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The Role of Lysogenic Bacteriophage in Virulence and Survival of Streptococcus Pyogenes

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THE ROLE OF LYSOGENIC BACTERIOPHAGE IN VIRULENCE AND SURVIVAL OF *STREPTOCOCCUS PYOGENES*

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Chad W. Euler

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This thesis investigates the interactions of lysogenic bacteriophage with *Streptococcus pyogenes*. We were specifically interested in elucidating novel ways in which the prophage influence GrAS virulence and survival either within the host cell or within the bacterial population. In turn, we also hoped to determine how the bacteria (and possibly other integrated phage) might influence prophage activity or gene expression. Our studies focused on two poly-lysogenized GrAS strains, a M6 serotype (MGAS10394) and a M1 serotype (SF370).

In our M6 studies, we chose a streptococcal strain lysogenized by a chimeric bacteriophage element (Φ10394.4), which contains the erythromycin resistance gene *mefA*, in order to elucidate why genomes of certain erythromycin-resistant isolates of group A streptococci are resistant to SmaI endonuclease cleavage. In the work presented here, we identified a restriction modification system contained on the bacteriophage chimeric-element and successfully characterized the first methyltransferase (M.SpyI) encoded in *S. pyogenes*.

In the M1 strain, allelic recombination techniques were used to begin to analyze the role and regulation of a bacteriophage-encoded potential superantigen, SpeH. Next, we designed a novel system to specifically select for the loss of integrated phages entirely from the genome. This technique allowed us to study the novel role that two SF370 phage play in regulating GrAS chromosomal genes involved in bacterial metabolism, DNA repair and mutagenesis. We were also able to begin to elucidate the effects that individual and multiple phage loss have on SF370 virulence and survival, as we successfully constructed the first strain of GrAS that is completely devoid of bacteriophages.

In the course of these studies, we also manipulated lytic bacteriophage genes from two different *Staphylococcus* phage genomes to genetically engineer a novel chimeric endolysin (ClyS). We demonstrated the antimicrobial lytic activity of ClyS against methicillin-resistant *Staphylococcus aureus* (MRSA) in both in vivo colonization and septicemia models, and were also able to show its synergistic activity with oxacillin in in vitro and in vivo models. This work highlights the potential of ClyS as a novel therapeutic agent for the treatment of MRSA and other staphylococcal infections.

To my Pop Pop, Tom Hardman,

Your life and teachings inspired me to look at the world analytically

while not forgetting there is magic in the world around us.

To my wife, Tara Morris Euler,

You are the magic in my world!

1 Acknowledgements

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5 Introduction

Describing bacterial pathogenesis, Felix d'Herelle wrote, "… the actions and reactions are not solely between these two beings, man and bacterium, for the bacteriophage also intervenes; -a third living being and, hence, a third variable is introduced" (d'Herelle 1930).

5.1 Bacteriophages

Bacteriophages (phages) are ubiquitous viruses that can infect bacteria. Independently discovered by Felix d'Herelle and Fredrick Twort in the early 1920's, bacteriophage research has made many contributions to the fields of genetics and molecular biology, including an understanding of genetic mutation (Luria and Delbruck 1943; Lederberg and Lederberg 1952), the discovery of a genetic code (Crick, Barnett et al. 1961), and the detection and manipulation of restriction enzymes (Luria and Human 1952). Phage research has also included therapeutic applications of phage as treatments against bacterial infections, as well as the role phage play in many diseases caused by bacterial pathogens (Waldor, Friedman et al. 2005).

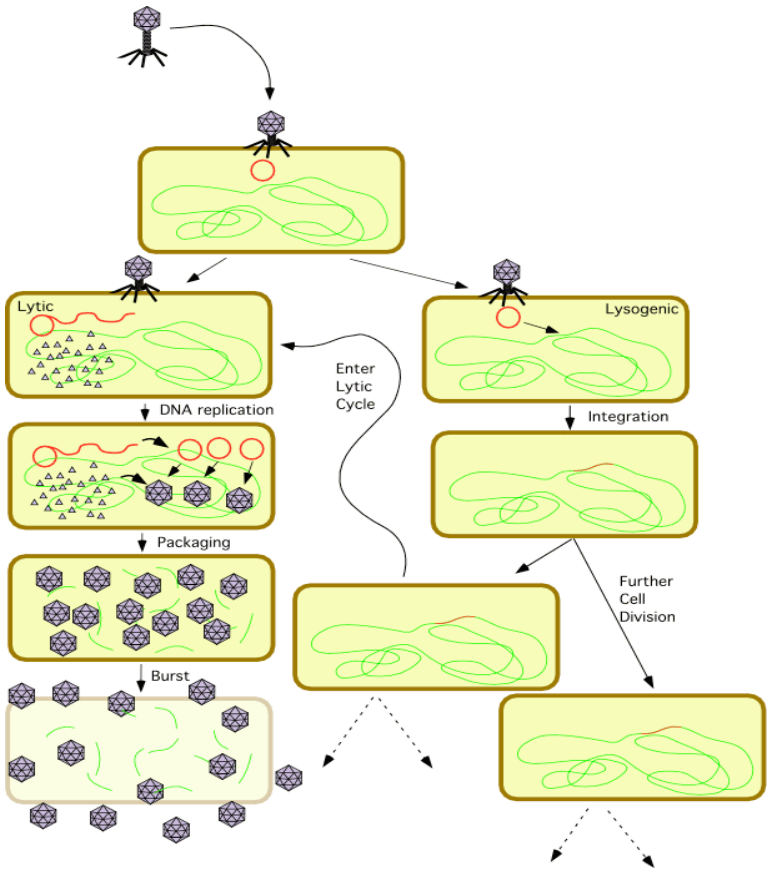


Figure 5.1. Bacteriophage lifecycle has two phases: Lytic and lysogenic. Adapted from http://inst.bact.wisc.edu/inst with permission.

5.2 Lytic Phage in Treatment of Bacterial Disease

Phages exhibit two distinct lifecycles: a lytic phase and a lysogenic phase (Figure 5.1). The lytic phase consists of phage infection of the host bacteria, replication of the viral genome, production and assembly of new viral particles, and then the rupture of the host bacterial cell to release the phage progeny. This phase, which ends with the death of the bacteria and further spread of the virus,

has been the focus of using “lytic phage” as therapeutic agents. d’Herelle’s own work throughout the early 1900’s focused on using phage preparations isolated from sterile culture supernatants of phage-infected bacteria to treat numerous bacterial diseases. His initial work focused on the use of phage to treat livestock animals suffering from avian typhosis (*Salmonella gallinarum*) and bovine hemorrhagic septicemia (*Pasteurella multocida*). He later used “shiga-bacteriophage” and “plague-phage” to successfully treat human patients suffering from dysentery and bubonic plague, respectively (d'Herelle 1930; Summers 2001). These studies, together with successful phage treatment regimes by other scientist increased interest in “phage therapy” to such an extent that several US pharmaceutical companies, including Eli-Lilly, commercialized products containing phage lysates for several target diseases (Summers 2001; Deresinski 2009). But since the discovery of antibiotics during World War II, interest in phage therapy fell in the United States as the use of antibiotics, which were cheaper to produce and could act against a wider spectrum of bacterial pathogens, dramatically increased throughout the decades. Since then, the misappropriate overuse of antibiotics has selected for the emergence of several bacterial pathogens that are now resistant to a majority of the current antibiotic treatments (Fluit, Verhoef et al. 2001; Gill, Fouts et al. 2005). The current state of antibiotic resistant bacteria has encouraged researchers in academia and biotechnology to look for alternative antimicrobials, and has stimulated a renewed interest in using lytic phages or phage-lytic enzymes, termed “lysins”,

(Figure 5.2) as a treatment against a number of different pathogens and drug resistant bacteria (Fischetti, Nelson et al. 2006; Fischetti 2008; Deresinski 2009; Daniel, Euler et al. 2010).

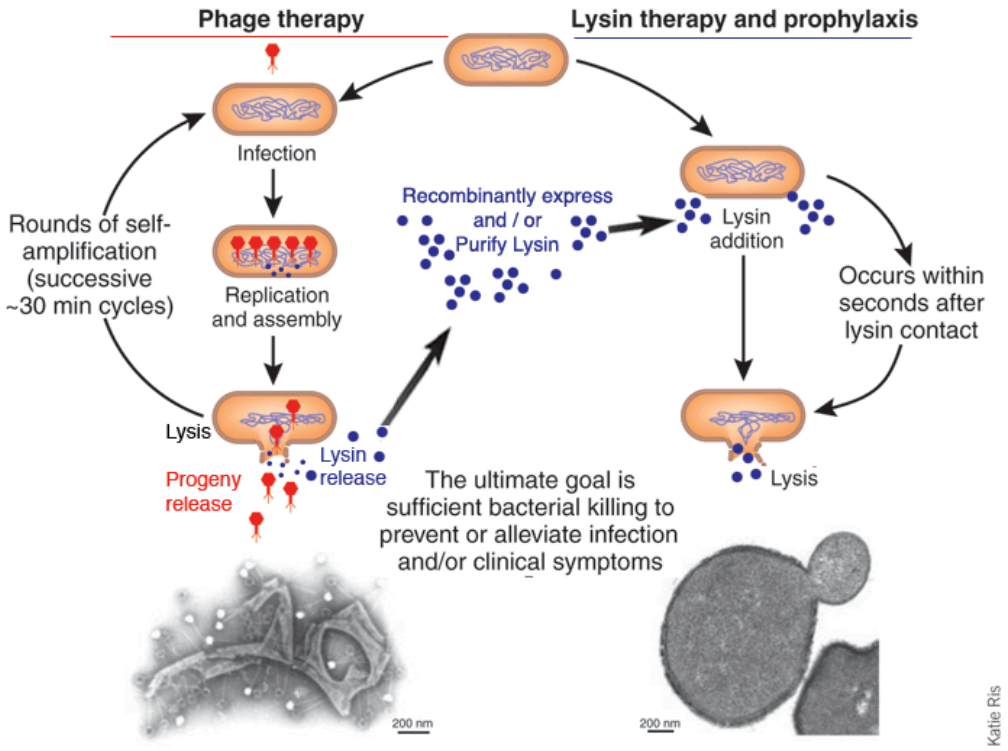

Phage therapy
Lysin therapy and prophylaxis
Infection
Rounds of self-amplification (successive ~30 min cycles)
Replication and assembly
Recombinantly express and / or Purify Lysin
Lysin addition
Occurs within seconds after lysin contact
Lysis
Progeny release
Lysin release
The ultimate goal is sufficient bacterial killing to prevent or alleviate infection and/or clinical symptoms
Lysis
200 nm
200 nm
Katie Ris

Figure 5.2. Bacterial lysis in phage therapy and lysin therapy.

Phage therapy (on the left) exploits a natural phage lytic cycle, which occurs over 30 min and is divided into three major steps, including the release of new virions (in red) into the environment. Subsequent infection of new hosts illustrates the process of self-amplification. The electron micrograph depicts phage particles adhering to the debris of a lysed streptococcal cell. In comparison, lysin therapy and prophylaxis (on the right) is defined by only two steps, in which purified or recombinant lysin (in blue) binds to, and rapidly kills through osmotic lysis, the target pathogen. The electron micrograph depicts a cross-section of Bacillus anthracis treated with the purified PlyG lysin showing an externalized cytoplasmic membrane just before lysis. Scale bar, 200 nm. Adapted with permission from (Fischetti, Nelson et al. 2006).

5.3 Bacteriophage Lysins

Phage lysins are cell wall hydrolases that are expressed late in the lytic cycle. With the help of phage encoded holins, lysins are translocated across the bacterial membrane and cleave the peptidoglycan bonds of the bacterial cell wall resulting in hypotonic lysis of the bacteria and phage progeny release (Young 1992). Fortuitously, the killing effect of lysins can also be elicited when their purified recombinant forms are directly applied to susceptible Gram-positive bacteria from the outside (Fischetti, Nelson et al. 2006). Gram-negative bacteria are protected from this lysis by their outer membrane, as the lysin cannot make contact with the peptidoglycan cell wall. The lytic spectrum of each lysin is usually narrow (species or even strain specific) and is constrained by the peptidoglycan-associated ligands, often carbohydrates, that serve as lysin binding targets, which themselves are often distributed in a species or strain specific manner. These moieties are often essential for bacterial viability, making it difficult for the bacteria to mutate these epitopes to become resistant to lysin binding (e.g. choline for *Streptococcus pneumoniae* or polyrhamnose in *Streptococcus pyogenes*) (Fischetti 2005). The catalytic activities of lysins generally fall into three classes based on bond specificity within the peptidoglycan: glycosidases that hydrolyze linkages within amino-sugar moieties, amidases that hydrolyze amide bond between the glycan and the pentapeptide, and endopeptidases that cleave within the pentapeptide or the

peptide cross-bridge. Significantly these bonds are invariant in all bacterial cell walls. A very important feature of lysins, with respect to their development as antibacterials, is that they have structural versatility. That is, the peptidoglycan hydrolysis activity and the surface carbohydrate binding activity are encoded in two distinct functional domains. These domains remain active after separation and can be recombined with complementary domains of other lysins to alter their specificity and/or catalytic functions (Donovan, Dong et al. 2006). In this manner a number of distinct lysins can be recombined to generate a highly variable pool of antimicrobials (Croux, Ronda et al. 1993; Lopez, Garcia et al. 1997; Daniel, Euler et al. 2010). Currently, some of the potential applications include prophylactically decolonizing or treating infections of the mucous membranes, skin and wounds with lysins that target *Staphylococcus aureus*, *Streptococcus agalactiae*, or *Streptococcus pyogene*s (Nelson, Loomis et al. 2001; Cheng, Nelson et al. 2005; Daniel, Euler et al. 2010), or treating systematic bacterial infections with lysins designed against anthrax, pneumococcal septicemia and enterococcal septicemia (Schuch, Nelson et al. 2002; Loeffler and Fischetti 2003; Yoong, Schuch et al. 2004).

5.4 Lysogenic Phage in Bacterial Pathogenesis

The lysogenic phase of the phage lifecycle also involves infection of the host bacteria, but instead of producing new viral particles, the phage genome is integrated by site-specific recombination into the bacterial chromosome

(Figure 5.1), at which time it is termed a prophage. Expression of a phage lytic repressor, encoded on the phage genome, allows the bacteriophage DNA to remain integrated and to be stably replicated as part of the host chromosome. The resulting prophage and host bacteria together form a lysogen. Sometimes lysogens can be induced to produce full phage particles once again, usually as a result of DNA damage (e.g. exposure UV light or mitomycin-C in the laboratory setting) or other environmental signals that result in the inactivation of the lytic phage repressor (Broudy, Pancholi et al. 2001; Banks, Lei et al. 2003). Integrated viral DNA then excises from the genome and enters lytic cycle replication, resulting in the production and assembly of viral components and the eventual rupture of the host bacteria. While lysogenic phages have the potential to kill their host bacteria, their stable integration often provides evolutionary benefits to host. For along with the genes necessary for the viral lifecycle, phages often encode virulence factors that increase the pathogenic potential and survival of the lysogen. Genomic analysis of most human pathogenic bacteria (gram positive and gram negative) shows that they can contain a number of phages that encode virulence factors (Table 5.1). These virulence proteins provide additional mechanisms for bacterial attachment and colonization, invasion into host tissue, antimicrobial resistance, damage to host cells (toxins), and evasion or alteration of the host immune response (Brussow, Canchaya et al. 2004). Furthermore, integrated phages also encode factors such as phage repressors, super infection exclusion proteins, and restriction enzyme systems

that prevent other lytic phage from infecting and killing its host. These phage-associated attributes give lysogens an advantage for survival in the host and over other bacteria, and thus increase the selection of bacteria that maintain the DNA of a lysogenic phage. Such selection is evident in the large number of lysogenic bacteriophages that are integrated into the genomes of members of the Genus *Streptococcus* (Beres and Musser 2007).

Table 5.1. Examples of bacteriophage encoded virulence factors

Virulence Protein	Gene	Phage	Bacterial host
Extracellular toxins			
Diphtheria toxin	*tox*	β-Phage	*C. diphtheriae*
Neurotoxin	C1	Phage C1	*C. botulinum*
Shiga toxins	*stx1, stx2*	H-19B	*E. coli*
Enterohaemolysin	*hly2*	ϕFC3208	*E. coli*
Cytotoxin	*ctx*	ϕCTX	*P. aeruginosa*
Enterotoxin	*see, sel*	NA	*S. aureus*
Enterotoxin P	*sep*	ϕN315	*S. aureus*
Enterotoxin A	*entA*	ϕ13	*S. aureus*
Enterotoxin A	*sea*	Mu50A	*S. aureus*
Exfoliative toxin A	*eta*	ϕETA	*S. aureus*
Leukocidin	*pvl*	ϕPVL	*S. aureus*
Cholera toxin	*ctxAB*	CTXϕ	*V. cholerae*
Superantigen	*speA*	T12	*S. pyogenes*
Superantigen	*speC*	CS112	*S. pyogenes*
Superantigens	*speA1* , *speA3* , *speC* , *spel* , *speH* , *speM* , *speL* , *speK* , *ssa*	8232.1, 315.5, 370.1, 370.2, 8232.3, 315.4, 315.2	*S. pyogenes*
Cytolethal distending toxin	*cdt*	Unnamed	*E. coli*
Proteins altering antigenicity			
Membrane proteins	Mu-like	Pnm1	*N. meningitidis*
Glucosylation	*rfb*	ε34	*S. enterica*
Glucosylation	*gtr*	P22	*S. enterica*
O-antigen acetylase	*oac*	Sf6	*S. flexneri*
Glucosyl transferase	*gtrII*	SfII, SfV, SfX	*S. flexneri*
Proteins involved in invasion			
Type III effector	*sopE*	SopEϕ	*S. enterica*
Type III effector	*sseI (gtgB)*	GIFSY-2	*S. enterica*
Type III effector	*sspH1*	GIFSY-3	*S. enterica*
Enzymes			
Superoxide dismutase	*sodC*	Sp4, 10	*E. coli* O157
Superoxide dismutase	*sodC-I*	GIFSY-2	*S. enterica*
Superoxide dismutase	*sodC-III*	Fels-1	*S. enterica*
Neuraminidase	*nanH*	Fels-1	*S. enterica*
Hyaluronidase	*hylP*	H4489A	*S. pyogenes*
Leukocidin	*pvl*	ϕPVL	*S. aureus*
Staphylokinase	*sak*	ϕ13	*S. aureus*
Phospholipase	*sla*	315.4	*S. pyogenes*
DNase/streptodornase	*sdn, sda, spd1,mf3, mf4*	315.6, 8232.5, 370.1, 370.2, 315.3	*S. pyogenes*
Serum resistance			
Outer membrane protien (OMP)	*bor*	λ	*E. coli*
OMP	*eib*	λ-like	*E. coli*
Adhesions for bacterial attachment			
Vir	*vir*	MAV1	*M. arthritidis*
Phage coat proteins	*pblA* , *pblB*	SM1	*S. mitis*
Others			
Mitogenic factor	*toxA*	Unnamed	*P. multocida*
Mitogenic factor	Unnamed	phisc 1	*S. canis*
Virulence	*gtgE*	GIFSY-2	*S. enterica*
Antivirulence	*grvA*	GIFSY-2, Fels-1	*S. enterica*

* Table adapted from Brussow, Canchaya *et al.* 2004 and Boyde and Brussow 2002 with permissions.

5.5 Genus Streptococcus

Streptococci are gram-positive, non-motile bacteria that are spherical or oval in shape and grow in chains or pairs. For clinical purposes they are delineated by their ability to lyse mammalian red blood cells, resulting in three hemolytic patterns on sheep blood agar: i) alpha or partial hemolysis with greening; ii) beta or total hemolysis with clearing; iii) gamma or no lysis of blood cells (Figure 5.3) (Brown 1919).

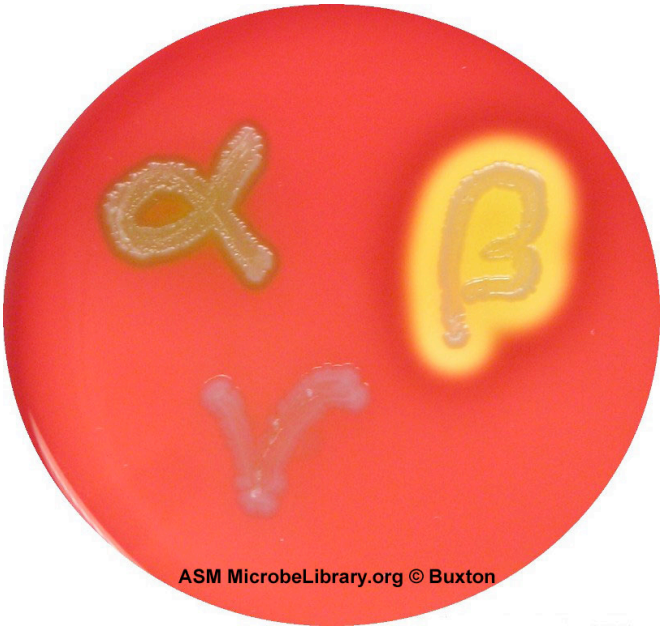


Figure 5.3. Example of the three hemolytic patterns of streptococci.

Alpha (α), beta (β), and gamma (γ), on sheep blood agar plates. Reproduced with permission from a photo taken by Rebecca Buxton, University of Utah.

With the exception of the alpha-hemolytic human pathogen *Streptococcus pneumoniae*, most alpha- and gamma-hemolytic streptococci are associated with the normal flora of the upper respiratory and intestinal tracts, and as such, most infections are usually opportunistic in nature, needing a predisposing factor such as an immunocompromised host, tissue injury, or an anatomical abnormality. Examples of such infections include sepsis in neutropenic patients; secondary wound infections, urinary tract infections and endocarditis. Conversely, beta-hemolytic streptococci are pathogens that cause a variety of human and animal diseases. Beta-hemolytic streptococci can be further divided into Lancefield serological groups (A-O) based on antigenic reactivity to group specific cell wall-associated carbohydrates (Lancefield 1933). While Lancefield groups, B, C, D, and G are associated with a number of animal and human infections, group A streptococci cause the majority and widest range of human infections amongst the various streptococcal species (Fischetti 2006).

5.6 Group A Streptococcus

Beta-hemolytic group A streptococcus (GrAS) or *Streptococcus pyogenes* is one of the most prevalent obligate human pathogens worldwide. It has the ability to asymptomatically colonize the human upper respiratory tract and skin (up to 25% of the population), as well as cause a wide variety of human diseases (Table 5.2). There is a stronger association of GrAS carriage and infection in children than in the adult population (Breese and Hall 1978). Mild infections and diseases

include: i) superficial infections of the skin, such as impetigo, polyderma, and erysipelas; ii) relatively uncomplicated infections of the mucosa, such as otitis media, sinusitis, and pharyngitis or “Strep throat”. GrAS are the main bacterial cause of acute pharyngitis and account for 15-30% of all pharyngitis cases in children and 5-10% of adult cases around the world (Schroeder 2003; Carapetis, Steer et al. 2005; Steer, Danchin et al. 2007). While these diseases seem relatively innocuous, it is estimated that there are over 111 million cases of GrAS polyderma in children and over 616 million cases of streptococcal pharyngitis per year worldwide (Carapetis, Steer et al. 2005). These rates of illness cause a significant burden on national healthcare systems and are a financial drain on families due to loss of work hours and the cost of antibiotics or medications to treat the disease symptoms. Additionally, these initial infections have the possibility of leading to more invasive streptococcal infections, such as cellulitis, pneumonia, and meningitis (Brussow, Fremont et al.). Occasionally, GrAS cause severe invasive infections that can kill a human within a few hours, such as streptococcal toxic shock syndrome, sepsis, and necrotizing fasciitis (reviewed in (Cunningham 2000)). If left untreated, GrAS infections can also lead to the development of post-streptococcal sequelae, such as glomerulonephritis and rheumatic fever (Cunningham 2000). Both are discussed below in more detail.

Table 5.2. Spectrum of clinical diseases caused by GrAS

Asymptomatic colonisation	**Superficial infection**
Throat	Pharyngitis and pharyngotonsillitis
Skin (immediately preceding infection)	Pyoderma
Also vagina, anus, scalp	
	Invasive disease
Suppurative respiratory disease	Bacteraemia/septicaemia
Peritonsillar abscess	Skin/soft tissue suppurative disease
Retropharyngeal abscess	Erysipelas
Cervical lymphadenitis	Cellulitis (including perianal cellulitis)
Sinusitis	Wound infection
Otitis media	Varicella superinfection
Pneumonia	Necrotising fasciitis
Empyema	Pyomyositis
	Puerperal sepsis
Genitourinary	Neonatal omphalitis
Urinary tract infection	
	Musculoskeletal
Central nervous system	Osteomyelitis
Meningitis	Septic arthritis
Brain abscess	
	Post-infectious sequelae
Cardiac	Rheumatic fever
Endocarditis	Acute post-streptococcal glomerulonephritis
	Reactive arthritis
Gastrointestinal	Erythema nodusum
Peritonitis	P.A.N.D.A.S. Pediatric autoimmune neuropsychiatric disorder
Hepatic	
Liver abscess	**Toxin-mediated disease**
	Scarlet fever
	Streptococcal toxic shock syndrome

5.7 Post-streptococcal Glomerulonephritis

Acute post-streptococcal glomerulonephritis (APSGN) is an autoimmune renal disease, which involves inflammation of the glomeruli or small blood vessels of the kidney. Symptoms usually appear within one to five weeks after a GrAS infection, and are often associated with GrAS polyderma rather than pharyngitis (Dillon 1979). Accompanied by fever and general malaise, the major manifestations of APSGN include: hematuria (blood in the urine), oliguria (reduced urine output), edema (accumulation of fluid in the body), and hypertension (high blood pressure) (reviewed in (Yoshizawa 2000)). The exact mechanism of disease progression after a streptococcal infection is unknown; however, studies indicate that kidney damage is often associated with autoreactive antibodies that can cross-react to both GrAS bacterial epitopes and different constitutes of the kidney (Wannamaker 1970; Goroncy-Bermes, Dale et al. 1987). These antibody-antigen immune complexes deposit in the kidney glomeruli, and together with subsequent complement pathway activation, lead to localized inflammation and tissue destruction (Westberg, Naff et al. 1971; Lange, Seligson et al. 1983). Further tissue damage and increased inflammatory responses have been linked to a number of GrAS virulence factors including the M-protein, the cysteine protease (SpeB), and the GrAS plasminogen activator proteins like streptokinase (Cu, Mezzano et al. 1998; Nordstrand, Norgren et al.

1998; Luo, Kuo et al. 2007) (see descriptions below). It is estimated that 500,000 cases of APSGN occur annually worldwide, with the majority of cases seen in less developed countries. Of these cases, 1% lead to death due to chronic renal failure or other hypertension associated diseases (Carapetis, Steer et al. 2005).

5.8 Post-streptococcal Rheumatic Fever

As with APSGN, rheumatic fever (Hirschman, Gerber et al.) is thought to be an autoimmune disease that occurs, on average, three weeks after GrAS pharyngitis (Cunningham 2000). Disease symptoms can vary depending on severity and location in the body. The major manifestations of ARF can include: carditis (inflammation of the heart tissue), chorea (inflammation of the central nervous system), migratory arthritis (inflammation of the joints), erythema marginaum (inflammation of the skin), and subcutaneous nodules on joints or bones (World Health Organization 2001). While the exact triggers and mechanisms of ARF have not been proven, studies indicate that an aberrant immune responses (consisting of the production of autoreactive antibodies and cross-reactive T-cells) develop against host tissues that share cross-reactive antigens with components of the GrAS bacteria, a phenomenon known as molecular mimicry (Kaplan and Svec 1964; Husby, van de Rijn et al. 1976; Stollerman 1991; Cunningham 2000; Ellis, Li et al. 2005; Martins, Hoffman et al. 2008). The most severe pathology of ARF takes place in the heart, where infiltrating immune cells, cytokines and auto-antibodies cause destruction of the

heart muscle and permanent damage to the heart valves and the surrounding tissue (Kaplan, Bolande et al. 1964; Quinn, Kosanke et al. 2001; Roberts, Kosanke et al. 2001). This inflammatory response can become chronic, i.e. rheumatic heart disease, causing pancarditis and deformative scaring of the heart valves that reduces blood flow in the host (Kaplan 1992). Patients who contract rheumatic fever are highly susceptible to recurrent bouts of disease following new GrAS infections, and are often prescribed continuous prophylactic antibiotics for years (Manyemba and Mayosi 2002; Cilliers 2006; Gerber, Baltimore et al. 2009). Eventually, patients may need heart valve replacement surgery and/or suffer congestive heart failure, which often leads to death (Carapetis, Steer et al. 2005; Cilliers 2006).

On a global scale, rheumatic heart disease causes the greatest burden of GrAS disease, affecting an estimated eighteen million people (Carapetis, Steer et al. 2005). It is the leading cause of preventable cardiovascular morbidity and mortality in children worldwide (Agarwal 1981; Carapetis, Steer et al. 2005). While today ARF occurs mainly in less developed countries (Agarwal 1981; Stollerman 1997; Carapetis, Steer et al. 2005), an unexplained resurgence in rheumatic outbreaks occurred in the United States in the previous two decades (Veasy, Wiedmeier et al. 1987; Bisno 1990; Veasy, Tani et al. 1994). In accordance with this, there has also been a continuing rise in the number and severity of invasive streptococcal diseases in North America, Europe, and Japan

(Schwartz, Facklam et al. 1990; Hoge, Schwartz et al. 1993; Gunzenhauser, Longfield et al. 1995; Holm 1996; Nakashima, Ichiyama et al. 1997; Eriksson, Andersson et al. 1998; Svensson, Oberg et al. 2000).

5.9 Severe GrAS Invasive Infections

GrAS can disseminate and cause numerous invasive infections in different areas of the body (Brussow, Fremont et al.). Reports in the late 1980's showed an increase in two of the more severe life-threatening GrAS diseases: streptococcal toxic shock syndrome (STSS) and necrotizing fasciitis (Cone, Woodard et al. 1987; Stevens, Tanner et al. 1989). Streptococcal toxic shock syndrome is characterized by fever, rash, and intense localized pain, which progresses to hypotension, shock, and multiple organ failure, and can cause death of the host within hours or a few days (McCormick, Yarwood et al. 2001). As with other GrAS diseases, a complete understanding of the mechanisms behind STSS is not yet available, but streptococcal superantigens are thought to play a role (discussed below). These toxins nonspecifically activate the host immune system causing a massive liberation of host cytokines. The cytokines in turn, induce a systemic inflammatory response, which results in increased vasodilation and capillary permeability, that leads to internal bleeding, hypotension and decreased perfusion to host organs (Kotb, Fraser et al. 2007).

STSS is often accompanied by necrotizing fasciitis (NF), termed “flesh eating disease” due to rapid and progressive tissue destruction involving the skin, subcutaneous tissue, deep fascia, and muscle (Sonnenfeld, Nowak et al.). This rapidly disseminating GrAS infection is so dramatic that in a matter of hours a small benign looking lesion can become a life threatening infection. Necrotizing fasciitis often requires high doses of intravenous antibiotics, rapid surgical debridement and even limb amputation in order to assure patient survival (Hasham, Matteucci et al. 2005). Like STSS, necrotizing fasciitis tissue destruction and bacterial spread is thought to be caused by a combination of an aggressive host immune response (possibly superantigen induced) and numerous GrAS secreted virulence factors that disrupt host tissue, such as secreted proteases; SpeB, SpyCEP, Mac1/IdeS, lipases; SlaA, and esterases; Sse. (Reviewed in Olson and Musser, 2009 and summarized in GrAS Virulence Factors below).

Altogether the estimated number of GrAS invasive deaths in the world is estimated to be greater 163,000 per year (Carapetis, Steer et al. 2005). The clinical severity of STSS and necrotizing fasciitis, along with their high mortality rates (30-60%), and increased incidences of these infections in the United States and around the world, has raised public health concerns (Stevens 2000; McCormick, Yarwood et al. 2001). This has served as an impetus for scientists

and epidemiologists to study the mechanisms that can lead to an increase of virulence in streptococcal populations.

5.10 Epidemiology: M-types and T-types of *Streptococcus pyogenes*

GrAS isolates can be classified by either M-type or T-type serotypic differences (Lancefield 1928; Swift, Wilson et al. 1943; Lancefield and Dole 1946). M-type is based on specific antigenic differences in M protein, the major antigenic determinant and surface exposed virulence factor on GrAS (see Virulence factor section below). There are currently more than 80 M-types, but with the progression of molecular biology, serological typing of the M-protein is being replaced by sequencing of the 5' end of the gene encoding the M protein (*emm),* known as *emm* sequence typing (Beall, Facklam et al. 1996; Facklam, Beall et al. 1999). There are currently over 124 such *emm* sequence types recognized by the CDC (http://www.cdc.gov/ncidod/biotech/strep/strepindex.htm).

T-typing was originally classified by the serologic variability of the trypsin "T" resistant antigen on the surface a large number of GrAS isolates (Lancefield and Dole 1946). Unlike M typing, the genetic basis for T-typing was not fully known until recently when the first T protein gene (*tee6*) was cloned and characterized (Schneewind, Jones et al. 1990). This was followed by localization of the *tee* genes to a highly variable region in the streptococcal chromosome known to contain genes associated with extracellular matrix (ECM) binding proteins. This

region was subsequently termed the fibronectin-binding collagen-binding T antigen (FCT) region (Bessen and Kalia 2002). In 2005, Mora *et. al.* made a landmark discovery that streptococci produce pili (once only thought to be associated with gram negative bacteria), and such pili are composed of the gene products encoded by the FCT region. Therefore, T antigens actually correspond to trypsin resistant pili (Mora, Bensi et al. 2005). Pili have since been shown to play a role in GrAS adherence and biofilm formation (Manetti, Zingaretti et al. 2007; Ryan, Kirk et al. 2007). To date, approximately twenty-five T-types have been identified (Jones, Schneewind et al. 1991) and the relationship between T type and pili sequence variation has led to the proposal of *tee* gene sequence typing (Falugi, Zingaretti et al. 2008). While there is an association between certain T-types and M-types, the use of T-typing, together with *emm* sequence typing allows for a better estimation of GrAS diversity (Beall, Facklam et al. 1997; Beall, Facklam et al. 1998).

5.11 Epidemiology of Diseases Caused by *S. pyogenes*

Epidemiological studies based on M and T-typing are crucial for associating certain serological types with tissue tropism and GrAS disease. As such, some M-types of (M1, 3, 5, 6, 14, 18, 19, 24) are associated with throat infections and rheumatic fever. Other M-types (M2, 49, 57, 59, 60, 61) are more commonly associated with skin infections and acute glomerulonephritis (summarized in (Cunningham 2000). Of note, the association of M-type and streptococcal

disease can vary with time, geography, population density, socio-economic status, and access to healthcare (Bessen, Carapetis et al. 2000; Shulman, Stollerman et al. 2006; McDonald, Brown et al. 2007; Steer, Law et al. 2009). Thus serological typing together with newer molecular biology classification methods (i.e. pulse field gel electrophoresis and multilocus sequence typing) allow for more accurate surveillance of changes in diversity and virulence in GrAS populations that may arise over time and geographical location (Chung, de Lencastre et al. 2000; Enright, Spratt et al. 2001).

Recent epidemiological studies have shown that certain M protein serotype strains are associated with the resurgence of rheumatic fever (M18 & M3) and severe invasive diseases (M1T1 & M3) (Schwartz, Facklam et al. 1990; Johnson, Stevens et al. 1992; Stollerman 1997). While some studies suggested that the latest increases in invasive disease could be linked temporally or geographically to specific “virulent clones” of certain M-types (Cleary, Kaplan et al. 1992; Johnson, Stevens et al. 1992; Musser, Kapur et al. 1995; Aziz and Kotb 2008), other studies that surveyed more diverse streptococcal populations could not support this hypothesis. Instead, the latter studies suggested that the change in disease might be due to either an increase in virulence in the overall GrAS population or to more complex relationships between GrAS clones, host factors, undiscovered bacterial virulence genes, and/or differences in bacterial genome regulation (Eriksson, Andersson et al. 1999; Enright, Spratt et al. 2001; Johnson,

Wotton et al. 2002; Vlaminckx, Schuren et al. 2007). These different hypotheses, each supported by differing sets of data, emphasize the need for alternative methods to elaborate the true mechanisms behind changes in GrAS pathogenesis. An analysis of the virulence factors harbored within different serological types has further elaborated novel potential mechanisms that could be responsible for different streptococcal diseases and the resurgences of particular types of infections (Yu and Ferretti 1991; Musser, Nelson et al. 1993; Bessen, Izzo et al. 1999; Hoe, Vuopio-Varkila et al. 2001; Sitkiewicz, Nagiec et al. 2006).

5.12 GrAS Virulence Factors

Streptococci produce a large number of secreted and surface exposed virulence factors that allow the bacteria to colonize and cause disease in humans. The number of virulence factors that GrAS encodes varies, even between strains of the same serotype. The combination of different virulence factors in each strain is thought to play a role in tissue tropism, virulence and the different disease manifestations caused by GrAS. These virulence factors can be encoded on the streptococcal chromosome or located on lysogenic bacteriophage. Some of the major virulence factors of each group are discussed below. Others are summarized in (Table 3) and are extensively reviewed in (Cunningham 2000; Hynes 2004; Nobbs, Lamont et al. 2009).

Table 5.3. Examples of group A streptococcal virulence factors.

Chromosome encoded surface-associated

Attachment, Invasion, Immune evasion

GrAS cell wall carbohydrates & Lipoteichoic acid
Hyaluronic acid capsule

emm gene super-family
M protein
Emm-like (Mrp, Arp, FcrA, ProtH)
Enn and others
Immunoglobulin binding proteins

Non-emm like immunoglobulin binding proteins
Collagen binding proteins, Scl1, Cpa
R-proteins, R28, Alp3

Fibronectin binding proteins:
Pili Proteins:
Protein F , Prtf1, SfbI
PrtF2, FbaA, PFBP
Fbp54
SfbX
FbaB
GAPDH / SDH
C5a peptidase (SCP)

Streptococcal protective antigen
Heme-binding proteins, Shr, Shp
Protein G-related alpha2-M-binding protein (GRAB)
Lipoprotein of *S. pyogenes*
CD15s-related antigen
Plasmin(ogen) binding proteins

Chromosome encoded secreted

Cytotoxins, Immune evasion, Proteases, Spreading factors

Streptolysin O (SLO)
Streptolysin S (SLS)
CAMP factor (Cfa)
NAD glycohydrolase
Diphosphopyridine Nucleotidase (DPN-ase)
Immunogenic secreted protein (Isp)
Superantigenic exotoxins: SmeZ, SpeG, and SpeJ
Streptococcal inhibitor of complement (Sic)
Mac1 / IdeS
SPyCEP / ScpC
SpeB (cysteine protease)
Chromosomally encoded hyaluronidase (Hly)
Streptokinase
Other Plasminogen binding proteins
Streptococcal Esterase

Lysogenic bacteriophage encoded

Cytotoxins, Immune Evasion, Spreading Factors, Antibiotic Resistance

Phospholipase (SlaA2)
Superantigenic exotoxins: Ssa, SpeA, SpeC, SpeI, SpeH, SpeM, SpeL, speK
DNAses & Streptodornases: Sdn, Sda, Spd1, Sda1, SdaD, Spd3 (MF3), Spd4
Phage encoded hyaluronidases: Hlyp1, Hlyp2, Hlyp3
MefA
R6

*Not an exhaustive list. Data was derived from multiple literature reviews (Cunningham 2000; Hynes 2004; Nobbs, Lamont et al. 2009) .

5.12.1 Chromosomally Encoded Surface-Associated Virulence Factors

The successful establishment of a GrAS infection depends largely on the many cell-associated determinants that are displayed on the bacterial surface (Table 5.3). These molecules, for example, allow GrAS to adhere to host cells, spread further into host tissue, and subvert host defenses. Two important surface-exposed virulence factors are discussed below.

5.12.1.1 M-protein

The most abundant protein attached to the cell surface, the M-protein, is the major antigenic determinant of GrAS (Fischetti, Jones et al. 1988). As such, it is one of the most studied, and best characterized streptococcal virulence factors. The M protein has been shown to play a role in most aspects of GrAS infection, including resistance to phagocytosis, adherence, and intracellular invasion (Cunningham 2000). The structure of the protein has also been extensively studied and is diagramed in Figure 5.4 (Fischetti 1989).

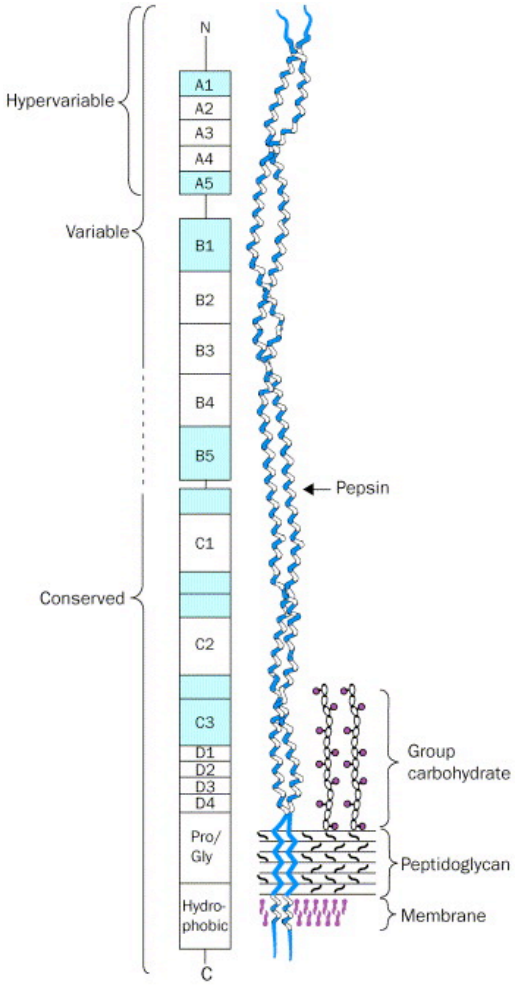


Figure 5.4. Characteristics of the complete M6 protein sequence.

Blocks A, B, C, and D designate the location of the sequence repeat blocks. Shaded blocks indicate those in which the sequence diverges from the central consensus sequence. Pro/Gly denotes the proline-rich and glycine-rich region likely located in the peptidoglycan. Pepsin identifies the position of the pepsin-sensitive site after amino acid 228. The C-terminal end is located within the cell wall and membrane. Reprinted from (Fischetti 2006), with permission.

The cytoplasmic and cell anchored C-terminal portion of the M-protein consists of a cytosolic charged tail and a hydrophobic region that spans the cell membrane. This is followed by a conserved gram-positive LPXTG (LPSTGE) cell wall anchoring motif. The surface exposed region of the protein is composed of 4 sets of repeated amino acids in blocks (A-D) that differ in size and sequence. These repeat blocks have a seven-residue amino acid periodicity, which allows the protein to form on the surface of streptococci as two polypeptide chains in an alpha-helical coiled-coil conformation (Manjula and Fischetti 1980; Phillips, Flicker et al. 1981). These M protein structures appear as fibrils on the surface of GrAS when viewed by electron microscopy (Fischetti 1989). Whereas the C-terminal region of the exposed protein is conserved among GrAS serotypes, the N-terminal region of the M-protein is highly variable (hypervariable) in amino acid sequence between different serotypes (Jones, Manjula et al. 1985). Antibodies in immune patient sera that are directed against the hypervariable N-terminal domain provide opsonic protection against strains of the same M-type through the classical complement and phagocytic pathways. This phenomenon forms the basis for Lancefield's serological M-typing of GrAS (Lancefield 1959; Lancefield 1962). In non-immune hosts, the negative charge of the distal amino acids in the N-terminus of the M-protein provides basic protection from macrophage and neutrophil phagocytosis through electrostatic repulsion. Phagocytosis is also inhibited by M protein binding to host proteins (fibrinogen, factor H, factor H-like protein, and C4BP) that are inhibitory or regulatory to the alternative complement

pathway (Horstmann, Sievertsen et al. 1988; Accardo, Sanchez-Corral et al. 1996; Johnsson, Berggard et al. 1998; Kotarsky, Hellwage et al. 1998). The decoration of these factors on the M-proteins of GrAS prevents deposition of the alternative pathway protein C3b and thus inhibits opsonic recognition and engulfment by phagocytic cells (Peterson, Schmeling et al. 1979).

Studies have shown that some M-types can exhibit different binding affinities to host proteins and therefore have varying functions during GrAS infection (Schmidt, Mann et al. 1993). Certain M-types, such as M1, M3, M6, and M18 are involved in adherence and internalization of cells at the mucosal surface and skin (Cue, Dombek et al. 1998; Fluckiger, Jones et al. 1998; Berkower, Ravins et al. 1999; Dombek, Cue et al. 1999). This is achieved either through the direct interaction of the M-protein with host cell receptors (i.e. CD46) or through the binding of fibronectin, which forms a cross-bridge between the M-protein and integrin receptors (Okada, Pentland et al. 1994; Okada, Liszewski et al. 1995). The formation of the complex of M protein, fibronectin, and integrin receptors on the host cell surface can cause a conformation change in the host cell, which then facilitates GrAS internalization (Molinari, Rohde et al. 2000).

Additionally, GrAS may contain other surface proteins that are structurally related to M-Protein, which are encoded for by genes in close proximity to the M protein gene (*emm*) in the *mga* regulon. These M-like proteins (i.e. Enn, Mrp, FrcA, Arp)

bind a range of other human plasma proteins (albumin, fibrinogen, plasminogen, complement, and IgG or IgA) and can share similar antiphagocytic, adherent, and/or invasive properties (Thern, Stenberg et al. 1995; Podbielski, Schnitzler et al. 1996; Ji, Schnitzler et al. 1998; Thern, Wastfelt et al. 1998; Sanderson-Smith, Dinkla et al. 2008).

In addition to M and M-like proteins, other surface proteins of some GrAS strains such as protein F, SfbI, SfbII, PFBP, FbaA, FbaB, and SOF, also bind fibronectin with high affinity and serve as both adhesive and invasive factors (Kreikemeyer, Talay et al. 1995; Okada, Tatsuno et al. 1998; Molinari and Chhatwal 1999; Rocha and Fischetti 1999; Timmer, Kristian et al. 2006).

5.12.1.2 Capsule

The polysaccharide capsule of GrAS is composed of hyaluronic acid (HA), a high molecular weight polymer containing repeating units of glucuronic acid and N-acetylglucosamine. This chemical structure is similar to the hyaluronic acid found in human connective tissue, and therefore may be recognized as “self” and is poorly immunogenic (Bisno, Brito et al. 2003). HA production by GrAS is produced from a highly conserved three-gene operon (*hasABC*). The *hasA* gene encodes a hyaluron synthase, *hasB* encodes an UDP-glucose dehydrogenase, and *hasC* encodes an UDP-glucose pyrophosphorylase. Only *hasA* and *hasB* have been shown to be required by GrAS for capsular synthesis (Ashbaugh,

Alberti et al. 1998). Individual strains of GrAS differ greatly in the amount capsule they produce. On agar plates, high levels of HA production are associated with a large mucoid or matte colony morphology, while lower levels of HA produce a smaller, glossy colony (Wilson 1959). The presence of the mucoid or matte colony morphology has been associated with GrAS isolates from the more severe infections, (i.e. ARF, STSS, and NF). More virulent mucoid strains have also been isolated after passage in human blood and murine infection models (Wessels and Bronze 1994; Wessels, Goldberg et al. 1994; Ashbaugh, Warren et al. 1998). Studies comparing mucoid strains to isogenic acapsular mutants have proven that the capsule plays a major role in GrAS resisting complement-mediated phagocytic killing (Wessels, Moses et al. 1991; Wessels and Bronze 1994; Wessels, Goldberg et al. 1994). Additionally, the HA capsule modulates attachment to host epithelial cells by either interfering with M-protein mediated adherence or by promoting GrAS attachment through direct interaction of the capsule as a ligand with CD44 on epithelial cell surfaces (Schrager, Rheinwald et al. 1996). Furthermore, although the presence of capsule reduces intracellular invasion of human keratinocytes, the binding of capsule to the CD44 protein also has been shown to cause disruption of intercellular junctions in the epithelium, allowing GrAS to invade further into tissues (Cywes and Wessels 2001).

5.12.2 Chromosomally Encoded Secreted Virulence Factors

GrAS produce a wide variety of extracellular factors that are capable of diffusing away the bacteria to aid in GrAS invasion and host immune subversion (Table 5.3). These factors include: i) Toxins, such as streptolysins O and S, which lyse host cells and are responsible for the β-hemolytic phenotype of GrAS; ii) Degradative enzymes, such as streptokinase and hyaluronidase, which dissolve blood clots and hydrolyze the extracellular matrix allowing GrAS to spread through tissue barriers; iii) Immune modulators, such as SIC, MAC, SPyCEP, and IdeS, which bind or degrade immune components to interfere with the clearance of streptococcal infections (Hynes 2004). One of the most studied secreted virulence factors encoded on the chromosome, SpeB has been grouped in to all three categories above.

