acid metabolism	-0.34	-1.68	0.00
Alanine dehydrogenase	LMO1579	Amino acid metabolism	-0.38	-0.25	0.00
IscU protein	LMO2412	Cofactor-coenzyme metabolism	-1.39	-1.00	0.00
Cysteine desulfurase	LMO2413	Cofactor-coenzyme metabolism	-2.04	-0.40	0.10
SufD protein	LMO2414	Cofactor-coenzyme metabolism	-1.50	-0.94	0.00
Pyridoxine biosynthesis protein	LMO2101	Cofactor-coenzyme metabolism	-1.82	-0.40	0.00
Pyridoxine biosynthesis amidotransferase	LMO2102	Cofactor-coenzyme metabolism	-0.15	-0.83	0.00
AA3-600 quinol oxidase subunit I	LMO0014	Respiration and oxidative phsphorylation	-0.91	-0.09	0.10

AA3-600 quinol oxidase subunit III	LMO0015	Respiration and oxidative phsphorylation	-0.73	-0.50	0.03
AA3-600 quinol oxidase subunit IV	LMO0016	Respiration and oxidative phsphorylation	-0.52	-0.37	0.17
H+-transporting ATP synthase chain alpha, *atpA*	LMO2531	Respiration and oxidative phsphorylation	-0.58	0.11	0.29
H+-transporting ATP synthase C chain, *atpE*	LMO2534	Respiration and oxidative phsphorylation	-1.14	0.32	0.00
UDP-N-acetylglucosamine 1-carboxyvinyltransferase, *murA*	LMO2526	Cell wall metabolism	-1.10	-0.21	0.00
Peptidoglycan anchored protein (LPXTG motif)	LMO2714	Cell wall metabolism	-0.03	-0.98	0.00
Transcriptional regulator, *MarR* family	LMO0266	Transcription regulator	-0.25	0.89	0.29
Transcriptional regulator, *MarR* family	LMO2200	Transcription regulator	0.62	-2.07	0.00
Transcriptional regulator, *LytR* family	LMO0433	Transcription regulator	-0.53	-0.80	0.01
Heat-inducible transcription repressor, *hrcA*	LMO1475	Transcription regulator	-0.56	-0.16	0.00
Peroxide operon regulator, *perR*	LMO1683	Transcription regulator	0.28	-2.71	0.00
Negative regulator of genetic competence *mecA*	LMO2190	Transcription regulator	0.24	-0.62	0.02
RNA polymerase (alpha subunit),*rpoA*	LMO2606	Transcription	-0.31	-0.30	0.01
RNA polymerase (beta subunit),*rpoB*	LMO0258	Transcription	-0.47	-0.76	0.02
RNA polymerase (beta' subunit), *rpoC*	LMO0259	Transcription	-0.12	-0.46	0.00
Ribosomal protein S6, *rpsF*	LMO0044	Protein biosynthesis	-0.47	-0.70	0.00
Ribosomal protein S18, *rpsR*	LMO0046	Protein biosynthesis	0.15	-0.67	0.00
Ribosomal protein S21, *rpsU*	LMO1468	Protein biosynthesis	1.37	-2.22	0.00
Ribosomal protein L16, *rplP*	LMO2625	Protein biosynthesis	-0.16	-0.56	0.00
Ribosomal protein L2, *rplB*	LMO2629	Protein biosynthesis	-0.52	-0.29	0.00
Ribosomal protein S2, *rpsB*	LMO1658	Protein biosynthesis	-0.26	-1.04	0.02
Ribosomal protein L27, *rpmA*	LMO1540	Protein biosynthesis	-0.21	-0.60	0.03
Hypothetical ribosome-associated protein	LMO1541	Protein biosynthesis	0.00	-0.59	0.03
Ribosomal protein L15, *rplO*	LMO2613	Protein biosynthesis	-0.62	0.06	0.23

Ribosomal protein L23, *rplW*	LMO2630	Protein biosynthesis	-0.38	-0.19	0.29
Translation elongation factor G	LMO 2654	Protein biosynthesis	-1.00	-0.35	0.02
Translation elongation factor EF-Tu	LMO 2653	Protein biosynthesis	-2.50	-1.13	0.00
Ribosomal-protein-alanine acetyltransferase	LMO1301	Protein biosynthesis	-0.18	-0.40	0.23
Ribosome associated factor Y (global translation inhibitor)	LMO2511	Protein biosynthesis	1.41	-3.85	0.00
Acetyltransferase	LMO0624	Post translational modification	0.39	-0.82	0.00
ATP-dependent endopeptidase clp ATP-binding subunit, *clpE*	LMO0997	Protein degradation	-0.70	-1.23	0.00
ATP-dependent endopeptidase clp ATP-binding subunit, *clpX*	LMO1268	Protein degradation	-0.78	-0.41	0.17
ATP-dependent clp endopeptidase Clp ATP-binding chain B, *ClpB*	LMO2206	Protein degradation	-0.72	-0.07	0.10
Oxidoreducatse involved in TATpathway secreted proteins	LMO0737	Secretion	0.61	-2.64	0.00
60 kDa inner membrane protein *yidC*	LMO1379	Secretion	-0.59	-0.57	0.00
Protein translocase subunit *secY*	LMO2612	Secretion	-0.79	-0.03	0.02
1-phosphatidylinositol phosphodiesterase precursor, *plcA*	LMO0201	Virulence	-0.33	-0.92	0.00
listeriolysin O precursor, *hly*	LMO0202	Virulence	1.31	-2.70	0.00
Invasion associated protein, i*ap*	LMO0582	Virulence	-0.99	-0.58	0.00
Fibronectin-binding protein	LMO0721	Virulence	0.76	-1.44	0.00
General stress protein	LMO1601	Stress	0.16	-0.73	0.00
Universal stress protein family	LMO1580	Stress	0.66	-0.19	0.00
Toxic ion resistance proteins	LMO1967	Stress	-0.74	-0.29	0.00
Arsenate reductase	LMO2230	Stress	0.59	-1.03	0.00
Superoxide dismutase, *sod*	LMO1439	Stress	-1.39	-4.35	0.00
Non-heme iron-binding ferritin, *fri*	LMO0943	Stress	1.23	-3.65	0.00
Peptide methionine sulfoxide reductases, *msrA*	LMO1860	Stress	-1.68	-0.25	0.00
Peptide methionine sulfoxide reductase, *msrB*	LMO1859	Stress	0.19	-0.90	0.00

