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Studies of a Novel Phage Lytic Enzyme, PlySs2

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STUDIES OF A NOVEL PHAGE LYTIC ENZYME, PlySs2

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STUDIES OF A NOVEL PHAGE LYTIC ENZYME, PlySs2

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Streptococcus suis infects pigs worldwide and may be zoonotically transmitted to humans with a mortality rate of up to 20%. Methicillin-resistant *Staphylococcus aureus* (MRSA) and *Streptococcus pyogenes* (group A streptococci – GrAS) cause potentially fatal human diseases. These are just three of the many Gram-positive pathogens for which resistance to leading antibiotics has emerged. The goal of this work was to develop a novel antimicrobial treatment to combat these and other antibiotic-resistant pathogens.

We identified a novel bacteriophage lysin, derived from an *S. suis* phage termed PlySs2 (**p**hage **ly**sin from *S. suis* **2**). This thesis is divided into four main sections detailing PlySs2: characterization (chapter 2); activity against *S. suis* (chapter 3); broad lytic activity (chapter 4); and efficacy *in vivo* (chapter 5).

PlySs2 has an N-terminal CHAP catalytic domain and a C-terminal SH3b binding domain. It is stable at 50°C for 30 min, 37°C for >24 h, 4°C for 15 days, and -80°C for >7 months; it maintained full activity after 10 freeze-thaw cycles.

PlySs2 displays potent lytic activity against most strains of *S. suis* including the type strain S735, the pathogenic serotype 2, strain 10, and the pathogenic serotype 9 strain 7997. At 64 μg/ml, PlySs2 reduced multiple strains of *S. suis* by 6-logs within 1 hour *in vitro*. PlySs2 exhibited a minimum inhibitory concentration (MIC) of 32 μg/ml for *S. suis* strain S735 and 64 μg/ml for strain 7997. While resistance to gentamicin was

observed after systematically increasing levels of gentamicin in an *S. suis* culture, the same protocol resulted in no observable resistance to PlySs2.

The bacteriophage lysin PySs2 also exhibits broad lytic activity against MRSA, vancomycin-intermediate *S. aureus* (VISA)*, Streptococcus suis, Listeria, Staphylococcus simulans, Staphylococcus epidermidis, Streptococcus equi, Streptococcus agalactiae* (group B streptococci – GBS), *S. pyogenes, Streptococcus sanguinis,* group G streptococci (GGS), group E streptococci (GES), and *Streptococcus pneumoniae*. PlySs2 at 128 μg/ml *in vitro* reduced MRSA and *S. pyogenes* by 5-logs and 3-logs within 1 hour respectively, and exhibited a minimum inhibitory concentration (MIC) of 16 μg/ml for MRSA. Serially increasing exposure of MRSA and *S. pyogenes* to PlySs2 or mupirocin resulted in no observed resistance to PlySs2 and resistance to mupirocin.

The relevance of our *in vitro* work was confirmed with multiple *in vivo* experiments. Using a single 0.1-mg dose of PlySs2, the colonizing *S. suis* strain 7997 was reduced from the murine intranasal mucosa by >4 logs; a 0.1-mg dose of gentamicin reduced *S. suis* by <3-logs. A combination of 0.05 mg PlySs2 + 0.05 mg gentamicin reduced *S. suis* by >5-logs. In protecting against mixed infections, a single, 2-mg dose of PlySs2 protected 92% (22/24) of the mice in a bacteremia model of dual MRSA and *S. pyogenes* infection.

This is the first known lysin with broad activity against multiple serotypes and strains of *S. suis,* making it a vital tool in the treatment and prevention of *S. suis* infections in pigs and humans. To date, no other lysin has shown such notable broad lytic activity, stability, and efficacy against multiple, leading, human bacterial pathogens; PlySs2 has all the characteristics to be an effective therapeutic.

To my wife, Chanel

To my children

To my parents

ACKNOWLEDGEMENTS

Most of all, I would like to thank my wife, Chanel Gilmer, who has journeyed with me through every day of this doctoral work. From applying to graduate school to writing my thesis, she has been an unwavering source of passionate encouragement and insightful perspective. My children have been wonderful joys throughout my study bringing meaning to my every day. And finally, I am forever indebted to my parents who taught me that my purpose comes before my profession in light of Matthew 6:33.

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LIST OF ABBREVIATIONS

Abbreviation	Descriptor
B. anthracis	*Bacillus anthracis*
B. cereus	*Bacillus cereus*
B. subtilis	*Bacillus subtilis*
B. thuringiensis	*Bacillus thuringiensis*
BCA	bicinchoninic acid (assay)
BD	binding domain
BHI	brain heart infusion (media)
Buffer A	15 mM PB, pH 8.0
Buffer B	15 mM PB, pH 6.7
Buffer C	50 mM PB, pH 7.4
Buffer D	20 mM PB, pH 7.4
Buffer E	20 mM glycine-NaOH, pH 9.3
CA-MRSA	community-acquired methicillin-resistant *S. aureus*
CD	catalytic domain
CFU	colony forming unit
CHAP	cysteine-histidine amidohydrolase/peptidase (domain)
ClyS	chimeric lysin for staphylococci
CM	carboxymethyl

Abbreviation	Descriptor
DEAE	diethylaminoethyl
DTT	dithiothreitol
E. coli	*Escherichia coli*
E. faecalis	*Enterococcus faecalis*
E. faecium	*Enterococcus faecium*
EDTA	ethylenediaminetetraacetic acid
FDA	Food and Drug Administration (United States)
FL	full-length (enzyme)
GBS	*Streptococcus agalactiae*
GCS	group C streptococci
GES	group E streptococci
GGS	group G streptococci
GrAS	group A streptococci, *S. pyogenes*
kD	kilodaltons
L. acidophilus	*Lactobacillus acidophilus*
L. gasseri	*Lactobacillus gasseri*
L. monocytogenes	*Listeria monocytogenes*
L. rhamnosus	*Lactobacillus rhamnosus*
LB	Luria-Bertani (media)

Abbreviation	Descriptor
Log fold kill	-log[(number of cells surviving under test condition)/(number of cells surviving under control condition)]
MH	Mueller-Hinton (media)
MIC	minimum inhibitory concentration
MRS	de Man, Rogosa, and Sharpe (media)
MRSA	methicillin-resistant *S. aureus*
MSSA	methicillin-sensitive *S. aureus*
OD	optical density
OD ratio	Treated OD_{600} / Untreated OD_{600}
P. aeruginosa	*Pseudomonas aeruginosa*
PBS	phosphate buffered saline
PlyC	a multimeric lysin of the C1 bacteriophage
PlySs2	**P**hage **ly**sin from *Streptococcus suis* 2
Rcf	relative centrifugal force
Rpm	revolutions per minute
S. agalactiae	*Streptococcus agalactiae*
S. aureus	*Staphylococcus aureus*
S. dysgalactiae	*Streptococcus dysgalactiae*
S. dysgalactiae equisimilis	*Streptococcus dysgalactiae equisimilis*

Abbreviation	Descriptor
S. epidermidis	*Staphylococcus epidermidis*
S. equi	*Streptococcus equi*
S. equi zooepidemicus	*Streptococcus equi zooepidemicus*
S. gordonii	*Streptococcus gordonii*
S. mutans	*Streptococcus mutans*
S. oralis	*Streptococcus oralis*
S. pneumoniae	*Streptococcus pneumoniae*
S. pyogenes	*Streptococcus pyogenes*
S. rattus	*Streptococcus rattus*
S. sanguinis	*Streptococcus sanguinis*
S. simulans	*Staphylococcus simulans*
S. sobrinus	*Streptococcus sobrinus*
S. suis	*Streptococcus suis*
ST	serotype
VISA	vancomycin-intermediate *S. aureus*
VRSA	vancomycin-resistant *S. aureus*

1   CHAPTER 1 – INTRODUCTION

1.1   Gram-positive pathogens

Gram-positive pathogens such as *Streptococcus pyogenes* (group A streptococci – GrAS)*, Staphylococcus aureus, Streptococcus suis, Streptococcus agalactiae* (group B streptococci – GBS)*,* and *Listeria monocytogenes* are responsible for millions of serious and sometimes fatal infections worldwide. Additionally, resistance to conventional antibiotics has been on the rise, resulting in increased rates of infection, morbidity, mortality, and treatment costs. Consequently, new therapeutic methods need to be developed to reduce the antibiotic pressure on these pathogens. Our laboratory studies many different genus and species of these bacteria in hopes of finding novel therapeutics

1.1.1   *Streptococcus suis*

1.1.1.1   Pathogenesis

The zoonotic Gram-positive pathogen *S. suis* causes sepsis and meningitis in pigs and humans. *S. suis* was first isolated from septicemic pigs and subsequently found to be biochemically distinct from other streptococci (de Moor 1963). After recent reclassification (Hill et al. 2005), there are 33 serotypes distinguished by variations in the capsular polysaccharide synthesis (*cps*) locus (Liu et al. 2013). Serotype 2, strain 10 and serotype 9, strain 7997 of *S. suis* have been most often associated with disease, with strain 7997 causing an increasing proportion of the infections worldwide (Silva et al. 2006, Wu, Zhang, and Lu 2008, Gottschalk et al. 2010). Pigs intranasally colonized with *S. suis* may transmit the pathogen to humans and piglets causing pneumonia, septicemia, or meningitis within days (Dekker et al. 2013, Arends and Zanen 1988, Gottschalk et al. 2010). While the pig carriage rate can reach 100% (Rasmussen et al. 1999), only a subset

of pigs develop meningitis or septicemia.

1.1.1.2   Zoonosis

In particular, the serotype 9 strain 7997 has been associated with increasing reports of zoonotic transmission from pigs to humans (Sriskandan and Slater 2006). The earliest human case was reported in 1968; since then, over 700 human cases have been reported in multiple continents with a 5-20% mortality rate (Figure 1.1) (Perch, Kristjansen, and Skadhauge 1968, Sriskandan and Slater 2006, Yu et al. 2006, Trottier et al. 1991). In a 2-month 2005 outbreak in China, among 203 human cases reported, the median time from exposure to infection was 2.2 days, but ranged from 3 hours to 14 days (Yu et al. 2006).
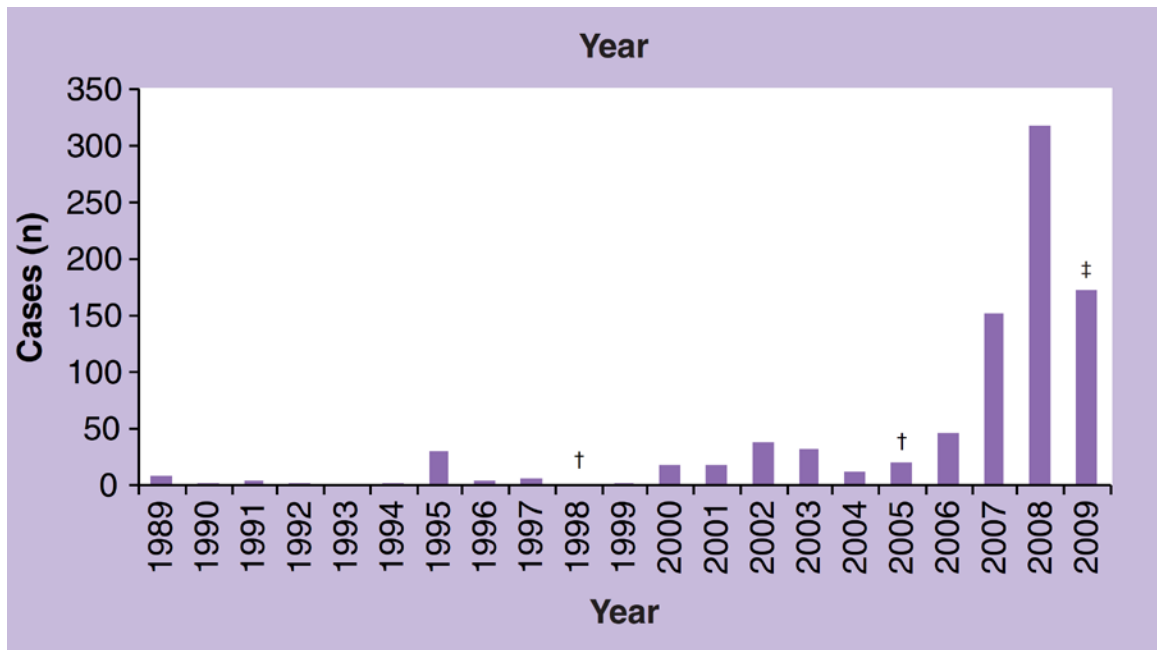
†The 215 cases officially reported during the 2005 outbreak in Sichuan Province and the 25 cases of 1998 outbreak in Jiangsu Province, both in China, were excluded from the analysis to accurately follow the evolution of *S. suis* human disease.

‡Only 11 months of 2009 were included.

Figure 1.1 Reports of individual human *S. suis* infections have recently increased.

Annual, confirmed, reported human cases of *S. suis* infection have increased over the past decade. This may be due, in part, to increased reporting rather than an actual increase in *S. suis* infection. Adapted from Future Microbiology (Gottschalk et al. 2010) with permission of Future Medicine Ltd.

Humans and pigs can be topically and systemically treated with penicillin or gentamicin with success, but *S. suis* isolates resistant to these antibiotics have emerged (Cantin et al. 1992, Varela et al. 2013, Gottschalk et al. 2010). Further, there is no vaccine for *S. suis* (Fittipaldi et al. 2012). As such, *S. suis* appears to be developing a more consistent presence in human populations and these infections may become more difficult to treat.

1.1.2   *Streptococcus pyogenes* - GrAS

1.1.2.1   Colonization

Streptococcus pyogenes is a group A beta hemolytic streptococci. Over 30% of the human population may be colonized with *Streptococcus pyogenes* in the upper respiratory tract – the only known site of benign colonization (Mandell et al. 2005). Colonized individuals are much less likely than severely sick persons to transmit illness (Mandell et al. 2005).

1.1.2.2   Pathogenesis

S. pyogenes annually infects over 750 million people (Carapetis et al. 2005), resulting in 25% mortality among the ~650,000 cases that progress to severe infection (Carapetis et al. 2005, Cunningham 2000, Bessen et al. 2011). This pathogen is responsible for a broad range of infections such as pharyngitis, impetigo, scarlet fever, erysipelas, cellulitis, toxic-shock syndrome, and necrotizing fasciitis; and it can lead to serious sequelae such as rheumatic fever, and acute glomerulonephritis (Swedo et al. 1997, Parker, Tomlinson, and Williams 1955, Bisno, Brito, and Collins 2003).

1.1.2.3 Mortality

The mortality rates can be very high for these infections, including 20% for necrotizing fasciitis, and 50% for toxic-shock syndrome (Bisno, Brito, and Collins 2003). Rheumatic fever, acute glomerulonephritis, and forms of obsessive-compulsive disorder are non-suppurative sequelae associated with an *S. pyogenes* infection (Swedo et al. 1997). Invasive streptococcal infections and rheumatic fever outbreaks have seen a rise worldwide since the 1980's (Kavey and Kaplan 1989).

1.1.3 *Staphylococcus aureus*

1.1.3.1 Pathogenesis

Of all the Gram-positive pathogens, *Staphylococcus aureus* has become the most difficult to treat. *S. aureus* is a Gram-positive facultative anaerobe that causes most *Staphylococcus* infections in man. Human anterior nares (nostrils) are typically the primary sites of *S. aureus* colonization, along with other moist openings on the body serving as additional sites for entry (White and Smith 1963, Kluytmans, van Belkum, and Verbrugh 1997, von Eiff et al. 2001, Wertheim et al. 2005a).

S. aureus is capable of producing severe, secondary infections in immunocompromised individuals, as well as causing disease in otherwise healthy people. Besides skin and soft tissue infections (SSTIs), *S. aureus* can cause sepsis, pneumonia, necrotizing fasciitis, pyomyositis, endocarditis, toxic shock syndrome, and scalded skin syndrome (White and Smith 1963, Wertheim et al. 2005b). These infections are usually treated with methicillin, mupirocin, or vancomycin. Unfortunately, many *S. aureus* strains, such as methicillin-resistant *S. aureus* (MRSA) and (less often) vancomycin-resistant *S. aureus* (VRSA), have acquired resistance to one or more antibiotics used as

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standard treatment (Howden et al. 2010), making them even more difficult to treat with available antimicrobials (Howden et al. 2010).

1.1.3.2   Transmission

Further exacerbating the problem, MRSA is readily transmitted between patients in hospitals (Coates, Bax, and Coates 2009). MRSA account for more than 50% of hospital isolates causing pneumonia and septicemia (Klein, Smith, and Laxminarayan 2007), particularly in intensive care units, resulting in 30-40% mortality (Tiemersma et al. 2004, Laupland, Ross, and Gregson 2008). It is the primary cause of lower respiratory tract infections, surgical site infections, and ~19,000 deaths/year in the US alone (Fischetti 2008, Klein, Smith, and Laxminarayan 2007).

While health-care-associated MRSA strains usually infect susceptible patients, community-associated MRSA (CA-MRSA) can infect healthy individuals (CDC 1999, Herold et al. 1998, Zetola et al. 2005, David and Daum 2010). CA-MRSA strains are often more virulent and are capable of causing more severe diseases in humans and animal models (Adem et al. 2005, Miller et al. 2005, Li et al. 2009, Voyich et al. 2005). Distinct strains of CA-MRSA are epidemic in Europe, North America, Oceania, and other regions (Herold et al. 1998, Vandenesch et al. 2003, Tristan et al. 2007). The MW2 strain (pulsed-field type USA400) is the prototypical CA-MRSA, having contributed to the incipient outbreak of CA-MRSA in the USA, which led to an epidemic (CDC 1999, Deleo et al. 2010). There is currently no vaccine for *S. aureus* (Middleton 2008).

1.1.4   Other Gram-positive pathogens

Zoonotic Gram-positive pathogens include: *Streptococcus equi* (strangles – an upper respiratory tract infection – in equines, e.g. horses); and *Streptococcus suis* (sepsis

and meningitis in pigs and humans). The pathogenic *S. suis* serotype 9 strain 7997 has been associated with increasing reports of zoonotic transmission from pigs to humans (Sriskandan and Slater 2006). Humans and pigs have been treated with penicillin or gentamicin, but *S. suis* isolates resistant to these antibiotics exist (Cantin et al. 1992). *S. suis* may develop a consistent presence in human populations in years to come.

There are many other Gram-positive human pathogens, including: *Streptococcus sanguinis* (dental *plaque* and caries); *S. sanguinis* (endocarditis); Group G *Streptococcus*; group E *Streptococcus*; and *S. pneumoniae* (pneumonia, otitis media, meningitis, bacteremia, sepsis, endocarditis, peritonitis, and cellulitis).

1.1.4.1 *Streptococcus agalactiae* – GBS

Another beta-hemolytic Gram-positive streptococcus, *Streptococcus agalactiae* (Group B streptococci – GBS) contains an antiphagocytic capsule as its primary virulence factor (Yeung and Mattingly 1984, Rubens et al. 1987). *S. agalactiae* can exist in the human gastrointestinal system, occasionally colonizing secondary sites like the vagina in over 33% of women (Boyer et al. 1983, Meyn, Krohn, and Hillier 2009). The colonizing *S. agalactiae* can infect a neonate during birth resulting in bacterial septicemia, making early-onset *S. agalactiae* the primary cause of death in newborns for over 4 decades (Lancefield and Hare 1935, Fry 1938, Hare and Colebrook 1934),(Zangwill, Schuchat, and Wenger 1992). The current standard of practice exposes the mother to antibiotics that further the likelihood of resistance.

1.1.4.2 *Listeria monocytogenes*

A recent Gram-positive pathogen outbreak involving *Listeria monocytogenes* killed 30 in the United States from July to December 2011 making it the most deadly

food-borne illness outbreak in the US since the 1970's (Baertlein 2011). Most individuals contract listeriosis after consumption of contaminated food, facing a mortality rate of 20-30%, even with antibiotic therapy (Schuppler and Loessner 2010, Hof, Szabo, and Becker 2007). *Listeria* survives well in food processing systems and the human gastrointestinal tract, readily adjusting to swift changes in pH, salinity, and temperature (Schuppler and Loessner 2010, Ramaswamy et al. 2007, Dieterich et al. 2006).

1.2   Antibiotics

1.2.1   Treatment

Many bacteria interact with molds, plants, or other bacteria. As a defense, these hosts have developed antibiotics – relatively small molecules that impede bacterial proliferation. Antibiotics may be bacteriostatic or bactericidal. A minimum inhibitory concentration (MIC) assay determines the bacteriostatic concentration of an antibiotic. A colony forming unit assay evaluates log fold killing to determine bactericidal efficacy (MBC).

Derivatives of molds and plants have been used to treat bacterial infections for millennia. The antiseptic properties of these antibiotic molecules began to be formally described and empirically evaluated throughout the late 19th century. Scientists began to focus on a small molecule excreted by mold that seemed to inhibit or kill bacteria. In 1923, Sir Alexander Fleming worked with a chemist to successfully purify penicillin. By 1945, in his Nobel Lecture, Fleming was already warning of antibiotic resistance.

Antibiotics can be used to treat a wide range of bacterial pathogens in humans. Nonetheless, antibiotics can present undesirable side-effects. An example of antibiotic-

associated illnesses is *Clostridium difficile*-associated enteritis ([Kuijper et al. 2006](#)). Further, antibiotics can be rendered ineffective when bacteria develop resistance.

1.2.2   Resistance

Today, there are hundreds of antibiotics available to treat numerous pathogens. Nonetheless, over-prescription of these drugs where they are not needed or beneficial is an issue. For example, viruses account for the vast majority of pharyngitis (sore throat) infections. Nonetheless, doctors prescribe antibiotics for sore throat nearly 60% of the time ([Barnett and Linder 2014](#)). While wiser use of antibiotics may decrease rates of resistance, antibiotic resistance existed before widespread use of antibiotics ([Lederberg and Lederberg 1952](#)). Unfortunately, the US Centers for Disease Control and Prevention did not begin tracking antibiotic resistance in a national report until 2013 ([CDC 2013](#)). Therefore, it is still difficult to evaluate just how severe antibiotic resistance has become and for how long.

Through mutation and horizontal gene transfer, bacteria are consistently acquiring resistance to leading antibiotics. In the case of *S. aureus*, there has been a consistent acquisition of resistance over time (Figure 1.2). Antibiotics act by inhibiting the synthesis of the bacterial cell wall, proteins, or nucleic acids. They may also alter the plasma membrane or metabolite activity to assert a lethal effect. Bacteria may be impervious to antibiotics due to innate, acquired, or adaptive resistance.
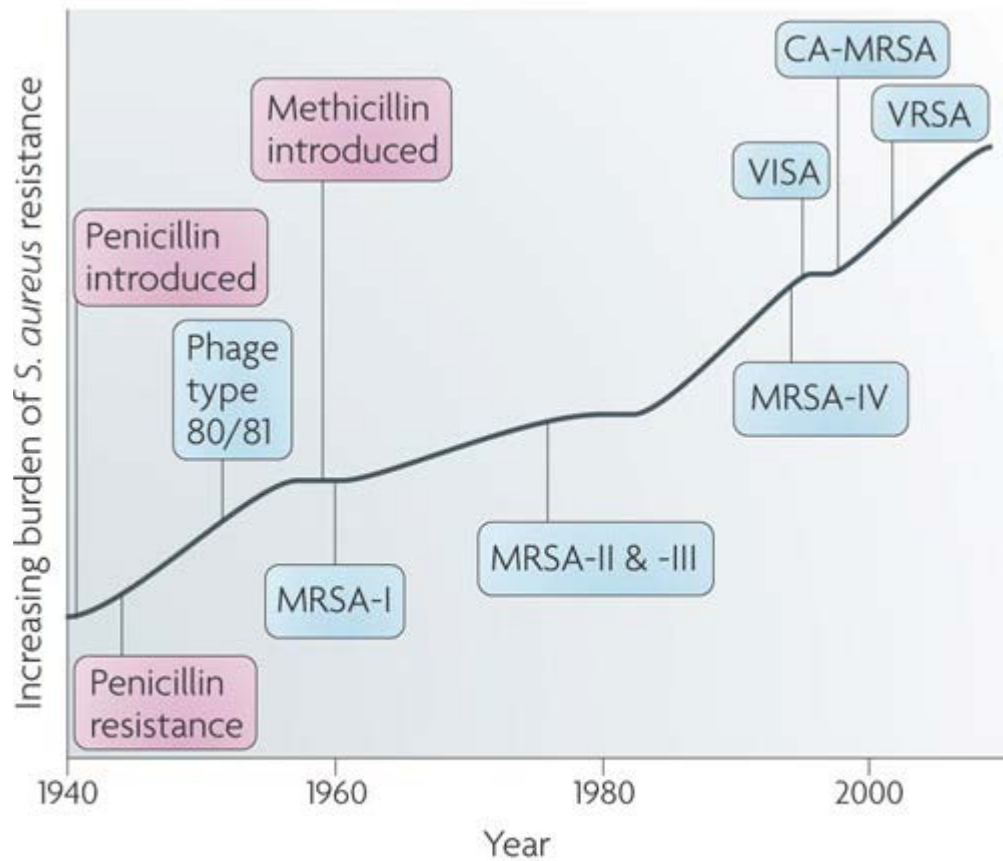
Figure 1.2 Periodic emergence of *S. aureus* antibiotic resistance.

For *S. aureus,* clinical resistance tends to emerge 5-10 years after the introduction of a novel antibiotic. There has been a steady decrease in the number of effective antibiotics to treat both community- and hospital-associated strains. *S. aureus* clone "phage type 80/81" was the most prevalent of the penicillin-resistant strains (which expressed a plasmid-encoded penicillinase and caused the first wave of resistance). Adapted from (Chambers and Deleo 2009) with permission from Nature Publishing Group.

Innate resistance refers to the inherent characteristics of a species of bacteria that enable it to resist the action of an antibiotic. For instance *Pseudomonas aeruginosa* and *Acinetobacter baumannii* have uniquely impervious outer membranes that decrease the influx of many antibiotics (Fernandez and Hancock 2012).

Acquired resistance occurs when bacteria incorporate new genetic sequences by acquiring new DNA (via plasmids, integrons, etc.) or mutations. If bacteria acquire genetic material encoding an antibiotic inhibitor, they will express that inhibitor, and thus be resistant.

Adaptive resistance occurs when bacteria change the expression of genes and/or proteins to withstand the presence of an antibiotic. For instance, up-regulating the expression of efflux pumps may keep intracellular concentrations of the antibiotic below bacteriostatic levels.

Antibiotic resistant bacteria could cause pandemics similar to those seen before the advent of antibiotic therapeutics. Compounding the problem, fewer systemic antibiotics are being approved by the US FDA (Figure 1.3). There are three main approaches to address the development of resistant bacteria: preventing antibiotic-resistant infections; tracking resistant bacteria with improved diagnostics and surveillance; and developing new antimicrobials. One new antimicrobial approach involves the oldest infectious agents discovered, bacteriophages, along with their enzymatic components – novel antimicrobial enzyme-based antibiotics termed "enzybiotics".
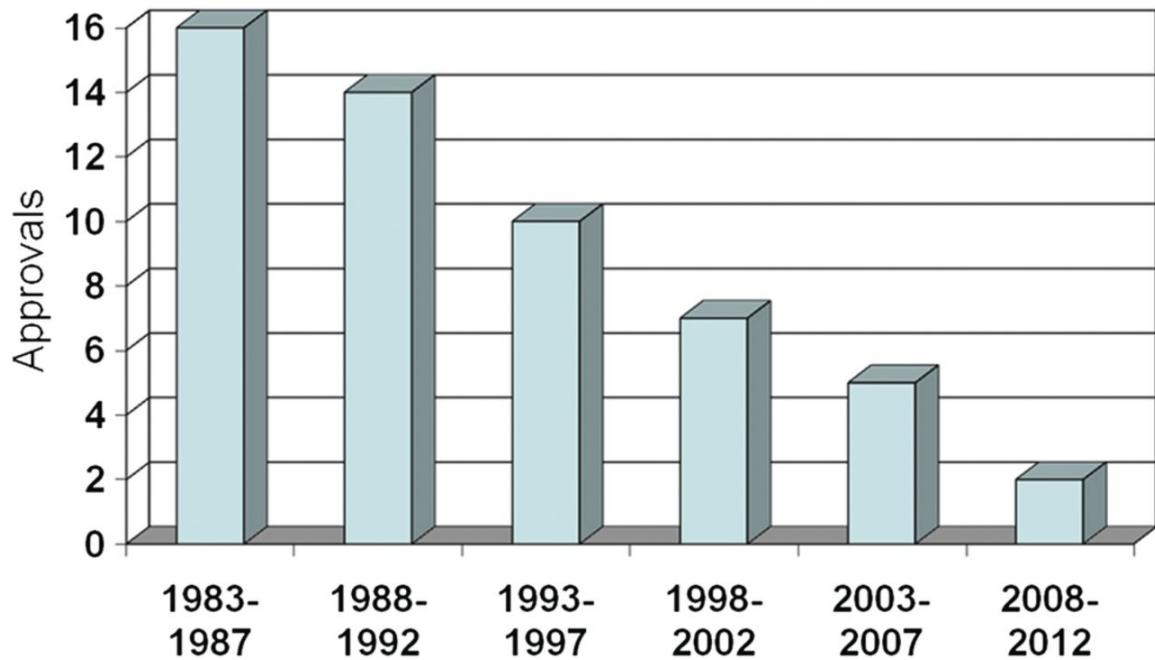
Figure 1.3 New systemic antibacterial agents approved by the US FDA.

There has been a steady decrease in the number of approved systemic antimicrobials over the past 5 years. This is partially due to a decreasing reservoir of antimicrobials, but economics and regulation have also affected recent output. Image taken from (Boucher et al. 2013) with permission from Oxford University Press.

1.3   Phage Lytic Enzymes

1.3.1   Phage

Bacteriophages (phages) are viruses that infect bacteria. They are also the most numerous genetic elements on Earth, numbering 10^{31}. Every 48 hours, half of the bacteria on Earth are destroyed by phages (Rohwer, Prangishvili, and Lindell 2009, Hendrix 2002). Having evolved over eons to infect and lyse bacteria, phages have been proposed as therapeutics ever since they were observed by Frederick Twort in 1915 and fully discovered by Félix d'Hérelle in 1917. Bacteria have evolved numerous defenses against viruses, exhibiting resistance to phages so rapidly that a cocktail of phages is required for treatment. Further, the cocktail must be changed during a treatment regimen. Nevertheless, the therapeutic effect of phages is found in their ability to lyse bacteria. This involves molecules to which bacteria are not able to easily evolve resistance.