5.12.2.1 SpeB, Streptococcal Proteinase

SpeB was originally described as two different proteins: i) the streptococcal pyogenic exotoxin B and ii) the streptococcal proteinase. Both were later shown to have the same sequence (Bohach, Hauser et al. 1988; Hauser and Schlievert 1990). SpeB is thought to be a cysteine protease that is encoded on the chromosome of almost all GrAS strains (Kapur, Topouzis et al. 1993). While the *speB* gene is highly conserved, expression of SpeB can be highly variable even between clonally related strains (Kansal, McGeer et al. 2000; Svensson, Scaramuzzino et al. 2000). SpeB is secreted as a 42 kDa zymogen that is self-

cleaved and reduced to an active 28 kDa enzyme (Elliott and Dole 1947). The active protease is capable of cleaving a large number of substrates from the GrAS bacteria as well as the host. The broad-spectrum protease activity has lead researchers to associate SpeB with numerous GrAS virulence phenotypes and diseases. SpeB can cleave host tissues and extracellular matrix proteins such as fibrin, fibronectin and vitronectin (Kapur, Topouzis et al. 1993). Additionally, SpeB activates host matrix metalloproteinases (MMP), which also remodel the host matrix (Burns, Marciel et al. 1996). These activities cause local tissue damage as well as allow GrAS to penetrate host barriers and become disseminated infections. As such, a number of studies have linked the expression of SpeB to invasive infections, STSS, and NF (Talkington, Schwartz et al. 1993; Eriksson, Andersson et al. 1999; Lukomski, Montgomery et al. 1999; Falugi, Zingaretti et al.).

SpeB is also capable of modulating the host immune system. SpeB induces a proinflammatory response by cleaving interleukin-1β and plasma kininogen precursors to their biologically active forms and can stimulate release of histamine and degranulation by mast cells (Kapur, Topouzis et al. 1993; Herwald, Collin et al. 1996; Watanabe, Todome et al. 2002). On the other hand, SpeB can reduce the effectiveness of the immune system response to streptococci by cleaving and inactivating antibodies, chemokines, C3B complement protein, and antimicrobial peptides such as human cathelicidin LL-37 (Collin and Olsen 2001;

Schmidtchen, Frick et al. 2002; Kuo, Lin et al. 2008; Terao, Mori et al. 2008; Egesten, Olin et al. 2009).

Another important activity for SpeB is the modulation of GrAS encoded virulence factors. The protease is capable of degrading most of the GrAS surface and secreted proteins, such as M-protein, protein F1, C5a peptidase, strepotolysin O, streptokinase, numerous streptococcal superantigens and DNases (Brussow, Fremont et al.) (Elliott 1945; Aziz, Pabst et al. 2004; Nooh, Aziz et al. 2006; Walker, Hollands et al. 2007). In accordance with this, the regulation and expression of SpeB in different strains or at different times during infection has a major impact on GrAS adherence, invasion, and host immune subversion (Burns, Lukomski et al. 1998; Kuo, Wu et al. 1998; Lukomski, Burns et al. 1998). This, and the fact that all streptococcal isolates have a *spe*B gene, has lead most researchers to postulate that SpeB is a major virulence factor involved in tissue tropism and outcome of GrAS disease (Lukomski, Sreevatsan et al. 1997; Kansal, McGeer et al. 2000; Svensson, Scaramuzzino et al. 2000; Sumby, Whitney et al. 2006; Walker, Hollands et al. 2007; Hollands, Aziz et al. 2008).

5.12.3 Lysogenic Bacteriophage Encoded Virulence Factors

A majority of the secreted virulence factors associated with GrAS are encoded by integrated prophage (Table 5.1). Often a streptococcal strain will contain multiple phages, with each phage containing one or more putative virulence factors

(Beres and Musser 2007). Since these factors are encoded on phage the virulence genes have the possibility of being mobilized and horizontally transferred to other streptococci or, with no selective pressure, lost by recombinational events (Desiere, McShan et al. 2001; Vlaminckx, Schuren et al. 2007). For this reason, one of the greatest variations in virulence between GrAS strains is thought to be the phage virulence factors they encode. Often secreted and capable of aiding in GrAS infection and host immune subversion, these factors also include: i) Toxins, such as phospholipase A_2 (Sla), which shares homology to snake toxin and can enhance GrAS colonization and tissue destruction (Sitkiewicz, Nagiec et al. 2006); ii) Degradative enzymes, such as phage-associated hyaluronidase, which may hydrolyze the extracellular matrix allowing bacterial or other toxins to spread through tissue barriers (Benchetrit, Gray et al. 1977); iii) Immune modulators and iv) Antibiotic resistant genes, as well as, other examples of phage encoded virulence factors are discussed below and in future chapters of this thesis.

5.12.3.1 Streptococcal Superantigens

Superantigens were first identified in the early 1920's as scarlet fever toxins, which were isolated from GrAS culture filtrates and capable of causing the scarlet fever rash when injected subcutaneously into subjects (Dick and Dick 1983). GrAS superantigens have had many names: erythrotoxins, mitogenic factors (MFs) and/or streptococcal pyrogenic exotoxins (SPEs). As the various names

suggest, the functions of superantigens have classically been associated with three different types of activities: T-cell mitogenesis, pyrogenicity, and enhancement of susceptibility to endotoxic shock (Kim and Watson 1970; Schuh, Hribalova et al. 1970). All three manifestations are now thought to be the result of exotoxin stimulation of lymphocytes and monocytes, which in turn, release cytokines to further activate a massive systematic host inflammatory response (Fast, Schlievert et al. 1989; Hackett and Stevens 1992). Stimulation of these immune cells results from the exotoxin binding to, and cross-linking, the Vβ region on T-cell receptors with the major histocompatability II (MHC II) receptors on antigen presenting cells (APCs) (Figure 5.5). Thus, these exotoxins were termed “superantigens” for their novel mechanism of T-cell stimulation and were shown to be similar in both structure and function to the staphylococcal enterotoxins (Kappler, Kotzin et al. 1989; Marrack and Kappler 1990). Unlike normal antigens, superantigens do not require internalization, processing, or presentation by APCs, nor do they rely on TCR specificity for the antigen peptide–MHC complex to activate specific T-cell clones (Figure 5.5). Instead, the crosslinking of the two receptors non-specifically activates up to twenty-five percent of all the resting T-cell population; this action is based on the ability of the superantigens to bind different TCR Vβ regions (reviewed in (Herman, Kappler et al. 1991).

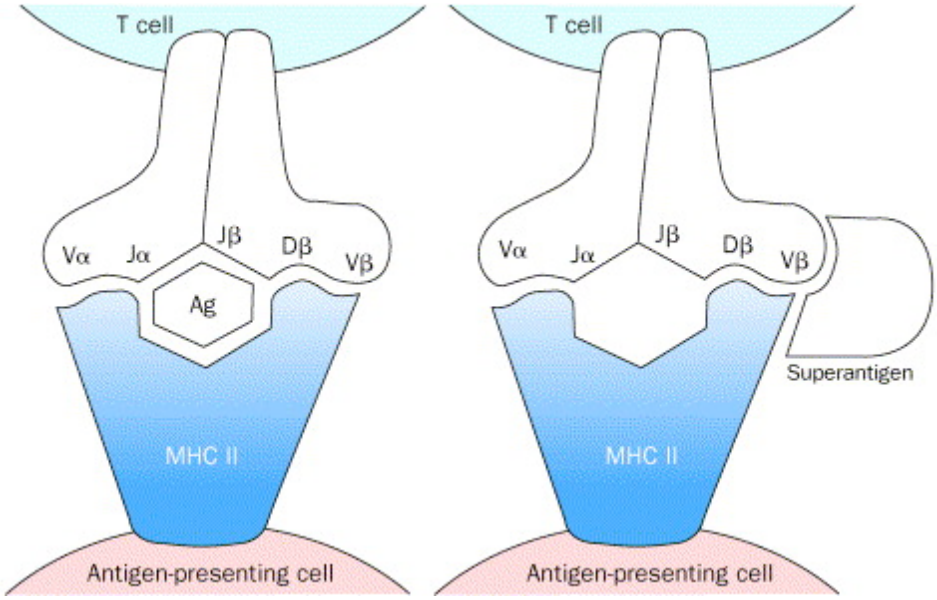


Figure 5.5. Schematic of the interactions of antigen or superantigen with the antigen presenting cell and the T lymphocyte.

Antigen is processed internally and presented by the APC to the T-cell (left). While superantigens bind and crosslink the MHCII and TCR Vβ region outside of the antigen peptide groove, activating both cells (right). Adapted from (Bisno, Brito et al. 2003), with permission.

The stimulation of large amounts T-cells and macrophages produces massive amounts of inflammatory cytokines and chemokines (i.e. TNFα, TNFβ, IL-1,IL-2 IL-8 and INFγ) (Unnikrishnan, Altmann et al. 2002). This "cytokine storm" increases vascular permeability and other non-specific immune and autoimmune responses, often leading to tissue damage, multiple organ failure, and even death. Thus, superantigenic effects have been associated with the symptoms of GrAS invasive diseases like STSS and NF, and may play a role in the post streptococcal autoimmune diseases of ARF and APSGN (Kotb 1995; McCormick, Yarwood et al. 2001). Three streptococcal pyrogenic exotoxins (termed SpeA, SpeB and SpeC) were first cloned from GrAS scarlet fever isolates. The *speA* and *speC* genes were found to be located on bacteriophages, supporting earlier evidence by Zabriskie that the SpeA toxin could be transferred between GrAS strains by lysogenic phage (Zabriskie 1964; Weeks and Ferretti 1986; Goshorn, Bohach et al. 1988). The cloning of the *speB* gene confirmed that it was identical to the streptococcal cysteine proteinase (Bohach, Hauser et al. 1988; Hauser and Schlievert 1990). Subsequently, its mitogenic effect was later proven to be the result of contamination with other superantigens (Gerlach, Reichardt et al. 1994; Gerlach, Fleischer et al. 2000). Analysis of the published genomes of GrAS along with biochemical and structural studies of the SPEs allowed the identification and cloning of a number of other GrAS superantigens including: Spe- G, H, I, J, K, L, M, SSA, SMEZ-1, SMEZ-2, SMEZ-3. Further sequence analyses have revealed

that these toxins can have minor allelic variations that may modulate their virulence (Proft, Moffatt et al. 2000) (Nelson, Schlievert et al. 1991; Kapur, Nelson et al. 1992).

Besides allowing for the spread of a localized infection, by causing cytokine induced vascular permeability and tissue damage, other mechanisms by which superantigens increase GrAS virulence are still unanswered. It has been hypothesized that superantigens allow GrAS to avert the immune system in three ways. First, they may be involved in superantigen-dependent cellular cytoxicity (SDCC), in which superantigens induce cytotoxic T-cells to kill APCs expressing MHC II-superantigen complexes (Dohlsten, Lando et al. 1990). Second, when superantigens bind T-cells only, and do not crosslink the TCR to MHC II, an absence of co-stimulatory signals from APCs can cause depletion of specific Vβ T-cells through anergy or apoptosis (Hewitt, Lamb et al. 1992; McCormack, Callahan et al. 1993). Third, the generation of a massive nonspecific immune response may divert attention away from a specific immune response to GrAS (Kotb 1995). Thus, in each of these possible scenarios, the actions of the superantigen eliminate immune cells, and/or limit a proper specific immune response to streptococci. Interestingly, while the genes for *smeZ*, *speG*, and *speJ* are chromosomally encoded, the genes encoding the remaining GrAS superantigens are located on lysogenic bacteriophage (summarized in (Beres

and Musser 2007; Kotb, Fraser et al. 2007)), providing further evidence that phage can potentially spread streptococcal virulence traits.

5.12.3.2 DNases

Historically GrAS have been known to secrete four antigenically distinct deoxyribonucleases, (A, B, C, and D), which were characterized based on immunological and biochemical properties (McCarty 1948; Wannamaker 1958; Wannamaker, Hayes et al. 1967; Wannamaker and Yasmineh 1967). Sera from GrAS infected patients were found to react and neutralize DNAse activity in a type (i.e. A, B, C, or D) specific manner. Immunoreactiviy against DNase B was most prominently found and has therefore been used diagnostically as an indicator for recent GrAS infections (Tiesler and Beck 1976; Gerber, Gray et al. 1980). DNase B was shown to be chromosomally encoded and was also originally classified as both a mitogenic factor (MF) (Arraiano, Bamford et al.) and SpeF because it was thought to have both mitogenic activity and cytokine-inducing properties (Yutsudo, Murai et al. 1992; Norrby-Teglund, Newton et al. 1994; Eriksson, Eriksson et al. 1999), but this was later linked to contamination with the SpeX/SMEZ or SpeC superantigens (Gerlach, Schmidt et al. 2001). Recently, molecular cloning techniques allowed the isolation and characterization of new GrAS DNases: streptodornase D (SdaD), streptococcal DNase α (Sdα), and streptococcal phage-encoded DNase (Spd1) (Podbielski, Zarges et al. 1996; Broudy, Pancholi et al. 2002; Hasegawa, Torii et al. 2002). A sequence

comparison of these DNase genes to the published streptococcal genomic DNA sequences elucidated additional DNase coding sequences, which showed that all GrAS contain multiple conserved DNases. The additional DNases identified (MF2/spd1= DNase A, spd3/Mf3= DNase C, SdaD= DNAse D, Sdn, Sda, MF4/Spd4, and Sda1) have since been localized to prophage elements integrated into the GrAS genome (Beres, Sylva et al. 2002; Broudy, Pancholi et al. 2002; Hasegawa, Torii et al. 2002; Smoot, Barbian et al. 2002; Nakagawa, Kurokawa et al. 2003; Aziz, Ismail et al. 2004; Sumby, Barbian et al. 2005).

Because most DNases are encoded by bacteriophage, it has been hypothesized that DNases may specifically aid in the in the spread of bacteriophage (Broudy, Pancholi et al. 2002). It has also been postulated that DNases contribute to the dissemination of GrAS into the surrounding tissue by liquefying pus at the site of infection (Tillett, Sherry et al. 1948). However, despite the extensive characterization of streptococcal DNases, no direct evidence has proven that DNAses are GrAS virulence factors. This may be partly due to the fact that GrAS strains can simultaneously express multiple DNases, so inactivation of a single DNase gene would not eliminate all GrAS DNase expression and thus may not attenuate virulence in mutants compared to the wild-type strains (Podbielski, Zarges et al. 1996).

In 2004, Brinkman *et al.* showed that neutrophils form neutrophil extracellular traps (Knetsch, Schafers et al.), which trap and kill bacteria and fungi extracellularly (Brinkmann, Reichard et al. 2004). NETs are composed of neutrophil granule components (antimicrobial peptides and enzymes) linked to a backbone of chromatin DNA and histones. Treatment with exogenous DNases destroys the NETs (Brinkmann, Reichard et al. 2004), thus, suggesting a role for secreted extracellular DNases in evasion of an innate immune response. This information combined with the knowledge that GrAS encode multiple DNases, led to the development of a triple-mutant strain devoid of all secreted DNAses (MF/Spd, SdaD, Spd3) (Sumby, Barbian et al. 2005). Compared to wild-type, the GrAS triple-mutant showed decreased survival in an in vitro neutrophil extracellular killing assay. Furthermore, the lack of all DNases reduced GrAS virulence in both a murine invasive disease model, as well as a non-human primate model of pharyngitis (Sumby, Barbian et al. 2005). Buchanan et al. further clarified the role of GrAS DNases by correlating virulence attenuation of GrAS *sda1* mutants in a murine NF model with lack of degradation of NETs in vitro and in vivo (Buchanan, Simpson et al. 2006). It has even been postulated that expression of DNase Sda1 serves a selective pressure for GrAS to mutate to a more hyper-virulent phenotype (Walker, Hollands et al. 2007). The fact that Sda1 and most of the secreted GrAS DNases are encoded on bacteriophage gives another example of how phage encoded virulence factors may modulate streptococcal pathogenesis.

5.13 GrAS Genomic Sequences in Relation to Lysogenic Phage

The genome sequences of thirteen different strains of GrAS have now been published, making it one of the most sequenced human pathogens to date (Ferretti, McShan et al. 2001; Beres, Sylva et al. 2002; Smoot, Barbian et al. 2002; Nakagawa, Kurokawa et al. 2003; Banks, Porcella et al. 2004; Green, Zhang et al. 2005; Beres and Musser 2007; Holden, Scott et al. 2007; McShan, Ferretti et al. 2008). The sequences represent 10 M types that make up more than 70% of the serotypes associated with different GrAS diseases in the western hemisphere including: invasive diseases (M1, M3, M3, M28), noninvasive diseases (M1, M2, M12), ARF (M5, M18), APGN (M12, M49) and erythromycin resistant isolates (M4, M6) (Beres and Musser 2007; McShan, Ferretti et al. 2008). Having genome sequences from different M types associated with multiple diseases allows investigators to study the contribution of strain genotype to the type and severity of streptococcal infection.

A comparison of the GrAS genomes showed that the overall chromosomal content of the different M types is 90% similar in coding sequence (Beres and Musser 2007). The majority of the genetic variation results from numerous lysogenic bacteriophage and bacteriophage-like elements or ICES (integrative and conjugative elements, (Burrus, Pavlovic et al. 2002)), which have been incorporated into the GrAS chromosomes (Beres and Musser 2007). The genome of each strain contains two to eight putative prophage elements for a

total of 57 phages and 12 ICEs in the GrAS metagenome (Figure 5.1). A majority of the prophage elements contain genes encoding numerous virulence factors including: superantigens (Spes, Ssa), phospholipases (Sla), DNases (Spds, MFs, Sda, Sdn), and hyaluronidases (Beres and Musser 2007; McShan, Ferretti et al. 2008). Taken together, these data suggest that differences in disease manifestation and severity of infection between different M types might be affected by prophage-encoded virulence factors (Sitkiewicz, Nagiec et al. 2006).

As comparative genomic studies have demonstrated that prophages account for most of the differences between M serotypes, comparative DNA microarray analyses proved the same holds true for strains within the same M serotype (Smoot, Barbian et al. 2002). Furthermore, a retrospective analysis of the bacteriophage content of M3-serotypes between 1920 and 2002 suggests that the successive gain of prophages over time has lead to a contemporary M3 strain with increased virulence (Beres, Sylva et al. 2002). Similarly, independent studies that assessed the emergence of contemporary M1T1 isolates responsible for severe invasive infections, have suggested that the acquisition of two phages (containing *speA2* and *sdaD2*) played a role in the emergence and persistence of a more virulent GrAS strain during last 20 years (Cleary, LaPenta et al. 1998; Sumby, Porcella et al. 2005; Aziz and Kotb 2008). These analyses highlight the role that GrAS bacteriophages play in interstrain diversity and provide evidence

for a mechanism by which certain “virulent clones” of GrAS could evolve through horizontal transfer of prophage-like elements.

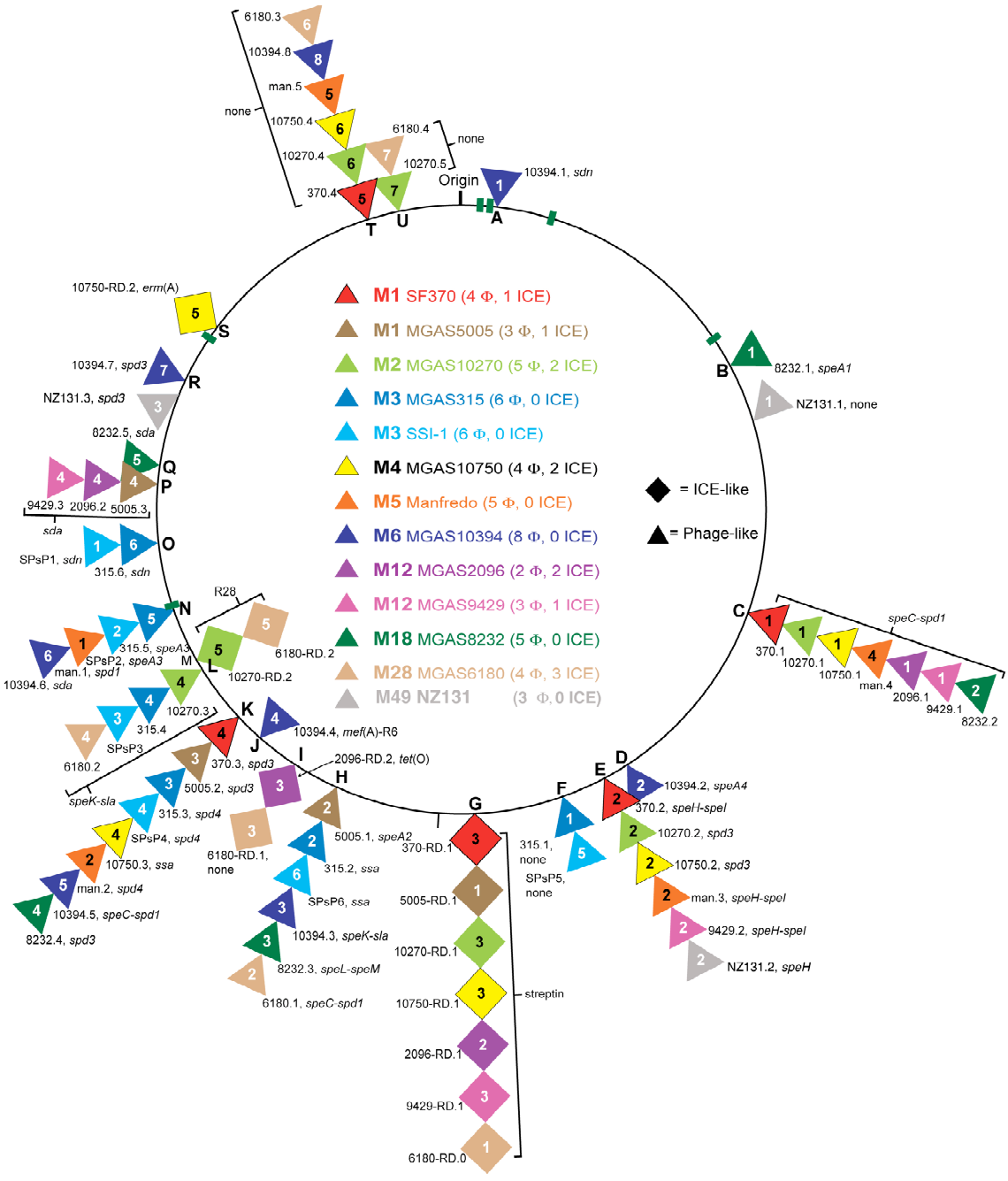

M1 SF370 (4 Φ, 1 ICE)
M1 MGAS5005 (3 Φ, 1 ICE)
M2 MGAS10270 (5 Φ, 2 ICE)
M3 MGAS315 (6 Φ, 0 ICE)
M3 SSI-1 (6 Φ, 0 ICE)
M4 MGAS10750 (4 Φ, 2 ICE)
M5 Manfredo (5 Φ, 0 ICE)
M6 MGAS10394 (8 Φ, 0 ICE)
M12 MGAS2096 (2 Φ, 2 ICE)
M12 MGAS9429 (3 Φ, 1 ICE)
M18 MGAS8232 (5 Φ, 0 ICE)
M28 MGAS6180 (4 Φ, 3 ICE)
M49 NZ131 (3 Φ, 0 ICE)
= ICE-like
= Phage-like
Origin
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T
U
10394.1, sdn
8232.1, speA1
NZ131.1, none
speC-spd1
370.1
10270.1
10750.1
man.4
2096.1
9429.1
8232.2
10394.2, speA4
370.2, speI-speI
10270.2, spd3
10750.2, spd3
man.3, speH-speI
9429.2, speH-speI
NZ131.2, speH
315.1, none
SPsP5, none
370-RD.1
5005-RD.1
10270-RD.1
10750-RD.1
streptin
2096-RD.1
9429-RD.1
6180-RD.0
10394.4, mef(A)-R6
2096-RD.2, tet(O)
5005.1, speA2
315.2, ssa
SPsP6, ssa
10394.3, speK-sla
8232.3, speL-speM
6180.1, speC-spd1
6180-RD.1, none
5005.2, spd3
315.3, spd4
SPsP4, spd4
10750.3, ssa
man.2, spd4
10394.5, speC-spd1
8232.4, spd3
speK-sla
6180.2
SPsP3
315.4
10270.3
370.3, spd3
R28
6180-RD.2
10270-RD.2
315.5, speA3
SPsP2, speA3
man.1, spd1
10394.6, sda
SPsP1, sdn
315.6, sdn
9429.3
2096.2
5005.3
sda
8232.5, sda
NZ131.3, spd3
10394.7, spd3
10750-RD.2, erm(A)
6180.3
10394.8
man.5
10750.4
10270.4
370.4
none
6180.4
10270.5
none

Figure 5.6. GAS metagenome exogenous elements.

Illustrated are loci of integration of phages and ICEs into the core chromosome. Prophages are indicated with triangles and ICEs with squares. Stacked triangles and squares indicate a common integration site. Elements are color-coded to indicate the source strain. Prophages and ICEs are numbered as they occur clockwise around the core chromosome for each strain. Integration loci are lettered alphabetically as they occur clockwise around the core chromosome. The six rRNA operons are shown as green bars. Gene designations are as follows: 1) secreted superantigens: *speA*, *speC*, *speH*, *speI*, *speK*, *speL*, *speM*, and *ssa*; 2) secreted DNAses: *sda*, *sdn*, *spd1*, *spd3*, and *spd4*; 3) secreted phospholipase: *sla*; 4) antimicrobial resistance: *erm*(A), *mef*(A), and *tet*(O); 5) cell surface adhesins: R6 and R28; 6) none, these elements lack a known or obvious virulence gene. Adapted from (Beres and Musser 2007) with permission.

5.14 Other Lysogenic Phage Effects on GrAS Virulence

Along with demonstrating the role of lysogeny in GrAS evolution, numerous studies have demonstrated that within the human host, lysogenic phage can infect non-toxigenic GrAS strains, and that these strains are lysogenically converted to toxin producing strains. Thus, lysogeny may increase streptococcal pathogenesis by directly increasing the number of virulent bacteria at the site of infection or colonization (Broudy and Fischetti 2003). Lysogenic phage induction also may serve as a way for GrAS to coordinate the production and release of multiple virulence factors at specific stages of host cell contact. Human pharyngeal cells produce a soluble phage-inducing factor, termed SPIF (streptococcal phage inducing factor), which stimulates the production of both streptococcal phages and their toxins (Broudy, Pancholi et al. 2001; Broudy, Pancholi et al. 2002). In addition to the potential to lysogenize neighboring streptococci, the induced bacteriophage particles could directly contribute to increased pathogenesis because they contain hyaluronidases and other virulence factors on their surface (Benchetrit, Gray et al. 1977; Bensing, Siboo et al. 2001; Starr and Engleberg 2006). Furthermore, lysogenic and even lytic phages can carry GrAS chromosomal encode genes that they have acquired during inaccurate excision or recombination events and they can transfer these potential virulence factors though a method of generalized transduction.

Besides carrying genes for virulence factors, prophage may also influence GrAS pathogenesis by encoding or interacting with regulatory factors that influence the expression of chromosomally encoded virulence genes. Spanier et al. showed that prophage integration increases the expression of the M-protein, thus increasing the bacteria's ability to resist phagocytosis (Spanier and Cleary 1980). Recently, Scott et al. showed that integration of a phage into a DNA repair operon increases the mutation rate of an M1 strain (Scott, Thompson-Mayberry et al. 2008). There are also many examples of how bacteriophage modify transcriptional regulation and the enzymatic modification of host bacterial components to influence virulence in other types pathogenic bacteria (Wagner and Waldor 2002). This suggests that the complete role of lysogenic phage in modulating GrAS virulence has yet to be fully elucidated.

6 Objectives

The overall aim of this study was to more comprehensively investigate the many ways in which bacteriophage interact with *Streptococcus pyogenes* in order to elucidate novel phage-specific mechanisms that affect bacterial pathogenesis and/ or survival. In an M6 serotype of GrAS, we identified a restriction modification (RM) system on a chimeric bacteriophage element (ϕ10394.4), which encodes the erythromycin resistance gene, *mefA*. Through a combination of cloning, heterologous expression and allelic replacement techniques, we characterized the activity of the methyltransferase (M.SpyI) from this RM system. We found that M.SpyI specifically recognizes and methylates the DNA sequence: 5'-CC^{m}NGG. To our knowledge, this is the first methyltransferase gene from *S. pyogenes* to be cloned and characterized. Our findings also revealed the reason why PFGE analysis of SmaI digested genomic DNA cannot be used to analyze the clonality of streptococci containing Φ10394.4, and explains the inability of previous epidemiological studies to use SmaI to analyze DNA from macrolide resistant streptococci. We speculate that the M.SpyI-RM cassette contained on Φ10394.4 could impart a selective advantage on host strain survival and may provide another explanation for the observed increase in macrolide resistant streptococci.

We also used allelic recombination techniques to analyze the role that a phage-encoded superantigen, SpeH, may play in colonization of the host with a M1 serotype of GrAS (SF370). In order to characterize additional phage genes that might function during the initial interaction between GrAS and the host, we developed novel techniques to specifically manipulate bacteriophage content in the streptococcal genome. Using this methodology, we systematically cured the genome of strain SF370 of all lysogenic phage, which resulted in the creation of the first strain of *S. pyogenes* that is devoid of all the major bacteriophage elements. The resulting mutants have allowed us to study the effects that individual and multiple phage deletions have on streptococcal virulence and survival. We used these phage knockout mutants to further investigate the role that two of the phage in this M1 strain play in regulating GrAS chromosomal genes involved in bacterial metabolism, DNA repair and mutagenesis.

We also manipulated lytic bacteriophage genes to genetically engineer a novel chimeric endolysin (ClyS) from the genomes of two different *Staphylococcus* phages. We demonstrated the lytic activity of ClyS against methicillin-resistant *Staphylococcus aureus* (MRSA) in both in vivo colonization and septicemia models, and determined its synergistic activity with oxacillin in in vitro and in vivo models. Our work highlights the potential of ClyS as a novel therapeutic agent for the treatment of MRSA and other staphylococcal infections.

7 Chapter 1

7.1 INTRODUCTION

The treatment of choice for streptococcal pharyngitis infections is penicillin V (Bisno, Gerber et al. 2002), but in recent years macrolide antibiotics such as azithromycin and erythromycin have been increasingly prescribed as an alternative treatment (Cizman, Pokorn et al. 2001; Gonzales, Malone et al. 2001; Linder and Stafford 2001; Coenen, Ferech et al. 2006). Concomitantly, there has been an increase of macrolide resistant strains of *S. pyogenes* (Cha, Lee et al. 2001; Cizman, Pokorn et al. 2001; Hsueh, Teng et al. 2002; Martin, Green et al. 2002; Bergman, Huikko et al. 2004; Green, Martin et al. 2004), posing a global health problem for patients that are hyper-sensitive to β-lactam antibiotics (Bisno, Gerber et al. 2002; Romano, Viola et al. 2002; Viola, Quaratino et al. 2005).

Macrolide resistance in streptococci is usually associated with the presence of one of three genes: i) *ermB* and ii) *ermTR*, both encode methyltransferases that confer resistance to macrolides, lincosamide and streptogramin B (MLS_B) antibiotics by modifying a conserved adenine residue on the drug target site of the 23S rRNA (Leclercq and Courvalin 1991; Seppala, Skurnik et al. 1998), and iii) *mefA*, which encodes a macrolide efflux pump that imparts resistance to 14- and 15- membered macrolides, such as erythromycin and azithromycin, but not to the 16–membered MLS_B antibiotics (classified as “M-phenotype” of macrolide resistance) (Clancy, Petitpas et al. 1996). The *mefA* gene is present in a number

of phylogenetically unrelated serotypes of *S. pyogenes* (Cha, Lee et al. 2001; Hsueh, Teng et al. 2002; Martin, Green et al. 2002; Bergman, Huikko et al. 2004) and in some regions of the world is becoming the predominant erythromycin resistance determinant in streptococcal isolates (Kataja, Huovinen et al. 1999; Ripa, Zampaloni et al. 2001; Hsueh, Teng et al. 2002; Silva-Costa, Ramirez et al. 2006). Earlier studies suggested that erythromycin resistance and the *mefA* gene may be acquired through horizontal gene transfer; however, at that time, the mechanisms were not clearly identified in *S. pyogenes* (Kataja, Huovinen et al. 1999; Luna, Coates et al. 1999; Del Grosso, Iannelli et al. 2002).

Recently, the *mefA* gene was identified on three different DNA chimeric elements, which have both transposon and prophage characteristics. Santagati et al. partially characterized a 52 kb chimeric element carrying the *mefA* gene, which was integrated into the *comEC* gene of an erythromycin resistant strain of *S. pyogenes* (Santagati, Iannelli et al. 2003). They described 7.2 kb of the 52 kb chimeric element as 100% identical to a defective *mefA* conjugative transposon, Tn1207.1, from *S. pneumoniae* (Santagati, Iannelli et al. 2000; Santagati, Iannelli et al. 2003). Additionally, they demonstrated that the *S. pyogenes* 52 kb chimeric element could be transferred by filter conjugation to different strains of *S. pyogenes, S. pneumoniae,* and *S. gordonii,* conferring erythromycin resistance to these species. For these reasons, the 52 kb chimeric element was classified as conjugative transposon Tn1207.3 (Santagati, Iannelli et al. 2003).

Concurrently, during genomic sequencing of a *S. pyogenes* strain, MGAS10394, isolated from an outbreak of erythromycin-resistant pharyngitis in elementary school children in Pittsburgh, PA (Martin, Green et al. 2002), Banks et al. identified a similar 58.8kb chimeric element (Banks, Porcella et al. 2003). The element was inserted into the same *comEC* site as Tn1207.3 and also contained *mefA* on the 7.2 kb defective transposon Tn1207.1. Nucleotide sequence analysis of the entire chimeric element revealed that it was also composed of conserved lysogenic bacteriophage genes. Additionally, chimeric element DNA was detected in bacteriophage particles released into the culture supernatant after induction with mitomycin C (Banks, Porcella et al. 2003). These results suggested that this chimeric element was composed of the transposon Tn1207.1, inserted into a functional prophage and thus it was classified as Φ10394.4 (Banks, Porcella et al. 2004). Further sequence analysis revealed that Φ10394.4 and Tn1207.3 are essentially identical in nucleotide sequence except that Φ10394.4 contains an additional 6 kb variable region upstream of the Tn1207.1 transposon (Banks, Porcella et al. 2004; Pozzi, Iannelli et al. 2004). Lately, a third chimeric element containing *mefA* and *tetO*, a tetracycline resistance gene, was described to have both conjugative transposon and bacteriophage characteristics (Giovanetti, Brenciani et al. 2003). These *tetO* -*mefA* chimeric elements are different from the other two elements, as they are composed of a varying number of genes similar to those of Tn1207.1 that have combined with a different set of bacteriophage genes, resulting in elements of various sizes (52 to

60 kb), which are integrated into chromosomal locations outside of the *comEC* gene (Giovanetti, Brenciani et al. 2003; Brenciani, Ojo et al. 2004; Giovanetti, Brenciani et al. 2005).

All of the above studies suggest that recent increases in the "M phenotype" of macrolide resistance may have been influenced by the acquisition and dissemination of these *mefA* chimeric elements within the streptococcal population (Giovanetti, Brenciani et al. 2003; Pozzi, Iannelli et al. 2004; Giovanetti, Brenciani et al. 2005). One of the most common methods to examine the clonality of such macrolide resistant and sensitive streptococcal isolates involves restriction of genomic DNA with SmaI endonuclease, followed by Pulse Field Gel Electrophoresis (PFGE) analysis (Tenover, Arbeit et al. 1995; Bert, Branger et al. 1997; Ripa, Zampaloni et al. 2001). Recently, however, a number of epidemiological studies have reported that the DNA from a diverse group of erythromycin resistant streptococci could not be digested with SmaI (Ripa, Zampaloni et al. 2001; Bingen, Leclercq et al. 2002; Martin, Green et al. 2002; Giovanetti, Brenciani et al. 2005; Malhotra-Kumar, Lammens et al. 2005). This finding prompted the use of alternative restriction enzymes, such as ApaI, EagI, and SfiI in the analysis, subsequently making it difficult to directly compare the clonality of strains that differ in macrolide susceptibility and to relate the results of different epidemiological studies (Bingen, Leclercq et al. 2002; Martin, Green et al. 2002; Malhotra-Kumar, Lammens et al. 2005).

While analyzing the clonality of erythromycin resistant *S. pyogenes* strains from the pharyngitis outbreak in Pittsburgh, PA, referenced above (Martin, Green et al. 2002), we found that the presence of Φ10394.4 in the genome was associated with streptococcal genomic DNA being refractory to SmaI restriction. Since SmaI cleavage is blocked by CpG methylation at the enzyme recognition site (REBASE: http:// rbase.neb.com) (Roberts, Vincze et al. 2005), we hypothesized that a 5-methylcytosine methyltransferase (MTase) encoded on Φ10394.4 may be methylating the genomic DNA, thereby inhibiting SmaI restriction. In the work presented here, we confirmed this hypothesis through the allelic replacement, cloning and characterization of the gene encoding the MTase from Φ10394.4. We classified this MTase gene as *spyIM* and its gene product as M.SpyI following the updated nomenclature of Roberts et al. (Roberts, Belfort et al. 2003)

7.2 MATERIALS AND METHODS

7.2.1 Bacterial strains and growth conditions.

S. pyogenes isolates obtained from a longitudinal study on the epidemiology of streptococcal infections of elementary school children in Pittsburgh, PA (Martin, Green et al. 2002; Martin, Green et al. 2004) are described in (Table 7.1). These isolates were previously characterized based on *emm*-type, macrolide susceptibility, mechanism of macrolide resistance, and clonality (Martin, Green et al. 2002; Martin, Green et al. 2004). In subsequent analyses the isolates were screened for the presence of the *mefA* gene, the Φ10394.4 element, and its insertion into the *comEC* gene by PCR and Southern blot hybridization. The primers used for this analysis are described under the chimeric element heading in Table 7.2. *E. coli* One Shot Top10 (Invitrogen) was used as the host strain for plasmid construction and recombinant protein expression.

E. coli was cultured in Luria-Bertani (LB) broth and on LB agar at 37°C. *S. pyogenes* strains were grown at 37°C in Brain Heart Infusion broth (Difco) and on Proteose Peptone Agar (Difco) supplemented with 4% defibrinated sheep blood (Cleveland Scientific). When required, media was supplemented with antibiotics at the following concentrations: ampicillin at 100 μg/ml for *E. coli*, erythromycin at 2 μg/ml for *S. pyogenes*, kanamycin at 50 μg/ml for *E. coli* and 250 μg/ml for *S. pyogenes*, and spectinomycin at 20 μg/ml for *E. coli* and 100 μg/ml for *S. pyogenes.*

Table 7.1. Bacterial strains and plasmids for M.SpyI studies.

TABLE 1. Strain and plasmid table

Strain or plasmid	Characteristics[a]	Erythromycin susceptibility[b]	Presence of Φ10394.4[c]	Cleaved by SmaI	Reference or source
Streptococcus pyogenes					
1PM6	M6 Pittsburgh isolate	Sensitive	-	+	(36)
2PM6	M6 Pittsburgh isolate	Resistant	+	-	(36)
3PM6	M6 Pittsburgh isolate	Resistant	+	-	(36)
4PM6	M6 Pittsburgh isolate	Resistant	+	-	(36)
5PM6	M6 Pittsburgh isolate	Sensitive	+	-	(36)
6PM6	M6 Pittsburgh isolate	Sensitive	+	-	(36)
7PM6	M6 Pittsburgh isolate	Sensitive	-	+	(36)
8PM6	M6 Pittsburgh isolate	Sensitive	-	+	(36)
9PM6	M6 Pittsburgh isolate	Sensitive	+	-	(36)
10PM6	M6 Pittsburgh isolate	Resistant	+	-	(36)
3PM6ΔRM	3PM6 with R-M replaced with *aacA/aphD* gene; Kan^r	Resistant	+	+	This study
3PM6ΔRM-C	3PM6ΔRM with pLZ12spec-*spyIM* plasmid; $Spec^r$	Resistant	+	-	This study
Escherichia coli					
Oneshot Top10	Used for cloning and recombinant protein expression				Invitrogen
Plasmids					
pFW13	*E. coli* -streptococcal shuttle vector with *E. coli* origin of replication				(42)
pLZ12spec	*E. coli* -streptococcal shuttle vector with gram-positive origin of replication				(10)
pBADTOPO	For recombinant expression of proteins under control of L-arabinose promoter				Invitrogen
pFW13EX	pFW13 with region upstream of R-M inserted into MCS I				This study
pFW13AA	pFW13 with region downstream of R-M inserted into MCS II				This study
pFW13EA	Combined pFW13EX + pFW13AA for R-M allelic replacement vector				This study
pLZ12spec-*spyIM*	pLZ12spec with *spyMI* gene and promoter region				This study
pBADTOPO-*spyIM*	pBADTOPO with *spyMI* gene under control of L-arabinose promoter				This study

[a] Abbreviations used: Kan^r, kanamycin-resistant; $Spec^r$, spectinomycin-resistant; R-M, restriction-modification cassette; MCS, multiple cloning site.

[b] Erythromycin susceptibility determined by Martin et al. and confirmed by methods described in text.

[c] Presence of Φ10394.4 in the streptococcal genomes was determined by PCR and Southern blot hybridization as described in methods.

Table 7.2. PCR and sequencing primers for M.SpyI studies.

Amplified region	Primer name	Primer sequence[a], 5' → 3'	Primer type[b]	Product length[c]	Reference or source
Chimeric element					
mefA gene	mefA-M2F mefA-M2R	AGTATCATTAATCACTAGTGC TTCTTCTGGTACTAAAAGTGG	PCR	346	(3)
across attpR	attpR-M5F attpR-M5R	GCCTGATGAAGCAAAGGTTG ACGCGTATGGAAGTTCTGGT	PCR	4781	(3)
Intact *comEC*	comECF comECR	GCAGGTGCGACAACGAGT TGTCCGACCAGTCAAACGAA	PCR	816	This study
and M.SpyI expression primers below					
M.SpyI *E. coli* expression					
spyIM gene	MetR MetFI	**A**TGACCTTTGATAGACACATTTTAG CTATTTTTTTAGGACGGCTAGTAT	PCR	1227	This study
R-M[d] allelic replacement					
Adjacent region up-stream of R-M	UpFEagI UpRXhoI	ATTCTC**CG**GCCGCTCTTCAAAGT AATTGC**T**C**G**A**G**AAATACTAGCCGTCC	PCR	944	This study
Adjacent region down-stream of R-M	DoFAvrII DoRAgeI	TTGTATCC**T**AG**G**TTAGAGGCAATTATTAAG TAGTA**A**CCGGTTCTAATGCATACCCT	PCR	1016	This study
M.SpyI *S. pyogenes* expression					
spyIM gene + promoter region	MetxBamHI MetxSphI	GATAGA**GGA**TTCGATGCTATTCTGC ATTTTGCA**TGC**AGAATTAAAAAAGTCTAT	PCR	1409	This study
Bisulfite reaction					
Sense strand of bisulfite modified DNA, 16S rRNA gene region	bi-F bi-R	TTGAAAGTGTGGGGAGTAAATA CTAACTCCTAATAAATTACCTCACC	PCR / Seq	701	This study
	bi-Fs1	AGTAAATAGGATTAGATATTTTGGTAGTTT	Seq		This study
	bi-Fs2	TGTTTTTTTATGATTTGGGTTATATA	Seq		This study
	bi-Rs1	TAACAACAACCATACACCACCATC	Seq		This study
Anti-sense strand of bisulfite modified DNA, 16S rRNA gene region	bi-botF bi-botR	TTGGTTTTTAATAGGTTATTTTATTGATTT AAAACTCAAAAACATAAAAAACAAACAA	PCR / Seq	705	This study
	bi-botFs1	GAGTTGATGATAATTATGTATTATTTGTTA	Seq		This study

[a] Mutated nucleotides are in boldface and introduced restriction sites are underlined.

7.2.2 DNA manipulations.

Streptococcal genomic DNA was isolated with either the DNeasy Tissue Kit or the Blood & Cell Culture DNA Kit (Qiagen) following the manufacturer's protocols, except for the substitution of a modified lysis buffer (50 mM Tris-Cl pH 6.6, 50 mM EDTA, 0.5% Tween-20, 0.5% TritonX-100) supplemented with 500U of plyC, a streptococcal bacteriophage lysin (Nelson, Schuch et al. 2006) and 250 ng/ml of RNase A (Qiagen). Plasmid DNA was isolated from *E.coli* using the QIAprep Spin Miniprep Kit or HiSpeed Plasmid Midi Kit (Qiagen). DNA fragments were gel purified from 1% Agarose gels using the QIAquick Gel Extraction Kit (Qiagen). T4 DNA ligase, M.HpaII MTase, and all restriction enzymes were purchased from New England Biolabs and used according to the manufacturer's instructions. Oligonucleotides were obtained from Sigma-Genosys. PCR was performed using AmpliTaq Gold DNA polymerase, Gold Buffer, 1.5 mM $MgCl_2$, and 200 μM dNTPS (Applied Biosystems) following standard protocols with the Eppendorf Mastercycler. DNA sequencing was performed by GENEWIZ, Inc. (North Brunswick, NJ). DNA sequence analysis, comparison, and manipulation required Lasergene software modules (DNASTAR Inc.) and the CLUSTALX (Thompson, Gibson et al. 1997) and BOXSHADE (http://www.ch.embnet.org/software/BOX_form.html) programs. DNA primers were designed with MacVector software (Accelrys Inc.).

7.2.3 PFGE molecular analysis.

Agarose discs of genomic DNA from the Pittsburgh isolates (Table 7.1) were prepared according to a protocol modified from Chung et al. (Chung, de Lencastre et al. 2000), in which 500U of plyC was substituted for lysozyme and lysostaphin enzymes in the cell lysis solution (Chung, de Lencastre et al. 2000; Nelson, Schuch et al. 2006). DNA was digested with SmaI or XmaI (40U) overnight at 25°C or 37°C, respectively, and subjected to PFGE with the CHEF-DR II system (Bio-Rad) as previously described (Chung, de Lencastre et al. 2000). DNA bands were visualized by staining the gel with ethidium bromide and the images captured by an Alpha imager (Alpha Innotech Corp.). DNA banding patterns were analyzed by previously described methods (Tenover, Arbeit et al. 1995; Chung, de Lencastre et al. 2000).

7.2.4 Construction of the pBadTOPO-*spyIM E.coli* expression plasmid.

The *spyIM* gene was PCR amplified from genomic DNA of strain 3PM6 using the MetR and MetFI primers described under the M.SpyI *E. coli* expression heading in Table 7.2. The 1227 bp PCR product was gel purified, ligated into pBAD-TOPO vector, and transformed into *E.coli* One Shot TOP10 using the pBAD TOPO TA Expression Kit (Invitrogen). Transformants were screened for proper insert orientation by colony PCR and DNA sequencing. Positive clones were screened for MTase expression by the SmaI protection assay below.

7.2.5 SmaI endonuclease protection assay.

Expression and activity of the recombinant M.SpyI was tested using a combination of protocols described previously (Card, Wilson et al. 1990; Klimasauskas, Steponaviciene et al. 1990) and the instructions of the pBADTOPO TA Expression Kit. Briefly, recombinant M.SpyI expression was induced by the addition of 0.2% or 2% L-arabinose to cultures at an OD_{600} of 0.5, and growth then continued for 2 or 4 hours at 37°C, respectively. L-arabinose concentrations and induction times were varied to determine the most efficient expression conditions. The cultures were centrifuged at 3000 x g for 10 min. The cell pellets were washed once in 50mM Tris-HCl pH 7.5, resuspended in MTase reaction buffer (50 mM Tris-HCl pH 7.5, 5 mM 2-mercaptoethanol, 10 mM EDTA) and frozen at -20°C overnight. Cells were lysed by two freeze-thaw cycles, followed by sonication for 5 min with a W-380 Sonicator (Heatsystems-Ultrasonics Inc.). The crude lysate was centrifuged at 16,000 x g for 20 min at 4°C to collect the supernatant. The lysate supernatant (10 ul), supplemented with 80 μM of S-adenosylmethionine (SAM) (New England Biolabs), was then incubated for 2 hours at 37°C with 1 μg of λ phage DNA (New England Biolabs) as the substrate for the methylation reaction. The reaction was heat inactivated for 20 min at 65°C and then the λ DNA was subjected to SmaI digestion for 2 hours at 25°C by the addition of NEBuffer 4 (40 μl), supplemented with 10 mM $MgCl_2$ and 20U SmaI. Digestion or protection of λDNA was analyzed by 1% Agarose gel electrophoresis.

