Characterization of the Bacteriophage Felix O1 Endolysin and Potential Application for *Salmonella* Bioremediation

Lori L. Settle

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F. William Pierson, co-chairman
Nammalwar Sriranganathan, co-chairman
Timothy J. Larson
Dr. Xiang-Jin Meng

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Abstract

There is an increasing incidence of antimicrobial-resistant organisms isolated from food and food products. Coupled with that rising incidence is increased media scrutiny and coverage of outbreaks of foodborne illnesses. Consequently, consumers increasingly demand safer food, and that the antimicrobial measures used be other than antimicrobial drugs. A possible solution is to use bacteriophages, or the purified holin and endolysin proteins that make them lethal and lytic, as antimicrobial food treatments or additives. The bacteriophage Felix O1 is a promising candidate for development as an anti-*Salmonella* food treatment. This dissertation describes the work done to determine if these proteins could be of value as bioremedial agents.

Endolysin treatments of Gram negative bacteria require two agents: the lytic endolysin, and a second agent to permeabilize the outer membrane of the bacterium. The holin protein was proposed as an outer membrane permeabilization agent. Methods used to locate the holin gene included BLAST analysis, analysis of putative Felix O1 proteins for transmembrane domains, and examination of the lysin sequence for an *N*-terminal signal sequence. Analyses did not reveal a promising candidate. Cloning of *rIIA* as a potential holin was attempted without success. Results of various analyses are discussed, as are chemical alternatives to the use of purified holin as a permeabilization agent.

The endolysin, LysO1, was successfully cloned and characterized. PHYRE analysis predicted that the enzyme structure is composed of α helices arranged into two lobes, with the active site in a cleft between them. The enzyme lysed all tested strains of *Salmonella* and a tested strain of the foodborne pathogen *Escherichia coli*. *Campylobacter jejuni* susceptibility remains ambiguous, and the enzyme had no effect on *Listeria monocytogenes* or *Micrococcus luteus*. LysO1 was most active at alkaline pH and low ionic strength; optimal activity was observed in 25 mM buffer at pH 10. If removed from frozen storage, the enzyme was most thermostable at 30 °C. Lytic activity was adversely affected by the presence of the divalent cations calcium, magnesium, and zinc, and by high ionic strength. Considerable time was devoted to development of the activity assay used to further characterize the enzyme, and details of those experiments are provided. Logical extensions of the research project, such as further characterization and testing needed to obtain government approval for widespread use of the treatment, and possible pursuit of treatment based on an enzyme derivative such as an antimicrobial peptide, are discussed.

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ABBREVIATIONS AND ACRONYMS

ΔOD_{600}/min	change in optical density at 600 nm per time in minutes
aa	amino acid
AMP	antimicrobial peptide
ANOVA	analysis of variance
APD2	Antimicrobial Peptide Database 2
ATCC	American Type Culture Collection
bp	base pair
CAMP	Collection of Anti-Microbial Peptides
CDC	Centers for Disease Control and Prevention
CEWL	chicken egg white lysozyme
CHL	chloroform-saturated Tris buffer
CMMID	Center for Molecular Medicine and Infectious Diseases
DA	discriminate analysis
dsDNA	double-stranded DNA
ECM	extracellular matrix
FDA	Food and Drug Administration
FSIS	Food Safety and Inspection Services
GRAS	generally recognized as safe
HEWL	hen egg white lysozyme, see "CEWL"
I	ionic strength
IPTG	isopropyl β-D-1- thiogalactopyranoside
kb	kilobase
LIN	lysis inhibition
LMS	least mean squares
LPS	lipopolysaccharide
MIC	minimum inhibitory concentration
NAG	*N*-acetylglucosamine
NAM	*N*-acetylmuramic acid
NMR	nuclear magnetic resonance
Ni-NTA	nickel-nitrilotriacetic acid
OD	optical density
OM	outer membrane
ON	overnight
PBS	phosphate-buffered saline
PHYRE	Protein Homology/analogy Recognition Engine
RF	random forest
RT	room temperature
RTE	ready to eat
SAR	signal arrest release
SPPS	solid phage protein synthesis
SVM	support vector machine
Tris	tris(hydroxymethyl)aminomethane
TMD	transmembrane domain
TSA	tryptic soy agar
TSB	tryptic soy broth
TSP	trisodium phosphate

Nucleotides

A	adenine
C	cytosine
G	guanine
T	thymine

Amino Acids

Ala	A	alanine
Arg	R	arginine
Asn	N	asparagine
Asp	D	aspartic acid (aspartate)
Cys	C	cysteine
Gln	Q	glutamine
Glu	E	glutamic acid (glutamate)
Gly	G	glycine
His	H	histidine
Ile	I	isoleucine
Leu	L	leucine
Lys	K	lysine
Met	M	methionine
Phe	F	phenylalanine
Pro	P	proline
Ser	S	serine
Thr	T	threonine
Trp	W	tryptophan
Tyr	Y	tyrosine
Val	V	valine

Chapter 1

Characterization of the Bacteriophage Felix O1 Endolysin and Potential Application for *Salmonella* Bioremediation: Overview of the Work Performed

Scope of the Work Performed

The research described here has been inspired by the widespread detection of drug-resistant organisms, as well as the possibility that, despite current anti-*Salmonella* measures taken during processing, such organisms may be found in poultry products at market. Despite those measures, salmonellosis remains a costly problem in the U.S. and around the world. Phages and phage proteins may provide solutions to the increasing problem of drug-resistant bacteria. The drawbacks of using whole phage as a an antimicrobial treatment have been described, but using the proteins that make phage antibacterial may be a viable option. As endolysins can be relatively species specific, use of an endolysin from a *Salmonella*-specific phage for an anti-*Salmonella* application may be a viable option. This research was conducted to test both hypotheses.

Hypotheses

Based on published research and preliminary homology data, I hypothesize that:

1. The Felix O1 endolysin:
    a. is a soluble enzyme with a structure, optimum temperature, and pH range similar to that of the P22 lysozyme.
    b. is lytic for *Salmonella* when applied externally, provided it is used in conjunction with the holin protein or suitable outer membrane permeabilization agent.
2. The Felix O1 holin:
    a. is a type II holin, located upstream of the *lys* gene.
    b. can permeabilize the outer membrane of *Salmonella* when applied externally.

3. The combination treatment of the Felix O1 endolysin and holin will reduce *Salmonella* by a significant amount in an artificially contaminated frankfurter model compared to control treatments.

Specific Aims

In order to test the above hypotheses, the following goals were pursued:

1. Characterize the purified endolysin, including:
    a. cloning, expression, and purification of the Felix O1 endolysin
    b. determination of optimal pH, ion concentration, and temperature for enzyme activity
    c. determination of the effect of divalent cations and sodium on enzyme activity
    d. determination of the effectiveness against other serotypes of *Salmonella* and other bacterial agents of foodborne illness
2. Identify and characterize the holin protein, including:
    a. pblast search of the Felix genome using known holin sequences
    b. cloning, expression, and purification of the holin in a suitable host organism
    c. determination of membrane permeabilization activity
    d. determination of pH, temperature, and ion concentration optimal for protein function
3. Determination of suitability of the holin-endolysin combination as an anti-*Salmonella* food treatment for processed poultry products using a turkey frankfurter model.

The search for the holin gene, and attempts to clone a potential holin, are described in chapter 3. Chapter 4 explains the characterization of the endolysin, LysO1, and the many attempts to develop an activity assay to measure activity of the enzyme are described and discussed in Appendix. I. Chapter 5 discusses future research projects suggested by the results obtained from this research project. Appendix II describes the preliminary results of one of those future projects – using the endolysin sequence as a source of antimicrobial peptides.

Chapter 2

An Endolysin-Based Therapy for the Reduction of *Salmonella* in Poultry Products: A Review of the Literature

1.0 Introduction

Salmonella bacteria are the agents of typhoid fever and foodborne illness in humans, and of significant animal diseases. Members of the genus are motile, facultatively anaerobic, Gram negative rods divided into two species, *S. enterica* and *S. bongori*. *S. enterica* is divided into six subspecies, which are further classified into serovars. Isolates are identified biochemically and subtyped with serotype reagents and typing phages (98). Over 2500 serovars have been identified, but most that cause illness are of *S. enterica* subspecies *enterica* (98).

Salmonella serovars that cause illness are of two types: host-adapted and nonhost-adapted. Host-adapted serovars have a predilection for a particular host, such as *S*. Typhi does for humans (70). Within that host they have the ability to cause systemic illness and may persist in the system for long periods of time. Nonhost-adapted serovars such as *S*. Typhimurium can cause illness in a wider range of hosts, but the illness is typically a less severe localized gastroenteritisis and is seldom fatal (70). Recovery from either condition can result in a carrier state and prolonged, intermittent shedding of the organism (98).

Non-typhoid, non-host-adapted *Salmonella enterica* serovars pose a significant threat to the health of people around the world. In the United States alone, *Salmonella* is the second most common cause of foodborne illness, and the Centers for Disease Control and Prevention estimates 1.4 million cases a year, causing nearly 15,000 hospitalizations and about 400 deaths (110). The annual economic burden of medical costs is estimated to be about 14.6 billion U.S. dollars when quality-of-life losses are included in the model (91). This estimate is much higher than a previous estimate by the World Health Organization (WHO) of $3 billion, and does not include industry or government expenses (125). Infection occurs by ingestion of contaminated food, and though outbreaks have been linked to raw fruits,

vegetables, and nuts, most cases are caused by contaminated animal products, particularly poultry meat and eggs (16). It is estimated that 50% of common-vector outbreaks originate from contaminated poultry products (20).

Officials and consumers are justifiably concerned about the link between salmonellosis and animal products. In 2010, the U.S. Department of Agriculture Food Safety and Inspection Service (FSIS) determined that about 6.7% of sampled whole, raw, broiler carcasses , 18.8% of ground chicken, 10.2% of ground turkey samples, and 4.6% of whole, raw turkeys from randomly selected poultry production sites tested positive for *Salmonella* (Table 2.1) (108). The most common serovars isolated that can also cause illness in humans were *S*. Typhimurium and *S*. Enteritidis, though frequencies varied from study to study. Data supported by the CDC list Enteritidis and Typhimurium as the most common causes of salmonellosis in humans (16).

Table 2.1. Percentage of HACCP samples testing positive for *Salmonella*, 2006 – 2010.

		2006		2007		2008		2009		2010	
Sample Type	**Target**	**n**	**% pos**	**n**	**% pos**	**n**	**% pos**	**n**	**% pos**	**n**	**% pos**
broilers	20	10,206	11.4	9,408	8.5	6,514	7.3	6,439	7.2	6,829	6.7
turkeys	19.6	2,785	7.1	1,744	6.2	129	6.2	1,432	3.8	1,444	4.6
ground chicken	44.6	222	45	506	26.3	411	25.5	374	18.2	426	18.8
ground turkey	49.9	444	20.3	820	17.4	876	15.4	608	10.7	873	10.2
market hogs	8.7	7,242	4	7,308	2.8	4,244	2.6	4,747	2.3	4,224	2.4
cows/bulls	2.7	2.246	0.8	3,969	1.1	2,301	0.5	2,036	0.6	1,764	0.5
steers/heifers	1	3,674	0.3	4,355	0.2	4,965	0.2	4,939	0.2	4,918	0.1
ground beef	7.5	17,849	2	13,695	2.7	16,736	2.4	8,541	1.9	9,256	2.2

Samples were collected for PR/HACCP testing by USDA-FSIS, the % positive shown includes all categories of testing. "Target" refers to the FSIS-mandated baseline percentage of acceptable *Salmonella*-positive samples per set of samples collected from one processing facility at any given testing time. The number of samples from a single plant that test positive for *Salmonella* must be at or below the target value for the plant to pass inspection.

Despite the higher-than-desired prevalence of positive samples, the actual number of viable *Salmonella* per carcass may be quite low. A study of whole broiler carcasses found that 68% of those sampled with a whole carcass wash carried fewer than ten viable salmonellae per carcass and only 2% carried more than 1,100 organisms per bird (25). There is some debate as to what constitutes an infectious

dose of *Salmonella*. Volunteer studies suggest an inoculum of 10^5 or more organisms is necessary, but analyses of actual outbreaks have suggested ingesting as few as ten organisms may be sufficient to cause illness (5, 50, 102). Regardless, the chance of illness increases as the number of ingested organisms increases, and a small number of organisms in a contaminated product can multiply very quickly under optimal conditions, such as mishandling of food during storage or preparation (16).

Risk factors for salmonellosis include increased exposure to the organism through internation travel or owning a pet bird or reptile, GI ailments, or being very young, elderly, pregnant, or immune-compromised (72). An additional, commonly known, risk factor is the consumption of insufficiently cooked or refrigerated poultry or eggs. If the handler is careless, contamination can also spread from raw poultry or eggs to fruits or vegetables during food preparation (16). Many cases of salmonellosis could be avoided if food were prepared and stored using methods that minimize the risk of bacterial growth and cross-contamination (16).

However, the ultimate cause of illness is the contaminated food. It is generally accepted, and data from FSIS sampling confirm, that the most common sources of salmonellosis are contaminated poultry products. *Salmonella* contamination is much more prevalent in poultry and eggs than pork or beef. FSIS reported that in 2010 only 2.4% of hog carcasses, 0.5% of cow/bull carcasses, 0.1% of steer/heifer carcasses, and 2.2% of ground beef tested positive for *Salmonella* (Table 2.1) (108). The stringent antimicrobial measures taken during food processing are not enough to completely eliminate the bacteria present, and those few organisms present may very quickly multiply to an infectious dose if the product is stored incorrectly.

1.1 Risk factors contributing to *Salmonella* contamination of poultry

Salmonella invasion and colonization of the gastrointestinal tract is a complex process. Birds may become infected *in ovo*, at the hatchery, on the farm, or during transport to the processing facility. Initial infection typically occurs in young birds as they are most susceptible; the number of intestinal organisms peaks during the second or third week of life and then declines until slaughter (20). Organisms from

hatchery equipment, bedding, food, or water are ingested and colonize the crop and/or cecum at low levels (20). Infected birds shed bacteria despite being asymptomatic, and the bacteria spread easily from bird to bird through the fecal-oral route, perpetuating the spread of the infection (9).

The proposed reason for the higher incidence of *Salmonella* in poultry is the difference in slaughter practices; only processing of poultry uses a common chill tank, which make cross contamination much more likely than cross-contamination of pork or beef. Several risk factors have been identified both immediately prior to and during slaughter. In addition to previously mentioned routes of infection, live poultry may be infected during transport to the processing facility by dirty crates and by high levels of dust at the plant (40, 93). In both studies, the serovar of contamination after slaughter was identical to that found in dirty transport crates, but not to the serovar identified at the hatchery of origin or on the farm. The order of processing is also a factor; one infected bird can contaminate equipment, and the contaminated equipment can spread the bacteria to uninfected birds processed later (89). Defeathering is a substantial source of contamination because it is difficult to thoroughly clean and disinfect the fingers on the mechanical defeatherer (77). Evisceration is also a contamination risk due to potential intestinal rupture and subsequent spread of intestinal contents during immersion chilling (89). The chill tank is also a factor in the spread of *Salmonella*. Because poultry are cooled in the same water, organisms washed from one infected carcass may cling to others passing through that water (59, 95, 99).

1.2 Current antimicrobial interventions during poultry processing

One of the most effective methods for decreasing the number of organisms per carcass is the countercurrent chiller. Water in the chiller runs opposed to the movement of the processing line, so birds exiting the chiller are rinsed by the cleanest water. The countercurrent is quite effective at reducing the number of organisms per carcass (95). However, organisms rinsed from one carcass may cling to another, as demonstrated by the fact that, though the number of organisms per bird decreases in the chiller, the number of positive carcasses increases (59, 95, 99).

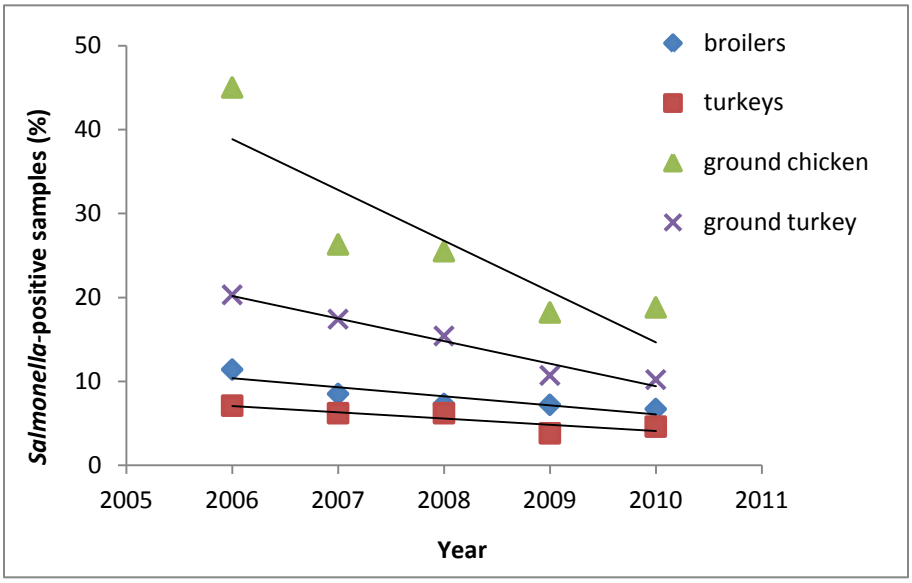
To further reduce the number of viable organisms per carcass, antimicrobial compounds are added to the chill water or the carcasses are sprayed with them during processing. Trisodium phosphate (TSP) has become an industry standard; a 10% solution is used as an antimicrobial spray or rinse (13). Chlorine is a common additive to chill water, but as it is quickly inactivated by organic material, the efficacy of chlorine dioxide and organic acids are also being evaluated (4, 73, 101).

Another antimicrobial measure is the temperature at which the products are processed, packaged, and stored. Although chiller water contains antimicrobial treatments, the main purpose of the chiller is to decrease carcass temperature to the required 4.4 °C. *Salmonella* do not grow at temperatures below 7 °C, and cold temperatures are likewise detrimental to the growth of a number of important foodborne pathogens and spoilage organisms, such as *E. coli* and *Campylobacter* species (96-98). The products are kept at that temperature (or colder if sold frozen) during packaging, shipment, and retail.

1.3 Federal oversight

Due to consistently high levels of bacterial contamination in raw and ready-to-eat (RTE) meat products, the USDA Food Safety and Inspection Services (FSIS) adopted the Pathogen Reduction; Hazard Analysis and Critical Control Point (HACCP) Systems in 1996; implementation began in 1997 (107). The Final Rule established baseline standards for certain foodborne pathogens in different types of raw and RTE meat products, and processing plants must meet or exceed requirements to pass inspection (107). Performance standards have been reevaluated and lowered every year (109). Although there have been setbacks, the number of samples testing positive for *Salmonella* has trended downward since HACCP was implemented (109) (Figure 2.1). There have also been some adjustments to the program: Young Chicken and Young Turkey standards were established in 2006 (106).

Figure 2.1. Five-year trend of HACCP data of the number of poultry samples testing positive for *Salmonella*, 2006-2010.



Percentage of samples of broilers, turkeys, ground chicken, and ground turkey testing positive for *Salmonella* from 2006 to 2010. Data are from the USDA-FSIS Progress Report on *Salmonella* Testing of Raw Meat and Poultry Products, 1998-2010, and were graphed using Microsoft Excel.

In addition, due to a slow and uncoordinated response to recent widely publicized outbreaks of foodborne illness, President Barack Obama created the Food Safety Working Group (FSWG) in March of 2009. The purpose of the group was to "coordinate federal efforts and develop short- and long-term agendas to make food safer" (30, 106). Their initial report outlined a set of three principles to develop a modern, coordinated system of food safety regulations and includes suggestions for implementing those principles, though details have not yet been finalized (30). The Food Safety Modernization Act (FSMA) was signed into law on Jan. 4, 2011. The act aims to prevent outbreaks of foodborne illness. It gives the FDA the ability to initiate recalls of foods suspected to be unsafe, and more powers of oversight and greater responsibility in food inspections (113). It also expands HACCP to include all types of food.

2.0 Bacteriophage as an Intervention Strategy

It has been estimated that most contamination found in raw poultry products occurs during slaughter by cross-contamination from a small number of colonized birds. Consequently, extremely

stringent anti-*Salmonella* measures are taken during and directly after poultry processing, and have been recently discussed in excellent reviews (24, 123).

A method long researched and recently approved is the addition of bacteriophages to ready-to-eat (RTE) lunchmeats. Bacteriophages are viruses that infect bacteria. Credit for discovery is jointly given to Frederick W. Twort in 1915 and Felix d'Herelle in 1917 (21, 100). d'Herelle was the first to see the therapeutic potential of bacteriophages, and used them to treat both human and animal patients (100). In the 1920s and 30s, d'Herelle was at the forefront of the therapeutic phage research being conducted in Europe and the United States. With the discovery of penicillin, the U.S. and Western Europe concentrated on antibiotic research. However, headed by the Tbilisi Institute of Phage Research in Georgia, Russia and Eastern Europe continued to fund new phage studies (100). Due to the increasing frequency of isolation of multidrug-resistant strains of pathogenic bacteria, some scientists regard bacteriophages as a potential answer to problems such as multidrug-resistant *Staphylococcus aureus* (MRSA) and *Salmonella* Typhimurium DT104 (39).

There are two types of phage infection. Lytic phages, also called virulent phages, lyse the host soon after infection. In a lysogenic infection, the phage genome is incorporated into the host genome and the prophage is then replicated along with the host genome. If the bacterium encounters adverse conditions prophage gene expression is triggered, which leads to phage replication and host cell lysis (81). Three pathways to lysis have been identified to date. The most common and well-characterized pathway requires two proteins: holin protein subunits in the cell membrane oligomerize to create large holes; lysin enzymes in the cytoplasm diffuse through those holes, break down peptidoglycan, and release any progeny phage (126). In this pathway lysin is necessary for dissemination of progeny phage but is not lethal to the host.

2.1 Phage therapy

The therapeutic possibilities of bacteriophages were seen soon after discovery and today, many researchers have proposed phages as an answer to the increasing number of multidrug-resistant infections

reported every year. However, phages are large enough for the immune system to recognize and remove them from systemic circulation, so systemic use is impractical (41). Possibilities explored thus far include treatment for gastric ailments, topical applications such as phage-impregnated bandages for burn victims or ointment for MRSA skin infections, and as antibacterial food additives (88). The treatments are quite safe; phages occur in nature wherever their hosts are found, but as they can not infect eukaryotic cells, the phages on our skin and in our food do not harm us.

In 2006 LMP-102 (now ListShield™, produced by Intralytix), a cocktail of six *Listeria*-specific phages, was approved by the FDA for addition to lunchmeats (6, 48). Advantages of using whole phage include magnification of the initial dose of phage and lack of effect on appearance, taste, or smell of the meat. The effects of phage on normal flora after ingestion are minimal, even when flora would be otherwise susceptible to the phage (19, 52, 58, 62). A disadvantage is that bacteria can become resistant to phage infection, so a clearly defined cocktail of several phages must be used. Since phage can be fairly host-specific, an individual cocktail would be needed for each organism targeted (6).

Despite the promise in phage therapy, some problems with development and implementation remain. Many of the most potent bacterial toxins are actually derived from phage proteins (8). Consequently, potentially therapeutic phages are never lysogenic and are screened carefully for any hint that a protein can be incorporated by the intended target organism as a virulence factor. Still, some detractors believe the precautions are not sufficiently rigorous (88). Another drawback is that bacteria can develop resistance to a specific phage, so a cocktail must be used. The anti-*Listeria* cocktail includes six distinct phages, all carefully characterized (6). A cocktail must be assembled from individual components and the process is labor-intensive; the phages must be propagated individually and mixed in the correct proportion. These drawbacks are a few of the reasons some phage therapy advocates are researching the lethal proteins phage genomes encode.

2.2 Government regulations

The FDA regulates all antimicrobial used in or on processed food, raw agricultural commodities, food processing water, and food-contact substances (111). If the food additive in question is also an enzyme, it would be subject FDA regulations regarding enzyme production and use. The information necessary to evaluate a new additive includes the identity of the additive, the manufacturing process, specifications for identity and purity, intended technical effect and use, and the intake estimation (112). For example, catalase used to eliminate hydrogen peroxide during cheese production must be derived from *M. lysodeikticus* that has been demonstrated to be a pure and nonpathogenic culture. The bacteria must be removed from the catalase prior to use of the catalase, and the catalase must be used at a concentration that will be effective (1).

3.0 Phage Holin Proteins

The holin is an integral membrane protein that serves two functions. As mentioned above, it forms large pores in the cell membrane that allow diffusion of endolysin to the periplasm. Intrinsic in that function is another – the holin controls lysis timing, so it determines the length of the phage replication cycle (17, 126). The group of proteins generically identified as 'holins' currently includes over 250 proteins in nearly fifty protein families (126). That diversity, along with lack of sequence identity among different holin families, suggests families within the group evolved from different origins to perform a similar function (120). Despite the heterogeneity, holins of the dual-component lysis systems, i.e., phage lysis systems using both a holin and an endolysin, have the same purpose and, it is thought, are triggered by similar mechanisms (126).

3.1 Structure

Three types of holin subunits have been identified. Type I holins are 90 to 125 amino acids (aa) in length and have three transmembrane domains (TMDs) (32). The model type I holin, phage λ S, is arranged with the *N*-terminus in the periplasm and the *C*-terminus in the cytoplasm (*N*-out, *C*-in

topology). Type II holins average 65 to 85 amino acids in length and have two TMDs. The model type II, phage 21 S, is arranged with both termini in the cytoplasm (*N*-in, *C*-in topology). The T4 holin, T, is the model and until recently the only known type III holin (120). T is arranged *N*-in, *C*-out, and has one TMD and a large, charged, periplasmic *C*-terminus (84). At 218 amino acids in length T is also significantly larger than other known holins. Despite variation in conformation, all holins thus far identified are integral membrane proteins with TMDs composed mainly of α helices. *C*-termini, whether periplasmic or cytoplasmic, are rich in basic residues and may act as regulatory domains (85).

3.2 Expression

During phage λ replication, lysin and holin proteins are translated from a single polycistronic mRNA. During T4 replication, lysin and holin are translated from separate polycistronic mRNAs. In both systems, expression of the holin and endolysin commences about eight minutes after infection and continues until host cell death (117). Holin subunits accumulate as randomly in the host cell membrane as dimers, but have no effect on the membrane until hole formation and host cell death (124). At an unknown signal, the dimers dimerize and the resulting tetramers oligomerize into large rings (90). In studies of the λ holin, it has been determined that each hole is comprised of 72 subunits that form a ring approximately 4 nm high, with an 8.5-nm inner diameter and an outer diameter of 23 nm, the largest such opening of its type yet characterized. The ring borders a large, nonspecific hole in the cell membrane (90). Due to the large size and lack of specificity of the multiple openings in the membrane, the cell cannot maintain the membrane potential necessary for ATP production, and dies.

3.3 Determination of Lysis Timing

The timing mechanism and trigger for holin oligomerization and cell death remain unknown (90). Studies using energy poisons have determined that a 40% reduction of the proton motive force – about 80mV – triggers cell lysis at the end of a replication cycle of phages that use a holin-endolysin system (34). Small changes to the amino acid sequence of a holin can have drastic effects on timing; the λ S105

mutant A52G lyses too early for successful phage reproduction. Conversely, the mutant A52V does not lyse (32). Similar changes have been traced to mutations of Ala48 and Ala55, which are on the same side of the α helix TM domain as Ala52 when the protein is correctly folded. In fact, most "clock" mutants of λ S – those mutants that affect lysis timing – map to the first or second TMD or to the connecting loop between them (83). Altered lysis timing is thought to be due to a change in the size of the side chain (120). Mutational studies with T4 T have determined that point mutations conveying delayed acute lysis occur in two main clusters: one near the center of the peptide, and a second at the extreme *C*-terminal end of the protein (85). Point mutations causing early lysis of T4 hosts often map to mutations in T4 *r* genes that control lysis inhibition, not to the holin protein itself(120). Thus, despite different morphology and lysis time, point mutations in any class of holin proteins are capable of significantly altering lysis timing.

There is some debate as to the length of replication cycle that maximizes production of viable progeny phage. Optimal lysis time is defined as that time when 'instantaneous rate of progeny production equals long-term rate of progeny production" (119). Using a mathematical model, Wang et al. determined that the important factors for a given phage and bacterial host are the density and quality of available hosts, and that host density is more important than host quality when selecting for a shorter lysis time (119). Experimentation with S mutants of phage λ, phages carrying mutations in the holin gene, indicated that optimal phage fitness is achieved with an intermediate, as opposed to long or short, lysis time, but the authors also admitted that the exact optimal lysis time involves numerous variables and so would be difficult to pinpoint (117). The implications are that one may select for a long- or short-lysis phage variant if host quantity and quality are adequately controlled (85).

Until recently, the accepted model of holin expression was that late in infection, holin subunits accumulated in lipid rafts in the cell membrane in such density that the lipid was effectively squeezed out of the mat of holin dimers (118). The close packing was thought to be possible because of associations between transmembrane domains of the dimerized subunits. As the number of dimers increased and lipid was eliminated from the cluster of proteins, the dominant interactions in the membrane changed from protein-lipid to protein-protein interactions, which are slightly repellent (118). The model speculated that

at some point, either through critical density or by random thermodynamic fluctuation, a portion of the raft formed a small hole, which caused localized depolarization of the cell membrane. That depolarization caused the entire mat of dimerized subunits to submit to the repellent forces between the individual subunits, which triggered conformational change, oligomerization, and the formation of more holes (126). It had been speculated that the conformational change was due to tertiary rearrangement of the α helices of the holin TMDs and quaternary rearrangement of holin subunits, rather than any change of secondary protein structures (118). An advantage of the model was that it allowed for the sequential diversity of the holin proteins. Holin function in that model relied on secondary protein structure. If the protein contained closely packed transmembrane domains composed of α helices then the model was functional, and the same secondary structure may result from a wide variety of primary protein sequences. Specific conserved amino acids were not necessary to explain holin function. (118).

However, new information was recently published regarding holin placement in a membrane after expression. The study used an S^{105}-GFP chimera to track the location of expressed holin in the cell membrane prior to lysis, and determined that holin subunits accumulate randomly in the host cell membrane, not in aggregates in lipid rafts as the model above described (124). In light of the new information, it is unknown how much of the model is accurate.

3.4 Control of Holin Function

As holins are the determinants of lysis timing and thus of how many progeny phage are produced, function is tightly controlled. One control mechanism described above is the critical limit of holin subunits. Some bacteriophages also carry genes for antiholins – proteins that bind holin subunits and prevent hole formation until a specific lysis time (35). At that time, antiholins then act as additional holin subunits helping form membrane holes. Different phages have different strategies for producing antiholins. Common in the lambdoid phages is the dual start motif of the holin gene. In phage λ, the first three codons of gene *s* are for the amino acid sequence Met-Lys-Met (MKM). Antiholin S^{107} results from translation initiation at the first start condon, initiation at codon three results in S^{105} (33). S^{105} and S^{107} are

produced in a 1:2 ratio, determined by an mRNA stem loop structure overlapping the translation initiation region (35). S^{107} and S^{105} form heterodimers in the host cell membrane, and the more common S^{107} inhibits S^{105} function if cell membrane potential is intact.

The function of the lysine reside at position 2 of S^{107} is unknown. If membrane potential decreases or the basic lysine is replaced with an uncharged or acidic residue, S^{107} becomes a lethal holin subunit and participates in hole formation (35).Conversely, if additional positively-charged or basic resides are added to the S^{107}*N*-terminus between the two start codons, lysis is delayed (35). It is also known that for lysis to occur, the *N*-termini of both S^{107} and S^{105} must be periplasmic (31). It is currently suspected that Lys2 of S^{107}, which has a positive charge at biological pH, prevents translocation of the *N*-terminus by interacting with the inner surface of the cell membrane. The bulk of the *N*-terminal domain lies horizontally in the inner membrane space, and the bulk prevents close association between dimers of S subunits. Local depolarization permits the *N*-terminus of the positive lysine residue to traverse the hydrophobic inner membrane space, which is thought to cause a conformational change in the protein subunits, allow closer interactions between dimers, and result in hole formation (31).

Antiholins of other phages are active only under specific circumstances. The T4 antiholin RI is a 95-amino acid protein active only during lysis inhibition (LIN), which follows superinfection of the host with another phage (84, 103). Like S^{107}binding to S^{105}, RI binds to T and inhibits its function. Given the near uniformity of S^{107} and S^{105}, and the penchant of S^{105} to form homodimers, it is unsurprising that S^{105} also dimerizes with S^{107}. However, unlike S^{107} and S^{105}, RI has no sequence similarity to T (103). Mutational analysis has determined that if superinfection occurs, the RI produced during primary infection interacts with the periplasmic *C*-terminal domain of T to initiate LIN (103).

The current model of T4 lysis inhibition proposes that RI possesses an *N*-terminal signal anchor release (SAR) domain that initially anchors it to the periplasmic surface of the cell membrane (104). RI is not a stable protein, and after release to the periplasm it either quickly binds to the periplasmic domain of T or is inactivated and degraded. Studies suggest RI binding to T sends a signal that stabilizes the pool of

periplasmic RI proteins. Thus, if superinfection does not occur, antiholin RI is quickly degraded and there is no lysis inhibition (104).

4.0 Endolysins

Most of the phages characterized to date use a two-protein lysis system; the lethal holin protein and the the progeny-releasing endolysin, or "lysin". Both proteins are expressed late in the phage infection cycle (117). Most endolysins lack a signal sequence and remain in the cytoplasm; holin subunits accumulate randomly in the cell membrane (34). At an as yet undetermined signal, the subunits oligomerize to form large, non-specific holes in the cell membrane, killing the cell (90). The lysin diffuses through the holes to the periplasm where it degrades peptidoglycan, which lyses the cell and releases any progeny phage.

Phage endolysins are members of the lysozyme-like superfamily of enzymes; similar proteins are found in such diverse places as egg white and human tears (12). Most are globular proteins with two domains. Endolysins can be relatively specific to broad-spectrum; a lysin lethal to one genus or species of bacteria may have no effect on another (12).

4.1 Classification of lysozymes

A lysozyme, by definition, cleaves the β-glycosidic bond between *C*-1 of *N*-acetylmuramic acid (NAM) and *C*-4 of *N*-acetylglucosamine (NAG) of bacterial peptidoglycan (49).The term "lysozyme" should be used to refer only to true lysozymes. Because not all phage endolysins are lysozymes, the term "endolysin" refers to any phage enzyme that has muramidase activity; any enzyme encoded by the phage genome that acts from within the host cell (with or without the presence of a holin) to degrade peptidoglycan and release progenyphage after replication is an endolysin. That group includes endopeptidases and *N*-acetylmuramoyl-L-alanine amidases as well as lysozymes.

Most phage endolysins, also called lysins, muramidases, and murein hydrolases, are members of an enzyme superfamily of lysozyme-like proteins, though there is some disagreement as to how the

superfamily is divided. One classification scheme, based on sequence similarity, divides the superfamily into six types: chicken-type (C), goose-type (G), virus-type (V), chalaropsis-type (CH), invertebrate-type (I), and λ-type lysozymes (27, 28). Although there is no statistically significant sequence similarity between the different types, four (C, G, V, and I) share a similar structure, prompting some to suggest that all lysozymes evolved from a common ancestral protein (27).

Most phage lysins are classified as V-type, but some have been identified as G- or CH-type, and λ-type lysozymes are grouped separately due to their transglycosylase activity. Endolysins are instead commonly classified according to the type of peptidoglycan bond they cleave and the products that are formed, rather than by which type within the enzyme superfamily they are most similar to. *N*-acetylglucosaminadases such as T4 E cleave the β-1,4 glycosidic bond between the NAM and NAG subunits of bacterial peptidoglycan. Transglycosylases such as λ R cleave the same β-1,4 bond but form a cyclic product (27). As such, those two groups are the only true lysozymes among the phage endolysins. Amidases such as the T7 lysozyme cleave the bond between NAM and L-alanine, and endopeptidases cleave peptide bonds in the peptidoglycan cross-links of Gram positive bacteria (18).

4.2 Structure

Initial structural studies of phage endolysins revealed two types of proteins, those with binding domains and those without. Lysins such as Cpl-1, from phages that infect Gram positive hosts, have two (or more) separate domains and each domain has a distinct function. The *C*-terminal domain recognizes a specific structure of peptidoglycan, the *N*-terminal domain cleaves the bond at that site (7). The lysins of phages that infect Gram negative hosts have a slightly different structure.

Endolysins of phages of Gram negative hosts include lysins of phages T4, λ, T7, and P22. T4 E has become the model enzyme, as it is the most often studied of the group and was the first to have its structure elucidated; other enzymes have been classified as T4-like as their similar structures were identified.T4 E is a globular protein with two lobes. The enzyme is roughly divided into *N*-terminal and *C*-terminal lobes of a single domain, with the active site found in the cleft between the lobes (71). The

lobes are joined by a long α helix .The *C*-terminal lobe consists of only short α-helices that form a rough cylinder with a hydrophobic core. The *N*-terminal lobe contains the only β sheet of the enzyme, consisting of three antiparallel β strands (71).

A residue necessary for enzyme activity is identified as such if replacement of that amino acid decreases or eliminates enzyme activity. For lysozymes, such residues include Glu 11, Asp 20, Glu 22, Glu 105, Trp 138, Asn 140, and Glu 141 (105). When the enzyme is properly folded, all residues critical for enzyme activity are located within the active site cleft. Glu 11 is of particular importance, as a glutamic acid residue at that general position is conserved in most V-type lysozymes (28). Access to the active site is restricted by the side chains of Thr 21-Glu 22 and Glu 141-Thr 142. Although the protein backbone is 8 Å wide at the active site, the side chains of the above amino acids restrict the opening to 3-5 Å (71). The hollow is filled and stabilized by a water molecule, which is also instrumental in catalytic activity.

The structures of endolysins of Gram positive- and Gram negative-specific phages were first believed to be very similar. However, most endolysins of Gram positive-specific phage have two separate domains, each with a distinct function. If separated, each domain may or may not retain its binding or catalytic function, and separation in some cases actually enhances enzymatic activity (41, 65). This arrangement is quite different from that of Gram negative-specific endolysins, as many of them lack the peptidoglycan recognition domain.

Proposed reasons for the observed structural differences are related to the morphological differences in Gram positive and Gram negative bacteria. The peptidoglycan of Gram positive bacteria is much thicker than that of Gram negatives and degradation requires more enzyme activity. It has been suggested that the peptidoglycan-binding module provides solid anchoring for the catalytic domain and thus increases enzyme activity (7). A second benefit is that Gram positive bacteria do not have an outer membrane. It has been observed that endolysins after host cell lysis are released to the environment and lyse nearby bacteria, which deprives newly released progeny phage of potential hosts (64). The strong

binding domain may bind the enzyme to the substrate and reduce enzymatic activity after host cell lysis (7).

4.3 Mechanism of cleavage

N-acetylglucosaminidases are the type of endolysin most commonly identified. They function via a general-acid-base mechanism; water is added across the β-1,4 glycosidic bond between NAM and NAG and individual NAM and NAG subunits are produced (55). Important residues of the *N*-terminal domain include a conserved glutamic acid residue and, in some cases, an aspartic acid and/or a threonine residue, which are necessary for catalysis (54). The proton-donating acid in the mechanism is usually a water molecule (54).The mechanism of lytic transglycosylases differs slightly: λ R uses the hydroxyl group of the C6 of the substrate as the nucleophile instead of a water molecule, which results in a cyclic product (27).

Catalytically important restudies of the *N*-terminal domain of the model T4 E *N*-acetylglucosaminidase include the conserved glutamic acid at position 11 and the conserved aspartic acid at position 20. The threonine residue at position 26 has also been demonstrated to be necessary for function (55). The mechanism has not been completely elucidated. It is known that a water molecule bonded to Asp 20 and Thr 26 acts as a general acid and attacks the C1 carbon of NAM. Glu 11, on the other side of the active site, acts as a general base (54). The net result of the *N*-acetylglucosaminidase action is cleavage of the β-1,4 glycosidic linkage, though other types of endolysins cleave different bonds. Endolysins are efficient – complete lysis of a culture may be observed in minutes after addition of the enzyme.

4.4 Non-enzymatic antimicrobial activity

While most studies of lysozyme antimicrobial activity have focused on the muralytic properties of the enzyme, a growing body of research suggests that lysozyme antibacterial activity may be due to more than enzyme activity. Hen egg white lysozyme (HEWL, also called chicken egg white lysozyme, or

CEWL) denatured by dithiothreitol demonstrated decreased enzymatic activity, but its antimicrobial activity against *S. sanguinis* was equal to that of the native-state lysozyme (56). The effect may have been incredibly specific – the same study found that the denatured lysozyme was not as effective against *S. faecalis*. A series of studies of heat-denatured HEWL determined that the enzyme denatured at 80 °C and pH 6 for 20 min (HLz80/6) was equally bactericidal as native enzyme, but its activity extended to some Gram negative bacteria (42-44). Heat-treated T4 lysozyme produced similar results (26).

It has been suggested that some of the observed bactericidal effect could be due to residual enzyme activity. The enzymes in several studies were only partially denatured and some retained up to 50% of enzyme activity. However, further research has questioned if enzymatic activity is at all necessary for lethal activity. Studies of site-directed mutagenesis that replaced the HEWL catalytically necessary Asp 52 with serine, or the T4 lys Asp 20 with Asn, were catalytically inactive, but their antibacterial activity was equal to that of functioning enzyme; HEWL on tested strains of *S. aureus* and *B. subtilis*, T4 Lys on *M. luteus* (formerly *lysodeikticus*) (26, 44, 68). Some researchers have questioned if use of the entire enzyme sequence is necessary: when HEWL was digested with clostrapain, a 15-aa peptide displayed higher antimicrobial activity against tested Gram positive organisms than the intact enzyme (82). When digested with pepsin, certain fragments inhibited growth of *E. coli* K12 and *S. aureus* (74). It may even be possible to construct such fragments *in vitro* – a constructed model of the α4 helix of the *C*-terminal region of the T4 lysozyme had increased bactericidal activity compared to native T4 lysozyme against tested *E. coli* (26).

Observations have revealed that denatured or fragmented lysozyme will kill bacteria, but cell death is not always accompanied by lysis. Laible and Germaine suggested that electrostatic interactions between the charged, denatured enzyme and the teichoic and lipoteichoic acids of the cell wall activate the cell's autolytic pathway (56). Ibrahim et al. agreed that the autolytic pathway is the cause of observed lysis of *B. subtilis*, which, like oral *Streptococcus*, is not terribly susceptible to lysozyme (45). However, they suggest the cause is actually a structural motif, rather than the cationic nature of HEWL. A second proposed mechanism suggests the non-lytic results could be due to membrane disruption by electrostatic

interactions among parts of the cell wall (26, 42-45, 74). Activity of denatured HEWL decreases if divalent cations are added to the reaction (44). It is postulated that the cations stabilize the negatively charged outer membrane.

However, both studies have been questioned. A trial that treated six Gram negative species with denatured HEWL or with a combination of HEWL and high hydrostatic pressure reported the HEWL had no antimicrobial effect at atmospheric pressure and suggested the earlier reported phenomenon may be incredibly strain-specific (69). In a further trial, peptides derived from T4 lysozyme and HEWL had no effect on tested bacteria samples at atmospheric pressure, though both displayed some antimicrobial activity under high-pressure conditions.

4.5 Antibacterial uses of endolysins

Usually, phage-mediated lysis is triggered by enzyme activity inside the cell. However, there are two mechanisms by which lysis can be triggered externally. Delbruck first observed "lysis from without" in bacteria suspended in a liquid culture medium that had been infected with a very high number of phage; there was a gradual clearing of the culture indicating lysis of the bacteria, coupled with a decrease in phage concentration. He suspected that an overabundance of phage binding to a single bacterium caused lysis of the bacterium without productive infection by and replication of the phage (23). Since his publication, it has been discovered that the distal tip of the protein complex that is the phage genome injection mechanism, which penetrates the cell wall to insert phage DNA into the host cytoplasm, has lytic activity similar to lysin proteins. An overabundance of phage binding to a single host and injecting their genomes degrades peptidoglycan more quickly than the host can replace it, and lyses the cell (3).

The endolysin active site recognizes both the inner and outer surfaces of the peptidoglycan substrate, and a second and potential therapeutically useful trigger of lysis from without is to apply purified endolysin to the surface of susceptible bacteria. Trials *in vitro* and *in vivo* have demonstrated endolysin efficacy against species of *Staphylococcus*, *Streptococcus, Bacillus*, and *Clostridia.* A minute amount of the purified endolysin Cpl-1, encoded by the pneumococcal-specific phage Cp-1, effected a 6

log reduction of a 10^7 CFU/ml culture of *Streptococcus pyogenes* seconds after application (78). A 100-µg/ml addition of the endolysin PlyG from the *Bacillus anthracis*-specific gamma-phage effected a 3 log reduction of 10^7 CFU/ml cultures of various *B. anthracis* strains, and was also effective against strains lacking a capsule or toxin-associated plasmids (92).

Although the lysozyme is large enough to stimulate an immune response, systemic therapy with phage lysins may be possible. Intravenous administrations of 2 mg of the Cpl-1 lysozyme reduced an artificially induced pneumococcal bacteremia from 10^4 CFU/ml to fewer than 10^2 CFU/ml in fifteen minutes and 100% survival after 48 hr, compared to 20% survival of the control group (63). Results indicated that antibodies slowed lysozyme activity, but did not decrease the overall effectiveness of the treatment (63). Corroborating results were found in mice infected with multi-drug resistant *S. aureus* using a phiMR11 endolysin (86). The enzymes have short half-lives; estimates of the half-life of the T4 lysozyme range from two to ten hours in eukaryotic cells, that of lysostaphin is less than one hour, and there has been some discussion of modification to extend the effective period of the treatment (29, 46, 47, 116).

In addition to the promising results from studies of infectious diseases and studies of whole phage as foodborne pathogen control mechanisms, there is a growing body of literature exploring the antimicrobial effects of endolysin alone as a food additive. One study reported that LysH5 from phage φH5 could reduce the number of actively multiplying *S. aureus* in a highly contaminated milk sample (10^7 CFU/ml), and reduce it to undetectable levels in a slightly contaminated sample (10^3 CFU/ml), in only four hours (80). A combination of bacteriophage λ lysozyme and high-pressure treatment was successful at reducing numbers of *Escherichia coli*, *Shigella flexneri*, *Yersinia enterocolitica*, or *Salmonella* Typhimurium in artificially contaminated milk and banana juice (75).

One potential advantage of endolysin therapy is the potential lack of pathogen ability to become resistant to the enzyme as they are becoming resistant to antibiotics. Resistance studies of different strains of *B. anthracis* repeatedly exposed to low levels of PlyG found no spontaneously resistant organisms after ten passages in liquid or forty passages on agar (92). The authors of the study speculated that the lack of

resistance is due to evolutionary pressure. Endolysins adapted over millions of years to release progeny phage from a peptidoglycan cage. As such, it would be most advantageous to use a receptor that is a necessary to peptidoglycan and thus always present (29). However, development of resistance when exposed to higher concentrations of the enzyme remains to be determined.

Another advantage of endolysin therapy for active infections is specificity. As discussed earlier, some endolysins are only effective against the host organism of the phage they originate from, so therapy using proteins with that specificity would have little to no effect on normal flora that consists of unrelated organisms (29). However, as a food application, specificity of the application for a particular genus or species could be a detriment; as with whole phage additives, one would need an individual treatment for each organism one wished to eliminate from food.

There is also one limitation to endolysin-only therapy. Preliminary studies of function indicate that in the absence of holin or other membrane-permeabilizing agents, purified endolysins are lytic only for Gram positive cells. The enzymes are unable to access peptidoglycan of Gram negative target bacteria because it is protected by the outer membrane. Endolysins are too large to pass through nonspecific outer membrane transport proteins (29). As many of the most important foodborne bacterial pathogens – *Salmonella, E. coli,* and *Campylobacter* – are Gram negative organisms, the inability to target Gram negative organisms influences the use of endolysins alone as a food safety application.

4.6 Membrane permeabilization

It has been demonstrated that external application of purified phage endolysin can be lethal to Gram positive organisms. The enzymes are also effective against Gram negative bacteria if the endolysin is combined with some manner of permeabilization of the outer membrane of the target bacteria. Any permeabilizing agent used must be safe for human consumption and have no adverse organoleptic effects on the product. Proposed candidates include the Felix O1 holin protein, trisodium phosphate (TSP), organic acids, polyethyleneimine (PEI), or a combination of high-concentration Tris buffer and EDTA.

The membrane-permeabilization properties of externally applied purified holin proteins have been demonstrated (94). Separate studies pairing holins with endolysins from different phages have indicated that there are no interactions between the two proteins during bacteria lysis; any holin could potentially serve as the second protein in the two-protein antibacterial system (118).

Trisodium phosphate is currently used by the poultry industry as an antimicrobial treatment (22). Although the exact mechanism of antimicrobial activity is unknown, it is speculated that a combination of detergent activity and increased pH disrupts bacterial cell membranes (13). TSP has also been demonstrated to improve bacterial susceptibility to lysozymes and nisin, presumably due to its membrane-disrupting activity (14). It is also classified as Generally Regarded as Safe for human consumption by the FDA (31).

Organic acids such as lactic acid have long been used as preservatives and flavoring agents (87). It has recently been determined that organic acids may also act as outer membrane permeabilization agents. Lactic acid increases cell uptake of the hydrophobic probe NPN and increases cell susceptibility to detergents and lysozyme (2). It has been hypothesized that at low pH, undissociated lactic acid protonates anionic compounds of the outer membrane, which weakens the interactions between the various membrane components (2).

PEI is a polycationic polymer that has also been demonstrated to permeabilize the outer membranes of Gram negative bacteria (37). Thin-section electron microscopy of *Salmonella* treated with PEI revealed vesicular structures on the surface of the bacteria but no membrane fragments (38). Further research determined that the solubility of rough LPS is strongly affected, but the effect on a mutant, more cationic form of LPS was only weakly affected. That weaker effect suggests that the electrostatic interactions between the positively charged PEI and negatively charged LPS are important factors in the interaction (38).

At high concentrations, Tris (tris(hydroxymethyl)aminomethane) is a membrane permeabilizer and removes lipopolysaccharide (LPS) from the cell wall. Tris at 100 mM [pH 7.2] released 20% of the LPS of *S*. Typhimurium, and *Pseudomonas aeruginosa* treated with 200 mM Tris [pH 7.4] became

susceptible to lysozyme (36, 114). Tris is thought to bind to LPS and replace divalent calcium and magnesium ions, which decreases the interactions among LPS components and destabilizes the outer membrane (79). EDTA is a chelating agent; like Tris, it is thought to remove divalent cations from the outer membrane, and it works particularly well in Tris buffer, though not in HEPES buffer or nutrient broth (114). Although not certified as GRAS, it is currently used in such food products as salad dressing, margarine, sandwich spreads, mayonnaise, processed fruits and vegetables, canned shellfish, and soft drinks, and the FDA has flagged it for further study pending certification (15).

4.7 Bacteriophage Felix O1

All Gram negative bacteria are resistant to externally applied lysozymes due to the presence of the outer membrane, which necessitates pretreatment with a permeabilizing agent if the added enzyme is to be effective. Several recent studies suggest *Salmonella* are more resistant to lysozyme activity than other Gram negatives (10, 11, 76). A possible solution is to use an endolysin from a *Salmonella*-specific bacteriophage, such as P22 or Felix O1. The advantage is that such an endolysin has been evolutionarily selected through millennia to function despite the anti-endolysin defenses of the host bacterium.

Bacteriophage Felix O1, also called 01, 0-1, and 0-I, is an excellent candidate due to its specificity for nearly every serovar of the genus *Salmonella* and lack of non-*Salmonella* hosts. The specificity for salmonellae is due to the binding affinity the tail fibers possess for the terminal *N*-acetylglucosamine residue of the core antigen of *Salmonella* LPS, which nearly all serovars possess (60, 61). *Salmonella* resistance to Felix O1 has been traced to mutation in the *rfa* cluster of genes that control LPS core structure. The resistant strains identified are either nonpathogenic rough mutants, or, if still pathogenic, have defects in LPS structure that make them susceptible to more antibiotics than non-resistant strains (67).

Unlike P22, Felix O1 is a lytic phage; there is no chance of the prophage-mediated development of immunity to the phage, or of toxin transfer to the host. Felix is classified as a member of the family *Myoviridae* (51). It has a large, icosahedral head containing a circular genome of dsDNA, 86,155 base

pairs (bp) in length (122). Six tail fibers are attached to the distal end of the long, contractile tail. Felix was classified as a member of *Myoviridae* due to its morphology, but until quite recently was thought to be a relative orphan within the family. Felix's isolation ended with the discovery of two similar phages: wV8, specific for *E. coli* O157:H7, and PhiEa21-4, specific for *Erwinia amylovora*, the agent responsible for fireblight (57, 115). The three phages have been informally grouped into the "Felix O1-like" myoviral genus.

Several studies have already attempted to take advantage of Felix's unique specificity; developing it for use as a *Salmonella* detection agent or as an antimicrobial treatment. In an attempt to create a specific and sensitive agent for *Salmonella* detection, *lux* genes A and B encoding a bacterial luciferase were inserted into the Felix O1 genome (53). Molecular manipulation was successful, but efficacy of the new anti-*Salmonella* reagent was not reported.

In addition to applications for detection, experimental evidence indicates that whole Felix would be an effective anti-*Salmonella* treatment on raw or RTE poultry products. In the only study available, wild-type and large-plaque variants of Felix achieved, respectively, a 1.8 and 2.1 log reduction on chicken frankfurter samples artificially inoculated with a 8.2×10^6 culture of *S.* Typhimurium DT104 when applied at an MOI of 1 (121). Although the results were promising, as yet there has been no further study reported, and no commercial adaptation of the application.

There is some evidence that orally administered bacteriophage would be a viable treatment for gastrointestinal infections if administered early enough after infection. The phage would need to be protected from the low pH of the stomach and upper intestine. To that effect, there has been some investigation of encapsulation of whole Felix for oral administration. Preliminary studies were promising but incomplete; encapsulation into a chitosan-alignate matrix was deemed successful; i.e, microspheres successfully protected the phage from the low pH of the stomach for delivery to the infection site, and also survived refrigeration for an extended time (66). However, no results from live animal studies have been reported, and results from such a trial would be a necessary part of any feasibility study for further

development. Despite their promise, there has been little investigation into whether purified lysis proteins would be an effective food safety measure, and none into the endolysin of Felix O1.

5.0 Research Summary

5.1 Hypothesis

Based on published research and preliminary homology data, the Felix endolysin is expected to be a soluble enzyme with a structure, optimum temperature, and pH range similar to that of the P22 lysozyme. The enzyme is expected to be lytic for *Salmonella* when applied externally, provided it is used in conjunction with the holin protein or suitable outer membrane permeabilization agent.

Based on the structure of phage lysis cassettes and location of holin genes in relation to endolysin genes in many characterized phages, the Felix O1 holin gene is expected to be upstream of the *lys* gene. The purified holin protein is expected to be a Type II holin and to permeabilize the outer membrane of *Salmonella* when applied externally.

5.2 Rationale

The hypotheses are based on results from three areas of study:

1. Previous studies show bacteriophage Felix O1 is lethal to *Salmonella*, and that it can effectively control the growth of *S.* Typhimurium in food samples stored under conditions of temperature abuse.
2. Studies of purified endolysins demonstrate that purified endolysins are bactericidal when applied to the surface of a Gram positive organism, or to the surface of a Gram negative organism pretreated with a membrane permeabilization agent.
3. Purified holins have successfully permeabilized cell membranes when applied externally.

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Chapter 3

Identification of the Holin of Bacteriophage Felix O1

Abstract

Holins are bacteriophage proteins that permeabilize the bacteria cell membrane during the phage replication cycle to allow a second phage protein, the endolysin, to access host bacterium peptidoglycan. Holins control lysis timing, and as a result of that control they also determine the length of the replication cycle. Purified holins also demonstrate permeabilization activity when applied to the external surface of a membrane. To characterize the holin of bacteriophage Felix O1, we first determined to locate the holin gene within the phage. When a BLAST search did not identify any promising candidates, we analyzed the putative Felix O1 proteins for transmembrane domains. Considering the possibility that the holin might not exist, we also analyzed the endolysin sequence for the presence of a secretory signal peptide. The putative RIIA protein was selected as a possible candidate for the holin gene, due to small sequence similarity over a short portion of its sequence with a known holin protein, and due to the role R proteins are known to play in bacteriophage-mediated cell lysis. The RIIA sequence was also analyzed to learn more of its secondary and tertiary protein structure. Attempts to clone *rIIA*, and what was learned about that protein and the suitability of holins for membrane permeabilization, are discussed.

1.0 Introduction

According to a recent study, foodborne illnesses cost the United States an estimated $150 billion a year in immediate and long-term medical care, lost productivity, pain and suffering (25). Salmonellosis, the illness caused by ingesting foodborne *Salmonella*, accounts for an estimated $14.6 billion of that total (25). Despite well-publicized outbreaks linked to produce, the most common vectors of salmonellosis are poultry products such as meat and eggs (23). One cause for the relatively high incidence of illness, despite the government's low tolerance to *Salmonella* in the nation's food supply, is that current protocols can

only reduce the number of organisms on a carcass or in a further processed product; they cannot eliminate the organism entirely (32). If the food is mishandled, the small number of organisms that remain can very quickly multiply to an infectious dose.

The consumer is increasingly antagonistic to the use of antibiotics in food animals, so a number of researchers are exploring alternatives, including the use of endolysins: bacteriophage-encoded enzymes that hydrolyze and destroy bacterial peptidoglycan (10, 19, 29). Research using lysins to control Gram positive organisms in food seems promising. However, if the purpose of the application is peptidoglycan degradation, any application of a lysin to a Gram negative organism must also include a way for the lysin to penetrate the outer membrane to gain access to the periplasm. It is possible that another phage protein, the holin, may be suitable for membrane permeabilization. Holin proteins are membrane proteins coded for by many large, tailed phages with genomes of double-stranded DNA. During the phage replication cycle, expressed holin subunits accumulate randomly in the cell membrane (37). At signal as yet undetermined, they aggregate and organize themselves into oligomers which form large, nonspecific holes in the host cell membrane. The holes result in collapse of membrane potential and host cell death (24). The holes also allow the endolysin to diffuse from the cytoplasm to the periplasm or extracellular matrix (ECM), where it degrades peptidoglycan. The loss of structural support, coupled with water diffusing into the dead cell, lyses the cell and releases any progeny phage.