1.3.1.1   Life cycles

As viruses, all phages are structured with nucleic acid packaged in protein. In order to replicate and assemble progeny, they must infect a host. Replication and the release of viral progeny can take place without killing the bacterial host (e.g. filamentous bacteriophages called inoviruses) (Waldor, Friedman, and Adhya 2005). Nonetheless, nearly all discovered viruses exhibit two life cycles - lysogenic and lytic. Phages have the potential to enter a particular life cycle upon initial infection based on the number of viral particles, the state of those particles, and the metabolic state within the host (Zeng et al. 2010).

1.3.1.1.1 Lysogenic cycle

In the lysogenic (i.e. temperate or benign) phase, the proviral nucleic acid remains in the host as a dormant episome or as a sequence integrated into the host's genomic DNA. Two important processes are required for lysogeny: integration into a specific location in the bacterial genome (Van Duyne 2005), and repression of lytic-pathway protein transcription (Waldor, Friedman, and Adhya 2005).

Prophage may replicate along with the host for many cycles (with copies of the prophage being passed on to host progeny) during the lysogenic phase. Reactivation of the phage occurs when it excises from the genome and lytic pathway proteins commence translation. Phages reactivate when their host is compromised due to DNA damage, environmental stress, or other host factors (Broudy, Pancholi, and Fischetti 2002). However, phages may spontaneously reactivate without stressors at a rate of $\sim 10^{-4}$ to 10^{-5} cells (Waldor, Friedman, and Adhya 2005).

If they remain integrated for a long period, prophage can be damaged or attenuated. If prophage lose the ability to reactivate, they may interminably persist as a part of the bacterium's prophage genome (Casjens 2003). Strains of bacteria will carry and express attenuated prophage genes if those genes prove advantageous. Although this is deleterious to the original phage, it represents another way that phages play a vital role in the evolution of bacteria.

1.3.1.1.2 Lytic cycle

The lytic (i.e. virulent) phase includes phage replication, viral assembly, and progeny release. In order to replicate, the phage takes over its host's transcriptional processes. After replication, the phage induces the host to transcribe proteins to package

the replicated phage nucleic acids. In most characterized pathways, after assembly, the phage induces lysis of the bacterial host, releasing phage progeny. Macroscopically, this appears as a clearing zone, or *plaque*, when phages are overlaid with susceptible bacteria on agar.

1.3.1.2 Role in nature

As discussed above phages play numerous, vital roles in the global microbiome. As the largest reservoir of genomic information, phages are responsible for most of the genetic exchange between living organisms. As previously alluded, phages encode many genes including virulence factors that can be advantageous to the host during viral lysogeny (Breitbart et al. 2004). Examples of such virulence factors are the *S. pyogenes* superantigens causing toxic shock, which are derived from prophage (Novick, Christie, and Penades 2010).

1.3.1.3 Shaping molecular biology

The ability of phages to transmit DNA has enabled experiments involving phages to guide the field of molecular biology from its inception as a discipline. The process of genetic mutation was elucidated by observing the development of phage-resistant *E. coli* (Luria and Delbruck 1943, Lederberg and Lederberg 1952). Further, in 1952, Hershey and Chase used T2 phage to confirm Avery's finding that DNA is the heritable genetic macromolecule for all life (Avery, Macleod, and McCarty 1944). Phage subcomponents currently play a daily role in molecular biology as ligases, kinases, and polymerases. Due to their narrow host-specificity, phages are currently used to identify and type many different bacterial isolates (Brown and Cherry 1955, Abshire, Brown, and Ezzell 2005).

1.3.1.4   Therapeutic application

Phages have been used to treat infections in humans since their discovery in 1917. Due to a variety of factors, biologists and clinicians had trouble optimizing phage therapy for widespread use. There were successful trials against pathogens in Eastern Europe, but the results did not gain traction in the West (Sulakvelidze, Alavidze, and Morris 2001). With the arrival of antibiotics in the 1940's, US research in therapeutic phages subsided.

The pervasive emergence of antibiotic-resistant bacteria forced a reevaluation of phage therapy application toward the end of the 20th century. Early work in the US included animal models of *E. coli* and *Pseudomonas aeruginosa* infections (Smith and Huggins 1982, 1983, Soothill 1994). Phages have now been tested through double-blinded phase II clinical trials against *P. aeruginosa* causing chronic otitis (Wright et al. 2009) with safety in all patients and efficacy in those treated. A single, 2.4 ng dose of 6 bacteriophages were able to improve the condition of 92% of the patients over 6 weeks, and led to the infection subsiding altogether in 25% of the patients (Wright et al. 2009). Phase I safety trials have also begun in the US (Rhoads et al. 2009, Bruttin and Brussow 2005, Merabishvili et al. 2009).

It has also been shown that the same benefits of phage therapy in humans translate to veterinary applications as well (Johnson et al. 2008). Finally, phage may also treat plant pathogens, which the phage already target and lyse in nature (Balogh et al. 2010). The US EPA has already approved phage cocktails to control Xanthomonas and Pseudomonas infections in tomatoes and pepper. A cocktail of phages has been approved by the US FDA to be included in packaged meat and cheese to prevent/control contamination by listeria (FDA Code of Federal Regulations 21CFR172.785).

Interestingly, phages are the only antimicrobial agent that amplifies itself during therapeutic delivery.

Phage specificity is also a limitation, as physicians rarely know the pathogen causing an infection before recommending treatment. Also, bacteria readily develop resistance to a single phage lineage, necessitating a cocktail for rapid bacterial treatment. The US FDA prefers to approve homogenous treatments rather than heterogeneous cocktails.

Bacteria have co-evolved with phages, building up multiple defenses against them. Alternatively, exogenous bacteriophage lysins mediate lysis in a pathway that differs from phages (as discussed below), so bacteria should not readily develop resistance. This has been confirmed in (Loeffler, Nelson, and Fischetti 2001, Schuch, Nelson, and Fischetti 2002, Pastagia et al. 2011).

1.3.2 Lysins

Alternative therapies must be developed to mitigate the sharp increase in antibiotic resistance among Gram-positive bacteria including *S. suis* and *S. aureus*. Novel antimicrobial sources include enzyme-based antibiotics ("enzybiotics") such as phage lytic enzymes (endolysins, or simply "lysins") (reviewed in (Fischetti 2010) and (O'Flaherty, Ross, and Coffey 2009). These peptidoglycan hydrolases (catalyzing a variety of specific bonds) are encoded by virtually all double-stranded DNA phages.

Bacteriophages encode lysins to hydrolyze the peptidoglycan bonds in the bacterial cell wall after phage progeny replicate inside the infected host bacterium (Wang, Smith, and Young 2000). Disruption of the cell wall leads to osmotic lysis of the bacteria and release of viral progeny (Fischetti 2008).

When applied exogenously, purified lysins are able to access and degrade the bonds in the cell wall of Gram-positive bacteria, because they lack the outer membrane found in Gram-negative bacteria (Fischetti 2008). Besides chemical agents, lysins kill bacteria more rapidly than any known biological compound (Nelson, Loomis, and Fischetti 2001, Loeffler, Nelson, and Fischetti 2001, Fischetti 2005). Lysins typically demonstrate high specificity, with lethal activity directed against the species that the lysin-encoding phage infects (Fischetti 2008, Nelson, Loomis, and Fischetti 2001, Daniel et al. 2010, Loeffler, Nelson, and Fischetti 2001, Cheng et al. 2005). Therefore, lysins should not perturb the host's normal flora as would broader-acting antibiotics (Fischetti 2008).

1.3.2.1   Discovery

It was nearly a century after their initial identification and extraction before lysins were tested *in vivo*. This delay is due primarily to the emergence of antibiotics and technological challenges. The identification, purification, and production of enough highly active lysin for *in vivo* trials took decades (Table 1.1).

Table 1.1 Timeline of lysin discovery

Discovery	Reference
Phage lytic agent first extracted	Felix d'Hérelle 1921
Phage virolysin differs from host lytic enzyme	(Ralston et al. 1955)
Isolated antigenically-distinct lytic enzymes	(Ralston and McIvor 1964)
First lysin amino acid sequence	(Tsugita and Inouye 1968)
Lysin purified by ion exchange chromatography	(Doughty and Mann 1967)
First lysin nucleotide sequence cloned	(Owen et al. 1983)
First therapeutic use of a lysin in an animal model	(Nelson, Loomis, and Fischetti 2001)

1.3.2.2 Structure

Gram-positive lysins usually have two domains, a catalytic N-terminal domain, and a C-terminal binding domain. This structural orientation is discussed in more detail in section 2.2.1 below. Some lysins have multiple units made of one subcomponent, such as PlyC. Others, usually staphylococcal lysins, will have multiple catalytic domains linked to one binding domain (Navarre et al. 1999). In most characterized lysins, the binding domain confers specificity, making the first interaction with cell wall components. This enables the catalytic domain to hydrolyze bonds in the cell wall. The full structure for Cpl-1 free and bound to choline indicate that the binding domain attaching to choline induces a conformational change orienting the catalytic domain for efficient peptidoglycan hydrolysis (Hermoso et al. 2003).

1.3.2.3 Activity

Today, lysins can be cloned from viral prophage sequences within bacterial genomes, recombinantly expressed in *Escherichia coli*, purified, and used for treatment (Beres and Musser 2007, Nelson, Loomis, and Fischetti 2001, Fischetti 2010). With advances in sequencing, one can screen the published bacterial sequences and identify candidate lysin sequences found in lysogens through homology analysis with known lysin sequences with standard algorithms (e.g. Blast, Pfam). Furthermore, complete phage genomes are being published more frequently than ever totaling 600+ in the NCBI database as of early 2014. As described by (Schmitz 2011), bioinformatic analyses can utilize metadata from public databases for lysin gene localization, identification, mechanism, phylogeny, and catalytic residues.

When applied exogenously, these enzymes are able to access the peptidoglycan layer in the Gram-positive cell envelope (due to the lack of an outer membrane) and produce the same lytic effect as when they are expressed inside the bacterial cell for phage progeny release (Figure 1.4) (Fischetti 2008).

Figure 1.4 Lytic cycle versus lysin treatment.

(A) Left: Phage-mediated host lysis enables the virions (in red) to escape after replication. Right: Recombinant lysins are able to exogenously lyse Gram-positive bacteria just as lysins expressed within their host. (B) Electron micrograph (EM) of cells after phage-mediated lysis. (C) EM of cells after lysin-mediated lysis. Image modified from (Fischetti, Nelson, and Schuch 2006) with permission from Nature Publishing Group.

A <u>Lytic cycle</u>　　　<u>Lysin treatment</u>

Infection

Replication
and assembly

Lysin
addition

Lysis

B

200 nm
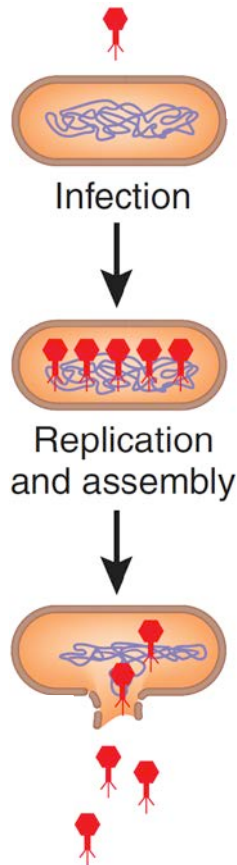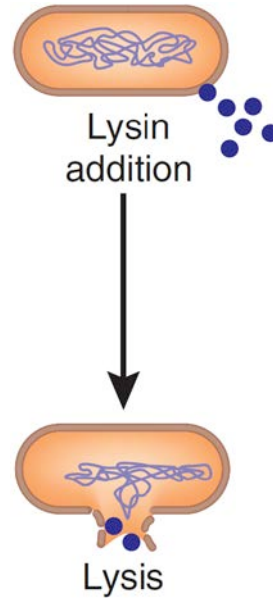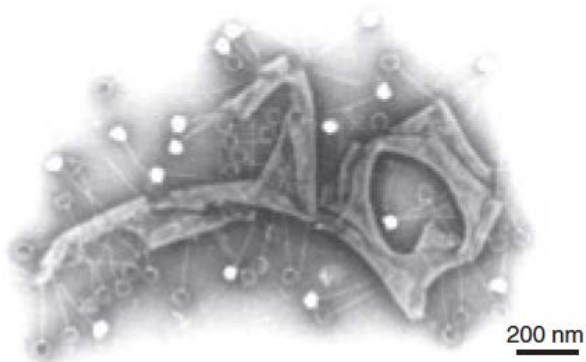
C
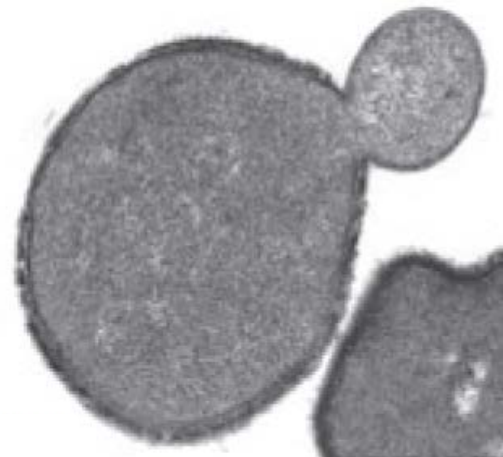
Unlike antibiotics, an important feature of phage lysins is their rapid, lethal effect on bacteria (Nelson, Loomis, and Fischetti 2001, Loeffler, Nelson, and Fischetti 2001, Fischetti 2005). Lysins are notable for the potency and specificity they demonstrate – generally, toward the species that the encoding phage infects or closely related organisms (Daniel et al. 2010, Nelson, Loomis, and Fischetti 2001, Loeffler, Nelson, and Fischetti 2001, Cheng et al. 2005, Fischetti 2008). As such, they presumably exert a less-dramatic affect on the normal nonpathogenic flora in the host than broad spectrum antibiotics (Fischetti 2008). Lysins kill bacteria quicker than any known non-chemical agents. While no lysin has yet been FDA-approved, these enzymes could be used to treat antibiotic-resistant bacteria. To date, no lysin has shown broad *in vivo* activity against multiple species of bacterial pathogens.

1.3.2.4   Current Lysins

Numerous lysins have been tested *in vitro*. They've shown activity against *Bacillus anthracis*, *Bacillus cereus*, *Listeria monocytogenes*, *Staphyloccous aureus*, *Streptococcus agalactiae*, *Streptococcus pneumonia*, *Streptococcus pyogenes*, *Streptoccus suis*, and *Streptococcus uberis* among other species (Garcia et al. 1987, Navarre et al. 1999, Gaeng et al. 2000, Loeffler, Nelson, and Fischetti 2001, Nelson, Loomis, and Fischetti 2001, Schuch, Nelson, and Fischetti 2002, Pritchard et al. 2004, Cheng et al. 2005, O'Flaherty et al. 2005, Korndorfer et al. 2006, Pritchard et al. 2007, Porter et al. 2007, Celia, Nelson, and Kerr 2008, Daniel et al. 2010, Schmelcher, Tchang, and Loessner 2011, Donovan et al. 2006, Donovan, Lardeo, and Foster-Frey 2006, Mao et al. 2013, Rodriguez et al. 2011). Most are N-acetylmuramoyl-L-alanine amidases and/or endopeptidases.

Of these, many have been tested *in vivo* including PlyC, PlyGBS, PlyPH, Cpl-1, PAL, MV-L, CHAP_K, LysGH15, ClyS, P-27/HP, and a few chimeras (Nelson, Loomis, and Fischetti 2001, Cheng et al. 2005, Schuch, Nelson, and Fischetti 2002, Yoong et al. 2006, Loeffler, Nelson, and Fischetti 2001, Loeffler and Fischetti 2003, Rashel et al. 2007, Fenton et al. 2011, Daniel et al. 2010, Gu et al. 2011, Gupta and Prasad 2011, Schmelcher et al. 2012). Most lysins have been tested in murine decolonization models. Further, Cpl-1 (a pneumococcal lysin) has been shown to be synergistic with another pneumococcal lysin Pal *in vivo* (Loeffler and Fischetti 2003, Jado et al. 2003). Likewise, ClyS, a staphylococcal lysin displays synergy with the antibiotic oxacillin (Daniel et al. 2010).

It has been shown that lysins delivered to animals systemically remain active for approximately 20 minutes (Loeffler, Djurkovic, and Fischetti 2003). Delivering a foreign protein to the bloodstream of animals will elicit an immunogenic effect. Nonetheless, rabbit hyperimmune serum raised against Cpl-1 does not inhibit Cpl-1 lytic activity (Loeffler, Djurkovic, and Fischetti 2003). The activity of Cpl-1 does not decrease as it remains active within the highly immune serum. This was also seen in experiments using a staphylococcal-specific lysin (Rashel et al. 2007). Loessner (Loessner et al. 2002) found that the binding domain of a listeria-specific phage enzyme has the nanomolar substrate affinity of an IgG molecule. This may partially explain why the action of the enzyme is not inhibited even in highly immune serum.

1.3.2.4.1 LySMP – *S. suis* phage enzyme

Two phages (Ss1 and SMP) infecting *S. suis* have been previously isolated. The first isolated from *S. suis* was Ss1, a siphoviral prophage induced from the genome of

serotype 2 strain 89-999 (Harel et al. 2003). An Ss1 lysin has not been identified, but the lysin of another *S. suis* phage has been developed. Ma and Lu isolated a lytic phage (SMP) after sequencing the 36 kb genome of *S. suis* retrieved from nasal swabs of healthy pigs (Ma and Lu 2008). SMP displayed narrow specificity, targeting just 2/24 *S. suis* serotype 2 strains. From SMP, Ma and Lu cloned and recombinantly expressed the SMP lysin (LySMP), which contains five cysteine residues thought to form intramolecular disulfide bridges. LySMP displayed *in vitro* bacteriolytic activity against many *S. suis* serotypes. Unfortunately, the recombinant LySMP only folds properly after the addition of reducing agents, which may limit its potential for *in vivo* trials (Wang, Sun, and Lu 2009). Since then, it has been tested against biofilms *in vitro*, but not *in vivo* (Meng et al. 2011).

Of the currently reported *S. suis* lysins, none have been shown to have activity against more than 3 serotypes of *S. suis*, nor have they been shown to decolonize animals *in vivo* (Harel et al. 2003, Ma and Lu 2008, Wang, Sun, and Lu 2009, Meng et al. 2011). Recently, our lab discovered a phage lytic enzyme from an *S. suis* prophage with broad activity against various pathogenic Gram-positives, which was named PlySs2 (**P**hage **ly**sin from *S. suis* **2**) (Gilmer et al. 2013). In this thesis, we characterize the activity of PlySs2 against *S. suis* and test this lysin's ability to decolonize *S. suis* from murine nasal passages. Further, we show that this enzyme protected mice from a mixed bacteremic infection of methicillin-resistant *Staphylococcus aureus* (MRSA) and *Streptococcus pyogenes*, neither of which were found to develop resistance to PlySs2 *in vitro* (Gilmer et al. 2013).

1.3.2.4.2 ClyS – staphylococcal phage enzyme

Several lysins have also been developed against MRSA ([Daniel et al. 2010](#), [O'Flaherty et al. 2005](#), [Rashel et al. 2007](#)). A staphylococcal-specific chimeric lysin, ClyS was previously developed in our lab from the bacteriophages Twort and Φ NM3 lysins ([Daniel et al. 2010](#)). ClyS displays activity against *S. aureus*, *MSSA*, *MRSA*, *S. epidermidis*, *S. simulans*, and *S. sciuri*. It is very specific to staphylococci, as it displays no activity against species of streptococci or bacilli. *In vivo*, it has been used to remove MRSA from C57BL/6J mice nasally colonized with ~5×10^9 CFU by ~2 logs. It has also been shown to protect mice from septicemia-induced death alone and synergistically with oxacillin.

1.3.2.4.3 PlyC – streptococcal phage lysin

A lytic agent from C_1 phage ([Krause 1957](#)), PlyC is one of the most effective lysins discovered. PlyC displays lytic activity against groups A, C, and E streptococci, but registers essentially no lytic activity against other bacteria. Just 10 ng is able to sterilize ~10^7 live *S. pyogenes* ([Nelson, Loomis, and Fischetti 2001](#)). Further, it has been shown to protect mice inoculated with 10^7 CFU *S. pyogenes* from colonization ([Nelson, Loomis, and Fischetti 2001](#)). This lysin also differs from all other published lysins in its 114 kD size – much larger than the 25-40 kD size of most phage lysins. Eight PlyCB (~8 kD) subunits form a nonamer with a single, distinct PlyCA (~50 kD) gene product to form the active PlyC molecule ([Nelson et al. 2006](#)).

To date, however, no lysin has shown high lytic activity against multiple species of different bacterial pathogens. While developing a lysin with activity against the zoonotic pathogen *Streptococcus suis*, we discovered that the enzyme PlySs2 has activity

against a wide range of Gram-positive pathogens, and *in vivo* efficacy against *S. suis*, MRSA and *S. pyogenes*.

1.4   Peptidoglycan

Bacterial cell walls provide shape, size, and defense. All of the nutrients that come into a bacterial cell traverse the plasma membrane, so an amenable surface area to volume ratio is essential. If the cell is too large, there is not enough surface area for the exchange of nutrients to maintain metabolic equilibrium inside the cell. If the cell is too small, there is too much surface area without enough volume for intracellular processes. The role of the cell wall in maintaining cell shape also affects surface area to volume ratio. A circular shape favors volume, and an elongated shape favors surface area. Bacteria are often hypotonic to their surroundings. This creates up to 10-25 atm of pressure, which would easily destroy the fluid plasma membrane if the cell were not encased by a wall. Finally, the cell wall acts as a physical barrier protecting the cell from physical and chemical stress.

Bacteria are divided into two, large, taxonomic categories according to their cell wall layers. Gram-negative bacteria have a plasma membrane, peptidoglycan layer, cell envelope and possibly a capsule. Gram-positive bacteria lack an outer envelope, so their thicker peptidoglycan is exposed to the outer milieu (Figure 1.5).
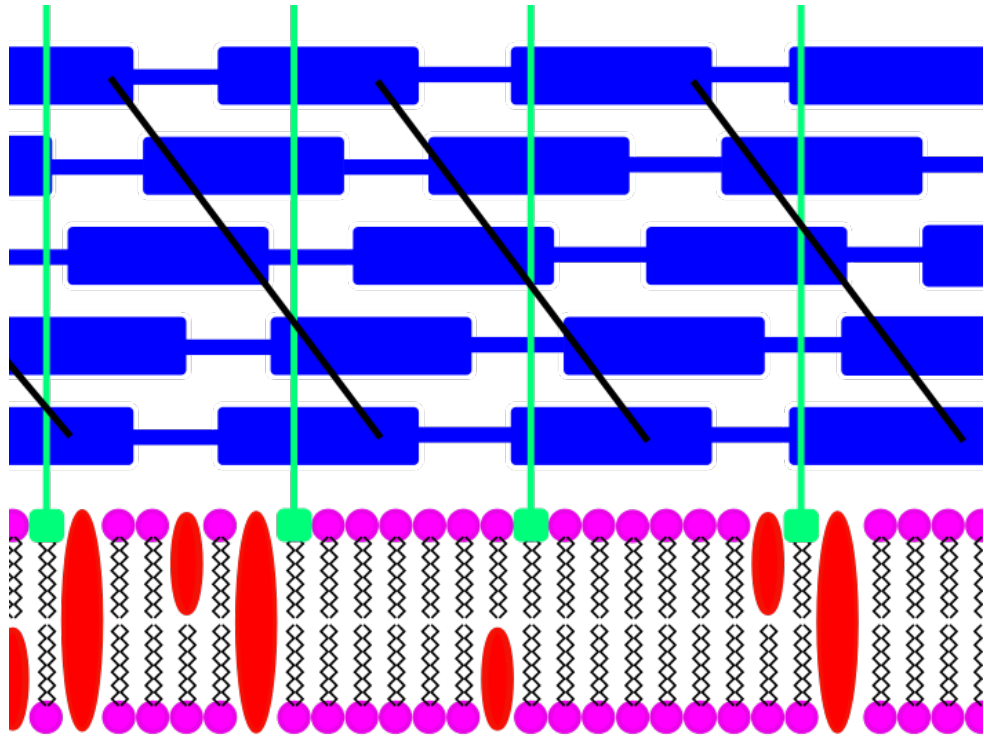
Figure 1.5 Gram-positive cell wall cross-section diagram.

From the extracellular space to the cytoplasm, the Gram-positive cell wall includes layers of peptidoglycan (blue) covering the plasma membrane composed of phospholipids (heads in pink, tails in black spirals). This organization contrasts with the Gram-negative cell wall, which has a thinner layer of peptidoglycan and a cell envelop that shields the peptidoglycan from the extracellular space. Image modified and used with permission from Wikimedia Commons.

The strongest (most rigid) structural feature of the bacterial cell wall is made of peptidoglycan. This macromolecule is composed of a polymeric carbohydrate backbone of repeating N-Acetylglucosamine (GlcNAc) N-Acetylmuramic acid (MurNAc) disaccharides (Vollmer, Blanot, and de Pedro 2008). Penta-/tetra-peptide stems connect to MurNAc on the backbone (Figure 1.6A). These stems can form cross-links or cross-bridges adding structural stability to the peptidoglycan cell wall (Figure 1.6B). The backbone is highly conserved among most bacteria, but the cross-bridge can widely vary even within species.

Figure 1.6 Peptidoglycan molecular composition.

(A) The *E. coli* peptidoglycan structure repeats through the cell wall. The yellow highlight inscribes the monomer, a disaccharide tetrapeptide. The red lettering highlights a cross-linked peptide. (B) Comparison between *E. coli* (left) and *S. aureus* (right) peptidoglycan dimers linked by their stems. From top to bottom, there is a disaccharide backbone, tetrapeptide stem, cross-link (for *E. coli*) or cross-bridge (pentaglycine for *S. aureus*), tetrapeptide stem, and disaccharide backbone. Images modified from (Vollmer, Blanot, and de Pedro 2008) with permission from publisher John Wiley and Sons.

A

GlcNAc-MurNAc
|
L-Ala
|
D-Glu
|
meso-A₂pm
|
D-Ala

B

32
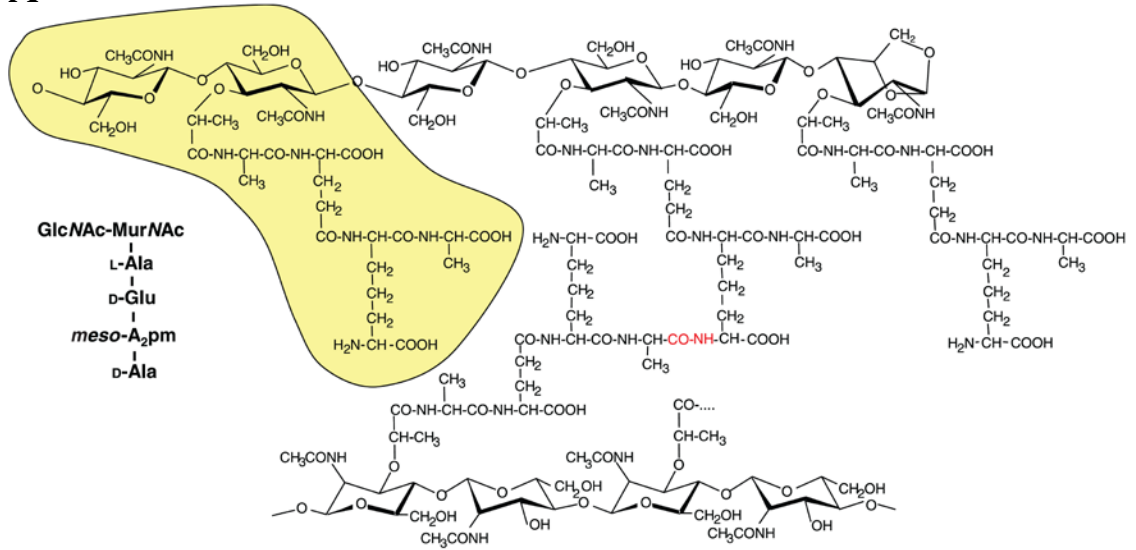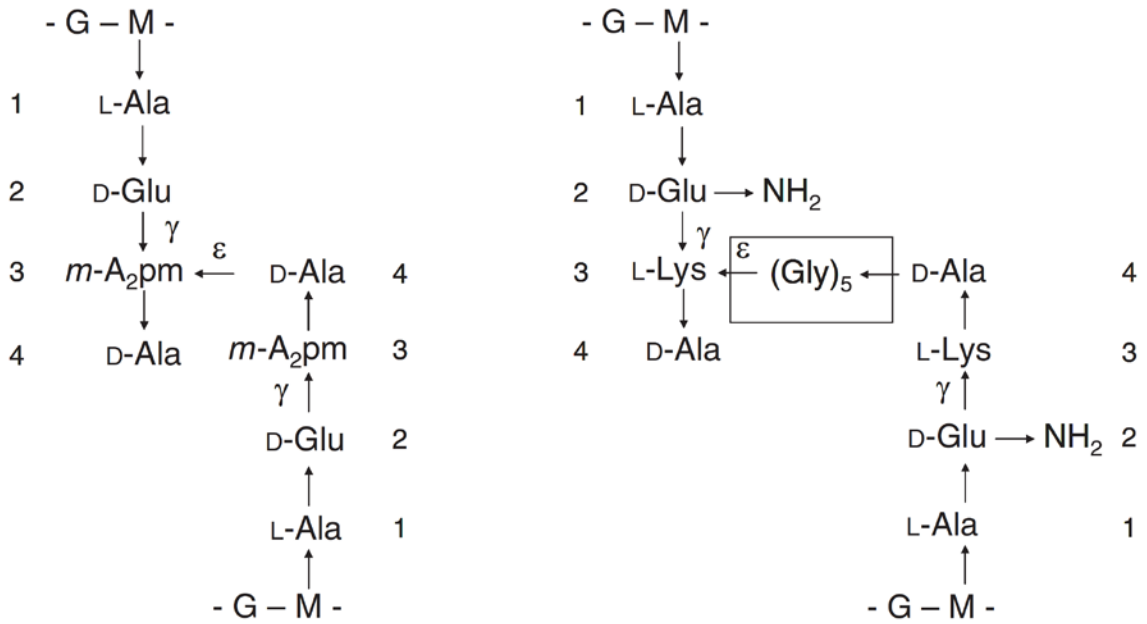
Lysins can catalyze any of the bonds in Figure 1.6. Endo-**β**-N-acetylglucosaminidases and N-acetylmuramidases catalyze bonds between the carbohydrate subunits. Endopeptidases hydrolyze stem or cross-bridge peptides, and N-acetylmuramoyl-L-alanine amidases cut the bond joining the disaccharide to the peptide stem ((Loessner 2005, Young 1992, Fischetti 2010)).

