Abstract

Title of dissertation: AN INVESTIGATION ON A BACTERIOPHAGE ENDOLYSIN POSSESSING ANTIMICROBIAL ACTIVITY AGAINST ANTIBIOTIC-RESISTANT *STAPHYLOCOCCUS AUREUS*

Sara Beth Linden, Doctor of Philosophy, 2016

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Staphylococcus aureus is one of the most common causes of nosocomial (i.e. hospital-acquired) infection. Significantly, over 90% of *S. aureus* strains are resistant to penicillin, and since the mid-1980's, methicillin-resistant *S. aureus* (MRSA) strains have become prevalent in hospitals worldwide, with resistance rates approaching 70%. In the U.S. alone, MRSA is responsible for over 100,000 invasive life threatening infections, such as necrotizing fasciitis, and causes 20,000 deaths annually. More worrisome, a variant known as community-acquired MRSA (CA-MRSA) is spreading in schools, gymnasiums, and even professional sports teams, where it infects otherwise healthy adolescents and young adults. Vancomycin is often considered the last antibiotic of choice against MRSA and other Gram-positive pathogens. However, rates of vancomycin-resistant enterococci (VRE) have already reached 30% and it is widely believed that emergence of vancomycin-resistant *S. aureus* (VRSA) is due to gene

transfer during co-colonization of MRSA and VRE. Thus, alternative antimicrobial approaches are desperately needed. Endolysins, or peptidoglycan hydrolases, are phage-derived enzymes that actively lyse bacterial cells upon direct contact and may be considered such an alternative option. Moreover, the inability of bacteria to evolve resistance to endolysins is due to the specificity of the N-terminal catalytic domain, which cleaves a conserved peptidoglycan bond, and the C-terminal cell wall binding domain, which binds a cell surface moiety. This thesis represents an investigation into the endolysin PlyGRCS, which displays potent bacteriolytic activity against all antibiotic-resistant strains of *S. aureus* tested. This enzyme is active in physiologically relevant conditions (pH, NaCl, temperature), and its activity is greatly enhanced in the presence of calcium. PlyGRCS is the first endolysin with a single catalytic domain that cleaves two distinct sites in the peptidoglycan. Unlike antibiotics, PlyGRCS displays anti-biofilm activity, preventing, removing, and killing biofilms grown on abiotic and biotic surfaces. Engineering efforts were made to create an enzyme with a variable binding domain, which unfortunately displayed less activity than the wild type endolysin in the conditions tested. The antimicrobial efficacy of PlyGRCS was validated in a mouse model of *S. aureus* septicemia. The results from this study indicate that the endolysin PlyGRCS is a revolutionary therapeutic that should be further pursued for subsequent translational development.

AN INVESTIGATION ON A BACTERIOPHAGE ENDOLYSIN POSSESSING ANTIMICROBIAL ACTIVITY AGAINST ANTIBIOTIC-RESISTANT *STAPHYLOCOCCUS AUREUS*

By

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List of Abbreviations

AA	Amino acid
Amp^r	Ampicillin-resistant
AMPs	Antimicrobial peptides
CA-MRSA	Community-associated methicillin-resistant *Staphylococcus aureus*
CBD	Cell wall binding domain
CD	Circular dichroism
CDC	Center for Disease Control and Prevention
CFU	Colony forming unit
CHAP	Cysteine, histidine-dependent amidohydrolase/peptidase
DNA	Deoxyribonucleic acid
DRSA	Daptomycin-resistant *Staphylococcus aureus*
dsDNA	Double-stranded deoxyribonucleic acid
EAD	Enzymatically active domain
ECD	Enzyme catalytic domain
EDTA	Ethylenediaminetetraacetic acid
EPS	Extracellular polymeric substance
FDA	United States Food and Drug Administration
GFP	Green fluorescent protein
GlcNAc	*N*-acetylglucosamine
GRAS	Generally recognized as safe
HAI	Hospital-acquired infection
HA-MRSA	Hospital-associated methicillin-resistant *Staphylococcus aureus*
HGT	Horizontal gene transfer
IACUC	Institutional Animal Care and Use Committee
IDSA	Infectious Disease Society of America
IMAC	Immobilized metal affinity chromatography
ip	Intraperitoneal
iv	Intravenous
LA-MRSA	Livestock-associated methicillin-resistant *Staphylococcus aureus*
LB	Luria-Bertani
LGT	Lateral gene transfer
LRSA	Linezolid-resistant *Staphylococcus aureus*
MBEC	Minimum biofilm elimination concentration
MDT	Maggot debridement therapy
MIC	Minimum inhibitory concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSSA	Methicillin-susceptible *Staphylococcus aureus*
MurNAc	*N*-acetylmuramic acid
NCBI	National Center for Biotechnology Information
NIST	National Institute of Standards and Technology
OD	Optical density
PBS	Phosphate buffered saline
PBP	Penicillin binding protein
PCR	Polymerase chain reaction

PIA	Polysaccharide intercellular adhesin
PTD	Protein transduction domain
QS	Quorum sensing
QSI	Quorum sensor inhibitor
RNA	Ribonucleic acid
SDM	Site-directed mutagenesis
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SNP	Single nucleotide polymorphism
T_m	Melting temperature
VISA	Vancomycin-intermediate *Staphylococcus aureus*
VGT	Vertical gene transfer
V_{max}	Maximum velocity
VRSA	Vancomycin-resistant *Staphylococcus aureus*
WHO	World Health Organization
WT	Wild-type

Chapter I: Introduction and Literature Review

Though much progress has been made in the last century towards combatting bacterial infections through the discovery of modern antibiotics, these types of maladies still account for the majority of the world's morbidity and mortality (Woodford and Livermore, 2009). Furthermore, the advances that have been made in the field seem to have contributed to the current predicament of increasingly antibiotic resistant bacterial infections and may be responsible for the undoing of the last 100 years of progress, compounding the situation even further (Livermore, 2009). Possibly the most infamous story related to antibiotic-resistant bacteria involves *Staphylococcus aureus*, which has the ability to cause a diverse array of life-threatening infections and the capacity to adapt to different environmental conditions. With this impending crisis upon us, researchers are turning to alternative methods for preventing and treating such bacterial infections.

Staphylococcus aureus History and Characteristics

Humankind has been constantly fighting the war against pathogenic bacteria, but it wasn't until the 19th century that we were able to establish a link between these organisms and infectious disease. This scientific progress led to an ability to identify and classify particular organisms and thus begin to understand our interactions with them.

Staphylococcus was first discovered as the causative agent of a wound infection by Sir Alexander Ogston in 1880 (Ogston, 1881), although the official naming of *Staphylococcus aureus* four years later is credited to Anton Julius Rosenbach (Rosenbach, 1884). *S. aureus* is classified as a facultative anaerobic Gram-positive coccal

bacterium, commonly observed forming grape-like clusters under the microscope and gold colonies on agar plates (both characteristics from which its name is derived).

The hardy nature of *S. aureus* enables it to survive in the environment on many types of surfaces. *S. aureus* has been found to survive, on glass, wood, vinyl plastic, and cloth; for some of these materials, bacteria were isolated up to 90 days after inoculation (Neely and Maley, 2000). This persistence, combined with its commensal behavior with the host, make it a potentially frightening pathogen. By some estimates, up to 20% of healthy individuals constantly asymptomatically carry this bacterium in the anterior nares of their nasal passages, throats, and on their skin; in addition, 60% of the population is suspected to be transiently asymptomatically carrying at any particular time (Williams, 1963). In normal healthy people, colonization usually does not lead to any symptoms or illness. However, *S. aureus* is a classic opportunistic pathogen in that it will take advantage of a compromised host to cause disease.

Staphyloccocal Infections

S. aureus has the capability to cause a wide range of illnesses, including skin, soft-tissue, ocular, brain, respiratory, bone, gastrointestinal, and endovascular disorders (Lowy, 1998) . While many groundbreaking medical advances have been made in the last century, non-healing wounds caused by *S. aureus*- induced skin and soft tissue infections still represent a significant burden to society. *S. aureus* skin and tissue infections can manifest themselves as the common pimple or can progress to the much more severe boil, abscess, folliculitis, impetigo, mastitis, cellulitis, or even necrotizing fasciitis; it is also the causative agent of scalded skin syndrome (Tong et al., 2015). Ocular infections

caused by *S. aureus* include endophthalmitis, conjunctivitis, and keratitis, and usually occur after refractive or cataract surgery or LASIK (Chuang et al., 2012). While uncommon, *S. aureus* induced meningitis has a poor prognosis, with mortality rates up to 80% (Durand et al., 1993). Staphylococcal pneumonia is becoming increasingly prevalent in hospitals (20%–40% of all nosocomial-acquired and ventilator-associated infections) and the community (Rubinstein et al., 2008). Staphylococcal food poisoning, caused by consuming food contaminated with enterotoxin, results in gastrointestinal distress and can be severe enough to necessitate hospitalization (Argudin et al., 2010). *S. aureus* is also a leading cause of bacteremia, which has a high mortality rate, especially in patients who are older, not treated with antibiotics, or have a medical device that has not been removed; *S. aureus* bloodstream infections allow for metastasis to other sites in the body, causing endocarditis, osteomyelitis, or septic arthritis, all of which can be further complicated by the presence of an implant (Mylotte et al., 1987). Notably, *S. aureus* causes the life-threatening toxic shock syndrome, which was associated with super absorbent tampons in the 1980s allowing for the accumulation of staphylococcal toxins (Hanrahan, 1994); non-menstrual causes include localized infections, surgery, or insect bites (Lowy, 1998).

S. aureus infections are not just limited to humans; other animals are susceptible to staphylococcal diseases as well. Household companion animals, such as cats and dogs, manifest *S. aureus* skin and soft tissue infections in the same way as humans do. Interestingly, it was found that the same genetic strain of *S. aureus* was carried in humans and their pets, and researchers suggest that it likely can be passed between species. (Harrison et al., 2014). Rodents (rabbits, mice, and rats) can also be colonized and

infected; rats may even play a role in spreading *S. aureus* on farms (van de Giessen et al., 2009; Weese, 2010). Livestock are also not immune to *S. aureus* infections. Cows, and other milking animals such as buffalo and camels, are subject to intramammary infections (Schmidt et al., 2015). In sheep and goats, *S. aureus* commonly causes dermatitis, and in pigs, *S. aureus* may cause botryomycosis, urinary tract infections, or mastitis. In chickens, *S. aureus* is responsible for infections such as bumblefoot, gangrenous dermatitis and bacterial chrondronecrosis with osteomyelitis (Lowder et al., 2009). Horses are susceptible to the same wide range of infections as those observed in humans (Weese, 2010).

Antibiotic Resistant *S. aureus*

In the years between the discovery of *S. aureus*, but before the discovery of modern day antibiotics, *S. aureus* infections had a nearly 80% mortality rate (Dancer, 2008). Upon the introduction and mass production of these traditional antibiotics, the mortality rate was dramatically reduced to 25% (Cosgrove et al., 2003; Fridkin et al., 2003; Rubin et al., 1999). However, in 2005, methicillin-resistant *Staphylococcus aureus* (MRSA) was responsible for an estimated 94,000 life-threatening infections and 18,650 deaths in the U.S. alone, more than double the national estimate only five years earlier (Klevens et al., 2007).

MRSA, which are also resistant to cephalosporins, was originally just a hospital acquired infection (HAI), due to the prevalence of elderly and immunocompromised individuals in this environment. Hospital-associated MRSA (HA-MRSA) represents about 8% of reported nosocomial infections (Hidron et al., 2008). However, in the past

20 years, MRSA has now become a greater problem in our communities, affecting children and healthy individuals including athletes, and especially thriving in areas where individuals are in close proximity with one another, such as prisons (David and Daum, 2010). Community-associated MRSA (CA-MRSA) is genetically distinct from HA-MRSA (Fey et al., 2003) and is endemic in some U.S. cities, reaching up to 50% of all *S. aureus* infections reported (Dukic et al., 2013). Further complicating the MRSA situation is the emergence of livestock-associated MRSA (LA-MRSA), which has resulted in transmission of a new strain from animals to humans (Ballhausen et al., 2014; Fluit, 2012; Hetem et al., 2013; Price et al., 2012)

The increasing prevalence of MRSA led to greater use of vancomycin (originally a last resort antibiotic) in the 1980s; this, in turn led to the emergence of vancomycin-resistant *S. aureus* (VRSA) in 2002 (Centers for Disease and Prevention, 2002). While there have only been 14 cases of VRSA (and no deaths) in the U.S., the CDC has labeled it a concerning threat. Reports of VRSA have emerged from around the world, indicating that this is a severe global issue (Melo-Cristino et al., 2013; Palazzo et al., 2005; Tiwari and Sen, 2006).

S. aureus has also developed resistance to the newer antibiotics; linezolid-resistant *S. aureus* (LRSA) and daptomycin-resistant *S. aureus* (DRSA) outbreaks have been reported, and while they are currently uncommon, it may only be a matter of time until their frequency reaches MRSA levels (Endimiani et al., 2011; Marty et al., 2006; Sanchez Garcia et al., 2010).

As one can imagine, this antibiotic resistance crisis has had an immeasurable impact not only on public health, but on economic outcomes. Antibiotic-resistant

infections are associated with prolonged treatments, extended hospital stays, and require additional doctor visits and healthcare use compared to antibiotic-susceptible bacteria, all leading to a costly outcome. One study estimated average cost per MRSA patient to be $34,657, compared with $15,923 for patients with methicillin-sensitive *S. aureus* (MSSA) (Filice et al., 2010). MRSA infections have been suggested to cost up to a total of $10 billion per year in the U.S. healthcare system alone (Klein et al., 2007).

Biofilms

S. aureus has an arsenal of virulence factors that allow it to cause the multitude of various illnesses described, hence its notoriety as a fearsome "nightmare bacteria" (Gordon and Lowy, 2008). One of the most important aspects of its virulence is the ability to form biofilms (Fig. 1-1). Biofilms are a microbial community of bacterial cells that are attached to a surface and each other, and are encased in extracellular polymeric substance (EPS) made up of extracellular DNA, proteins, and carbohydrates. They exhibit behaviors divergent from bacteria in the planktonic state of growth, due to the complex nature of physiologically distinct cells present in the population of a bacterial biofilm (Kiedrowski and Horswill, 2011; Parsek and Singh, 2003). Biofilms are notoriously difficult to eradicate, as they are resistant to antibiotics and are resilient against the host immune system (Scherr et al., 2014). Furthermore, antibiotics have been shown to induce changes in planktonic bacteria that result in an increased proclivity for forming biofilms (de la Fuente-Nunez et al., 2014). Additionally, biofilms provide a site for cells to disperse from and colonize other areas or cause acute infections (Costerton et al., 1999). Staphylococcal

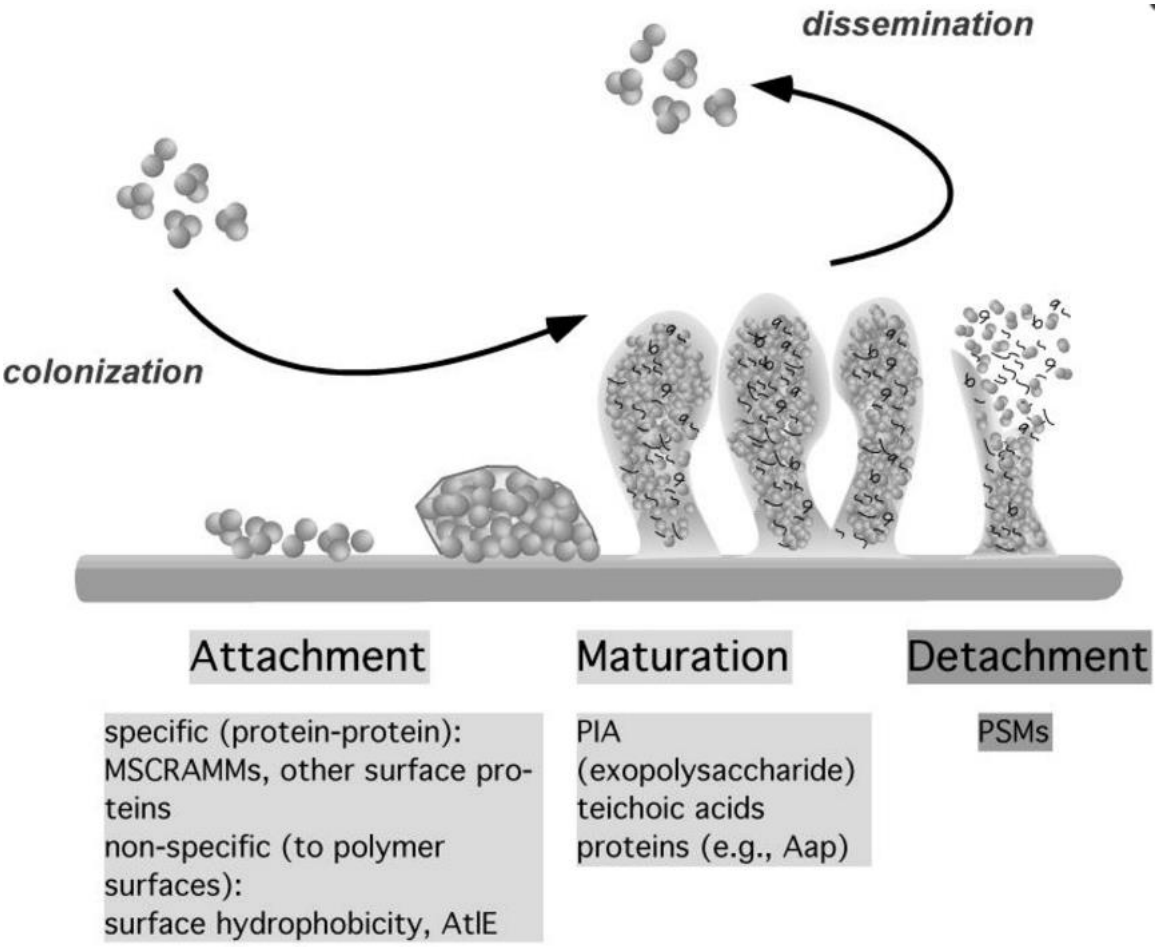

Figure 1-1. Staphylococcal biofilm lifecycle. Biofilms form by initial attachment of bacteria, through specific, protein-protein interactions with an abiotic surface or non-specifically to an abiotic surface. The maturation process consists of the production of molecules including exopolysaccharide, teichoic acids, extracellular DNA, and proteins that connect the cells and provide structural integrity. The final step of detachment occurs through expression of quorum-sensing systems and surfactant-like molecules and aids in the dissemination of an infection. Figure from (Otto, 2008)

biofilms are associated with many different types of chronic infections; furthermore, the ability of *S. aureus* to form biofilms on implanted medical devices complicates these infections and the only option is removal of the affected material (Darouiche, 2004; Francois et al., 1996).

Management of *S. aureus*

Throughout the last century scientists have developed numerous techniques to prevent and treat *S. aureus* infections; however, the current techniques lack the desired degree of efficacy. Furthermore, the overuse of the most commonly used treatment option, antibiotics, has resulted in the selection of antibiotic-resistant bacteria, which further compounds the problem.

Antibiotics

After all these years, the gold standard for treatment of bacterial infections remains traditional antibiotics. The drug of choice for treatment of *S. aureus* infections is still penicillin, one of the first commercially available antibiotics, even though 90% of isolates are now resistant (Lowy, 2003; Tong et al., 2015; Vardakas et al., 2014). In the likely case that resistance to penicillin is encountered, penicillinase-resistant β-lactams such as methicillin, oxacillin, dicloxacillin or flucloxacillin are available as second options (Rayner and Munckhof, 2005). However, the ubiquity of *S. aureus* resistant to methicillin resulted in physicians having to use "last resort" antibiotics, such as the glycopeptide vancomycin, as a first course of action. Once strains with partial or total resistance to vancomycin emerged, new drugs had to be developed; ceftobiprole has been

found to be effective against vancomycin-intermediate *Staphylococcus aureus* (VISA) and trimethoprim/sulfamethoxazole was shown to have efficacy in treating VRSA. Other new treatment options for invasive MRSA infections include linezolid, daptomycin, tigecycline, and quinupristin/dalfopristin. Additionally, a number of new anti-MRSA compounds have been recently approved by the FDA: novel lipoglycopeptides telavancin in 2009, dalbavancin and oritavancin in 2014, and the oxazolidinone tedizolid phosphate in 2014 or are under development (iclaprim) (Higgins et al., 2005; Krievins et al., 2009; Louie et al., 2011; Schwalbe et al., 1996; Steiert and Schmitz, 2002). However, if the lessons of methicillin- and vancomycin-resistant *S. aureus* are any indication, replacing one antibiotic treatment with another will just perpetuate the cycle. It is only a matter of time before resistance to these new treatments is observed as well. Additionally, these new antibiotics often come with undesirable side effects. Linezolid has been associated with thrombocytopenia, vision problems and serotonin toxicity (Garazzino et al., 2007; Lawrence et al., 2006), quinupristin/dalfopristin causes a high rate of adverse venous events (Nichols et al., 1999), and televancin causes adverse renal effects (Stryjewski et al., 2008).

Vaccine development

The holy grail in the war against staphylococcal infections would be a vaccine; however, there is currently no safe and effective vaccine despite the numerous attempts at developing one. Many of these vaccines have failed in late stage clinical trials, after much time, effort, and money had already been spent. A majority of the failures have been due to a monovalent approach, which can be overcome by the ability of *S. aureus* to utilize its

functional redundancy to downregulate the targeted virulence factor and upregulate compensatory factors. In clinical trials, vaccines against the capsule polysaccharide (StaphVAX® and Altastaph® from Nabi) did not provide protection against *S. aureus* bacteremia (Shinefield et al., 2002). Two vaccines against clumping factor A from Inhibitex (Veronate® and Aurexis®) resulted in no difference in clinical end points in infants and adults (Shah and Kaufman, 2009; Weems et al., 2006). However, when vaccines against capsular polysaccharide and clumping factor A were given in conjunction with each other, a greater level of protection was afforded, indicating the necessity of a multivalent vaccine (Tuchscherr et al., 2008). Based off of those preclinical results, Pfizer developed SA3Ag®, which performed well in a phase I clinical trial, proving to be safe and immunogenic, and will proceed to phase II (Nissen et al., 2015). A vaccine from Merck (V710®) against iron surface determinant B was assessed for its ability to prevent *S. aureus* infections, and it was found that patients actually fared worse upon receiving this vaccine (Allen et al., 2014). Aurograb® from NeuTec, a vaccine against an ATP-binding cassette transporter, failed to treat MRSA infections in a phase II clinical trial (Otto, 2010). Pagibaximab® by Biosynexus, a vaccine against lipoteichoic acid, shows great promise having passed three rounds of clinical trials; results showed reduced rates of sepsis in infants and so far it is safe and well-tolerated (Patel and Kaufman, 2015). Nabi recently conducted phase I/II clinical trials using vaccines against α-toxin and LukS-PV and showed good immunogenicity and safety profiles (Lalani et al., 2013). Integrated Biotherapeutics just completed phase I clinical trials using STEBVax® against enterotoxin B, and results are pending (Larkin et al., 2010). Novadigm's candidate NDV-3® against agglutinin-like sequence 3 protein was safe and immunogenic

in phase I/Ib clinical trials (Schmidt et al., 2012). In all, the efforts to create safe and effective vaccines for *S. aureus* are numerous and represent a promising approach to combating these infections. Furthermore, many of the pitfalls experienced in the earlier iterations have allowed scientists to learn from these mistakes and strategically improve upon future vaccine candidates.

Metals and metal chelation

The use of metals against multiple species of bacteria has been exploited since ancient times, and more recently with some basic understanding of mechanism. Referred to as the oligodynamic effect, the mechanism of action is thought to be that the metal ion (especially from heavy metals) binds to the reactive (thiol) groups of proteins involved in metabolism, resulting in precipitation and thereby inactivation (Gibbard, 1937). While many different types of metals have been utilized for their antimicrobial characteristics, silver and copper are most widely used due to their lower toxicity against eukaryotes (Singh et al., 2011). Metal ions have an application in treating burn wounds and in impregnation of medical devices (Church et al., 2006), and also as disinfectants in non-medical appliances (Jung et al., 2008). Metal ion therapy has been particularly useful in prevention and treatment of biofilm bacteria (Nan et al., 2015; Roe et al., 2008). While research into metal ions as antimicrobials has been somewhat promising, resistance has been observed. Even more discouraging is the observation of cross- and co-resistance to traditional antibiotics upon exposure to metal ions, indicating that this therapy may be doing more harm than good (Baker-Austin et al., 2006).

At the opposite end of the spectrum, removal of metals from the bacterial environment has also been somewhat successful in treatment of infections. Because metal acquisition is important during pathogenesis, scientists have experimented with the use of metal chelators as antimicrobials (Hammer and Skaar, 2012). Several compounds have shown antimicrobial efficacy against *S. aureus* (Prachayasittikul et al., 2013; Qiu et al., 2011; Short et al., 2006) and may also have a role in biofilm disruption (Abraham et al., 2012). However, as metal chelators could potentially be cytotoxic, can interfere with immunodefense, and some may not be able to be used systemically, they may not be the most ideal choice for treatment of bacterial infections (Baggiolini, 1984; Sangvanich et al., 2014).

Maggot therapy

A cure not for the slight of heart, maggot debridement therapy (MDT) (biodebridement, larval therapy, therapeutic myiasis), the intentional application of live "medical-grade" fly larvae to wounds in order to effect debridement, disinfection, and ultimately wound healing, is entering a renaissance period of sorts (Sherman, 2009). While this technique was used quite frequently in the first half of the 1900s, the widespread use of antibiotics eliminated its usage. However, now that society is faced with the emergence of antibiotic-resistant bacteria, some earlier technologies are being reexamined with the knowledge gained over the past century. The technical challenges of maggot therapy 100 years ago are now able to be overcome due to the advances in dressings that confine maggots to the wound and the ability to obtain germ-free maggots quickly and cheaply. Maggot therapy is effective because maggots can debride and

disinfect the wound and stimulate healing. These mechanisms of action are thought to occur most effectively in the presence of the animal, but attempts to isolate the "magic molecule" have been only somewhat successful. While some antibacterial factors have been discovered, they have not been compared head to head against use of the whole maggot (Arora et al., 2011). Maggot therapy may be most effective when there is a synergistic effect of multiple antimicrobial components or the physical movement of the maggot itself may aid in the removal of the necrotic tissue. Importantly, biofilm inhibition and eradication was able to be achieved with just the excretions and secretions from the maggot (Cazander et al., 2009). While results appear to be positive, this therapy faces major hurdles to becoming widely used. The number one aversion to maggot therapy is the obvious "yuk factor;" patients must overcome the thought of live insects crawling on their bodies (Steenvoorde et al., 2005). Furthermore, some users reported pain. On the doctor's end, they must contend with keeping the maggots alive, a problem not normally dealt with when considering typical antimicrobials. Another issue is proper containment and eventual disposal of the maggots, as they are essentially "mobile fomites" and can transfer whatever infection they are trying to eradicate around a hospital or treatment facility.

Other treatments

In addition to the above mentioned therapies, there are numerous other treatments for *S. aureus* infections that are in various stages of development or not as commonly used in the clinic. A somewhat intriguing *S. aureus* control strategy is the use of the commensal organism *S. epidermidis* as a competitive colonizer of the anterior nares (Park

et al., 2011). In another approach, researchers have recently discovered that magnetotactic bacteria that possess magnetic nanocrystals could kill *S. aureus* by a magnetic hyperthermia mechanism (Chen et al., 2016). There has also been a push toward more in depth understanding of "natural" therapies in recent years, despite some of these treatments being used successfully for centuries. These include the 1000-year-old remedy known as an eye salve, consisting of garlic, onion, wine, and bile, honey, and various extracts from medicinal plants such as tea tree oil (Brudzynski and Lannigan, 2012; Carson et al., 2002; Chusri et al., 2013; Harrison et al., 2015). Other anti-staphylococcal options that are being investigated include small molecules, antimicrobial peptides (AMPs), and quorum sensing inhibitors (Haisma et al., 2016; Jin et al., 2015; Tan et al., 2015). In addition to therapeutics, there is a need for disinfecting surfaces contaminated with *S. aureus*. Currently, alcohols, chlorhexidine, hexachlorophene, cetrimide, and triclosan are commonly used in places such as hospitals and gyms, and additional sanitizers are being investigated (Wootton et al., 2009; Yuen et al., 2015).

Antibiotic Discovery and Resistance Development

The identification of infectious agents and their associated illnesses, along with the beginnings of a basic understanding of mechanisms of infection, led to an ability to more systematically approach methods of battling pathogenic bacteria. While antimicrobial agents have been used throughout history, the advent of the golden age of antibiotics is considered to have begun in 1928 with the discovery of the β-lactam penicillin by Alexander Fleming (Fleming, 1929). It was introduced for widespread public use a decade later and hailed as a lifesaver, especially for staphylococcal

infections; however, the first resistance to penicillin was observed by 1945, an ominous sign of things to come (Lewis, 2013). In the 1940s, several translation inhibitors were discovered and soon thereafter were available to the public; the same resistance patterns were observed and interestingly, for tetracyclines, resistance was noted before going to market. Two other classes of 50S ribosomal subunit inhibitors, oxazolidinones and streptogramins, were discovered in 1955 and 1963, respectively, but were not introduced until 2000 and 1998. Frighteningly, resistance to streptogramin B was observed one year after its discovery and it was still allowed to come to market 35 years later. This period of intense discovery was rounded out by three other classes of drugs, glycopeptides (vancomycin in 1953), rifamycins (rifampicin in 1957) and quinolones (ciprofloxacin in 1961), all of which bacteria developed resistance to before or very soon after becoming available. After that, a new class of antibiotics was not discovered until 1986 (the lipopeptide, daptinomycin). Resistance was observed only a year later, yet daptinomycin was still approved for use by the FDA in 2003. Other newly approved antibiotics are derivatives from existing classes of drugs, such as tigecycline in 2005 (a glycylcycline derived from tetracycline) and ceftaroline in 2010 (a fifth generation cephalosporin), both of which have been met with the emergence of resistant strains. The last new class of antibiotics to be approved were the diarylquinolines for treatment of multidrug resistant tuberculosis in 2012, six years after resistance had been observed. Clearly, new antibiotics cannot be developed quickly enough or smartly enough to be considered a viable therapeutic option (Boucher et al., 2013).

Antibiotic Mechanisms of Action

Antibiotics are effective because they target processes or structures that are essential for bacterial survival (Newton, 1965). Antibiotics can be bactericidal (resulting in bacterial death) or bacteriostatic (preventing growth and reproduction). There are 5 distinct mechanisms that are employed by antibiotics to achieve these goals. The largest class of antibiotics hinders peptidoglycan biosynthesis by preventing cell wall cross-linking directly or interacting with precursors (e.g. penicillins, cephalosporins, glycopeptides, carbapenems, monobactams, bacitracin, cycloserine, fosfomycin, isoniazid, ethambutol). A second mechanism by which antibiotics function is by inhibiting translation; they directly interact with the 30S (e.g. aminoglycosides, tetracyclines) or 50S (e.g. macrolides, chloramphenicol, lincosamides, oxazolidinones, streptogramins) ribosomal subunits to suppress protein synthesis. Another group of antibiotics disrupt cell membrane permeability (e.g. polymyxin, ionophores). Next, antibiotics can block DNA (e.g. fluoroquinolones, novobiocin, nitrofurans, metronidazole) or RNA (e.g. rifampin, bacitracin) synthesis. Finally, they can possess antimetabolite activity that blocks enzyme-catalyzed reactions of the bacterial cell metabolism, such as mycolic acid synthesis inhibitors (e.g. isoniazid), folic acid synthesis inhibitors (e.g. sulfonamides, dapsone, trimethoprim), and ATP synthase inhibitors (e.g. diarylquinolines). The lipopeptide daptomycin is unique in that it has multiple mechanisms of action (Pogliano et al., 2012). It initially inserts into the cell membrane, where it aggregates, thereby altering membrane curvature. In turn, this change in curvature results in the formation of holes in the membrane, causing leakage of ions and massive depolarization. This loss of membrane potential then results in the inhibition of

protein, DNA, and RNA synthesis and ultimately cell death. Excitingly, the first in the newest class of antibiotics discovered in 20 years (yet still several years away from consumer use), teixobactin, utilizes a new mechanism of inhibition of cell wall synthesis, by binding to precursors of both peptidoglycan and teichoic acid (Ling et al., 2015). Importantly, the method by which this antibiotic was identified, the iChip, resulted in the growth of previously uncultivable bacteria, opening the door for many more antimicrobials to be found (Nichols et al., 2010).

Bacterial Resistance to Antibiotics

Although scientists have developed traditional antibiotics that use many different mechanisms by which to combat pathogens, they underestimated the ways in which these drugs would fail through evolutionary advantages obtained by the bacteria. Bacterial resistance to antibiotics can occur in two distinct ways (Blair et al., 2015). Inherent resistance is the ability to resist activity of a particular antimicrobial through intrinsic structural or functional characteristics. On the other hand, while some bacteria may be naturally resistant to certain agents, acquired bacterial resistance is caused by the selective pressure imposed by the introduction of an antibiotic. Bacteria acquire these mechanisms through mutations, horizontal gene transfer, or vertical gene transfer. Mutation involves the modification of the native DNA, which results in the production of altered bacterial proteins. These mutations can be nucleotide(s) base substitutions/ single nucleotide polymorphisms (SNPs), insertions, deletions, or frameshifts. Horizontal/ lateral gene transfer (HGT/LGT) is the acquisition of new DNA from other bacteria, either through transformation (uptake of naked DNA), transduction (acquisition of

bacteriophage DNA during infection), or conjugation (transfer of DNA by direct bacteria-bacteria contact). Vertical gene transfer is the transmission of spontaneously generated mutations from the parental generation to offspring.

The same four mechanisms are utilized in both inherent and acquired resistance: the antibiotic may lack affinity for the bacterial target, be unable to enter the cell, be exported from the cell, or be inactivated or degraded by enzymes. *S. aureus* makes use of each one of these mechanisms of resistance, by both innate and acquired means. *S. aureus* is naturally resistant to only two commonly used antibiotics: metronidazole and aztreonam. Metronidazole inhibits nucleic acid synthesis, only in its reduced form, which must occur in anaerobic conditions (Lofmark et al., 2010). While *S. aureus* can grow in anaerobic environments, under typical aerobic conditions, metronidazole has no effect. Aztreonam has a high affinity for the penicillin-binding protein 3 (PBP-3) of aerobic Gram-negative bacteria, and binding causes inhibition of this enzyme to catalyze cross linking of the cell wall (Davies et al., 2008). The weaker binding of aztreonam to Gram-positive PBP-3 results in no inhibitory effect against *S. aureus*. While *S. aureus* only possesses natural resistance to these two antibiotics, it has developed acquired resistance to almost every other antibiotic currently available to treat *S. aureus*-induced infections.

The β-lactam antibiotics (penicillins, cephalosporins, monobactams, carbapenems) inhibit cell wall synthesis by binding PBPs, and thereby disallowing crosslinking of the peptidoglycan. This weakened cell wall results in eventual lysis due to osmotic stress. The most common mechanism of staphylococcal resistance to β-lactam antibiotics is through the production of a β-lactamase enzyme (BlaZ), encoded by a gene

carried on a plasmid, which degrades and inactivates these drugs (Sabath, 1982). When researchers developed modified β-lactams, in which the β-lactam ring core was protected from degradation, staphylococci acquired a new mechanism of resistance via horizontal gene transfer from a still unknown source: the mobile genetic element staphylococcal cassette chromosome *mec* (SCC*mec*) (Katayama et al., 2000). This element carries the *mec* complex composed of *mecA*, encoding PBP2a (PBP2'), and regulatory elements, as well the *ccr* site specific recombinase genes, and may contain other resistance genes, such as those against aminoglycosides, macrolides, tetracycline, or heavy metals. PBP2a is a unique penicillin binding protein in that it has much weaker affinity than other PBPs for the β-lactam antibiotics, especially methicillin; therefore transpeptidation of the cell wall can still occur in the presence of these antibiotics.