Organic hydroperoxide resistance protein	LMO2199	Stress	0.79	-1.51	0.00
Thioredoxin reductase, *trxB*	LMO2478	Stress	-1.61	-0.63	0.00
Heat shock protein, *grpE*	LMO1474	Stress	-0.87	-0.03	0.04
Class I heat-shock protein (chaperonin), *groEL*	LMO2068	Stress	-0.88	-0.44	0.03
Single-stranded DNA-binding protein, *ssb*	LMO0045	Stress	0.53	-3.84	0.00
Excinuclease ABC (subunit A), *uvrA*	LMO2488	Stress	-0.53	-0.84	0.04
Endonuclease involved in recombination	LMO1502	Stress/Cell division	-0.48	-0.86	0.03
DNA gyrase subunit B, *gyrB*	LMO0006	Cell division	-0.85	-0.29	0.00
DNA gyrase subunit A, *gyrA*	LMO0007	Cell division	-0.51	-0.14	0.02
ATPase associated with chromosome architecture/replication	LMO2759	Cell division	0.46	-0.77	0.00
Cell division initiation protein DivIVA	LMO1888	Cell division	-0.65	-0.28	0.03
Hypothetical Protein	LMO0377	Unknown	-0.08	-0.73	0.00
Hypothetical Protein	LMO0393	Unknown	0.87	-0.95	0.00
Hypothetical Protein	LMO0647	Unknown	1.00	-2.42	0.00
Hypothetical Protein	LMO1380	Unknown	-0.63	-0.07	0.00
Hypothetical Protein	LMO1423	Unknown	-1.04	-1.28	0.00
Hypothetical Protein	LMO1612	Unknown	1.20	-1.69	0.00
Hypothetical Protein	LMO2257	Unknown	0.74	-0.75	0.00
Hypothetical Protein	LMO2432	Unknown	3.52	-3.23	0.00
Hypothetical Protein	LMO2828	Unknown	0.79	-1.05	0.00
Hypothetical Protein	LMO2156	Unknown	0.72	-0.88	0.01
Hypothetical Protein	LMO1893	Unknown	-0.06	-0.51	0.03
Hypothetical Protein	LMO1980	Unknown	0.58	-0.39	0.07
Hypothetical membrane spanning protein	LMO0625	Unknown	0.12	-1.29	0.00

Hypothetical membrane spanning protein	LMO0653	Unknown	-0.52	-0.31	0.01
Hypothetical membrane associated protein	LMO2119	Unknown	-0.62	-0.54	0.00
Hypothetical membrane spanning protein	LMO1690	Unknown	-1.04	-0.63	0.00
GatB/Yqey domain protein	LMO1468	Unknown	0.25	-0.92	0.00
Hypothetical cytosolic protein	LMO1501	Unknown	0.05	-1.45	0.00
Hypothetical cytosolic protein	LMO2472	Unknown	-0.58	0.21	0.00
Hypothetical cytosolic protein	LMO0964	Unknown	-3.18	-0.84	0.00
Cytosolic protein containing multiple CBS domains	LMO1576	Unknown	-1.18	-0.16	0.00
Stage V sporulation protein G	LMO0197	Others	-0.38	-0.31	0.23
Stage V sporulation protein G	LMO0196	Others	-0.72	-0.27	0.23
Acetyl esterase	LMO2089	Others	-0.30	-1.19	0.00
Protease I	LMO2256	Others	0.83	-2.28	0.00
Glyoxalase family protein	LMO2437	Others	2.91	-4.22	0.00
Predicted hydrolases or acyltransferases (alpha/beta hydrolase superfamily)	LMO2453	Others	-0.80	0.13	0.10
Hydrolase (HAD superfamily)	LMO1399	Others	-0.87	-0.38	0.17
Putative transcriptional regulator, *AraC* family	LMO0109	Others	-0.50	-0.14	0.10
Putative ranscriptional regulator, *ArsR* family	LMO0101	Others	-0.30	0.58	0.03
Putative transcription regulator	LMO0740	Others	0.13	0.76	0.29
Phosphoesterase, DHH family protein	LMO1575	Others	-0.56	-0.37	0.00
Carboxylesterase	LMO2452	Others	-0.92	0.03	0.00
Glycerol uptake facilitator protein	LMO1539	Others	-0.56	-0.37	0.07
Permease	LMO2148	Others	0.15	0.64	0.17