1.5 AIMS

For most of the 20th century, antibiotics effectively treated bacterial infections. With the emergence of widespread antibiotic resistance, bacteriophages provide an alternative antimicrobial. Rather than using the entire phage, lysins provide a simpler, more effective treatment.

Our first objective was to find a novel lysin with activity against *S. suis*. In characterizing this lysin *in vitro*, we discovered its efficacy against a variety of disparate species including *S. aureus, S. pyogenes*, and other Gram-positive pathogens. We biochemically characterized the lysin over ranges of temperature, salt, and pH. We also tested this lysin to ensure that resistance would not readily emerge.

Given its utility, we proceeded to develop novel *in vivo* models to expand lysin applications for decolonization, with antibiotics, treatment of bacteremia, against two infections, and with two lysins against two infections. To lysin technology, we hope to contribute a novel lysin candidate and novel *in vivo* models in light of emerging antibiotic resistance.

2 CHAPTER 2 – LYSIN CHARACTERIZATION

2.1 MATERIALS AND METHODS

2.1.1 Discovery

2.1.1.1 Genomic sequence analysis

There are over 11 completed *S. suis* genomes in the NCBI database. Dr. Jonathan Schmitz analyzed the published sequences of *S. suis* strains to locate potential phage lysins within prophage regions. The theoretical translation of each open reading frame (ORF) was evaluated with BlastP and Pfam, revealing a single candidate of interest. Through sequence analysis and functional screening, we confirmed a new phage lysin from *S. suis* (termed PlySs2).

2.1.1.2 Cloning

The candidate lysin gene (*PlySs2* from *S. suis* strain 89/1591) was PCR-cloned from genomic DNA with the following primers: AAT<u>GCTAGC</u>CTGATACACAGTTAG AGACC (forward) and CCT<u>AAGCTT</u>CTTTTCACAAATCATAATCCCCAG (reverse). The underlined nucleotides represent engineered restriction sites (NheI and HindIII), which were cut with the corresponding enzymes (NEB, Ipswich, MA) to clone *PlySs2* into the pBAD24 expression plasmid (pBAD24_*PlySs2*) encoding ampicillin-selection and arabinose-induction. The pBAD24_*PlySs2* vector was transformed into *E. coli* TOP10 cells (Invitrogen).

2.1.1.3 Candidate assay

The aforementioned *E. coli* clone was grown as a patch on LB-agar supplemented with 0.2% arabinose, permeabilized by a 10-minute exposure to chloroform vapor, and overlaid with soft-agar containing heat-killed *S. suis* bacteria. A streptococcal clearing

34

zone around the *E. coli* patch confirmed active recombinant expression of PlySs2 ([Wang, Sun, and Lu 2009](#)).

2.1.2   Expression

For PlySs2 expression and purification, the above clone was propagated in LB broth (37°C, 220 rpm aeration) with 100 μg/ml ampicillin. Recombinant expression was induced at OD_{600} ~0.8 by addition of arabinose (0.2%, final concentration). Following overnight incubation, the cells were pelleted at 10,722 rcf for 20 mins and resuspended in 15 mM phosphate buffer (PB), pH 8.0 (buffer A) supplemented with protease inhibitor cocktail tablets (Roche). Cells were lysed with an EmulsiFlex C-5 homogenizer. The homogenate was centrifuged at 1,723 rcf for 20 mins. After debris removal via ultracentrifugation (35,000× G, 1 hr), the supernatant was adjusted to pH 7.4 with the addition of ~4 volumes of buffer A.

2.1.3   Purification

The sample was passed through a HiTrap Fast Flow diethylaminoethyl (DEAE) anion-exchange column (General Electric) equilibrated with 15 mM PB, pH 7.4. The flow-through (which contained the desired PlySs2) was subjected to ammonium sulphate precipitation at 225 g/l (40% saturation). The precipitated protein was resolubilized in 40 ml of 15 mM PB, pH 6.7 (buffer B) for every liter of initial *E. coli* culture to prevent precipitation. This solution was dialyzed extensively against buffer B with 12-14 kD dialysis tubing (Spectrum Laboratories) to remove any remaining ammonium sulphate. Finally, the dialysate was passed through a HiTrap Fast Flow carboxymethyl (CM) cation-exchange column (General Electric). The CM column was washed in buffer B + 17 mM NaCl, which resulted in an elution peak containing the purified PlySs2.

The presence of PlySs2 was confirmed by lytic activity (clearing zones on agar plates containing embedded, autoclaved *S. suis*) and verified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis with Coomassie stain. All fractions containing PlySs2 were pooled and stored at -80°C. For *in vivo* tests requiring higher concentrations of 20 mg/ml, purified PlySs2 was dialyzed (into 15 mM NaCl, 5 mM PB, pH 7.4), frozen to -80°C, lyophilized overnight, and resuspended in approximately one tenth of the initial volume before lyophilization. A bicinchoninic acid (BCA) assay (Sigma) was used to determine the protein concentration.

2.1.4   Characterization

2.1.4.1   Optimization

The optimal biochemical conditions for PlySs2 enzymatic activity were screened using spectrophotometric analysis. Log-phase *S. suis* strain 7997 were adjusted with buffer to an OD_{600} of 0.8 to 1.0 in 96-well microtiter plates (Falcon). PlySs2, at 32 μg/ml, or control vehicle was added to each sample well. At room temperature, a Spectramax Plus 384 (Molecular Devices) took spectrophotometric readings (at $\lambda = 600$ nm, i.e., OD_{600}) of each well every minute over an hour. The degree of *S. suis* turbidity reduction (OD_{600}) in the test wells indicated the amount of lysin activity. The pH-dependence of the enzyme was first addressed using two buffer sets with overlapping pH ranges, citrate/phosphate (4.6 – 8.0) and bis-Tris propane (7.0 – 9.7). Concentrations of NaCl, ethylenediaminetetraacetic acid (EDTA), and dithiothreitol (DTT) were also varied.

2.1.4.2   Stability

The thermal stability of PlySs2 was studied by pre-exposing the enzyme to temperature conditions for defined scales of time: various high temperatures for 30

minutes, 37°C for hours, 4°C for days, and -80°C for months. The activity of each aliquot against *S. suis* 7997 was determined spectrophotometrically as described above. PlySs2 activity was also tested after consecutive freeze-thaw cycles between -80°C and room temperature.

2.2 RESULTS

2.2.1 Identification

PlySs2 was identified in a prophage region of an *S. suis* serotype 2 strain 89/1591 (the ORF was originally annotated in GenBank as *SH3-type 5 domain protein*, ZP_03625529 (Lucas 2004, Schmitz, Schuch, and Fischetti 2010, Schmitz 2011)). The putative *PlySs2* lysin had the greatest homology of only 35% identity over 53% coverage with an E-value of $<10^{-7}$ – this was to an *S. suis* surface antigen. *E. coli* transformed with pBAD24_PlySs2 (a plasmid for PlySs2 expression) were grown on *S. suis* overlay plates. The formation of clearing zones, or *plaques*, around these *E. coli* colonies confirmed successful cloning and soluble expression.

According to a National Center for Biotechnology Information Basic Local Alignment Search Tool (BLAST) search, PlySs2 likely encodes an N-terminal CHAP catalytic domain (cysteine-histidine amidohydrolase/peptidase, PF05257) and a C-terminal SH3-type 5 binding domain (PF08460) (Figure 2.1). By primary sequence, the PlySs2 CHAP domain is divergent from other database CHAP domains (Figure 2.2, (Schmitz 2011), data not shown).

37

A
```
MTTVNEALNN VRAQVGSGVS VGNGECYALA SWYERMISPD ATVGLGAGVG WVSGAIGDTI
SAKNIGSSYN WQANGWTVST SGPFKAGQIV TLGATPGNPY GHVVIVEAVD GDRLTILEQN
YGGKRYPVRN YYSAASYRQQ VVHYITPPGT VAQSAPNLAG SRSYRETGTM TVTVDALNVR
RAPNTSGEIV AVYKRGESFD YDTVIIDVNG YVWVSYIGGS GKRNYVATGA TKDGKRFGNA
WGTFK
```

B
CHAP   **SH3-5**
1   245
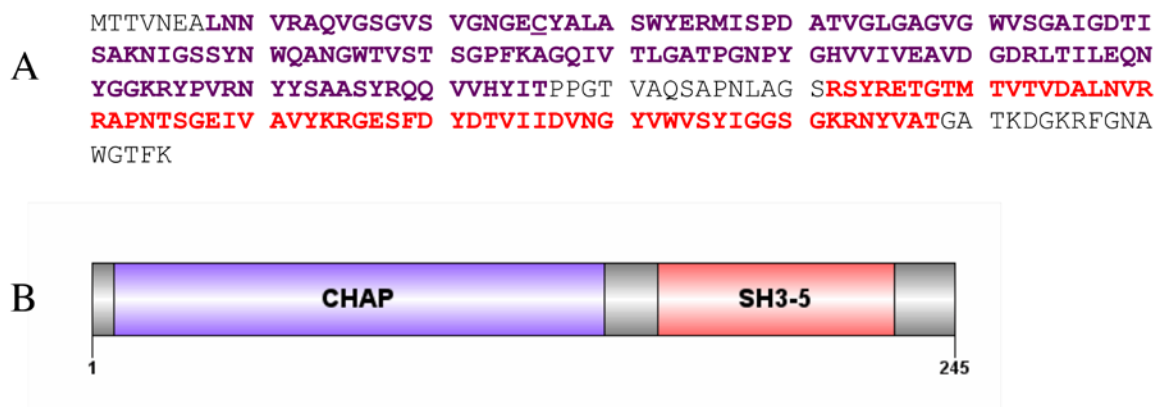
Figure 2.1 PlySs2 amino acid sequence and structure.

With a molecular mass of ~26 kD, PlySs2 contains an amine-terminal catalytic domain and a carboxy-terminal binding domain. (A) PlySs2 amino acid sequence. (B) The catalytic domain corresponds to residues 8-146. The binding domain spans residues 162-228.

38
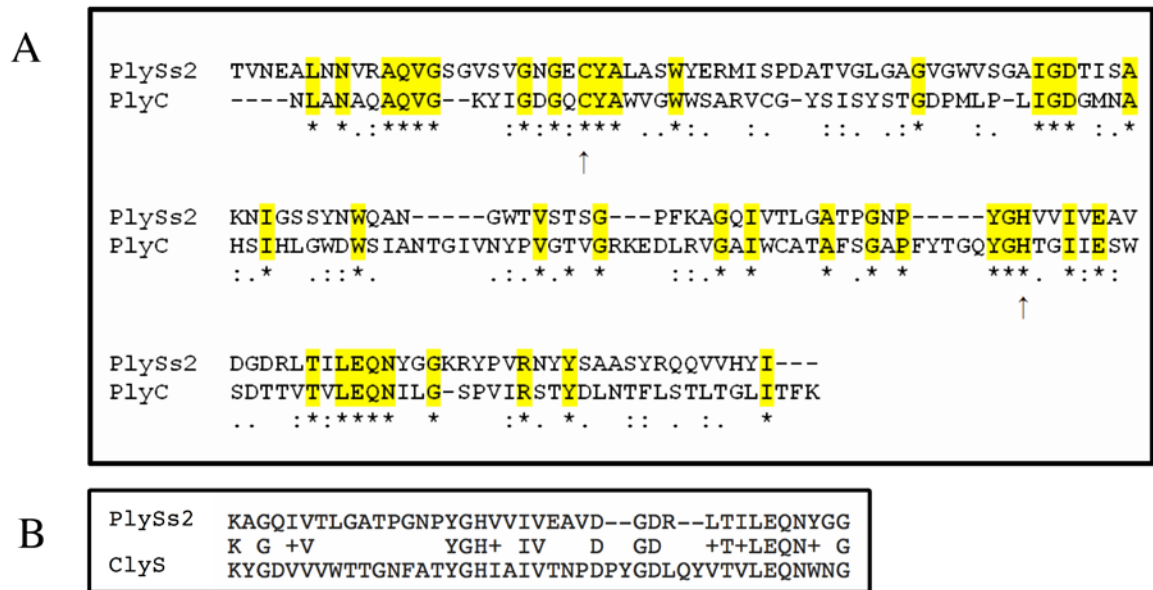
Figure 2.2 PlySs2 enzymatic domain alignment.

(A) The CHAP domains of the streptococcal lysins PlySs2 and PlyC (subunit A, GenBank no. AAP42310) are aligned here. Amino-acid identities are indicated with underlying asterisks and highlighting. The positions of the presumptive catalytic residues (cysteine and histidine, for which the domain is named) are indicated with arrows. (B) The aligned catalytic domains of the staphylococcal lysins PlySs2 and ClyS. Adopted with permission from (Schmitz 2011).

2.2.2 Purification

The aforementioned preparation led to a highly pure lysin preparation in just two chromatographic steps. With a predicted pI of 9.01, PlySs2 flowed directly through a DEAE column at pH 7.4 (Figure 2.3, lane 4) leaving the bulk of the contaminant proteins on the DEAE column (compare Figure 2.3, lanes 3 and 4). Following an ammonium sulfate-precipitation step, PlySs2 eluted cleanly both in the shoulder of the flow through peak of a CM column and in the 17 mM NaCl wash (see methods for details), being purified from proteins that rapidly flowed through the CM resin. The preparation yielded ~60 mg of protein per liter of *E. coli* culture at ~1.5 mg/ml with >99% purity (Figure 2.3, lane 6). The yield increased to ~150 mg per liter of *E. coli* culture at ~2.0 mg/ml with >90% purity when the CM column step was omitted (Figure 2.3, lane 5). All experiments were performed with this preparation. Concentrating PlySs2 via lyophilization and reconstitution generated a soluble solution of PlySs2 at ~20 mg/ml, which retained the concentration-adjusted activity of the lower concentration starting material.
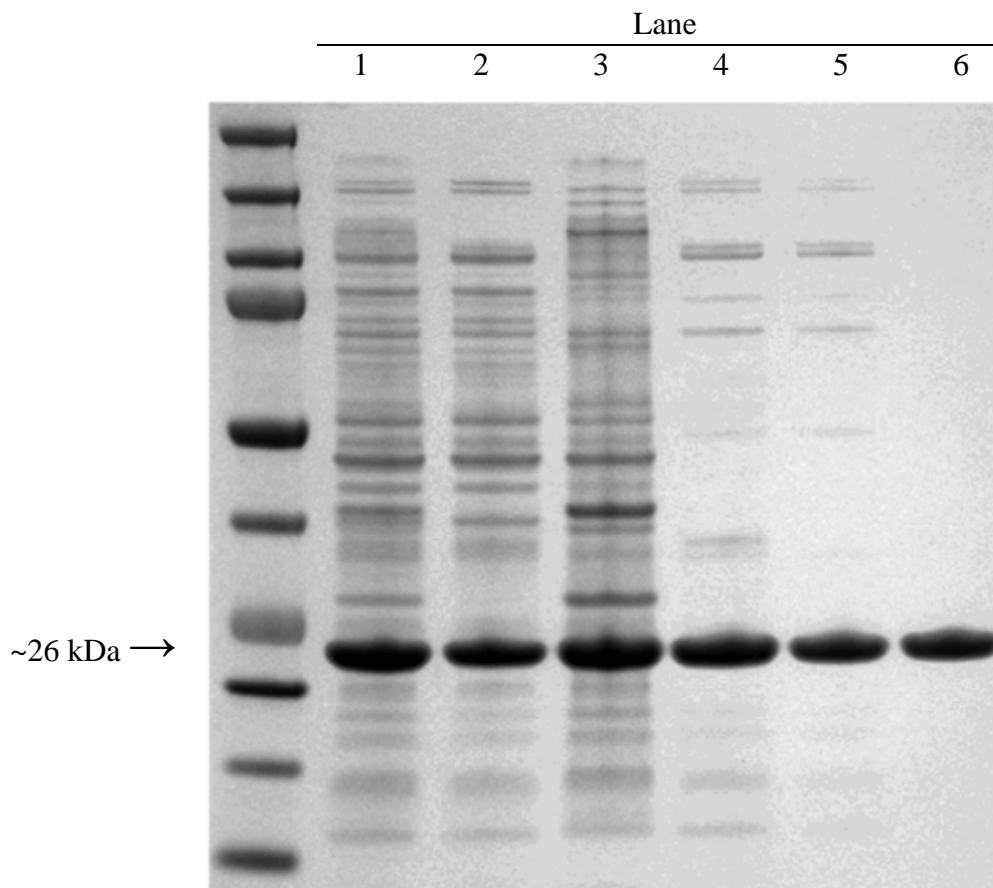
Figure 2.3 PlySs2 corresponded to bands at ~26 kDa on SDS-PAGE.

All PlySs2 purification samples were run on 4-12% Bis-Tris gels at 200 V for ~40 mins and stained with Coomassie. Lane 1: Whole cell lysate from *E. coli*. Lane 2: Supernatant from lysed *E. coli*. Lane 3: Pellet from lysed *E. coli*. Lane 4: The DEAE column flow through containing PlySs2. Lane 5: Resuspended pellet from 40% ammonium sulphate precipitation. Lane 6: A single band at ~26 kDa indicating the purity of PlySs2 after the CM column. Note: PlySs2 has a predicted molecular mass of ~26 kDa.

2.2.3   Characterization

2.2.3.1   Optimization

The optimal biochemical conditions for PlySs2 activity were determined by evaluating PlySs2 activity against log-phase pathogenic *S. suis* strain 7997 under different conditions. The *S. suis* cells were prepared for spectrophotometric analysis. The activity was determined by the degree of *S. suis* strain 7997 turbidity reduction (OD_{600}) following the addition of PlySs2 at 32 μg/ml. PlySs2 activity was tested through a range of pH values to determine its optimum physiological buffering conditions. The lysin was most active in citrate/phosphate buffer at pH 8.0 (Figure 2.4A) and in bis-Tris propane buffer at pH 9.7 (Figure 2.4B). PlySs2 could be optimally active at higher pH levels, but those levels would not be physiologically relevant. In the acidic range, there was strong activity to pH 6.0. Unlike certain other lysins, salt did not augment PlySs2 activity (Figure 2.5). Conversely, DTT did not inhibit PlySs2 function (Figure 2.6). Treatment with >4 μM EDTA had an inhibitory effect on PlySs2-induced lysis of *S. suis* (Figure 2.7).

Figure 2.4 PlySs2 was found to have the most acute activity in basic pH levels.

To test the optimal pH for PlySs2 activity, 32 μg/ml PlySs2 was mixed with log-phase *S. suis* 7997 suspended to a final OD_{600} of 1.0 in phosphate/citrate buffer (A) or bis-tris propane (B) at various pH levels. In controls (-), ddH_2O replaced PlySs2. Spectrophotometric readings were taken at OD_{600} every minute for an hour.
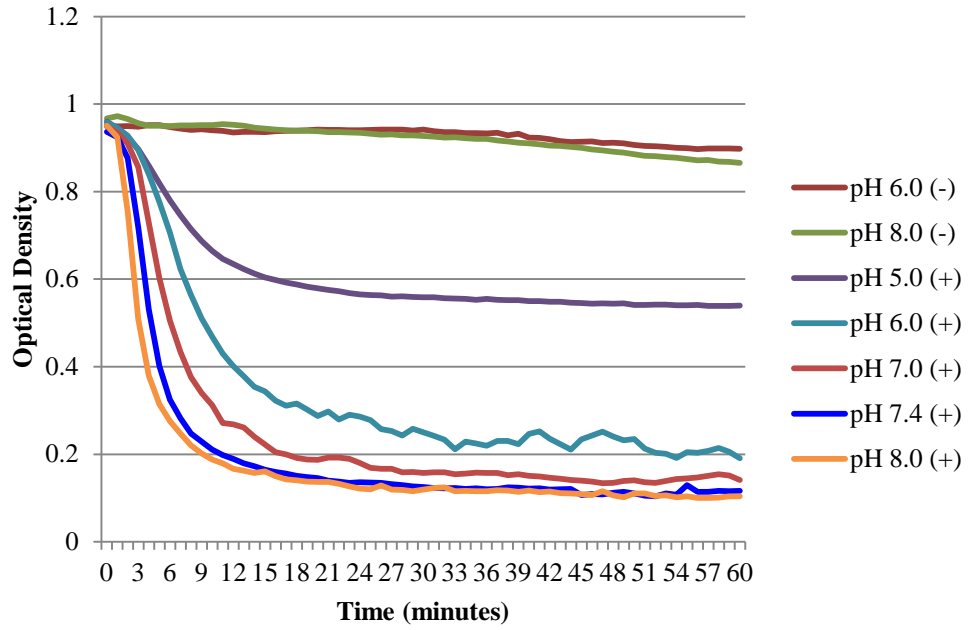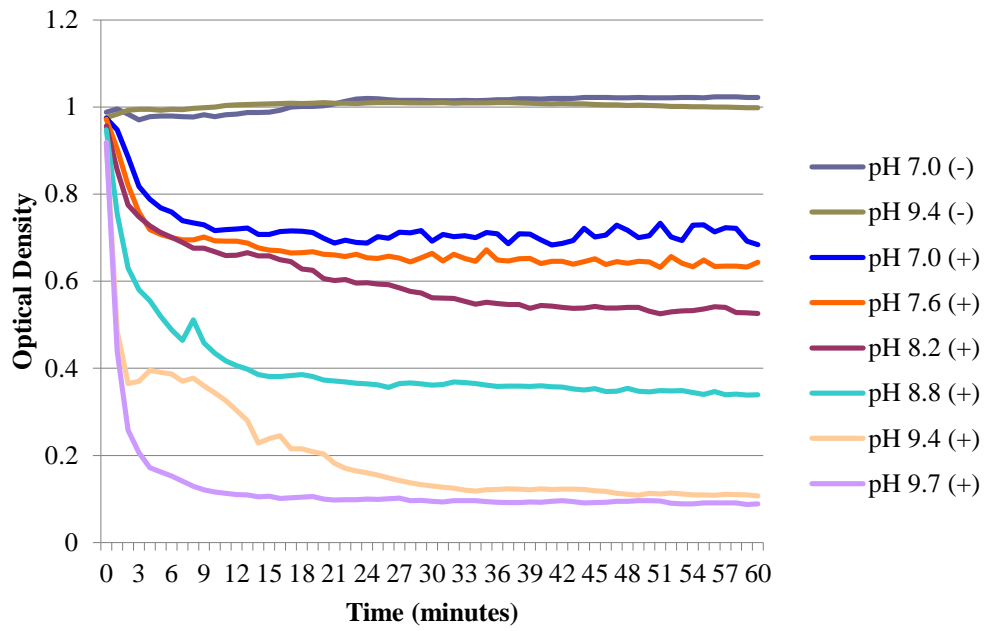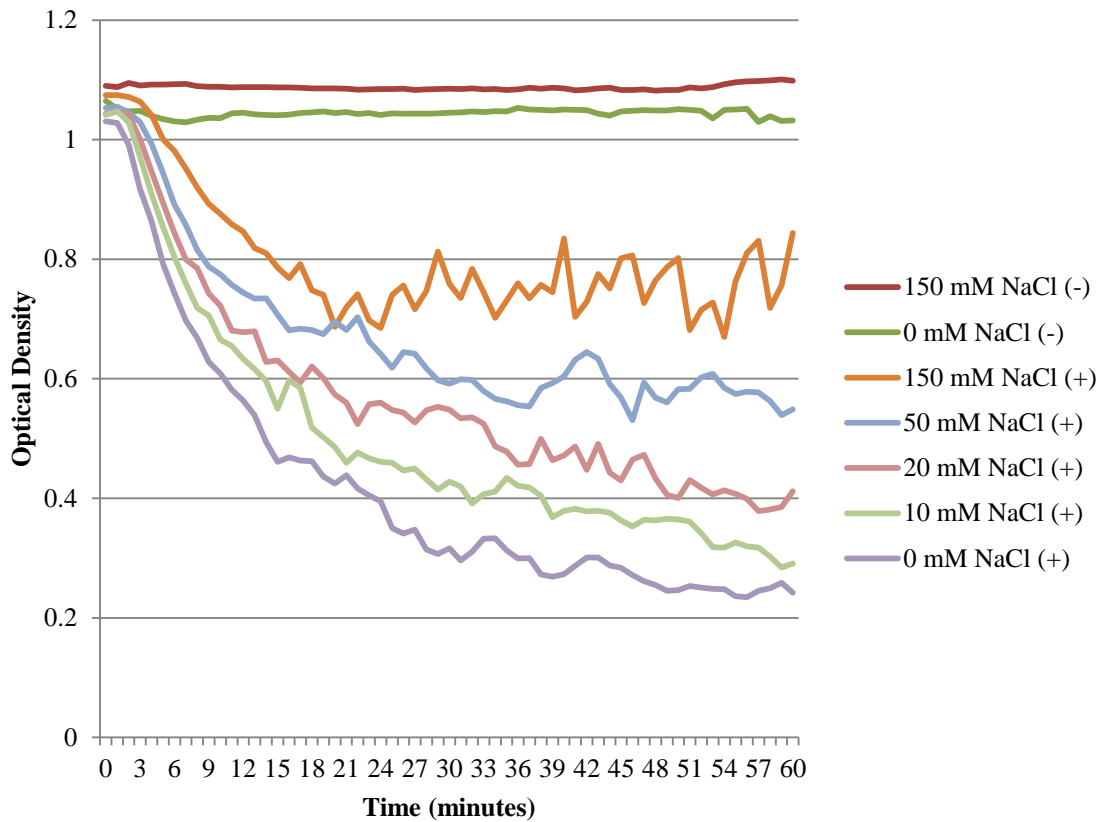
Figure 2.5 NaCl does not augment PlySs2 activity.

Log-phase *S. suis* 7997 were centrifuged and resuspended in PB to OD_{600} ~1.0. To test the optimal salinity for PlySs2 activity, 32 μg/ml PlySs2 was mixed with *S. suis* strain 7997 in phosphate buffer at different NaCl concentrations. PlySs2 is most active in the absence of NaCl. In controls (-), PB replaced PlySs2. Spectrophotometric readings were taken at OD_{600} every minute for an hour.
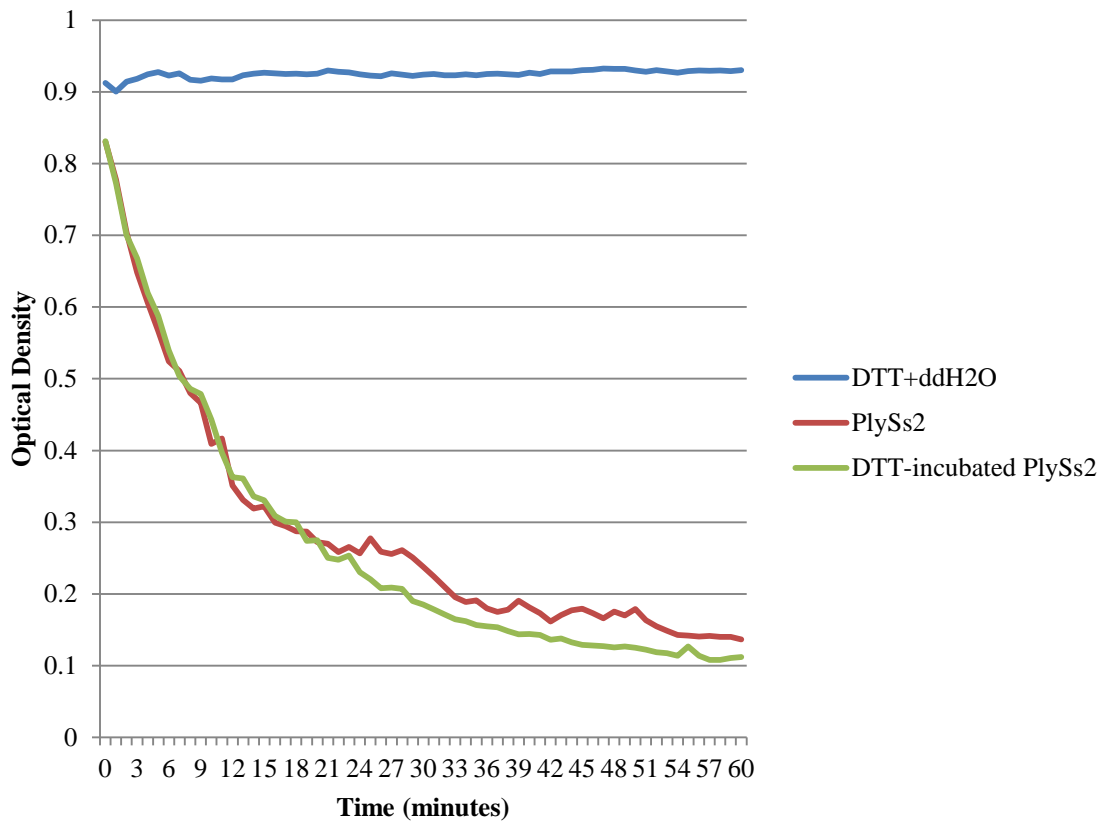
Figure 2.6 Dithiothreitol (DTT) does not inhibit PlySs2 activity.

Log-phase *S. suis* 7997 were centrifuged and resuspended in PB to OD_{600} ~1.0. PlySs2 was incubated in 5 mM DTT at room temperature for 60 minutes. Incubated or unincubated PlySs2 was added at 32 μg/ml. In controls (-), PB replaced PlySs2. Spectrophotometric readings were taken at OD_{600} every minute for an hour.
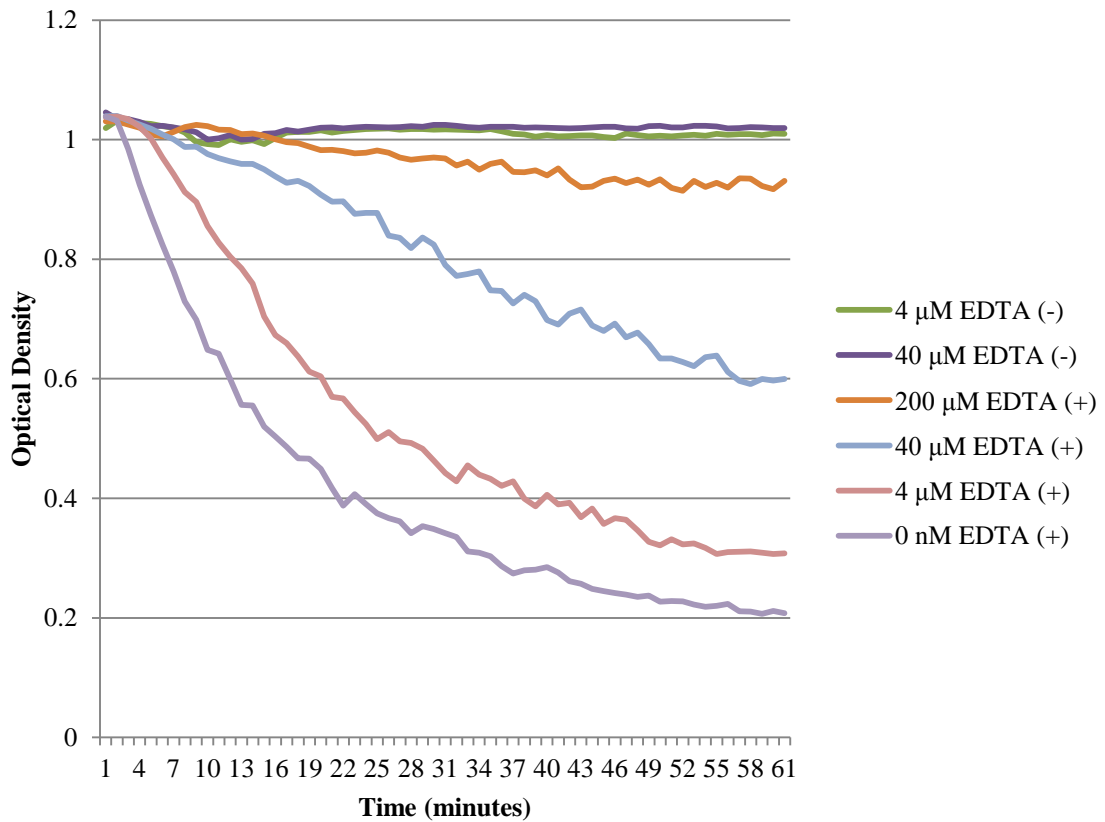
Figure 2.7 Minimal ion depletion from EDTA inhibits PlySs2 activity.