Vancomycin inhibits cell wall synthesis by binding to the acyl-D-alanyl-D-alanine in the peptidoglycan precursor lipid II and thereby sterically hindering transglycosylation and transpeptidation. Resistance to vancomycin occurs in two distinct manners. The first is through the thickening of the cell wall, through an as yet undetermined mechanism. These thicker cell walls make it harder for vancomycin to diffuse to its target (Hiramatsu et al., 1997). In addition, these so called VISA strains (MIC (minimum inhibitory concentration) from 4-8 μg/ml) display reduced crosslinking, which results in more D-ala-D-ala residues available to trap vancomycin. The second method is by acquisition of the *vanA* operon via an enterococcal plasmid, causing the emergence of VRSA strains (MIC ≥16 μg/ml). This results in the production of peptidoglycan precursors in which the peptide stem ends in D-alanyl-D-lactate, which vancomycin binds with 1000x less affinity than the native D-alanyl-D-alanine.

Acquisition of *vanA* also results in strains that are resistant to teicoplanin (Showsh et al., 2001). Though resistance has yet to be observed for the newest glycopeptides, dalbavancin and oritavancin, they utilize the same mechanism of action as vancomycin, suggesting that they may also be subject to the same mechanism of resistance. The lipoglycopeptide televancin (a semi synthetic derivative of vancomycin) utilizes the same mechanism of inhibition of cell wall synthesis as vancomycin, so it is subject to development of resistance via acquisition of *vanA* (Karlowsky et al., 2015). However it additionally disrupts membrane integrity via depolarization and increased permeabilization. Despite this dual mechanism of action, low level resistance has been observed in the laboratory and once in the clinic; however it is currently not fully understood how *S. aureus* are able to overcome the secondary action of this antibiotic.

Alternative Antimicrobials Needed

The efforts to control antibiotic use to counteract the emergence of antibiotic resistant bacteria have been numerous and somewhat effective, but may be difficult to enforce. Only completely withdrawing specific antibiotics from use in the clinic led to a decrease in antibiotic-resistant *S. aureus* (Aubry-Damon et al., 1997; Barber et al., 1960; Ridley et al., 1970). One area in which antibiotic use can be lessened or eradicated is on the farm; Denmark, one of the world's largest producers of pork, has cut antibiotic usage on farms by 40% without affecting production (Aarestrup et al., 2010). The European Union has already implemented strict guidelines on antimicrobial use in food animal production (Maron et al., 2013). In the United States, the CDC has obtained $160 million from Congress for implementation of the Antibiotics Resistance Solutions Initiative

(CDC, 2016). This approach is outlined in the National Action Plan for Combating Antibiotic-Resistant Bacteria and the National Strategy for Combating Antibiotic-Resistant Bacteria (CARB); the overall vision is: "The United States will work domestically and internationally to prevent, detect, and control illness and death related to infections caused by antibiotic-resistant bacteria by implementing measures to mitigate the emergence and spread of antibiotic resistance and ensuring the continued availability of therapeutics for the treatment of bacterial infections." Faced with this crisis of an impending post-antibiotic era, alternatives to these traditional antibiotics must be identified. As discussed, scientists are developing vaccines, quorum sensing inhibitors, antimicrobial peptides, iron chelators, and other unique therapies to combat bacterial infections. Excitingly, a both old and new field of antimicrobial research that is generating much interest is the use of bacteriophages and their lytic enzymes.

Bacteriophage

Bacteriophages are viruses that infect and replicate within bacteria (Drulis-Kawa et al., 2012). One way in which bacteriophages are classified is based upon their replication cycle; lysogenic (temperate) phages integrate their DNA with that of the host, while lytic (virulent) phages destroy the host immediately after replication of the virion. For the double-stranded DNA (dsDNA) phage lytic cycle, the host cell infection process consists of four steps. First, is the adsorption and penetration step. The bacteriophage randomly encounters the host bacterium, then reversibly adheres to a specific receptor via its tail fibers. The viral genetic material is injected into the host through the tail core, a process that is either mechanical or enzymatic in nature, or both. Second, during the

synthesis of proteins and nucleic acid phase, the early proteins are transcribed and translated, resulting in the production of many copies of the phage DNA; this is followed by the transcription and translation of the late proteins, mostly structural in function. Third, virion assembly occurs. The structural proteins are then assembled into the mature virus, which is composed of the capsid head encapsulating a copy of the viral DNA and the tail components. During this time, endolysin also being produced to degrade the peptidoglycan and allow release of the mature bacteriophages. Lastly, the virion is released. Once the peptidoglycan is degraded, the viral particles are liberated by host cell lysis, thus completing the phage lytic cycle.

After being discovered by Francis Twort in 1915 (Twort, 1915) and Felix d'Herelle in 1917 (d'Herelle, 1917), bacteriophages were briefly considered as potential therapeutic agents (d'Herelle, 1931); however, the discovery of the modern day antibiotic a decade later pushed them into the background in the Western world, mainly to be used as research tools. In the former Soviet Union and Eastern Europe, however, phage therapy, the use of bacteriophage for the prevention and treatment of bacterial infections, has been pursued with much success throughout the last century (Sulakvelidze et al., 2001; Sulakvelidze and Morris, 2001). The earliest reported use of bacteriophage treatment of staphylococcal skin disease in humans resulted in clearance of infection within 2 days after bacteriophage were injected into and around surgically opened lesions (Bruynoghe R., 1921). At the Eliava Institute of Bacteriophage, Microbiology, and Virology (EIBMV) of the Georgian Academy of Sciences, Tbilisi, Georgia, and the Hirszfeld Institute of Immunology and Experimental Therapy (HIIET) of the Polish Academy of Sciences, Wroclaw, Poland, researchers have been actively and successfully

utilizing bacteriophage therapy against numerous bacterial infections, including those caused by *S. aureus* (Meladze et al., 1982; Slopek et al., 1984; Slopek et al., 1983a, b; Slopek et al., 1985a, b, c). In fact, the HIIET is currently conducting a clinical trial utilizing bacteriophage preparations against 15 different pathogens (Experimental Phage Therapy of Drug-resistant Bacterial Infections, Including MRSA Infections NCT00945087) (Vandenheuvel et al., 2015).

A reinvigorated worldwide interest in bacteriophage therapy has led to many groups using bacteriophage to both treat and prevent *S. aureus* in different types of small mammal models of infection, as staphylococcal infections can present in a multitude of ways. The nasal passage is the primary route of infection; as mentioned, *S. aureus* colonizes the anterior nares of 20% of the population persistently and 60% carry intermittently, leading to an increased risk of surgical site infections, foreign body infections and bacteremia, as well as transmission to non-colonized, especially immunocompromised, individuals. To evaluate the efficacy of bacteriophage as a nasal decolonization agent, intranasal administration of bacteriophage MR-10 was utilized in a mouse colonization model (Chhibber et al., 2014). Bacteriophage MR-10 was able to reduce the bacterial load by day 2 as compared to untreated mice, and by day 10, mice were completely sterile. Combinatorial treatment with mupirocin resulted in bacterial clearance by day 5. Myeloperoxidase activity was also lowered in bacteriophage and dual therapy; histopathogical analysis of excised nasal tissue showed reduced inflammation when compared to untreated samples. Taken together, these results validate the efficacy of bacteriophage (and combinatorial therapy with antibiotic) for nasal decolonization of *S. aureus*.

As *S. aureus* is often a causative agent of wounds and soft tissue infections, several groups have set out to investigate the therapeutic efficacy of bacteriophage against skin infections. Phage LS2a injected subcutaneously demonstrated the ability to prevent and treat abscess formation in a rabbit model of infection (Wills et al., 2005). As *S. aureus* is one of the most common pathogens isolated from diabetic foot wounds, Chhibber, et al. developed a mouse model to study bacteriophage treatment during a diabetic hindpaw infection (Chhibber et al., 2013). Treatment with bacteriophage MR-10 was able to reduce the bacterial burden starting on day 1; sterile paws and resolution of infection occurred by day 7, whereas untreated mice resolved the infection on day 12, yet still had a low bacterial load. Utilizing both MR-10 and linezolid further reduced the bacterial counts and resulted in lower oedema and lesion scores, highlighting the benefit of combinatorial therapy, as the emergence of resistant mutants can be staved off due to differing mechanisms of action.

Because *S. aureus* is also a major causative agent of bloodstream-associated infections, such as septicemia, invasive endocarditis, and septic arthritis, several groups have evaluated the efficacy of bacteriophage treatment against systemic MRSA infection. Bacteriophage M^{Sa} given intravenously was able to protect 93% of mice from bacteremia induced death, reduced the proinflammatory response during infection, and was effective even when given 10 days after the start of the infection (Capparelli et al., 2007). Subcutaneously injected bacteriophage P-27/HP sufficiently protected mice from bacteremia and death and lowered the bacterial load in the spleen by 6 logs (Gupta and Prasad, 2011a). In a mouse model of MRSA induced bacteremia, phiMR11 was able to

successfully prevent death (Matsuzaki et al., 2003), as was the morphologically similar ΦMR25 (Hoshiba et al., 2010).

As mentioned, staphylococcal infections can be exacerbated by the formation of a biofilm, especially in the present of a medical device, and because antibiotics are ineffective, the only solution is removal of the implant. Several groups have developed biofilm and implant-mediated biofilm models to study the effectiveness of bacteriophage in biofilm removal. Sb-1 was able to decrease the bacterial load of MRSA in a tibial implant model and, when used in conjunction with antibiotics, prevented the formation of a biofilm (Yilmaz et al., 2013). In a rabbit model of central venous catheter-related infection, bacteriophage K, given as an antimicrobial lock therapy, reduced the ability of bacteria to colonize and form biofilms (Lungren et al., 2014). A cocktail of *S. aureus* bacteriophage was shown to effectively reduce the biofilm mass in a sheep model of sinusitis (Drilling et al., 2014).

As most of the bacteriophage treatment studies utilize an intraperitoneal (ip) method of infusion, the results may be somewhat artificial. To understand if bacteriophage would be just as effective when given orally, as they might be in a hospital setting, mice were given *S. aureus* bacteremia and treated with bacteriophage A5. Results showed that both ip and oral administration were able to reduce the bacterial load in the liver to the same extent, indicating the validity of the ip model and also that bacteriophages could enter the circulatory system via the oral route (Zimecki et al., 2008).

Because some individuals are carriers of *S. aureus*, prophylactic treatment before a hospital stay or treatment might be desired. In the same vein, preventative treatment

may also be valuable for immunocompromised individuals. The need to steer away from using antibiotics as prophylactics due to the promotion of resistance, led researchers to study the use of bacteriophage as an alternative. In one study, immunosuppressed mice that were given bacteriophage A5/L 30 minutes before infection with *S. aureus* bacteremia had lower bacterial cell counts in their organs and lower cytokine levels in their blood than those that did not receive pretreatment (Zimecki et al., 2009). In addition, bacteriophage seemed to stimulate myelocytic and lymphocytic cell renewal and antibody production, indicating a beneficial effect on the immune system. This group also determined that this bacteriophage extended the survival and had the same beneficial effects on immunosuppressed mice that underwent a bone marrow transplant, bolstering the case for using bacteriophages as prophylactic measures (Zimecki et al., 2010).

To study bacteriophage therapy on respiratory infection induced *S. aureus* septicemia, a common result of healthcare-associated, hospital-acquired, or ventilator-associated staphylococcal pneumonia, mice were intranasally inoculated with *S. aureus* at a dose effective to cause lethality at 3 days, primarily caused by fibrosis, bleeding, and neutrophil infiltration (Takemura-Uchiyama et al., 2014). Mice that were treated with bacteriophage S13' 6 hours post-infection had a 70% survival rate 2 weeks later, had significantly lower concentrations of bacteria in their livers and spleens, and had lower concentrations of TNF-a and IL-6 in their blood.

While rodents provide a satisfactory model for studying staphylococcal infection and resolution by bacteriophage treatment, scientists have developed an invertebrate model to alleviate some of the hassle associated with mammalian studies. The use of insects of the order Lepidoptera, in particular *Bombyx mori* (the silkworm) and *Galleria*

mellonella (the waxworm) are excellent model organisms because no Institutional Animal Care and Use Committee (IACUC) protocol is necessary and they are inexpensive. Furthermore, they are a proven model used to study bacterial pathogenicity and pharmacokinetics, pharmacodynamics, and toxicology of drugs, as they provide comparable data to mouse models of infection. The therapeutic effects of two bacteriophages were examined in a silkworm model of *S. aureus* infection (Takemura-Uchiyama et al., 2013). Treatment with bacteriophage 10 minutes after the start of the infection was able to rescue approximately 80% of the silkworms by day 2; in the mouse model, 100% of the mice survived when given bacteriophage S25-3, while 50% of the mice remained alive when treated with bacteriophage S13'. This study indicates that valid information on the therapeutic efficacy of bacteriophages can be gleaned from this invertebrate model and can be extrapolated to mammalian systems.

Bacteriophage-Derived Peptidoglycan Hydrolases: Endolysins

Phage therapy is effective because, as mentioned, late in the bacteriophage lytic cycle, two enzymes are produced: a holin, which oligomerizes to create holes in the cytoplasmic membrane, and an endolysin (Young et al., 2000). Endolysins are peptidoglycan hydrolases produced by bacteriophage to degrade the now accessible peptidoglycan "from within" for release of progeny phage. Researchers have taken advantage of this peptidoglycan-degrading mechanism by applying endolysins to the outside of Gram-positive bacteria, which lack an outer membrane, resulting in lysis "from without" (Fig. 1-2). Due to the internal turgor pressure of 20-50 atmospheres, the cytoplasmic membrane alone is no longer effective at maintaining the structure of the cell

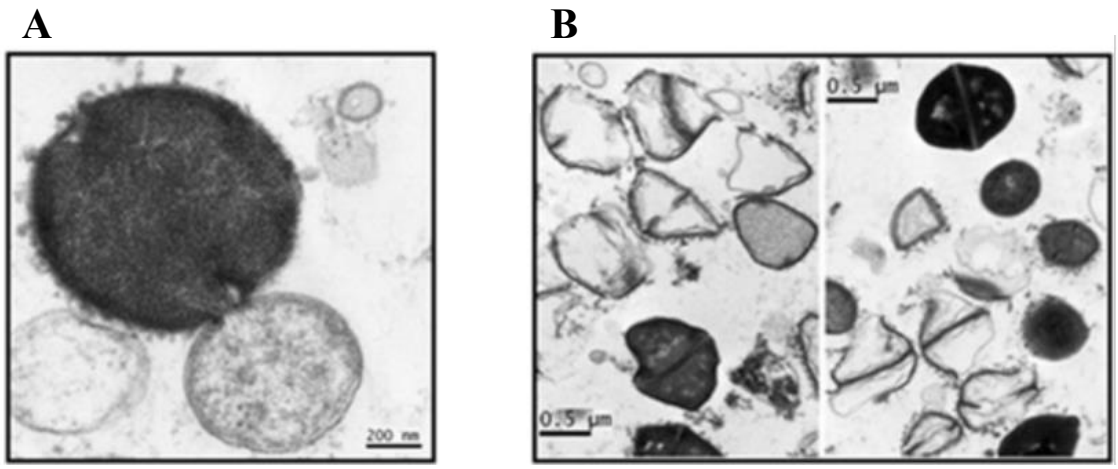

Figure 1-2. Thin-section transmission electron micrographs of ClyS-treated *S. aureus*. (A) After exposure of *S. aureus* to 250 µg of ClyS for just 3 minutes, cells displayed localized degradation of the cell wall, resulting in osmotic stress-induced lysis and visualized by extrusion of the cytoplasm. (B) The bacteriolytic activity of the endolysin ultimately results in cell death and the presence of "ghosts" can be observed. Figure from (Daniel et al., 2010)

and ultimately the result is death due to osmotic stress. This phenomenon means that the whole bacteriophage is not necessary to achieve antimicrobial efficacy; use of the endolysin alone causes the same result. The use of only the endolysin (a single protein entity) makes endolysin therapy a much more attractive option, as there are numerous benefits to eliminating the whole phage from a preventative or treatment regimen.

The main benefit to the use of an endolysin over the whole bacteriophage is the simplification of a complex system. From both a health care and regulatory standpoint, use of a controlled single protein is an improvement over the use of a complicated self-replicating virus (Gill and Hyman, 2010; Pirnay et al., 2011). The stripping of the bacteriophage to just the bare essentials needed for antimicrobial activity also allows for future improvement efforts. As evolved as endolysins are, scientists are always trying to improve and adapt them for use as antimicrobials. While it is not impossible to genetically engineer a bacteriophage to improve lytic efficacy (Westwater et al., 2003), it is certainly much easier to alter individual proteins. Engineering desirable properties in enzymes can be achieved using either rationale-based (e.g. sequence comparison, structure-guided site directed mutagenesis (SDM), or *in silico* computational modeling) or random techniques (e.g. random mutagenesis, gene shuffling, or directed evolution) (Schmelcher et al., 2012a). These proteins can be easily engineered for increased lytic activity, enhanced or alternative binding, or other improvements in protein characteristics such as thermostability. Importantly, bacteriophage resistance has been observed (Labrie et al., 2010), while multiple efforts to isolate endolysin resistant bacteria have been unsuccessful. Lastly, bacteriophages are associated with strain-specificity, which is not ideal for a viable antimicrobial therapy (Sulakvelidze et al., 2001). Sick patients would

have to be strain-typed, a process that takes longer and is more expensive than just a species identification. In order to deal with this problem, proponents of bacteriophage therapy have suggested the use of cocktails; however, the regulatory issues are magnified in a multi-component system. Endolysins are typically species-specific and can even be genus-specific; in rare cases, they have a broader host range. The not-too-narrow spectrum of endolysins makes them preferable as they can be used as a general treatment against a particular bacterial infection without having to know the exact strain.

Endolysin Structure

Endolysins from bacteriophages that infect Gram-positive hosts are typically composed of two modular domains: an N-terminal catalytic domain (also known as an enzymatically active domain (EAD) or enzyme catalytic domain (ECD)) and a C-terminal cell wall binding domain (CBD) (Fig. 1-3) (Borysowski et al., 2006). The CBD is responsible for bringing the protein in contact with the bacterial cell, typically through an interaction with a carbohydrate, although the receptor is choline in the case of pneumococcal endolysins or the peptidoglycan directly for staphylococcal endolysins (Fischetti, 2003; Garcia et al., 1983; Lu et al., 2006). After the protein has bound to its target, the business end can accomplish its enzymatic task. The catalytic domain is responsible for the hydrolytic cleavage of highly conserved bonds in the peptidoglycan. Peptidoglycan is a polymer made of repeating sugar components (β-(1,4) linked N-acetylmuramic acid (MurNac) and N-acetylglucosamine (GlcNac)), with a peptide chain (L-alanine, D-glutamic acid, L-lysine, D-alanine in non-bacilli Gram-positive bacteria) attached to the MurNac, and crosslinked (either directly or through an interpeptide bridge

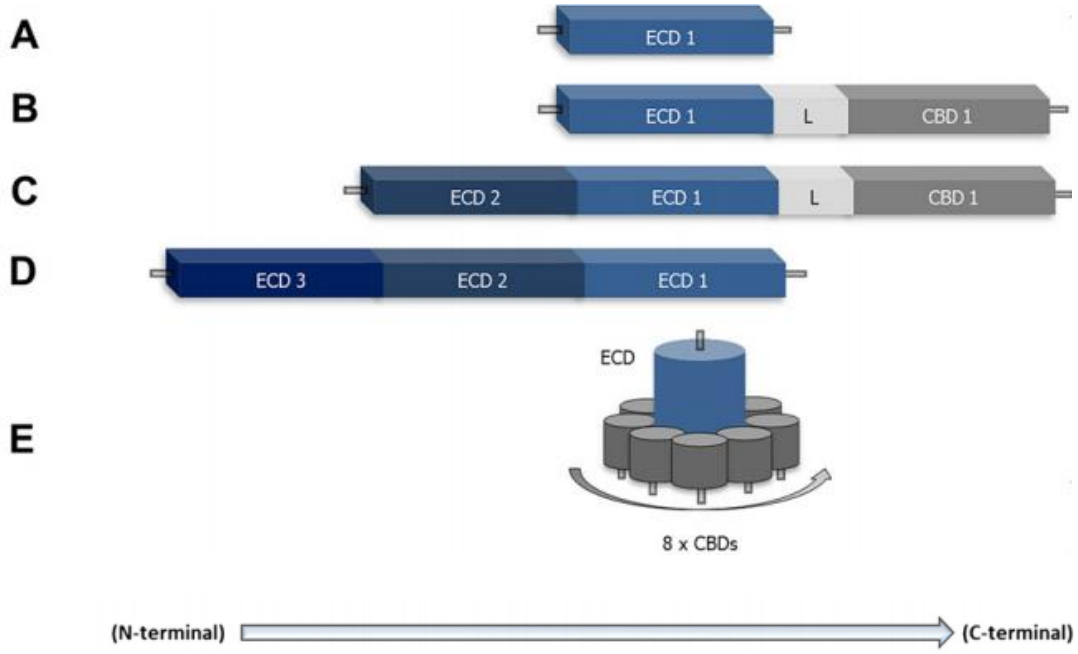

Figure 1-3. Modular architectures of Gram-positive endolysins. (A) A typical globular enzyme consisting of ECD only. (B) Typical bimodular N-terminal ECD and C-terminal CBD joined by a linker region. Trimodular structures of (C) dual catalytic domains and 1 CBD or (D) 3 ECDs. (E) The unique multimeric configuration of the streptococcal endolysin PlyC. Figure from (Oliveira H et al., 2012)

(pentaglycine for *S. aureus*)) to other peptide chains to create a meshlike structure (Fig. 1-4). Peptidoglycan provides important structural integrity for the bacterial cell and is an ideal target for an antimicrobial as it is essential for bacterial survival. The catalytic domains of endolysins can be classified into four groups depending on the target bond to be cleaved. In the glycan component, *N*-acetylmuramidases are responsible for the cleavage on the reducing end of the *N*-acetylmuramic acid, while *N*-acetyl-β-D-glucosaminidases cleave at the reducing end of *N*-acetylglucosamine. *N*-acetylmuramoyl-L-alanine amidases cleave the bond connecting the glycan component and the peptide stem. Lastly, endopeptidase is a broad term encompassing any endolysin that cuts between two amino acids found in the stem peptide or interpeptide bridge. While there are multiple catalytic domains that have been associated with staphylococcal endolysins, the most commonly found class is the cysteine, histidine-dependent amidohydrolase/ peptidase (CHAP) domain. These domains have been associated with both *N*-acetylmuramoyl-L-alanine amidase and endopeptidase activity. A sequence comparison of endolysins in the same enzyme class shows that the catalytic domains are highly conserved, whereas the binding domains are more variable (Oliveira et al., 2013).

Interestingly, the domains of endolysins interact not only with the bacterial cell, but it has been shown that they interact with each other. Structural evidence obtained using the pneumococcal endolysin Cpl-1 crystallized in free and choline-bound states showed that, in the absence of the binding substrate, the endolysin formed a hairpin, rendering the catalytic domain inactive; however, choline recognition by the CBD allowed for the undoing of the hairpin and the proper positioning of the catalytic domain to access and cleave the peptidoglycan (Hermoso et al., 2003).

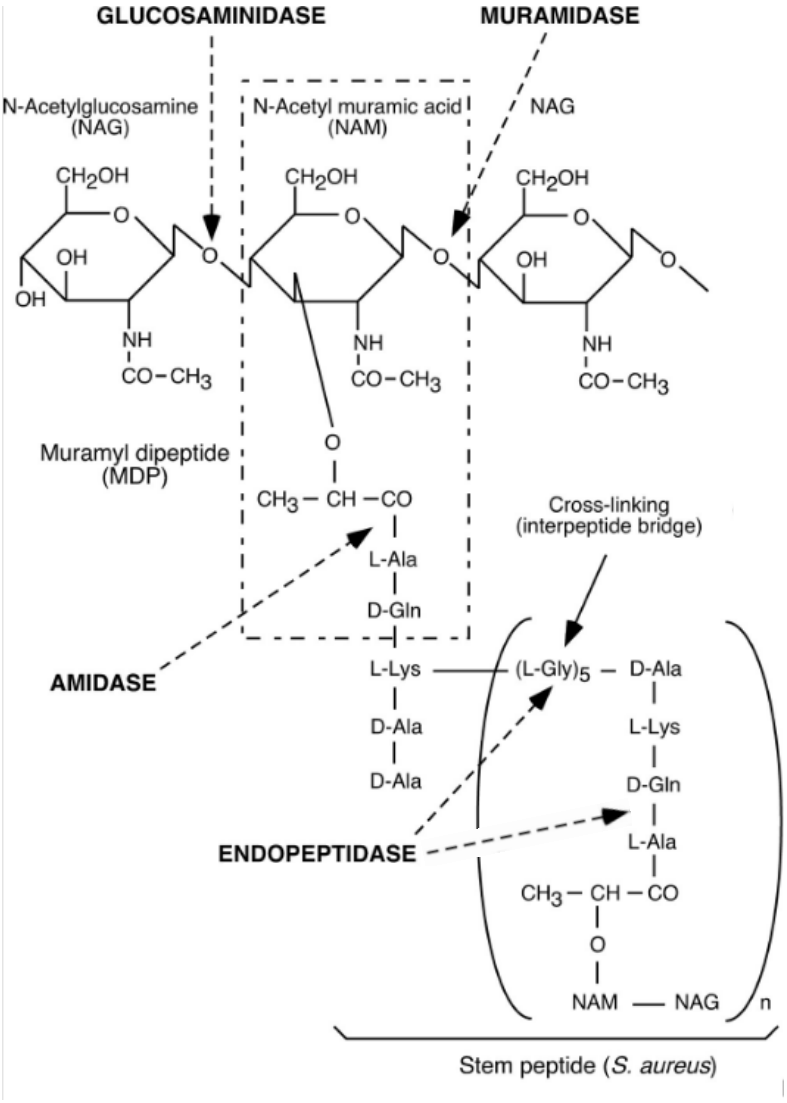

Figure 1-4. *S. aureus* peptidoglycan and endolysin cleavage sites. The structure of *S. aureus* peptidoglycan consists of sugar components (β-(1,4) linked *N*-acetylmuramic acid and *N*-acetylglucosamine) and a peptide stem of 4 amino acids (L-alanine, D-glutamic acid, L-lysine, D-alanine) crosslinked through an interpeptide bridge (pentaglycine) to another unit. The dotted arrows denote the sites targeted for cleavage by the catalytic domains of endolysins. Figure from (Fournier and Philpott, 2005)

Staphylococcal Endolysin Cell Wall Binding Domains

SH3b CBD

All endolysins displaying activity against staphylococcal species (except one, described further below) possess an SH3b cell wall binding domain. SH3 (src homology 3) domains in eukaryotes and viruses are implicated in mediating protein-protein binding through interactions with proline-rich sequence motifs (Weng et al., 1995); however, the SH3b domain in prokaryotes, while displaying sequence similarity to the SH3 domain, shows an alternative folding in the RT loop (Whisstock and Lesk, 1999). The SH3b domain is found in both staphylococcal and streptococcal endolysins, usually in combination with a CHAP domain or a CHAP plus another catalytic domain. The staphylococcal endolysins Twort, LysH5, ΦSh2, Φ11, Sal1, and LysK contain both CHAP and Amidase-2 EADs with SH3b CBD, staphylococcal endolysin 2638A contains M23 and Amidase-2 EADs with SH3b, and the GBS (Group B streptococcus) endolysin B30 has CHAP and Acm glycosidase EADs with SH3b (Abaev et al., 2013; Becker et al., 2008; Becker et al., 2015; Donovan et al., 2006b; Donovan et al., 2006c; Jun et al., 2011; Obeso et al., 2008; Schmelcher et al., 2012b). SH3b-containing endolysins against other species of bacteria utilize various other catalytic domains.

In addition to endolysins, SH3b domains have also been found in association with other classes of proteins, such as other enzymes, adaptor proteins, or peptidoglycan binding proteins. SH3b encompasses such a broad group of proteins because this domain is actually further classified into SH3b1 and SH3b2 and divided even more into the subgroups SH3_1-9; as of yet only SH3_3 and SH3_5 have been found to be associated with endolytic enzymes. The endolysin SH3b is unique in that most endolysin binding

domains interact with a carbohydrate moiety embedded in the cell wall (or choline in the case of pneumococci), whereas SH3b domains are predicted to bind peptidoglycan, specifically the peptide component (Schmelcher et al., 2012a). For staphylococcal SH3b domains, that peptide component is proposed to be the pentaglycine crossbridge, unique to *S. aureus* (Grundling and Schneewind, 2006; Lu et al., 2006); the streptococcal SH3b ligand is currently unknown, but may be the free amino group of the N-terminal alanine in the stem peptide (Xu et al., 2010).

There are currently many discrepancies in our understanding of the endolysin SH3b domains, mainly having to do with the residues important for the binding interaction. Although the staphylococcal SH3_5 has been extensively investigated structurally and bioinformatically, there are conflicting reports about the amino acids responsible for binding. For example, the ALE-1 endolysin contains an SH3_5 domain that was shown to specifically recognize the pentaglycine crossbridge of *S. aureus*; however, some of the amino acid residues implicated in being responsible for this binding were outside of the canonical 63 amino acid SH3b domain and were variable from the residues determined to be important in the SH3_5 domain from LysGH15, despite the almost 50% identity between these two enzymes (Becker et al., 2009b; Gu et al., 2014; Lu et al., 2006). Additionally confounding is the classification of both streptococcal and staphylococcal endolysin SH3b as SH3_5, yet these CBDs are distinctly different from each other in sequence; while both contain the consensus Y(6-8x)G(xx)W(6-8x)G, they have virtually nothing else in common.

Further complicating our understanding of these domains, some SH3b domains do not display species specificity, and it is unknown whether the lack of specificity is due to

the catalytic domain or the SH3b domain, as binding studies have not been performed. A catalytic domain that can cleave a bond shared by multiple species could perhaps act upon many different bacteria regardless of the binding domain. Examples include LysB4 (VanY L-alanoyl-D-glutamate endopeptidase + SH3_5) which shows activity against *Bacillus cereus, Bacillus subtilis,* and *Listeria monocytogenes*, LysBPS13 (PGRP + SH3_5) and Ply21 (*N*-acetylmuramoyl-L-alanine amidase + SH3_5) effectively lyses several bacilli species, and CP25L (*N*-acetylmuramoyl-L-alanine amidase + SH3_3) which is active against clostridial and bacilli species (Gervasi et al., 2014; Park et al., 2012; Son et al., 2012). One endolysin (PlySs2) containing an SH3_5 domain does not display genus/species specificity and maintains activity against both staphylococci and streptococci (and some other species). In this case, however, it has been determined that this phenomenon is due to the promiscuity of the catalytically diverse CHAP catalytic domain and not due to the SH3b binding domain (Gilmer et al., 2013; Yang et al., 2015). This SH3b domain has been shown to directly bind streptococci, but does not interact with staphylococci (Huang et al., 2015). Despite this, because this SH3b domain, in both its natural and chimeric forms, is linked to a CHAP domain (which can cleave bonds present in both staphylococci and streptococci), the full length endolysin possesses activity against multiple bacterial species.

ΦNM3 CBD

As mentioned, all discovered CBDs from endolysins against *S. aureus* have been classified as SH3b domains, except for one. The CBD of the endolysin from the ΦNM3 prophage from *S. aureus* Newman has no known identity to any domains in the database

and is described in the literature as “non-SH3b like” (Bae et al., 2006). The native ΦNM3 CBD is linked to a CHAP catalytic domain and it specifically binds staphylococci (Daniel et al., 2010). The ΦNM3 CBD has been the subject of engineering studies, due to its divergence from the typical staphylococcal cell wall binding domain (SH3b). ClyS, composed of the PlyTW endopeptidase EAD and the ΦNM3 CBD, displayed potent *in vivo* efficacy against *S. aureus*, decolonizing mouse nasal cavities, providing protection against systemic infection, and reducing the bacterial load in a skin infection after being topically applied (Daniel et al., 2010; Pastagia et al., 2011). ClyH (Ply187 CHAP + ΦNM3 CBD) also eliminated MRSA in a mouse model of systemic infection (Yang et al., 2014b)

Endolysin Advantages

The use of endolysins is not only preferred over using whole bacteriophage, as mentioned, but has many benefits over the use of classical antibiotics as well. Most importantly, no resistance to endolysins has been observed, despite efforts to isolate resistant mutants (Pastagia et al., 2011). Whereas antibiotics act against easily mutable targets (Spratt, 1994), the activity of an endolysin is due its binding and cleavage of highly conserved substrates. Furthermore, there is no chance of non-target resistance due to this high specificity. The usage of broad spectrum antibiotics may allow for the overgrowth of certain species of normal flora, resulting in a state of dysbiosis; thus, they could also encourage the development of resistance in populations of bacteria that normally occupy a small niche (Nelson et al., 2012; Schmelcher et al., 2012a). This selective nature of endolysins also alleviates some of the non-desirable side effects

caused by antibiotic-induced disruption of normal flora. The use of antibiotics can cause diarrhea and nausea/vomiting or vaginal and oral yeast infections due to the depletion of typical species that live in these environments and the overgrowth of infectious bacteria (Tedesco et al., 1974), whereas an endolysin would only eliminate the targeted pathogen. The most common side effect associated with antibiotics is an allergic reaction, ranging from the mild (red, itchy, flaky, or swollen skin rash) to the severe (peeling and blistering skin or eye problems) and life-threatening (anaphylaxis) (Anderson, 1992). Despite the proteinaceous nature of endolysins, they have been shown to be non-immunogenic and do not cause any of the allergic reactions associated with antibiotics. Endolysins also hold promise in the elimination of biofilms (Sass and Bierbaum, 2007; Son et al., 2010), whereas antibiotics are ineffective against bacteria in this lifestyle (Fig. 1-5) (Hoiby et al., 2010). Additionally, antibiotics can only be utilized in the field of medicine, whereas endolysins can not only be used for public health, but have other applications, such as disinfectants or detection agents in the field of food safety or as biotechnological tools.

From an economic standpoint, pharmaceutical companies do not want to spend money developing a traditional antibiotic when the payoff is so low, due to the fact that their product may soon be obsolete, when resistance emerges. Therefore, hardly any new antibiotics are coming through the pipeline; this, combined with the inability to use certain current antibiotics, puts society in a frightening situation (Boucher et al., 2013). The promise of endolysins' inability to develop resistance, along with the potential of extending the use of antibiotics formerly thought to be ineffective through combinatorial therapy with endolysins, means that the pharmaceutical industry can continue to invest in preventing and treating bacterial infections.

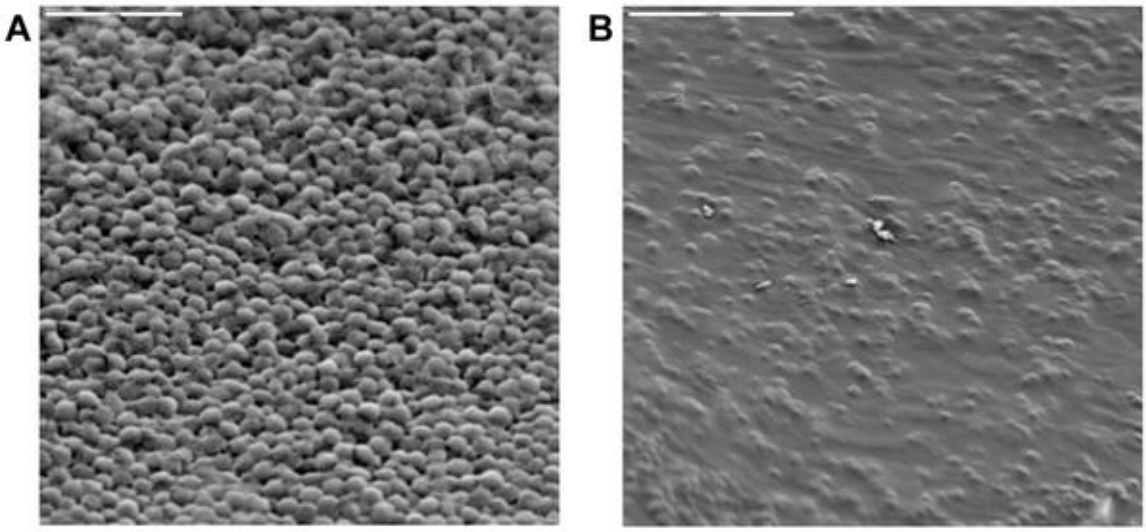

Figure 1-5. Scanning electron micrographs of LysH5-treated *S. aureus* biofilms. (A) Biofilms formed by *S. aureus* 15981 after 24 hours show cells attached to each other and the surface and covered in a layer of EPS. (B) After endolysin treatment, bacteria in the biofilm have been lysed and all material has been removed from the surface. Figure from (Gutierrez et al., 2014)

S. aureus Endolysins

Many laboratories have been searching for endolysins effective against *S. aureus* for the last two decades. Difficulties in obtaining staphylococcal endolysins in particular include issues with solubility, yield, and stability. Despite these problems, scientists have identified or constructed approximately 50 endolysins with anti-staphylococcal activity (Table 1-1). Measurements of endolysin activity can be obtained using many different methods (zymogram, plate lysis assay, turbidity reduction, minimum inhibitory concentration (MIC), colony forming unit (CFU) counts, etc.), and experiments have been performed on numerous strains under variable conditions; thus it is notoriously difficult to compare one endolysin to another. Standardization of the determination of endolysin activity has been attempted, but needs to be effectively enforced (Briers et al., 2007; Mitchell et al., 2010).