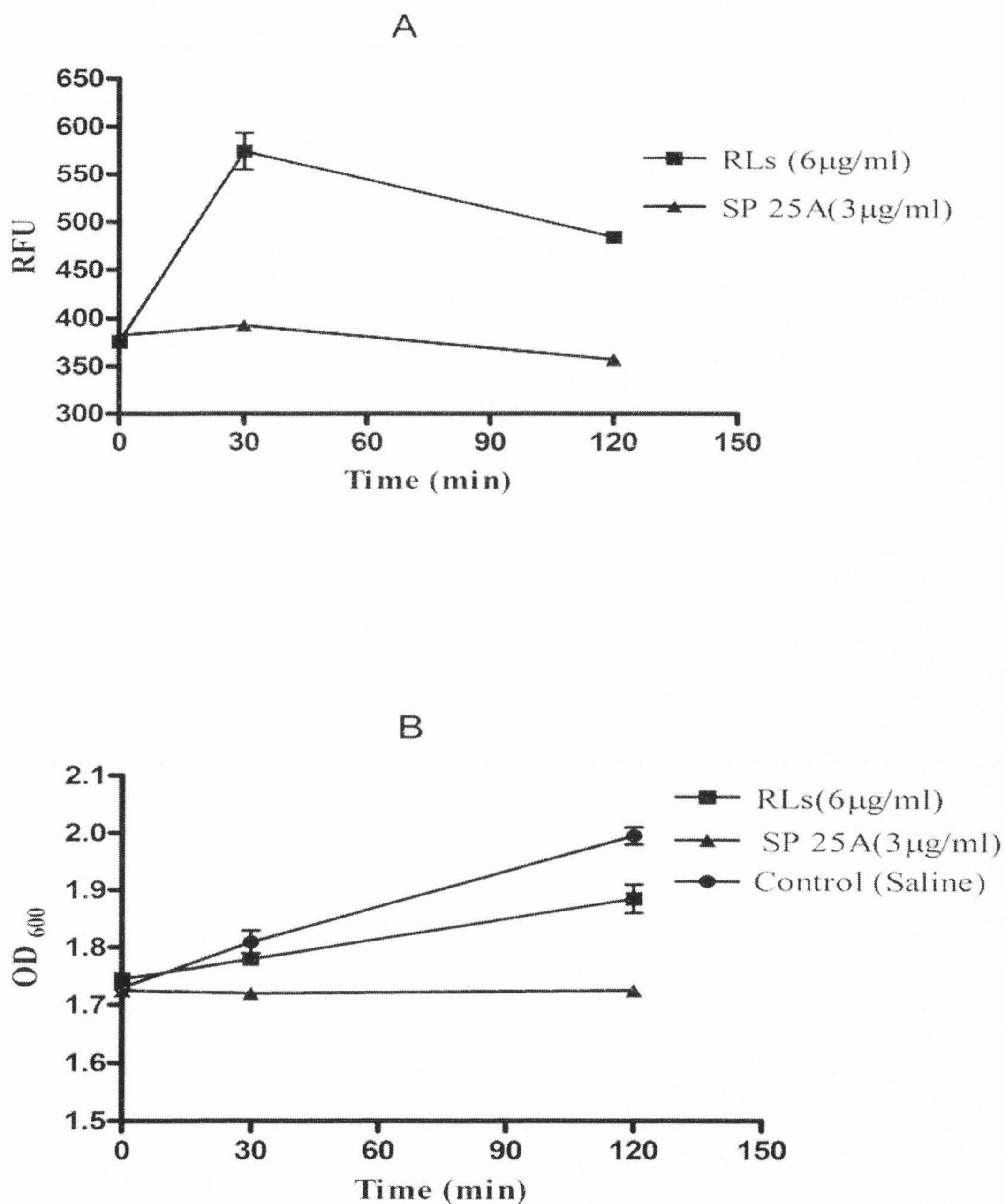

Fig 4.1. Membrane permeabilization determined by the increase in fluorescence (RFU) with PI uptake during treatment of *L. monocytogenes* with SP 25A and RLs (A), and cell growth when treated with SP 25A, RLs and saline (control) over a period of 120 min (B).

CHAPTER 5

SUMMARY

Development of antibiotic resistance in bacteria is one of the most troublesome issues facing health sciences today. There is no certain way to circumvent this problem, but discovery and development of novel compounds that inhibit bacteria by different mechanisms is required on a consistent basis. Secondary metabolites from microorganisms have been the most abundant source of new antimicrobials in the past. This study used two secondary metabolites from fluorescent pseudomonads, syringopeptin 25A (SP 25A) and rhamnolipids (RLs), a lipodepsipeptide and a surfactant, respectively, to determine the potential for their use as antimicrobial agents.

Several criteria must be met before a compound can be added to the arsenal of antibiotics in daily use: 1) the range of activity against bacteria must be known, 2) the MIC must be low enough to permit active doses that are not toxic, 3) an estimation of the mechanism of action must be determined, 4) the pharmacokinetics of the compound fate must be known, and 5) the compound must be approved for human use. This study focused on the first three criteria as a measure of the need to pursue criteria four and five.

Both the compounds, though from different chemical classes, are thought to act on the bacterial cell membrane to cause cell lysis and death. RLs have a very low critical micelle concentration (1-10μg/ml) and are thought to have a detergent-like action on the bacterial cell membrane causing it to dissolve (2). SP 25A, and syringopeptins generally,

on the other hand is thought to form transmembrane pores in the cell membrane; thereby, disrupting the permeability barrier of the bacteria ultimately leading to lysis (4, 7). Previous studies with these compounds showed that they inhibit a number of bacteria, but the range of organisms used was somewhat limited (1, 2, 5, 6). However, an ever-increasing data set is emerging that is challenging these models for antimicrobial action. These studies screened a large number of diverse bacteria and mold to determine the activity range of these two compounds, subsequently, used gene expression arrays during exposure of *Listeria monocytogenes* to sub-MIC doses of these compounds to determine non-membrane targets associated with growth inhibition.

Hypothesis

Syringopeptin 25A (SP 25A) and rhamnolipids (RLs) inhibit many bacterial species by disrupting the cellular membrane.

Objectives

1. Screen candidate compounds for microbial inhibition and determine the minimum inhibitory concentration for these compounds against selected bacteria
2. Determine the cellular responses to sub-MIC levels of SP 25A and RLs using gene expression arrays.