Log-phase *S. suis* 7997 were centrifuged and resuspended in PB to OD_{600} ~1.0. PlySs2 was added at 32 µg/ml to cells with various concentrations of EDTA. In controls (-), PB replaced PlySs2. Spectrophotometric readings were taken at OD_{600} every minute for an hour.

2.2.3.2   Stability

The activity of each PlySs2 aliquot incubated at different temperatures was tested (at 32 μg/ml) against *S. suis* strain 7997 and spectrophotometrically evaluated, as outlined above. When PlySs2 was incubated at different temperatures from 22°C – 85°C for 30 min, its activity was principally unaffected until 60°C (Figure 2.8A). Activity was retained during incubation at 37°C for 24 hours, with activity starting to diminish after 48 hours (Figure 2.8, B). There was no observable decrease in activity after 15 days at 4°C (Figure 2.8, C) or storage at -80°C for >7 months (Figure 2.8, D). The lysin also endured 10 consecutive freeze-thaws cycles (-80°C to room temperature) without any observable effects (Figure 2.9).

Figure 2.8 PlySs2 is stable under a variety of conditions.

PlySs2 was incubated: (A) for 30 minutes at various temperatures, then cooled; (B) at 37°C for different increments of time; (C) at 4°C for different numbers of days; (D) at -80°C for different numbers of months. For each test, 32 μg/ml PlySs2 was added to *S. suis* 7997 cells at an OD_{600} of ~1.0. Spectrophotometric readings were taken at OD_{600} every minute for an hour.

A

B
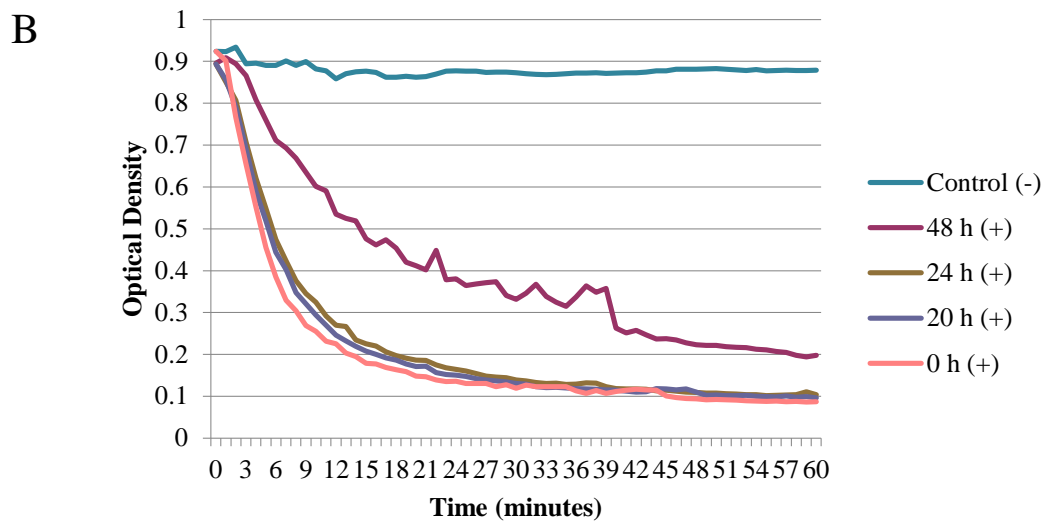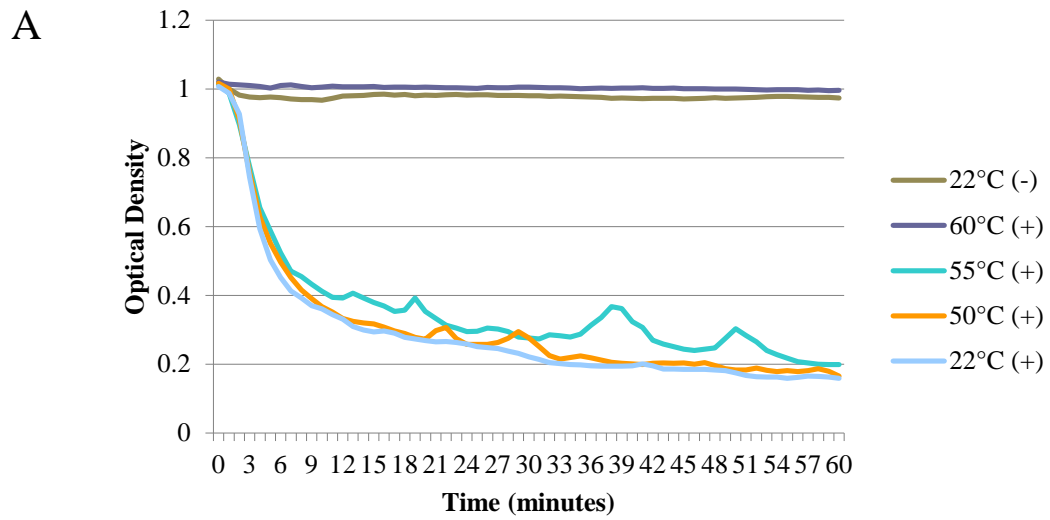
C
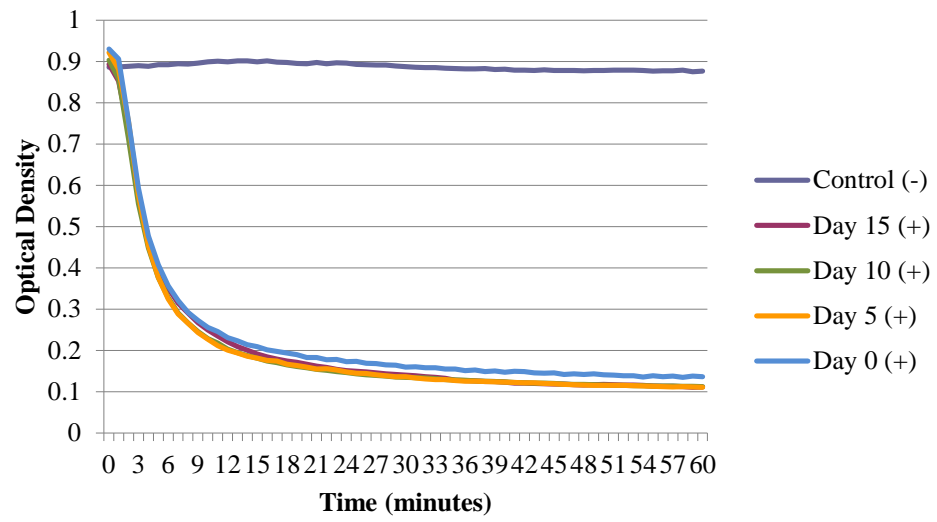
D
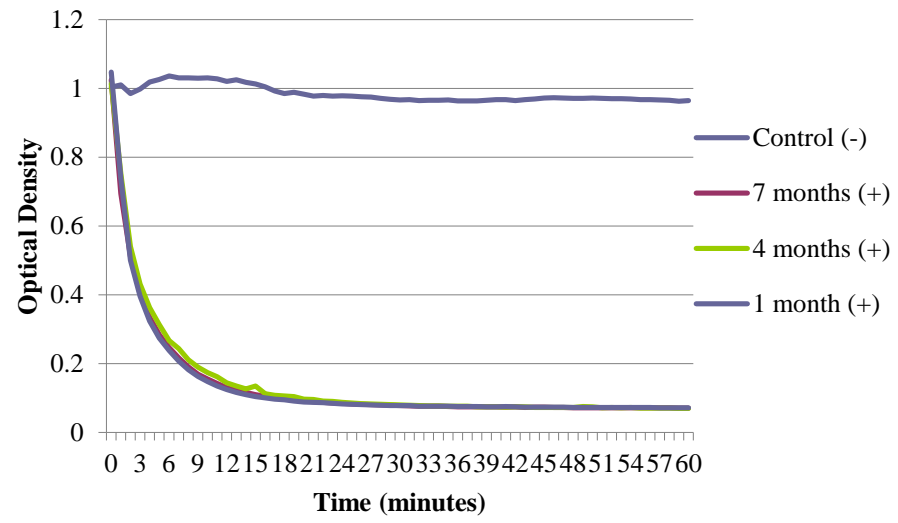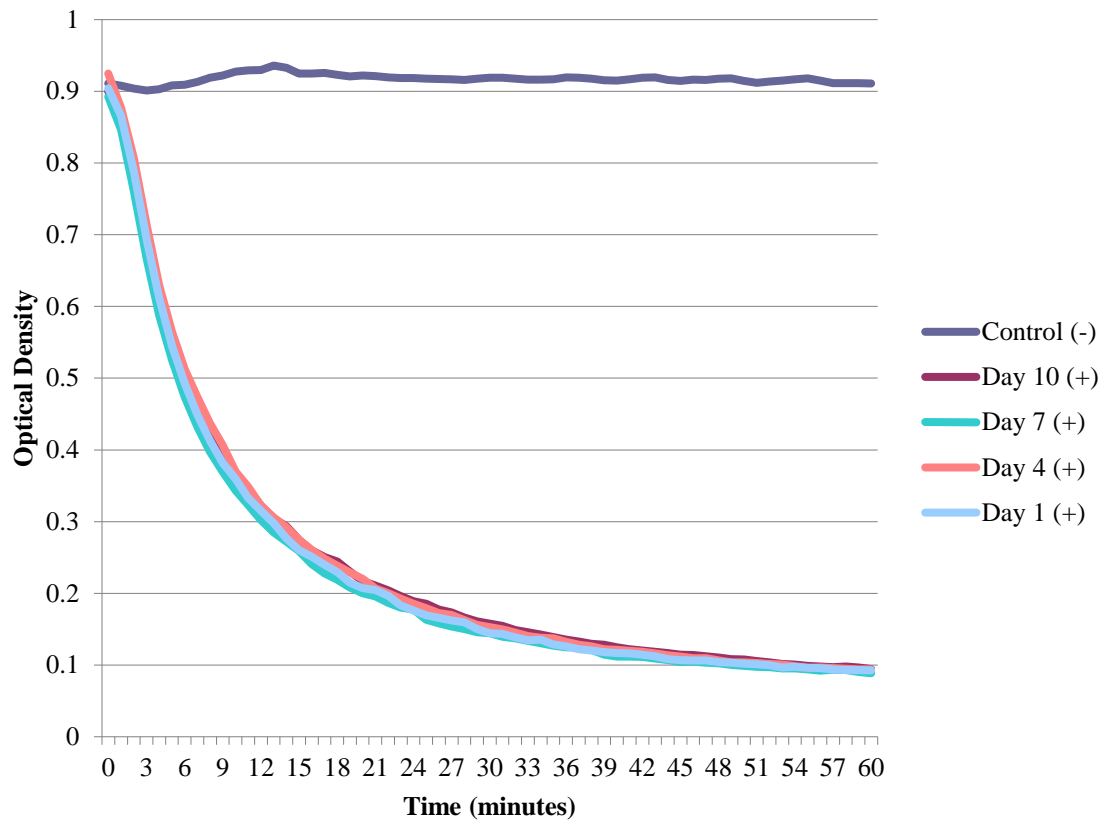
Figure 2.9 PlySs2 is stable and active after ten, consecutive freeze-thaws.

Log-phase *S. suis* 7997 were centrifuged and resuspended in PB to OD_{600} ~1.0. PlySs2 was taken from -80°C to room temperature and back to -80°C on subsequent days from Day 1 to Day 10. A sample from each day was added to cells at 32 µg/ml. In controls (-), PB replaced PlySs2. Spectrophotometric readings were taken at OD_{600} every minute for an hour.

2.3   ACKNOWLEDGEMENTS

3   CHAPTER 3 – *STREPTOCOCCUS SUIS* SUSCEPTIBILITY

3.1   MATERIALS AND METHODS

3.1.1   Bacterial strains

All *S. suis* strains were stored at -80°C (Table 3.1). The strains were grown in brain heart infusion (BHI) broth at 37°C for all tests. Luria Bertani (LB) broth was used to cultivate *Escherichia coli*. All media was from Becton Dickinson, and Company (Sparks, MD).

Table 3.1 Strains used in the *S. suis* susceptibility study

Species	Serotype	Strain	ATCC	Source[a]
Escherichia coli		TOP10		1
Streptococcus suis	1			2
Streptococcus suis	1	6112		2
Streptococcus suis	1	6388		2
Streptococcus suis	2			2
Streptococcus suis	2	10		2
Streptococcus suis	2	S735	43765	2
Streptococcus suis	3			2
Streptococcus suis	4			2
Streptococcus suis	5			2
Streptococcus suis	6			2
Streptococcus suis	7			2
Streptococcus suis	7	7197		2
Streptococcus suis	7	7711		2
Streptococcus suis	8			2
Streptococcus suis	9			2
Streptococcus suis	9	7997		2
Streptococcus suis	9	8067		2
Streptococcus suis	10			2

Species	Serotype	Strain	ATCC	Source[a]
Streptococcus suis	11			2
Streptococcus suis	12			2
Streptococcus suis	13			2
Streptococcus suis	14			2

[a] 1, The Rockefeller University Collection; 2, Jaap A. Wagenaar, Utrecht University, Utrecht, The Netherlands.

3.1.2 Lytic activity against *S. suis*

Strains of bacteria were grown to log-phase at 37°C and brought to an optical density (OD_{600}) of ~1.0 with 15 mM phosphate buffer (PB), pH 8.0 (buffer A) as measured in 96-well microtiter plates (Falcon). From these bacterial stocks, 245 µl were added to each well of a 96-well microtiter plate. In triplicate for every strain, each well received 5 µl of PlySs2 at 1.6 µg/µl (8 µg, thus a final concentration of 32 µg/ml). [In preliminary experiments, 32 µg/ml provided the best resolution in determining PlySs2 activity.] Corresponding triplicate wells received 5 µl of 15 mM PB, pH 6.7 (buffer B) control vehicle. *S. suis* 7997 served as a positive control for each trial. As detailed above, spectrophotometric readings were taken of each well every minute over an hour. The degree of turbidity reduction indicated lysin activity.

3.1.3 Bactericidal assay

Log-phase bacteria were centrifuged and washed 1× in buffer A and adjusted to an OD_{600} of 0.1 (= 0.5 McFarland, ~10^8 CFU/ml) in buffer A with a SmartSpec™ Plus Spectrophotometer (Bio-Rad). A 100 µl aliquot of the cell suspension was distributed in 96-well polypropylene microtiter plate (Costar). PlySs2, at 64 µg/ml, or buffer B control vehicle was added to wells in triplicate for each strain. Plates were sealed and shaken at 37°C every minute for 1 hour. At this time, 10-fold serial dilutions of each well were plated on BHI agar and incubated at 37°C. Resultant colonies were enumerated after 18 h. The bactericidal effect was calculated as the difference between vehicle-treated and PlySs2-treated CFU for each strain.

3.1.4 MIC assay

The protocol of Wiegand, et al. (Wiegand, Hilpert, and Hancock 2008) was used with adjustments to determine minimum inhibitory concentrations (MICs). Briefly, each strain was grown in BHI and adjusted to ~5×10^5 cells/ml in BHI and distributed into 4 wells of a 96-well round bottom polystyrene microtiter plate. In each of two wells corresponding to each strain, either sterile-filtered lysin or control vehicle was added (Wiegand, Hilpert, and Hancock 2008). The lysin concentration varied from 0.5 – 1,024 μg/ml PlySs2. The plates were then incubated for 18 h at 37°C. The MIC was the highest concentration of lysin that prevented the formation of a visible cell pellet (a measure of growth) on the bottom of the wells. The MICs were also colorimetrically confirmed by staining the bacteria in the wells with alamarBlue® vital dye following the manufacturer's protocol (Invitrogen).

3.1.5 Resistance

A published protocol to test the *in vitro* development of antibiotic-resistance was followed (Rouse et al. 2005, Pastagia et al. 2011, Gilmer et al. 2013). Briefly, *S. suis* was grown in the presence of doubling concentrations of PlySs2 over 8 days in BHI broth, and the PlySs2 MIC was tested daily to determine if resistance was acquired. On the first day, bacteria at ~5×10^8 CFU/ml were grown overnight at 37°C in 10 ml BHI containing $1/32\times$ the PlySs2 MIC for the given strain. On the second day, the culture was split into two equal portions. The cells of one aliquot were pelleted and resuspended in 10 ml fresh BHI media with double the concentration of PlySs2 (i.e., $1/16\times$ the PlySs2 MIC on day 2). This aliquot was re-incubated at 37°C overnight. Over 8 days, the concentration of

PlySs2 was serially doubled from $1/32\times$ the initial MIC (on day 1) to $4\times$ the initial MIC (on day 8).

A sample of the second half of the aliquot was spread on BHI agar containing the PlySs2 MIC for that strain. After incubation at $37°C$, 4 colonies were selected from the BHI agar plate to determine if a 4-fold increase in PlySs2 MIC was achieved for that strain, indicating the emergence of resistance. The protocol was repeated with gentamicin as an antibiotic resistance control for each *S. suis* strain, because *S. suis* strains develop resistance to gentamicin *in vitro* ([Varela et al. 2013](#)).

3.2 RESULTS

3.2.1 Lytic activity against *S. suis*

Purified PlySs2 was tested against 22 strains representing 8 serotypes of *S. suis* to assess its range of lytic activity. Over 30 minutes, 14 of 22 strains were reduced to an OD_{600} ratio of ≤ 0.2 from a starting OD_{600} of ~1.0 (Figure 3.1). Readings taken after 60 minutes showed the same relative reduction in OD_{600} (Figure 3.2). This group of PlySs2-sensitive *S. suis* strains included the type strain S735, and the pathogenic strains 10 and 7997. The reduction in OD_{600} ratio of other *S. suis* strains was reduced to ≤ 0.6. Serotype 12 was the only strain that exhibited a negligible decrease in optical density.
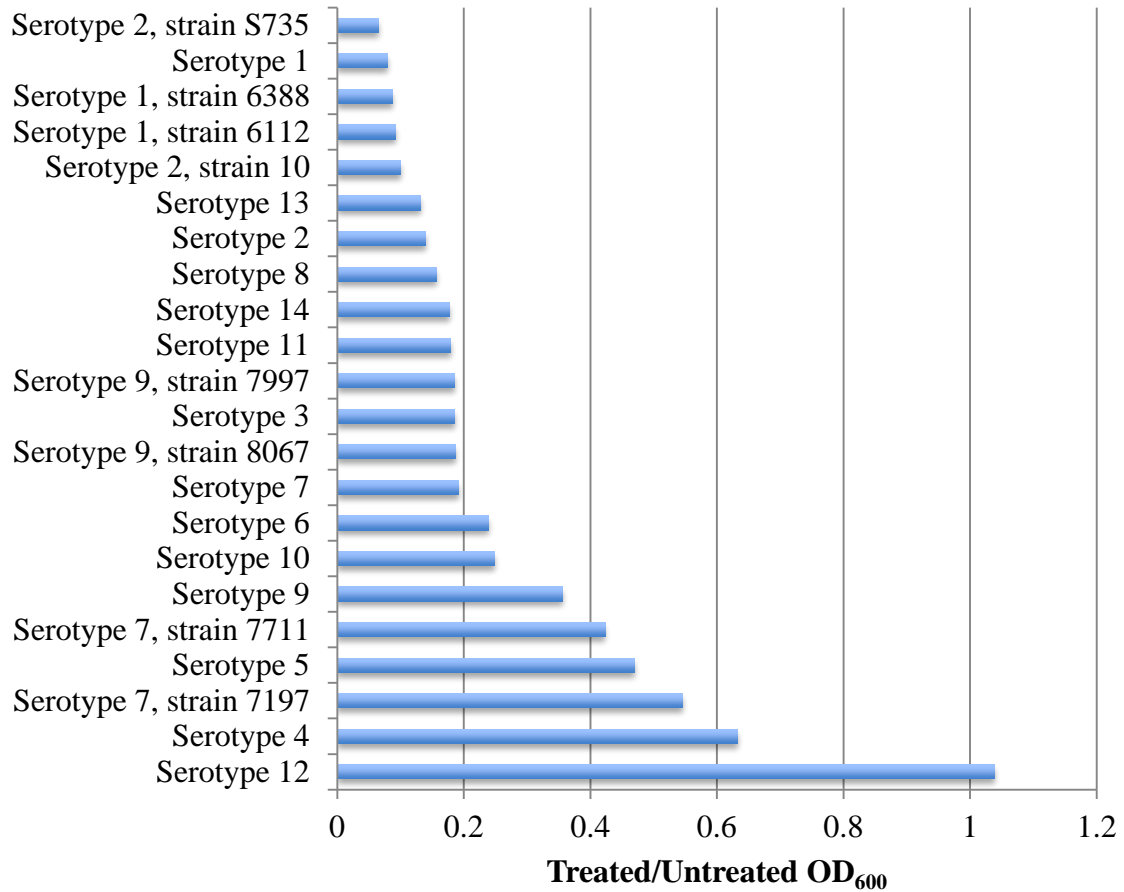
Figure 3.1 PlySs2 lysed almost all strains of *S. suis* over 30 minutes.

Bacteria in logarithmic growth were exposed to 32 µg/ml PlySs2 for 30 minutes in PB (for 60-minute readings, see Figure 3.2). The activity was measured by OD_{600} reduction. To normalize and combine values from multiple tests, the final OD_{600} of the treated samples was divided by the final OD_{600} of the untreated samples. An OD_{600} ratio of 1.0 indicates no lysis, while an OD_{600} ratio of ~0.02 indicates complete lysis.
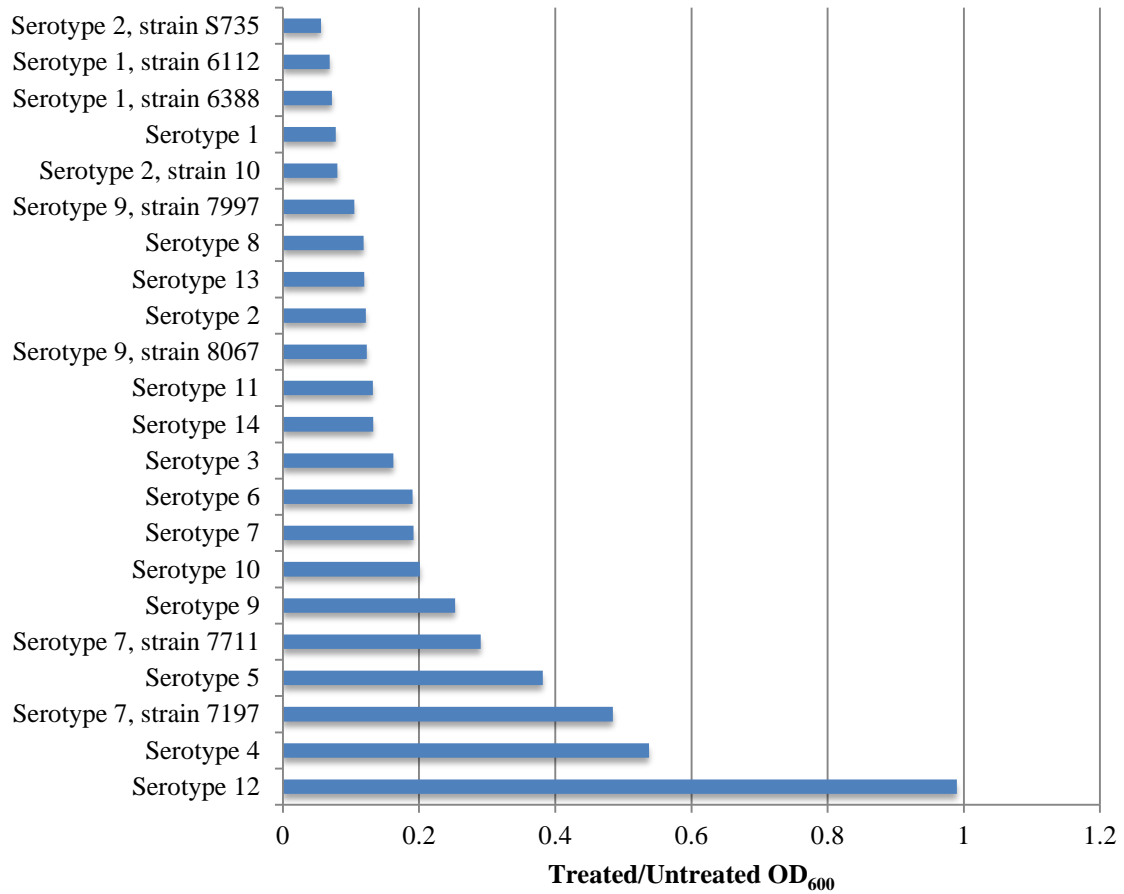
Figure 3.2 PlySs2 lysed almost all strains of *S. suis* over 60 minutes.

Bacteria in logarithmic growth were exposed to 32 μg/ml PlySs2 for 60 minutes in PB (for 30-minute readings, see Figure 3.1). The activity was measured by OD_{600} reduction. To normalize and combine values from multiple tests, the final OD_{600} of the treated samples was divided by the final OD_{600} of the untreated samples. An OD_{600} ratio of 1.0 indicates no lysis, while an OD_{600} ratio of ~0.02 indicates complete lysis.

S. suis strains S735 and 7997 were also tested in a time-dependent lytic assay at various PlySs2 dosages (Figure 3.3 and Figure 3.4), showing a significant drop in OD_{600} at >4 µg/ml for both strains. These time-dependent lytic assay results led us to use 32 µg/ml for the best resolution in testing all strains.
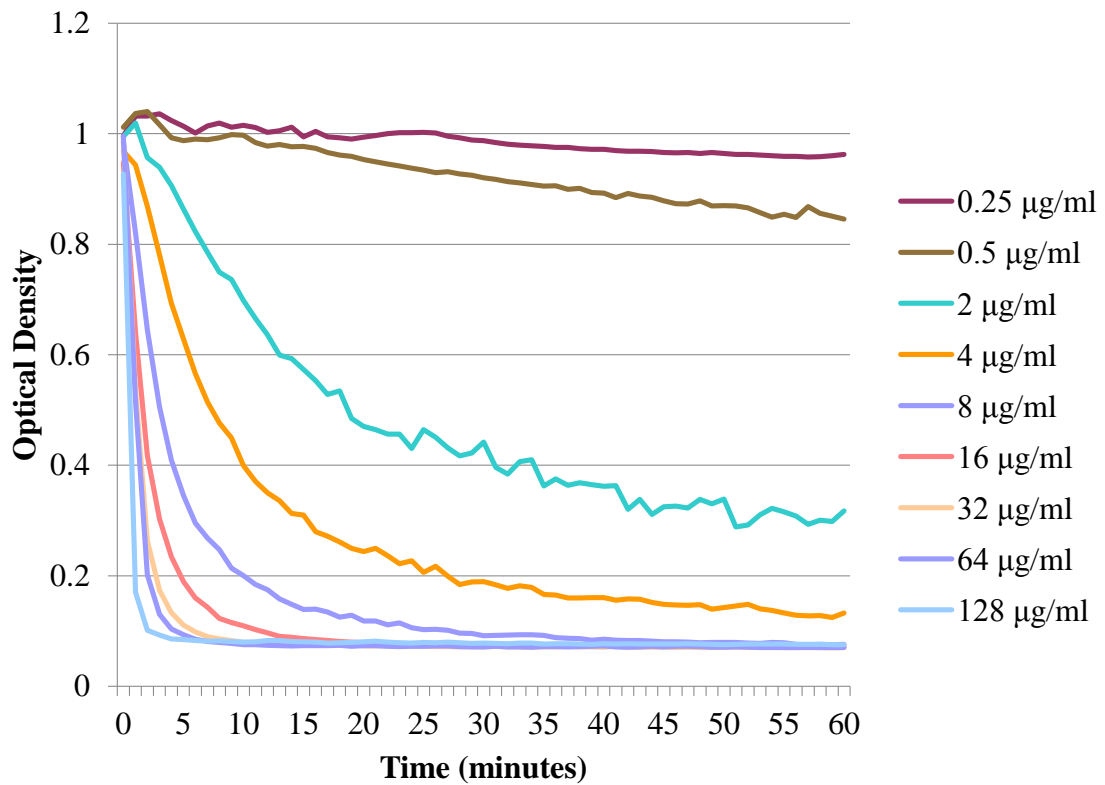
Figure 3.3 *S. suis* **strain S735 exposed to various concentrations of PlySs2.**

S. suis strain S735 is sensitive to concentrations as low as 0.5 µg/ml PlySs2. *S. suis* strain S735 in logarithmic growth was exposed to various concentrations of PlySs2 ranging from 0.25 µg/ml – 128 µg/ml for 60 minutes in PB. Readings at OD_{600} were taken every minute.
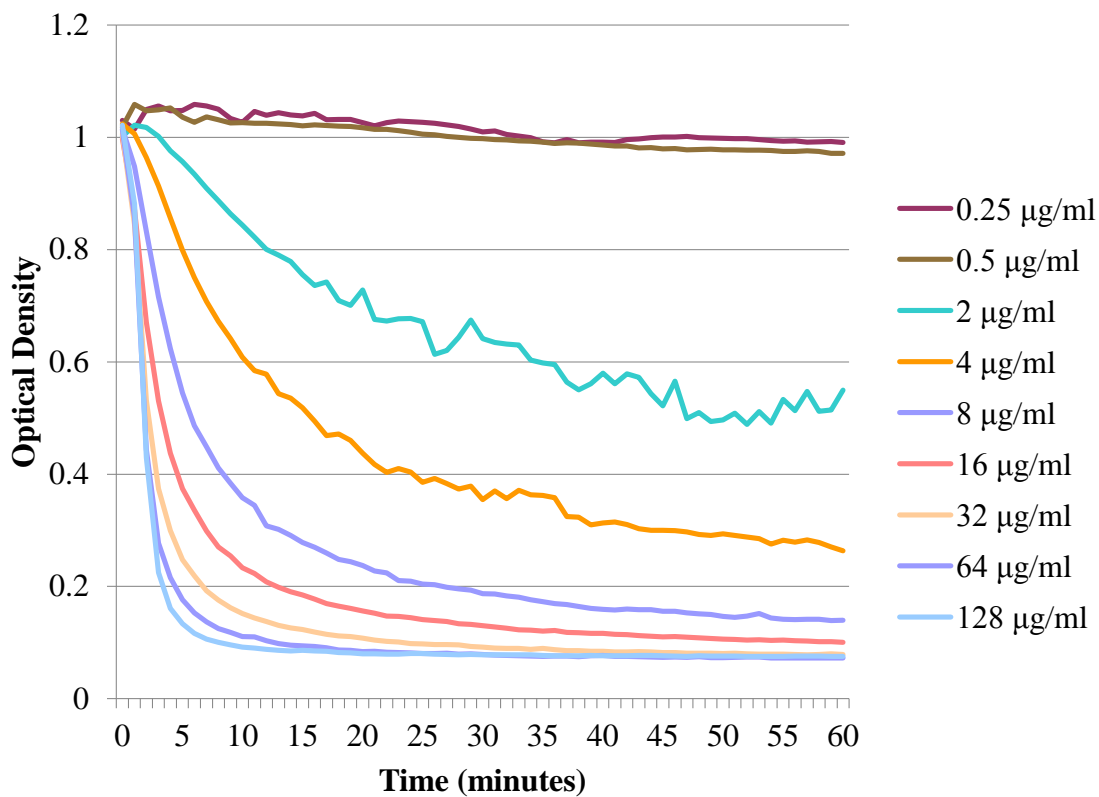
Figure 3.4 *S. suis* **strain 7997 exposed to various concentrations of PlySs2.**

S. suis strain 7997 is sensitive to concentrations as low as 1.0 μg/ml PlySs2. *S. suis* strain 7997 in logarithmic growth was exposed to various concentrations of PlySs2 ranging from 0.25 μg/ml – 128 μg/ml for 60 minutes in PB. Readings at OD_{600} were taken every minute.