Antimicrobial Potential

As mentioned, a major benefit to using an endolysin over a traditional antibiotic is the pathogen's inability to develop resistance. Despite many attempts to isolate resistant mutants, researchers have been unsuccessful. LysH5 and chimeric endolysins composed of HydH5 fused to lysostaphin or just the lysostaphin CBD were unable to select for resistant mutants after 10 days in both solid and liquid media, while lysostaphin MICs increased 100-fold and 1000-fold, respectively (Rodriguez-Rubio et al., 2013b). Similarly, when exposed to increasing amounts of PlySs2 over 8 days, neither a MSSA nor a MRSA strain developed resistance (defined as a four-fold increase from the original

Table 1-1. *S. aureus* endolysins.

Endolysin	Activity
PlyTW	shows *N*-acetylmuramoyl-L-alanine amidase activity via HPLC analysis (Loessner et al., 1998), has specific activity of 0.2 OD_{600}/min/mg, increases 3 fold when amidase-2 domain is removed (Becker et al., 2015)
Lys16	crude lysate lyses 12 strains of *S. aureus* (Takac et al., 2005)
P68 Protein 17	50 ng/ml results in 2 log killing (Takac and Blasi, 2005)
LysWMY	displays activity on zymogram (Yokoi et al., 2005)
LysK	crude lysate causes 2 log killing (O'Flaherty et al., 2005), purified LysK has specific activity of 0.04 OD_{600}/min/mg, MIC = 32 µg/ml (Becker et al., 2008)
443-Lyso, 182-Lyso	has specific activity of 0.127 and 0.059 OD_{600}/min/mg, respectively (Donovan et al., 2006a)
Φ11	has specific activity of 1.5 OD_{600}/min/mg (Donovan et al., 2006c), 20 µg/ml reduces OD_{600} 60% in 20 minutes, 10 µg removes biofilms (Sass and Bierbaum, 2007)
MV-L	5 µg reduces CFU counts by 2-5 logs in 30 minutes, 50 µg protects 100% of mice in MRSA nasal colonization model (Rashel et al., 2007)
λSA2	digests cell walls as per mass spectrometry analysis (Pritchard et al., 2007), displays weak activity in plate lysis assay (Donovan and Foster-Frey, 2008) 10 µM reduces OD_{600} by >75% in 30 minutes (Roach et al., 2013)
LysH5	5 U/ml reduces OD_{600} by 1.25 in 600 seconds, 160 U/ml decreases bacterial counts by 6 logs in 4 hours in pasteurized milk (Obeso et al., 2008), MIC = 50 U/ml (Garcia et al., 2010), has specific activity of 166.85 U/mg (Rodriguez-Rubio et al., 2012a), 0.15 µM reduces *in vitro* biofilm bacterial counts by 1-3 logs (Gutierrez et al., 2014)
P16-17	displays activity on zymogram, 10 µg/ml reduces bacterial counts by 95% in 1 hour (Manoharadas et al., 2009)
$CHAP_K$	0.5 nmol reduces OD_{600} by 90% in 30 minutes (Horgan et al., 2009), 5 µg/ml causes 3 logs killing, MIC = 31.25 µg/ml,

	(Fenton et al., 2011b) 925 µg/60 µl decreases bacterial counts in nares of mice by 2 logs (Fenton et al., 2010a), 500 µg/ml reduces biofilm bacterial count by 4 logs, 200 µg/ml reduces bacterial count on pig skin by 2 logs (Fenton et al., 2013)
SAL-2	5 µg displays activity on plate lysis assay, 20µg removes biofilms (Son et al., 2010)
ClyS	250 µg results in OD_{600} decrease of 0.6 and 3 log killing in 30 minutes, 1 mg reduces the bacterial load in the nasal cavities of mice by 2 logs and protects 88% from MRSA septicemia (Daniel et al., 2010), 10% (wt/wt) dose causes a 3 log reduction in a mouse skin infection model (Pastagia et al., 2011)
SAL-1	MIC = 0.2 – 3.2 µg/ml, 5 µg reduces OD_{600} by 0.5 in 15 minutes (Jun et al., 2011), 10 µg/ml removes biofilms, 12.5 mg/kg rescues mice in iv septicemia model (Jun et al., 2013)
P-27/HP	10 µg/ml reduces CFU counts by 4 logs in 2 hours, 250 µg/ml rescues 100% of mice from bacteremia (Gupta and Prasad, 2011b)
LysAB2	displays activity on zymogram, 500 µg/ml kills 82% of bacteria (Lai et al., 2011)
PRF-119	MIC = 0.024–0.780 µg/ml (MSSA), MIC = 0.024–1.563 µg/ml (MRSA) (Idelevich et al., 2011)
HydH5, CHAP, LYZ2	HydH5 and catalytic domains alone (20 µg) reduce bacterial counts by 40%, 25%, and 23%, respectively (Rodriguez et al., 2011)
LysGH15	40 µg/ml reduces OD_{600} 80% in 30 minutes, 50 µg protects 100% of mice from MRSA induced bacteremia and reduces the bacterial load in the spleen 2 logs (Gu et al., 2011a; Gu et al., 2011b)
P128	50 µg/ml reduces OD_{600} by 0.8 in 30 minutes, 100 µg decolonizes 44% of rat nares (Paul et al., 2011), MIC = 1- 64 µg/ml, MBC = 1-64 µg/ml, 1.5 µg/ml reduces bacterial counts by 5 logs in simulated nasal fluid (Vipra et al., 2012), 10 µg/ml kills 3-5 logs in serum, plasma, and whole blood (George et al., 2012), 6 µg reduces OD_{600} by 65-75% in 30 minutes (Saravanan et al., 2013), ≥12.5 µg/mL reduced biofilm

	biomass by up to 95.5% (Drilling et al., 2016)
λSA2-E-Lyso-SH3b, λSA2-E-LysK-SH3b	have specific activities of ~.01 OD_{600}/min/mg, display activity on plate lysis assay (Becker et al., 2009b), reduces bacterial counts in mouse intramammary infection (0.63 and 0.81 log for λSA2-E-Lyso-SH3b and λSA2-E-LysK-SH3b), λSA2-E-LysK-SH3b combinatorial treatment with lysostaphin reduces bacterial counts 3.36 logs (Schmelcher et al., 2012c)
Lys168-87, Lys170-87	10 µg displays activity on plate lysis assay, 10 µg/ml reduces OD_{600} by 40% in 1 hour (Fernandes et al., 2012)
ΦSH2	has specific activity of 0.02 OD_{600}/min/mg, increases 3 fold when amidase and SH3b domain are deleted (Schmelcher et al., 2012b)
HydH5, HydH5Lyso, HydH5SH3b, CHAPSH3b	display activity on zymogram and plate lysis assay, have specific activity of 0.03-0.1 OD_{600}/min/mg (Rodriguez-Rubio et al., 2012b), reduce CFU counts by 2 logs after 6 hours, 1.65 µM CHAPSH3b kills up to 3 logs in 30 minutes in raw and pasteurized milk (Rodriguez-Rubio et al., 2013a), cleaves peptidoglycan as per mass spectrometry analysis (Rodriguez-Rubio et al., 2013b)
2638A	0.5 µM yields specific activity of 0.07 OD_{600}/min/mg (Abaev et al., 2013)
LysSA4	5 µl produces clearing zone via plate lysis assay (Mishra et al., 2013)
CF301 (PlySs2)	32 µg/ml reduces OD_{600} >70% in 30 minutes, 128 µg/ml reduces CFU counts by 2-5 logs, 1 mg protects 90% of mice from MRSA bacteremia (Gilmer et al., 2013), MIC = 2-8 µg/ml, removes biofilms, 5 mg/kg rescues 70% of mice from MRSA-induced bacteremia (Schuch et al., 2014)
Ply187AN-KSH3b	has specific activity of 1.2 OD_{600}/min/mg (Mao et al., 2013), MIC = 4-8 µg/ml, reduces *in vitro* biofilms counts by 5 logs, 1 µg/µl administered intravitreally at 6 h or 12 h postinfection reduces bacterial counts by 2 or 1 log, respectively, and attenuates symptoms of endophthalmitis (Singh et al., 2014)
LysDW2	displays activity on zymogram (Keary et al., 2014)
ClyH	MIC = 0.05-1.61 µg/ml, 360U protects 100% of mice (Yang et al., 2014b)

Ply187N-V12C	MIC = 2 μM, 50 pmol results in a clearing zone (Dong et al., 2015)
ClyR	25 μg/ml reduces OD_{600} by 0.05-0.4 in 20 minutes (Yang et al., 2015)
MR-10	18 μg/ml and 36 μg/ml reduces biofilm bacterial counts by ~1 log in 6 hours in *ica*-negative and *ica*-positive MRSA, respectively (Chopra et al., 2015)
80α, Φ11, LysK, P68, Twort, 2638A, ΦSH2, WMY	200 μg protects 100% of mice (80α, Φ11, LysK, 2638A, WMY), 60% of mice (ΦSH2), 50% of mice (Twort) from death induced by MRSA septicemia (Schmelcher et al., 2015)
LysDB	displays activity on zymogram, 10 μg/ml reduces the OD_{600} by 60% in 100 minutes, constitutive expression by a starter culture reduces the bacterial counts by 4 logs after 6 weeks compared to control cheese (Guo et al., 2016)
Hy133	MIC = 0.125 μg/ml - 0.5 μg/ml (Idelevich et al., 2016)
K-L	has specific activity of 5 OD_{600}/s/mg (Filatova et al., 2016), reduces intracellular bacterial load by ~50% in MAC-T cells and ~1 log in a mouse model of osteomyelitis, 1.4 μM reduces bacteria in a dynamic biofilm by 76% (Becker et al., 2016)
Trx-SA1	displays activity on plate lysis assay, 20 mg given once per day intramammary reduces bacterial counts over 3 days (Fan et al., 2016)

A summary of endolysins possessing activity against *S. aureus* and results obtained from these studies.

MIC) to this endolysin, while both strains developed resistance to mupirocin (Gilmer et al., 2013).

While technically not an endolysin, lysostaphin has peptidoglycan hydrolase activity and its M23 lytic domain has often been utilized in chimeric engineering studies. Importantly, as mentioned, lysostaphin resistant *S. aureus* has been isolated both *in vitro* and *in vivo* (Climo et al., 1998; Stranden et al., 1997). One mechanism by which this occurs involves mutations in *femA* or *femB*, which are responsible for adding the second and third or fourth and fifth glycines to the staphylococcal cross bridge, respectively (Ehlert et al., 1997). A mono- or triglycine cross bridge is therefore more resistant to recognition and cleavage by lysostaphin. The other mechanism of lysostaphin resistance involves acquisition via shuttle vector of the lysostaphin immunity factor (*lif*)/ endopeptidase resistance gene (*epr*) (Thumm and Gotz, 1997). *S. aureus* that express this protein display serines instead of glycines in their peptidoglycan cross bridges, which cannot be recognized and therefore cleaved by lysostaphin. However, lysostaphin resistance is not a terrible acquisition as it is incompatible with resistance to β-lactam antibiotics (Climo et al., 2001), even in strains that are originally MRSA. Thus, the acquisition of lysostaphin resistance by MRSA renders these strains now susceptible to β-lactams; it has also been shown that antibiotic treatment used concurrently with lysostaphin prevented lysostaphin resistant mutants from arising. Additionally, lysostaphin resistant mutants are not as fit, as shown by reduced growth rate, increased susceptibility to high temperatures, and less virulence in a mouse kidney infection model (Kusuma et al., 2007).

The high specificity of endolysins for their bacterial targets means that they can be safely used in medical applications; because the cleavage and binding targets are not present in eukaryotic cells, endolysins will not negatively interact with them. Endolysins have been used effectively in many animal models without causing harmful, irritating, or abnormal side effects (Fenton et al., 2010b). Additonally, endolysins have not displayed toxicity in tissue culture; PlyC did not compromise the membrane integrity of A549 lung epithelial cells, even though this endolysin has the unique capability to become internalized (Shen et al., 2016). Similarly, endolysins that were engineered to target intracellular staphylococci by adding a protein transduction domain (PTD) did not harm cells in tissue culture (Becker et al., 2016). These studies show that an endolysin can interact with the eukaryotic cell membrane without negatively impacting the health of the cell. Co-cultures of bacteria with eukaryotic cells lines have shown that the endolysin (whether it can go intracellular or not) will specifically lyse the target bacteria without harming non-bacterial cells. A major concern, however, is the fallout from massive and rapid bacterial lysis: an influx of proinflammatory cellular debris (teichoic acids, lipoteichoic acids and peptidoglycan), leading to life-threatening complications, such as septic shock and multiple organ failure (Nau and Eiffert, 2002). The use of interval dosing instead of continuous infusion may alleviate this issue (Entenza et al., 2005; Witzenrath et al., 2009).

Because endolysins are proteins, they have the ability to elicit an immune response. Mice that were injected with the pneumococcal endolysin Cpl-1 did in fact generate antibodies, and the resulting hyperimmune serum slightly decreased the efficacy of Cpl-1 *in vitro*; however *in vivo* these antibodies did not cause a reduced ability of Cpl-

1 to protect mice during an *S. pneumoniae* challenge (Loeffler et al., 2003). No adverse immunogenic side effects or inactivation of endolysin were also reported in several other studies (Jado et al., 2003; Rashel et al., 2007). It has been proposed that the affinity of the CBD for the bacterial surface is higher than that of IgG for the endolysin, thus explaining the maintenance of potent activity *in vivo* (Loessner et al., 2002). The short half-life of endolysins (Loeffler et al., 2003) can be compensated for by addition of polyethylene glycol to the protein (Walsh et al., 2003). In the case of abolished or severely decreased enzymatic activity (Resch et al., 2011a), it has been shown that dimerization can improve the pharmacokinetics of endolysins (Resch et al., 2011b).

Excitingly, endolysins have displayed synergistic behavior when used in combination with each other or antibiotics, resulting in lower doses, increased efficacy, and reduced risk of resistance development. Synergy when using multiple endolysins can be explained by the cleavage of multiple different bonds at the same time, or cleavage by one endolysin could facilitate access to the cleavage target of a second endolysin (Becker et al., 2008; Schmelcher et al., 2012c). Synergy between antibiotics and endolysins is important because it can potentially reinvigorate the use of antibiotics that were previously thought to be ineffective. Staphylococcal resistance to vancomycin and daptomycin was suppressed in the presence of the endolysin CF-301, which was shown to accelerate binding of these antibiotics to the bacterial cell, and utilization of combinatorial therapy proved to be more effective that any of the individual treatments (Schuch et al., 2014).

Endolysin-Related Applications

The antimicrobial potential of these enzymes has recently resulted in the formation of endolysin-specific companies that are trying to harness their commercialization power. In fact, large corporations took interest in bacteriophage as commercial entities immediately after their initial discovery, but as mentioned, the antibiotic era pushed them into the background; L'Oréal marketed five preparations including Bacté-staphy-phage, and in the U.S., the Eli Lilly Company had seven phage products for human use, including Staphylo-lysate (Sulakvelidze et al., 2001).

An obvious application for the use of endolysins is in the medical field. Utilization of endolysins as preventative or treatment options has been highlighted in this age of increasing antibiotic resistance. The Dutch company Micreos has been marketing Staphefekt™ since 2013, when it became the first endolysin product for human use against *S. aureus* skin conditions with an infectious component, such as acne, eczema, rosacea and skin irritation. Staphefekt™ is a chimeolysin composed of the M23 glycyl glycyl endopeptidase domain from lysostaphin, the amidase domain from 2638, and the SH3b domain from 2638 that displays potent anti-staphylococcal activity (Offerhaus and Eichenseher, 2015). Speaking to the potential of this product, Micreos secured $13 million in early 2016 for further clinical development and declared using Staphefekt™ to treat diabetic wound infections, ulcers, and burn wounds is in their pipeline. In 2015, Contrafect Corp. became the first company in the U.S. have an endolysin (CF-301 for the treatment of *S. aureus* bacteremia) enter and complete a phase I clinical trial in healthy volunteers and no adverse effects were observed. It was granted Fast Track Designation from the Food and Drug Administration (FDA), due to its 70% protection in a mouse

model of MRSA-induced bacteremia, and will be proceeding to phase II clinical trials (Gilmer et al., 2013). The numerous successful animal studies conducted utilizing endolysins against *S. aureus* (and other pathogens) indicate that this is just the beginning in the application of these enzymes for human medicine.

Not only can human health be improved by endolysins, but animal health stands to benefit as well. A veterinary or animal agriculture application of endolysins could improve the lives of animals and humans and could pave the way for a human health application, as the regulatory hurdles for product use in animals are greatly lowered. As mentioned, *S. aureus* is one of the four causative agents of bovine mastitis. Thus, this represents an opportunity for the introduction of endolysins for the treatment or prevention of staphylococcal-induced bovine mastitis. Several laboratories have investigated the activity of staphylococcal endolysins in milk and milk-simulating conditions, as a preliminary step in determining their efficacy as potential anti-mastitis agents; while activity was reduced in milk when compared to buffer, several endolysins displayed specific lytic activity against *S. aureus* (Donovan et al., 2006a; Donovan et al., 2006c; Mao et al., 2013). The efficacy of chimeric endolysins λSA2-E-Lyso-SH3b and λSA2-E-LysK-SH3b was validated in a mouse model of staphylococcal mastitis (Schmelcher et al., 2012c). The ultimate test of the application of endolysins as anti-mastitis agents was in transgenic cows expressing lysostaphin; protection against mastitis was effectively achieved, indicating the viability of this option (Wall et al., 2005). While lysostaphin is technically not an endolysin, its structural and behavioral similarity indicate that this technique could be replicated with endolysins. Treating staphylococcal-induced bovine mastitis is just one potential use for endolysins as animal therapeutics; as

many species are affected by *S. aureus* infections, staphylococcal endolysins could have a broad veterinary application.

An additional animal agricultural use for endolysins is as a disinfectant. An endolysin could be sprayed around animal housing facilities or on equipment as a safer alternative to the harsh chemicals currently used to decolonize surfaces and prevent the transmission of bacteria from surface to animal (or human). A proof of concept showed that a streptococcal endolysin, PlyC, could be aerosolized and effectively eliminate *Streptococcus equi* on surfaces (nylon, cotton, leather, neoprene, polyester, wood, stainless steel, glass) and under conditions (detergents, chelators, serum, hard water) found in horse stables (Hoopes et al., 2009).

Food safety is one area that could benefit greatly from the usage of endolysins, both as antimicrobial agents and as detection devices. Just as endolysins could be used on the farm to decontaminate surfaces, they could additionally be used in food processing plants. As *S. aureus* is a major causative agent of food borne illness, utilizing an endolysin to eliminate this pathogen on equipment or on the food itself would save both in food waste and in trips to the hospital. The activity of endolysins against biofilms makes them an ideal choice for use in decontaminating the many surfaces that food products touch in a processing facility (Schmelcher and Loessner, 2016). Additionally, they can be added directly to the product; food items that *S. aureus* has been associated with include meat products, poultry products, salads, bakery products, and it is a common contaminant during processing of milk into cheeses and other dairy products. Endolysins (Lysdb and LysH5) have been engineered to be secreted from starter culture *Lactobacillus* strains during milk processing and have been shown to effectively decrease

S. aureus contamination (Garcia et al., 2010; Guo et al., 2016; Obeso et al., 2008). A major obstacle in using endolysins against bovine mastitis or during milk processing is the reduction of activity in milk. Additionally, many studies have employed the use of pasteurized and/or homogenized milk, not raw milk, thereby not replicating the endolysin's application in the real world. Raw milk is a complex substance, containing eukaryotic cells and bacteria, and a lipid, protein, and carbohydrate profile different from processed milk (Jenness, 1974). If these enzymes have already shown decreased activity in milk free of contaminating factors and displaying different attributes, they may perform even more poorly when applied in the desired environment.

Endolysins could also be utilized in food safety as agents for the detection of pathogens. For example, CBDs bound to paramagnetic beads have been shown to effectively and quickly detect specific pathogens in food by polymerase chain reaction (PCR), fluorescent imaging, or surface plasmon resonance (SPR) (Kong et al., 2015; Kretzer et al., 2007; Walcher et al., 2010). CBDs have also been utilized in a chemical impedance sensor to identify bacteria in milk (Tolba et al., 2012).

Currently, companies such as Micreos and Intralytix have bacteriophage products targeting pathogens, including *Listeria, Salmonella, and E. coli*, available for use on food equipment and products that are approved by the FDA as "generally recognized as safe" (GRAS). As of yet, there are no GRAS endolysin products for use in the food processing industry, but the wealth of publications supporting their efficacy against many different pathogens on surfaces and food items indicates that they have the potential for this application.

While not relevant to staphylococcal endolysins, another application includes the use of endolysins as alternatives to antibiotics in the aquaculture industry (Richards, 2014). Additionally, endolysins could be utilized in plant agriculture; for example, transgenic potatoes expressing lysozyme were shown to be resistant to soft rot induced by *Erwinia carotovora* (During, 1993). One agricultural use that could be applied to staphylococcal endolysins is the use of plants as bioreactors; the pneumococcal endolysins Cpl-1 and Pal and the GBS endolysin PlyGBS were able to be produced in tobacco plant chloroplasts at enormously high levels (Oey et al., 2009a; Oey et al., 2009b).

Endolysins also have an application as biotechnological tools. They can be used as rapid and specific agents for obtaining nucleic acids or proteins (Loessner et al., 1995). They could be used to gently generate empty bacterial cell envelopes ("ghosts") for use as vaccines (Panthel et al., 2003). They could also be used in an auto-inducible lytic system in the development of bacterium based vaccines (Zhang et al., 2009). CBDs could be used as anchors to surface display proteins that could be used for live vaccine development, library screening, biocatalysis, and bioadsorption (Lee et al., 2003).

Phage GRCS and PlyGRCS

In an effort to identify a novel bacteriophage therapy to combat *S. aureus*-associated bacteremia, staphylococcal phage GRCS was isolated from raw sewage taken from a municipal sewage treatment system (Sunagar et al., 2010). Phage GRCS was classified as a member of the Podoviridae family based upon the observation of short noncontractile tails via electron microscopy (unpublished data). This bacteriophage

exhibited lytic activity *in vitro* against MSSA and MRSA. Because of the pressing need for new treatments against staphylococcal infections, phage GRCS was tested in a mouse model of bacteremia. Administration of phage GRCS 30 minutes post-infection protected 100% of the mice and outperformed oxacillin, even when given in multiple doses (Fig. 1-6). The bactericidal effect of phage GRCS was confirmed by observation of lower CFU of *S. aureus* in blood obtained from the mice 24 hours post infection, as compared to both PBS control and those treated with oxacillin. Delay of administration of phage GRCS post infection up to 4 hours resulted in 100% protection, and even postponing treatment 20 hours still rescued 20% of mice. Importantly, although titers of IgG and IgM mildly increased over 4 weeks after multiple injections of phage GRCS, it appears to be non-adversely immunogenic, as no anaphylactic reactions, changes in core body temperature, or other adverse events were observed.

Due to the high association of diabetes and multi drug resistant bacteremia, phage GRCS was also tested for its protective ability against *S. aureus* in a mouse model of streptozotocin-induced diabetes. While the diabetic mice were slightly more susceptible than non-diabetic mice to the *S. aureus* bacteremia, a 90% survival rate was achieved when phage GRCS was administered 30 minutes post infection. Furthermore, although a delay in phage treatment of diabetic mice led to less protection than that observed in non-diabetic mice at the same time points, it was still able to rescue 20% of mice when given 16 hours post infection.

The *in vivo* efficacy of phage GRCS suggests that it is a viable antimicrobial option for *S. aureus*-associated infections in both diabetic and non-diabetic models.

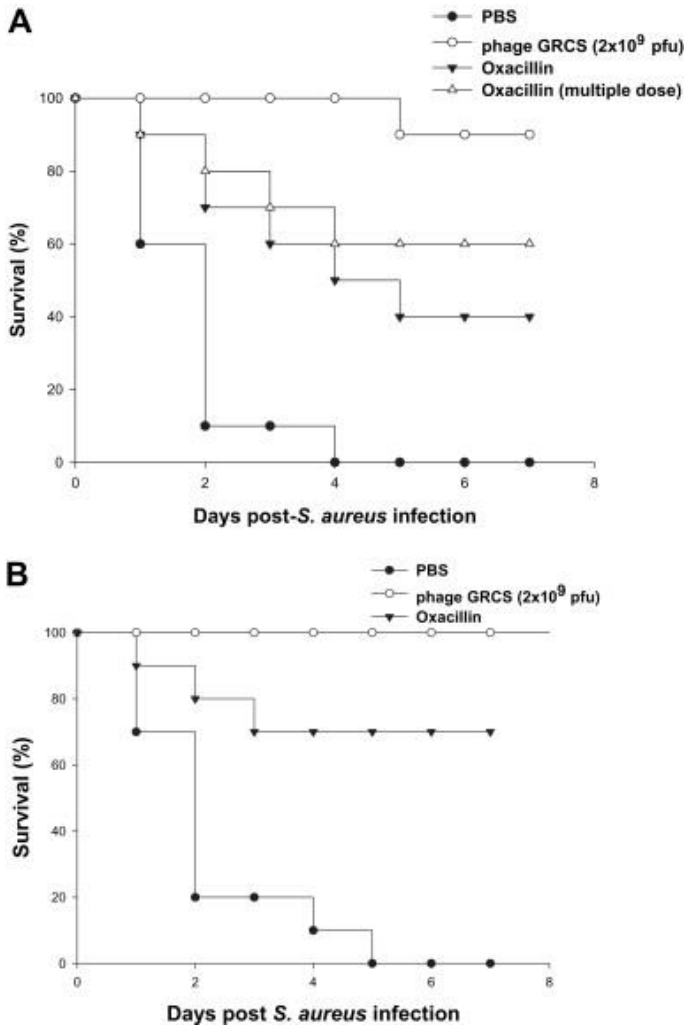

Figure 1-6. Bacteriophage GRCS efficacy in a mouse model of *S. aureus* bacteremia. Survival curves of (A) diabetic and (B) non-diabetic mice indicate that treatment with bacteriophage GRCS can protect mice from death by lethal bacteremic infection induced by *S. aureus* Figure from (Sunagar et al., 2010).

However, the desire to steer away from the use of whole phage for aforementioned reasons led us to investigate the bactericidal potential of the GRCS endolysin. To this end, the phage linear double-stranded DNA genome was sequenced (Swift and Nelson, 2014), an endolysin-like ORF was identified by bioinformatics analysis, and PlyGRCS was cloned, expressed, and tested for antimicrobial activity. This thesis represents the investigation into the antimicrobial potential of PlyGRCS against *S. aureus*.

Chapter II: Biochemical and biophysical characterization of PlyGRCS, a bacteriophage endolysin active against methicillin-resistant *Staphylococcus aureus*

This chapter, in part, is published as:

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Abstract

The increasing rate of resistance of pathogenic bacteria, such as *Staphylococcus aureus*, to classical antibiotics has driven research towards identification of other means to fight infectious disease. One particularly viable option is the use of bacteriophage-encoded peptidoglycan hydrolases, called endolysins or enzybiotics. These enzymes lyse the bacterial cell wall upon direct contact, are not inhibited by traditional antibiotic resistance mechanisms, and have already shown great promise in the areas of food safety, human health, and veterinary science. We have identified and characterized an endolysin, PlyGRCS, which displays dose-dependent antimicrobial activity against both planktonic and biofilm *S. aureus*, including methicillin-resistant *S. aureus* (MRSA). The host range for this enzyme includes all *S. aureus* and *S. epidermidis* strains tested, but not other Gram-positive pathogens. The contributions of the PlyGRCS putative catalytic and cell wall binding domains were investigated through deletion analysis. The cysteine, histidine-dependent amidohydrolase/peptidase (CHAP) catalytic domain displayed activity by itself, though reduced, indicating the necessity of the binding domain for full activity. In contrast, the SH3_5 binding domain lacked activity but was shown to interact directly with the staphylococcal cell wall via fluorescent microscopy. Site-directed mutagenesis studies determined that the active-site residues in the CHAP catalytic domain were C29 and H92, and its catalytic functionality required calcium as a co-factor. Finally, biochemical assays coupled with mass spectrometry analysis determined that PlyGRCS displays both *N*-acetylmuramoyl-L-alanine amidase and D-alanyl-glycyl endopeptidase hydrolytic activities despite possessing only a single catalytic domain.

These results indicate that PlyGRCS has the potential to become a revolutionary therapeutic option to combat bacterial infections.

Introduction

It has been estimated that 70% of the bacteria that cause hospital-acquired infections are now resistant to one or more antibiotics (Taubes, 2008). One of the most alarming antibiotic-resistant bacterial species is *Staphylococcus aureus*. Specifically, methicillin-resistant *S. aureus* (MRSA) are the group of *S. aureus* strains resistant to the entire class of β-lactam antibiotics. Hospital-acquired MRSA (HA-MRSA) often leads to severe and life-threatening infections, such as those at surgical sites, in the bloodstream, or pneumonia, while community-acquired MRSA (CA-MRSA) typically leads to superficial skin infections that can ultimately progress to induce severe invasive complications, such as necrotizing fasciitis (Lowy, 1998) (Tang and Stratton, 2010). Approval of new antibiotics, including linezolid (oxazolidinone class) in 2000, daptomycin (cyclic lipopeptide class) in 2003, and tigecycline (glycylcycline class) in 2005, provides alternatives to vancomycin, which was formerly the only antibiotic treatment for MRSA (Micek, 2007). These new antibiotics, along with increased awareness and adherence to universal decolonization practices have led to a significant decrease in the incidence of MRSA in intensive care units (Huang et al., 2013). Nonetheless, the most recent Centers for Disease Control report indicates there are still over 80,000 severe MRSA infections per year in the United States resulting in over 11,000 deaths (CDC, 2013). The same report labeled MRSA as a "serious" public health threat and vancomycin-resistant *S. aureus* (VRSA) as a "concerning" threat, underscoring the need for development of alternative therapeutics.

To counteract bacterial resistance and ameliorate the problems caused by *S. aureus* infections, endolysin therapy is one such avenue that is being pursued

(Borysowski et al., 2011; Nelson et al., 2012). Endolysins are enzymes released by bacteriophages during the lytic cycle of viral infection. Once produced within the bacterial cytoplasm by replicating bacteriophage, these enzymes hydrolyze bonds in the bacterial cell wall (i.e. peptidoglycan) until lysis is complete. The idea of utilizing endolysins therapeutically is based on the phenomenon of "lysis from without", a phrase used to describe the destruction of the bacterial envelope without production of phage virions (Abedon, 2011).

The classical structure of endolysins that act on Gram-positive cell walls employs a modular architecture consisting of an N-terminal catalytic domain linked to a C-terminal cell wall binding domain (CBD). The catalytic domain is responsible for cleaving specific covalent bonds in the peptidoglycan structure that are essential for maintaining its intrinsic structural integrity. The CBD confers endolysin specificity by recognizing and noncovalently binding to species- or strain-specific epitopes associated with the cell envelope. It is the high specificity derived by the combined actions of the catalytic and CBD domains that cause endolysins to be highly refractory to the resistance commonly observed upon treatment with classical antibiotics (Fischetti, 2005; Schuch et al., 2002). This is due to the evolution of bacteriophage to target specific, conserved bonds in the peptidoglycan of a bacteria cell wall, ensuring that the progeny phage will survive (Low et al., 2011). Even if resistance were to develop, endolysins can be engineered through domain shuffling or used in combination with other endolysins or antibiotics to prolong the use of these enzymes (Shen et al., 2012). Thus, they are promising candidates to help prevent or treat bacterial infections.

This study investigates PlyGRCS, the endolysin from the GRCS bacteriophage which was isolated from sewers in India (Sunagar et al., 2010). While a phage therapy study was performed on this bacteriophage, this analysis is the first of its kind to investigate the PlyGRCS endolysin. We have found that PlyGRCS exhibits strong activity against *S. aureus* and have conducted a biochemical and biophysical characterization of this enzyme.

Materials and Methods

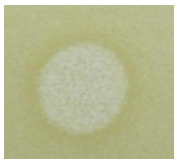
Bacterial Strains

Bacterial species, strains, and any associated antimicrobial resistance phenotypes are shown in (Table 2-1). All staphylococci containing the NRS strain designations were provided by the Network on Antimicrobial Resistance in *Staphylococcus aureus* (NARSA) which is distributed by BEI Resources depository in Manasas, VA, USA, under the direction of the National Institute of Allergy and Infectious Diseases and the National Institutes of Health. A *Streptococcus suis* clinical isolate was obtained from Dr. Randy Shirbroun at Newport Laboratories in Worthington, MN, USA. *Streptococcus pyogenes* and *Enterococcus facealis* were obtained from Drs. Vincent Fischetti and Alexander Tomasz, respectively, at The Rockefeller University, USA. A *Bacillus pumulis* clinical isolate was obtained from Dr. John Mayo at Louisiana State University, USA. The remaining strains, *Streptococcus pneumonia*, *Streptococcus uberis*, and *Streptococcus equi*, were obtained from the American Type Culture Collection (ATCC) as indicated in Table 2-1. All strains were stored at -80°C and routinely grown at 37°C.

Table 2-1. PlyGRCS spectrum of lytic activity.

Bacterial species, strains tested	PlyGRCS	$CHAP_{GRCS}$
MRSA NRS-385	+	-
MRSA NRS-382	+	-
MRSA NRS-384	+	+
MRSA NRS-71	++	+
VISA NRS-14	++	-
Staphylococcus epidermidis NRS-101	++	+
Streptococcus suis 730082	-	-
Streptococcus pyogenes D471	-	-
Streptococcus pneumococcus TIGR4	-	-
Streptococcus uberis 700407	-	-
Streptococcus equi 9528	-	-
Bacillus pumulis BJ0055	-	-
Enterococcus facealis EF24	-	-

Activity of PlyGRCS (6 µg) or $CHAP_{GRCS}$ (6 µg) against various species was evaluated via plate lysis assays. The strength of lytic zones was defined qualitatively: strong lytic zone = ++, weak lytic zone = +, no lytic zone = -. Examples of strong and weak lytic zones are shown below:

Strong lytic zone = 

Weak lytic zone = 

Streptococcal strains were grown in Todd-Hewitt broth, supplemented with 1% yeast extract (THY) (Alpha Bioscience), or on THY plates; staphylococcal strains, *B. pumulis*, and *E. facealis* were grown in trypticase soy broth (TSB) (Becton-Dickinson), or on TSB plates; *Escherichia coli* was cultivated in Luria Broth (LB) (Alpha Bioscience), or on LB plates. Unless otherwise indicated, all chemicals were purchased from Sigma and were of the highest purity available.

Cloning, Domain Constructs, and Site Directed Mutagenesis

The phage GRCS genome has recently been elucidated (GenBank Accession KJ210330) (Swift and Nelson, 2014). Bioinformatic analysis using BLAST and PFAM programs [both from the National Center for Biotechnology Information (NCBI)] predicted a putative endolysin for ORF15 (AHJ10590), which we named PlyGRCS, that contains an N-terminal cysteine, histidine-dependent amidohydrolase/peptidase (CHAP) catalytic domain and a C-terminal bacterial src-homology 3 (SH3_5) binding domain. As such, individual domain clones for CHAP (i.e. $CHAP_{GRCS}$) and SH3_5 (i.e. $SH3\_5_{GRCS}$) were amplified using the primer pairs shown in Table 2-2. For the full-length PlyGRCS, the CHAP-F and SH3_5R primers were utilized. All reverse primers incorporated a 6XHis purification tag. Specific point mutations to putative active-site residues (C29S and H92A) were made with phosphorylated primers (Table 2-2) using the Change-IT Multiple Mutation Site Directed Mutagenesis Kit (Affymetrix USB) according to the manufacturer's instructions. All PCR products were cloned into pBAD24, transformed into *E.coli* BL21 (DE3) cells and were consequently sequenced (Macrogen, Rockville,

Table 2-2. Primers.