7.2.6 Allelic replacement of the restriction-modification (R-M) cassette in Φ10394.4.

The strategy for allelic replacement of the R-M cassette is outlined in Figure 7.1. First, DNA regions flanking *spyIM* and the adjacent restriction enzyme genes (944 bp upstream and 1016 bp downstream) were PCR amplified from the genomic DNA of strain 3PM6 using the two primer sets described under the R-M KO heading in Table 7.2. The PCR products were treated with a combination of either EagI and XhoI or AvrII and AgeI and were purified by Qiaquick PCR Purification Kit (Qiagen). The upstream and downstream fragments were individually cloned into shuttle vector pFW13 (Podbielski, Spellerberg et al. 1996). The two resulting vectors, pFW13EX and pFW13AA, were then digested with NheI and NcoI, and religated to produce the R-M allelic replacement vector, pFW13EA. The pFW13EA vector was treated with the M.HpaII MTase, following the manufacturer suggestions (New England Biolabs), to methylate 5'CCGG residues and to prevent vector digestion by the cognate restriction enzyme of the R-M cassette within the streptococci. The methylated vector was electroporated into 3PM6 following the streptococcal transformation protocol of Kimoto et al. (Kimoto and Taketo 2003) and transformants were selected on proteose peptone blood agar supplemented with kanamycin. Allelic replacement of the R-M cassette with the Kan^R gene (*aacA/aphD*) was confirmed by PCR, Southern blot

analysis and DNA sequencing. The resulting mutant strain (3PM6ΔRM) lacked the genes that encoded the M.SpyI and the adjacent restriction enzyme subunit.

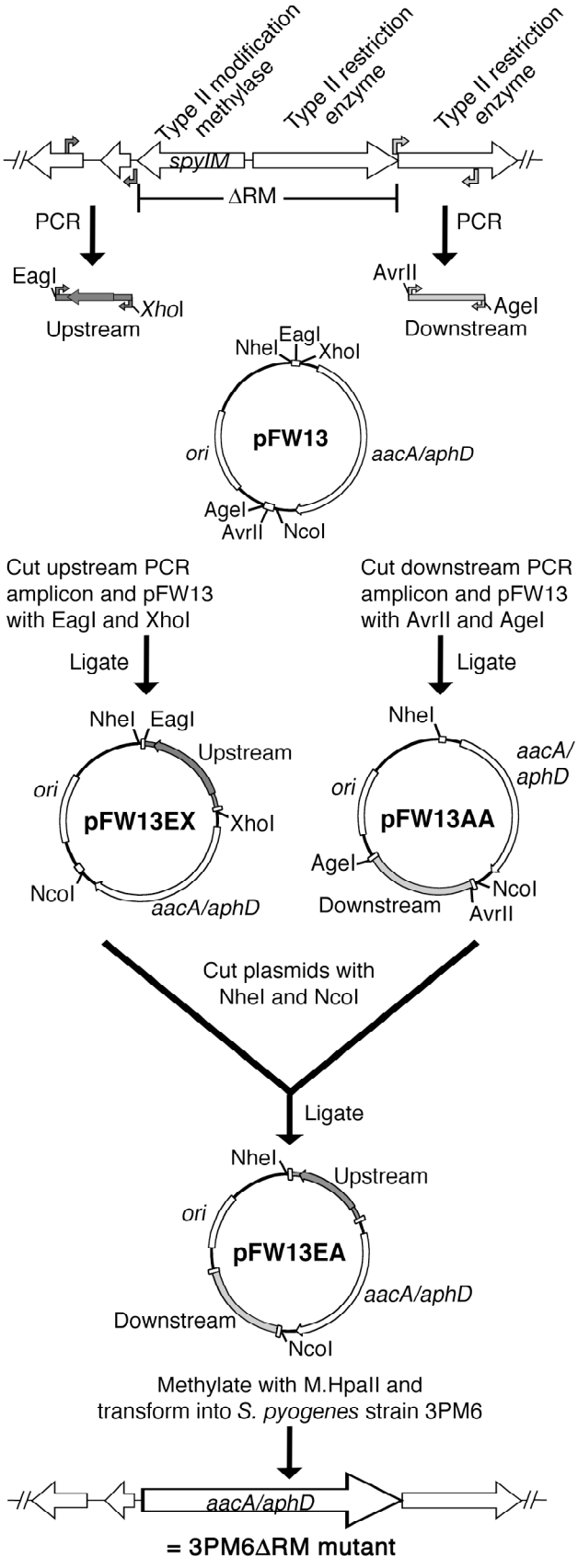

Type II modification methylase
Type II restriction enzyme
Type II restriction enzyme
spyIM
ΔRM
PCR
PCR
EagI
XhoI
Upstream
AvrII
AgeI
Downstream
NheI
EagI
XhoI
pFW13
ori
aacA/aphD
AgeI
AvrII
NcoI
Cut upstream PCR amplicon and pFW13 with EagI and XhoI
Cut downstream PCR amplicon and pFW13 with AvrII and AgeI
Ligate
Ligate
NheI
EagI
Upstream
ori
pFW13EX
XhoI
NcoI
aacA/aphD
NheI
aacA/ aphD
ori
pFW13AA
AgeI
NcoI
Downstream
AvrII
Cut plasmids with NheI and NcoI
Ligate
NheI
Upstream
ori
pFW13EA
aacA/aphD
Downstream
NcoI
Methylate with M.HpaII and transform into S. pyogenes strain 3PM6
aacA/aphD
= 3PM6ΔRM mutant

Figure 7.1. Construction of the allelic replacement vector pFW13EA.

This diagram outlines the steps to construct vector pFW13EA. Top panel shows the location of the adjacent regions upstream and downstream of the R-M cassette that were PCR amplified and digested with the appropriate restriction enzymes by methods detailed in the text. Each amplicon was separately ligated into plasmid pFW13 to create pFW13EX and pFW13AA. Both plasmids were subsequently digested with NheI and NcoI and ligated to produce pFW13EA, the allelic replacement vector, which contains the kanamycin resistance gene (*aacA/aphD*) flanked by the regions adjacent to the R-M cassette. Grey arrows and blocks indicate the regions PCR amplified for insertion into the pFW13 vector. ΔR-M bracket indicates the region of the restriction-modification cassette that was replaced with the kanamycin resistance gene (*aacA/aphD*). Plasmid diagrams indicate relevant restriction sites, kanamycin resistance gene (*aacA/aphD*) and *E. coli* origin of replication (*ori*).

7.2.7 Construction of pLZ12spec-*spyIM GrAS* expression plasmid.

A 1409 bp region encompassing both the *spyIM* gene and upstream promoter region was PCR amplified from the genomic DNA of strain 3PM6 using the primers described under the M.SpyI *S. pyogenes* expression heading of (Table 7.2.) The amplicon was then digested with BamHI and SphI and cloned into pLZ12spec, an *E. coli* -streptococcal shuttle vector (Caparon and Scott 1991), to produce pLZ12spec-*spyIM* (Table 7.1). This construct was electroporated into the mutant strain 3PM6ΔRM to produce the complemented strain 3PM6ΔRM-C (Table 7.1). Trans-complementation of the MTase knockout by pLZ12spec-*spyIM* was tested by PFGE molecular analysis described above.

7.2.8 Bisulfite analysis of 5-methylcytosine residues.

Streptococcal genomic DNA (500 ng) was modified by bisulfite treatment with the EZ DNA Methylation-Gold kit (Zymo Research) according to manufacturer instructions. The bisulfite-modified DNA was used as the template for PCR using primer pairs specific for the sense (bi-F and bi-R) or anti-sense (bi-botF and bi-botR) strands of the modified DNA, as described in (Table 7.2) under the bisulfite reaction heading. The amplicons were then purified and sequenced in both the 5' and 3' directions using the primers listed under bisulfite reaction heading in (Table 7.2). The DNA sequences were aligned with the published sequence of *S. pyogenes* strain MGAS10394 using the MegAlign program (DNAstar Inc.) to help identify methylated cytosine residues.

7.3 RESULTS

7.3.1 Genomic DNA harboring Φ10394.4 is resistant to SmaI restriction.

To further differentiate the clonality of the *S. pyogenes* strains isolated from an outbreak of erythromycin resistant pharyngitis in Pittsburgh school children (Martin, Green et al. 2002), PFGE was performed on SmaI digested genomic DNA from isolates that were positive or negative for the chimeric element Φ10394.4 (Table 7.1 & Figure 7.2). Analysis of the PFGE patterns revealed that SmaI was able to digest DNA from strains that did not contain Φ10394.4. Conversely, genomic DNA from strains containing Φ10394.4 was resistant to SmaI digestion (Table 7.1 & Figure 7.2). Furthermore, SmaI did not digest the genomic DNA of three erythromycin susceptible isolates (Table 7.1 & Figure 7.2). PCR and sequence analysis showed these strains also contained the Φ10394.4 chimeric-element but had mutations in the *mefA* gene (Table 7.1 & data not shown). These observations suggested that resistance to SmaI cleavage of genomic DNA was not directly related to erythromycin resistance, but rather the presence of the Φ10394.4 chimeric element.

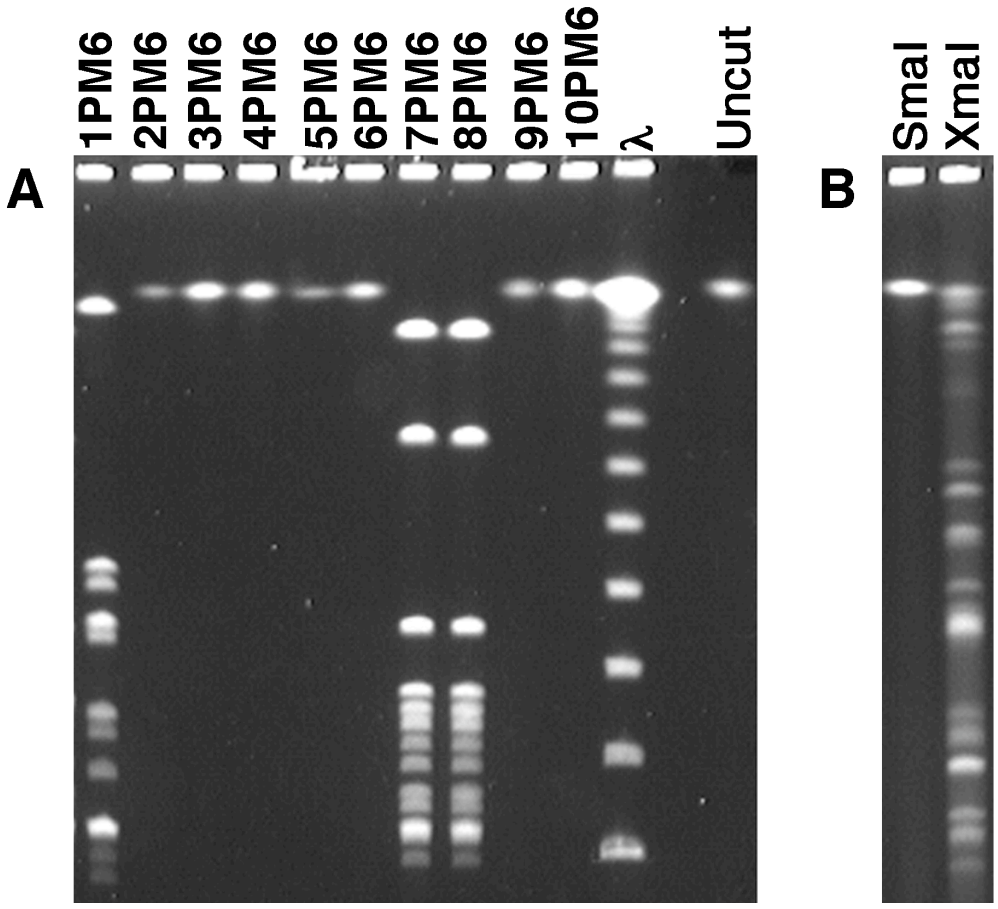


Figure 7.2. Pulse field gel electrophoresis (PFGE) analysis of *S. pyogenes* M6 isolates from school children in Pittsburgh, PA.

(A) PFGE patterns of SmaI digested genomic DNA from isolates that were negative (strains 1, 7, and 8PM6) or positive (strains 2, 3, 4, 5, 6, 9, and 10PM6) for the 58 kb *mefA*-encoding chimeric element Φ10394.4 (Table 7.1). λ, Lambda Ladder PFG Marker (New England Biolabs). (B) Comparison of PFGE patterns of SmaI and XmaI digested genomic DNA from the Φ10394.4 positive isolate 3PM6.

7.3.2 Genomic DNA harboring Φ10394.4 is partially digested by XmaI.

SmaI restriction of DNA can be blocked by CpG methylation at the restriction enzyme cleavage site, 5'-CCC ↓ GGG, [REBASE: http:// rbase.neb.com] (Roberts, Vincze et al. 2005). To determine if this type of methylation was protecting the genomic DNA of Φ10394.4–containing isolates, DNA preparations were treated with XmaI, an isoschizomer of SmaI that cleaves DNA at the sequence: 5'-C ↓ CCGGG. Unlike SmaI, XmaI is not fully blocked, but partially impaired, by CpG methylation overlapping the SmaI recognition site [REBASE: http:// rbase.neb.com] (Roberts, Vincze et al. 2005). PFGE analysis showed that while SmaI was inhibited, XmaI partially cleaved the genomic DNA from the Φ10394.4-containing strain 3PM6 (Figure 7.2). Taken together, the difference in digestion patterns between XmaI and SmaI may be explained by the location of their distinct cleavage sites within the same methylated recognition sequence. These results suggest that the partial digestion by XmaI and the inability of SmaI to cleave 3PM6 DNA may indeed be caused by methylation.

7.3.3 5-methylcytosine MTase identified on Φ10394.4.

Analysis of the published sequence of Φ10394.4 revealed that the bacteriophage-like region of this chimeric element contains a putative type II restriction-modification (R-M) cassette encoding a type II restriction

endonuclease (in two subunits) and an adjacent MTase gene (*spyIM*) (Figure 7.3) (Banks, Porcella et al. 2003; Banks, Porcella et al. 2004).

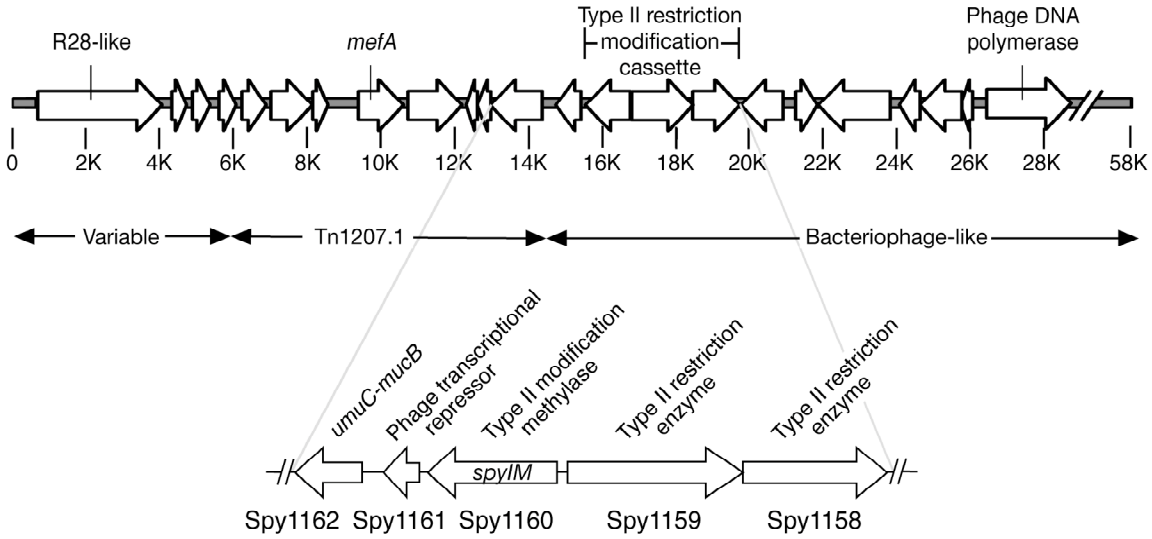

R28-like
mefA
Type II restriction
modification
cassette
Phage DNA
polymerase
0
2K
4K
6K
8K
10K
12K
14K
16K
18K
20K
22K
24K
26K
28K
58K
Variable
Tn1207.1
Bacteriophage-like
umuC-mucB
Phage transcriptional
repressor
Type II modification
methylase
Type II restriction
enzyme
Type II restriction
enzyme
spyIM
Spy1162
Spy1161
Spy1160
Spy1159
Spy1158

Figure 7.3. Schematic of a 28 kb segment of the chimeric element Φ10394.4.

Top panel shows the positions of the *mefA* gene and the restriction-modification cassette, which contains the *spyIM* gene, within the predicted ORFs of Φ10394.4. Numbers below the sequence indicate relative size in kb. Black arrows denote regions that have transposon and bacteriophage-like characteristics, as detailed in the text. Bottom panel is a close-up of the predicted ORFs and their corresponding Spy numbers from the genome sequence of *S. pyogenes* MGAS10394, GenBank accession number CP000003.

A BLASTP comparison of the predicted amino acid sequence of this MTase (M.SpyI) to proteins in the REBASE database [http://tools.neb.com/~vincze/rebhit/blast.php3] (Altschul, Madden et al. 1997; Roberts, Vincze et al. 2005), revealed high sequence similarities to other 5-methylcytosine MTases that recognize the DNA sequence: 5'-CCNGG (Table 7.3).

Table 7.3. BlastP comparison of M.SpyI with other cytosine MTases.

MTase	Recognition sequence[a]	MTase[b]	E-Value	GenBank accession number
M.SpyI[c]	CCNGG	C5	0.0	YP_060478.1
M.Spy1207ORFAP[d]	CCNGG	C5	0.0	AAT72355.1
M1.ScrFI	CCNGG	C5	0.0	AAB66696.1
M.StyD4I	CCNGG	C5	e-168	BAA11167.1
M.Ecl18kI	CCNGG	C5	e-168	CAA76526.1
M.SenPI	C**C**NGG	C5	e-168	AA645971.1
M.SsoII	C**C**NGG	C5	e-167	AAA98279.1

[a] When known, the sites of methylation are indicated by bold letters in the recognition sequence.

[b] C5, 5-methylcytosine MTase.

[c] We reclassified M.Spy10394ORF1160P (YP_060478.1) as M.SpyI.

[d] M.Spy1207ORFAP is identical to M.SpyI and is the uncharacterized MTase in the chimeric element Tn1207.3.

The comparison also identified M.Spy1207ORFAP, which is located in the DNA sequence of Tn1207.3, as an identical ORF to M.SpyI (Santagati, Iannelli et al. 2003). Of note, no corresponding ORF to M.SpyI has yet been identified in the *tetO-mefA* chimeric elements (D'Ercole, Petrelli et al. 2005). A CLUSTALW alignment of the amino acid sequences from the BLASTP search showed M.SpyI contained the 10 conserved motifs that are present in the 5-methylcytosine MTases, in the proper order, and significant homology within the variable region making up the target recognition domain (TRD) of these enzymes (Figure 7.4). These conserved features suggested that *spyIM* may encode a 5-methylcytosine MTase that could recognize the DNA sequence: 5'-CCNGG. This recognition sequence is included within the DNA sequence recognized by SmaI (5'-CCCGGG). Therefore, we were interested in determining if M.SpyI methylates CpG sites on the streptococcal chromosome, such as those contained in the SmaI DNA recognition sequence.

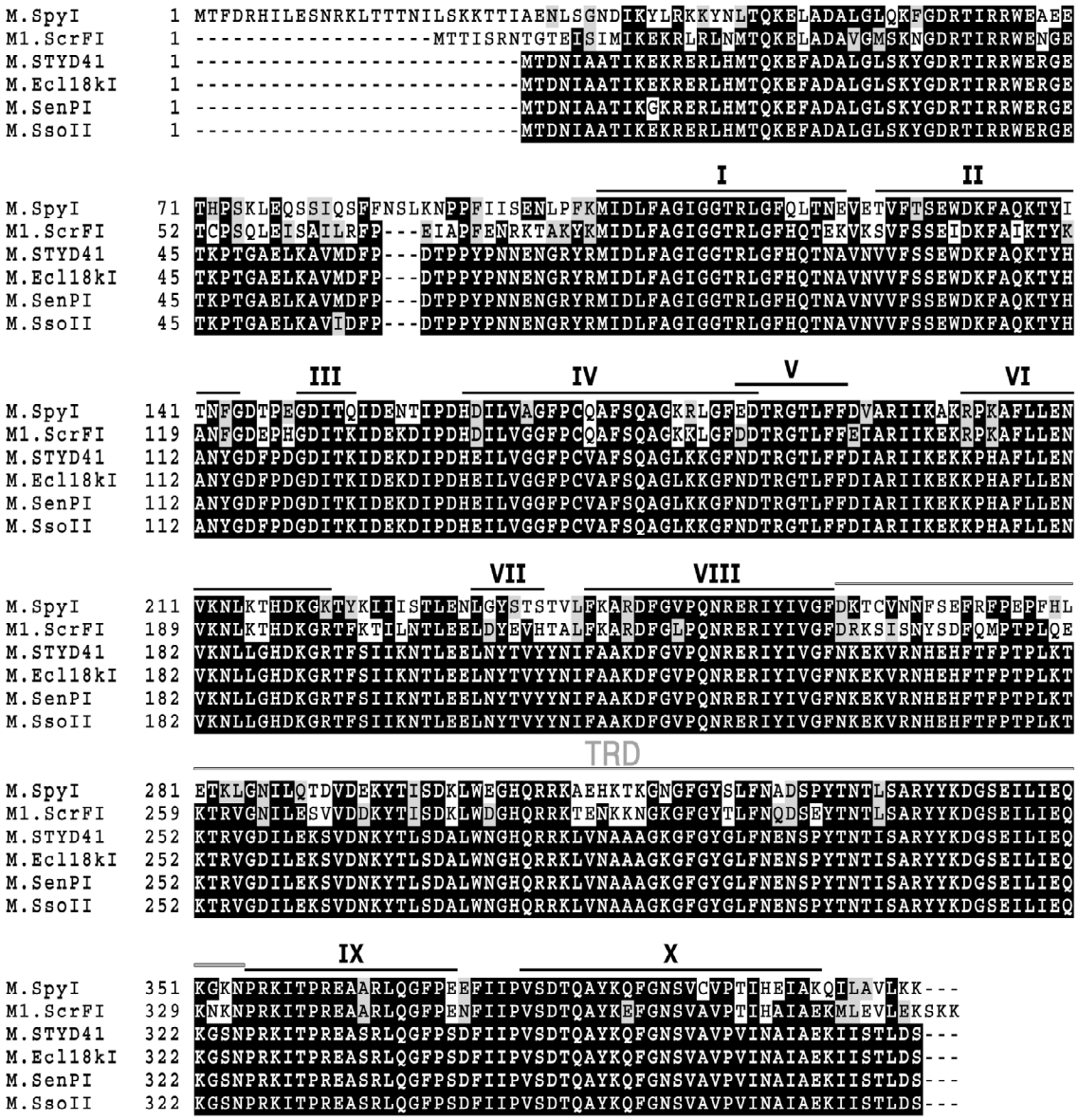

M.SpyI 1 MTFDRHILESNRKLTTTNILSKKTTIAENLSGNDIKYLRKKYNLTQKELADALGLQKFGDRTIRRWEAEE
M1.ScrFI 1 ----------MTTISRNTGTEISIMIKEKRLRLNMTQKELADAVGMSKNGDRTIRRWENGE
M.STYD41 1 ----------MTDNIAATIKEKRERLHMTQKEFADALGLSKYGDRTIRRWERGE
M.Ecl18kI 1 ----------MTDNIAATIKEKRERLHMTQKEFADALGLSKYGDRTIRRWERGE
M.SenPI 1 ----------MTDNIAATIKGKRERLHMTQKEFADALGLSKYGDRTIRRWERGE
M.SsoII 1 ----------MTDNIAATIKEKRERLHMTQKEFADALGLSKYGDRTIRRWERGE
I
II
M.SpyI 71 THPSKLEQSSIQSFFNSLKNPPFIISENLPFKMIDLFAGIGGTRLGFQLTNEVETVFTSEWDKFAQKTYI
M1.ScrFI 52 TCPSQLEISAILRFP---EIAPFENRKTAKYKMIDLFAGIGGTRLGFHQTEKVKSVFSSEIDKFAIKTYK
M.STYD41 45 TKPTGAELKAVMDFP---DTPPYPNNENGRYRMIDLFAGIGGTRLGFHQTNAVNVVFSSEWDKFAQKTYH
M.Ecl18kI 45 TKPTGAELKAVMDFP---DTPPYPNNENGRYRMIDLFAGIGGTRLGFHQTNAVNVVFSSEWDKFAQKTYH
M.SenPI 45 TKPTGAELKAVMDFP---DTPPYPNNENGRYRMIDLFAGIGGTRLGFHQTNAVNVVFSSEWDKFAQKTYH
M.SsoII 45 TKPTGAELKAVIDFP---DTPPYPNNENGRYRMIDLFAGIGGTRLGFHQTNAVNVVFSSEWDKFAQKTYH
III
IV
V
VI
M.SpyI 141 TNFGDTPEGDITQIDENTIPDHDILVAGFPCQAFSQAGKRLGFEDTRGTLFFDVARIIKAKRPKAFLLEN
M1.ScrFI 119 ANFGDEPHGDITKIDEKDIPDHDILVGGFPCQAFSQAGKKLGFDDTRGTLFFEIARIIKEKRPKAFLLEN
M.STYD41 112 ANYGDFPDGDITKIDEKDIPDHEILVGGFPCVAFSQAGLKKGFNDTRGTLFFDIARIIKEKKPHAFLLEN
M.Ecl18kI 112 ANYGDFPDGDITKIDEKDIPDHEILVGGFPCVAFSQAGLKKGFNDTRGTLFFDIARIIKEKKPHAFLLEN
M.SenPI 112 ANYGDFPDGDITKIDEKDIPDHEILVGGFPCVAFSQAGLKKGFNDTRGTLFFDIARIIKEKKPHAFLLEN
M.SsoII 112 ANYGDFPDGDITKIDEKDIPDHEILVGGFPCVAFSQAGLKKGFNDTRGTLFFDIARIIKEKKPHAFLLEN
VII
VIII
M.SpyI 211 VKNLKTHDKGKTYKIIISTLENLGYSTSTVLFKARDFGVPQNRERIYIVGFDKTCVNNFSEFRFPEPFHL
M1.ScrFI 189 VKNLKTHDKGRTFKTILNTLEELDYEVHTALFKARDFGLPQNRERIYIVGFDRKSISNYSDFQMPTPLQE
M.STYD41 182 VKNLLGHDKGRTFSIIKNTLEELNYTVYYNIFAAKDFGVPQNRERIYIVGFNKEKVRNHEHFTFPTPLKT
M.Ecl18kI 182 VKNLLGHDKGRTFSIIKNTLEELNYTVYYNIFAAKDFGVPQNRERIYIVGFNKEKVRNHEHFTFPTPLKT
M.SenPI 182 VKNLLGHDKGRTFSIIKNTLEELNYTVYYNIFAAKDFGVPQNRERIYIVGFNKEKVRNHEHFTFPTPLKT
M.SsoII 182 VKNLLGHDKGRTFSIIKNTLEELNYTVYYNIFAAKDFGVPQNRERIYIVGFNKEKVRNHEHFTFPTPLKT
TRD
M.SpyI 281 ETKLGNILQTDVDEKYTISDKLWEGHQRRKAEHKTKGNGFGYSLFNADSPYTNTLSARYYKDGSEILIEQ
M1.ScrFI 259 KTRVGNILESVVDDKYTISDKLWDGHQRRKTENKKNGKGFGYTLFNQDSEYTNTLSARYYKDGSEILIEQ
M.STYD41 252 KTRVGDILEKSVDNKYTLSDALWNGHQRRKLVNAAAGKGFGYGLFNENSPYTNTISARYYKDGSEILIEQ
M.Ecl18kI 252 KTRVGDILEKSVDNKYTLSDALWNGHQRRKLVNAAAGKGFGYGLFNENSPYTNTISARYYKDGSEILIEQ
M.SenPI 252 KTRVGDILEKSVDNKYTLSDALWNGHQRRKLVNAAAGKGFGYGLFNENSPYTNTISARYYKDGSEILIEQ
M.SsoII 252 KTRVGDILEKSVDNKYTLSDALWNGHQRRKLVNAAAGKGFGYGLFNENSPYTNTISARYYKDGSEILIEQ
IX
X
M.SpyI 351 KGKNPRKITPREAARLQGFPEEFIIPVSDTQAYKQFGNSVCVPTIHEIAKQILAVLKK---
M1.ScrFI 329 KNKNPRKITPREAARLQGFPENFIIPVSDTQAYKEFGNSVAVPTIHAIAEKMLEVLEKSKK
M.STYD41 322 KGSNPRKITPREASRLQGFPSDFIIPVSDTQAYKQFGNSVAVPVINAIAEKIISTLDS---
M.Ecl18kI 322 KGSNPRKITPREASRLQGFPSDFIIPVSDTQAYKQFGNSVAVPVINAIAEKIISTLDS---
M.SenPI 322 KGSNPRKITPREASRLQGFPSDFIIPVSDTQAYKQFGNSVAVPVINAIAEKIISTLDS---
M.SsoII 322 KGSNPRKITPREASRLQGFPSDFIIPVSDTQAYKQFGNSVAVPVINAIAEKIISTLDS---

Figure 7.4. Alignment of the amino acid sequence of M.SpyI with the five closest 5-methylcytosine MTases from a REBASE BLASTP search.

Alignment of the sequences: M.SpyI, M1.ScrFI, M.StyD41, M.Ecl18kI, M. SenPI, and M.SsoII was carried out using the ClustalX program and amino acids shaded with the Box Shade program. Black boxes highlight identical residues, while grey boxes highlight conserved residues. Gaps are indicated by dashes. The locations of the 10 conserved amino acid motifs present in 5-methylcytosine MTases are indicated by black lines above the amino acid sequences and numbered I to X. The variable region, making up the target recognition domain (TRD) of 5-methylcytosine MTases is indicated by a grey line above the amino acid sequences. M.Spy1207ORFAP was not included in the alignment because it is the identical ORF to M.SpyI in the Tn1207.3 chimeric element.

7.3.4 Recombinant M.SpyI protects λ DNA from SmaI digestion.

To investigate whether M.SpyI was capable of modifying DNA to inhibit SmaI, recombinant M.SpyI was expressed in *E. coli* and tested for MTase activity. Cell lysates from cultures in which M.SpyI was either induced or not induced were separately incubated with λDNA as a substrate and then subsequently subjected to SmaI digestion. This analysis revealed that SmaI did not cleave λDNA that was incubated with recombinant M.SpyI induced under various conditions (Figure 7.5) Conversely, λDNA incubated with non-induced cell lysates (i.e. M.SpyI not present) was cleaved by the action of SmaI (Figure 7.5). Taken together these results indicated that the M.SpyI was able to modify the DNA to render it resistant to SmaI digestion. Furthermore, cell lysates containing M.SpyI that were not supplemented with SAM in the methylation reaction did not protect λDNA from SmaI digestion (Figure 7.5). Since SAM is an essential co-factor in Type II methyltransferase reactions (Wilson and Murray 1991; Roberts, Belfort et al. 2003), this result suggests that DNA methylation is the modification responsible for SmaI inhibition.

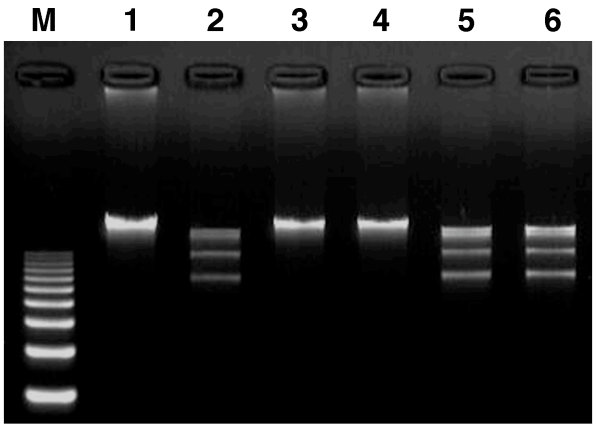


Figure 7.5. Recombinant M.SpyI protects λDNA from digestion with the SmaI endonuclease.

λ DNA was first incubated with the crude lysate of *E.coli* cells that expressed recombinant M.SpyI, and then was subjected to SmaI digestion. Digestion patterns were analyzed by electrophoresis on a 1% agarose gel. Lane 1, undigested l DNA; lane 2, non-lysate treated l DNA alone with SmaI; lanes 3 and 4, l DNA incubated with crude lysate (supplemented with 80 μM of SAM) in which M.SpyI was induced with 0.2% arabinose for 2 hr or 2% arabinose for 4 hr, respectively; lane 5, λ DNA incubated with M.SpyI induced crude lysate without SAM; lane 6, λ DNA + crude lysate from a non-induced culture, 0% arabinose for 4hr; M, 1 kb Plus DNA Ladder (Invitrogen).

7.3.5 Allelic replacement of the R-M cassette allows cleavage of genomic DNA by SmaI and XmaI.

Deletion of the *spyIM* gene in Φ10394.4 would confirm the role of the MTase in methylation of the genomic DNA and provide a useful tool to further dissect the functions of the R-M system genes from the rest of those encoded on the chimeric element. Since an active MTase enzyme is usually necessary to protect host DNA from being digested by the cognate restriction enzyme of the R-M cassette (Kobayashi 2001), we sought to inactivate both enzyme activities by allelic replacement of part of the R-M cassette with a kanamycin resistance gene from vector pFW13EA (Figure 7.1). This resulted in the mutant strain 3PM6ΔRM, which lacked the *spyIM* gene and the adjacent ORF encoding one of the restriction enzyme subunits (Figure 7.1 and Table 7.1). PFGE analysis of digested genomic DNA from this strain showed that it was completely cleaved by both SmaI and XmaI (Figure 7.6). These results indicated that methylation of the genomic DNA by M.SpyI is responsible for the inhibition of SmaI digestion and the partial inhibition of XmaI digestion of the genomic DNA.

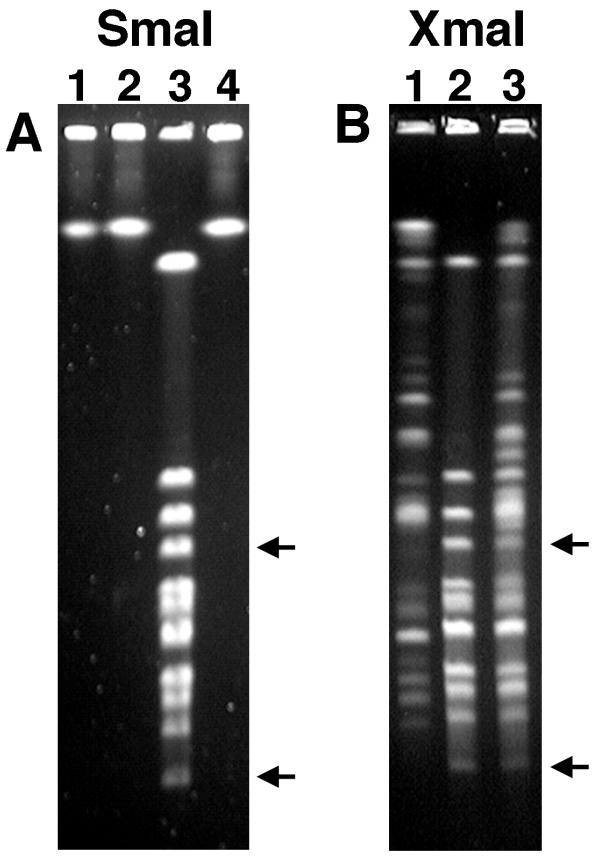


Figure 7.6. PFGE patterns of digested genomic DNA.

From 3PM6 (*spyIM* positive strain), 3PM6ΔRM (*spyIM* deletion mutant), and 3PM6ΔRM-C (trans-complemented *spyIM* mutant). Panel (A) shows the restriction patterns of SmaI digested genomic DNA: Lane 1, undigested DNA (3PM6); lane 2, 3PM6; lane 3, 3PM6ΔRM; lane 4, 3PM6ΔRM-C. Panel (B) shows the restriction patterns of XmaI digested genomic DNA: Lane 1, 3PM6; lane 2, 3PM6ΔRM; lane 3, 3PM6ΔRM-C. Arrows in both panels identify fragments that are different from the predicted digestion pattern of *S. pyogenes* strain MGAS10394 due to the allelic replacement of part of the RM cassette with the kanamycin resistance gene (*aacA/aphD*).

To confirm that the digestion of 3PM6ΔRM DNA was due to a lack of M.SpyI activity in the mutant, the *spyIM* gene was trans-complemented into 3PM6ΔRM on the pLZ12spec-*spyIM* vector and the genomic DNA was tested for inhibition of the restriction enzymes. Both the inhibition and the partial inhibition of genomic DNA digestion by SmaI and XmaI, respectively, were restored in the M.SpyI complemented mutant (Figure 7.6). These results confirmed that M.SpyI was responsible for protecting the DNA from the actions of these restriction enzymes in vitro.

7.3.6 M.SpyI recognizes the genomic DNA sequence CCNGG.

Bisulfite modification of DNA converts nonmethylated cytosine residues to uracil, while 5-methylcytosine residues are protected and remain unaltered in the reaction. Subsequent PCR amplification of the modified DNA changes the uracil residues into thymine so that only the 5-methylcytosine residues are identified as cytosine when the amplicon is sequenced (Frommer, McDonald et al. 1992). To determine the nucleotide sequence that was methylated by M.SpyI, we used the bisulfite reaction to modify the genomic DNA from the Φ10394.4-containing strains 3PM6 and 10PM6, the 3PM6ΔRM deletion mutant, and 8PM6, a Φ10394.4 negative strain (Table 7.1). A 700 bp DNA sequence inside the 16S rRNA genes of these strains was chosen to be PCR amplified and sequenced because it contained all combinations of the 5'-CCNGG DNA sequence,

including the SmaI DNA recognition site and other potentially methylated CpG sequences.

The modified sequences were aligned to 600 bp of the published genome sequence of MGAS10394 to identify the methylation patterns of each strain. The alignment in Figure 7.7 shows that in the DNA sequences of strains that do not contain the *spyIM* gene (3PM6ΔRM mutant and 8PM6), all cytosines were converted to thymine. Conversely, DNA from *spyIM* positive strains (3PM6 and 10PM6) contained unchanged cytosines on the inner C residue of the 5'-CCNGG DNA sequences (denoted in boxes, Figure 7.7), suggesting that M.SpyI methylates this specific cytosine residue (5'-CC^{m}NGG). Additionally, both inner C residues were methylated in the SmaI recognition sequence, 5'-CC^{m}C^{m}GGG (dashed box in Figure 7.7). This methylation pattern fits with the above results because the SmaI recognition sequence actually consists of two overlapping 5'-CCNGG DNA sequences (5'-CCCGG and 5'-CCGGG). Analysis of the DNA sequence from the opposite strand of the same 700 bp showed an equivalent cytosine methylation pattern (data not shown), indicating that these sites have the same cytosine residue methylated on both strands of the DNA, a finding consistent with most type II restriction-modification MTases (Wilson and Murray 1991; Roberts, Vincze et al. 2005). The specificity and activity of M.SpyI was also confirmed by bisulfite analysis of two other regions outside the 16S rRNA genes and with λ DNA methylated with recombinant M.SpyI (data not shown).

Taken together, these results confirm that the DNA sequence modified by M.SpyI is 5'-CCNGG, as predicted by the BLASTP comparison of M.SpyI to other 5-methylcytosine MTases above. These results further indicate that M.SpyI methylation of the DNA is responsible for the inhibition of the of SmaI cleavage of the genomic DNA from the Φ10394.4-containing isolates.

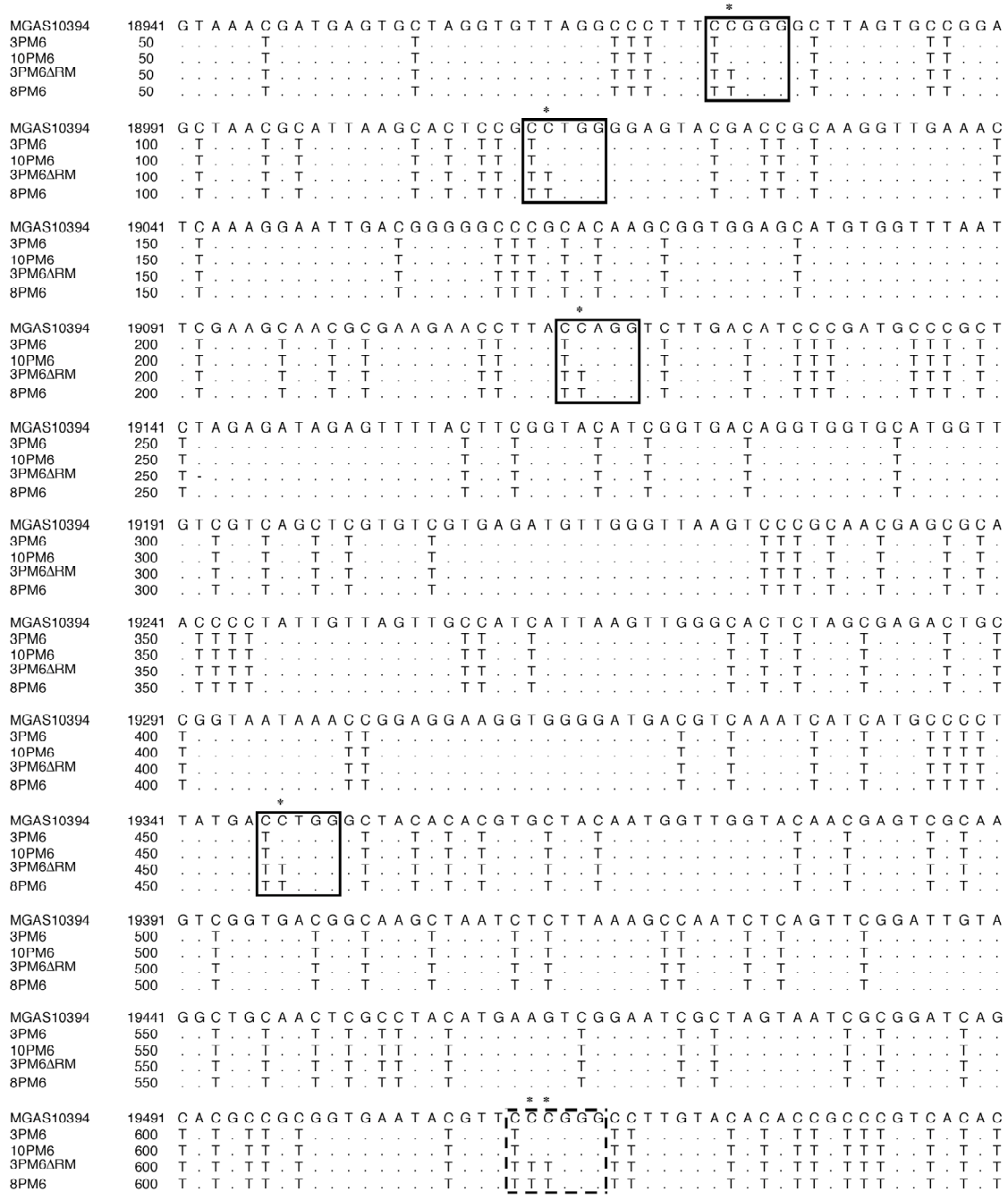

MGAS10394 18941 GTAAACGATGAGTGCTAGGTGTTAGGCCCTTTCCGGGGCTTAGTGCCGGA
3PM6 50 ..... T ..... T ..... TTT ... T ..... T ..... TT ...
10PM6 50 ..... T ..... T ..... TTT ... T ..... T ..... TT ...
3PM6ΔRM 50 ..... T ..... T ..... TTT ... TT ..... T ..... TT ...
8PM6 50 ..... T ..... T ..... TTT ... TT ..... T ..... TT ...
MGAS10394 18991 GCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAAC
3PM6 100 .T...T.T.....T.T.TT.T..... T..TT.T..... T
10PM6 100 .T...T.T.....T.T.TT.T..... T..TT.T..... T
3PM6ΔRM 100 .T...T.T.....T.T.TT.TT.... T..TT.T..... T
8PM6 100 .T...T.T.....T.T.TT.TT.... T..TT.T..... T
MGAS10394 19041 TCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAAT
3PM6 150 .T.....T.....TTT.T.T...T.....T.....T.....
10PM6 150 .T.....T.....TTT.T.T...T.....T.....T.....
3PM6ΔRM 150 .T.....T.....TTT.T.T...T.....T.....T.....
8PM6 150 .T.....T.....TTT.T.T...T.....T.....T.....
MGAS10394 19091 TCGAAGCAACGCGAAGAACCTTACCCAGGTCTTGACATCCCGATGCCCGCT
3PM6 200 .T...T.T.T.....TT...T.....T...T..TTT...TTT.T.
10PM6 200 .T...T.T.T.....TT...T.....T...T..TTT...TTT.T.
3PM6ΔRM 200 .T...T.T.T.....TT...T.....T...T..TTT...TTT.T.
8PM6 200 .T...T.T.T.....TT...TT.....T...T..TTT...TTT.T.
MGAS10394 19141 CTAGAGATAGAGTTTTACTTCGGTACATCGGTGACAGGTGGTGCATGGTT
3PM6 250 T.....T.T.T.T.....T.....T.....
10PM6 250 T.....T.T.T.T.....T.....T.....
3PM6ΔRM 250 T-.....T.T.T.T.....T.....T.....
8PM6 250 T.....T.T.T.T.....T.....T.....
MGAS10394 19191 GTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA
3PM6 300 ..T..T..T.T....T.....TTT.T..T...T.T.
10PM6 300 ..T..T..T.T....T.....TTT.T..T...T.T.
3PM6ΔRM 300 ..T..T..T.T....T.....TTT.T..T...T.T.
8PM6 300 ..T..T..T.T....T.....TTT.T..T...T.T.
MGAS10394 19241 ACCCCTATTGTTAGTTGCCATCATTAAGTTGGGCACTCTAGCGAGACTGC
3PM6 350 .TTTT.....TT..T.....T.T.T...T....T..T
10PM6 350 .TTTT.....TT..T.....T.T.T...T....T..T
3PM6ΔRM 350 .TTTT.....TT..T.....T.T.T...T....T..T
8PM6 350 .TTTT.....TT..T.....T.T.T...T....T..T
MGAS10394 19291 CGGTAATAAACCGGAGGAAGGTGGGGATGACGTCAAAATCATCATGCCCCT
3PM6 400 T.....TT.....T..T....T..T...TTTT.
10PM6 400 T.....TT.....T..T....T..T...TTTT.
3PM6ΔRM 400 T.....TT.....T..T....T..T...TTTT.
8PM6 400 T.....TT.....T..T....T..T...TTTT.
MGAS10394 19341 TATGACCTGGGCTACACACGTGCTACAATGGTTGGTACAACGAGTCGCAA
3PM6 450 .....T.....T..T.T.T...T..T.....T..T....T.T..
10PM6 450 .....T.....T..T.T.T...T..T.....T..T....T.T..
3PM6ΔRM 450 .....TT.....T..T.T.T...T..T.....T..T....T.T..
8PM6 450 .....TT.....T..T.T.T...T..T.....T..T....T.T..
MGAS10394 19391 GTCGGTGACGGCAAGCTAATCTCTTAAAGCCAATCTCAGTTCGGATTGTA
3PM6 500 ..T.....T..T...T....T.T.....TT...T.T....T.....
10PM6 500 ..T.....T..T...T....T.T.....TT...T.T....T.....
3PM6ΔRM 500 ..T.....T..T...T....T.T.....TT...T.T....T.....
8PM6 500 ..T.....T..T...T....T.T.....TT...T.T....T.....
MGAS10394 19441 GGCTGCAACTCGCCTACATGAAGTCGGAATCGCTAGTAATCGCGGATCAG
3PM6 550 ..T..T..T.TT..T.....T.....T.T.....T.T....T..
10PM6 550 ..T..T..T.TT..T.....T.....T.T.....T.T....T..
3PM6ΔRM 550 ..T..T..T.TT..T.....T.....T.T.....T.T....T..
8PM6 550 ..T..T..T.TT..T.....T.....T.T.....T.T....T..
MGAS10394 19491 CACGCCGCCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACAC
3PM6 600 T.T.TT.T.....T..T.....TT.....T.T.TT.TTT..T.T.T
10PM6 600 T.T.TT.T.....T..T.....TT.....T.T.TT.TTT..T.T.T
3PM6ΔRM 600 T.T.TT.T.....T..TTT...TT.....T.T.TT.TTT..T.T.T
8PM6 600 T.T.TT.T.....T..TTT...TT.....T.T.TT.TTT..T.T.T

Figure 7.7. Sequence comparison of PCR products from bisulfite modified streptococcal genomic DNA to identify cytosines methylated by M.SpyI.