Potential therapeutic uses for holin remain relatively unexplored. Several studies have concluded that holin proteins are lethal to host cells if expressed in a eukaryotic cancer cell line. Viability of cell cultures decreased by 98% when MCF-7 or HeLa cells were transfected with a plasmid engineered to express λ S^{105} (2). Growth of tumor MCF-7 xenografts in immunoincompetent mice and in murine mammary adenocarcinoma cell-derived tumors in syngeneic BALB/c mice was significantly decreased when S^{105} expression was induced. It was determined that the proteins localized in the endoplasmic reticulum and mitochondrial membranes, resulting in the loss of mitochondrial membrane potential (1). Purified holins are membrane-permeable if applied externally; an increase in fluorescence was measured

in the suspension medium of liposomes filled with fluorescent dye after the addition of purified λ holin carrying an internal tag, but not after addition of the same protein carrying a missense mutation. (28).

Current understanding of endolysin therapy suggests using an endolysin from a phage specific for the target bacteria. The lysins encoded by such phages have been evolutionarily optimized to degrade that particular peptidoglycan. Bacteriophage Felix O1 was selected because it is relatively specific for the *Salmonella* genus but quite indiscriminate within that genus, which makes it unique among phages (18). The phage has been sequenced and the lysin and holin genes were selected for further development as an anti-*Salmonella* food additive. The purpose of this study is to identify the holin gene and isolate and characterize the holin protein.

2.0 Materials and Methods

2.1 Identification of the holin gene

2.1.1 Homology search

A homology search for the Felix O1 holin gene was conducted using the blastx algorithm. The translated nucleotide Felix O1 genome (GenBank access number AF320576) was used, with the Entrez query "holin". Any Felix protein that the program identified as having sequence identity to known or putative holins was analyzed with the blastp algorithm and examined for potential transmembrane domains (described below).

2.1.2 Transmembrane domain prediction of Felix O1 protein sequences

To screen for the two TMDs of Type III holins, the list of potential protein coding sequences of the Felix genome was accessed through GenBank and all potential Felix proteins 60-89 amino acids (aa) in length were screened with both TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0/) and DAS (http://www.sbc.su.se/~miklos/DAS/) . Any sequence that both programs indicated had two transmembrane domains was then analyzed with the blastp program. To screen for the three TMDs of type II holins, all potential proteins 90-119 aa in length were examined with both programs, and any

sequence predicted to have three TMDs and the *N*-terminus in the cytoplasm was analyzed with blastp against known holin sequences. Despite the rarity of type III holins, the few proteins of a size similar to the T4 type III holin, 195-240 aa, were also analyzed. Later, every putative Felix protein was analyzed using the same software. Any sequence that both programs predicted had one, two, or three TMDs was analyzed with blastp algorithm for sequence identity to know holin proteins.

2.1.3 Screen of putative Felix O1 proteins for a dual start motif

The *N*-terminus of the translated protein sequence of each putative protein in the Felix O1 proteome was examined for a possible dual start motif: methionine, lysine, and methionine at the first, second, and third positions from the *N*-terminus, respectively. Sequences beginning Met-Lys-Met were analyzed with TMD prediction software as described above and analyzed with the blastp algorithm.

2.1.4 Search for a lysin signal sequence

Some lysins are secretory proteins, and thus the phages that encode them are lytic without the permeabilization activity of a holin protein. Acting on the possibility that the Felix lysin is a secretory protein that is functional without a holin, the BLAST alignment algorithm was used to align the P1 Lyz protein sequence with that of the Felix O1 LysO1 endolysin sequence. The query used was the Felix endolysin (NP_944846.1; the subject was the P1 Lyz protein (YP_006484.1).

The Felix genome was examined for a protein homologous to known phage amurins. ΦX174 E (NP_040709), MS2 L (NP_040649.1), and Qβ A_2 (NP_046749.1) were compared to Felix O1 known and putative proteins with blastp, using the GenBank reference sequence as the sequence query and enterobacteria phage Felix 01 (taxid 77775) as the organism.

In the event the lysin contains a signal sequence nonhomologous to known secretory endolysins or amurins, the first ten amino acids of the Felix *lys* sequence were examined with SPdb, and the entire protein sequence was examined with the Signal Peptide Website (http://www.signalpeptide.de/), which compares the query sequence to known signal sequences (5, 14). Both programs compare the peptide

sequence of interest to databases of known signal sequences from all three domains of life. The SPdb program compares the sequence of interest to a database of signal sequences based on information from SwissProt release 55.0. The Signal Peptide Website database is based on UniProt Knowledge Release 14.7 released Jan. 20, 2009, from SwissProt release 56.7.

2.1.5 Secondary and tertiary structural analysis of the putative Felix O1 RIIA protein by PHYRE

PHYRE (http://www.sbg.bio.ic.ac.uk/~phyre/) is a freely available program that predicts the secondary structures of a given protein sequence, and uses sequence homology to suggest proteins of known tertiary structure that may be similar to the protein of interest. To predict the secondary structures of RIIA and determine if the selective tag could have any effect on holin function, and to gain some idea of the tertiary structure of the protein, the putative RIIA protein sequence (NP_944777.1) and the sequence with the addition of the pRSET A *N*-terminal polyhistidine tag (MRGSHHHHHH GMASMTGGQQ MGRDLYDDDD KDRWGS) were submitted to PHYRE.

2.1.6 Cloning of Felix O1 rIIA, a potential holin gene

The gene *rIIA* was PCR-amplified from whole phage. Primers used were O1rIIAF (5'-CCC**GGATCC**ATGACACCTGAAATGTTCAGCCTT-3') and O1rIIAR (5'-CCC**GAATTC**TTAATCGTAAACTGTTGTAACTTC-3') (Invitrogen, cleavage sites in bold, start codon underlined). To amplify *rIIA*, a 5-min initiation step at 95 °C was followed by 40 cycles of a 30-s melting step at 94 °C, a 30-s annealing step at 59 °C, and a 2.5-min elongation step at 68 °C in an iCycler (Bio-Rad, Hercules, CA). The reaction was then held for 10 min at 72 °C before being cooled to 4 °C. The PCR product was gel-purified, digested with *Bam*HI and *Eco*RI (Invitrogen, Life Technologies, Carlsbad, CA) overnight at 37 °C, then directionally cloned into pRSET A (Invitrogen) that had been digested with the same enzymes. The ligation reaction was performed with DNA ligase (Promega, Fitchburg, WI) by alternating 2 min at 30 °C and 2 min at 10 °C for 4 hr. The ligated plasmid was transformed into Mach 1

E. coli (Invitrogen), which was spread-plated on TSA (Difco) containing 100 µg/ml ampicillin and incubated overnight at 37 °C. The experiment was then repeated with an overnight ligation step.

3.0 Results

3.1.1 Homology Search

The homology search performed immediately after genome sequencing did not identify a potential holin gene (36). As some time had passed since the original search, another search was initiated. The search used the list of putative Felix O1 proteins and the Entrez query "holin". Results with an Expect value (E-value) of 0.01 or higher were given a lower priority, as higher E-values indicate an increasing likelihood that sequence identity is due to random chance rather than homology (34).

The whole-genome homology search with blastx revealed two putative Felix O1 proteins that displayed sequence identity to putative holin proteins through part of their lengths (summarized in Table 3.1). The program matched the *S. pyogenes* bacteriophage MGAS10394 holin (YP_060445.1, 760 aa) and different parts of two predicted Felix O1 proteins, both putative tail fiber proteins. A blastp analysis of the first Felix O1 protein (NP_944921.1) revealed similarities both between itself and the aforementioned MGAS10394 holin and between itself and the conserved tail fiber protein Gp37 (AAR83237.1, 760 aa). Each match displayed identical characteristics: sequence identity stretched from position 151 to 226 of the Felix O1 query sequence (490-566 of the subject sequence), and both had an E-value of approximately 7.0×10^{-8}. The second Felix O1 protein (NP_944923), also a putative tail fiber protein, was similar to the *S. pyogenes* putative phage holin from position 541 to position 656 of a 782-aa sequence (443-561of the phage MGAS10394 protein sequence), with an E-value of 0.15.

Table 3.1. blastp analysis of Felix O1 putative proteins with sequence identity to holins or putative holin proteins.

Query Sequence				Subject Sequence				Results				
Access #	Protein	Organism	Length	Access #	Protein	Organism	Length	Sequence Identity (%)	Region of Similarity	Length of Similarity	Evalue	Notes
NP_944921.1	putative tail fiber protein	Felix O1	388 aa	YP_060445.1	putative holin	*S. pyogenes* bacteriophage MGAS10394	760 aa	38	Q151/S490 - Q226/S566	79	$7 * 10^{-8}$	Also displayes 91% sequence identity to *Staphylococcus* phage SA1 putative tail fiber protein ACZ55517.1, with an E value of 0.
				AAR83237.1	tail fiber protein Gp37	*S. pyogenes* bacteriophage MGAS10394	760 aa	38	Q151/S490 - Q226/S566	79	$6.8 * 10^{-8}$	
NP_944923	putative tail fiber protein	Felix O1	782 aa	YP_060445.1	putative holin	*S. pyogenes* bacteriophage MGAS10394	760 aa	25	Q541/S433 - Q656/S561	133	0.15	Also displayes 80% sequence identity to *Staphylococcus* phage SA1 putative tail fiber protein ACZ55517.1, with an E value of 0.
NP_944777.1	putative rIIA	Felix O1	763 aa	NP_839939.1	holin	*Lactococcus* phage P335	153 aa	23	Q629/S49 - Q713/S129	85	0.058	Also displayes 94% sequence identity to the *Staphylococcus* phage SA1 rIIA protein, with an E value of 0.

As mentioned above, the first putative Felix O1 protein, NP_944921.1, displayed sequence identity to two different proteins of *S. pyogenes* phages. It was thought unusual that both proteins contained the same number of amino acids and the sequence similarity of the Felix protein was to the same part of each *S. pyogenes* phage protein. A review of available sequence information for phage MGAS10394 revealed the two proteins, identified respectively as holin and Gp37, have the same sequence and are the same identified protein. The sequence in question contains five overlapping putative conserved domains. The largest, DUF859, is the *N*-terminal 570 amino acids of the sequence. The function of the domain is unknown at this time. The second, third, and fourth domains are the *C*-terminal end of the protein and overlap to a large extent. Phage_holin_4 (positions 647-754), COG4824 (650-757), and holin_tox_secr (647-760) have all been tentatively identified as domains of holin proteins, with one member from *Clostridium* species active in toxin secretion. The fifth domain, 10 (491-557), has been provisionally identified as a domain of phage base plate and tail wedge proteins. The sequence similarity between the putative Felix protein and the MGAS10394 holin was to a region identified not as a conserved holin domain but as a structural protein. As the putative Felix protein NP_944921.1 has been tentatively identified as a tail fiber protein (91% sequence identity to *Staphylococcus* phage SA1 tail fiber protein ACZ55517.1, E-value of 0), the sequence similarity to a domain common in base plate and tail fiber proteins further indicates that the protein is question is not the Felix holin.

A third possible match was also identified through the homology search, though it displayed sequence identity through a short sequence. The sequence identity was between the putative holin protein of *Lactococcus* phage P335 (NP_839939.1) and the putative Felix O1 RIIA, a protein thought to be necessary for host cell lysis during phage replication. Sequence identity stretched from position 629 to713 of the RIIA sequence (49-129 of phage P335), with an E-value of 0.058 (Figure 3.1). Although the E-value is higher than the value accepted as significant, the lack of identification of any more likely candidate, as well as the role R proteins are reported to play in lysis, convinced us to attempt to clone *rIIA* as a potential holin gene and express and purify RIIA as a potential holin.

Figure 3.1. Alignment of the putative Felix O1 RIIA and the putative *Lactococcus* phage P335 holin protein sequences.

```
Query  629  SWIIARNFTFNNRKISRGYCYSRDTSKTIFLEGNEEAVEAIFGKIQYVAAPFAYTYTISV  688
            SW   +NF  N  K+S    Y  + +K + ++ N   +EA   +I+   A     +T
Sbjct  49   SWA-QKNFNENPEKLSEAINYVTEEAKKLKIKTNPAQIEA---QIEASLAQLKKNFTADP  104

Query  689  LQTLKGCLDNNTKLYKKIRKAGDRM  713
             +T+K      +++ + + K  D +
Sbjct  105  AKTIKDVAQATSEVAQSVSKTADTI  129
```

The query is Felix RIIA, the subject is the phage P335 holin. Sequence identity is 23 %, The E-value of 0.058 suggests that the similarity may be due to random chance rather than to homology.

3.1.2 Predicted transmembrane domains of putative Felix O1 proteins

Putative Felix O1 proteins were analyzed by both prediction algorithms, and any putative TMD had to be identified as such by both programs. The protein sequences that were predicted by both programs to contain one, two, or three TMDs were analyzed with blastp with and without the Entrez query "holin". Among the proteins 60-89 aa in length screened for two TMDs, DAS and TMHMM agreed that two, NP_ 944998.1 and NP_945001.1, both tentatively identified as phage membrane proteins, each had two TMDs, each had a predicted topology placing both termini in the cytoplasm, similar to type III holins (Table 3.3). The holin-specific BLAST search of both proteins identified matches with high E-values; the lowest was 1.0. A second, non-specific BLAST search revealed that only two organisms possess similar putative proteins: Felix O1 and the recently identified Felix-like phage WV8 (Table 3.3)

Of the screened proteins 90-120 aa in length, none indicated potential for two or three TMDs, and only one had potential for one TMD. A blastp analysis of the potential protein sequence, NP_944790.1, revealed a large number of similar proteins exist in various phages but none has been definitively identified (Table 3.2). A blastp analysis against known holin proteins identified several proteins with possible sequence identity to the query sequence, notably to the T1 holin and the family of holins similar to the putative holin of bacteriophage LL-H, which infects *Lactobacillus lactis*. However, the E-values of those matches, 0.042 and 0.02 respectively, were high enough to be due to random chance.

Table 3.2. Transmembrane domain prediction summary of results; potential Type I holins.

Type I Screen (90 - 119 aa)				
Protein	**Length**	**TMHMM software, v. 2.0 Results**	**DAS Results (1.7 cutoff)**	**BLAST Results**
NP_944790.1	**115**	**outside 1-9, TM helix 10-27, inside 28-115**	**TMD 9-22**	**T1 (.046) LL-H family (.02, 0.7)**
NP_944793.2	102	no TM domain, external protein	no	
NP_944795.1	108	no TM domain, external protein	TMD 34-38	
NP_944810.1	98	no TM domain, internal protein	no	
NP_944811.1	110	external protein, but 0.4 prob of TM domain 0-25	TMD 7-16	
NP_944813.1	105	external protein, no TM domain	no	
NP_944817.1	134	external protein, no TM domain	no	
NP_944820.1	113	internal protein, no TM domain	no	
NP_944838.1	114	internal protein, no TM domain	no	
NP_944840.1	97	external protein, no TM domain	no	
NP_944842.1	131	internal protein, no TM domain	no	
NP_944844.1	132	external protein, no TM domain	no	
NP_944851.1	123	internal protein, no TM domain	TMD 71-85	
NP_944856.1	123	internal protein, no TM domain	no	
NP_944866.1	130	external protein, no TM domain	no	
NP_944887.1	110	external protein, no TM domain	TMD 15-22, 100-102	
NP_944890.1	125	external protein, no TM domain	no	
NP_944894.1	133	external protein, no TM domain	no	
NP_944899.1	132	external protein, no TM domain	no	
NP_944909.1	113	external protein, no TM domain	TMD 21-24	
NP_944920.1	100	external protein, no TM domain	no	
NP_944927.1	**121**	**inside 1-6, TM helix 7-29, outside 30-121**	**TMD 12-26**	**High E-value**
NP_944933.1	119	external protein, no TM domain	no	
NP_944934.1	109	internal protein, no TM domain	TMD 80-84, 87-88	
NP_944938.1	127	external protein, no TM domain	TMD 8-14	
YP_001504374.1	93	external protein, no TM domain	TMD 60-70, 76-78	
NP_944987.2	107	internal protein, no TM domain	TMD 79-85	
NP_944989.1	112	external protein, no TM domain	no	
NP_945005.1	125	external protein, no TM domain	TMD 27-34	
NP_945007.1	106	internal protein, no TM domain	TMD 12-16	
NP_945013.1	92	external protein, no TM domain	no	
NP_945021.1	111	internal protein, no TM domain	TMD 45-53	

ORFs of Felix O1, identified by GenBank accession numbers, were analyzed by TMHMM and DAS using default parameters. Bolding entries are those that TMHMM and DAS indicated consensus in the location of predicted TMDs. Bolded entries were further analyzed by BLAST and results are included. Lengths of the putative protein are given in number of amino acids (aa).

Table 3.3. Transmembrane domain prediction summary of results; potential Type II holins.

Type II Screen (60 - 89 aa)				
Protein	**Length**	**TMHMM software, v. 2.0 Results**	**DAS Results (1.7 cutoff)**	**BLAST Results**
NP_944791.1	89	external protein, no TM domain	TMD 7-19	
NP_944792.1	89	internal protein, no TM domain	TMD 35-42	
NP_944797.1	75	internal protein, no TM domain	no	
NP_944800.1	66	external protein, no TM domain	TMD 37-41	
NP_944804.1	70	internal protein, no TM domain	no	
NP_944806.1	68	external protein, no TM domain	no	
NP_944825.1	82	internal protein, no TM domain	no	
NP_944828.1	74	external protein, no TM domain	TMD 9-13, 38-45	
NP_944832.1	72	internal protein, no TM domain	no	
NP_944854.1	77	external protein, no TM domain	no	
NP_944860.1	76	external protein, no TM domain	TMD 35-43	
NP_944868.1	65	external protein, no TM domain	TMD 20-30	
NP_944880.1	75	external protein, no TM domain	TMD 40-50	
NP_944882.1	66	external protein, no TM domain	TMD 24-32	
NP_944900.1	79	external protein, no TM domain	TMD 45-53	
NP_944925.1	64	external protein, no TM domain	TMD 10-25	
NP_944930.1	86	internal protein, no TM domain	no	
NP_944937.1	70	internal protein, no TM domain	TMD 20-29	
NP_944945.1	72	internal protein, no TM domain	no	
NP_944947.1	68	internal protein, no TM domain	no	
NP_944949.1	85	internal protein, no TM domain	TMD 10-22	
NP_944952.2	72	external protein, no TM domain	no	
NP_944953.1	69	internal protein, no TM domain	no	
NP_944966.1	66	internal protein, no TM domain	no	
NP_944983.1	82	external protein, no TM domain	no	
NP_944992.2	85	external protein, no TM domain	no	
NP_944996.1	80	external protein, no TM domain	no	
NP_944998.1	**68**	**inside 1-4, TMD 5-27, outside 28-31, TMD 32-54, inside 55-68**	**TMD 13-25, 35-47**	**only matches are Felix and WV8**
NP_945001.1	**66**	**inside 1-8, TMD 9-28, outside 29-42, TMD 43-60, inside 61-66**	**TMD 8-26, 50-58**	**only matches are Felix and WV8**
NP_945006.1	85	internal protein, no TM domain	TMD 13-27	

ORFs of Felix O1, identified by GenBank accession numbers, were analyzed by TMHMM and DAS under default parameters. Bolding entries are those that TMHMM and DAS indicated consensus in the location of predicted TMDs. Bolded entries were further analyzed by BLAST and results are included. Lengths of the putative protein are given in number of amino acids (aa).

Table 3.4. Transmembrane domain prediction summary of results; potential Type III holins.

Type III Screen (195 - 240 aa)				
Protein	**Length**	**TMHMM software, v. 2.0 Results**	**DAS Results (1.7 cutoff)**	**BLAST Results**
NP_944782.1	231	external protein, no TMD	TMD 136, 138-140, 196-204	
NP_944836.1	193	external protein, no TMD	TMD 112-120	
NP_944853.1	196	external protein, no TMD	no	
NP_944879.1	**244**	**inside 1-20, TMD 21-43, outside 44-57, TMD 58-80, inside 81-244**	**TMD 27-46, 62-71, 213-214**	**Compliments red mutants of λ**
NP_944895.1	199	external protein, no TMD	TMD 16-19, 83-92, 149-154	
NP_944912.1	207	external protein, no TMD	TMD 87-90, 116-122	

ORFs of Felix O1, identified by GenBank accession numbers, were analyzed by TMHMM and DAS under default parameters. Bolding entries are those that TMHMM and DAS indicated consensus in the location of predicted TMDs. Bolded entries were further analyzed by BLAST and results are included. Lengths of the putative protein are given in number of amino acids (aa).

Table 3.5. Transmembrane domain prediction summary of results; all other putative protein sequences.

Other				
Protein	**Length**	**TMHMM software, v. 2.0 Results**	**DAS Results (1.7 cutoff)**	**BLAST Results**
NP_944777.1	763	external protein, no TMD	TMD 251-259, 684-688	
NP_944778.1	369	external protein, no TMD	TMD 353-357	
NP_944779.1	182	external protein, no TMD	TMD 6-17, 50-57	
NP_944787.1	154	external protein, no TMD	no	
NP_944801.1	261	external protein, no TMD	no	
NP_944808.1	170	external protein, no TMD	no	
NP_944822.1	176	external protein, no TMD	TMD 54-72	
NP_944826.2	182	external protein, no TMD	no	
NP_944827.1	154	external protein, no TMD	TMD 38-42, 126-131	
NP_944829.2	**167**	**in 1-83, TMD 84-106, out 107-120, TMD 121-140, in 141-167**	**TMD 85-108, 125-140**	**not a holin match**
NP_944833.1	181	external protein, no TMD	no	
NP_944846.1	154	external protein, no TMD	no	
NP_944848.1	294	external protein, no TMD	TMD 142-148, 202-208, 271-274	
YP_001504372.1	53	interior protein, no TMD	TMD 30-37	
NP_944857.1	471	external protein, no TMD	TMD 292-303	
NP_944863.2	161	external protein, no TMD	no	
NP_944871.1	137	external protein, no TMD	no	
NP_944875.1	**185**	**inside 1-8, TMD 9-31, outside 32-185**	**TMD 8-32, 135-142, 175-178**	**no holin matches**
NP_944884.1	533	external protein, no TMD	no	
NP_944885.2	488	external protein, no TMD	TMD 60-64, 112-117, 335-339, 359-368	
NP_944886.1	166	external protein, no TMD	no	
NP_944888.1	488	external protein, no TMD	TMD 63-71, 171-174	
NP_944891.1	368	external protein, no TMD	no	
NP_944892.1	149	external protein, no TMD	TMD 55-57	
NP_944893.1	160	external protein, no TMD	no	
NP_944896.1	450	external protein, no TMD	TMD 430-439	
NP_944897.1	148	external protein, no TMD	no	
NP_944901.1	742	external protein, no TMD	243-252,475-484,530-560,592-600	
NP_944906.1	265	external protein, no TMD	TMD 67-78	
NP_944910.1	325	external protein, no TMD	no	
NP_944914.1	139	external protein, no TMD	no	
NP_944915.1	489	external protein, no TMD	43-48, 171-174, 215-223, 241-246, 289-299	
NP_944918.1	285	external protein, no TMD	TMD 123-129, 149-160	
NP_944921.1	388	external protein, no TMD	no	
NP_944923.1	782	external protein, no TMD	no	
NP_944928.1	299	external protein, no TMD	TMD 174-176, 200-216	

Table 3.5 cont.

NP_944929.1	181	external protein, no TMD	TMD 106-126	
NP_944932.1	171	external protein, no TMD	TMD 135-139, 161-163	
NP_944942.1	366	external protein, no TMD	TMD 18-22, 148-159, 310-314	
YP_001504373.1	48	interior protein, no TMD	no	
NP_944955.1	167	external protein, no TMD	TMD 57-63	**only matches are Felix and WV8**
NP_944958.1	906	external protein, no TMD	TMD 609-615, 722-724	
NP_944959.1	140	external protein, no TMD	no	
NP_944960.1	266	external protein, no TMD	68-81, 116-124, 183-201, 228-231	
NP_944963.1	247	external protein, no TMD	no	
NP_944967.2	661	external protein, no TMD	TMD 455-472	
YP_001504375.1	49	interior protein, no TMD	TMD 13-15	
NP_944970.1	285	external protein, no TMD	TMD 180-193, 210-215	
NP_944975.1	348	external protein, no TMD	TMD 245-246	
NP_944978.1	174	external protein, no TMD	no	
NP_944980.1	166	external protein, no TMD	TMD 30-35	
NP_944984.1	251	external protein, no TMD	TMD 68-71, 173-178	
NP_944991.1	744	external protein, no TMD	76-83, 95-96, 426-430, 454-463, 478-485	
NP_944994.1	357	external protein, no TMD	TMD 82-86, 178-199	
NP_944999.1	714	external protein, no TMD	TMD 170-180, 206-216, 295-301, 508-509	
NP_945002.1	131	external protein, no TMD	no	
NP_945003.1	161	external protein, no TMD	TMD 19-23, 52-56	
NP_945009.1	171	external protein, no TMD	TMD 153-161	
NP_945016.1	293	external protein, no TMD	TMD 113-117	
NP_945017.1	163	external protein, no TMD	TMD 18-28	
NP_945019.1	593	external protein, no TMD	TMD 50-56, 483-485	
NP_945023.1	**58**	**outside 1-3, TMD 4-23, inside 24-58**	**TMD 7-24**	**only matches are Felix and WV8**

ORFs of Felix O1, identified by GenBank accession numbers, were analyzed by TMHMM and DAS under default parameters. Bolding entries are those that TMHMM and DAS indicated consensus in the location of predicted TMDs. Bolded entries were further analyzed by BLAST and results are included. Lengths of the putative protein are given in number of amino acids (aa).

When proteins not fitting the length requirements for Type III or Type I holins were screened, several more proteins with predicted membrane domains were identified. NP_944927.1, NP_944875.1, and NP_945023 all displayed a high likelihood of containing a single TMD, which would classify them as Type III holins. However, blastp analysis did not detect any similarity to known holin proteins. The analysis identified numerous proteins with possible homology to NP_944875.1 but the lowest E-value for an identified protein, a "putative heme lyase subunit, cytochrome c-type biogenesis" from *Salmonella*

Cholerasuis, was 0.055, which suggests the match could be due to random chance rather than homology. Analysis of NP_944879.1 and NP_944829.2 revealed two potential TMDs. A blastp analysis suggested that the first, NP_944879.1, is similar to proteins that compliment λ red mutants (Table 3.4). The second, NP_944829.2, is similar to a phage WV8 protein but unidentified at this time (Table 3.5).

Of note is that the protein RIIA (NP_944777.1), previously considered a potential holin gene, was removed from consideration due to a lack of consensus regarding the presence of transmsmbrane domains (Table 3.5). All holins identified to date have at least one TMD. The program TMHMM predicted RIIA to be an external protein with no TMDs, which agrees with what is currently known of RIIA proteins. DAS suggested RIIA possesses two TMDs. The first includes the residues 251-259, and a second includes the residues 684-688. The lack of consensus of the two prediction algorithms regarding the presence of TMDs in RIIA removed the protein from further consideration as a holin candidate.

3.1.3 Search of the endolysin for a dual start motif

When homology searches and TMD analysis did not reveal a likely holin candidate, another method of identification was sought. The λ dual start motif is recognizable, so each translated nucleotide sequence of each putative Felix O1 protein not otherwise tentatively identified was visually examined at the N-terminus for the Met-Lys-Met motif seen in positions 1-3 of the bacteriophage λ holin protein sequence. Examination of the *N*-terminus of each sequence and putative sequence revealed two potential sequences: NP_944966.1 begins with the sequence MKM, YP_001504373.1 begins with MKKM. Subsequent review of the TMD prediction results revealed that neither was expected to have transmembrane domains (Table 3.3, 3.5). Due to holin's known identity as a membrane protein, the lack of a TMD eliminated both proteins from consideration as a potential holin.

3.1.4 Search for an endolysin signal sequence

There is one known phage, coliphage P1, in which the genome encodes a secretory endolysin, *lyz*, spatially separated from its holin gene (39). A comparison of the Felix O1 Lys sequence to the P1

lysozyme Lyz, which possesses a signal arrest release (SAR) domain, revealed that sequence identity between the two begins at position 1 of the Felix O1 protein and extends to position 98, which corresponds to positions 28-122 of the P1 lysozyme. As there is no similarity between the Felix protein and the portion of Lyz that is the signal sequence, it suggests that Felix LysO1 does not possess a SAR domain similar to that of P1 Lyz.

Lack of a SAR domain does not negate the possibly of LysO1 being a secreted enzyme, so a search was initiated for a signal sequence at the *N*-terminus of the lysin protein sequence. Neither search revealed any portion of the sequence predicted to be a signal peptide.

A blastp analysis comparing known amurins ΦX174 E, MS2 L, or Qβ A_2 to known and putative Felix proteins revealed no sequence identity between any of the secreted proteins and any identified or putative protein in the Felix proteome. Later, a third party analysis of the endolysin gene sequence prior to cloning and expression also concluded that the Felix O1 endolysin was not a secretory protein (chapter 4, section 3.2).

3.1.5 PHYRE prediction

The **P**rotein **H**omology/analog**Y** **R**ecognition **E**ngine, or PHYRE, is a freely available program that predicts structures of a primary protein sequence. It uses three algorithms to predict secondary structures of a query protein sequence, then takes a consensus of the three algorithms. The program also uses fold recognition and sequence identity to other proteins of known tertiary structure to suggest proteins that may have a similar tertiary structure (15). After analysis, PHYRE determined that RIIA most likely consists of a combination of α helices and β sheets linked by connecting sequences (Table 3.6). The polyhistidine tag had no secondary structure (Table 3.7). One algorithm predicted a small (3-aa) helix, but was in disagreement with the other two prediction algorithms and the helix was not included in the consensus sequence.

Table 3.6. Summary of secondary structures of RIIA as predicted by PHYRE.

structure	start	end	length	structure	start	end	length	structure	start	end	length
alpha	3	11	9	beta	347	350	4	beta	551	553	3
alpha	18	35	18	beta	356	361	6	beta	556	557	2
beta	42	47	6	beta	367	370	4	alpha	561	572	12
beta	54	59	6	beta	377	382	6	beta	575	580	6
alpha	66	76	11	alpha	385	389	4	alpha	589	601	13
beta	88	88	1	alpha	394	398	5	alpha	604	619	16
alpha	93	94	2	beta	399	402	4	alpha	628	635	8
beta	95	97	3	alpha	403	410	8	beta	641	649	9
alpha	98	98	1	alpha	416	421	6	beta	656	659	4
beta	99	102	4	beta	424	430	7	alpha	662	670	9
beta	104	110	7	alpha	444	450	7	beta	675	676	2
beta	116	123	8	alpha	452	452	1	alpha	679	694	16
beta	128	133	6	alpha	456	456	1	alpha	700	709	10
beta	143	149	7	alpha	458	462	5	alpha	712	712	1
alpha	152	167	15	beta	468	472	5	beta	713	717	5
beta	174	178	5	alpha	475	484	10	alpha	721	721	1
alpha	195	197	3	alpha	489	496	8	alpha	724	729	6
beta	213	217	5	alpha	506	514	9	beta	730	730	1
beta	221	221	1	beta	515	519	5	alpha	731	732	2
alpha	234	242	9	alpha	529	529	1	beta	733	733	1
beta	243	243	1	beta	530	530	1	alpha	734	734	1
beta	248	253	6	alpha	531	532	2	alpha	741	747	7
alpha	264	268	5	alpha	535	537	3	beta	752	754	3
alpha	271	325	55	beta	542	547	6	beta	760	761	2
alpha	336	341	6								

The start and end positions of each predicted secondary structure are noted. Length of the structure is given in number of amino acids.

Table 3.7. Summary of secondary structures of the pRSET A polyhistidine tag predicted by PHYRE.

tag	structure	start	end	length
pRSET A	alpha	20	20	1
	alpha	32	33	2

Length is given in number of amino acids.

The predicted tertiary structure is based on sequence identity to proteins of known tertiary structure, and the results are summarized in Table 3.8. The possible structures fall into two broad categories. The prediction algorithm suggested that, based on sequence identity to proteins of known

tertiary structure, RIIA most likely has a tertiary structure similar to heat shock and chaperone proteins. A structure similar to DNA repair proteins is possible but less likely.

Table 3.8. Proteins with sequence identity to the Felix O1 *rIIA* translated nucleotide sequence

PDB ID	E-value	% ID to RIIA	Name	Classification	Source
c1y4uB_	$2.7 * 10^{-25}$	12	HtpG	chaperone	*E. coli*
c2ioqB_	$6.3 * 10^{-25}$	12	HtpG	chaperone	*E. coli*
c2o1uB_	$1.4 * 10^{-24}$	12	GRP94	chaperone - endoplasmic reticulum	*C. lupus familiaris*
c2cg9B_	$1.2 * 10^{-23}$	12	Hsp 90	chaperone	*S. cerevisiea*
c2o1wA_	$2.5 * 10^{-22}$	13	GRP94	chaperone - endoplasmic reticulum	*C. lupus familiaris*
c1nhiA_	$4.2 * 10^{-13}$	11	MutL (fragment)	DNA mismatch repair	*E. coli* K12
c1ea6B_	$7.3 * 10^{-13}$	11	MutLalpha (fragment)	DNA mismatch repair	*H. sapiens*
d1qy5a_	$2.9 * 10^{-12}$	13	Hsp 90	chaperone	*C. familiaris*
c2o1tC_	$3.0 * 10^{-12}$	11	GRP94	chaperone - endoplasmic reticulum	*C. lupus familiaris*
d1uyla_	$3.5 * 10^{-12}$	14	Hsp 90	chaperone	*H. sapiens*

3.1.6 Cloning of putative rIIA – *a potential holin gene*

The first attempts to clone *rIIA* into pRSET A were unsuccessful. While the transformed cells grew on selective media, subsequent testing revealed that *rIIA* was not present in the plasmids – antibiotic resistance was due the presence of the pRSET A plasmid without the inserted gene. As *rIIA* is 2292 nucleotides in length, a second insertion attempt was made using a longer ligation step at a lower temperature. Again, testing of the growth on selective media after transformation revealed that the gene had not inserted into the plasmid.

4.0 Discussion

4.1 Holin identification

Despite their heterogeneity, holin proteins share several characteristics: they are small, membrane proteins, and found in genomes of bacteriophages with large, dsDNA genomes. Many holin genes also

possess a dual start motif and are part of a lysis cassette. These known characteristics provided the initial parameters for the search for the Felix O1 holin.

The homology search did not identify a holin candidate. While a homology search did reveal two putative proteins potentially homologous to a known holin, there were several inconsistencies between the characteristics of the putative proteins and those of known holins. The first was size. The potential matches, Felix NP_944921.1 and NP_944923, are predicted to be 388 and 782 aa in length, respectively, which is 1.7 and 3.6 times as large as the largest known holin, the 218-aa T4 T. A second inconsistency is that holins are membrane proteins, and transmembrane domain (TMD) prediction software analysis of both putative sequences indicated that neither protein is expected to have transmembrane domains. The third inconsistency is that the additional results of the homology search revealed that each putative protein displayed much greater sequence identity to a superfamily of tail fiber proteins. It is possible that the initial search of the sequenced Felix O1 genome for open reading frames made some small errors predicting the size of the putative protein and the actual proteins are much smaller. But sequence identity with such a well-known superfamily of proteins, coupled with the lack of predicted TMDs, makes it unlikely either NP_944921.1 or NP_944923 is the elusive holin.

The notable features of recognized holin protein subunits are their small size and multiple transmembrane domains. There is software available to analyze a protein sequence and identify potential TMDs; the program scans the input sequence for amino acid sequences that are known to form hydrophobic α helices. To ensure as high a certainty as possible regarding the presence, location, and general length of the predicted TMD, two programs with different stringencies were selected: DAS and TMHMM (4, 6). The programs were selected for their high prediction accuracy. DAS is 90-95% accurate (6). TMHMM is 97-98% accurate, and can differentiate between soluble and membrane proteins with an accuracy of greater than 99% (4).

As the homology search did not reveal a holin candidate, the next search method used was analysis of putative protein sequences with transmembrane domain prediction software. Every putative open reading frame of Felix was analyzed. The analysis revealed several proteins predicted to have one or

two TMDs; none was predicted to have three. Subsequent blastp analysis of those protein sequences revealed that the protein in question had been tentatively identified as another protein, with greater certainty, or that nothing similar had yet been identified.

Homology search and transmembrane domain analysis, the two techniques thought most likely to reveal a potential holin gene, did not. The next searches were based on less common characteristics of holin genes. The first was identification of a dual start motif, which not all holins possess. A visual examination of the *N*-terminus of each protein revealed two that might have the necessary Met-Lys-Met dual start motif: the first three amino acids of NP_944966.1 are Met-Lys-Met; the first four of YP_001504373.1 are Met-Lys-Lys-Met. Experiments have determined that additional lysine residues between the methionine start codons prolongs lysis (12). The Felix replication cycle is estimated to last approximately one hour , unlike the estimated 25 min of the T4 cycle, or the λ estimated replication time of 35 min (30). A dual start motif containing extra lysine residues would help explain the increased duration of the replication cycle. However, blastp revealed that both proteins have been tentatively identified as other proteins, with higher certainty, so the likelihood of either protein being the holin is quite low.

There are a few phages that use an alternate lysis strategy; they encode secretory endolysins or amurins rather than the two-protein system of holin and endolysin. Although the phages known to use that mechanism are often much smaller than Felix O1 and other tailed phages, with correspondingly smaller genomes, the possibility of Felix using an amurin or a secretory endolysin could not be overlooked. Two approaches were used. The first used blastp to identify homology between known secretory endolysin and the Felix endolysin or putative proteins. The second used available software programs to predict signal sequences of the Felix endolysin LysO1.

To date, three families of bacteriophages have been identified that do not use the dual-protein lysis system. It is known that two of these small-genome phages, ΦX174 and Qβ, use an amurin – a secreted protein that inhibits peptidoglycan synthesis. Although given the size of the Felix genome and that fact that it is composed of double-stranded DNA, the possibility could not be ignored that Felix might

employ a similar mechanism. However, blastp analysis of each protein compared to the Felix putative proteome revealed that ΦX174 E, MS2 L, and Qβ A_2were not homologous to any known or putative Felix protein. The lack of similarity to the identified amurins was not unexpected. Of the phages possessing the amurin genes in question, ΦX174 has a single-stranded DNA genome of roughly 5.4 kb, the MS2 genome is single-stranded RNA of 3.5 kb, and the Qβ genome is single-stranded RNA of 4.2 kb (41). Felix is a large, tailed phage, with a genome of double-stranded DNA 86.6 kb in size. The literature to date suggests that phages with those characteristics possess dual-protein lysis systems consisting of a holin and a nonsecretory endolysin (41).

As a further check on the possibility that Felix LysO1 could be a secretory endolysin, two programs were used to identify potential signal sequences in the protein. Both were based on information obtained from SwisProt, though different updates of that information, so it may have been possible that the Felix lysin contained a signal sequence unrecognized by SwisProt and consequently by both analysis programs. However, later experiments with lysin expression confirmed the absence of a signal sequence. As discussed in depth in Chapter 4, the cloned endolysin remained in the expression host cytoplasm until cell lysis. Despite repeated attempts, no endolysin was found in the culture media during expression or at any stage prior to cell lysis. Finally, third party confirmation that the enzyme in question is not a secretory enzyme has removed any possibility of the Felix endolysin being a secretory endolysin without need of a holin protein.

4.2 Prediction of transmembrane domains

Analysis of the sequence of the Felix putative RIIA sequence was inconclusive – TMHMM predicted the sequence to be an external protein with no TMDs, DAS predicted it to be a membrane protein with two TMDs. It has been determined that other RIIA proteins are membrane proteins, so the lack of consensus between the prediction methods could mean that the identification criteria of one algorithm are not optimal for prediction of TMDs of that particular protein sequence. Though RIIA is thought to be integral to the membrane, there is still the possibility that the protein is instead a peripheral

membrane protein and thus does not possess transmembrane domains (8). The Felix O1 *rIIA* was originally identified through sequence identity to T4 *rIIA*. It is possible that despite that similarity, mutations have altered the Felix O1 RIIA protein an its location in the bacteria host has changed as a result of those mutations. It is also possible that the putative gene has been misidentified and not actually the *rIIA* gene. However, a blastp analysis of the putative protein sequence reveals similarity to numerous proteins identified as RIIA, so the last scenario is unlikely. Also, the gene *rIIB* is consistently found immediately downstream of *rIIA*, and in the Felix genome, the gene immediately downstream of *rIIA* is the putative *rIIB* (36).

4.3 PHYRE analysis of secondary and tertiary structure

Little is known about RIIA proteins. They are membrane proteins. Mutation studies suggest they are active in several pathways of phage replication relating to cell energetics, host cell membrane integrity, or DNA metabolism, though available information is insufficient to more specifically determine protein function (8, 13, 21). Structurally, the estimated length of the peptide, 763 aa, suggests the presence of more than one protein domain (9). The protein sequence was submitted to PHYRE to predict the secondary structures, and to observe proteins of suggested similar tertiary structure.

Analysis of the putative RIIA protein sequence by software designed to predict the type and location of secondary structures suggests a combination of α helices and β sheets. Secondary structure of only α helices or only β sheets has an observable effect on the tertiary structure, and thus the function, of the protein in question. Proteins composed mainly of α helices tend to be tough and durable, such as the keratin in hair and feathers. Proteins composed mainly of β sheets, such as silk, tend to be soft and flexible. (9). However, at this time any correlation between secondary structure consisting of both α helices and β sheets of a protein and its tertiary structure remains unknown.

Three-dimensional structure is unknown for any RIIA protein, though there are multiple algorithms available to predict tertiary structure based on protein sequence (comparative modeling) or homology to other proteins (fold recognition) (26). PHYRE uses a fold recognition algorithm to match

stretches of protein sequences with unknown structure to those of similar sequence whose structure has been determined. Instead of building a model, it then displays the proteins with known three-dimensional structure most similar to that of the query sequence (15). As the accuracy of the model generated by comparative modeling depends on the degree of sequence similarity between the query and the model, less than 30% sequence identity may lead to significant errors in the generated model (26). Fold recognition algorithms may generate a more accurate three-dimensional model with lower sequence identity – accurate models can be generated despite sequence identity as low as 15-25% (15).

Experimental evidence suggests RIIA function is related to DNA metabolism, it is perhaps surprising that the proteins identified by PHYRE as having similar structures were not chaperone proteins that do not bind DNA. However, the information available for fold recognition prediction algorithms states that the minimum sequence identity to build an accurate model of a query proteins is 15% (15). The highest sequence identity between the RIIA query sequence and any of the proteins identified by PHYRE is 14%. The low sequence identity suggests that, while the two proteins may have similar structures, the identified structure would not be an accurate three-dimensional model of the query sequence (15). The extremely low sequence identity between RIIA and other proteins of known three-dimensional structure also calls into question the current availability of the information and current modeling capabilities necessary to construct an accurate model.

4.4 Cloning of a potential holin gene

The lack of successful cloning is thought to be due to the expressed RIIA protein being detrimental to the *E. coli* host. The function of the protein has not been definitely identified. Mutants of *rII* display rapid lysis and will not plate on K-12 strains of *E. coli* lysogenized with bacteriophage λ (21). It has been proposed that RII proteins are not active in lysis inhibition, but that the *lack* of RII proteins results in cell lysis when other prophages are present in the genome (21). The protein has also been hypothesized to be a membrane protein that adversely affects function of *E. coli* membrane ATPase (8). That effect may be fatal to any host if the protein is overexpressed in even small amounts, such as those

produced by leaky expression. Although in theory expression of the inserted gene should be low without the presence of lactose or IPTG, in practice there is always some basal expression, and the low amount may have been enough to kill any cells that received a plasmid in which *rIIA* had inserted in the correct orientation.

Other obstacles to successful cloning include unsuccessful or incomplete restriction of the plasmid or the amplified gene. Restriction could fail for a number of reasons: nonfunctioning enzymes, inadequate length of the restriction reaction, incubation of the reaction at a temperature in in a buffer at which the enzyme is not active. In this study, problems with digestion were unlikely to be the cause of the lack of cloning success. The pairs of restriction enzymes used, *Bam*HI and *Hin*DIII to cleave the *lys* gene and plasmid, and *Bam*HI and *Eco*RI to cleave the *rIIA* gene and plasmid, were selected because each pair of enzymes is functional in the same restriction buffer. The selection allowed digestion with each gene of the pair to be run simultaneously in the same reaction tube (22). A restriction of the amplified *lys* gene was run in parallel to that of *rIIA*, and each reaction was analyzed with gel-electrophoresis and purified from the gel before ligation. The gel analysis revealed bands of three different sizes for each digested plasmid. The bands correspond to the three forms the digested plasmid could take: cut, uncut, and uncut spiral, and suggested that all enzymes were functional under the conditions used. While it is possible that cleavage of *rIIA* or the plasmid was unsuccessful, our data indicate that it is unlikely.

4.5 Conclusions

All approaches to identify a holin gene that are currently available and practical have been exhausted. Not all holin genes are adjacent to the phage lysin gene, so we looked elsewhere in the genome. Not all holins are similar to the λ holin, so we searched for homology to other known families of holin proteins. As not all lysins require a holin to release them from the host cell, we also examined the endolysin sequence for evidence of a signal sequence.

A possible answer is to use the holin from another phage. There is no interaction between the holin and endolysin in a two-component lysis system; phages that have had their holin gene replaced with

the holin from an unrelated phage are equally efficient at replication and host cell lysis (31, 33, 40). However, concurrent with our inability to identify a potential holin, further review of the literature has revealed the unsuitability of the holin protein for the function originally intended. Cloning and expression of holin genes is difficult due to expression of the protein being fatal to the bacteria used to express the protein (28). After purification, reinsertion of the holin into a membrane is possible and does result in membrane permeabilization, but energetically unfavorable and would not be an ideal candidate for membrane permeabilization or for bioremediation (9, 28). The availability of simple chemical compounds with known outer membrane-permeabilizing activity has added additional support to the conclusion that the holin is not the ideal candidate for an outer membrane permeabilization agent of Gram negative bacteria.

However, the study has raised some very interesting questions. There are fifty protein families currently known to be holins, yet despite the heterogeneity of the group, the gene remained unidentifiable by a homology search. The relative isolation of Felix O1on the phylogenic family tree raises the question of whether Felix O1 possesses yet another novel holin, a new type in a new location, and what its eventual identification and characterization will reveal about holin proteins and about the Felix O1 replication strategy.

4.6 Future Work

4.6.1 Existence of the Felix O1 holin

The simplest explanation of our inability to find a holin gene in the Felix O1 genome is that Felix O1 does not have one, and that is possible. There is no hard evidence that the genome of bacteriophage Felix O1 contains a holin gene. Lack of a holin is rare among phages, but not unheard of: ΦX174, MS2, and Qβ do not use holin proteins. Instead, their lysis proteins (E, L, and A_2, respectively) inhibit the enzymes active in peptidoglycan synthesis and repair (36, 37, 40, 41). However, the phages in question are quite small compared to Felix O1, and do not possess genomes of double-stranded DNA (dsDNA)

(41). Phages with genomes of dsDNA the size of Felix O1 – 86.6 kb – include identified or putative holin genes (41).

A second reason to hypothesize that Felix O1 uses a holin are the characteristics of the endolysin it carries. LysO1 has been identified as a lysozyme, which are specific for peptidoglycan, but no signal sequence was identified (this study). There is a cell membrane between the enzyme and its substrate, and the enzyme itself does not carry the means to cross that membrane. Lysis will not occur unless something gives it access to the periplasm, and among complex phages, the method of access is a hole in the membrane formed by holin proteins (41).

A possible method to confirm the existence of the holin protein is to infect *Salmonella* with whole Felix O1 and use electron microscopy to examine the inner membranes of the lysed cells for holes approximately 8.5 nm in diameter – the size of the pores formed by holin proteins (24).

4.6.2 Location of the holin gene

Another possible method to locate the holin gene is to reexamine the homology search results and reanalyze proteins that are similar to a known holin through their entire length, even if that similarity is low. The protein we selected as a holin candidate, the putative RIIA, was not similar to known holins. It was selected because phage R proteins are known to play a role in host cell lysis, though it was later learned that RII proteins are not active in cell lysis except under very specific conditions (21). The homology search that identified a small segment of the translated *rIIA* sequence as similar to the phage λ holin S also identified several putative FelixO1 proteins similar to phage proteins identified as known or putative holins. We did not pursue these leads at the time because the E-value of each sequence identity was quite high, suggesting the match could be due to random chance (34). However, in light of the diversity of holin proteins, they merit a further examination, first *in silico* to determine if they possess the physical characteristics of a holin, followed by cloning, expression, purification, and characterization.

The follow-up searches focused on the putative proteins identified in the original open reading frame (17) analysis done when the genome was sequenced, the physical characteristics of transmembrane

domains (TMDs) and the dual start motif. Assuming the analysis accurately identified all open reading frames, the holin gene may remain elusive not because it does not exist, but because it is not similar enough to any other currently known holin protein. However, it may be that the ORF analysis used omitted the holin gene. The original analysis by PEDANT (later repeated with Kodon) to identify open reading frames did not include nested ORFs in the final data set (ORFs) (35, 36). Nested genes are not unheard of in bacteriophage genomes, and the ORF containing the holin gene may have been omitted (41). A solution would be to conduct a new ORF analysis of the Felix O1 genome and examine any nested ORFs for sequence similarity or physical similarities to known holin proteins.

Alternatively, the tblastn search can find protein homologues in unannotated genome information (11). It does so by comparing a query protein sequence to every possible reading frame in the genome of interest. ATG is not the only known start codon, and the parameters may be widened to include alternate start codons such as TTG. Occasional protein sequences begin with valine or leucine rather than methionine (16). Once open reading frames have been identified, that variety may be taken into account by including VKM and LKM sequences as potential dual start motifs.

An approach that does not rely on *in silico* work is to perform a microarray of the Felix O1 mRNA at different time points during the replication cycle to see which proteins are upregulated at the time the holin mRNA is thought to be transcribed. Additionally, gel electrophoresis may be used to separate proteins expressed during Felix O1 replication, and likely bands can be removed from the gel and analyzed with mass spectrometry. i.e., matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) (3, 20).

4.6.3 Characterization of the holin protein

Characterization of holin proteins is made more difficult by the protein's demonstrated lethality to *E. coli* and yeast expression hosts (27). However, production by overexpression of subunits in an *E. coli* host, with the gene under the control of a strong promoter such as the *lac* promoter, is two orders of magnitude higher than during the phage replication cycle (28). Alternate expression methods for lethal

proteins include expression in a baculovirus system and or expression in a cell-free system (38). Purification methods vary, but the chaperone protein GroEL has been shown to effectively solubilize λ S^{105} (though not its antiholin counterpart S^{107}), when the holin and antiholin were overproduced in *E. coli* (7, 28). The ring structure form by the protein subunits in bacteria cell membranes may then be determined by allowing the subunits to oligomerize and examining the rings with transmission electron microscopy (24).

5.0 References

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Chapter 4

Purification and Characterization of the Bacteriophage Felix O1 Endolysin

Abstract

The increasing incidence of isolation of antimicrobial-resistant foodborne organisms has intensified research into drug-free food additives and treatments. We have characterized the endolysin of bacteriophage Felix O1 in part to evaluate its potential as an anti-*Salmonella* food additive. The enzyme structure is predicted to be composed of α helices arranged into two lobes, with the active site between them. The enzyme was lytic for all tested strains of *Salmonella*, as well as a tested strain of the foodborne pathogen *E. coli*. *C. jejuni* susceptibility remains ambiguous; the enzyme had no effect on *L. monocytogenes* or *M. luteus*. The enzyme is most active at basic pH and low ion concentration; optimal activity was observed in 25 mM buffer at pH 10. If removed from frozen storage, the enzyme was most thermostable at 30 °C. Activity is adversely affected by the presence of divalent cations. Based on demonstrated lytic activity against multiple species of foodborne pathogens, and high optimum pH, we suggest LysO1 is a good candidate for development as an anti-*Salmonella* treatment or food additive if the antibacterial activity can be established and quantified.

1.0 Introduction

Recently published models estimate that the total yearly cost of foodborne illness in the United States totals $152 billion (37). Of that total, the cost due to salmonellosis, the illness caused by foodborne *Salmonella* serovars, is an estimated $14.6 billion U.S. dollars. Contaminated poultry products are a major vector of salmonellosis, particularly in outbreaks; it is estimated that half of common-vector outbreaks originate from contaminated poultry products (12, 15).

Processing incorporates antimicrobial measures, but it is currently only feasible to reduce the number of pathogenic microorganisms present, not eliminate them. Sanitary handling and proper cooking

eliminate disease-causing bacteria from the food we eat, but if food is handled improperly a few organisms can quickly multiply to an infectious dose, or can cross-contaminate other items in the kitchen (12). Products such as lunchmeats are sold ready-to-eat (21), and as such foodstuffs are not heated again before consumption, any viable organisms in the packaged product may cause illness, especially if the product is incorrectly stored (12).

Consumers are increasingly requesting food with was raised and processed without the use of antibiotics, and processing plants are limited in treatment options by safety standards and cost. A possible approach is to use bacteriophages – viruses that infect and kill bacteria – as an additional antibacterial treatment for raw and RTE poultry products. Previous research has indicated that whole phage reduce the number of targeted organisms when used as additives or applied to the surface of a product (20, 22, 45, 50). A six-phage anti-*Listeria* cocktail was recently approved by the FDA for addition to lunchmeats (43).

Despite the advantages, there are several drawbacks to using whole phage preparations as food additives. Lysogenic phages, those that insert their genome into the bacterial genome after infection, have been known to harbor virulence genes and transport them between bacteria. Consequently, only lytic phages, those that begin the replication cycle and lyse the host immediately after infection, should be used, and they are carefully screened for lack of virulence genes (7). To target a single organism, a cocktail of several phages should be used to minimize the possibility of resistance to any one phage (14). To make the cocktail, the necessary phages must be determined to be infective for the target organism under the conditions the phage will be used. Then each phage must be propagated, purified, and mixed in the correct proportions, which can be both expensive and time-consuming (4).

It has been demonstrated that the purified lytic enzymes encoded in bacteriophage genomes, referred to by the generic term “endolysins” or the shorter “lysins”, can be lethal if applied to the surface of Gram positive bacteria (30). Lysins display antimicrobial activity against Gram negative bacteria if used in combination with an outer membrane permeabilizer (6, 31). Using the purified lysin as a bioremediation for *Salmonella* on food surfaces would have several advantages. Such a preparation would have only one active ingredient instead of many, so regulatory approval may be more easily obtained (9).

The purified endolysin may have a broader range of lytic activity than its phage of origin and would be broader-spectrum that a single phage preparation or cocktail (9, 31). There is also some evidence that combining the use of such an endolysin with existing antimicrobial measures would enhance the efficiency of both treatments (10).

Bacteriophage Felix O1 is a lytic myovirus, a phage with an icosahedral head and a long, contractile tail. Felix O1 is unique for its specificity for and limitation to infection of the *Salmonella* genus (26). Previous studies have determined that a preparation of whole phage can control foodborne *Salmonella* DT104 in artificially contaminated chicken frankfurters (45). We have theorized that the purified endolysin of Felix O1 used in combination with a suitable membrane permeabilization agent will be active against foodborne *Salmonella* species. Before testing, the enzyme must be cloned from the phage, expressed, purified, and characterized.

2.0 Materials and Methods

2.1 Identification of the Felix O1 *lys* gene and prediction of protein structure

To confirm identification of LysO1 from a previous study and to identify active site residues, the protein sequence was analyzed with pblast, and Clustal was used to align LysO1 with known lysozymes CEWL, T4 E, and P22 Lyz, and the putative endolysins from phages similar to Felix O1: WV8 and phiEa21-4. Protein sequences or putative sequences from GenBank were entered into ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and default alignment parameters were selected.

The protein sequence was copied from GenBank and submitted to PHYRE as previously described (chapter 3, section 2.1.5). Sequences including *N*-terminal polyhistidine tags from expression vectors pRSET A (MRGSHHHHHH GMASMTGGQQ MGRDLYDDDD KDRWGS), used by our lab, and E1 (MGSSHHHHHH ENLYFQG), used by GenScript, were also submitted.

2.2 Cloning of the Felix O1 *lys* gene

Lys was amplified by PCR using primers O1LysF (5'-CCC**GAATTC**ATGCAACTCTCAAG AAAAGGTTTA-3') and O1LysR (5'-CCC**AAGCTT**TTACTTTGGATATACACTGTCAAG-3') (Invitrogen, Life Technologies, Carlsbad, CA; the restriction site in each primer sequence is shown in bold; the start codon and stop codon, in the F and R primer, respectively, are underlined). The PCR reaction was amplified in an iCycler (Bio-Rad, Hercules, CA) as follows. The reaction was incubated at 94 °C for 2 min, then 40 cycles of 94 °C for 30 s, 59 °C for 30 s, and 68 °C for 2.5 min. The reaction was incubated at 72 °C for 10 min, then held at 4 °C until needed. The product was gel-purified and the amplified gene and pRSET A expression vector (Invitrogen, Life Technologies, Carlsbad, CA) were restricted with BamHI and HinDIII (Promega, Fitchburg, WI) overnight at 37 °C. Each product was purified, and the gene was ligated into pRSET A. The ligated product was transformed into Mach 1 *E. coli* (Invitrogen, Life Technologies, Carlsbad, CA), spread-plated on tryptic soy agar (TSA; Difco, BD, Franklin Lakes, NJ) containing 100 µg/ml ampicillin, and incubated overnight (ON) at 37 °C.