Primer	Sequence (5' → 3')
CHAP-F	GGGGAATTCATTATGAAATCACAACAACAAGCAAAAGAATGGATATA
CHAP-R	AAATCTAGATTAATGATGATGATGATGATGACTAGCAGAAAATTTAG
SH3_5-F	GGGGAATTCATTATGAATACATTTGGAAATTGGAAACAAAACCAATAC
SH3_5-R	AAATCTAGATTAATGATGATGATGATGATGTGAGAACACCCCCCAAG
C29S	[Phos]-GCATATGGTTTTCAAAGCATGGACTTAGCTGTT
H92A	[Phos]-AATTCTCAATATGGTGCGATTCAATGTGTAATA

Primers utilized in this study to amplify the full-length PlyGRCS, each domain individually, and make site-directed mutations.

MD). The ApE program (University of Utah) was utilized for DNA sequence analysis and manipulations.

Expression and Purification

E. coli were grown at 37°C in baffled flasks to an OD_{600} = 1 in LB supplemented with 100 µg/ml ampicillin. Expression was induced with 0.25% arabinose overnight at 18°C. Crude protein extracts were purified by a Bio-Scale Mini Profinity IMAC Cartridge (Bio-Rad) and eluted in 10 ml fractions of 20 mM, 50 mM, 100 mM, 250 mM, and 500 mM imidazole, followed by SDS-PAGE analysis. Fractions containing proteins of the correct predicted molecular weight were pooled and dialyzed against PBS pH 7.4 with 300 mM NaCl.

Quantification of Lytic Activity

Lytic activity was based on turbidity reduction assay, as previously described (Nelson et al., 2012). Briefly, bacterial cells were centrifuged (4,000 RPM, 5 minutes, 4°C), resuspended in buffer and mixed 1:1 (v/v) with endolysin to a final OD_{600} = 1. OD_{600} readings were taken every 15 seconds for 20 minutes at 37°C. Endolysin activity was equated to the V_{max} dictated by the linear portion of the resulting killing curve. Each experiment was performed in triplicate.

Characterization of PlyGRCS

To determine dose response, PlyGRCS was serially diluted and each dosage (100 µl) was added in triplicate to a 96-well polystyrene microtiter plate (Nest Biotech Co, Ltd) just before addition of bacterial cells (100 µl) according to the turbidity reduction assay described above. For optimum pH determination, bacterial cells were suspended in 40 mM boric acid/phosphoric acid (BP) buffer, pH 3–11, and were challenged against PlyGRCS. The influence of NaCl on PlyGRCS activity was tested in BP buffer at the experimentally determined pH optimum using the same assay. The effect of divalent cations was determined using the turbidity reduction assay with several modifications. First, PlyGRCS was incubated at room temperature in PBS or PBS supplemented with 5 mM EDTA for 10 minutes. Secondly, the EDTA-treated samples received either no further treatment, or were supplemented with 6 mM $CaCl_2$ or 6 mM $MgCl_2$. Finally, the lytic active of the samples was then immediately assayed and compared to PlyGRCS in PBS prior to EDTA inactivation. Kinetic stability was evaluated as described (Son et al., 2012), with minor modifications. Lytic assays were performed in optimal conditions after PlyGRCS was incubated at indicated temperatures for 30 minutes and subsequently recovered on ice for 5 minutes.

Cell Wall Binding

An overnight culture of *S. aureus* NRS-14 was concentrated 5X in BP buffer and was incubated at room temperature with 10 µg $SH3\_5_{GRCS}$ containing the 6XHis tag for 10 minutes. A control without $SH3\_5_{GRCS}$ was also utilized. The samples were washed with PBS and incubated for 10 minutes at room temperature with 1 µl mouse anti-His

antibody (Gen Script). After washing with PBS, AlexaFluor-488 conjugated goat anti-mouse IgG (H+L) antibody (1 µl) (Invitrogen) was incubated with samples for an additional 10 minutes. Samples were washed again with PBS before being visualized via fluorescence and bright field microscopy. An Eclipse 80i epifluorescent microscope workstation (Nikon) with X-Cite 120 illuminator (EXFO) and Retiga 2000R CCD camera was used. NIS-Elements software (Nikon) was used for image analysis.

Host Range Analysis

Host range analysis was performed as described (Schmelcher et al., 2012c), with minor modifications. Bacterial cells were diluted in sterile PBS to an OD_{600} = 1 and spread on each plate. 10 µl spots (600 µg/ml) of PlyGRCS or $CHAP_{GRCS}$ were applied. Plates were incubated overnight at 37°C. Strength of lytic zones was defined qualitatively.

Biofilm Assay

An overnight culture of *S. aureus* NRS-14 (1 ml per well) was placed into 24-well CELLBIND plates (Corning) containing 500 µl of TSB per well. After an additional 24 hour incubation at 37°C, media was aspirated and samples were washed with PBS to remove unattached cells. Two-fold serial dilutions of PlyGRCS in triplicate were added in 1 ml BP buffer pH 7 and incubated at 37°C for one hour. Liquid was aspirated and samples were washed with distilled water before drying. Biofilms were stained with .01% crystal violet for 10 min at room temperature. After removing the excess crystal violet,

samples were washed with PBS and dried before the addition of 1 ml 10% SDS to extract the crystal violet from the biomass for quantification at OD_{595}.

Bactericidal Analysis

Sterile-filtered PlyGRCS was 2-fold serially diluted in PBS supplemented with 1 mM $CaCl_2$ and an equal volume of either various concentrations of enzyme or buffer only was added to 10^5 *S. aureus* NRS-14 in a microtiter plate. Samples were incubated at 37ºC for 1 hour, then serially diluted, plated on TSB agar, and incubated overnight at 37ºC to obtain CFU counts. The MBC (minimum bactericidal concentration) was determined as the minimum concentration of enzyme that killed ≥99.9% of bacteria.

Circular Dichroism (CD) Spectropolarimetry

CD experiments for wild-type (WT) and active-site mutants were performed on a Chirascan CD spectrometer (Applied Photophysics) equipped with a thermoelectrically controlled cell holder. CD spectra were obtained in the far-UV range (190-260 nm) in a 1 mm path length quartz cuvette at 1 nm steps with 5 second signal averaging per data point. Spectra were collected in triplicate, followed by averaging, baseline subtraction, smoothing and conversion to mean residue ellipticity (MRE) by the Pro-Data software (Applied Photophysics). Secondary structure prediction was performed using the Provencher and Glockner method (Provencher and Glockner, 1981) provided by DICHROWEB (Whitmore and Wallace, 2004). Melting experiments were performed by heating PlyGRCS at a 0.1 mg/ml concentration in 20 mM sodium phosphate buffer pH 7

from 20°C to 95°C using a 1°C/min heating rate. MRE was monitored at 218 nm in a 1 mm path length quartz cuvette at 0.5°C °C steps with 5 second signal averaging per data point. The melting data was smoothed, normalized and fit with a Boltzmann sigmoidal curve. The first derivative of the melting curve was then taken to determine the temperature (T_m) at which the folded and unfolded protein species in solution were at equilbrium (Fallas and Hartgerink, 2012).

Biochemical Assays

For analysis of reducing sugars released from the peptidoglycan, the dinitrosalicylic acid (DNSA) assay was used (Danner et al., 1993). *S. aureus* NRS-14 peptidoglycan was purified as previously described (Pritchard et al., 2004; Schmelcher et al., 2012d) and was treated for one hour at 37ºC with 50 µg/ml of PlyGRCS in optimal conditions. Samples were centrifuged and the supernatant was added to an equal volume of 87.7 mM DNSA (20 g/L in .7 M NaOH). After boiling for 5 minutes, samples were allowed to cool and the absorbance was read at OD_{535}. Known concentrations of glucose were used to create a standard curve. To determine an increase in free amine groups, the trinitrophenylation reaction originally described by Satake et al. and modified by Mokrasch was used (Mokrasch, 1967; Satake et al., 1960). Purified peptidoglycan (OD_{600}=1) was treated with PlyGRCS (50 µg/ml) for one hour at 37ºC. Samples were pelleted and the supernatant was filtered through a 0.22 µM filter. The sterile filtrate was added to sodium tetraborate and trinitrobenzenesulfonic acid and incubated for 30 minutes at room temperature. Samples were read at OD_{420}. Lysine was used as a standard.

Cleavage Analysis by Mass Spectrometry

For determination of cut sites within the staphylococcal peptidoglycan, purified cell walls were digested with PlyGRCS and the resulting fragments were analyzed via mass spectrometry as previously described (Becker et al., 2009a; Pritchard et al., 2004). Briefly, SA113 Δ*tagO* cell walls (Atilano et al., 2010; Weidenmaier et al., 2004) were digested in 25 mM Tris, 200 mM NaCl, pH 7.4 at 37°C for 18 hours with 50 µg/ml of PlyGRCS, filtered through 5000-MW cutoff Vivaspin 500 units (Sartorius North America Inc., Bohemia, NY), and desalted using C18 Zip Tips (Millipore, Zug, Switzerland). Controls included peptidoglycan digested with the amidase domain of 2638A, a known *N*-acetylmuramoyl-L-alanine amidase (M. Schmelcher, unpublished data), or undigested peptidoglycan. To further define the PlyGRCS cut site, double digests with PlyGRCS and a truncation construct containing only the CHAP domain of LysK (CHAP-K), a known D-alany-glycyl endopeptidase (Becker, Dong et al. 2009), were performed. The samples were eluted from the Zip Tips with 50:50:0.01 (v/v/v) CH_3OH:H_2O:HCOH (pH ~2), and NanoESI-MS analysis was performed on a Q-TOF Ultima API mass spectrometer (Micromass, UK).

Results

Expression of PlyGRCS and Domain Constructs

The phage GRCS genome was recently sequenced (KJ210330) (Swift and Nelson, 2014) and bioinformatic analysis predicted an endolysin for ORF15 (AHJ10590), which we named PlyGRCS. This enzyme contains a putative N-terminal CHAP domain, which has been shown to encompass bacteriolytic activity in other characterized endolysins, and

a C-terminal bacterial src-homology 3 (SH3_5) domain that functions as a CBD in many staphylococcal and streptococcal endolysins (Nelson et al., 2012). The closest homologs to PlyGRCS are a hypothetical protein from *S. aureus* 2011-60-1490-31 (EZV76040.1, 98% identity), an amidase from *Staphylococcus* phage 44AHJD (NP_817310.1, 96% identity), ORF009 of *Staphylococcus* phage 66 (YP_239469.1, 97% identity), the SAL-2 amidase from *Staphylococcus* phage SAP-2 (YP_001491539.1, 96% identity), and an unnamed protein product of *Staphylococcus* phage S24-1 (YP_004957430.1, 92% identity). To study the full-length enzyme and elucidate the contributions of each domain, we cloned the full length PlyGRCS, as well as its isolated CHAP domain ($CHAP_{GRCS}$, amino acids 1-140) and SH3_5 domain ($SH3\_5_{GRCS}$, amino acids 150-250) into expression vectors. All three constructs were expressed as soluble proteins and purified to homogeneity by nickel affinity chromatography via the C-terminal 6XHis tags on each protein.

Characterization of PlyGRCS

PlyGRCS displayed a dose-response curve from 28 to 1.75 µg/ml when tested in a turbidity reduction assay using stationary phase *S. aureus* NRS-14 cells (Fig. 2-1A). The highest dose corresponded to a 70% decrease in optical density in just 15 minutes (50% decrease in < 10 minutes). When tested at equimolar concentrations, $CHAP_{GRCS}$ displayed ~8% of full-length PlyGRCS activity (Figure 2-2). In contrast, $SH3\_5_{GRCS}$ did not display any lytic activity. However, this domain alone possessed the ability to specifically bind staphylococci as detected by antibody recognition of the 6XHis

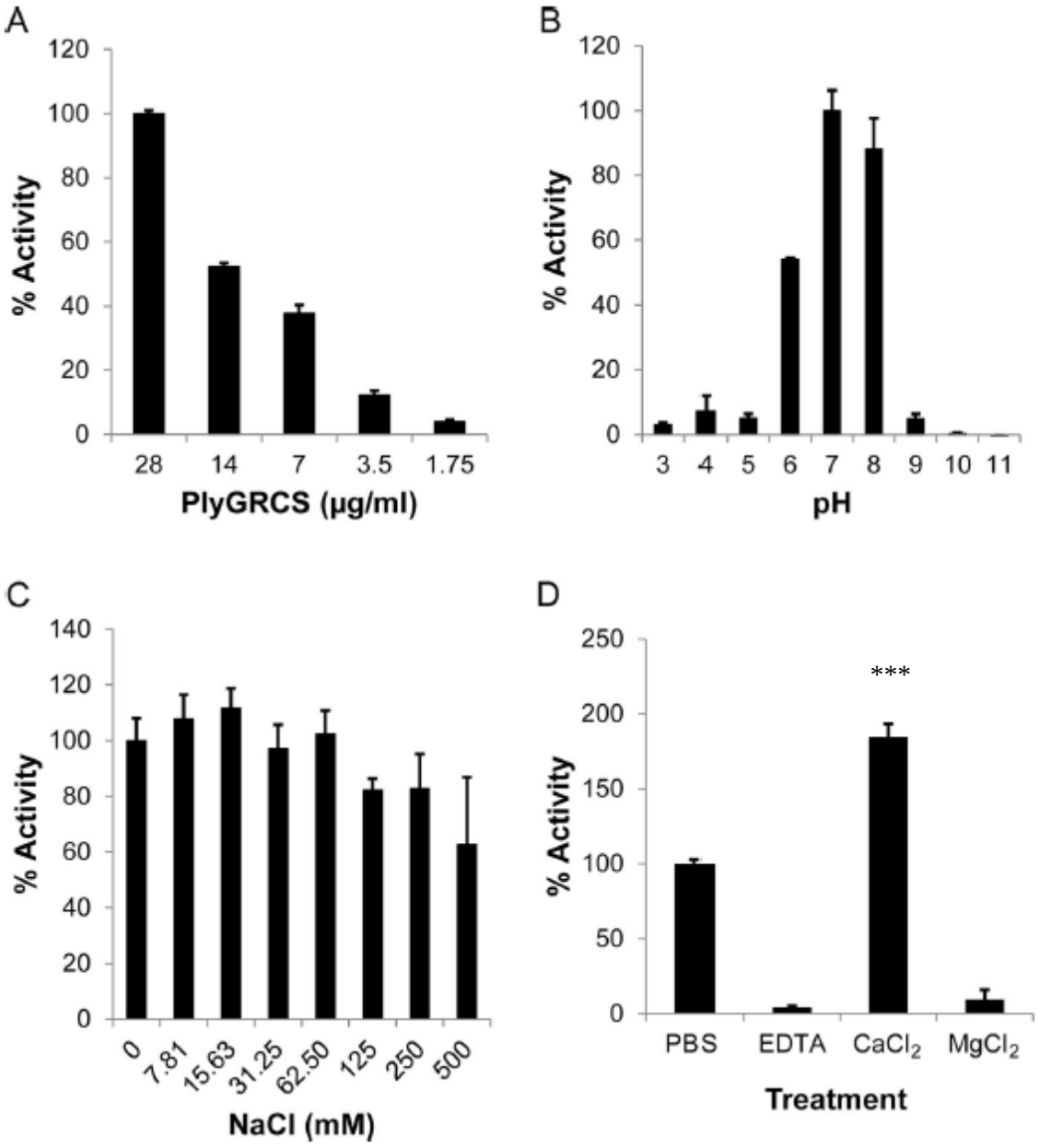


Figure 2-1. Biochemical characterization of optimal conditions for PlyGRCS activity. The influence of (A) dose, (B) pH, (C) NaCl, and (D) divalent cations on PlyGRCS activity against stationary phase *S. aureus* NRS-14. Error bars represent the standard deviation, and all experiments were done in triplicate. Statistical analysis was performed by unpaired *t* test. *** $P < 0.0001$

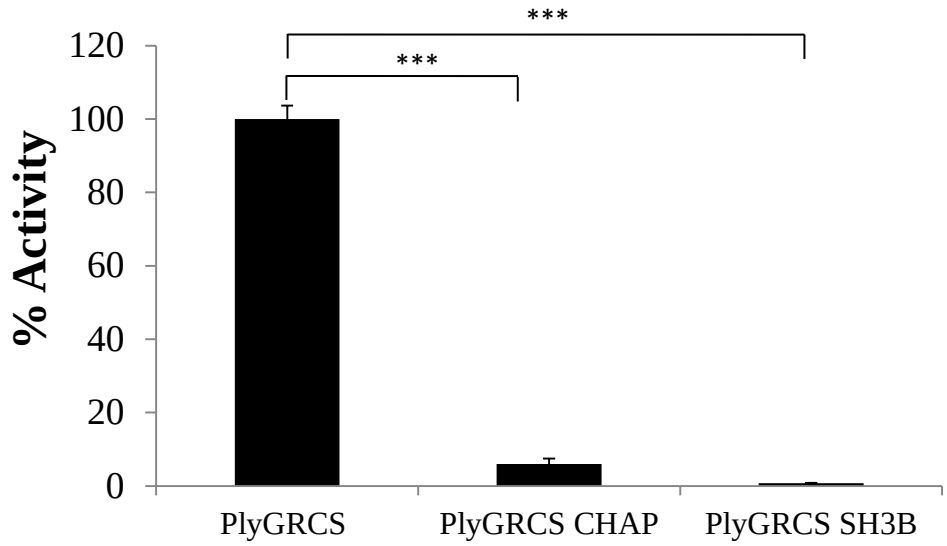


Figure 2-2. PlyGRCS contains an N-terminal catalytic domain. Turbidity reduction analysis reveals $CHAP_{GRCS}$ displays ~8% of full-length PlyGRCS activity, while $SH3\_5_{GRCS}$ does not display any lytic activity, indicating the importance of both domains for full activity. Error bars represent the standard deviation, and all experiments were done in triplicate. Statistical analysis was performed by unpaired *t* test. *** $P < 0.0001$

purification tag on the staphylococcal surface (Fig. 2-3). Control experiments without SH3_5$_{GRCS}$ did demonstrate binding of the antibody (data not shown). Therefore, while the CHAP domain is independently capable of lysing *S. aureus*, the full antimicrobial efficacy of the endolysin is dependent on the simultaneous presence of both the CHAP and SH3_5$_{GRCS}$ domains.

Lytic activity of PlyGRCS was then tested in BP buffer with a pH range from 3.0 to 11.0 to determine optimum conditions. Optimal pH was determined to be 7.0, with an active range between 6.0 and 8.0 (Fig. 2-1B). PlyGRCS activity was markedly reduced at pH extremes. Based on the above observations, subsequent turbidity reduction and antimicrobial assays were performed in BP buffer pH 7.0. Because the activity of many endolysins, including various staphylococcal endolysins (Becker et al., 2008; Garcia et al., 2010), is enhanced by the addition of NaCl, we investigated the activity of PlyGRCS in the presence of NaCl ranging from 0 to 500 mM. Surprisingly, NaCl had little effect (±10%) on PlyGRCS activity up to 125 mM and only slightly inhibited activity at higher concentrations (~35% decrease at 500 mM) (Fig. 2-1C). Several CHAP domain-containing staphylococcal endolysins (Donovan et al., 2006c; Fenton et al., 2011b), as well as streptococcal endolysins (Celia et al., 2008; Pritchard et al., 2004), have been shown to require calcium for activity. Furthermore, the structure of the staphylococcal LysGH15 CHAP domain shows calcium in an EF-hand-like structure (Gu et al., 2014) and the CHAP domain of PlyGRCS shares identity in three critical aspartic acid residues known to complex this cation in LysGH15 and other calcium binding proteins, although it only shows 42% in overall identity with the LysGH15 CHAP domain. With this in

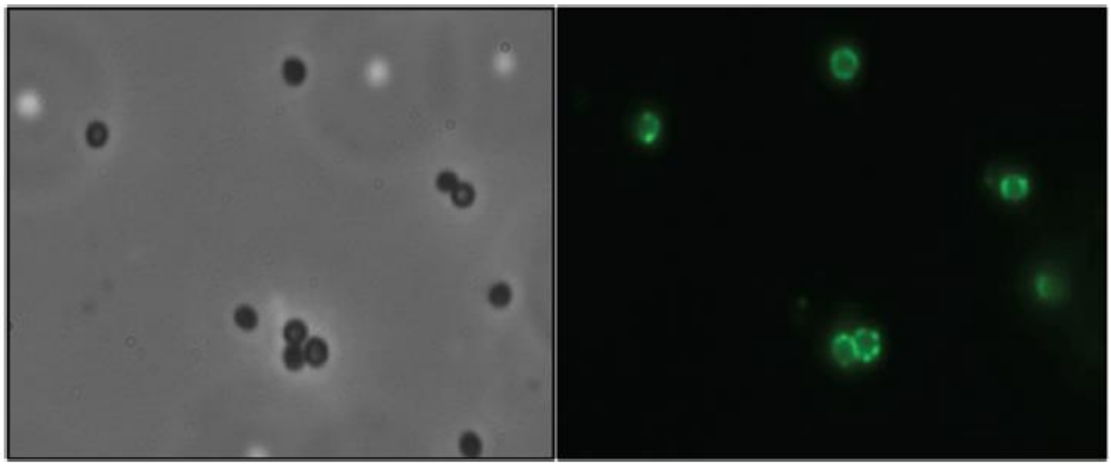


Figure 2-3. PlyGRCS contains a C-terminal cell wall binding domain. SH3_5$_{GRCS}$ directly interacts with *S. aureus* NRS-14. Images represent brightfield (left) and fluorescent (right) images viewed at 1000X magnification. Cell wall binding was detected via mouse anti-His and goat anti-mouse IgG AlexaFluor 488.

mind, the activity of PlyGRCS was analyzed in either the presence or absence of calcium. PlyGRCS was first incubated with EDTA to remove all divalent cations from solution. EDTA-treated PlyGRCS was devoid of lytic activity (Fig. 2-1D). Next, EDTA-treated PlyGRCS was incubated with excess $CaCl_2$. Calcium-treated PlyGRCS displayed nearly twice the lytic activity when compared to PlyGRCS prior to EDTA treatment. To determine divalent metal dependence of PlyGRCS is specific to calcium, the activity of the EDTA-treated endolysin was measured after the addition of an alternative divalent metal, magnesium. The activity of magnesium-treated PlyGRCS mimicked that of the EDTA-treated sample, suggesting that the divalent metal dependence of PlyGRCS is calcium-specific.

Finally, the kinetic and thermodynamic stability of PlyGRCS was investigated. PlyGRCS displayed > 90% residual lytic activity after incubating at temperatures ranging from 4°C to 37°C for a total of 30 minutes. At temperatures of ≥ 40°C, lytic activity was not observed (Fig. 2-4A). Melting experiments performed on a CD spectrophotometer show cooperative unfolding of PlyGRCS with a T_m of 43.5°C (Fig. 2-4B), which further confirms the lack of activity at ≥ 40°C observed during the kinetic stability experiment. $CHAP_{GRCS}$ (Fig. 2-4C) and $SH3\_5_{GRCS}$ (Fig. 2-4D) had similar T_m values of 44.8°C and 44.5°C, respectively. The observed PlyGRCS stability profile is consistent with that of other phage lysins. For example, the *S. aureus* endolysin LysK is kinetically inactivated at 42.0°C and the *Streptococcus pneumoniae* endolysin Cpl-1 displays a T_m of 43.5°C (Filatova et al., 2010; Sanz et al., 1993).

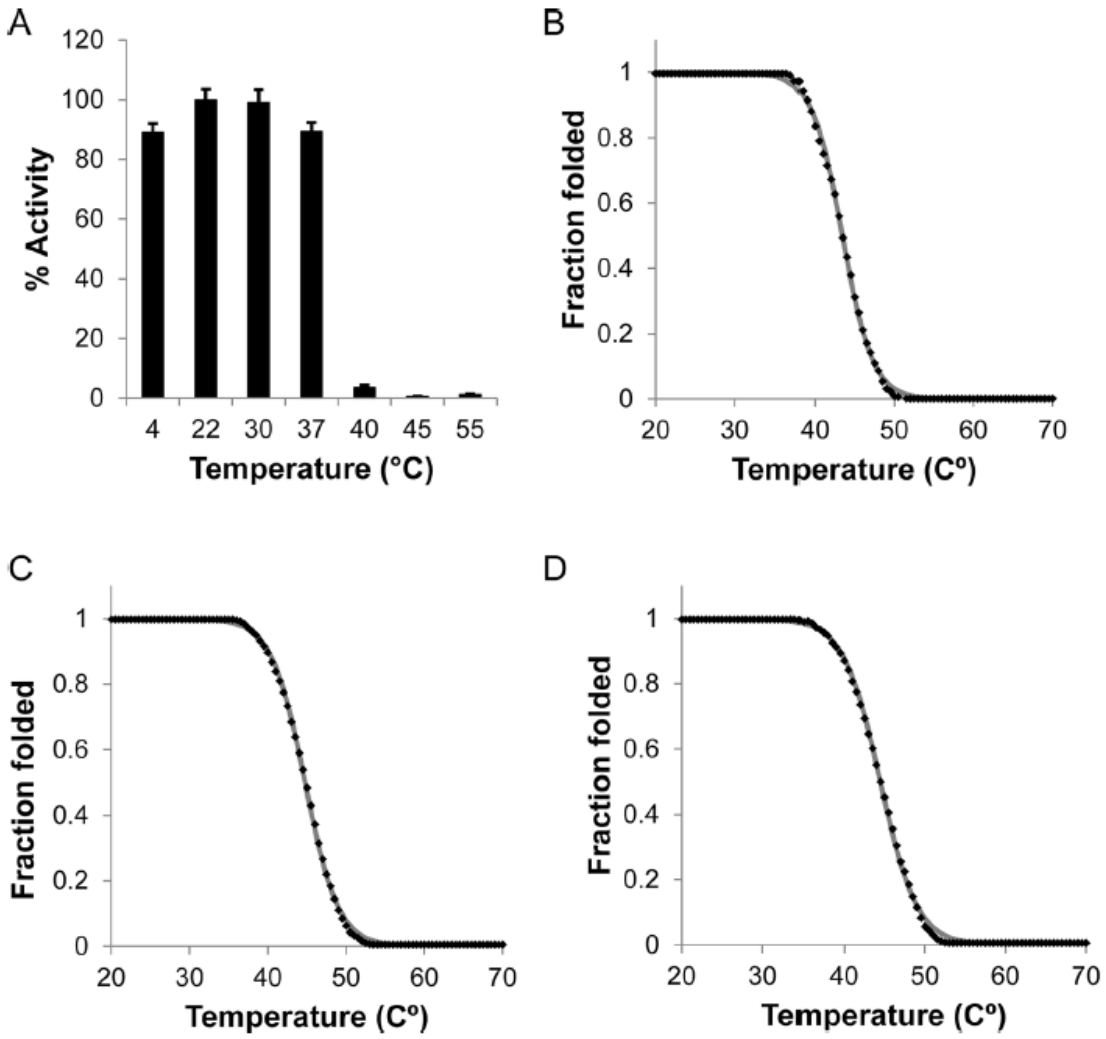


Figure 2-4. PlyGRCS temperature stability. (A) Stationary phase *S. aureus* NRS-14 treated with 25 μg/ml of PlyGRCS after being held at indicated temperatures for 30 min and recovered on ice for 5 min. Error bars represent the standard deviation, and all experiments were done in triplicate The thermal stability of (B) full-length PlyGRCS as well as (C) $CHAP_{GRCS}$ and (D) $SH3\_5_{GRCS}$ was determined by means of CD melting experiments. Samples were heated from 20 to 95 °C at 1 °C/min in 20 mM sodium phosphate buffer pH 7 using a protein concentration of 0.1 mg/ml.

PlyGRCS Host Range

In order to determine the host range of PlyGRCS, activity was tested against 13 different bacterial strains including methicillin-resistant and vancomycin-intermediate resistant *S. aureus*, methicillin-resistant *S. epidermidis*, and several other representative Gram-positive pathogens (Table 2-1). At 6 µg, lytic activity was seen against all staphylococcal strains, with PlyGRCS exhibiting the greatest strength against *S. aureus* strains NRS-71 and NRS-14 and *S. epidermidis* NRS-101. As expected, $CHAP_{GRCS}$ did not exhibit as much activity, causing only weak clearing zones on plates of *S. aureus* strains NRS-384 and NRS-71 and *S. epidermidis* NRS-101. No lytic activity was observed on any other strains. Thus, PlyGRCS has an activity spectrum confined to staphylococcal species, as no activity was observed against streptococci or representative bacilli and enterococci species listed in Table 2-1.

Biofilm Assay

Considering the ability of *S. aureus* to form biofilms and thus present a further barrier to traditional treatments, we investigated the anti-biofilm properties of PlyGRCS. When 1 day biofilms were treated with PlyGRCS for 1 hour, a dose response decrease in the amount of biofilm was visualized, with as little as 6.25 µg/ml affecting a ~50% decrease in biofilm biomass (Fig. 2-5).

Bactericidal Effects of PlyGRCS

It has been noted that the minimal inhibitory concentration (MIC) assay may not be the most appropriate assay to measure endolysin efficacy due to the speed at which the

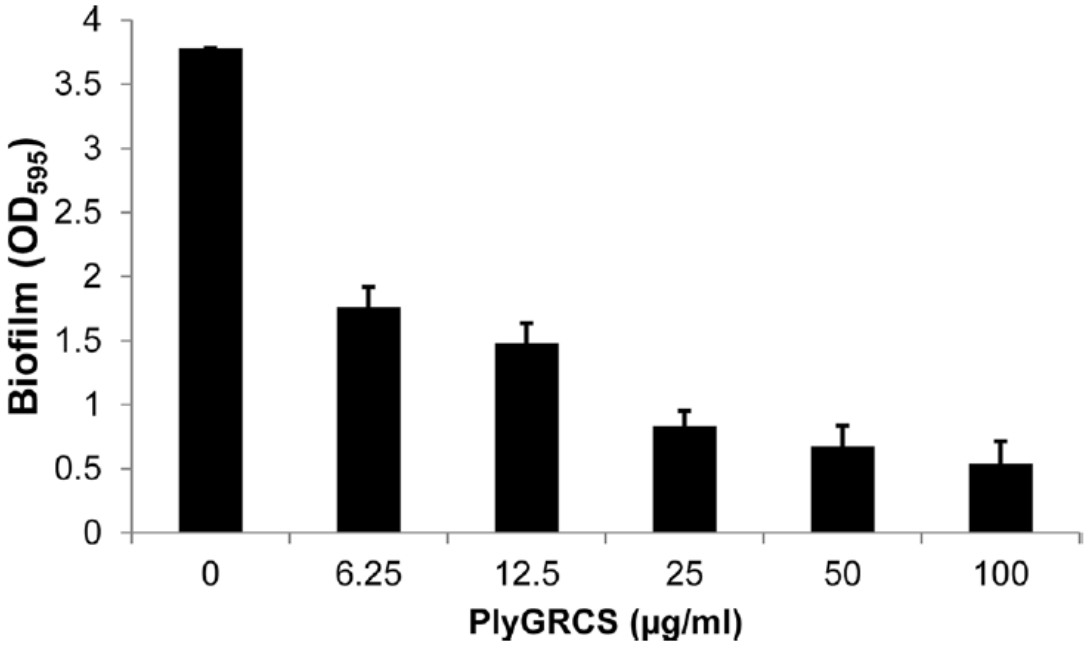


Figure 2-5. Antibiofilm activity of PlyGRCS. *S. aureus* NRS-14 was allowed to form static biofilms for 24 h and treated with PlyGRCS at indicated concentrations for 1 h. The amount of biofilm is represented by the quantification of crystal violet staining of biomass at OD_{595}. Error bars represent the standard deviation, and all experiments were done in triplicate.

enzyme acts (Kusuma and Kokai-Kun, 2005). Therefore, we employed the minimum bactericidal concentration (MBC) assay, which is the lowest concentration of enzyme that kills ≥99.9% (i.e. 3 logs) of the test inoculum (Jones et al., 1985). When tested against a VISA strain in stationary phase, 25 µg/ml PlyGRCS resulted in 3 log killing, 12.5 µg/ml yielded a 2.5 log decrease, and 6.25 µg/ml reduced bacterial counts by 2 logs (data not shown). Of note, VISA strains possess thicker cell walls than other *S. aureus* strains. This phenotype may cause the bacteria to be more resilient to endolysin treatment, and hence require higher than normal MBC values (Howden et al., 2010; Sieradzki and Tomasz, 2003). Nonetheless, our results compare favorably to other anti-staphylococcal endolysins. PlySs2 represents the only other staphylococcal endolysin with reported bactericidal activity against a VISA strain, requiring 128 µg/ml to decrease the colony counts of mid-log phase cells by 2 logs (Gilmer et al., 2013).

Confirmation of N-terminal CHAP Catalytic Domain

By definition, CHAP domains contain two invariant residues, a cysteine and a histidine (Bateman and Rawlings, 2003; Rigden et al., 2003). Presumably, the cysteine acts as a catalytic nucleophile and the histidine may function as a general base to deprotonate the thiol group of the cysteine. To determine the contributions of these putative critical residues in PlyGRCS, we used site-directed mutagenesis to alter C29 and H92, the residues identified by a PFAM alignment of PlyGRCS to archetypical CHAP domains. Circular dichroism analysis demonstrated that both the C29S and H92A point mutants had similar secondary structures to WT PlyGRCS (data not shown). No lytic activity was observed when the C29S mutant was used against *S. aureus* NRS-14 in a

turbidity reduction assay (Fig. 2-6); however, H92A still exhibited lytic activity, although reduced to 40% compared to WT. At present, it is not clear why activity is associated with the H92A mutant, although similar mutagenesis of active-site histidine residues in cysteine proteases have likewise displayed reduced, but measurable activity(Ekici et al., 2008; Khayat et al., 2001). While not proven, it is possible that other residues near the active-site residues could substitute for the histidine as an electron acceptor during the nucleophilic attack by the cysteine.

Cleavage Specificity of the CHAP Domain

CHAP domains are associated with *N*-muramoyl-L-alanine amidase (amidase) or endopeptidase activity (Bateman and Rawlings, 2003). Specifically, CHAP domains of staphylococcal endolysins have been characterized as amidases or D-alanyl-glycyl endopeptidases (Schmelcher et al., 2012a). To determine the specific catalytic nature of the PlyGRCS CHAP domain, two biochemical assays were employed to analyze the reducing sugar (indicative of glycosidase activity) or amine (indicative of amidase/endopeptidase activity) release upon PlyGRCS treatment. As predicted, PlyGRCS did not show any glycosidase activity. However, free amines were detected when *S. aureus* cell walls were treated with PlyGRCS, revealing that the catalytic activity is indeed an amidase or endopeptidase (Fig. 2-7A).