3.2.2 Bactericidal assay

The lethal effect of PlySs2 was quantified for a select set of *S. suis* serotypes and strains. After 60 minutes of exposure to 64 µg/ml of PlySs2, all *S. suis* strains were reduced by 5-6 logs, except serotype 5 (~2-logs) (Figure 3.5). The relative lethal effect from one strain to another correlated with the lytic activity reported above. Strain 7197 was an outlier in that it was reduced by ~5.5 logs in the bactericidal assay, but was only reduced in the lytic assay to an OD_{600} ratio of ~0.5.
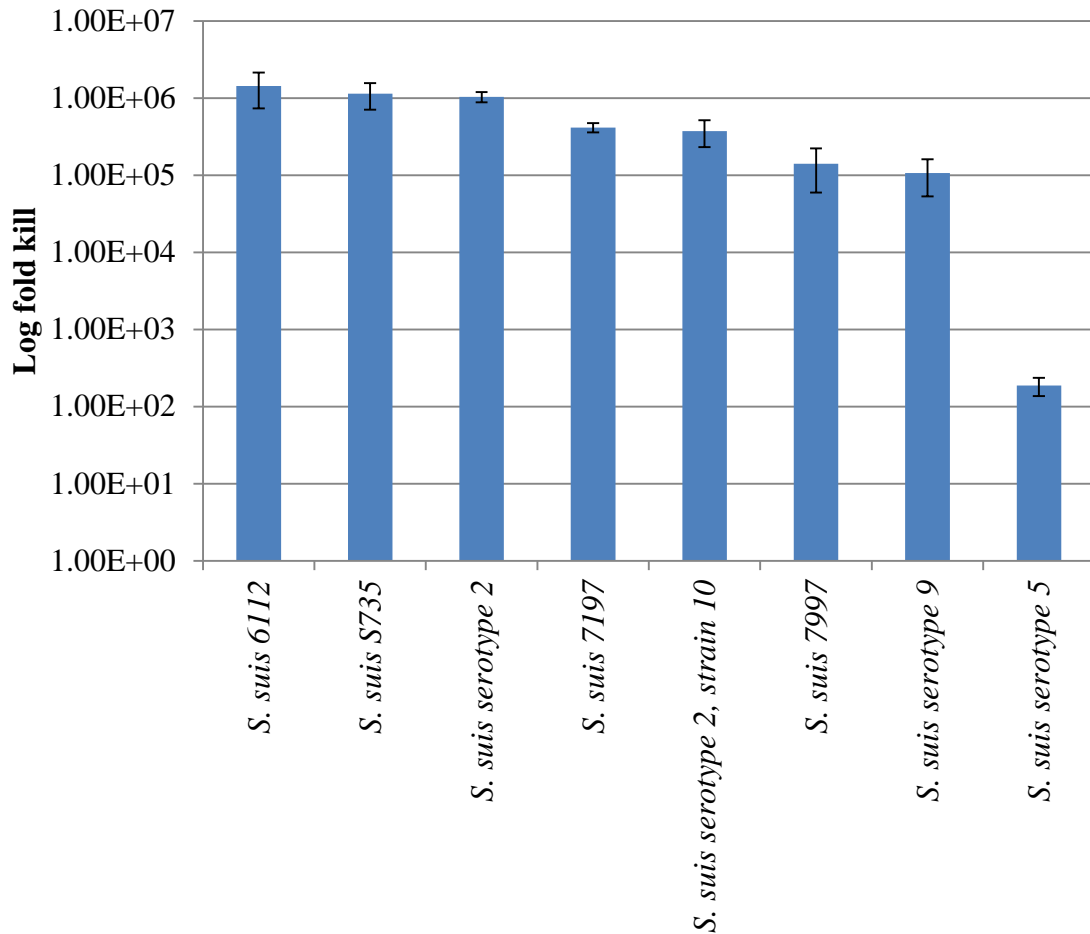
Figure 3.5 PlySs2 was bactericidal to nearly all strains of *S. suis*.

Bacteria were grown to log-phase. After exposure to 64 µg/ml PlySs2 in buffer A for 60 min in 96-well plates, bacteria were serially diluted and plated to BHI agar for CFU enumeration. The CFU numbers of most *S. suis* strains dropped by 5 to 6 logs after PlySs2 treatment including the type strain S735 and the pathogenic strains 10 and 7997. Log fold kill was calculated as -log[(# cells surviving test condition)/(# cells surviving control condition)] and averaged together from multiple trials.

3.2.3 MIC assay

The MIC of PlySs2 against these serotypes also qualitatively correlated with the lytic and bactericidal assays. The PlySs2 MIC ranged from 32 to 512 μg/ml for all PlySs2-sensitive strains. As expected, from earlier data, serotype 12 was not inhibited at >1,024 μg/ml (Table 3.2).

Table 3.2 The MIC of PlySs2 for *S. suis* serotypes and strains[a]

Species	Serotype	Strain	MIC (µg/ml)
S. suis	2	S735	32
	2		64
	7	7197	128
	9	7997	128
	9		128
	1	6112	256
	2	10	256
	5		512
	12		>1,024
S. aureus		MW2	16
S. pyogenes	M1	MGAS 5005	128

[a] Bacteria were examined for growth at each concentration of PlySs2 from 0.5 - 1,024 µg/ml. The lowest concentration preventing growth is the PlySs2 MIC (column 4) for each strain (columns 2 and 3) of each species (column 1). Consistent with other tests, *S. suis* type strain S735 registered a low MIC while there was a higher MIC observed for *S. suis* strain 7997. The MIC of PlySs2 for *S. suis* serotype 12 was above the assay parameters. The previously published PlySs2 MICs for *S. aureus* and *S. pyogenes* are included for reference (Gilmer et al. 2013).

3.2.4 Resistance

According to an established protocol ([Rouse et al. 2005](#), [Pastagia et al. 2011](#), [Gilmer et al. 2013](#)), both *S. suis* strains S735 and 7997 were challenged with incrementally doubling concentrations of PlySs2 to determine if they would develop resistance. Neither strain developed PlySs2 resistance – defined as exhibiting a >4× increase from the original PlySs2 MIC for each strain (Figure 3.6). Using the antibiotic gentamicin in the same procedure led to both *S. suis* strains S735 and 7997 developing resistance (Figure 3.6).
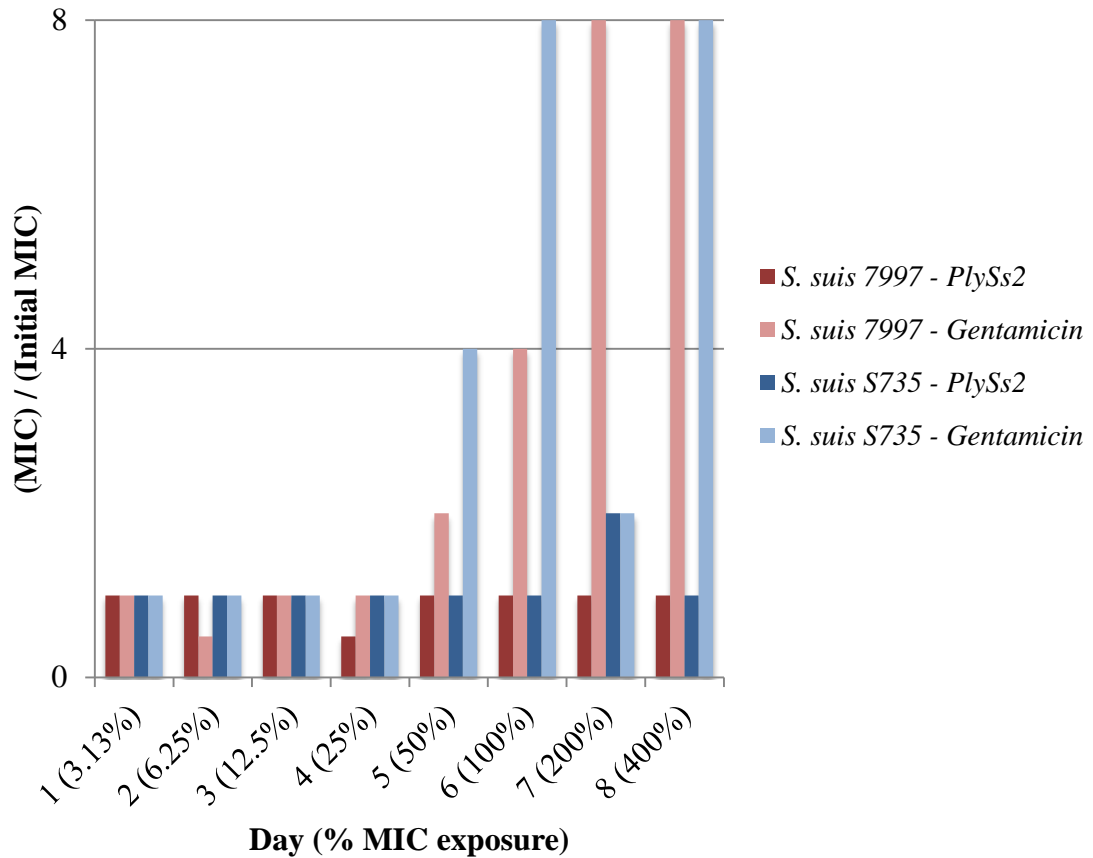
Figure 3.6 *S. suis* **7997 and S735 did not develop resistance to PlySs2**
in vitro.

S. suis S735 and *S. suis* 7997 grew in media containing $1/32\times$ (3.13%) to $4\times$ (400%) the MIC of PlySs2 or gentamicin over 8 days. Comparing the MICs of PlySs2 after each day to the initial MIC of PlySs2 for each strain determined resistance. Neither developed resistance to PlySs2. Both *S. suis* strain S735 and *S. suis* strain 7997 developed resistance to the positive control, gentamicin.

3.3 ACKNOWLEDGEMENTS

4 CHAPTER 4 – BROAD GRAM-POSITIVE SUSCEPTIBILITY

4.1 MATERIALS AND METHODS

4.1.1 Bacterial strains

All strains were stored at -80°C (Table 1.1). *Staphylococcus*, *Streptococcus*, *Listeria*, *Enterococcus*, *Pseudomonas, Bacillus* strains were cultivated in brain heart infusion (BHI) broth unless substituted with Mueller Hinton (MH) medium during the minimum inhibitory concentration (MIC) determination below. *Lactobacillus* strains were cultivated in de Man, Rogosa and Sharpe (MRS) broth (Sigma). *Escherichia coli* were grown in Luria Bertani (LB) broth. All media were acquired from Becton, Dickinson, and Company (Sparks, MD), unless otherwise stated. Bacteria were propagated at 37°C and shaken at 200 rpm, if necessary.

Table 4.1 Strains used in this study

Organism	Serotype	Strain	ATCC	Source[a]	Notes
Bacillus anthracis		Δ Sterne		1	
Bacillus cereus		14579		1	
Bacillus subtilis		SL4		1	
Bacillus thuringiensis		HD-73		1	
Enterococcus faecalis		V583		1	
Enterococcus faecium				1	EFSK-2
Escherichia coli		TOP10		1	
Group E streptococci	2	K131	123191	2	
Lactobacillus acidophilus		*Pak*	*4357*	3	
Lactobacillus acidophilus			*11975*	3	
Lactobacillus acidophilus			*4356*	3	
Lactobacillus gasseri			*19992*	3	
Lactobacillus rhamnosus		*LMS2-1*		3	
Lactobacillus rhamnosus			*21052*	3	

Organism	Serotype	Strain	ATCC	Source[a]	Notes
Listeria monocytogenes		HER 1184		1	
Listeria monocytogenes	4b	N3013		1	
Listeria monocytogenes	3b	FSLJ 1		1	
Listeria monocytogenes				1	RS823
Listeria monocytogenes				1	RS820
Listeria monocytogenes		HER1083		1	
Listeria monocytogenes			BAA-680	2	
Pseudomonas aeruginosa		RS1		1	
Staphylococcus aureus				4	vancomycin intermediate resistance IV
Staphylococcus aureus				4	vancomycin intermediate resistance III
Staphylococcus aureus		RN4220		1	
Staphylococcus aureus		Newman		5	methicillin sensitive - mutant LyrA
Staphylococcus aureus		Newman		5	methicillin sensitive
Staphylococcus aureus		MW2	BAA-1707	2	methicillin resistant - community acquired
Staphylococcus aureus		192		1	methicillin resistant
Staphylococcus aureus				1	methicillin resistant from patient DS
Staphylococcus aureus				1	highly mupirocin resistant
Staphylococcus aureus				1	D712 - daptomycin resistant

Organism	Serotype	Strain	ATCC	Source[a]	Notes
Staphylococcus aureus				1	0325 - daptomycin resisitant
Staphylococcus epidermidis		HER 1292		6	
Staphylococcus simulans				5	TNK3
Streptococcus agalactiae	Type II			1	Group B streptococcus
Streptococcus agalactiae		090R		1	Group B streptococcus
Streptococcus dysgalactiae				1	Group G streptococcus
Streptococcus dysgalactiae equisimilis		26RP66		1	Group C streptococcus
Streptococcus equi			9528	2	
Streptococcus equi zooepidemicus			700400	2	
Streptococcus gordonii			10558	2	
Streptococcus mutans		U159		1	
Streptococcus oralis		35037		1	

Organism	Serotype	Strain	ATCC	Source[a]	Notes
Streptococcus pneumoniae	9V	DCC1335		1	
Streptococcus pneumoniae	6	DCC1850		1	
Streptococcus pneumoniae	15	DCC1476		1	
Streptococcus pneumoniae	11			1	
Streptococcus pneumoniae				1	mutant Lyt 4-4
Streptococcus pyogenes	M6	D471		1	
Streptococcus pyogenes	M-neg	D471		1	mutant JRS75
Streptococcus pyogenes	M6	MGAS 10394	BAA-946	2	
Streptococcus pyogenes	M49	NZ131		7	
Streptococcus pyogenes	M4	SmR		1	streptomycin resistant - mucoid
Streptococcus pyogenes	M3	MGAS 315	BAA-595	2	
Streptococcus pyogenes	M18	MGAS 8232	BAA-572	2	
Streptococcus pyogenes	M1	CEM1ΔΦ		1	mucoid – mouse passaged
Streptococcus pyogenes	M1	CEM1ΔΦ		1	
Streptococcus pyogenes	M1	SF370		1	mucoid – mouse passaged

Organism	Serotype	Strain	ATCC	Source[a]	Notes
Streptococcus pyogenes	M1	SF370		7	
Streptococcus pyogenes	M1	MGAS 5005	BAA-947	2	
Streptococcus rattus		BHT		1	
Streptococcus sanguinis			10556	2	
Streptococcus sobrinus		6715		1	
Streptococcus suis	9	7997		8	

[a] 1, The Rockefeller University Collection; 2, ATCC; 3, ContraFect Corporation, Yonkers, NY; 4, Alexander Tomasz, The Rockefeller University; 5, Olaf Schneewind, University of Chicago, Chicago, IL; 6, Barry Kreiswirth, Public Health Research Institute, New Jersey, NJ; 7, Joseph Ferretti, University of Oklahoma Health Science Center, Oklahoma City, OK; 8, Jaap A. Wagenaar, Utrecht University, Utrecht, The Netherlands.

4.1.2 Lytic activity

Log-phase bacteria were adjusted with buffer A to an OD_{600} of 0.8 to 1.0 in 96-well microtiter plates (Falcon). PlySs2, at 32 μg/ml, or buffer B control vehicle was added to each sample well. In each run, *S. suis* 7997 was included as a positive control. Spectrophotometric readings (OD_{600}) of each well were taken by a Spectramax Plus 384 (Molecular Devices) every minute over 60 minutes at room temperature. Lysin activity was gauged by the degree of turbidity reduction (OD_{600}) following enzyme addition.

4.1.3 Bactericidal assay

Log-phase bacteria were resuspended in buffer A to an OD_{600} of 0.1 (= 0.5 McFarland, ~10^8 CFU/ml) and aliquoted into wells of a polypropylene microtiter plate (Costar). Actual inoculum titers for each experiment were derived from plating serial dilutions of each inoculum. For each organism, buffer B control vehicle or PlySs2 was added at 128 μg/ml to wells in triplicate. Plates were sealed and incubated at 37°C with agitation every 5 minutes for 1 hour. After incubation, cells were serially diluted in 10-fold increments and plated on BHI agar. Death (log fold kill) was calculated as -log[(CFUs in the test condition) ÷ (CFUs in the control condition)].

4.1.4 MIC assay

The protocol from Wiegand, *et al*. was followed to determine MICs, with adjustments as detailed below (Wiegand, Hilpert, and Hancock 2008). Briefly, a final suspension of ~5×10^5 cells/ml in MHB (or BHI for *S. pyogenes*) plus sterile-filtered lysin or control vehicle was distributed within a 96-well microtiter plate in triplicate (Wiegand, Hilpert, and Hancock 2008). Cells were challenged with 0.5 – 1,024 μg/ml PlySs2 in triplicate. MICs were determined by detection of cell pellet formation in the

bottom of rounded polysterene plate wells; they were corroborated colorimetrically with alamarBlue® vital dye (Invitrogen) following the manufacturer's protocol.

4.1.5 Resistance

According to an established protocol for the *in vitro* development of mupirocin-resistance ([Rouse et al. 2005](#), [Pastagia et al. 2011](#)), *S. aureus* CA-MRSA MW2, *S. aureus* MSSA 8325, and *S. pyogenes* MGAS 5005 were grown in the presence of PlySs2 in liquid culture. Initially, bacterial cells at 5×10^8 CFU/ml were grown overnight in the presence of $1/32\times$ the MIC of PlySs2 against each strain (37°C, BHI broth for *S. pyogenes* with cap secured during gentle shaking, MHB broth for *S. aureus* with 220 rpm aeration). The cells were pelleted by centrifugation for 10 min at 900 rcf and sub-divided into two aliquots.

One aliquot was diluted 10-fold into fresh MHB media with double the concentration of PlySs2; a portion of the other was spread onto the surface of MHA agar containing the PlySs2 MIC for that species. The MIC of 4 resultant colonies was recalculated by microdilution for each serial passage, as described above ([Wiegand, Hilpert, and Hancock 2008](#)). This was done to determine if a resistant clone had emerged (defined as a 4-fold increase in MIC). The above procedure was repeated over an 8-day period, and the concentration of PlySs2 in the liquid culture was serially doubled from $1/32\times$ to $4\times$ the original MIC. This process was also conducted with mupirocin for each MRSA strain, to serve as an antibiotic resistance positive control.

4.1.6 PlySs2 catalytic domain lytic assay

The PlySs2 catalytic domain sequence (*PlySs2-CD*, ~0.49 kb), containing some residues from the linker, was inserted into pBAD24 vector with HindIII and NheI. This

vector was transformed into TOP10 *E. coli* (Invitrogen). For purification, *E. coli* cells induced to express *pBAD24_PlySs2-CD* were pelleted and re-suspended in 20 mM PB, pH 7.4 (buffer D). EDTA-free protease inhibitor tablets were added before homogenizing the cells, which were subsequently spun at 16,000 rpm for 60 min. The volume was adjusted with buffer D to raise pH.

The soluble lysate was run through a CM Sepharose® column equilibrated to buffer D. The flow through containing PlySs2-CD was collected. This was dialyzed overnight at 4ºC into 20 mM glycine-NaOH, pH 9.3 (buffer E). This material was run over on Mono Q column equilibrated to buffer E. The material was eluted gradually from 1-100 mM NaCl, buffer E. This was repeated with elution by 30-100 mM NaCl, buffer E. Pure fractions dialyzed into buffer D and pooled. Concentration was confirmed to be 1.7 mg/ml.

Following the aforementioned protocol for lytic assay (page 59), log-phase *S. suis* strain 7997 cells were exposed to full length PlySs2 (PlySs2-FL) at 32 μg/ml, and PlySs2-CD at 850 μg/ml. As before, the degree of turbidity reduction (OD_{600}) in the test wells indicated the degree of lysin activity.

4.1.7 Fluorescent binding assay

The PlySs2 binding domain sequence (*PlySs2-BD*, ~0.26 kb) was cloned from *S. suis* strain 89/1591 with the forward primer (containing NheI site, underlined) AAT<u>GCTAGC</u>ATGCGTTCCTATCGCGAGAC, and the reverse primer (containing HindIII site, underlined) CCT<u>AAGCTT</u>CTTTTCACAAATCATAATCCCCAG. This was cloned into the pBAD24 vector and transformed into TOP10 *E. coli* (Invitrogen). The resultant protein, PlySs2-BD is expected to be 9.5 kD and have a theoretical pI 9.57.

E. coli cells induced to express *pBAD24_PlySs2-BD* were pelleted and re-suspended them in 20 mM PB, pH 7.4 (buffer D). EDTA-free protease inhibitor tablets were added before homogenizing the cells, which were subsequently spun at 15,000 rpm for 80 min. The pH was reduced by adding 30 mM PB, pH 6.0. The lysate was run over a CM Sepharose® FF column where it eluted at 250 mM NaCl, buffer D. The pure fractions were pooled and dialysed overnight in 4 l of buffer D. A BCA protein assay (Sigma) confirmed the concentration to be 8.9 mg/ml.

This PlySs2-BD was labeled with AlexaFluor 488 as per the manufacturer's instructions (Becton, Dickinson and Company – Sparks, MD). Subject strains were grown overnight in BHI at 37°C, and subsequently pelleted and resuspended in PBS to OD_{600} = 0.1. Of this dilution, 1 ml was pelleted and resuspended in 200 μl of 2% paraformaldehyde in PBS for 30 minutes.

After washing with PBS, pellets were resuspended in 500 μl PBS + 5% blocking solution for 1 hour to which 500 μl PBS + 0.5 μl labeled PlySs2-BD was added for another hour. After three washes with PBS, pellets were resuspended in 50 μl PBS. Cells (5 μl) were then pipetted onto a slide sealed with a cover slip. Fluorescence photographed on an Eclipse E400 microscope (Nikon) using the QCapture Pro version 5.1 imaging software. Images displayed high, medium, and low intensity.

4.2 RESULTS

4.2.1 Lytic activity

Purified PlySs2 was tested against a wide range of bacterial species and strains to determine the range of lytic activity. Starting at an OD_{600} of ~1.0, all tested strains of *S. aureus* – including strains resistant to methicillin, vancomycin, daptomycin, mupirocin,

and lysostaphin – were reduced to an OD_{600} ratio ≤ 0.3 after lysis by PlySs2 over 30 minutes of exposure (Figure 4.1). Readings were also taken after 60 minutes (Figure 4.2). The OD_{600} ratio of other staphylococci, including *Staphylococcus simulans* and *Staphylococcus epidermidis*, was reduced to ~0.2.

Figure 4.1 PlySs2 displayed activity against various species.

PlySs2 displayed activity against various species. Multiple strains of staphylococci (including MRSA, MSSA, and VISA), streptococci, enterococci, Listeria, bacilli, and lactobacilli were tested for susceptibility to PlySs2 activity. *Escherichia* and *Pseudomonas* were tested as Gram-negative controls. Log-phase cultures were exposed to 32 μg/ml PlySs2 for 30 min in PB (for 60-min readings) (see also Figure 4.2). The final OD_{600} of the treated samples was divided by the final OD_{600} of the untreated samples to generate the normalized values. Complete lysis registered a ratio of ~0.02. ST, serotype.

Treated/Untreated OD$_{600}$

0   0.2   0.4   0.6   0.8   1   1.2

- S. aureus - mupirocin resistant
- MSSA 8325
- MRSA MW2
- S. aureus 0325
- S. aureus D712
- S. aureus RN4220
- MRSA 192
- VISA IV
- VISA III
- MSSA Newman
- MSSA LyrA Newman
- GrAS MGAS 315
- GrAS MGAS 5005
- GrAS SF370 - mucoid
- GrAS CEM1ΔΦ - mucoid
- GrAS MGAS 10394
- GrAS NZ131
- GrAS SF370
- GrAS ST M4 - SmR - mucoid
- GrAS CEM1ΔΦ
- GrAS D471 JRS75
- GrAS D471
- GrAS MGAS 8232
- S. pneumoniae Lyt 4-4
- S. pneumoniae DCC 1476
- S. pneumoniae DCC 1335
- S. pneumoniae DCC 1850
- S. pneumoniae DCC 1811
- S. equi zooepidemicus ATCC 700400
- S. equi ATCC 9528
- S. simulans TNK3
- GBS type II
- S. epidermidis HER 1292
- S. sanguinis ATCC 10556
- S. gordonii ATCC 10558
- GBS 090R
- GGS
- GES
- E. faecalis V583
- GCS
- S. mutans U159
- S. oralis ATCC 35037
- S. rattus BHT
- S. sobrinus 6715
- E. faecium EFSK2
- L. monocytogenes HER 1083
- L. monocytogenes HER 1184
- L. monocytogenes RS822
- L. monocytogenes FSLJ 1
- L. monocytogenes ATCC BAA-680
- L. monocytogenes N3013
- L. monocytogenes RS823
- B. anthracis
- B. subtilis SL4
- B. cereus ATCC 14579
- B. thuringiensis
- L. rhamnosus ATCC 21052
- L. rhamnosus LMS2-1
- L. acidophilus ATCC 4356
- L. acidophilus ATCC 11975
- L. gasseri ATCC 19992
- L. acidophilus Pak
- P. aeruginosa
- E. coli

Figure 4.2 PlySs2 displayed activity against various species over 60 minutes.

Multiple strains of staphylococci (including MRSA, MSSA, and VISA), streptococci, enterococci, *Listeria,* bacilli, and lactobacilli were tested for susceptibility to PlySs2 activity. *Escherichia* and *Pseudomonas* were tested as Gram-negative controls. Log-phase cultures were exposed to 32 μg/ml PlySs2 for 60 minutes in PB. The final OD_{600} of the treated samples was divided by the final OD_{600} of the untreated samples to generate the normalized values. Complete lysis registers a ratio of ~0.02.

Treated/Untreated OD$_{600}$

0   0.2   0.4   0.6   0.8   1   1.2

S. aureus - mupirocin resistant
S. aureus D712
MRSA MW2
MSSA 8325
MRSA 192
S. aureus RN4220
S. aureus 0325
VISA III
VISA IV
MSSA Newman
MSSA LyrA Newman
GrAS MGAS 315
GrAS MGAS 5005
GrAS SF370 - mucoid
GrAS ST M4 - SmR - mucoid
GrAS CEM1ΔΦ - mucoid
GrAS MGAS 10394
GrAS NZ131
GrAS SF370
GrAS CEM1ΔΦ
GrAS D471 JRS75
GrAS D471
S. pneumoniae Lyt 4-4
S. pneumoniae DCC 1476
S. pneumoniae DCC 1335
S. pneumoniae DCC 1850
S. pneumoniae DCC 1811
S. simulans TNK3
S. equi zooepidemicus ATCC 700400
S. epidermidis HER 1292
S. equi ATCC 9528
GBS type II
GBS 090R
S. sanguinis ATCC 10556
S. gordonii ATCC 10558
GrAS MGAS 8232
GGS
GES
E. faecalis V583
GCS
S. oralis ATCC 35037
S. rattus BHT
S. sobrinus 6715
S. mutans U159
E. faecium EFSK2
L. monocytogenes HER 1083
L. monocytogenes HER 1184
L. monocytogenes RS822
L. monocytogenes FSLJ 1
L. monocytogenes ATCC BAA-680
L. monocytogenes N3013
L. monocytogenes RS823
B. subtilis SL4
B. cereus ATCC 14579
B. thuringiensis
B. anthracis
L. acidophilus ATCC 11975
L. acidophilus ATCC 4356
L. rhamnosus LMS2-1
L. rhamnosus ATCC 21052
L. acidophilus Pak
L. gasseri ATCC 19992
E. coli
P. aeruginosa

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With streptococci, PlySs2 lysed most of the tested M-protein serotypes of *S. pyogenes,* including M1, M3, M4, M6, M18, M49, an M-negative variant, as well as unencapsulated and highly encapsulated strains, decreasing their OD_{600} ratio to ≤ 0.4. PlySs2 also exhibited strong lytic activity against *S. suis*, *Streptococcus equi zooepidemicus*, *Streptococcus equi*, *S. agalactiae* Type II (encapsulated), and *S. agalactiae* 090R. The pathogenic *Streptococcus sanguinis*, and groups G and E streptococci were moderately sensitive to PlySs2. *Streptococcus mutans*, group C streptococci, *Streptococcus oralis*, *Streptococcus rattus*, and *Streptococcus sobrinus* were only reduced to an OD_{600} ratio between 0.7 and 0.9. PlySs2 did not reduce the OD_{600} ratio for any *Streptococcus pneumoniae* strains below 0.5. *Streptococcus gordonii* was the only commensal against which PlySs2 exhibited activity (Figures 4.1 and 4.2).