Insertion efficacy was tested using three methods. Six ampicillin-resistant colonies were inoculated into TSB containing selection antibiotics and grown ON at 37 °C with shaking. The plasmids, designated pRSETA/LysVT-1 – pRSETA/LysVT-6, were repurified from those cultures using a QIAgen miniprep kit (QIAgen, Hilden, Germany) according to package instructions. Each purified plasmid was analyzed by PCR using *lys*-specific primers. PCR-positive clones were restricted with *Bam*HI and *Hin*dIII, and the reaction was analyzed by gel electrophoresis using a 1% agar gel. Plasmids positive for both PCR and restriction were sent to the Virginia Bioinformatics Institute (VBI, Blacksburg, VA) for sequencing.

2.3 Expression

pRSETA/Lys1 or the empty vector pRSET A used as a control were purified from Mach 1 *E. coli* and transformed into BL21 Star (DE3) pLysS *E.coli* (Invitrogen, Life Technologies, Carlsbad, CA). The transformed culture was plated on TSA containing 100 µg/ml ampicillin and 34 µg/ml chloramphenicol

selection antibiotics and incubated overnight at 37 °C. The plate was scraped with 1 ml tryptic soy broth (TSB; Difco, BD, Franklin Lakes, NJ) and the fluid was collected and diluted 1:60 in 25 ml TSB containing the same selection antibiotics, incubated at 37 °C with shaking to a turbidity of 60-70 Klett units (OD_{600} of approximately 0.35), and induced for two hours with isopropyl β-D-1-thiogalactopyranoside (IPTG) to 0.5 mM. Cells were collected by centrifuging in a Centra GP8R centrifuge (Thermo Fisher Scientific; Waltham, MA) at 1,860 x *g* for 30 min at 4 °C. Purification was done immediately after expression.

Optimal expression time was determined by western blot. One-milliliter samples of the expression and control cultures were taken just before induction and every hour after induction for four hours. Samples were centrifuged in a tabletop microcentrifuge 5415 R (Eppendorf) at 5,000 x *g* for 2 min at room temperature. The supernatant was removed and pellets were stored at -20 °C overnight. Pellets were resuspended in 100 µl sterile PBS and boiled 5 min. A 30-µl sample of each was taken, 30 µl Laemmli buffer was added, and samples were boiled 5 min, then centrifuged 15 min at 15,300 x *g*. Ten microliters of supernatant from samples taken before induction and 2, 3, and 4 hr after inducation were loaded on a 12% Tris·HCl gel and 200 V direct current was applied for 30 min. The gel was removed from the casing and soaked in western transfer buffer 40 min, then blotted onto a nitrocellulose membrane (pore size 0.45 um, Bio-Rad, Hercules, CA) for 30 min using 10 V direct current. The membrane was pre-blocked with 2% milk in sterile PBS at 4 °C overnight. It was then rinsed 3 times with PBS-Tween 20 and treated with 10 µl 0.1 mg/ml mouse anti-pentaHis antibodies (QIAgen) diluted in 10 ml PBS-Tween 20 for 1 hr at room temperature with swirling. The primary antibody was removed, the membrane was rinsed 3 times with PBS-Tween 20, and 100 µl 0.1 mg/ml goat anti-mouse horseradish peroxidase was added. It was then swirled 1 hr at room temperature, the secondary antibody was removed and the membrane was treated with a small amount of peroxide substrate. When color developed, the membrane was rinsed with deionized water to halt the reaction.

2.4 Solubility

An initial test to determine protein solubility was conducted as follows: 30-µl samples of the lysed expression cultures were centrifuged in an Eppendorf 5415 R tabletop microcentrifuge (Eppendorf; Hamburg, Germany) at 15,700 x *g* and 24 °C for 0, 1, 2, 5, or 10 min, and supernatants were analyzed by SDS-PAGE on a 12% Tris·HCl polyacrylamide gel (Remel, Thermo Fisher Scientific; Waltham, MA). The protocol was then repeated with alterations: 4 1-ml samples of lysate of induced culture were centrifuged 25 min at 15,700 x *g* at 24 °C. The supernatants were transferred to another tube, a 30-µl sample was taken of each, and then ammonium sulfate powder was added to 0, 40, 60, or 80% saturation and incubated 1 hr at room temperature (RT) with occasional inversion. After 1 hr, the suspensions were centrifuged 15 min at 10,000 x *g*. When centrifugation under those conditions failed to pellet the precipitated protein, the suspensions were centrifuged again for 20 min at 13,400 x *g*, pellets and supernatants were separated, and all pellets and supernatants were analyzed by sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE).

As an alternative method to determine the relative amount of expressed soluble protein, after a 2-hr expression the culture was divided into two aliquots of equal volume and purified in parallel under both denaturing and native state conditions by a QIAquick Ni-NTA kit (QIAgen, Hilden, Germany). Samples of all steps were analyzed by SDS-PAGE. Sterile glycerol to 20% total volume was added to the purified endolysin and it was stored at -20 °C until needed.

2.5 Purification

The endolysin was purified under denatured and native state conditions with a QIAquick Ni-NTA spin kit, according to manufacturer's instructions with minor changes. Briefly, cells of the expression culture were harvested by centrifugation at 1,890 x *g* for 35 min at 4 °C, resuspended in 630 µl lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8) with 70 µl 10 mg/ml chicken egg white lysozyme (CEWL; lysozyme from chicken egg white, 10 mg/ml, > 90% pure; Sigma-Aldrich, St. Louis, MO and freeze-thawed through 3 cycles, alternating -80 °C for 20 min and cold tap water for 20

min. The lysed culture was clarified by centrifugation (15,700 x *g*, 30 min, room temperature (RT)) and the supernatants applied to equilibrated nickel-nitrilotriacetic acid (Ni-NTA) 1 ml spin columns. To bind the enzyme to the column, columns were centrifuged 5 min with lids open at 300 x *g*, then washed twice with 600 µl wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8) to remove impurities and eluted twice with 200 µl elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8). All flow-throughs were saved for SDS-PAGE analysis. Sterile glycerol (Sigma-Aldrich, St. Louis, MO) was added to eluted protein to 20% and purified endolysin samples were stored at -20°C.

Denaturing conditions followed similar steps, but Buffers B (7 M urea, 0.1 M NaH_2PO_4;0.01 M Tris·HCl, pH 8), C (8 M urea, 0.1 M NaH_2PO_4;0.01 M Tris·HCl, pH 6.3), and E (8 M urea, 0.1 M NaH_2PO_4;0.01 M Tris·HCl, pH 4.5) were used instead of the lysis, wash, and elution buffers used for native state purification. The harvested cells were resuspended in 700 µl of Buffer B without added CEWL, and the pellet was incubated 1 hr at RT with mild agitation instead of being freeze-thawed. Expressed cells containing the vector control were included in all tests.

After purification, all products were analyzed by SDS-PAGE.

2.5.1 Alternative expression and purification - GenScript[1]

The endolysin gene as found in GenBank was synthesized *de novo* by GenScript with a tag in the construct, and the sequence was inserted into three expression vectors: E1, E3, and pGS21a (GenScript Corp., Piscataway, NJ). Plasmids were transformed into BL21 (DE3) *E. coli*. A single colony was inoculated into LB medium containing ampicillin or kanamycin; cultures were incubated at 37 °C with shaking at 200 rpm. Once cell density reached OD_{600} of 0.6-0.8, cultures were induced with isopropyl β-D-1- thiogalactopyranoside (IPTG). SDS-PAGE was used to monitor the expression. After a review of the preliminary expression data, construct E1 was selected for further expression. Endolysin was purified

[1] The characterization of LysO1 was done using LysO1 expressed and purified by GenScript. Here, both the GenScript-expressed enzyme and the enzyme expressed and purified in our lab are used. To avoid confusion, the GenScript-purified enzyme is referred to as "LysO1" and the enzyme expressed in our lab is referred to as LysO1-VT.

from the supernatant of 1 L of culture grown in LB medium and analyzed by western blot using antihistidine antibodies and by SDS-PAGE.

2.6 Determination of LysO1 activity

A plate of *S. enterica* Typhimurium grown overnight at 37 °C on TSA was flooded with 2 ml of 0.01 M Tris·HCl buffer [pH 8] and the fluid was scraped with a sterile plate scraper and collected. It was diluted 1:10 into 4 aliquots of the same Tris buffer and 4 aliquots of chloroform-saturated buffer (CHL) for a total volume of 1.5 ml per aliquot, and incubated at room temperature for 30 min with occasional inversion. The cells were centrifuged 5 min at 4 °C and 5,000 x *g*, then the pretreatment buffer was removed by washing the pellet twice in half volumes of the same Tris buffer and resuspending it in the same volume of 0.025 M Tris·HCl [pH 8]. Six 149-µl aliquots of each suspension were added to a 96-well plate, then three wells of each were treated with CEWL and three with LysO1, each enzyme was added to a final concentration of 0.1, 1.0, or 10 µg/ml. An equal volume of the respective enzyme storage buffers were used as negative controls (CEWL buffer: 0.025 M sodium acetate, pH 4.5, glycerol added to 50%; LysO1 buffer: 0.05 M Tris·HCl [pH 8] 0.15 M NaCl, glycerol added to 10% final volume). Optical density at 600 nm (OD_{600}) was measured at 26 °C at 1.5, 2, 3, 4, 5, 7, 10, and 15 min after addition of the enzymes (SpectraMax 340PC^{384} microplate reader, SoftMax Pro Data Acquisition and Analysis software, Molecular Devices, LLC, Sunnyvale, CA) with a 5-s plate mix before each measurement.

The experiment was then repeated with CEWL omitted, LysO1 added to 0.001, 0.01, or 0.1 µg/ml, and OD_{600} was measured before treatment and every minute after treatment for 10 min. LysO1 storage buffer was used as a control.

2.7 Characterization of the Felix O1 endolysin

2.7.1 Determination of optimal pH and ion concentration of antimicrobial activity

Ten 1-ml suspensions of *S.* Typhimurium were pretreated with 0.01 M Tris·HCl [pH 8] or the same chloroform-saturated Tris·HCl (CHL) and washed as described above. One of each group was

resuspended in twice the original volume of 0.01, 0.025, 0.05, 0.1, or 0.25 M Tris·HCl [pH variable]. Treated cells were aliquotted into a 96-well plate and treated as described. Optical density at 600 nm was measured before treatment and every minute after treatment for 10 min. The experiment was conducted using buffers of pH 5, 6, 7, 8, 9, and 10. Each was done in triplicate and repeated.

2.7.2 Determination of thermal stability of LysO1

Thermal stability of LysO1 was determined as follows. Ten-microliter aliquots of LysO1 were incubated at 0, 4, 22, 30, 37, and 40 °C for 30 min, then residual activity was determined using CHL-pretreated *S.* Typhimurium as previously described. LysO1 that had been stored at -80 °C was used as a control.

2.7.3 Effect of metal cations on LysO1 function

The effect of divalent cations on enzyme function was determined as follows. Eight aliquots of *S.* Typhimurium were pretreated with 0.01 M Tris·HCl buffer [pH 8] or CHL as previously described, with one exception. After the second wash, cells were resuspended in twice the original volume of 0.025 M Tris·HCl [pH 8] to which $ZnCl_2$, $MgCl_2$, or $CaCl_2$ had been added to 0.1, 1.0, or 5.0 mM. Six 149-µl aliquots of each suspension were added to a 96-well plate, and OD_{600} was measured. LysO1 to a final concentration of 0.1 µg/ml or the same volume of sterile storage buffer (1.0 µl) was added to three aliquots of each suspension. Optical density at 600 nm was measured just before enzyme treatment and every minute for 10 min after addition of the enzyme.

The effect of sodium on LysO1 activity was determined as follows. *S.* Typhimurium was pretreated with buffer or CHL as described, then washed twice and resuspended in twice the original volume of 0.025 M Tris·HCl [pH 8], or buffer to which NaCl had been added to 10, 25, 50, 100, and 250 mM. Six 149-µl aliquots of each suspension were added to a 96-well plate and OD_{600} was measured. Cells were treated with storage buffer or with LysO1 to a final concentration of 0.1 µg/ml, and OD_{600} was measured every minute for 10 min.

2.7.4 Effect of LysO1 on non-Typhimurium Salmonella *serovars*

The effect of LysO1 on *Salmonella* serovars Agona, Montevideo, Muenster, Newport, Reading, Seftenberg, and Uganda was determined using the activity assay described in section 2.6, by including the indicated serovars in the assay described for *S.* Typhimurium. *S.* Typhimurium was included in the assay as a positive control.

*2.7.5 Effect of LysO1 on non-*Salmonella *foodborne pathogenic bacteria*

The effect of LysO1 on *Escherichia coli* V517, *Campylobacter jejuni* subsp. *jejuni* (ATCC strain 43446), and *Listeria monocytogenes* (ATCC strain BAA-769) was determined using the activity assay described above, by substituting the indicated organism for the *S.* Typhimurium in the original assay. *S.* Typhimurium was included in the assay as a control. *C. jejuni* and *L. monocytogenes* were propagated 48 hr on Colombia blood with 5% sheep's blood (Thermo-Fisher Scientific, Waltham, MA). *C. jejuni* was incubated in reduced atmosphere conditions. *E. coli* and *S.* Typhimurium were propagated overnight on TSA. All organisms were incubated at 37 °C.

2.7.6 Lethality of the LysO1 against S. Typhimurium

2.7.6.1 Cells pretreated with chloroform-saturated buffer

S. Typhimurium was pretreated with Tris or CHL, washed, and resuspended as described previously. Six 149-µl aliquots each of two 1-ml suspensions pretreated with Tris and 2 with CHL were added to a sterile 96-well plate with lid, and treated with LysO1 or buffer control as described previously. Optical density at 600 nm was measured at 12.5 and 17.5 min after treatment. Samples were stamped onto TSA using a Repliplater (FCM Corporation, Madison, WI) before treatment and immediately after measurements of optical density. Each sample was taken in duplicate. Plates were allowed to dry and incubated at 37 °C overnight.

The experiment was repeated with the following changes: A 150-µl aliquot of *S.* Typhimurium was diluted in 1350 µl CHL pretreatment, incubated and washed as described in section 2.6, and

resuspended in 1 ml 0.025 M buffer after final wash. Optical density at 600 nm was measured before and at 1 and 5.5 min after treatment. After each measurement, samples were diluted ten-fold into cold, sterile PBS. After all samples had been collected, 100 µl of each sample was ten-fold serially diluted in sterile PBS to 10^{-8}, and 5 10-µl aliquots of each dilution were plated on TSA plates to determine number of viable cells per ml of suspension medium.

The experiment was repeated again with the following changes: three plates of *S.* Typhimurium were incubated overnight at 37 C. Cells were collected by flooding each plate with 2 ml 0.01 M Tris·HCl [pH 8], allowing the buffer to incubated for one minute, then scraping the plate with a sterile plate scraper and collecting the fluid. Two 100 µl aliquots of each culture were diluted into 900 µl 10 mM Tris or CHL and all diluted suspensions were incubated 30 min at room temperature with occasional inversion. Suspensions were washed twice in half volumes of 0.01M Tris and resuspended in 1 ml 0.025 M Tris, and treated with LysO1 to a final concentration of 0.1 µg/ml or an equal volume of storage buffer (1.7 µl). A 100-µl sample of each suspension was taken before and 5 min after treatment, spread plated on TSA, and incubated overnight at 37 °C. The experiment was then repeated with one alteration: instead of pipetting to mix suspensions before sampling, suspensions were pulse-vortexed 3 s.

To increase the number of viable cells after CHL pretreatment, the experiment was altered as follows. An overnight broth culture of *S.* Typhimurium was diluted 1:500 in 1 L fresh TSB and incubated with shaking 6.5 hr at 37 °C. The culture was collected by dividing into 180-ml aliquots in six 250-ml centrifuge bottles and centrifuging in a Sorvall RC5C centrifuge (Thermo Fisher Scientific, Waltham, MA) 15 min at 5000 x *g* at 10 °C using a GSA rotor. Pellets were decanted and cells were resuspended in 150 ml CHL and incubated 30 min at room temperature with occasional swirling. Cells were washed twice in half volumes of 0.01 M Tris·HCl [pH 8], resuspended in minimal volumes of 0.025M Tris·HCl [pH 8], and pooled into two suspensions. Each was removed to a sterile glass test tube and the volume was adjusted to 10 ml using 0.025 M Tris·HCl [pH 8]. One tube was treated LysO1 to a final concentration of 10 µg/ml, the other tube was treated with an equal volume of a buffer control (26 µl). A Klett meter (Klett Manufacturing Corporation, Inc., New York, NY) was used to measure turbidity before

and 1 and 10 min after treatment. Samples of 10, 100, and 1000 µl were taken from each suspension immediately after turbidity measurements, spread-plated on TSA and incubated overnight at 37 °C.

The experiment was repeated with the following alterations: a 400-ml culture of *S.* Typhimurium was grown with shaking for 6 hr. It was divided into two 250-ml RC5C centrifuge bottles and centrifuged as described for 10 min. The supernatant was decanted and pellets were resuspended in 10 ml 0.01M Tris·HCl [pH 8], then 130 ml CHL was added to each bottle. Suspensions were incubated 30 min at room temperature with occasional swirling, then washed twice in 75 ml 0.01 M Tris·HCl [pH 8], and resuspended in 0.025 M Tris·HCl to 10 ml total volume. Each suspension was removed to sterile glass test tube. Suspensions were treated with LysO1 to a final concentration of 11 µg/ml or an equal volume storage buffer (30 µl), and turbidity was measured and samples taken as described above, just before treatment, and at 5 and 30 min after treatment.

To determine the effect of CHL on the *S.* Typhimurium outer membrane, the experiment was repeated with the following changes: *S.* Typhimurium was propagated as before, but divided into four 100-ml aliquots. Cells were collected, resuspended, and treated with CHL as described. The additional two aliquots were pretreated with Tris buffer without added chloroform. Cells were washed and resuspended in 0.01 M Tris·HCl [pH 8] as described and removed to sterile glass test tubes. One suspension of each pretreatment group was treated with enzyme, the other with a buffer control, and samples were collected and turbidity measured before and 5 min after enzyme treatment.

Additional 500-µl samples of each cell suspension were collected at all sample times, diluted 1:1 in Karnovsky's fixative, and stored overnight at 4 °C. Samples were washed twice in 0.1 M Na Cacodylate for 15 min each, and post-fixed 1 hr in 1% OsO4 in 0.1 M Na Cacodylate. Samples were dehydrated 15 min per solution in graded ethanol solutions increasing in concentration as follows: 15%, 30%, 50%, 70%, 95%, and 100%, then submersed in propylene oxide 15 min. Samples were then infiltrated with a 50:50 solution of propylene oxide:Poly/Bed 812 for 12 hr and infiltration was completed by immersing in 100% Poly/Bed 812 for 12 hr. Samples were cured at 60 °C for 12 hr, then sectioned to a thickness of 60-90 nm, placed on nickel grids and viewed on a JEM 1400 electron microscope (JEOL,

Ltd., Tokyo, Japan) at a magnification of 10,000. The number of intact and lysed cells was determined for three pictures of different areas of each sample. Each picture was of the same magnification and contained at least 100 cells in the frame.

2.7.6.2 Cells pretreated with Tris(hydrosymethyl)aminomethane·EDTA (Tris·EDTA)

Three 100-µl aliquots of an overnight culture of *S*. Typhimurium were diluted into three 25-ml volumes of fresh TSB in 250-ml Klett flasks and grown to 70 Klett units. Cells were transferred to 50-ml screw-cap tubes and collected by centrifugation at 1,870 x *g* for 30 min. The supernatant was decanted and each pellet was resuspended in 1 ml Tris-EDTA (0.2 M Tris·HCl [pH 8]; 5 mM EDTA) and transferred to a 2-ml snapcap centrifuge tube. Cells were incubated 30 min at room temperature with occasional inversion, then washed twice in half volumes of 0.01 M Tris·HCl [pH 8] and resuspended in 1 ml 0.025 M Tris·HCl [pH 8]. Two 100-µl aliquots of each suspension were serially diluted ten-fold in 25 mM Tris buffer, from an initial concentration of 10^{10} CFU/ml to a final concentration of 10^{0} CFU/ml. Suspensions of 10^{4} to 10^{0} CFU/ml were treated with LysO1 to a final concentration of 0.1 µg/ml or an equal volume of storage buffer (1.7 µl) and inverted several time. A 100-µl sample of each treated suspension was spread-plated on TSA before and 5 min after treatment of enzyme. Plates were incubated overnight at 37 °C and examined the next morning. The experiment was then repeated using suspensions of $10^{7} – 10^{3}$ CFU/ml.

Three cultures of *S*. Typhimurium grown overnight on TSA were collected by flooding each plate with 2 ml 10 mM Tris buffer, allowing the plate to incubate 1 min at room temperature, then scraping the plate with a sterile plate scraper and collecting the fluid. Each suspension was diluted 1:10 in 0.2 M Tris; 5.0 mM EDTA, and incubated 30 min at room temperature with occasional inversion. Suspensions were washed twice in half volumes 0.01 M Tris and resuspended in 1 ml 0.025 M Tris. LysO1 to 0.1 µg/ml or an equal volume of storage buffer (1.7 µl) was added to each tube, tubes were inverted several times, and incubated at room temperature 7 min. Samples of 100 µl were taken from each tube before and after treatment, ten-fold serially diluted in sterile PBS, and plated on TSA. The experiment was then repeated

with the following change: after pretreatment, suspensions were diluted 100-fold in 0.025 M Tris buffer, then sampled, treated, and sampled again.

S. Typhimurium was propagated and diluted as described, into 0.01 M and 0.2 M Tris buffer, or the same buffer containing 1.0, 3.0, or 5.0 mM EDTA. Suspensions were incubated 30 min at room temperature, washed twice, and resuspended in 2 ml 0.025 M Tris. Nine 149-µl aliquots of each suspension were added to a sterile 96-well plate. Optical density at 600 nm was measured, a 100-µl sample of each pretreatment group was ten-fold serially diluted in 0.025 M Tris and plated, OD_{600} was measured again, then cells were treated with LysO1 to a final concentration of 0.1µg/ml or an equal volume of storage buffer (1 µl). Optical density at 600 nm was measured immediately after addition of the enzyme, then every minute for five minutes. After OD_{600} measurements, 100-µl samples of the buffer- and Lys-treated cells from each pretreatment group were ten-fold serially diluted in 0.025 M Tris buffer and plated on TSA. All plates were incubated overnight at 37 °C.

2.8 Statistical analysis

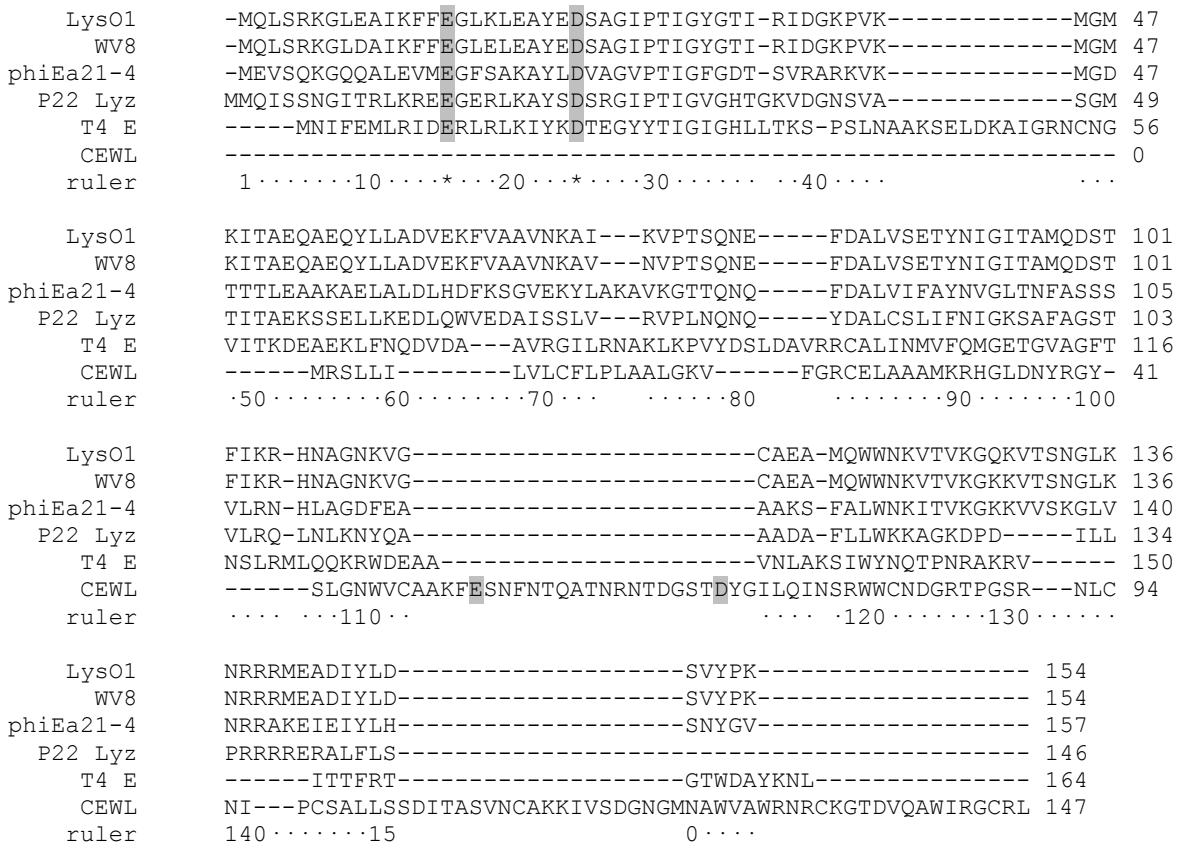
The calculation to determine rate of reaction of LysO1 and of the control treatments was adapted from Rao and Burma, 1971 (35). Briefly, the second OD reading taken after treatment was subtracted from the first reading taken after treatment. The difference was divided by the elapsed time in minutes, and the absolute value of the result was used as the rate of reaction. The data points were analyzed using JMP 9.0 (SAS Institute Inc., Cary, NC). The importance of individual experimental factors and the interactions among experimental factors was analyzed using a full factorial Least Mean Squares (LMS) analysis. The importance of factors and interactions among them was based on the calculated F Ratio. Factors and interactions that had no effect on experimental outcomes were removed from the analysis. The effect of specific variables on LysO1 activity was analyzed using an analysis of variance (ANOVA) and Student's t-test. Data that were not normally distributed were transformed using a BoxCox transformation and reanalyzed.

3.0 Results

3.1 Sequence alignment and structure prediction

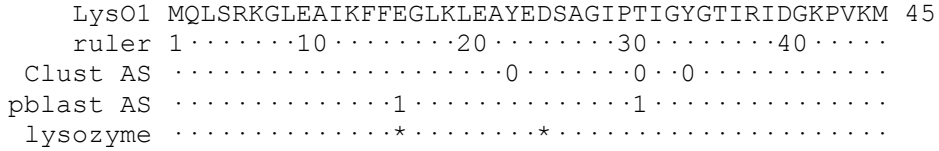
The Felix O1 endolysin was identified in a previous study by homology to the phage AB2 endolysin (46). To identify the amino acids necessary for enzyme activity, a sequence alignment and pblast were used to analyze the sequence. A protein sequence alignment of LysO1 with putative lysins of closely related phages and known lysozymes indicated that LysO1 possessed significant sequence identity to the putative lysins of phages WV8 (97% identity) and phiEa21-4 (44% identity), and to *Salmonella*-specific P22 Lyz (40% identity) (Figure 4.1). Clustal identified essential active site residues for all six aligned sequences as tyrosine, threonine, and glycine. In the LysO1 protein sequence, they are at positions 22, 30, and 32, respectively. A pblast search suggested that the active site residues are at positions 15 and 30, which are glutamic acid and threonine (Figure 4.1 b).

Figure 4.1. Sequence alignment of LysO1 with known and putative lysozymes.



Sequence alignment of LysO1 with known and putative lysozymes. Highlighted amino acids and asterisks at positions 15 and 24 mark amino acids glutamic acid and aspartic acid, the two residues thought to be essential to enzyme activity.

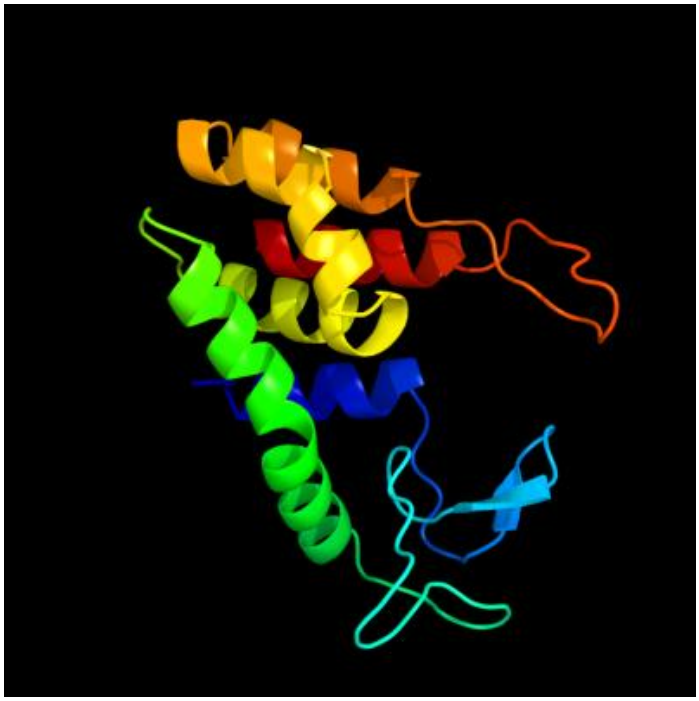
Figure 4.1 b. Active site residues



Active site residue of LysO1 predicted by clustalW2, pblast, and comparison with other lysozymes. Clustal predicted tyrosine, threonine, and glycine at positions 22, 30, and 32 as the active site residues (positions marked with a "0" on the Clust AS ruler). Pblast predicted glutamic acid and threonine at positions 15 and 30 (positions marked with a "1" on the pblast AS ruler). The lysozyme ruler marks glutamic acid and aspartic acid at positions 15 and 24 as active site residues based on sequence identity and presumed homology between LysO1 and the lysozyme superfamily, and the known active site residues of other lysozymes.

PHYRE predicted six secondary structures of the untagged LysO1 sequence: six α helices and one β sheet (Table 4.1a). The tertiary structure, shown in Figure 4.2, was predicted by fold recognition and most similar to the structure of phage P22 Lyz. PHYRE predicted very short (1-5 aa) α and β sequences in the pRSET A tag, and a 10-aa α helix formed by the E1 tag (Table 4.1b).

Figure 4.2. Model of the tertiary structure of LysO1, as predicted by PHYRE, v. 2.0



The primary protein sequence of LysO1 was obtained from GenBank (access number NP_944846.1) and submitted to PHYRE, v. 2.0 (25). The model is based on the P22 lysozyme template. The positions of 146 of 164 residues (95%) were predicted with 100% confidence, and the sequence identity was 40%. The model is rainbow-coded; the *N*-terminus is red, the *C*-terminus is blue.

Table 4.1. Summary of secondary structures of **a.**LysO1 and **b.** polyhistidine tags predicted by PHYRE.

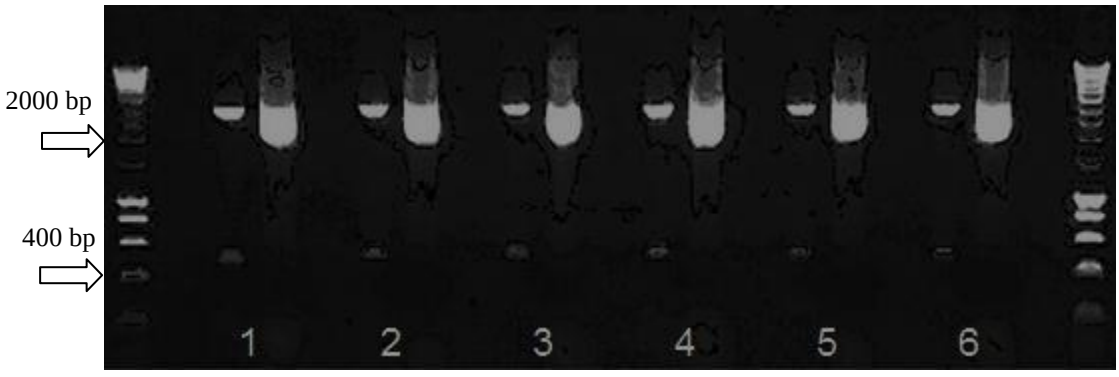
Predicted Secondary Structures - Endolysin			
structure	**start**	**end**	**length**
alpha	5	14	10
beta	29	36	8
alpha	51	72	22
alpha	79	91	13
alpha	100	107	8
alpha	111	124	14
alpha	134	148	15

Predicted Secondary Structures - Tags				
tag	**structure**	**start**	**end**	**length**
pRSET A endolysin	beta	7	7	1
	alpha	8	9	2
	beta	12	16	5
	beta	20	20	1
pRSET A rIIA E1	alpha	20	20	1
	alpha	32	33	2
	alpha	8	17	10

3.2 Cloning

In six tested samples, the uncut plasmid control was visible as a single band, and a sample of the plasmid after restriction showed two bands – one between 2200 and 2300 bp, the size of the plasmid, and one just over 400 bp in length (Figure 4.3). Samples of all six plasmids were sent to VBI for sequencing, and results revealed one of the samples, pRSETA/Lys5, was missing the N-terminal methionine start codon. Sequencing data of the remaining five plasmids confirmed the gene was inserted into the plasmid in the correct orientation with no deletions or mutations.

Figure 4.3. Gel of the restriction reaction of the six plasmids pRSETA/LysO1.

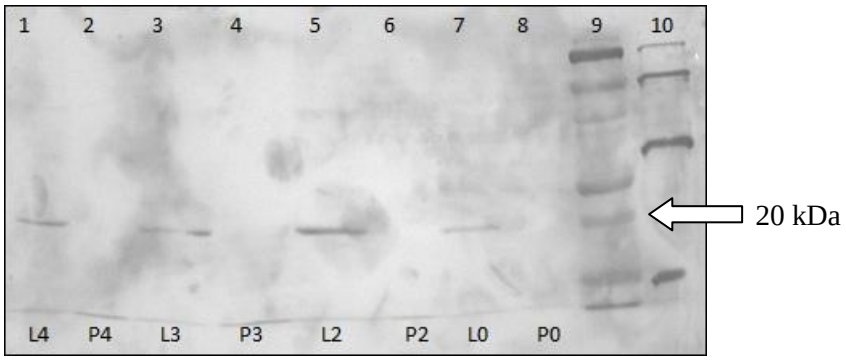


The six clones of pRSETA/LysO1 were digested with *Bam*HI and *Hin*DIII and analyzed on a 1% agarose gel. The uncut plasmid is the right lane of each pair, the cut plasmid is the left. Two distinct bands are clearly visible in each of the samples of restricted plasmid. At just over 400 bp in size, the smallest band is the correct size to be the 464-bp endolysin gene.

3.3 Expression of LysO1-VT and LysO1.

As sequencing data indicated that all constructs except pRSETA/LysVT-5 were identical, one plasmid, pRSETA/LysVT-11, was chosen for further development. Initial expression attempts induced with 1.0 mM IPTG at a turbidity of 60 Klett units for four hours, with samples taken every hour for analysis. SDS-PAGE and western blot analysis both indicated that optimal expression occurred at 2 hr after induction, and the amount of expressed protein had decreased by 4 hr after induction (Figure 4.4).

Figure 4.4. Western blot of expressed Felix O1 endolysin LysO1-VT.



Expression of LysO1-VT was induced for four hours. Mouse anti-pentaHis was used as the primary antibody and goat anti-mouse horseradish peroxidase was the secondary antibody. The membrane was then treated with peroxide substrate until color developed. Lane 10 is the 6xHis protein ladder (15 – 100 kDa) that served as the positive control, lane 9 contains the protein standards ladder (10 – 250 kDa). Samples labeled P were taken from a culture containing pRSET A with no insert. Samples labeled L were taken from a culture containing pRSETA/LysVT-1. The number indicates the time in hours after induction of expression that the sample was taken.

The amount of expressed protein was lower than expected and various methods were attempted to increase the yield: induction at 180 Klett units instead of 60, incubation at room temperature with no shaking after induction instead of 37°C with shaking, use of Overnight Express™ autoinduction medium (Merck KGaA, Darmstadt, Germany) instead of TSB. None was successful. Induction with varying concentrations of IPTG indicated adding IPTG to 0.5 mM yielded equal amounts of the desired protein as the protocol's suggestion concentration of 1.0 mM.

Samples were taken during expression to determine if the expressed protein could be lethal to the host bacteria. Determination of viable cell counts of each culture determined that after induction, culture P decreased from an initial count of 2.16 x 10^8 CFU/ml at induction to 3.6 x 10^7 CFU/ml 1 hr post induction, and decreased to 8.0 x 10^5 CFU/ml 2 hr post-induction. Just before induction, L-60 (induced at 60 Klett units) had 1.98 x 10^8 CFU/ml. At 1 hr post induction that had decreased to 1.56 x 10^7 CFU/ml, and at 2 hr post induction it remained almost stationary, at 1.6 x 10^7 CFU/ml. Culture L-180 decreased from an initial count of 5.4 x 10^9 CFU/ml to 5.2 x 10^7 CFU/ml 1hr post-induction, then increased slightly, to 9.6 x 10^7 CFU/ml at 2 hr post-induction.

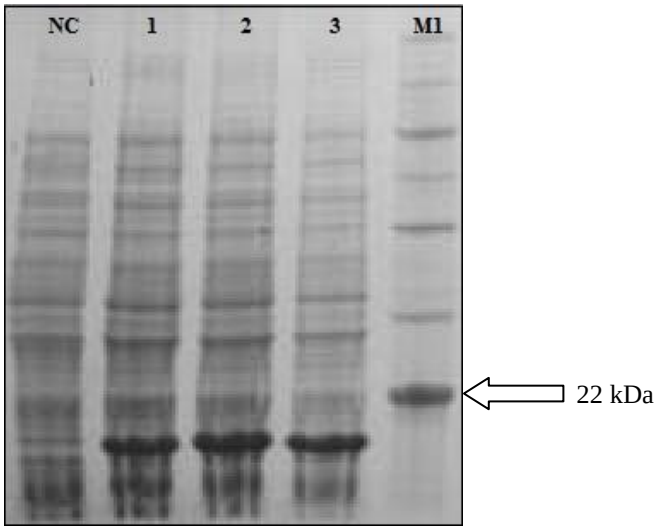
Three freeze/thaw cycles resulted in a reduction of approximately two orders of magnitude of viable cells for each culture, which equates to 99% mortality and presumed lysis. The number of viable cells in culture P (control culture containing a plasmid with no insert) decreased from 1.0 x 10^8 to 2.34 x 10^6 CFU/ml. The number of viable cells culture L (culture containing the plasmid with the gene of interest) induced at 60 Klett units decreased from 3.4 x 10^8 to 6.5 x10^6 CFU/ml. The culture L induced at 180 Klett units decreased from 5.2 x 10^8 to 5.4 x 10^5 CFU/ml, a decrease of three orders of magnitude.

The GenScript (Piscataway, NJ) pilot expression revealed optimal expression conditions (Figure 4.5). Subsequent expression in 1 L of LB broth produced an estimated 6.5 mg of LysO1 protein, obtained from the culture supernatant at a concentration of 3.48 mg/ml. SDS-PAGE and western blot analysis by GenScript revealed that the purified protein is between 15 and 22 kDa (Figure 4.6, 4.7). The molecular mass and isoelectric point were estimated from the sequence to be 19.2 kDa and 9.26, respectively. The protein was shipped on dry ice, and per instructions was aliquotted and stored at -80 °C upon arrival.

LysO1 expressed by our lab was slightly larger than the enzyme expressed by GenScript. From the sequence, LysO1 was calculated to be 17.2 kDa by Protein Molecular Weight (http://www.bioinformatics.org/sms/prot_mw.html). Our expressed protein was calculated to be 21.3 kDa by the same program, and 20 kDa from the molecular standards ladder used with SDS-PAGE. The protein from GenScript was calculated to be 19 kDa, and between 15 and 22 kDa from the standards used during

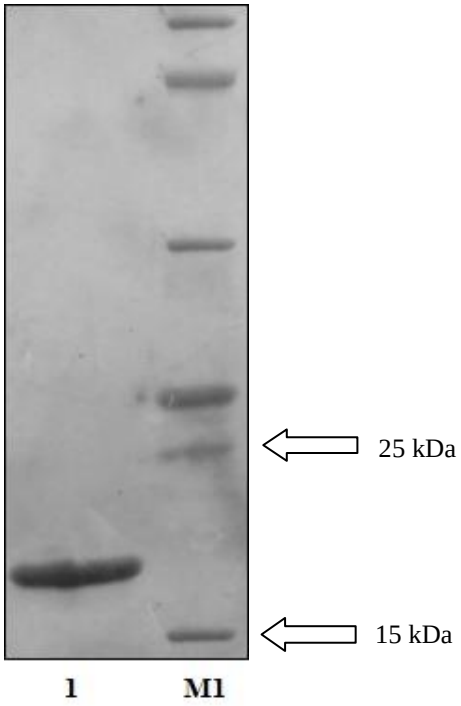
SDS-PAGE and western blot. The reason for the discrepancy is that the expression vectors incorporated different polyhistidine tags. The one used by our lab was 4 kDa, the one used by GenScript was 2 kDa.

Figure 4.5. SDS-PAGE analysis of LysO1 expression from GenScript Construct E1



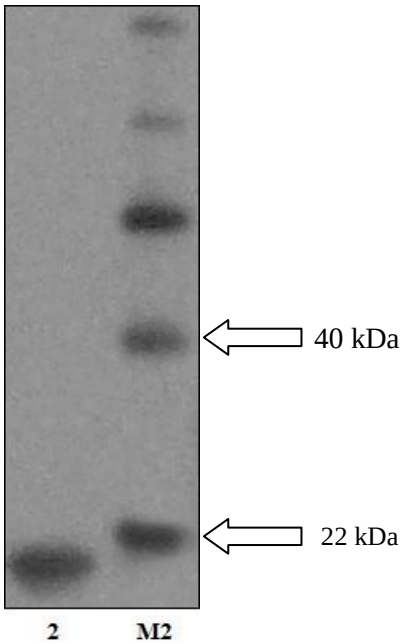
Expression of LysO1 (GenScript) under different conditions and followed by Coomassie Brilliant Blue Staining. Lane NC is the uninduced cell lysate, Lane 1 is the cell lysate after overnight induction with 1.0 mM IPTG at 15 °C. Lanes 2 and 3 are the cell lysate after induction for 4 hours by 1.0 mM IPTG at 37 °C. Lane M1 is the low-weight marker (figure from GenScript)

Figure 4.6. SDS-PAGE of GenScript-expressed LysO1.



LysO1 was purified from the supernatant of a 1 L culture of BL21 DE3 *E. coli* incubated at 37 °C that had been transformed with the E1 expression construct containing the *de novo*-synthesized LysO1 gene, grown to an OD_{600} of 0.6, and induced with IPTG. The SDS-PAGE was run on a 4%~20% gradient gel. Lane 1: SDS-PAGE analysis LysO1 (2 μg) followed by Coomassie Blue staining (figure from GenScript).

Figure 4.7. Western blot of analysis of GenScript-expressed Felix O1 endolysin using antihistidine antibodies.

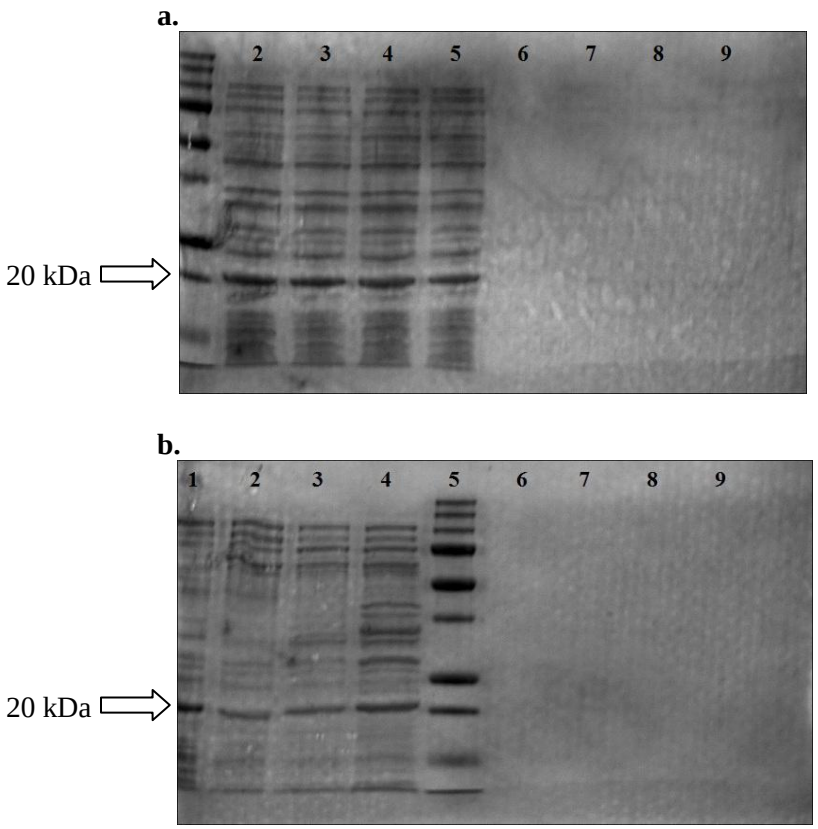


Lane 2 is the expressed LysO1; Lane M2 is the protein marker (figure from GenScript).

3.4 Solubility of expressed LysO1-VT

The results of the solubility test are shown in Figure 4.8, a and b. Results from the SDS-PAGE analysis of solubility were inconclusive. If the expressed enzyme were soluble, a 20 kDa band should be visible in the first supernatant sample and in the second pellet sample. If the expressed enzyme were aggregated into inclusion bodies, those aggregates should be large enough to easily pellet in the first centrifugation, so a 20 kDa band should be visible in the first pellet and nothing of that size should be present in the samples of the first supernatant, second pellet, or second supernatant. There was a moderate band at 20 kDa in the first set of pellets, indicating some of the protein aggregates into inclusion bodies after expression. However, there was also a moderate band in each sample from protein precipitation with ammonium sulfate, including the sample that was not precipitated. Precipitation with ammonium sulfate would indicate at least some of the protein was expressed in soluble form. A band of expressed protein from cell lysate that had already been clarified indicated that most of the protein is insoluble. There was no visible protein band in the samples of supernatant run.

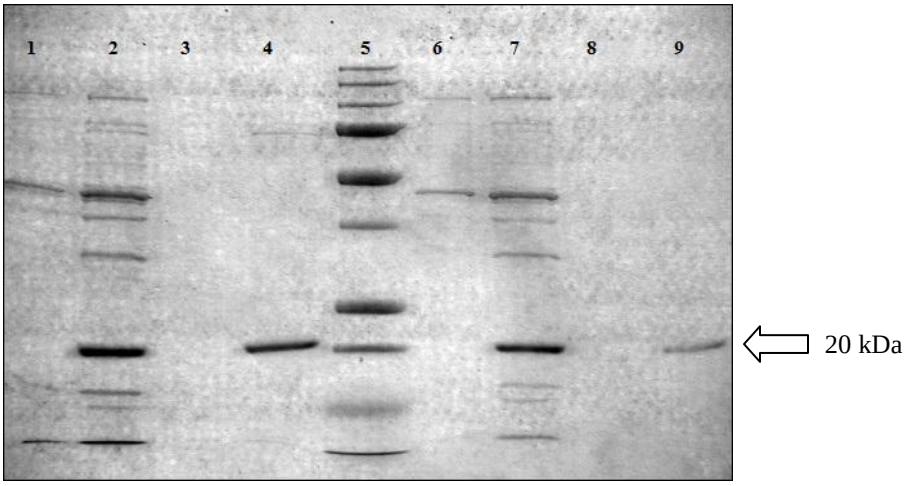
Figure 4.8 a, b. SDS-PAGE of solubility of LysO1-VT.



Samples of an expression culture of LysO1-VT. **A.** depicts samples of pelleted cell debris (lanes 2 – 5) and samples of clarified cell lysate (lanes 6 – 9) before precipitation with ammonium sulfate. The bands of 20 kDa protein visible are insoluble. **B.** depicts samples of pelleted cell debris after precipitation with, respectively, 0 (lane 1), 40 (lane 2), 60 (lane 3), and 80% (lane 4) ammonium sulfate. The 20 kDa protein is soluble protein precipitated by the ammonium sulfate treatment. Lanes 6-9 are samples of clarified cell lysate.

Results from parallel purification under denaturing and native state conditions and subsequent SDS-PAGE analysis revealed a band 20 kDa in size in samples of the denatured protein, and a smaller band in samples of the enzyme purified under native conditions (Figure 4.9).

Figure 4.9. SDS-PAGE of purification of LysO1-VT under denaturing or native state conditions.



LysO1-VT and the expression control were purified using a QIAgen Ni-NTA column under denaturing and native state conditions. Each column was eluted twice, and the first (lanes 1 – 4) and second (lanes 6 – 10) elutions were saved and tested separately. The expressed protein is 20 kDa in size. Lanes 2 and 7 are the expressed endolysin purified under denaturing conditions; lanes 4 and 9 are the expressed endolysin purified under native state conditions. Lanes 1, 3, 6, and 8 are the purification results from the expression control culture. Yield was lower than expected for purification under native state conditions.

3.5 Activity of LysO1

LysO1 activity was first assayed using *S*. Typhimurium pretreated with Tris buffer or chloroform-saturated Tris buffer as the substrate. The activity of CEWL was measured in parallel as an additional comparison. LysO1 and CEWL were used at the same concentrations: 0, 0.1, 1.0, and 10 µg/ml. A preliminary LMS analysis and ANOVA were conducted using LysO1 at a concentration of 0.1 µg/ml to determine the enzyme's effect on CHL-pretreated cells, compared to cells pretreated with Tris. A LMS analysis revealed that each factor (pretreatment with Tris or CHL, treatment with buffer or LysO1) and the interaction between them contributed to the experimental outcome (R^2adj 0.9252, Table 4.2).

Table 4.2. LMS analysis of LysO1 or buffer on *S*. Typhimurium pretreated with Tris or CHL.

R^2adj	0.9252	
Factor	F Ratio	Prob > F
1	64.8	< 0.0001
2	114.9	< 0.0001
1*2	108.0	< 0.0001

LMS analysis determines the relative importance of individual experimental factors and the interactions among them. Factor 1 is the pretreatment used (Tris or CHL), factor 2 is the treatment (buffer or LysO1). The F Ratio is the relative importance of that factor or interaction to the experimental results, and Prob > F is the p-value of the importance.

The group of cells pretreated with Tris and then treated with the storage buffer control had an observed rate of reaction of 0.0175 ΔOD_{600}/min; the group treated with LysO1 had an average rate of reaction of 0.0194 ΔOD_{600}/min (Table 4.3). An ANOVA revealed that the difference was not statistically significant (Prob > F = 0.7252). The LysO1 storage buffer used as the control treatment had no effect on CHL-pretreated *S*. Typhimurium. An ANOVA of CHL-pretreated cells revealed that there was a highly significant difference (Prob > F < 0.0001) in the rate of reaction of cells treated with buffer (0.0036 ΔOD_{600}/min) or with LysO1 (0.1293 ΔOD_{600}/min).

Table 4.3. Effect of LysO1 or buffer on *S*. Typhimurium pretreated with Tris or CHL.

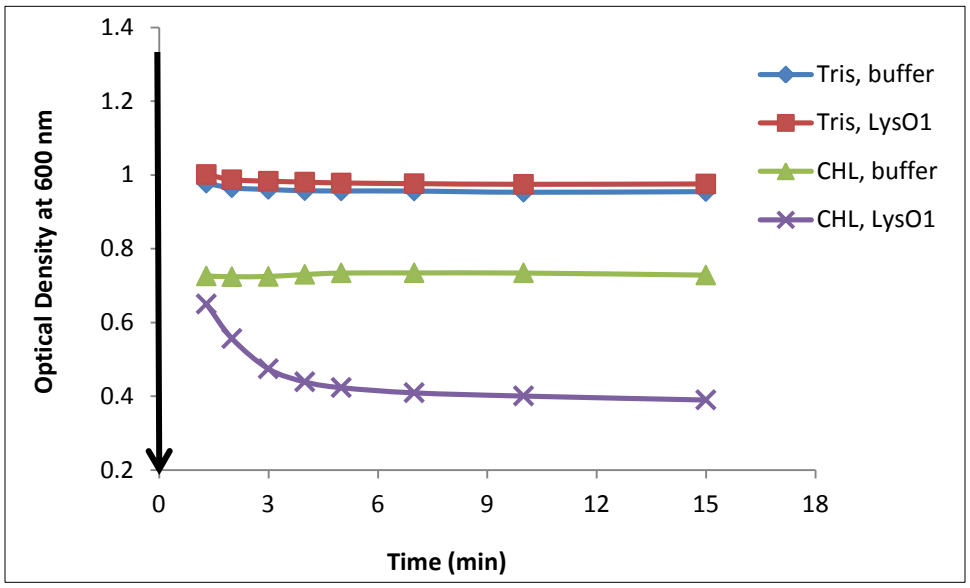
	Tris			**CHL**		
Enzyme	Rate	St. Dev.	Prob > F	Rate	St. Dev.	Prob > F
0	0.0175	0.0088	0.7252	0.0036	0.0017	< 0.0001
0.1	0.0194	0.0082		0.1293*	0.0237	

Enzyme concentration is given in µg/ml, rate is average rate of reaction, and is in ΔOD_{600}/min. Rates of reaction marked with an * are statically different from the buffer-treated control.

The results are further demonstrated in Figure 4.10: optical density of the Tris-pretreated cells did not decrease after treatment. The lower initial optical density of CHL-pretreated cells confirmed that

optical density of the cell suspension ass affected by CHL pretreatment, but an additional effect was visible only in cells that were then treated with LysO1.

Figure 4.10. Effect of LysO1 or buffer control on *S.* Typhimurium pretreated with Tris or CHL.



S. Typhimurium was pretreated with Tris or chloroform-saturated Tris (CHL). Cells were washed twice, resuspended in fresh 25 mM Tris buffer, then treated with LysO1 at a concentration of 0.1 µg/ml or the same volume of LysO1 storage buffer. The arrow indicates addition of the treatment; OD at 600 nm was measured at intervals after treatment. Tris and CHL denote the pretreatment used, buffer and LysO1 denote the treatment. Tests were done in triplicate and repeated.

A second analysis was conducted whereby each enzyme treatment was analyzed separately by LMS (R^2Adj CEWL = 0.5217, R^2Adj LysO1 = 0.9416). Results of the LMS analysis are summarized in Table 4.4. Both analyses revealed that the interaction between the chloroform pretreatment and the enzyme treatment had an effect on the experimental outcome (CEWL F Ratio = 11.3, LysO1 F Ratio = 223.4; Prob > F < 0.0001).

Table 4.4. LMS analysis of the effect of CEWL or LysO1 on *S*. Typhimurium.

	CEWL		LysO1 (0.1 - 10 µg/ml)		LysO1 (0.001 - 0.1 µg/ml)	
R^2adj	0.5217		0.9416		0.9678	
Factor	F Ratio	Prob > F	F Ratio	Prob > F	F Ratio	Prob > F
pretrt	18.1	0.0001	301.7	< 0.0001	153.2	< 0.0001
trt	2.12	0.1124	75.2	< 0.0001	199.4	< 0.0001
pretrt*trt	11.3	< 0.0001	79.3	< 0.0001	223.4	< 0.0001

Two experiments are depicted. One used CEWL or LysO1 to a final concentration of 0.1, 1.0 or 10 µg/ml. The second used LysO1 to a final concentration of 0.001, 0.01, or 0.1 µg/ml. Pretrt is the pretreatment used (Tris buffer or CHL), trt is the concentration of enzyme used. The F Ratio is an estimation of the importance of that factor or interaction to the observed results; Prob > F is the p-value of the F Ratio.

Table 4.5. Effect of CEWL or LysO1 on CHL-pretreated *S*. Typhimurium.

	CEWL				LysO1				Effective Units	
[Enzyme]	Rate	St. Dev.	%	p-value	Rate	St. Dev.	%	p-value	CEWL	LysO1
0	0.0036	0.0024	NA	NA	0.0036	0.0017	NA	NA	NA	NA
0.1	0.0039	0.0019	3.02	0.8645	0.1293*	0.0237	100	< 0.0001	3.9 ± 1.9	129.3 ± 23.7
1	0.0058	0.0043	4.49	0.3094	0.1534*	0.0136	118.6	< 0.0001	5.8 ± 4.3	153.4 ± 13.6
10	0.0152*	0.0041	11.75	< 0.0001	0.0609*	0.0134	47.10	< 0.0001	15.2 ± 4.1	60.9 ± 13.4

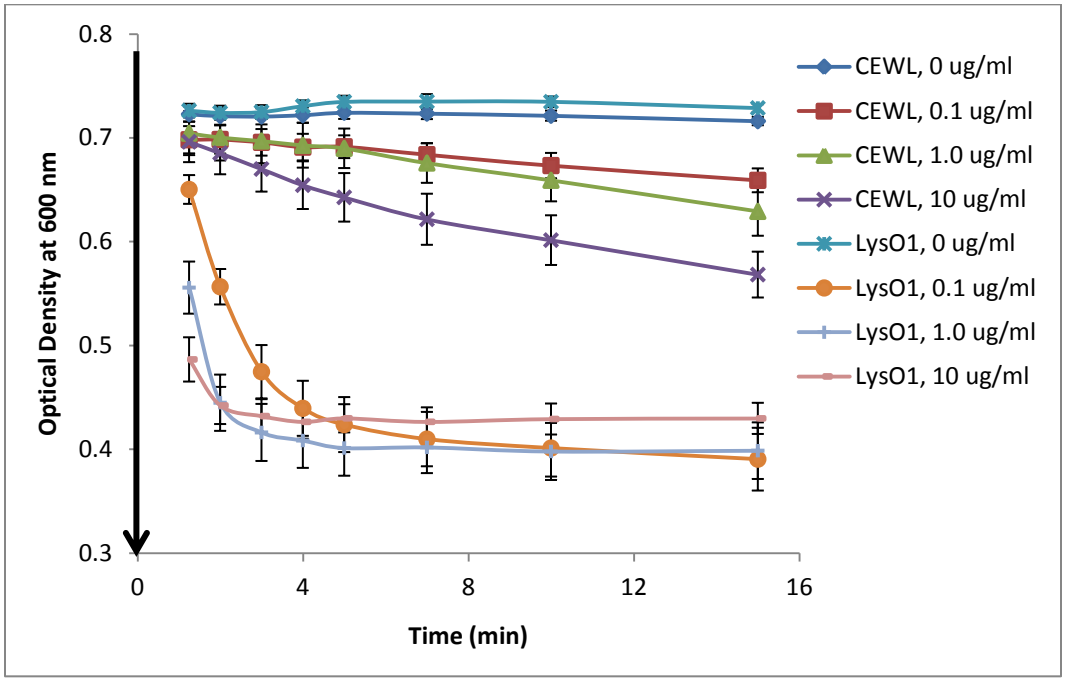
Enzyme concentration is given in µg/ml, rate of reaction is in ΔOD_{600}/min. A p-value less than 0.05 indicates a rate of reactions statistically different from the control. % gives the rate of reaction as a percentage of the rate of 0.1 µg/ml LysO1, which is 100%. One unit of lysozyme causes a decrease of 0.001 OD units in 1 min, Effective Units gives the units of each enzyme used in each assay.