To further elucidate which hydrolytic activity PlyGRCS possesses, enzymatically digested *S. aureus* peptidoglycan preparations were subjected to electron spray ionization-mass spectrometry (ESI-MS). Unexpectedly, the PlyGRCS digest (Fig. 2-7B, top spectrum) revealed a peak at *m/z*=702.35, which could only be produced by the

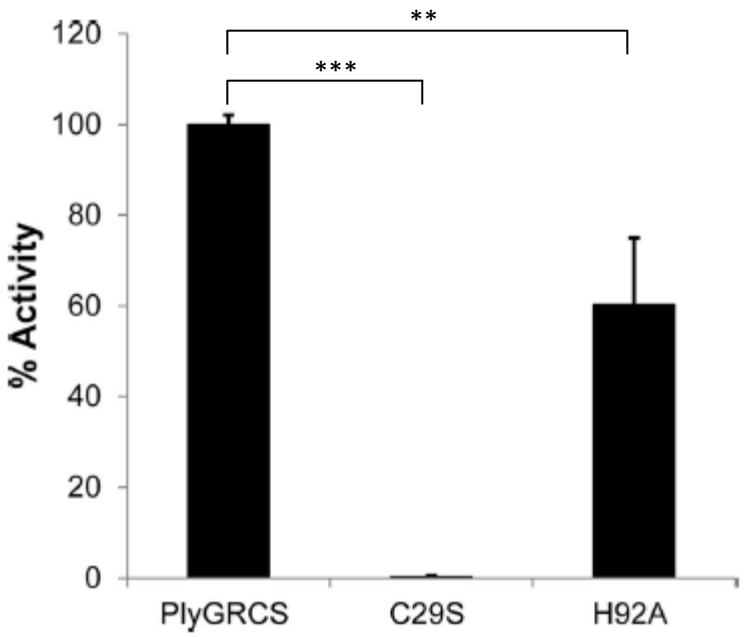
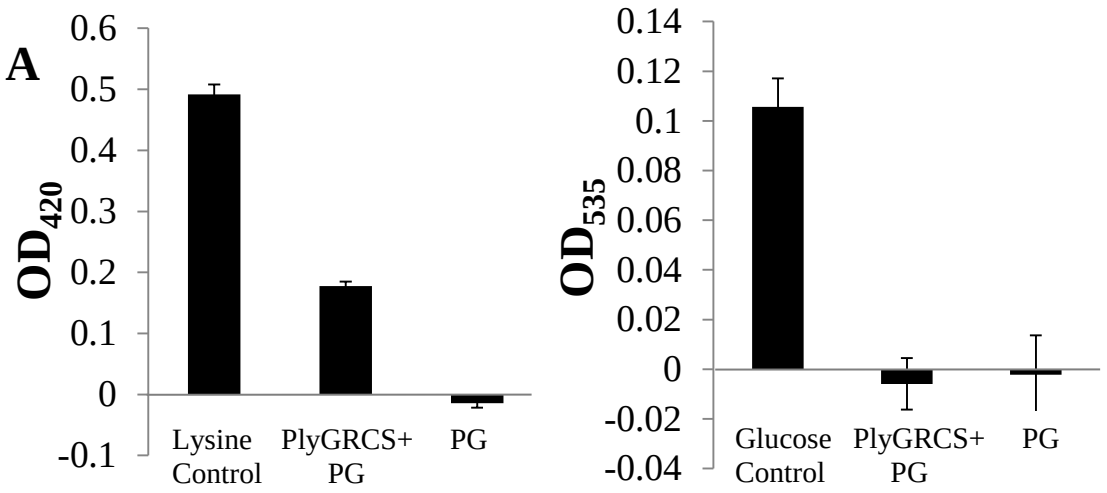
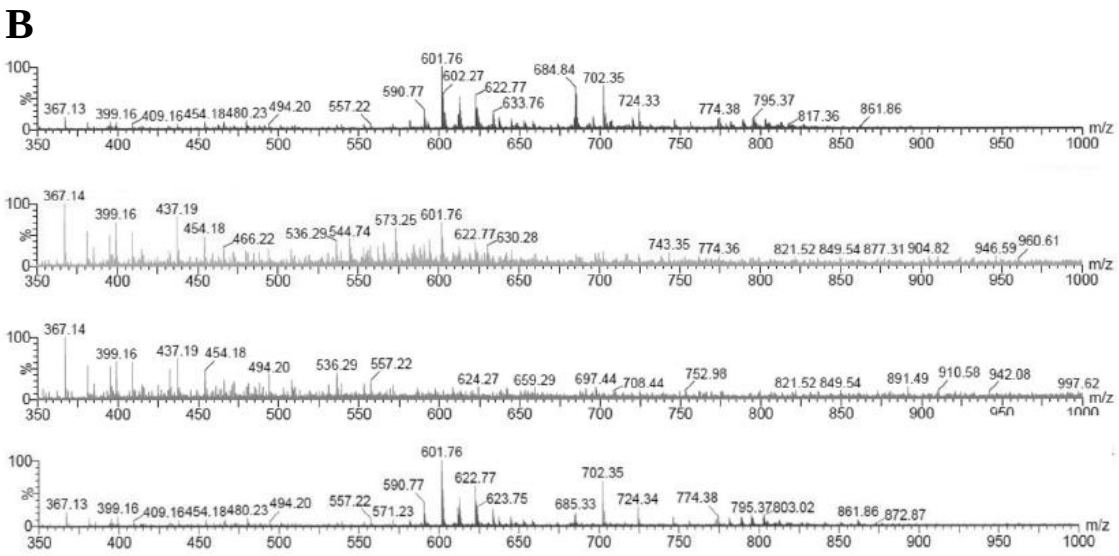
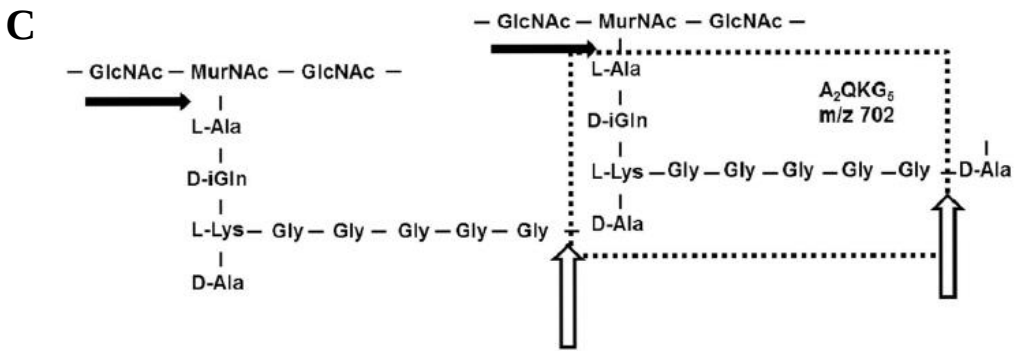


Figure 2-6. PlyGRCS contains an N-terminal catalytic domain with an active site cysteine and histidine. Stationary phase *S. aureus* NRS-14 was treated with 25 μg/ml PlyGRCS, PlyGRCS-C29S, or PlyGRCS-H92A. The reduction in activity of PlyGRCS-C29S and PlyGRCS-H92A indicates that these are the active site residues. Error bars represent the standard deviation, and all experiments were done in triplicate. Statistical analysis was performed by unpaired *t* test. *** $P < 0.0001$, ** $P < 0.01$.


A
OD420
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
Lysine Control
PlyGRCS+ PG
PG
OD535
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
-0.02
-0.04
Glucose Control
PlyGRCS+ PG
PG


B
m/z


C
— GlcNAc — MurNAc — GlcNAc —
L-Ala
D-iGln
L-Lys — Gly — Gly — Gly — Gly — Gly
D-Ala
A2QKG5
m/z 702

D

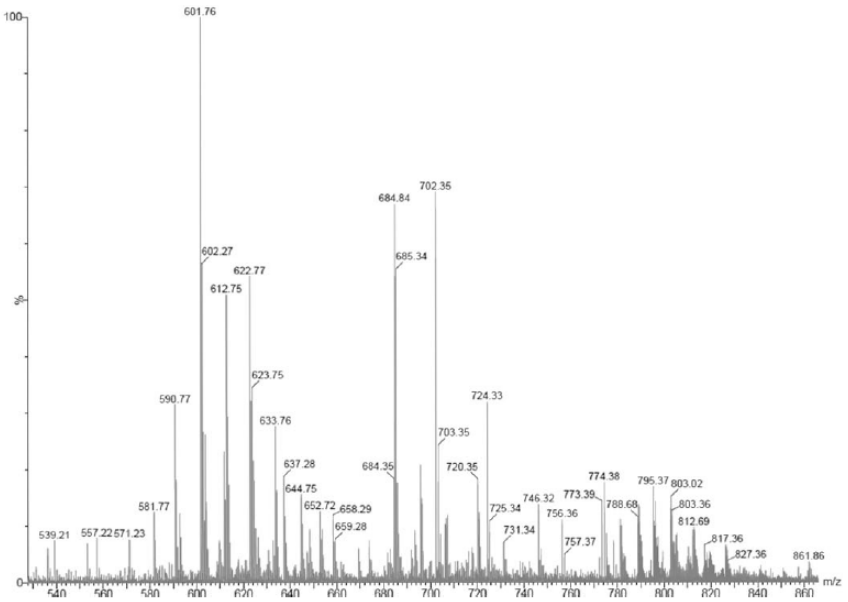


Figure 2-7. Catalytic Mechanism of PlyGRCS. (A) The TNBS assay (left) and DNSA assay (right) performed on PlyGRCS-digested peptidoglycan reveal that PlyGRCS displays amidase or endopeptidase activity, but not glycosidase activity. Error bars represent the standard deviation, and all experiments were done in triplicate. (B) ESI-MS analysis of PlyGRCS digested peptidoglycan results in a spectrum (top) containing a peak at *m/z* =702.35, indicating that PlyGRCS possesses endopeptidase and amidase activities. This peak is absent in peptidoglycan digested with a known *N*-acetylmuramoyl-L-alanine amidase (second spectrum), or undigested peptodiglycan (third spectrum). Double digest with PlyGRCS and CHAP-K (bottom spectrum) yields a spectrum identical to that of PlyGRCS alone. (C) Schematic showing the A_2QKG_5 fragment corresponding the 702.35 peak generated by both an *N*-acetylmuramoyl-L-alanine amidase activity (black arrows) and a D-alanyl-glycyl endopeptidase activity (white arrows). (D) PlyGRCS peptidoglycan digest data showing both the A_2QKG_5 (702.35 *m/z* peak) and the larger, doubly charged $A_4Q_2K_2G_{10}$ moiety (684.84 *m/z* peak). Analysis performed by M. Schmelcher and F. Eichenseher.

presence of two enzymatic activities, an *N*-acetylmuramoyl-L-alanine amidase and either a D-alanyl-glycyl endopeptidase or a glycyl-glycyl endopeptidase, to yield the fragment A_2QKG_5 (single letter amino acid code) (Fig. 2-7C). Moreover, a larger double-charged ion (*m/z*=684.84) was also observed that likely corresponds to the fragment $A_4Q_2K_2G_{10}$ (without a water molecule), resulting from incomplete peptidoglycan digest (Fig. 2-7B and D). Presence of the 702.35 and 684.84 peaks was reproducible on independent digests and ESI-MS experiments. Control experiments with peptidoglycan digested with the 2638A amidase domain, a known *N*-acetylmuramoyl-L-alanine amidase (Fig. 2-7B, second spectrum), or undigested peptidoglycan (Fig. 2-7B, third spectrum) did not contain the 702.35 or 684.84 peaks suggesting that generation of the 702.35 and 684.84 fragments by PlyGRCS was not an artifact of a single enzymatic activity acting on uncrosslinked or partially cleaved peptidoglycan. Furthermore, a double digest with PlyGRCS and CHAP-K, which reportedly has D-alanyl-glycyl endopeptidase activity (Becker et al., 2009a), was performed to elucidate the specific nature of the endopeptidase activity. Because this spectrum was identical to that of the PlyGRCS alone digested peptidoglycan, it was determined that PlyGRCS possesses a D-alanyl-glycyl endopeptidase activity, as a glycyl-glycyl endopeptidase activity would have yielded a different fragment pattern. Taken together, these data imply that PlyGRCS, which has a single catalytic CHAP domain, can cleave two distinct bonds in the staphylococcal peptidoglycan.

Discussion

The use of endolysins provides a targeted treatment for bacterial infections that circumvents traditional antibiotic resistance mechanisms (Spratt, 1994). In this study, the novel endolysin PlyGRCS was characterized and demonstrated bacteriolytic activity against MRSA successfully. The endolysin dosage used in this study demonstrates that the efficacy of PlyGRCS is comparable to or better than other published staphylococcal endolysins (Gilmer et al., 2013; Jun et al., 2011; Sass and Bierbaum, 2007) and since the optimal conditions for PlyGRCS activity were determined to be in the physiological range, this enzyme has the capability to be used as an antimicrobial agent. Even more impressive is the ability of PlyGRCS to act against stationary phase staphylococci as well as medically relevant biofilms, a further hindrance to traditional antibiotic therapy. The ability of endolysins, like PlyGRCS to disrupt biofilms may lead to their use in conjunction with classical antibiotics. In this scenario, the endolysin would provide the initial disturbance to the biofilm structure, thereby allowing the antibiotic to subsequently access the now susceptible target bacteria. It has already been shown that antibiotics applied in combination with endolysins bind more efficiently to their planktonic target bacterial cells; this same phenomenon may also be observed in biofilms as well (Schuch et al., 2013).

Identification of the PlyGRCS cleavage sites is a critical finding. To our knowledge, this is the first reported case of a single CHAP domain, or any individual endolysin catalytic domain, that possesses the ability to cleave two disparate bonds in the bacterial peptidoglycan. Initially we thought the results could be attributed to a single cleavage of uncrosslinked peptidoglycan resulting in a fragment that appeared to be

created by two cleavage events. However, spectra from repeated experiments on undigested control peptidoglycan and control digests with enzymes of known specificity collectively indicate that PlyGRCS is capable of liberating the fragment A_2QKG_5 from the staphylococcal peptidoglycan. This would necessitate cleavage of the amide bond formed between MurNAc and Ala residues as well as the hydrolysis of the amide bond formed between D-Ala and Gly residues or one of the Gly-Gly bonds. Further experiments with a double digest, including PlyGRCS and CHAP-K, a D-alanyl-glycyl endopeptidase, showed an identical pattern to the PlyGRCS only spectrum, indicating that the endopeptidase activity of PlyGRCS is identical to CHAP-K. While these findings indicating both amidase and endopeptidase activities associated with the single CHAP domain containing PlyGRCS were surprising, it is noteworthy that CHAP domains have been associated with an *N*-acetylmuramoyl-L-alanine amidase activity in the streptococcal PlyC endolysin (McGowan et al., 2012) as well as D-alanyl-glycyl endopeptidase activity in multiple staphylococcal endolysins (Schmelcher et al., 2012a). Moreover, the recently crystallized CHAP domain from the staphylococcal endolysin LysGH15 shows highest structural homology to the aforementioned CHAP domain of PlyC, with a root-mean-square deviation (RMSD) = 2.32 Å (Gu et al., 2014), further supporting our interpretation that these domains can exhibit multiple activities. Finally, consistent with the findings of our biochemical assays, both amidase and endopeptidase activities would yield free amine groups via cleavage of peptide moieties and additionally would not liberate reducing sugars, which requires the cleavage of at least one of the two glycosidic bonds responsible for maintaining the glycan backbone of peptidoglycan.

The implications of a single catalytic domain with two cleavage specificities are numerous for bioengineering efforts. First, it is well known that endolysins display synergy with other endolysins of different cleavage specificities. For example, killing of pneumococci is enhanced when the endolysins Cpl-1, an *N*-acetylmuramidase, and PAL, an *N*-acetylmuramoyl-L-alanine amidase, are used together compared to twice the concentration of either enzyme alone (Loeffler and Fischetti, 2003). Likewise, mutagenesis of active-site residues was used to show synergy between two catalytic domains, an *N*-acetylmuramoyl-L-alanine amidase and a glycosyl hydrolase, within the PlyC endolysin (McGowan et al., 2012) . While not proven, it is believed that synergy arises from cleaving the peptidoglycan at two different locations, which is more destabilizing to the superstructure than repetitive cleavages at one location and would result in accelerated osmolysis of the bacterial cell. Additionally, cleavage of one bond may facilitate access to the second target, further contributing to this synergistic effect.

A second benefit of a catalytic domain with dual activities is that it would be less susceptible to development of resistance. While there are currently no specific reports of bacterial strains developing resistance to phage-encoded endolysins, resistance to peptidoglycan hydrolases as a general class has been reported. Notably, modifications to the peptidoglycan backbone can render *N*-acetylmuramidases (i.e. lysozymes) ineffective (Davis and Weiser, 2011; Vollmer, 2008). More specific to the staphylococcal peptidoglycan, resistance to lysostaphin, a bacterial derived glycyl-glycine endopeptidase, can be achieved by simple modification of the pentaglycine crossbridge in these species (Nelson et al., 2012). It is thus anticipated that endolysins naturally evolved

or engineered to have more than one catalytic activity would circumvent resistance development targeting the specificity of one activity.

This study is only the beginning in understanding PlyGRCS. As protein therapeutics, PlyGRCS and other endolysins are amenable to domain shuffling, directed evolution, and bioengineering approaches to further enhance efficacy and/or specificity. The unique dual substrate activity of the PlyGRCS catalytic domain offers an ideal starting point for chimeragenesis studies with other domains from staphylococcal-specific endolysins.

Acknowledgements

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Chapter III: Anti-Biofilm Activity of PlyGRCS: Removal, Prevention, and Bacteriolytic Efficacy of a Bacteriophage Endolysin Against Multi-Drug Resistant *Staphylococcus aureus* Biofilms

This chapter is being edited and formatted for imminent submission.

Abstract

The ability of *Staphylococcus aureus* to form biofilms represents a major virulence factor and contributes to its ability to cause chronic infections. Furthermore, *S. aureus* can form biofilms on medical devices, leading to colonization of areas of the body where it would not normally be able to persist. Biofilms are notoriously difficult to eradicate, as they are resistant to antibiotics and are resilient against the host immune system. Currently, the treatment for *S. aureus* biofilm infections is debridement or removal of the affected implant, which is not a desirable outcome, and as such, alternative therapies are needed. PlyGRCS, an endolysin which has shown antimicrobial activity against planktonic *S. aureus,* represents such an alternative option. In this study, we investigate the use of PlyGRCS against staphylococcal biofilms. PlyGRCS displays the ability to remove biofilms from abiotic surfaces at concentrations much lower than its minimum inhibitory concentration. In addition to its ability to disperse biofilms, as shown by crystal violet staining, *in vivo* imaging, and confocal microscopy, PlyGRCS also kills bacteria within biofilms. PlyGRCS removes *S. aureus* biofilms formed under dynamic conditions in medical-grade catheters, mimicking an *in vivo* infection. Finally, PlyGRCS possesses the ability to kill bacteria within biofilms grown on epithelium, without harming the eukaryotic cells. In summary, we show that PlyGRCS has a potential application an anti-biofilm therapeutic.

Introduction

Staphylococcus aureus is one of the most commonly isolated antibiotic-resistant bacteria in both the community and in the hospital setting (Lowy, 1998). It is particularly frightening as methicillin-resistant *S. aureus* (MRSA) infections alone have a 15% morbidity rate just in the United States (CDC, 2013), and as we enter a post-antibiotic era, that number may increase drastically. Further complicating this situation is the ability of S. aureus to participate in the biofilm lifestyle; the biofilm mode of growth is an important key to pathogenesis, as biofilms are resistant to host defense and the most commonly used treatment for bacterial infections, antibiotics (Otto, 2008). Bacterial biofilms are microbial communities of physiologically and metabolically diverse cells attached to each other and a surface, encased in a matrix of extracellular polymeric substance (EPS), and formed by a highly regulated process, influenced by environmental and genetic factors (Archer et al., 2011).

While not necessary for *S. aureus* biofilm formation, the presence of medical devices such as catheters, heart valves, stents, cosmetic, dental, and prosthetic implants, and additional indwelling devices allows *S. aureus* to persist in areas of the body in which it would not normally be able to colonize and cause diseases that are infinitely harder to treat (Costerton et al., 2005). Infections caused by *S. aureus* biofilms include urinary tract infections, endocarditis, peri-implantitis, osteomyletis, and other persistant infections. Implant mediated biofilm infections caused by non-*S. aureus* (non-coagulase staphylococci, Gram-negative bacilli) may be attempted to be treated by antibiotics (at 100-1000X MIC) via antimicrobial lock therapy (ALT); however, in the case of *S.*

aureus, the protocol in every hospital in the U.S is to remove the affected device (Mermel et al., 2009).

Because the widespread use of antibiotics has contributed to the development of bacterial resistance and due to the inability of antibiotics to disperse and kill biofilm bacteria, alternative methods to combat biofilm-associated bacterial infections must be investigated. One promising technique is the use of endolysins, peptidoglycan hydrolases that are released by bacteriophages during the lytic cycle of viral infection (Nelson et al., 2012). While naturally produced in the cytoplasm, resulting in lysis from the inside out, researchers have found that endolysins can be applied externally to the bacterial cell to achieve the same end goal. The canonical structure of an endolysin consists of an N-terminal catalytic domain responsible for cleavage of a specific peptidoglycan bond and a C-terminal cell wall binding domain that interacts with a ligand on the bacterial surface. Endolysins display several traits that make them ideal for treatment of bacterial infections. The specificity for both cleavage site and binding site ensures that resistance is unlikely to be observed. However, even if resistance were to develop, the modular domain architecture makes endolysins amenable to engineering efforts.

Moreover, use of endolysins is a better option for treating biofilm-associated bacterial infections because the reasons for the ineffectiveness of antibiotics against biofilm bacteria do not apply to endolysins. First, the formation of a biofilm prevents an antibiotic from reaching its target, whereas endolysins can easily penetrate and can even be attracted to the bacterial cell surface (Keren et al., 2004). Second, the heterogeneous nature of a biofilm results in subpopulations of persister cells and metabolically inactive cells, thereby not allowing for an antibiotic that targets active cell processes to be

effective; however, endolysins are effective regardless of bacterial metabolic state or phase of growth. Antibiotics have even been shown to induce changes in planktonic bacteria that result in an increased propensity for forming biofilms (Kaplan et al., 2012), a phenomenon that does not occur upon application of endolysins (Gutierrez et al., 2014).

We have previously identified and characterized PlyGRCS, the endolysin from the *S. aureus* bacteriophage GRCS, and have shown that it possesses activity against static *S. aureus* biofilms. This study is an in depth investigation into the further application of PlyGRCS as an anti-biofilm therapeutic.

Materials and Methods

Bacterial Strains

All bacterial strains were routinely grown at 37°C. Staphylococcal strains were grown in trypticase soy broth (TSB) (Becton-Dickinson), or on TSB plates; *S. aureus* AH1350 was grown in TSB with 1000 µg/ml spectinomycin (TSBspec) for plasmid maintenance; *Escherichia coli* was grown in Luria Broth (LB) (Alpha Bioscience), or on LB plates, with 100 µg/ml ampicillin for plasmid maintenance. AH1350, a GFP derivative of RN4220, was a kind gift of Alex Horswill (via Mark Shirtliff). Unless otherwise denoted, all chemicals were obtained from Sigma and were of the highest purity available.

Expression and Purification

PlyGRCS was expressed and purified as in (Linden et al., 2015). Briefly, *E. coli* were grown at 37°C in baffled flasks to an $OD_{600} = 1$ in LB supplemented with 100 µg/ml

ampicillin. Arabinose (0.25%) was used for induction of expression for 4 hours at 37°C and then overnight at 18°C. Cells were harvested, lysed by sonication, and crude protein extracts were purified by a Bio-Scale Mini Profinity IMAC Cartridge (Bio-Rad). Fractions containing the desired protein were then pooled and dialyzed against PBS pH 7.4 plus 300 mM NaCl and 0.25 mM $CaCl_2$.

Minimum Inhibitory Concentration

Serial dilutions of PlyGRCS or antibiotics in PBS 1 mM $CaCl_2$ were added to an equal volume of bacteria (100 μl) (overnight cultures diluted 1:10000 in 2X TSB (2X TSBspec for AH1350)). Samples were incubated at 37ºC overnight, and the MIC (minimum inhibitory concentration) was determined to be the lowest concentration of enzyme that allowed the medium to remain clear.

Minimum Bactericidal Concentration

Serially diluted PlyGRCS or antibiotics in PBS 1 mM $CaCl_2$ or buffer control (100 μl) were added to an equal volume of bacteria (~10^5 CFU/ml). Samples were incubated at 37ºC for 60 minutes, then serially diluted, plated, and incubated overnight, whereupon CFU counts were obtained. While the MBC (minimum bactericidal concentration) is often defined as the lowest amount of antibacterial agent required to totally kill an organism, we define the MBC as the minimum concentration of enzyme that caused a 3 log (≥ 99.9%) decrease in the amount of bacteria as compared to the untreated sample (Jones et al., 1985).

Static Biofilm Eradication Assay

For treatment of established static biofilms, aliquots of overnight *S. aureus* AH1350 cultures (1 ml per well) were placed into 24-well CELLBIND plates (Corning) containing 500 µl of TSBspec and 2.5 mM glucose. After allowing for formation of biofilms (24 hr), media was aspirated and samples were washed with PBS to remove unattached cells. PlyGRCS at indicated concentrations or controls were added in 1 ml PBS plus 0.25 mM $CaCl_2$ and incubated at 37°C for one hour. Liquid was aspirated, at which point samples were imaged using the In Vivo Imaging System (IVIS) Lumina XR (Caliper Life Sciences/ Perkin Elmer). Biofilms were washed with PBS and, after drying, were stained with 0.1% crystal violet for 10 min at room temperature. Upon removal of excess crystal violet, samples were washed with PBS and dried before the addition of 1 ml 1% SDS to extract the crystal violet from the biomass for quantification at OD_{595} on a spectrophotometer.

Microscopy of Static Biofilms

For observation of cell death via fluorescent microscopy, static *S. aureus* AH1350 biofilms were grown as described above for 24 hours in poly-L-lysine coated 8-well Nunc™ Lab-Tek™ Chambered Coverglass slides. Medium containing planktonic cells was discarded and the remaining biofilm-associated cells were washed with PBS, then treated with 200 µl PlyGRCS (100 µg/ml) in PBS with 0.25 mM $CaCl_2$ or controls and 30 µM propidium iodide (PI) for 15 min. Biofilms were examined with an Eclipse 80i fluorescence microscope with a 20x/1.3 objective lens (Nikon, Melville, NY, USA) using an X-cite 120 illuminator (EXFO, Quebec, Canada). Images were acquired using a Retiga

2000R camera with Q Capture Pro software (both from Q-imaging, Surrey, Canada). To quantify the viable cells, the mean fluorescent intensity of live (green channel) and dead (red channel) cell populations was calculated using NIS-Elements software (Nikon) and compared to a fluorescence calibration curve generated from a known ratio of live and dead cells stained with PI.

For observation of biofilm degradation via confocal laser scanning microscopy, images were acquired using a Carl Zeiss 710 inverted microscope in combination with the Zeiss Argon laser scanning confocal imaging system. Images and z-stack analysis were obtained with a 20x/1.3 objective lens and analyzed by Zen 2010 digital imaging software (Carl Zeiss). In order to visualize the degradation of the biofilm matrix by PlyGRCS, *S. aureus* AH1350 biofilms were grown as described in 4-well Nunc™ Lab-Tek™ Chambered Coverglass slides, coated with 0.1% gelatin, for 48 hours. Medium containing planktonic cells was discarded and the remaining biofilm-associated cells were washed with PBS, then treated with 1ml PlyGRCS (100 µg/ml) or oxacillin (100 µg/ml) in PBS 0.25 mM $CaCl_2$. Three dimensional image stacks were recorded at indicated times.

Determination of Cell Death

Static *S. aureus* AH1350 biofilms were grown as described above for 24 or 48 hours on poly-L-lysine coated black 96 well plates, then were treated for 30 minutes with 100 µl PlyGRCS (100 µg/ml) in PBS 0.25 mM $CaCl_2$ or controls and 30 µM propidium iodide (PI). Fluorescence of live (530 nm) and dead (630 nm) cell populations was monitored via a SpectraMax M5 spectrophotometer (Molecular Devices). Using a

fluorescence calibration curve generated from a known ratio of live and dead cells stained with PI, the percentage of viable cells was obtained from the ratio of mean fluorescent intensities.

Dynamic Biofilm Eradication Assay

For treatment of established dynamic biofilms, the Stovall flow cell system was used, as per manufacturer instructions, with the flow cell chambers replaced by catheters (Excel International siliconized arteriovenous fistula, tube length 12", tube i.d. 0.14"). An overnight culture of VISA NRS-14 was diluted 1:10 into fresh media (TSB with 2.5 mM glucose) and 3 ml was injected into each catheter via a 27 G syringe. The system was incubated statically for 1 hour to allow for adherence, after which flow was initiated and continued for 18 hours at 0.5 ml/min. PBS with 2.5 mM glucose and 0.25 mM $CaCl_2$ was allowed to flow through the system for 20 minutes after which 3 ml PlyGRCS (1 mg/ml) in PBS with 2.5 mM glucose and 0.25 mM $CaCl_2$ or controls lacking PlyGRCS were injected and incubated statically for 24 hours. Then, PBS with 2.5 mM glucose and 0.25 mM $CaCl_2$ was flowed at 0.5 ml/min for 20 minutes. For quantification of bacteria survival after treatment, catheters were sonicated to disrupt biofilm, then were flushed and samples were serially diluted, plated, and incubated overnight, whereupon CFU counts were obtained.

Biofilm Induction/Inhibition Assays

Two-fold serially diluted PlyGRCS in PBS and 0.25 mM $CaCl_2$ (100 µl) or controls were added to 200 µl of *S. aureus* NRS-14 (in TSB with 2.5 mM glucose) in 96-

well CELLBIND plates (Corning). Samples were incubated at 37°C overnight after which liquid was aspirated and biofilms were stained with crystal violet as described above.

PlyGRCS Toxicity on MAC-T Cells

MAC-T bovine mammary epithelial cells were grown in a 2 well chamber slide in DMEM-F12 and 10% FBS until 80% confluent. Media was removed and cells were washed 3X with HBSS. Serum-free media with 1 µg/ml PI (1 ml per well) and PlyGRCS (100 µg/ml) or 0.02% Triton X 100 was added and cells were incubated for 30 minutes at 37 ºC. Samples were washed with HBSS and cells were fixed with 4% PFA at room temperature for 15 min. Samples were mounted with Prolong Gold DAPI antifade and imaged on an Eclipse 80i fluorescence microscope with a 20x/1.3 objective lens (Nikon, Melville, NY, USA) using an X-cite 120 illuminator (EXFO, Quebec, Canada).

Biofilm Formation of S. aureus on MAC-T and Treatment

MAC-T cells were grown until 100% confluent as described above in an 8 well chamber slide (approximately 10^6 cells in each well). Media was removed and cells were washed with HBSS. DMEM/F12 with 10% FBS (500 µl) and 5 µl DAPI was added to the wells and incubated for 30 min at 37 ºC. Media was removed and samples were washed with HBSS. An overnight culture of *S. aureus* AH1350 diluted 1:100 in DMEM-F12 with 10% FBS and 1 mg/ml spectinomycin and 500 µl was added to the wells and incubated for 1 hour at 37 ºC to allow for attachment Media was removed and samples were washed with HBSS to remove non-adhered bacteria. DMEM/F12 with 10% FBS

and 1 mg/ml spectinomycin was added to the wells to allow biofilm formation for 5 hours at 37 ºC. Media was removed and samples were washed with HBSS. DMEM/F12 with 1 mg/ml spectinomycin and 1 mg/ml PlyGRCS (or no PlyGRCS control) was added to the wells and one section in the center of the wells was imaged on a Carl Zeiss 710 inverted microscope in combination with the Zeiss Argon laser scanning confocal imaging system at several time points. At the end of the experiment, the supernatant was plated, along with bacteria recovered from a wash step. The remaining adherent biofilm bacteria were plated after treatment with Triton X 100 (.025%) and Trypsin (.25%) to remove the MAC-T cells from the wells.

Results

Minimum Inhibitory and Bactericidal Concentration

To set a benchmark for PlyGRCS activity against *S. aureus*, the minimum inhibitory concentration for endolysin and antibiotics was determined. It was found that planktonic *S. aureus* (MSSA, MRSA, and VISA) are susceptible to both traditional commonly used antibiotics and PlyGRCS at concentrations ranging from 128-256 µg/ml (Table 3-1). Of note, due to the mass differences between antibiotics and PlyGRCS (~29 kDa), on a molar basis of comparison, 20-85X more antibiotic than PlyGRCS would be needed at comparable molar concentrations.

Because of the speed at which endolysin activity occurs, the minimum bactericidal concentration (MBC) may be a more accurate measurement of antimicrobial efficacy (Kusuma and Kokai-Kun, 2005). Significantly, at only 16 µg/ml, the MBC for PlyGRCS was less than the MIC, while the MBC for all antibiotics could not be

Table 3-1. Antimicrobial susceptibility.

Strain	Minimum Inhibitory Concentration				
	PlyGRCS	PEN	OXA	VAN	CAM
	29.3 kDa	.34 kDa	.40 kDa	1.45 kDa	.32 kDa
AH1350	>256 μg/ml	>64 μg/ml (R)	.125 μg/ml	2 μg/ml	4 μg/ml
	>8.74 μM	>188.24 μM	.312 μM	1.38 μM	12.5 μM
NRS-14	128 μg/ml	.5 μg/ml	.5 μg/ml	4 μg/ml (I)	4 μg/ml
	4.37 μM	1.47 μM	1.25 μM	2.76 μM	12.5 μM
ATCC27217	128 μg/ml	<.0625 μg/ml (S)	.125 μg/ml	1 μg/ml	8 μg/ml
	4.37 μM	<.183 μM	.312 μM	.690 μM	25 μM
NRS-71	256 μg/ml	64 μg/ml (R)	>64μg/ml (R)	1 μg/ml	2 μg/ml
	8.74 μM	188.24 μM	160 μM	.690 μM	6.25 μM

The minimum inhibitory concentration (MIC) obtained for PlyGRCS and indicated antibiotics against several strains of *S. aureus*. (R) = Resistant, (S) = Sensitive, (I) = Intermediate.

determined during the same time frame (Fig. 3-1). This is indicative of the quick bactericidal and lytic activity of endolysins.

Disruption of Static Biofilms

Having determined that PlyGRCS possesses inhibitory and bactericidal activity against planktonic *S. aureus*, we wished to evaluate its efficacy against bacteria in the biofilm mode of growth. After one hour of treatment, PlyGRCS at even the lowest dose tested (6.25 μg/ml), was able to disrupt static biofilms as visualized by both fluorescent imaging using IVIS and crystal violet staining (Fig. 3-2). Importantly, PlyGRCS was more effective than even a high dose of oxacillin, even though AH1350 is a methicillin-sensitive *S. aureus* and indeed was inhibited by approximately 1000X less oxacillin in the MIC assay. Notably, the PlyGRCS doses needed for biofilm disruption were less than the MIC and MBC for this strain (both of which could not be achieved at 256 μg/ml, the maximum concentration tested for this strain).

After observation of biofilm disruption at the macroscopic level, we assessed the ability of PlyGRCS to destroy the three dimensional structure of the biofilm matrix by confocal laser scanning microscopy (Fig. 3-3). Upon treatment with PlyGRCS, the fluorescence decreased over time (a middle slice is shown as a representative image), and visualization of the entire Z-axis showed diffusion of the fluorescence away from the structured biofilm as early as 15 minutes post-treatment, indicating that the biofilm was being degraded and perhaps that lysis was occurring. At 30 minutes, the biofilm superstructure was completely degraded. In contrast, treatment with oxacillin resulted in maintenance of an ordered compact structure throughout the experiment.

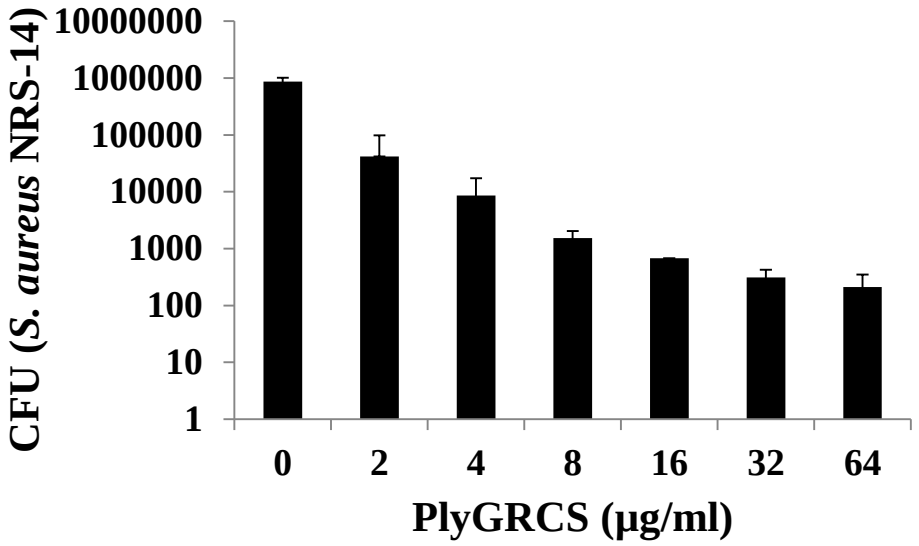

Figure 3-1. Bactericidal activity of PlyGRCS. PlyGRCS kills *S. aureus* in a dose dependent manner. The minimum bactericidal concentration (16 µg/ml) against *S. aureus* NRS-14 was determined after incubating endolysin and bacteria for 1 hour. Error bars represent the standard deviation, and all experiments were done in triplicate.