PlySs2 showed some activity against genera outside *Staphylococcus* and *Streptococcus*. While two strains of *Listeria* were sensitive to PlySs2, other strains were not. In the *Enterococcus* genus, which is associated with high levels of antibiotic resistance, *E. faecalis* was sensitive to PlySs2 (although less than staphylococci or streptococci), but *E. faecium* was not. No activity was seen against any of the different species of *Bacilli*, strains of lactobacilli, or Gram-negatives.

4.2.2   Bactericidal assay

PlySs2 was tested for log-fold killing of several species and strains of susceptible pathogenic organisms tested by OD_{600} decrease. At 128 µg/ml PlySs2 and 60 minutes exposure, the vancomycin-intermediate *S. aureus* (VISA) strain was only reduced by 2-logs. However, PlySs2 reduced the viability of *L. monocytogenes, S. agalactiae*, *S.*

aureus, and *S. pyogenes* from ~3 to >6-logs (Figure 4.3). The negative control *E. coli* was

not reduced in OD_{600} after PlySs2 treatment.
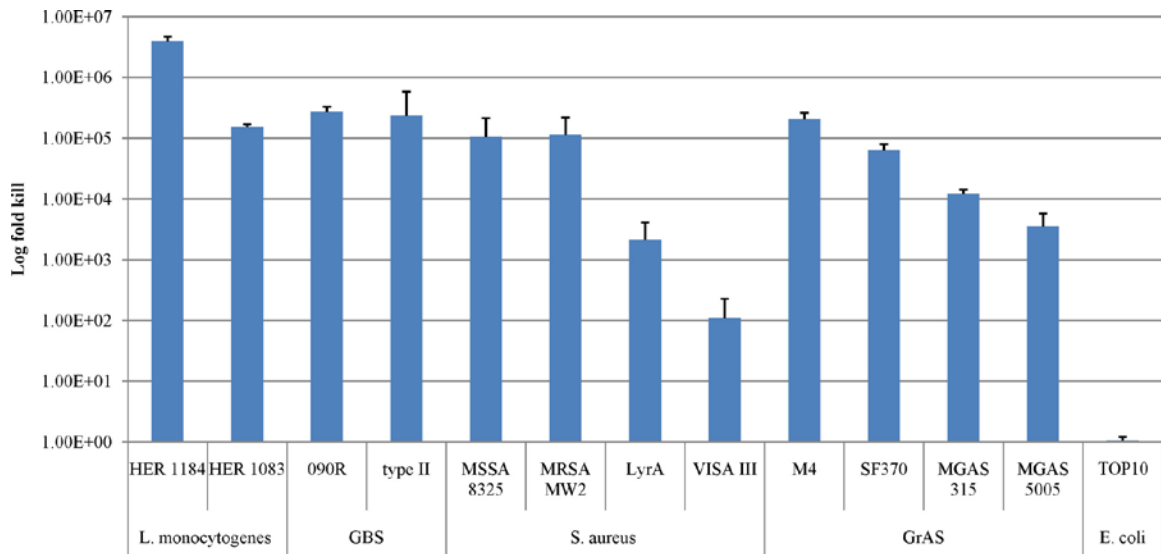
Figure 4.3 PlySs2 was bactericidal across multiple species of bacteria.

Log-phase bacteria were treated in 96-well plates with 128 μg/ml PlySs2 in buffer A for 60 min, then serially diluted and plated onto BHI agar for CFU enumeration. The log kill was calculated by comparing the difference between vehicle-treated and PlySs2-treated CFU results. Of note, PlySs2 dramatically reduced *S. agalactiae* and *L. monocytogenes*. There was no reduction in number of the negative control *E. coli*.

4.2.3 MIC Assay

When the MIC of PlySs2 was tested against strains of *S. aureus*, *S. pyogenes*, *L. monocytogenes*, *S. agalactiae*, and *E. coli*, most of the values qualitatively correlated directly to the lytic and killing activity. MICs ranged from 8 to 256 µg/ml for all strains, except the VISA strain, which was not inhibited at >1,024 µg/ml (Table 4.2). The MIC of PlySs2 was relatively low for *L. monocytogenes* and *S. aureus*. *S. pyogenes* and *S. agalactiae* registered similar PlySs2 MICs. The negative control *E. coli* did not register a measurable PlySs2 MIC.

Table 4.2 The MIC of PlySs2 for various Gram-positive species[a]

Species	Strain	MIC µg/ml	
		Visual	Colorimetric
L. monocytogenes	HER 1184	8	16
	HER 1083	8	16
S. aureus	MSSA 8325	16	16
	MRSA MW2	16	32
	LyrA	32	32
	VISA III	32	64
GrAS	SF370	128	128
	MGAS 5005	128	256
GBS	090R	256	256
	type II	512	512
E. coli	Top10	>1,024	>1,024

[a]All MICs were evaluated visually for bacterial growth and with alamarBlue® vital dye (colorimetrically) at concentrations from 0.5 to 1,024 µg/ml of PlySs2 for each strain of each species listed. There was a low MIC for MRSA MW2, as expected, and a higher MIC for GrAS strain MGAS 5005. The MIC of PlySs2 for the negative control, *E. coli*, was above the limits of the assay. For a reference, ClyS, LysK, and CHAP$_K$ lysins have registered MICs against *S. aureus* strains from 30 to 80 µg/ml (Pastagia et al. 2011, Daniel et al. 2010, Fenton et al. 2011, Becker et al. 2009).

4.2.4   Resistance

Using a published, standardized method for calculating resistance, both staphylococcal and streptococcal strains were analyzed for the development of resistance against PlySs2 by serial exposure to incrementally doubling concentrations of the lysin. Under these testing conditions, none of the *S. aureus* or *S. pyogenes* strains exposed to PlySs2 over 8 days developed resistance (defined as a 4-fold increase from the original MIC) (Figure 4.4). Following the same procedure, both *S. aureus* strains MW2 and 8325 developed resistance to the antibiotic mupirocin (Figure 4.4).
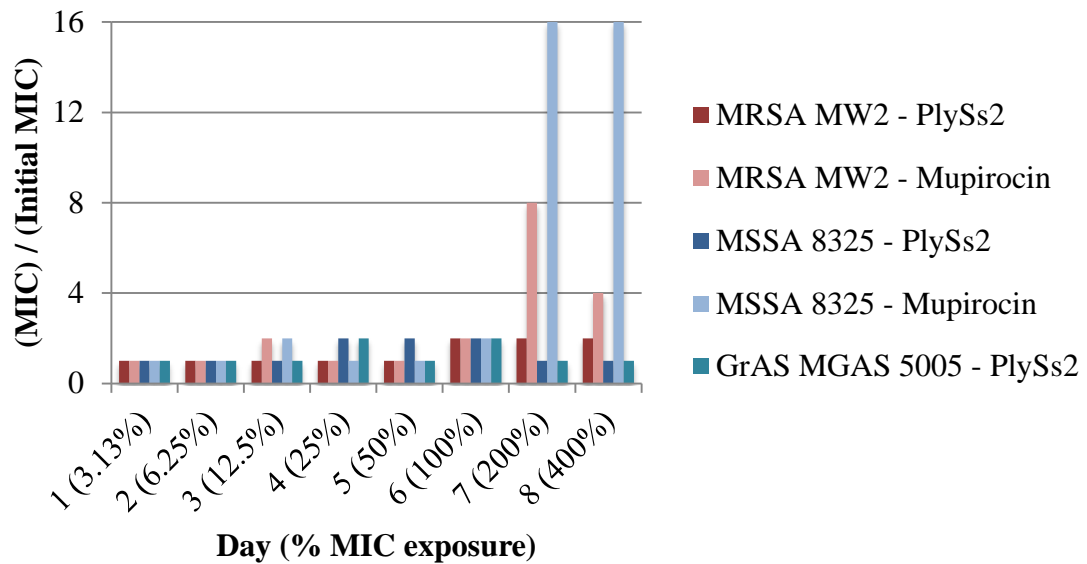
Figure 4.4 MRSA, MSSA, and GrAS did not acquire resistance to PlySs2 *in vitro*.

MRSA strain MW2, MSSA strain 8325, and GrAS strain MGAS 5005 were exposed to $1/32\times$ to $4\times$ the MIC of PlySs2 and mupirocin (*S. aureus* strains) over 8 days. The daily MICs of PlySs2 were compared to the starting MIC of PlySs2 for each strain of bacteria to ascertain resistance. None developed resistance to PlySs2. Both MW2 and 8325 developed resistance to the positive control, mupirocin.

4.2.5 PlySs2 catalytic domain lytic assay

The catalytic domain is much less effective at mediating lysis of *S. suis* strain 7997 compared to the full length PlySs2 lysin. Therefore, we used 850 μg/ml in order to establish enough resolution within its activity profile among various species and strains. Nevertheless, at a high concentration, it is able to lyse certain strains similar to the effect of PlySs2-FL at 32 μg/ml. The specificity of PlySs2-CD at high concentrations is somewhat consistent with the specificity of PlySs2 at lower concentrations against most streptococci. PlySs2-CD does not seem to target staphylococci even at high concentrations.
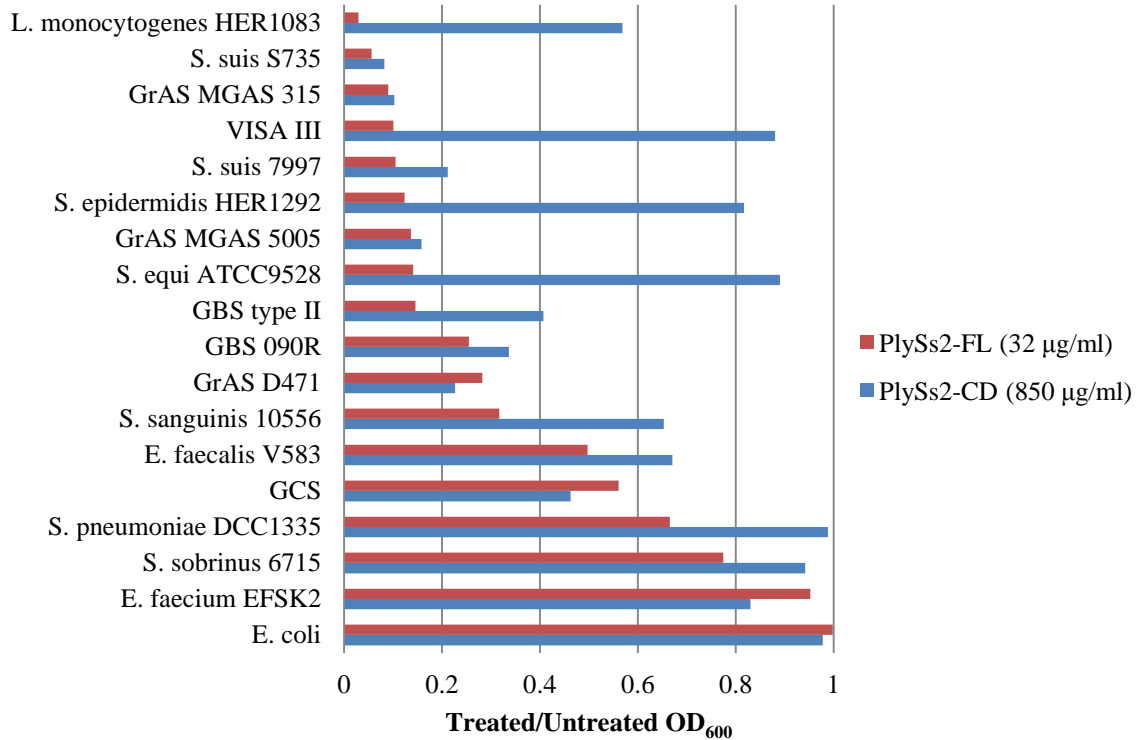
Figure 4.5 Lytic effect of PlySs2 Full Length VS Catalytic Domain

Strains of staphylococci (including MRSA, MSSA, and VISA), streptococci, enterococci, *Listeria,* bacilli, and lactobacilli were tested for susceptibility to PlySs2-FL or PlySs2-CD activity. *Escherichia* was tested as a Gram-negative control. Log-phase cultures were exposed to lysin for 60 minutes in PB. The final OD_{600} of the treated samples was divided by the final OD_{600} of the untreated samples to generate the normalized values. Complete lysis registers a ratio of ~0.02.

4.2.6 Fluorescent binding assay

Cells were tested for binding fluorescent PlySs2-BD. Many species were tested, but only three are presented here as representative results. PlySs2-BD bound best to *S. agalactiae* type II, although PlySs2-FL has less activity against this strain than against *S. pyogenes* strain 5005 or *S. suis* strain 7997.

There was little correlation between the species that PlySs2-BD bound and the *in vitro* activity of PlySs2-FL or PlySs2-CD. This may be attributed to each domain requiring the other to mediate their effect. Nevertheless, this preliminary study will require further investigation to elucidate the specificity of PlySs2.

Figure 4.6 PlySs2-BD Flurescence and comparison to PlySs2-CD activity.

1 – *S. suis* strain 7997

2 – *S. pyogenes* strain 5005

3 – *S. agalactiae* type II [1× exposure time (left bottom); ½× (right bottom)]

(A) This PlySs2-BD was labeled with AlexaFluor 488 as per the manufacturer's instructions (Becton, Dickinson and Company – Sparks, MD). Subject strains were resuspended in 500 μl PBS + 5% blocking solution for 1 hour to which 500 μl PBS + 0.5 μl labeled PlySs2-BD was added for another hour. Cells (5 μl) were pipetted onto a slide sealed with a cover slip. Fluorescence photographed on an Eclipse E400 microscope (Nikon) using the QCapture Pro version 5.1 imaging software. Scale bars, 1 μm. (B) Results for these three strains taken from Figure 4.5.

4.3   ACKNOWLEDGEMENTS

5 CHAPTER 5 – *IN VIVO* MODELS

5.1 MATERIALS AND METHODS

5.1.1 Intranasal mucosa model

5.1.1.1 *S. suis* colonization

A nasal mucosal colonization model described by Seitz, M., et al. ([Seitz et al. 2012](#)), was used to test the *in vivo* efficacy of PlySs2 to decolonize *S. suis* strain 7997. Each strain was made spontaneously resistant to 200 μg/ml streptomycin, through passage of bacteria in media containing increasing concentrations of antibiotic, to distinguish it from other organisms found in the murine nasal mucosa. Next, 4-week old female CD-1® mice were obtained from Charles River (Wilmington, MA). After a week of acclimation, mice were given water with 5 mg/ml streptomycin. After two days, mice were anesthetized and 12.5 μl of 1% acetic acid was delivered to each nostril. An hour later, mice were administered 10 μl (~1 × 10⁹ CFU) of mid log-phase (OD$_{600}$ of ~0.5) bacteria in 50 mM PB, pH 7.4 (buffer C) in each nostril. An aliquot of each inoculation stock was serially diluted and plated to Columbia blood agar plates to confirm the actual bacterial inoculation titer.

5.1.1.2 Treatment

Twenty-four hours post-colonization, the animals were randomly divided into 4 treatment groups. To each nostril, we delivered 10 μl of either: buffer C alone (group 1), 5 mg/ml PlySs2 in buffer C (group 2), 5 mg/ml gentamicin in buffer C (group 3), or a combination of 2.5 mg/ml PlySs2 and 2.5 mg/ml gentamicin in buffer C (group 4). Twenty-four hours after treatment, all mice were euthanized by CO_2-inhalation. The nasal passage of each mouse was surgically removed post-mortem, bisected to expose the

sinuses, and vortexed in 500 μl of buffer C. Serial dilutions were then streaked on 5% sheep blood plates (containing 200 μg/ml streptomycin) and incubated at 37°C for final colony counts. All protocols in this *in vivo* study were approved by The Rockefeller University's Institutional Animal Care and Use Committee.

5.1.2   Mixed bacteremia model

5.1.2.1   MRSA + GrAS Infection

The Rockefeller University's Institutional Animal Care and Use Committee approved all *in vivo* protocols. A systemic infection model described by Daniel, A. *et al.*, was used to test for the *in vivo* efficacy of PlySs2 against multiple Gram-positive bacteria (Daniel et al. 2010). Briefly, 4-5 week old female FVB/NJ mice (weight range 15 to 20 g) were obtained from The Jackson Laboratory (Bar Harbor, ME). After a period of acclimation, mice were injected intraperitoneally (IP) with 0.5 ml of mid log-phase (OD_{600} of 0.5) bacteria diluted with 5% hog gastric mucin (Sigma) in saline. Bacterial suspensions contained ~5×10^5 CFU/ml of MW2, a PVL toxin-encoding MRSA strain, ~1×10^7 of MGAS 5005, an M1 serotype of *S. pyogenes* that is virulent in humans and mice, or a simultaneous combination of both bacteria at the above concentrations for the mixed infection experiments. Actual bacterial inoculation titers were calculated by serial dilution and plating to Columbia blood agar plates for each experiment.

5.1.2.2   Treatment

Mice became bacteremic within 1-3 hours and contained MRSA and/or *S. pyogenes* within multiple organs, including spleen, liver, kidney, and heart/blood (reference (Daniel et al. 2010), and unpublished observations). Three hours post-infection, the animals were divided into 4 to 5 treatment groups per infection type and

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were administered IP 0.5 ml of either 20 mM phosphate buffer, 2 mg/ml of the streptococcal-specific lysin PlyC ([Nelson et al. 2006](#)), 2 mg/ml of the staphylococcal-specific lysin ClyS ([Daniel et al. 2010](#)), 2-4 mg/ml PlySs2, or a combination of 2 mg/ml PlyC and 2 mg/ml ClyS. A PlySs2 stock of 4 mg/ml was used for *S. pyogenes*-infected mice to increase survival over an initial 70% survival rate with treatment of 2 mg/ml of PlySs2 (data not shown). While this dosage was possible with PlySs2, it was above our obtainable PlyC or ClyS stock concentration at the time.

The survival rate for each experimental group was monitored every 12 hours for the first 24 hours, then every 24 hours up to 10 days post-infection. The data were statistically analyzed by Kaplan-Meier Survival curves with standard error, 95% confidence intervals, and significance (Log-rank / Mantel-Cox test) calculated using the Prism computer program (GraphPad Software; La Jolla, CA).

5.2   RESULTS

5.2.1   Intranasal mucosa decolonization

To determine if the *in vitro* activity of PlySs2 against *S. suis* predicts its ability to remove *S. suis* colonizing the nasal passages *in vivo*, mice were intranasally colonized with *S. suis* strain 7997, and subsequently treated intranasally with a single dose of PlySs2 or buffer. The number of CFUs remaining in the nasal mucosa was determined by plating serial dilutions on blood agar. The results from multiple, separate experiments were combined and plotted (Figure 5.1). Relative to the buffer-treated control, the nasal mucosa of mice were decolonized of *S. suis* by >3 logs after gentamicin treatment, >4 logs after PlySs2 treatment, and >5 logs after treatment with gentamicin + PlySs2.

Figure 5.1 PlySs2 and gentamicin may act additively to reduce *S. suis* *in vivo*.

PlySs2 removed *S. suis* from the murine intranasal mucosa. CD-1® mice were nasally colonized with the pathogenic *S. suis* strain 7997. Twenty-four hours after colonization, in each nostril, mice received 10 μl of either 50 mM PB, pH 7.4 (buffer C), 5 mg/ml PlySs2 in buffer C, 4 mg/ml gentamicin in buffer C, or a combination of 2.5 mg/ml PlySs2 and 2.5 mg/ml gentamicin in buffer C. *S. suis* CFU counts were calculated for the anterior nasal passage of each mouse.
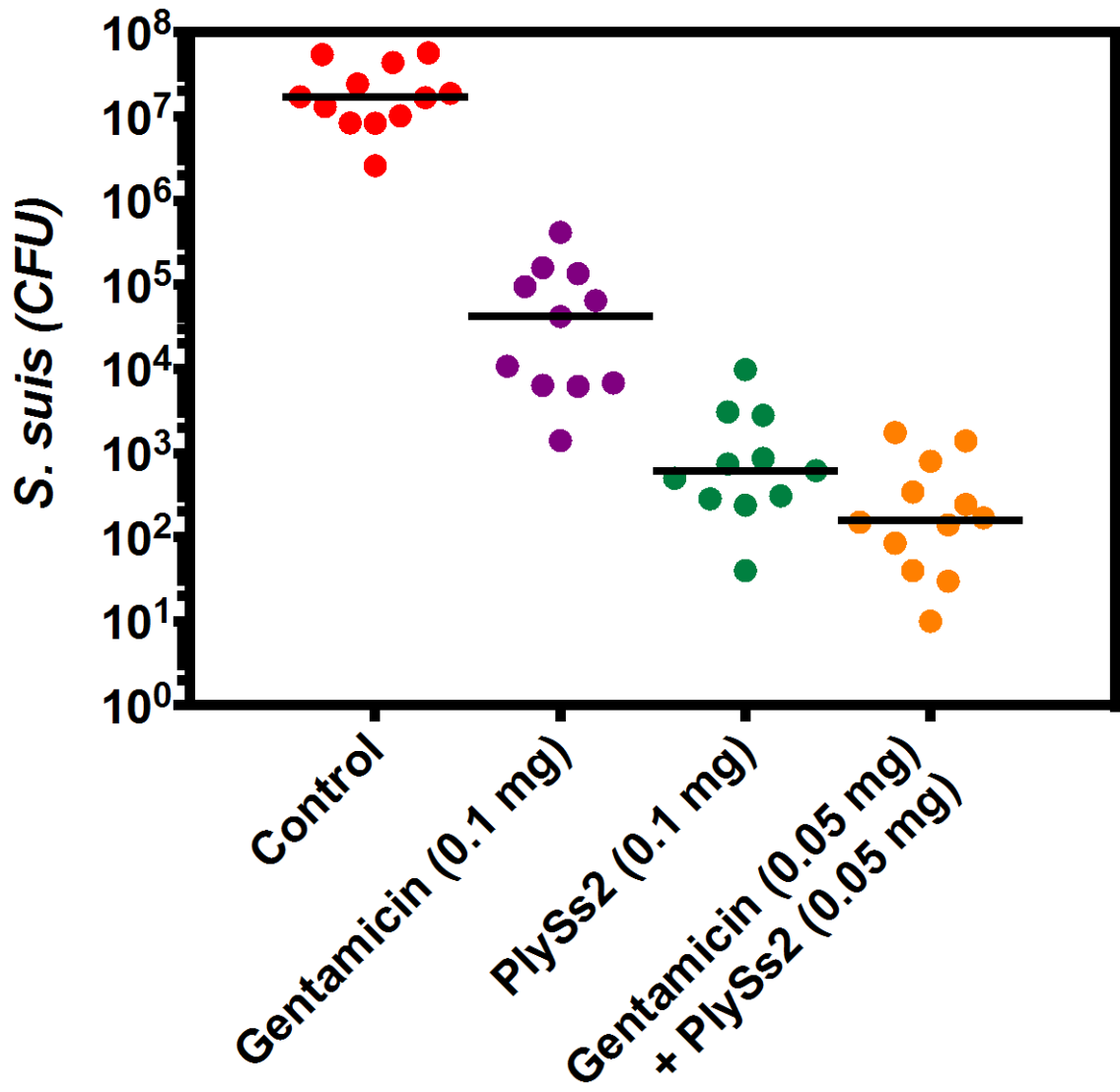
5.2.2 Mixed bacteremia protection

To determine if the broad lytic activity of PlySs2 could provide *in vivo* protection from Gram-positive pathogens, mice were infected IP with either MRSA (MW2) or *S. pyogenes* (MGAS 5005) independently or simultaneously, and subsequently treated with lysin(s). The results from 4 separate experiments were combined and mouse survival data plotted with a Kaplan-Meier survival curve (Figure 5.2).

Figure 5.2 PlySs2 protected mice from mixed MRSA and GrAS infection.

FVB/NJ mice were injected i.p. with 5% mucin containing the pathogen of interest. Three hours post-infection, mice received one i.p. injection of either 20 mM phosphate buffer (control) or lysin treatment. (A) Survival data for the MRSA infection. Mice were infected with -5 × 10^5 CFU of MRSA strain MW2 and treated with either 1 mg of ClyS, 1 mg of PlyC, or 1 mg of PlySs2. (B) Survival data for the GrAS infection. Mice were infected with -1 × 10^7 GrAS strain MGAS 5005 and treated with either 1 mg of ClyS, 1 mg of PlyC, or 2 mg of PlySs2. (C) Survival data for the mixed MRSA and GrAS infection. Mice were infected with a combination of both bacteria from the above inoculums at the same concentrations. Mice were treated with either 1 mg of ClyS, 1 mg of PlyC, a combination of 1 mg of ClyS plus 1 mg of PlyC, or 2 mg of PlySs2. In all tests (A to C), mice were monitored for survival over 10 days. The results from 4 independent experiments were combined, and the data are plotted as a Kaplan-Meier survival curve; bars indicate standard errors. All the PlySs2 treatment groups (A to C) showed statistically significant differences ($P < 0.0001$) compared to the nonspecific single lysin or buffer controls, based on the log rank (Mantel-Cox) test.

C

Percent survival

Days

Mix-PlySs2 n=24
Mix-ClyS+PlyC n=20
Mix-ClyS n=18
Mix-PlyC n=18
Mix-Buffer n=23
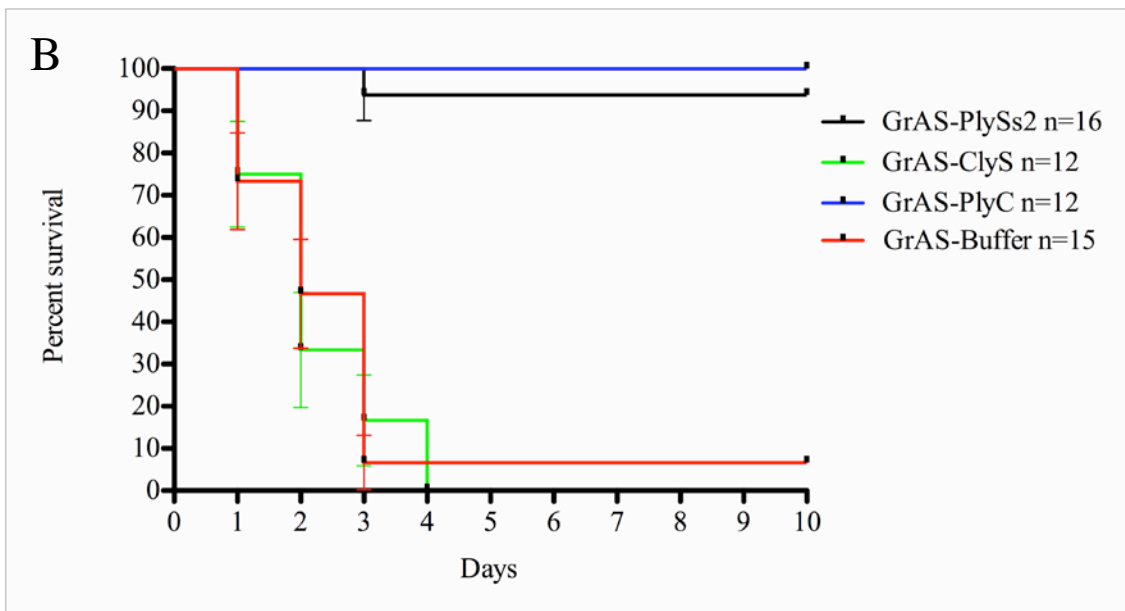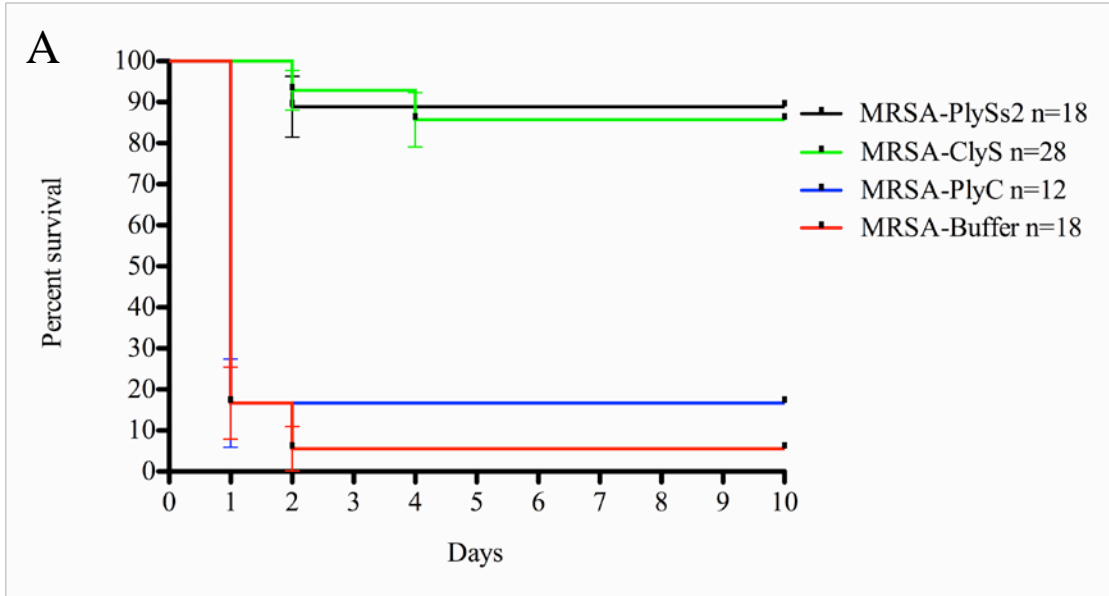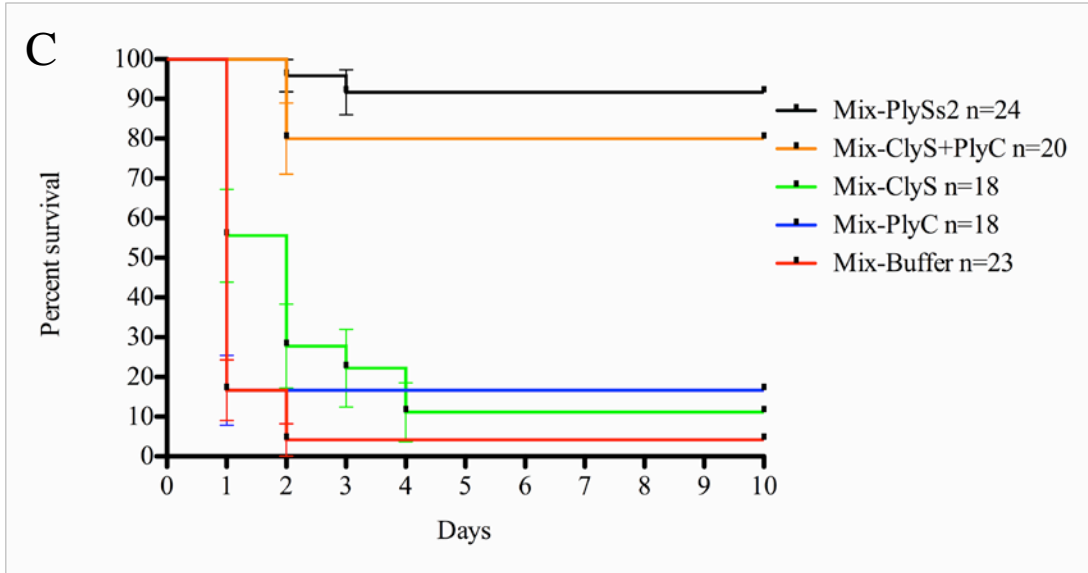
Mice infected with MRSA alone could be protected from death with either PlySs2 (89%; 16/18) or ClyS (86%; 24/28) lysins, but not the *Streptococcus*-specific PlyC (17%; 2/12) (Figure 5.2A); while mice infected with *S. pyogenes* alone could only be protected with either PlySs2 (94%; 15/16) or PlyC (100%; 12/12), but not the *Staphylococcus*-specific ClyS (0%; 0/12) (Figure 5.2B). Mice infected with *S. pyogenes* alone tended to succumb at a slower rate. All deaths from the MRSA infection occurred within the first two days; deaths from the *S. pyogenes* infection continued into the fourth day. In the first 24 hours of infection: 4/18 (22%) control mice survived MRSA and 11/15 (73%) control mice survived *S. pyogenes*; with only 1/18 (6%) and 1/15 (7%) surviving to the end of the experiment, respectively.