To screen the antimicrobial potential of these compounds they were tested against 27 different organisms, which included Gram-positive and Gram-negative bacteria and mold that are multiple drug resistant human pathogens, food spoilage organisms, bacterial spores, and fermentative bacteria. The initial screening was done using a rapid technique to determine the membrane permeabilization with a fluorescent DNA-binding dye that is blocked from entering the cell by an intact membrane. Subsequently, the MIC for each compound with each inhibited organism. Both the compounds inhibited growth of all the Gram-positive organisms. *Mycobacterium smegmatis*, the surrogate test organisms for *Mycobacterium tuberculosis*, was also inhibited. SP 25A also inhibited the. Interestingly, SP 25A inhibited two multiple antibiotic resistant strains of *Staphylococcus aureus* and *Enterococcus faecalis* and spore germination of bacterial spores. For all the organisms tested SP 25A showed similarly lower MIC values as compared to RLs with a range from 3 μg/ml to 16 μg/ml; reasonable concentrations for therapeutic use.

While the MICs were reasonable, we sought to reduce it further by combining the compounds to determine if they were synergistic in their activity. We demonstrated a synergistic activity against *Listeria monocytogenes*, which allowed the concentration of SP 25A to be reduced to below the MIC level, yet increase the cellular inhibition. Neither compound was toxic to three mammalian cell lines at the concentration of the effective MICs. Hence, taking into account the lower MICs, anti-spore activity, antimycobacterial activity, inhibition of multiple antibiotic resistant strains and no toxicity to mammalian cell lines SP 25A appears to be a promising therapeutic agent.

During the course of this study we observed a lack of correlation between membrane permeabilization and growth inhibition for SP 25A. When *L. monocytogenes* was challenged with sub-MIC doses of both the compounds, RLs induced PI uptake while SP 25A did not, yet it completely inhibited cell growth. A number of other groups have also observed that membrane-active antimicrobial peptides exert effects on cell growth beyond their ability to interact with the membrane (3, 8). This apparent lack of correlation between membrane permeabilization and inhibition of cell growth led us to suspect that the pore forming model alone may not be responsible for all of the antimicrobial properties of SP 25A. Therefore, we hypothesized that SP 25A represses gene expression to cause growth inhibition. To test this we challenged *L. monocytogenes* with sub-MIC concentrations of SP 25A and RLs and monitored gene expression profiles before treatment with antimicrobials, 30 min after treatment, and 120 min after treatment using a custom commercial high-density, whole genome oligonucleotide arrays (Chapter 4).

The gene expression profile was distinct between the two antimicrobials. SP 25A repressed genes required for cell division, chromosome replication and segregation, intermediary metabolism, transcription, translation, and virulence genes. Conversely, RLs induced a broad set of genes that were related to energy production. Hence, these data indicate that even though both the antimicrobials may interact with a common cellular structure (i.e. the cell membrane) their mode of action is different, with SP 25A having little ability to damage the membrane, but substantial ability to inhibit production of proteins critical to cellular replication. This supports the concept that antimicrobial

peptides do not entirely rely upon their membrane permeabilizing ability to exert their antibacterial action. This study brings forth SP 25A as a promising therapeutic agent that has a unique cellular target (e.g. DivIVA) to inhibit cell growth of a pathogenic bacterium at a reasonable MIC.

Future work with this compound needs to demonstrate the other criteria for antibiotics. Additional work needs to be done to assess the antimycobacterial potential and the efficacy in vivo for the inhibition of other Gram-positive pathogens.