When individual pretreatment/treatment groups were analyzed by ANOVA to determine the effects of enzyme or buffer on pretreated cells, it revealed that CEWL had no effect on cells pretreated with Tris (F Ratio = 2.8, Prob > F = 0.0635). Analysis suggested LysO1 had some effect (F Ratio = 6.73, Prob > F = 0.0026). The effect was concentration-dependent; the calculated rate of reaction decreased as the final concentration increased, and the effect was noticeable only at the highest concentration of LysO1 used. The rate of reaction of the buffer treated control was 0.0175 ΔOD_{600}/min. The rates of reaction of enzyme added to final concentrations of 0.1 and 1.0 µg/ml did not differ from the control (0.0194 ΔOD_{600}/min, 0.0104 ΔOD_{600}/min, p = 0.0803, p = 0.7214). The rate of reaction of LysO1 added to Tris-

pretreated cells to 10 µg/ml was 0.004 ΔOD_{600}/min, statistically different from the buffer-treated control ($p = 0.0022$).

An ANOVA and Student's t-test suggested that both enzymes reacted with the CHL-pretreated *S.* Typhimurium substrate in a concentration-dependent manner; results are summarized in Table 4.5. Over the observation period, the OD_{600} of cells treated with CEWL declined in a linear fashion (Figure 4.11). In contrast, the OD_{600} of cells treated with LysO1 exhibited a pattern of exponential decay; optical density declined rapidly through the initial part of the observation period, then remained steady through the end of the experiment. The rate of reaction of CEWL added to a final concentration of 10 µg/ml was 0.0152 ΔOD_{600}/min, which differed from the control rate of 0.0034 ΔOD_{600}/min ($p < 0.0001$). When CEWL was added to a final concentration of 0.1 and 1.0 µg/ml, the rate of reaction did not differ from that of the buffer-treated control. The tested concentrations of LysO1 were out of linear range of the enzyme. The highest rate of reaction of LysO1 was observed using a moderate amount of enzyme; LysO1 added to a final concentration of 1 µg/ml had a rate of reaction of 0.1534 ΔOD_{600}/min, compared to the rate of reaction of 0.0609 ΔOD_{600}/min observed when enzyme was added to a final concentration of 10 µg/ml, and 0.1293 ΔOD_{600}/min when added to 0.1 µg/ml. Rates of reaction of each concentration of enzyme differed from the rate of reaction of the buffer control (0.0036 ΔOD_{600}/min, $p < 0.0001$), and also from each other.

Figure 4.11. Comparison of the activity rates of CEWL and LysO1 on *S.* Typhimurium pretreated with CHL.



S. Typhimurium was pretreated with CHL 30 min at room temperature with occasional inversion, then cells were washed twice in Tris·HCl and resuspended in 0.025 M Tris·HCl [pH 8]. CEWL or LysO1 was added to 0.1, 1.0, or 10ug/ml final concentration, and optical density at 600 nm was measured at intervals. "0 µg/ml" denotes treatment with a volume of storage buffer equal to the volume of enzyme used in other treatment groups. The CEWL storage buffer was used as the CEWL control; the LysO1 storage buffer was used as the LysO1 control.

The assay was later repeated with the CEWL omitted, and with LysO1 used at final concentrations of 0, 0.001, 0.01, and 0.1 µg/ml. LMS analysis revealed that the interaction between the pretreatment and the enzyme treatment was an important element of the observed results (F Ratio = 223.4, Prob > F < 0.0001). When individual pretreatment/treatment groups were analyzed by ANOVA, LysO1 again reacted with the *S.* Typhimurium substrate in a concentration-dependent manner; the highest rate of reaction was observed in replicates that were treated with the highest concentration of enzyme. The rate of reaction on LysO1 at 0.1 µg/ml was 0.0872 ΔOD_{600}/min, higher than the rate of reaction of the buffer-treated control (0.0018 ΔOD_{600}/min, $p < 0.0001$). The rate of reaction of LysO1 at a final concentration of 0.01µg/ml was 0.0153 ΔOD_{600}/min, also different from the control ($p < 0.0001$). LysO1

added to 0.0001 µg/ml, while higher than the control rate, was not statistically different (0.0053 ΔOD_{600}/min, p = 0.1346).

3.6 Characterization of LysO1 activity

3.6.1 Optimal conditions

To determine the effect of pH on LysO1 activity, assays were performed at pH 5, 6, 7, 8, 9, and 10. The results of the LMS analysis of the pH study are summarized in Table 4.6. The effect of the pretreatment on the experimental outcome increased as pH increased; at pH 5, the F Ratio was 1.97 (Prob > F < 0.0001), at pH 10 it was 337.8 (Prob > F < 0.0001). The importance of enzyme treatment, as well as the interaction with the pretreatment, remained consistently high regardless of pH. As pH increased, the importance of pH as an experimental factor declined, as did the importance of the interaction of pH with other factors. The importance of the concentration of the cell suspension buffer increased as pH increased from 5 to 7, then declined as pH continued to increase. Results are summarized in Table 4.6.

Table 4.6. LMS analysis of the effect of pH on LysO1 activity.

	pH 5		**pH 6**		**pH 7**		**pH 8**		**pH 9**		**pH 10**	
R^2adj	0.9332		0.9552		0.9676		0.8924		0.9718		0.9573	
Factor	F Ratio	Prob > F	F Ratio	Prob > F	F Ratio	Prob > F	F Ratio	Prob > F	F Ratio	Prob > F	F Ratio	Prob > F
pretrt	1.97	< 0.0001	61.2	< 0.0001	48.9	< 0.0001	1.00	0.3199	159.3	< 0.0001	337.8	< 0.0001
trt	350.1	< 0.0001	661.2	< 0.0001	588.3	< 0.0001	419.7	< 0.0001	628.0	< 0.0001	648.8	< 0.0001
pretrt*trt	356.3	< 0.0001	399.7	< 0.0001	510.7	< 0.0001	273.7	< 0.0001	617.5	< 0.0001	566.0	< 0.0001
pH	421.9	< 0.0001	339.0	< 0.0001	152.5	< 0.0001	NA	NA	3.85	0.0521	2.52	0.1152
pretrt*pH	124.3	< 0.0001	175.6	< 0.0001	10.5	0.0015	NA	NA	12.3	0.0006	7.54	0.0069
trt*pH	225.3	< 0.0001	252.9	< 0.0001	35.4	< 0.0001	NA	NA	5.55	0.0201	4.82	0.0301
pretrt*trt*pH	347.4	< 0.0001	235.2	< 0.0001	55.2	< 0.0001	NA	NA	1.0	0.322	5.37	0.0222
buf	10.3	< 0.0001	16.1	< 0.0001	32.7	< 0.0001	21.5	< 0.0001	24.7	< 0.0001	17.1	< 0.0001
pretrt*buf	38.2	< 0.0001	67.8	< 0.0001	90.9	< 0.0001	13.6	< 0.0001	2.27	0.0655	10.1	< 0.0001
trt*buf	9.39	< 0.0001	28.8	< 0.0001	76.3	< 0.0001	23.4	< 0.0001	16.3	< 0.0001	10.5	< 0.0001
pretrt*trt*buf	15.5	< 0.0001	31.8	< 0.0001	68.4	< 0.0001	19.4	< 0.0001	7.44	< 0.0001	4.55	0.0019

LMS analysis reveals the importance of individual experimental factors and interaction between and among the factors in an experiment. A Prob > F value of 0.05 or below indicates that factor or interaction influenced the experimental results. Pretrt is pretreatment used (buffer or CHL), trt is treatment (LysO1 or buffer control), buf is the concentration of the cell suspension buffer. Two factors joined by an asterisk indicated the analysis of the effect of the interaction between those factors on the experimental results. Shaded cells denote that buffer of pH 8 was the control, so the importance of pH as a factor of that study could not be calculated.

Table 4.7. Effect of increasing pH and ion concentration on LysO1 activity.

pH	[Tris]	Rate	St. Dev.	%	p-value	pH	[Tris]	Rate	St. Dev.	%	p-value
5	10	0.0004*	0.0003	0.57	< 0.0001	6	10	0.0011*	0.0008	1.53	< 0.0001
5	25	0.0008*	0.0004	1.16	< 0.0001	6	25	0.0035*	0.0022	5.02	< 0.0001
5	50	0.0021*	0.0003	3.11	< 0.0001	6	50	0.0145*	0.0008	21.1	< 0.0001
5	100	0.0131*	0.0007	19.0	< 0.0001	6	100	0.0243*	0.0016	35.3	< 0.0001
5	250	0.0012*	0.0011	1.80	< 0.0001	6	250	0.0011*	0.0009	1.67	< 0.0001
8	25	0.0687	0.0021	100	NA	8	25	0.0688	0.0036	100	NA

pH	[Tris]	Rate	St. Dev.	%	p-value	pH	[Tris]	Rate	St. Dev.	%	p-value
7	10	0.0173*	0.0033	20.7	< 0.0001	8	10	0.0520	0.0079	83.9	0.1068
7	25	0.0381*	0.0033	45.7	< 0.0001	8	25	0.0620	0.0120	100	NA
7	50	0.0550*	0.0088	65.9	< 0.0001	8	50	0.0552	0.0150	89.1	0.2664
7	100	0.0375*	0.0030	44.9	< 0.0001	8	100	0.0389*	0.0102	62.8	0.0007
7	250	0.0004*	0.0003	0.42	< 0.0001	8	250	0.0012*	0.0007	1.88	< 0.0001
8	25	0.0834	0.0039	100	NA						

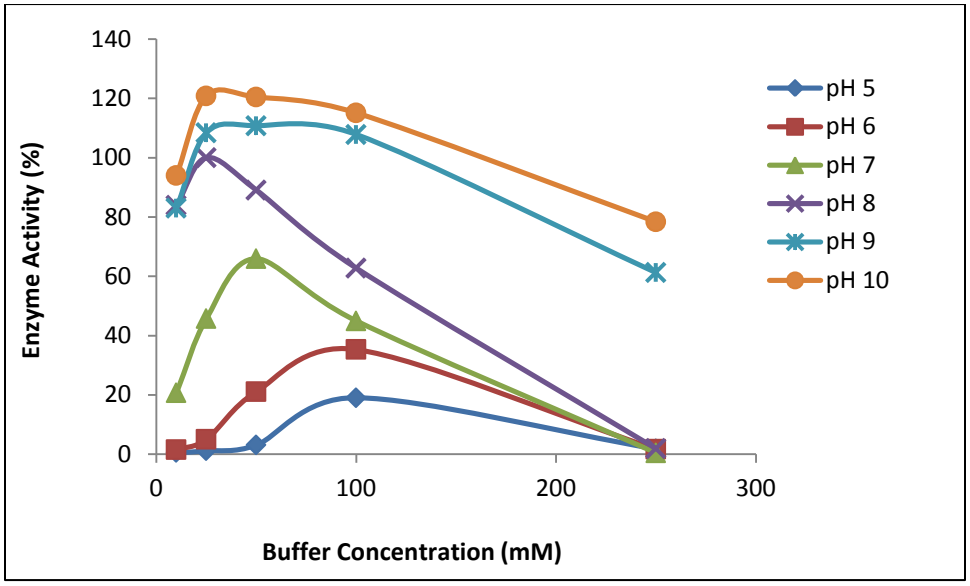
pH	[Tris]	Rate	St. Dev.	%	p-value	pH	[Tris]	Rate	St. Dev.	%	p-value
9	10	0.0603*	0.0029	83.0	< 0.0001	10	10	0.0765	0.0025	94.0	0.0974
9	25	0.0787*	0.0009	108.4	0.0008	10	25	0.0983*	0.0037	120.8	< 0.0001
9	50	0.0804*	0.0039	110.8	< 0.0001	10	50	0.0980*	0.0039	120.5	< 0.0001
9	100	0.0738*	0.0017	107.8	0.0016	10	100	0.0937*	0.0059	115.1	0.0002
9	250	0.0445*	0.0030	61.3	< 0.0001	10	250	0.0638*	0.0069	78.4	< 0.0001
8	25	0.0726	0.0035	100	NA	8	25	0.0814	0.0054	100	NA

Rate of reaction of LysO1 was measured at variable pH and buffer concentration. Concentration of Tris buffer is given in mM; the concentration that displayed the highest rate of reaction at each pH is shaded. Cells suspended in 25 mM Tris·HCl [pH 8], were used as controls and used to determine the % of activity observed at other pHs and buffer concentrations. A p-value less than 0.05, or an asterisk by the rate of reaction, indicates a rate of reaction different from the rate of the control. % gives the rate of reaction as a percentage of the rate observed in 0.025 M Tris·HCl [pH 8], which is 100%.

When the rate of reaction of LysO1 was measured under conditions of variable pH and ion concentration, the highest rates of activity were observed at basic pH and low ion concentration (Figure 4.12, 4.13). Very little activity was observed at the acidic pHs tested (Figure 4.12). The highest rate of reaction observed at pH 5 was 1.3% of the rate measured at pH 8, and at pH 6 the highest activity observed was 2.4% of the control rate. Both were observed in 0.1 M buffer. At neutral pH, the optimum observed was higher: 65.9% of the rate of pH 8 control, and occurred in 0.05M buffer. The highest

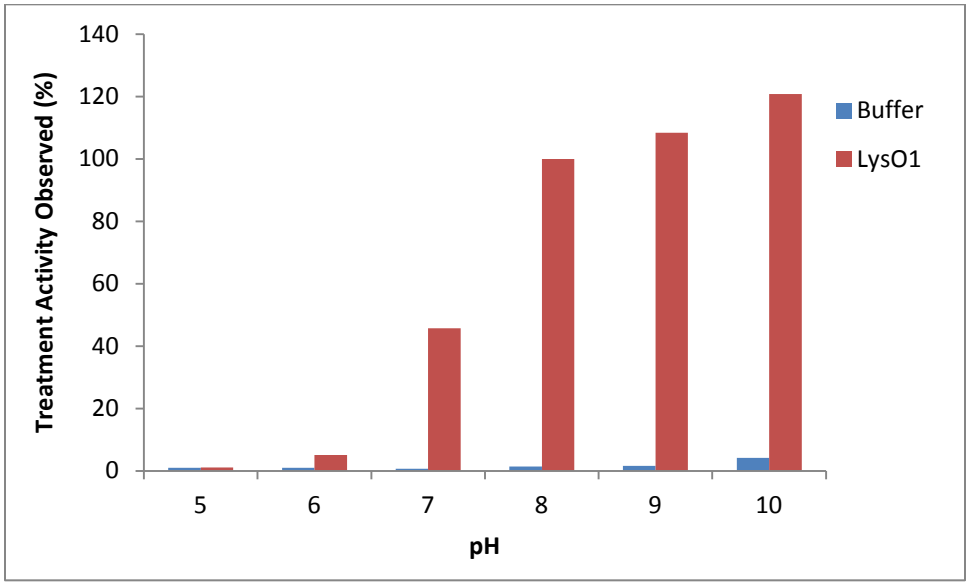
activity observed was at basic pH. At pH 9, the highest rate observed was 110% of the pH 8 control, and at pH 10 the observed rate was 120.7% of the pH 8 control, the highest rate observed (Figure 4.13). The optimal rate at pH 9 was in 0.05M buffer (the rate was not statistically different from the rate in 0.025 M buffer: p = 0.302) and the optimal rate at pH 10 was in 0.025 M buffer. Results are summarized in Table 4.7.

Figure 4.12. LysO1 activity as a function of buffer concentration at variable pH.



Activity was measure at pH 5, 6, 7, 8, 9, and 10. The comparison is based on the activity observed in 25 mM Tris·HCl [pH 8]. The concentration of the buffer is equal to the ionic strength *I* of the solution.

Figure 4.13. Observed LysO1 activity in 25 mM Tris·HCl at variable pH.



The observed activity of each treatment at each pH was calculated by comparing the observed rate in 25 mM buffer with rate of activity of LysO1 in 25 mM Tris·HCl [pH 8]. Results are displayed as a percentage.

To determine the thermostability of LysO1, the endolysin was diluted in storage buffer and aliquots were stored at 0, 4, 22, 30, 37, and 40 °C for 30 min, then residual activity of LysO1 was measured using *S.* Typhimurium as the substrate. When interactions were analyzed, the most important factors were chloroform pretreatment, enzyme treatment, and the interaction between them (F Ratio = 524.2, 2039.8, 1885.7, respectively; Prob > F < 0.0001, R^2Adj = 0.9638). Other factors and interactions were not significant contributors to the observed results; results are summarized in Table 4.8. However, when temperature-treated enzymes were analyzed on CHL-pretreated cells, there was a temperature-related difference in the performance of the enzyme. The highest rate of reaction was observed in the control enzyme kept under storage conditions until needed for the assay: 0.0702 ΔOD_{600}/min. The enzyme was most stable at 30 °C (0.0625 ΔOD_{600}/min, 89.0% of activity retained) and least stable at 40 °C (0.0549 ΔOD_{600}/min, 78.2% of activity retained). Each rate was statistically different from the control rate (p_{30} = 0.0035, p_{40}< 0.0001), and from each other (p = 0.0038). The activity after storage at 22 °C was 0.0605, no different from storage at 30 °C. Rate of reaction after storage at 0, 4, and 37 °C (0.059, 0.0592,

0.0579ΔOD_{600}/min) was different from the control ($p < 0.0001$), though not different from activity after storage at 30 or 40 °C. Results are summarized in Table 4.9 and Figure 4.14.

Table 4.8. LMS analysis of the thermostability of LysO1.

Thermostability		
R^2adj	0.9638	
Factor	F Ratio	Prob > F
pretrt	524.2	< 0.0001
trt	2039.7	< 0.0001
pretrt*trt	1885.7	< 0.0001
temp	0.63	0.7061
pretrt*temp	1.02	0.4129
trt*temp	1.29	0.2641
pretrt*trt*temp	0.68	0.6676

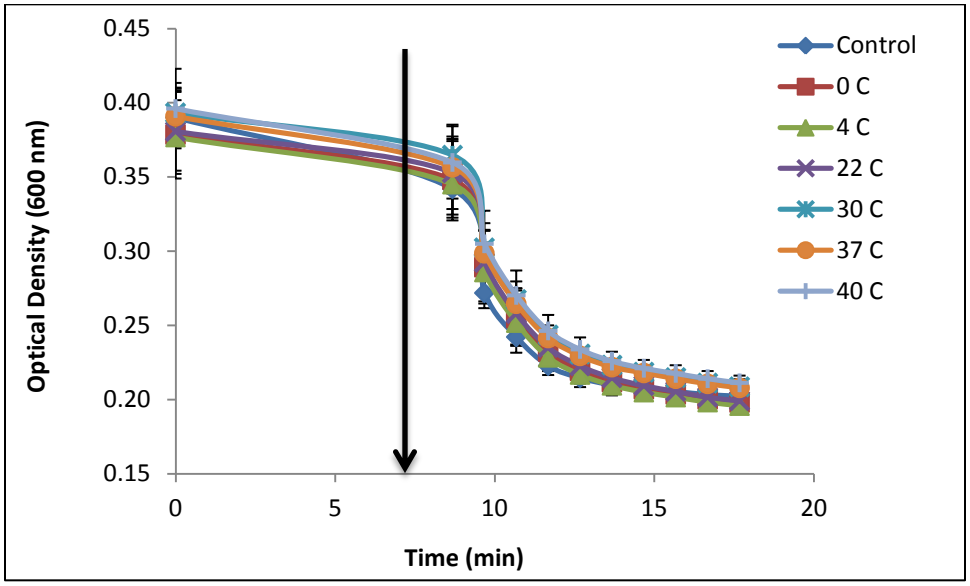
LMS analysis reveals the importance of individual experimental factors and interaction between and among the factors in an experiment. A Prob > F value of 0.05 or below indicates that factor or interaction influenced the experimental results. Pretrt is pretreatment used (buffer or CHL), trt is treatment (LysO1 or buffer control), temp is the temperature at which the enzyme was incubated prior to use. Two factors joined by an asterisk indicate the the effect of the interaction between those factors on the experimental results.

Table 4.9. Thermostability of LysO1

Temp.	Rate	St. Dev.	%	p-value
Control	0.0702	0.0077	100	NA
0	0.0590*	0.0035	84.0	< 0.0001
4	0.0592*	0.0033	84.4	< 0.0001
22	0.0604*	0.0030	86.0	0.0003
30	0.0625*	0.0045	89.0	0.0035
37	0.0579*	0.0030	82.6	< 0.0001
40	0.0549*	0.0027	78.2	< 0.0001

Rate of reaction was measured after LysO1 had been incubated at non-storage temperatures for 30 min. Enzyme concentration is given in μg/ml, rate of reaction is in ΔOD_{600}/min. A p-value less than 0.05, also denoted by as asterisk by the rate of reaction, indicates a rate of reaction statistically different from the control. % gives the rate of reaction as a percentage of the rate observed in LysO1 that has remained at storage conditions (-80 °C) until the assay.

Figure 4.14. Thermostability of LysO1.



Aliquots of dilute endolysin were incubated at the given temperature for 30 min, then residual activity was measured. Temperatures are in degrees Celsius.

3.6.2 Effect of divalent cations and sodium on LysO1 activity

When $CaCl_2$ was added to the reaction buffer, analysis of the interactions between the factors indicated that the interaction between pretreatment and enzyme treatment, and among pretreatment, enzyme treatment, and the concentration of calcium in the suspension buffer, were important to the experimental outcome (F Ratio = 207.6, 32.3; Prob > F< 0.0001; R^2Adj = 0.8878). When the effects of calcium on enzyme activity were analyzed, calcium had some effect on the optical density of cells pretreated with CHL (F Ratio = 27.7, Prob > F < 0.0001, results are summarized Table 4.10). The change in optical density was higher for cells in 5 mM $CaCl_2$ than for cells in 0, 0.1, or 1 mM ($p < 0.0001$). $CaCl_2$ added to 0.1 or 1 mM had no effect ($p = 0.9767$, $p = 0.5997$, Figure 4.15). The rate of reaction of LysO1 in 0.1 mM $CaCl_2$ had no effect compared to the control, the rate of reaction increased slightly compared to the control (0.0693 compared to 0.0639), but the two rates were not statistically different ($p = 0.4636$). $CaCl_2$ inhibited LysO1 activity at tested concentrations higher than 0.1 mM: rate of reaction in 1 mM

$CaCl_2$ was 54% of the rate in buffer control (0.035, p = 0.0005), and rate of reaction in 5 mM $CaCl_2$ was 6% of the control rate (0.004 ΔOD_{600}/min, p < 0.0001).

Table 4.10. LMS analysis of the effect of divalent cations or sodium on LysO1 activity.

	Calcium		**Magnesium**		**Zinc**		**Sodium**	
R^2adj	0.8788		0.9591		0.8463		0.9477	
Factor	F Ratio	Prob > F	F Ratio	Prob > F	F Ratio	Prob > F	F Ratio	Prob > F
pretrt	14.2	0.0003	340.7	< 0.0001	6.36	0.0136	183.2	< 0.0001
trt	235.0	< 0.0001	650.0	< 0.0001	42.4	< 0.0001	540.9	< 0.0001
pretrt*trt	207.6	< 0.0001	603.1	< 0.0001	32.0	< 0.0001	545.4	< 0.0001
ion	9.95	< 0.0001	55.5	< 0.0001	19.0	< 0.0001	114.8	< 0.0001
pretrt*ion	8.74	< 0.0001	53.0	< 0.0001	58.8	< 0.0001	33.1	< 0.0001
trt*ion	31.5	< 0.0001	51.0	< 0.0001	34.4	< 0.0001	63.3	< 0.0001
pretrt*trt*ion	32.3	< 0.0001	57.8	< 0.0001	40.2	< 0.0001	57.3	< 0.0001

LMS analysis reveals the importance of individual experimental factors and interaction between and among the factors in an experiment. A Prob > F value of 0.05 or below indicates that factor or interaction influenced the experimental results. Pretrt is pretreatment used (buffer or CHL), trt is treatment (LysO1 or buffer control), ion is the importance of the cation in use. Two factors joined by an asterisk indicate the effect of the interaction between those factors on the experimental results.

Table 4.11. Effect of divalent cations on LysO1 activity

	Calcium				**Magnesium**				**Zinc**			
Conc.	Rate	St. Dev.	%	p-value	Rate	St. Dev.	%	p-value	Rate	St. Dev.	%	p-value
0	0.0640	0.0117	100	NA	0.0724	0.0099	100	NA	0.0759	0.0046	100	NA
0.1	0.0694	0.0151	108.4	0.7163	0.0765	0.0093	105.7	0.421	0.0020*	0.0017	2.66	< 0.0001
1	0.0349*	0.0157	54.6	0.0005	0.0043*	0.0104	61.2	< 0.0001	0.0048*	0.0061	6.36	< 0.0001
5	0.0044*	0.0034	6.90	< 0.0001	0.0122*	0.0034	16.8	< 0.0001	0.0066*	0.0039	8.64	< 0.0001

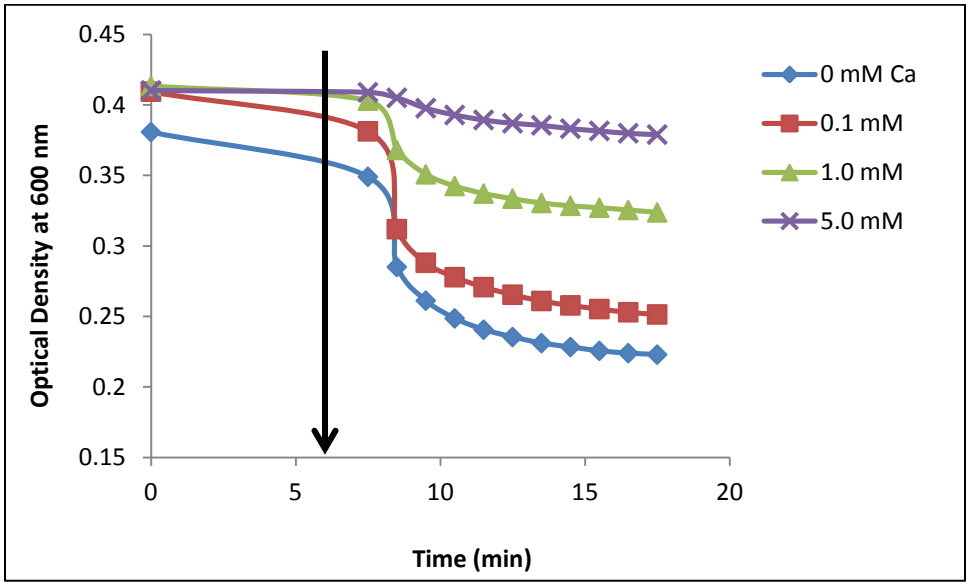
Rate of reaction of LysO1 was measured in buffer to which $CaCl_2$, $MgCl_2$, or $ZnCl_2$ had been added to 0.1, 1.0, or 5.0 mM. Concentrations are given in mM, all ion buffers were made using 25 mM Tris·HCl [pH 8]. Rate is the average rate of reaction of LysO1 observed at a given ion concentration. A p-value of less than 0.05 indicates a rate of reactions statistically different from the control. . % gives the rate of reaction as a percentage of the rate observed in LysO1in Tris buffer with no additional cations.

Table 4.12. Effect of sodium cations on LysO1 activity

Sodium	Rate	St. Dev.	%	p-value
0	0.0784	0.0041	100	NA
10	0.0747	0.0020	95.2	0.1407
25	0.0619*	0.0080	79.0	< 0.0001
50	0.0346*	0.0047	44.1	< 0.0001
100	0.0066*	0.0013	8.39	< 0.0001
200	0.0016*	0.0013	2.06	< 0.0001

Rate of reaction of LysO1 was measured in buffer to which NaCl had been added to the indicated concentrations. Concentrations are given in mM, all experimental buffers were made by adding NaCl to 25 mM Tris·HCl [pH 8]. Rate is the average rate of reaction of LysO1 observed at a given ion concentration, % is the percentage of enzyme activity observed comparted to the control (0 mM sodium). A p-value less than 0.05 indicates a rate of reaction statistically different from the control. . % gives the rate of reaction as a percentage of the rate observed in LysO1 in buffer with no NaCl added.

Figure 4.15. Effect of calcium cations on LysO1 activity

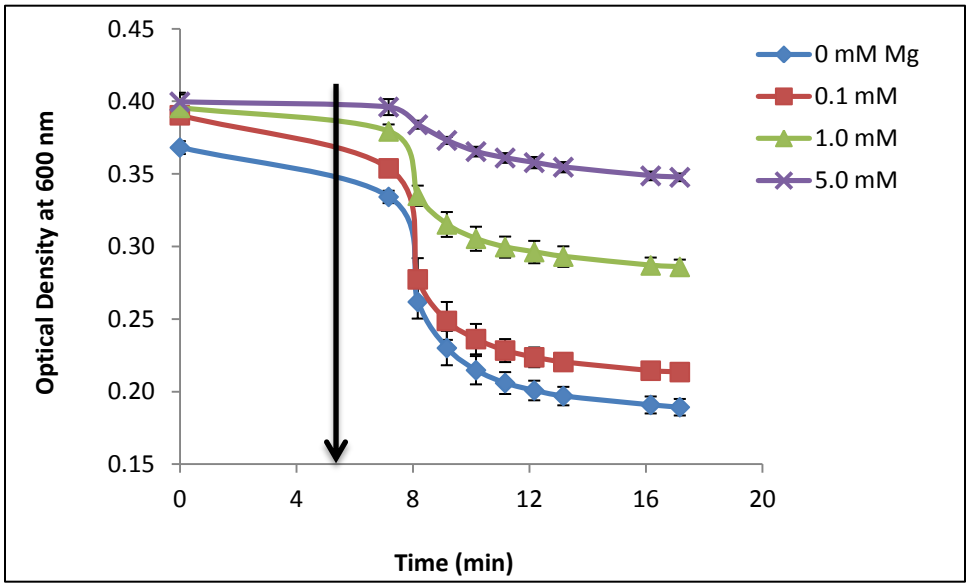


S. Typhimurium was pretreated with Tris or CHL, then washed twice and resuspended in 25 mM Tris·HCl [pH 8] or the same buffer with $CaCl_2$ added to 0.1, 1.0, or 5.0 mM. Cells were treated with LysO1 to a final concentration of 0.1 µg/ml, indicated by the arrow, and OD at 600 nm was measured before treatment and every minute after treatment for 10 min. The experiment was done in triplicate and repeated.

In the presence of Mg^{2+}, analysis of the interactions between experimental factors indicated that the interaction between pretreatment and enzyme treatment, and among pretreatment, enzyme treatment,

and the concentration of calcium in the suspension buffer, were important to the experimental outcome (F Ratio = 603.0, 57.8; Prob > F< 0.0001; R^2Adj = 0.9591). Results are summarized in Table 4.11. The rate of reaction of LysO1 in 0.1 mM $MgCl_2$ increased slightly compared to the control (0.072 compared to 0.076 ΔOD_{600}/min), but the two rates were not statistically different (p = 0.421). Magnesium inhibited LysO1 activity at tested concentrations higher than 0.1 mM; the enzyme rate of reaction in 1 mM $MgCl_2$ was 61.2% of the rate in buffer control (0.044 ΔOD_{600}/min, p < 0.0001), and rate of reaction in 5 mM $MgCl_2$ was 16.8% of the control rate (0.012 ΔOD_{600}/min), which was different from both the control rate and the rate of reaction on 1 mM (p < 0.0001) (Figure 4.16, Table 4.12). $MgCl_2$ had no effect on cells treated with buffer (F Ratio = 1.30, Prob > F = 0.3005).

Figure 4.16. Effect of magnesium cations on LysO1 activity

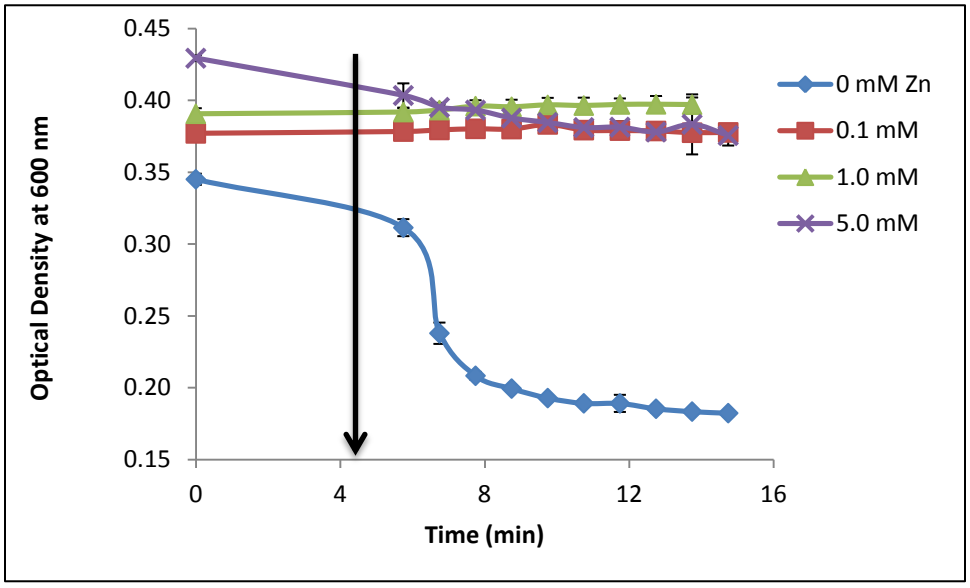


S. Typhimurium was pretreated with Tris or CHL, then washed twice and resuspended in 25 mM Tris·HCl [pH 8] or the same buffer with $MgCl_2$ added to 0.1, 1.0, or 5.0 mM. Cells were treated with LysO1 to a final concentration of 0.1 µg/ml, indicated by the arrow, and OD at 600 nm was measured before treatment and every minute after treatment for 10 min. The experiment was done in triplicate and repeated.

In the presence of Zn^{2+}, LMS analysis indicated interaction between pretreatment and treatment and among pretreatment, treatment, and zinc concentration was important (F Ratio = 31.9737, 40.2143;

Prob > F < 0.0001; R^2Adj = 0.8463). When the effects of zinc on LysO1 activity were analyzed, zinc inhibited LysO1 activity at the lowest tested concentration, 0.1 mM (Figure 4.17, Table 4.12). The rate of reaction in $ZnCl_2$ was 0.007 ΔOD_{600}/min; the control rate was 0.076 ΔOD_{600}/min ($p < 0.0001$). The rates of reaction in 1 mM and 5 mM $ZnCl_2$ were 0.005 ΔOD_{600}/min and 0.002 ΔOD_{600}/min, respectively, which were also different from the control ($p < 0.0001$). $ZnCl_2$ had a small but significant concentration-dependent effect on the OD_{600} of buffer-treated cells (F Ratio = 3.6838, P > F = 0.0292).

Figure 4.17. Effect of zinc cations on LysO1 activity

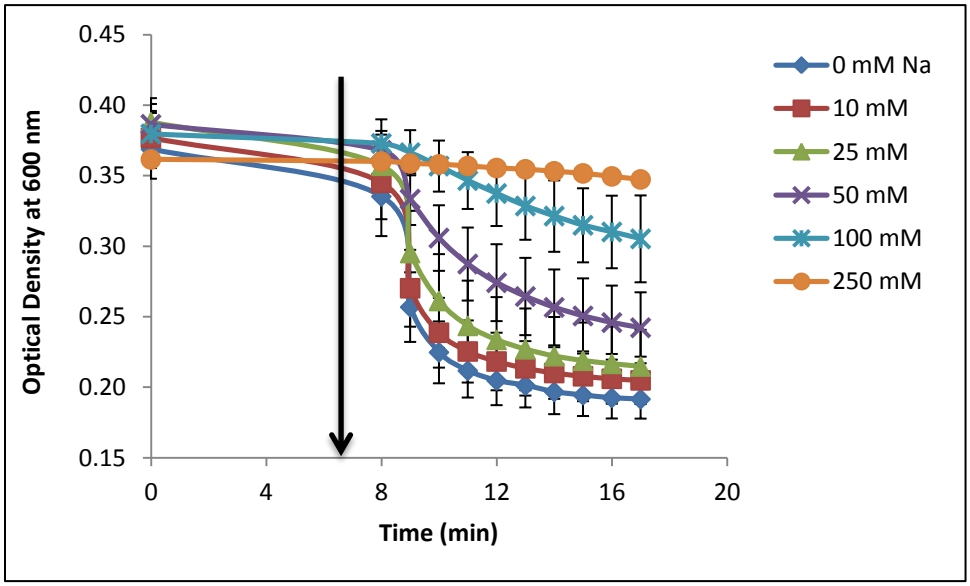


S. Typhimurium was pretreated with Tris or CHL, then washed twice and resuspended in 25 mM Tris·HCl [pH 8] or the same buffer with $ZnCl_2$ added to 0.1, 1.0, or 5.0 mM. Cells were treated with LysO1 to a final concentration of 0.1 µg/ml, indicated by the arrow, and OD at 600 nm was measured before treatment and every minute after treatment for 10 min. The experiment was done in triplicate and repeated.

Analysis of the interactions between experimental factors in the presence of sodium indicated that interaction between pretreatment and treatment, and among pretreatment, treatment, and sodium concentration, were important to the experimental outcome (F Ratio = 545.4, 57, 3; Prob > F < 0.0001) (Table 4.11).Analysis by ANOVA and Student's t-test indicated that addition of sodium had a concentration-dependent effect on LysO1 activity (Figure 4.18, Table 4.12), and the importance of

interactions with other factors, while also significant, was also less of a contributing factor to the observed results. The rate of reaction on 10 mM NaCl was lower than the control rate, but not different (0.078 ΔOD_{600}/min, 0.074 ΔOD_{600}/min, $p = 0.1407$). The rate of reaction on 25 mM NaCl was lower than the control rate, and different (0.062 ΔOD_{600}/min, $p < 0.0001$). The rate of reaction in 50 mM NaCl was lower than and different from both the control rate and the rate in 25 mM NaCl (0.035 ΔOD_{600}/min, $p < 0.0001$). The rate in 100 mM NaCl was lower than and different from the control rate and the rate in 25 or 50 mM NaCl (0.007 ΔOD_{600}/min, $p < 0.0001$). The rate of reaction in 250 mM NaCl was the lowest rate of reaction measured, and was different from rates of LysO1 in the control buffer, buffer with 10, 25, and 50 mM NaCl added, but not 100 mM (0.0016 ΔOD_{600}/min, $p < 0.0001$, $p = 0.0542$). Sodium had no effect on buffer-treated cells (F Ratio = 0.75, Prob > F = 0.594).

Figure 4.18. Effect of sodium cations on LysO1 activity



S. Typhimurium was pretreated with Tris or CHL, then washed twice and resuspended in 25 mM Tris·HCl [pH 8] or the same buffer with NaCl added to 10, 25, 50, 100, or 250 mM. Cells were treated with LysO1 to a final concentration of 0.1 µg/ml, indicated by the arrow. Optical density at 600 nm was measured before treatment and every minute after treatment for 10 min. The experiment was done in triplicate and repeated.

3.6.3 Effect of LysO1 on other Salmonella *serovars*

Analysis revealed interaction between pretreatment and treatment, and among pretreatment, treatment, and the *Salmonella* serovar used (F Ratio = 2958.0, 8.92; Prob > F < 0.0001; R^2Adj. = 0.978) (Table 4.13). When LysO1 activity was analyzed on chloroform-pretreated *Salmonella* serovars, it was found that addition of the enzyme caused lysis of all serovars tested (Figure 4.19). However, susceptibility to the enzyme was not the same for all strains. The observed rate of reaction ranged from the high of 0.1297 ΔOD_{600}/min against *S.* Agona (150% of the rate seen against *S.* Typhimurium), to a low of 0.08 ΔOD_{600}/min against *S.* Reading (92.6% of the rate observed against *S.* Typhimurium). Results are summarized in Table 4.14. The buffer added as a control treatment had some effect on cells (F Ratio = 2.91, Prob > F = 0.0148), but the highest observed rate of cell lysis in buffer-treated cells, 0.004 ΔOD_{600}/min, is less than one tenth that of the lowest rate of reaction observed in LysO1-treated cells (0.08 ΔOD_{600}/min). Consequently, although statistically significant, the effect of the buffer is thought to be minimal.

Table 4.13. LMS analysis of the effect of LysO1 on various serovars of *Salmonella enterica*.

Serovars		
R^2adj	0.978	
Factor	F Ratio	Prob > F
pretrt	2126.5	< 0.0001
trt	3240.9	< 0.0001
pretrt*trt	2958.0	< 0.0001
ser	7.21	< 0.0001
pretrt*ser	8.03	< 0.0001
trt*ser	4.21	0.0003
pretrt*trt*ser	8.92	< 0.0001

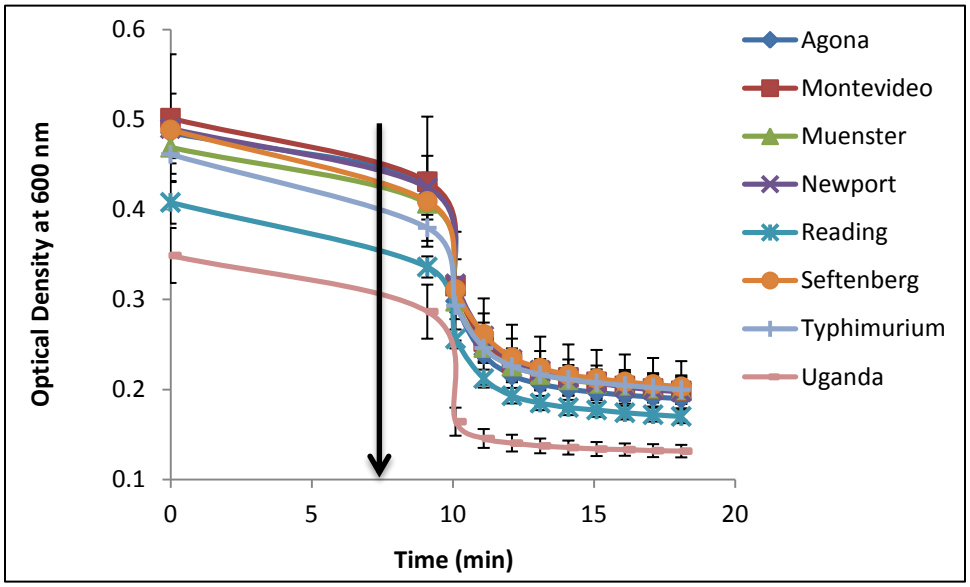
LMS analysis reveals the importance of individual experimental factors and interaction between and among the factors in an experiment. A Prob > F value of 0.05 or below indicates that factor or interaction influenced the experimental results. Pretrt is pretreatment used (buffer or CHL), trt is treatment (LysO1 or buffer control), ser is the serovar used as the substrate. Two factors joined by an asterisk indicate the effect of the interaction between those factors on the experimental results.

Table 4.14. Effect of LysO1 on various serovars of *Salmonella enterica*.

Serovar	Rate	St. Dev	%	p-value
Typhimurium	0.0864	0.0024	100	NA
Agona	0.1297*	0.0056	150.1	< 0.0001
Montevideo	0.1162*	0.0129	134.5	< 0.0001
Muenster	0.1089*	0.0053	126.0	< 0.0001
Newport	0.1069*	0.0108	123.7	0.0002
Reading	0.0800	0.0042	92.6	0.2807
Seftenberg	0.0981*	0.0015	113.5	0.0245
Uganda	0.1224*	0.0151	141.7	< 0.0001

Serovars of *S. enterica* subsp. *enterica* were pretreated with 10 mM Tris·HCl [pH 8] or the same buffer saturated with chloroform, then LysO1 activity was measured using the pretreated cells. Rate indicates the average rate of reaction, St. Dev. is the standard deviation. A p-value of less than 0.05 indicates a rate of reactions statistically different from the control. % gives the rate of reaction as a percentage of the observed rate of reaction of LysO1 on *S.* Typhimurium.

Figure 4.19. Effect of LysO1 on various serovars of *Salmonella enterica*.



Serovars of *S. enterica* subsp. *enterica* were pretreated 30 min with Tris buffer or CHL, then washed twice and resuspended in 25 mM Tris·HCl [pH 8]. Cells were treated with 0.1 µg/ml LysO1, indicated by the arrow. Optical density was measured before treatment and every minute after treatment for 10 min. *S.* Typhimurium was used as a control, the experiment was done in triplicate and repeated.

3.6.4 Effect of LysO1 on other foodborne bacterial pathogens

The interactions between pretreatment and treatment, and among the pretreatment, treatment, and species of the substrate, were also important (F Ratio = 255.6, 51.3; Prob > F < 0.0001; R^2Adj = 0.9365) (Table 4.15). When the effect of LysO1 on species was compared, the rate of reaction observed on an *S.* Typhimurium substrate was 0.1022 ΔOD_{600}/min. The reaction rate was significantly different when the enzyme was added to *L. monocytogenes*, or *C. jejuni*. When added to *E. coli*, the observed rate of reaction was 0.0934 ΔOD_{600}/min, significantly lower than the reaction rate on a *S.* Typhimurium substrate (p = 0.0489). The rate of reaction when added to *L. monocytogenes* (0.0012 ΔOD_{600}/min) or *C. jejuni* (0.0024 ΔOD_{600}/min) was highly significantly different (p < 0.0001). Effectively, there was no lysis observed when LysO1 was added to those substrates. Results are summarized in Figure 4.20 and Table 4.16.

Table 4.15. LMS analysis of the effect of LysO1 on non-*Salmonella* foodborne pathogens

Species		
R^2adj	0.9365	
Factor	F Ratio	Prob > F
pretrt	0.18	0.6756
trt	359.2	< 0.0001
pretrt*trt	255.6	< 0.0001
sp	117.8	< 0.0001
pretrt*sp	61.2	< 0.0001
trt*sp	37.0	< 0.0001
pretrt*trt*sp	51.3	< 0.0001

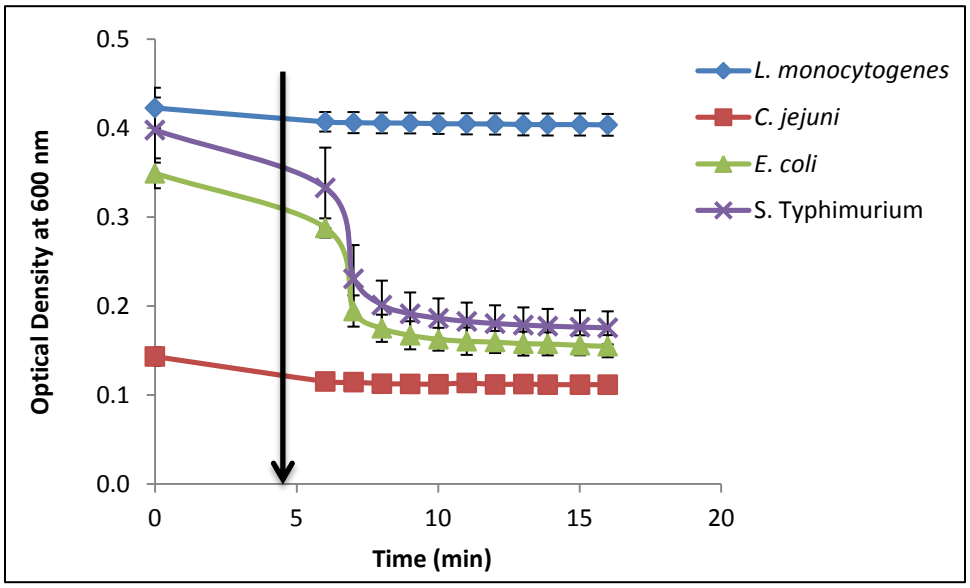
LMS analysis reveals the importance of individual experimental factors and interaction between and among the factors in an experiment. A Prob > F value of 0.05 or below indicates that factor or interaction influenced the experimental results. Pretrt is pretreatment used (buffer or CHL), trt is treatment (LysO1 or buffer control), sp is the species used as the substrate. Two factors joined by an asterisk indicate the effect of the interaction between those factors on the experimental results.

Table 4.16. Effect of LysO1 on non-*Salmonella* foodborne pathogens.

Species	Rate	St. Dev.	%	p-value
L. monocytogenes	0.0012*	0.0006	1.17	< 0.0001
C. jejuni	0.0024*	0.0014	2.35	< 0.0001
E. coli	0.0934	0.0124	91.4	0.0489
S. Typhimurium	0.1022	0.0074	100	NA

The rate of reaction of LysO1 was measured using foodborne pathogenic bacteria as the substrate, bacteria had been pretreated with buffer or chloroform-saturated buffer and washed as described. *S*. Typhimurium was included as a control. Rate indicates the average rate of reaction observed with that organism as the LysO1 substrate. A p-value of less than 0.05, or a rate of reaction marked with an asterisk, indicates a rate of reaction statistically different from the control. % is the observed rate of reaction as a percentage of the observed rate of reaction of LysO1 on *S*. Typhimurium.

Figure 4.20. Effect of LysO1 on non-*Salmonella* foodborne pathogens



Foodborne pathogens were pretreated 30 min with 10 mM Tris buffer or CHL, then washed twice and resuspended in 25 mM Tris·HCl [pH 8]. Cells were treated with 0.1 µg/ml LysO1, indicated by the arrow. Optical density was measured before treatment and every minute after treatment for 10 min. *S*. Typhimurium was used as a control, the experiment was done in triplicate and repeated.

3.6.5 Measurement of lethal activity of LysO1

In the first iteration of the experiment, there was no growth of samples taken from cell suspensions pretreated with CHL, so it was impossible to measure the lethality of LysO1 on the pretreated cells. When the experiment was repeated, a decrease in OD_{600} of 0.17 units was observed in cells treated

with LysO1 compared to a decrease in the buffer-treated control of 0.02 units, but again, none of the plated samples produced viable growth.

Visible clearing was observed in the suspensions treated with enzyme compared to those treated with buffer, but there was no appreciable difference in number of viable cells between the two groups. Results are summarized in Table 4.17. Upon repetition of the experiment, visible clearing of the enzyme-treated culture compared to the buffer-treated control was observed, but there no visible growth of spread-plated samples.

Table 4.17. Effect of LysO1 or buffer control on the viability of *S*. Typhimurium

	Buffer			**LysO1**		
Culture	pre-CHL	Pre-treat	Post-trt	pre-CHL	Pre-treat	Post-trt
1	220	0	78	33	279	296
2	73	99	160	7	3	4
3	11	312	TNTC	4	4	2

All cells were pretreated with CHL. Following pretreatment, one all replicates of one group were treated with buffer and replicates of the other group were treated with LysO1. Samples of 100 µl were taken before CHL pretreatment, after pretreatment, and after treatment with LysO1 or the control. Samples were spread-plated, numbers indicate colonies observed after overnight incubation.

When the experiment was altered to use a suspension concentrated from a much larger volume of cell culture, there was no decrease in turbidity observed after enzyme treatment – the culture was too turbid for the Klett meter to obtain an accurate reading. Samples taken before Tris or CHL pretreatment and before and after enzyme treatment all produced a lawn of cells when spread-plated, suggesting pemeabilization was ineffective and LysO1 was not able to access its substrate.

Upon repetition, partial clearing was observed in the culture treated with LysO1, after addition of the enzyme. Prior to treatment, the turbidity of both suspensions of cells was too high to measure accurately with the Klett meter. Five minutes after treatment, the buffer-treated culture B had decreased to 650 and the LysO1-treated culture L to 425, and 25 min later, turbidity of the buffer-treated control had decreased to 620 and LysO1-treated suspension to 400. Examination of plated samples revealed no

treatment-dependent decrease in number of viable cells after treatment. Results are summarized in Table 4.18.

Table 4.18. Effect of LysO1 or buffer control on the viability of *S*. Typhimurium.

	Buffer				**LysO1**			
Time	Klett	10 µl	100 µl	1000 µl	Klett	10 µl	100 µl	1000 µl
0	max	1	6	87	max	3	80	TNTC
5	650	1	12	76	425	14	189	TNTC
30	620	2	14	TNTC	400	5	115	TNTC

Two groups of cells were pretreated with CHL, then one was pretreated with buffer and the other with LysO1. Samples were taken and turbidity (in Klett units) was measured before treatment and five and thirty minutes after treatment.

Upon the third repetition, the turbidity of all suspensions was again too high to be measured by a Klett meter prior to treatment. After treatment, of the two suspensions pretreated with Tris, the turbidity of suspension treated with the buffer control was 700 Klett units and the turbidity of the suspension treated with LysO1 was 720. Of the two suspensions pretreated with chloroform, the turbidity of the buffer-treated control was 660 Klett units and turbidity of the LysO1-treated suspension was 530 Klett units. Examination of plates revealed no significant decrease in the number of viable cells after enzyme treatment. Results are summarized in Table 4.19.

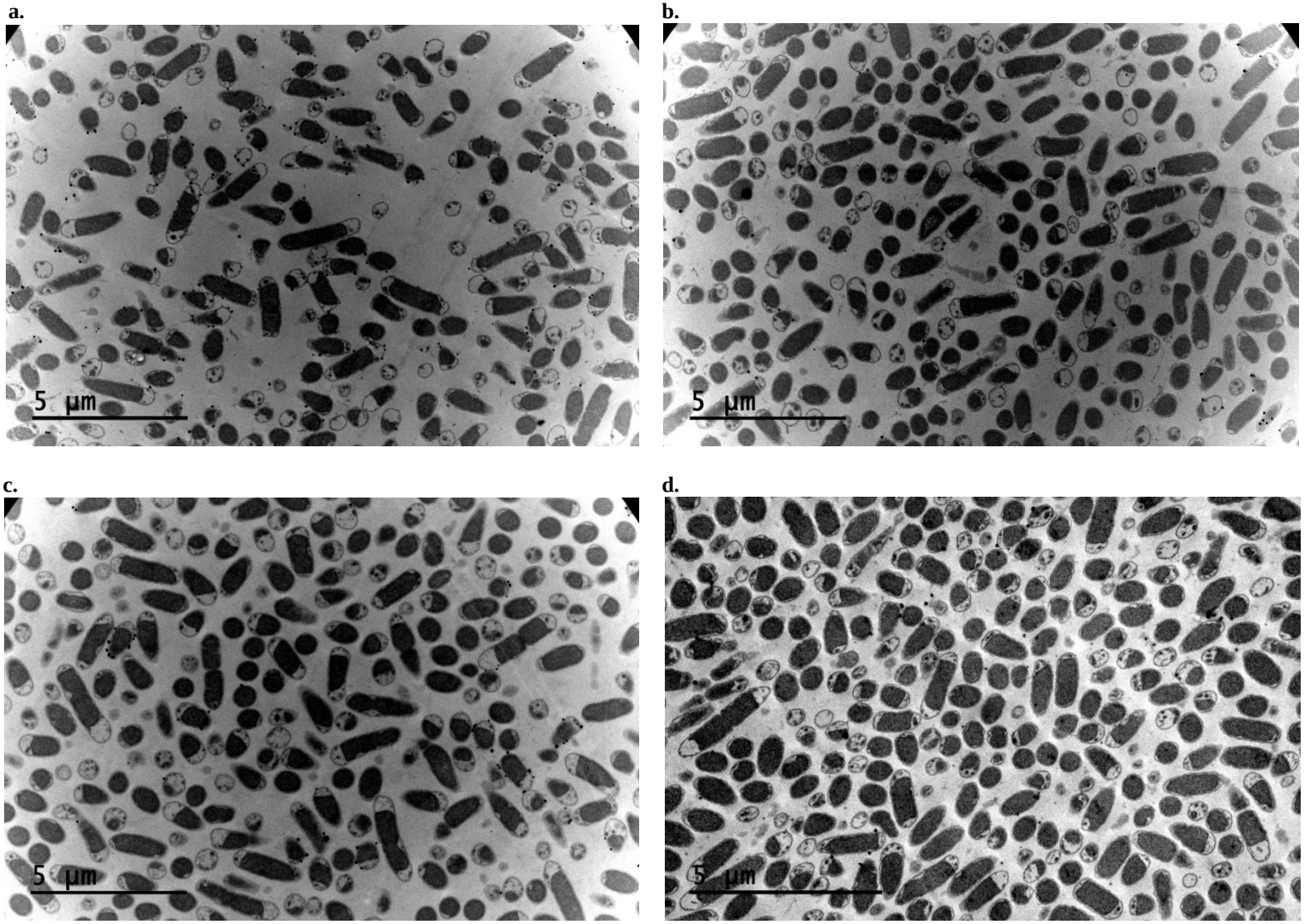
Table 4.19. Effect of LysO1 or buffer control on the viability of *S*. Typhimurium.