Sequences were aligned to the published genomic sequence of the S. pyogenes M6 strain MGAS10394 on the top line of each row. Unmodified nucleotides that were identical to the residues in the MGAS10394 reference sequence (GenBank accession number CP000003) have been illustrated with a dot. Methylated-cytosines have been defined as cytosine nucleotides that were not converted to thymine residues by bisulfite treatment and are highlighted by an asterisk above the DNA sequences. Boxes indicate the putative DNA recognition sequences for M.SpyI, with the dashed box highlighting the overlapping sequence recognized by SmaI. Line 1, MGAS10394 reference sequence; Line 2-3, modified DNA from Φ10394.4-containing isolates 3PM6 and 10PM6, respectively; Line 4, modified DNA from the spyIM deletion mutant 3PM6ΔRM; Line 5, modified DNA from the Φ10394.4 negative isolate 8PM6. Numbering of the sequence in line 1 corresponds to the published numbered DNA sequence of MGAS10394. Numbering of the other lines corresponds to the position of the nucleotides from the start of the bi-F primer in the sense strand amplicon of the bisulfite reaction (Table 7.2).

7.4 DISCUSSION

PFGE analysis of SmaI digested genomic DNA has been used in many epidemiological studies to successfully characterize the clonality of multiple serotypes of *S. pyogenes* (Tenover, Arbeit et al. 1995; Stanley, Desai et al. 1996; Nakashima, Ichiyama et al. 1997; Nguyen, Levy et al. 1997). However, numerous studies have reported that the DNA from some macrolide resistant isolates are refractory to SmaI digestion (Ripa, Zampaloni et al. 2001; Bingen, Leclercq et al. 2002; Martin, Green et al. 2002; Giovanetti, Brenciani et al. 2005; Malhotra-Kumar, Lammens et al. 2005); an observation recently associated with the "M phenotype" of erythromycin resistance, which is imparted by the Tn1207.3 or Φ10394.4 chimeric elements (Ripa, Zampaloni et al. 2001; Giovanetti, Brenciani et al. 2005). The results of our study agree with these previous reports. Here, we also found that SmaI could not digest the genomic DNA from streptococcal isolates containing Φ10394.4; this led to the identification of a type II R-M cassette on Φ10394.4, which encodes two subunits of a restriction endonuclease and an adjacent MTase gene (*spyIM*).

We cloned, expressed and characterized the *spyIM* gene, encoding the 5-methylcytosine MTase, which we named M.SpyI. Through the heterologous expression of M.SpyI, we provided evidence that it is capable of protecting DNA from SmaI digestion. The allelic replacement of the *spyIM* gene in *S. pyogenes* combined with bisulfite methylation analysis confirmed that the DNA sequence

that was methylated was CC^{m}NGG and that M.SpyI alone was responsible for the methylation leading to the partial digestion by XmaI and the inhibition of SmaI digestion of streptococcal DNA in vitro. Although *spyIM* is encoded on a mobile element, to our knowledge, this is the first MTase gene to be cloned and characterized from *S. pyogenes*.

The possible in situ roles for the MTase and the adjacent restriction enzyme subunits of the type II R-M system are currently being investigated. However, previous studies have shown that type II MTases act in concert with their cognate type II restriction enzymes, which usually recognize and cleave the overlapping DNA sequence that is acted upon by the MTase, to protect the bacteria from foreign DNA (reviewed in (Wilson and Murray 1991)). Methylated genomic DNA is protected from the action of the cognate restriction enzyme, while nonmethylated DNA from other lytic or lysogenic bacteriophages are restricted so that they cannot kill or sabotage the survival of the bacteria (Wilson and Murray 1991). This suggests another example of how Φ10394.4 may increase host strain survival in the population and provides another reason why erythromycin resistant isolates encoding *mefA* are increasing in the streptococcal population (Kataja, Huovinen et al. 1999; Ripa, Zampaloni et al. 2001; Hsueh, Teng et al. 2002; Silva-Costa, Ramirez et al. 2006). In fact, analysis of DNA sequences from 23 of the published lysogenic bacteriophages of *S. pyogenes* show that they contain an average of 17 CCNGG sites in their genomes (data not shown)

(Ferretti, McShan et al. 2001; Beres, Sylva et al. 2002; Smoot, Barbian et al. 2002; Banks, Porcella et al. 2004). Interestingly, all seven of the other bacteriophages present in the published sequence of the MGAS10394 strain, in which Φ10394.4 was classified, contain 5'-CCNGG sites (Banks, Porcella et al. 2004). The presence of these sites could have allowed the restriction enzyme to cleave their incoming bacteriophage DNA if the R-M cassette was already integrated into the genome, suggesting that Φ10394.4 might have been the most recent element acquired by this streptococcal strain.

Another potential role for the R-M cassette might be to promote maintenance of the *mefA* chimeric element in the bacterial chromosome, through the post-segregational killing of strains that have had a recombinational event, which cured the strain of Φ10394.4 (Handa, Nakayama et al. 2001; Kobayashi 2001; Robinson, Sutcliffe et al. 2006). Strains that have lost Φ10394.4 and its associated MTase may initially contain residual restriction enzyme that could digest any nonmethylated regions of genomic DNA, resulting in the suicidal death of the bacteria (Kulakauskas, Lubys et al. 1995; Naito, Kusano et al. 1995; Ichige and Kobayashi 2005). This process would select for isolates that have preserved Φ10394.4 and the *mefA* gene in the population, and thus possibly maintain macrolide resistance even when the selective antibiotic is not present in the bacterial environment.

Our results also provide an explanation for a finding from past epidemiological studies that the genomic DNA of some streptococcal isolates with the "M-phenotype" of macrolide resistance cannot be digested with SmaI (Ripa, Zampaloni et al. 2001; Bingen, Leclercq et al. 2002; Martin, Green et al. 2002; Giovanetti, Brenciani et al. 2005; Malhotra-Kumar, Lammens et al. 2005). We have shown that the M.SpyI MTase, encoded by Φ10394.4 and other similar chimeric elements, is directly responsible for this phenotype. In the future, we recommend the use of alternative restriction enzymes, which recognize identical DNA sequences to SmaI but are not blocked by this type of methylation; such as the Crf9I endonuclease (Fermentas Inc.), recently used to compare the clonality of macrolide resistance isolates of *S. pyogenes* from Portugal (Silva-Costa, Ramirez et al. 2006). We are currently examining another role that the Φ10394.4 chimeric element may play in regulating the increased invasiveness observed in some erythromycin resistant isolates of streptococci. To further explore this phenotype we are utilizing the phage counter-selection and curing method described below to create an isogenic GrAS mutant lacking the Φ10394.4.

8 Chapter 2

8.1 INTRODUCTION

Starting in the 1930's it was shown that group A streptococci contain lysogenic bacteriophage (Evans 1934; Evans 1940), however it was not until the early 1960's that it was discovered that these prophage are associated with streptococcal virulence factors or "toxins" (Zabriskie 1964), and that the frequency of GrAS lysogeny is quite high (>90%) (Yu and Ferretti 1989; Yu and Ferretti 1991). In 2001, Ferretti *et al.* reported the first complete genome sequence of an M1 serotype strain, SF370, isolated from a wound infection (Ferretti, McShan *et al.* 2001). The SF370 genomic sequence confirmed the intimate relationship between prophage and GrAS by revealing that this streptococcal strain is poly-lysogenized, and that the integrated prophages encode a number of putative virulence factors.

The genome of SF370 contains four major bacteriophage elements (Φ370.1, Φ370.2, Φ370.3 and Φ370.4). Only one prophage (Φ370.1) has been reported to be induced upon mitomycin C treatment, whereas the other three appear to be defective prophages (Ferretti, McShan et al. 2001; Canchaya, Desiere et al. 2002). Phage Φ370.1 has a 41 kb genome and encodes the *speC* superantigen gene, the *spd1* DNase gene, and the *hylp1* hyaluronidase gene. Phage Φ370.2 has a 43 kb genome and encodes two potential superantigen genes (*speH* and *speI*) and a hyaluronidase gene (*hylp2*). Initial sequence analysis indicated that

Φ370.2 is a complete phage, but closer inspection revealed two inactivating mutations, one in the replisome organizer (involved in initiating induced phage DNA replication) and the other in the portal protein (involved in the packaging of phage chromosome into the prohead). Phage Φ370.3 has a 33 kb genome and contains another DNase gene (*spd3*) and hyaluronidase gene (*hylp3)*. It also contains a stop mutation in the replisome organizer. Phage Φ370.4 has a 13kb genome that has undergone extensive deletion, eliminating any identifiable structural, lysis, or virulence genes (Ferretti, McShan *et al.* 2001; Canchaya, Desiere *et al.* 2002).

Subsequently, the genome sequences of thirteen other GrAS genomes have been completed, representing 10 different serotypes (M1, M2, M3, M4, M5, M6, M12, M18, M28, and M49) from a variety of streptococcal diseases (Ferretti, McShan *et al.* 2001; Beres, Sylva *et al*. 2002; Smoot, Barbian *et al.* 2002; Nakagawa, Kurokawa *et al.* 2003; Banks, Porcella *et al.* 2004; Green, Zhang *et al.* 2005; Beres and Musser 2007; Holden, Scott *et al.* 2007; McShan, Ferretti *et al.* 2008). Like SF370, all sequenced GrAS genomes are poly-lysogenized, containing from two to eight phage-like elements. These prophages encode multiple novel virulence factors including: superantigens (e.g. SpeH, SpeK, and SpeM), DNases (e.g. Spd4, Sda, and Sdn), phage-encoded hyaluronidases (Hlyp), antibiotic resistant cassettes (MefA) and phospholipases (Sla) (summarized in (Beres and Musser 2007; McShan, Ferretti et al. 2008).

Including these virulence factors, the prophage open reading frames (ORFs) represent an average of 10% of the streptococcal genome. But while they represent a minor fraction of the total genome, they account for up to 74% of the variation in gene content between GrAS strains (Banks, Beres *et al.* 2002). This data clearly shows that the differences in the combinations of integrated prophage and the types of virulence factors they encode could have an impact on the variety of diseases caused by these different strains.

The fact that integrated prophage account for the greatest genetic variation between genomes of *S. pyogenes* strains (both inter and intra-serotype differences) suggests that the creation of a strain in which the prophages could be manipulated would be a useful tool to further understand the role bacteriophage play in streptococcal pathogenicity and evolution. A comparison of isogenic strains of *S. pyogenes* that differ in specific bacteriophage genes or in overall bacteriophage content would allow us to study the direct effects that individual bacteriophage have on streptococcal virulence and may provide insight into the evolution of pathogenicity of specific strains before and after prophage integration events occurred.

In this study, two different approaches were used to manipulate the SF370 strain in order to produce isogenic mutants that differ in bacteriophage gene content. First, individual bactriophage genes encoding potential GrAS virulence or

regulatory factors were targeted for allelic replacement using previously established methods and protocols (Podbielski, Spellerberg *et al.* 1996; Euler, Ryan *et al.* 2007). Second, we developed a novel approach to select for isolates of the SF370 strain that were cured of lysogenic bacteriophage. To accomplish this, we used allelic replacement techniques to exchange an individual bacteriophage gene with a two gene cassette containing: 1) the (*aacA-aphD*) gene, responsible for kanamycin resistance (Kan^R) (Podbielski, Spellerberg *et al.* 1996), which provides the first level of selection to insert the cassette into the bacteriophage. 2) The wild-type (WT) *rpsL* gene, encoding the ribosomal subunit protein S12, the target of the antibiotic, streptomycin (Reyrat, Pelicic *et al.* 1998).

While mutations in the *rpsL* gene provide a high level of resistance to streptomycin (Sm^R), the resistance is recessive if a wild-type copy of the *rpsL* gene (such as on the above cassette) is also expressed in the same cell (Lederberg 1951; Reyrat, Pelicic *et al.* 1998). Therefore, in a bacterial strain that harbors a chromosomal Sm^R *rpsL* mutation, it is possible to select for clones that have subsequently lost the inserted copy of the WT *rpsL* allele ($rpsL^{WT}$) by plating the bacteria to media containing streptomycin (Reyrat, Pelicic *et al.* 1998). Isolates that retain the $rpsL^{WT}$ copy will be streptomycin sensitive (Sm^S), while strains that lose the $rpsL^{WT}$ gene will be Sm^R. This counter-selectable WT *rpsL* gene marker has been used before in *S. pyogenes* strains to select against transformed recombinant shuttle vectors or single crossover integration events

(McIver and Myles 2002), and similar methods have been applied to delete pathogenicity islands from other bacteria (Rajakumar, Sasakawa *et al.* 1997).

In our approach, the cassette is inserted into a bacteriophage gene, allowing for selection of bacteriophage presence and absence based on streptomycin sensitivity and resistance, respectively. Using this method, which allowed for a more precise manipulation of phage lysogeny, we have been able to create mutants of the modern *S. pyogenes* strain SF370 that have been cured of some or all of the integrated bacteriophage. In total we have created 15 different mutants that encompass every permutation of SF370 phage knockout (KO): single, double, and triple knockouts, as well as a mutant strain that is completely cured of all bacteriophage elements (a loss of ~10% of the genome). To our knowledge, this is the first time such mutants of *S. pyogenes* have been successfully created.

These isogenic mutants were compared to SF370 for phenotypes that may affect virulence and survival of *S. pyogenes* and were further characterized to investigate bacteriophage-GrAS interactions at the molecular level. The derived phage KO strains should be useful tools to study the dynamics of phage lysogeny, to allow for a more thorough analysis of the role phage play both in virulence and in the regulation of chromosomal genes, and to provide insight into the evolution of streptococcal pathogenicity.

8.2 METHODS

8.2.1 Bacterial strains and growth conditions.

S. pyogenes strain SF370 (an M1 serotype) was originally isolated from a wound infection and kindly provided by J. Ferretti, University of Oklahoma Health Sciences Center. Unless otherwise stated, a spontaneous streptomycin-resistant derivative of SF370 (discussed below) will be referred to as wild type or SF370 throughout these studies and all isogenic phage-KO mutants were derived from this GrAS background (Table 8.1). *E. coli* strain One Shot DH5α (Invitrogen) was used as the host strain for plasmid construction and vector propagation.

E. coli was cultured in Luria-Bertani (LB) broth and on LB agar at 37°C. *S. pyogenes* strains were grown at 37°C in Brain Heart Infusion (BHI) broth or Todd Hewitt broth + 0.5% Yeast Extract (THY) and on Proteose Peptone Blood Agar (i.e. supplemented with 4% defibrinated sheep blood (Cleveland Scientific)) or Colombia Blood Agar plates. When required, media was supplemented with antibiotics at the following concentrations: erythromycin at 200 μg/ml for *E. coli* and 15 μg/ml for *S. pyogenes*; kanamycin at 50 μg/ml for *E. coli* and 250 μg/ml for *S. pyogenes*; streptomycin at 200 μg/ml for *S. pyogenes*. All antibiotics were supplied by Sigma and all media was supplied by Difco (BD, Becton, Dickinson and Company) unless stated otherwise.

Table 8.1. Bacterial strains for phage deletion mutants.

Strain[a]	Bacteriophage and/or gene deleted	Antibiotic resistance phenotype[b]	Refererence or source[c]
Streptococcus pyogenes			
SF370	None	Sensitive	(1)
SF370Δ*speH*	*speH*	Erm^R	This study
SF370SM^R , SF370, WT	None	Sm^R	This study
CEM1KRΔ*speC*	*speC* (370.1 superantigen)	Kan^R SM^S	This study
CEM1KRΔ*speH*	*speH* (370.2 superantigen)	Kan^R SM^S	This study
CEM1KRΔ*spd3*	*spd3* (370.3 DNase)	Kan^R SM^S	This study
CEM1KRΔ*SPy2136*	*SPy2136* (370.4 primase)	Kan^R SM^S	This study
Single phage KOs			
CEM1Δ1	370.1	Kan^s SM^R	This study
CEM1Δ2	370.2	Kan^s SM^R	This study
CEM1Δ3	370.3	Kan^s SM^R	This study
CEM1Δ4	370.4	Kan^s SM^R	This study
CEM1KRΔ2-*speC*	370.2, *speC*	Kan^R SM^S	This study
CEM1KRΔ1-*spd3*	370.1, *spd3*	Kan^R SM^S	This study
CEM1KRΔ1-*SPy2136*	370.1, *SPy2136*	Kan^R SM^S	This study
CEM1KRΔ2-*spd3*	370.2, *spd3*	Kan^R SM^S	This study
CEM1KRΔ2-*Spy2136*	370.2, *SPy2136*	Kan^R SM^S	This study
CEM1KRΔ4-*spd3*	370.4, *spd3*	Kan^R SM^S	This study
Double phage KOs			
CEM1Δ1,2	370.1, 370.2	Kan^s SM^R	This study
CEM1Δ1,3	370.1, 370.3	Kan^s SM^R	This study
CEM1Δ1,4	370.1, 370.4	Kan^s SM^R	This study
CEM1Δ2,3	370.2, 370.3	Kan^s SM^R	This study
CEM1Δ2,4	370.2, 370.4	Kan^s SM^R	This study
CEM1Δ3,4	370.3, 370.4	Kan^s SM^R	This study
CEM1KRΔ1,2-*spd3*	370.1, 370.2, *spd3*	Kan^R SM^S	This study
CEM1KRΔ1,2-*Spy2136*	370.1, 370.2, *SPy2136*	Kan^R SM^S	This study
CEM1KRΔ1,4-*spd3*	370.1, 370.4, *spd3*	Kan^R SM^S	This study
CEM1KRΔ2,3-*spd3*	370.2, 370.4, *spd3*	Kan^R SM^S	This study
Triple phage KOs			
CEM1Δ1,2,3	370.1, 370.2, 370.3	Kan^s SM^R	This study
CEM1Δ1,2,4	370.1, 370.2, 370.4	Kan^s SM^R	This study
CEM1Δ1,3,4	370.1, 370.3, 370.4	Kan^s SM^R	This study
CEM1Δ2,3,4	370.2, 370.3, 370.4	Kan^s SM^R	This study
CEM1KRΔ1,2,4-*spd3*	370.1, 370.2, 370.4, *spd3*	Kan^R SM^S	This study
Quadruple phage KO			
CEM1ΔΦ	370.1, 370.2, 370.3, 370.4	Kan^s SM^R	This study
Re-Lysogenized strain			
CEM1ΔΦC1	370.2, 370.3, 370.4	Kan^s SM^R	This study
Escherichia coli			
One Shot DH5α	Used for cloning and propagation of the counter-selection vectors		Invitrogen

[a] SF370Sm^R is referred to throughout this study as wild type of SF370. Phage number or gene to the right of the Δ symbol have been deleted; KR, contains counter-selection cassette (*(aacA-aphD)* / *rpsL* ^{WT}).

[b] Abbreviations used: Erm^R, Erythromycin resistant; Kan^R, kanamycin-resistant; Kan^S , Kanamycin-sensitive; Sm^R, streptomycin-resistant; Sm^S, streptomycin-sensitive.

[c] Source (1); (Ferretti, McShan et al. 2001).

8.2.2 DNA manipulations.

Streptococcal genomic DNA was isolated with either the DNeasy Tissue Kit or the Blood & Cell Culture DNA Kit (Qiagen) following the manufacturer's protocols, except for the substitution of a modified lysis buffer (50 mM Tris-Cl pH 6.6, 50 mM EDTA, 0.5% Tween-20, 0.5% TritonX-100) supplemented with 500U of plyC, a streptococcal bacteriophage lysin (Nelson, Schuch *et al.* 2006) and 250 ng/ml of RNase A (Qiagen). Plasmid DNA was isolated from *E.coli* using the QIAprep Spin Miniprep Kit or HiSpeed Plasmid Midi Kit (Qiagen). DNA fragments were gel purified from 1% Agarose gels using the QIAquick Gel Extraction Kit (Qiagen). T4 DNA ligase and all restriction enzymes were purchased from New England Biolabs and used according to the manufacturer's instructions, unless otherwise stated. Oligonucleotides were obtained from Fisher Scientific-Operon. PCR was performed using AmpliTaq Gold DNA polymerase, Gold Buffer, 1.5 mM $MgCl_2$, and 200 μM dNTPS (Applied Biosystems) following standard protocols with the Eppendorf Mastercycler. DNA sequencing was performed by GENEWIZ, Inc. (North Brunswick, NJ). DNA and RNA sequence analysis, comparison, and manipulation required Lasergene software modules (DNASTAR Inc.). DNA primers were designed with MacVector software (Accelrys Inc.).

8.2.3 PFGE molecular analysis.

Agarose discs of genomic DNA from WT and mutant isolates (Table 8.1) were prepared according to a protocol modified from Chung *et al.* (Chung, de Lencastre *et al.* 2000), in which 500U of PlyC was substituted for lysozyme and lysostaphin enzymes in the cell lysis solution (Chung, de Lencastre *et al.* 2000; Nelson, Schuch *et al.* 2006). DNA was digested with *Sma*I overnight at 25°C and subjected to PFGE with the CHEF-DR II system (Bio-Rad) as previously described (Chung, de Lencastre *et al.* 2000; Euler, Ryan *et al.* 2007). DNA bands were visualized by staining the gel with ethidium bromide and the images captured by an Alpha imager (Alpha Innotech Corp.). DNA banding patterns were analyzed by previously described methods (Tenover, Arbeit *et al.* 1995; Chung, de Lencastre *et al.* 2000).

8.2.4 Allelic replacement of the *speH* gene in SF370.

The strategy for allelic replacement of *speH* gene was followed as previously described (Euler, Ryan *et al.* 2007). Briefly, upstream and downstream DNA regions flanking *speH* (662 bp upstream and 888 bp downstream) were separately amplified using the primer sets listed in (Ryan, Kirk *et al.* 2007). PCR products were treated with the appropriate restriction enzymes, according to manufacturer instructions. Fragments were gel purified and the respective upstream and downstream regions of *speH* were sequentially ligated into the allelic replacement vector pFW15 (Podbielski, Spellerberg *et al.* 1996), creating

plasmid pFWΔ*speH*. To construct deletion mutants of the *speH* gene the vector was electroporated into *S. pyogenes* SF370 following the transformation protocol of Kimoto *et al.* (Kimoto and Taketo 2003). Transformants were selected on proteose peptone blood agar supplemented with erythromycin. Allelic replacement was confirmed by both PCR and RT-PCR analyses of total RNA extracted (as described above) from both mid-logarithmic (A_{600} 0.4) and stationary phase (A_{600} 1.0) bacterial cultures using gene specific primers. Total RNA from strain SF370 served as control. The resulting strain, SF370Δ*speH,* lacked the *speH* gene (Table 8.1).

8.2.5 Isolation of SF370 with a genomic mutation conferring streptomycin resistance.

Spontaneous streptomycin-resistant mutants were selected for by serial passage of strain SF370 in brain heart infusion broth (BHI) containing increasing concentrations of the antibiotic (0 – 200 μg/ml). A streptomycin resistant (Sm^R) colony was picked from proteose peptone agar containing 200 μg/ml streptomycin and 4% defibrinated sheep blood. Genomic DNA isolated from this mutant served as a template for the PCR amplification of the *rpsL* gene, in which the amplicon was subsequently purified from a 1% agarose gel using the QIAquick Gel Extraction Kit (Qiagen) and then sequenced. The nucleotide sequences of the mutant *rpsL* gene were compared to the published *rpsL* sequences from both the Wild type SF370 genome and from other types of Sm^R

bacteria, in order to confirm that a mutation in the *rpsL* gene caused the Sm^R phenotype (Funatsu and Wittmann 1972; Finken, Kirschner *et al.* 1993; Ferretti, McShan *et al.* 2001; Martin-Galiano and de la Campa 2003). The resulting Sm^R strain was used in all subsequent bacteriophage deletion experiments and is referred to as wild type or SF370 in these studies.

8.2.6 Construction of bacteriophage counter-selection vector, pFWKR.

To generate the counter-selection vector pFWKR, the pFW13 streptococcal shuttle vector, which contains an *E. coli* origin of replication (*ori*) and a kanamycin resistance gene (*aacA-aphD*) flanked by two multiple cloning sites (MCSI and MCSII) (Podbielski, Zarges *et al.* 1996) was modified to include a WT streptococcal *rpsL* gene between the kanamycin gene and the MCSII. To accomplish this modification, a 676 bp fragment containing both the WT *rpsL* gene and its upstream promoter region was PCR amplified from SF370 genomic using the following primers: rpsLF1; 5'-AGGTTCCACCAGGTATCTAAGTGATGCATTC-3', and rpsLR1; 5'-TTACGACTCATGAGCTCTTATCCCCCTTCTA-3'. The amplicon was digested with restriction enzymes *Mab*I (SibEnzyme) and *Eco*ICRI (Promega), and the pFW13 plasmid was digested with enzymes *Mab*I and *Swa*I. After gel purification of both digestion reactions, the amplicon was ligated into the plasmid. The recombinant vector was transformed into *E. coli* cells, and transformants were selected on LB agar containing kanamycin. Plasmid DNA was isolated

from the kanamycin resistant transformants and sequenced to verify proper insertion of the WT *rpsL* gene into the vector. This plasmid (pFWKR) was used as the backbone to make the rest of the phage counter-selection vectors.

8.2.7 Counter-selection Step 1. Allelic replacement of the *speH* gene using pFWKR-*speH*.

The strategy for the allelic replacement of the *speH* gene (encoded by Φ370.2) essentially followed the protocol described above and by Ryan *et al.* (Ryan, Kirk *et al.* 2007), which was used to replace the *speH* gene in SF370. In these experiments, however, the genomic *speH* gene was replaced with the ((*aacA-aphD*) / *rpsL*^{WT}) cassette contained in the pFWKR-*speH* vector. Briefly, the DNA regions flanking *speH* were separately PCR amplified from the genomic DNA of strain SF370 (662 bp upstream and 888 bp downstream) using the two primer sets described by Ryan *et al.* (Ryan, Kirk *et al.* 2007). The amplicons and pFWKR vector were digested with the corresponding restriction enzymes, gel purified, and the individual upstream and downstream fragments were cloned into the MCSI and MCSII, respectively. The resulting plasmid DNA was PCR amplified and sequenced to confirm the creation of the pFWKR-*speH* vector. The pFWKR-*speH* vector was then electroporated into the SF370 strain following the protocol of Kimoto and Taketo (Kimoto and Taketo 2003), and transformants were selected on proteose peptone blood agar containing kanamycin. Next, kanamycin (Kan^{R}) resistant colonies were transferred to PPB agar containing

kanamycin (250 μg/ml) and streptomycin (200 μg/ml) to verify streptomycin sensitivity, which was conferred by the presence of the WT *rpsL* gene in the replacement cassette. (Kan^R / Sm^S) mutants were then screened by PCR and Southern blot analyses for the proper allelic replacement of the *speH* gene with the ((*aacA-aphD*) / ($rpsL^{WT}$) cassette. The resulting mutant strain CEM1KRΔ*speH* was then used to screen for the loss of the entire Φ370.2 bacteriophage (Table 8.1).

8.2.8 Counter-Selection Step 2. Selection for the loss of the Φ370.2 phage from CEM1KRΔ*speH*.

To allow the bacteria to cure itself of the Φ370.2 phage, strain CEM1KRΔ*speH* was inoculated into antibiotic free BHI and grown overnight at 37°C. The overnight culture was then streaked onto PPB agar containing streptomycin (200 μg/ml), and incubated overnight at 37°C to select for colonies that had lost the counter-selection cassette. As a confirmation, Sm^R colonies were replica plated to proteose peptone blood agar containing kanamycin to identify mutants that had also become Kan^S due to the loss of the (*aacA-aphD*) kanamycin resistance gene of the counter-selection cassette, which further indicated potential loss of bacteriophage. (Sm^R / Kan^S) mutants identified in this screen were then verified for the complete loss of the Φ370.2 phage by PCR (using primer sets that span the bacteriophage attachment sites), Southern blot hybridization analysis (with Φ370.2 specific gene probes), DNA sequence analysis, and PFGE analysis. The

resulting mutant strain, CEM1Δ2, no longer contained the integrated Φ370.2 DNA in the streptococcal chromosome (Table 8.1).

8.2.9 Selection for the loss of the remaining bacteriophage from SF370.

Unless otherwise stated in the results, the counter-selection and loss of the other three integrated bacteriophage elements from the genome of the SF370 strain was selected for and screened by following the two-step protocol described above for the deletion of the Φ370.2 bacteriophage. The ((*aacA-aphD*) / (*rpsL* ^{WT}) cassette was targeted to replace putative virulence factor genes in each of the remaining phage. Adjacent DNA regions (600 bp – 800 bp) from each prophage were PCR amplified and individually inserted into the either side (MCSI and MCSII) of the counter-selection cassette on plasmid pFWKan^{R}. The resulting vectors were individually transformed into SF370, CEM1Δ2, and each subsequent bacteriophage deletion mutant, to insert the counter-selection cassette into the respective bacteriophage. These Kan^{R}/ Sm^{S} recombinant mutants were grown without antibiotics, then were plated to streptomycin plates to select for loss of the associated bacteriophage (Sm^{R} / Kan^{S} phenotype). As described above loss of each phage was confirmed by PCR (using primer sets that span the bacteriophage attachment sites), Southern blot hybridization analysis (with phage specific gene probes, e.g. integrases and virulence factor genes), DNA sequence analysis, and PFGE analysis. In the multiple phage deletion mutants, the experimental order of phage counter-selection and phage

loss always followed: Φ370.2, Φ370.1, Φ370.4, Φand 370.3, first to last respectively. This process was repeated until every permutation of phage loss (15 in all) was obtained, including a strain that was devoid of all major bacteriophage-like DNA sequences named CEM1ΔΦ (Table 8.1).

8.2.10 In vitro pharyngeal cell adherence and internalization assays.

Assays on streptococcal adherence and internalization with human pharyngeal cell line Detroit 562 (D562) were performed as described previously (Ryan, Pancholi *et al.* 2001), with the modifications described in (Ryan, Kirk *et al.* 2007). Briefly streptococcal stock cultures (of both wild type and mutant strains) were pre-incubated at 37 °C for thirty minutes in minimal essential media (MEM) without serum. Aliquots of streptococcal stock cultures (1 ml containing 1 $x10^7$ CFUs) were added in triplicate to confluent monolayers of D562 cells grown in twenty-four well tissue culture plates (1 $x10^5$ D562 cells / well for an M.O.I. of 100). Co-cultures were incubated for 2.5 hours at 37 °C plus 5% CO_2 and then monolayers were washed three times with PBS (1 ml/well/wash) to remove associated (non-adherent) streptococci.

Internalization assay wells were treated with 1ml of MEM containing 100 μg/ml penicillin and 600 μg/ml of gentamicin. Aliquots of MEM without antibiotics were added to wells used to assay adherent bacteria. Plates were incubated for one hour at 37°C to allow sufficient time for the antibiotics in the internalization wells

to kill any non-internalized bacteria. Wells were then treated with 100 μl of 0.25% trysin-0.02% EDTA for 15 min at 37 °C to desorb the eukaryotic monolayer and streptococci from the tissue culture wells. Next 400 ul of 0.025% Triton X-100, containing 100 units of Benzonase Nuclease (Novagen), in PBS at 4 °C was added to the wells and incubated at room temperature for five minutes to lyse the pharyngeal cells and release adherent and internalized bacteria. Aliquots of the cell suspensions were then serially diluted and plated to Colombia blood agar plates. Plates were incubated at 37°C plus 5% CO_2 overnight and streptococcal colonies enumerated the next day.

The number of adherent streptococcal cells was calculated as the difference between the average number of colony forming units (CFU) from the adherence assay wells and the average number of CFU from the internalization assay wells. For each comparison the results from three independent experiments were tabulated and then averaged for each isolate. Both data sets were expressed as the percentage of cells from the bacterial inoculum that adhered to the pharyngeal cells or were internalized by the pharyngeal cells.

8.2.11 Comparison of DNase activity in bacteriophage cured strains.

Overnight cultures of SF370 and phage cured mutants were streaked onto DNase Test Agar with Methyl Green (Difco). After overnight incubation at 37°C, the size of clearing zones surrounding colonies (which indicated DNase activity

on the DNA-methyl green substrate in the agar) were compared for qualitative DNAse activity of each strain.

8.2.12 RNA isolation for RT-PCR.

S. pyogenes strain SF370 and the Φ370.1 phage knockout mutant (CEM1Δ1) were grown to early log-phase (A_{600} 0.25), mid-log phase (A_{600} 0.5) and late log-phase (A_{600} 0.7) in Brain Heart Infusion broth as described above. Recovered streptococci were washed three times in PBS. PlyC lysin was added to the bacterial samples (2U / $1x10^8$ CFU) and incubated for 15 min at room temperature, which preliminary experiments indicated to be optimum for complete streptococcal lysis (Nelson, Schuch *et al.* 2006). Total RNA was isolated immediately after lysis using the PureLink Total RNA Purification System (Invitrogen). RNA was digested with TURBO™ DNase I (Ambion Applied Biosystems) following the manufacturer's instructions and quantitated using the Nanodrop UV-Vis Spectrophotometer (Thermo Fisher Scientific).

8.2.13 RT-PCR of *pepD* RNA transcript.

Forward (F) and reverse (R) primers to amplify the *pepD* transcript from the genomes of SF370 (PepD-SF370-F and -R) and CEM1Δ1 phage knockout mutant (PepD-KO-F and –R) were constructed. Four different forward primers (PepD-KO-F1, 2, 3 and 4) were constructed for the Φ370.1 knock-out mutant, to

represent the primer associated with the ATG start codon (F1) as well as three additional primers (F2, F3 and F4) to amplify the RNA transcript beginning at three different sites upstream of the start codon. The primer sequences are as follows: PepD-S370-F (5'-ATGGATAAAAAAATACAGCG-3') and PepD-SF370-R (5'-AAACGATTGGTCATTAGATT); PepD-KO-F1 (5'-ATGGCATGTACAACTATACTC-3'); PepD-KO-F2 (5'-GACTGATAATGGCATGTAC-3') PepD-KO-F3 (5'-GACTGATAATGGCATGTACAAC-3'); PepD-KO-F4 (5'-TAACGTTTGTAAAGGAGACTG-3'); PepD-KO-R (5'-AAACGATTGGTCATTAGATT-3'). The generation of amplicons by RT-PCR was performed with the SuperScript III One-Step RT-PCR system with Platinum *Taq* DNA polymerase (Invitrogen) in reaction mixtures (50 μl) containing 0.2 μM of each gene specific forward and reverse primer and 0.5 μg of DNase-treated, purified total RNA. All remaining components were added as per manufacturer specifications. We included control reactions, in which *Taq* DNA polymerase was substituted for the reverse transcription enzyme mixture, to confirm that genomic DNA was not present in the RNA preparations. RNA was converted to cDNA (48-50°C depending on the primer set, for 30 min), which was then PCR amplified in the same tube (45 cycles of the following conditions: 94°C for 15 sec, 52°C for 30 sec, and 68°C for 2 min). Resulting DNA fragments were separated on 1% agarose gels in TAE buffer and visualized by ethidium bromide staining.

8.2.14 Responses of CEM1Δ4 and SF370 to UV light irradiation.

Bacteria with defects in RuvA have increased sensitivity to UV irradiation (Iwasaki, Shiba *et al.* 1989). To assess such increased sensitivity in the Φ370.4 KO mutant, CEM1Δ4, both this mutant and strain SF370 were grown overnight in THY broth at 37° C. After dilution in fresh THY to an A_{600} 0.05, cells were harvested at mid-logarithmic phase (A_{600} 0.3-0.4) and stationary phase (A_{600} > 0.6). The latter time point coincides with the integrative state of the Φ370.4 prophage (Scott, Thompson-Mayberry *et al.* 2008). The cells were harvested by centrifugation (6,000 x g, 5 minutes) and resuspended in sterile 0.1 M $MgSO_4$ to a final absorbance of 0.5 at 600 nm. A calibrated 254 nm germicidal lamp (120 $\mu W/cm^2$) was pre-warmed for 30 minutes prior to strain exposure. For each strain, 10-fold serial dilutions of each were made in 0.1 M $MgSO_4$ and 2 μl of each dilution was spotted in multiple columns to proteose peptone agar. Since a homolog of photolyase is present in the *S. pyogenes* genome, the UV irradiation was carried out in a darkened room. Cell dilutions were exposed to UV light for increasing periods of time (30, 60, 90, or 120 seconds), and then the plate was incubated in the dark at 37° C for 24 hours. Comparisons between SF370 and the CEM1Δ4 mutant were based on the highest dilutions of each strain that contained multiple surviving CFUs.

8.2.15 Sensitivity of CEM1Δ4 and SF370 to Ethidium Bromide Killing.

Strains were grown overnight in THY broth at 37°C. After dilution in fresh THY to an A_{600} 0.05, cells were grown to early-logarithmic phase (A_{600} 0.15-0.2), mid-logarithmic phase (A_{600} 0.3), or stationary phase ($A_{600} > 0.6$) to account for the different integrative states of the Φ370.4 prophage (Scott, Thompson-Mayberry *et al.* 2008). Cultures were then divided and ethidium bromide (EtBr) added in increasing final concentrations (0μM, 1.25μM, 2.5μM, 5.0μM, 7.5μM, 10μM and 20μM). Bacteria were incubated overnight at 37°C then plated to proteose peptone blood agar plates to enumerate surviving CFUs from each growth phase and EtBr concentration. Results were tabulated as the average of three independent experiments and were expressed as the percentage of CFU that survived treatment compared to the control without EtBr.

8.2.16 Mitomycin C induction of prophage.

Mitomycin C induction of prophage was performed by using a modification from McShan et al. (McShan, Tang et al. 1997). Briefly, a single colony of SF370 (Sm^S) or CEM1ΔΦ was picked from proteose peptone plates supplemented with 5% sheep blood and used to inoculate a 10 ml aliquot of BHI, which was subsequently incubated overnight at 37°C. The overnight culture was diluted 1:30 (v/v) into 100 ml of pre-warmed BHI at 37°C, and the culture was incubated at 37°C until early logarithmic growth (A_{600} 0.2). This culture was divided into two 50-ml cultures, and one cultures received mitomycin C (Sigma) at a final

concentration of 0.2 μg/ml. The cultures were incubated at 37°C and after two hours the absorbance readings for the mitomycin C induced culture of SF370 began to drop (this effect was absent in the non-induced cultures and the mitomycin C treated CEM1ΔΦ culture). Cells were pelleted by centrifugation at 4,000 rpm for 10 min at 4°C. Supernatants were collected and filtered twice through 0.22 μm filters. Filtrates (15 mls x 2) were applied separately to two 50,000 MWCO Amicon Ultra-15 centrifugal filter devices (Millipore), which were spun at 4,000 rpm at 25°C for 45 minutes to achieve a final volume of 500 μl in each concentrator. Next, 10 mls of prophage suspension buffer (0.15 M NaCl, 10 mM Tris HCl [pH 7.5], 5 mM $MgCl_2$, 1 mM $CaCl_2$) described in (Vlaminckx, Schuren *et al.* 2007), was added to the concentrators, which were then centrifuged at 4,000 rpm at 25°C for 45 minutes to again achieve a final volume of 500 μl in the concentrator. The concentrated supernatants were stored at 4°C until further use.

8.2.17 Analysis of bacteriophage plaques and selection for lysogens.

As potential indicator strains, SF370, CEM1ΔΦ, and the single phage KO mutants from (Table 8.1) were grown overnight at 37°C in BHI containing 200 μg/ml of streptomycin. Aliquots (100 ul) of the overnight cultures were added to 3 ml of melted BHI soft agar (7.5%) at 50°C, mixed, and poured on top of proteose peptone agar plates. Plates were allowed to dry and then 10 μl serial dilutions (in prophage suspension buffer) of the concentrated mitomycin C supernatants

(described above) were spotted onto the plates. As a control for bacterial contamination, supernatants were also spotted to proteose peptone agar plates alone. After allowing sufficient time for the spots to dry, the plates were incubated overnight at 37°C plus 5% CO_2. The following day, the plates were analyzed for growth inhibition or bacteriophage plaques in the location of the spots and any bacterial growth in the spots from the non-overlaid plates. If plaques or growth inhibition was observed, colonies in or around the edge of these areas were picked and streaked for single colonies on proteose peptone blood agar plates containing 200 μg of streptomycin to inhibit potential contaminating bacteria. After overnight incubation at 37°C plus 5% CO_2, streptomycin resistant colonies were screened for potential lysogeny by PCR analysis using primers specific for bacteriophage genes from each of the four prophage of SF370. Colonies that were positive by this initial PCR screen for phage genes were further screened by PCR with primers specific for phage attachment sites to ascertain proper phage integration. Potential lysogen genomic DNA was subjected to PFGE analysis as described above.

8.3 RESULTS

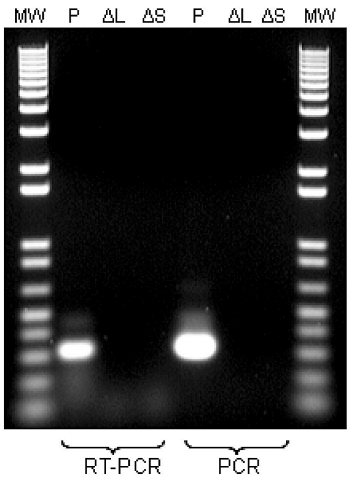
8.3.1 Effect of SpeH on GrAS interactions with pharyngeal cells.

While analyzing the global transcriptional response of *S. pyogenes* strain SF370 during *in vitro* adherence to Detroit 562 human pharyngeal epithelial cells, we found eleven prophage genes that were differentially expressed with significance

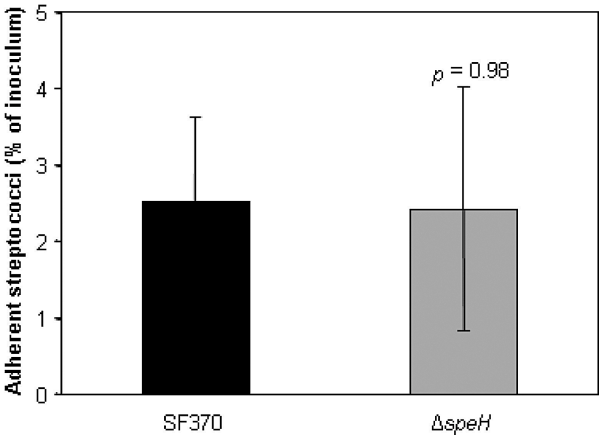
during adherence as compared to non-adherent (associated) bacteria (Ryan, Kirk *et al.* 2007). These eleven genes were localized to the defective bacteriophage Φ370.2, suggesting that this defective phage is not transcriptionally silent (Ferretti, McShan *et al.* 2001; Ryan, Kirk *et al.* 2007). One of the phage genes, *speH*, was induced, while the remaining ten phages genes (located in the phage replication and regulation modules) were down-regulated during cell adherence. The *speH* gene encodes a functional superantigen (Proft, Moffatt *et al*. 1999) reportedly induced in other strains of GrAS during polymorphonuclear leukocyte phagocytosis (Voyich, Sturdevant *et al.* 2003) but has not been implicated previously in adherence.

To determine if SpeH plays a direct role in the initial events of GrAS colonization, a deletion mutant (SF370Δ*speH*) was created by replacing the *speH* gene in SF370 with an erythromycin resistance gene (*ermAM*). The absence of SpeH was confirmed by PCR (not shown) and RT-PCR (Figure 8.1). The ability of SF370Δ*speH* to adhere to and be internalized by human pharyngeal cells was tested in a number of *in vitro* co-culture assay experiments (Ryan, Kirk *et al.* 2007). There was no significant difference in adherence or internalization between the wild type (SF370) and SF370Δ*speH* (Figure 8.1).

A


MW P ΔL ΔS P ΔL ΔS MW
RT-PCR
PCR

B


Adherent streptococci (% of inoculum)
0
1
2
3
4
5
p = 0.98
SF370
ΔspeH

C

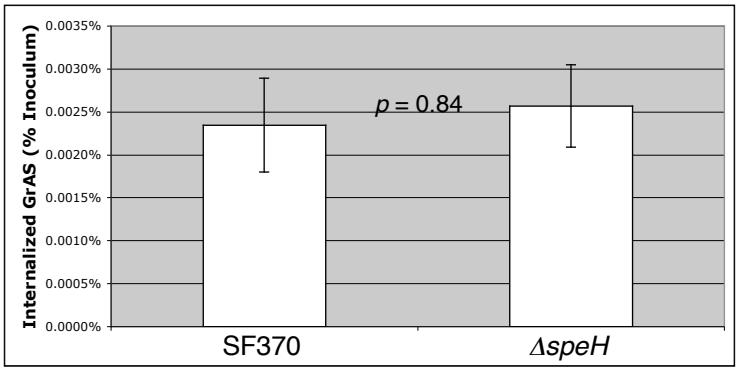

Internalized GrAS (% Inoculum)
0.0035%
0.0030%
0.0025%
0.0020%
0.0015%
0.0010%
0.0005%
0.0000%
p = 0.84
SF370
ΔspeH

Figure 8.1. Confirmation of *speH* deletion mutant and pharyngeal cell adherence and internalization assays.

(A) Results of RT-PCR and PCR analyses of total RNA preparations isolated from mid-log (A_{600} 0.4) and stationary phase (A_{600} 1.0) cultures of the Δ*speH* deletion mutant (ΔL and ΔS, respectively), and stationary phase cultures of the SF370 parental strain (P). RNA was reverse-transcribed as described in Methods. To assess genomic DNA contamination, control reactions containing Taq DNA polymerase instead of reverse transcriptase were included. The cDNA products were separated on a 1% agarose gel and visualized by ethidium bromide staining. Lanes containing products from either the RT-PCR or PCR analysis are designated at the bottom of the panel. Lanes labeled MW contain 1 kb Plus DNA ladder (Invitrogen). (B) Results of the pharyngeal cell adherence and, (C) internalization assays (detailed in Methods), comparing parental strain SF370 with the deletion mutant SF370Δ*speH* (abbreviated Δ*speH*). Adherent and internalized streptococci are reported as the percentage of total number of streptococci added as inoculum to pharyngeal cell monolayers. Statistical significance (reported as *p* value) was determined by Student's *t*-test.

8.3.2 Loss of the Φ370.2 bacteriophage from SF370.

The Φ370.2 phage encodes three virulence factors, a hyaluronidase (hlyP1) and two superantigen genes *speH* and *speI*. The phage is integrated into the 3' end of a dTDP-glucose-4,6-dyhydrase leaving the gene intact. As a proof of principle for our phage counter-selection technique, we first chose to eliminate the Φ370.2 prophage sequence from the SF370 genome to assess the role of *speH* and the other ten Φ370.2 genes differentially expressed during GrAS adherence to pharyngeal cells (Ryan, Kirk *et al.* 2007). Our first step was to isolate a mutant strain of SF370 that had become spontaneously resistant to streptomycin. DNA sequence analysis of the *rpsL* region in the genome of this mutant showed a single point mutation in the *rpsL* gene (lysine 56 to arginine (K56R)), which was consistent with other *rpsL* mutations that confer streptomycin resistance in different bacteria species (Funatsu and Wittmann 1972; Finken, Kirschner *et al.* 1993; Martin-Galiano and de la Campa 2003). This streptomycin resistant mutant was transformed with the counter-selection vector, pFWKR-*speH*, to replace the *speH* gene and create mutant CEM1KRΔ*speH* (Table 8.1). This mutant was then passed in media without antibiotics and plated on streptomycin agar plates to select for a recombinational event that resulted in the loss of the Φ370.2 prophage. Confirmation of the entire Φ370.2 DNA loss and absence of gross chromosomal alterations were confirmed by sequencing PCR products generated across the *attB* phage integration site and PFGE analysis (Figure 8.2).

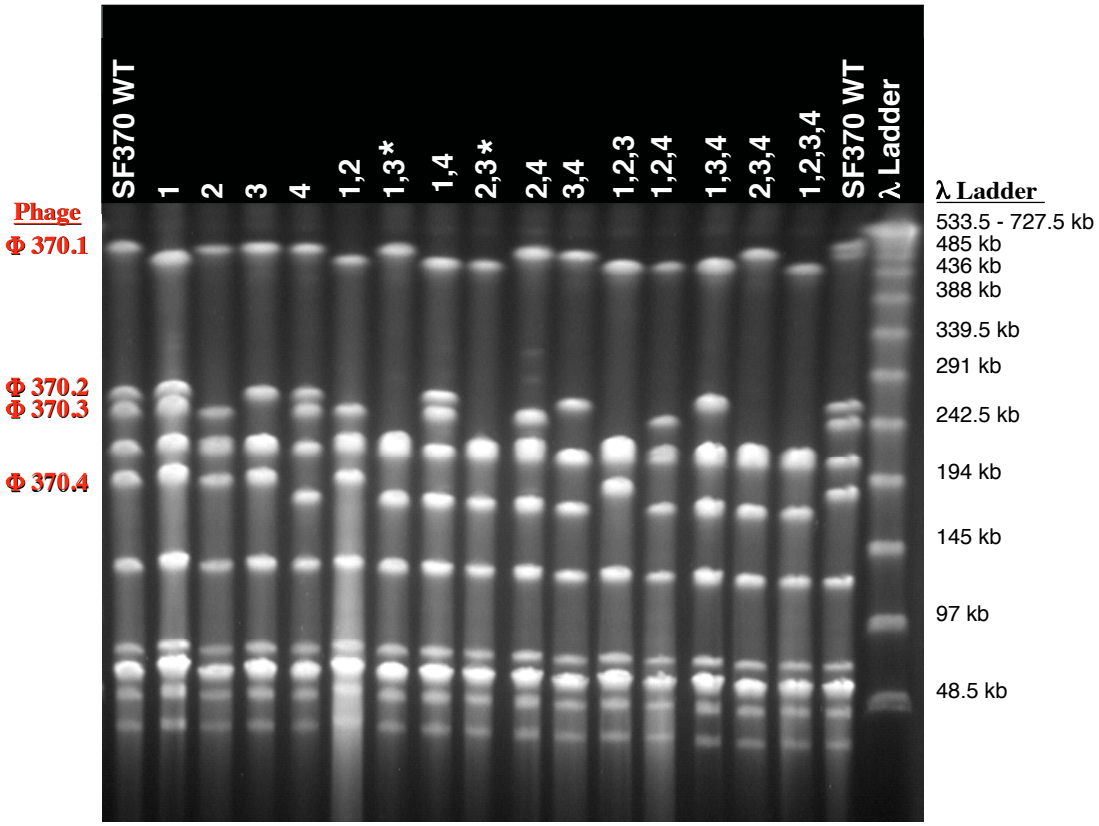

SF370 WT
1
2
3
4
1,2
1,3*
1,4
2,3*
2,4
3,4
1,2,3
1,2,4
1,3,4
2,3,4
1,2,3,4
SF370 WT
λ Ladder
Phage
Φ 370.1
Φ 370.2
Φ 370.3
Φ 370.4
λ Ladder
533.5 - 727.5 kb
485 kb
436 kb
388 kb
339.5 kb
291 kb
242.5 kb
194 kb
145 kb
97 kb
48.5 kb

Figure 8.2. Pulse field gel electrophoresis (PFGE) analysis of *S. pyogenes* SF370 phage deletion mutants.