References

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APPENDICES

APPENDIX A

COMPOUND PURITY

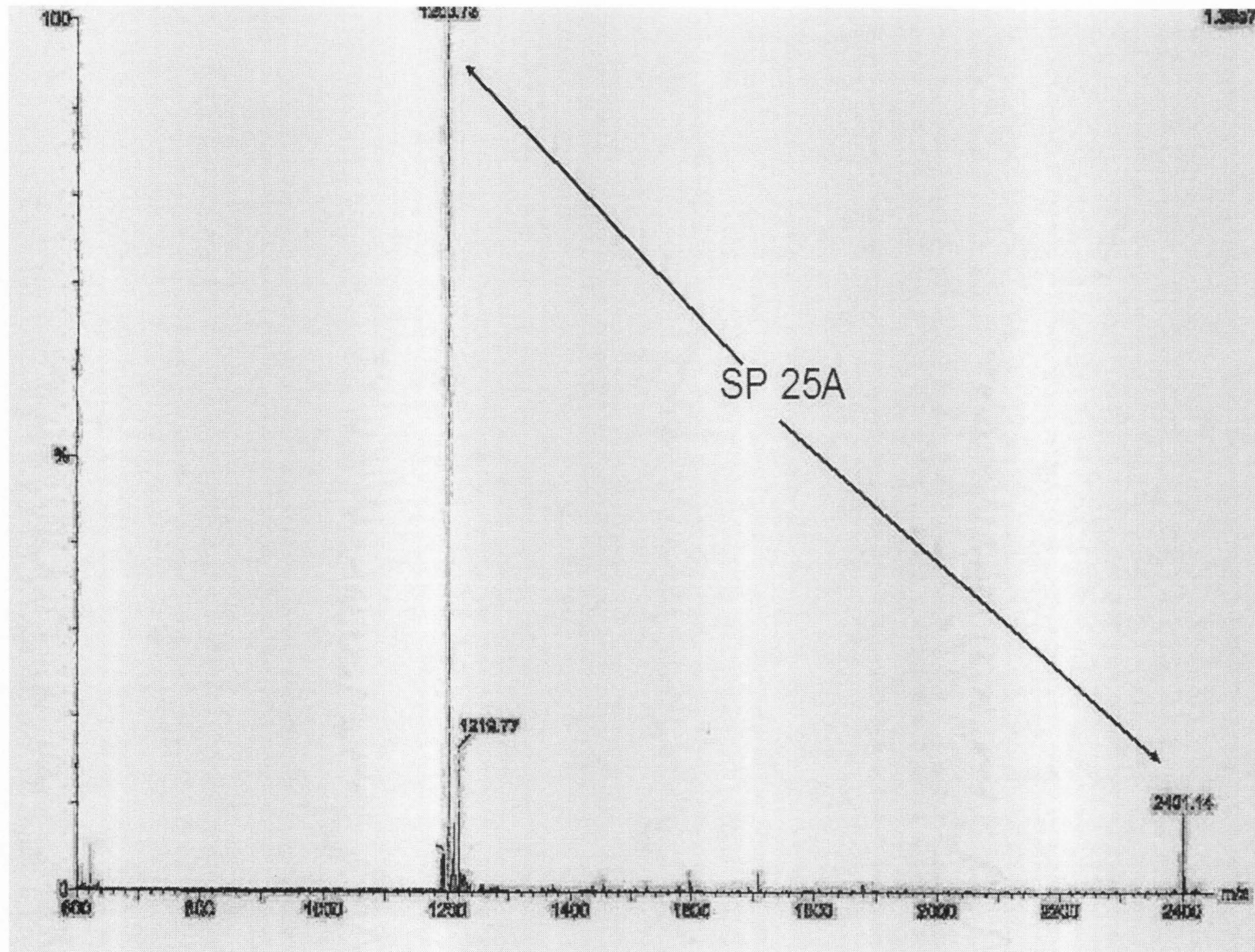


Fig A1. Massspectrogram of SP 25A purified from *Pseudomonas syringae* pv *syringae* M1 cultures.

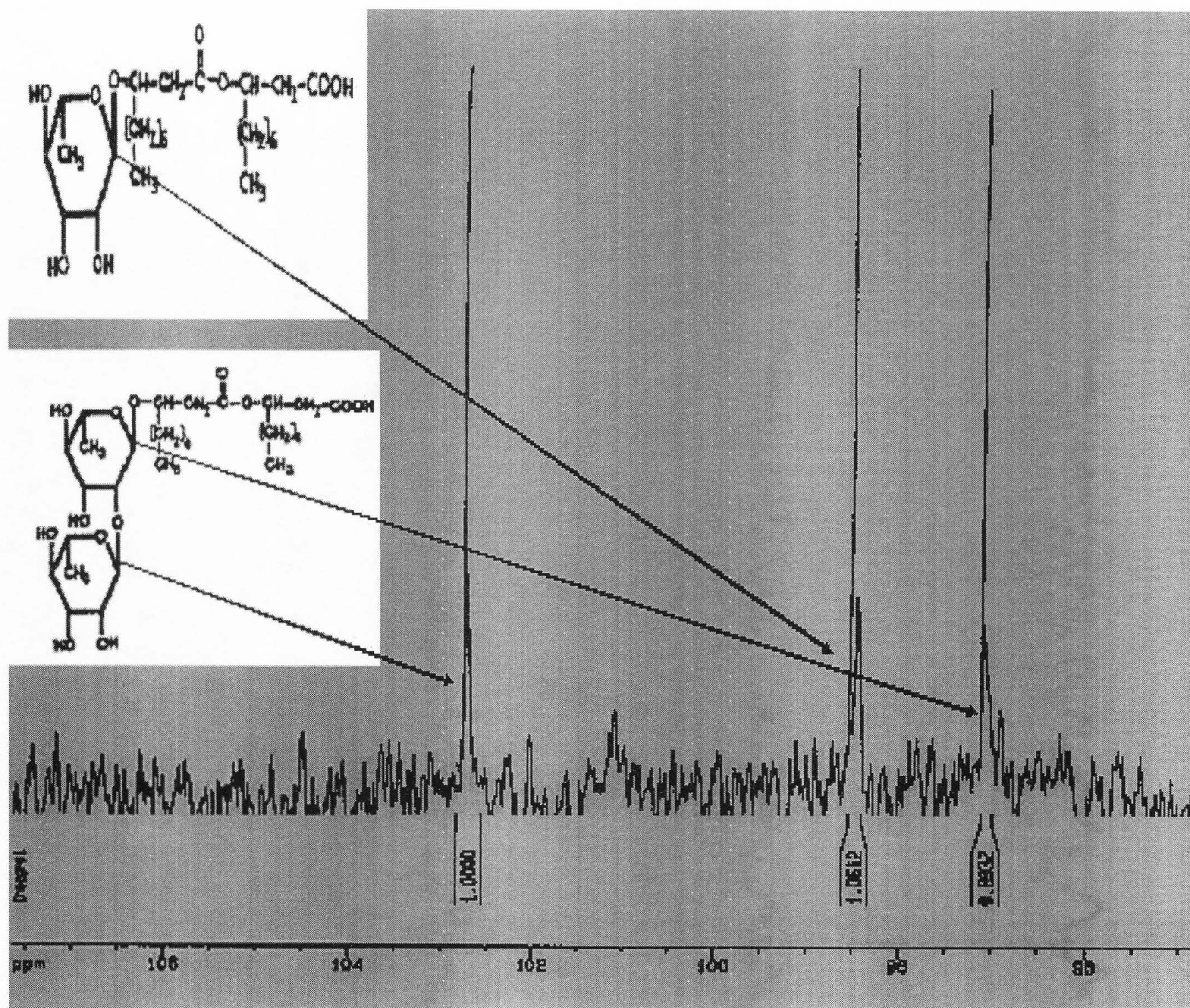


Fig A2. ^{13}C NMR spectra of RL mixture obtained from Jeniel Biotech.