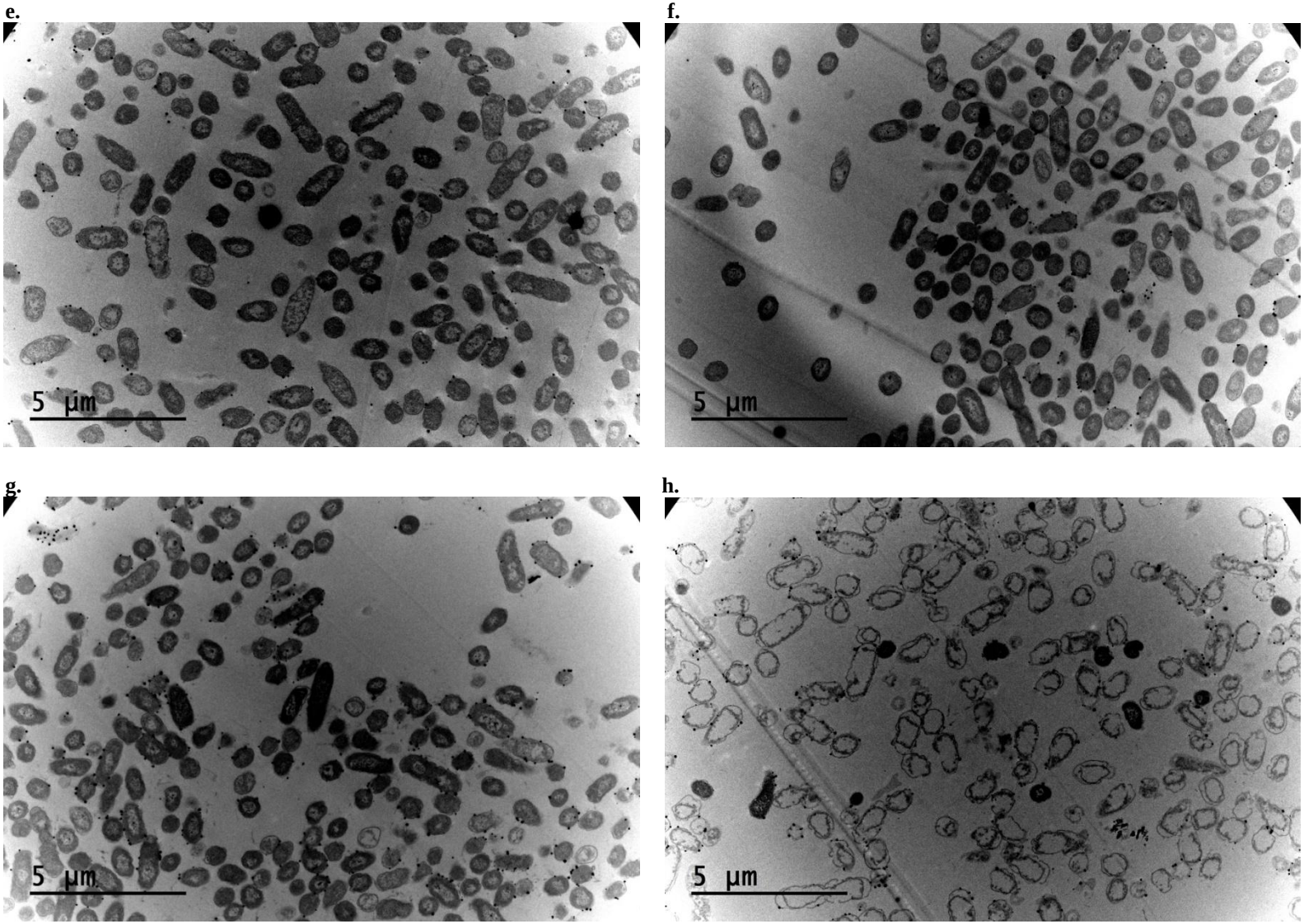
	Tris Pretreatment				**CHL pretreatment**			
	Buffer		LysO1		Buffer		LysO1	
	before	after	before	after	before	after	before	after
Turbidity (Klett units)	max	700	max	720	max	660	max	530
CFU/10 µl	lawn	lawn	lawn	lawn	41	11	0	1
CFU/100 µl	lawn	lawn	lawn	lawn	616	221	14	13
CFU/1000 µl	lawn	lawn	lawn	lawn	TNTC	TNTC	228	272

Two groups of cells were pretreated with Tris buffer, two were pretreated with CHL. Then one of each pretreatment groups was treated with buffer, the other was treated with LysO1. Samples and turbidity readings were taken before and after treatment.

Samples of pretreated *S*. Typhimurium taken before and after LysO1 or control treatment were viewed with transmission electron microscopy (TEM) at a magnification of 10,000 (Figure 4.21). Three pictures of each samples were viewed, the intact and lysed cells (cells depleted of cytoplasmic contents; sometimes referred to as ghost cells) in each group were counted, and the results were analyzed using LMS and ANOVA. Results are summarized in Table 4.20. Briefly, of cells pretreated with Tris buffer, 1 out of 511 total cells counted were lysed before treatment (0.2%) and 1 out of 731 total cells were lysed after treatment. Cells treated with LysO1 averaged 0 cells lysed before treatment out of 543 counted, and 0 of 595 lysed after treatment. Of cells pretreated with CHL, 26 of 355 of control-treated cells (6.98%) were lysed before treatment and 16 out of 411 total cells were lysed after treatment (3.89%). Of cells treated with LysO1, 11.6 of 652 total cells were lysed before enzyme treatment (5.54%) and 397 of 444 total cells were lysed after treatment (89.4%).

Figure 4.21. Effect of LysO1 or control on *S*. Typhimurium pretreated with buffer or CHL – thin-section ultramicroscopy





To determine lytic activity of LysO1, *S*. Typhimurium was pretreated with Tris or CHL, then cells were washed twice and resuspended in fresh buffer. Samples of pretreated cells were taken before and after treatment. Tris-pretreated samples are shown **a**. before and **b**. after control treatment, **c**. before and **d**. after LysO1 treatment, and CHL-pretreated cells and shown **e**. before and **f**. after control treatment, and **g**. before and **h**. after LysO1 treatment.

Table 4.20. Effect of LysO1 on *S*. Typhimurium, measured from TEM micrographs.

a.

Group		Pre-treatment						Post-treatment					
Pretrt	**Trt**	**Replicate**	**Intact**	**Lysed**	**Unsure**	**Total**	**% Lysis**	**Replicate**	**Intact**	**Lysed**	**Unsure**	**Total**	**% Lysis**
Tris	buffer	TB 1-1	175	0	3	178	0	TB 2-1	248	0	6	254	0
		TB 1-2	152	0	3	155	0	TB 2-2	243	1	2	246	0.41
		TB 1-3	176	1	1	178	0.56	TB 2-3	228	0	3	231	0
Tris	LysO1	TL 1-1	189	0	0	189	0	TL 2-1	229	0	3	232	0
		TL 1-2	168	0	0	168	0	TL 2-2	165	0	4	169	0
		TL 1-3	186	0	0	186	0	TL 2-3	192	0	2	194	0
CHL	buffer	CB 1-1	145	14	0	159	8.81	CB 2-1	153	7	1	161	4.35
		CB 1-2	93	8	0	101	7.92	CB 2-2	95	3	0	98	3.06
		CB 1-3	91	4	0	95	4.21	CB 2-3	146	6	0	152	3.95
CHL	LysO1	CL 1-1	259	8	0	267	3.00	CL 2-1	18	136	4	158	86.1
		CL 1-2	211	18	0	229	7.86	CL 2-2	13	128	0	141	90.8
		CL 1-3	147	9	0	156	5.77	CL 2-3	12	133	0	145	91.7

b.

Time of Sample	Tris Pretreatment				CHL Pretreatment			
	buffer		LysO1		buffer		LysO1	
	Av. (%)	St. Dev.	Av. (%)	St. Dev.	Av. (%)	St. Dev.	Av. (%)	St. Dev.
before trt	0.20	0.32	0	0	7.32	2.44	5.37	2.44
after trt	0.14	0.23	0	0	3.89	0.66	89.4	3.03
difference	-0.06	0.49	0	0	-3.43	2.55	84.0	1.71

a. Total numbers of intact and lysed cells (cells depleted of cytoplasmic contents) observed in each replicate of the TEM study, and **b**. a summary of the results, with the average lysis given as a percentage of cells counted.

An LMS analysis revealed that that each experimental factor (pretreatment and treatment) and the interaction between them was highly significant (R^2adj = 0.9984, Prob > F < 0.0001), and that the change in the percentage of lysed cells in each group after enzyme or control treatment was not the same for all groups (Table 4.21). An ANOVA was performed to determine the effect of the treatment factor. The effect of LysO1 was no different from that of the control treatment on cells pretreated with Tris buffer (Prob > F = 0.8627). The effect of LysO1 was highly significantly different from that of the control treatment on cells pretreated with CHL (Prob > F < 0.0001).

Table 4.21. LMS analysis of the effect of LysO1 on *S.* Typhimurium, measured from TEM micrographs.

Lysis measured by TEM		
R^2adj	0.9984	
Factor	F Ratio	Prob > F
pretrt	2035.3	< 0.0001
trt	2369.6	< 0.0001
pretrt*trt	2363.9	< 0.0001

LMS measures the relative importance of experimental factors and the interactions among them. Pretrt is pretreatment, trt is treatment. A Prob > F value of 0.05 or lower indicates that the factor or interaction in question is significant to the experimental outcome.

The lethality of the chloroform pretreatment made the measurement of lethality due to LysO1 difficult, so a different pretreatment was selected. A previous study indicated that short-term exposure to Tris-EDTA did not have a significant effect on viability of *S.* Typhimurium. However, of the sixty samples taken from suspensions that should contain 1 to 1000 organisms per ml, only six samples produced colonies, and of those, five samples held only a single colony, which was insufficient to determine the if LysO1 is lethal to live cells. The same results were observed upon repetition with suspensions containing 10^4 to 10^7 organisms per ml.

As Tris-EDTA is not known to be lethal to *Salmonella* over short duration, the pH of the Tris-EDTA buffers was measured, and was found to be ~ 11 instead of the desired pH of 8. The EDTA stock and working buffers were remade and the pH of each was measured to ensure it was within the same range as the Tris control. When the experiment was repeated using the new buffers there was measurable growth, but no appreciable difference in number of viable cells was observed between the enzyme-treated group compared to the control group treated with buffer (Table 4.22). The same results were observed when suspensions were vortexed before sampling to ensure homogenous suspension (Table 4.23).

Table 4.22. Effect of LysO1 or buffer control on the viability of *S*. Typhimurium

	Pre-treatment		**Post-treatment**		**Log decrease**	
Culture	buffer	LysO1	buffer	LysO1	buffer	LysO1
1	2.4 * 10^9	3.6 * 10^9	1.36 * 10^9	1.3 * 10^9	-0.25	-0.44
2	1.9 * 10^9	1.64 * 10^9	1.48 * 10^9	1.68 * 10^9	-0.11	0.01
3	1.18 * 10^9	9.2 * 10^8	1.5 * 10^9	1.06 * 10^9	-0.1	0.06

Cells were pretreated with 200 mM Tris; 5 mM EDTA buffer. Cell suspensions were mixed by pipetting before samples were taken.

Table 4.23. Effect of LysO1 or buffer control on the viability of *S*. Typhimurium.

	Pre-treatment		**Post-treatment**		**Log decrease**	
Culture	buffer	LysO1	buffer	LysO1	buffer	LysO1
1	1.26 * 10^7	1.3 * 10^7	5.2 * 10^6	3.4 * 10^6	-0.38	-0.58
2	9.4 * 10^6	6.6 * 10^6	1.58 * 10^6	2.6 * 10^6	-0.77	-0.4
3	9.8 * 10 ^6	1.14 * 10^7	3.2 * 10^6	3.56 * 10^6	-0.49	-0.51

Cells were pretreated with 200 mM Tris; 5 mM EDTA buffer. Cell suspensions were mixed by vortexing before samples were taken.

Finally, the experiment was altered to measure optical density at 600 nm and obtain and accurate cell count both before and after enzyme treatment. The expected decrease in optical density of enzyme-treated cells compared to controls was observed (summarized in Table 4.24), but there was no decrease in number of viable *Salmonella* concurrent with the decrease in optical density.

Table 4.24. Effect of LysO1 or buffer control on the viability of *S*. Typhimurium.

	Buffer		LysO1	
Tris/EDTA	Rate	log Δ	Rate	log Δ
10/0	0.0068	0.23	0.0074	0.07
10/1	0.0111	-0.18	0.0318	-0.11
10/3	0.0083	-0.44	0.0339	-0.07
10/5	0.0111	0.18	0.0326	0.07
200/0	0.0142	0.23	0.0172	0.07
200/1	0.0103	-0.17	0.0595	-0.29
200/3	0.0068	-1.04	0.0542	-1.16
200/5	0.0064	-0.77	0.0514	-0.62

An activity assay of LysO1 was done as described previously, with two exceptions. *S*. Typhimurium was pretreated with 10 or 200 mM Tris containing 0, 1, 3, or 5 mM EDTA, instead of CHL. Samples were taken before treatment and 5 min after treatment to determine the number of viable cells in each sample. Rate is the rate of reaction of LysO1 or the storage buffer control. Log Δ is the change in viable cell numbers observed before and after treatment of Tris- or Tris-EDTA pretreated cells.

4.0 Discussion

4.1 Sequence alignment and structure prediction

ClustalW2 predicted the catalytic residues to be at positions 22, 30, and 32 which are tyrosine, threonine, and glycine; pblast predicted catalytic residues at positions 15 (glutamic acid) and 30 (threonine). However, evidence suggests that LysO1 is a lysozyme like P22 Lyz; that family of enzymes is known to have only two active site residues, glutamic acid and aspartic acid (17). The sequence alignment indicated that glutamic acid at position 15 and aspartic acid at position 24 residues in LysO1 align with the known active site residues of P22 Lyz, and we suggest that these two residues are the essential residues for LysO1 activity.

The predicted secondary structures of the pRSET A polyhistidine tag revealed through PHYRE analysis of the primary structure were quite small. The three prediction algorithms were in general agreement regarding the placement and type of the secondary structures in the endolysin sequence, differing only occasionally, and then only by one or two residues in the length of the structure. Addition of a tag sequence to the protein was not expected to reveal any changes to secondary or tertiary structure

of the untagged sequence, as prediction of structural changes based on such small sequence differences is beyond PHYRE's capabilities (25).

In predicting the secondary structures of the pRSET A tag, the three algorithms disagreed not only on placement, but in some cases in the type of secondary structure. As the minimum number of residues required for a single turn is three, it is possible that the 1- and 2-aa structures predicted in the tag are artifacts of the prediction algorithms, and the tag does not actually assume those structures (18).

In contrast to the ambiguity of prediction of the pRSET A tag, the prediction algorithms were consistent in predicting an α helix in the E1 tag: one of 12 aa, one of 11 aa, and one 4 aa in length; the consensus sequence helix was 10 aa. As the helix in question was only separated from the first helix of the enzyme by 4 residues, it may be close enough to the folded protein to affect enzyme function if it occludes the active site.

The purpose of submitting the tagged sequences was to detect any predicted secondary structures of the tags and their relative positions to the main sequence. Although inhibition of enzyme activity due to a purification tag is rare, it is not unheard of, and we wished to determine if the tags used in this study could possibly be in positions to physically inhibit the active site of the enzyme (49). To definitively answer the question, it would be necessary to cleave the tag and repurify and retest the enzyme for activity, but several observations make the premise of enzyme inhibition due to the polyhistidine tag unlikely. It has been demonstrated that LysO1 tagged at the *N*-terminus with E1 is active. Also, the high rates of reaction observed under optimal conditions and with different *Salmonella* serovar substrates make it unlikely that the E1 tag at all hinders enzyme activity.

Based on sequence similarity, PHYRE analysis suggests that the tertiary structure of LysO1 is most similar to P22 Lyz. PHYRE predicts LysO1 to have two lobes composed of α helices, with the active site in the cleft between the lobes.

4.2 Expression of LysO1

Induction of protein expression is often detrimental to the expression host; a decrease in the number of viable cells by an order of magnitude decrease is not unheard of (39). Determination of viable cell counts was an attempt to see if induction caused a severe post-induction reduction in the number of viable cells in the cultures expressing the protein of interest. Of greater concern was the post-induction three-orders-of-magnitude decrease in culture P, which did not contain the protein of interest and so should not be as strongly affected by protein expression as culture L. The most likely cause of the decrease is that induction of protein expression if expression of the gene of interest in controlled by a strong promoter redirects the cell's resources to production of the protein. The redirection depletes stores of ATP, which is fatal to the cell.

4.3 Solubility analysis of expressed LysO1

Results from precipitation with ammonium sulfate were ambiguous. By definition, a soluble protein is one that does not pellet when centrifuged under the most stringent conditions of a differential centrifugation (48). Inclusion bodies pellet during the first step of differential centrifugation under the least stringent conditions (48). If the protein were insoluble, it would be expected to pellet along with the cellular debris, thus the band at 20 kDa of the first set of samples should decrease in intensity with increased centrifugation intensity, and it did not. Subsequent centrifugation after ammonium sulfate precipitation again revealed a 20 kDa band in all samples, including the one that received no ammonium sulfate. The presence of that band may be due to denaturation of the enzyme during the hour-long incubation at room temperature. The denatured enzyme then would aggregate into inclusion bodies large enough to pellet during centrifugation. As results were ambiguous, a different method to determine solubility was attempted.

SDS-PAGE analysis of the enzyme purified under denaturing conditions showed the expected 20 kDa band; the band of enzyme purified under native conditions was not as heavy as the band from the same volume of sample purified under denaturing conditions. The difference can be explained by the

different conditions under which the two samples were purified. For affinity column purification, the affinity tag must be close enough to the surface of the protein to bind to the resin in the column. Purification under denaturing conditions often purifies more of the expressed protein, because denaturation of the enzyme improves the ability of the tag to bind to the matrix. Denaturation also solubilizes any inclusion bodies of expressed enzyme that were present in aggregates of misfolded protein, and those are purified as well. Purification under native state conditions does not net as much purified product because does not expose the affinity tag if buried and does not solubilize aggregates of poorly folded enzyme. Consequently, it is expected that the enzyme purified under denaturing conditions is a combination of active soluble and inactive aggregated LysO1. The enzyme purified under native state conditions is soluble and presumably active, and difference between the two is the fraction that is insoluble.

4.4 Lytic activity of LysO1

All trials contained the pretreatment groups Tris and CHL, and the treatment groups buffer and LysO1 (some also contained CEWL as an additional treatment). Two things enabled LysO1 characterization. First, the pretreatment buffers were removed and the cells were washed twice and resuspended in fresh buffer to remove any residue of the pretreatment before they were treated with enzyme or buffer. Therefore, although CHL had some effect on optical density compared to Tris-pretreated controls, any effect on optical density observed after resuspension of the cells in fresh buffer and addition of the treatment was due to the effect of the buffer and/or to enzyme activity, not to the continuing effect of the CHL pretreatment. Second, a statistical analysis revealed that LysO1 had no significant effect on buffer-pretreated cells compared to the buffer control treatment, and had an additional effect beyond that of the chloroform on CHL-pretreated cells, the effect of the CHL on optical density could be explained as background effect. As a result, the effects of ionic strength, pH, divalent cations, and sodium on LysO1 activity could be measured.

For an endolysin to effectively lyse a Gram negative organism, the outer membrane (OM) of the bacteria must be permeabilized. In addition, it is becoming increasingly clear that, as bacteria have evolved proteins that inhibit lysozyme function, those proteins must be removed or their function inhibited (1, 8, 13, 27). Chloroform acts by dissolving phospholipids that compose the outer membrane. That activity has the effect of permeabilizing the outer membrane and may remove it entirely (32). In addition, when the chloroform fraction is removed from pretreated cells the lysozyme inhibitor is removed as well. Evidence to support the removal of lysozyme inhibitors as a reason for subsequent observed lysozyme activity on chloroform-treated cells was demonstrated in separate studies using extracts from *E. coli*, *S.* Typhimurium, *Y. enterocolitica*, *P. aeruginosa*, and *S. flexneri*(32, 35). In both studies, HEWL, the phage λ lysozyme, and the phage P22 lysozyme affected optical density of *M. luteus* suspensions, but had no effect if the chloroform fraction from Gram negative cells pretreated with chloroform was added to the reaction (5, 33).

The chloroform-dependent removal of lysozyme inhibitors from cell suspension may also explain why TSP, PEI, and the organic acids tested were not suitable outer membrane permeabilization agents for *S.* Typhimurium (details of those experiments are in Appendix B). Although TSP, PEI, and lactic acid are known membrane permeabilizers, interaction with known lysozyme inhibitors has not been reported (2, 10, 19). Membrane permeabilization without lysozyme inhibitor removal or inhibition does not predispose the cell to endolysin activity.

CEWL and LysO1, added in the same concentration to the same *S.* Typhimurium substrate, had markedly different rates of reaction; LysO1 was more efficient at lysing *Salmonella*. The results were not unexpected; CEWL is optimized for degradation of the peptidoglycan of Gram positive bacteria, and lysis of *M. luteus* by CEWL is highly efficient (36). LysO1 is an enzyme from a phage that selectively infects species of the genus *Salmonella*, and any mutation that resulted in less efficient lysis would be detrimental to the phage (28, 29). Consequently, unlike CEWL, LysO1 has been evolutionarily selected to lyse *Salmonella* peptidoglycan. Unexpectedly, LysO1 had no effect on the standard lysozyme substrate,

M. luteus. The lack of observed enzyme activity is thought to be due to different peptidoglycan structures of Gram positive and Gram negative bacteria.

The results currently available regarding the lethality of LysO1 are inconclusive. Lysis of *Salmonella* serovars and at least one other genus was observed, and has been demonstrated to be due to the enzymatic activity of LysO1 and not to lingering effects of the chloroform pretreatment. It is possible that the enzyme is not lethal to living cells and that the decrease in OD_{600} is due to lysis of cells already dead. However, CEWL treatment of *M. luteus* results in nearly complete lysis, as measured by OD decreasing to nearly undetectable levels. That complete clearing was not observed during LysO1 treatment of *S.* Typhimurium – only partial clearing was observed. It may be that the permeabilization treatment is incomplete, and viable cells observed before and after sampling are cells that were not adequately permeabilized and were protected from lytic activity.

The electron microscopy study provided visual confirmation that LysO1 causes lysis of *S.* Typhimurium that were pretreated with CHL. Although CHL is lethal to the cells and some lysis was visible before the enzyme treatment, the number of lysed cells after enzyme treatment was significantly higher than the number of lysed cells treated with the buffer control. The CHL was removed before the enzyme was added, and the pretreated cells show no evidence of blebbing that would indicate a weakened peptidoglycan layer. As the evidence suggests the peptidoglycan is intact before enzyme or control treatment, and cells are overwhelming lysed after LysO1 treatment but not after the control treatment, is further evidence of the lytic nature of LysO1. It is also evidence that the lytic activity observed during the characterization of LysO1 was a result of enzyme activity, and not delayed results of the CHL pretreatment.

An additional concern is that the lysed cells in the EM samples look largely intact, but empty. However, the samples were thin-sectioned before viewing, so degraded areas of the cell wall due to LysO1 activity were not visible if they were not in the small cross-section of the sample that was viewed.

Lysozyme function depends on ion concentration, pH, and temperature. Optimal ion concentration for activity of LysO1 at pH 8 was determined to be 0.025 M Tris·HCl. At high ion

concentrations, increased ionic interactions with charged groups of enzyme alter the structure of the protein, which adversely affects enzyme function (47). Our findings reflected this: LysO1 was less active at high ion concentrations.

A BLAST search revealed that LysO1 was most similar to the lysozyme of phage P22. Based on that sequence similarity, it was expected that the pH range for optimal LysO1 activity would be similar to that of Lyz, which is pH 7-8, and the control pH of 8 was selected based on that expectation (35). Like P22 Lyz, LysO1 was more active at alkaline pH, but unlike Lyz, the optimal pH for LysO1 activity was determined to be 10, much higher than expected. Lysozymes with high optimal pH are rare, but not unheard of; lytic activity of CEWL is optimal at pH 9.2 (40). A possible reason for the high optimum is that *S*. Typhimurium is a gastrointestinal organism, and optimal growth is observed at acidic to neutral pH. The high rate of reaction observed at pH 9 and 10 may be due in part to Salmonella being easier to lyse due to the high pH, and the slightly higher rate of activity of the control treatment at higher pH supports the idea. However, the experiment also demonstrates that LysO1 is active at basic pH. An optimal pH in the alkaline range could be useful; some antimicrobial treatments used during processing, such as TSP, increase pH, and LysO1 would not be inactivated by the conditions.

LMS analysis suggests that as pH increased, the importance of that factor and its interactions with other factors decreased compared to a pH 8 control. Observed activity at pH 7 was only 65% of the rate of reaction at pH 8, a difference of 35%. Rate of reaction at pH 9 was 110%, a difference of only 10%. The difference may be due to LysO1 having an alkaline optimal pH; the effect of increasing already alkaline pH on rate of enzyme reaction is less than increasing acidic or neutral pH to alkaline, because the rate of reaction is already close to the maximum rate of reaction possibly for the enzyme. In that case, temperature and substrate concentration would have a greater effect on rate of reaction than a change in pH that was already near optimal.

It is accepted that the interaction between a lysozyme and the cell wall containing its substrate is driven by electrostatic forces – the positively charged lysozyme is attracted to the negatively charged cell wall (16). Increasing ion concentration interferes with that attractive force, and a net decrease in enzyme

activity is observed as the result. The reason for activity being pH-dependent is two-fold. First, lysozyme activity depends on unprotonated glutamic acid and protonated aspartic acid (17). Decreasing pH increases the likelihood that both residues will be protonated, which interferes with catalytic activity. Also, decreasing pH decreases the charge on the cell wall, weakening the electrostatic force between the lysozyme and the cell wall (16).

LysO1 is most thermostable at temperatures ranging from room temperature to slightly below body temperature. Although incubation at temperatures ranging from 0 °C to 40 °C resulted in a net decrease in enzyme activity compared to enzyme stored at -80 °C, the results demonstrate that prolonged incubation at body temperature or slightly above does not inactivate the enzyme. The effects of prolonged exposure to cooking temperatures on LysO1 activity have not been determined. Lysozymes are typically inactived by exposure to cooking temperatures at alkaline pH, though the length of time necessary for denaturation also depends on the food matrix (24). Consequently, the effects of cooking temperatures on LysO1 activity would need to be experimentally determined in the food system of interest.

The lack of complete inhibition when exposed to ambient temperature may be explained by evolution: the host of the enzyme's phage source is an enteric bacterium which lives at ambient temperature or body temperature. LysO1 is necessary for successful replication, yet is produced early in the infection cycle (46). The replication cycle of FelixO1 is about an hour, so any mutation that resulted in LysO1 completely denatured by short exposure to ambient temperature would be a disadvantage.

Enzyme activity was not affected by the lowest concentration of calcium tested (0.1 mM); the rate of reaction and change in optical density of LysO1 in 0.1 mM Ca^{2+} were not significantly different from the control lacking calcium. However, gradual increase of calcium concentration resulted in a decrease in observed enzyme activity – the rate of reaction was lower in the presence of 1.0 mM calcium, and lower still when calcium was added to 5.0 mM. A similar pattern was seen when magnesium was added – as magnesium concentration increased, rate of reaction and net change in optical density decreased.

The explanation for the observed effects could be one or a combination of several mechanisms. Divalent cations are known to bind to and stabilize cell membranes, and the increased charge could also affect the electrostatic interaction between the enzyme and the cell surface (23, 34). Some lysozymes also show decreased activity at higher ion concentrations, and addition of the divalent cations could increase ion concentration enough to adversely affect activity (16, 35). Tris·HCl buffer at a concentration of 0.05 M has an ionic strength (*I*) of 0.05 M. The *I* of 0.025 M Tris buffer with 5.0 mM $CaCl_2$ or $MgCl_2$ added is slightly lower (0.04 M), but the rate of reaction of the enzyme is much lower in buffer with magnesium or calcium added than in Tris buffer alone. Essentially, the difference in enzyme activity between buffer and buffer with added calcium or magnesium is larger than can be explained by increased ionic strength from the added ions alone. Consequently, the observed decrease in enzyme activity is thought to be due to a combination of reinforcement of the cell surface, and electrostatic interference of the enzyme binding to the peptidoglycan.

Date from the pH and divalent cations studies also suggest potential difficulties problem in the development of LysO1 as a food additive. Chicken skin is slightly acidic, with a pH of about 6.41 (3). Consequently, LysO1 would need to be combined with an additional treatment to raise the pH of the skin, or would need to used during a processing step that already incorporates agents that raise the pH to alkaline levels for the treatment to be an effect antimicrobial measure. Like low pH, the presence of divalent cations decreased enzyme activity. The surface of poultry skin during processing may be rich with cations due to plasma leakage, and could decrease the effectiveness of a surface application or wash of LysO1. Combining the LysO1 with a small amount of ETDA may protect enzyme activity: EDTA acts as a chelator to remove metal cations, and is already in use as an antimicrobial food additive (11).

The effect of sodium on enzyme activity was more subtle than that of the tested divalent cations, and similar to that observed in P22 Lyz. Similarity to Lyz was expected – that enzyme is the most similar to LysO1 according to a BLAST search, and sodium was reported to have an inhibitory effect on its activity: Lyz retained 32% of its normal activity in 0.025 M Tris buffer containing 51 mM sodium (*I* = 0.076) (35) . Similarly, LysO1 retained 44% activity in Tris buffer with 50 mM sodium added (I = 0.075).

Comparison of the rate of reaction of LysO1 in sodium buffer to the rate in Tris buffer of a similar *I*-value clarified the inhibitory effect: LysO1 retained 88.7% of normal function in 0.05mM Tris buffer ($I = 0.05$), and 79% in 25 mM Tris with 25 mM NaCl added ($I = 0.05$). At high concentrations the effect is more noticeable: LysO1 in 100 mM Tris ($I = 0.1$) retained 62.7% of activity, but LysO1 in 25 mM Tris with 100 mM NaCl added ($I = 0.125$) retained only 8.4% of activity.

The results of the sodium study have implications for the use of LysO1. The amount of sodium in TSP is inherent in the name of the compound; there are three moles of sodium in each mole of TSP. The amount used during poultry processing as an antimicrobial measure is between 8 and 12% wt./vol. The molarity of an 8% solution of TSP is 0.49 M. The effective concentration of sodium in that solution is 1.47 M, and that concentration would render LysO1 nonfunctional. Although we had thought LysO1 might be a suitable antimicrobial agent to combine with TSP during processing, the detrimental effects of sodium on enzyme activity would require any processing facility that adopted the treatment to use a sodium-free antimicrobial method in the chill tank. Of additional note is that many processed foods contain sodium, so the treatment would be limited to use in low-sodium or sodium-free products.

Activity in the presence of zinc did not follow the pattern set by calcium and magnesium. Instead of the gradual decrease in LysO1 activity seen with increasing Mg^{+2} or Ca^{+2} concentration, there was no detectable enzyme activity at the lowest concentration of zinc tested. Although some enzymes are known to require zinc as a cofactor, others, including lysozymes, are inhibited by the presence of the metal (35). Zinc binds proteins, and by preference binds to residues with negative charges, including aspartate and glutamate (41). As those residues are necessary for lysozyme function, zinc binding would render the enzyme completely inactive.

LysO1 lysed all tested strains of *Salmonella*. Though our results indicate that the enzyme worked with varying efficiency depending on the strain tested, it is known that enzyme rate of reaction depends in part on the concentration of the substrate, and the differences may be due in part to the different amounts of substrate used in the individual tests (18). However, the average initial optical density of serovar Agona was among the highest of the trial, but the reaction rate of LysO1 against Agona was the highest

observed. The average initial optical density of serovar Reading was the lowest observed, and the reaction rate of LysO1 against it was also the lowest measured. The rate of enzyme reaction may be affected by several things – among them is the thickness of the peptidoglycan layer and arrangement of the glycan strands, which can vary with the growth phase and turgor pressure of the organism (44). However, as all cells were grown and harvested under the same conditions, it is inferred that the different rates of LysO1 reaction observed are due in part to small differences in the *Salmonella* cell wall structure among the different serovars. Also, the difference implies that effect was not influenced by CHL, but by the addition of LysO1.

When tested with other foodborne pathogens, LysO1 lysed *E. coli* and had no effect on Gram positive *L. monocytogenes*. Lytic activity on a *C. jejuni* substrate could not be definitively determined from the data gathered. The results were not unexpected – *Escherichia* and *Salmonella* are closely related and their peptidoglycan structures are similar (38). It is thought that the difference in lytic activity between *Listeria* and the *S.* Typhimurium control is due to variations in peptidoglycan structures of Gram positive and Gram negative bacteria. Although peptidoglycan of Gram positive and Gram negative organisms contains the same components, there are differences in length and arrangement of the glycan strands, and additions and substitutions of individual peptides in the crosslinks (44). Active sites of lysozymes recognize a sequence of three disaccharides of the glycan strand, and cleave between the fourth and fifth saccharide molecules (17). If the series of disaccharides is blocked or altered by any of these different arrangements, additions, or substitutions, the cleavage site will not fit the enzyme active site.

The CHL pretreatment did not have the effect on the OD of *Listeria* that it had on *E. coli*, *S.* Typhimurium, and *C. jejuni*. That lack of effect may also be explained by the Gram positive cell structure. The layer of peptidoglycan of Gram positive organisms is much thicker than that of Gram negatives. CFU studies have determined that CHL pretreatment is lethal to *S.* Typhimurium, and TEM results indicate that pretreatment does not often results in lysis, but does cause visible breakdown and thinning of the cytoplasm. The Gram positive cell wall may be thick enough to offset the effect the

decreased optical density caused by the thinning cytoplasm, and so the optical density of *Listeria* would not be adversely affected by CHL pretreatment as the Gram negative organisms.

LMS analysis consistently reported that interaction between the pretreatment and enzyme treatment had a measurable effect on the experimental outcome. The LMS results highlight what is already known: Gram negative organisms need some form of permeabilization and anti-lysozyme negation for lysozyme treatment to work. When a third factor was added to the experimental design, LMS analysis reported that interaction among pretreatment, enzyme treatment, and that third factor had a consistently lower F Ratio than that of the interaction between the pretreatment and treatment factors, suggesting the magnitude of that effect on experimental outcome was not as great as the effect of the interaction between pretreatment and treatment. As the focus of this study has been the determination of conditions optimal for enzyme activity of LysO1, ANOVA analyses of single sub-groups of cells has been discussed more in-depth than the results of the LMS analysis. The ANOVA analyzes only the cells that have been pretreated the same way and treated with the same amount of enzyme (or buffer). The differences of a single variable (divalent cation concentration, for example) on LysO1 function within those parameters are often quite stark, but LMS analysis reminds us that the most important factor is the reaction of the enzyme on permeabilized cells, and underscores the importance of finding a GRAS pretreatment if foodborne application is to be truly effective.

4.5 Implications for development of LysO1 as an anti-*Salmonella* food treatment

Any enzyme developed as an antimicrobial food treatment must be lethal for the target bacteria, and we were unable to demonstrate lethal activity under the conditions tested. If lethality can be determined, the characterization of LysO1 revealed that it possesses a number of characteristics that make it a good candidate for further development as food-safe anti-*Salmonella* treatment:

Broad lytic spectrum within the *Salmonella* genus – the observed decrease in OD_{600} after addition of LysO1 has been demonstrated to be due to LysO1 enzymatic activity and not to continuing activity of the CHL pretreatment. LysO1 lyses all tested strains of *Salmonella*. Although *S.* Typhimurium is an

important pathogenic serovar, it is not the only foodborne *Salmonella* serovar capable of causing illness, and the broad-spectrum activity displayed by LysO1 against other serovars of the genus, suggests it is a good candidate for development as an anti-*Salmonella* food additive.

Rapid activity – the vast majority of lytic activity observed occurred in the first five minutes after addition of the enzyme. This rate of activity is compared to CEWL, which, when added to the same concentration and observed for the same length of time, was only a fraction as effective.

Spectrum of activity - In addition to being lytic for numerous *Salmonella* serovars, LysO1 is also lytic for the tested strain of *E. coli*. If that susceptibility to LysO1 is widespread in the *Escherichia* genus as it is in the *Salmonella* genus, LysO1 May be useful in controlling, in particular, the "big six", non-O157:H7 serotypes of *E. coli*. The serotypes in question produce Shiga toxin, are associated with serious foodborne illness, The six serotypes are O26, O45, O103, O111, O121, and O145; they were recently classified as food contaminants by the FDA, and raw ground beef and beef scraps must test negative for them before sale and distribution (42).

Thermostability – during processing, poultry is chilled to 4 °C and held at that temperature. Experimental evidence indicates LysO1 is not inactivated by the low temperature. However, should enzyme treatment be used during the chill step, the rate of reaction at that temperature would need to be determined.

Use of the enzyme does possess certain disadvantages. Activity is inhibited by low pH, high concentrations of sodium, and divalent cations. Poultry skin pH is slightly acidic, and may contain both monovalent and divalent cations. Many food additives contain sodium. These conditions may be circumvented by using LysO1 in conjunction with an alkaline permeabilization agent, a chelator such as EDTA to remove metal cations, and/or a permeabilization agent that does not contain sodium.

A second disadvantage is that the enzymatic activity of LysO1 is inhibited by a protein, PliC, that is naturally produced by *Salmonella* species (8). The disadvantage may be circumvented by co-treatment with an agent that neutralizes PliC. Should that prove impractical, it may be possible to use LysO1 as the

basis of an antibacterial treatment that is not dependent on enzyme activity such as an antimicrobial peptide, a possibility further discussed in Appendix A.

5.0 References

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Chapter 5

Conclusions and Future Work

1.0 Introduction

This body of work discusses the steps taken to locate the bacteriophage Felix O1 holin gene and to characterize the holin and endolysin proteins. We have successfully characterized the endolysin, LysO1, and demonstrated proof of concept: that the enzyme has potential as an anti-*Salmonella* food treatment if a suitable permeabilization agent can be identified. BLAST studies suggest it is a member of the lysozyme superfamily of proteins that cleaves peptidoglycan at the β-1,4 bond between *N*-acetylglucosamine and *N*-acetylmuramic acid. The enzyme appeared to cleave the peptidoglycan of all *Salmonella* strains tested, as well as *E. coli*, though it had no effect on tested Gram positive organisms. The enzyme was most active at basic pH (pH 10) and low ionic strength *I* (0.025 M). The divalent cations calcium, magnesium, and zinc had an inhibitory effect on enzyme activity. Monovalent sodium was also inhibitory, though a much higher concentration was necessary for the effect to be observable. When removed from frozen storage, the enzyme was most stable between 22 and 30 °C.

An additional research goal was to identify the holin gene and characterize the holin protein, but a homology search, transmembrane domain analysis, and signal sequence analysis did not reveal a likely candidate. Attempts to clone Felix *rIIA*, thought at the time to be a possible holin, were not successful, and later review of the literature revealed that although RIIA is active in host lysis, its role is distinct from that of the holin protein.

These findings suggest a number of additional research questions regarding LysO1, RIIA, and the still-elusive holin protein. Although we have demonstrated proof of concept regarding LysO1, development as a food treatment or additive requires us to understand the enzyme's mechanism of action and to demonstrate lethal activity against the target bacteria. Conditions optimal for LysO1 activity must also be fully determined. Although the holin is no longer under consideration for development as a

permeabilization agent, the lack of observed homology to any known holin is puzzling, and a way to determine the reason for that lack is to identify the gene and characterize the protein. RIIA proteins are known to play a role in host lysis under certain conditions, but that role has not yet been fully elucidated. Further study of the RIIA protein may answer those questions.

2.0 Endolysin

2.1 Structural studies

Very little has been determined about the structure and biochemistry of LysO1, with the exception of work done *in silico*. If the enzyme is to be further developed as part of a food additive, it will be subject to FDA approval before use, and it is therefore necessary to know the details of the structure and mechanism of action of the enzyme, as well as potential adverse health effects (40). Understanding the mechanism of action could also help understand ways to optimize LysO1 production and application

The structure of the enzyme has been predicted, but those predictions must be confirmed. The most accurate method of confirmation is X-ray crystallography, which has been used to determine protein structures to within about 1.8 Å, and the generated data can be further refined using software (22, 28). A common method of crystal formation is hanging drop vapor diffusion, in which purified protein and buffer are combined in a reservoir and crystals form over time as the protein precipitates. Crystals of the *B. anthracis* phage endolysin PlyL catalytic domain were obtained using 2 µl protein and 1 µl buffer (0.6 M NaH_2PO_4, 1.0 M K_2HPO_4, 0.1 M acetate at pH 6.7), but the crystallization conditions of LysO1 may be different (25).

Another method for determination of protein structure is nuclear magnetic resonance spectroscopy (NMR), which can be used in conjunction with or in place of X-ray crystallography (45). One advantage of the method is that crystallization of the protein of interest – often the most difficult step in protein crystallography – is not necessary for NMR. Studies are conducted in two phases. The first uses ^{15}N- and ^{13}C-labeled recombinant protein to determine the positions of atoms in the backbone of the structure, the second determines the positions of the side chain residues (35).

The mechanism of action may be learned by pursuing two different lines of inquiry: identification of the active site residues and the exact site of cleavage of the substrate. Lysozyme activity depends on a glutamic acid residue and an aspartic acid residue at the *N*-terminus of the enzyme (11). It is thought that the amino acids essential for LysO1 activity are glutamic acid at position 15, and aspartic acid, either at position 24 or 39. Point mutations of the individual residues in question would be able to determine if the particular residue is important to enzyme function (26). Mutations from one residue to another of a similar size and charge would remove the amino acid without changing the shape of the active site; and it is possible by site-directed mutagenesis to alter each residue of interest to one with similar characteristics: glutamic acid at position 15 to glutamine, and the aspartic acid residues at positions 24 and 39 to asparagine residues. Genes with individual or combinations of these alterations could be expressed and the proteins purified and characterized to determine which residue, or combination of residues, is necessary for enzyme activity.

A second part of determining the mechanism of action is to identify exactly where the enzyme cuts. By definition, a lysozyme cleaves the β-1,4 glycosidic bond between *N*-acetylmuramic acid and *N*-acetylglucosamine. Sequence homology identified LysO1 as a lysozyme and it is expected that it would cut that same bond (11). Confirmation of the location of the cleavage site may be achieved by treating purified peptidoglycan with LysO1 and analyzing the fragments with mass spectrometry to ascertain which bond of the substrate the enzyme breaks.

2.2 Potential use as an antimicrobial food additive

We have shown that LysO1 is a good candidate for further development as an anti-*Salmonella* food treatment or additive. It lyses permeabilized *S*. Typhimurium and has demonstrated lytic activity for all tested strains of *Salmonella enterica*, was not denatured after incubation at a range of temperatures including the temperature at which poultry are chilled, and was only partially inhibited by low concentrations of sodium.

However, additional questions must be answered for development to proceed. The initial objective is to quantify the lethality of the enzyme. Although results from activity assays and from transmission electron microscopy indicate that LysO1 is lytic to *Salmonella*, the methods employed were unable to demonstrate that the enzyme is also lethal to the target bacteria. The lack of a nonlethal permeabilization treatment preempted any attempts to confirm the data collected through optical density measurements with experiments that measured numbers of viable bacteria before and after treatment with LysO1.

Any food additive should be classified as generally recognized as safe (13). The use of chloroform as a permeabilization agent was effective to establish proof of concept but is not safe for human consumption. Tris·EDTA also permeabilizes *Salmonella* to endolysin activity, and derivatives of EDTA (calcium disodium EDTA and ferric disodium EDTA) are currently FDA-approved as food additives (41). However, Tris is not GRAS, so one possibility is to combine EDTA with a high-concentration buffer that is safe for consumption. Also, the approved derivatives of EDTA incorporate metal cations. The effects of iron on LysO1 activity have not been tested, but we now know that calcium is inhibitory. Consequently, the EDTA derivatives would need to be non-inhibitory for LysO1 to have any antimicrobial effect on the treated product.

Studies suggest that the lysozyme treatment may be effective if combined with an essential oil. Essential oils such as carracrol (oregano oil) have permeabilizing effects on bacterial membranes, and increase the shelf life of artificially contaminated poultry products when used in conjunction with lysozyme (4, 31). However, it has been reported that essential oils function better at low temperature and low pH, so studies would have to be done to see if they are also functional at the pH and temperature necessary for optimal lysozyme activity. Alternatively, they might be combined with inactivated lysozyme that retains its antimicrobial properties without enzyme activity (4, 9).

After the pretreatment combination has been determined, the next step would be to test it using a food-based model. A model developed in our lab used artificially contaminated samples of chicken frankfurters to demonstrate the efficacy of bacteriophage Felix O1 to suppress the growth of *S.*

Typhimurium DT104 (43). One problem with the model is that it uses a higher concentration of *Salmonella* that would occur under natural conditions, as it was thought a lower inoculum would not be sufficiently reproducible to allow rigorous statistical analysis (43). The model also did not address the question of the minimum concentration of phage necessary for growth suppression. However, the model was an excellent tool to determine the efficacy of phage treatment and could be adapted to test and compare several treatments at once. It could be easily adapted to test the efficacy of LysO1 instead of phage, and the problems could be circumvented by further testing. Testing more replicates of a lower initial inoculum of *Salmonella* would ensure reproducible results, and using the same amount of inoculum to test the efficacy of varying concentrations of LysO1 could determine the lowest amount necessary.

After demonstrating efficacy in a food-based model, production of the enzyme would need to be scaled up, which can be difficult even if small-scale production has optimal yield. Our protocol has another disadvantage: production as described in Chapter 4 was lower than expected.

The pRSET A expression vector we used for the expression of LysO1 incorporates a T7-*lac* promoter, which is known for its high yield (38). Our yield was lower than expected, although we attempted a number of different expression conditions to increase productivity. One option to increase LysO1 production may be to insert the gene into another plasmid under the control of a different promoter. Moderately high expression has been reported in plasmids that incorporate the *tac* promoter (8). Another option is to place the gene under control of the P_{BAD} promoter, which is expression-variable depending on the amount of inducer used (14). A disadvantage of using the *tac* and T7-*lac* promoters is that the IPTG necessary for induction is relatively expensive (38). While the cost may be negligible for the small amounts of protein produced in a laboratory setting, it would be prohibitive for the production of commercially useful quantities. L-arabinose, used to induce expression of genes under control of the P_{BAD} promoter, may be more economical (38).

Two expression hosts commonly used to increase recombinant protein expression for industrial applications are *E. coli*, often used for prokaryotic proteins, and yeast such as *Saccharomyces cerevisiae*, for eukaryotic proteins (42). Each system has disadvantages: proteins expressed in *E. coli* are retained in

the cytoplasm, so cells must be lysed to obtain the protein. While proteins expressed in yeast are secreted, they are often secreted to the periplasmic space, not the culture medium. Proteins expressed in *E. coli* may be in inclusion bodies and require reconstitution, and expression levels in yeast are low compared to those achievable using an *E. coli* host. However, each system has advantages, as well. Both systems are well characterized, and the fermentation technology is well established. In addition, *E. coli* can produce large amounts of the target protein, while the expression level in yeast is not as high. LysO1 is a phage protein, and its natural host is a Gram-negative bacterium closely related to *E. coli*.

An alternative to expression and purification is to synthesize the enzyme. Solid phase protein synthesis (SPPS) allows the growth of peptides by addition of individual amino acids to the growing peptide chain. However, two characteristics of the process make it less than ideal for industrial-scale expression of LysO1. Although the cost has decreased since the technology was pioneered, it remains expensive. Also, there is an upper limit of peptide length of about 60 aa that can be practically expressed using SPPS (30). These limitations currently make *de novo* synthesis a viable option only for small-scale use. However, as synthesis costs decrease and the process is optimized, SPPS may become a viable option, particularly if alternatives to LysO1, such as a minimized sequence that retains enzyme activity or a sequence-derived antimicrobial peptide, are developed (9, 21). A further consideration concerns the ultimate use of the expressed enzyme as a food additive. Proteins expressed in *E. coli* may have LPS contamination of the final product, so the enzyme must be expressed in a nonpathogenic GRAS host and carefully purified (42).

LysO1 activity is dependent on four factors: temperature, pH, ion concentration, and the concentration of substrate. To be most effective, LysO1 would be limited to basic environments of relatively low ion concentration. As the enzyme had no effect on *M. luteus* or *L. monocytogenes*, its use would likely be limited to elimination of Gram negative contaminants.

2.3 Improvement of a LysO1-based antibacterial food treatment

Concerns with the adaptation of any new process are related to safety, efficacy, cost, and ease of use. A new food antimicrobial additive or treatment must be safe for human consumption, effective for the purpose for which it is intended, and economical. In addition, the method of application should be uncomplicated and easy to incorporate into existing processes.

2.3.1 Treatments dependent on enzyme activity

A disadvantage of the LysO1-based antibacterial food additive is that the enzyme displays little to no activity against Gram positive bacteria. One of the four most important foodborne pathogens, *Listeria monocytogenes*, is Gram positive, and the effects of listeriosis can be severe, especially if the patient is pregnant, very old, very young, or immune-compromised (6). Unlike the single-domain arrangements of endolysins from phages specific for Gram negative bacteria, many of the endolysins from Gram positive-specific phages that have been characterized to date contain two domains; a *C*-terminal recognition domain to find the cleavage site of the substrate, and an *N*-terminal domain that cuts the bond at that site (11). Studies indicate that a recognition domain attached to the *C*-terminus of the activity domain of a different endolysin expands the activity spectrum of the endolysin (2). Studies in question suggest that the recognition domain is not necessary for activity; it may be possible to attach a recognition domain from an endolysin of a *Listeria*-specific phage such as PSA to the *C*-terminus of LysO1 to expand its spectrum of activity (22). It is possible the altered enzyme would no longer recognize its original *Salmonella* substrate due to the specificity of the recognition domain, but it is equally plausible that it may be incorporated into a combination therapy to serve as a broad-spectrum antibacterial treatment. Of course, the altered enzyme would need to be characterized to determine the effects of addition of the new domain on activity, i.e., optimal temperature range, pH, and ion effects.

A way to improve the cost effectiveness of a LysO1-based treatment is to develop a reusable treatment. A novel method for detection of *Listeria* in food products involved the attachment of a protein derived from the endolysin of a *Listeria*-specific phage and the beads used were paramagnetic (29). The

beads were recovered following application of a magnetic field. However, enzyme function after bead attachment was unreliable. (Also, if paramagnetic beads are determined to be a viable methods of treatment recovery and re-use, they must also be FDA approved a food treatment). In another study, CEWL was functional, but not as active as unbound enzyme (44). A third study reported that attachment to positively-charged polystyrene beads increased enzyme activity; it was speculated that the positive charge of the bead enhanced interaction of the enzyme with the negatively charged cell wall (34). As enzyme activity is critical to the effectiveness of the antimicrobial treatment and bead attachment may decrease the effectiveness, an alternative to development of a reusable treatment might be to use a denatured enzyme that is not dependent on enzyme activity for antimicrobial activity, rather than an enzyme that must be functional to be effective (9).

A potential obstacle to the development of a reusable enzyme-based treatment is that, in the case of LysO1, the enzyme loses some lytic activity when removed from frozen storage. However, combining paramagnetic beads with a stabilized mutant of LysO1 may be effective, thought the beads would also need to be approved as a food additive. Lysozyme is tolerant of mutations, provided changes are not to residues essential for enzyme activity (1). The T4 lysozyme has been successfully mutated to incorporate disulfide bridges which have been shown to stabilize protein structure. Addition of two bridges (Cys 3-97 and Cys 9-164) resulted in an enzyme with a melting temperature 15 °C higher than that of the wild type, but lytic activity was unaltered (27). However, sites for addition of cysteine residue mutation would need to be carefully selected to avoid conformational changes at the active site (27).

Another method to decrease the overall cost of the treatment might be to engineer the enzyme to be shorter while retaining activity. Minimization of protein structure has been demonstrated to be feasible with small antimicrobial and binding proteins; length can be reduced by as much as 50%, and NMR analysis suggests the minimized sequences have three-dimensional structures very similar to the parent

protein (21, 37, 39). Minimization, either through design, phage display-based selection of mutants[2], or a combination of the two methods, involves the removal of stretches of the sequence not directly involved in antimicrobial or binding activity and stabilizes the shorter sequence through addition of disulfide or other internal bonds (7). More recently, it has been shown that these methods may be used on large proteins and enzymes (15). As the cost of protein expression is often determined per gram of purified protein, engineering a smaller enzyme that retains function would decrease the cost of the treatment.

2.3.2 Treatments independent of enzyme activity

Some heat-denatured lysozymes retain antimicrobial activity, though the mechanism of action changes (9, 18, 19). There are advantages to relying on a physical mechanism of action instead of an enzymatic mechanism. The processing temperature of certain food is often regulated; meat and poultry are chilled to 4 °C during processing and all subsequent processing steps must be conducted at that temperature or lower. Lysozyme activity is temperature-dependent, and activity decreases as temperature decreases (33). Most lysozymes are much less active at colder temperatures, so a treatment incorporating lysozymes during or post-chill would require more enzyme and a longer contact time, to achieve the same effect as an enzymatic reaction conducted at room temperature (12). The antimicrobial activity of denatured lysozymes is thought to be due to membrane perturbation of the bacterium. The mechanism is dependent on increased hydrophobicity of denatured lysozyme and is unaffected by temperature, so taking advantage of such a mechanism would increase the range of conditions over which the treatment could be effective (18-20).

Another possibility is to explore the mechanism of action of the denatured enzyme to see if the reported membrane perturbation results in permeabilization of the outer membrane and would be an adequate pretreatment for LysO1 (18-20). There is the possibility that the denatured lysozyme will also inactive the lysozyme inhibitor produced by S. Typhimurium, PliC. However, the inhibitor acts by

[2] Phage display is a method of protein expression whereby protein-coding DNA is cloned into a phage genome, and tagged such that the protein is expressed in the exterior of the phage capsid. To generate mutants of a single protein, mutated DNA sequences are inserted instead of the wild-type gene (7).

binding across the lysozyme active site and inserting a loop of its structure into the active site cleft (23). To adequately bind to and neutralize the inhibitor, denatured LysO1 would need an active site that was still similar in structure to active LysO1.

Denatured enzymes have been reported to display antimicrobial activity over an increased host range, which would be a second advantage to their use (19). There are four bacterial pathogens of major importance to the food industry. A limitation of phage endolysin-based treatments is that a treatment might only be effective against the genus of bacteria that is the phage's natural host. A disadvantage of phage therapy is that the phage is active against a very narrow range of bacteria.

A second possibility is to develop an antimicrobial peptide (AMP) from the LysO1 sequence. Antimicrobial peptides are short peptide sequences, generally less than 150-200 aa in length, cationic, with an amphipathic structure, produced by most living organisms (3, 17). Some are gene-encoded and transcribed in active form, others are cleaved in active form from a larger, inactive precursor (3). An antimicrobial food additive based on an AMP could in some ways be more advantageous than an additive based on a functional enzyme, because AMPs are smaller and therefore less expensive to produce. They also often have a broad-spectrum antimicrobial activity, and work by inserting themselves into and disrupting cell membranes (3, 17). Resistance would require restructuring of the cell membrane (46).

There are different approaches to producing an antimicrobial peptide. The LysO1 protein may be digested with one or more common peptidases and the crude digest tested for antimicrobial activity against the organism of interest using a standard plate or broth assay. If the crude digest displays antimicrobial activity, the individual fragments may be purified from the digest and tested individually. Another approach would be to take advantage of antimicrobial peptide prediction software available to screen known protein sequences for peptides that display appropriate characteristics. We used AntiBP2, APD2, and CAMP to screen the RIIA and LysO1 protein sequences for fragments that might have antimicrobial activity (Appendix A). The segments generated by *in silico* protease cleavage suggest that LysO1 possesses segments with antimicrobial potential. Cleavage could be achieved using chymotrypsin, and segments not easily obtainable from proteolytic cleavage could be cloned and purified for further

analysis. Additionally, de novo synthesis of short sequences could be a viable method to produce enough of the AMP for initial laboratory analysis.

The fourth α helix of the T4 lysozyme (aa 143 to 155) has been determined to have antimicrobial activity (5, 9). Many antimicrobial peptides, both gene-encoded and cleaved, have α helical conformations, so another method of AMP production could be to focus on the α helices of LysO1, identified in Chapter 4 (16).

3.0 Further Characterization of RIIA

To date, the function of RII proteins remains unknown, but several possibilities have been suggested. Mutants of RII display rapid lysis and will not plate on K-12 strains of *E. coli* lysogenized with bacteriophage λ (32). It has been proposed that RII proteins are not active in lysis inhibition, but that the lack of RII proteins results in cell lysis when other prophages are present in the genome (32). The protein has also been hypothesized to be a membrane protein that adversely affects function of *E. coli* membrane ATPase (10). That effect may be fatal to any host if overexpressed in even small amounts, such as those produced by leaky expression.

The first steps of the preferred method of protein characterization are to insert the gene of interest into a plasmid, then to use a bacterial host to grow the plasmid to high numbers so it can be purified, sequenced, and check to ensure that the gene inserted correctly. However, the lethal properties of RIIA in even the small amounts resulting from basal expression have made obtaining a plasmid containing the gene of interest difficult. As basal expression is the suspected cause of our lack of success in obtaining a host with the plasmid containing the gene of interest, a plasmid with lower levels of basal expression may make cloning *rIIA* possible. The expression vector pQE-80L is a low-copy plasmid that incorporates cis-lacIq, which overexpresses LacI and keeps expression very tightly repressed. It has also been a successful vehicle for cloning and expression of toxic genes (24). An expression host containing the plasmid pREP4, which constitutively expresses LacI, would decrease basal expression of RIIA from any experimental plasmid transformed into the host organism. That repression would have two

advantages. It would enable the host bacteria to grow and propagate enough of the plasmid-RIIA construct to sequence and ensure that the gene inserted correctly and would yield a protein of the correct sequence. During expression, strong repression until specific induction would enable the host cells containing the expression construct to grow to optimal conditions before expression of the protein (36).

4.0 Conclusions

While the course of study examining the lysis proteins of Felix O1 has answered many questions, it has generated even more. Despite the diversity of known holin proteins and the multiple methods used to identify the Felix holin, we did not find a suitable candidate gene. That lack in the face of such incredible holin diversity has raised questions about holin proteins in general and what new information about the family regarding location of the gene, protein structure, and sequence homology the eventual identification of the Felix O1 holin will reveal.