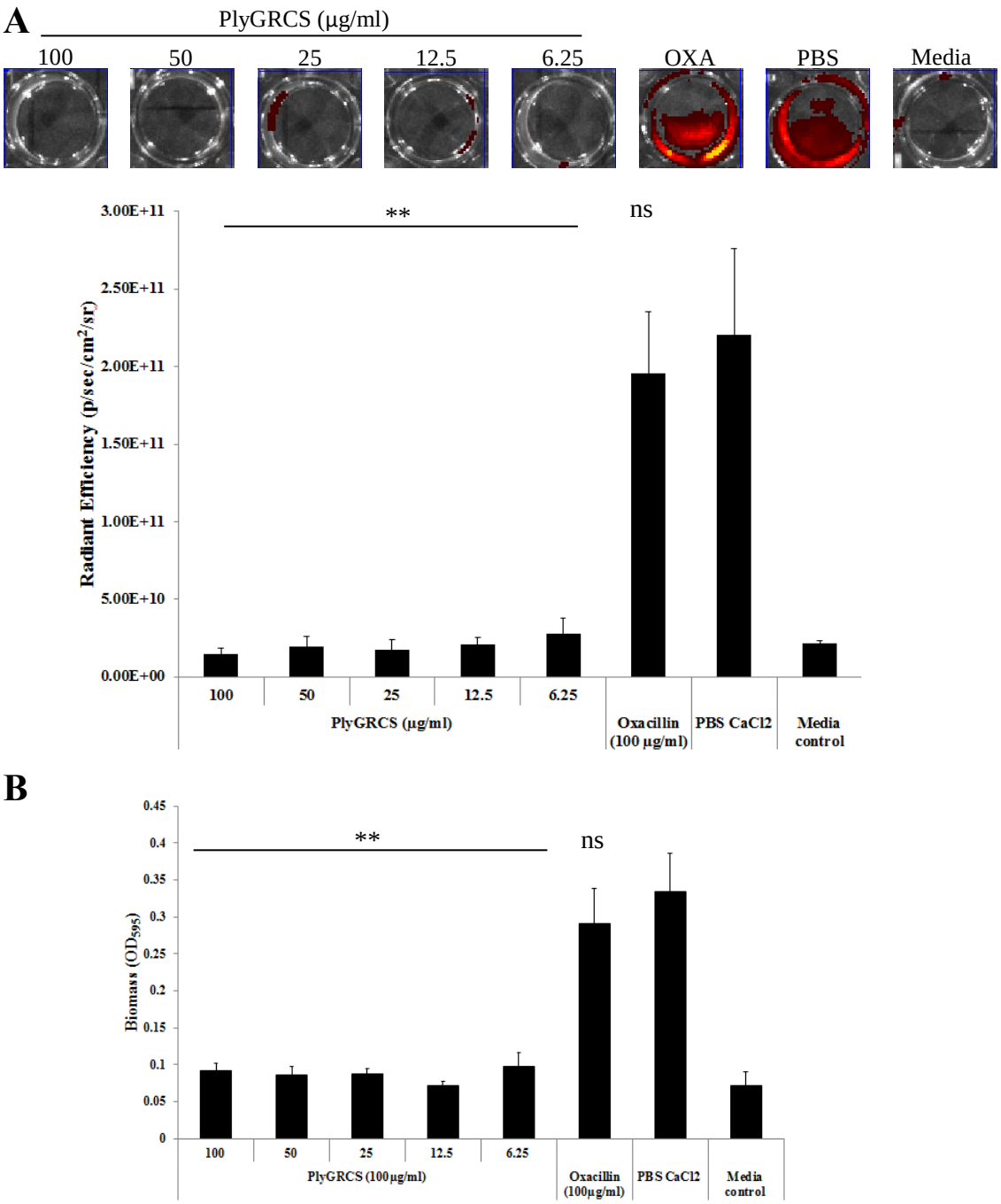

Figure 3-2. Removal of static biofilms by PlyGRCS. Disruption of *S. aureus* AH1350 biofilms can be visualized by measuring (A) fluorescence via IVIS or (B) crystal violet staining. Error bars represent the standard deviation, and all experiments were done in triplicate. Statistical analysis was performed by unpaired *t* test. ** $P < 0.01$.

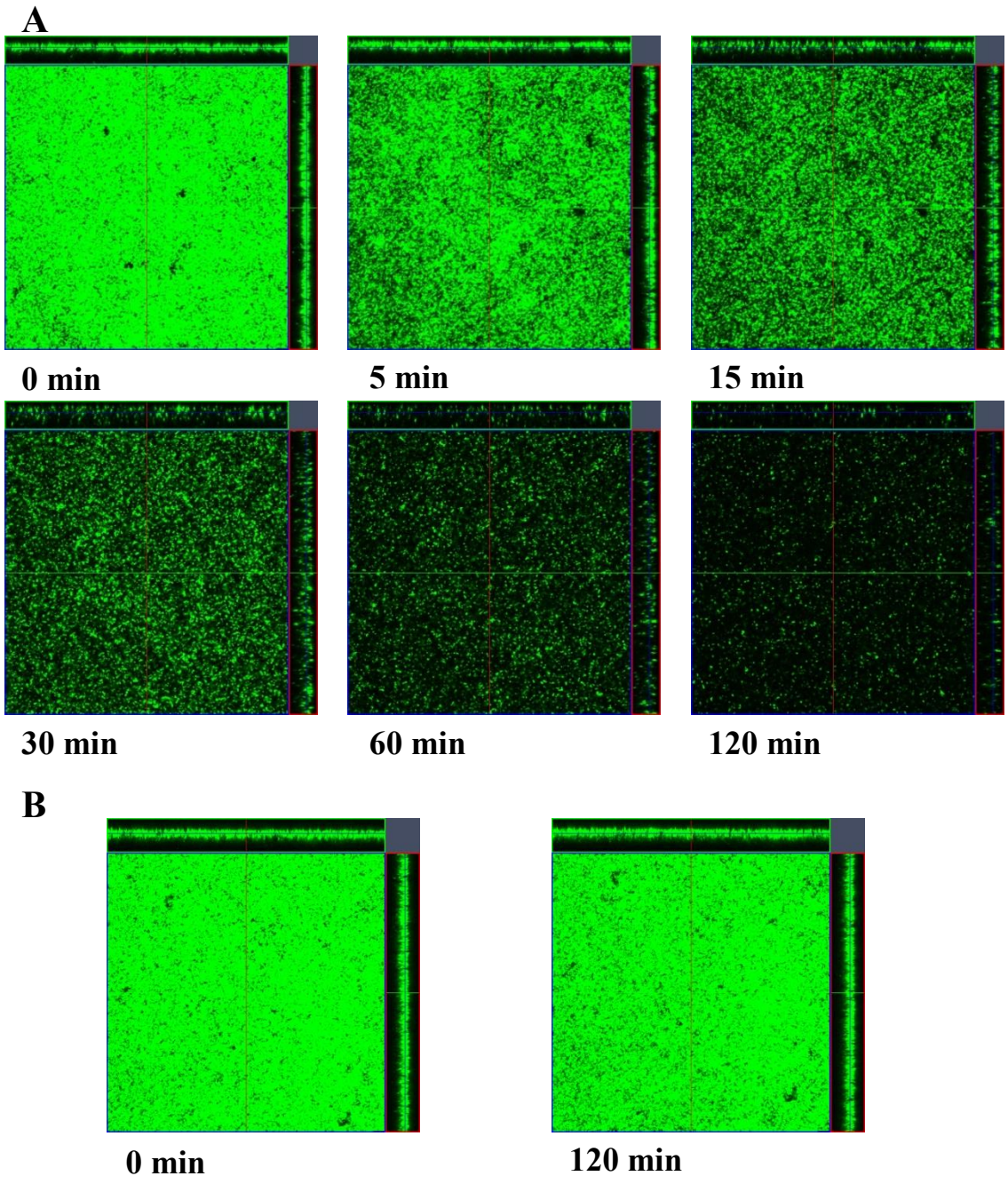

Figure 3-3. Degradation of staphylococcal biofilms. Confocal microscopy (200X magnification) shows the complete disruption of *S. aureus* AH1350 biofilms upon addition of (A) PlyGRCS (100 µg/ml), while (B) oxacillin (1000X MIC) does not disturb the structure.

Killing of S. aureus in Static Biofilms

The limits of crystal violet staining and biofilm disruption assays are that these techniques only allow for the determination of biomass. As the previous assays only established that PlyGRCS was able to disrupt static biofilms, we wished to determine if the bacteria in the biofilm were actually being killed or if the structural integrity was simply being destroyed. To this end, we assessed the killing ability of PlyGRCS in *S. aureus* biofilms, by staining with propidium iodide (Fig. 3-4A). Prior to treatment, biofilms were mostly green, indicating viable bacteria; however dead cells, visible by red staining by propidium iodide, naturally occur in a low but measurable frequency in biofilm populations. When biofilms were treated with 100 µg/ml oxacillin, the percentage of viable bacteria did not decrease when compared to control biofilms as quantified by fluorescent signals; in contrast, 100 µg/ml of PlyGRCS was able to eliminate ~60% of the bacteria in 24 hour biofilms and ~30% of the bacteria in 48 hour biofilms. We were also able to visualize this phenomenon microscopically (Fig 3-4B); control biofilms were composed predominately of viable bacteria, with few dead cells, while biofilms that were treated with PlyGRCS were mostly removed from the surface and most of the cells that did remain attached were killed on contact.

Treatment of Dynamic Biofilms

The environment in which a biofilm forms is often not static, but rather involves a dynamic flow, especially in medical devices. Importantly, these dynamic biofilms are much harder to eradicate due to the increased shear force exerted upon them (Shaw et al., 2004). To more accurately represent the conditions a *S. aureus* biofilm would encounter

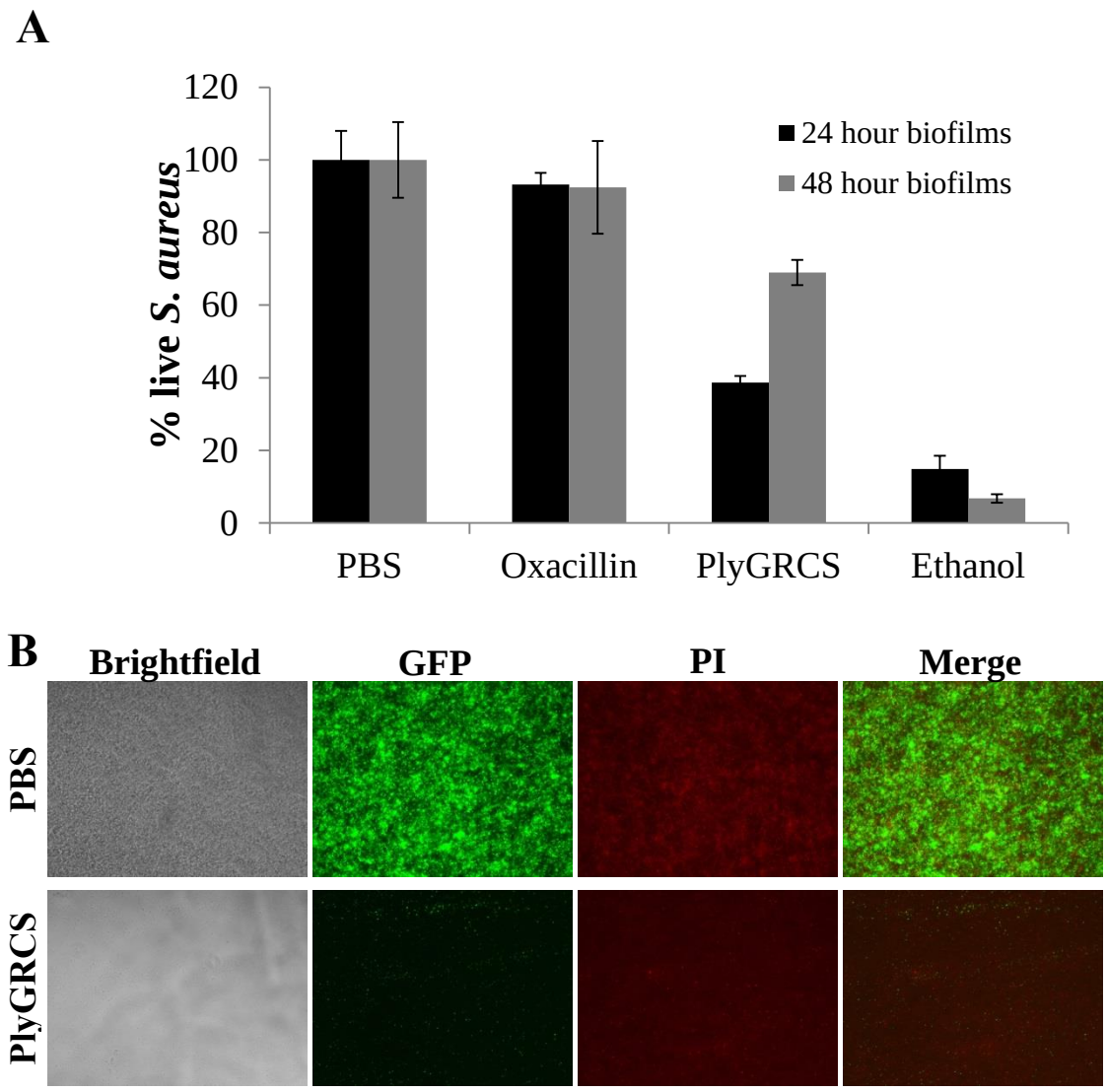

Figure 3-4. Killing of bacteria in staphylococcal biofilms. PlyGRCS kills *S. aureus* AH1350 grown in static biofilms as visualized by (A) spectrophotometric readout of fluorescence and (B) fluorescent microscopy. Live cells are green (GFP) and dead cells are red (propidium iodide). Overlay image is shown on the right and brightfield image is shown on the left. Images were obtained at 200X magnification. Error bars represent the standard deviation, and all experiments were done in triplicate.

in a medically relevant setting, we constructed a modified Stovall flow cell system to include catheters. *S. aureus* biofilms were grown in the catheters under flow for 18 hours, then treated with PlyGRCS for a dwell time of 24 hours, after which the detached biofilms were flushed out of the catheter and the remaining attached bacteria were quantified. PlyGRCS was able to efficiently remove on average 10^3 cfu/ml (Fig. 3-5). This is the first reported use of an endolysin against a dynamic staphylococcal biofilm grown in a medical grade device.

Biofilm Prevention

Important considerations when using a typical antimicrobial treatment are the notions that a subpopulation of persister cells may be selected and biofilm formation may be induced. Notably, antibiotics have been shown to be ineffective towards preventing biofilms or may actually induce biofilm formation. To determine if PlyGRCS would induce biofilm formation, *S. aureus* were exposed to sub-MIC concentrations of PlyGRCS and then assessed for their ability to form biofilms. When *S. aureus* was allowed to form biofilms in the presence of low concentrations of oxacillin or penicillin, an increase in the amount of biomass was observed (Fig. 3-6). Treatment with vancomycin slightly decreased the amount of biofilm formation at very low concentrations; however, approaching the MIC caused a slight upward trend in biofilm formation (a phenomenon observed with other VISA strains). In contrast to the increase in biofilm formation observed when *S. aureus* was incubated with select antibiotics, PlyGRCS treatment greatly diminished the ability of *S. aureus* to form biofilms.

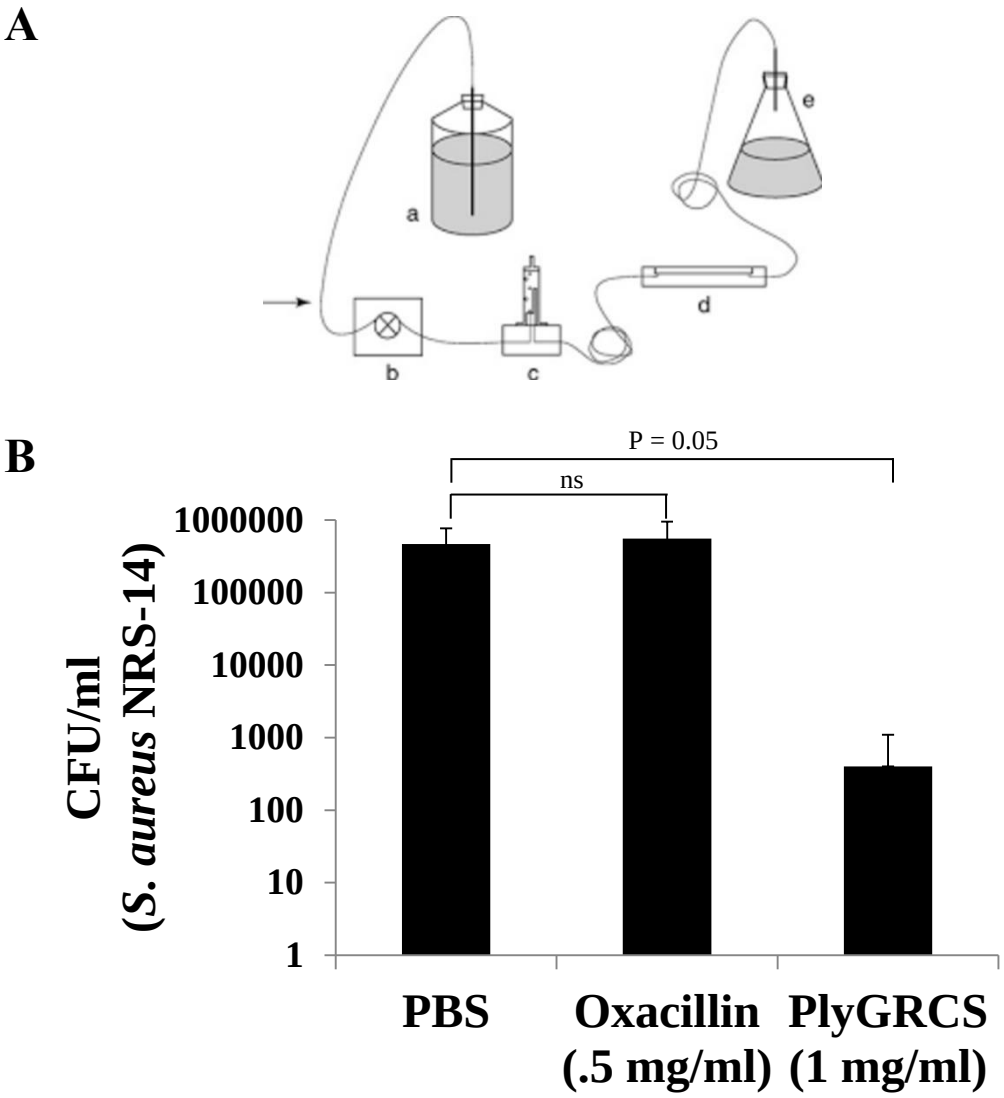

Figure 3-5. Eradication of dynamic biofilms. (A) Schematic of biofilm flow setup (Sternberg and Tolker-Nielsen, 2006). Arrow indicates direction of flow. (a) Input media (b) Peristaltic pump (c) Bubble trap (d) Catheter (e) Waste (B) Lock treatment with PlyGRCS removes *S. aureus* NRS-14 biofilms formed in a catheter under flow conditions as measured by the amount of bacteria recovered from the catheter after a 24-hour incubation. Error bars represent the standard deviation, and all experiments were done in triplicate. Statistical analysis was performed by unpaired *t* test.

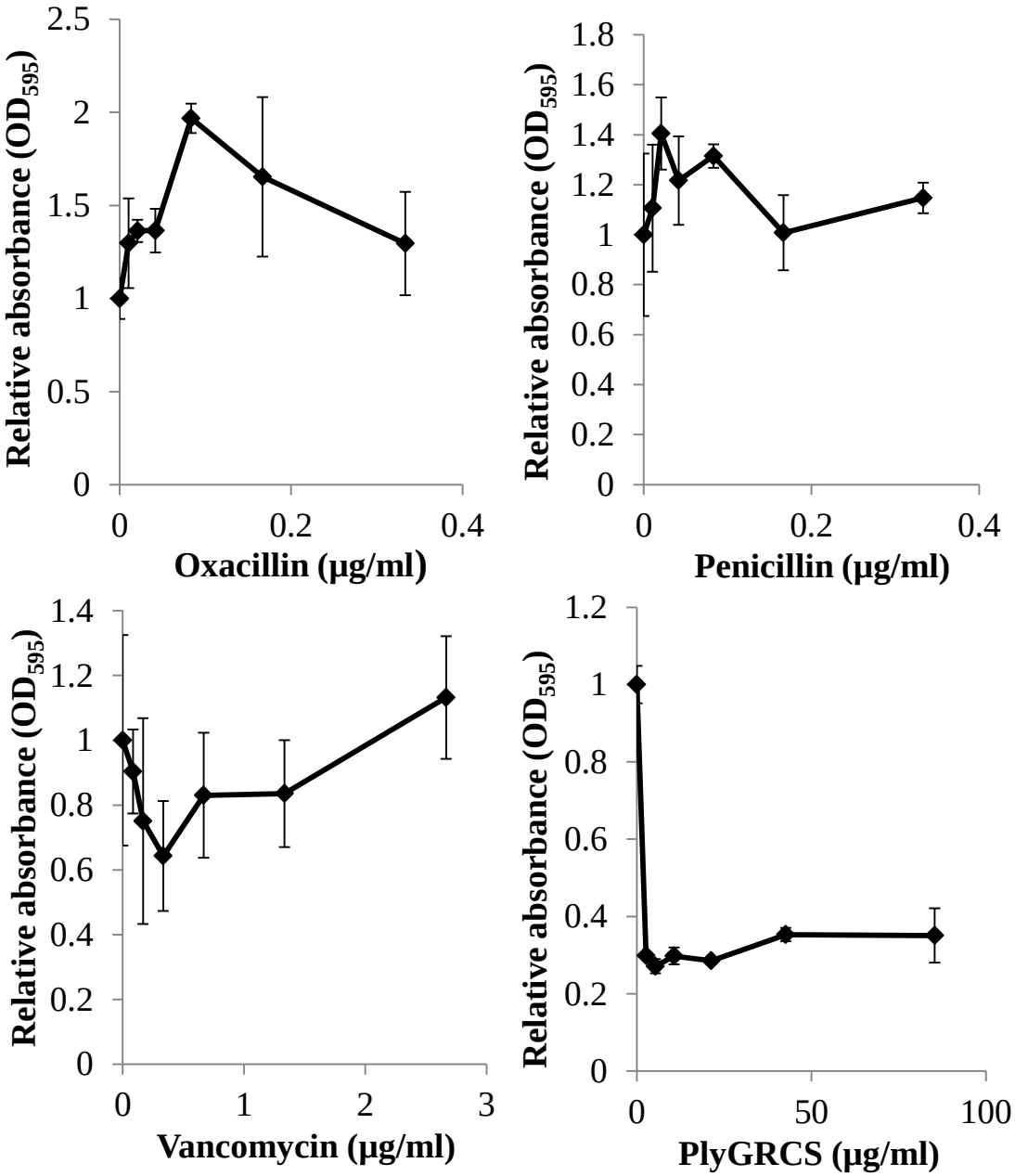

Figure 3-6. Biofilm behavior in the presence of sub-inhibitory concentrations of antimicrobials. Biofilm formation was induced in the presence of low concentrations of typical antibiotics, such as oxacillin (top left), penicillin (top right), and vancomycin (bottom left), while PlyGRCS (bottom right) prevents the formation of *S. aureus* NRS-14 biofilms, as shown by crystal violet staining. Error bars represent the standard deviation, and all experiments were done in triplicate.

PlyGRCS Interaction with MAC-T Cells and Removal of Biofilms

In addition to formation of biofilms on abiotic material, the ability of *S. aureus* to form biofilms directly on the epithelium led us to question whether PlyGRCS could also be an effective treatment in this scenario. First, to ensure the safety of PlyGRCS on eukaryotic cells, we incubated the endolysin and MAC-T bovine epithelial cells and assessed the membrane integrity. PlyGRCS-treated MAC-T cells did not show any propidium iodide staining, indicating that their membranes had not been compromised by the addition of the enzyme (Fig. 3-7). Finally, we determined the ability of PlyGRCS to eliminate *S. aureus* biofilms that had been formed on MAC-T cells. As visualized by confocal microscopy, there was a marked reduction in the amount of green fluorescence, indicating that the bacteria expressing GFP were being lysed (Fig. 3-8). Quantification of *S. aureus* from the PlyGRCS-treated samples showed an approximately 1 log reduction as compared to the untreated biofilms.

Discussion

In the age of increasing resistance to traditional antibiotics, identifying the next generation of antimicrobial agents, such as endolysins, is a crucial and pressing matter. Furthermore, the inability of antibiotics to be effective against bacteria in biofilms highlights the importance of finding alternative therapeutics. Even more alarming, is that the recommended treatment for infections caused by implant-mediated *S. aureus* biofilms is removal of the device, which is clearly not an acceptable solution.

In this study, the endolysin PlyGRCS successfully demonstrated biofilm dispersing and killing activity against *S. aureus*. While activity against biofilms seems to

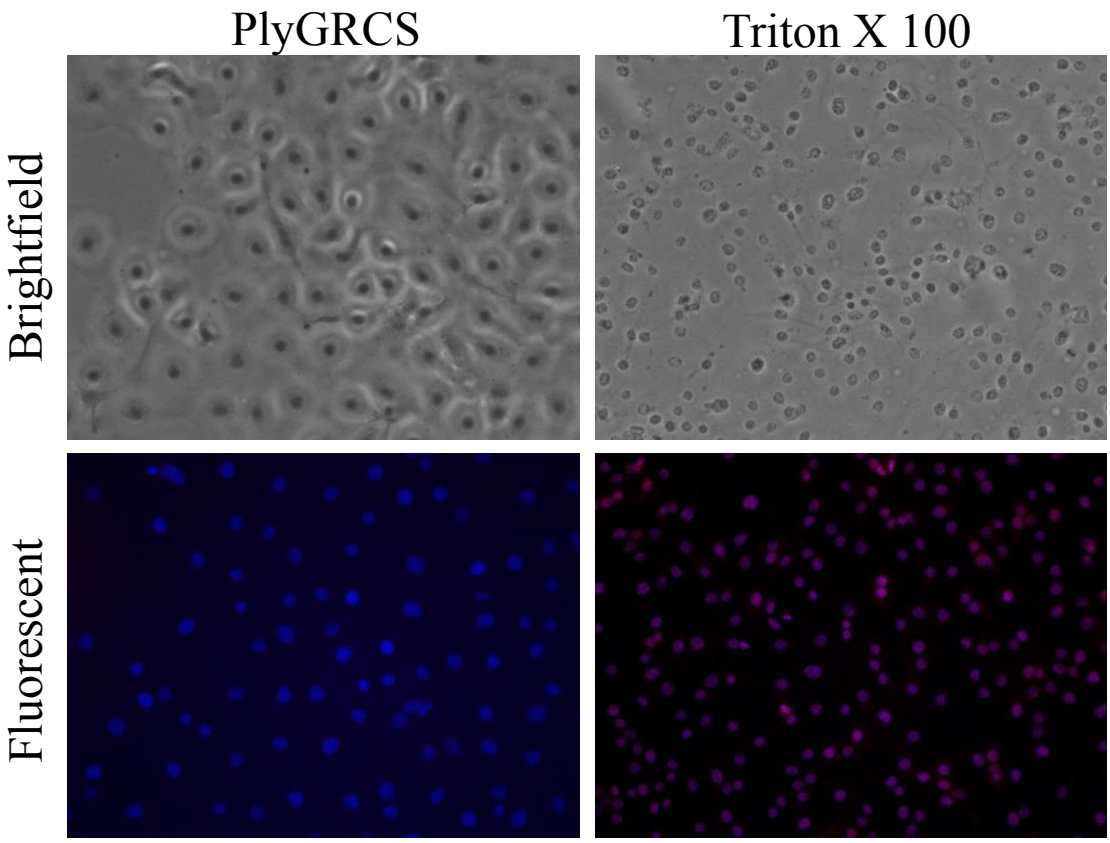

Figure 3-7. PlyGRCS interaction with eukaryotic cells. PlyGRCS-treated MAC-T bovine epithelial cells (nuclei stained with DAPI) do not show membrane permeabilization (bottom left panel), while the Triton X 100-treated control shows compromised cell membranes, as evidenced by red propidium iodide staining (bottom right panel). Brightfield images are shown in the top panels. Images were obtained at 200X magnification.

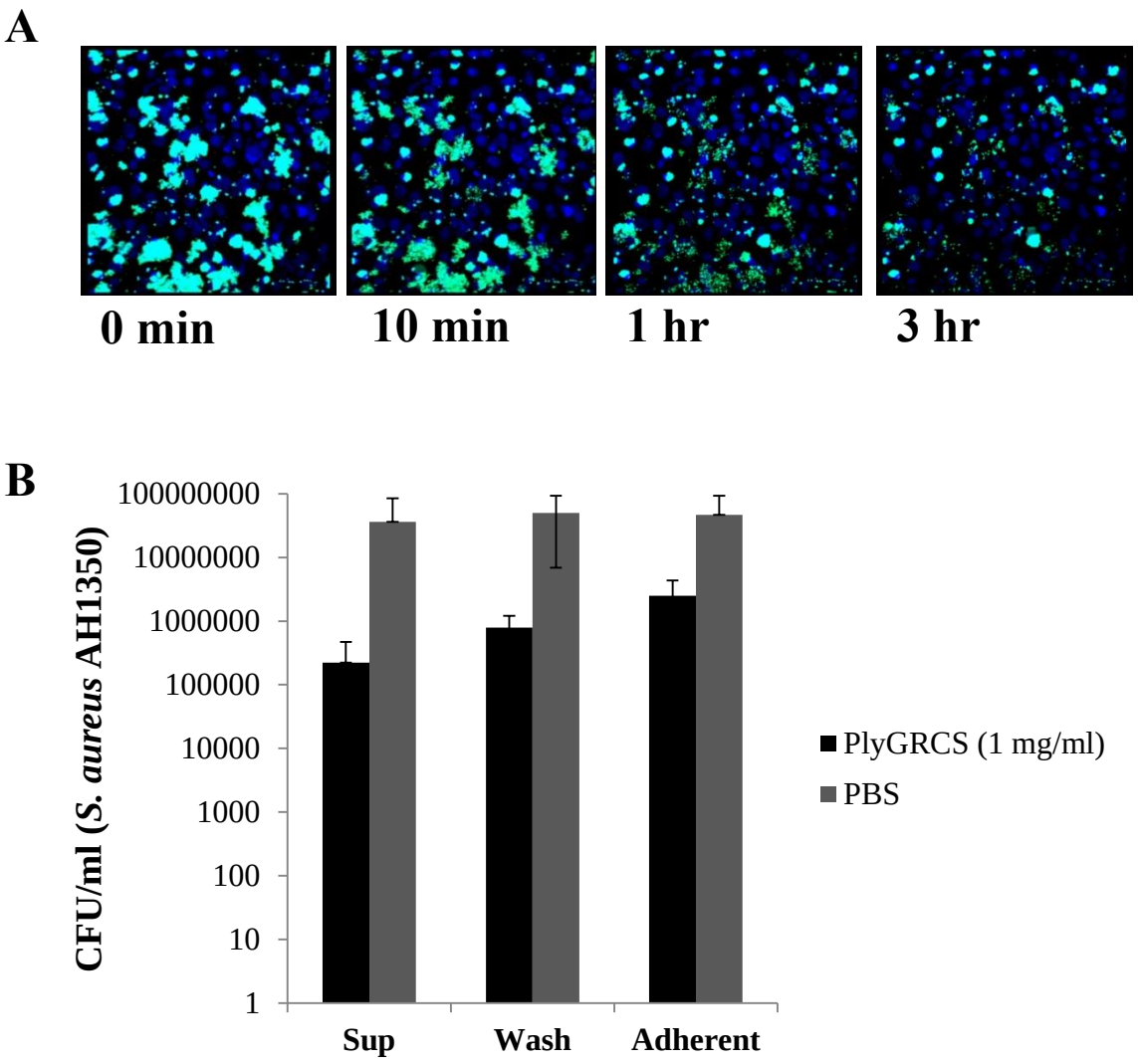

Figure 3-8. PlyGRCS treatment of *S. aureus* biofilms on MAC-T cells. (A) Confocal microscopy (maximum intensity projections) showing the reduction of biofilm bacteria (*S. aureus* AH1350-GFP) after treatment with PlyGRCS. MAC-T nuclei are stained with DAPI (blue). Images were obtained at 200X magnification. (B) Quantification of *S. aureus* recovered after treatment with PlyGRCS vs. PBS. Bacteria were taken from the supernatant (sup), after two washes (wash), and still adhered to the MAC-T cells (adherent). Error bars represent the standard deviation, and all experiments were done in triplicate.

be a property shared by other endolysins, the endolysin dosage used in this study demonstrates that the efficacy of PlyGRCS is comparable to or better than other published staphylococcal endolysins (Drilling et al., 2016; Fenton et al., 2013; Gilmer et al., 2013; Gutierrez et al., 2014; Jun et al., 2013). As of yet, the only *in vivo* applications of endolysins against staphylococcal biofilm related infections are Ply187, which reduced the intravitreal bacterial load and attenuated symptoms of endophthalmitis, and chimeric endolysins λSA2-E-Lyso-SH3b and λSA2-E-LysK-SH3b, which reduced bacterial counts in a mouse model of mastitis (Schmelcher et al., 2012c; Singh et al., 2014); our initial *in vitro* work shows that PlyGRCS has promise to succeed in utilization in a biofilm infection model.

Importantly, PlyGRCS was able to efficiently remove staphylococcal biofilms formed in a catheter under dynamic conditions representative of a medically relevant scenario. During attempts to remove implant mediated non-*S. aureus* biofilms from infected patients, healthcare practitioners may utilize antimicrobial lock therapy, in which the affected device is filled with highly concentrated antibiotic and closed off for up to 48 hours, after which the solution is removed, hopefully removing the biofilm along with it; however it is not always effective (40-70% catheter salvage rate, depending on the organism), and as mentioned the first line of action against implants containing *S. aureus* biofilms is to bypass ALT and just remove the implant (Justo and Bookstaver, 2014; Poole et al., 2004). The ability of lock therapy using PlyGRCS to remove dynamically formed *S. aureus* biofilms from a catheter represents a major breakthrough in treatment of implant mediated biofilm infections.

As *S. aureus* can form biofilms in the absence of a medical device, and these biofilms are implicated in both human and animal infections such as mastitis, having a safe and efficacious treatment for such infections is necessary. PlyGRCS activity was displayed not only against biofilms grown on abiotic surfaces, but also on a monolayer of bovine epithelial cells. Importantly, this activity was specific against the bacteria and did not harm the eukaryotic cells. While no adverse effects have been observed in animal models so far, the rapid bacteriolytic activity of endolysins may require some fine-tuning of dosage before use in the clinic, especially in bloodstream related infections, as a sudden influx of proinflammatory cellular debris (teichoic acids, lipoteichoic acids, toxins, and peptidoglycan) could lead to life-threatening complications, such as septic shock and multiple organ failure (Nau and Eiffert, 2002). However, for decolonization of the skin as a prophylactic measure before surgery or on a cow's udder before milking, the fallout from a lytic therapy may be less severe. As one of the four major causative agents of bovine mastitis, *S. aureus* biofilms play a major role in chronic intramammary infections that result in significant losses for the dairy industry, and as a result, a viable anti-biofilm agent is critically needed (Oliveira et al., 2007).

While biofilms are clearly a problem in the medical field, the formation of biofilms on food and food processing equipment represents a major safety issue in the food industry. Food-borne illness caused by *S. aureus* results in hundreds of thousands of emergency room visits per year and economic losses in the billions of dollars from recalls and healthcare related costs (Kadariya et al., 2014). Additionally, biofilms on food processing equipment can interfere with proper function by impeding liquid flow and heat transfer, damaging the equipment by corrosion, and thereby leading to economic

losses due to machine repairs and downtime. The current chemical cleaners and disinfectants are not 100% effective, need to be completely removed from surfaces after use, and furthermore, a shift toward "green" biological products is underway. In fact, bacteriophage preparations against *Listeria, E. coli,* and *Salmonella* are already labelled GRAS (do not need to be removed from equipment and food after use and can be consumed by humans) and are being utilized in food processing facilities (Sharma, 2013). The fact that endolysins, including PlyGRCS, can remove bacterial biofilms from surfaces indicates that they too can find an application in this industry.