APPENDIX B

VALIDATION OF PI ASSAY

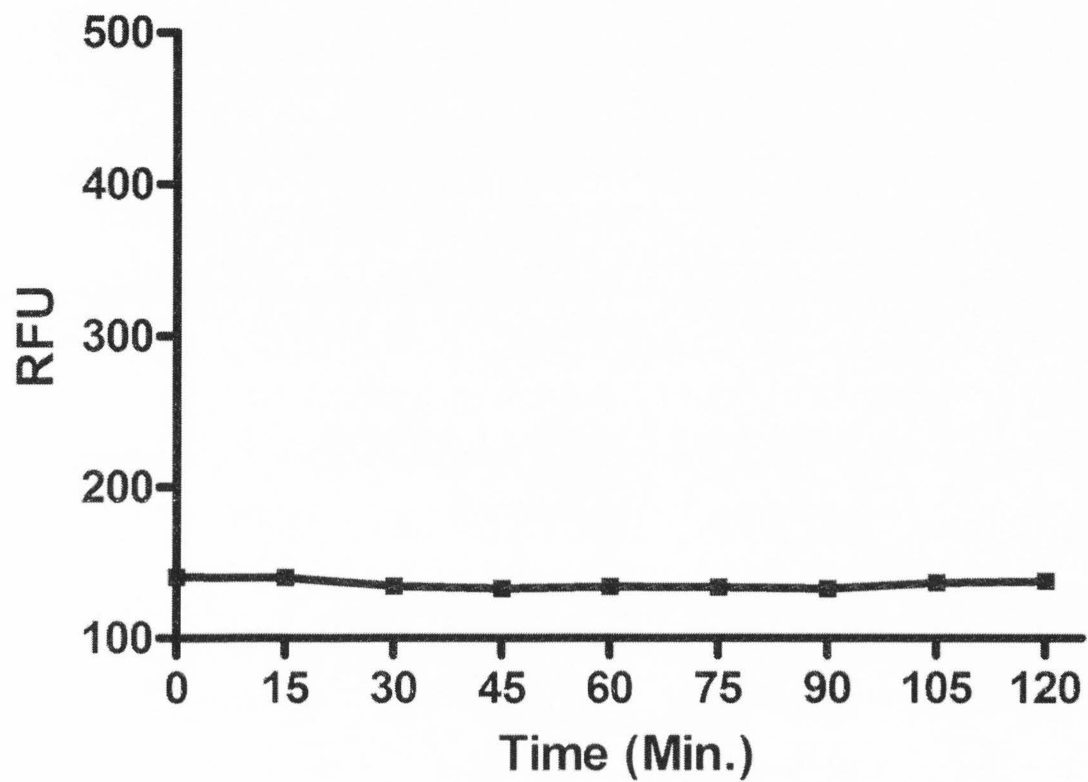


Fig B1. Mean PI uptake by *L. lactis* IL1403 treated with saline (negative control for the PI assay) over a period of 120 min.

APPENDIX C

R CODE FOR DATA NORMALIZATION

Code For RMA Normalization In R Of Raw Data Received From NimbleGen Systems:

```
Library (affy)

Data=read.table("lmono.txt",nrows=400000,sep="\t",check.names=false,header=true)

Corrected.data<-apply(as.matrix(data[,3:14]),2,bg.adjust)

Normalized.data<-normalize.quantiles(corrected.data)

Probe.data<-cbind(data[,1:2],normalized.data)

Expression.data<-data.frame()

Seq.ids<-unique(as.vector(probe.data$seq_id))

Expression.data<-data.frame()

For (seq.id in seq.ids) { probes<-

new("probeset",id=seq.id,pm=as.matrix(subset(probe.data[,3:14],probe.data$seq_id==se

q.id)))

Ev= express.summary.stat(probes,summary="medianpolish",pmcorrect="pmonly")

Expression.data<-rbind(expression.data,t(ev$exprs))

}

Expression.data<-as.data.frame(cbind(seq.ids,expression.data))

Names(expression.data)<-names(data)[c(1,3:15)]

write.table(expression.data,file="nfile.txt",col.names=na,quote=f,sep="\t")
```

APPENDIX D.