PFGE patterns of *Sma*I digested genomic DNA from the wild type (WT) SF370 strain and fifteen different permutations of phage deletion mutants are shown. The DNA bands representing each individual (intact) phage genome in SF370 are indicated in red on the left side of the figure. The lanes corresponding to each phage deletion mutant is indicated at the top of the figure. The specific phages deleted in each mutant are indicated with numbers (1-4), which corresponding to the individual phages in SF370 (Φ370.1-4). The absence or change in size of a phage genome band represents a phage deletion. The asterisks (*) indicate incorrect phage banding patterns, which were found to be correct on additional PFGE gels (data not shown). λ, indicates the Lambda Ladder PFG Marker, and DNA fragment sizes are indicated in the right column (New England Biolabs).

The resulting mutant strain, CEM1Δ2 (Table 8.1), lacked the integrated Φ370.2 DNA and recapitulated the *attB* bacterial attachment site seen in the genome sequence of the modern M1 *S. pyogenes* strain, MGAS5005, which does not contain bacteriophage integrated into the homologous genome location (Data not shown) (Sumby, Porcella *et al.* 2005). Gross colony morphology and growth curve comparisons of CEM1Δ2 versus SF370 showed no difference in colony morphology or growth rate between the phage deletion mutant and the wild type strain (data not shown and Figure 8.3).

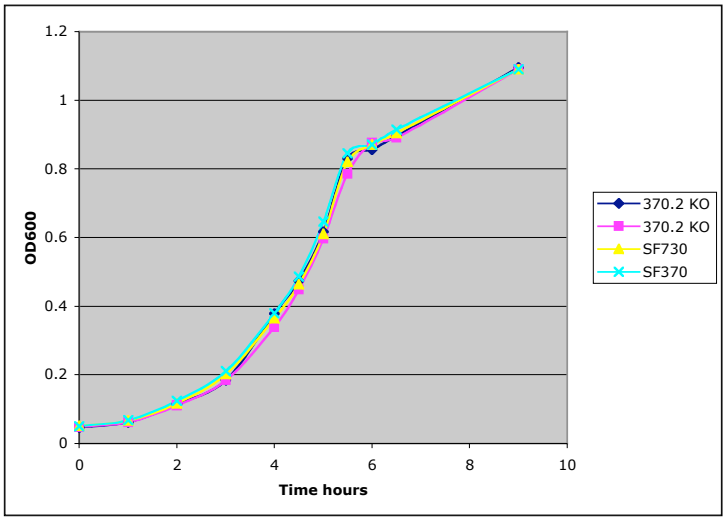


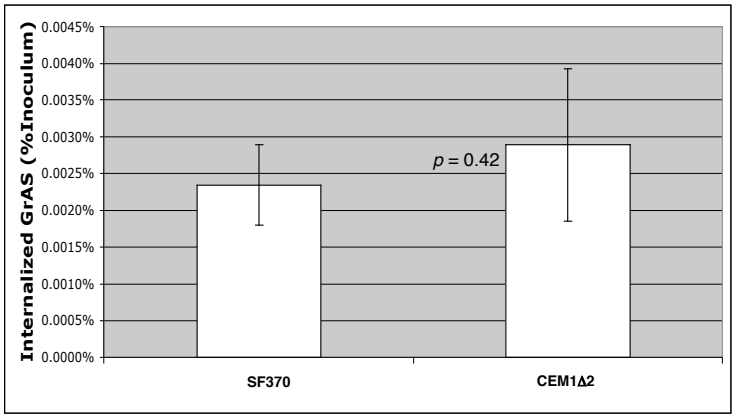
Figure 8.3. Growth curve comparisons of two different cultures of the wild type SF370 and the Φ370.2 phage deletion mutant (370.2 KO).

Overnight cultures were diluted 1:50 in 10 ml of pre-warmed BHI and incubated at 37°C. Culture absorbance (A_{600}) was measured at varying time points using a spectronic 20D spectrophotometer (Thermo Electron Corporation) and the results were plotted.

8.3.3 Analysis of Φ370.2 in GrAS interactions with pharyngeal cells.

To assess the role of the Φ370.2 genes products, including the 10 ORFs on Φ370.2 previously identified as down regulated during streptococcal adherence to human pharyngeal cells (Ryan, Kirk *et al.* 2007), CEM1Δ2 was compared to SF370 in an *in vitro* co-culture assay described above. As shown in (Figure 8.4), the lack of the Φ370.2 genome in CEM1Δ2 had no significant effect on either GrAS adherence to, or subsequent internalization by, pharyngeal epithelial cells.

A


Internalized GrAS (%Inoculum)
0.0045%
0.0040%
0.0035%
0.0030%
0.0025%
0.0020%
0.0015%
0.0010%
0.0005%
0.0000%
p = 0.42
SF370
CEM1Δ2

B

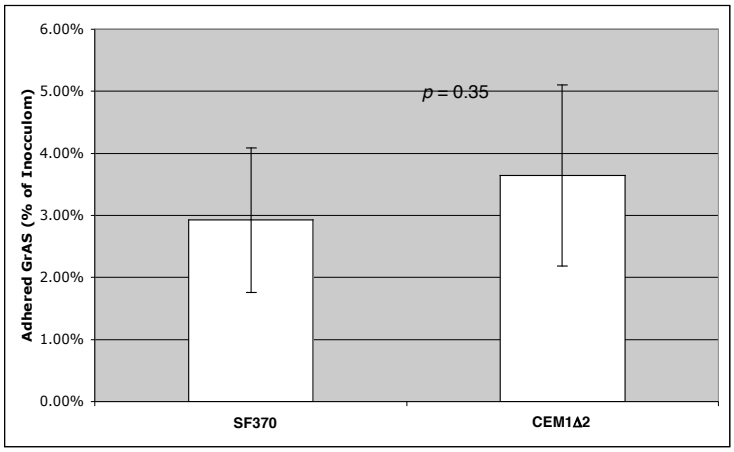

Adhered GrAS (% of Inocculom)
6.00%
5.00%
4.00%
3.00%
2.00%
1.00%
0.00%
p = 0.35
SF370
CEM1Δ2

Figure 8.4. Pharyngeal cell adherence and internalization assays with Φ370.2 KO mutant (CEM1Δ2).

(A) Results of the pharyngeal cell adherence and (B) internalization assays (detailed in Methods), comparing parental strain SF370 with the Φ370.2 KO phage deletion mutant (CEM1Δ2). Adherent and internalized streptococci are reported as the percentage of total number of streptococci added as inoculum to pharyngeal cell monolayers. Statistical significance (reported as *p* value) was determined by Student's *t*-test.

8.3.4 Loss of the Φ370.1 bacteriophage from SF370.

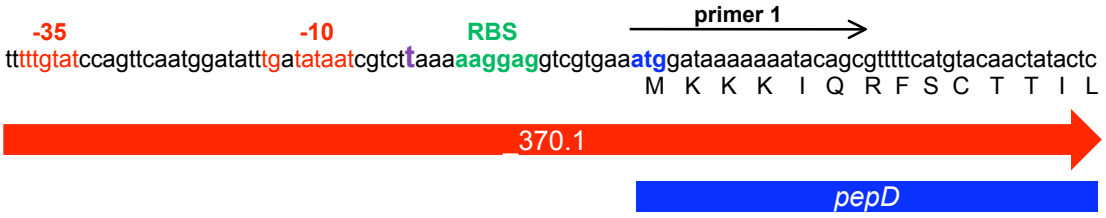
The mitomycin C inducible prophage Φ370.1 encodes three putative virulence factors, a hyaluronidase gene (*hlp1*) and a two-gene cassette containing the *speC* superantigen gene and the *spd1* DNase gene. To select for loss of the Φ370.1 phage, the *speC* gene was replaced with the counter-selection cassette to create mutant PFWKRΔ*speC*. This mutant was passaged in BHI media and plated to streptomycin agar plates. Sm^R/ Kan^S colonies were screened as above and PFGE analysis confirmed that Φ370.1 was lost and that there were no aberrant recombination events for the mutant CEM1KRΔ1 (Figure 8.2 and Table 8.1). Using this procedure Φ370.1 was also cured from CEM1KRΔ2 to make the Φ370.1and Φ370.2 double phage deletion mutant CEM1KRΔ1,2 (Figure 8.2 and Table 8.1).

8.3.5 Expression of the peptidase gene, *pepD,* from an alternate bacteriophage promoter.

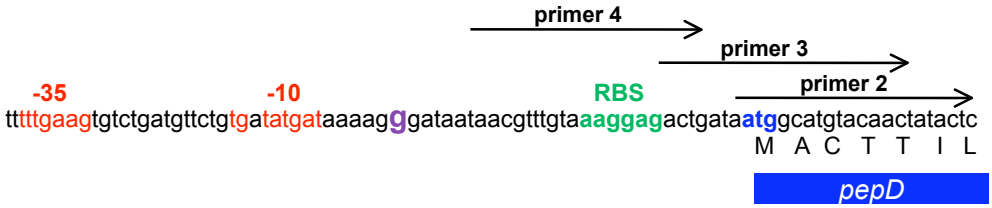
Prophage Φ370.1 is integrated into the 5' end of the dipeptidase gene (*pepD*) replacing the first nine N-terminal amino acids and the upstream promoter sequence with bacteriophage-specific DNA (Figure 8.5) (Canchaya, Desiere *et al.* 2002). This suggests that Φ370.1 integration may affect the expression of the *pepD* gene or overall activity of the peptidase. In a preliminary analysis, RT-PCR

was used to screen the expression profiles of *pepD* during growth of the SF370 wild type (WT) strain and the Φ370.1 deleted mutant, CEM1Δ1. Using primers specific for either the WT type *pepD* transcript or the phage KO *pepD,* each was individually tested for expression from their respective cDNA during early-log, mid-log, and late-log phases of growth. As shown in (Figure 8.5) *pepD* is expressed during all growth phases in both the SF370 and CEM1Δ1, suggesting that the Φ370.1 specific DNA sequences in the SF370 wild type strain also contain a functional promoter sequence for *pepD*. To further explore the dynamics of Φ370.1 integration, excision, and pepD expression, we performed RT-PCR on the cDNA of the SF370 wild type strain using primers specific for the upstream region of *pepD* when the Φ370.1 phage is deleted or excised. Results showed that both transcripts of *pepD* (when phage Φ370.1 is integrated in the genome and when it is excised) are expressed in SF370 during all phases of growth in (Figure 8.5). This suggests that Φ370.1 is excising from the genome in a certain population of SF370 cells throughout the bacterial growth cycle.

A. *pepD* genomic region:SF370



B. *pepD* genomic region: CEM1Δ1 = Φ370.1KO



C.

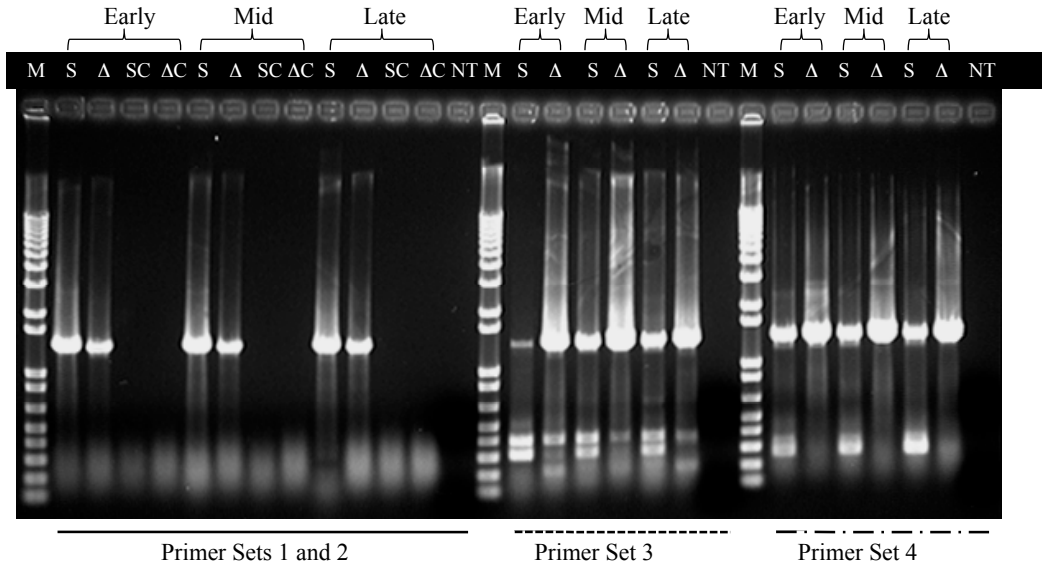


Figure 8.5. RT-PCR analysis of the *pepD* transcript in SF370 and phage knock-out mutant.

Panels (A) and (B) are schematic representations of the *pepD* genomic region in SF370 and the phage knock-out mutant, respectively. Putative promoter regions (-35 and -10 sites) are designated and the putative transcriptional start site is labeled in large, bold purple font. Both regions were predicted by programs available for prokaryotic genomes on the Berkeley Drosophila Genome Project website (www.fruitfly.org). The predicted Shine-Delgarno ribosomal binding site (RBS) was determined as described previously (Ryan, Kirk et al., 2007). Start codons (ATG) are appropriately labeled. The translated amino acids are listed below the sequence. The red block arrow shows the region encoded on bacteriophage Φ370.1 and the blue rectangle shows the ORF of the *pepD* gene in both genomes. Positions of the Forward (5') primers for RT-PCR are indicated by arrows above nucleotide sequences.

Panel (C) shows the results of RT-PCR analysis on mRNA from WT SF370 (S) and the full-phage knock out mutant (Δ). mRNA was isolated from early-log phase (Early), mid-log phase (Mid) or late-log phase (Late) cells (indicated above panel) and reverse transcribed with various primer combinations, which are indicated below the panel as primer sets 1, 2, 3 and 4. Primer set 1 (PepD-SF370-F and -R) was used to amplify SF370 RNA from all growth stages. Primer set 2 (PepD-KO-F1 and PepD-KO-R), set 3 (PepD-KO-F3 and PepD-KO-R) and set 4 (PepD-KO-F4and PepD-KO-R) were used to amplify RNA from the knock-out mutant at different sites along the transcript. The 5' forward primer was different in each such primer set, while the 3' reverse primer remained the same. Primer sequences are listed in Methods. cDNA products were separated on a 1% agarose gel and visualized by ethidium bromide staining. The expected sizes of resulting cDNAs from SF370 using primer set 1 is 1403 bp, primer set 2 is 1382 bp, primer set 3 is 1390 bp, and primer set 4 is 1406 bp. Control reactions (C) containing mRNA and Taq DNA polymerase instead of reverse transcriptase are indicated. Additional controls (NT) containing primers alone with no template are indicated. Lane M contains 1-kb Plus DNA ladder (1 μg, Invitrogen).

8.3.6 Loss of the Φ370.4 bacteriophage from SF370.

Prophage Φ370.4 is integrated between the *mutS* gene and the 5' region of the *mutL* gene in a methyl-directed mismatch repair (MMR) operon that includes several other DNA repair genes. The dynamic nature of prophage Φ370.4 excision, replication, and reintegration in response to growth was previously described by Scott et al. (Figure 8.6) (Scott, Thompson-Mayberry et al. 2008).

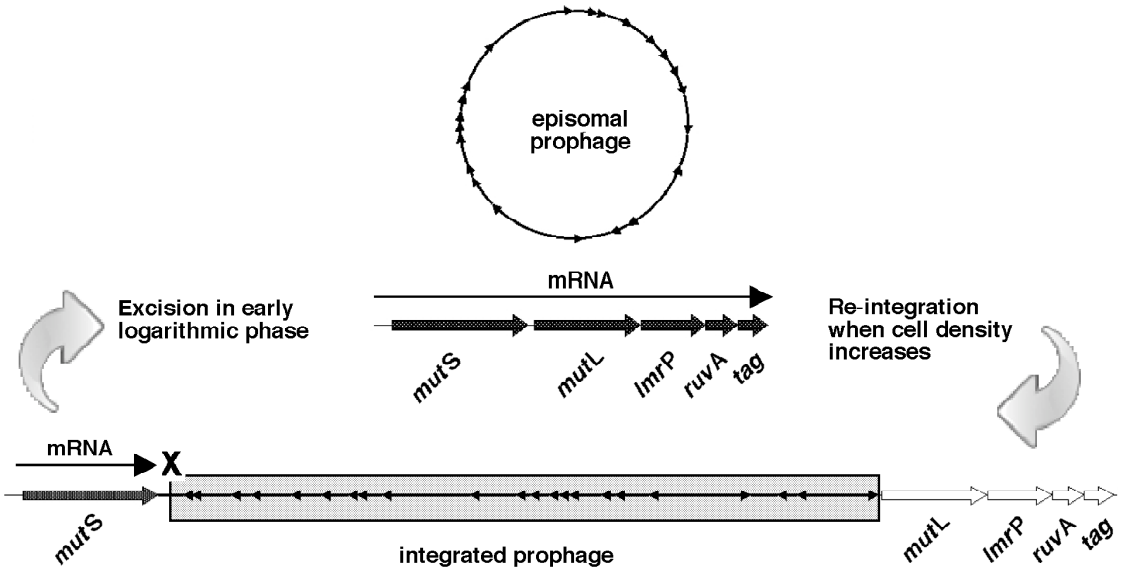


Figure 8.6. Prophage regulation of the MMR operon in GAS.

Integration of prophage SF370.4 separates *mutL*, *lmrP, ruvA,* and *tag* from the promoter, which is upstream of *mutS*, and prevents expression of these genes. Loss of *mutL* inactivates MMR, allowing mutations to accumulate, resulting in a mutator phenotype. Loss of *ruvA* expression leaves cells unable to repair DNA damage from shortwave UV light, increasing cell sensitivity to UV irradiation. Phage excision from the host chromosome restores transcription of the polycistronic message from *mutS* to *tag*. The excised phage circularizes and replicates in host cell as an episome. Prophage Φ370.4 is defective and does not contain genes for phage particle production or cell lysis. Abbreviations: *mut*S and *mutL* = DNA mismatch repair proteins, *lmrP* = multidrug resistance transporter, tag = base excision repair glycosylase, *ruvA* = Holliday Junction DNA helicase. Figure was designed by Michael McShan, and reproduced with permission.

By applying our counter-selection method we hoped to cure SF370 of prophage Φ370.4 to better measure the phenotypes under the dynamic control of this phage. Since the Φ370.4 does not encode a putative virulence factor, we chose to insert the counter-selection cassette in place of the phage primase gene (*Spy2136*) to create the CEM1KRΔ*Spy2136* mutant (Table 8.1). Deletion of the primase gene was thought to interfere with replication of the excised circular form of Φ370.4, potentially promoting loss of the phage during cell division. When this mutant was grown without antibiotics overnight and plated to proteose peptone blood agar containing streptomycin (to select for isolates that had lost the Φ370.4 prophage) all of the resistant colonies that grew (> 500 CFU) were Sm^R/ Kan^R, suggesting a rate of recombination or mutation in the *rpsL* ^{WT} that was signifigantly higher than the loss of the Φ370.4 prophage (Sung, Li *et al.* 2001).

To further increase the odds of isolating a cured mutant, CEM1KRΔ*Spy2136* was plated directly to streptomycin plates from antibiotic free cultures at multiple times during early exponential growth (A_{600} of 0.1, 0.2, 0.3, 0.4) since this when Φ370.4 was shown to excise from the chromosome (Scott, Thompson-Mayberry *et al.* 2008). All the resulting streptomycin resistant colonies screened (>1000 CFU) were also Sm^R/ Kan^R. This confirmed previous results (using recombinational and conventional methods) that Φ370.4 was difficult to cure from SF370 (McShan, Personal communication). Curing of 370.4 was finally achieved by growing CEM1KRΔ*Spy2136* in BHI overnight at an increased temperature of

42°C and plating to streptomycin plates, following an adapted protocol from (Verheust, Fornelos *et al.* 2005). Of the 650 streptomycin resistant colonies screened, 13 were Sm^R/ Kan^S and all were confirmed to have lost Φ370.4 by PCR, Southern blot, and PFGE analysis with one mutant designated as CEM1Δ4 (Table 8.1 and Figure 8.2). Of note Φ370.4 was cured much easier from the CEM1Δ1, CEM1Δ2, and CEM1Δ1,2 mutants using the normal counter-selection protocol above with growth conditions at 37°C to produce the double and triple phage cured mutants listed in (Table 8.1).

8.3.7 Sensitivity to UV irradiation is associated with phage 370.4.

The operon containing *mutS* and *mutL* is also predicted to contain the downstream gene *ruvA* (Figure 8.6). Transcription of this gene would also be interrupted by the presence of Φ370.4, resulting in increased sensitivity to killing by UV irradiation (Iwasaki, Shiba *et al.* 1989; Scott, Thompson-Mayberry *et al*. 2008). As shown in (Figure 8.7), the SF370 wild-type strain displays increased resistance to killing during exposure to 254-nm irradiation when actively dividing in mid-log phase as compared to stationary phase when Φ370.4 is integrated. Conversely, the mutant strain (CEM1Δ4) lacking Φ370.4 shows an increased resistance to UV light in either phase of growth and is at least 100 times more resistant than stationary SF370 cells at a 30 second UV exposure (data not shown and Figure 8.7).

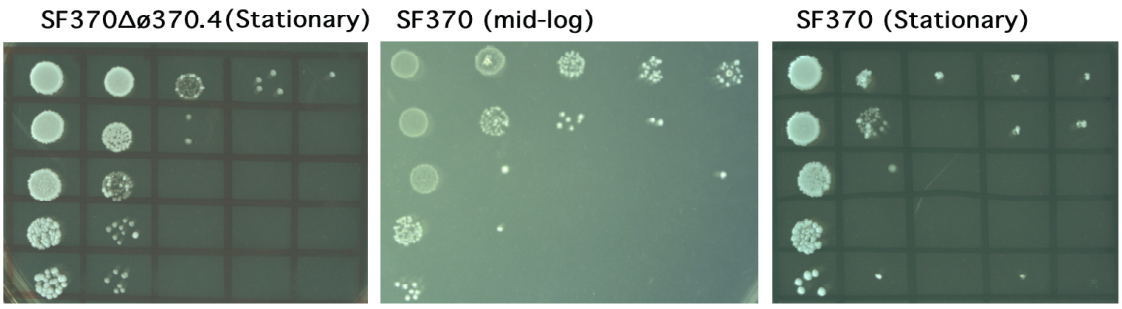


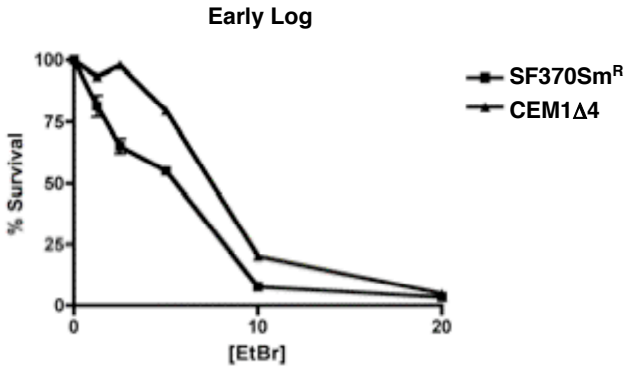
Figure 8.7. Growth dependent UV sensitivity in SF370 is lost when Φ370.4 is deleted.

WT Strain SF370 shows a growth dependent increase in resistance to UV irradiation when the cells are rapidly dividing (mid-log, middle panel) as compared to cells in stationary phase (right panel). The mutant deleted for Φ370.4 by contrast is equally resistant at either phase of growth (left panel and not shown). For each assay, replicates of ten-fold serial dilutions (top to bottom) of cells were spotted onto a THY agar plate and exposed to increasing doses of UV light (0 to 120 seconds, left to right). Prior to dilution, bacterial cells densities (A 600) were equalized.

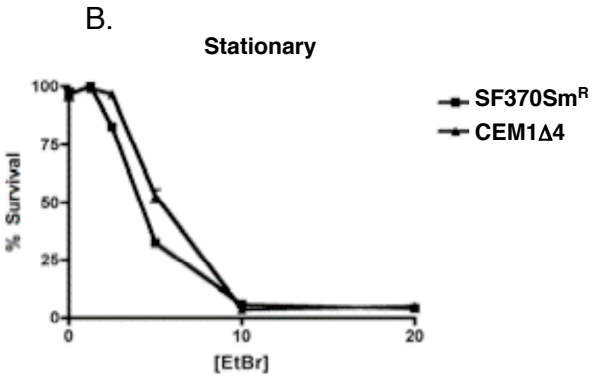
8.3.8 Sensitivity to EtBr is associated with phage Φ370.4.

Encoded in the MMR operon, LmrP is a proton motive force-dependent drug transporter that confers resistance to lipophilic antimicrobial agents, such as ethidium bromide (EtBr) (Bolhuis, Poelarends *et al.* 1995). We found that SF370 is more sensitive to EtBr killing than strain CEM1Δ4 in both early logarithmic growth (A_{600} 0.35) and stationary phases ($A_{600} > 0.6$), although the distinction is less pronounced in stationary phase (Figure 8.8 A, B). The difference in the overall sensitivity to EtBr in the log phase cells of the two strains is significant ($p < 0.01$). Alternatively, at a very early log phase of growth (A_{600} 0.15 - 0.20), when the SF370.4 prophage is thought to excise from the chromosome, the percentage of bacterial survival is almost identical between the SF370 and the CEM1Δ4 (Figure 8.8 C).

A.


Early Log
SF370Sm^R
CEM1Δ4
% Survival
100
75
50
25
0
0
10
20
[EtBr]

B.


Stationary
SF370Sm^R
CEM1Δ4
% Survival
100
75
50
25
0
0
10
20
[EtBr]

C.

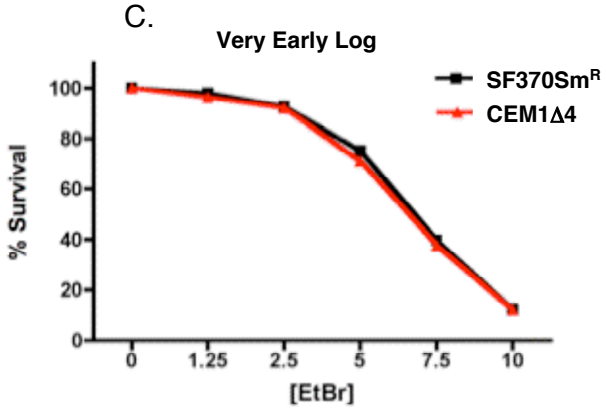

Very Early Log
SF370Sm^R
CEM1Δ4
% Survival
100
80
60
40
20
0
0
1.25
2.5
5
7.5
10
[EtBr]

Figure 8.8. Sensitivity to EtBr killing is associated with presence of Φ370.4.

Cultures of SF370 and CEM1Δ4 were exposed to increasing concentrations of EtBr (0μM, 1.25μM, 2.5μM, 5.0μM, 7.5μM, 10μM and 20μM) for 12 hours at 37°C and then plated to measure CFUs. Results are expressed as the percent survival of the non-treated cultures. The panels represent the results from each growth phase: (A) Early logarithmic growth (A_{600} 0.3 – 0.4); (B) Stationary phase ($A_{600} > 0.6$); (C) Earlier log phase of growth (A_{600} 0.15 - 0.200). The results were tabulated as the average and SD of three replicate experiments. Certain error bars are not visible as SD values were extremely small, indicating good reproducibility. The difference in the overall sensitivity to EtBr in the log phase cells of the two strains is significant ($p < 0.01$).

8.3.9 Curing of prophage Φ370.3 to make the full phage KO CEM1ΔΦ.

Phage 370.3 has a 33 kb genome and contains another DNase gene (*spd3*) and hyaluronidase gene (*hylp3)*. To select for loss of the Φ370.3 phage, the *spd3* gene was chosen for allelic replacement with the counter-selection cassette. Using our two-step allelic replacement technique, we successfully created a knockout mutant of this phage in SF370 to derive strain CEM1Δ3. Of note, the same vector and methods were used to delete Φ370.3 from the triple KO mutant CEM1Δ1,2,4 to derive the full phage KO CEM1ΔΦ (Table 8.1 and Figure 8.2). We examined the basic phenotypes of CEM1ΔΦ compared to the SF370 wild type strain and found no differences in microscopic analysis of cell shape or chain length, colony morphology, blood hemolytic pattern, or in growth characteristics in liquid culture (data not shown and Figure 8.9).

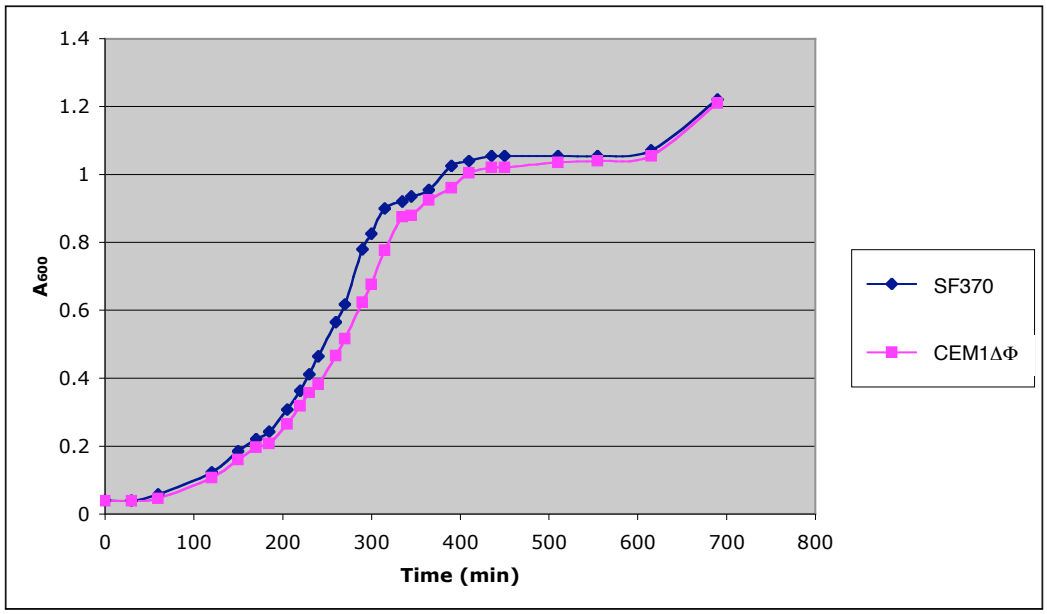

1.4
1.2
1
0.8
0.6
0.4
0.2
0
A600
0
100
200
300
400
500
600
700
800
Time (min)
SF370
CEM1ΔΦ

Figure 8.9. Growth comparison of the wild type SF370 strain and the full phage deletion mutant (CEM1$\Delta\Phi$).

Overnight cultures where diluted 1:50 in 10 ml of pre-warmed BHI and incubated at 37°C. Absorbance of cultures (A_{600}) was measured at different time points using a spectronic 20D spectrophotometer (Thermo Electron Corporation) and the results were plotted.

8.3.10 Comparison of CEM1ΔΦ to SF370 interactions with pharyngeal cells.

To assess if any of the lysogenic bacteriophage of SF370 play a role in streptococcal adherence to, and internalization by, pharyngeal epithelial cells, the fully phage cured strain (CEM1ΔΦ) was compared to SF370 in the co-culture adherence assay. As shown in (Figure 8.10), no significant differences were observed between CEM1ΔΦ and SF370 in adherence to pharyngeal cells or subsequent internalization.

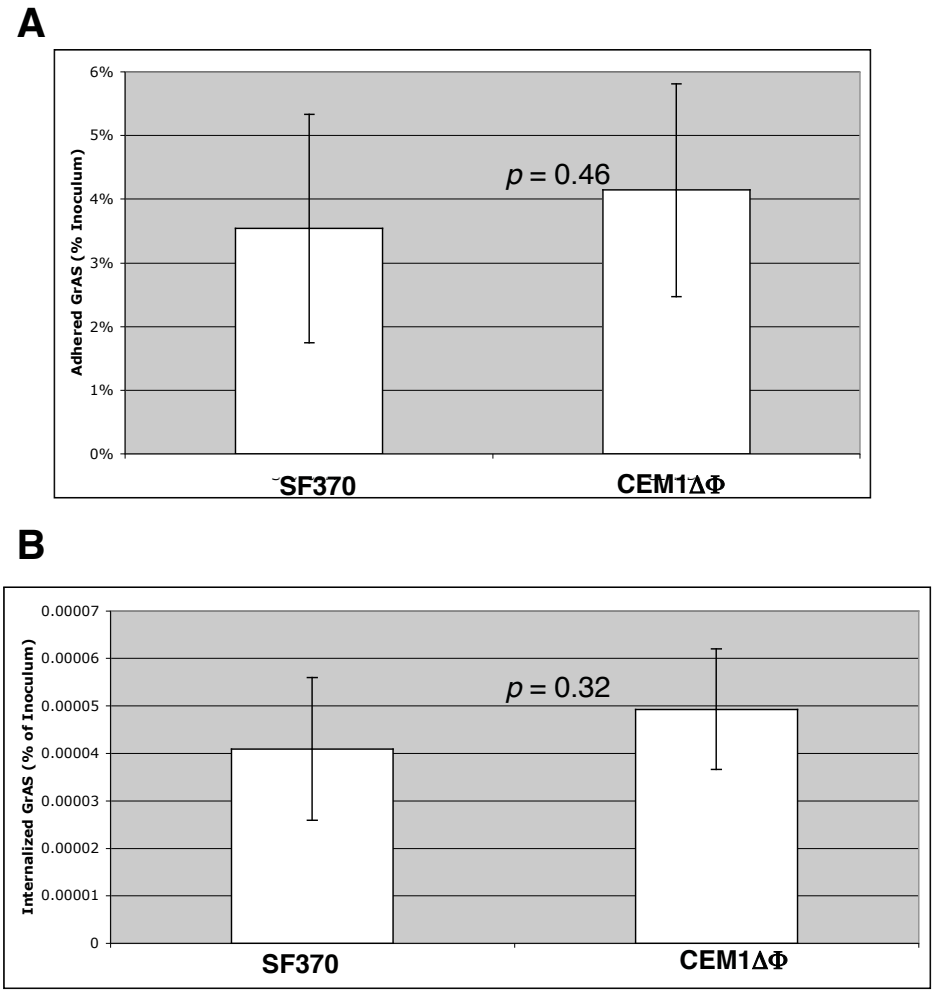


Figure 8.10. Pharyngeal cell adherence and internalization assays on the full phage KO.

(A) Results of the pharyngeal cell adherence and (B) internalization assays (detailed in Methods), comparing parental strain SF370 with the phage free mutant (CEM1ΔΦ). Adherent and internalized streptococci are reported as the percentage of total number of streptococci added as inoculum to pharyngeal cell monolayers. Statistical significance (reported as *p* value) was determined by Student's *t*-test.

8.3.11 Decreased expression of DNase in bacteriophage-free mutant CEM1ΔΦ.

The genome of SF370 contains three genes encoding secreted DNases: one chromosomally encoded (*spd/mf1*) and two phage-encoded (*spd1* on Φ370.1 and *spd3* on Φ370.3). To begin to assess the effects of the phage encoded DNases, the prophage-cured strain CEM1ΔΦ and the wild-type strain SF370 were plated to DNase Test Agar with Methyl Green. After overnight growth, colonies from SF370 produced larger and more distinct clearing zones of DNA lysis than the CEM1ΔΦ colonies (Figure 8.11). This suggests that phage DNases contribute significantly to the total activity of DNases secreted from SF370.

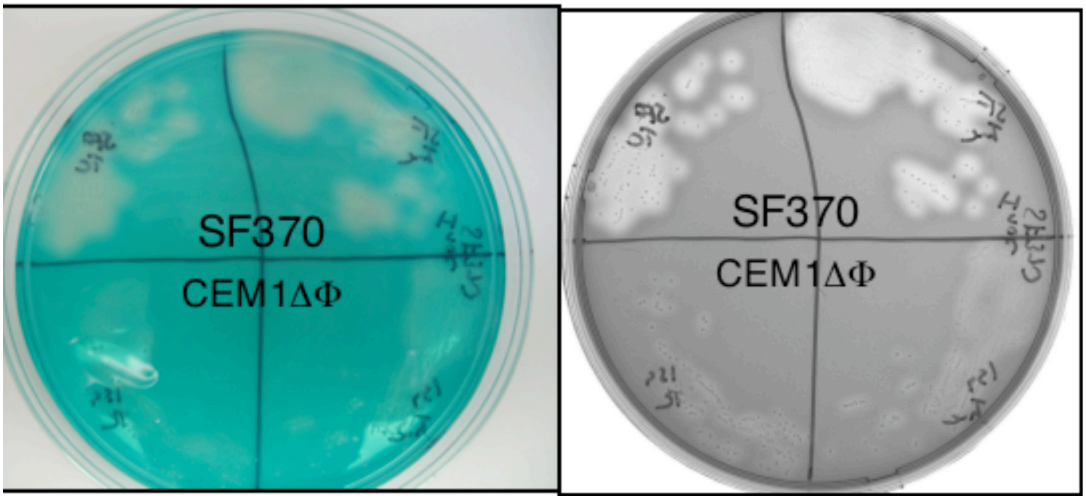


Figure 8.11. DNase activity of WT SF370 and the full-phage knock-out mutant CEM1ΔΦ.

Both strains were cultured on DNase Test Agar with Methyl Green for 17 h. The upper half of the plate contains WT colonies and the lower half of the plate contains the full phage knock-out mutant CEM1ΔΦ colonies. DNase activity is indicated by a clearing zone around the colonies, which represents hydrolysis of the DNA and methyl green substrate. The figure includes both a color and a black and white photograph of the same plate taken at the same time, to aid in discerning colonies and DNAse activiy.

8.3.12 Interactions of CEM1ΔΦ with SF370 induced bacteriophage.

To test if the CEM1ΔΦ phage-cured mutant could be lysed and / or lysogenized by induced SF370 bacteriophages, concentrated supernatants of induced or non-induced cultures were spotted onto BHI soft agar overlays containing the SF370

or CEM1ΔΦ as indicator strains. No growth inhibition or plaque formation was observed for any supernatant applied to the SF370 indicator plate (Figure 8.12). Additionally, no growth inhibition or plaque formation was observed on the CEM1ΔΦ indicator strain from any non-induced culture or the mitomycin C induced CEM1ΔΦ supernatant. Conversely, the supernatant of the mitomycin C induced SF370 culture produced growth inhibition and plaque formation on the lawn of the CEM1ΔΦ indicator strain (Figure 8.12)

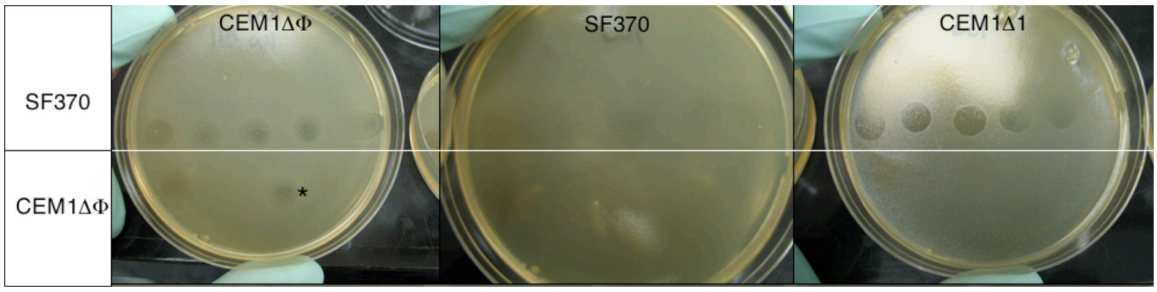


Figure 8.12. Analysis of bacteriophage plaque formation on phage deletion mutants.

Concentrated supernatants from mitomycin C induced cultures of either the WT SF370 (top half of plates) or CEM1ΔΦ full phage deletion mutant (bottom half of plate) were spotted (10μl) across BHI soft agar overlays containing either CEM1ΔΦ, SF370, or CEM1Δ1. Plates were incubated overnight at 37°C and then observed for plaque formation and/or growth inhibition. An asterisk (*) indicates an area where a drop of SF370 supernatant accidentally fell on the plate.

To begin to assess which SF370 bacteriophage may be involved in the plaque formation, mitomycin C induced supernatants were dropped onto soft agar overlays containing the single phage deleted mutants (CEM1Δ1, CEM1Δ2, CEM1Δ3, and CEM1Δ4) or CEM1ΔΦ. Growth inhibition was only observed in response to the SF370 mitomycin C induced supernatant on the lawns of the CEM1ΔΦ and CEM1Δ1 mutants (Data not shown and Figure 9.1). This suggests that Φ370.1 is the functional phage responsible for the majority of initial plaques on the lawns of CEM1ΔΦ.

To assay for lysogeny, CEM1ΔΦ colonies were picked from inside the plaque zones from the SF370 mitomycin C-induced supernatants and an isolate was identified that had the Φ370.1 *speC* phage gene. Confirmation of proper insertion into the original Φ370.1 integration *attB* site was achieved by sequencing across the phage-bacterium junction and PFGE (Data not shown).

8.4 DISCUSSION

Comparative genomic studies of the published sequences of *S. pyogenes,* isolated from different streptococcal infections and diseases, have confirmed the important relationship between *S. pyogenes* and the many lysogenic bacteriophages contained within the genome of this species. While phage have been shown to play a role in streptococcal pathogenesis because of the virulence

factors they encode, the molecular interactions between the phage and the host need further dissection. As such, a comparison of isogenic strains of *S. pyogenes* that differ in specific bacteriophage genes or in bacteriophage content would allow us to study the direct effects that individual bacteriophage have on streptococcal genome and /or virulence.

In this study, we first used allelic replacement techniques to target bacteriophage genes encoding potential GrAS virulence or regulatory factors for allelic replacement. In previous microarray studies on SF370, we observed that 11 genes from the Φ370.2 prophage were differentially expressed when in contact with human pharyngeal epithelial cells (Ryan, Kirk et al. 2007). One of these genes (*speH)* encoding a putative superantigen was up-regulated, suggesting that it may play a novel role in streptococcal colonization. Adherence-mediated up-regulation of *speH* was likely not simply the result of phage induction, as the remaining phage Φ370.2 genes identified in that analysis were down-regulated. Instead, increased expression of *speH* during pharyngeal cell adherence suggested that the SpeH exotoxin is either necessary for adherence or is a component of a downstream infection process. To answer this question, the *speH* gene was replaced with an erythromycin resistant gene, and the mutant strain was subsequently compared to the wild-type SF370 strain in various in vitro co-culture assays with human pharyngeal cells. No discernable effects on adherence, internalization or intracellular survival were seen between SF370 and

the isogenic mutant lacking the *speH* gene in the pharyngeal cell assay. These results suggest that SpeH is not involved directly in attachment, internalization or survival during contact with pharyngeal cells. The significant up-regulation of the *speH* gene seen by Ryan et al. during adherence suggests that the gene product may function instead during a subsequent stage of infection (Ryan, Kirk et al. 2007).

To discriminate whether the 10 other genes from Φ370.2, which were differentially expressed during pharyngeal cell adherence (Ryan, Kirk et al. 2007), had a role in GrAS pharyngeal cell adherence or internalization, we decided to delete the entire Φ370.2 bacteriophage from the streptococcal genome. In order to accomplish a precise phage deletion, a second approach was developed to select for isolates of SF370 that were cured of lysogenic bacteriophage. Using allelic replacement techniques to exchange individual bacteriophage genes with the counter-selection cassette ((*aacA-aphD*) / *rpsL*^{WT}), it was possible to select for strains that had lost the various phage of interest.

This counter-selection technique was applied to the Φ370.2 prophage, resulting in a mutant strain (CEM1Δ2) that had excised and deleted the Φ370.2 phage and recapitulated the original *attB* phage integration site in the chromosome. A comparison of CEM1Δ2 and SF370 in the pharyngeal co-culture assays showed that the absence of Φ370.2 from the streptococcal genome had no effect on

GrAS adherence to, or internalization by, pharyngeal epithelial cells. This suggests that the 10 other Φ370.2 genes that were differentially expressed during adherence have alternative functions during the interaction of *S. pyogenes* with epithelial cells (Ryan, Kirk et al. 2007).

Although we were unable to detect a difference in adherence or internalization in the Φ370.2 knock-out mutant, the successful curing of Φ370.2 from SF370 served as proof of principle that we could specifically manipulate prophage gene content in *S. pyogenes* using our phage counter-selection technique. This methodology was then applied to the remaining bacteriophages in the SF370 genome and we successfully created a total of fifteen mutants encompassing every permutation of phage knockout (KO): single, double, and triple knockouts, as well as a mutant strain that was completely cured of all bacteriophage elements, CEM1ΔΦ. These isogenic mutant strains have given us the tools necessary to further analyze the role that phage may play in streptococcal survival, and to examine the molecular interactions of the lysogenic bacteriophage within GrAS.

Since 10% of the genome of SF370 had been deleted in the phage-free mutant, we first compared the basic phenotypes of the CEM1ΔΦ mutant to the wild type SF370 strain. Despite the fact that a large portion of the total genomic content of SF370 had been removed, we found no discernable differences in bacterial cell

shape, chain length, colony morphology, hemolytic patterns on blood agar, or on the growth curves of cultures cultivated in liquid media. These results showed that the phage of SF370 do not affect the basic growth properties or colony characteristics of SF370, which caused us to look for alternative phenotypes that may indeed be phage-related.

To assess the role that all phages of SF370 play in the initial events of GrAS infection, we compared the full phage knock out CEM1ΔΦ to SF370 in the pharyngeal cell co-culture assay. No difference was observed in either adherence or internalization between the wild type and the mutant strains, suggesting that in this particular in vitro model, lysogenic bacteriophage genes do not facilitate the initial attachment or internalization of GrAS at the pharyngeal epithelial surface. These results led us to explore other ways in which the lysogenic phage of SF370 may contribute to pathogenesis.

Recently, others have shown that bacteriophage DNases can protect streptococci from neutrophil-formed extracellular traps (NETs), which are composed of neutrophil granule components linked to DNA and which function to trap and kill extracellular bacteria and fungi (Knetsch, Schafers et al. 2001; Brinkmann, Reichard et al. 2004; Sumby, Barbian et al. 2005). The genome of SF370 contains three genes encoding secreted DNases: one chromosomally encoded (*spd/mf1*) and two phage-encoded (*spd1* on Φ370.1 and *spd3* on

Φ370.3). A comparison of activity on DNase Test Agar showed that the full phage deletion mutant CEM1ΔΦ had lower DNase activity then did the wild type SF370 strain. This suggests that although the chromosomal *spd/mf1* gene is present in the knock-out genome, the two phage DNases may contribute significantly to the total DNase activity secreted from SF370. We hypothesize that such DNase activity may play a role in GrAS survival during streptococcal infection by allowing the organism to escape entrapment within the NETs. Future experiments will use more quantitative assays to compare the DNase activity of CEM1ΔΦ and SF370 to that of the single phage deletion mutants (CEM1Δ1 and CEM1Δ3), so that the activity of the individual DNases in SF370 can be analyzed. The phage deletion mutants will be also tested for their ability to survive during in vitro neutrophil extracellular killing assays and murine models of infection (Sumby, Barbian et al. 2005; Buchanan, Simpson et al. 2006).