RIIA proteins are active in the phage lytic cycle under very specific conditions. Although they have been identified as membrane proteins and are known to be lethal to many expression vectors, little else is known about them; their role in lysis has not been fully determined. Our results add impetus to the questions regarding the role and mechanism of action of RIIA proteins during host cell lysis.

LysO1 is a functional enzyme, is lytic for tested serovars of *Salmonella*, and is a good candidate for development as an anti-*Salmonella* food treatment provided a suitable permeabilization agent can be identified. Several possibilities have been proposed, and EDTA appears to have potential in that regard. In order for development to be successful, the mechanism of action and lethality must also be determined. Once that information is available, there are a number of avenues of research to pursue to develop a reusable treatment, or an antimicrobial treatment based on the endolysin protein sequence.

In short, our work has answered some questions regarding Felix O1 RIIA, holin, and LysO1, but the conclusions drawn from the research revealed just how much remains to be discovered, and the incredible potential in this line of research for topics as diverse as phage lysis, protein science, and food safety.

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Appendix A

Detection of Antimicrobial Peptides in the RIIA and LysO1 Protein Sequences

1.0 Introduction

Antimicrobial peptides (AMPs) are naturally occuring peptide sequences with antimicrobial properties, produced by most living organisms. They are generally less than 150-200 amino acids (aa) in length, cationic, and have an amphipathic structure (4, 11). Some are gene-encoded and are translated in active form, others are translated in inactive forms and activated by cleavage (4). They are effective as broad-spectrum antibiotics against both Gram positive and Gram negative bacteria, including strains that are resistant to current antibiotics (8). They are also effective against mycobacteria, enveloped viruses, fungi, and cancerous cells (8).

The AMP mechanism of action against bacteria is dependent on electrostatic interactions between the cationic AMP and the negatively charged outer leaflet of the cell membrane. The interaction between the AMP and the membrane displaces membrane lipids, which alters the membrane structure (23). In some cases, the AMP enters the cell. Cholesterol decreases the effectiveness of AMPs, though it is unknown if the decrease is due to cholesterol stabilization of the membrane or to interaction with the AMP (23). Increasing ionic strength also decreases effectiveness of AMPs, thought to be due to decreasing electrostatic interactions between the AMP and the membrane (22).

An advantage of AMPs over traditional antibiotics is that, while resistance is possible, it is more difficult to develop than resistance to antibiotics (16, 23). To develop resistance, a bacterium would have to remodel its cell membrane to alter the lipid composition or configuration (23).

A wide variety of naturally-occurring antimicrobial peptides has been documented, but observed antimicrobial activity is not limited to those peptides that occur naturally (4-6).Two sources of artificial AMPs have been reported. AMP design, either *de novo* or by minimalization of an existing protein, is an area of intense research due to increased prevalence of antimicrobial-resistant bacteria, and has met with

some success (1, 9, 15, 19). Another source of AMPs is those peptides that are derived from larger proteins, such as aa 96-116 of hen egg white lysozyme (HEWL), and α helix 4 of the T4 lysozyme (6, 17). Given that peptide fragments of functional proteins may display antimicrobial activity independent of the function of the protein they are derived from, we decided to analyze the Felix O1 RIIA and LysO1protein sequences for any stretches that might display antimicrobial activity.

2.0 Materials and Methods

To predict antimicrobial peptides in a longer protein sequence, the RIIA sequence was copied from GenBank (accession number NP_944777.1) and divided into 100-aa segments that overlapped by 50 aa. The LysO1 sequence (GenBank, NP_944846.1) was divided two ways: into segments aa 1-100 and aa 61-154, and into segments aa 1-80, aa 51-120, and aa 81-154. Each segment was submitted to AntiBP2 (http://www.imtech.res.in/raghava/antibp2/submit.html) for analysis using the "NC-termin" terminus option of a Support Vector Machines (SVM) search algorithm, with a default threshold value of 0. A positive score assigned to an individual peptide by AntiBP2 indicated that peptide had potential antimicrobial activity. The sequences with antimicrobial potential were examined to ensure they were accurate sequences of LysO1 or RIIA, corrected if necessary, and analyzed for antimicrobial potential using APD2 (http://aps.unmc.edu/AP/prediction/prediction_main.php) and the Collection of Antimicrobial Peptides (CAMP, http://www.bicnirrh.res.in/antimicrobial/def/). Before submission to CAMP, sequences were converted to FASTA format using fmtseq (http://www.bioinformatics.org/JaMBW/1/2/). APD2 uses a single algorithm, CAMP uses three: SVM, random forest (RF), and discriminate analysis (DA). Per publications explaining the algorithms, default settings were used for all analyses (13, 18, 20).

To determine if peptides derived from protein digestion with a peptidase had antimicrobial potential, the LysO1 and RIIA sequences were submitted to Expasy PeptideCutter (http://expasy.org/tools/peptidecutter/). Proteins were cleaved *in silico* with chymotrypsin (high and low specificity), trypsin, pepsin (pH 1.3 and >2), proteinase K, thrombin, and enterokinase. Analysis of

fragments from cleavage with kallikrein and plasmin was not possible, as cleavage with those enzymes was not an option in PeptideCutter. Predicted fragments 15 aa in length or longer were converted to FASTA format if necessary and submitted to APD2 and CAMP for antimicrobial peptide prediction analysis as described above.

Sequences of LysO1 predicted by PHYRE to be α helices (Chapter 4, section 3.6) were submitted to APD2 and CAMP for analysis of antimicrobial potential.

3.0 Results

The analyzed peptide sequences were generated from a longer protein sequence using three different methods: software identification of smaller peptides in the longer sequence, cleavage with a protease, and location of possible α helices. All were analyzed by both APD2 and CAMP. For a sequence to be considered a potential AMP, three of the four algorithms used must predict that the sequence possessed antimicrobial activity.

3.1 Individual analysis of peptides

3.1.1 AntiBP2

AntiBP2 generated 155 potential AMPs from NTCT15 analysis of segments of the RIIA sequence, and 47 potential AMPs from analysis of segments of the LysO1 protein sequence. Full results are given in Tables A-1 and A-2. Briefly, of the 155 peptides from RIIA that were predicted by AntiBP2 to have antimicrobial activity, that prediction was supported for 29 peptides by further analysis using APD2 and CAMP. The corrected sequences ranged in length from 24 to 100 aa. Of the 47 peptides from LysO1 predicted by AntiBP2 to have antimicrobial activity, analysis with APD2 and CAMP supported that potential for four peptides, ranging in length from 18 to 34 aa.

Table A-1. Antimicrobial peptides identified from AntiPB2 analysis of the RIIA protein sequence.

AntiBP2 Sequence	Score	Corrected Sequence	Length	APD2	CAMP Prediction		
					(SVM)	(RF)	(DA)
Segment 1 (aa1-100)							
LSSGVYTFKERAVIRAST KNDSNDYIGAMG	0.267	LSSGVYTFKERAVIRELSCNAVDAQKEAGKENIPFHVHLPTRF EPYFEVRDFGTGLTHDKVMSLYLNGYASTKNDSNDYIGAM	84	maybe - 20	no - 0.970	yes - 0.634	no - 0.76
SNDYIGAMGIGSKSPPE MFSLLSSGVYTFK	0.227	PEMFSLLSSGVYTFKERAVIRELSCNAVDAQKEAGKENIPFHV HLPTRFEPYFEVRDFGTGLTHDKVMSLYLNGYASTKNDSNDY IGAMGIGSKSP	96	maybe - 22	no - 0.870	yes - 0.932	no - 0.715
GVYTFKERAVIRELSNYG ASTKNDSNDYIG	0.191	GVYTFKERAVIRELSCNAVDAQKEAGKENIPFHVHLPTRFEPY FEVRDFGTGLTHDKVMSLYLNGYASTKNDSNDYI	78	maybe - 18	no - 0.982	no - 0.998	no - 0.586
LPTRFEPYFEVRDFGNIPF HVHLPTRFEPY	0.138	NIPFHVHLPTRFEPYFEVRDFG	22	doubtful - 2	no - 0.995	no - 0.98	no - 0.904
VYTFKERAVIRELSCLNY GASTKNDSNDYI	0.117	VYTFKERAVIRELSCNAVDAQKEAGKENIPFHVHLPTRFEPYF EVRDFGTGLTHDKVMSLYLNGYASTKNDSNDY	76	maybe - 17	no - 0.990	no - 0.994	no - 0.776
RDFGTGLTHDKVMSLA VDAQKEAGKENIPF	0.112	AVDAQKEAGKENIPFHVHLPTRFEPYFEVRDFGTGLTHDKV MSL	44	maybe - 8	no - 0.993	no - 0.986	no - 0.953
LTHDKVMSLYLNYGARE LSCNAVDAQKEAG	0.055	RELSCNAVDAQKEAGKENIPFHVHLPTRFEPYFEVRDFGTGLT HDKVMSLYLNGYA	56	maybe - 13	no - 0.988	no - 0.998	no - 0.685
EAGKENIPFHVHLPTEPY FEVRDFGTGLTH	0.023	EAGKENIPFHVHLPTRFEPYFEVRDFGTGLTH	32	maybe - 3	no - 0.991	no- 0.982	no - 0.697
Segment 2 (aa51-150)							
FGTGLTHDKVMSLYLPQ VTKLTTNPTNEPN	0.562	FGTGLTHDKVMSLYLNYGASTKNDSNDYIGAMGIGSKSPFAI AQSFTVSSYVDGVVNKYSVYLENGIPQVTKLTTNPTNEPN	82	maybe - 16	no - 0.951	yes - 0.772	no - 1.176
NDYIGAMGIGSKSPFAQS FTVSSYVDGVVN	0.365	NDYIGAMGIGSKSPFAIAQSFTVSSYVDGVVN	32	very little chance	no - 0.944	no - 0.69	no - 1.104
SYVDGVVNKYSVYLEAST KNDSNDYIGAMG	0.267	ASTKNDSNDYIGAMGIGSKSPFAIAQSFTVSSYVDGVVNKYSV YLE	46	very little chance	no - 0.985	no - 0.844	no - 1.268
KSPFAIAQSFTVSSYMGI GSKSPFAIAQSF	0.239	MGIGSKSPFAIAQSFTVSSY	20	maybe - 3	no - 0.947	no - 0.818	no - 0.819

The AntiBP2 sequence is the sequence from the larger protein identified as an AMP, Score is the AntiBP2 score of antimicrobial activity. The corrected sequence is the AntiBP2 sequence after comparison to the protein and sequence corrections. Numbers after the APD2 prediction indicate the number of hydrophobic residues predicted to be on the same plane of an α helix; a "C" after the prediction indicates an even number of cysteine residues and the possibility of β sheet formation. Numbers in SVM and RF predictions are the likelihood of the prediction being true; numbers in DA predictions are the discriminate scores for the peptides. Bolded sequences indicate the corrected sequence received a positive score from three of the four prediction algorithms and is a potential AMP.

Table A-1 cont.

EVRDFGTGLTHDKVMKLTTNPTNEPNGLAV	0.199	EVRDFGTGLTHDKVMSLYLNYGASTKNDSNDYIGAMGIGSKSPFAIAQSFTVSSYVDGVVNKYSVYLENGIPQVTKLTTNPTNEPNGLAV	90	very little chance	no - 0.931	yes - 0.864	no - 0.928
DGVVNKYSVYLENGINYGASTKNDSNDYIG	0.191	NYGASTKNDSNDYIGAMGIGSKSPFAIAQSFTVSSYVDGVVNKYSVYLENGI	52	very little chance	no - 0.948	no - 0.754	no - 1.093
DFGTGLTHDKVMSLYQVTKLTTNPTNEPNG	0.186	DFGTGLTHDKVMSLYLNYGASTKNDSNDYIGAMGIGSKSPFAIAQSFTVSSYVDGVVNKYSVYLENGIPQVTKLTTNPTNEPNG	84	very little chance	no - 0.947	yes - 0.85	no - 1.109
GVVNKYSVYLENGIPLNYGASTKNDSNDYI	0.177	LNYGASTKNDSNDYIGAMGIGSKSPFAIAQSFTVSSYVDGVVNKYSVYLENGIP	54	very little chance	no - 0.985	no - 0.828	no - 1.141
PFAIAQSFTVSSYVDGAMGIGSKSPFAIAQ	0.113	GAMGIGSKSPFAIAQSFTVSSYVD	24	maybe - 5	no - 0.869	no - 0.638	no - 1.355
AMGIGSKSPFAIAQSSPFAIAQSFTVSSYV	0.054	AMGIGSKSPFAIAQSFTVSSYV	22	maybe - 12	no - 0.883	no - 0.718	no - 0.624
NPTNEPNGLAVRVAVEPYFEVRDFGTGLTH	0.023	EPYFEVRDFGTGLTHDKVMSLYLNYGASTKNDSNDYIGAMGIGSKSPFAIAQSFTVSSYVDGVVNKYSVYLENGIPQVTKLTTNPTNEPNGLAVRVAV	98	very little chance	no - 0.819	no - 0.904	no - 0.957
Segment 3 (aa101-200)							
FTVSSYVDGVVNKYSLADMNVIAREKGVYD	0.562	FTVSSYVDGVVNKYSVYLENGIPQVTKLTTNPTNEPNGLAVRVAVADHRISKFFEEAGNVYSYFAVKPESNIVYDDVLADMNVIAREKGVYD	92	very little chance	no - 0.866	yes - 0.954	no - 0.567
EAGNVYSYFAVKPESPQVTKLTTNPTNEPN	0.562	PQVTKLTTNPTNEPNGLAVRVAVADHRISKFFEEAGNVYSYFAVKPES	48	maybe - 11	no - 0.971	no - 1	no - 1.033
EPNGLAVRVAVADHRAVADHRISKFFEEAG	0.538	EPNGLAVRVAVADHRISKFFEEAG	24	maybe - 9	no - 0.899	no - 0.894	no - 0.412
MNVIAREKGVYDAMIAQSFTVSSYVDGVVN	0.365	AQSFTVSSYVDGVVNKYSVYLENGIPQVTKLTTNPTNEPNGLAVRVAVADHRISKFFEEAGNVYSYFAVKPESNIVYDDVLADMNVIAREKGVYDAMI	98	very little chance	no - 0.781	yes - 0.954	no - 0.804
KFFEEAGNVYSYFAVKLTTNPTNEPNGLAV	0.199	KLTTNPTNEPNGLAVRVAVADHRISKFFEEAGNVYSYFAV	40	maybe - 11	no - 0.934	no - 0.998	no - 0.539
EEAGNVYSYFAVKPEQVTKLTTNPTNEPNG	0.186	QVTKLTTNPTNEPNGLAVRVAVADHRISKFFEEAGNVYSYFAVKPE	46	maybe - 13	no - 0.964	no - 1	no - 0.849
SSYVDGVVNKYSVYLDDVLADMNVIAREKG	0.043	SSYVDGVVNKYSVYLENGIPQVTKLTTNPTNEPNGLAVRVAVADHRISKFFEEAGNVYSYFAVKPESNIVYDDVLADMNVIAREK	86	very little chance	no - 0.901	yes - 0.922	no - 0.462
VDGVVNKYSVYLENGIVYDDVLADMNVIAR	0.029	VDGVVNKYSVYLENGIPQVTKLTTNPTNEPNGLAVRVAVADHRISKFFEEAGNVYSYFAVKPESNIVYDDVLADMNVIAR	80	very little chance	no - 0.930	no - 0.868	no - 0.429
LENGIPQVTKLTTNPYSYFAVKPESNIVYD	0.001	LENGIPQVTKLTTNPTNEPNGLAVRVAVADHRISKFFEEAGNVYSYFAVKPESNIVYD	58	very little chance	no - 0.964	no - 0.998	no - 0.828

Table A-1 cont.

Segment 4 (aa151-250)							
RSSGNRTDFNVVMGNLADMNVIAREKGVYD	0.562	LADMNVIAREKGVYDAMIHKQSWRSSGNRTDFNVVMGN	38	maybe - 9	no - 0.949	no - 0.996	no - 0.962
MNVIAREKGVYDAMIQSWRSSGNRTDFNVV	0.3	MNVIAREKGVYDAMIHKQSWRSSGNRTDFNVV	32	maybe - 5	no - 0.943	no - 0.984	no - 0.779
IAREKGVYDAMIHKQIHKQSWRSSGNRTDF	0.199	IAREKGVYDAMIHKQSWRSSGNRTDF	26	maybe - 3	no - 0.819	no - 0.848	no - 0.566
VYDAMIHKQSWRSSGGVYDAMIHKQSWRSS	0.069	GVYDAMIHKQSWRSSG	16	maybe - 3	no - 0.853	no - 0.762	no - 0.188
VIAREKGVYDAMIHKHKQSWRSSGNRTDFN	0.06	VIAREKGVYDAMIHKQSWRSSGNRTDFN	28	maybe - 3	no - 0.828	no - 0.868	no - 0.411
REKGVYDAMIHKQSWAMIHKQSWRSSGNRT	0.05	REKGVYDAMIHKQSWRSSGNRT	22	maybe - 3	no - 0.675	no - 0.634	yes - -0.489
GNRTDFNVVMGNIAYDDVLADMNVIAREKG	0.043	DDVLADMNVIAREKGVYDAMIHKQSWRSSGNRTDFNVVMGNIAY	44	maybe - 12	no - 0.951	no - 0.964	no - 0.734
VKPESNIVYDDVLADMGNIAYPVNMEALLG	0.037	VKPESNIVYDDVLADMNVIAREKGVYDAMIHKQSWRSSGNRTDFNVVMGNIAYPVNMEALLG	62	very little chance	no - 0.973	no - 0.998	no - 1.186
TDFNVVMGNIAYPVNIVYDDVLADMNVIAR	0.029	IVYDDVLADMNVIAREKGVYDAMIHKQSWRSSGNRTDFNVVMGNIAYPVN	50	maybe - 15	no - 0.964	no - 1	no - 0.756
YPVNMEALLGDDFFKYSYFAVKPESNIVYD	0.001	YSYFAVKPESNIVYDDVLADMNVIAREKGVYDAMIHKQSWRSSGNRTDFNVVMGNIAYPVNMEALLGDDFFK	78	very little chance	no - 0.989	no - 1	no - 1.507
Segment 5 (aa201-300)							
TEAITKDVIKRVNSQQSWRSSGNRTDFNVV	0.3	QSWRSSGNRTDFNVVMGNIAYPVNMEALLGDDFFKVLPEFFRRSVDLVNIYMPIGSVAIAASREALQMNDTTKNVIIEATKKITEAITKDVIKRVNSQ	98	maybe - 30	no - 0.623	yes - 0.94	no - 0.226
LVNIYMPIGSVAIAAPEFFRRSVDLVNIYM	0.206	PEFFRRSVDLVNIYMPIGSVAIAA	24	maybe - 9	no - 0.912	no - 0.956	no - 0.412
GDDFFKVLPEFFRRSGSVAIAASREALQMN	0.14	GDDFFKVLPEFFRRSVDLVNIYMPIGSVAIAASREALQMN	40	very little chance	no - 0.962	no - 0.996	no - 0.954
SVDLVNIYMPIGSVAFRRSVDLVNIYMPIG	0.129	FRRSVDLVNIYMPIGSVA	18	maybe - 6	no - 0.797	no - 0.644	no - 0.116
GNIAYPVNMEALLGDMNDTTKNVIIEATKK	0.102	GNIAYPVNMEALLGDDFFKVLPEFFRRSVDLVNIYMPIGSVAIAASREALQMNDTTKNVIIEATKK	66	very little chance	no - 0.980	no - 0.988	no - 0.756
TDFNVVMGNIAYPVNVIIEATKKITEAITK	0.092	TDFNVVMGNIAYPVNMEALLGDDFFKVLPEFFRRSVDLVNIYMPIGSVAIAASREALQMNDTTKNVIIEATKKITEAITK	80	very little chance	no - 0.985	no - 0.772	no - 0.832
VLPEFFRRSVDLVNINIYMPIGSVAIAASR	0.091	VLPEFFRRSVDLVNIYMPIGSVAIAASR	28	maybe - 9	no - 0.895	no - 0.95	no - 0.200

Table A-1 cont.

VNIYMPIGSVAIAASLPEFFRRSVDLVNIY	0.063	LPEFFRRSVDLVNIYMPIGSVAIAAS	26	maybe - 9	no - 0.9	no - 0.956	no - 0.527
MEALLGDDFFKVLPEAASREALQMNDTTKN	0.043	MEALLGDDFFKVLPEFFRRSVDLVNIYMPIGSVAIAASREALQMNDTTKN	50	very little chance	no - 0.99	no - 1	no - 1.496
NDTTKNVIIEATKKIMGNIAYPVNMEALLG	0.037	MGNIAYPVNMEALLGDDFFKVLPEFFRRSVDLVNIYMPIGSVAIAASREALQMNDTTKNVIIEATKKI	68	very little chance	no - 0.99	no - 1	no - 0.999
KKITEAITKDVIKRVRSSGNRTDFNVVMGN	0.007	RSSGNRTDFNVVMGNIAYPVNMEALLGDDFFKVLPEFFRRSVDLVNIYMPIGSVAIAASREALQMNDTTKNVIIEATKKITEAITKDVIKRV	92	maybe - 30	no - 0.7	yes - 0.868	no - -0.012
Segment 6 (aa251-350)							
DTTKNVIIEATKKITSREMLNAVCPKLEWG	0.583	DTTKNVIIEATKKITEAITKDVIKRVNSQPTLMDAAQAYAELRLNSREMLNAVCPKLEWG	60	maybe - 17	no - 0.9	no - 0.968	no - 0.246
IEATKKITEAITKDVYAELRLNSREMLNAV	0.178	IEATKKITEAITKDVIKRVNSQPTLMDAAQAYAELRLNSREMLNAV	46	maybe - 16	no - 0.946	no - 0.98	no - 0.849
GVKLDTLEEELLNIRGSVAIAASREALQMN	0.14	GSVAIAASREALQMNDTTKNVIIEATKKITEAITKDVIKRVNSQPTLMDAAQAYAELRLNSREMLNAVCPKLEWGGVKLDTLEEELLNIR	90	very little chance	no - 0.522	yes - 0.912	yes - -0.484
VNSQPTLMDAAQAYADVIKRVNSQPTLMDA	0.105	DVIKRVNSQPTLMDAAQAYA	20	maybe - 8	no - 0.867	no - 0.97	no - 0.820
EMLNAVCPKLEWGGVMNDTTKNVIIEATKK	0.102	MNDTTKNVIIEATKKITEAITKDVIKRVNSQPTLMDAAQAYAELRLNSREMLNAVCPKLEWGGV	64	maybe - 17	no - 0.934	no - 0.994	no - 0.446
IYMPIGSVAIAASRETLEEELLNIRRGIIH	**0.096**	**IYMPIGSVAIAASREALQMNDTTKNVIIEATKKITEAITKDVIKRVNSQPTLMDAAQAYAELRLNSREMLNAVCPKLEWGGVKLDTLEEELLNIRRGIIH**	**100**	**maybe - 31**	**yes - 0.621**	**yes - 0.954**	**yes - -0.754**
ELRLNSREMLNAVCPVIIEATKKITEAITK	0.092	VIIEATKKITEAITKDVIKRVNSQPTLMDAAQAYAELRLNSREMLNAVCP	50	maybe - 17	no - 0.944	no - 0.988	no - 0.441
KLEWGGVKLDTLEEEAASREALQMNDTTKN	0.043	DTTKNVIIEATKKITEAITKDVIKRVNSQPTLMDAAQAYAELRLNSREMLNAVCPKLEWGGVKLDTLEEE	80	very little chance	no - 0.839	no - 0.94	no - 0.027
NDTTKNVIIEATKKIREMLNAVCPKLEWGG	0.029	NDTTKNVIIEATKKITEAITKDVIKRVNSQPTLMDAAQAYAELRLNSREMLNAVCPKLEWGG	62	maybe - 17	no - 0.866	no - 0.986	no - 0.190
KKITEAITKDVIKRVAAQAYAELRLNSREM	0.006	KKITEAITKDVIKRVNSQPTLMDAAQAYAELRLNSREM	38	maybe - 11	no - 0.947	no - 0.998	no - 0.876
Segment 7 (aa301-400)							
KGNNIPKVAYLYNPVSREMLNAVCPKLEWG	**0.583**	**NAVCPKLEWGGVKLDTLEEELLNIRRGIIHAEDGSVIYERDGKGNIKVDSKGNNIPKVAYLYNPV**	**70**	**maybe - 12**	**yes - 0.637**	**no - 0.902**	**yes - -0.912**

Table A-1 cont.

TLEEELLNIRRGIIHAEDGSVIYERDGKGN	0.558	TLEEELLNIRRGIIHAEDGSVIYERDGKGN	30	very little chance	no - 0.810	no - 0.96	no - 0.577
GIIHAEDGSVIYERDELLNIRRGIIHAEDG	0.45	ELLNIRRGIIHAEDGSVIYERD	22	very little chance	no - 0.796	no - 0.944	no - -0.017
LRLNSREMLNAVCPKIPKVAYLYNPVAYVK	**0.278**	**LRLNSREMLNAVCPKLEWGGVKLDTLEEELLNIRRGIIHAEDGSVIYERDGKGNIKVDSKGNNIPKVAYLYNPVAYVK**	**78**	**maybe - 17**	**yes - 0.636**	**no - 0.652**	**yes - -1.065**
VAYLYNPVAYVKFNSYAELRLNSREMLNAV	**0.178**	**YAELRLNSREMLNAVCPKLEWGGVKLDTLEEELLNIRRGIIHAEDGSVIYERDGKGNIKVDSKGNNIPKVAYLYNPVAYVKFNS**	**84**	**maybe - 17**	**yes - 0.705**	**yes - 0.922**	**yes - -0.862**
AAQAYAELRLNSREMYNPVAYVKFNSLESK	**0.122**	**AAQAYAELRLNSREMLNAVCPKLEWGGVKLDTLEEELLNIRRGIIHAEDGSVIYERDGKGNIKVDSKGNNIPKVAYLYNPVAYVKFNSLESK**	**92**	**maybe - 19**	**yes - 0.871**	**yes - 0.922**	**yes - -0.834**
CPKLEWGGVKLDTLEKGNIKVDSKGNNIPK	**0.111**	**CPKLEWGGVKLDTLEEELLNIRRGIIHAEDGSVIYERDGKGNIKVDSKGNNIPK**	**54**	**maybe - 8**	**yes - 0.632**	**no - 0.892**	**yes - -0.903**
EELLNIRRGIIHAEDIIHAEDGSVIYERDG	0.104	EELLNIRRGIIHAEDGSVIYERDG	24	very little chance	no - 0.858	no - 0.93	no - -0.10
LEEELLNIRRGIIHAHAEDGSVIYERDGKG	0.064	LEEELLNIRRGIIHAEDGSVIYERDGKG	28	very little chance	no - 0.638	no - 0.812	no - 0.159
VKLDTLEEELLNIRRSVIYERDGKGNIKVD	0.058	VKLDTLEEELLNIRRGIIHAEDGSVIYERDGKGNIKVD	38	very little chance	no - 0.600	no - 0.842	no - -0.058
SKGNNIPKVAYLYNPREMLNAVCPKLEWGG	0.029	REMLNAVCPKLEWGGVKLDTLEEELLNIRRGIIHAEDGSVIYERDGKGNIKVDSKGNNIPKVAYLYNP	68	maybe - 14	no - 0.555	no - 0.928	yes - -0.816
Segment 8 (aa351-450)							
NGTEKTVGRNQILRGAEDGSVIYERDGKGN	**0.558**	**AEDGSVIYERDGKGNIKVDSKGNNIPKVAYLYNPVAYVKFNSLESKIRATALSYTQEASMFNIFGAMRKSQIEQFLFVINDRRNKNGTEKTVGRNQILRG**	**100**	**maybe - 20**	**yes - 0.913**	**yes - 0.966**	**yes - -0.656**
AYVKFNSLESKIRATALSYTQEASMFNIFG	0.504	AYVKFNSLESKIRATALSYTQEASMFNIFG	30	maybe - 8	no - 0.957	no - 0.906	no - 1.348
FNSLESKIRATALSYIRATALSYTQEASMF	0.334	FNSLESKIRATALSYTQEASMF	22	maybe - 6	no - 0.990	no - 0.982	no - 2.087
NIFGAMRKSQIEQFLIPKVAYLYNPVAYVK	0.278	IPKVAYLYNPVAYVKFNSLESKIRATALSYTQEASMFNIFGAMRKSQIEQFL	52	maybe - 18	no - 0.986	no - 0.994	no - 1.410
YNPVAYVKFNSLESKTQEASMFNIFGAMRK	0.276	YNPVAYVKFNSLESKIRATALSYTQEASMFNIFGAMRK	38	maybe - 12	no - 0.967	no - 0.914	no - 1.144
YERDGKGNIKVDSKGINDRRNKNGTEKTVG	**0.17**	**YERDGKGNIKVDSKGNNIPKVAYLYNPVAYVKFNSLESKIRATALSYTQEASMFNIFGAMRKSQIEQFLFVINDRRNKNGTEKTVG**	**86**	**maybe - 20**	**yes - 0.543**	**yes - 0.896**	**no - 0.076**

Table A-1 cont.

QFLFVINDRRNKNGTKG NIKVDSKGNNIPK	0.111	KGNIKVDSKGNNIPKVAYLYNPVAYVKFNSLESKIRATALSYT QEASMFNIFGAMRKSQIEQFLFVINDRRNKNGT	76	maybe - 20	no - 0.685	no - 0.664	no - 0.079
SKGNNIPKVAYLYNPMR KSQIEQFLFVIND	0.108	SKGNNIPKVAYLYNPVAYVKFNSLESKIRATALSYTQEASMFN IFGAMRKSQIEQFLFVIND	62	maybe - 20	no - 0.978	no - 0.986	no - 1.195
NIPKVAYLYNPVAYVIFG AMRKSQIEQFLF	0.08	NIPKVAYLYNPVAYVKFNSLESKIRATALSYTQEASMFNIFGA MRKSQIEQFLF	54	maybe - 18	no - 0.987	no - 1	no - 1.413
RRNKNGTEKTVGRNQS VIYERDGKGNIKVD	**0.058**	**S VIYERDGKGNIKVDS KGNNIPKVAYLYNPVAYVKFNS LES KI RATALS YTQEAS MFNIFGAMRKS QIEQFLFVINDRRNKNGTE KTVGRNQ**	**92**	**maybe - 20**	**yes - 0.696**	**yes - 0.938**	**yes - -0.269**
EDGS VIYERDGKGNIKN GTEKTVGRNQILR	**0.051**	**EDGS VIYERDGKGNIKVDS KGNNIPKVAYLYNPVAYVKFNS L ES KIRATALS YTQEAS MFNIFGAMRKS QIEQFLFVINDRRNK NGTEKTVGRNQILR**	**98**	**maybe - 20**	**yes - 0.878**	**yes - 0.92**	**yes - -0.638**
NPVAYVKFNSLESKIYTQ EASMFNIFGAMR	0.022	NPVAYVKFNSLESKIRATALSYTQEASMFNIFGAMR	36	maybe - 9	no - 0.977	no - 0.976	no - 1.245
Segment 9 (aa401-500)							
KNGTEKTVGRNQILRCR DYASESSLFHRYN	0.835	KNGTEKTVGRNQILRGACRDYASESSLFHRYN	32	maybe - 3	no - 0.546	no - 0.728	no - -0.222
DRRNKNGTEKTVGRNA SESSLFHRYNGIVF	**0.639**	**DRRNKNGTEKTVGRNQILRGACRDYASESLFHRYNGIVF**	**40**	**maybe - 5**	**yes - 0.612**	**no - 0.614**	**yes - -1.005**
NKNGTEKTVGRNQILRD YASESSLFHRYNG	0.588	NKNGTEKTVGRNQILRGACRDYASESSLFHRYNG	34	maybe - 3	no - 0.597	no - 0.772	no - -0.203
KLDKSLLKIVKMSDKAL SYTQEASMFNIFG	**0.504**	**ALS YTQEAS MFNIFGAMRKS QIEQFLFVINDRRNKNGTEKTV GRNQILRGACRDYAS ES S LFHRYNGIVFVFS TEKELDDLINLH KLDKSLLKIVKMS DK**	**100**	**maybe 25**	**yes - 0.895**	**yes - 0.968**	**yes - -0.336**
INLHKLDKSLLKIVKTQE ASMFNIFGAMRK	**0.276**	**TQEAS MFNIFGAMRKS QIEQFLFVINDRRNKNGTEKTVGRN QILRGACRDYAS ES S LFHRYNGIVFVFS TEKELDDLINLHKLD KSLLKIVK**	**92**	**maybe - 24**	**yes - 0.844**	**yes - 0.946**	**yes - -0.570**
RKSQIEQFLFVINDRVFVF STEKELDDLIN	0.251	RKSQIEQFLFVINDRRNKNGTEKTVGRNQILRGACRDYASESS LFHRYNGIVFVFSTEKELDDLIN	66	maybe - 14	no - 0.575	no - 0.904	yes - -0.387
ESSLFHRYNGIVFVFINDR RNKNGTEKTVG	0.17	INDRRNKNGTEKTVGRNQILRGACRDYASESSLFHRYNGIVFV F	44	maybe - 6	no - 0.667	no - 0.752	yes - -0.590
ACRDYASESSLFHRYNGT EKTVGRNQILRG	0.157	NGTEKTVGRNQILRGACRDYASESSLFHRY	30	maybe - 3	no - 0.816	no - 0.856	no - 0.156
FVFSTEKELDDLINLMRK SQIEQFLFVIND	0.108	MRKSQIEQFLFVINDRRNKNGTEKTVGRNQILRGACRDYASE SSLFHRYNGIVFVFSTEKELDDLINL	68	maybe - 15	no - 0.707	no - 0.948	no - 0.925
TEKELDDLINLHKLDIFG AMRKSQIEQFLF	0.08	IFGAMRKSQIEQFLFVINDRRNKNGTEKTVGRNQILRGACRD YASESSLFHRYNGIVFVFSTEKELDDLINLHKLD	76	maybe - 18	no - 0.549	no - 0.926	yes - -0.346

Table A-1 cont.

RRNKNGTEKTVGRNQYASESSLFHRYNGIV	**0.068**	**RRNKNGTEKTVGRNQILRGACRDYASESSLFHRYNGIV**	**38**	**maybe - 3**	**yes - 0.558**	**no - 0.67**	**yes - -0.948**
FLFVINDRRNKNGTEFHRYNGIVFVFSTEK	0.053	FLFVINDRRNKNGTEKTVGRNQILRGACRDYASESSLFHRYNGIVFVFSTEK	52	maybe - 8	no - 0.657	no - 0.834	yes - -0.346
NLHKLDKSLLKIVKMYTQEASMFNIFGAMR	**0.022**	**YTQEASMFNIFGAMRKSQIEQFLFVINDRRNKNGTEKTVGRNQILRGACRDYASESSLFHRYNGIVFVFSTEKELDDLINLHKLDKSLLKIVKM**	**94**	**maybe - 25**	**yes - 0.777**	**yes - 0.962**	**no - -0.224**
GTEKTVGRNQILRGAGACRDYASESSLFHR	0.015	GTEKTVGRNQILRGACRDYASESSLFHR	28	maybe - 3	no - 0.761	no - 0.876	no - 0.120
Segment 10 (aa451-550)							
LDTIEEPQLYIKAVGCRDYASESSLFHRYN	**0.835**	**CRDYASESSLFHRYNGIVFVFSTEKELDDLINLHKLDKSLLKIVKMSDKEHHYQRKEAVRGVVKLWKAVPAESVASYTEVSEDLDTIEEPQLYIKAVG**	**98**	**maybe - 27**	**yes - 0.797**	**yes - 0.96**	**yes - -0.381**
VSEDLDTIEEPQLYIASESSLFHRYNGIVF	0.639	ASESSLFHRYNGIVFVFSTEKELDDLINLHKLDKSLLKIVKMSDKEHHYQRKEAVRGVVKLWKAVPAESVASYTEVSEDLDTIEEPQLYI	90	maybe - 25	no - 0.753	yes - 0.942	no - 0.113
DLDTIEEPQLYIKAVRDYASESSLFHRYNG	**0.588**	**RDYASESSLFHRYNGIVFVFSTEKELDDLINLHKLDKSLLKIVKMSDKEHHYQRKEAVRGVVKLWKAVPAESVASYTEVSEDLDTIEEPQLYIKAV**	**96**	**maybe - 27**	**yes - 0.776**	**yes - 0.912**	**no - -0.228**
KSLLKIVKMSDKEHHSDKEHHYQRKEAVRG	0.308	KSLLKIVKMSDKEHHYQRKEAVRG	24	maybe - 5	no - 0.810	no - 0.746	no - -0.039
KAVPAESVASYTEVSVFVFSTEKELDDLIN	0.251	VFVFSTEKELDDLINLHKLDKSLLKIVKMSDKEHHYQRKEAVRGVVKLWKAVPAESVASYTEVS	64	maybe - 19	no - 0.842	no - 0.948	no - 0.022
FHRYNGIVFVFSTEKVASYTEVSEDLDTIE	0.174	FHRYNGIVFVFSTEKELDDLINLHKLDKSLLKIVKMSDKEHHYQRKEAVRGVVKLWKAVPAESVASYTEVSEDLDTIE	78	maybe - 25	no - 0.810	no - 0.734	no - -0.004
QRKEAVRGVVKLWKAINLHKLDKSLLKIVK	**0.123**	**INLHKLDKSLLKIVKMSDKEHHYQRKEAVRGVVKLWKA**	**38**	**maybe - 14**	**yes - 0.574**	**yes - 0.512**	**yes - -0.814**
EHHYQRKEAVRGVVKKLDKSLLKIVKMSDK	0.111	KLDKSLLKIVKMSDKEHHYQRKEAVRGVVK	30	maybe - 9	no - 0.624	no - 0.614	yes - -0.550
SEDLDTIEEPQLYIKYASESSLFHRYNGIV	0.068	YASESSLFHRYNGIVFVFSTEKELDDLINLHKLDKSLLKIVKMSDKEHHYQRKEAVRGVVKLWKAVPAESVASYTEVSEDLDTIEEPQLYIK	92	maybe - 25	no - 0.518	yes - 0.978	no - 0.062
GIVFVFSTEKELDDLVPAESVASYTEVSED	0.014	GIVFVFSTEKELDDLINLHKLDKSLLKIVKMSDKEHHYQRKEAVRGVVKLWKAVPAESVASYTEVSED	68	maybe - 22	no - 0.813	no - 0.916	no - -0.113

Table A-1 cont.

Segment 11 (aa501-600)							
SYTEVSEDLDTIEEPSPEDVAKSVANVIGK	0.479	SYTEVSEDLDTIEEPQLYIKAVGDTVDSECFCSSPEDVAKSVANVIGK	48	very little chance	no - 0.953	no - 1	no - 0.888
EDLDTIEEPQLYIKASECFCSSPEDVAKSV	0.182	EDLDTIEEPQLYIKAVGDTVDSECFCSSPEDVAKSV	36	very little chance	no - 0.933	no - 0.97	no - 1.416
EDVAKSVANVIGKTVVASYTEVSEDLDTIE	0.174	VASYTEVSEDLDTIEEPQLYIKAVGDTVDSECFCSSPEDVAKSVANVIGKTV	78	very little chance	no - 0.944	no - 0.966	no - 0.807
DLDTIEEPQLYIKAVDSECFCSSPEDVAKS	0.124	DLDTIEEPQLYIKAVGDTVDSECFCSSPEDVAKS	34	very little chance	no - 0.959	no - 0.984	no - 1.602
KEAVRGVVKLWKAVPRKANWKKIPEDWIEV	**0.088**	**KEAVRGVVKLWKAVPAESVASYTEVSEDLDTIEEPQLYIKAVGDTVDSECFCSSPEDVAKSVANVIGKTVYVFRKANWKKIPEDWIEV**	**88**	**very little chance**	**yes - 0.672**	**yes - 0.902**	**yes - -0.477**
EAVRGVVKLWKAVPAFRKANWKKIPEDWIE	**0.065**	**EAVRGVVKLWKAVPAESVASYTEVSEDLDTIEEPQLYIKAVGDTVDSECFCSSPEDVAKSVANVIGKTVYVFRKANWKKIPEDWI**	**86**	**very little chance**	**yes - 0.563**	**yes - 0.894**	**yes - -0.275**
RKEAVRGVVKLWKAVKANWKKIPEDWIEVD	**0.04**	**RKEAVRGVVKLWKAVPAESVASYTEVSEDLDTIEEPQLYIKAVGDTVDSECFCSSPEDVAKSVANVIGKTVYVFRKANWKKIPEDWIEVD**	**90**	**very little chance**	**yes - 0.731**	**yes - 0.898**	**yes - -0.661**
SVANVIGKTVYVFRKVPAESVASYTEVSED	0.014	VPAESVASYTEVSEDLDTIEEPQLYIKAVGDTVDSECFCSSPEDVAKSVANVIGKTVYVFRK	32	very little chance	no - 0.944	no - 0.99	no - 0.582
Segment 12 (aa551-650)							
TSSWIIARNFTFNNRSPEDVAKSVANVIGK	0.479	SPEDVAKSVANVIGKTVYVFRKANWKKIPEDWIEVDEKLLNDSLTDVHWINHNRYMTRIYMNGVLDLTSSWIIARNFTFNNR	82	maybe - 26	no - 0.904	yes - 0.548	no - 0.174
ARNFTFNNRKISRGYSECFCSSPEDVAKSV	**0.182**	**SECFCSSPEDVAKSVANVIGKTVYVFRKANWKKIPEDWIEVDEKLLNDSLTDVHWINHNRYMTRIYMNGVLDLTSSWIIARNFTFNNRKISRGY**	**94**	**maybe - C**	**yes - 0.520**	**yes - 0.944**	**yes - -0.402**
DVAKSVANVIGKTVYLDLTSSWIIARNFTF	0.127	DVAKSVANVIGKTVYVFRKANWKKIPEDWIEVDEKLLNDSLTDVHWINHNRYMTRIYMNGVLDLTSSWIIARNFTF	76	maybe - 26	no - 0.891	no - 0.986	no - 0.287
RNFTFNNRKISRGYCDSECFCSSPEDVAKS	**0.124**	**DSECFCSSPEDVAKSVANVIGKTVYVFRKANWKKIPEDWIEVDEKLLNDSLTDVHWINHNRYMTRIYMNGVLDLTSSWIIARNFTFNNRKISRGYC**	**96**	**maybe - 26**	**yes 0.682**	**yes - 0.9**	**yes - -0.575**
DEKLLNDSLTDVHWIIEVDEKLLNDSLTDV	0.101	IEVDEKLLNDSLTDVHWI	18	very little chance	no - 0.724	no - 0.896	no - 1.008
FRKANWKKIPEDWIEWINHNRYMTRIYMNG	0.09	FRKANWKKIPEDWIEVDEKLLNDSLTDVHWINHNRYMTRIYMNG	44	maybe - 10	no - 0.984	no - 0.984	no - 0.585
HWINHNRYMTRIYMNRKANWKKIPEDWIEV	0.088	RKANWKKIPEDWIEVDEKLLNDSLTDVHWINHNRYMTRIYMN	42	maybe - 10	no - 0.979	no - 0.984	no - 0.335

Table A-1 cont.

CFCSSPEDVAKSVANIIARNFTFNNRKISR	**0.043**	**CFCSSPEDVAKSVANVIGKTVYVFRKANWKKIPEDWIEVDEKLLNDSLTDVHWINHNRYMTRIYMNGVLDLTSSWIIARNFTFNNRKISR**	**90**	**maybe - C**	**no - 0.544**	**yes - 0.932**	**yes - -0.562**
VHWINHNRYMTRIYMKANWKKIPEDWIEVD	0.04	KANWKKIPEDWIEVDEKLLNDSLTDVHWINHNRYMTRIYM	40	maybe - 10	no - 0.986	no - 0.972	no - 0.603
EDWIEVDEKLLNDSLLLNDSLTDVHWINHN	0.018	EDWIEVDEKLLNDSLTDVHWINHN	24	very little chance	no - 0.943	no - 0.918	no - 0.548
NRYMTRIYMNGVLDLTVYVFRKANWKKIPE	0.013	TVYVFRKANWKKIPEDWIEVDEKLLNDSLTDVHWINHNRYMTRIYMNGVLDL	52	maybe - 17	no - 0.994	no - 0.998	no - 1.091
Segment 13 (aa601-700)							
HWINHNRYMTRIYMNFAYTYTISVLQTLKG	0.971	HWINHNRYMTRIYMNGVLDLTSSWIIARNFTFNNRKISRGYCYSRDTSKTIFLEGNEEAVEAIFGKIQYVAAPFAYTYTISVLQTLKG	88	maybe - 20	no - 0.961	yes - 0.908	no - 0.197
NRKISRGYCYSRDTSYCYSRDTSKTIFLEG	0.761	NRKISRGYCYSRDTSKTIFLEG	22	doubtful	no - 0.740	no - 0.586	no - 0.399
ISRGYCYSRDTSKTISRGYCYSRDTSKTIF	0.493	ISRGYCYSRDTSKTIF	16	doubtful	no - 0.946	no - 0.662	no - 0.398
TFNNRKISRGYCYSRSRDTSKTIFLEGNEE	0.385	TFNNRKISRGYCYSRDTSKTIFLEGNEE	28	doubtful	no - 0.981	no - 0.966	no - 0.641
VHWINHNRYMTRIYMAYTYTISVLQTLKGC	0.185	VHWINHNRYMTRIYMNGVLDLTSSWIIARNFTFNNRKISRGYCYSRDTSKTIFLEGNEEAVEAIFGKIQYVAAPFAYTYTISVLQTLKGC	90	maybe - C	no - 0.937	yes - 0.908	no - -0.009
LTSSWIIARNFTFNNGNEEAVEAIFGKIQY	0.18	LTSSWIIARNFTFNNRKISRGYCYSRDTSKTIFLEGNEEAVEAIFGKIQY	50	maybe - 9	no - 0.899	no - 0.758	no - 0.141
EEAVEAIFGKIQYVALDLTSSWIIARNFTF	0.127	LDLTSSWIIARNFTFNNRKISRGYCYSRDTSKTIFLEGNEEAVEAIFGKIQYVA	54	maybe - 12	no - 0.909	no - 0.92	no - 0.126
TDVHWINHNRYMTRITYTISVLQTLKGCLD	0.091	TDVHWINHNRYMTRIYMNGVLDLTSSWIIARNFTFNNRKISRGYCYSRDTSKTIFLEGNEEAVEAIFGKIQYVAAPFAYTYTISVLQTLKGCLD	94	maybe - C	no - 0.938	yes - 0.928	no - 0.307
PFAYTYTISVLQTLKWINHNRYMTRIYMNG	0.09	WINHNRYMTRIYMNGVLDLTSSWIIARNFTFNNRKISRGYCYSRDTSKTIFLEGNEEAVEAIFGKIQYVAAPFAYTYTISVLQTLK	86	maybe - 20	no - 0.970	yes - 0.894	no - 0.240
TIFLEGNEEAVEAIFIIARNFTFNNRKISR	0.043	IIARNFTFNNRKISRGYCYSRDTSKTIFLEGNEEAVEAIF	40	maybe - 7	no - 0.888	no - 0.952	no - 0.072
SKTIFLEGNEEAVEAARNFTFNNRKISRGY	0.013	ARNFTFNNRKISRGYCYSRDTSKTIFLEGNEEAVEA	36	maybe - 3	no - 0.928	no - 0.972	no - 0.439

Table A-1 cont.

Segment 14 (aa651-750)							
RKAGDRMVIKVTNYLFAYTYTISVLQTLKG	0.971	FAYTYTISVLQTLKGCLDNNTKLYKKIRKAGDRMVIKVTNYL	42	maybe - 12	no - 0.656	no - 0.726	no - -0.019
KIQYVAAPFAYTYTIIKVTNYLSKRKQENF	0.508	KIQYVAAPFAYTYTISVLQTLKGCLDNNTKLYKKIRKAGDRMVIKVTNYLSKRKQENF	58	maybe - 15	no - 0.547	no - 0.554	no - -.105
SKTIFLEGNEEAVEAHLDWNKVSPIEVSKF	**0.306**	**SKTIFLEGNEEAVEAIFGKIQYVAAPFAYTYTISVLQTLKGCLDNNTKLYKKIRKAGDRMVIKVTNYLSKRKQENFLLSHLDWNKVSPIEVSKF**	**94**	**maybe - 25**	**yes - 0.849**	**yes - 0.96**	**yes - -0.411**
RDTSKTIFLEGNEEAWNKVSPIEVSKFLGF	**0.255**	**RDTSKTIFLEGNEEAVEAIFGKIQYVAAPFAYTYTISVLQTLKGCLDNNTKLYKKIRKAGDRMVIKVTNYLSKRKQENFLLSHLDWNKVSPIEVSKFLGF**	**100**	**maybe - 27**	**yes - 0.938**	**yes - 0.93**	**yes - -0.649**
IRKAGDRMVIKVTNYAYTYTISVLQTLKGC	0.185	AYTYTISVLQTLKGCLDNNTKLYKKIRKAGDRMVIKVTNY	40	maybe - 11	no - 0.602	no - 0.604	no - -0.029
QENFLLSHLDWNKVSGNEEAVEAIFGKIQY	**0.18**	**GNEEAVEAIFGKIQYVAAPFAYTYTISVLQTLKGCLDNNTKLYKKIRKAGDRMVIKVTNYLSKRKQENFLLSHLDWNKVS**	**80**	**maybe - 25**	**yes - 0.736**	**yes - 0.592**	**yes - -0.444**
NEEAVEAIFGKIQYVKQENFLLSHLDWNKV	**0.169**	**NEEAVEAIFGKIQYVAAPFAYTYTISVLQTLKGCLDNNTKLYKKIRKAGDRMVIKVTNYLSKRKQENFLLSHLDWNKV**	**78**	**maybe - 25**	**yes - 0.599**	**yes - 0.552**	**yes - -0.327**
YVAAPFAYTYTISVLRMVIKVTNYLSKRKQ	0.114	YVAAPFAYTYTISVLQTLKGCLDNNTKLYKKIRKAGDRMVIKVTNYLSKRKQ	52	maybe - 14	no - 0.520	no - 0.582	yes - -0.264
APFAYTYTISVLQTLAGDRMVIKVTNYLSK	0.112	APFAYTYTISVLQTLKGCLDNNTKLYKKIRKAGDRMVIKVTNYLSK	46	maybe - 12	no - 0.622	no - 0.522	no - -0.124
KKIRKAGDRMVIKVTTYTISVLQTLKGCLD	0.091	TYTISVLQTLKGCLDNNTKLYKKIRKAGDRMVIKVT	36	maybe - 11	no - 0.581	no - 0.584	no - -0.200
LQTLKGCLDNNTKLYNNTKLYKKIRKAGDR	**0.012**	**LQTLKGCLDNNTKLYKKIRKAGDR**	**24**	**maybe - 5**	**no - 0.668**	**yes - 0.556**	**yes - 0.280**
Segment 15 (aa701-763)							
DWNKVSPIEVSKFLGIKVTNYLSKRKQENF	0.508	IKVTNYLSKRKQENFLLSHLDWNKVSPIEVSKFLG	35	maybe - 8	no - 0.831	no - 0.854	no - 0.312
YKKIRKAGDRMVIKVFLGFNVKCVPEGTTV	0.423	YKKIRKAGDRMVIKVTNYLSKRKQENFLLSHLDWNKVSPIEVSKFLGFNVKCVPEGTTV	53	maybe - 14	no - 0.575	no - 0.792	no - -0.243
VTNYLSKRKQENFLLHLDWNKVSPIEVSKF	0.306	VTNYLSKRKQENFLLSHLDWNKVSPIEVSKF	31	maybe - 5	no - 0.961	no - 0.982	no - 0.680
VIKVTNYLSKRKQENWNKVSPIEVSKFLGF	0.255	VIKVTNYLSKRKQENFLLSHLDWNKVSPIEVSKFLGF	37	maybe - 9	no - 0.831	no - 0.802	no - 0.174
KQENFLLSHLDWNKVKQENFLLSHLDWNKV	0.169	KQENFLLSHLDWNKV	15	maybe - 5	no - 0.837	no - 0.928	yes - -0.283

Table A-1 cont.

KVSPIEVSKFLGFNVRMV IKVTNYLSKRKQ	0.144	RMVIKVTNYLSKRKQENFLLSHLDWNKVSPIEVSKFLGFNV	41	maybe - 11	no - 0.894	no - 0.864	no - 0.200
PIEVSKFLGFNVKCVAGD RMVIKVTNYLSK	0.112	AGDRMVIKVTNYLSKRKQENFLLSHLDWNKVSPIEVSKFLGF NVKCV	47	maybe - 13	no - 0.745	no - 0.768	no - -0.214
DRMVIKVTNYLSKRKVS PIEVSKFLGFNVK	0.083	DRMVIKVTNYLSKRKQENFLLSHLDWNKVSPIEVSKFLGFNV K	43	maybe - 11	no - 0.920	no - 0.754	no - -0.062

Table A-2. Antimicrobial peptides identified from AntiPB2 analysis of the LysO1 protein sequence.

AntiBP2 Sequence	Score	Corrected Sequence	length	APD2	CAMP		
					(SVM)	(RF)	(DA)
Segment 1 (aa1-80)							
MKITAEQAEQYLLADEAYEDSAGIPTIGYG	0.586	EAYEDSAGIPTIGYGTIRIDGKPVKMGMKITAEQAEQYLLAD	42	very little chance	no - 0.959	no - 0.924	no - 1.070
TIRIDGKPVKMGMKIGYGTIRIDGKPVKMG	**0.432**	**GYGTIRIDGKPVKMGMKI**	**18**	**unknown**	**yes - 0.750**	**yes - 0.612**	**yes 0.581**
KPVKMGMKITAEQAEAGIPTIGYGTIRIDG	0.3	AGIPTIGYGTIRIDGKPVKMGMKITAEQAE	30	unknown	no - 0.916	no - 0.778	no - 0.482
GLKLEAYEDSAGIPTAEQAEQYLLADVEKF	0.293	GLKLEAYEDSAGIPTIGYGTIRIDGKPVKMGMKITAEQAEQYLLADVEKF	50	very little chance	no - 0.920	no - 0.998	no - 0.706
RIDGKPVKMGMKITATIGYGTIRIDGKPVK	0.291	TIGYGTIRIDGKPVKMGMKITA	22	unknown	no - 0.563	yes - 0.54	no - -0.167
KITAEQAEQYLLADVLEAYEDSAGIPTIGY	0.262	LEAYEDSAGIPTIGYGTIRIDGKPVKMGMKITAEQAEQYLLADV	44	very little chance	no - 0.966	no - 0.998	no - 1.133
QAEQYLLADVEKFVAFEGLKLEAYEDSAGI	0.055	FEGLKLEAYEDSAGIPTIGYGTIRIDGKPVKMGMKITAEQAEQYLLADVEKFVA	54	very little chance	no - 0.934	no - 0.992	no - 0.770
FFEGLKLEAYEDSAGAEQYLLADVEKFVAA	0.052	FFEGLKLEAYEDSAGIPTIGYGTIRIDGKPVKMGMKITAEQAEQYLLADVEKFVAA	56	very little chance	no - 0.941	no - 0.994	no - 0.748
Segment 2 (aa51-120)							
LLADVEKFVAAVNKADSTFIKRHNAGNKVG	0.523	LLADVEKFVAAVNKAIKVPTSQNEFDALVSETYNIGITAMQDSTFIKRHNAGNKVG	56	maybe 15	no - 0.745	no - 0.946	no - -0.046
TSQNEFDALVSETYNNEFDALVSETYNIGI	0.352	TSQNEFDALVSETYNIGI	18	very little chance	no - 0.958	no - 0.954	no - 1.546
HNAGNKVGCAEAMQWAEQAEQYLLADVEKF	0.293	AEQAEQYLLADVEKFVAAVNKAIKVPTSQNEFDALVSETYNIGITAMQDSTFIKRHNAGNKVGCAEAMQW	70	very little chance	no - 0.892	no - 0.98	no - 0.811
DVEKFVAAVNKAIKVAMQDSTFIKRHNAGN	0.225	DVEKFVAAVNKAIKVPTSQNEFDALVSETYNIGITAMQDSTFIKRHNAGN	50	maybe - 11	no - 0.891	no - 0.97	no - 0.298
KVPTSQNEFDALVSEDALVSETYNIGITAM	0.203	KVPTSQNEFDALVSETYNIGITAM	24	very little chance	no - 0.953	no - 1	no - 1.398

The AntiBP2 sequence is the sequence from the larger protein identified as an AMP, Score is the AntiBP2 score of antimicrobial activity. The corrected sequence is the AntiBP2 sequence after comparison to the protein and sequence corrections. Numbers after the APD2 prediction indicate the number of hydrophobic residues predicted to be on the same plane of an α helix; a "C" after the prediction indicates an even number of cysteine residues and the possibility of β sheet formation. Numbers in SVM and RF predictions are the likelihood of the prediction being true; numbers in DA predictions are the discriminate scores for the peptides. Bolded sequences indicate the corrected sequence received a positive score from three of the four prediction algorithms and is a potential AMP.

Table A-2 cont.