PlyGRCS is a robust enzyme and has many beneficial properties that warrant further investigation into development of this enzyme as a potential antimicrobial agent against staphylococcal biofilms. Future studies should be performed to determine the synergistic ability of PlyGRCS with other endolysins and antibiotics. Engineering approaches to enhance PlyGRCS activity should also be performed to increase thermostability properties for long-term storage, augment catalytic activity, or modulate binding to allow for a greater turnover rate. Finally, *in vivo* activity analysis of PlyGRCS in animal models of (implant-mediated) biofilm formation should be conducted.

The use of endolysins provides a more effective treatment for bacterial biofilm infections than antibiotics. The usage of sub-inhibitory concentrations of many antibiotics has been shown to enhance staphylococcal biofilm formation, thought to be mediated by an overall stress response, whereas the quick acting lytic activity of endolysins does not allow time for such a response to be mounted (Poole, 2012). We have shown here that PlyGRCS is actually inhibitory to biofilm formation. Endolysins also have a potential longer lifespan than antibiotics, with properties such as engineering and synergy to

prolong the use of these enzymes. Additionally, they may breathe new life into dying antibiotics, in a combinatorial therapy approach, where the endolysin would provide the initial disturbance to the biofilm structure, providing the antibiotic access to now susceptible target bacteria (Schuch et al., 2014). Most importantly, no research has been able to demonstrate the development of bacterial resistance; PlyGRCS may be even more refractory to resistance as it has two catalytic activities despite its single catalytic domain (Linden et al., 2015). Additionally, as antibiotics can only be utilized during an active infection (and are ineffective against biofilms anyway) and not for decontamination of an abiotic surface, having an agent such as an endolysin that can have a two-pronged application as a medical therapeutic and a surface disinfectant represents a superior antimicrobial option.

In conclusion we show that PlyGRCS displays specific bacteriolytic activity against *S. aureus* biofilms, indicating that this endolysin has the potential to be utilized as a skin decolonizing agent to prevent nosocomial and livestock-associated infections, a treatment for (implant-mediated) biofilm infections, and decontamination of food and food processing equipment

Chapter IV: Unpublished Dissertation-Related Data

Introduction

The previous two chapters represent our current understanding of the biochemical and biophysical characteristics of PlyGRCS, as well as its anti-biofilm behavior. However, this is only the beginning in our comprehensive knowledge of and experimentation on this endolysin. Toward our ultimate goal of utilizing PlyGRCS as a therapeutic against *S. aureus* infection, we have begun *in vivo* testing, as well as engineering experiments aimed at optimizing its activity. The unpublished, preliminary results from these studies are presented in this chapter.

The dearth of new traditional antibiotics, along with rising antimicrobial resistance rates highlights the application of endolysins as alternative antibacterial therapeutics. Because of the approximately 150,000 cases of *S. aureus* bacteremia per year in the U.S. alone, resulting in a 20% morbidity rate, this type of infection represents an opportunity to benefit from endolysin treatment (van Hal et al., 2012). However, to date, there have been few *in vivo* studies on endolysin treatment of *S. aureus* bacteremia (Gilmer et al., 2013; Gu et al., 2011a; Jun et al., 2013; Schmelcher et al., 2015; Schuch et al., 2014) . Importantly, the only endolysin that has attempted and completed Phase I clinical trials in the U.S. is CF-301 (Gilmer et al., 2013; Schuch et al., 2014). ContraFect Corporation intends to utilize this endolysin as a treatment against *S. aureus* bloodstream infections. However, all of the mouse experiments on this endolysin (and all other *S. aureus* endolysins, except for Sal-1) utilize an intraperitoneal infusion of both the bacterial load and the endolysin treatment. While some of the ip infused components eventually make their way to the bloodstream, we feel as though this is an artificial

model, as the peritoneal cavity provides a buffer of protection, not allowing the rapidly lysed components to quickly circulate in the bloodstream and cause a massive cytokine storm (Nau and Eiffert, 2002). In the clinic, the endolysin treatment would be administered intravenously, and therefore the dosage has to be completely verified using the same method in the mouse model. In fact, during the phase I clinical trial, human patients were given CF-301 over a single 2- hour intravenous (iv) infusion, and no major adverse events were observed (Cassino et al., 2016). However, as this was in the absence of an active *S. aureus* infection and there have been no mouse studies conducted on iv infusion of both CF-301 and *S. aureus*, so it is unknown what may happen when bacteria and this endolysin meet in the bloodstream. Alarmingly, the planned dose of CF-301 for phase II trials is 0.25 mg/kg, which resulted in a 100% death rate of mice within 18 hours when *S. aureus* and CF-301 were infused ip. To more accurately represent the real world application of an endolysin treatment against *S. aureus* septicemia, we have decided to use a mouse model in which both bacteria and endolysin are administered intravenously.

While we felt that the potent activity of PlyGRCS warranted pursuing *in vivo* studies utilizing the native enzyme, we also wanted to subject PlyGRCS to chimeragenesis to see if we could create an endolysin with improved efficacy. Engineering endolysins through chimeragenesis has proved to be an easy (due to the modular architecture of these enzymes) and effective technique of modification (Schmelcher et al., 2012a). Chimeric endolysins have been constructed to display characteristics such as increased catalytic activity, modified cleavage specificity, altered binding (strength and target), enhanced solubility and thermostability, and other desirable properties (Becker et al., 2009b; Donovan et al., 2006a; Manoharadas et al., 2009;

Schmelcher et al., 2011). Because the PlyGRCS CHAP domain already displayed such potency due to its dual catalytic activities, the SH3b CBD was chosen for replacement. All other staphylococcal endolysins possess SH3b domains, except for the ΦNM3 endolysin; thus, the ΦNM3 CBD was chosen as the desired replacement domain (Bae et al., 2006). The ΦNM3 CBD lacks homology to any annotated domains in the database, but has been shown to specifically bind *S. aureus* and has been successful in previous chimeragenesis experiments (Daniel et al., 2010; Pastagia et al., 2011; Yang et al., 2014a; Yang et al., 2014b). Furthermore, the substrate for the staphylococcal SH3b binding domain is predicted to be the pentaglycine crossbridge of the *S. aureus* peptidoglycan, and exposure of *S. aureus* to the SH3b containing lysostaphin has been shown to select for lysostaphin-resistant mutants with altered crossbridges (Grundling and Schneewind, 2006; Stranden et al., 1997). While the ΦNM3 CBD epitope is unknown, desire to steer away from a binding domain that may induce resistance prompted us to investigate this alternative CBD. We felt that these engineering efforts might result in an endolysin with enhanced activity or other improved characteristics.

Materials and Methods

Murine Bacteremia Model

PlyGRCS was purified as previously described (Linden et al., 2015), with an added 0.1% Triton X-114 purification step after application of lysate to the column for removal of endotoxin (Reichelt et al., 2006). Therapeutic treatment after septicemia induced by *S. aureus* infection was performed by TICRO at Trudeau Institute (Saranac

Lake, NY). As per the protocol, 8 week-old C57Bl6 female mice were obtained from Jackson Laboratories (Bar Harbor, ME) and acclimatized to the facility for two weeks. Mice were handled according to all applicable institutional, state, and Federal animal care guidelines under animal care protocols approved by the Institutional Animal Care and Use Committee (IACUC). Mice were injected intravenously with approximately $5x10^7$ CFU of *S. aureus* (ATCC 27217), an LD_{90}, which induced septicemia and death in mice after 2 to 3 days. Thirty minutes after infection the first group of mice was injected intravenously with 200 µl of the control vehicle (PBS) and the second group was injected with 200 µl of the therapeutic compound (PlyGRCS 200 µg/ml). Mice were monitored twice daily for weight loss and clinical signs defined as follows:

0 = no visible signs of disease

1 = slight ruffling of fur

2 = ruffled fur, reduced mobility

3 = ruffled fur, reduced mobility, rapid breathing

4 = ruffled fur, minimal mobility, huddled appearance, rapid and/or labored breathing

5 = death/euthanize

Animals displaying moribund signs were humanely euthanized and recorded as dead on that particular day. All animals found dead in the cage were recorded as dead on that day. Mice, once infected, were monitored twice daily for weight changes and euthanized immediately if they became recumbent, failed to move upon stimulation, exhibited an inability to eat or drink, or if they lost >20% of their initial body weight as stipulated and approved in the IACUC application 15-002. All surviving animals from the study were euthanized on Day 7 after the infection, by the administration of CO_2 asphyxiation and

cervical dislocation in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institute of Health.

Chimeragenesis of PlyGRCS

The ΦNM3 CBD was cloned into pBAD24 from pET21-$CBD_{ΦNM3}$ using the primers $CBD_{ΦNM3}$-F (5'GGGTCTAGAATCGGCAAATCAGCAAGC3') and $CBD_{ΦNM3}$-R (5'GGGAAGCTTTTAATGATGATGATGATGATGAAAAACTTCTTTCAC3'), and thus named pBAD-$CBD_{ΦNM3}$. The PlyGRCS CHAP domain with a linker region was then cloned from pBAD24-PlyGRCS using the primers CHAP-F (5'GGGGAATTCATTATGAAATCACAACAACAAGCAAAAGAATGGATATA3') and CHAPlink-R (5'TCTAGAGTTTTGTTTCCAATTTCC3'), and subcloned into pBAD24-$CBD_{ΦNM3}$ using the restriction enzymes *Eco*RI and *Xba*I, making the final chimeric construct (pBAD24-$CHAP_{GRCS}$-$CBD_{ΦNM3}$. All clones were confirmed by sequence analysis (Macrogen, Rockville, MD). Protein was expressed, purified, and tested for activity via the turbidity reduction assay and bactericidal assay, as previously described (Linden et al., 2015).

Results

In vivo Efficacy of PlyGRCS

Having validated the antimicrobial activity of PlyGRCS *in vitro*, we wished to determine if this endolysin would provide protection against an active infection in an animal model. First, to verify the safety of PlyGRCS, healthy uninfected mice were

injected with an intravenous bolus of enzyme and monitored for adverse effects over several days. Mice did not display any outwardly visible intolerance towards PlyGRCS. Next, mice were challenged in a model of *S. aureus* septicemia (Fig. 4-1). After 60 hours, 90% of the mice that were left untreated had died, while PlyGRCS treatment resulted in rescuing 30% of the mice. While these results were non-significant, these initial experiments are encouraging for the further development of PlyGRCS as a therapeutic against *S. aureus* infections.

Lytic Activity of PlyGRCS Chimera

The activity of the PlyGRCS chimera ($CHAP_{GRCS}$-$CBD_{\Phi NM3}$) was first compared to that of the WT endolysin via the turbidity reduction assay. On all *S. aureus* strains tested, $CHAP_{GRCS}$-$CBD_{\Phi NM3}$ displayed lytic activity; however, somewhat disappointingly, it did not perform as successfully as WT PlyGRCS (Fig. 4-2A-B). Interestingly, both enzymes displayed preferences for the same strains, indicating that it is in fact the change in binding domains that caused the difference in activity.

Next, the bactericidal capability of $CHAP_{GRCS}$-$CBD_{\Phi NM3}$ was assessed. It was determined that 32 µg/ml of endolysin reduced the bacterial count of VISA NRS-14 by 1 log in 1 hour (Fig. 4-2C). In comparison, only 2 µg/ml PlyGRCS resulted in the same bactericidal activity and increasing the dosage to 32 µg/ml caused a ~3.5 log reduction in CFU. As experimental conditions were optimized for the WT enzyme, the weaker activity of the chimera could be attributed to necessitating variable buffer components for enhanced activity. Further optimization of both conditions and enzyme are ongoing, as well as understanding the ideal binding for enhanced activity.

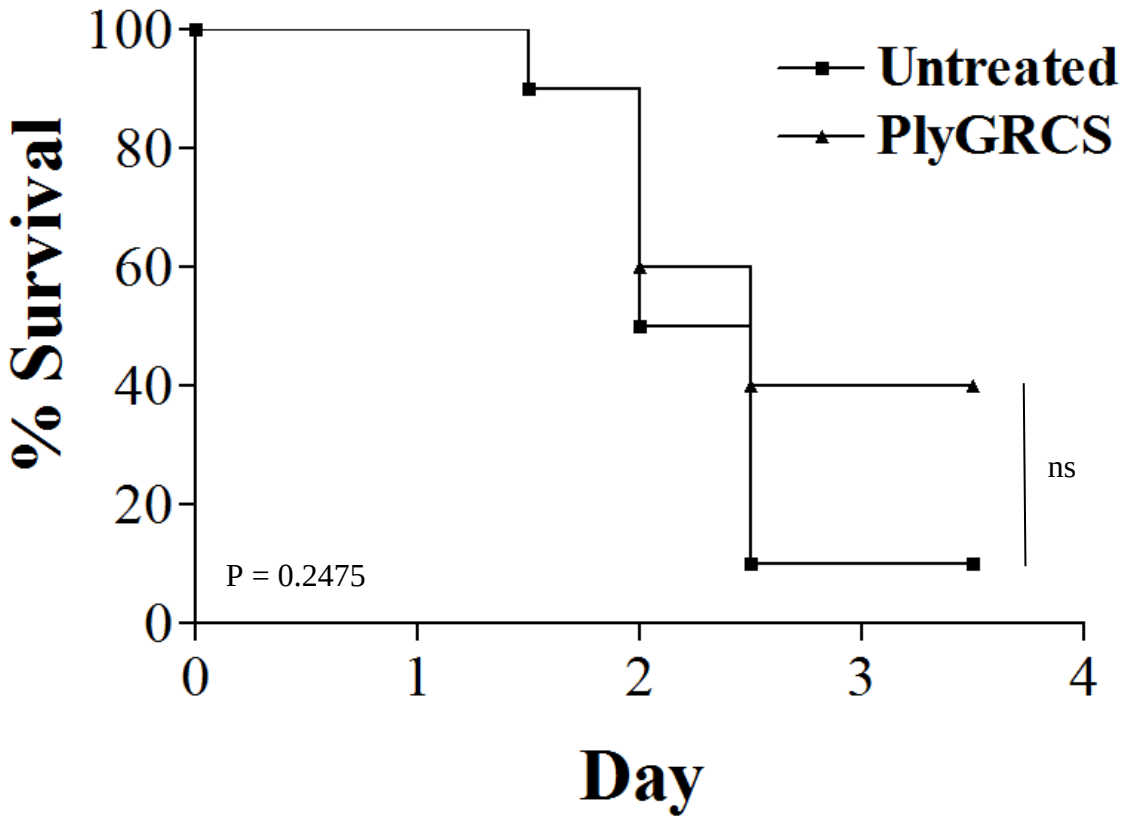

Figure 4-1. *In vivo* efficacy of PlyGRCS. Mice were injected intravenously with *S. aureus* (ATCC 27217), followed by PlyGRCS or control (PBS), and evaluated for survival (n=10 per group). PlyGRCS was effective at rescuing 30% of the mice from death induced by *S. aureus* bacteremia after 3.5 days. Data was analyzed using Kaplan-Meier survival estimator log-rank test.

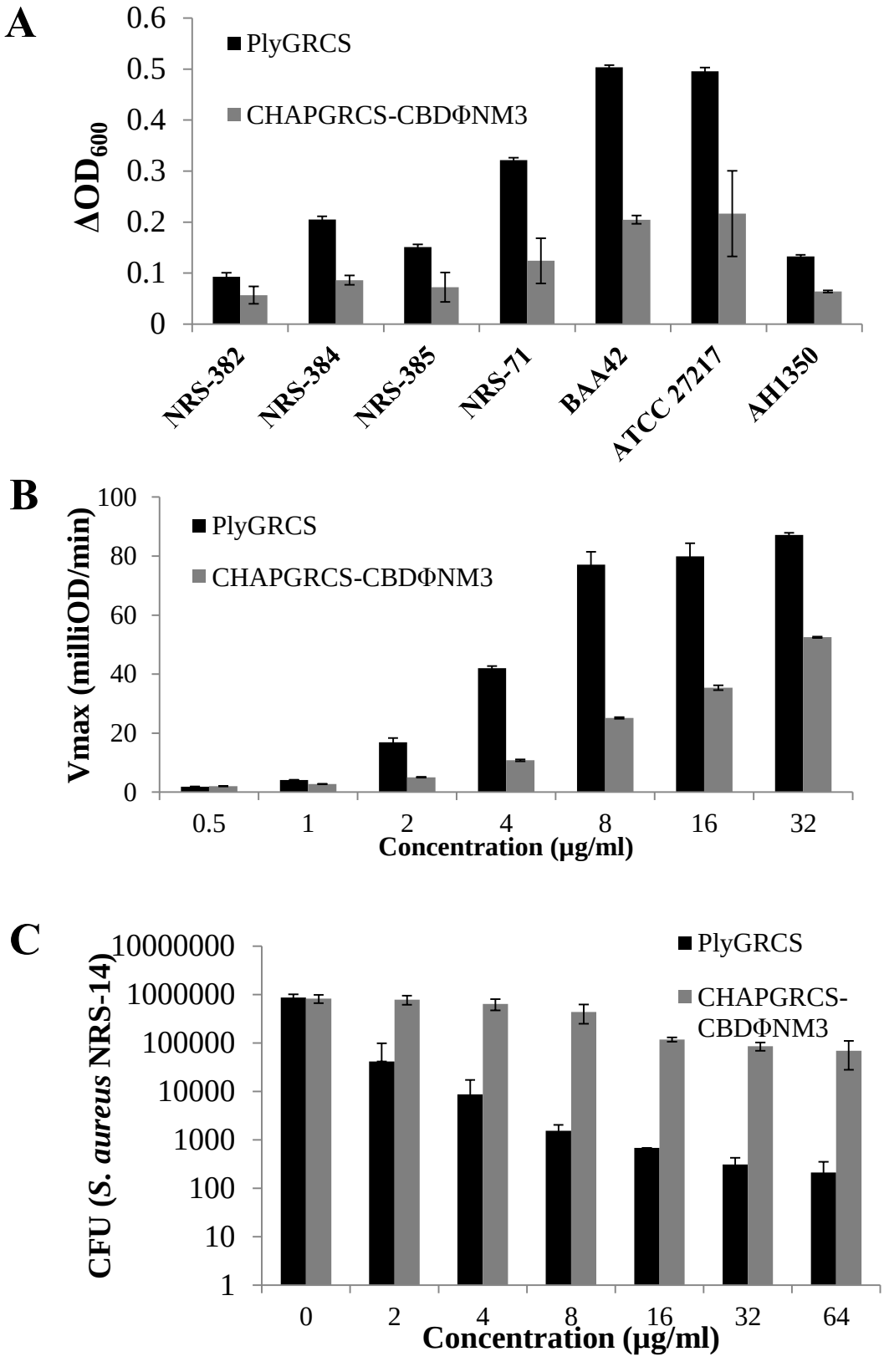
A
0.6
0.5
0.4
0.3
0.2
0.1
0
ΔOD600
PlyGRCS
CHAPGRCS-CBDΦNM3
NRS-382
NRS-384
NRS-385
NRS-71
BAA42
ATCC 27217
AH1350
B
100
80
60
40
20
0
Vmax (milliOD/min)
PlyGRCS
CHAPGRCS-CBDΦNM3
0.5
1
2
4
8
16
32
Concentration (µg/ml)
C
10000000
1000000
100000
10000
1000
100
10
1
CFU (S. aureus NRS-14)
PlyGRCS
CHAPGRCS-CBDΦNM3
0
2
4
8
16
32
64
Concentration (µg/ml)

Figure 4-2. Comparison of PlyGRCS WT and chimera. (A) Turbidity reduction assay analysis of PlyGRCS WT and chimera (16 μg/ml) against multiple strains of *S. aureus*. (B) Turbidity reduction assay showing dose response of PlyGRCS WT and chimera against *S. aureus* NRS-14 (C) Bactericidal activity of both endolysins against *S. aureus* NRS-14 Error bars represent the standard deviation, and all experiments were done in triplicate.

Discussion

The experiments in this chapter represent an initial foray into further understanding PlyGRCS and important first steps into implementing PlyGRCS as a therapeutic option. However, there are many more experiments to perform, troubleshooting to undertake, and challenges to overcome before getting to this next level.

Although endolysin therapy possesses many attributes that make it preferred over treatment with traditional antibiotics, it also could have some potential drawbacks. The first is the nature of endolysin therapy. The fact that this treatment is a lytic therapy may prove to be a challenge, especially if we wish to use it directly in the bloodstream to combat *S. aureus*-induced bacteremia. Indeed, the lower success rate of our mouse model when compared to other models may be due to the more real-world application of intravenous infusion (Schmelcher et al., 2015; Schuch et al., 2014). To study if death was caused by the bacteremia or the fallout from the immense amount of bacterial lysis, future experiments will include pathology analysis of the organs, as well as an investigation of the immune response. If these results show that mice are dying from the massive release of bacterial cell components, a lower dose or interval dosing of endolysin may need to be used in order to avoid such a robust response. Intravenous infusion of PlyGRCS also paves the way for future pre-investigational new drug enabling studies, such as safety, tolerability, and pharmacokinetics/pharmacodynamics (Cassino et al., 2016). Alternatively, targeting other *S. aureus*-associated diseases may prove to be a better route than attempting to pursue a treatment for bloodstream infections. Endolysins against *S. aureus* have shown great efficacy in decolonizing the nasal passage, topically

treating wounds, and decreasing the bacterial load in mastitis infections, and PlyGRCS may perform better in one of these models (Pastagia et al., 2011; Rashel et al., 2007; Schmelcher et al., 2012c).

Another potential problem with using endolysins is the emergence of resistant mutants. While several studies have compared the abilities of both antibiotics and endolysins to select for resistant bacteria and results have shown a lack of endolysin-resistant mutants, this does not mean it is impossible for endolysins to induce resistance (Gilmer et al., 2013; Pastagia et al., 2011). Futhermore, the use of lysostaphin, which contains a similar SH3b binding domain, has been shown to select for lysostaphin-resistant mutants, arising from modification of the pentaglycine crossbridge (Climo et al., 1998). While we have not shown that the SH3b binding domain of PlyGRCS interacts with the pentaglycine crossbridge, the possibility that this potential target could be modified is cause for concern.

In contrast to antibiotics, the ability of endolysins to elicit an immune response along with their short half-lives could be additional downsides. Although endolysins have been shown to generate antibodies, these antibodies are non-neutralizing and therefore do not affect the efficacy of the endolysins (Loeffler et al., 2003). Additionally, these antibodies did not cause anaphylaxis or other adverse effects in healthy subjects. However, the production of a hyperimmune response in some individuals could result in a potentially deadly scenario, so patients will have to be carefully screened to ensure the safety of using endolysins in their treatment. The short half-life of endolysins (about 20 minutes, comparable to other proteins) means that multiple doses would need to be used to totally eradicate the bacterial infection (Loeffler et al., 2003). It has also been

suggested that PEGylation, which has been used to increase the half-life of other biological molecules, be applied to endolysins; however, addition of PEG severely reduced the activity of Cpl-1 (Resch et al., 2011a). Excitingly, dimerization of Cpl-1 both increased its activity and half-life, indicating that this approach may be successful for overcoming fast clearance of other endolysins (Resch et al., 2011b).

Lastly, the extreme specificity of endolysins as compared to antibiotics could be viewed as a negative. In many cases, there is not enough time to identify a pathogen, so a broad-spectrum antibiotic is necessarily utilized. While many researchers are currently devising methods to more quickly identify bacteria, the current turn-around time of 1-2 days could mean life or death for some individuals. Having to wait to identify the species that is causing an infection to determine which endolysin to use for treatment could lead to a preference for antibiotics over endolysins.

In our efforts to create an endolysin against *S. aureus* with an atypical binding domain, we unfortunately resulted in producing a chimera with less activity than the wild-type. However, this does not mean that this chimeric derivative has no future application. In fact, endolysins with varying levels of *in vitro* activity have shown equal levels of *in vivo* activity (Schmelcher et al., 2015). Furthermore, the chimeric endolysins ClyS and ClyH, which share the ΦNM3 binding domain, have shown great efficacy in multiple *in vivo* applications (Daniel et al., 2010; Pastagia et al., 2011; Yang et al., 2014b). Similar doses of ClyH and the chimeric PlyGRCS showed similar results in the turbidity reduction assay, and ClyH protected 100% of mice in a MRSA model of infection, indicating the same could be achieved with our endolysin. ClyS, at a dose of 250 µg reduced cell counts by 3 logs *in vitro*, whereas we utilized a much lower dose (3.2

μg) to reduce CFU counts by 1 log. Even so, ClyS went on to perform quite well *in vivo*, eliminating nasal colonization, protecting 85% of mice from death caused by a systemic MRSA infection, and reducing the bacterial counts on infected skin. In all, this indicates that the chimeric PlyGRCS, despite lower *in vitro* activity than the wild-type endolysin, could potentially still be an i*n vivo* success.

Chapter V: Discussion

Summary of Dissertation

S. aureus is a significant pathogen, with an arsenal of virulence factors, including the propensity to form biofilms. Currently, the emergence of antibiotic-resistant strains represents a major pharmaceutical challenge and societal burden, causing millions of deaths and illnesses. Thus, the urgent need for alternative antimicrobial approaches prompted us to investigate the use of a bacteriophage-encoded endolysin against *S. aureus*.

PlyGRCS was derived from the bacteriophage GRCS, which had already shown activity against *S. aureus* in a mouse model of infection. We found that this endolysin was active under physiological conditions (pH and NaCl). Activity was greatly enhanced by the addition of calcium, and sequence analysis showed the presence of three critical aspartic acid residues shown in other endolysins to complex this cation. PlyGRCS displayed lytic activity against all strains of *S. aureus* tested, including MRSA and VISA, and also against *S. epidermidis*. Assays were performed using stationary phase bacteria, indicating the potent activity of this enzyme; 16 µg/ml reduced the OD_{600} of VISA NRS-14 by ~90% in 20 minutes and caused a 3 log decrease in CFU in 1 hour. PlyGRCS enzymatic activity was stable at temperatures up to 37°C; thermodynamic stability experiments showed cooperative unfolding of PlyGRCS with a T_m of 43.5°C.

Several techniques were utilized to understand the mechanistic behavior of PlyGRCS. Fluorescence microscopy showed that the C-terminal SH3_5 domain specifically bound staphylococci, while the N-terminal domain was confirmed to be catalytically active and possessed an active-site cysteine and histidine, defining it as a

CHAP domain. Biochemical assays detected free amine release (indicative of amidase/endopeptidase activity) upon PlyGRCS treatment; mass spectrometry analysis determined that, despite the single catalytic domain, PlyGRCS was able to cleave two distinct sites in the peptidoglycan (between the *N*-acetylmuramic acid and L-alanine and between the D-alanine in the peptide stem and the glycine in the interpeptide bridge).

As alternatives to traditional antibiotics, endolysins display the added benefit of activity against biofilms. PlyGRCS was able to disrupt static biofilms at concentrations much lower than the MIC, while antibiotics at 1000X MIC had no effect on biofilms. PlyGRCS not only disrupted these biofilms, but killed the bacteria within them as well. Additionally, PlyGRCS removed dynamic biofilms from medical grade catheters. Furthermore, unlike antibiotics, which were shown to promote biofilm formation, PlyGRCS prevented the formation of biofilms.

To study the effects of PlyGRCS treatment in the presence of a eukaryotic system, we first established *S. aureus* biofilms on bovine mammary epithelial cells. PlyGRCS was able to remove and kill biofilms grown on these epithelial cells. Importantly, PlyGRCS was non-toxic toward MAC-T cells. Our preclinical evaluation of PlyGRCS in a mouse model showed that intravenous application did not result in any adverse effects. A single treatment of PlyGRCS (200 µg/ml) was able to protect 30% of mice from death induced by *S. aureus* septicemia until day 3.5 post-infection.

Lastly, we have begun subjecting PlyGRCS to chimeragenesis to create the most ideal endolysin. Engineering efforts showed that replacing the native SH3_5 domain with the ΦNM3 CBD resulted in an endolysin that was less active as measured by turbidity reduction and CFU counts. The WT PlyGRCS caused a 1 log decrease at only 2 µg/ml,

while 32 µg/ml of the chimeric $CHAP_{GRCS}$-$CBD_{\Phi NM3}$ was necessary to achieve the same effect. However, it is well documented that *in vivo* efficacy of staphylococcal endolysins does not necessarily correlate with *in vivo* efficacy. Indeed, the endolysin 2638A performed poorly in several *in vitro* lytic and anti-biofilm assays, yet protected all mice in a lethal septicemia assay (Schmelcher et al., 2015). Thus, future *in vivo* testing will be needed to fully evaluate the chimeric $CHAP_{GRCS}$-$CBD_{\Phi NM3}$.

In conclusion, we have shown that PlyGRCS has potent antimicrobial activity against planktonic and biofilm antibiotic resistant *S. aureus*. Our initial work on tissue cultured cells and in mice, along with the low likelihood of developing resistance due to its dual catalytic activities, specific binding, and the possibility of future engineering indicate that PlyGRCS is a promising alternative antibacterial agent for both human and animal uses.

Discussion and Future Directions

Therapeutic Potential

The ultimate goal of this project was to investigate an endolysin against *S. aureus* that could eventually be used translationally as a new antimicrobial therapy. All *in vitro* experiments performed suggested that PlyGRCS would be a good candidate for *in vivo* testing. The preclinical trial in our mouse model of treatment against *S. aureus* septicemia indicated that PlyGRCS provides some protection; however, there is still obviously a long way to go before proceeding to human subjects.

Despite this, we have already made some progress toward understanding how to use PlyGRCS *in vivo*. In an earlier experiment, 1 mg/ml of PlyGRCS was used, and

surprisingly, mice that received endolysin treatment fared worse than mice that were left untreated; 8/10 mice died within the first 24 hours and the remaining two were moribund and had to be euthanized within the next 24 hours. Interestingly, the mice that received PlyGRCS displayed hind leg paralysis, an indication that they died due to septic shock. Our theory was that the PlyGRCS dosage used was too high, causing rapid lysis of bacteria too quickly, releasing cellular debris and triggering a massive inflammatory response ultimately resulting in death of the animals. The initial dosage we used was based on several other models of *S. aureus* bacteremia; however, the typical model for MRSA septicemia utilizes ip infusion of both bacteria and endolysin, thus providing a cavity of protection, preventing the majority of lysed components from entering the bloodstream and thereby not promoting as robust of a response. When we lowered the dose to 200 µg/ml, we were able to save 40% of the mice until day 3.5, supporting our theory. Therefore, more experiments need to be performed to optimize dosage of PlyGRCS. The dosage may need to be lowered even more or perhaps we could give multiple injections of a very low dose over an extended period to minimize the amount of bacterial lysis at one time. Another possibility is utilizing less bacteria to establish infection (LD_{50} instead of LD_{90}), as there will be a lower amount of bacterial contents upon lysis. In fact, the only other example of intravenous endolysin treatment (Sal-1) of *S. aureus* septicemia utilized an amount of bacteria to give a lower mortality rate than what we used in our study (Jun et al., 2013). Alternatively, as *S. aureus* causes a wide variety of infections, PlyGRCS may perform better in a different model, such as in a topical application against a skin infection or in a nasal decolonization model.

Based on our finding that PlyGRCS can remove dynamic biofilms from medical grade devices, an implant-associated biofilm model is also worth investigating. As of yet, there have been no *in vivo* studies on the use of endolysins to treat staphylococcal biofilms mediated by medical implants and only recently has a staphylococcal endolysin been tested and found effective against an *in vitro* dynamic biofilm (Becker et al., 2016). In fact, the only application of endolysin treatment against an implant mediated biofilm appears to be PlyF307, which reduced the *A. baumanii* load of indwelling subcutaneous catheters by 2 logs and destroyed much of the EPS (Lood et al., 2015). This seminal experiment indicates that other endolysins, including PlyGRCS, could efficaciously treat infections associated with medical devices. Directly treating the implant with an endolysin would be much desired over the alternative removal method. Our laboratory has acquired a Perkin Elmer *In vivo* Imaging System (Lumina XR), which we can utilize to monitor the infection and treatment in real time, making this both the first investigation of an endolysin against an implant-associated *S. aureus* infection and the first use of the IVIS to observe endolysin treatment of an implant mediated infection (Fenton et al., 2010a; Kadurugamuwa et al., 2003).

In addition to the above mentioned experimental variables, adjusting the chemical formulation of the PlyGRCS treatment may improve *in vivo* efficacy. Sal-1, which bears about 50% identity in both its CHAP and SH3b domains to PlyGRCS, successfully protected mice from intravenously induced MRSA septicemia (Jun et al., 2013). The Sal-1 formulation included 10 mM $CaCl_2$ for enchancement of activity; we have already included calcium in our PlyGRCS formula. However, the presence of calcium can be associated with destabilization and aggregation of proteins, which may explain some of

the difficulties observed in the *in vivo* experiments. The final formulation of Sal-1 included stabilizing components 0.01 M L-histidine (pH 6.0), 5% (w/v) sorbitol, and 0.1% (w/v) Poloxamer 188, the major contributor to stabilization. Before addition of these components, Sal-1 aggregation occurred after five minutes of agitation or two hours in storage at 4°C; in the presence of these elements, four hours of agitation was required to induce aggregation and the formulation was stable for eight weeks at 4°C, while maintaining activity. In this formulation, the first Good Laboratory Practice compliant safety evaluation of an endolysin, including general toxicology and safety pharmacology tests, determined that Sal-1 did not cause any adverse effects in dogs or rats (Jun et al., 2014). If PlyGRCS stability is not enhanced by the Sal-1 formulation, there are alternative ingredients that may allow for an improved *in vivo* outcome. Other amino acids, poloxamers, and polyols display stability enhancing abilities and are commonly acknowledged by the pharmaceutical industry as acceptable and safe (Kamerzell et al., 2011). Additionally, salts, hydrophilic polymers (PEGs, polysaccharides and inert proteins), and surfactants are all currently used as stabilizing components in protein therapeutic formulations.

Finally, as *in vitro* experiments are not always the best indicators of *in vivo* efficacy, $CHAP_{GRCS}$-$CBD_{\Phi NM3}$, despite its lower activity in the turbidity reduction and bactericidal assays, may actually prove to be a better enzyme when placed into a mouse (Henry et al., 2013; Schmelcher et al., 2015). Furthermore, an enzyme that is less active *in vitro* might be more desirable *in vivo*, since it may avoid the massive bacteriolytic fallout seen when using the high dose of PlyGRCS.

Structural Analysis

Obtaining structural knowledge helps us to better understand the function of a protein and also may pave the way for future design of improved enzymes. As of yet, only two *S. aureus* endolysin crystal structures have been solved: the CHAP domain of LysK and all 3 domains of LysGH15 (Gu et al., 2014; Sanz-Gaitero et al., 2014). Excitingly, these endolysins are almost identical to each other (only differing by four amino acids), and their CHAP and SH3b domains share about 50% identity to PlyGRCS, which means they can give us important structural insight into our endolysin.