HEATMAPS OF EXPRESSION DATA

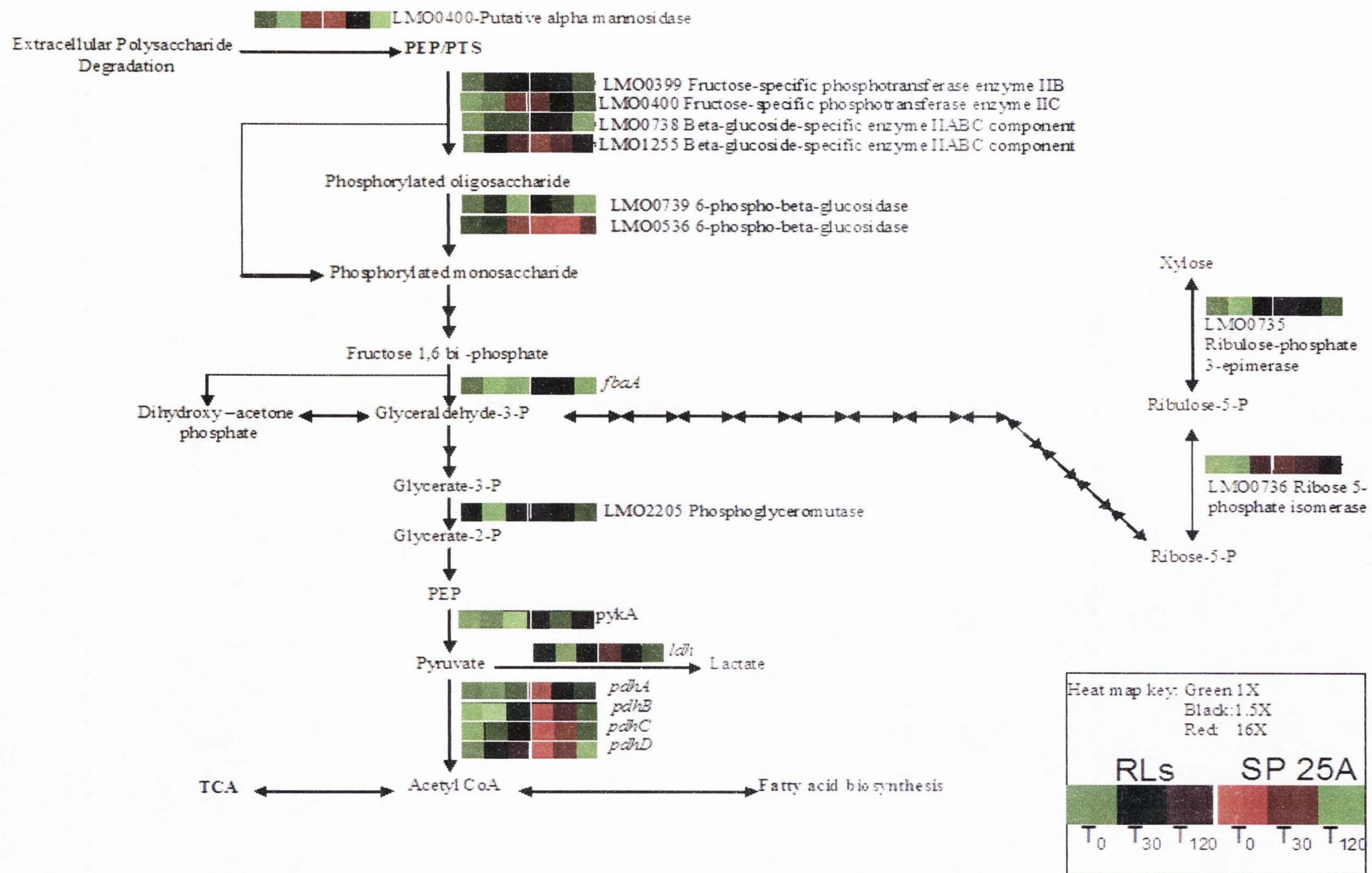
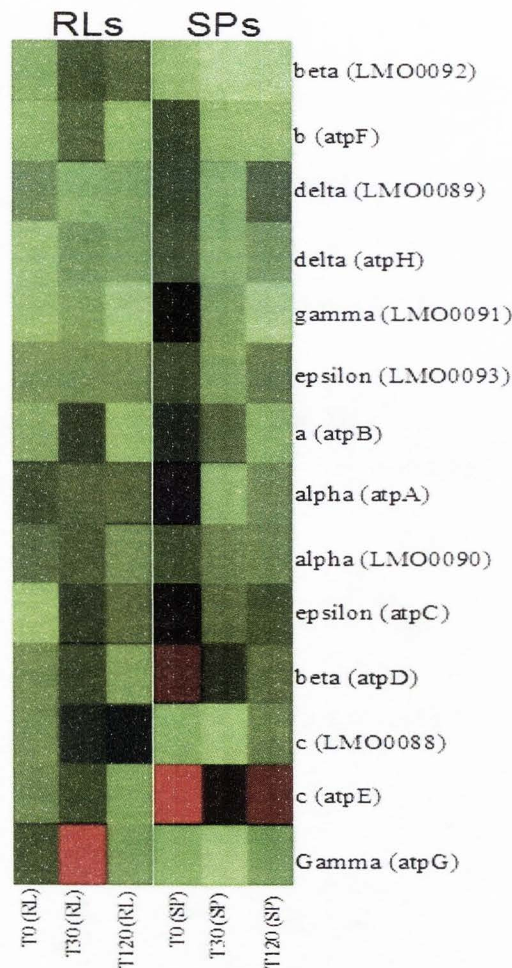
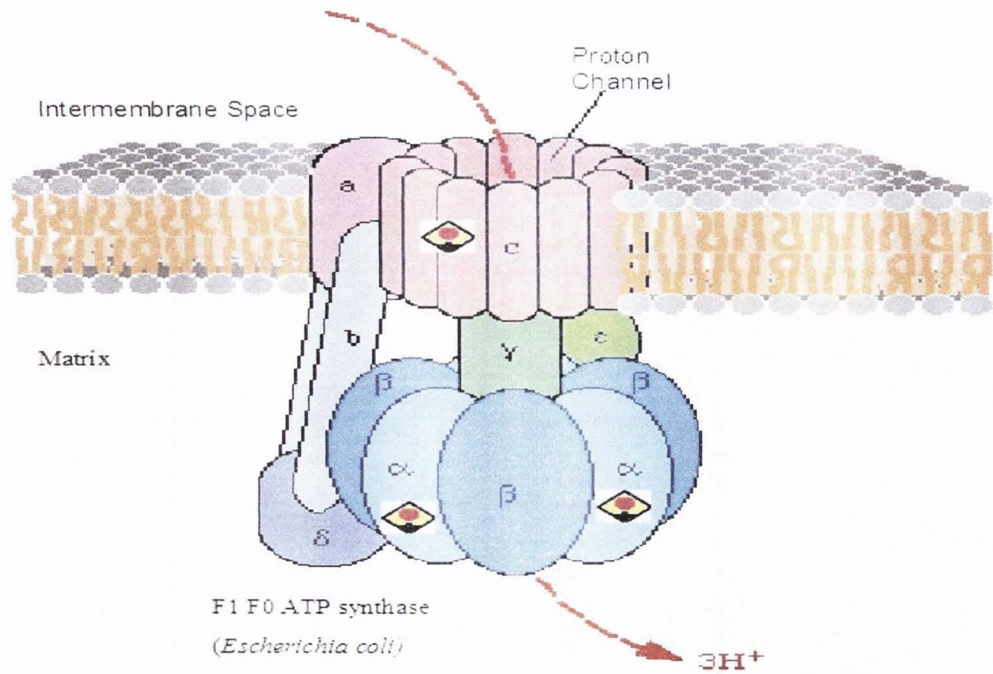
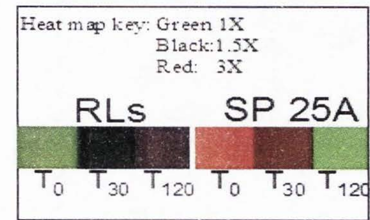


Fig D1. Heatmap of differentially expressed genes in *L monocytogenes* affecting intermediatery metabolism when treated with sub-MIC doses SP 25A and RLs over a period of 120 min.