One example of how bacteriophages interact with GrAS can be classified as integrational or positional effects, and is associated with the chromosomal location of phage integration and the method by which such integration occurs. Prophage integration occurs through homologous recombination between a common sequence shared between the phage and bacterial DNA (*attP* and *attB*, respectively), which inserts the prophage into the bacterial chromosome between adjacent duplications of the attachment site (*attL* and *attR*) (Campbell 1992). Most bacteriophages integrate into the 3' end of a host gene, leaving the ORF

intact either by duplication or by providing an alternative carboxy terminal sequence (Campbell, Schneider et al. 1992; Campbell 1992). However, GrAS prophages frequently integrate into the 5' end of genes or into gene promoters (Fischetti 2006), and thus, could potentially disrupt or alter host gene expression. In the genome of SF370, two of the four bacteriophages (Φ370.1 and Φ370.4) integrate into the 5' region of particular genes. We decided to further explore how prophage integration may regulate the expression of these particular disrupted genes by comparing their transcriptional profiles or putative phenotypes in wild-type SF370 and in single phage deletion mutants.

Prophage Φ370.1 is integrated into the 5' end of the dipeptidase gene (*pepD*), which replaces the first nine N-terminal amino acids and the upstream promoter sequence with bacteriophage specific DNA (Canchaya, Desiere et al. 2002). The altered amino terminal sequence and promoter element suggests that Φ370.1integration into this gene may directly affect or disrupt the expression of the *pepD* gene or the activity of its gene product. In our preliminary analysis, we screened *pepD* expression by RT-PCR at different stages of growth in the SF370 wild type (WT) strain and the Φ370.1 KO mutant, CEM1Δ1. Interestingly, while one might expect *pepD* to be inactivated by the insertion of Φ370.1 directly into the promoter region and into the sequence encompassing the start site of this gene, the *pepD* transcript was present during all phases of growth in both CEM1Δ1 and the wild-type strain SF370. These results suggested that there

was a functional promoter for *pepD* encoded within the Φ370.1 DNA itself. Additional sequence examination identified a strong gram-positive promoter sequence and Shine-Delgarno sequence in the bacteriophage DNA (Figure 8.5). While there are multiple dipeptidases encoded in the genome of SF370 (Ferretti, McShan et al. 2001), this PepD peptidase might be important to the growth and survival of *S. pyogenes* since evolution has selected for the integrated phage to retain a promoter sequence within its 3' region, presumably to not disrupt *pepD* function. A conserved domain search of the PepD amino acid sequence (http://www.ncbi.nlm.nih.gov/Structure) indicated that PepD belongs to the peptidase C69 super-family, which encompasses a large group of dipeptidases with numerous substrates. A substrate has not yet been identified, so we could not compare activities of the PepD peptidases expressed by CEM1Δ1 and SF370. Once a substrate is identified, we hope to determine (in future experiments) if both *pepD* gene products are equally active, given that the 5' end of the *pepD* gene sequence is different when the phage is integrated and when it is not. Experiments to delete *pepD* from the streptococcal genome are also underway to ascertain its function in *S. pyogenes*.

Because we had found in this analysis that both the presence and absence of Φ370.1 within the SF370 genome allows for a *pepD* RNA transcript to be produced, we were interested in analyzing if both *pepD* transcripts were produced in the WT strain. The reverse transcription analysis allowed us to

differentiate the two transcripts, and we found that during early, mid, and late log growth phases both the WT type *pepD* (pepDΦ) and the phage KO *pepD* (*pepD*KO) were being expressed. The finding that the two different transcripts were produced by SF370 indicates that Φ370.1 is actually excising from the genome in a certain population of cells during all phases of growth, and that the presence of prophage Φ370.1 in the streptococcal genome may be more dynamic than previously thought. Prophage Φ370.1 is a functional phage, and as such, is able to make viral particles (Ferretti, McShan et al. 2001), which leads us to further speculate about the dynamics and outcome of the excision event. Specifically we are interested in determining if the *pepD*KO transcript observed in SF370 cells indicates that the cell is entering the lytic cycle, or if the Φ370.1 DNA is excising from the chromosome, replicating episomally and then reintegrating into the genome. This latter mechanism is similar to excision and reintegration dynamics of the Φ370.4, which is discussed in more detail below (Scott, Thompson-Mayberry et al. 2008). We plan to use more sensitive transcript analysis (i.e. QRT PCR) to calculate the concentrations of the pepDΦ and *pepD*KO transcripts, in addition to quantitating the transcripts of any lytic and lysogenic regulatory genes in the wild type SF370. These future studies will likely provide a better understanding of the dynamics of Φ370.1 and the regulation of the *pepD* gene during phage integration and excision.

While Φ370.4 appears to be a defective lytic phage, its genome has retained intact direct sequence repeats (*attL and attR*) at the junction between the phage and bacterial DNA sequences and also encodes ORF modules homologous for integration, lysogeny control, replication and regulation (Canchaya, Desiere *et al.* 2002). These findings suggest that this phage-element may be able to perform some phage functions within the GrAS genome. Prophage Φ370.4 is integrated between the *mutS* gene and the 5' region of the *mutL* gene in the methyl-directed mismatch repair (MMR) operon that includes several other DNA repair genes. Phage integration separates *mutL* from its shared promoter upstream of *mutS,* and thus is predicted to inactivate *mutL* expression and disrupt MMR in SF370, resulting in a fixed mutator phenotype. Recently, Scott *et al.* reported that in rapidly dividing cells or following DNA damage, SF370 expresses both *mutS* and *mutL*, but only expresses *mutS* in stationary phase cells. (Scott, Thompson-Mayberry et al. 2008). The authors concluded that the differential expression of *mutL* results from excision, circularization and reintegration of the Φ370.4 prophage in a growth phase dependent manner (summarized in Figure 8.6). Furthermore, they showed Φ370.4-containing strains in stationary phase had a higher mutation rate and increased sensitiviy to UV irradiation then non-homologous strains with an intact MMR operon (Scott, Thompson-Mayberry et al. 2008). Unfortunately, in these experiments they were not able to compare the direct effect of the Φ370.4 prophage on MMR phenotypes because they could

not cure SF370 of the Φ370.4 prophage to compare isogenic strains (McShan personal communication and (Scott, Thompson-Mayberry *et al.* 2008).

When we applied our counter-selection technique to cure SF370 of prophage Φ370.4, we also experienced difficulties in deleting this phage from the genome of SF370. Although the phage excises from the genome during early log growth, which would suggest that the chance of getting a cell division event that causes a loss of phage might be good, we speculate that this phage is difficult to cure because of its ability to excise and replicate episomally (similar to that of a high copy number plasmid). In fact, the Φ370.4 replicase and primase genes are homologous to DNA replication genes from plasmid pST106 of *Streptococcus thermophillus* (Scott, Thompson-Mayberry et al. 2008). Interestingly, we created a deletion mutant of the Φ370.4 primase gene, but found that the genome was still able to replicate episomally, thus preventing its elimination. This finding suggested that another primase may be complementing the Φ370.4 primase mutant. A search for primases in the genome sequence of SF370 showed that Φ370.2 (and possibly Φ370.1) contain hypothetical phage primases, which may be "helping" Φ370.4 replicate its genome. This hypothesis is supported by our later results that showed that the Φ370.4 phage was easier to cure (following the normal counter-selection protocol) in mutants that had Φ370.1 or Φ370.2 previously deleted from the SF370 genome. Future experiments will examine these relations between the phage using additional primase deletion mutants and

QRT-PCR to look at primase and episomal phage concentrations in our KO mutants. We were finally successful at curing SF370 of Φ370.4 by increasing the temperature of the overnight incubation to 42°C (a method often used to cure bacteria of plasmids), which may have disrupted the replication efficiency of the episomal phage (Verheust, Fornelos *et al.* 2005).

With the creation of the isogenic Φ370.4 phage deletion mutant (CEM1Δ4) we were able to compare the direct effects of the Φ370.4 phage genes on the MMR operon in SF370. Our results support previous studies that showed that SF370 has a growth-dependent increase in resistance to UV irradiation when cells are rapidly dividing compared to cells in stationary phase (Scott, Thompson-Mayberry et al. 2008). Additionally, we show that the absence of Φ370.4 restores UV resistance to CEM1Δ4 at any stage of growth. Confirming that the excision (or deletion) of Φ370.4 restores transcription of the MMR polycistronic message, which subsequently allows for the expression of the *ruvA* gene.

To explore other phenotypic changes that might be under the dynamic control of Φ370.4, we examined the activities of another gene product, LmrP, in the MMR operon. Protein LmrP is a proton motive force-dependent drug transporter that was originally described in the gram-positive bacterium *Lactococcus lactis* (Bolhuis, Poelarends *et al.* 1995). It is a predicted membrane protein with 12 transmembrane regions, which confers resistance to lipophilic antimicrobial

agents, such as ethidium bromide (EtBr) and daunomycin in *L. lactis*. Inactivation of the *lmrP* gene or the use of a proton pump inhibitor results in increased killing of *L. lactis* following antimicrobial treatment in a concentration dependent manner (Bolhuis, Poelarends *et al.* 1995; Mazurkiewicz, Poelarends *et al.* 2004). According to the model of prophage Φ370.4 regulating genes of the MMR operon, LmrP should not be expressed when the prophage is integrated into the bacterial chromosome at *mutL* (Scott, Thompson-Mayberry *et al.* 2008). This inhibition of LmrP should result in an increased sensitivity to EtBr and similar agents (Bolhuis, Poelarends *et al.* 1995). Accordingly, we found that compared to CEM1Δ4, the wild type SF370 strain had a significant increase in EtBr induced death during early log and stationary phases. However, we observed that during very early log growth, when Φ370.4 is excised from the genome, the survival of the SF370 and the Φ370.4 deletion mutant were essentially identical. We interpret these results to indicate that the LmrP protein is turned over rapidly, so that its expression in SF370 occurs mainly in very early log growth, when Φ370.4 is excised, and continually diminishes over time. Expression of LmrP also appears to be diminished in stationary phase cells of CEM1Δ4, as these organisms experience increased sensitivity to EtBr. This phenomenon may also result from a decreased level expression of the polycistronic message from the *mutS* promoter.

We are currently examining other potential roles of Φ370.4 in the control of LmrP, and the effect that such regulation may have on streptococcal pathogenesis. Preliminary evidence from other groups suggests that expression of LmrP increases survival of GrAS at low pH values (McShan, personal communication), which may aid in increasing streptococcal survival inside phagosomes of human leukocytes (Beck, Bergner-Rabinowitz et al. 1969; Bassoe and Bjerknes 1985), and that this phenotype may also be under the control of phage integration and excision. We hope to use our phage knock out mutants to determine the basis for this observation. We are also examining if the Φ370.4 phage-controlled mutator phenotype affects mutation rates of a two-component regulatory system (*covR* / *covS*), which has been shown to influence GrAS survival in a murine model of subcutaneous infection (Kansal, Datta et al. ; Graham, Virtaneva et al. 2005; Graham, Virtaneva et al. 2006; Sumby, Whitney et al. 2006).

We have demonstrated, in the work presented here, that the phage-cured mutants are useful tools to analyze the role of phage in GrAS pathogenesis and to study the molecular interactions of phage genes and the streptococcal chromosome. These mutants can also be used to study the mechanisms of lysogeny and phage-phage interactions, as well as to identify phenotypes associated with additional novel lysogenic phages that insert into the SF370 genome. We hypothesized that our phage-free mutant strains would likely be more amenable to future lysogenization by other bacteriophage since there are

no prophage at the potential bacteriophage integration sites or other bacteriophage encoded proteins that could interfere with subsequent bacteriophage infection (e.g. bacteriophage CI-repressors and superinfection exclusion genes) (Canchaya, Desiere et al. 2002). To test this hypothesis, we exposed the mutant strains to supernatants from mitomycin C induced cultures of SF370, and found that the phage cured CEM1ΔΦ mutant was capable of being infected and subsequently lysed by lytic bacteriophage. We then used the single phage deletion mutants to identify which infecting phages may be responsible for the lytic effect. Plaque formation was only seen in the CEM1Δ1 mutant, suggesting that Φ370.1 was the only induced phage. This was confirmed by isolating a lysogen of the CEM1ΔΦ mutant that contained Φ370.1 re-integrated into the original attB site on the chromosome. These findings are in agreement with previous studies that suggested Φ370.1 is the only inducible phage after mitomycin C treatment (Ferretti, McShan et al. 2001), but are still novel because the authors of that study did not examine if the inducible phage particles were actually capable of re-infecting and/or lysogenizing GrAS bacteria. Thus we were able to show, for the first time, that Φ370.1 from SF370 is a fully functional lysogenic bacteriophage and proved that our phage knock out mutants derived from our novel counter-selection method are capable of being infected and lysogenized. We will continue to study the lysogenic potential of the additional phage deletion mutants using both SF370 induced cultures, as well as cultures of other strains of *S. pyogenes* that contain novel phage not found in SF370.

We are also currently using microarray analyses to compare the gene expression profiles of the full phage deletion mutant CEM1$\Delta\Phi$ to the wild type SF370 strain during different growth stages in liquid culture. These experiments will assess the effects that both bacteriophage integration and phage genes have on chromosomal gene expression. These studies will establish a foundation for future studies, in which the global transcriptional effects of phages can be analyzed in both in vitro and in vivo models of GrAS infection.

This is the first report of a method that systematically cures *S. pyogenes* of all phage elements. Our counter–selection technique allows for precise manipulation of lysogeny to analyze the role phage play in both virulence and the regulation of chromosomal genes. The derived KO strains have allowed us to begin to directly examine novel bacteriophage interactions with GrAS and elucidate their specific roles in streptococcal survival, pathogenicity, and evolution. The techniques developed in this study could easily be modified for other bacterial-bacteriophage systems, and thus could be beneficial to the study of not just streptococcal-phage interactions but to other areas of research in microbial pathogenesis, ecology or biotechnology.

9 Chapter 3

9.1 INTRODUCTION

Staphylococcus aureus is an opportunistic pathogen inhabiting human skin and mucous membranes and is the causative agent of variety of skin and soft tissue infections as well as serious infections such as pneumonia, meningitis, endocarditis, and osteomyelitis. *S. aureus* exotoxins also cause disease syndromes such as bullous impetigo, scalded skin syndrome, and toxic shock syndrome. While outbreaks of cutaneous infections in otherwise healthy people can be managed well without antibiotics (Rajendran, Young et al. 2007), in compromised individuals staphylococci are an important cause of life-threatening nosocomial infections such as ventilator-acquired pneumonia (VAP) (Kollef, Morrow et al. 2006). The spread of methicillin resistant *S. aureus* (MRSA) has been of critical concern to health care providers, and the further distribution of Panton-Valentine Leucocidin (PVL) carrying community acquired MRSA strains to the hospital (Popovich, Weinstein et al. 2008) poses a threat that is even more serious than that of MRSA alone due to the striking pathogenicity of this toxin (Boyle-Vavra and Daum 2007).

Currently, 40-60% of nosocomial infections of *S. aureus* are resistant to oxacillin (Massey, Horsburgh et al. 2006) and greater than 60% of the isolates are resistant to methicillin (Gill, Fouts et al. 2005). Treating infections caused by the drug-resistant *S. aureus* has become increasingly difficult and is therefore a

major concern among healthcare professionals. To combat this challenge, development of new and effective antibiotics belonging to different classes are being aggressively pursued. A number of new antimicrobial agents such as linezolid, quinupristin–dalfopristin, daptomycin, telavancin, new glycopeptides and ceftobiprole have been introduced or are under clinical development (Aksoy and Unal 2008). However, clinical isolates of MRSA with resistance to these new classes of antibiotics have already been reported (Tsiodras, Gold et al. 2001; Mangili, Bica et al. 2005; Skiest 2006). Consequently, there is an urgent need to develop novel therapeutic agents or antibiotic alternatives that are active against MRSA. As an option, current antibiotics for which MRSA are resistant may be resurrected as viable candidates in the treatment of MRSA when used in combination with other agents, offering a new dimension to a dwindling list of potential anti-infectives for this pathogen.

Carriage of both MSSA and MRSA in the human anterior nares is the major reservoir for *S. aureus* infection. Studies have shown that roughly 80% of the population could be nasally colonized by *S. aureus*, and that colonization increases the risk factor for developing other more serious *S. aureus* infections (Kluytmans, van et al. 1997). Elimination of nasal carriage in the community or in the hospital setting could possibly reduce the risk of infection and slow the spread of drug resistant *S. aureus* (Kluytmans, van et al. 1997). Only one agent, intranasal mupirocin, has been approved in this indication. Mupirocin ointment

must be applied twice daily to the anterior nares for 3-5 days. When used in this manner, mupirocin ointment has been shown to significantly reduce the risk of post-operative staphylococcal infection in patients who were *S. aureus* carriers (Perl, Cullen et al. 2002). However, the value of mupirocin is compromised by a relatively high rate of resistance mutations (Coates, Bax et al. 2009) and by potential compliance issues associated with its method of use. Despite these issues, pre-operative prophylaxis with mupirocin is already mandated for cardiovascular surgery (Engelman, Shahian et al. 2007) and has also been recommended for orthopedic surgery (Wilcox, Hall et al. 2003). Clearly a superior product for intranasal prophylaxis in at-risk patients would be valuable.

Bacteriophage endolysins (lysins) are a new class of novel antimicrobial agents that are emerging as effective agents for the prophylactic and therapeutic treatment of bacterial infections. Lysins are cell wall hydrolases that are produced during the infection cycle of double-stranded DNA bacteriophages enabling the release of progeny virions. When applied exogenously, native or recombinant lysins are able to cleave the integral peptidoglycan bonds of susceptible gram-positive bacteria resulting in rapid cell lysis (Nelson, Loomis et al. 2001). Lysins have been developed against a number of Gram-positive pathogens including *S. pyogenes* (Nelson, Loomis et al. 2001), *S. pneumoniae (Loeffler, Nelson et al. 2001), Bacillus anthracis (Schuch, Nelson et al. 2002)*, enterococci (Yoong, Nelson et al. 2004), Group B streptococci (Cheng, Nelson et

al. 2005), and *Staphylococcus aureus* (O'Flaherty, Coffey et al. 2005; Rashel, Uchiyama et al. 2007). The efficacy of most of these lysins has been demonstrated in *in vivo* models. Several unique characteristics of lysins make them attractive antibacterial candidates against Gram-positive pathogens. These include i) rapid antibacterial activity both in vitro and in vivo; ii) narrow lytic spectrum (species-specific); iii) strong receptor-binding affinity, typically in the nanomolar range; iv) very low probability of developing resistance since the binding epitopes on the bacteria are essential for viability; v) safe; and vi) relative ease of modification by genetic engineering (Fischetti, Nelson et al. 2006).

Development of a highly active *S. aureus*-specific lysin has been challenging either due to lack of expression in a heterologous host, insolubility of the expressed protein, or poor expression, except one report to our knowledge of a *S. aureus*-specific lysin from phage phiMR11 (Rashel, Uchiyama et al. 2007). To circumvent these issues a few studies have reported the construction of truncated (Horgan, O'Flynn et al. 2009) or chimeric versions of lysins (Manoharadas, Witte et al. 2009).

Typically, lysins have two distinct functional domains consisting of an N-terminal catalytic domain for peptidoglycan hydrolysis and a C-terminal binding domain for recognition of surface moieties on the bacterial cell walls. The catalytic domains are relatively conserved among lysins and the activities can be classified into

three basic groups based on peptidoglycan bond specificity: i) glycosidases that hydrolyze linkages within the amino sugar moieties; ii) endopeptidases that cleave the peptide moiety; iii) and amidases that hydrolyzes the amide bond connecting the glycan strand and stem peptide. The binding domains however are not conserved among lysins. Hence the binding domain often imparts species-specificity because the binding targets, often carbohydrates associated with the peptidoglycan, display species-specific distribution (Fischetti 2007). The modular architecture of lysins is an important feature with respect to their development as antimicrobial agents. This enables creation of chimeric enzymes by swapping lysin domains and thereby altering binding specificity or enzymatic activity or both (Croux, Ronda et al. 1993; Sheehan, Garcia et al. 1996; Lopez, Garcia et al. 1997; Donovan, Dong et al. 2006).

In this chapter we describe the genetic engineering of a novel chimeric lysin constructed by fusing the catalytic domain of a *Staphylococcus*-specific phage lysin with a unique binding domain from a different *Staphylococcus*-specific lysin. This engineered lysin called ClyS (for chimeric lysin for staphylococci) was soluble, highly active and displayed rapid and specific lytic activity against susceptible and drug-resistant staphylococci. We demonstrate the lytic activity of ClyS in in vivo colonization and septicemia models as well as its synergistic activity with oxacillin in in vitro and in vivo models. These results highlight the

potential of ClyS as a novel therapeutic agent for the treatment of MRSA and other staphylococcal infections.

9.2 MATERIALS AND METHODS

9.2.1 Bacterial strains.

Bacterial strains (Table 9.1) were stored at -80°C and routinely grown at 37°C. Staphylococcal strains used in this study were grown in Trypticase Soy Broth (TSB) media, streptococcal strains were grown in THY (Todd–Hewitt broth, 1% wt/vol yeast extract) media, *B. cereus* and *P. aeruginosa* were grown in BHI (Brain Heart Infusion) media while *E. coli* was cultivated in LB (Luria Bertani) media. Unless stated otherwise, all media was supplied by Becton, Dickinson and Company (Sparks, MD).

Table 9.1. Bacterial strains for ClyS lysin studies.

Bacteria	Strain	Source
Staphylococcus aureus	8325-4	1
Staphylococcus aureus	RN4220	1
Staphylococcus aureus	191-streptomycin^{R}	1
Staphylococcus aureus	lyrA del2335	2
Staphylococcus aureus	Newman	2
MRSA	COL	4
MRSA	E2125	4
MRSA	HPV107	4
MRSA	HDE288	4
MRSA	BK2529	4
MRSA	HAR24	4
MRSA	MW2	5
VISA	HIP 11714	4
VISA	Mu50	4
VISA	JH9	4
VISA	PC3	4
VISA	MI	4
VISA	NJ	4
Staphylococcus epidermidis	RP62A	3
Staphylococcus epidermidis	HER1292	3
Staphylococcus simulans	TNK3	2
Staphylococcus sciuri subsp. *sciuri*		3
Group A streptococcus	D471	1
Group C streptococcus	2GRP66	1
Group E streptococcus	K131	1
Group B streptococcus	NCTC 11234	1
Streptococcus gordonii	PK488	1
Streptococcus salivarius	ATCC 9222	1
Streptococcus uberis		1
Bacillus cereus	ATCC 4342	1
Pseudomonas aeruginosa	RS1	1
Escherichia coli	Top10	1

MRSA: (methicillin resistant *Staphylococcus aureus*), VISA (Vancomycin intermediate *Staphylococcus aureus*). 1, The Rockefeller University Collection; 2, Olaf Schneewind, University of Chicago, Chicago, Il.; 3, Barry Kreiswirth, Public Health Research Institute, New Jersey, NJ; 4. Alexander Tomasz, The Rockefeller University; 5. ATCC.

9.2.2 Construction of ClyS.

The chimeric lysin was constructed by amplifying and ligating individual domains from respective genes. For this, the gene fragment encoding the Twort endopeptidase domain was PCR amplified from plasmid pCR2.1plyTW, which contains the entire lysin (*plyTW*) gene using primers TW-Endo-NcoI-F: 5'-CTAGCCATGGAAACCCTGAAACAAGCAG-3' and TW-Endo-PstI-R: 5'-ACATGCTGCAGAACCATATTGTAATTAATATTAGTTCTATC-3'. The gene fragment encoding the cell wall targeting (CWT) domain was PCR amplified from *S. aureus* strain 8325 genomic DNA containing the phiNM3 bacteriophage using primers NM3-CBD-PstI-F: 5'-ACATGCTGCAGGGTAAA TCTGCAAGTAAAATAACAG-3' and NM3-CBD-Hind-R: 5'-CCCAAGCTTAAAACACTTC TTTCACAATCAATCTC-3'. The two PCR amplicons were ligated using the PstI restriction endonuclease site. The ligated product was cloned into pBAD24 vector using the NcoI-HindIII cloning sites to generate recombinant plasmid pAD127. In the second step, the entire DNA fragment corresponding to *clyS* was PCR amplified from pAD127 using primers NM3-Lys-Xba-F: 5'-CTAGTCTAGAGGTGGAATAATGAAAA CATACAGTGAAGCAAG-3' and primer NM3-CBD-Hind-R. The PCR product was cloned into expression vector pJML6 to generate pAD138. The DNA sequence of *clyS* was confirmed through DNA sequencing of the pAD138 plasmid by Genewiz, Inc. (South Plainfield, NJ). The recombinant plasmid

pAD138 was transformed into *E. coli* DH5α cells (Invitrogen Corporation, Carlsbad, CA).

9.2.3 Over-expression and purification of ClyS.

ClyS was induced overnight from *E. coli* DH5α (pAD138) cells with lactose (10g/500ml final concentration) at 30°C. Cells were harvested by centrifugation, resuspended in buffer A (20 mM phosphate buffer (PB), 1 mM DTT) and lysed at 4°C by an EmulsiFlex-C5 high-pressure homogenizer (Avestin). The lysates were cleared by centrifugation (2x 50,000xg) for 30 min. at 4°C and the supernatant applied to a CM-sepharose column (Amersham Pharmacia, Piscataway, N.J.). ClyS was eluted with buffer A + 1M NaCl using a linear gradient of 0-50% B in 15 columns volumes. Fractions were analyzed for lytic activity as previously described (Nelson, Loomis et al. 2001). Fractions displaying lytic activity were pooled and dialyzed overnight against buffer B (PB, 1 mM DTT, 50mM NaCl). The dialyzed sample was applied to a hydroxylapatite (MacroPrep TypeII 40μm, BioRad) column and eluted with elution buffer (500 mM PB + 50 mM NaCl+ 1 mM DTT) using a linear gradient of 0-100% B in 20 columns volumes. The fractions were analyzed by SDS-PAGE and for lytic activity. Active and pure fractions of ClyS were pooled and dialyzed against buffer B. Protein concentration was determined with the BCA method (Sigma, St. Louis, MO).

9.2.4 Quantification of ClyS activity.

ClyS activity was measured as previously described (Nelson, Loomis et al. 2001; Coates, Bax et al. 2009), with some modifications. Briefly, *S. aureus* strain 8325-4 was grown to an OD_{600} of 0.25-0.3, centrifuged, and resuspended in PB to a final OD_{600} of 0.8-1.0. Two-fold serial dilutions of purified ClyS (100μl) were added to 100μl of bacterial suspension in 96-well plates (Costar) and the decrease in OD_{600} was monitored by a Spectramax Plus 384 spectrophotometer (Molecular Devices) over 30 min at 37°C. A unit of ClyS activity per milliliter was defined as the reciprocal of the highest dilution of lysin that decreased the absorbance by 50% in 15 min.

9.2.5 In vitro ClyS activity.

The effect of ClyS on bacterial viability was tested as previously described (Nelson, Loomis et al. 2001). Briefly, log phase cultures of an *S. aureus* strain 8325-4 were resuspended in PB to an OD_{600} of 0.8-1.0. 250 μg of ClyS or the corresponding volume of PB was added to bacterial cells and aliquots were removed, serially diluted, and plated at 1, 5, 10, 30, and 60 min to assess the viability of the treated and control cells. All experiments were performed in triplicate. The activity of ClyS was also tested on various bacterial strains as described previously (Schuch, Nelson et al. 2002). Briefly, log phase bacterial cells were treated with 250 μg of ClyS at 37°C for 15 min. The samples were

serially diluted and plated. Control experiments with the addition of phosphate buffer (pH 7.0) were performed under the same conditions.

9.2.6 Electron Microscopy.

S. aureus strain 8325-4 was grown to log-phase, centrifuged and resuspended in PBS to an absorbance at 600 nm of 1.0. The bacterial suspension was incubated with 250 μg of ClyS at room temperature. The lytic reaction was terminated after 1 min and 5 min by adding glutaraldehyde (final concentration 2.5%). The suspension was pelleted by centrifugation and overlaid with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4). The samples were then post-fixed in 1% osmium tetroxide, block stained with uranyl acetate and processed according to standard procedures by The Rockefeller University Electron Microscopy Service.

Fluorescent labeling and binding analyses of phiNM3 CWT. *S. aureus* strain 8325-4 genomic DNA was used to amplify the gene fragment encoding the putative CWT of phiNM3 lysin using primers NM3-FWD 5'-CATGCCATGGGTAAATCTGCAAGTAAAATAACAG-3' and NM3-REV 5'-CCCAAGCTTAAAACACTTCTTTCACAATCAATCTC-3'. The resulting amplicon was cloned into the arabinose-inducible expression vector pBAD24 (ATCC, Manassas, VA). Positive clones containing the insert were confirmed by sequencing. The ~10-kDa phiNM3 CWT protein was expressed in *E. coli* DH5α, which were lysed as above, and the protein was purified in one step by cation-

exchange chromatography. The purified protein (1mg/ml) was incubated for 1 hr with 10 μl of FITC (1 mg/ml, Sigma). Excess FITC was removed on a desalting column. The labeled-protein (50 μg) was incubated with bacterial cells for 10 min, washed 3x with phosphate-buffered saline (pH 7.4) and observed under an Eclipse E400 microscope (Nikon) using the QCapture Pro® version 5.1 imaging software.

9.2.7 Immunological assays.

To test whether ClyS-specific antibodies would neutralize the activity of ClyS, two rabbits were hyperimmunized with purified ClyS using a standard protocol: primary immunization with complete Freund's adjuvant and three monthly boosts in incomplete Freund's adjuvant. ELISA titers were >100,000 for each animal (reciprocal of the highest dilution with an OD_{405} of ≥1.0). ClyS was diluted in 2-fold dilutions through 11 wells in a microtiter plate beginning at 800 μg. 10 μl of either hyperimmune rabbit serum, preimmune serum or PBS was added to each well and incubated at 21°C for 15 minutes. An equal volume of staphylococci (prepared as described above) was then added to each well and immediately assayed as described above. The rate of OD_{600} decrease by ClyS was measured in each well for the hyperimmune serum and compared to PBS and preimmune serum for the same dilution of ClyS.

9.2.8 Synergy testing of ClyS with oxacillin or vancomycin.

ClyS and oxacillin/vancomycin interactions were assessed by the standard checkerboard broth microdilution assay as described before (Loeffler and Fischetti 2003). Briefly, ClyS and antibiotic were diluted two-fold horizontally and vertically, respectively, in a final volume of 50 μl with an inoculum of $3x10^5$ to $5x10^5$ CFU per well. MRSA strain COL was used to test ClyS - oxacillin interaction while VISA strain Mu50 was used to test ClyS – vancomycin interaction. The plates were incubated at 37°C with intermittent shaking in a spectrophotometer. Growth was determined by reading the plates at OD_{600} over 20 hrs. The fractional inhibitory concentrations (FICs) of ClyS and antibiotics were determined and plotted in an x/y plot called an isobologram. The FIC index (Σ) was calculated as the MIC of the drug when used in combination with ClyS divided by the MIC of the drug when used alone (Loeffler and Fischetti 2003). In all cases the ΣFICs were found to be <0.5. Synergy was defined as an ΣFIC of $\leq$0.5.

9.2.9 In vivo murine infection models.

All in vivo protocols were approved by The Rockefeller University's Institutional Animal Care and Use Committee. A modified method from Kiser et al., (Kiser, Cantey-Kiser et al. 1999) was used for the intranasal colonization model. Briefly, MRSA strain 191-SM^R was grown overnight at 37°C, with shaking at 250 rpm, in TSB media plus 200 μg/ml of streptomycin. The culture was then diluted 1:50

and grown as above to mid log-phase (OD_{600} 0.5), centrifuged and re-suspended in 0.9% saline for injection (Hospira, Inc., Lake forest, IL) to a predefined titer ($5x10^9$ CFU/ml) for mouse infection. Actual inoculum titers were derived from plating serial dilutions of each inoculum onto Spectra MRSA agar plates (a selective chromogenic medium developed to diagnostically detect MRSA nasal colonization, Remel, Lenexa, KS) and Columbia blood agar (Becton, Dickinson & Co., Sparks, MD). Six-week-old female C57BL/6J mice (weight range 22 to 24 g, Charles River Laboratories, Wilmington, MA) were fed water that contained 5 g/L of streptomycin for 48 hours prior to infection. Mice were anesthetized with a mixture of ketamine (Fort Dodge Animal Health, Fort Dodge, IA, 1.2 mg/animal) and xylazine (Miles Inc., Shawnee Mission, KS, 0.25 mg/animal), and were inoculated with two consecutive doses of 15 μl per nostril of the 191-SM^R bacterial suspensions. Twenty-four hours after infection the animals were divided into 2 groups (n = 20) and administered 60 μl (20μl per nostril and 20 μl orally) of either 20 mM phosphate buffer or 960 μg ClyS. Mice were sacrificed one hour after treatment and subjected to nasal dissection. The excised nasal cavities were aseptically bisected, suspended in 500 μl of phosphate buffered saline (PBS) and vortexed for 60 seconds to suspend adherent bacteria and dilutions of the bacterial suspensions were plated to Spectra MRSA agar plates and Columbia Sheep's Blood agar plates for CFU determination. No significant differences in CFU were obtained between plating to Spectra MRSA agar or Columbia blood agar (Data not shown). Three independent experiments were

performed to evaluate a total 20 mice for each treatment group. Nasal colonization rates of the treatment groups were analyzed statistically by an ANOVA test (http://www.danielsoper.com/statcalc/) and Student's T-test (program).

For the systemic infection model, 4-5 week old female FVB/NJ mice (weight range 15 to 20 g) were obtained from The Jackson Laboratory (Bar Harbor, ME). The PVL toxin encoding MRSA strain MW2 was initially passaged twice through the FVB/NJ mice to select for isolates that might be more capable of causing systematic infection in mice. Briefly, MW2 was grown in TSB media to mid log-phase, centrifuged and suspended in saline to a predefined titer of $1x10^9$ CFU/ml. 0.5 ml of the bacteria suspension was injected intraperitoneally and bacteria were isolated, on Spectra MRSA plates, from the spleens and hearts of the mice upon death 18-20 hours later. The bacterial isolates were then grown in TSB overnight and aliquots frozen in 30% glycerol at - 80°C. For intraperitoneal (IP) infection, a 10 μl loop of MRSA from a frozen aliquot the mouse passaged MW2 was inoculated into 10 ml of TSB media and grown overnight at 37°C with shaking. This culture was then diluted 1:50 in TSB media and grown for 2 hours at 37°C to mid log-phase (OD_{600} 0.5). The bacteria were then centrifuged, washed once with saline for injection, and re-suspended in saline to a predefined titer of 1 $x10^8$ to $5x10^8$ CFU/ml. This suspension was serially diluted with 5% hog gastric mucin (Sigma) in saline to a bacterial titer of $5x10^5$ to $1x10^6$ CFU/ml

and 0.5 ml of the bacterial suspension was injected intraperitoneally into each mouse. For each experiment, actual bacterial inoculation titers were calculated by serial dilution and plating to Columbia blood agar plates.

For the ClyS in vivo efficacy experiments, three hours post infection the animals were divided into 2 treatment groups and were intraperitoneally administered 0.5 ml of either 20 mM phosphate buffer (*n* =14) or 2 mg/ml ClyS. For the in vivo synergy experiments, three hours post IP infection the animals were divided into 3 to 8 treatment groups and were intraperitoneally administered a combination of 0.5 ml of 0.33 mg/ml ClyS (166 μg) or 20 mM phosphate buffer along with a 100 μl intramuscular (IM) injection of 10 μg to 100 μg oxacillin in saline or saline alone control. The survival rate for each experimental group was monitored every 4 hours for the first 24 hours then every 12– 24 hours up to 10 days post infection. The data was statistically analyzed by Kaplan Meier Survival curves and a Logrank test performed for 95% confidence intervals using the Prism computer program (GraphPad Software; La Jolla, CA).

9.3 RESULTS

9.3.1 Identification of a unique cell wall targeting (CWT) domain in Staphylococcus lysins.

Previous studies have indicated that the penta-glycine peptide cross-bridge within the staphylococcal peptidoglycan functions as the receptor for the CWT

domain of lysostaphin, a staphylolytic enzyme produced by *Staphylococcus simulans* (Grundling and Schneewind 2006). The CWT domain of lysostaphin has homology with the bacterial SH3-like (SH3b) domain, suggesting that lysins with SH3b domains might also utilize the peptide cross-bridge as their receptor (Grundling and Schneewind 2006; Lu, Fujiwara et al. 2006). Since resistance to lysostaphin can be due to small alterations within the peptide cross-bridges (Grundling and Schneewind 2006), we sought to identify a CWT domain within staphylococcal lysins that was unique and not homologous to SH3b domains. Our hypothesis was that such a CWT domain would bind to alternative epitope(s) such as cell wall-associated carbohydrates in the staphylococcal cell wall instead of the peptide cross-bridges, thereby reducing the likelihood of becoming the target of resistance.

Conserved domain searches of *Staphylococcus*-specific phage and prophage lysin sequences in the GenBank database (http://www.ncbi.nlm.nih.gov/Genbank/index.html) identified a few lysins that did not display homology to a C-terminal SH3b domain. These included the *S. aureus* phage phiNM3 lysin (protein accession #: YP_908849), *S. aureus* prophage phi13 amidase (accession #: NP_803402) and *S. aureus* prophage MW2 amidase (accession #: NP_646703.1). These three lysins share 100% sequence identity with each other (data not shown). We thus characterized the

region corresponding to the putative CWT domain of the phiNM3 lysin with respect to its biochemical and functional properties.

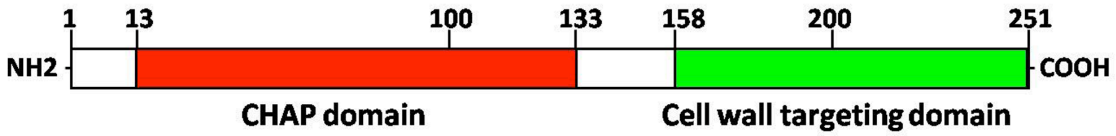


Figure 9.1. Schematic diagram of phiNM3 lysin showing the putative CHAP and the CWT domains.

The numbers represent the amino acid positions and the domain limits. The CWT domain of ClyS is shown as green box.

9.3.2 The phiNM3 CWT binds specifically to staphylococci.

The putative CWT domain corresponding to amino acid residues 158-251 of phiNM3 lysin, (Figure 9.1) was cloned and expressed in *E. coli*. The ~10-kDa protein was highly soluble and was purified to homogeneity (data not shown). To determine the binding specificity of this domain toward bacterial cells, the purified protein was labeled with FITC and exposed to log-phase *S. aureus*, *S. epidermidis* and a mixed population of *S. aureus* and *Bacillus cereus*. *S. pyogenes*, *E. coli* and *B. cereus* served as controls. The FITC-labeled phiNM3 CWT domain displayed species-specificity by binding specifically to *S. aureus* (Figure 9.2-1) and *S. epidermidis* (Figure 9.2-3) cells and was able to bind

specifically to the staphylococcal cells in mixed populations (Figure 9.2-6). No binding was observed to *Bacillus* (Figure 9.2-2), *E. coli* (Figure 9.2-4), or streptococci (Figure 9.2-5), a characteristic of the specificity of many phage lysins (Loeffler, Djurkovic et al. 2003; Mangili, Bica et al. 2005). Binding of the labeled-CWT domain was generally over the entire cell surface (Figure 9.2-1 and Figure 9.2-3) with more intense localization near the polar and septal regions.

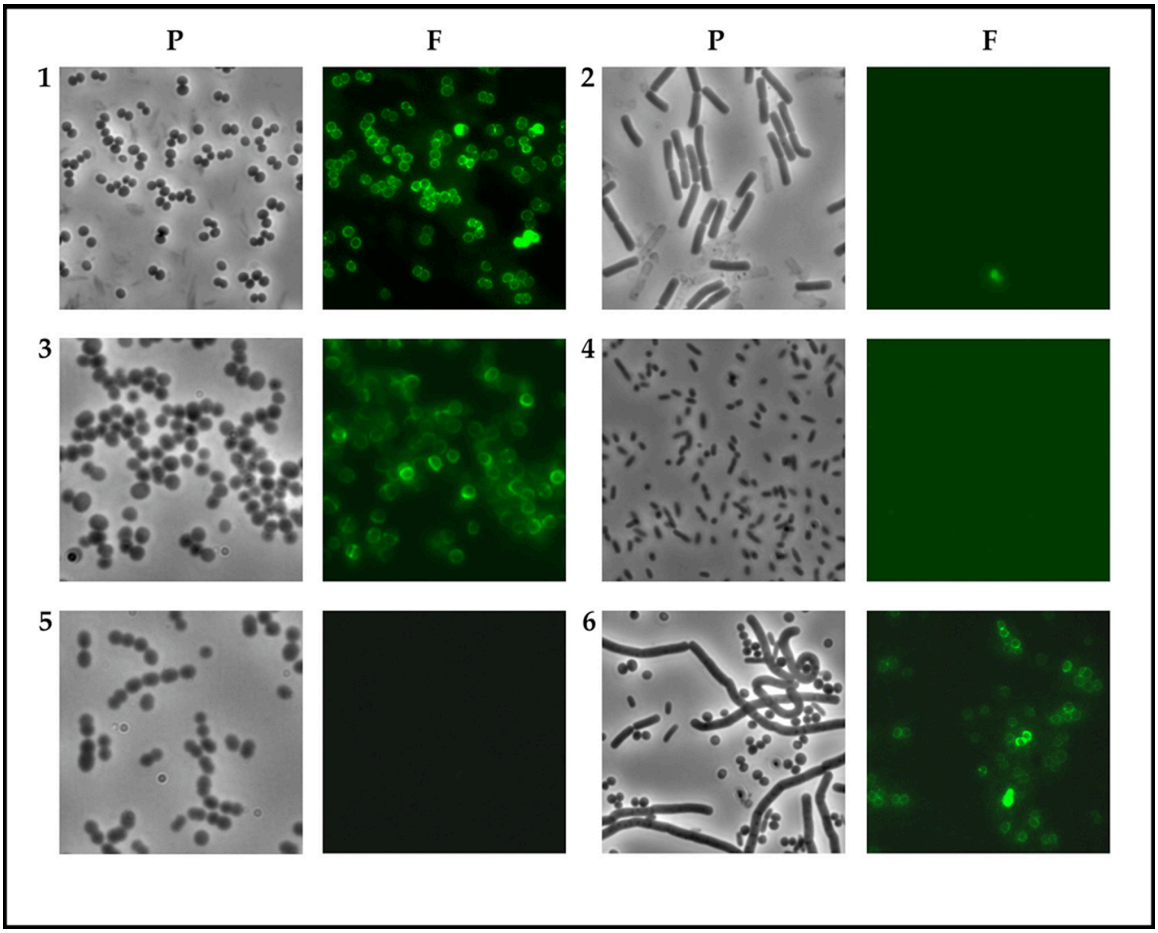
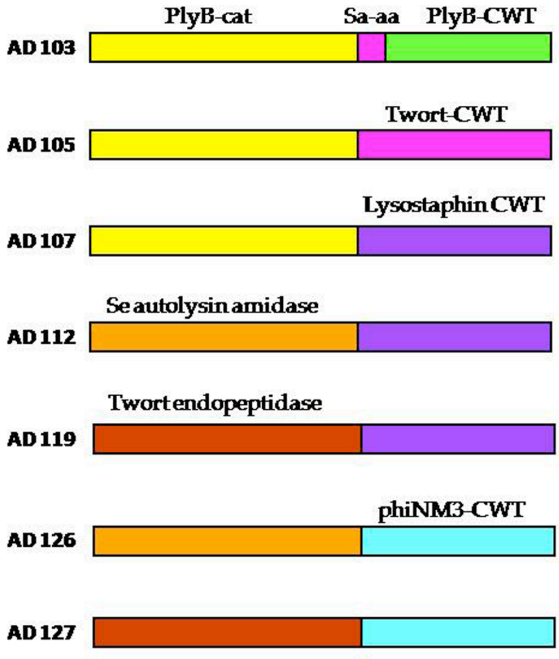


Figure 9.2. PhiNM3 CWT binds specifically to staphylococci.

Purified phiNM3 CWT was labeled with FITC and exposed to 1) *S. aureus*; 2) *B. cereus*; 3) *S. epidermidis*; 4) *E. coli*; 5) *S. pyogenes* and 6) mixed suspension of *S. aureus* and *B. cereus* cells. P: phase-contrast image and F: fluorescent image.

9.3.3 Function-guided construction of chimeric lysin ClyS.

Previous attempts in our laboratory to clone and express a soluble and active native staphylococcus-specific phage lysin have been unsuccessful. Therefore, we attempted to circumvent these problems by taking advantage of the modular nature of lysins and develop lysin chimeras. Many inactive chimeras were generated in a series of logical but progressive steps (Figure 9.3), the most successful of which was chimera AD127 composed of the lysin endopeptidase domain of phage Twort fused amino-terminally of the phiNM3 CWT domain. The AD127 chimera was soluble and highly active against staphylococcal cells but suffered from poor expression in *E. coli*. To overcome this, the ORF encoding AD127 was cloned into expression plasmid pJML6 to get pAD138. Plasmid pJML6 was previously used to over express *S. pneumoniae* lysin Cpl-1 and PAL in *E. coli* (Loeffler, Djurkovic et al. 2003). Chimera AD138 was named 'ClyS' for Chimeric lysin for *Staphylococcus*. ClyS is a 280 amino acid protein with a deduced molecular mass of 31,956 Da and a theoretical isoelectric point of 9.17. It was purified by a two-step column chromatography method to >90% homogeneity (data not shown).



	Expression	Solubility	*S. aureus* activity
AD 103	+/-	+	-
AD 105	+	+	-
AD 107	+	-	-
AD 112	+++	+++	-
AD 119	+/-	+/-	+/-
AD 126	-	-	-
AD 127	+/-	+	+++

Figure 9.3. Chimeric lysin development.

A. schematic diagram of various chimeric lysins showing their respective domains and the corresponding expression, solubility and activity of the protein against *S. aureus* cells. Similar domains are depicted in the same colors and are labeled. PlyB-cat: catalytic domain of Bacillus-specific lysin PlyB; Sa-aa: 16 amino acid residues specific for staphylococcal lysins; PlyB-CWT: CWT domain of PlyB; Twort-CWT: CWT domain of *S. aureus* phage Twort lysin; Lysostaphin CWT: CWT domain of lysostaphin; Se autolysin amidase: amidase domain of *S. epidermidis* autolysin.

9.3.4 In vitro activity of ClyS.

In preliminary experiments using varying quantities of ClyS with staphylococci it was determined that 250 μg was an effective dose. For example, when 250 μg of ClyS was added to exponential phase of *S. aureus* 8325-4 cells, the OD_{600} dropped 3-4 fold within ~15 min (Figure 9.4). To confirm that the observed loss of turbidity corresponded to a decrease in viable cell counts, aliquots from the spectrophotometric lysis assay were plated to TSB agar at various time points and CFU were enumerated. A decrease in viability of ~3-logs was observed in 30 min (Figure 9.4).

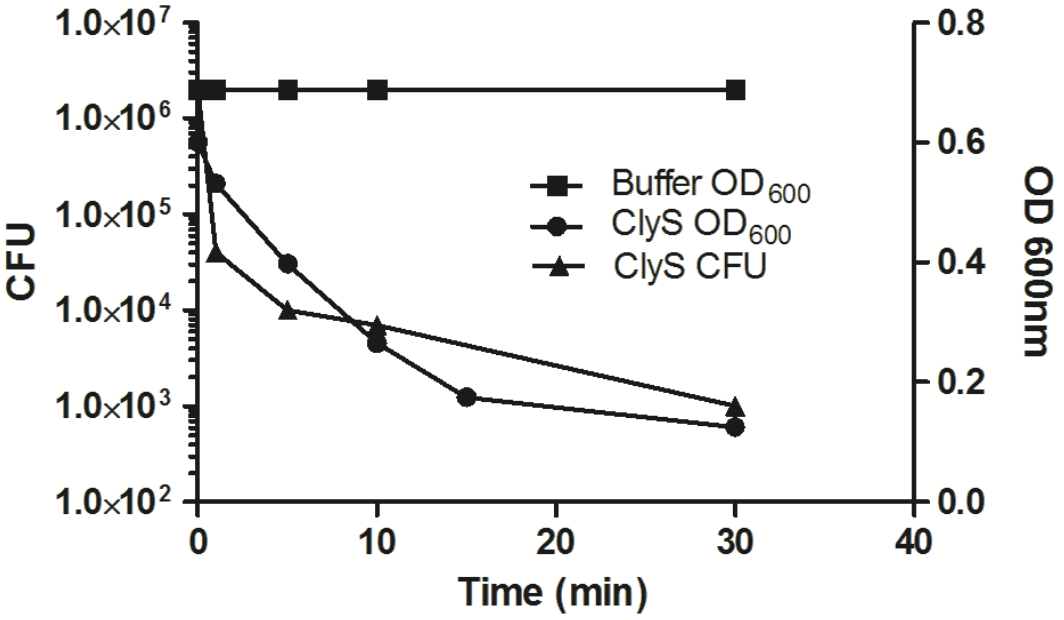


Figure 9.4. Activity of ClyS against *S. aureus* in vitro.