ADVEKFVAAVNKAIKM QDSTFIKRHNAGNK	0.173	ADVEKFVAAVNKAIKVPTSQNEFDALVSETYNIGITAMQDST FIKRHNAGNK	52	maybe - 11	no - 0.841	no - 0.976	no - 0.136
VEKFVAAVNKAIKVPTA MQDSTFIKRHNAG	0.108	VEKFVAAVNKAIKVPTSQNEFDALVSETYNIGITAMQDSTFI KRHNAG	48	maybe - 9	no - 0.882	no - 0.984	no - 0.345
ALVSETYNIGITAMQIKV PTSQNEFDALVS	0.073	IKVPTSQNEFDALVSETYNIGITAMQ	26	very little chance	no - 0.967	no - 0.998	no - 1.485
IKRHNAGNKVGCAEAAE QYLLADVEKFVAA	0.052	AEQYLLADVEKFVAAVNKAIKVPTSQNEFDALVSETYNIGIT AMQDSTFIKRHNAGNKVGCAEA	64	very little chance	no - 0.849	no - 0.988	no - 0.376
Segment 3 (aa81-154)							
NKVTVKGQKVTSNGLDS TFIKRHNAGNKVG	0.523	DSTFIKRHNAGNKVGCAEAMQWWNKVTVKGQKVTSNGL	38	maybe - 6	no - 0.577	no - 0.51	yes - -0.308
KRHNAGNKVGCAEAMA MQWWNKVTVKGQKV	**0.331**	**KRHNAGNKVGCAEAMQWWNKVTVKGQKV**	**28**	**maybe - 6**	**yes - 0.590**	**yes - 0.724**	**yes - -1.084**
NAGNKVGCAEAMQWW CAEAMQWWNKVTVKG	**0.284**	**NAGNKVGCAEAMQWWNKVTVKG**	**22**	**maybe - 6**	**yes - 0.689**	**yes - 0.682**	**yes - -0.839**
TAMQDSTFIKRHNAGV KGQKVTSNGLKNRR	0.246	TAMQDSTFIKRHNAGNKVGCAEAMQWWNKVTVKGQKVT SNGLKNRR	46	maybe - 6	no - 0.810	no - 0.742	no - -0.233
TVKGQKVTSNGLKNRA MQDSTFIKRHNAGN	0.225	AMQDSTFIKRHNAGNKVGCAEAMQWWNKVTVKGQKVTS NGLKNR	44	maybe - 6	no - 0.772	no - 0.676	no - -0.168
NRRRMEADIYLDSVYDA LVSETYNIGITAM	0.203	DALVSETYNIGITAMQDSTFIKRHNAGNKVGCAEAMQWWN KVTVKGQKVTSNGLKNRRRMEADIYLDSVY	70	maybe - 10	no - 0.853	no - 0.972	no - 0.393
VTVKGQKVTSNGLKNM QDSTFIKRHNAGNK	0.173	MQDSTFIKRHNAGNKVGCAEAMQWWNKVTVKGQKVTSN GLKN	42	maybe - 6	no - 0.805	no - 0.864	no - 0.023
VKGQKVTSNGLKNRRT AMQDSTFIKRHNAG	0.108	TAMQDSTFIKRHNAGNKVGCAEAMQWWNKVTVKGQKVT SNGLKNRR	46	maybe - 6	no - 0.810	no - 0.742	no - -0.233
TFIKRHNAGNKVGCAW WNKVTVKGQKVTSN	**0.009**	**TFIKRHNAGNKVGCAEAMQWWNKVTVKGQKVTSN**	**34**	**maybe - 6**	**no - 0.655**	**yes - 0.612**	**yes - -0.332**
Segment 1a (aa1-100)							
AAVNKAIKVPTSQNEEA YEDSAGIPTIGYG	0.586	EAYEDSAGIPTIGYGTIRIDGKPVKMGMKITAEQAEQYLLAD VEKFVAAVNKAIKVPTSQNE	62	very little chance	no - 0.872	no - 0.982	no - 0.497
EQYLLADVEKFVAAVGY GTIRIDGKPVKMG	0.432	GYGTIRIDGKPVKMGMKITAEQAEQYLLADVEKFVAAV	38	maybe - 10	no - 0.906	no - 0.99	no - 0.634

Table A-2 cont..

GLEAIKFFEGLKLEANEFDALVSETYNIGI	0.352	GLEAIKFFEGLKLEAYEDSAGIPTIGYGTIRIDGKPVKMGMKITAEQAEQYLLADVEKFVAAVNKAIKVPTSQNEFDALVSETYNIGI	88	very little chance	yes - 0.490	yes - 0.918	no - 0.051
DVEKFVAAVNKAIKVAGIPTIGYGTIRIDG	0.3	AGIPTIGYGTIRIDGKPVKMGMKITAEQAEQYLLADVEKFVAAVNKAIKV	50	maybe - 13	no - 0.750	no - 0.784	no - -0.102
IRIDGKPVKMGMKITAEQAEQYLLADVEKF	0.293	IRIDGKPVKMGMKITAEQAEQYLLADVEKF	30	maybe - 7	no - 0.938	no - 0.976	no - 0.928
YLLADVEKFVAAVNKTIGYGTIRIDGKPVK	0.291	TIGYGTIRIDGKPVKMGMKITAEQAEQYLLADVEKFVAAVNK	42	maybe - 10	no - 0.902	no - 0.978	no - 0.348
AVNKAIKVPTSQNEFLEAYEDSAGIPTIGY	0.262	LEAYEDSAGIPTIGYGTIRIDGKPVKMGMKITAEQAEQYLLADVEKFVAAVNKAIKVPTSQNEF	64	very little chance	no - 0.917	no - 0.992	no - 0.693
SRKGLEAIKFFEGLKDALVSETYNIGITAM	0.203	SRKGLEAIKFFEGLKLEAYEDSAGIPTIGYGTIRIDGKPVKMGMKITAEQAEQYLLADVEKFVAAVNKAIKVPTSQNEFDALVSETYNIGITAM	94	very little chance	yes - 0.570	yes - 0.938	no - 0.101
FEGLKLEAYEDSAGIIKVPTSQNEFDALVS	0.073	FEGLKLEAYEDSAGIPTIGYGTIRIDGKPVKMGMKITAEQAEQYLLADVEKFVAAVNKAIKVPTSQNEFDALVS	74	very little chance	no - 0.857	no - 0.992	no - 0.460
IKVPTSQNEFDALVSFEGLKLEAYEDSAGI	0.055	AYEDSAGIPTIGYGTIRIDGKPVKMGMKITAEQAEQYLLADVEKFVAAVNKAIKVPTSQNEFDALVS	67	very little chance	no - 0.885	no - 0.988	no - 0.064
YGTIRIDGKPVKMGMAEQYLLADVEKFVAA	0.052	YGTIRIDGKPVKMGMKITAEQAEQYLLADVEKFVAA	36	maybe - 9	no - 0.949	no - 0.992	no - 0.823
Segment 2a (aa61-154)							
FIKRHNAGNKVGCAEDSTFIKRHNAGNKVG	0.523	DSTFIKRHNAGNKVGCAE	18	maybe - 3	no - 0.557	no - 0.824	yes - -0.329
WNKVTVKGQKVTSNGNEFDALVSETYNIGI	0.352	NEFDALVSETYNIGITAMQDSTFIKRHNAGNKVGCAEAMQWWNKVTVKGQKVTSNG	56	maybe - 11	no - 0.770	no - 0.984	no - 0.369
ALVSETYNIGITAMQAMQWWNKVTVKGQKV	0.331	DALVSETYNIGITAMQDSTFIKRHNAGNKVGCAEAMQWWNKVTVKGQKVT	50	maybe - 8	no - 0.757	no - 0.89	no - 0.270
SETYNIGITAMQDSTCAEAMQWWNKVTVKG	0.284	SETYNIGITAMQDSTFIKRHNAGNKVGCAEAMQWWNKVTVKG	42	maybe - 8	no - 0.794	no - 0.74	no - 0.591
VPTSQNEFDALVSETVKGQKVTSNGLKNRR	0.246	VPTSQNEFDALVSETYNIGITAMQDSTFIKRHNAGNKVGCAEAMQWWNKVTVKGQKVTSNGLKNRR	66	maybe - 11	no - 0.844	no - 0.974	no - 0.029
RHNAGNKVGCAEAMQAMQDSTFIKRHNAGN	0.225	AMQDSTFIKRHNAGNKVGCAEAMQ	24	maybe - 4	no - 0.780	no - 0.97	no - 0.948

Table A-2 cont.

KRHNAGNKVGCAEAM MQDSTFIKRHNAGNK	0.173	MQDSTFIKRHNAGNKVGCAEAM	22	maybe - 4	no - 0.876	no - 0.996	no - 0.978
HNAGNKVGCAEAMQW TAMQDSTFIKRHNAG	0.108	TAMQDSTFIKRHNAGNKVGCAEAMQW	26	maybe - 4	no - 0.853	no - 0.99	no - 1.164
GQKVTSNGLKNRRRMIK VPTSQNEFDALVS	0.073	IKVPTSQNEFDALVSETYNIGITAMQDSTFIKRHNAGNKVGC AEAMQWWNKVTVKGQKVTSNGLKNRRRM	70	maybe - 11	no - 0.8	no - 0.87	no - -0.129
EFDALVSETYNIGITWWN KVTVKGQKVTSN	0.009	EFDALVSETYNIGITAMQDSTFIKRHNAGNKVGCAEAMQW WNKVTVKGQKVTSN	54	maybe - 11	no - 0.823	no - 0.964	no - 0.393

3.1.2 Analysis of peptides derived from digestion with peptidases

The RIIA and LysO1 sequences of FelixO1 were cleaved *in silico* with chymotrypsin (high and low specificity), trypsin, pepsin (pH 1.3 and >2), proteinase K, thrombin, or enterokinase. Fragments 15 aa in length or longer were analyzed for potential antimicrobial activity. Full results, including all amino acid sequences analyzed and numerical results of APD2 and CAMP analysis, are displayed in Tables B-3 and B-4. Of 58 sequences generated from RIIA and 15 from LysO1, three were predicted to have antimicrobial activity. Of the two potentially antimicrobial sequences generated from RIIA, one resulted from cleavage with high-specificity chymotrypsin at position 720, and is 17 aa long. The other is from cleavage at position 357 with pepsin at pH 1.3, and is 15 aa in length. The only predicted AMP from LysO1 is from cleavage with pepsin at pH > 2, at position 35, and is 33 aa in length.

Table A-3. Peptides derived from cleavage of RIIA.

Cleavage Site	Peptide Sequence	Length	APD2	CAMP		
				(SVM)	(RF)	(DA)_
chymotrypsin high (C-term to [FYW], not before P)						
43	KERAVIRELSCNAVDAQKEAGKENIPF	27	maybe - 7	no - 0.903	no - 0.746	yes - -0.810
157	LENGIPQVTKLTTNPTNEPNGLAVRVAVADHRISKF	36	maybe - 9	no - 0.855	no - 0.994	no - 0.548
195	DDVLADMNVIAREKGVY	17	very little chance	no - 0.887	no - 0.964	no - 0.083
234	NVVMGNIAYPVNMEALLGDDF	21	very little chance	no - 0.941	no - 0.984	no - 1.198
309	MPIGSVAIAASREALQMNDTTKNVIIEATKKITEAITK DVIKRVNSQPTLMDAAQAY	57	maybe - 20	no - 0.948	no - 0.998	no -0.734
329	AELRLNSREMLNAVCPKLEW	20	maybe - 3	no - 0.891	no - 0.888	no - 0.978
358	GGVKLDTLEEELLNIRRGIIHAEDGSVIY	29	very little chance	no - 0.585	no - 0.948	no - -0.061
380	ERDGKGNIKVDSKGNNIPKVAY	22	unknown	no - 0.749	no - 0.518	yes - -1.309
455	VINDRRNKNGTEKTVGRNQILRGACRDY	28	maybe - 3	no - 0.567	no - 0.52	yes - -0.587
504	STEKELDDLINLHKLDKSLLKIVKMSDKEHHY	32	maybe - 10	no - 0.986	no - 0.968	no - 0.781
544	TEVSEDLDTIEEPQLY	16	very little chance	no - 0.594	no - 0.908	no - 3.515
577	CSSPEDVAKSVANVIGKTVY	20	maybe - 7	no - 0.896	no - 0.62	no - 0.146
608	IEVDEKLLNDSLTDVHW	17	very little chance	no - 0.718	no - 0.93	no - 1.194
720	**KKIRKAGDRMVIKVTNY**	**17**	**maybe - 6**	**yes - 0.550**	**yes - 0.538**	**yes - -1.420**
chymotrypsin low (C-term to [FYWML], not before P						
43	SCNAVDAQKEAGKENIPF	18	very little chance	no - 0.696	no - 0.88	no - 0.453
267	MPIGSVAIAASREAL	15	maybe - 4	no - 0.961	no - 0.884	no - 0.739
302	NDTTKNVIIEATKKITEAITKDVIKRVNSQPTL	33	maybe - 9	no - 0.858	no - 0.968	no - 0.092
380	ERDGKGNIKVDSKGNNIPKVAY	22	unknown	no - 0.749	no - 0.518	yes - -1.309
448	VINDRRNKNGTEKTVGRNQIL	21	unknown	no - 0.688	no - 0.594	yes - -0.582
577	CSSPEDVAKSVANVIGKTVY	20	maybe - 7	no - 0.896	no - 0.62	no - 0.146
trypsin						
17	MTPEMFSLLSSGVYTFK	17	maybe - 6	no - 0.993	no - 0.994	no - 1.936
146	LTTNPTNEPNGLAVR	15	unknown	no - 0.994	no - 0.99	no - 1.622
190	FFEEAGNVYSYFAVKPESNIVYDDVLADMNVIAR	34	very little chance	no - 0.992	no - 1.0	no - 1.071
236	TDFNVVMGNIAYPVNMEALLGDDFFK	26	very little chance	no - 0.992	no - 0.976	no - 1.239
264	SVDLVNIYMPIGSVAIAASR	20	maybe - 8	no - 0.894	no - 0.534	no - 0.111

313	VNSQPTLMDAAQAYAELR	18	very little chance	no - 0.997	no - 0.974	no - 2.000
418	ATALSYTQEASMFNIFGAMR	20	maybe - 4	no - 0.958	no - 0.772	no - 1.505
546	AVPAESVASYTEVSEDLDTIEEPQLYIK	28	very little chance	no - 0.988	no - 0.984	no - 2.013
566	AVGDTVDSECFCSSPEDVAK	20	very little chance	no - 0.883	no - 0.946	no - 1.564
613	LLNDSLTDVHWINHNR	16	maybe - 4	no - 0.888	no - 0.984	no - 0.554
634	IYMNGVLDLTSSWIIAR	17	maybe - 6	no - 0.898	no - 0.7	no - 0.635
672	TIFLEGNEEAVEAIFGK	17	very little chance	no - 0.759	no - 0.902	no - 0.560
693	IQYVAAPFAYTYTISVLQTLK	21	maybe - 5	no - 0.973	no - 0.702	no - 0.517
pepsin pH 1.3						
42	LSCNAVDAQKEAGKENIP	18	very little chance	no - 0.564	no - 0.824	no - 0.649
104	IGAMGIGSKSPFAIAQS	17	maybe - 2	no - 0.53	no - 0.608	yes - -0.278
267	YMPIGSVAIAASREAL	16	maybe - 6	no - 0.962	no - 0.74	no - 0.797
302	QMNDTTKNVIIEATKKITEAITKDVIKRVNSQPTL	35	maybe - 9	no - 0.916	no - 0.986	no - 0.583
357	**NIRRGIIHAEDGSVI**	**15**	**maybe - 4**	**yes - 0.596**	**no - 0.824**	**yes - -0.697**
380	ERDGKGNIKVDSKGNNIPKVAY	22	maybe - 0	no - 0.749	no - 0.518	yes - -1.309
447	VINDRRNKNGTEKTVGRNQI	20	maybe - 0	no - 0.780	no - 0.578	yes - 0.518
577	CSSPEDVAKSVANVIGKTVY	20	maybe - 7	no - 0.896	no - 0.62	no - 0.146
719	YKKIRKAGDRMVIKVTN	17	maybe - 6	no - 0.872	no - 0.54	no - -0.226
pepsin pH > 2						
42	LSCNAVDAQKEAGKENIP	18	very little chance	no - 0.564	no - 0.824	no - 0.649
104	NYGASTKNDSNDYIGAMGIGSKSPFAIAQS	30	maybe - 4	no - 0.598	no - 0.78	no - 0.767
121	TVSSYVDGVVNKYSVY	16	maybe - 4	no - 0.992	no - 0.756	no - 0.169
213	ADMNVIAREKGVYDAMIHKQSWRSSGNRTDF	31	maybe - 6	no - 0.931	no - 0.994	no - 1.136
228	NVVMGNIAYPVNMEA	15	very little chance	no - 0.582	no - 0.886	no - 0.913
267	VNIYMPIGSVAIAASREAL	19	maybe - 7	no - 0.612	yes - 0.526	no - 0.532
302	QMNDTTKNVIIEATKKITEAITKDVIKRVNSQPTL	35	maybe - 9	no - 0.916	no - 0.986	no - 0.583
380	NIRRGIIHAEDGSVIYERDGKGNIKVDSKGNNIPKVAY	38	maybe - 4	no - 0.542	no - 0.548	yes - -0.856
447	VINDRRNKNGTEKTVGRNQI	20	maybe - 0	no - 0.780	no - 0.578	yes - 0.518
515	KIVKMSDKEHHYQRKEAVRGVVK	23	maybe - 6	no - 0.826	no - 0.626	yes - -0.489
534	WKAVPAESVASYTEVSED	18	very little chance	no - 0.993	no - 0.992	no - 1.742
578	CSSPEDVAKSVANVIGKTVYV	21	maybe - 7	no - 0.882	no - 0.554	no - 0.078
597	RKANWKKIPEDWIEVDEK	18	maybe - 3	no - 0.961	no - 0.844	yes - -0.552
623	TDVHWINHNRYMTRIYMNGV	20	maybe - 6	no - 0.972	no - 0.948	no - 0.522
658	NNRKISRGYCYSRDTSKTIF	20	maybe - 0	no - 0.627	yes - 0.512	no - -0.104
720	YKKIRKAGDRMVIKVTNY	18	maybe - 6	no - 0.829	no - 0.548	no - -0.21

Peptides were generated by *in silico* cleavage with the indicated peptidase, and antimicrobial potential is predicted by APD2 or CAMP. APD2 predictions are the numbers of hydrophobic residues predicted to be on the same plane of an α helix. SVM and RF predictions are the likelihood of the prediction being true; DA predictions are the discriminate scores for the peptides. Bolded peptides are predicted AMPs, based on results from APD2 and CAMP.

Table A-4. Peptides derived from cleavage of LysO1.

Cleavage Site	Peptide Sequence	Length	APD2	CAMP		
				(SVM)	(RF)	(DA)_
chymotrypsin high						
57	GTIRIDGKPVKMGMKITAEQAEQY	24	unknown	no - 0.934	no - 0.92	no - 1.041
82	VAAVNKAIKVPTSQNEF	17	maybe - 3	no - 0.757	no - 0.73	no - -0.128
147	NKVTVKGQKVTSNGLKNRRRMEADIY	26	unknown	no - 0.848	no - 0.66	no - 0.495
chymotrypsin low						
82	VAAVNKAIKVPTSQNEF	17	maybe - 3	no - 0.757	no - 0.73	no - -0.128
136	NKVTVKGQKVTSNGL	15	unknown	yes - 0.645	no - 0.538	yes - -0.601
trypsin						
37	LEAYEDSAGIPTIGYGTIR	19	very little chance	no - 0.902	no - 0.832	no - 0.421
64	ITAEQAEQYLLADVEK	16	very little chance	no - 0.904	no - 0.886	no - 1.802
104	VPTSQNEFDALVSETYNIGITAMQDSTFIK	30	very little chance	no - 0.997	no - 0.996	no - 2.009
pepsin pH 1.3						
56	GTIRIDGKPVKMGMKITAEQAEQ	23	maybe - 2	no - 0.905	no - 0.916	no - 0.964
18	VAAVNKAIKVPTSQNE	16	maybe - 3	no - 0.839	no - 0.692	yes - -0.324
119	IKRHNAGNKVGCAEAMQ	17	maybe - 4	no - 0.672	no - 0.578	no - -0.081
pepsin pH > 2						
57	EAYEDSAGIPTIGYGTIRIDGKPVKMGMKITAEQAEQY	38	very little chance	no - 0.93	no - 0.946	no - 1.094
81	VAAVNKAIKVPTSQNE	16	maybe - 3	no - 0.839	no - 0.692	yes - -0.324
101	VSETYNIGITAMQDST	16	very little chance	no - 0.982	no - 0.946	no - 2.372
135	**IKRHNAGNKVGCAEAMQWWNKVTVKGQKVTSNG**	**33**	**maybe - 6**	**no - 0.586**	**yes - 0.668**	**yes - -0.532**

Peptides were generated by *in silico* cleavage with the indicated peptidases, and their antimicrobial potential as predicted by APD2 or CAMP. Numbers in APD2 predictions are the numbers of hydrophobic residues predicted to be on the same plane of an α helix. The cleavage site and peptide sequence are given. Numbers in SVM and RF predictions are the likelihood of the prediction being true; numbers in DA predictions are the discriminate scores for the peptides. Bolded peptides are predicted AMPs, based on results from APD2 and CAMP.

3.1.3 Analysis of α helices of LysO1

PHYRE predicted six α helices in the LysO1 sequence (Chapter 4, section 3.6, 4.6). The lengths of the six helices varied from eight to 22 amino acids in length; all were analyzed for antimicrobial potential. Sequences of the helices and results of the analyses are given in Table A-5. Analysis identified two of the six sequences, helix 1 (aa 5-14) and helix 4 (aa 100-107), as potentially antimicrobial.

Table A-5. Antimicrobial potential of the predicted α helices of LysO1.

Peptide Information				APD2	CAMP		
sequence	start	end	length		SVM	RF	DA
RKGLEAIKFF	**5**	**14**	**10**	**maybe - 3**	**no - 0.934**	**yes - 0.636**	**yes - -1.783**
AEQAEQYLLADVEKFVAAVNKA	51	72	22	very little chance	no - 0.734	no - 0.694	no - 0.374
QNEFDALVSETYN	79	91	13	very little chance	no - 0.759	no - 0.898	no - 1.614
STFIKRHN	**100**	**107**	**8**	**not long enough**	**yes - 0.85**	**yes - 0.57**	**yes - -0.92**
KVGCAEAMQWWNKV	111	124	14	maybe - 5	no - 0.804	no - 0.636	yes - -1.099
NGLKNRRRMEADIYL	134	148	15	not long enough	no - 0.808	no - 0.752	no - 0.337

Location of α helices was predicted by PHYRE, antimicrobial potential was predicted using APD2 and three algorithms of CAMP. Bolded peptides are predicted to be AMPs.

3.2 Length of potential antimicrobial peptides

Peptides derived from RIIA that were identified as possessing antimicrobial activity were longer peptides. Thirty-two peptides derived from the RIIA protein sequence were determined to have antimicrobial potential, 29 from AntiBP2 and 3 from PeptideCutter. Of those 32 potential AMPs, only six are less than 50 aa in length. Of the remaining 26, one is 54 aa in length, the remaining 25 peptides are 70 aa in length or longer. Seven peptides derived from the LysO1 protein sequence were determined to have antimicrobial potential. Of those sequences, none is longer than 40 aa and two are 10 aa or shorter. A summary of the data arranged to highlight peptide length and AMP length is in Table A-6.

For a more stringent comparison, peptides were divided into categories by length, in 10-aa increments, and the percentage of AMPs from the total peptide fragments of each category was calculated and compared (Table A-6, Figure A-1). Figure 1 highlights the anomaly of the length of AMPs derived by an AntiBP2 search. From the graph, it is clear that only a small percentage of peptide fragments of any

length category have been determined to have AMP potential, and for fragments derived from all three methods the ratio of AMPs per the total number of peptides of that length analyzed declines as the peptide length approaches 60 aa. However, for peptide fragments derived by AntiBP2 from the RIIA protein, as the length increases to 70-79 aa, the percentage of fragments identified as AMPs increases, and the ratio increases further as the length increases. Of the four 100-aa peptides derived from RIIA by AntiBP2 analysis, APD2 and CAMP confirmed antimicrobial potential in every one.

Table A-6. Length of potential antimicrobial peptides.

	rIIA			LysO1		
length	**total seq.**	**AMPs**	**%**	**total seq.**	**AMPs**	**%**
AntiBP2						
10	5	0	0	3	1	0.333
20	22	1	0.045	8	2	0.25
30	24	2	0.083	6	1	0.167
40	21	1	0.048	9	0	0
50	17	1	0.059	10	0	0
60	12	0	0	5	0	0
70	11	3	0.273	4	0	0
80	14	5	0.357	1	0	0
90	24	12	0.5	1	0	0
100	4	4	1	0	0	N/A
Peptide Cutter						
10	28	2	0.071	9	0	0
20	28	0	0	3	0	0
30	15	1	0.067	5	1	0.2
40	1	0	0	0	0	N/A
50	2	0	0	0	0	N/A
60	1	0	0	1	0	0
70	0	0	N/A	0	0	N/A
80	1	0	0	0	0	N/A
90	0	0	N/A	0	0	N/A
100	0	0	N/A	0	0	N/A
Phyre						
10				5	2	0.4
20				1	0	0
30				0	0	N/A
40				0	0	N/A
50		N/A		0	0	N/A
60				0	0	N/A
70				0	0	N/A
80				0	0	N/A
90				0	0	N/A
100				0	0	N/A
Totals						
10	33	2	0.06061	17	3	0.17647
20	50	1	0.02	12	2	0.16667
30	39	3	0.07692	11	2	0.18182
40	22	1	0.04545	9	0	0
50	19	1	0.05263	10	0	0
60	13	0	0	6	0	0
70	11	3	0.27273	4	0	0
80	15	5	0.33333	1	0	0
90	24	12	0.5	1	0	0
100	4	4	1	0	0	N/A

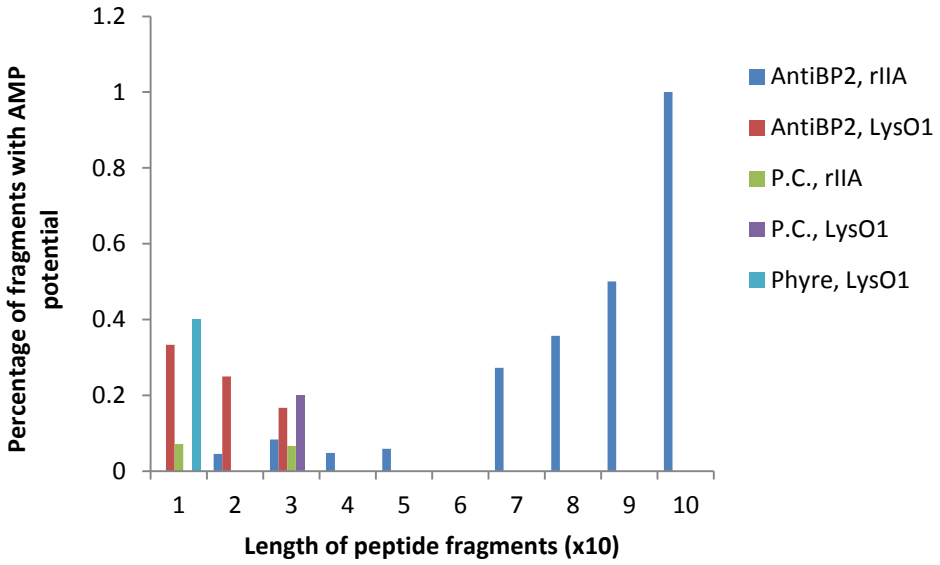
Peptides were divided into categories according to length, then analyzed according to protein of origin and the software used to derive the fragments. Total seq. is the number of sequences of that length. AMP is the number that had AMP potential % is the percentage of peptide fragments that had antimicrobial potential.

Table A-7. Summary of the average lengths of the analyzed peptide fragments and the peptides thought to have antimicrobial potential.

	rIIA			LysO1		
Source	**All Seq.**	**AMPs**	**%**	**All Seq.**	**AMPs**	**%**
AntiBP2	56.6	81.7	144.3	46.1	25.5	55.3
P.C.	25.5	23.3	91.4	25.4	34	133.9
Phyre	N/A	N/A	N/A	13.7	9	65.7
Total	46.3	76.25	164.7	38.1	22	57.7

Peptides are sorted by protein of origin and the software used to derive the fragment. The difference in the average length between all sequences derived from the protein and the sequences predicted to have antimicrobial potential is given as a percentage.

Figure A-1. Comparison of peptide fragment length and percentage of fragments of that length identified as AMPs.



Peptides generated *in silico* were divided into ten categories based on length. The single peptide fragment less than 10 aa was grouped with the peptides 10-19 aa in length.

3.3 Location of antimicrobial peptides in the parent protein sequence

Identified AMPs derived from RIIA tended to originate from the *C*-terminal end of the protein sequence. Of the 32 AMPs identified, none is found in the N-terminal 250 aa of the rIIA protein sequence, and 21 of the 32 originate for the *C*-terminal half of the protein. A similar trend was observed

in AMPs derived from LysO1. Of the seven identified, only one originated from the *N*-terminal half of the sequence. Data are summarized in Tables A-8 and A-9.

Table A-8. Summary of the position of each RIIA-derived AMP in the protein of origin.

AMP Sequence	Start	Length	Software
IYMPIGSVAIAASREALQMNDTTKNVIIEATKKITEAITKDVIKRVNSQPTLMDAAQAYAELRLNSREMLNAVCPKLEWGGVKLDTLEEELLNIRRGIIH	251	100	AntiBP2
AAQAYAELRLNSREMLNAVCPKLEWGGVKLDTLEEELLNIRRGIIHAEDGSVIYERDGKGNIKVDSKGNNIPKVAYLYNPVAYVKFNSLESK	305	92	AntiBP2
YAELRLNSREMLNAVCPKLEWGGVKLDTLEEELLNIRRGIIHAEDGSVIYERDGKGNIKVDSKGNNIPKVAYLYNPVAYVKFNS	309	84	AntiBP2
LRLNSREMLNAVCPKLEWGGVKLDTLEEELLNIRRGIIHAEDGSVIYERDGKGNIKVDSKGNNIPKVAYLYNPVAYVK	312	78	AntiBP2
NAVCPKLEWGGVKLDTLEEELLNIRRGIIHAEDGSVIYERDGKGNIKVDSKGNNIPKVAYLYNPV	321	70	AntiBP2
CPKLEWGGVKLDTLEEELLNIRRGIIHAEDGSVIYERDGKGNIKVDSKGNNIPK	324	54	AntiBP2
AEDGSVIYERDGKGNIKVDSKGNNIPKVAYLYNPVAYVKFNSLESKIRATALSYTQEASMFNIFGAMRKSQIEQFLFVINDRRNKNGTEKTVGRNQILRG	351	100	AntiBP2
EDGSVIYERDGKGNIKVDSKGNNIPKVAYLYNPVAYVKFNSLESKIRATALSYTQEASMFNIFGAMRKSQIEQFLFVINDRRNKNGTEKTVGRNQILR	352	98	AntiBP2
SVIYERDGKGNIKVDSKGNNIPKVAYLYNPVAYVKFNSLESKIRATALSYTQEASMFNIFGAMRKSQIEQFLFVINDRRNKNGTEKTVGRNQ	355	92	AntiBP2
NIRRGIIHAEDGSVI	357	15	PeptideCutter
YERDGKGNIKVDSKGNNIPKVAYLYNPVAYVKFNSLESKIRATALSYTQEASMFNIFGAMRKSQIEQFLFVINDRRNKNGTEKTVG	358	86	AntiBP2
DGKGNIKVDSKGNNIPKVAYLYNPVAYVKFNSLESKIR	398	38	PeptideCutter
ALSYTQEASMFNIFGAMRKSQIEQFLFVINDRRNKNGTEKTVGRNQILRGACRDYASESSLFHRYNGIVFVFSTEKELDDLINLHKLDKSLLKIVKMSDK	401	100	AntiBP2
YTQEASMFNIFGAMRKSQIEQFLFVINDRRNKNGTEK	404	94	AntiBP2
TQEASMFNIFGAMRKSQIEQFLFVINDRRNKNGTEKTVGRNQILRGACRDYASESSLFHRYNGIVFVFSTEKELDDLINLHKLDKSLLKIVK	405	92	AntiBP2
DRRNKNGTEKTVGRNQILRGACRDYASESLFHRYNGIVF	431	40	AntiBP2
RRNKNGTEKTVGRNQILRGACRDYASESSLFHRYNGIV	432	38	AntiBP2
CRDYASESSLFHRYNGIVFVFSTEKELDDLINLHKLDKSLLKIVKMSDKEHHYQRKEAVRGVVKLWKAVPAESVASYTEVSEDLDTIEEPQLYIKAVG	452	98	AntiBP2
RDYASESSLFHRYNGIVFVFSTEKELDDLINLHKLDKSLLKIVKMSDKEHHYQRKEAVRGVVKLWKAVPAESVASYTEVSEDLDTIEEPQLYIKAV	453	96	AntiBP2

Table A-8 cont.

INLHKLDKSLLKIVKMSDKEHHYQRKEAVRGVVKLWKA	482	38	AntiBP2
RKEAVRGVVKLWKAVPAESVASYTEVSEDLDTIEEPQLYIKAVGDTVDSECFCSSPEDVAKSVANVIGKTVYVFRKANWKKIPEDWIEVD	506	90	AntiBP2
KEAVRGVVKLWKAVPAESVASYTEVSEDLDTIEEPQLYIKAVGDTVDSECFCSSPEDVAKSVANVIGKTVYVFRKANWKKIPEDWIEV	507	88	AntiBP2
EAVRGVVKLWKAVPAESVASYTEVSEDLDTIEEPQLYIKAVGDTVDSECFCSSPEDVAKSVANVIGKTVYVFRKANWKKIPEDWIE	508	86	AntiBP2
DSECFCSSPEDVAKSVANVIGKTVYVFRKANWKKIPEDWIEVDEKLLNDSLTDVHWINHNRYMTRIYMNGVLDLTSSWIIARNFTFNNRKISRGYC	553	96	AntiBP2
SECFCSSPEDVAKSVANVIGKTVYVFRKANWKKIPEDWIEVDEKLLNDSLTDVHWINHNRYMTRIYMNGVLDLTSSWIIARNFTFNNRKISRGY	554	94	AntiBP2
CFCSSPEDVAKSVANVIGKTVYVFRKANWKKIPEDWIEVDEKLLNDSLTDVHWINHNRYMTRIYMNGVLDLTSSWIIARNFTFNNRKISR	556	90	AntiBP2
RDTSKTIFLEGNEEAVEAIFGKIQYVAAPFAYTYTISVLQTLKGCLDNNTKLYKKIRKAGDRMVIKVTNYLSKRKQENFLLSHLDWNKVSPIEVSKFLGF	651	100	AntiBP2
SKTIFLEGNEEAVEAIFGKIQYVAAPFAYTYTISVLQTLKGCLDNNTKLYKKIRKAGDRMVIKVTNYLSKRKQENFLLSHLDWNKVSPIEVSKF	654	94	AntiBP2
GNEEAVEAIFGKIQYVAAPFAYTYTISVLQTLKGCLDNNTKLYKKIRKAGDRMVIKVTNYLSKRKQENFLLSHLDWNKVS	662	80	AntiBP2
NEEAVEAIFGKIQYVAAPFAYTYTISVLQTLKGCLDNNTKLYKKIRKAGDRMVIKVTNYLSKRKQENFLLSHLDWNKV	663	78	AntiBP2
LQTLKGCLDNNTKLYKKIRKAGDR	689	24	AntiBP2
KKIRKAGDRMVIKVTNY	720	17	PeptideCutter

Table A-9. Summary of the position of each LysO1-derived AMP in the protein of origin

AMP Sequence	Start	Length	Software
RKGLEAIKFF	5	10	Phyre
GYGTIRIDGKPVKMGMKI	32	18	AntiBP2
STFIKRHN	100	8	Phyre
TFIKRHNAGNKVGCAEAMQWWNKVTVKGQKVTSN	101	34	AntiBP2
KRHNAGNKVGCAEAMQWWNKVTVKGQKV	104	28	AntiBP2
NAGNKVGCAEAMQWWNKVTVKG	107	22	AntiBP2
HNAGNKVGCAEAMQWWNKVTVKGQKVTSNGLKNR	139	34	PeptideCutter

4.0 Discussion

Studies have proposed AMPs as an answer to the problem of microbes that are increasingly drug-resistant. Advantages of AMP therapy include broad spectrum, high activity, and low likelihood of the pathogen developing resistance to the AMP (23). One of the difficulties of AMP research is that potential sequences can be generated much faster than they can be produced for antimicrobial activity and minimum inhibitory concentration (MIC) studies. To mitigate that difficulty, computer programs are trained to prescreen sequences; to examine them for possible antimicrobial activity as a way to focus attention on those sequences with the most antimicrobial potential. However, different algorithms may reach different conclusions about the same peptide.

Three prediction programs were used to identify and confirm AMP potential. All three used some form of SVM, a machine learning algorithm which uses pattern recognition and regression to identify AMPs, though varying degrees of accuracy were achieved (13, 14, 18, 20). Accuracy is determined by the success of the algorithm at identifying known antimicrobial peptides. AntiBP2 is 92.14% accurate when the NTCT15 search option, named because it identifies potential AMPs by analyzing 15 aa at both the *N*- and *C*-termini, is used. The SVM algorithm of CAMP is 91.5% accurate. CAMP includes two additional prediction algorithms: RF and DA. RF is based on the FORTRAN language and uses decision trees to reach a conclusion about a given peptide sequence, and achieves 93% accuracy. DA uses a linear combination of factors to separate one group into two or more classes and is 87% accurate (18). APD2 also uses SVM, though accuracy was not available.

Despite the different approaches used by different algorithms, the occasionally contradictory conclusions they reach, and the varying accuracy they are capable of, all were developed in essentially the same way. Machine learning uses sets of training data to teach a computer program to look for specific characteristics in data (10). The developers of AntiBP2 used four sets of training data, including AMPs taken from Swiss-Prot (Swiss Institute of Bioinformatics, Geneva, Switzerland) and sequences identified as definitely not AMPs, to train the algorithm to find AMPs in a set of testing data (13, 14). CAMP and APD2 used similar techniques, though the AMP sequences were acquired from NCBI and a literature review, respectively (18, 21). As the training sets included AMPs of all structural types, with different specificities, from different sources, recognition of AMPs was not limited to those sequences possessing specific secondary structures or from a single animal (14, 18, 21).

When potential AMP sequences identified by AntiBP2 were compared to the parent segment to check the accuracy of the sequence, every potential AMP was centered on the midpoint of the parent segment. A possible cause for this phenomenon is that AntiBP2 was not intended to identify shorter sequences in a longer sequence; it was intended to detect AMP potential of an input peptide sequence, the same as CAMP and APD2 (13, 14). The analysis algorithm examines the *N*-terminal and *C*-terminal 15 amino acids of a peptide sequence and compares them with the sequences of known AMPs (13). When a peptide segment was analyzed using "NC-termin" rather than the "full sequence composition" terminus option, the algorithm identified two 15-aa stretches of the query sequence that most closely matched the *N*-terminal and *C*-terminal peptide biases of known AMPs. The identified sequences were not always contiguous, might overlap, and the *C*-terminal 15 aa of some peptides was actually upstream of the *N*-terminal 15 aa. Several of the corrected sequences of the AntiBP2 results did have potential antimicrobial activity confirmed by other algorithms. It is possible that in addition to prediction of antimicrobial activity, AntiBP2 could be used in the detection of new potential AMP sequences from a longer peptide sequence if such detection is done cautiously. All peptides identified using such a method should be further screened and antimicrobial activity should be confirmed in laboratory studies.

A difference between APD2, CAMP, and AntiBP2 is that the output of APD2 includes information about the peptide and the characteristics that classified it as a potential AMP. The output of CAMP and AntiBP2 is a single number. APD2 gives information about potential α helices and β sheets, and the number of hydrophobic residues in a possible α helix, indicating amphipathic potential. If the overall charge of the query sequence was negative, APD2 listed that as a reason for rejecting the peptide sequence as a potential AMP. Two anomalies were noticed in the prediction results. First, APD2 was more likely to predict α helices with amphipathic potential (70.2% of peptides derived from RIIA and 54.4% of peptides derived from LysO1) than to predict β structures (1.9% of peptides derived from RIIA, and 0 derived from LysO1). Nearly a third of AMPs known have been determined to have some sort of β structure. Only 14.67% have α structures, and 2.47% have both; the number of fragments predicted by APD2 to have α structures is much higher than expected (21). Second, some predictions were contrary to what is understood about helix structure. The length of an α helix averages about four turns (about 15 aa), but can vary from fewer than two to more than eight (about seven to about 30 aa) (7). A peptide sequence of 82 aa was predicted to have 26 hydrophobic amino acids on the same plane of a single helix, and thus be amphipathic. Assuming the most common helix formation of 3.6 aa per turn, the length of the helix would be approximately 93 aa, which is much longer than the observed upper limit of about 30 aa. It may be that the identification algorithms for the two characteristics are not linked, and that analysis for helix formation is independent of detection of amphipathic potential. As the length of the analyzed sequence increases, the probability that the algorithm will detect a part that could be a helix, or hydrophobic residues evenly spaced to be on the same surface of a helix, may also increase, which could add a length bias to the results.

Beta structures rank as the most commonly observed structures of known AMPs. Of AMPs in the APD database, β structures alone are present in 33.14%, and another 2.47% have been determined to possess both α and β structures (4, 21). Of the AMPs that have only β structures, 97.7% of those are due to disulfide bonds between pairs of cysteine residues. In this study APD2 predicted that four of 231 peptides derived from RIIA (about 1.7%), and none of the 71 derived from LysO1, form β structures. The

lack of AMPs with predicted β structures from LysO1 is understandable, i.e., of the seven secondary structures predicted for LysO1, six are helices. However, prediction of β structures in the RIIA sequence was more numerous. APD2 determines the potential for β structures in peptide fragments by looking for an even number of cysteine residues. As not all cysteine residues participate in the formation of disulfide bonds, the method may underestimate the number of AMPs with β structures by overlooking those with an odd number of cysteine residues.

An additional reason to use more than one AMP identification algorithm is that certain sequences were rejected by APD2 on the basis of having a net negative charge. This classification is understandable, as most AMPs do have a net positive charge (4, 8, 22). However, ovine pulmonary surfactant (APD number AP00528) has a net charge of -6 and displays antibacterial activity against both Gram positive and Gram negative organisms (3). Maximin H5 (APD number AP00497) has a net charge of -3 and displays both antibacterial and antiviral activity (12). The two negatively charged peptides demonstrate that effective AMPs with net negative charges exist. Also, antimicrobial domains isolated from larger proteins may have a net negative charge (2). Although rejecting negatively charged peptides may simplify the search algorithm, there is also a possibility that such rejection based solely on charge overlooks effective AMPs.

CAMP uses three different algorithms to predict the probability of a given peptide sequence having antimicrobial activity, so analysis of the same peptide with each algorithm generated either of two different responses (yes or no), and the response was accompanied by varying degrees of uncertainty (18). For that reason, if all three algorithms (or two and APD2) reached the same "yes" conclusion, the sequence was marked as a potential AMP. Since CAMP algorithms did not include information about charge or possible secondary structures, is it not possible to use structure information to contemplate the effectiveness and possible bias of the CAMP analysis algorithms.

Analysis by CAMP and APD2 revealed that many of the peptides from RIIA that have AMP potential are longer than average AMPs. The average length of all AMPs in the APD database is 27.97 aa (14). Yet, the average length of AntiBP2-derived RIIA fragments identified by CAMP and APD2 analysis

as potential AMPs is 81.7 aa. As with APD2 analysis, the discrepancy may be explained by the analysis algorithms, that is, as the length of the peptide increases, the probability of the algorithm detecting somewhere in the sequence the characteristics of an AMP may also increase, which would bias the analysis in favor of longer peptides.

Perfect accuracy in peptide prediction is not expected when using AMP prediction algorithms. AMPs are a diverse class of peptides with variable secondary structure and very little sequence similarity. That diversity makes building a search algorithm difficult, and perfect accuracy may never be achieved. What is possible is to generate potential AMP sequences, then to screen those generated sequences to determine the sequences that have a high likelihood of being AMPs. Although each prediction algorithm has some bias in its prediction methods, using more than one to determine antimicrobial potential of a given peptide sequence could reduce the number of false positives in the output data and may be a way to quickly screen large numbers of sequences to find potential antimicrobial peptides.

5.0 References

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Appendix B

Development of a Lysozyme Activity Assay using Chicken Egg White Lysozyme and LysO1

1.0 Introduction

Enzyme characterization required a function activity assay to assess enzyme activity. Development of the assay to assess LysO1 activity against *Salmonella* required two pieces of information: if LysO1 were functional, and the conditions that would adequately permeabilize the *Salmonella* outer membrane to expose the peptidoglycan and allow LysO1 to function. Original tests of the function of LysO1 used the indicator organism *Micrococcus luteus* due to its susceptibility to most characterized lysozymes. The effectiveness of pretreatments designed to permeabilize the *Salmonella* outer membrane to lysozyme activity was tested by treating the bacteria with chicken egg white lysozyme (CEWL). This section is a record of the activity assays and attempts at membrane permeabilization that did not work as intended, and a discussion of why each was ineffective.

2.0 Materials and Methods

2.1 Determination of LysO1 activity

2.1.1 Effect of LysO1-VT on pretreated S. *Typhimurium*

To determine the lytic activity of a crude purification of the expressed LysO1-VT[3], tryptic soy agar (TSA, Difco, BD, Franklin Lakes, NJ) plates were spread-plated with 200 µl of a McFarland 1 suspension of *S*. Typhimurium suspended in phosphate-buffered saline (PBS). Plates were incubated 45 min at 37 °C to allow the bacteria time to adhere to the plate surface. To treat plated bacteria with chloroform vapor, 200 µl chloroform (Acros Organics, Thermo Fisher Scientific, Waltham, MA) was added to the inside of a glass petri plate lid and the plate of *S*. Typhimurium was inverted over the

[3] The characterization of the enzyme described in Chapter 4 was done using LysO1 expressed and purified by GenScript. Here both the GenScript-expressed enzyme and enzyme that was expressed and purified in our lab were used. To avoid confusion and maintain continuity with Chapter 4, the GenScript-purified enzyme will be referred to as "LysO1", and the enzyme expressed in our lab will be referred to as "LysO1-VT".

chloroform for 1 hr. To treat plated bacteria with trisodium phosphate dodecahydrate (TSP, Sigma-Aldrich, St. Louis, MO), 4 ml of a 9.3% solution of TSP in dH_2O was added to the plate surface, allowed to incubate 15 s, and then removed. TSP-treated plates were then incubated 1 hr at 37 °C. Two expression culture lysates were thawed in tepid water 10 min and centrifuged to remove cell debris, yielding crude purifications of LysO1-VT and the expression control. When a 30-min centrifugation step in a Thermo IEC centrifuge (Thermo Fisher Scientific, Waltham, MA) at 1,860 x *g* was insufficient to pellet cell debris, each lysate was divided into two aliquots in 1.5-ml snapcap tubes and centrifuged 10 min in an Eppendorf 5415 R tabletop microcentrifuge (Hamburg, Germany) at 15,700 x *g*. The supernatant was removed and used further. Twelve plates (4 each pretreated with chloroform, TSP, or no pretreatment) were divided into quarters. Three aliquots each of 15, 20, or 50 µl of the control were applied a quarter of each plate. The other 6 plates were treated with 3 aliquots each of 15, 20, or 50 µl of LysO1-VT crude purification. One plate was not inoculated and served as a control. Two plates were spread-plated but not further treated, and two plates each were pretreated with chloroform or TSP but not treated with cell lysate. Aliquots of cell lysate were allowed to dry and plates were incubated inverted overnight at 37 °C.

The experiment was repeated with the following modifications: chloroform pretreatment was omitted, concentration of TSP in the pretreatment wash was reduced to 1%, plates were incubated longer before additions of the enzyme, and CEWL (Sigma-Aldrich, St. Louis, MO) was included as a positive control. TSA plates were spread-plated with 200 µl of a McFarland 1 suspension of *S*. Typhimurium and incubated 4 hr at 37 °C until growth was visible. After incubation, experimental plates were divided into pretreatment and control groups. Plates in the pretreatment group were washed with 1% TSP for 15 s and allowed to dry. A 10-µl aliquot of LysO1-VT or CEWL was applied to the surface of both pretreated and control plates. Aliquots were allowed to dry, then plates were incubated at 37 °C and checked for lysis or growth inhibition after 30, 60, and 120 min and again after overnight incubation. Additional controls included *S*. Typhimurium with and without the pretreatment TSP wash, and *S*. Typhimurium to which 3 10-µl aliquots each of Buffer E and Elution Buffer from the QIAgen purification kit had been applied.

2.1.2 Effect of LysO1-VT and LysO1 on Micrococcus luteus

The activity of LysO1-VT was tested in a plate-based assay using *M. luteus* ATCC strain 4698 as the indicator organism and CEWL as a positive control. Five TSA plates were dried and labeled. Four were spread-plated with 200 µl of a McFarland 1 suspension of *M. luteus* and allowed to dry. Three of the plates containing *M. luteus* were then divided into quadrants numbered Q1-Q4. A 10-mg/ml concentration of CEWL was diluted 1:1000 in sterile PBS. Purified LysO1-VT and elution from the expression control culture P were thawed. Q1 received 10 µl CEWL, Q2 10 µl P, Q3 10 µl LysO1-VT, and Q4 10 µl sterile PBS. Aliquots were allowed to dry 5 min and plates were incubated overnight at 30°C.

The activity of LysO1 (GenScript Corp., Piscataway, NJ) was tested in both plate- and broth-based assays using *M. luteus* as the indicator organism and CEWL as a positive control. Seven aliquots of 150 µl of a McFarland 1 suspension of *M. luteus* in sterile PBS were spread-plated onto TSA plates and allowed to dry 15 min. Plates were divided into eight sections. CEWL and LysO1 (thawed on ice ten minutes) were serially diluted ten-fold in sterile PBS to $1{:}10^{-7}$. A 10-µl aliquot of each dilution of each enzyme was added to the surface of the appropriate section of each plate. Tests were done in triplicate. Spots were allowed to dry and plates were incubated overnight at 30 °C.

To determine the activity of LysO1 in broth and at a higher concentration, one plate of *M. luteus* grown 48 hr at 30 °C was scraped with 2.5 ml TSB and the fluid was collected. The culture was subjected to two ten-fold serial dilutions using 1 part culture and 9 parts sterile TSB to obtain suspensions B (1:10) and C (1:100). Nine 145-µl aliquots of each culture were added to a sterile 96-well plate with lid. Three of each concentration were untreated controls, three received 5.0 µl LysO1 that had been thawed on ice 10 min (final concentration 0.116 mg/ml), and three of each received 1.8 µl CEWL as a positive control (final concentration 0.12 mg/ml). Optical density at 600 nm was measured at 0, 2, 5, 10, 15, 30, 60, 90, and 120 min after addition of the enzyme on a SpectraMax 340PC^{384} microplate reader (SoftMax Pro Data Acquisition and Analysis software, Molecular Devices, LLC, Sunnyvale, CA), with a 5-s plate mix step before each measurement. The plate was held at room temperature with swirling between measurements.

2.2 Membrane permeabilization of *S*. Typhimurium

2.2.1 Effect of CEWL on a plate or broth culture of Micrococcus luteus

2.2.1.1 Determination of the functionality of CEWL using plate- and broth-based assays

To confirm that CEWL was functional, TSA plates were dried, labeled, divided into quadrants, and spread-plated with 200 µl of a McFarland 1 suspension of *M. luteus* in sterile PBS. Stock CEWL (10 mg/ml) was serially diluted ten-fold in sterile PBS to 1:1000, and a 10-µl aliquot of each dilution was applied to one quadrant of each plate. The fourth quadrant was treated with a 10-µl aliquot of sterile PBS as a negative control. Plates were incubated at 30 °C or 37 °C overnight.

The effect of CEWL on *M. luteus* in broth was determined as follows: *M. luteus* was grown 48 hr on TSA at 30 °C. A volume of 2.8 ml sterile TSB was added, and the plate was scraped and fluid collected after incubation at room temperature for 2 min (undiluted suspension). This suspension was further diluted 1:10 (dilution B) and 1:100 (dilution C) in sterile TSB, then 9, 150-ul aliquots of each suspension were added to a sterile 96-well plate and OD_{600} was measured. CEWL was diluted ten-fold in sterile dH_2O. CEWL stock was added to three wells of each bacterial suspension, an equal volume of dilute CEWL was also added to three wells of each suspension (final concentration 0.1 mg/ml or 0.01 mg/ml, respectively), and the remaining three wells of each suspension were used as untreated controls. Optical density at 600 nm was measured at 0, 2, 5, 10, 15, 30, 60, 90, and 120 min after addition of the enzymes, with a 5-s plate mix before each measurement. The plate was held at room temperature between measurements. During long intervals the plate was swirled on a Thermolyne Roto Mix plate mixer (Thermo Fisher Scientific, Waltham, MA) at speed 2. Additionally, the number of viable cells in each dilution of *M. luteus* was determined by ten-fold serial dilution and plating.

2.2.1.2 Effect of CEWL on plated *M. luteus*

To determine the zone of inhibition of CEWL on *M. luteus*, a 150-µl aliquot of a McFarland 1 suspension of *M. luteus* (approximately 1.5 x 10^8 CFU/ml) was spread-plated onto TSA plates and allowed to dry 15 min. CEWL was serially diluted ten-fold in sterile PBS to 1:10^{-8}. A 10-µl aliquot of

each dilution of CEWL was added to one of eight sections on each plate. A 10-µl aliquot of PBS was included as a negative control. Plates were done in triplicate. Aliquots were allowed to dry, and plates were incubated overnight at 30 °C.

To more accurately determine the zone of inhibition of CEWL on spread-plated *M. luteus*, a 150-µl aliquot of a McFarland 1suspension of *M. luteus* was spread-plated onto each plate and allowed to dry 15 min. A 1:1000 dilution of CEWL was serially diluted two-fold to 1:8, then a 10-µl aliquot of each dilution was applied to one of eight sections on each plate. Plates were done in triplicate. Aliquots were allowed to dry and plates were incubated overnight at 30 °C.

2.2.1.3 Function of CEWL in high concentrations of TSP

M. luteus was grown 48 hr on TSA, then the plate was scraped with 2 ml sterile TSB and the fluid collected. Three 150-ul aliquots were centrifuged 1 min at 15,700 x *g*, supernatant was removed, and pellets were resuspended in 1.5 ml TSB, TSB with 5% TSP, or TSB with 10% TSP, then diluted ten-fold in the resuspension medium. Six 149-ul aliquots of each diluted suspension were added to a 96-well plate, CEWL was added to three wells of each to a final concentration of 6.67 µg/ml, and optical density at 600 nm was measured at 0, 2.5, 5, 10, 15, 30, 60, 90, and 120 min after treatment.

2.2.2 Effect of outer membrane-permeabilizing agents on viability of S. Typhimurium

To determine the appropriate chloroform or TSP pretreatment for *S*. Typhimurium, TSA plates were spread-plated with 200 µl of a McFarland 1 suspension of *S*. Typhimurium in sterile PBS, then incubated 3 hr at 37 °C to allow the bacteria time to adhere to the plate. To pretreat with chloroform, a piece of sterile filter paper was placed in a glass petri plate lid, a 50-µl aliquot of chloroform was added, and the plate of *S*. Typhimurium was inverted over the chloroform for 0, 1, 2, or 5 min. To pretreat with TSP, plates were treated with 4 ml 0, 1, 2 or 5% TSP for 15 s by pouring the TSP solution onto the plate, tilting the plate to ensure the entire surface was covered, then removing the solution after 15 s. The

procedure was then repeated with plates treated as before and rinsed 15 s with sterile PBS after TSP treatment. All plates were done in duplicate, allowed to dry, and incubated overnight at 37 °C.

After analysis of the results of the previous experiment, the amount of TSP necessary was more precisely determined as follows. Fresh TSB was inoculated with one colony of *S.* Typhimurium and incubated with shaking at 37 °C until mid-log phase, and then 8 1-ml aliquots were centrifuged at 15,700 x *g* for 1 min. The supernatants were removed and the pellets were resuspended in 1 ml of the following: fresh TSB, TSB saturated with chloroform, or TSB to which TSP had been added to 1.0, 1.25, 1.5, 1.75, or 2.0%. A sterile 96-well plate with lid was inoculated with 3 150-µl aliquots of each resuspended culture, and OD_{450} was measured at 0, 15, 30, 45, and 60 min.

When it became necessary to alter the growth protocol *S.* Typhimurium from a broth culture to a plate culture, the previous experiment was repeated to determine if bacteria grown under different conditions responded differently to the pretreatment conditions. A 2.7-ml aliquot of sterile TSB was added to two plates of *S.* Typhimurium grown overnight at 37 °C. Plates were held at room temperature for 1 min before scraping, then the fluid was collected and pooled. Ten 100-µl aliquots were centrifuged 1 min at 15,700 x *g*, the supernatant was removed, and pellets resuspended in 1 ml TSB, TSB with 1.25, 1.5, 1.75, 2.0, or 2.25% TSP, or TSB with 50, 75, 100, 125, or 150 mM Tris·HCl [pH 8.7]. Three 150-µl aliquots of each culture were added to a 96-well plate and OD_{600} was measured at 0, 2, 5, 10, 15, 30, 60, 90, and 120 min with a 5-s plate shaking step before each measurement. After data were collected, a 100-µl sample of each culture was serially diluted ten-fold to 10^{-8} and plated to determine the number of viable bacteria per ml. The experiment was then repeated with TSB with 2.5, 3.0, 3.5, 4.0, 4.5, and 5.0% TSP, the Tris buffer was omitted, and the observation time was increased to two hours.

2.2.3 Effectiveness of CEWL and outer membrane-permeabilizing treatments on S. *Typhimurium*

To determine a permeabilization treatment that would make *Salmonella* susceptible to lysozyme activity, *S.* Typhimurium was grown in TSB to mid-log phase at 37 °C with shaking. Three 3-ml aliquots were centrifuged in a Thermo IEC centrifuge (Thermo Fisher Scientific, Waltham, MA) at 1,870 x *g* for

20 min at 24 °C. The supernatant was removed and pellets were resuspended in fresh TSB, TSB saturated with chloroform, or TSB with TSP added to 1.25%. Three aliquots of 150 µl and 6 aliquots each of 148.5 µl and 142.5 µl were added to a 96-well plate. Optical density at 450 nm was measured, and the plate was incubated 15 min at room temperature. After incubation, aliquots of 1.5 µl or 7.5 µl CEWL or dH_2O were added for a total volume of 150 µl. Final CEWL concentrations were 0.1 mg/ml (1.5 µl) and 0.5 mg/ml (7.5 µl). Optical density at 450 nm was measured at 0, 5, 10, 15, 30, and 60 min after addition of the enzyme.