Likewise, only 1/23 (4%) of the buffer-treated control mice survived when simultaneously infected with both MRSA and *S. pyogenes*. In these mixed infection experiments, single-agent therapy with either ClyS (11%; 2/18) or PlyC (17%; 3/18) was also not significantly protective. While the mix infection animals treated with ClyS succumbed slower, with 14/16 dead by day 4, similar to mice infected with only *S. pyogenes*. However, mice could be protected from death with either a combination of ClyS and PlyC (80%; 16/20) or PlySs2 alone (92%; 22/24) over the 10-day course of the experiment (Figure 5.2C). Most of the PlySs2-treated mice survive the mixed infection yielding results comparable to, if not better than, the mice treated with both ClyS + PlyC at the same time.

5.3   ACKNOWLEDGEMENTS

model with the assistance of Nathan Franck. I am grateful to: Alexander Tomasz for MRSA, VISA, and other staphylococcal strains; ATCC for MRSA and streptococcal strains; and Jaap Wagenaar for *S. suis* strains S735 and 7997.

6   CHAPTER 6 – DISCUSSION

6.1   Lysin Characterization

6.1.1   Identification

Previous lysins have been identified from bacteriophage lysate, metagenomic screens, or shotgun-cloned phage DNA. After inspecting several *S. suis* genomic sequences, we found a putative open reading frame that resembled a phage lytic enzyme. This gene, *PlySs2*, was cloned, expressed, and found to have lytic activity against peptidoglycan.

6.1.2   Purification and Stability

PlySs2 is more tractable and stable than many previously reported lysins (Wang, Sun, and Lu 2009, Daniel et al. 2010, Nelson et al. 2006). PlySs2 preparation is relatively straightforward, yielding very pure, high yields of product in just a few steps. It remains soluble in concentrations exceeding 20 mg/ml (data not shown), and can be subjected to high or low temperatures for prolonged periods with little affect on its activity, even when repeatedly freeze-thawed. These features support PlySs2 as a suitable lysin for further development.

Biochemical experiments analyzing the *in vitro* activity of PlySs2 showed that DTT had no effect on PlySs2 activity. This indicates that: [1] the lysin does not form dimers via disulfide bridges, and [2] recombinant PlySs2 folded properly – LySMP requires reducing agents (Wang, Sun, and Lu 2009). The lysins may differ in this requirement, because they vary in cysteine residues – LySMP has five, while PlySs2 has just one. Alternatively, the EDTA-induced inhibition of PlySs2 suggests that it may rely upon a divalent cation as a cofactor.

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6.1.3 Catalytic Domain

The PlySs2 catalytic domain is less lytic than the full-length protein, but still retains specificity. We had to use a much greater concentration of PlySs2-CD compared to PlySs2-FL, to mediate lysis. Nonetheless, our results suggest that a degree of specificity lies in the catalytic domain alone. Other phage lysins have CHAP domains (Nelson et al. 2006, Baker et al. 2006, Daniel et al. 2010, Becker et al. 2009, Donovan et al. 2006), but PlySs2 has a distinct primary sequence from other database CHAP domains (all pairwise E-values $>10^{-15}$) (Schmitz 2011). In Figure 2.2, the CHAP domain of PlySs2 is aligned to PlyC with little identity (28% sequence identity, E-value = 10^{-8}) (Nelson et al. 2006). This indicates that there are novel features in the PlySs2 catalytic domain that may influence the lysins specificity, but the target is currently unknown.

CHAP domains may act as alanine-amidases (Nelson, Loomis, and Fischetti 2001) or cross-bridge endopeptidases (Daniel et al. 2010). Further, some strains of *S. suis* have no cross-bridge while others do (Kilpperbalz and Schleifer 1987). Therefore, while the PlySs2 CHAP domain mediates the catalytic activity of PlySs2, it probably does not confer the entire specificity of the enzyme. Going forward, the active site will need to be determined, possibly via the novel method involving mass spectrometry employed with the newly identified, anti-streptococcal lysin PlyPy (Lood et al. 2014).

6.1.4 Binding Domain

The promiscuity of PlySs2 casts even more uncertainty on the target for this binding domain. The cross-bridge varies greatly across all PlySs2-susceptible specimens, so it is an unlikely target for the PlySs2 binding domain. These cross-bridges can acquire variations leading to lysostaphin resistance, but PlySs2 displayed activity against both

lysostaphin-sensitive and lysostaphin-resistant (LyrA) *S. aureus* strains. Accordingly, experiments are in progress to determine the binding and cleavage substrates for PlySs2.

In our work studying the binding domain, we've found that it differentially binds species and even strains of the same species. While the activity of the binding domain does not seem to correlate with that of the full-length enzyme (again suggesting some specificity attributed to the catalytic domain), the binding domain of PlySs2 does seems to contribute to its unique activity profile. Previous phage lysin binding domains have been shown in general to determine lysin specificity (Hermoso et al. 2003, Grundling and Schneewind 2006). As shown, PlySs2 has an SH3b domain, which has been shown to increase endolysin lytic domain activity against staphylococci and streptococci (Donovan et al. 2006). SH3 domains are commonly seen in viral and bacterial cell wall-binding proteins, although the exact molecular target remains unknown (Xu et al. 2009). SH3b (bacterial homologues of SH3 domains) have been shown to bind metals and polypeptides (Pohl, Holmes, and Hol 1999, Wylie et al. 2005). As such, an SH3b domain of a *B. cereus* endopeptidase has been shown to bind the free amino group of the N-terminal alanine in the stem peptide of the peptidoglycan (Xu et al. 2010). The ubiquity of this amino acid domain in peptidoglycans, suggests this amine could also be the substrate for the broad binding of the PlySs2 SH3b domain.

6.2   *S. suis* susceptibility to PlySs2

6.2.1   Lytic activity

The majority of examined *S. suis* strains were very sensitive to the PlySs2 lysin as seen in lytic, bactericidal, and MIC assays. As such, PlySs2 could alter the way veterinarians and clinicians treat *S. suis* in pigs and humans. Neither the *S. suis* type strain

S735 nor the pathogenic *S. suis* strain 7997 developed resistance to PlySs2 when tested *in vitro*. A critical strength of PlySs2 is its wider specificity to a subset of Gram-positive bacterial pathogens, including *S. suis* (Gilmer et al. 2013), enabling broader protection from these organisms.

There was consistency among the lytic, bactericidal, and MIC assays; each returned qualitatively correlative results – i.e., the most sensitive strains displayed high susceptibility in each test. The MIC of other *S. suis* lysins have not been published, but the PlySs2 MIC for *S. aureus* and *S. pyogenes* are similar to those of *S. suis* (Table 1, (Gilmer et al. 2013)). For many clinical strains of *S. suis*, the MICs of ampicillin, amoxicillin, ciprofloxacin, kanamycin, and rifampin is >640 μg/ml (Meng et al. 2011). This MIC level is higher than the PlySs2 MIC against all but one of the *S. suis* strains we tested (Table 1). On a molar basis, with the molecular weight of PlySs2 being 26,060 g/mol, PlySs2 is several-fold more effective than antibiotics, which are usually ≤500 g/mol (e.g., gentamicin ~478 g/mol).

6.2.2   Implications of lytic activity

PlySs2 could have promise *in vivo* against nearly all *S. suis* strains (save serotype 12 – the only tested serotype that was not moderately susceptible to PlySs2) (Figure 3.1). The reason for this innate resistance is unknown at this time, but could be due to differences in the cell wall that prevent lysin access to the peptidoglycan. Modifications in the binding or catalytic substrates of the *S. suis* serotype 12 cell wall could also nullify PlySs2 activity. Nevertheless, all other strains including both pathogenic strains 10 and 7997 were highly sensitive to PlySs2. As with some of the *S. suis* strains, PlySs2 was previously shown to display moderate activity against *S. pyogenes* strain MGAS 5005 *in*

vitro ([Gilmer et al. 2013](#)). However, despite this lower efficiency against *S. pyogenes* 5005, PlySs2 is able to protect mice from bacteremia by strain MGAS 5005, suggesting that PlySs2 could also be protective *in vivo* to moderately susceptible *S. suis* strains ([Gilmer et al. 2013](#)). Alternative reasons for the various levels of activity among *S. suis* strains could also be due to a variety of differences including, but not limited to: i) encapsulation ii) cell wall thickness iii) catalytic domain specificity and iv) expression of the cell wall receptor to which the binding domain attaches.

6.3   Broad Gram-positive susceptibility

6.3.1   Lysin specificity

PlySs2, has demonstrated broad lytic activity against multiple Gram-positive pathogens, including *S. pyogenes* and *S. aureus*, *in vitro*. Most previously characterized lysins, by contrast, demonstrate activity against a narrow spectrum of species. Contrary to the broad activity of PlySs2, a noted strength of many lysins is their target-specificity. Antibiotics may kill commensal organisms along with target pathogens, potentially leading to adverse sequelae (e.g. diarrhea, or more serious *Clostridium difficile* complications) ([Kuijper et al. 2006](#)). By contrast, lysins might be used to treat a single pathogen without disrupting the normal bacterial flora ([Fischetti 2008](#)), although this specificity could admittedly be a limitation in treating multiple pathogens. Our results show it is possible that a single lysin like PlySs2 could be used to treat multiple Gram-positive pathogens, leaving many other Gram-positive and all Gram-negative commensals unaffected.

PlySs2 did not lyse any commensal lactobacilli and showed significant activity against other genera, killing some strains of *Listeria* and *E. faecalis*, but not *E. faecium* or

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strains of bacilli. It is unlikely that PlySs2 could be used therapeutically for *S. mutans*, *S. oralis*, *S. rattus*, and *S. sobrinus* as well as some strains of *S. pneumonia*, because of its low activity against these pathogens.

6.3.2   Broad PlySs2 lytic activity

PlySs2 exhibits activity against members of two distinct phylogenetic orders: *Bacillales* (*Staphylococcus*, *Listeria*, et al.) and *Lactobacillales* (*Streptococcus*, *Enterococcus*, et al.). The peptidoglycan structures of these two orders are quite similar except for their cross-bridges, which vary widely in composition and length (Schleifer and Kandler 1972, Vollmer, Blanot, and de Pedro 2008). Phage lysins rarely act on both *Bacillales* and *Lactobacillales* (Loeffler, Nelson, and Fischetti 2001, Schuch, Nelson, and Fischetti 2002, O'Flaherty et al. 2005, Zimmer et al. 2002, Loessner et al. 1997, Daniel et al. 2010). Furthermore, other lysins usually retain greater activity against the species infected by the phage from which the lysin was cloned (Yoong et al. 2004, 2006), whereas PlySs2 demonstrated more activity against *S. aureus* than *S. suis*. The lack of activity against Gram-negatives like *Escherichia coli* is expected, because their outer-membrane shields their peptidoglycan layer.

Lysing only 2 of 6 listeria strains tested, PlySs2 activity against listeria was less determinate. The activity of PlySs2 against *Staphylococcus simulans*, *Streptococcus equi zooepidemicus*, *Staphylococcus equi*, *S. suis* provides further evidence that the substrate for the binding domain exists outside of the cross-bridge, because their cross-bridges vary (with some having no cross-bridge at all). The polysaccharide capsule around *S. agalactiae* enhances its virulence. Type II *S. agalactiae* has a thicker capsule than most, and has a correspondingly higher level of virulence (Yeung and Mattingly 1984). *S.*

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agalactiae strains with thinner capsules have been shown to be less virulent ([Rubens et al. 1987](#), [Wessels et al. 1989](#)). Importantly, PlySs2 has comparable activity against *S. agalactiae* with and without a capsule, Type II, and 090R respectively.

6.3.2.1 *S. aureus* susceptibility to PlySs2

All tested strains of *S. aureus* were highly susceptible to lysis by PlySs2 (Figure 4.1), including strains resistant to methicillin, vancomycin, daptomycin, mupirocin, and lysostaphin. Its lytic activity against vancomycin-intermediate *S. aureus* (VISA) and Newman strains was somewhat less than its lytic activity against other staphylococcal strains. The reduced activity against the VISA strains could be the result of the thicker cell wall in these organisms ([Sieradzki and Tomasz 2003](#)), increasing the time necessary to result in lysis. The strong activity of PlySs2 against *S. simulans* and *S. epidermidis* supports its use to treat a wide array of other staphylococcal infections.

6.3.2.2 Streptococcal susceptibility to PlySs2

Many streptococci were susceptible to PlySs2 lysis *in vitro*. PlySs2 exhibited potent lytic activity against its native species, *S. suis*, as well as *S. equi*, *S. equi zooepidemicus*, and *S. pyogenes*. Of note, there was no difference in PlySs2 activity against unencapsulated or highly encapsulated variants of *S. pyogenes*. There are greater than 150 M types for *S. pyogenes* ([Bessen et al. 2011](#)). Remarkably, PlySs2 has activity against all the tested M-types. Moderate activity was observed against groups C, E, and G streptococci, suggesting *in vivo* experiments will be necessary to determine if PlySs2 could be used to treat infections by these organisms. PlySs2 activity against *S. sanguinis* also indicates its potential to treat dental *plaque*. Further studies must investigate its efficacy against *S. sanguinis* after biofilm development.

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Although the *in vitro* activity of PlySs2 is more robust against MRSA than *S. pyogenes*, our *in vivo* data demonstrates its efficacy as an effective therapeutic against each. This may be due to differences in the structure or composition of the *S. aureus* cell wall compared to that of *S. pyogenes* affecting substrate accessibility. It could also be a result of the pathogenesis of each species and how they interact with the murine host.

6.3.3   Broad bactericidal activity against streptococci and staphylococci

As with the activity against *S. suis*, we found that the bactericidal assays quantitatively confirm PlySs2's ability to effectively kill strains that vary in drug resistance and encapsulation; with correlations between the lytic, bactericidal, and MIC assays. Thus, even though they were not tested, it is likely that pathogens that were found to be more sensitive to *in vitro* PlySs2 lysis than *S. pyogenes* MGAS 5005, may also be sensitive to PlySs2 *in vivo*. Of note, the MIC of PlySs2 against several *S. aureus* strains was less than or equal to the MICs of other staphylococcal lysins including ClyS, LysK, and CHAP$_K$ (Pastagia et al. 2011, Daniel et al. 2010, Fenton et al. 2011, Becker et al. 2009).

6.4   Absence of *in vitro* resistance to PlySs2

6.4.1   No observable *in vitro* resistance to PlySs2 in *S. suis*

Using the same conditions that led to gentamicin resistance, the pathogenic *S. suis* strain 7997 and the type strain S735 were unable to establish resistance to PlySs2. This result is consistent with other lysins, such as ClyS and PlyG (Schuch, Nelson, and Fischetti 2002, Pastagia et al. 2011). Antibiotic resistance may occur when bacteria either inactivate the drug or alter the target site. No molecule has been described to extracellularly inactivate lysins before they cleave the cell wall. Because PlySs2 lyses

disparate bacterial species with either diverse peptidoglycan cross-bridge structures, or no cross-bridge at all (Robinson, Hardman, and Sloan 1979), the PlySs2 cleavage site is unlikely to be the cross-bridge, but a more common peptidoglycan structure. Since lysins have evolved to target essential cell wall structures (Fischetti 2008), it may be difficult for structural resistance to rapidly occur.

6.4.2 No observable *in vitro* resistance to PlySs2 in MRSA or *S. pyogenes*

All of the Gram-positive pathogens against which PlySs2 has activity have developed resistance against conventional antibiotics. Neither *S. pyogenes* nor MRSA were able to establish resistance to PlySs2 under conditions leading to mupirocin resistance. As mentioned, a PlySs2-susceptible pathogen may not easily alter the PlySs2 peptidoglycan target. To date, the only known resistance to a lysin-like molecule involves the insertion of a serine residue into an *S. aureus* pentaglycine cross-bridge to establish resistance to lysostaphin (a non-phage endopeptidase from *S. simulans*). However, it is unlikely that PlySs2 is a canonical cross-bridge endopeptidase, because of its activity against disparate bacterial species with diverse cross-bridge structures (Robinson, Hardman, and Sloan 1979), which included lysostaphin-resistant staphylococci.

6.5 Intranasal mucosa decolonization

PlySs2 is one of the most effective lysins used to decolonize the murine intranasal mucosa of pathogenic bacteria (Cheng et al. 2005, Loeffler, Nelson, and Fischetti 2001, Nelson, Loomis, and Fischetti 2001, Rashel et al. 2007, Schuch, Nelson, and Fischetti 2002, Daniel et al. 2010). It is also the first lysin tested in combination with an antibiotic against *S. suis in vivo* and shown to be more effective than each compound alone.

On a molar basis, our results show that PlySs2 can kill *S. suis in vivo* several-fold more effectively than gentamicin, without killing as broadly as gentamicin. During treatment of an *S. suis* infection with antibiotics, both commensal and pathogenic bacteria would be affected, causing deleterious sequelae in the treated individuals. However, PlySs2 used alone to treat an *S. suis* infections should have minimal effect on the normal bacterial flora.

6.5.1   Lysin alone or with antibiotic

Colonization by *S. suis* could be reduced by PlySs2 alone or in combination with gentamicin. An important finding in this thesis was that a single dose of PlySs2 could reduce the pathogenic *S. suis* strain 7997 from murine intranasal mucosa by >4 logs (Figure 5.1). This is significantly greater than the <3-log reduction of strain 7997 after treatment with gentamicin. PlySs2 + gentamicin resulted in a >5-log decrease in intranasal carriage of strain 7997 after a single dose, suggesting that such a combination can be used together for increased effectiveness. This builds on previous studies reporting beneficial interactions between lysins and antibiotics – the first being the *in vitro* synergy of Cpl-1 with gentamicin and penicillin (Djurkovic, Loeffler, and Fischetti 2005). *In vivo* synergy has been reported between ClyS and oxacillin (Daniel et al. 2010), and recently also between PlySs2 and daptomycin, vancomycin, or oxacillin (Schuch et al. 2013).

Other lysins have been shown to decolonize staphylococci, streptococci, or pneumococci in oral and nasal animal models (Cheng et al. 2005, Loeffler, Nelson, and Fischetti 2001, Nelson, Loomis, and Fischetti 2001, Rashel et al. 2007, Schuch, Nelson, and Fischetti 2002, Daniel et al. 2010); but these lysin decolonization models did not include antibiotic combinations, and none were tested against *S. suis*. Further, bacteria

have developed resistance to mupirocin, the main anti-infective used for mucous membrane decolonization or carriage reduction of staphylococci (Hudson 1994). Our results indicate that lysins could be used in combination with antibiotics for mucosal decolonization, capitalizing on the strengths of both lysins (rapid, specific killing) and antibiotics (longer half life).

6.5.2 Prophylaxis

A pregnant sow carrying *S. suis* in her nasal mucosa may transmit the pathogen to her piglets (Clifton-Hadley 1984, Robertson and Blackmore 1989), resulting in fatal *S. suis* infections (Robertson et al. 1991, Amass, Wu, and Clark 1996, Torremorell, Calsamiglia, and Pijoan 1998, Cloutier et al. 2003, Swildens et al. 2007). PlySs2 could be used to prophylactically remove or significantly reduce *S. suis* from the nasal mucosa of the pregnant or nursing sow until her offspring are beyond the age of acute *S. suis* susceptibility. This could be done with a minimally invasive topical treatment, as shown with our murine model, as lysins can be active in both liquid and gel formulations (Pastagia et al. 2011, Gilmer et al. 2013).

6.6 Mixed bacteremia protection

No lysin has been used to clear a bacteremic infection *in vivo* by more than one pathogenic organism; to our knowledge mixed infections have not been previously tested. An important finding in this study was our ability to clear mixed MRSA and *S. pyogenes* bacteremic infections from 92% of mice with a single dose of PlySs2. This survival rate is better than the 80% survival of mice treated with the combination of ClyS + PlyC (Figure 5.2C).

In a mixed infection, using either PlyC or ClyS alone eliminated only one of the infecting pathogens, resulting in death by the other; this death occurred within the same time frame and rate as the corresponding singly infected, non-treated controls, i.e., MRSA-infected animals died in 24 hours and *S. pyogenes* infected animals in 3 days. All three curves of the infection experiments (Figure 5.2A, B, and C) come from the same initial inoculums in 4 experiments, i.e. mixed infection experiments always paired with both types of single infection experiments to show that the single infection alone would kill animals if not treated properly. These results strongly support the idea that PlySs2 is curing the animals of their infection by simultaneously killing both pathogens. Further, stronger results may arise from increased or repeated dosage.

6.6.1   Implications

This study demonstrates that a combination of lysins (PlyC and ClyS) with different specificities could be used effectively to treat mixed infections (Figure 5.2C). The chimeric staphylococcal lysin ClyS, has been shown to clear MRSA from a murine model of septicemia (Daniel et al. 2010). To date, besides our work on PlySs2, no lysin has been shown to clear more than one organism from a septicemia infection, and none has cleared a mixed infection of any kind.

Recent studies have indicated that secondary infections caused by colonizing MRSA, *S. pyogenes*, or *S. pneumoniae* have accounted for up to 90% of deaths from influenza pandemics (Morens, Taubenberger, and Fauci 2009, Brundage and Shanks 2007, 2008, Hussell, Wissinger, and Goulding 2009). The same pathogens caused complications in nearly 30% of the 2010 H1N1 pandemic cases (2009). Prophylactic usage could decrease the rate of these fatalities.

The moderate PlySs2 specificity for certain pathogens, and the lack of resistance in those pathogens qualifies PlySs2 for prophylactic usage. Mupirocin and polysporin are typically given to treat *S. aureus* on mucous membranes, but *S. aureus* can develop resistance to each (Hudson 1994). Lysins with specific activities against either staphylococci, streptococci, or pneumococci have each been shown to decolonize these pathogens in animal models of oral and nasal mucosal colonization (Cheng et al. 2005, Loeffler, Nelson, and Fischetti 2001, Nelson, Loomis, and Fischetti 2001, Rashel et al. 2007, Schuch, Nelson, and Fischetti 2002, Daniel et al. 2010), however, a mixture of the three enzymes would need to be used to remove these pathogens. PlySs2 alone could be used to decolonize susceptible populations of staphylococci, streptococci, and perhaps certain pneumococcal isolates during flu season to reduce the possibility of a secondary infection.

Furthermore, PlySs2 may also serve as a viable treatment for other infections and diseases caused by *S. aureus* and/or *S. pyogenes* such as: scarlet fever, erysipelas, cellulitis, necrotizing fasciitis, endocarditis, bacteremia/sepsis, a variety of abscess and non-abscess forming SSTIs, and impetigo. It may also treat neonatal septicemia, because it displays more *in vitro* activity against *S. agalactiae* than it does against *S. pyogenes* 5005 (Figure 4.1, Figure 4.3, Table 4.2). *S. aureus* and *S. pyogenes* cause diseases with similar pathologies and sites of infection in man. Clinicians are often unsure which organism is causing disease, which could be a mixed infection in severe trauma cases. PlySs2 would be a valuable tool in their repertoire to treat such cases.

As a lysin, PlySs2 rapidly kills its target, far quicker than antibiotics. PlySs2 only has to contact the bonds of the cell wall to mediate its effect; conventional antibiotics

must remain in contact with the bacteria for 1-3 hours before any effect is seen on bacterial viability is observed. This feature has enabled phage lysins to treat systemic infections in animal models (Loeffler, Nelson, and Fischetti 2001, Schuch, Nelson, and Fischetti 2002).

6.6.2   PlySs2 applications

One could also use PlySs2 to prophylactically clear human mucous membrane reservoirs of pathogenic bacteria resistant to antibiotics. Antibiotics are not used in this manner, because resistant bacteria could emerge. Penicillin can be used to treat *S. pyogenes*, which remains acutely sensitive; but if the impetigo is caused by MRSA, penicillin may be ineffective. If PlySs2 were used prophylactically and resulted in even a moderate drop in colonization of 10% or more, it could reduce the severity of this disease.

PlySs2 may treat primary infections and prophylactically decrease the likelihood of secondary infections. Given its rapid clearance of MRSA and *S. pyogenes*, PlySs2 could be used in the case of complications. This would be especially necessary for high-risk individuals, which are allergic to leading antibiotics, have been exposed to a series of antibiotics, or are immunocompromised (which leads to a higher rate of mortality from bacterial infection). In the septic mice, PlySs2 acted quickly enough that it could have an effect before being cleared from the animal. The results also indicate that PlySs2 remains active in blood.

In humans, there are no known substances to which lysins bind. Neither antibodies formed by mice nor humans are able to inactivate lysins (Loeffler, Djurkovic, and Fischetti 2003, Loessner et al. 2002, Rashel et al. 2007, Jado et al. 2003). When

administered intraperitoneally, Cpl-1 (an anti-pneumococcal lysin) cured mice of pneumonia ([Grandgirard et al. 2008](), [Witzenrath et al. 2009]()). This provides further *in vivo* evidence that lysins can act quickly and effectively at different sites from administration.

6.7   Other tests

In this report, we show PlySs2 was effective against *S. suis*, MRSA, and GrAS in murine decolonization and bacteremia models. Other *in vivo* models have been executed using PlySs2. This includes a MRSA rat wound infection model, which we will publish soon. In the Appendix, we detail the preliminary work behind a set of swine *S. suis* colonization and infection experiments. Our collaborators at Novartis have also executed a rabbit MRSA endocarditis experiment, but there were technical issues, which obfuscated the results. Finally, there are multiple promising *in vivo* models currently underway, which were designed by ContraFect Corp. in an effort to enter Phase I trials with PlySs2 (termed "CF-301" by ContraFect Corp.).

6.8   Importance

In summary, we have discovered a novel approach for treatment and prophylaxis of *S. suis* infection and/or colonization, with remarkable activity against all but one tested strain of *S. suis*. Pregnant sows treated prophylactically with PlySs2 prior to delivery, and *S. suis* piglet infections treated with PlySs2 alone or in combination with antibiotic could help control this disease on farms. Since we found that neither *S. suis* strain 7997 nor S735 developed resistance to PlySs2 *in vitro*, PlySs2 could be developed as a vital addition to the current approaches controlling *S. suis* spread in pigs, and zoonotic transmission causing fatal infections in humans.

While pursuing a novel treatment for *S. suis* infection, we discovered a lysin with broad lytic activity against strains of MRSA, VISA, *S. suis*, *Listeria*, *S. simulans*, *S. equi zooepidemicus*, *S. equi, S. agalactiae*, *S. pyogenes, S. sanguinis*, *S. gordonii*, group G streptococci, group E streptococci, *E. faecalis*, and *S. pneumoniae*. PlySs2 was relatively simple to produce, tractable, and very stable. We have demonstrated here the ability of PlySs2 to protect mice with mixed infection by MRSA and *S. pyogenes*. Neither of these pathogens was observed to develop resistance to PlySs2 *in vitro*. PlySs2 could therefore become a vital addition to the armamentarium against multi-drug resistant *S. aureus, S. pyogenes*, and various other Gram-positive pathogens.

PlySs2 represents a novel breakthrough in the field of bacteriophage lysin technology. It is now possible to envision other lysins with broad therapeutic activity retaining specificity to a subset of mostly pathogenic Gram-positives. For PlySs2, this novel capability possibly lies in the divergent PlySs2 CHAP domain, or SH3 binding domain. PlySs2 occupies a vital space in the spectrum between strict lysin specificity and unselective antibiotic activity. Ideally, a therapeutic should have activity against major pathogens without affecting commensals; this report of PlySs2 is the first to indicate that a lysin could serve that function.

7   CHAPTER 7 – CONCLUSIONS

Bacteriophages have evolved lysins to infect and destroy bacterial hosts over a billion years. Lysins provide a novel source of antimicrobials as antibiotic resistance expands. With technological advances, numerous lysins are being discovered. This study expands the lysin armamentarium with a vital addition and new applications.

We have introduced one of the first lysins with *in vivo* efficacy against multiple, leading, human bacterial pathogens. This is the first indication that a lysin with activity against two pathogens could be used to treat a mixed or indistinguishable infection – rather than using a broad-spectrum antibiotic. This study reinforces the strength of lysin technology as none of the bacteria treated *in vivo* developed lysin resistance *in vitro*.

The results indicate that lysins could be used combinatorially or with antibiotics, capitalizing on the strengths of both lysins and antibiotics. The latter approach could reduce the rate of antibiotic resistance, extending antibiotic utility. Having used two lysins to clear a mixed infection, we can now envision the application of a lysin armamentarium. A repertoire of lysins targeted to specific pathogens could be used for each pathogen independently, or in concert.

Many challenges remain. At this time, no lysins have been approved by the FDA for human therapeutic use. Furthermore, there are no reports describing the clinical development of a phage lysin, however these activities have begun for some enzymes. Because of the narrow spectrum of activity for some lysins, diagnostics for rapid pathogen identification would be useful for selecting the therapeutically relevant lysin. Nevertheless, the need for novel antimicrobials and advances in technology should mitigate these obstacles. Ideally, the development of therapeutic lysins will increase

faster than the prevalence of antibiotic resistance. This work is a contribution to that effort.