S. aureus strain 8325-4 cells were resuspended in 20 mM phosphate buffer (pH 7.4), incubated with 250 μg of ClyS and OD_{600} (filled circles) monitored by a spectrophotometer. Control experiments (filled squares) were performed under the same conditions with buffer alone. Cell viability (filled triangles) measured as CFU/ml, was determined by serially diluting and plating the same cell suspensions to TSB agar plates.

The lytic effect on *S. aureus* 8325-4 cells exposed to 250 μg of ClyS for 1-3 min was visualized by transmission electron microscopy (Figure 9.5 A and B). Cells exposed to ClyS showed localized degradation of the cell wall at single or multiple sites, which is typical of lysin-mediated cell lysis. The sites of degradation on the cell were randomly distributed over the entire cell surface. This observation correlated with the binding of FITC-labeled CWT domain to the whole surface of the staphylococcal cells. The localized weakening of cell wall resulted in extrusion and rupture of the cell membrane (Figure 9.5) and subsequent loss of cytoplasmic contents and formation of cell-ghosts (Figure 9.5).

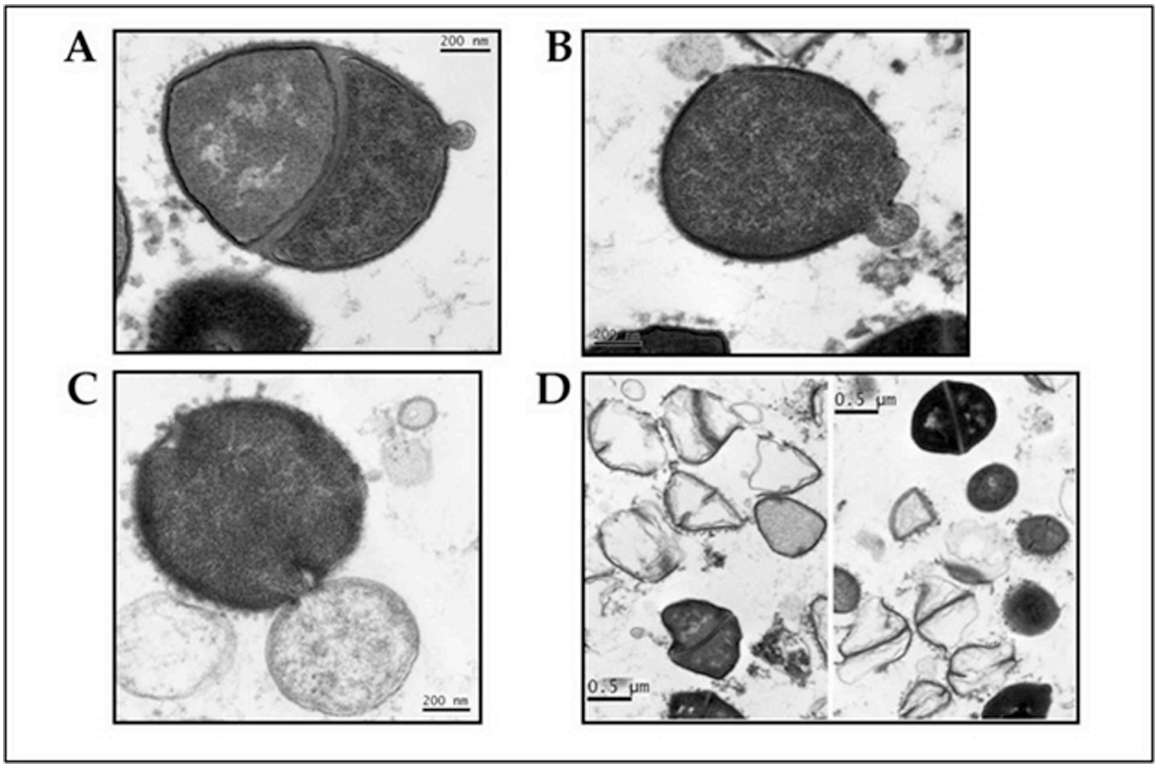


Figure 9.5. ClyS causes cell wall disruption and ultimately lysis of 8325-4 cells. (A-C) Thin-section transmission electron micrographs (bars, 200 nm) of *S. aureus* 3 minutes after exposure to 250 μg of ClyS. The arrows indicate cytoplasmic membrane extrusions through holes generated in the cell wall by ClyS. Ultimate lysis results in mostly “cell-ghosts” (D) after the loss of cytoplasmic contents (bar, 500 nm).

9.3.5 Specificity of ClyS.

Muralytic activity of ClyS was tested on a number of bacterial strains representing a variety of genus and species that were divided into several sets (Table 9.1 and Figure 9.6). Set I consisted of methicillin-sensitive (MSSA) and methicillin-resistant strains (MRSA) of *S. aureus*. ClyS was very active against both MSSA and MRSA although some differences were observed between *S. aureus* strains. ClyS lysin also effectively killed six vancomycin intermediate resistant strains of staphylococci (VISA) and the lysostaphin resistant *S. aureus* strain LyrA (Data not shown). Set II consisted of different species of staphylococci including *S. epidermidis*, *S. simulans* and *S. sciuri (subsp. sciuri)*. ClyS was not only active against *S. epidermidis* including the biofilm-forming strain RP62A (Tao, Fan et al. 2006) but was also active against *S. simulans* and *S. sciuri (subsp. sciuri).* Set III consisted of a mix of Gram-positive and Gram-negative bacteria including representatives of group A, B, C and E streptococci, the oral streptococcal species *S. gordonii*, and *S. salivarius,* as well as *S. uberis, Bacillus cereus, Pseudomonas aeruginosa* and *E. coli*. ClyS did not exhibit lytic activity against any of these bacteria (Figure 9.6).

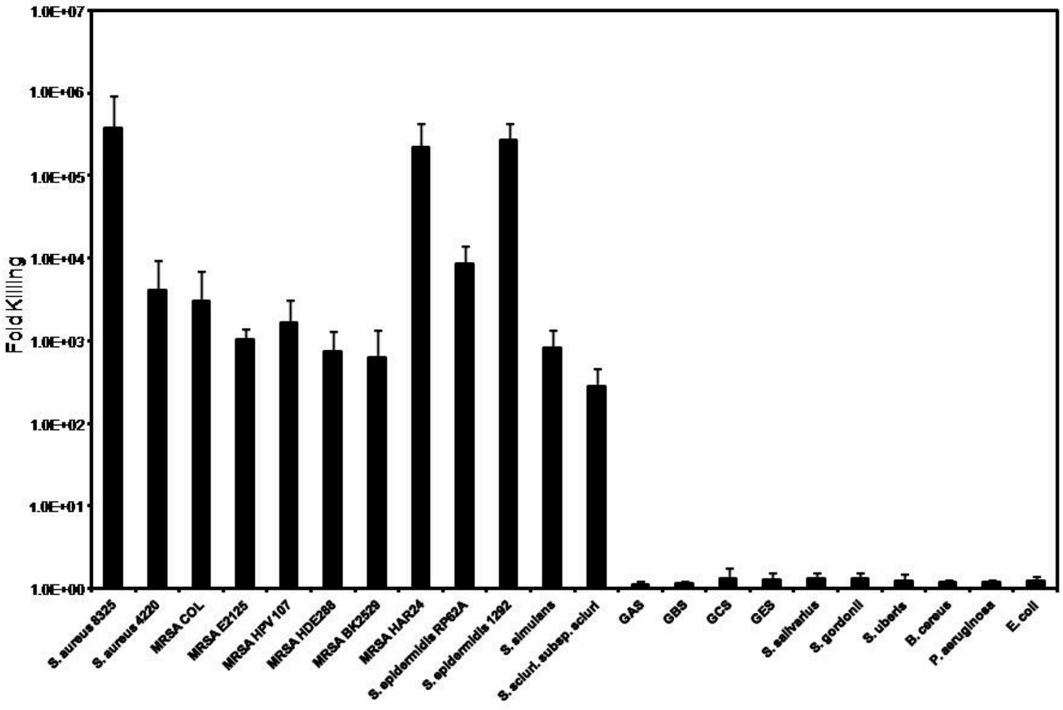


Figure 9.6. ClyS exerted specific killing of antibiotic-susceptible and resistant staphylococci.

Log-phase cultures of different bacteria were exposed to 250 μg of ClyS for 15 min. Fold killing was calculated by dividing the number (CFU) of viable bacteria after buffer treatment by the number (CFU) after exposure to ClyS enzyme. Several MSSA and MRSA strains along with *S epidermidis*, *S. simulans* and *S. sciuri* strains were tested for susceptibility to ClyS killing. Control strains GAS: group A streptococci; GBS: group B streptococci; GCS: group C streptococci and GES: group E streptococci along with enterococci, other streptococci, bacilli, pseudomonas and *E. coli* were also tested.

9.3.6 Immune response to ClyS.

It was shown previously that lysins have the unique capacity to resist neutralization by antibodies, both in vitro and in vivo assays (Loeffler, Djurkovic et al. 2003; Mangili, Bica et al. 2005). However, chimeric lysins have not evolved naturally and as such may not retain this unique capacity. To address this, rabbit hyperimmune serum raised against ClyS (ELISA titer of >100,000) was assayed for its effect on lytic activity. When the antibody was added to ClyS (100 μg) at a 1:10 dilution (10,000 final ELISA titer) and allowed to stand for 15 minutes before adding staphylococci, no effect on the lytic activity was observed (Figure 9.7). A similar result was obtained with a second hyperimmune rabbit serum to ClyS (data not shown).

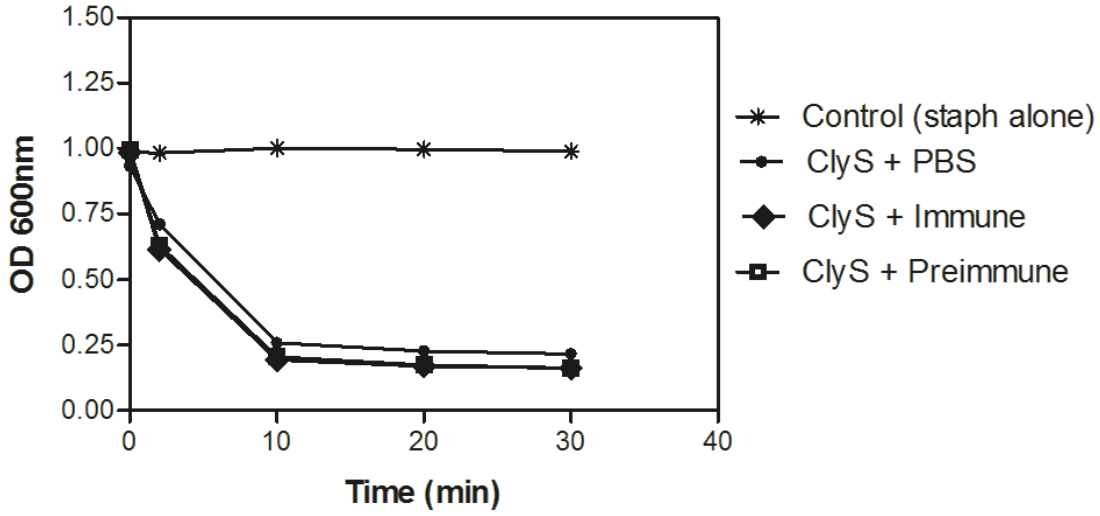


Figure 9.7. Effect of hyperimmune rabbit sera on ClyS activity.

10 μl of either hyperimmune rabbit serum (final ELSA titer of 10,000), pre-immune rabbit serum or PBS was added to 100 μl of ClyS (100 μg). After 15 min, staphylococci were added and the change in OD_{600} was measured for 30 min.

In vivo Nasal Decolonization of MRSA by ClyS. To study the potential of ClyS to reduce MRSA colonization of the nasal cavities, C57BL/6J mice were intranasally inoculated with ~$5x10^9$ of a spontaneously streptomycin resistant strain of MRSA (191-SM^R). Twenty-four hours post-infection mice were administered one dose of either buffer or ClyS into the nasal passages. An hour after the treatment, mice were sacrificed and bacterial colonies were enumerated on Spectra MRSA agar. Three independent experiments were performed to evaluate a total of 19 mice for each treatment group (Figure 9.8). Compared to the buffer alone control (Avg. 120,197 CFU/cavity), a single ClyS treatment (Avg. 731 CFU/cavity) significantly ($p<0.002$) reduced the mean CFU on the nasal mucosa by greater than 2 logs.

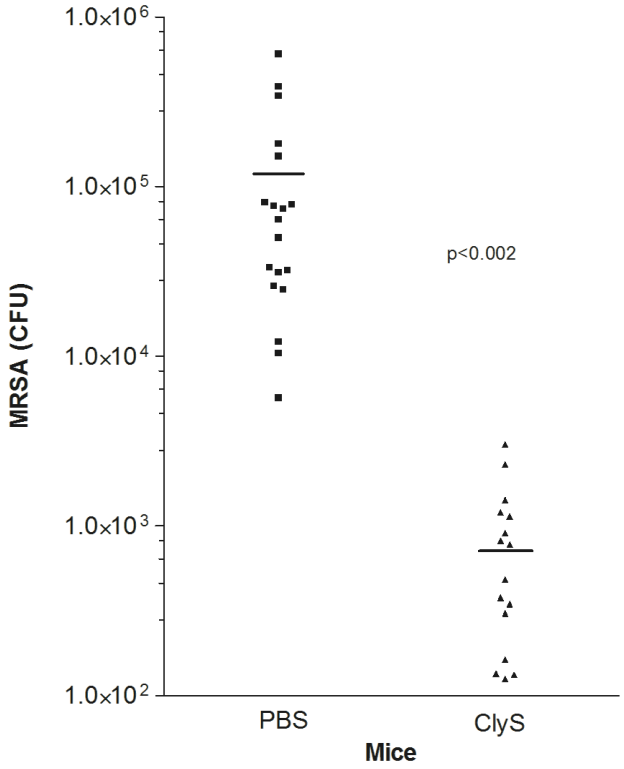


Figure 9.8. Effect of ClyS on nasal colonization by MRSA.

Nasal passages of C57BL/6J mice were inoculated with ~5x10^9 of MRSA strain 191-SM^R. After 24 hours, mice were treated by a single intranasal administration of 1 mg of ClyS or 20 mM phosphate buffer. One hour after treatment the bacteria from the excised nasal cavities were plated on Spectra MRSA agar to enumerate CFU. Data from three independent experiments were combined (19 mice per treatment group) and analyzed for statistical significance with the Student's T-test.

9.3.7 ClyS Treatment of Systemic MRSA Infections.

In order to assess whether ClyS treatment could prevent death resulting from systemic MRSA infections, 4 week old FVB/NJ mice were intraperitoneally injected with ~$5x10^5$ CFU of the community-acquired (PVL toxin-encoding) MRSA strain MW2 in 5% mucin. Preliminary experiments determined that $5x10^5$ CFU was 10 fold the LD_{100} dose for a twenty-four hour period and that within 1-3 hours of IP injection the MRSA infection was systemic, (i.e. >1,000 MRSA CFUs recovered from heart, liver, spleen, and kidney) (data not shown). Treatment occurred three hours post-infection, with either 20mM phosphate buffer or a single 1 mg dose of ClyS in 20mM phosphate buffer injected IP. Mice were then monitored for survival over ten days. The results from three independent experiments were combined (ClyS treatment, n =16; buffer treatment, n =14) and mouse survival data plotted with a Kaplan Meier Survival curve (Figure 9.9). Within twenty-four hours of MRSA infection all of the control mice died of bacterial sepsis, while only 2/16 of ClyS treated mice died at forty-eight hours. The remaining ClyS treated mice (14/16, 88%) survived over the 10-day course of the experiments (Figure 9.9). Other experiments showed that treatment with one dose of 1mg of ClyS / mouse at 1hr or 6hr post infection also rescued mice from death (data not shown).

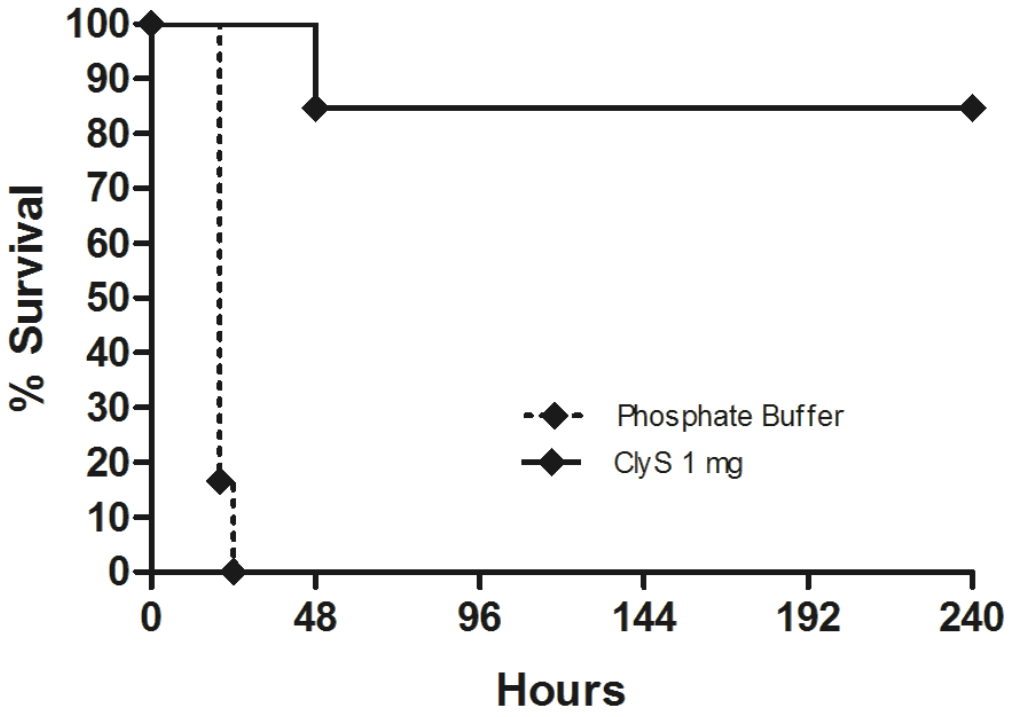


Figure 9.9. ClyS protected mice from death caused by MRSA septicemia.

FVB/NJ mice were intraperitonally injected with ~5x10^5 CFU of MRSA strain MW2 in 5% mucin. Three hours post-infection, mice received one intraperitoneal injection of 20 mM phosphate buffer control or 1 mg of ClyS. Mice were monitored for survival over ten days. The results from three independent experiments were combined (ClyS treatment, *n* =16; buffer treatment, *n* =14) and the mice survival data plotted with a Kaplan Meier Survival curve.

9.3.8 ClyS shows synergistic interaction with Vancomycin and Oxacillin.

A standard checkerboard broth-microdilution assay was used to test the interaction of ClyS with vancomycin and ClyS with oxacillin to determine synergy. The ClyS MIC was 30 to 40 μg/ml for both strains tested while the vancomycin MIC for VISA strain Mu50 was 16 μg/ml and the oxacillin MIC for MRSA strain COL was 64 μg/ml. Isobolograms were plotted for ClyS with vancomycin and with oxacillin by transcribing the enzyme concentrations along the inhibitory line on the microtiter plate in an *x*/*y* plot. The shape of the curves for both antibiotics were characteristic of highly synergistic interactions (Figure 9.10) and were confirmed by calculating the ΣFIC for both interactions which were <0.5).

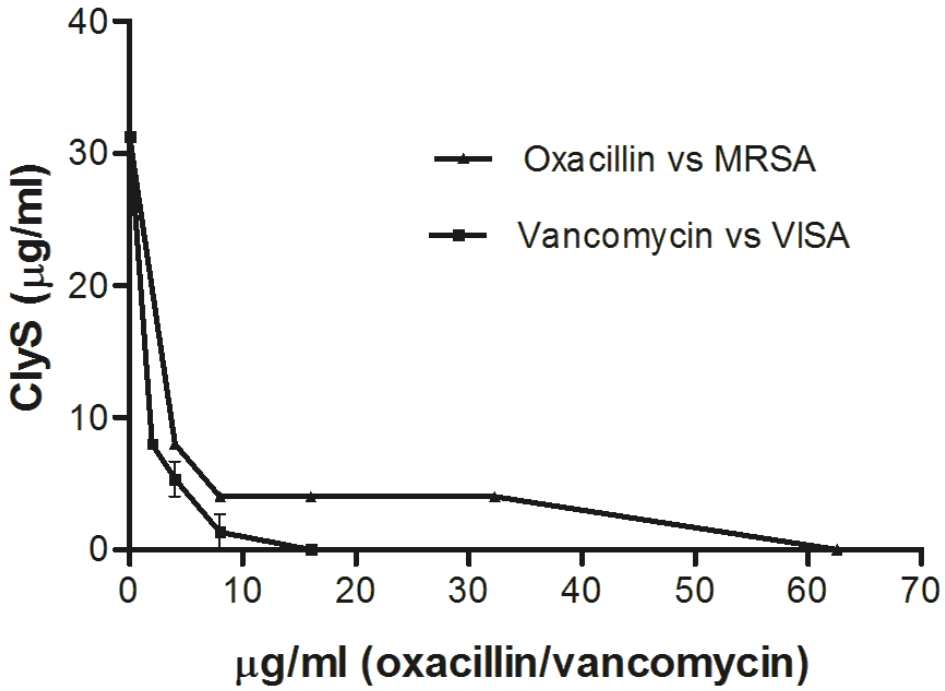


Figure 9.10. ClyS showed synergistic interaction with vancomycin or oxacillin.

Interactions between ClyS and antibiotics were tested by the checkerboard broth micro-dilution assay. Fractional concentrations of MICs along the inhibitory line for enzyme or antibiotic were plotted on the x/y plot to generate an isobologram. Error bars show standard errors of mean.

9.3.9 In vivo Synergy of Oxacillin and ClyS in MRSA Infections.

To determine if the in vitro synergy observed between ClyS and oxacillin could be translated in our MRSA septicemia model, FVB/NJ mice were intraperitoneally injected with ~$5x10^5$ CFU of MRSA strain MW2 as above. At three hours post infection, the bacteremic mice were treated in parallel, with a low IP dose of ClyS (166 μg/mouse) and different concentrations of oxacillin IM (ranging from 10–100 μg/mouse) or buffer controls by the same routes. Preliminary experiments determined that an ED_{30} dose of ClyS (166 μg/mouse) provided enough efficacy to offer partial protection to mice while being a low enough concentration to evaluate the effect of combinatorial treatment with oxacillin (data not shown). Mice were monitored for survival for 10 days and the results of 5 independent experiments were combined and plotted in a Kaplan Meier Survival curve (Figure 9.11). While only 30% (6/20 alive) to 35% (8/23 alive) of mice survived with individual treatments of either 166 μg/mouse of ClyS or 100 μg/mouse of oxacillin, respectively, neither differed significantly from the survival rate of the buffer alone (13%, 2/15 alive) control. Conversely, a single dose of the combined treatment of intraperitoneally injected ClyS with either 50 μg or 100 μg of oxacillin injected intramuscularly significantly increased mouse survival (80%, 8/10 alive; 82%, 18/22 alive respectively) compared to the individual treatments and buffer alone (Figure 9.11).

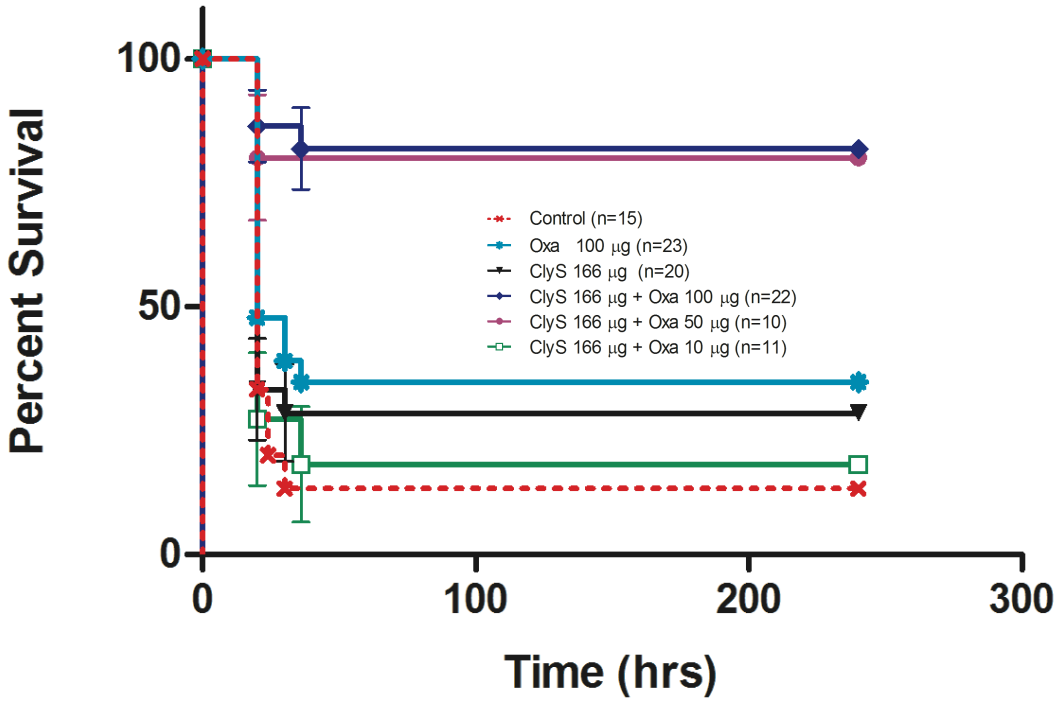


Figure 9.11. Synergistic effects of ClyS and oxacillin protected mice from MRSA septicemia induced death.

FVB/NJ mice were intraperitoneally injected with ~5x10^5 CFU of MRSA strain MW2 in 5% mucin. Three hours post-infection, mice received an IP injection of a suboptimal concentration of ClyS (166μg) or 20mM phosphate buffer along with an IM injection of oxacillin (10μg - 100μg) or saline control. Mice were monitored for survival for 10 days and the results of 5 independent experiments were combined and plotted in a Kaplan Meier Survival curve.

9.4 DISCUSSION

ClyS, a staphylococcal-specific lysin, has demonstrated synergistic interaction in vitro (ΣFIC <0.5) with two antibiotics commonly used to treat staphylococcal infections, namely oxacillin and vancomycin. These results are in agreement with previous studies suggesting a synergistic interaction between phage lysins with different classes of antibiotics (Rashel, Uchiyama et al. 2007; Manoharadas, Witte et al. 2009). More importantly however, we have demonstrated the synergistic interaction of ClyS with oxacillin in vivo, the first report to our knowledge. A suboptimal concentration of ClyS in combination with oxacillin was able to significantly increase survival of mice infected with MRSA strain MW2 compared to each compound individually. We speculate that the in vivo synergy between oxacillin and ClyS may be due to enhanced lysis of the bacteria, similar to what is observed with oxacillin treatment of the *mgrA/sarA* mutants of *S. aureus* (Trotonda, Xiong et al. 2009). Oxacillin inhibits cell wall assembly enzymes resulting in increased expression of internal peptidoglycan hydrolases and autolysins causing weakening of the cell wall peptidoglycan. As an endopeptidase, adding ClyS would elevate the total concentration of wall-degrading enzymes further shifting the balance of the cell wall repair and degrading machinery to cause increased degradation and thus lysis of the bacteria. These results could allow antibiotics now discontinued in the clinic, due

to increased resistance issues, to be reinstated when combined with an appropriate dose of ClyS.

Using ClyS alone may prove to be a viable treatment for more serious, *S. aureus* diseases, such as soft tissue infections, bacteremia, infective endocarditis, and pneumonia. Unlike antibiotics whose effects are only seen hours after administration, lysins work immediately, reducing the accumulation of toxic substances. This is illustrated in recent studies showing the effectiveness of phage lysins in treating systemic infections (Schuch, Nelson et al. 2002; Loeffler, Djurkovic et al. 2003) and in one case the successful treatment of an established pneumococcal pneumonia (Witzenrath, Schmeck et al. 2009). In our study, a single IP dose of ClyS in mice septic with MRSA resulted in significantly higher survival rates (88%) than untreated septic mice (0%). It is likely that the survival rates of the treated mice could be increased further by changing the dosing to either a higher dose of ClyS or by repeat dosing.

To accomplish these protection experiments, it was necessary to develop a chimeric lysin in order to circumvent the solubility problems inherent in staphylococcal phage lysins (Navarre, Ton-That et al. 1999; O'Flaherty, Coffey et al. 2005) and our own unpublished data). We took advantage of the modular nature of lysins, which has been exploited by researchers, to swap different catalytic and binding domains while retaining the activity and/or specificity,

respectively, of the original lysin (Donovan, Dong et al. 2006). Using biochemical and functional assays as guidance, more than 15 chimeras were strategically engineered to contain different combinations of catalytic and binding domains. From these constructs a novel lysin, ClyS, was engineered that had the desirable properties of a well expressed and highly soluble lysin that was active against staphylococci.

ClyS is a chimera consisting of the N-terminal endopeptidase domain of the lysin from phage Twort fused with the C-terminal CWT domain of phage phiNM3 lysin. The phiNM3 CWT domain by itself was highly soluble and displayed binding specificity toward staphylococcal cells. Additionally, the CWT domain met a second criterion wherein it did not have homology to the SH3b-like binding domain associated with lysostaphin and some lysins. SH3b binding domains are presumed to bind the peptide cross-bridges in the bacterial cell wall peptidoglycan (Grundling and Schneewind 2006). These peptide bridges can be readily altered by the bacteria rendering them resistant to these lytic enzymes including lysostaphin. This mechanism of resistance could presumably be circumvented by engineering a chimeric lysin that contained a novel CWT domain. As such, the phiNM3 CWT domain of ClyS does not share homology to any known domains in the GenBank database. The absence of homology in the binding domain is a common feature for lysins such as PlyG and Cpl-1 (Loeffler, Nelson et al. 2001; Schuch, Nelson et al. 2002). These lysins use carbohydrate

and choline moieties respectively in the bacterial cell wall as their receptors. The uniqueness of the binding domains of such lysins complements the unique structures present on the bacterial cell walls, thereby imparting to lysins their characteristically exquisite specificity. The binding domain of ClyS also presumably recognizes a cell wall-associated carbohydrate or another moiety. This in turn may make ClyS an attractive target for development as an alternative therapeutic because of the lack of resistance as seen for PlyG (Schuch, Nelson et al. 2002), Cpl-1, and other lysins (unpublished data).

Analysis of the spectrum of activity for ClyS supports the above assumptions for ClyS since lysostaphin-resistant *lyrA* mutants of *S. aureus*, as well as the lysostaphin-producer *S. simulans* were susceptible to ClyS (Grundling, Missiakas et al. 2006; Grundling and Schneewind 2006). Activity of ClyS against *S. simulans* indicates that the binding domain is unaffected by the presence of a serine residue in the peptide cross-bridges of *S. simulans* peptidoglycan, the reason for its resistance to lysostaphin. ClyS was also active against several *Staphylococcus sp.* including all tested MRSA and VISA strains, as well as, *S. sciuri* and biofilm-producing and non-producing strains of *S. epidermidis* suggesting that the binding moiety of ClyS probably is a common cell wall component found in all *Staphylococcus* species. Such broad-spectrum activity of ClyS seen against all staphylococci tested is uncommon among native phage lysins, which typically display species specificity (Loeffler, Nelson et al. 2001;

Schuch, Nelson et al. 2002). However, the lack of activity of ClyS against other Gram-positive or Gram-negative bacteria resembles the characteristic specificity seen in native phage lysins. The species specificity of ClyS and its ability to kill multi-drug resistant as well as biofilm-producing staphylococci in vitro makes ClyS a valuable treatment option for staphylococcal infection or decolonization of skin and mucous membranes.

In humans, the nasal mucous membranes are the major reservoir of staphylococci, including MRSA, and are an endemic risk factor for skin and soft tissue infections as well as bacteremia in the patient population (Coates, Bax et al. 2009). A decline in nosocomial infection is reported with the reduction or elimination of this reservoir. Lysins have been successfully used in several different animal models of mucosal colonization to decolonize a wide range of pathogenic bacteria including staphylococci, pneumococci, and group A and group B streptococci (Nelson, Loomis et al. 2001; Schuch, Nelson et al. 2002; Loeffler, Djurkovic et al. 2003; Cheng, Nelson et al. 2005; Rashel, Uchiyama et al. 2007). When administered intranasally to mice, ClyS efficiently eliminated MRSA by decolonizing the nasal passages. A greater than two-log drop was seen in an hour after a single treatment as compared to buffer alone. The currently accepted *S. aureus* decolonization regimen includes treatment with mupirocin ointment nasally for 5 days, but recolonization and inadequate clearance often results because of increasing resistance to the antibiotic or poor

patient compliance (Coates, Bax et al. 2009; Udo and Sarkhoo 2009). The fact that ClyS works as a potent decolonizing agent suggests that lysin therapy is a viable alternate treatment option in specific high-risk populations such as in hospitals and in nursing homes etc. and may aid in decreasing primary and secondary infection rates. Furthermore, unlike antibiotics, the specificity of ClyS to staphylococci may allow it to be used prophylactically, to reduce further carriage of *S. aureus* and MRSA in health-care employees or in community settings where MRSA resistance is becoming an increasing problem, such as military bases, prisons, and sports teams (Coates, Bax et al. 2009). Finally, recent evidence indicates that >90% of deaths as a result of influenza pandemics were caused from secondary infections caused by *S. aureus, S. pneumoniae,* and *S. pyogenes* (Brundage and Shanks 2007; Morens, Taubenberger et al. 2009), and 30% of deaths from the recent H1N1 was complicated due to the same causes (MMWR 2009). Thus, ClyS and other lysins may be used during flu season to reduce colonization and/or treat secondary infections caused by these pathogens in high-risk individuals.

The current standard of care for treatment of serious bacterial infections, pending identification of the organism, is to cover the most likely organisms based on the suspected site of infection, the patient's clinical condition, and any relevant environmental factors (Dellinger, Carlet et al. 2004; Cunha 2008). Since staphylococci are ubiquitous organisms, anti-staphylococcal coverage is included

in many empiric regimens. Treatment of serious infections caused by *S. aureus* has been a challenge for decades, because the bacteria rapidly develop resistance mutations to novel antibacterials. Beta lactam antibiotics are among the most widely used for treatment of serious infection, but no currently available beta lactam agent is effective against MRSA. Furthermore, MRSA have developed resistance to newer agents including glycopeptides (Huang, Hsiao et al. 2008) and oxazolidinones (Wilson, Andrews et al. 2003). Lysins, because of their low probability of bacterial resistance plus rapid activity and high specificity could be an important addition to the medical armamentarium.

In summary, we report the development of a novel chimeric lysin with improved biochemical properties and excellent lytic activity against all staphylococci including MRSA and VISA strains. We have demonstrated the effectiveness of ClyS when used alone or in combination with existing antibiotics for treatment of serious staphylococcal infections. This alternative therapeutic option will provide a viable tool to combat the increasing problem of infections caused by multidrug resistant *S. aureus*.

10 Conclusions

Although the existence of phage has been known and studied for over three quarters of a century, we are far from having a full understanding of the many interactions between bacteriophage and their hosts or the dynamic roles that both play in bacterial pathogenesis and survival. As more sophisticated molecular biology and sequencing techniques have been developed, our understanding and appreciation of the complex role that bacteriophage play in the life cycle of both non-virulent and pathogenic bacteria have increased accordingly. We believe that our studies on the manipulation of phage genes and the perturbation of phage-host interactions have further elaborated new and novel mechanisms by which phages and their hosts interact at the molecular level. We hope the novel techniques that we have developed throughout these studies will aid future scientists in the exploration of even more complex relationships between these organisms. For as our understanding of these interactions increase, so does the potential for novel antimicrobials to be developed in the treatment and prevention of bacterial diseases. After all...

" … the actions and reactions are not solely between these two beings, bacterium and phage, for the scientist also intervenes; a third living being and hence, a third variable is introduced" (Euler 2010).

11 References

Accardo, P., P. Sanchez-Corral, et al. (1996). "Binding of human complement component C4b-binding protein (C4BP) to Streptococcus pyogenes involves the C4b-binding site." J Immunol **157**(11): 4935-9.

Agarwal, B. L. (1981). "Rheumatic heart disease unabated in developing countries." Lancet **2**(8252): 910-1.

Aksoy, D. Y. and S. Unal (2008). "New antimicrobial agents for the treatment of Gram-positive bacterial infections." Clin.Microbiol Infect. **14**(5): 411-420.

Altschul, S. F., T. L. Madden, et al. (1997). "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs." Nucleic Acids Res **25**(17): 3389-402.

Arraiano, C. M., J. Bamford, et al. (2007). "Recent advances in the expression, evolution, and dynamics of prokaryotic genomes." J Bacteriol **189**(17): 6093-100.

Ashbaugh, C. D., S. Alberti, et al. (1998). "Molecular analysis of the capsule gene region of group A Streptococcus: the hasAB genes are sufficient for capsule expression." J Bacteriol **180**(18): 4955-9.

Ashbaugh, C. D., H. B. Warren, et al. (1998). "Molecular analysis of the role of the group A streptococcal cysteine protease, hyaluronic acid capsule, and M protein in a murine model of human invasive soft-tissue infection." J Clin Invest **102**(3): 550-60.

Aziz, R. K., S. A. Ismail, et al. (2004). "Post-proteomic identification of a novel phage-encoded streptodornase, Sda1, in invasive M1T1 Streptococcus pyogenes." Mol Microbiol **54**(1): 184-97.

Aziz, R. K. and M. Kotb (2008). "Rise and persistence of global M1T1 clone of Streptococcus pyogenes." Emerg Infect Dis **14**(10): 1511-7.

Aziz, R. K., M. J. Pabst, et al. (2004). "Invasive M1T1 group A Streptococcus undergoes a phase-shift in vivo to prevent proteolytic degradation of multiple virulence factors by SpeB." Mol Microbiol **51**(1): 123-34.

Banks, D. J., S. B. Beres, et al. (2002). "The fundamental contribution of phages to GAS evolution, genome diversification and strain emergence." Trends Microbiol **10**(11): 515-21.

Banks, D. J., B. Lei, et al. (2003). "Prophage induction and expression of prophage-encoded virulence factors in group A Streptococcus serotype M3 strain MGAS315." Infect Immun **71**(12): 7079-86.

Banks, D. J., S. F. Porcella, et al. (2004). "Progress toward characterization of the group A Streptococcus metagenome: complete genome sequence of a macrolide-resistant serotype M6 strain." J Infect Dis **190**(4): 727-38.

Banks, D. J., S. F. Porcella, et al. (2003). "Structure and distribution of an unusual chimeric genetic element encoding macrolide resistance in phylogenetically diverse clones of group A Streptococcus." J Infect Dis **188**(12): 1898-908.

Bassoe, C. F. and R. Bjerknes (1985). "Phagocytosis by human leukocytes, phagosomal pH and degradation of seven species of bacteria measured by flow cytometry." J Med Microbiol **19**(1): 115-25.

Beall, B., R. Facklam, et al. (1997). "Application of emm gene sequencing and T antigen serology for typing group A streptococcal systemic isolates. Survey of random and outbreak-related isolates." Adv Exp Med Biol **418**: 307-11.

Beall, B., R. Facklam, et al. (1996). "Sequencing emm-specific PCR products for routine and accurate typing of group A streptococci." J Clin Microbiol **34**(4): 953-8.

Beall, B., R. R. Facklam, et al. (1998). "Streptococcal emm types associated with T-agglutination types and the use of conserved emm gene restriction fragment patterns for subtyping group A streptococci." J Med Microbiol **47**(10): 893-8.

Beck, A., S. Bergner-Rabinowitz, et al. (1969). "Effect of pH on in vitro phagocytosis of Streptococcus pyogenes." J Bacteriol **100**(3): 1204-7.

Benchetrit, L. C., E. D. Gray, et al. (1977). "Hyaluronidase activity of bacteriophages of group A streptococci." Infect Immun **15**(2): 527-32.

Bensing, B. A., I. R. Siboo, et al. (2001). "Proteins PblA and PblB of Streptococcus mitis, which promote binding to human platelets, are encoded within a lysogenic bacteriophage." Infect Immun **69**(10): 6186-92.

Beres, S. B. and J. M. Musser (2007). "Contribution of exogenous genetic elements to the group A Streptococcus metagenome." PLoS One **2**(8): e800.

Beres, S. B., G. L. Sylva, et al. (2002). "Genome sequence of a serotype M3 strain of group A Streptococcus: phage-encoded toxins, the high-virulence phenotype, and clone emergence." Proc Natl Acad Sci U S A **99**(15): 10078-83.

Bergman, M., S. Huikko, et al. (2004). "Effect of macrolide consumption on erythromycin resistance in Streptococcus pyogenes in Finland in 1997-2001." Clin Infect Dis **38**(9): 1251-6.

Berkower, C., M. Ravins, et al. (1999). "Expression of different group A streptococcal M proteins in an isogenic background demonstrates diversity in adherence to and invasion of eukaryotic cells." Mol Microbiol **31**(5): 1463-75.

Bert, F., C. Branger, et al. (1997). "Pulsed-field gel electrophoresis is more discriminating than multilocus enzyme electrophoresis and random amplified polymorphic DNA analysis for typing pyogenic streptococci." Curr Microbiol **34**(4): 226-9.

Bessen, D. E., J. R. Carapetis, et al. (2000). "Contrasting molecular epidemiology of group A streptococci causing tropical and nontropical infections of the skin and throat." J Infect Dis **182**(4): 1109-16.

Bessen, D. E., M. W. Izzo, et al. (1999). "Genetic linkage of exotoxin alleles and emm gene markers for tissue tropism in group A streptococci." J Infect Dis **179**(3): 627-36.

Bessen, D. E. and A. Kalia (2002). "Genomic localization of a T serotype locus to a recombinatorial zone encoding extracellular matrix-binding proteins in Streptococcus pyogenes." Infect Immun **70**(3): 1159-67.

Bingen, E., R. Leclercq, et al. (2002). "Emergence of group A streptococcus strains with different mechanisms of macrolide resistance." Antimicrob Agents Chemother **46**(5): 1199-203.

Bisno, A. L. (1990). "The resurgence of acute rheumatic fever in the United States." Annu Rev Med **41**: 319-29.

Bisno, A. L., M. O. Brito, et al. (2003). "Molecular basis of group A streptococcal virulence." Lancet Infect Dis **3**(4): 191-200.

Bisno, A. L., M. A. Gerber, et al. (2002). "Practice guidelines for the diagnosis and management of group A streptococcal pharyngitis. Infectious Diseases Society of America." Clin Infect Dis **35**(2): 113-25.

Bohach, G. A., A. R. Hauser, et al. (1988). "Cloning of the gene, speB, for streptococcal pyrogenic exotoxin type B in Escherichia coli." Infect Immun **56**(6): 1665-7.

Bolhuis, H., G. Poelarends, et al. (1995). "The Lactococcal lmrP gene encodes a proton motive force-dependent drug transporter." J Biol Chem **270**(44): 26092-8.

Boyle-Vavra, S. and R. S. Daum (2007). "Community-acquired methicillin-resistant Staphylococcus aureus: the role of Panton-Valentine leukocidin." Lab Invest **87**(1): 3-9.

Breese, B. B. and C. B. Hall (1978). Beta hemolytic streptococcal diseases. Boston, Houghton Mifflin.

Brenciani, A., K. K. Ojo, et al. (2004). "Distribution and molecular analysis of mef(A)-containing elements in tetracycline-susceptible and -resistant Streptococcus pyogenes clinical isolates with efflux-mediated erythromycin resistance." J Antimicrob Chemother **54**(6): 991-8.

Brinkmann, V., U. Reichard, et al. (2004). "Neutrophil extracellular traps kill bacteria." Science **303**(5663): 1532-5.

Broudy, T. B. and V. A. Fischetti (2003). "In vivo lysogenic conversion of Tox(-) Streptococcus pyogenes to Tox(+) with Lysogenic Streptococci or free phage." Infect Immun **71**(7): 3782-6.

Broudy, T. B., V. Pancholi, et al. (2001). "Induction of lysogenic bacteriophage and phage-associated toxin from group a streptococci during coculture with human pharyngeal cells." Infect Immun **69**(3): 1440-3.

Broudy, T. B., V. Pancholi, et al. (2002). "The in vitro interaction of Streptococcus pyogenes with human pharyngeal cells induces a phage-encoded extracellular DNase." Infect Immun **70**(6): 2805-11.

Brown, J. H. (1919). The use of blood agar for the study of streptococci. New York,, The Rockefeller institute for medical research.

Brundage, J. F. and G. D. Shanks (2007). "What really happened during the 1918 influenza pandemic? The importance of bacterial secondary infections." J.Infect.Dis. **196**(11): 1717-1718.

Brussow, H., C. Canchaya, et al. (2004). "Phages and the evolution of bacterial pathogens: from genomic rearrangements to lysogenic conversion." Microbiol Mol Biol Rev **68**(3): 560-602.

Brussow, H., M. Fremont, et al. (1994). "Detection and classification of Streptococcus thermophilus bacteriophages isolated from industrial milk fermentation." Appl Environ Microbiol **60**(12): 4537-43.

Buchanan, J. T., A. J. Simpson, et al. (2006). "DNase expression allows the pathogen group A Streptococcus to escape killing in neutrophil extracellular traps." Curr Biol **16**(4): 396-400.

Burns, E. H., Jr., S. Lukomski, et al. (1998). "Genetic inactivation of the extracellular cysteine protease enhances in vitro internalization of group A streptococci by human epithelial and endothelial cells." Microb Pathog **24**(6): 333-9.

Burns, E. H., Jr., A. M. Marciel, et al. (1996). "Activation of a 66-kilodalton human endothelial cell matrix metalloprotease by Streptococcus pyogenes extracellular cysteine protease." Infect Immun **64**(11): 4744-50.

Burrus, V., G. Pavlovic, et al. (2002). "Conjugative transposons: the tip of the iceberg." Mol Microbiol **46**(3): 601-10.

Campbell, A., S. J. Schneider, et al. (1992). "Lambdoid phages as elements of bacterial genomes (integrase/phage21/Escherichia coli K-12/icd gene)." Genetica **86**(1-3): 259-67.

Campbell, A. M. (1992). "Chromosomal insertion sites for phages and plasmids." J Bacteriol **174**(23): 7495-9.

Canchaya, C., F. Desiere, et al. (2002). "Genome analysis of an inducible prophage and prophage remnants integrated in the Streptococcus pyogenes strain SF370." Virology **302**(2): 245-58.

Canchaya, C., F. Desiere, et al. (2002). "Genome analysis of an inducible prophage and prophage remnants integrated in the *Streptococcus pyogenes* strain SF370." Virology **302**(2): 245-58.

Caparon, M. G. and J. R. Scott (1991). "Genetic manipulation of pathogenic streptococci." Methods Enzymol **204**: 556-86.

Carapetis, J. R., A. C. Steer, et al. (2005). "The global burden of group A streptococcal diseases." Lancet Infect Dis **5**(11): 685-94.

Card, C. O., G. G. Wilson, et al. (1990). "Cloning and characterization of the HpaII methylase gene." Nucleic Acids Res **18**(6): 1377-83.

Cha, S., H. Lee, et al. (2001). "The emergence of erythromycin-resistant Streptococcus pyogenes in Seoul, Korea." J Infect Chemother **7**(2): 81-6.

Cheng, Q., D. Nelson, et al. (2005). "Removal of group B streptococci colonizing the vagina and oropharynx of mice with a bacteriophage lytic enzyme." Antimicrob Agents Chemother **49**(1): 111-7.

Cheng, Q., D. Nelson, et al. (2005). "Removal of group B streptococci colonizing the vagina and oropharynx of mice with a bacteriophage lytic enzyme." Antimicrobial Agents and Chemotherapy **49**(1): 111-117.

Chung, M., H. de Lencastre, et al. (2000). "Molecular typing of methicillin-resistant Staphylococcus aureus by pulsed-field gel electrophoresis: comparison of results obtained in a multilaboratory effort using identical protocols and MRSA strains." Microb Drug Resist **6**(3): 189-98.

Cilliers, A. M. (2006). "Rheumatic fever and its management." BMJ **333**(7579): 1153-6.

Cizman, M., M. Pokorn, et al. (2001). "The relationship between trends in macrolide use and resistance to macrolides of common respiratory pathogens." J Antimicrob Chemother **47**(4): 475-7.

Clancy, J., J. Petitpas, et al. (1996). "Molecular cloning and functional analysis of a novel macrolide-resistance determinant, mefA, from Streptococcus pyogenes." Mol Microbiol **22**(5): 867-79.

Cleary, P. P., E. L. Kaplan, et al. (1992). "Clonal basis for resurgence of serious Streptococcus pyogenes disease in the 1980s." Lancet **339**(8792): 518-21.

Cleary, P. P., D. LaPenta, et al. (1998). "A globally disseminated M1 subclone of group A streptococci differs from other subclones by 70 kilobases of prophage DNA and capacity for high-frequency intracellular invasion." Infect Immun **66**(11): 5592-7.

Coates, T., R. Bax, et al. (2009). "Nasal decolonization of Staphylococcus aureus with mupirocin: strengths, weaknesses and future prospects." J Antimicrob.Chemother. **64**(1): 9-15.