The above experiment was modified in the following way to include different membrane-permeabilizing agents. *S.* Typhimurium was grown to mid-log phase at 37 °C with shaking. Eight 1.6-ml aliquots were centrifuged, the supernatant was removed, and the pellets were resuspended in 1.5 ml fresh TSB, TSB saturated with chloroform, TSB with 1.25, 1.5, or 2.0% TSP added, TSB with 0.1% Triton X-100, TSB with 50 mM EDTA, or Tris-EDTA buffer (0.05 M Tris [pH 7.2]; 50 mM EDTA).Three 150-µl aliquots and six 142.5-µl aliquots of each culture were added to a 96-well plate, OD_{450} was measured, and the plate was incubated 10 min at room temperature with swirling. After 10 min, 7.5 µl dH_2O or CEWL (final concentration 0.5 mg/ml) was added to three wells of each pretreatment group, and OD_{450} was measured at 0, 5, 10, 15, 30, and 60 min after addition.

The protocol was modified again in the following ways. *S.* Typhimurium was grown overnight on TSA plates instead of in broth cultures. A high concentration of lysozyme can cause cells to clump, so the assay was adapted to use lower concentrations of enzyme. Optical density of concentrated bacterial cultures can be inaccurate at 450 nm, so the wavelength was increased to 600 nm. A 2.7-ml aliquot of TSB, TSB with 50 mM Tris, or TSB with 1.25% TSP was added to three plates of *S.* Typhimurium that had been incubated overnight at 37 °C. The liquid was allowed to soak for 1 min, then plates were scraped and the fluid was collected. Each culture was serially diluted ten-fold to 1:100 in the broth used to scrape the plate. Nine 150-µl aliquots of each culture were added to a 96-well plate. Three aliquots were untreated, and 3 each received CEWL to a final concentration of 0.1 mg/ml or 0.01 mg/ml. Optical density at 600 nm was measured at 0, 2, 5, 10, 15, 30, 60, 90, and 120 min after addition of the enzyme,

with a 5-s mix step before each measurement to homogenize the suspensions in each well of the plate. The plate was held at room temperature between measurements, and during intervals of 15 min or more it was mixed gently on a Thermolyne Roto Mix at speed 2. Replicates were averaged and standard deviation calculated in MS Excel. After data collection, a 100-µl aliquot of each culture was ten-fold serially diluted and plated out to determine the number of viable organisms present.

To determine the effect of CEWL on *S.* Typhimurium pretreated with higher concentrations of TSP, TSP was added to TSB to 2.5, 3.0, 3.5, 4.0, 4.5, and 5.0% wt/vol and broths were filter-sterilized. *S.* Typhimurium was grown on TSA overnight at 37°C. The next morning, the plate was scraped with sterile TSB and fluid was collected. Seven aliquots of 100 µl each were centrifuged 1 min at 15,700 x *g* in an Eppendorf microcentrifuge (Eppendorf, Hamburg, Germany). The supernatant was removed and pellets were resuspended in 1 ml of sterile TSB with TSP added (one pellet received sterile TSB only as a control). Six 150-µl aliquots of each suspension were added to a 96-well plate. Three wells each were treated with CEWL to a final concentration of 66.7 µg/ml, and OD_{600} was measured at 0, 2, 5, 10, 15, 30, 60, 90, and 120 min after addition of the enzyme. The experiment was later repeated with TSB containing 0, 8, 10, and 12% TSP, and OD_{600} was measured at 0, 2, 5, 10, 15, and 30 min after addition of the enzyme.

2.2.4 Effect of outer membrane permeabilization treatments on S. *Typhimurium in buffer*

2.2.4.1 Elimination of sodium chloride as the cause of lack of observable enzyme activity

After repeated observation of lack of CEWL activity on *S.* Typhimurium, it was suggested that bacteria be grown to early log phase instead of mid-log phase, and that the observation time be increased to two hours; the protocol was altered accordingly. *S.* Typhimurium was grown to early log phase, 4 1.4-ml aliquots were centrifuged 5 min at 15,700 x *g*, the supernatants were removed, and the pellets were resuspended in 1.4 ml of 0.01 M Tris buffer [pH 8.7], 0.01 M Tris; 25 mM EDTA, and Tris and Tris-EDTA buffers with 25 mM NaCl added. Three 150-µl aliquots, three 148.5-µl aliquots, and 3 142.5-µl aliquots were added to a 96-well plate. Then CEWL was added to 0.1 mg/ml (1.5 µl) or 0.5 mg/ml (7.5

µl) final concentration and OD_{600} were measured at 0, 2.5, 5, 10, 15, 30, 60, 90, and 120 min after addition.

2.2.4.2 Effect of CEWL on *S.* Typhimurium in high-concentration Tris and Tris-EDTA buffers

S. Typhimurium and *M. luteus* were grown on agar. Two ml of 0.2 M Tris·HCl [pH 7.2] was added to each plate and incubated 1 min, then plates were scraped and fluid collected. Suspensions of bacteria were serially diluted ten-fold to 1:100. Six 149-µl aliquots of each dilution were added to a sterile, 96-well plate. Two aliquots were untreated, two each were treated with CEWL or LysO1 to a final concentration of 6.67 µg/ml. Optical density at 600nm was measured at 0, 2, 5, 10, 15, 30, and 60 min after enzyme treatment, with a 5-s plate mix step before each measurement. The plate was held at room temperature between measurements. The experiment was then repeated with the following changes: 0.2 M Tris·HCl [pH 7.2]; 5.0 mM EDTA was used instead of 0.2 mM Tris buffer, and the most concentrated suspension of each bacteria species was omitted.

2.3 Effect of alternative membrane permeabilization after establishment of LysO1 activity

2.3.1 Pretreatment with organic acids

S. Typhimurium was propagated and collected as described above, the 100-µl aliquots were suspended in 900 µl 0.01 M Tris·HCl [pH8] or the same buffer with citric acid (CA, Sigma-Aldrich, St. Louis, MO), lactic acid (LA, Thermo Fisher Scientific, Waltham, MA), or proprionic acid (PA, Acros Organics, Thermo Fisher Scientific, Waltham, MA) added to 1.0, 2.0, or 5.0 mM. Cells were pretreated for 30 min at room temperature with inversion at 0 and 15 min of treatment. Aliquots were washed twice in half volumes of 0.01 M Tris buffer, and resuspended in twice the original volume of 0.025 M Tris·HCl [pH 8]. Six, 149-µl aliquots of each suspension were added to a 96-well plate, and OD_{600} was measured. LysO1 (final concentration 0.1 µg/ml) or the same volume of storage buffer was added to 3 aliquots of each suspension, and OD_{600} was measured every minute for 15 min. All measurements were preceded by

a 5-s plate mix step. Rate of reaction was calculated from the first minute of measurement after addition of the enzyme or control treatment.

2.3.2 Pretreatment with PEI

S. Typhimurium was propagated and collected as described above, the 100-µl aliquots were suspended in 900 µl 0.01 M Tris·HCl [pH 8] or the same buffer with polyethyleneimine (PEI, M.N. 60,000, Acros Organics, Thermo Fisher Scientific, Waltham, MA) added to a final concentration of 10 µg/ml. Cells were pretreated for 30 min at room temperature with inversion at 0 and 15 min of treatment. Aliquots were washed twice in half volumes of 0.01 M Tris·HCl [pH 8] and resuspended in twice the original volume of 0.025 M Tris·HCl, pH 8. Six 149-µl aliquots of each suspension were added to a 96-well plate, and OD_{600} was measured. LysO1 (final concentration 0.1 µg/ml) or the same volume of storage buffer was added to 3 aliquots of each suspension, and OD_{600} was measured every minute for 15 min. All measurements were preceded by a 5-s plate mix step. Rate of reaction was calculated from the first minute of measurement after addition of the enzyme or control treatment.

2.3.3 Pretreatment with TSP

S. Typhimurium was propagated overnight on TSA and collected as described in Chapter 4, then 100-µl aliquots were suspended in 900 µl 0.01 M Tris·HCl [pH 8] or the same buffer with TSP added to 2, 5, 10, or 50 mM. Cells were pretreated for 30 min at room temperature with inversion at 0 and 15 min of treatment. Aliquots were washed twice in half volumes of 0.01 M Tris buffer, and resuspended in twice the original volume of 0.025 M Tris·HCl [pH 8]. Six 149-µl aliquots of each suspension were added to a 96-well plate, and OD_{600} was measured. LysO1 (final concentration 0.1 µg/ml) or the same volume of storage buffer was added to three aliquots of each suspension, and OD_{600} was measured every minute for 15 min. All measurements were preceded by a 5-s plate mix step. Rate of reaction was calculated from the first minute of measurement after addition of the enzyme or control treatment.

2.3.4 Pretreatment with Tris or Tris-EDTA buffer

S. Typhimurium was propagated and collected as described above, the 100-µl aliquots were suspended in 900 µl Tris·HCl [pH 8] to a final concentration of 0.01, 0.1, or 0.2 M, or the same buffer with ethylenediamenetetraacetic acid (EDTA, Sigma-Aldrich, St. Louis, MO) added to a final concentration of 5.0 mM. Cells were pretreated for 5 min, then washed twice in half volumes of 0.01 M Tris·HCl buffer and resuspended in twice the original volume of 0.025 M Tris·HCl [pH 8]. Six 149-µl aliquots of each suspension were added to a 96-well plate, and OD_{600} was measured. LysO1 (final concentration 0.1 µg/ml) or the same volume of storage buffer was added to 3 aliquots of each suspension, and OD_{600} was measured every minute for 15 min. All measurements were preceded by a 5-s plate mix step. Rate of reaction was calculated from the first minute of measurement after addition of the enzyme or control treatment.

3.0 Results

3.1 Determination of activity of LysO1-VT and LysO1

3.1.1 LysO1-VT activity on pretreated S. *Typhimurium*

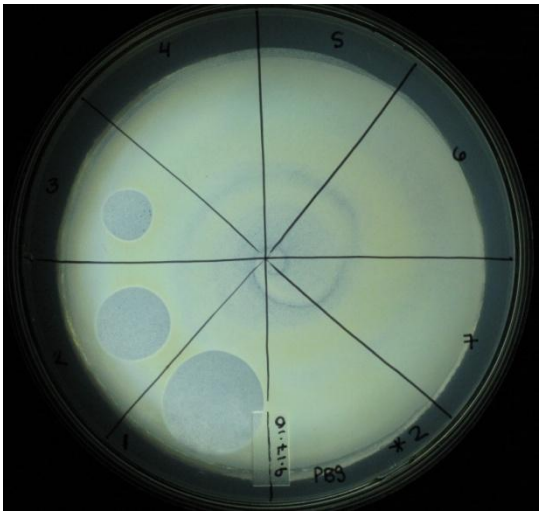
To test the functionality of LysO1-VT, clarified lysates of the expression and control cultures were applied to plates of *S.* Typhimurium, or *S.* Typhimurium pretreated with chloroform vapor or 9.3% TSP. Clarified lysates of the expression culture (L) containing LysO1-VT and the control culture (P) had no effect on untreated *Salmonella*. There was no growth at all on pretreated plates regardless of later application of crude-purified endolysin or the control culture, suggesting that pretreatment with both chloroform and 9.3% TSP was too harsh for the *Salmonella* to survive and needed to be moderated for experimental purposes. Upon repetition with 1% TSP, there was no visible zone of inhibition attributable to either enzyme after 120 min of observation or after further overnight incubation on either pretreated or non-pretreated plates. Buffer E from the purification kit, used under denaturing conditions, did inhibit growth of *S.* Typhimurium, but the purified LysO1-VT (results below) was purified under native conditions and the elution buffer used in the protocol had no effect on *S.* Typhimurium.

3.1.2 Effect of LysO1-VT and LysO1on M. luteus

In the assay used to determine activity of crude-purified LysO1-VT, addition of dilute CEWL to a lawn of *M. luteus* resulted in a zone of inhibition approximately 10 mm in diameter, while applications of expression culture (P) and PBS had no effect. The clarified lysate containing LysO1-VT (L) also had no effect on the indicator bacteria.

In the assay used to determine activity of LysO1 (GenScript Corp., Piscataway, NJ), the plates to which CEWL had been applied showed a zone of inhibition after overnight incubation; the size of the inhibition zone was dependent on the concentration of the applied enzyme. Ten microliters of a 1 mg/ml suspension of CEWL (1:10 dilution) inhibited an area 18 mm in diameter. Ten microliters of a 0.1 mg/ml enzyme suspension (1:100 dilution) inhibited an area 12.5 mm in diameter. Ten microliters of a 0.01 mg/ml suspension (1:1000 dilution) inhibited an area 8 mm in diameter, which was the most dilute enzyme solution to have a visible effect. The change in the size of the zones of inhibition relative to enzyme concentration suggests there is some diffusion of the enzyme across of the plate (Figure B-1). There were no visible zones of inhibition in the lawn of *M. luteus* treated with LysO1.

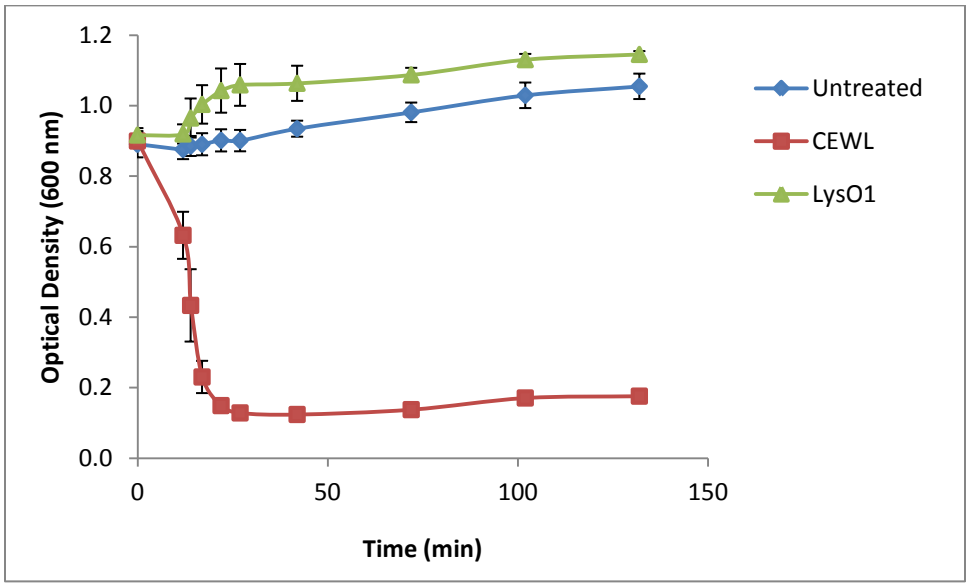
Figure B-1. Effect of CEWL on spread-plated *M. luteus*.



Ten microliters of each step of a ten-fold serial dilution of CEWL were spotted onto spread-plated *Micrococcus luteus* and incubated overnight at 30 °C. The largest circle (1:10 dilution, or 1.0 mg/ml) was 18 mm in diameter, the smallest (1:1000, 10 µg/ml) was 8 mm wide.

When repeated in broth, the expected decrease in optical density was observed in wells to which the control enzyme CEWL had been added. There was a noticeable decrease in optical density at 15 min after addition of the enzymes. Thirty minutes after addition of the enzyme the OD_{600} of an *M. luteus* suspension diluted 1:10 decreased from 0.63 to 0.12 (Figure B-2). An hour after addition, the OD_{600} had begun to increase slightly, and by the end of the two-hour experiment the optical density had increased to 0.17 from a low of 0.12. The same pattern was observed when *M. luteus* was diluted 1:100. A possible reason for the increase is that the enzyme was not completely effective against the *M. luteus*, and after 60 min the enzyme had become inactive and any surviving bacteria had begun to multiply. The initial sharp decline of OD was also observed when undiluted *M. luteus* was used as the enzyme substrate, but the OD continued to decline slightly until the end of the observation

Figure B-2. Effect of CEWL or LysO1 on *M. luteus* diluted 1:10 in TSB.



The assay tested the functionality of the LysO1 purchased from GenScript. *M. luteus* was used as the indicator organism, and CEWL was used as a positive control. The enzymes were added to approximately the same final concentration: LysO1 was added to 0.116 mg/ml and CEWL was added to 0.122 mg/ml. Decrease of OD of *M. luteus* treated with CEWL is indicative of enzyme function, lack of decrease in OD of cell suspensions to which LysO1 was added suggests a lack of lytic activity at those conditions. The test was done in triplicate.

Despite a relative increase in concentration of the LysO1 in the broth assay compared to the plate assays, there was no evidence of lysis in wells treated with LysO1; optical density did not decrease. There was an increase in optical density compared to the control in the first 15 min after addition of the enzyme. For the remaining time, the OD_{600} of the LysO1-treated and control cultures increase at the same rate at all concentrations of *M. luteus* tested (Figure B-2).

3.2 Membrane permeabilization of *S*. Typhimurium

3.2.1 Effect of CEWL on a plate or broth culture of Micrococcus luteus

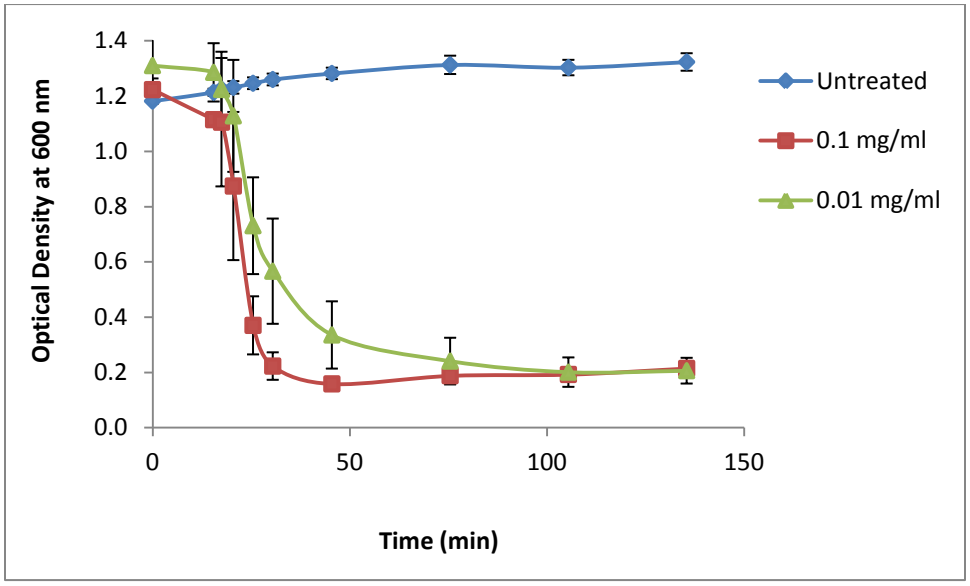
3.2.1.1 Determination of CEWL function

The initial plate-based activity assay using CEWL as the lysozyme and *M. luteus* as the indicator of enzyme activity determined that 10 µl of a 1:1000 dilution (10 µg/ml) effectively lyses *M. luteus*. On plates incubated at 30 °C, 10 µl of a 1:10 dilution (1.0 mg/ml) resulted in a zone of inhibition averaging 15.5 mm in diameter. Ten milliliters of a 1:100 dilution (0.1 mg/ml) resulted in a zone of inhibition 12 mm in diameter, and the 1:1000 dilution effected a zone of inhibition averaging 7.5 mm in diameter. On plates incubated at 37 °C the zones of inhibition averaged 18, 12.5, and 7.5 mm, respectively, and growth on those plates was translucent and not as thick as growth on plates incubates at 30 °C. The ideal incubation temperature for *M. luteus* is 30 °C; the difference in morphology of cultures grown at 30 and 37 °C may therefore be due to thermal stress. Stressed cells are more easily lysed, so the thermal stress of cells grown at 37 °C may also account for the larger zones of inhibition effected by the CEWL.

When the assay was repeated in broth, both concentrations of CEWL (0.1 and 0.01 mg/ml final concentration) affected the optical density of both dilutions of *M. luteus* (undiluted A; 1:10 B; and 1:100 C). The differences observed were of the same magnitude of the observed decrease in optical density and the rate at which it occurred. In dilution A (undiluted *M. luteus*), CEWL added to 0.1 mg/ml effected a larger and more rapid drop in optical density than CEWL added to 0.01 mg/ml. In dilution B (1:10 dilution of *M. luteus*), each concentration of enzyme effected the same decrease, but the more concentrated CEWL caused a more rapid decrease than the diluted enzyme (Figure B-3). In dilution C

(1:100 *M. luteus*), there was little difference in the rate of decrease. The optical density of untreated wells of culture A remained nearly stationary. The OD of wells of dilution B increased slightly, and the largest increase in OD was observed in the wells containing dilution C. The increase in OD is thought to be due to cell growth and multiplication, and as C was the least concentrated culture, those cells had the most available nutrients and grew the most rapidly.

Figure B-3. Effect of CEWL on *M. luteus* diluted 1:10 in TSB.



The assay measured whether CEWL was effective against *M. luteus* grown in a broth culture. CEWL was added at a concentration of 70 or 7 µg/ml.

A color change in undiluted suspension A was observed in an aliquot of undiluted *M. luteus* after addition of CEWL. Wells to which undiluted CEWL had been added were observed to partially clear during the course of the experiment, from a bright, opaque yellow to a translucent off-white. In wells to which dilute CEWL had been added, the color changed from bright yellow characteristic of *M. luteus* to dull brown over the observation period.

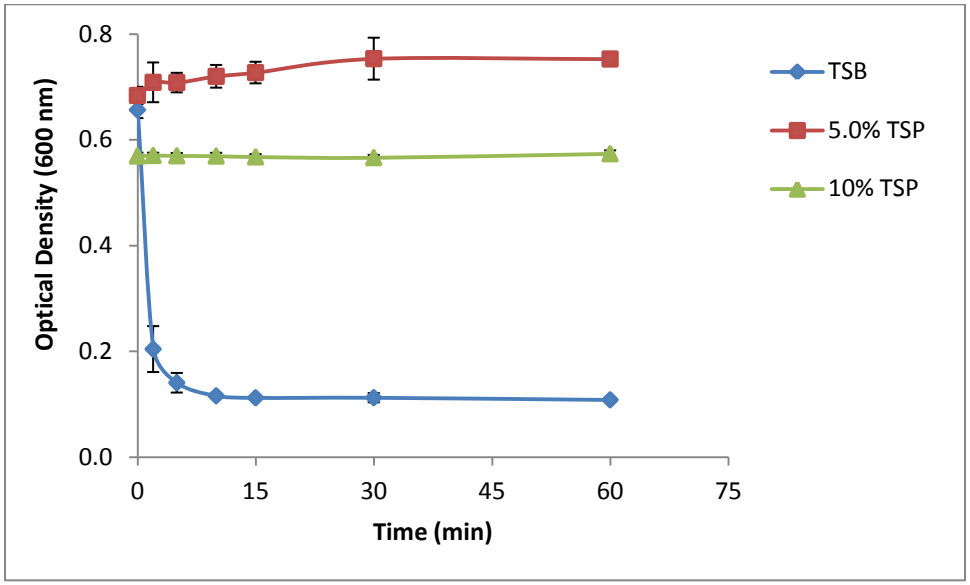
3.2.1.2 Determination of the minimum inhibitory concentration of CEWL

Determination of the size of the zone of inhibition of CEWL on spread-plated *M. luteus* was undertaken in two steps. The first step, plating each step of a ten-fold serial dilution, revealed that addition of a 10-µl aliquot of a 1:1000 dilution of CEWL to spread-plated *M. luteus* resulted in a zone of inhibition averaging 8 mm in diameter, but that 10 µl of a $1:10^{-4}$ dilution had no visible effect (Figure B-1). Concurring with the CEWL activity assay described previously, it was again observed that 10 µl of the 1:10 dilution resulted in a zone of inhibition 17 mm in diameter and the addition of an equal volume of the 1:100 dilution resulted in a zone of inhibition averaging 12 mm in diameter. As the surface area of the zone of inhibition was larger than the area covered by the diluted enzyme upon application to the plate, it suggests the enzyme diffuses through the agar after addition.

3.2.1.3 CEWL function in high-concentration TSP

M. luteus was diluted 1:10 in TSB with 0, 5, or 10% TSP, treated with CEWL, and observed over one hour. There was no observable CEWL activity against *M. luteus* suspended in 5% or 10% TSP. Fifteen minutes after addition of the enzyme, the OD_{600} of *M. luteus* had decreased from 0.6537 to 0.1119, (Figure B-4). At the end of the observation period, the OD had decreased further, to 0.1081. Of the cells in TSB with 5% TSP, 15 min after treatment with CEWL the OD had increased from 0.6836 to 0.7526. The OD continued to increase; at the end of one hour it was 0.7526. Of the cells in TSB with 10% TSP, 15 minutes after treatment with CEWL the OD had increased from 0.5691 to 0.5734. The same patterns were observed using when *M. luteus* diluted 1:100.

Figure B-4. CEWL function in high-concentration TSP



CEWL activity was measured in high-concentration TSP. *Micrococcus luteus* was diluted 1:10 and suspended in TSB with TSP added to 0, 5, or 10% wt/vol, then cells were treated with CEWL to a final concentration of 6.67 µg/ml and observed over 1 hr. Decrease of OD of *M. luteus* without added TSP is indicative of enzyme function, lack of decrease in suspensions with added TSP suggests the enzyme is nonfunctional in the indicated concentration of TSP.

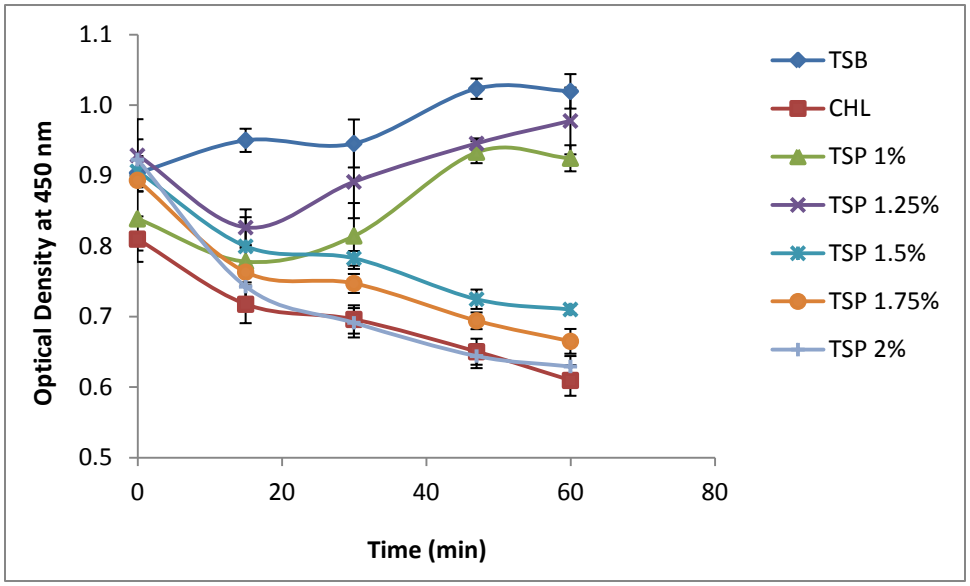
3.2.2. Effect of outer membrane-permeabilizing agents on the viability of S. *Typhimurium*

Exposure of plate cultures of *S*. Typhimurium to chloroform vapor for 1, 2, or 5 min had no noticeable effect on growth of *S*. Typhimurium. All lawns were smooth and appeared healthy, so it was decided to pretreat plates *S*. Typhimurium with chloroform for 5 min. One percent TSP had little effect on the bacteria compared to the untreated control. Growth was patchy on the plate treated with 2% TSP, and growth after exposure to 5% TSP was nonexistent. A second trial revealed that rinsing with PBS after TSP pretreatment had no noticeable effect on growth of the culture.

It was decided that an activity assay using bacteria suspended in a liquid medium was a more accurate representation of conditions during poultry processing than a plate-based assay. Determination of the percentage of TSP needed for pretreatment was continued using a broth-based assay, with the intention of using the highest percentage of TSP that did not inhibit growth of *S*. Typhimurium, and

inhibition was defined as a decrease in OD_{450} over the observation period compared to the OD of bacteria in unaltered TSB. *S*. Typhimurium was suspended in TSB, TSB saturated with chloroform, or TSB with 1.0, 1.25, 1.5, 1.75, or 2.0% TSP added. The optical density of *S*. Typhimurium in untreated broth, or broth to which TSP had been added to 1.0 or 1.25%, trended up over the hour the cultures were observed (Figure E-5). The optical density of the bacteria in broth saturated with chloroform or broth to which TSP had been added to 1.5, 1.75, or 2.0% trended downward over that hour. Consequently, 1.25% TSP was selected for further development of the endolysin activity assay.

Figure B-5. Effect of TSB or TSB saturated with TSP or chloroform on *S*. Typhimurium.



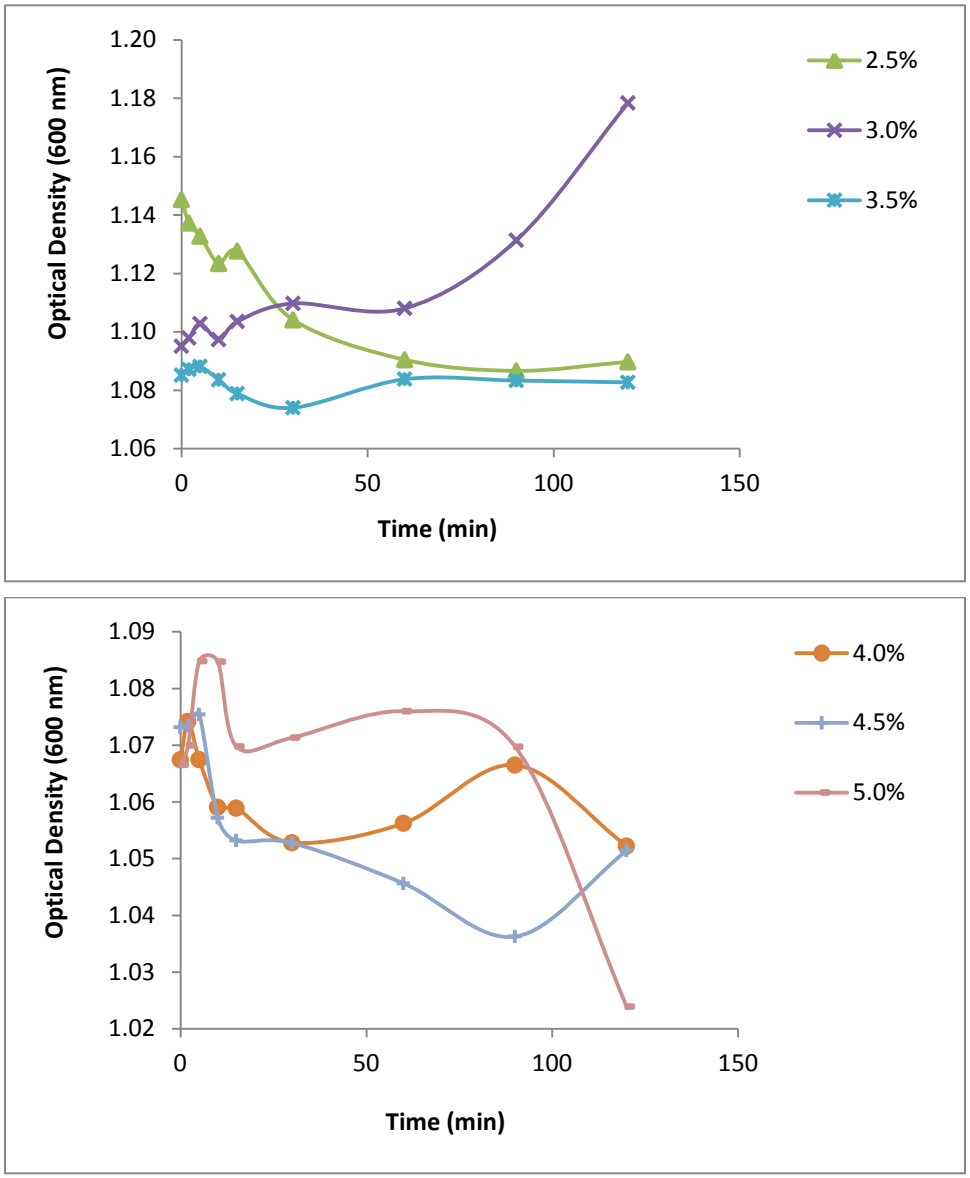
The effect of TSP on *S*. Typimurium was measured over 1 hr. Cells were suspended in TSB, TSB saturated with chloroform, or TSB to which TSP had been added to the indicated percentage (wt./vol.) and OD was observed over 1 hr. Results indicated a low concentration of TSP (1% or 1.25%) was least detrimental to cells.

After the protocol was altered to an assay of plate-propagated *S*. Typhimurium suspended in a liquid medium, it was necessary to repeat the study to ensure that bacteria grown on agar reacted the same way to 1.25% TSP as did bacteria grown in a broth culture. Tris was included, as it was currently under consideration as a pretreatment. *S*. Typhimurium was suspended in TSB with 0, 1.25, 1.5, 1.75, 2.0, or

2.25% TSP, or with Tris·HCl [pH 8.7] added to 50, 75, 100, 125, or 150 mM. The pretreatment had the same effect, regardless of concentration. The OD_{600} of bacteria suspended in TSB-Tris increased, regardless of concentration of the Tris, and increased at about the same rate. At the beginning of the observation period, the OD of all cultures was about the same, and it increased at about the same rate for each culture. The OD of cultures suspended in different concentrations of TSP remained stationary.

As the results from pretreatment with low concentrations of TSP were ambiguous, the experiment was repeated with higher concentrations of TSP: 2.5, 3.0, 3.5, 4.0, 4.5, and 5.0%. Tris was eliminated. The OD_{600} of *S*. Typhimurium in TSB treated with different concentrations of TSP was monitored over two hours. As the effect of the different concentrations on optical density was not clear, results were analyzed with trend lines (Figure B-6 a, b). The optical density of bacteria suspended in 3.0% TSP trended up. The trend line for 3.5% was ambiguous. The lines trending down and indicating conditions that had a negative effect on bacterial growth were, in order of magnitude least to greatest: 4.0%, 4.5%, 5.0%, and 2.5%. However, the effects on OD were small – the largest was an increase of 0.0833 units in bacteria suspended in TSB with 3% TSP. Also, the change in optical density of all suspensions over time was unpredictable. Such small and inconsistent changes may be due to random chance rather than the TSP in the medium.

Figure B-6 a, b. Optical density of *S.* Typhimurium in TSB-TSP



To measure the effects of higher concentrations of TSP on *Salmonella*, *S.* Typhimurium was diluted into TSB or TSB to which TSP had been added to the indicated percentage, and optical density was over two hours. A clear demarcation between low concentrations of TSP that permitted *Salmonella* growth, and higher concentrations that were detrimental, was not visible.

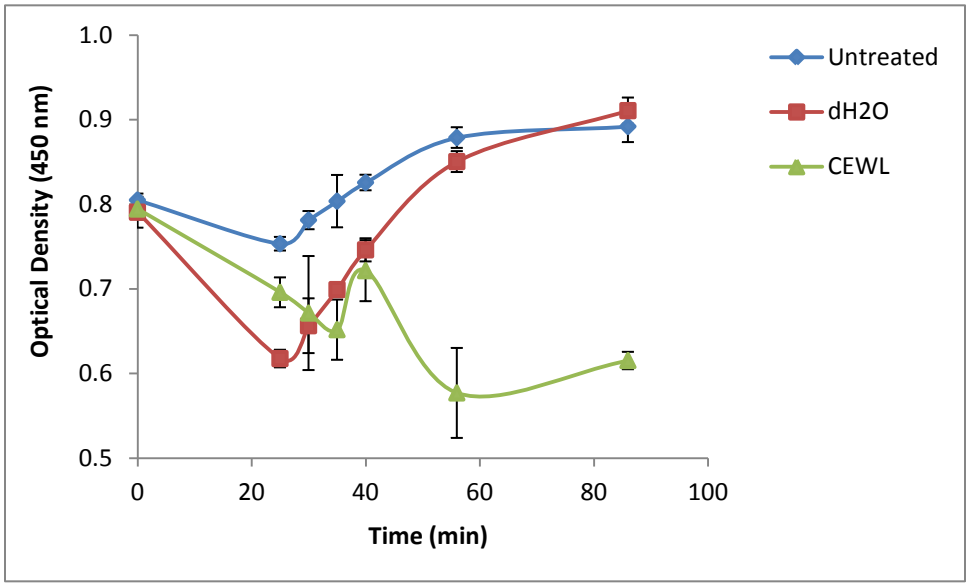
3.2.3 Effect of CEWL on S. *Typhimurium treated with outer membrane-permeabilizing agents in broth*

In the initial experiment using *S*. Typhimurium and CEWL in broth, addition of CEWL to bacteria in TSB, TSB saturated with chloroform, or TSB with TSP added to 1.25%had no easily discernible effect on pretreated *S*. Typhimurium compared to untreated controls.

In the second iteration of the assay, *S*. Typhimurium was treated with TSB, TSB saturated with chloroform, TSB with 1.25, 1.5, or 2.0% TSP, TSB with 0.1% Triton-X-100, TSB with 50 mM EDTA, or Tris-EDTA buffer (50 mM Tris·HCl [pH 7.2]; 50 mM EDTA). Addition of CEWL to pretreated *S.* Typhimurium had no clear effect compared to untreated controls. Observed at intervals over the hour after CEWL was added, there were some changes in OD, but with the exception of the culture suspended in Tris·EDTA buffer, all cultures in the same group followed the same growth pattern, regardless of whether or not they had subsequently been treated with CEWL.

Tris·EDTA buffer was included because some reports suggest that lysozyme activity is adversely affect by NaCl, which is present in TSB (14). Addition of CEWL to *S*. Typhimurium suspended in Tris buffer instead of media seemed to have some effect on optical density; OD_{450} declined during plate incubation before addition of the enzyme, but OD of the controls increased after CEWL addition and continued to increase until the end of the observation period (Figure B-7). After addition of the enzyme or dH_2O control the OD of the culture receiving the control treatment began to increase, but OD of the wells receiving CEWL continued to decrease for 10 min after addition of the enzyme. After 15 min OD appeared to have recovered, but after 30 min it had decreased again, to the lowest observed value. At the end of the hour OD had begun to increase slightly from the previous time point. However, the final OD was lower than that of any of the controls and was the only noticeable effect of CEWL addition on pretreated or untreated *S*. Typhimurium. Pretreatment of *S*. Typhimurium suspended in buffer was pursued through several more experiments, as discussed in the next section.

Figure B-7. Effect of CEWL or a control on *S.* Typhimurium in Tris·EDTA.



The permeabilization activity of Tris·EDTA (50 mM Tris·HCl [pH 7.2]; 50 mM EDTA) on *S.* Typhimurium was measured. Cells were suspended in the buffer, CEWL was added to a final concentration of 0.5 mg/ml, and optical density was observed over 90 minutes. A volume of sterile deionized water equal to the volume of CEWL was used as a control treatment.

After alteration of the protocol to include plate-propagated *S.* Typhimurium instead of bacteria grown in liquid medium, increase of OD wavelength of 450 nm to 600 nm as a better indication of lysis, treatment with a decreased amount of enzyme, and inclusion of Tris as a pretreatment, some CEWL-dependent decrease of optical density was expected. It had been observed in a previous experiment that less concentrated cultures of *M. luteus* lysed more quickly after addition of CEWL than more concentrated cultures. It was expected that the more dilute *S.* Typhimurium pretreated with Tris or TSP and then treated with CEWL would display some CEWL-related decrease in optical density. Despite the changes, there was no clearly observable difference between OD_{600} of *S.* Typhimurium treated with CEWL and the untreated controls, regardless of the concentration of bacteria.

At concentrations of 2.5% to 5% TSP, the OD of the samples treated with CEWL was expected to decrease, and decrease more rapidly in samples suspended in higher concentrations of TSP. The optical density of untreated samples and samples with lower concentrations of TSP in the broth was expected to

remain stationary or increase slightly due to multiplication of the bacteria. However, the added CEWL had no clearly observable effect on the optical density of any suspension of *Salmonella*, regardless of the TSP concentration in the media.

Upon repetition of the experiment with 8, 10, and 12% TSP, there was no noticeable difference in optical density during the 30-min observation period. The optical density of the suspension of *S.* Typhimurium with and without CEWL, with no TSP added to the media, increased very slightly, indicating the culture was healthy and that CEWL had no effect on untreated *S.* Typhimurium. The optical density of the suspensions of *S.* Typhimurium with TSP decreased very slightly with or without added CEWL, but there was no difference between the OD_{600} of the controls and those samples to which CEWL had been added.

3.2.4 Effect of CEWL on S. *Typhimurium pretreated with OM-permeabilizing agents in buffer*

To determine if the presence of NaCl was the cause of the continued lack of demonstrable enzyme activity, *S.* Typhimurium was suspended in Tris buffer and Tris-EDTA buffer, with 0 or 0.025 M NaCl. Lack of NaCl did not enhance CEWL activity.

It has been reported that 0.2 M Tris, or 0.2 M Tris with EDTA added to 5.0 mM, permeabilizes *P. aeruginosa* to enable lysozyme activity (18). When *S.* Typhimurium and *M. luteus* were suspended in 0.2 M Tris·HCl [pH 7.2], addition of CEWL to *M. luteus* resulted in a decrease in optical density of the suspension over the observation period, in a manner inversely proportional to the concentration of bacteria.

Treatment of *M. luteus* with LysO1 did not result in a decrease in OD compared to untreated controls, nor did treatment of *S.* Typhimurium with either enzyme compared to untreated controls. Similar results were observed when *S.* Typhimurium was suspended in 200 mM Tris·HCl [pH 7.2] with EDTA added to 5 mM.

3.3 Summary of effective activity assay for LysO1

In the course of development, the activity assay was altered in several significant ways. The method of propagation of the indicator organisms was changed from growth in broth with shaking to growth overnight on solid media. The indicator organisms were originally plated on solid media before treatment, but that changed to suspension in broth and finally in Tris buffer; cells suspended in buffer were easier to pretreat than plated cells. After establishment of a buffer-based assay, the first wavelength used to measure OD was 450 nm. That was later increased to 600 nm, which accurately measured any decrease in OD due to lysis but was less sensitive to small, random changes in the culture. Another adjustment was to use chloroform as a permeabilizing agent rather than a GRAS permeabilizer. The final change was to incorporate a wash step after pretreatment and to resuspend the pretreated cells in fresh buffer after washing twice. The activity assay used to characterize LysO1 used *S.* Typhimurium that had been pretreated with 10 mM Tris·HCl saturated with chloroform, washed twice, and resuspended in fresh 25 mM Tris·HCl buffer. Optical density was measured at 600 nm before and every minute for 10 min after treatment.

3.4 Effectiveness of pretreatment with organic acids, PEI, TSP, or Tris buffer

After it was determined that LysO1 was a functional enzyme and capable of lysing *S.* Typhimurium, some alternate pretreatments to chloroform-saturated buffer were revisited. Results were analyzed using JMP 9.0; the importance of individual factors and the interactions between them was determined using a Least Mean Squares (LMS) analysis, the effect of enzyme treatment on individual pretreatment groups was determined using an ANOVA and a Student's t-test if necessary.

3.3.1 Pretreatment with organic acids

S. Typhimurium was pretreated with 10 mM Tris·HCl [pH 8] or buffer to which citric, lactic, or proprionic acid had been added to 1, 2, or 5 mM. A LMS analysis revealed no interaction between the

treatment and pretreatment (Table E-1, F Ratio = 0.8339, Prob > F = 0.4780), suggesting that organic acids at the concentrations tested do not increase susceptibility of *S*. Typhimurium to LysO1.

Table B-1. LMS analysis of the effect of LysO1 on *S*. Typhimurium pretreated with organic acids, PEI, TSP, or Tris·EDTA.

	Organic Acid		**PEI**		**TSP**		**Tris-EDTA**	
R^2adj	-0.0096		0.0335		-0.0111		0.896	
Factor	F	Prob > F	F	Prob > F	F	Prob > F		
pre	0.3946	0.7572	0.4576	0.7135	1.6576	0.2126	9.3071	0.0003
trt	0.9567	0.3301	5.3881	0.0255	0.1167	0.7362	289.1947	< 0.0001
pre*trt	0.8339	0.478	0.6227	0.6045	0.9737	0.3355	9.7019	0.0002
EDTA							30.8321	< 0.0001
pre*EDTA							1.3032	0.2792
trt*EDTA							152.0066	< 0.0001
pre*trt*EDTA							2.1943	0.1203

LMS analysis reveals the importance of individual experimental factors and interaction between and among the factors in an experiment. A Prob > F value of 0.05 or below indicates that factor or interaction influenced the experimental results. Pre is pretreatment used (buffer or CHL), trt is treatment (LysO1 or buffer control), EDTA is the addition of EDTA to the reaction buffer. Two factors joined by an asterisk represent the effect of the interaction between those factors on the experimental results

3.3.2Pretreatment with PEI

Analysis by LMS of cells pretreated with 10 µg/ml PEI in 10 mM Tris·HCl, pH 8, or Tris buffer alone, revealed no interaction between the pretreatment and treatment, suggesting that PEI did not sensitize *S*. Typhimurium to LysO1 activity (Table B-1, F Ratio = 0.6227; Prob > F = 0.3355).

3.3.3 Pretreatment with buffer or buffer with TSP added

A LMS analysis of cells pretreated with 10 mM Tris·HCl, [pH 8], to which TSP had been added to 2, 5, or 10 mM revealed no interaction between the pretreatment and the enzyme treatment (Table B-1, F Ratio = 0.9737, Prob > F = 0.3355), suggesting that none of the tested concentrations of TSP pretreatment sensitized the cells to enzyme activity. Pretreatment with 50 mM TSP caused cells to

aggregate. Collection and subsequent resuspension in fresh buffer was not possible, so these cells were discarded.

3.3.4 Pretreatment with Tris buffer or Tris-EDTA

S. Typhimurium was pretreated with Tris·HCl buffer, pH 8, at 0.01, 0.1, and 0.2 M, or the same buffers with 5 mM EDTA. Least Mean Squares analysis revealed that an interaction between the presence of EDTA and the addition of LysO1 (Table B-1, F Ratio = 152.0066; Prob > F < 0.0001).

The magnitude of the effect of increasing the concentration of buffer pretreatment was analyzed by ANOVA and Student's t-test, as was the effect of pretreatment buffer concentration in the presence of EDTA. Increasing the concentrations of Tris buffer to 0.1 or 0.2 M had no effect on cells compared to the 0.01 M control (F Ratio = 0.01, Prob > F = 0.9987). Concentration of the buffer pretreatment did have an effect on the rate of reaction of LysO1. The rates of reaction of LysO1 against cells pretreated with 0.01, 0.1, and 0.2 M buffer were 0.013, 0.016, and 0.021 ΔOD_{600}/min, respectively. The rate of the enzyme against cells pretreated with 0.2 M Tris was different from cells pretreated with 0.01 M Tris, though not from cells pretreated with 0.1 M Tris (p = 0.0072, p = 0.0701). The rates of reaction of cells pretreated with 0.01 M and 0.1 M were not different (p = 0.266). Results are summarized in Table B-2.

Table B-2. Rate of reaction of LysO1 on *S.* Typhimurium pretreated with concentration-variable Tris or Tris-EDTA.

	Tris			Tris·EDTA		
Conc.	**Rate**	**St. Dev.**	**p-value**	**Rate**	**St. Dev.**	**p-value**
0.01	0.0128	0.0046	NA	0.0286	0.0068	NA
0.1	0.0160	0.0054	0.266	0.0422*	0.0062	0.0076
0.2	0.0214*	0.0043	0.0072	0.0462*	0.0095	0.0012

Concentration of Tris·HCl buffer (Conc.) is given in M. Rate is the rate of reaction of LysO1, given in ΔOD_{600}/min. P-values are in comparison to rate of reaction in of enzyme on cells pretreated with 0.01 M Tris buffer. Rates of reaction marked with an asterisk are different from the rate of reaction of the control treatment.

Pretreatment with 0.1 or 0.2 M Tris buffer also had no effect on buffer-treated cells when 5 mM EDTA was added, compared to 0.01 M Tris control (F Ratio = 0.068, Prob > F = 0.5222). However, when

cells were treated with LysO1, the rate of reaction of the enzyme was affected in a concentration-dependent manner. The reaction rate of LysO1 against cells pretreated with 0.01, 0.1, or 0.2 M Tris·HCl [pH 8]; 5 mM EDTA, was 0.029, 0.042, and 0.046 ΔOD_{600}/min, respectively. Although the rate of reaction of LysO1 was higher in cells pretreated with 0.2 M buffer, the rates of reaction of cells pretreated with 0.1 M and 0.2 M buffers were not statistically different ($p = 0.3774$). Each rate was different from the rate of reaction in cells pretreated with 0.01 M buffer ($p = 0.0076$, $p = 0.0012$). Results are summarized in Table B-2.

4.0 Discussion

Under many of the conditions described above, LysO1-VT and LysO1 had no effect on *S.* Typhimurium or *M. luteus*. The lack of observed lytic activity initially suggested one or a combination of five factors: that both LysO1-VT and LysO1 were nonfunctional, *S.* Typhimurium and *M. luteus* were not sensitive to the Felix endolysin, the enzyme concentration was not high enough, that the pretreatment was not effective, or the conditions were not suitable for enzyme activity.

An alternate pretreatment protocol for *S.* Typhimurium is described in Chapter 4 and revealed that LysO1 is functional, and negated all hypotheses regarding lack of function. *S.* Typhimurium is sensitive to LysO1 at a concentration of nanograms per milliliter, and to CEWL at a concentration of micrograms per milliliter. LysO1 had no effect on *M. luteus*. The enzyme was most active over a range of pH from 8 to 10, maintained at least partial activity after incubation at temperatures ranging from 0 to 40 °C, and had a rate of reaction significantly different from controls at concentrations as low as 50 ng/ml. Confirmation of LysO1 activity prompted a reexamination of the assays described here, to determine why the initial efforts were unsuccessful.

S. Typhimurium and *M. luteus* were used as indicators of conditions suitable for lysis. *S.* Typhimurium was used because it is an important foodborne pathogen in the target genus. *M. luteus* was used due to its susceptibility to most lysozymes. *M. luteus* was also used as a positive control in some experiments, to provide affirmation of enzyme activity when conditions necessary to permeabilize

Salmonella remained unknown. The use of two different indicators of enzyme activity highlighted two pieces of information. First, due to the presence of both the outer membrane and a recently identified lysozyme inhibition protein, *Salmonella* are difficult to lyse with externally applied lysozymes. In retrospect, the repeated failure of different permeabilization agents under different conditions to make cells susceptible to lysis emphasized that to be functional, any pretreatment of *Salmonella* must neutralize or eliminate the lysozyme inhibition protein, PliC, from *Salmonella* (4, 13). Second, LysO1 had no effect on *M. luteus*, which, as *M. luteus* is susceptible to most known lysozymes, is quite unusual. Implications of the lack of susceptibility are discussed in Chapter 4.

The initial tests of permeabilization and enzyme activity used plated bacteria as the indicator of lysis, because it was thought at the time that bacteria on a solid surface was a better model of processing conditions than bacteria suspended in a liquid. However, included in the initial protocols was a prolonged incubation step after treatment with the permeabilization agent but before application of the lytic enzyme, which would allow the bacteria to recover from the effects of the permeabilization. That step was eliminated in later versions of the activity assay. Even without the non-optimal protocol used, the plate-based assay had some disadvantages. It was difficult to pretreat large numbers of plates in a uniform and timely manner. The large number of plates needed also made the assay relatively resource-intensive compared to a broth-based assay. Upon encountering difficulties treating the plate surface and analyzing results, the focus switched to development of a liquid culture-based method. Advantages of this method included fewer resources necessary and ease of pretreatment of bacteria when suspended in broth or a buffer. Also, measurement of optical density over time made it possible to calculate the rate of the enzyme reaction, which would not have been possible using data obtained from a plate-based assay.

Chloroform, Triton X-100, TSP, PEI, lactic acid, and Tris are known membrane permeabilizers of Gram negative bacteria (2, 7, 10, 11, 16, 18). It is commonly known that organic solvents such as chloroform disrupt cell membranes. TSP and lactic acid are effective permeabilization agents due to their high and low pH, respectively, which disrupts the outer membrane by releasing LPS (2, 7, 9). PEI releases LPS from the outer membrane and binds it in insoluble form (10, 11). The permeabilization

activity of Triton X-100 is presumed to be due to detergent activity (16). High-concentration Tris is thought to bind to LPS and replace the stabilizing divalent cations, which reduces interactions between the LPS molecules and weakens the membrane (18) When the Tris-treated cells are then resuspended in buffer of a much lower molarity, osmotic shock may further weaken the cells. Addition of the chelating agent EDTA works synergistically with Tris by removing divalent cations (18). However, it is of note that only select agents made *Salmonella* susceptible to lysozyme activity, and only after the pretreated cells had been washed and suspended in fresh buffer. CEWL demonstrated lytic activity against *M. luteus* in 200 mM Tris and 200 mM Tris-EDTA, and the concentration of CEWL used, 66.7 µg/ml, was later demonstrated to have an effect, albeit diminished, on chloroform-permeabilized *Salmonella*. The lack of enzyme function was presumably due to the presence of PliC.

However, pretreatment with many of the agents described above did not make *Salmonella* susceptible to lysis by subsequent treatment with LysO1 or CEWL. The suspected reason is the presence of a protein. PliC is a member of one of several families of proteins that control the autolytic activity of transglycosylases in the periplasm of Gram negative cells that do not *O*-acetylate their peptidoglycan (8). An additional effect of the presence of such proteins in the periplasm is inhibition of the activity of added lysozymes, as lysozymes share a substrate with the lytic transglycosylases that the inhibitors are targeting (1, 4, 5). Four families of inhibitory proteins have been identified to date; Ivy was first purified from *E. coli* and identified as an inhibitory agent of vertebrate lysozymes of the *c*- and *g*-types (5). The MliC/PliC family was originally found in *P. aeruginosa* (MliC) and *S.* Enteritidis (PliC), and was determined to inhibit *c*-type lysozymes (4). The PliI and PliG families are found in *Proteobacteria*, and inhibit *i*-type and *g*-type lysozymes, respectively (13). For *Salmonella* species to be susceptible to externally applied lysozyme, not only must the outer membrane be permeabilized, the inhibitory protein PliC must be removed or inactivated (4).

As the lysozyme inhibitors have only been identified and characterized relatively recently, a treatment to selectively inactive them has not yet been reported. Studies that identified PliC as a lysozyme inhibitor did so by treating the bacteria with chloroform, then removing and analyzing the chloroform

fraction (4). Pretreatment with chloroform (and presumably Tris-EDTA buffer, per our results), followed by a wash step, removes the periplasmic inhibitor(3). Our results suggest that removal of PliC does not occur with treatment with those agents alone, or with pretreatment and washing with TSP, PEI, or other agents that failed to permeabilize cells. A possible explanation is that TSP and PEI are membrane permeabilizers, whereas treatment with chloroform-saturated buffer also extracts periplasmic proteins (3). Studies suggest that PliC is not tethered to the inner membrane or peptidoglycan, and therefore is released into the medium during treatment with chloroform and removed by subsequent washing, i.e., the chloroform fraction of pretreated cells containing the gene for PliC was found to inhibit enzyme activity, whereas the fraction from cells lacking *pliC* had no inhibitory effect (4). Permeabilization with TSP, PEI and organic acids may not sufficiently permeabilize the membrane to allow for enzyme access to the peptidoglycan, or it may allow LysO1 access but not be an adequate treatment for the removal of PliC.

Several studies have reported that TSP is an effective membrane permeabilization agent at low concentrations in addition to its known lytic activity at high concentrations(6).The research presented here suggests that at the concentrations tested (up to 10 mM, for 30 min), it does not make *S.* Typhimurium susceptible to lysozyme activity. Cells suspended in solutions of TSP of up to 12% total wt/vol were treated with CEWL and LysO1, yet there was no pattern of exponential decay observed in optical density measurements, suggesting that there was no lysozyme activity. One reason, described above, is the likely inhibitory activity of the lysozyme inhibitory protein PliC. Another reason is the presumed inactivation of the added lysozyme by high pH, as indicated by the fact that CEWL is inactive against *M. luteus* at 5% TSP. The optimal pH of CEWL activity is 9.2, and the pH of a 5% solution of TSP in TSB was later determined to be 11.5 (15). Consequently, TSP would need to be removed prior to addition of the enzyme for the TSP/enzyme combination to be effective. However, cells aggregated during a 30-min pretreatment with 50 mM TSP, so pretreatment with higher concentrations was not tested.

An additional reason for the lack of activity observed in high concentrations of TSP was revealed during characterization of LysO1. The enzyme was partially inhibited by moderate concentrations of sodium. As sodium concentration increased, the observed activity of LysO1 decreased, and at 250 mM

sodium the effect of the enzyme was indistinguishable from that of the control treatment. The amount of sodium in TSP is inherent in the name of the compound; there are three moles of sodium in each mole of TSP. The amount used during poultry processing as an antimicrobial measure is between 8 and 12% wt./vol. The molarity of an 8% solution of TSP is 0.49 M. The effective concentration of sodium in that solution is 1.47 M, and that concentration would render LysO1 nonfunctional.

Although effective, TSP may not be environmentally friendly. TSP was one in widespread use as a cleaning agent, but is falling out of favor due to ecological impacts. Algal blooms and shallow water eutrophication are attributed to high levels of phosphorus in agricultural runoff, and phosphorus additives in many cleaning agents have been banned (12, 17).

The permeabilization agents mentioned previously have not been reported to inhibit lysozyme activity unless they are used in quantities that alter pH and ion concentration of the medium from what is known to be optimal for the lysozyme in question. An exception to that lack of known inhibition is Triton X-100, a nonionic surfactant and known membrane permeabilizer (16). As with TSP, PEI, and organic acid pretreatment, there was no observed enzyme activity when CEWL was added to *S*. Typhimurium cells pretreated with Triton X-100, which could be due to inadequate permeabilization of the outer membrane or lack of inactivation of PliC. However, another possible reason for the lack of activity is that the cells were not removed from the pretreatment and washed before CEWL was added, and CEWL activity is inhibited by compounds that contain carbon chains of more than twelve atoms in length (15).

5.0 References

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