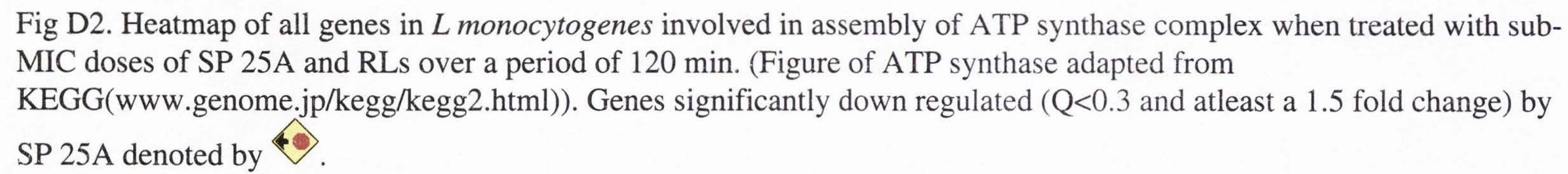


Fig D2. Heatmap of all genes in *L monocytogenes* involved in assembly of ATP synthase complex when treated with sub-MIC doses of SP 25A and RLs over a period of 120 min. (Figure of ATP synthase adapted from KEGG(www.genome.jp/kegg/kegg2.html)). Genes significantly down regulated (Q<0.3 and atleast a 1.5 fold change) by SP 25A denoted by ◆.

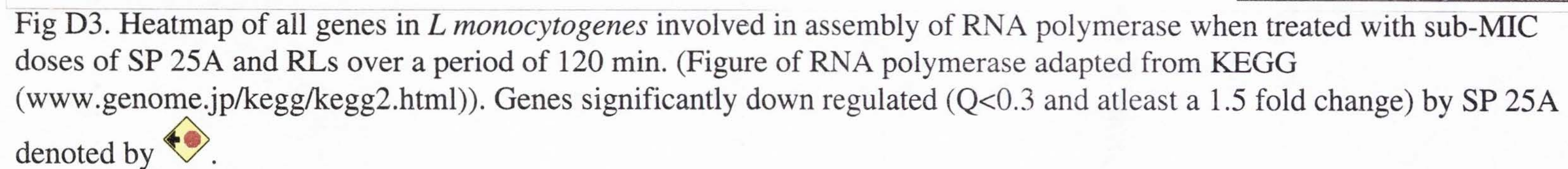


Fig D3. Heatmap of all genes in *L monocytogenes* involved in assembly of RNA polymerase when treated with sub-MIC doses of SP 25A and RLs over a period of 120 min. (Figure of RNA polymerase adapted from KEGG (www.genome.jp/kegg/kegg2.html)). Genes significantly down regulated (Q<0.3 and atleast a 1.5 fold change) by SP 25A denoted by ◈.

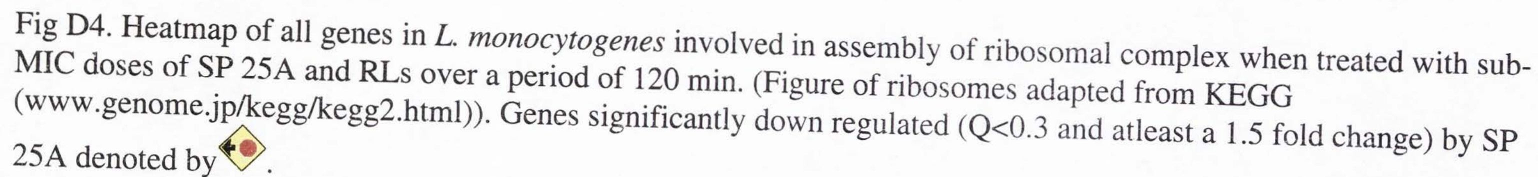


Fig D4. Heatmap of all genes in *L. monocytogenes* involved in assembly of ribosomal complex when treated with sub-MIC doses of SP 25A and RLs over a period of 120 min. (Figure of ribosomes adapted from KEGG (www.genome.jp/kegg/kegg2.html)). Genes significantly down regulated (Q<0.3 and atleast a 1.5 fold change) by SP 25A denoted by ◆.

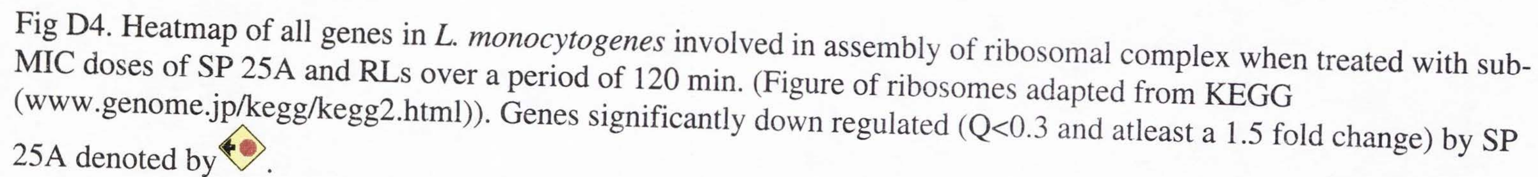


Fig D4. Heatmap of all genes in *L. monocytogenes* involved in assembly of ribosomal complex when treated with sub-MIC doses of SP 25A and RLs over a period of 120 min. (Figure of ribosomes adapted from KEGG (www.genome.jp/kegg/kegg2.html)). Genes significantly down regulated (Q<0.3 and atleast a 1.5 fold change) by SP 25A denoted by ◆.