The crystal structures of the CHAP domains of LysK and LysGH15 were obtained at the same time and yielded similar results, in terms of overall structural layout, residues implicated in interaction and catalysis, and the importance of metal cations. The overall structure of the proteins consists of two alpha helices connected by a loop containing the catalytic cysteine and a 3_{10}-helix which connects to six beta-strands arranged in an anti-parallel beta-sheet (with another 3_{10}-helix between beta strands 2 and 3 (Fig. 5-1A). They both display a long and deep hydrophobic groove with conserved residues lining its surface, providing structural evidence for the catalytic center and its reaction mechanism of the cleavage of peptidoglycan (Figure 5-1B). The typical CHAP Cys-His-Glu-Asn quartet was found to be close to the cleft and is responsible for a proteolytic relay event, resulting in the nucleophilic attack of a peptide bond. Most importantly, in and around the active site cysteine lays a classical 12-residue (positions1D, 3D, 5Y, 7H, and 12D) calcium-binding site. Calcium was found to be essential for catalytic activity (as in the case of PlyGRCS), and the most important

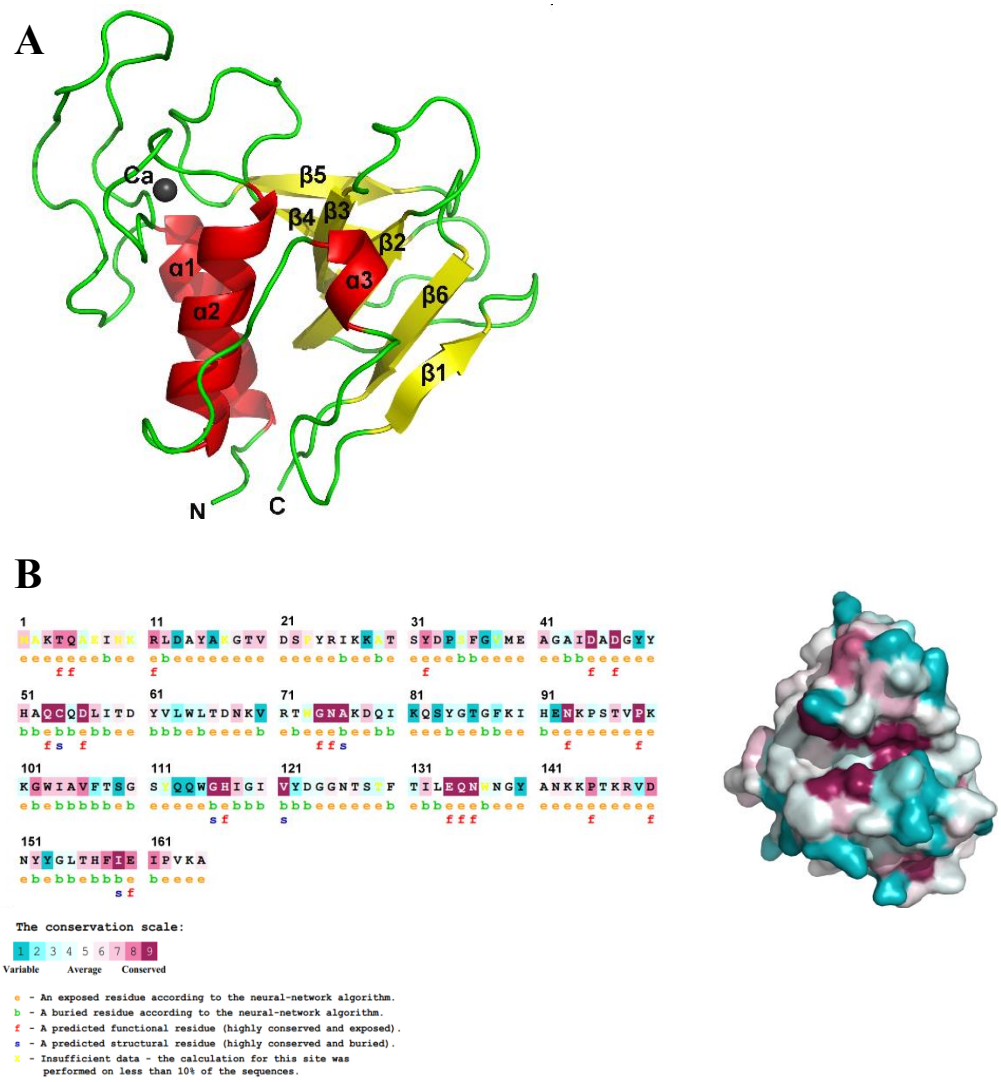

Figure 5-1. Structural and functional analysis of LysGH15 CHAP. (A) Overall structure of the CHAP domain (residues 1–164) coordinating a Ca^{2+} ion, showing its secondary structure composition and the globular nature of endolysin domains. (B) An alignment of sequences showing sequence conservation and predicted surface rendering of the catalytic groove. Figure from (Gu et al., 2014)

residues in coordination of the ion were the three aspartic acids, as determined by site directed mutagenesis. PlyGRCS contains these three aspartic acids, but has alanine and glycine in the 5^{th} and 7^{th} positions, respectively. The calcium ion is proposed to help maintain structure by assisting with positioning of the catalytic residues; additionally, the loop containing the calcium binding site contains residues that were shown to potentially interact with the peptidoglycan substrate, suggesting a role in determining substrate specificity. The importance of the calcium binding site was also demonstrated by an observed ~2°C shift in *Tm*, in the presence of Ca^{2+}, indicating its effect on thermostability. The approximately 50% identity and even higher level of homology between these two proteins and PlyGRCS means that information about the structures of the CHAP domains of these two enzymes can be applied to future engineering of PlyGRCS, discussed in detail in the next section.

Before the crystal structure of $CHAP_K$ was experimentally resolved, *in silico* modeling using I-TASSER and visualized via PyMol were utilized to somewhat accurately predict its 3D structure based upon comparison to an already solved protein (Fenton et al., 2011a). In the event of our inability to obtain a crystal structure through X-ray crystallography or NMR spectroscopy, this alternative technique could provide an approximation of the conformation of PlyGRCS and insight into interactions between its domains and their substrates.

While it has been determined that the lysostaphin and ALE-1 CBD substrate is the pentaglycine crossbridge of *S. aureus* peptidoglycan, the PlyGRCS SH3b belongs to a variable SH3b subgroup (which includes LysGH15 and LysK), indicating that it may have a distinctly different substrate (Fig. 5-2) (Becker et al., 2009b). In fact, the binding

between LysGH15 SH3b and the peptide "AGGGGG" was shown to be quite weak at only 3 mM; additionally, this SH3b and pentaglycine were never co-crystallized, further suggesting that they may not interact (Gu et al., 2014). This may also explain why there is much discrepancy about the LysGH15 and ALE-1 residues involved in binding, as well as slight differences observed in structure of the CBDs from these two endolysins. Because we cannot co-crystallize the PlyGRCS SH3b with its substrate if we aren't sure what that substrate is, a computational modelling approach may help to determine this ligand. However, we must have some potential candidates for this method, so we have constructed a partial list of *S. aureus*-specific molecules that are accessible to binding by the PlyGRCS CBD (Table 5-1). These include teichoic acids, surface anchored proteins, carbohydrates, and components of the staphylococcal peptidoglycan. RosettaLigand has been successfully used to model small molecule interactions with a protein receptor. In fact, this program defined phosphatidylserine as the interacting partner for the binding domain of PlyCB that facilitates internalization of the PlyC holoenzyme (Shen et al., 2016). Additionally, other ligand-receptor modeling programs have been utilized to study the interactions between CBDs and their binding targets, providing important structural insight and mechanistic understanding (Hirakawa et al., 2009; Perez-Dorado et al., 2007).

Engineering: Next Generation PlyGRCS

Despite the potent antimicrobial activity of PlyGRCS, we would like to alter some of its properties to make it a better enzyme. As mentioned, the modular domains of endolysins makes them highly amenable to chimeragenesis engineering efforts to

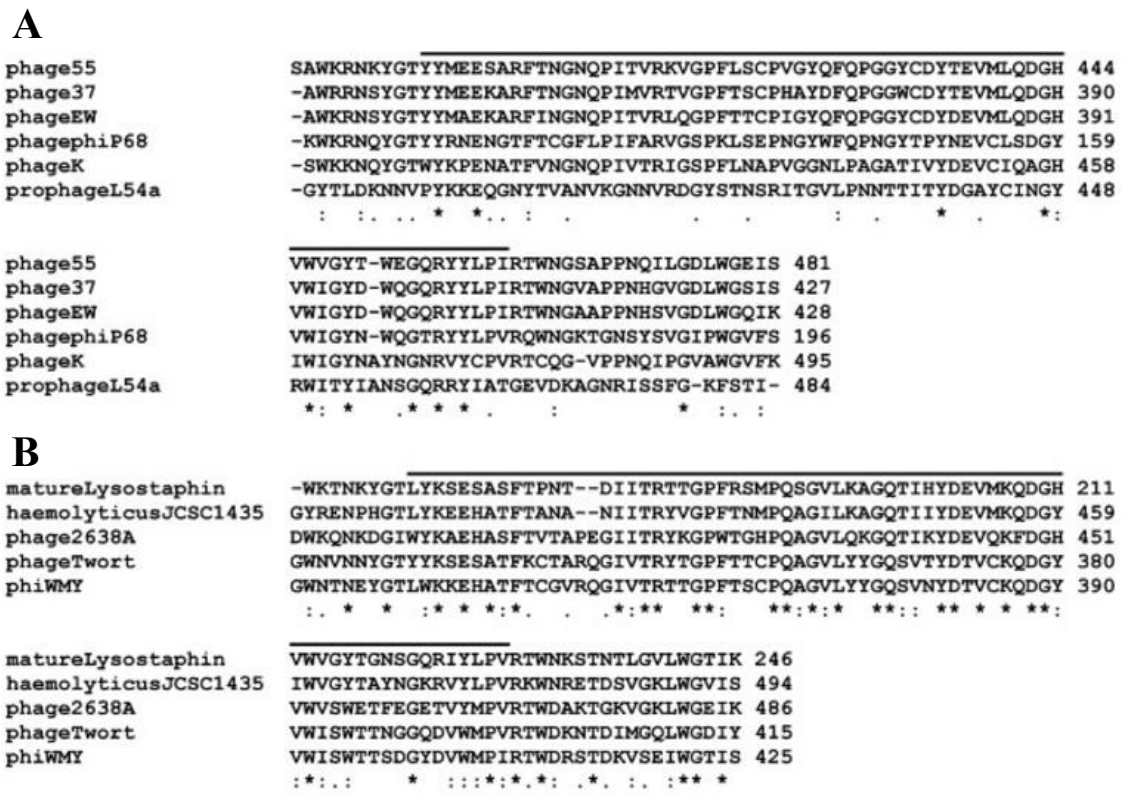

Figure 5-2. Analysis of SH3b domains. Sequence alignment of SH3b domains from 11 staphylococcal endolysins representing five homology groups and 6 stand-alone proteins, shows conserved residues that allow for the clustering into two subgroups. (A) Weakly conserved subgroup. (B) Highly conserved subgroup. Overlined residues constitute the formal SH3b_5 domain (Pfam database). * perfectly conserved residue, : highly conserved residue, . weakly conserved residue Figure from (Becker et al., 2009b)

Table 5-1. Candidates for PlyGRCS SH3b binding partners.

Molecule	Function	Ref.
Polyribitolphosphate wall teichoic acid	shape determination, regulation of cell division	(Vinogradov et al., 2006)
Polyglycerolphosphate lipotechoic acid	antigenicity, regulator of autolysis	(Endl et al., 1983)
Peptidoglycan components (GlcNac, MurNac, Peptide stem, 5Gly interpeptide bridge	structural integrity	(Tipper and Berman, 1969)
MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) (i.e. FnBPA, FnBPB, Cna, ClfA, ClfB, SdrC, SdrD, SdrE)	adhesion, immune evasion	(Foster and Hook, 1998)
NEAT motif family proteins (IsdA, IsdB, IsdH)	iron acquisition	(Grigg et al., 2007)
SasB, C, D, F, G, J, K, L, X	adhesion, biofilm formation	(Roche et al., 2003)
Protein A	immune evasion, antigenicity	(Forsgren and Sjoquist, 1966)
AdsA	immune evasion	(Thammavongsa et al., 2011)
Bap	biofilm formation, invasion	(Cucarella et al., 2001)
SraP	adhesion	(Siboo et al., 2005)

Select molecules present in only *S. aureus* that could be potential substrates for the PlyGRCS SH3b binding domain.

improve efficacy, change host range, or vary other characteristics. Additionally, rational design though structure guided site directed mutagenesis, along with random mutagenesis techniques including directed evolution, have all proven effective ways of modifying endolysins.

One possibility, which we have already started to investigate through our ΦNM3 CBD experiments, is replacing the native SH3b with other CBDs. This can be approached in two ways. The first requires changing the CBD but keeping the *S. aureus* species specifity. Staphylococcal endolysins containing SH3b domains are classified into five homology groups and 6 stand-alone proteins, leading us to believe that there might be 11 different SH3b domains that could be paired with the PlyGRCS CHAP (Becker et al., 2009b). However, most of the sequence conservation appears to be within the C-terminal SH3b domain, and a comprehensive bioinformatic analysis revealed there to be only 2 distinct SH3b subgroups (Fig. 5-2). As the native PlyGRCS SH3b domain belongs in the "weakly conserved subgroup," future studies would involve replacing it with an SH3b domain from the "highly conserved subgroup," one of whose members is the lysostaphin SH3b. It has already been determined that the λSa2 endopeptidase catalytic domain paired with the lysostaphin SH3b displayed approximately 2x greater activity than when paired with the LysK SH3b (from the weakly conserved group). As the λSa2 endopeptidase domain only has one catalytic activity, the dual catalytic activity of PlyGRCS CHAP combined with the improved binding of lysostaphin SH3b may create a super enzyme. An important note is that increased binding does not necessarily mean increased catalysis. In theory, a CBD that binds too tightly and does not release its target quickly or at all would make a worse enzyme than a CBD that displays weaker binding

and can rapidly move between substrates. While affinities of listerial CBDs were determined to be in the nanomolar range, there is currently no understanding of the "best" binding for optimal endolysin activity and few data are available on the K_d of staphylococcal CBDs (Gu et al., 2014; Schmelcher et al., 2010). In this vein, we would like to determine the affinities of the highly conserved SH3b, the weakly conserved SH3b, and the ΦNM3 CBD, and how the strength of their binding contributes to activity. Our facility has recently acquired the Octet RED96 system, which can determine kinetic binding constants in real time. These data, combined with information obtained from the crystal structure and the role of ionic interaction by way of charge, may help us to define an ideal binding domain, as well as identify future points of modification to modulate binding.

In addition to changing the *S. aureus*-specific CBD for another *S. aureus*-specific CBD, a non-*S. aureus* CBD could be fused to the PlyGRCS catalytic domain to change the target organism. As noted, the catalytic domain is highly active due to its dual activities; however, while one of the bonds cleaved by the PlyGRCS CHAP domain is present in all bacteria (the linkage between the *N*-acetylmuramic acid and the L-alanine of the peptide stem), the other bond is only present in staphylococcal species (between L-alanine and a glycine in the crossbridge). We could replace the PlyGRCS SH3b domain with a CBD from an endolysin against any other species, but the activity may be weakened with only one catalytic activity. Based on this, *S. epidermidis*, which is a causative agent of opportunistic nosocomial infections, may be a good target for modified PlyGRCS. We have already determined that PlyGRCS displays weaker activity against *S. epidermidis* than the activity observed against *S. aureus*, which may be due to its inability

of the CBD to recognize a non-*S. aureus* target. While there are other endolysins reported in the literature to have activity on both *S. aureus* and *S. epidermidis*, there are no *S. epidermidis*-specific endolysins. Additionally, there appear to only be 5 *S. epidermidis* bacteriophage genomes sequenced, leading to the identification of only 5 genes annotated as endolysins (with no data on their activity) (Aswani et al., 2014; Daniel et al., 2007; Melo et al., 2014a, b). Three of these endolysins have CBDs annotated as SH3b, while the other two have C-terminal regions that display no homology to any domains in the database. Creating a chimeric enzyme composed of the PlyGRCS CHAP domain and one of these predicted CBDs may prove to be extremely effective against *S. epidermidis*, due to dual catalytic activity combined with potential *S. epidermidis*-specific binding.

The chimeragenesis approach is just one method by which we could rationally design an improved endolysin. Additionally, information gleaned from obtaining the crystal structure of PlyGRCS, along with bioinformatic sequence analysis may help us understand how to modify the catalytic domain for enhanced activity (Fig. 5-3) (Di Tommaso et al., 2011; Notredame et al., 2000). A comparative analysis of Sal-1 and LysK found that Sal-1 was approximately twice as active as LysK, despite only differing from each other at 3 residues (Jun et al., 2011). It was determined that the Sal-1 glutamine at position 113 contributed to this enhanced activity; LysK has a glutamic acid at this point in the CHAP domain. The LysGH15 CHAP domain also contains a glutamine at position 113, and only differs from Sal-1 by one amino acid in the SH3b domain (LysGH15 D469; Sal-1 N469). Crystal structures of both the CHAP domains from LysK and LysGH15 identify W115 and H117 from the loop between beta-strands B and C as two of the residues participitating at the surface of a long and deep hydrophobic

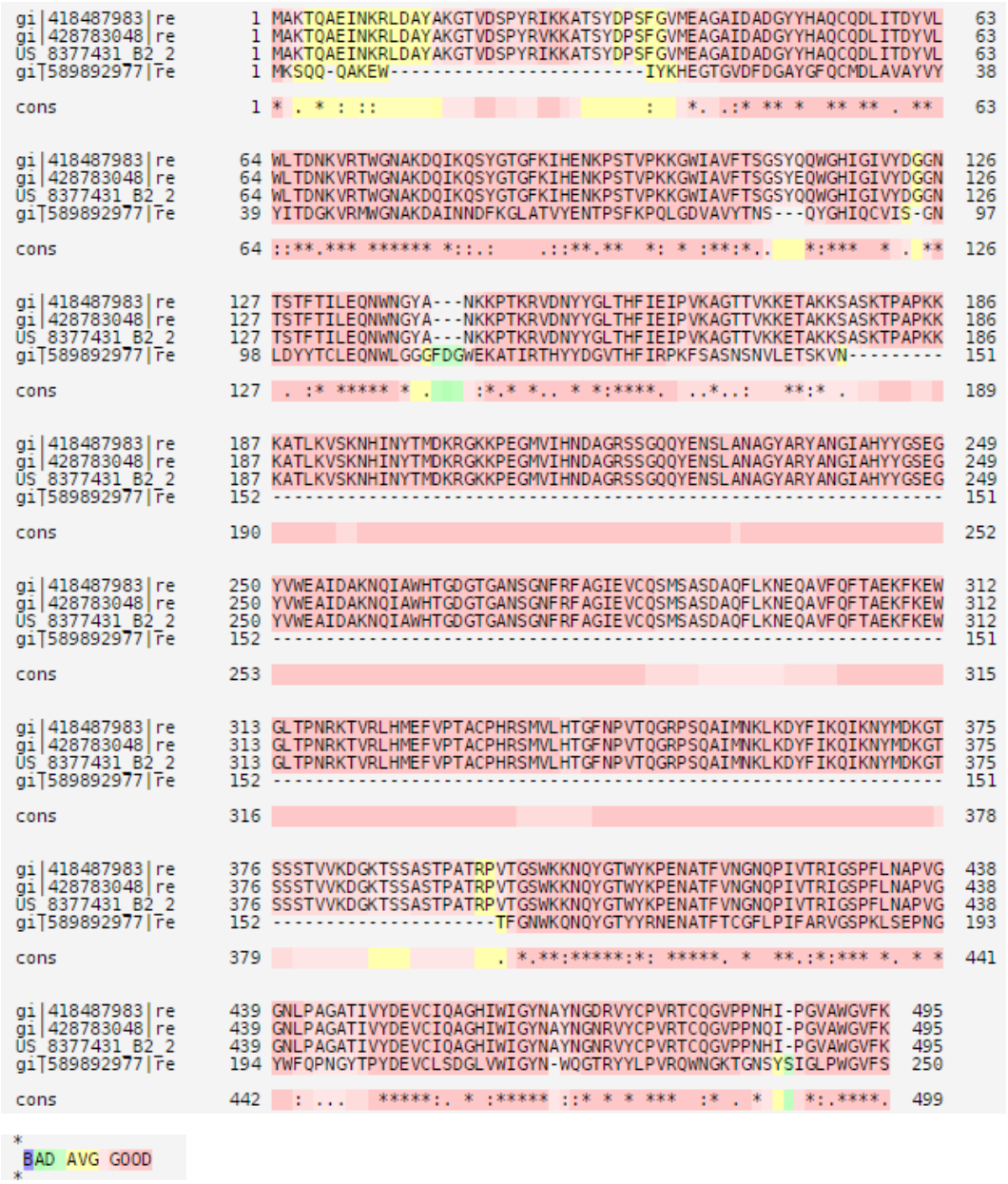

Figure 5-3. Alignment of LysGH15, LysK, Sal-1, and PlyGRCS. An alignment by T-COFFEE showing the CHAP domains of LysGH15, LysK, Sal-1 (residues 45-159) and PlyGRCS (residues 20-133) and SH3b domains of LysGH15, LysK, Sal-1 (residues 412-477) and PlyGRCS (residues 164-229). The three reference proteins contain an additional central Amidase_2 catalytic domain (residues 207-330). * perfectly conserved residue, : highly conserved residue, . weakly conserved residue

groove that possibly accomodates the peptidoglycan substrate (Gu et al., 2014; Sanz-Gaitero et al., 2014). The proximity of the 113th amino acid to this site and its effect on activity indicates that it may contribute to the structure of the groove. While PlyGRCS contains a similarly hydrophobic tyrosine (corresponding to the W115), as well as the histidine as part of its active site, the alignment of PlyGRCS with LysK, LysGH15, and Sal-1 shows that there is a gap in homology corresponding to amino acids 111-113 of the three reference proteins (beween S88 and Q89 in PlyGRCS). Based upon this, inserting three residues (SYQ) to fill in this gap may modify the activity of PlyGRCS. Analysis ofthe crystal structures of the CHAP domains of LysK and LysGH15 additionally identified F36, D47, Y49, Y50, Q53, C54, D56, T59, R71, W73, N75, N136 and W137 (for LysK) and D47, Q53, C54, D56, G74, N75, H117, E134, and N136 (for LysGH15) as residues in the groove that might contact peptidoglycan (Gu et al., 2014; Sanz-Gaitero et al., 2014). The alignment shows that PlyGRCS only differs at these suggested peptidoglycan-contacting amino acids corresponding to positions 36, 49, and 59. Therefore, making point mutations to PlyGRCS at these corresponding positions may prove to alter its activity. Perhaps the most important mutation involves the PlyGRCS residue corresponding to position 49 (position 5 in the calcium binding site), as this region has been shown to contribute to structural stability, activity, and thermostability. PlyGRCS contains an alanine at this position; it was previously shown that LysGH15 Y49A displayed ~80% of the bacteriolytic activity of the WT endolysin. Although not as critical as the residues in positions 1, 3, and 12 of the calcium binding site (which all displayed <5% activity when mutated), this 5th position does seem to have some significance. Although the main chain oxygens are responsible for the coordination of the

calcium ion, there seems to be an additional factor, such as size of the amino acid, which affects activity. Therefore, changing this alanine to a tyrosine may enhance the activity of PlyGRCS. Additionally, although not one of the suggested peptidoglycan contacting residues, the slight reduction in activity when H51 was mutated to alanine in LysGH15 and LysK indicates that the 7^{th} position in the calcium binding site may be more important than previously thought. In PlyGRCS, the amino acid at this position is glycine, so mutation to a much larger charged residue, such as histidine, may alter activity.

In addition to rationale design to improve the efficacy of endolysins, random mutagensis has also proven to be a valuable technique in the development of better enzymes and could be utilized to create a better PlyGRCS. Two methods have already been efficacious in producing randomly engineered endolysins with improved activity or other desirable characteristics and could easily be adapted to improving PlyGRCS. The first utilized an *E. coli* mutator strain and error-prone PCR to create a GBS endolysin that lysed more bacteria at a faster rate (Cheng and Fischetti, 2007). The other was the use of directed evolution to increase the thermostability of the streptococcal endolysin PlyC (Heselpoth and Nelson, 2012). In order to use directed evolution to improve the efficacy of PlyGRCS, a system would need to be created in which *E. coli* express and secrete PlyGRCS to kill *S aureus* in competitive growth. Models have been established to show that this scenario is theoretically possible (Bull et al., 2015). In the two proposed methods, mathematical and computational models show that the selective pressure could be achieved in order to force the *E. coli* host to produce a more efficient endolysin.

Appendix A: List of Non-Thesis Published, Submitted, and Planned Co-Authored Manuscripts

Catheter-associated urinary tract infection by *Pseudomonas aeruginosa* is mediated by exopolysaccharide independent biofilms.

Stephanie J. Cole, Angela R. Records, Mona W. Orr, Sara B. Linden, Vincent T. Lee.
Infect Immun. 2014 May; 82(5): 2048–2058.

Abstract

Pseudomonas aeruginosa is an opportunistic human pathogen that is especially adept at forming surface-associated biofilms. *P. aeruginosa* causes catheter-associated urinary tract infections (CAUTIs) through biofilm formation on the surface of indwelling catheters. *P. aeruginosa* encodes three extracellular polysaccharides, PEL, PSL, and alginate, and utilizes the PEL and PSL polysaccharides to form biofilms *in vitro*; however, the requirement of these polysaccharides during *in vivo* infections is not well understood. Here we show in a murine model of CAUTI that PAO1, a strain harboring *pel*, *psl*, and *alg* genes, and PA14, a strain harboring *pel* and *alg* genes, form biofilms on the implanted catheters. To determine the requirement of exopolysaccharide during *in vivo* biofilm infections, we tested isogenic mutants lacking the *pel*, *psl*, and *alg* operons and showed that PA14 mutants lacking these operons can successfully form biofilms on catheters in the CAUTI model. To determine the host factor(s) that induces the Δ*pelD* mutant to form biofilm, we tested mouse, human, and artificial urine and show that urine can induce biofilm formation by the PA14 Δ*pelD* mutant. By testing the major constituents of urine, we show that urea can induce a *pel*-, *psl*-, and *alg*-independent biofilm. These *pel*-, *psl*-, and *alg*-independent biofilms are mediated by the release of extracellular DNA. Treatment of biofilms formed in urea with DNase I reduced the biofilm, indicating that extracellular DNA supports biofilm formation. Our results indicate that the opportunistic pathogen *P. aeruginosa* utilizes a distinct program to form biofilms that are independent of exopolysaccharides during CAUTI.

A chimeolysin with robust bacteriolytic activity and extended-spectrum streptococcal host range found by an induced-lysis based rapid screening method.

Hang Yang, Sara B. Linden, Jing Wang, Junping Yu, Daniel C. Nelson, Hongping Wei.
Sci Rep. 2015 Nov; 5: 17257.

Abstract

The increasing emergence of multi-drug resistant streptococci poses a serious threat to public health worldwide. Bacteriophage lysins are promising alternatives to antibiotics; however, their narrow lytic spectrum restricted to closely related species is a central shortcoming to their translational development. Here, we describe an efficient method for rapid screening of engineered chimeric lysins and report a unique "chimeolysin", ClyR, with robust activity and an extended-spectrum streptococcal host range against most streptococcal species, including *S. pyogenes*, *S. agalactiae*, *S. dysgalactiae*, *S. equi*, *S. mutans*, *S. pneumoniae*, *S. suis* and *S. uberis*, as well as representative enterococcal and staphylococcal species (including MRSA and VISA). ClyR is the first lysin that demonstrates activity against the dominant dental caries-causing pathogen as well as the first lysin that kills all four of the bovine mastitis-causing pathogens. This study demonstrates the success of the screening method resulting in a powerful lysin with potential for treating most streptococcal associated infections.

A bacteriophage endolysin that eliminates intracellular streptococci.

Yang Shen, Marilia Barros, Tarek Vennemann, D. Travis Gallagher, Yizhou Yin, Sara B. Linden, Ryan D. Heselpoth, Dennis J. Spencer, David M. Donovan, John Moult, Vincent A. Fischetti, Frank Heinrich, Mathias Lösche, Daniel C. Nelson. Elife. 2016 Mar; 5. pii: e13152.

Abstract

PlyC, a bacteriophage-encoded endolysin, lyses *Streptococcus pyogenes* (*Spy*) on contact. Here, we demonstrate that PlyC is a potent agent for controlling intracellular *Spy* that often underlies refractory infections. We show that the PlyC holoenzyme, mediated by its PlyCB subunit, crosses epithelial cell membranes and clears intracellular *Spy* in a dose-dependent manner. Quantitative studies using model membranes establish that PlyCB interacts strongly with phosphatidylserine (PS) whereas its interaction with other lipids is weak, suggesting specificity for PS as its cellular receptor. Neutron reflection further substantiates that PlyC penetrates bilayers above a PS threshold concentration. Crystallography and docking studies identify key residues that mediate PlyCB-PS interactions, which are validated by site-directed mutagenesis. This is the first report that a native endolysin can translocate epithelial membranes, thus substantiating the potential of PlyC as an antimicrobial for *Spy* in the extra- and intracellular milieu and as a scaffold for engineering other functionalities.

Enzybiotics: Endolysins and Bacteriocins.

*In Press

Ryan D. Heselpoth, Steven M. Swift, Sara B. Linden, Michael S. Mitchell, Daniel C. Nelson. Bacteriophages: Biology, Technology, Therapy. 2016. Springer Publishers. Edited by David Harper, Steve Abedon, Ben Burrowes, Malcolm McConville

Abstract

The growing prevalence within community and healthcare settings of antibiotic resistant Gram-positive and Gram-negative bacterial pathogens is alarming. Particularly concerning are reports of bacteria that are resistant to last-resort antibiotics such as carbapenems and vancomycin. Thus, novel concepts are needed to face the serious challenge posed by multidrug resistant bacterial infections. A promising alternative antimicrobial approach to conventional antibiotics involves the use of bacteriophage-derived protein(s), generically known as "enzybiotics". Endolysins, one type of enzybiotic, are cell-wall, i.e., peptidoglycan hydrolases that act on the host bacterium late in the phage replication cycle. These enzymes hydrolyze critical covalent bonds essential for maintaining cell wall structural integrity. Due to the absence of an outer membrane, extrinsically applied recombinant endolysins have direct access to the bacterial cell wall to lyse susceptible Gram-positive pathogens. Highlighting their therapeutic potential, the efficacy of endolysins has been validated *in vitro* and/or *in vivo* against a variety of Gram-positive pathogens, and in the less than 15 years since their first documented use as an antimicrobial in 2001, endolysins are now being commercially developed and undergoing clinical trials. Alternatively, phage-like or particulate bacteriocins comprise a second class of enzybiotics that can be used therapeutically. These multi-protein structures resemble bacteriophage tail-like assemblies and are produced by both Gram-negative and Gram-positive bacteria. Unlike fully-functional bacteriophages, bacteriocins are incapable of replicating, though they nonetheless possess a pseudo-injection mechanism that results in loss of bacterial membrane integrity and subsequent bacterial death.

Anti-biofilm activities of a novel chimeolysin against *S. mutans* in physiological and cariogenic conditions.

*Submitted

Hang Yang, Yongli Bi, Xiaoran Shang, Mengyue Wang, Sara B. Linden, Yunpeng Li, Yuhong Li, Daniel C. Nelson, Hongping Wei.

Abstract

Streptococcus mutans, a common oral bacterium, often survives as a biofilm on the tooth surface and contributes to the development of dental caries. In this study, we investigated the efficacy of ClyR, a chimeolysin with an extended streptococcal host-range, against *S. mutans* biofilms in physiological and cariogenic conditions. Susceptibility tests showed that ClyR was active against all clinical *S. mutans* isolates tested as well as *S. mutans* biofilms that displayed resistance to penicillin. *S. mutans* biofilms formed on hydroxyapatite discs in physiological sugar conditions and cariogenic conditions were reduced ~2 logs and 3 logs after treatment with 100 μg/ml ClyR, respectively. In comparison, only 1 log reduction was observed in the chlorhexidine (ChX) treated group, and no killing effect was observed in the NaF treated group. A mouse dental colonization model showed that repeated use of ClyR for 3 weeks (5 μg/day) reduced the number of colonized *S. mutans* in the dental plaques significantly ($p<0.05$), without harmful effects on the body weight and the vitality of mice. Furthermore, toxicity was not noted at concentrations exceeding those used for these studies and ClyR-specific antibodies could not be detected in mice saliva after repeated use of ClyR in the oral cavity. Our data collectively demonstrates that ClyR is active against *S. mutans* biofilms both *in vitro* and *in vivo*, thus representing a preventative or therapeutic agent against dental caries.

Development of a bacteriophage lysin for therapeutic use against pathogenic streptococci infecting livestock.

*Submission Pending

Sara B. Linden, Parimala Sharma, Caren J. Stark, James Todd Hoopes, David E. Kerr, Vincent A. Fischetti, Kasey M. Moyes, Daniel C. Nelson.

Abstract

Bovine mastitis is an inflammation of a cow's mammary gland, usually due to a microbial infection originating from contaminated teats. Several bacterial species have the ability to cause bovine mastitis, but *Streptococcus uberis* is responsible for the majority of chronic cases. The current treatment of antibiotics is not only ineffective, but undesirable, due to concerns about the emergence of antibiotic resistant bacteria and the spillage of milk from cows until antibiotic clearance has occurred. As an alternative, we propose the use of the bacteriophage endolysin PlyC, which displays antimicrobial activity against select streptococcal species. This study investigates the use of PlyC as an antimicrobial enzyme against *S. uberis*. Our results show that PlyC possesses potent lytic activity against all strains of *S. uberis* tested, including 7 clinical isolates. Importantly, despite the ability of other endolysins to display activity against *S. uberis*, none have successfully functioned in raw cow's milk, presumably due to inactivation by native proteins and lipids; however, PlyC at a concentration of just 2 times the MIC affects 3 logs of killing in just 1 hour in raw milk from mastitic cows. We have also through fluorescent microscopy that the binding domain of PlyC selectively interacts with *S. uberis* in the presence of raw cow's milk. Furthermore, the lack of neutralization by antibodies specifically targeted against PlyC bolsters the potential of this enzyme as an antimicrobial treatment. Significantly, PlyC is non-irritating as observed on rabbit epidermis and mucous membrane, and non-toxic as observed on a bovine mammary cell line. Taken together, these microbiological, biophysical, and immunological results indicate that PlyC has the potential to be used as a novel therapeutic against *S. uberis*-associated bovine mastitis.

Analysis of the antimicrobial activity of PlySs9, a *Streptococcus suis* derived endolysin.

*In preparation

Sara B. Linden and Daniel C. Nelson.

Abstract

The crisis of increasing resistance of pathogenic bacteria to classical antibiotics has driven research towards identification of other means to fight infectious disease. One particularly attractive option is the use of bacteriophage-encoded peptidoglycan hydrolases (endolysins). These enzymes are able to lyse the bacterial cell wall upon direct contact when applied externally and lack the drawbacks of typical antimicrobials. Endolysins have already shown potential in the areas of food safety, human health, and veterinary science. One specific area that could benefit from endolysin application is the overwhelming problem of *Streptococcus suis* infections of pigs. While the economic toll on the swine industry can be devastating in the event of an outbreak, it is the zoonotic nature of *S. suis* that is particularly alarming. There are currently no effective approaches to eradicate *S. suis* from pig herds and preventing disease outbreaks has proven extremely difficult. Therefore, the overall objective of this proposal is to identify and evaluate novel *S. suis*-specific endolysins for antibacterial activity.

Recombinant bacteriophage endolysin, PlyC, is non-toxic and does not alter blood polymorphonuclear leukocytes or mammary cells in lactating dairy cows.

*In preparation

Cynthia M. Sholte, Sara B. Linden, T.H. Elsasser, S. Kahl, E.E. Connor, Y. Qu, Parimala Sharma, Daniel C. Nelson, Kasey M. Moyes

Abstract

As bacteria develop resistance to conventional antibiotics, it is relevant to explore alternate therapies to treat intramammary infections. A newly proposed treatment method utilizes endolysin PlyC, an enzyme produced by bacteriophages that causes targeted lysis of the plasma membrane of *Streptococcus uberis*. A relatively low concentration (1.0 μg/mL) of recombinant PlyC (rPlyC) can induce lytic activity, suggesting that a low dose may successfully eliminate infection. We evaluated the dose effect of rPlyC on cytotoxicity and oxidative response of bovine blood neutrophils. We hypothesized that rPlyC would be non-toxic and not alter the inflammatory response of neutrophils in vitro. Cells were isolated from plasma obtained from healthy, mid-lactation primiparous dairy cows (n=12) and incubated with various concentrations of rPlyC (0, 1, 10, and 50 μg/mL) for 0.5 and 2 hours. Following incubation, cytotoxicity was measured by non-radioactive, colorimetric assay to quantify lactate dehydrogenase. Oxidative response was measured by chemiluminescence assay of reactive oxygen species (ROS) production in response to 0 and 1.6 μg/mL phorbol 12-myristate-13-acetate (PMA) in addition to rPlyC during incubation. Data were analyzed as ANOVA using mixed model procedures in SAS (version 9.3). As expected, neutrophil cytotoxicity varied across incubation time with greater cell toxicity measured at 2 hours incubation as compared to 0.5 hours (P=0.01; 55 vs. 45±3%) and is primarily attributed to the short half-life of neutrophils. Oxidative response was affected by incubation time (P=0.04) and PMA concentration (P<0.01) with the greatest ROS production at 0.5 hour incubation in the presence of 1.6 μg/mL PMA. Concentration of rPlyC did not affect oxidative response (P=0.73) nor neutrophil cytotoxicity (P=0.41). In summary, varying doses of rPlyC are non-toxic and do not alter ROS production in bovine neutrophils. The use of rPlyC as an alternative intramammary therapy for *Streptococcus uberis* mastitis is promising as our data indicate that rPlyC may not interfere with immune response during mastitis.

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