8   APPENDIX

8.1   PlySs2 minipig protection from *S. suis*

8.1.1   BACKGROUND

The first *in vivo* PlySs2 test included PlySs1 and occurred in minipigs. Prior to the discovery of PlySs2, Dr. Jonathan Schmitz discovered PlySs1 – a LySMP homologue – by screening the unsequenced genome of clinical *S. suis* strain 7711 ([Schmitz 2011](#)). After cloning and expression, PlySs1 was purified through ammonium sulphate precipitation and DEAE anion-exchange column chromatography.

8.1.2   MATERIALS AND METHODS

8.1.2.1   PlySs1 + PlySs2 preliminary drug interaction

PlySs1 and PlySs2 were tested to determine whether they would have an antagonistic, additive, or synergistic effect on their respective activity against *S. suis* strain 7997. As in previous chapters, log-phase bacteria were adjusted with buffer A to an OD_{600} of 0.8 to 1.0 in 96-well microtiter plates (Falcon). PlySs1 at 8 or 16 μg/ml, PlySs2 at 2 or 4 μg/ml, PlySs1 + PlySs2 (at 8 μg/ml and 2 μg/ml, respectively), or buffer B control vehicle was added to each sample well. Spectrophotometric readings (OD_{600}) of each well were taken by a Spectramax Plus 384 (Molecular Devices) every minute over one hour at room temperature. Lysin activity was gauged by the degree of turbidity reduction (OD_{600}) following enzyme addition.

8.1.2.2   Minipig treatment

To test PlySs1 and PlySs2 against *S. suis in vivo*, 22 minipigs were colonized with ~1×10^{9} CFU *S. suis* strain 7997; 12 were colonized by contact; 10 were colonized by

direct nasal inoculation. After 1 week, each group was separated into two equal subgroups.

Twice daily, half of the contact-colonized, and half of the inoculation-colonized pigs were treated. These treatment subgroups received, both nasally and orally, 5 ml saline solutions containing 1,118 mg of PlySs1 and 430 mg of PlySs2 twice daily. A higher dose of PlySs1 was given, because PlySs1 has is less active against *S. suis* than PlySs2. The increased dose of PlySs1 compared to PlySs2 was inversely proportional to their different *S. suis* activity. The untreated subgroups received 5 ml saline solution nasally and orally twice daily. This fatal infection rate was tracked over two weeks.

8.1.3 RESULTS

8.1.3.1 PlySs1 + PlySs2 preliminary drug interaction

PlySs1 and PlySs2 seem to have a synergistic effect on *S. suis*. When used at half the concentration together, they register a greater activity than either of them at double those respective concentrations. This suggests that their divergent structures confer binding and/or catalytic activity on different substrates within the cell wall.
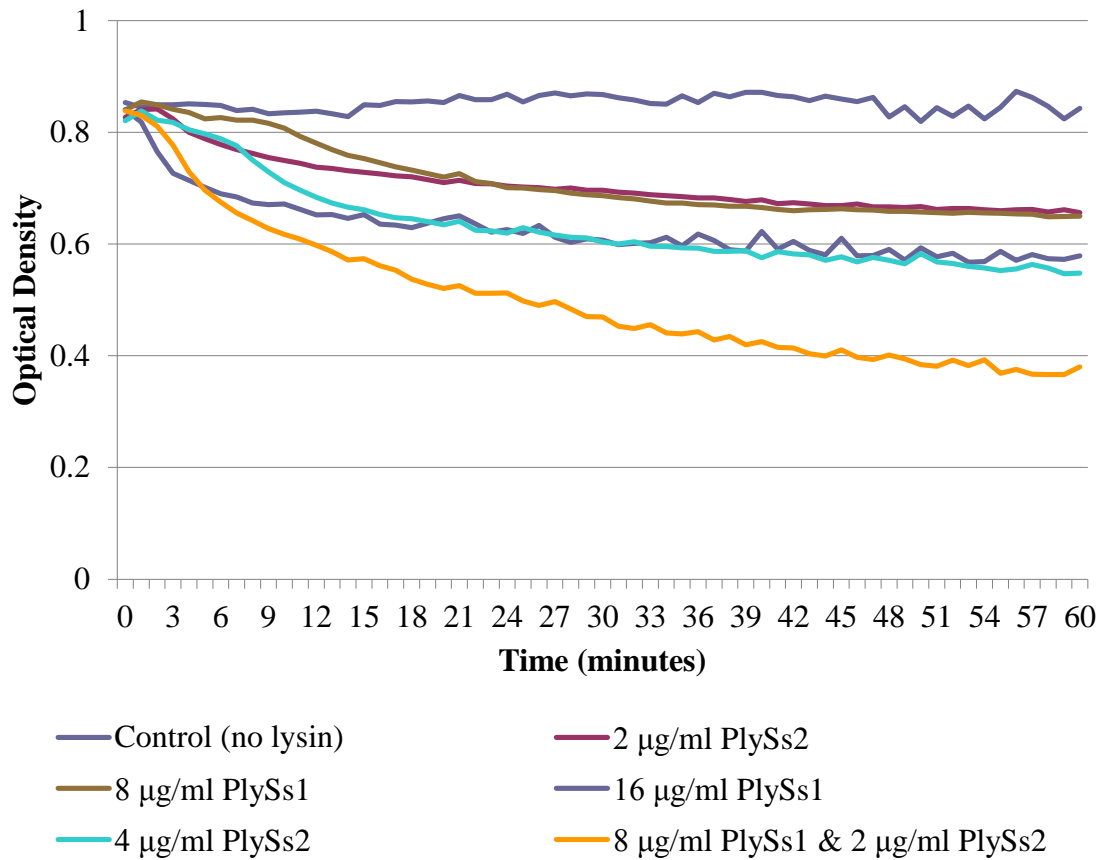
Figure 8.1 PlySs1 + PlySs2 drug interaction.

Log-phase *S. suis* 7997 were centrifuged and resuspended in PB to OD_{600} ~1.0. To test PlySs1 and PlySs2 interaction, 8 μg/ml PlySs1 and 2 μg/ml PlySs2 were mixed with *S. suis* strain 7997 in phosphate buffer. Tests were also run with 8-16 μg/ml PlySs1 and 2-4 μg/ml PlySs2 alone to indicate whether PlySs1 + PlySs2 combined activity was merely additive or synergistic. In controls (-), PB replaced lysin. Spectrophotometric readings were taken at OD_{600} every minute for an hour.

8.1.3.2 Minipig treatment

None of the contact inoculated pigs died over two weeks; neither in the control nor the test group. Of the minipigs directly inoculated with *S. suis*, 1/5 (20%) of the control pigs survived, while 4/5 (80%) of the treated pigs survived.
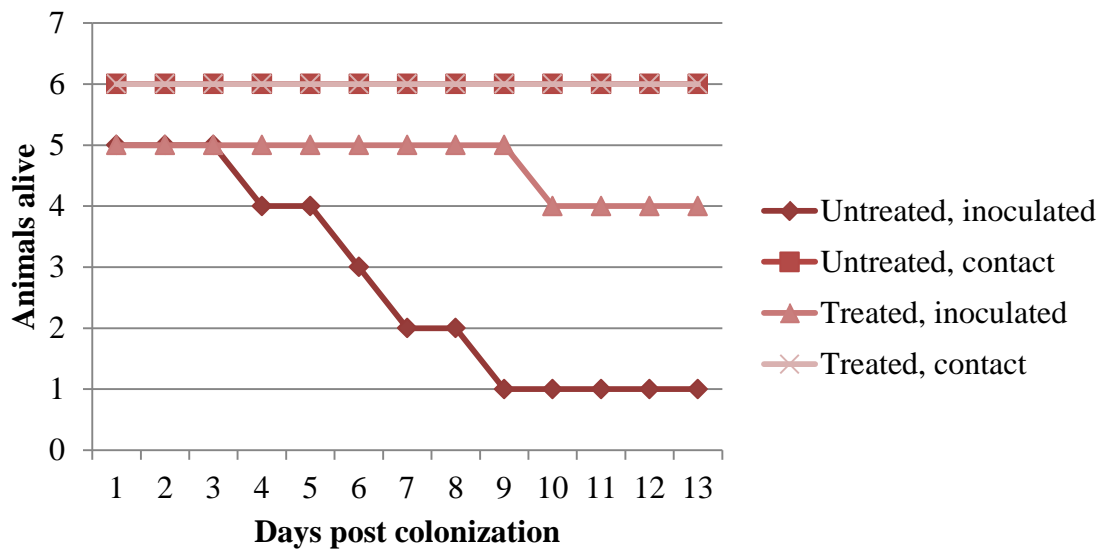
Figure 8.2 Pig deaths over two weeks.

Minipigs were either inoculated with ~1×10^9 CFU *S. suis* strain 7997 or put in contact with the inoculated pigs. The treated pigs received, both nasally and orally, 5 ml saline solutions containing 1,118 mg of PlySs1 and 430 mg of PlySs2 twice daily. Untreated animals received vehicle of the same volume at the same intervals.

8.1.4 DISCUSSION

The PlySs1 + PlySs2 drug interaction test was preliminary, because we did not execute a checkerboard broth microdilution assay to fully indicate synergism. Nonetheless, in this test, the combination of PlySs1 + PlySs2 was greater than either used at the same concentration individually (Figure 8.1), which is the definition of synergism.

From colony counts collected from individual minipigs (data not shown), it seems as though the inoculation-colonized minipigs carried higher levels of *S. suis* strain 7997 than the contact-inoculated animals. This is the best explanation for all of the minipigs contact-colonized surviving. Often, *in situ*, mother sows colonized with *S. suis* pass the pathogen to her piglets through contact. This transmission did not occur to the same extent in our study.

This study indicated very promising applications for PlySs1 + PlySs2 given their ability to protect 80% of minipigs inoculated with *S. suis* strain 7997 where only 20% of the controls survived. The results from this initial minipig study were very promising and led to further studies slated for submission to a journal later this year. Given PlySs2's novel structure, and higher activity against *S. suis* and other pathogens, we proceeded to focus on PlySs2 for all future *in vivo S. suis* studies after this minipig model.

8.1.5 ACKNOWLEDGEMENTS

Dr. Jonathan Schmitz purified all of the PlySs1 for the minipig study and aided in the shipping of all material. I am very thankful for Dr. Niels Dekker and Dr. Jaap Wagenaar of Utrecht University designed and executed the minipig *S. suis* infection model.

8.2   Rat oral cavity metagenomics

8.2.1 BACKGROUND

Animals contain symbiotic bacterial microbiomes composed of both commensal and pathogenic species. In humans, the cells of the gut microbiome outnumber human cells 10:1 ([Berg 1996](#)). Commensal bacteria assist in many of our bodily functions providing both digestive and immunologic benefits. They enable us to absorb nutrients from fibers our cells are unable to metabolize. Further, bacteria provide an intricate system of defenses within the body, suppressing pathogenic bacteria, and presenting antigens against which we develop antibodies.

Broad-spectrum, systemic antibiotics kill many commensals in addition to the pathogenic bacteria species causing the illness. This often causes diarrhea, which can lead to dehydration. Prolonged use can allow pathogenic bacteria (lying outside of the antibiotic's spectrum of efficacy) to flourish such as *C. difficile* ([Kuijper et al. 2006](#)).

A key benefit to using phage lysins is their specificity. This maximizes their rapid action against a single target. Lytic specificity should limit the perturbation to the microbiome of the patient. In order to test this hypothesis, we attempted to reduce a genus of bacteria and evaluate the effect on other bacterial levels in that environment.

ClyS was the best lysin for this study. All the other lysins are either too broad, or act on bacteria not typically found in the rat oral cavity.

8.2.2 MATERIALS AND METHODS

The rats were male Sprague Dawley rats (Strain Code 400 from Charles River), weighing 150-250 grams, born on the same date. Samples for culture were taken in preliminary experiments. These samples confirmed that a 2-log drop occurred *in vivo* among *S. aureus*. This was determined via CFU analysis of serial dilutions from swabs

taken before and after treatment (data not shows). Two morphologies were observed on staph-selective mannitol plates - a small white one, and the other yellow and smooth. Both the yellow and white staph were sensitive to PlySs2 (data not shown). I did a straight-forward lytic assay (reduced OD_{600} absorbance) taking readings every minute over an hour of exposure to varying concentrations of PlySs2 from 16-500 µg/ml. Each was suspended in BHI media with 25% glycerol. Aliquots were shipped to collaborators for sequencing to establish exactly which strains to expect to observe a reduction.

In the final test, all rats were given one week to acclimate in our animal facility and environment. Then, across two weeks, the oral cavities of 14 rats were sampled daily, day 1 through day 8. On day 8, nine rats were treated with lysin while the remaining rats received an equal volume of vehicle. All rats were housed individually and numbered. Each rat was treated with 200 µl of 1 mg/ml ClyS or its vehicle for 15 minutes (while anesthetized) twice, over 2 hours. After the first treatment, rats were placed in new bedding without food or water. Thirty minutes after the second treatment, fresh food and water were provided. After treatment the rats were additionally swabbed 4 hours after each being treated with ClyS or control vehicle. The oral cavities were then sampled daily until day 15. Thereafter, rats were sampled weekly, day 22, and day 29. Furthermore, at each sample collection, 1-2 fresh fecal pellets were also obtained with sterile tweezers for additional microbiological analysis.

Samples were collected from the oral cavity by inserting a single swab and rolling it over the back of the tongue (approx. 3-4 cm from the tip of the tongue). This single swab samples the tongue, cheeks, roof of the mouth, and top of the throat. Separate swabs were used for culture. Swabs for 16S rDNA profiling (deep sequencing) were tubed and

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frozen at -80°C. Following the standard protocols from the Human Microbiome Project, we employed sterile Catch-All™ sample collection swabs from Epicentre Biotechnologies (Madison, WI) for all oral sample collection ([Aagaard et al. 2013](#)).

All samples were collected in freezing vials that did not contain any preservatives. They were subsequently stored at -80°C immediately after collection, to minimize any growth that may alter the composition of the microbial samples. For transport, samples were placed on dry ice in a styrofoam container with the lid secured and shipped overnight by FedEx®. The samples never thawed during shipment. No culture samples were taken from the animals in the test to minimize disruption from multiple swabs.

Two rats of the 14 rats died during the study due to suffocation during oral treatment. Therefore, in the following results, the test rat numbers are 1, 2, 3, 4, 5, 7, 9, 10, 11. The controls rat numbers are 12, 13, 14. This nonlinear numbering comes from the deceased rats being previously labeled 6 and 8. In total, 232 swabs and 164 pellets were shipped; near 400 samples in total. To determine background DNA levels, our collaborators extracted DNA from devices used for sample acquisition, storage and extraction (e.g. tubes, swabs).

8.2.3 RESULTS

The test run yielded a 1-2 log drop (reducing the *S. aureus* to an average of approximately 1-10% its original levels in 5 rats). In a couple rats, there was about a 100-fold reduction, while a few had reduction was less than even 10-fold. In the three controls given buffer rather than ClyS, *S. aureus* CFU increased slightly in two and reduced in one.

The *S. aureus* 16S rDNA level was near the threshold of detection, so our collaborators decided to re-sequence some of the samples to obtain more sequences in total per sample. Our collaborators plan to set up a Staph qPCR assay. Interestingly, they found a non-staphyloccal strain of interest, in which the operational taxonomic unit (OUT) is significantly reduced within the oral microbiota 4 hours after treatment. It shows no sensitivity to ClyS. Past work indicates that this strain may have a metabolic dependence on other organisms such as *S. aureus*.

It seems as though ClyS has a subtle effect on the oral cavity. The two most dominant genera in the rat oral cavity before treatment are *Actinobacteria* (Rothia) and streptococci. Both are resistant to ClyS, and their relative levels do not seem to change after treatment. There may be significant changes among low-abundant taxa, but resequencing will be needed to provide more resolution. Initially, it seemed as though some commensal staph strains increased short-term after lysin treatment although there was no increase in *S. aureus*. Further testing will need to confirm whether these staph species occupy the niche vacated by *S. aureus* lysis. On average, there was an increase of *Veillonella* abundance after treatment.

Figure 8.3 There were two predominant phyla in the oral cavity.

Most of the bacteria, by genomic quantification, belong to the bacterial phyla *Actinobacteria* and *Firmicutes*. These two phyla compose approximately 70% and 20%, respectively, of the species by number. Combined, they accounted for at least 60% of the species in all samples.
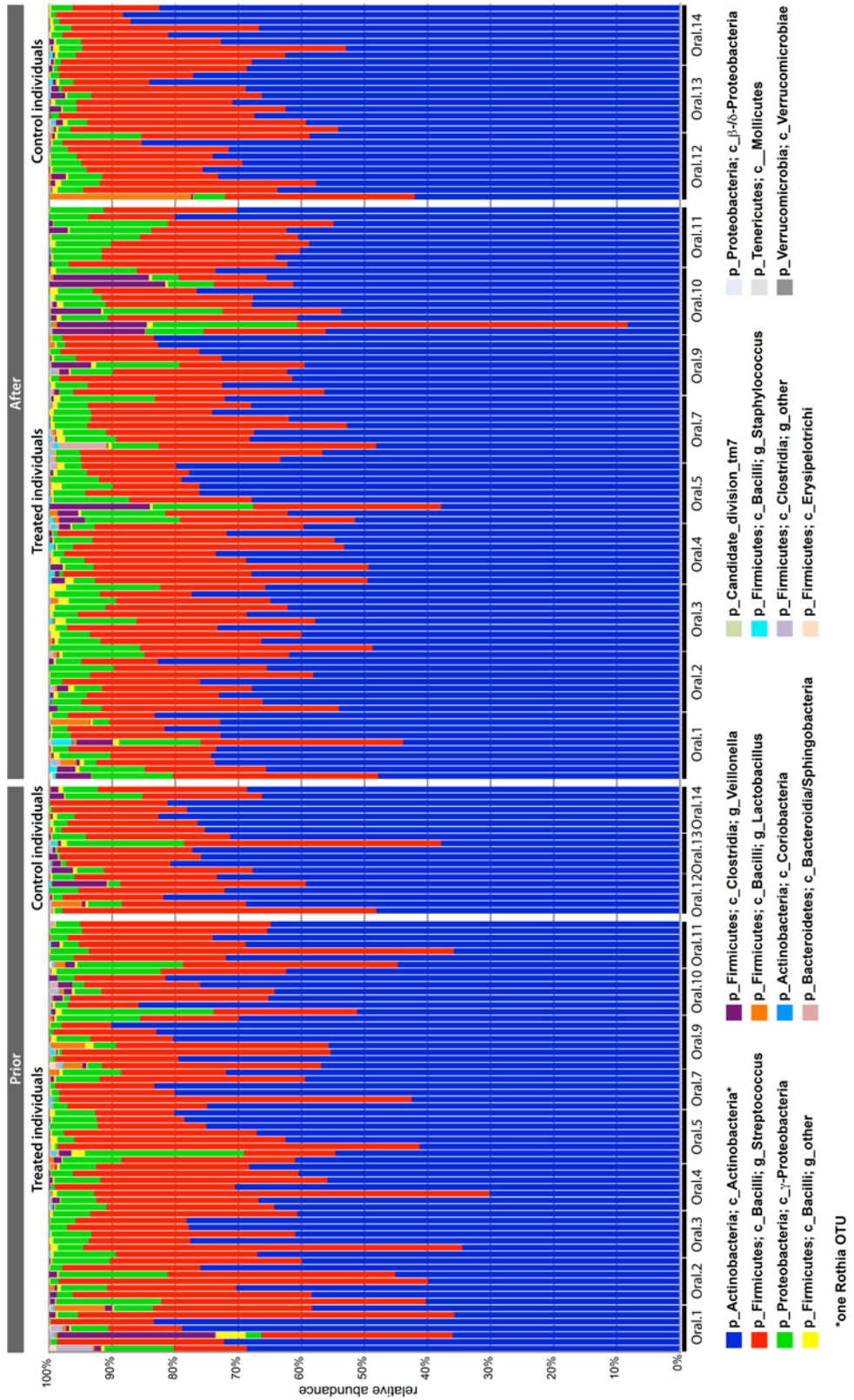
Our collaborators are currently re-sequencing samples for higher resolution to gather more subtle changes that may have occurred after treatment. The two main issues that must be addressed are: [1] if the staph in the test rats of the first test were significantly reduced, and [2] had some consistent affect on other species as compared to the controls.

8.2.4   DISCUSSION

Lysins should have less effect on any microbiome due to their specificity. ClyS seems to have had a minimal effect on the rat oral cavity. More sequencing will be necessary to confirm the degree to which ClyS removed *S. aureus*. Nonetheless, if *S. aureus* was dramatically decreased, the minimal effect would support the theory that lysins are less disruptive to a system than broad-spectrum antibiotics.

This is a very preliminary experiment, so there are a number of variables one would want to mitigate upon repeating a similar study. From the start, we planned to retroactively test ClyS against other species of interest after sequencing the rat oral microbiota. It may also be worth also examining isoflurane effects by swabbing a few new Sprague-Dawley rats before and after 15 minutes exposure to isoflurane. We could also test any affect of the swabs as well. However, any perturbations to the microbiome within the mouth due to daily swabbing, twice on day 7, and anesthetization should be seen or accounted for in the control as well. In the future, it would be good to get swabs before and after the test in order to test species for lysin sensitivity, morphology, or other characteristics.

Complete reduction of *S. aureus* was not necessary, just a couple logs so that it would show up in the sequencing of the microbiota in the test rats. One would expect there to always be some *S. aureus* persisting in crevices of the oral cavity. It would also be good to get all numbers (colony counts, sequencing reads, etc.) from the same experiment (rats). A strength of this study is that either result from *S. aureus* removal (other species affected OR not affected) would be important. A main concern i the possible challenge of knocking down *S. aureus* with certainty in the tests versus controls. It also seems that the coprophagous behavior of the rats contaminated a few of the oral swabs and thus other methods to reduce this variable may be necessary.

Since the most dramatic shifts in number seem to be among taxa of relatively low abundance, our collaborators have to execute very deep sequencing to resolve the changes in number among these species. This work also has the importance of surveying the microbiota of the rat oral cavity, which has not been well characterized to date (even if specific to this distributor and these rats).

In the future it may be worth trying the following experimental design. Infect mice with *S. aureus* as in the aforementioned bacteremia model. Then, treat with a very broad spectrum antibiotic or ClyS injected IP, daily for 14 days. Sample retrieval would be a challenge throughout the study, but collecting fecal pellets daily might reveal some gut microbiota changes. At the end of the 14 days, the mice could be killed and their entire intestinal tract could be removed (end to end, clamped, washed, and frozen, or even homogenized and counted).

8.2.5 ACKNOWLEDGEMENTS

Thank you to Dr. Sünje Pamp and Dr. David Relman of Stanford University for helping to design this study and for executing all of the sequencing.

8.3 Structural studies

8.3.1 BACKGROUND

A few lysin structures have been solved – there are approximately 10 in Protein Data Bank (PDB) as of early 2014. Given PlySs2's unique domain sequence, we sought to solve the structure of this novel phage lysin. So far, the results have not led to a diffraction pattern from which we can solve the structure. Nonetheless, extensive screening of crystallization conditions has yielded crystals in multiple conditions.

8.3.2 METHODS

Purified PlySs2 protein was buffer exchanged into 25 mM Tris / 50 mM NaCl during a polishing step using size exclusion chromatography (HiLoad 16/60 Superdex 75 pg, GE Healthcare). Fractions were analyzed using SDS-PAGE and those that contained protein corresponding to the MW of PlySs2 were pooled and concentrated to 15 mgs/ml for use in vapor-diffusion crystallization trials. Initial hits were obtained in sparse-matrix screens using the sitting drop method at a 1:1 mixture of protein and mother liquor in a drop volume of 0.6 μl. Subsequent fine screening of conditions and seeding experiments were carried out using the hanging drop technique and larger drop and mother liquor volumes (1-10 μl and 0.5-1 ml, respectively). Protein concentration was also screened as a variable (9-30 mgs/ml).

8.3.3 RESULTS

For crystallization, the most promising conditions contained 40-50% (v/v) 2-methyl-2,4-pentadiol and 0.1 M Tris buffer, in a pH range of 8-8.6 (Figure 8.4). Protein crystals, confirmed by UV light imaging, were mounted in fiber loops ranging in size from 0.05-0.5 mm, flash frozen in a nitrogen stream at 100 K, and subjected to diffraction experiments using either a rotating anode X-ray generator at our home source or synchrotron radiation at Brookhaven National Synchrotron Light Source beamline X29. Crystallization and diffraction experiments are ongoing. To date, more than 200 crystals have been screened, and although diffraction has been detected, none of the data has been suitable for indexing.
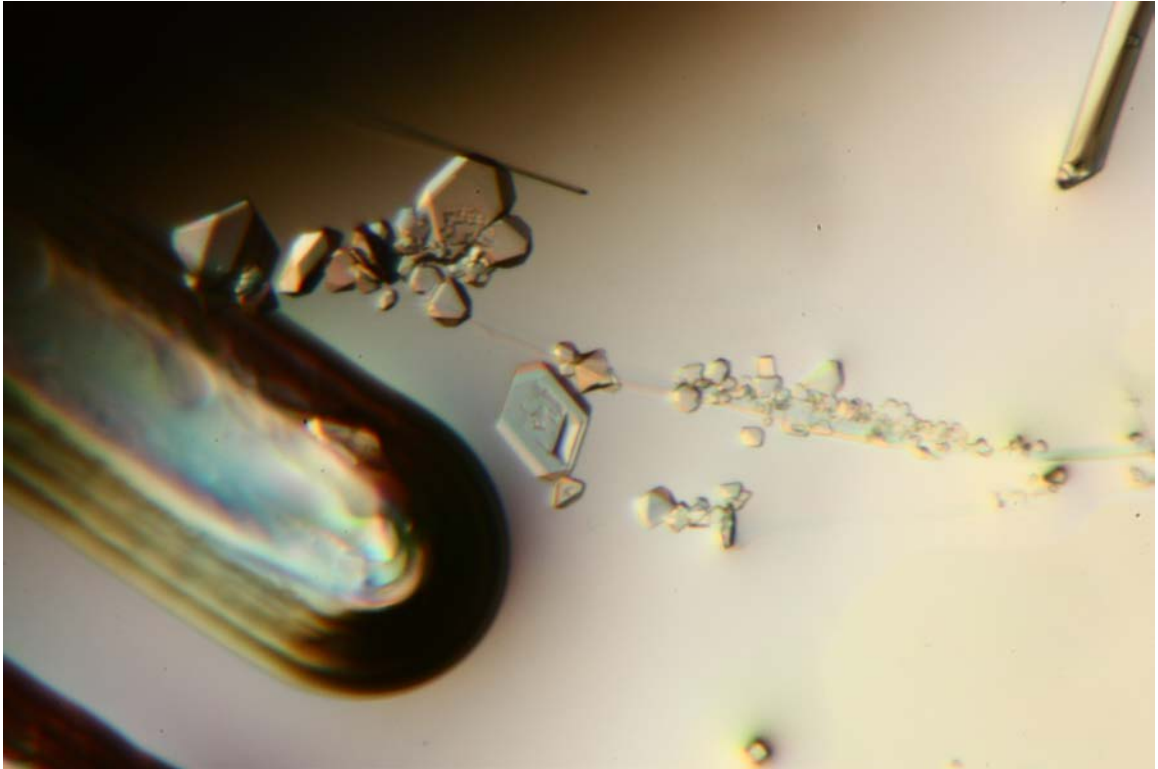
Figure 8.4 Crystal of PlySs2.

This crystal, which is approximately 100 μm, resulted from PlySs2 at 9 mg/ml mixed with a crystallization cocktail of 40-50% (v/v) 2-methyl-2,4-pentadiol and 0.1 M Tris buffer, pH 8-8.6.

8.3.4 ACKNOWLEDGEMENTS

8.4 DNA AND AMINO ACID SEQUENCES

PlySs2

- GenBank Accession Number: ZP_03625529 (from *S. suis* strain 89/1591)

- 738 base pairs; 245 amino acids; 26.06 kDa; Theoretical pI = 9.06

PlySs2 nucleotide sequence:

ATGACAACAG TAAATGAAGC ATTAAATAAT GTAAGAGCTC AGGTTGGGTC

CGGTGTGTCT GTTGGCAACG GCGAATGCTA CGCTTTGGCT AGTTGGTACG

AGCGCATGAT TAGTCCGGAT GCAACTGTCG GACTTGGCGC TGGTGTGGGC

TGGGTCAGCG GTGCAATCGG CGATACAATC TCTGCCAAAA ACATCGGCTC

ATCATACAAC TGGCAAGCTA ACGGCTGGAC AGTTTCCACA TCTGGTCCAT

TTAAAGCAGG TCAGATTGTG ACGCTTGGGG CAACACCAGG AAACCCTTAC

GGACATGTGG TAATCGTCGA AGCAGTGGAC GGCGATAGAT TGACTATTTT

GGAGCAAAAC TACGGCGGGA AACGTTATCC CGTCCGTAAT TATTACAGCG

CTGCAAGCTA TCGTCAACAG GTCGTGCATT ACATCACACC GCCTGGCACG

GTCGCACAGT CAGCACCCAA CCTTGCAGGC TCTCGTTCCT ATCGCGAGAC

GGGCACTATG ACTGTCACGG TCGATGCTCT CAATGTTCGC AGGGCGCCAA

ATACTTCAGG CGAGATTGTA GCAGTATACA AGCGTGGTGA ATCATTTGAC

TATGATACTG TCATCATCGA TGTCAATGGC TATGTCTGGG TGTCTTACAT

AGGCGGCAGC GGCAAACGTA ACTACGTTGC GACGGGCGCT ACCAAAGACG

GTAAGCGTTT CGGCAATGCT TGGGGTACAT TTAAATAA

PlySs2 amino acid sequence:

MTTVNEA**LNN** **VRAQVGSGVS** **VGNGECYALA** **SWYERMISPD** **ATVGLGAGVG**

WVSGAIGDTI **SAKNIGSSYN** **WQANGWTVST** **SGPFKAGQIV** **TLGATPGNPY**

GHVVIVEAVD **GDRLTILEQN** **YGGKRYPVRN** **YYSAASYRQQ** **VVHYIT**PPGT

VAQSAPNLAG S**RSYRETGTM** **TVTVDALNVR** **RAPNTSGEIV** **AVYKRGESFD**

YDTVIIDVNG **YVWVSYIGGS** **GKRNYVAT**GA TKDGKRFGNA WGTFK

- CHAP enzymatic domain (PF05257)

- SH3-Type 5 binding domain (PF08460)

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