Coenen, S., M. Ferech, et al. (2006). "European Surveillance of Antimicrobial Consumption (ESAC): outpatient macrolide, lincosamide and streptogramin (MLS) use in Europe." J Antimicrob Chemother **58**(2): 418-22.

Collin, M. and A. Olsen (2001). "Effect of SpeB and EndoS from Streptococcus pyogenes on human immunoglobulins." Infect Immun **69**(11): 7187-9.

Cone, L. A., D. R. Woodard, et al. (1987). "Clinical and bacteriologic observations of a toxic shock-like syndrome due to Streptococcus pyogenes." N Engl J Med **317**(3): 146-9.

Crick, F. H., L. Barnett, et al. (1961). "General nature of the genetic code for proteins." Nature **192**: 1227-32.

Croux, C., C. Ronda, et al. (1993). "Interchange of functional domains switches enzyme specificity: construction of a chimeric pneumococcal-clostridial cell wall lytic enzyme." Mol.Microbiol **9**(5): 1019-1025.

Cu, G. A., S. Mezzano, et al. (1998). "Immunohistochemical and serological evidence for the role of streptococcal proteinase in acute post-streptococcal glomerulonephritis." Kidney Int **54**(3): 819-26.

Cue, D., P. E. Dombek, et al. (1998). "Streptococcus pyogenes serotype M1 encodes multiple pathways for entry into human epithelial cells." Infect Immun **66**(10): 4593-601.

Cunha, B. A. (2008). "Sepsis and septic shock: selection of empiric antimicrobial therapy." Crit Care Clin. **24**(2): 313-34, ix.

Cunningham, M. W. (2000). "Pathogenesis of group A streptococcal infections." Clin Microbiol Rev **13**(3): 470-511.

Cywes, C. and M. R. Wessels (2001). "Group A Streptococcus tissue invasion by CD44-mediated cell signalling." Nature **414**(6864): 648-52.

D'Ercole, S., D. Petrelli, et al. (2005). "Distribution of mef(A)-containing genetic elements in erythromycin-resistant isolates of Streptococcus pyogenes from Italy." Clin Microbiol Infect **11**(11): 927-30.

d'Herelle, F. (1930). The bacteriophage and its clinical applications. Springfield, IL Baltimore, MD, Charles C Thomas.

Daniel, A., C. Euler, et al. (2010). "Synergism Between a Novel Chimeric Lysin and Oxacillin Protects Against Infection by Methicillin-Resistant Staphylococcus aureus." Antimicrob Agents Chemother.

Del Grosso, M., F. Iannelli, et al. (2002). "Macrolide efflux genes mef(A) and mef(E) are carried by different genetic elements in Streptococcus pneumoniae." J Clin Microbiol **40**(3): 774-8.

Dellinger, R. P., J. M. Carlet, et al. (2004). "Surviving Sepsis Campaign guidelines for management of severe sepsis and septic shock." Crit Care Med. **32**(3): 858-873.

Deresinski, S. (2009). "Bacteriophage therapy: exploiting smaller fleas." Clin Infect Dis **48**(8): 1096-101.

Desiere, F., W. M. McShan, et al. (2001). "Comparative genomics reveals close genetic relationships between phages from dairy bacteria and pathogenic Streptococci: evolutionary implications for prophage-host interactions." Virology **288**(2): 325-41.

Dick, G. F. and G. H. Dick (1983). "Landmark article Jan 26, 1924: The etiology of scarlet fever. By George F. Dick and Gladys Henry Dick." Jama **250**(22): 3096.

Dillon, H. C., Jr. (1979). "Post-streptococcal glomerulonephritis following pyoderma." Rev Infect Dis **1**(6): 935-45.

Dohlsten, M., P. A. Lando, et al. (1990). "Targeting of human cytotoxic T lymphocytes to MHC class II-expressing cells by staphylococcal enterotoxins." Immunology **71**(1): 96-100.

Dombek, P. E., D. Cue, et al. (1999). "High-frequency intracellular invasion of epithelial cells by serotype M1 group A streptococci: M1 protein-mediated invasion and cytoskeletal rearrangements." Mol Microbiol **31**(3): 859-70.

Donovan, D. M., S. Dong, et al. (2006). "Peptidoglycan hydrolase fusions maintain their parental specificities." Appl.Environ.Microbiol **72**(4): 2988-2996.

Egesten, A., A. I. Olin, et al. (2009). "SpeB of Streptococcus pyogenes differentially modulates antibacterial and receptor activating properties of human chemokines." PLoS One **4**(3): e4769.

Elliott, S. D. (1945). "A Proteolytic Enzyme Produced by Group a Streptococci with Special Reference to Its Effect on the Type-Specific M Antigen." J Exp Med **81**(6): 573-592.

Elliott, S. D. and V. P. Dole (1947). "An Inactive Precursor of Streptococcal Proteinase." J Exp Med **85**(3): 305-320.

Ellis, N. M., Y. Li, et al. (2005). "T cell mimicry and epitope specificity of cross-reactive T cell clones from rheumatic heart disease." J Immunol **175**(8): 5448-56.

Engelman, R., D. Shahian, et al. (2007). "The Society of Thoracic Surgeons practice guideline series: Antibiotic prophylaxis in cardiac surgery, part II: Antibiotic choice." Ann.Thorac.Surg. **83**(4): 1569-1576.

Enright, M. C., B. G. Spratt, et al. (2001). "Multilocus sequence typing of Streptococcus pyogenes and the relationships between emm type and clone." Infect Immun **69**(4): 2416-27.

Eriksson, A., B. Eriksson, et al. (1999). "Streptococcal DNase B is immunologically identical to superantigen SpeF but involves separate domains." Clin Diagn Lab Immunol **6**(1): 133-6.

Eriksson, B. K., J. Andersson, et al. (1998). "Epidemiological and clinical aspects of invasive group A streptococcal infections and the streptococcal toxic shock syndrome." Clin Infect Dis **27**(6): 1428-36.

Eriksson, B. K., J. Andersson, et al. (1999). "Invasive group A streptococcal infections: T1M1 isolates expressing pyrogenic exotoxins A and B in combination with selective lack of toxin-neutralizing antibodies are associated with increased risk of streptococcal toxic shock syndrome." J Infect Dis **180**(2): 410-8.

Euler, C. W. (2010). Personal Communication.

Euler, C. W., P. A. Ryan, et al. (2007). "M.SpyI, a DNA methyltransferase encoded on a mefA chimeric element, modifies the genome of Streptococcus pyogenes." J Bacteriol **189**(3): 1044-54.

Evans, A. C. (1934). "The Prevalence of Streptococcus Bacteriophage." Science **80**(2063): 40-41.

Evans, A. C. (1940). "The Potency of Nascent Streptococcus Bacteriophage B." J Bacteriol **39**(5): 597-604.

Facklam, R., B. Beall, et al. (1999). "emm typing and validation of provisional M types for group A streptococci." Emerg Infect Dis **5**(2): 247-53.

Falugi, F., C. Zingaretti, et al. (2008). "Sequence variation in group A Streptococcus pili and association of pilus backbone types with lancefield T serotypes." J Infect Dis **198**(12): 1834-41.

Fast, D. J., P. M. Schlievert, et al. (1989). "Toxic shock syndrome-associated staphylococcal and streptococcal pyrogenic toxins are potent inducers of tumor necrosis factor production." Infect Immun **57**(1): 291-4.

Ferretti, J. J., W. M. McShan, et al. (2001). "Complete Genome Sequence of an M1 Strain of *Streptococcus pyogenes*." Proceedings of the National Academy of Sciences of the United States of America **98**(8): 4658-4663.

Ferretti, J. J., W. M. McShan, et al. (2001). "Complete genome sequence of an M1 strain of Streptococcus pyogenes." Proc Natl Acad Sci U S A **98**(8): 4658-63.

Finken, M., P. Kirschner, et al. (1993). "Molecular basis of streptomycin resistance in Mycobacterium tuberculosis: alterations of the ribosomal protein S12 gene and point mutations within a functional 16S ribosomal RNA pseudoknot." Mol Microbiol **9**(6): 1239-46.

Fischetti, V. A. (1989). "Streptococcal M protein: molecular design and biological behavior." Clin Microbiol Rev **2**(3): 285-314.

Fischetti, V. A. (2005). "Bacteriophage lytic enzymes: novel anti-infectives." Trends Microbiol **13**(10): 491-6.

Fischetti, V. A. (2006). Gram-positive pathogens. Washington, D.C., ASM Press.

Fischetti, V. A. (2007). Controlling streptococci with phage lytic enzymes.

Fischetti, V. A. (2008). "Bacteriophage lysins as effective antibacterials." Curr Opin Microbiol **11**(5): 393-400.

Fischetti, V. A., K. F. Jones, et al. (1988). "Structure, function, and genetics of streptococcal M protein." Rev Infect Dis **10 Suppl 2**: S356-9.

Fischetti, V. A., D. Nelson, et al. (2006). "Reinventing phage therapy: are the parts greater than the sum?" Nat Biotechnol **24**(12): 1508-11.

Fischetti, V. A., D. Nelson, et al. (2006). "Reinventing phage therapy: are the parts greater than the sum?" Nat.Biotechnol. **24**(12): 1508-1511.

Fluckiger, U., K. F. Jones, et al. (1998). "Immunoglobulins to group A streptococcal surface molecules decrease adherence to and invasion of human pharyngeal cells." Infect Immun **66**(3): 974-9.

Fluit, A. C., J. Verhoef, et al. (2001). "Frequency of isolation and antimicrobial resistance of gram-negative and gram-positive bacteria from patients in intensive care units of 25 European university hospitals participating in the European arm of the SENTRY Antimicrobial Surveillance Program 1997-1998." Eur J Clin Microbiol Infect Dis **20**(9): 617-25.

Frommer, M., L. E. McDonald, et al. (1992). "A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands." Proc Natl Acad Sci U S A **89**(5): 1827-31.

Funatsu, G. and H. G. Wittmann (1972). "Ribosomal proteins. 33. Location of amino-acid replacements in protein S12 isolated from Escherichia coli mutants resistant to streptomycin." J Mol Biol **68**(3): 547-50.

Gerber, M. A., R. S. Baltimore, et al. (2009). "Prevention of rheumatic fever and diagnosis and treatment of acute Streptococcal pharyngitis: a scientific statement from the American Heart Association Rheumatic Fever, Endocarditis, and Kawasaki Disease Committee of the Council on Cardiovascular Disease in the Young, the Interdisciplinary Council on Functional Genomics and Translational Biology, and the Interdisciplinary Council on Quality of Care and Outcomes Research: endorsed by the American Academy of Pediatrics." Circulation **119**(11): 1541-51.

Gerber, M. A., E. D. Gray, et al. (1980). "Enzyme-linked immunosorbent assay of antibodies in human sera to streptococcal DNase B." J Lab Clin Med **95**(2): 258-65.

Gerlach, D., B. Fleischer, et al. (2000). "Purification and biochemical characterization of a basic superantigen (SPEX/SMEZ3) from Streptococcus pyogenes." FEMS Microbiol Lett **188**(2): 153-63.

Gerlach, D., W. Reichardt, et al. (1994). "Separation of mitogenic and pyrogenic activities from so-called erythrogenic toxin type B (Streptococcal proteinase)." Zentralbl Bakteriol **280**(4): 507-14.

Gerlach, D., K. H. Schmidt, et al. (2001). "Basic streptococcal superantigens (SPEX/SMEZ or SPEC) are responsible for the mitogenic activity of the so-called mitogenic factor (MF)." FEMS Immunol Med Microbiol **30**(3): 209-16.

Gill, S. R., D. E. Fouts, et al. (2005). "Insights on evolution of virulence and resistance from the complete genome analysis of an early methicillin-resistant Staphylococcus aureus strain and a biofilm-producing methicillin-resistant Staphylococcus epidermidis strain." J Bacteriol. **187**(7): 2426-2438.

Gill, S. R., D. E. Fouts, et al. (2005). "Insights on evolution of virulence and resistance from the complete genome analysis of an early methicillin-resistant Staphylococcus aureus strain and a biofilm-producing methicillin-resistant Staphylococcus epidermidis strain." J Bacteriol **187**(7): 2426-38.

Giovanetti, E., A. Brenciani, et al. (2003). "Presence of the tet(O) gene in erythromycin- and tetracycline-resistant strains of Streptococcus pyogenes and linkage with either the mef(A) or the erm(A) gene." Antimicrob Agents Chemother **47**(9): 2844-9.

Giovanetti, E., A. Brenciani, et al. (2005). "Prophage association of mef(A) elements encoding efflux-mediated erythromycin resistance in Streptococcus pyogenes." J Antimicrob Chemother **55**(4): 445-51.

Gonzales, R., D. C. Malone, et al. (2001). "Excessive antibiotic use for acute respiratory infections in the United States." Clin Infect Dis **33**(6): 757-62.

Goroncy-Bermes, P., J. B. Dale, et al. (1987). "Monoclonal antibody to human renal glomeruli cross-reacts with streptococcal M protein." Infect Immun **55**(10): 2416-9.

Goshorn, S. C., G. A. Bohach, et al. (1988). "Cloning and characterization of the gene, speC, for pyrogenic exotoxin type C from Streptococcus pyogenes." Mol Gen Genet **212**(1): 66-70.

Graham, M. R., K. Virtaneva, et al. (2005). "Group A Streptococcus transcriptome dynamics during growth in human blood reveals bacterial adaptive and survival strategies." Am J Pathol **166**(2): 455-65.

Graham, M. R., K. Virtaneva, et al. (2006). "Analysis of the transcriptome of group A Streptococcus in mouse soft tissue infection." Am J Pathol **169**(3): 927-42.

Green, M., J. M. Martin, et al. (2004). "Reemergence of macrolide resistance in pharyngeal isolates of group a streptococci in southwestern Pennsylvania." Antimicrob Agents Chemother **48**(2): 473-6.

Green, N. M., S. Zhang, et al. (2005). "Genome sequence of a serotype M28 strain of group a streptococcus: potential new insights into puerperal sepsis and bacterial disease specificity." J Infect Dis **192**(5): 760-70.

Grundling, A., D. M. Missiakas, et al. (2006). "Staphylococcus aureus mutants with increased lysostaphin resistance." J Bacteriol. **188**(17): 6286-6297.

Grundling, A. and O. Schneewind (2006). "Cross-linked peptidoglycan mediates lysostaphin binding to the cell wall envelope of Staphylococcus aureus." J Bacteriol. **188**(7): 2463-2472.

Gunzenhauser, J. D., J. N. Longfield, et al. (1995). "Epidemic streptococcal disease among Army trainees, July 1989 through June 1991." J Infect Dis **172**(1): 124-31.

Hackett, S. P. and D. L. Stevens (1992). "Streptococcal toxic shock syndrome: synthesis of tumor necrosis factor and interleukin-1 by monocytes stimulated with pyrogenic exotoxin A and streptolysin O." J Infect Dis **165**(5): 879-85.

Handa, N., Y. Nakayama, et al. (2001). "Experimental genome evolution: large-scale genome rearrangements associated with resistance to replacement of a chromosomal restriction-modification gene complex." Mol Microbiol **40**(4): 932-40.

Hasegawa, T., K. Torii, et al. (2002). "Cloning and characterization of the deoxyribonuclease sd alpha gene from Streptococcus pyogenes." Curr Microbiol **45**(1): 13-7.

Hasegawa, T., K. Torii, et al. (2002). "Cloning and characterization of two novel DNases from Streptococcus pyogenes." Arch Microbiol **177**(6): 451-6.

Hasham, S., P. Matteucci, et al. (2005). "Necrotising fasciitis." BMJ **330**(7495): 830-3.

Hauser, A. R. and P. M. Schlievert (1990). "Nucleotide sequence of the streptococcal pyrogenic exotoxin type B gene and relationship between the toxin and the streptococcal proteinase precursor." J Bacteriol **172**(8): 4536-42.

Herman, A., J. W. Kappler, et al. (1991). "Superantigens: mechanism of T-cell stimulation and role in immune responses." Annu Rev Immunol **9**: 745-72.

Herwald, H., M. Collin, et al. (1996). "Streptococcal cysteine proteinase releases kinins: a virulence mechanism." J Exp Med **184**(2): 665-73.

Hewitt, C. R., J. R. Lamb, et al. (1992). "Major histocompatibility complex independent clonal T cell anergy by direct interaction of Staphylococcus aureus enterotoxin B with the T cell antigen receptor." J Exp Med **175**(6): 1493-9.

Hirschman, S. Z., M. Gerber, et al. (1978). "Differential activation of hepatitis B DNA polymerase by detergent and salt." J Med Virol **2**(1): 61-76.

Hoe, N. P., J. Vuopio-Varkila, et al. (2001). "Distribution of streptococcal inhibitor of complement variants in pharyngitis and invasive isolates in an epidemic of serotype M1 group A Streptococcus infection." J Infect Dis **183**(4): 633-9.

Hoge, C. W., B. Schwartz, et al. (1993). "The changing epidemiology of invasive group A streptococcal infections and the emergence of streptococcal toxic shock-like syndrome. A retrospective population-based study." Jama **269**(3): 384-9.

Holden, M. T., A. Scott, et al. (2007). "Complete genome of acute rheumatic fever-associated serotype M5 Streptococcus pyogenes strain manfredo." J Bacteriol **189**(4): 1473-7.

Hollands, A., R. K. Aziz, et al. (2008). "A naturally occurring mutation in ropB suppresses SpeB expression and reduces M1T1 group A streptococcal systemic virulence." PLoS One **3**(12): e4102.

Holm, S. E. (1996). "Invasive group A streptococcal infections." N Engl J Med **335**(8): 590-1.

Horgan, M., G. O'Flynn, et al. (2009). "Phage lysin LysK can be truncated to its CHAP domain and retain lytic activity against live antibiotic-resistant staphylococci." Appl.Environ.Microbiol **75**(3): 872-874.

Horstmann, R. D., H. J. Sievertsen, et al. (1988). "Antiphagocytic activity of streptococcal M protein: selective binding of complement control protein factor H." Proc Natl Acad Sci U S A **85**(5): 1657-61.

Hsueh, P. R., L. J. Teng, et al. (2002). "Increased prevalence of erythromycin resistance in streptococci: substantial upsurge in erythromycin-resistant M phenotype in Streptococcus pyogenes (1979-1998) but not in Streptococcus pneumoniae (1985-1999) in Taiwan." Microb Drug Resist **8**(1): 27-33.

Huang, Y. T., C. H. Hsiao, et al. (2008). "Bacteremia and infective endocarditis caused by a non-daptomycin-susceptible, vancomycin-intermediate, and methicillin-resistant Staphylococcus aureus strain in Taiwan." J Clin.Microbiol **46**(3): 1132-1136.

Husby, G., I. van de Rijn, et al. (1976). "Antibodies reacting with cytoplasm of subthalamic and caudate nuclei neurons in chorea and acute rheumatic fever." J Exp Med **144**(4): 1094-110.

Hynes, W. (2004). "Virulence factors of the group A streptococci and genes that regulate their expression." Front Biosci **9**: 3399-433.

Ichige, A. and I. Kobayashi (2005). "Stability of EcoRI restriction-modification enzymes in vivo differentiates the EcoRI restriction-modification system from other postsegregational cell killing systems." J Bacteriol **187**(19): 6612-21.

Iwasaki, H., T. Shiba, et al. (1989). "Involvement in DNA repair of the *ruv*A gene of *Escherichia coli*." Molecular and General Genetics **219**(1-2): 328-31.

Ji, Y., N. Schnitzler, et al. (1998). "Impact of M49, Mrp, Enn, and C5a peptidase proteins on colonization of the mouse oral mucosa by Streptococcus pyogenes." Infect Immun **66**(11): 5399-405.

Johnson, D. R., D. L. Stevens, et al. (1992). "Epidemiologic analysis of group A streptococcal serotypes associated with severe systemic infections, rheumatic fever, or uncomplicated pharyngitis." J Infect Dis **166**(2): 374-82.

Johnson, D. R., J. T. Wotton, et al. (2002). "A comparison of group A streptococci from invasive and uncomplicated infections: are virulent clones responsible for serious streptococcal infections?" J Infect Dis **185**(11): 1586-95.

Johnsson, E., K. Berggard, et al. (1998). "Role of the hypervariable region in streptococcal M proteins: binding of a human complement inhibitor." J Immunol **161**(9): 4894-901.

Jones, K. F., B. N. Manjula, et al. (1985). "Location of variable and conserved epitopes among the multiple serotypes of streptococcal M protein." J Exp Med **161**(3): 623-8.

Jones, K. F., O. Schneewind, et al. (1991). "Genetic diversity among the T-protein genes of group A streptococci." Mol Microbiol **5**(12): 2947-52.

Kansal, R. G., V. Datta, et al. "Dissection of the molecular basis for Hypervirulence of an in vivo-selected phenotype of the widely disseminated M1T1 strain of group A Streptococcus bacteria." J Infect Dis **201**(6): 855-65.

Kansal, R. G., A. McGeer, et al. (2000). "Inverse relation between disease severity and expression of the streptococcal cysteine protease, SpeB, among clonal M1T1 isolates recovered from invasive group A streptococcal infection cases." Infect Immun **68**(11): 6362-9.

Kaplan, E. L. (1992). "The carditis/cardiomyopathy of rheumatic fever: relationship to pathogenesis." Postgrad Med J **68 Suppl 1**: S21-3.

Kaplan, M. H., R. Bolande, et al. (1964). "Presence of Bound Immunoglobulins and Complement in the Myocardium in Acute Rheumatic Fever. Association with Cardiac Failure." N Engl J Med **271**: 637-45.

Kaplan, M. H. and K. H. Svec (1964). "Immunologic Relation of Streptococcal and Tissue Antigens. Iii. Presence in Human Sera of Streptococcal Antibody Cross-Reactive with Heart Tissue. Association with Streptococcal Infection, Rheumatic Fever, and Glomerulonephritis." J Exp Med **119**: 651-66.

Kappler, J., B. Kotzin, et al. (1989). "V beta-specific stimulation of human T cells by staphylococcal toxins." Science **244**(4906): 811-3.

Kapur, V., K. Nelson, et al. (1992). "Molecular population genetic evidence of horizontal spread of two alleles of the pyrogenic exotoxin C gene (speC) among pathogenic clones of Streptococcus pyogenes." Infect Immun **60**(9): 3513-7.

Kapur, V., S. Topouzis, et al. (1993). "A conserved Streptococcus pyogenes extracellular cysteine protease cleaves human fibronectin and degrades vitronectin." Microb Pathog **15**(5): 327-46.

Kataja, J., P. Huovinen, et al. (1999). "Erythromycin resistance genes in group A streptococci in Finland. The Finnish Study Group for Antimicrobial Resistance." Antimicrob Agents Chemother **43**(1): 48-52.

Kim, Y. B. and D. W. Watson (1970). "A purified group A streptococcal pyrogenic exotoxin. Physiochemical and biological properties including the enhancement of susceptibility to endotoxin lethal shock." J Exp Med **131**(3): 611-22.

Kimoto, H. and A. Taketo (2003). "Efficient electrotransformation system and gene targeting in pyogenic streptococci." Biosci Biotechnol Biochem **67**(10): 2203-9.

Kiser, K. B., J. M. Cantey-Kiser, et al. (1999). "Development and characterization of a Staphylococcus aureus nasal colonization model in mice." Infect.Immun. **67**(10): 5001-5006.

Klimasauskas, S., D. Steponaviciene, et al. (1990). "M.Smal is an N4-methylcytosine specific DNA-methylase." Nucleic Acids Res **18**(22): 6607-9.

Kluytmans, J., B. A. van, et al. (1997). "Nasal carriage of Staphylococcus aureus: epidemiology, underlying mechanisms, and associated risks." Clin.Microbiol Rev. **10**(3): 505-520.

Knetsch, M. L., N. Schafers, et al. (2001). "The Dictyostelium Bcr/Abr-related protein DRG regulates both Rac- and Rab-dependent pathways." EMBO J **20**(7): 1620-9.

Kobayashi, I. (2001). "Behavior of restriction-modification systems as selfish mobile elements and their impact on genome evolution." Nucleic Acids Res **29**(18): 3742-56.

Kollef, M. H., L. E. Morrow, et al. (2006). "Clinical characteristics and treatment patterns among patients with ventilator-associated pneumonia." Chest **129**(5): 1210-1218.

Kotarsky, H., J. Hellwage, et al. (1998). "Identification of a domain in human factor H and factor H-like protein-1 required for the interaction with streptococcal M proteins." J Immunol **160**(7): 3349-54.

Kotb, M. (1995). "Bacterial pyrogenic exotoxins as superantigens." Clin Microbiol Rev **8**(3): 411-26.

Kotb, M., J. D. Fraser, et al. (2007). Superantigens : molecular basis for their role in human diseases. Washington, D.C., ASM Press.

Kreikemeyer, B., S. R. Talay, et al. (1995). "Characterization of a novel fibronectin-binding surface protein in group A streptococci." Mol Microbiol **17**(1): 137-45.

Kulakauskas, S., A. Lubys, et al. (1995). "DNA restriction-modification systems mediate plasmid maintenance." J Bacteriol **177**(12): 3451-4.

Kuo, C. F., Y. S. Lin, et al. (2008). "Degradation of complement 3 by streptococcal pyrogenic exotoxin B inhibits complement activation and neutrophil opsonophagocytosis." Infect Immun **76**(3): 1163-9.

Kuo, C. F., J. J. Wu, et al. (1998). "Role of streptococcal pyrogenic exotoxin B in the mouse model of group A streptococcal infection." Infect Immun **66**(8): 3931-5.

Lancefield, R. C. (1928). "The Antigenic Complex of Streptococcus Haemolyticus : I. Demonstration of a Type-Specific Substance in Extracts of Streptococcus Haemolyticus." J Exp Med **47**(1): 91-103.

Lancefield, R. C. (1933). "A Serological Differentiation of Human and Other Groups of Hemolytic Streptococci." J Exp Med **57**(4): 571-595.

Lancefield, R. C. (1959). "Persistence of type-specific antibodies in man following infection with group A streptococci." J Exp Med **110**(2): 271-92.

Lancefield, R. C. (1962). "Current knowledge of type-specific M antigens of group A streptococci." J Immunol **89**: 307-13.

Lancefield, R. C. and V. P. Dole (1946). "The Properties of T Antigens Extracted from Group a Hemolytic Streptococci." J Exp Med **84**(5): 449-471.

Lange, K., G. Seligson, et al. (1983). "Evidence for the in situ origin of poststreptococcal glomerulonephritis: glomerular localization of endostreptosin and the clinical significance of the subsequent antibody response." Clin Nephrol **19**(1): 3-10.

Leclercq, R. and P. Courvalin (1991). "Bacterial resistance to macrolide, lincosamide, and streptogramin antibiotics by target modification." Antimicrob Agents Chemother **35**(7): 1267-72.

Lederberg, J. (1951). "Streptomycin resistance; a genetically recessive mutation." J Bacteriol **61**(5): 549-50.

Lederberg, J. and E. M. Lederberg (1952). "Replica plating and indirect selection of bacterial mutants." J Bacteriol **63**(3): 399-406.

Linder, J. A. and R. S. Stafford (2001). "Antibiotic treatment of adults with sore throat by community primary care physicians: a national survey, 1989-1999." Jama **286**(10): 1181-6.

Loeffler, J. M., S. Djurkovic, et al. (2003). "Phage lytic enzyme Cpl-1 as a novel antimicrobial for pneumococcal bacteremia." Infect.Immun. **71**(11): 6199-6204.

Loeffler, J. M. and V. A. Fischetti (2003). "Synergistic lethal effect of a combination of phage lytic enzymes with different activities on penicillin-sensitive and -resistant Streptococcus pneumoniae strains." Antimicrob Agents Chemother **47**(1): 375-7.

Loeffler, J. M., D. Nelson, et al. (2001). "Rapid killing of Streptococcus pneumoniae with a bacteriophage cell wall hydrolase." Science **294**(5549): 2170-2172.

Lopez, R., E. Garcia, et al. (1997). "The pneumococcal cell wall degrading enzymes: a modular design to create new lysins?" Microb.Drug.Resist. **3**(2): 199-211.

Lu, J. Z., T. Fujiwara, et al. (2006). "Cell wall-targeting domain of glycylglycine endopeptidase distinguishes among peptidoglycan cross-bridges." J Biol.Chem. **281**(1): 549-558.

Lukomski, S., E. H. Burns, Jr., et al. (1998). "Genetic inactivation of an extracellular cysteine protease (SpeB) expressed by Streptococcus pyogenes decreases resistance to phagocytosis and dissemination to organs." Infect Immun **66**(2): 771-6.

Lukomski, S., C. A. Montgomery, et al. (1999). "Extracellular cysteine protease produced by Streptococcus pyogenes participates in the pathogenesis of

invasive skin infection and dissemination in mice." Infect Immun **67**(4): 1779-88.

Lukomski, S., S. Sreevatsan, et al. (1997). "Inactivation of Streptococcus pyogenes extracellular cysteine protease significantly decreases mouse lethality of serotype M3 and M49 strains." J Clin Invest **99**(11): 2574-80.

Luna, V. A., P. Coates, et al. (1999). "A variety of gram-positive bacteria carry mobile mef genes." J Antimicrob Chemother **44**(1): 19-25.

Luo, Y. H., C. F. Kuo, et al. (2007). "Streptococcal pyrogenic exotoxin B antibodies in a mouse model of glomerulonephritis." Kidney Int **72**(6): 716-24.

Luria, S. E. and M. Delbruck (1943). "Mutations of Bacteria from Virus Sensitivity to Virus Resistance." Genetics **28**(6): 491-511.

Luria, S. E. and M. L. Human (1952). "A nonhereditary, host-induced variation of bacterial viruses." J Bacteriol **64**(4): 557-69.

Malhotra-Kumar, S., C. Lammens, et al. (2005). "Macrolide- and telithromycin-resistant Streptococcus pyogenes, Belgium, 1999-2003." Emerg Infect Dis **11**(6): 939-42.

Manetti, A. G., C. Zingaretti, et al. (2007). "Streptococcus pyogenes pili promote pharyngeal cell adhesion and biofilm formation." Mol Microbiol **64**(4): 968-83.

Mangili, A., I. Bica, et al. (2005). "Daptomycin-resistant, methicillin-resistant Staphylococcus aureus bacteremia." Clin.Infect.Dis. **40**(7): 1058-1060.

Manjula, B. N. and V. A. Fischetti (1980). "Tropomyosin-like seven residue periodicity in three immunologically distinct streptococal M proteins and its implications for the antiphagocytic property of the molecule." J Exp Med **151**(3): 695-708.

Manoharadas, S., A. Witte, et al. (2009). "Antimicrobial activity of a chimeric enzybiotic towards Staphylococcus aureus." J Biotechnol. **139**(1): 118-123.

Manyemba, J. and B. M. Mayosi (2002). "Penicillin for secondary prevention of rheumatic fever." Cochrane Database Syst Rev(3): CD002227.

Marrack, P. and J. Kappler (1990). "The staphylococcal enterotoxins and their relatives." Science **248**(4956): 705-711.

Martin, J. M., M. Green, et al. (2002). "Erythromycin-resistant group A streptococci in schoolchildren in Pittsburgh." N Engl J Med **346**(16): 1200-6.

Martin, J. M., M. Green, et al. (2004). "Group A streptococci among school-aged children: clinical characteristics and the carrier state." Pediatrics **114**(5): 1212-9.

Martin-Galiano, A. J. and A. G. de la Campa (2003). "High-efficiency generation of antibiotic-resistant strains of Streptococcus pneumoniae by PCR and transformation." Antimicrob Agents Chemother **47**(4): 1257-61.

Martins, T. B., J. L. Hoffman, et al. (2008). "Comprehensive analysis of antibody responses to streptococcal and tissue antigens in patients with acute rheumatic fever." Int Immunol **20**(3): 445-52.

Massey, R. C., M. J. Horsburgh, et al. (2006). "The evolution and maintenance of virulence in Staphylococcus aureus: a role for host-to-host transmission?" Nat.Rev.Microbiol **4**(12): 953-958.

Mazurkiewicz, P., G. J. Poelarends, et al. (2004). "Facilitated drug influx by an energy-uncoupled secondary multidrug transporter." Journal of Biological Chemistry **279**(1): 103-8.

McCarty, M. (1948). "The occurrence of nucleases in culture filtrates of group A hemolytic streptococci." J Exp Med **88**(2): 181-8.

McCormack, J. E., J. E. Callahan, et al. (1993). "Profound deletion of mature T cells in vivo by chronic exposure to exogenous superantigen." J Immunol **150**(9): 3785-92.

McCormick, J. K., J. M. Yarwood, et al. (2001). "Toxic shock syndrome and bacterial superantigens: an update." Annu Rev Microbiol **55**: 77-104.

McDonald, M., A. Brown, et al. (2007). "Apparent contrasting rates of pharyngitis and pyoderma in regions where rheumatic heart disease is highly prevalent." Heart Lung Circ **16**(4): 254-9.

McIver, K. S. and R. L. Myles (2002). "Two DNA-binding domains of Mga are required for virulence gene activation in the group A streptococcus." Mol Microbiol **43**(6): 1591-601.

McShan, W. M., J. J. Ferretti, et al. (2008). "Genome sequence of a nephritogenic and highly transformable M49 strain of Streptococcus pyogenes." J Bacteriol **190**(23): 7773-85.

McShan, W. M., Y. F. Tang, et al. (1997). "Bacteriophage T12 of Streptococcus pyogenes integrates into the gene encoding a serine tRNA." Mol Microbiol **23**(4): 719-28.

MMWR (2009). Bacterial coinfections in lung tissue specimens from fatal cases of 2009 pandemic influenza A (H1N1) - United States M-August 2009**:** 1-4.

Molinari, G. and G. S. Chhatwal (1999). "Role played by the fibronectin-binding protein SfbI (Protein F1) of Streptococcus pyogenes in bacterial internalization by epithelial cells." J Infect Dis **179**(4): 1049-50.

Molinari, G., M. Rohde, et al. (2000). "Two distinct pathways for the invasion of Streptococcus pyogenes in non-phagocytic cells." Cell Microbiol **2**(2): 145-54.

Mora, M., G. Bensi, et al. (2005). "Group A Streptococcus produce pilus-like structures containing protective antigens and Lancefield T antigens." Proc Natl Acad Sci U S A **102**(43): 15641-6.

Morens, D. M., J. K. Taubenberger, et al. (2009). "The persistent legacy of the 1918 influenza virus." N.Engl.J Med. **361**(3): 225-229.

Musser, J. M., V. Kapur, et al. (1995). "Genetic diversity and relationships among Streptococcus pyogenes strains expressing serotype M1 protein: recent

intercontinental spread of a subclone causing episodes of invasive disease." Infect Immun **63**(3): 994-1003.

Musser, J. M., K. Nelson, et al. (1993). "Temporal variation in bacterial disease frequency: molecular population genetic analysis of scarlet fever epidemics in Ottawa and in eastern Germany." J Infect Dis **167**(3): 759-62.

Naito, T., K. Kusano, et al. (1995). "Selfish behavior of restriction-modification systems." Science **267**(5199): 897-9.

Nakagawa, I., K. Kurokawa, et al. (2003). "Genome sequence of an M3 strain of Streptococcus pyogenes reveals a large-scale genomic rearrangement in invasive strains and new insights into phage evolution." Genome Res **13**(6A): 1042-55.

Nakashima, K., S. Ichiyama, et al. (1997). "A clinical and bacteriologic investigation of invasive streptococcal infections in Japan on the basis of serotypes, toxin production, and genomic DNA fingerprints." Clin Infect Dis **25**(2): 260-6.

Navarre, W. W., H. Ton-That, et al. (1999). "Multiple enzymatic activities of the murein hydrolase from staphylococcal phage phi11. Identification of a D-alanyl-glycine endopeptidase activity. ." The Journal of Biological Chemistry **274**(22): 15847-15856.

Nelson, D., L. Loomis, et al. (2001). "Prevention and elimination of upper respiratory colonization of mice by group A streptococci by using a bacteriophage lytic enzyme." Proc Natl Acad Sci U S A **98**(7): 4107-12.

Nelson, D., L. Loomis, et al. (2001). "Prevention and elimination of upper respiratory colonization of mice by group A streptococci by using a bacteriophage lytic enzyme." Proc.Natl.Acad.Sci.U.S.A **98**(7): 4107-4112.

Nelson, D., R. Schuch, et al. (2006). "PlyC: A multimeric bacteriophage lysin." Proc Natl Acad Sci U S A **103**(28): 10765-70.

Nelson, K., P. M. Schlievert, et al. (1991). "Characterization and clonal distribution of four alleles of the speA gene encoding pyrogenic exotoxin A (scarlet fever toxin) in Streptococcus pyogenes." J Exp Med **174**(5): 1271-4.

Nguyen, L., D. Levy, et al. (1997). "Molecular epidemiology of Streptococcus pyogenes in an area where acute pharyngotonsillitis is endemic." J Clin Microbiol **35**(8): 2111-4.

Nobbs, A. H., R. J. Lamont, et al. (2009). "Streptococcus adherence and colonization." Microbiol Mol Biol Rev **73**(3): 407-50, Table of Contents.

Nooh, M. M., R. K. Aziz, et al. (2006). "Streptococcal mitogenic exotoxin, SmeZ, is the most susceptible M1T1 streptococcal superantigen to degradation by the streptococcal cysteine protease, SpeB." J Biol Chem **281**(46): 35281-8.

Nordstrand, A., M. Norgren, et al. (1998). "Streptokinase as a mediator of acute post-streptococcal glomerulonephritis in an experimental mouse model." Infect Immun **66**(1): 315-21.

Norrby-Teglund, A., D. Newton, et al. (1994). "Superantigenic properties of the group A streptococcal exotoxin SpeF (MF)." Infect Immun **62**(12): 5227-33.

O'Flaherty, S., A. Coffey, et al. (2005). "The recombinant phage lysin LysK has a broad spectrum of lytic activity against clinically relevant staphylococci, including methicillin-resistant Staphylococcus aureus." J.Bacteriol. **187**(20): 7161-7164.

Okada, N., M. K. Liszewski, et al. (1995). "Membrane cofactor protein (CD46) is a keratinocyte receptor for the M protein of the group A streptococcus." Proc Natl Acad Sci U S A **92**(7): 2489-93.

Okada, N., A. P. Pentland, et al. (1994). "M protein and protein F act as important determinants of cell-specific tropism of Streptococcus pyogenes in skin tissue." J Clin Invest **94**(3): 965-77.

Okada, N., I. Tatsuno, et al. (1998). "Streptococcus pyogenes protein F promotes invasion of HeLa cells." Microbiology **144 (Pt 11)**: 3079-86.

Perl, T. M., J. J. Cullen, et al. (2002). "Intranasal mupirocin to prevent postoperative Staphylococcus aureus infections." N.Engl.J Med. **346**(24): 1871-1877.

Peterson, P. K., D. Schmeling, et al. (1979). "Inhibition of alternative complement pathway opsonization by group A streptococcal M protein." J Infect Dis **139**(5): 575-85.

Phillips, G. N., Jr., P. F. Flicker, et al. (1981). "Streptococcal M protein: alpha-helical coiled-coil structure and arrangement on the cell surface." Proc Natl Acad Sci U S A **78**(8): 4689-93.

Podbielski, A., N. Schnitzler, et al. (1996). "M-related protein (Mrp) contributes to group A streptococcal resistance to phagocytosis by human granulocytes." Mol Microbiol **19**(3): 429-41.

Podbielski, A., B. Spellerberg, et al. (1996). "Novel series of plasmid vectors for gene inactivation and expression analysis in group A streptococci (GAS)." Gene **177**(1-2): 137-47.

Podbielski, A., I. Zarges, et al. (1996). "Molecular characterization of a major serotype M49 group A streptococcal DNase gene (sdaD)." Infect Immun **64**(12): 5349-56.

Podbielski, A., I. Zarges, et al. (1996). "Molecular characterization of a major serotype M49 group A streptococcal DNase gene (*sda*D)." Infection and Immunity **64**(12): 5349-56.

Popovich, K. J., R. A. Weinstein, et al. (2008). "Are community-associated methicillin-resistant Staphylococcus aureus (MRSA) strains replacing traditional nosocomial MRSA strains?" Clin.Infect.Dis. **46**(6): 787-794.

Pozzi, G., F. Iannelli, et al. (2004). "Genetic elements carrying macrolide efflux genes in streptococci." Curr Drug Targets Infect Disord **4**(3): 203-6.

Proft, T., S. L. Moffatt, et al. (1999). "Identification and characterization of novel superantigens from Streptococcus pyogenes." J Exp Med **189**(1): 89-102.

Proft, T., S. L. Moffatt, et al. (2000). "The streptococcal superantigen SMEZ exhibits wide allelic variation, mosaic structure, and significant antigenic variation." J Exp Med **191**(10): 1765-76.

Quinn, A., S. Kosanke, et al. (2001). "Induction of autoimmune valvular heart disease by recombinant streptococcal m protein." Infect Immun **69**(6): 4072-8.

Rajakumar, K., C. Sasakawa, et al. (1997). "Use of a novel approach, termed island probing, identifies the Shigella flexneri she pathogenicity island which encodes a homolog of the immunoglobulin A protease-like family of proteins." Infect Immun **65**(11): 4606-14.

Rajendran, P. M., D. Young, et al. (2007). "Randomized, double-blind, placebo-controlled trial of cephalexin for treatment of uncomplicated skin abscesses in a population at risk for community-acquired methicillin-resistant Staphylococcus aureus infection." Antimicrob.Agents Chemother. **51**(11): 4044-4048.

Rashel, M., J. Uchiyama, et al. (2007). "Efficient elimination of multidrug-resistant Staphylococcus aureus by cloned lysin derived from bacteriophage phi MR11." J.Infect.Dis. **196**(8): 1237-1247.

Reyrat, J. M., V. Pelicic, et al. (1998). "Counterselectable markers: untapped tools for bacterial genetics and pathogenesis." Infect Immun **66**(9): 4011-7.

Ripa, S., C. Zampaloni, et al. (2001). "SmaI macrorestriction analysis of Italian isolates of erythromycin-resistant Streptococcus pyogenes and correlations with macrolide-resistance phenotypes." Microb Drug Resist **7**(1): 65-71.

Roberts, R. J., M. Belfort, et al. (2003). "A nomenclature for restriction enzymes, DNA methyltransferases, homing endonucleases and their genes." Nucleic Acids Res **31**(7): 1805-12.

Roberts, R. J., T. Vincze, et al. (2005). "REBASE--restriction enzymes and DNA methyltransferases." Nucleic Acids Res **33**(Database issue): D230-2.

Roberts, S., S. Kosanke, et al. (2001). "Pathogenic mechanisms in rheumatic carditis: focus on valvular endothelium." J Infect Dis **183**(3): 507-11.

Robinson, D. A., J. A. Sutcliffe, et al. (2006). "Evolution and global dissemination of macrolide-resistant group a streptococci." Antimicrob Agents Chemother **50**(9): 2903-11.

Rocha, C. L. and V. A. Fischetti (1999). "Identification and characterization of a novel fibronectin-binding protein on the surface of group A streptococci." Infect Immun **67**(6): 2720-8.

Romano, A., M. Viola, et al. (2002). "Diagnosing nonimmediate reactions to penicillins by in vivo tests." Int Arch Allergy Immunol **129**(2): 169-74.

Ryan, P. A., B. W. Kirk, et al. (2007). "Novel algorithms reveal streptococcal transcriptomes and clues about undefined genes." PLoS Comput Biol **3**(7): e132.

Ryan, P. A., V. Pancholi, et al. (2001). "Group A streptococci bind to mucin and human pharyngeal cells through sialic acid-containing receptors." Infect Immun **69**(12): 7402-12.

Sanderson-Smith, M. L., K. Dinkla, et al. (2008). "M protein-mediated plasminogen binding is essential for the virulence of an invasive Streptococcus pyogenes isolate." FASEB J **22**(8): 2715-22.

Santagati, M., F. Iannelli, et al. (2003). "The novel conjugative transposon tn1207.3 carries the macrolide efflux gene mef(A) in Streptococcus pyogenes." Microb Drug Resist **9**(3): 243-7.

Santagati, M., F. Iannelli, et al. (2000). "Characterization of a genetic element carrying the macrolide efflux gene mef(A) in Streptococcus pneumoniae." Antimicrob Agents Chemother **44**(9): 2585-7.

Schmidt, K. H., K. Mann, et al. (1993). "Multiple binding of type 3 streptococcal M protein to human fibrinogen, albumin and fibronectin." FEMS Immunol Med Microbiol **7**(2): 135-43.

Schmidtchen, A., I. M. Frick, et al. (2002). "Proteinases of common pathogenic bacteria degrade and inactivate the antibacterial peptide LL-37." Mol Microbiol **46**(1): 157-68.

Schneewind, O., K. F. Jones, et al. (1990). "Sequence and structural characteristics of the trypsin-resistant T6 surface protein of group A streptococci." J Bacteriol **172**(6): 3310-7.

Schrager, H. M., J. G. Rheinwald, et al. (1996). "Hyaluronic acid capsule and the role of streptococcal entry into keratinocytes in invasive skin infection." J Clin Invest **98**(9): 1954-8.

Schroeder, B. M. (2003). "Diagnosis and management of group A streptococcal pharyngitis." Am Fam Physician **67**(4): 880, 883-4.

Schuch, R., D. Nelson, et al. (2002). "A bacteriolytic agent that detects and kills Bacillus anthracis." Nature **418**(6900): 884-9.

Schuch, R., D. Nelson, et al. (2002). "A bacteriolytic agent that detects and kills Bacillus anthracis." Science **418**: 884-889.

Schuh, V., V. Hribalova, et al. (1970). "The pyrogenic effect of scarlet fever toxin. IV. Pyrogenicity of strain C 203 U filtrate: comparison with some basic characteristics of the known types of scarlet fever toxin." Yale J Biol Med **43**(1): 31-42.

Schwartz, B., R. R. Facklam, et al. (1990). "Changing epidemiology of group A streptococcal infection in the USA." Lancet **336**(8724): 1167-71.

Scott, J., P. Thompson-Mayberry, et al. (2008). "Phage-Associated Mutator Phenotype in Group A Streptococcus." Journal of Bacteriology **190**: 6290–6301.

Scott, J., P. Thompson-Mayberry, et al. (2008). "Phage-associated mutator phenotype in group A streptococcus." J Bacteriol **190**(19): 6290-301.

Seppala, H., M. Skurnik, et al. (1998). "A novel erythromycin resistance methylase gene (ermTR) in Streptococcus pyogenes." Antimicrob Agents Chemother **42**(2): 257-62.

Sheehan, M. M., J. L. Garcia, et al. (1996). "Analysis of the catalytic domain of the lysin of the lactococcal bacteriophage Tuc2009 by chimeric gene assembling." FEMS Microbiol Lett. **140**(1): 23-28.

Shulman, S. T., G. Stollerman, et al. (2006). "Temporal changes in streptococcal M protein types and the near-disappearance of acute rheumatic fever in the United States." Clin Infect Dis **42**(4): 441-7.

Silva-Costa, C., M. Ramirez, et al. (2006). "Identification of macrolide-resistant clones of Streptococcus pyogenes in Portugal." Clin Microbiol Infect **12**(6): 513-8.

Sitkiewicz, I., M. J. Nagiec, et al. (2006). "Emergence of a bacterial clone with enhanced virulence by acquisition of a phage encoding a secreted phospholipase A2." Proc Natl Acad Sci U S A **103**(43): 16009-14.

Skiest, D. J. (2006). "Treatment failure resulting from resistance of Staphylococcus aureus to daptomycin." J Clin.Microbiol **44**(2): 655-656.

Smoot, J. C., K. D. Barbian, et al. (2002). "Genome sequence and comparative microarray analysis of serotype M18 group A Streptococcus strains associated with acute rheumatic fever outbreaks." Proc Natl Acad Sci U S A **99**(7): 4668-73.

Sonnenfeld, T., J. Nowak, et al. (1979). "LEg venous oxygen saturation in the evaluation of intra-operative blood flow during arterial reconstructive surgery." Scand J Clin Lab Invest **39**(6): 577-84.

Spanier, J. G. and P. P. Cleary (1980). "Bacteriophage control of antiphagocytic determinants in group A streptococci." J Exp Med **152**(5): 1393-406.

Stanley, J., M. Desai, et al. (1996). "High-resolution genotyping elucidates the epidemiology of group A streptococcus outbreaks." J Infect Dis **174**(3): 500-6.

Starr, C. R. and N. C. Engleberg (2006). "Role of hyaluronidase in subcutaneous spread and growth of group A streptococcus." Infect Immun **74**(1): 40-8.

Steer, A. C., M. H. Danchin, et al. (2007). "Group A streptococcal infections in children." J Paediatr Child Health **43**(4): 203-13.

Steer, A. C., I. Law, et al. (2009). "Global emm type distribution of group A streptococci: systematic review and implications for vaccine development." Lancet Infect Dis **9**(10): 611-6.

Stevens, D. L. (2000). "Streptococcal toxic shock syndrome associated with necrotizing fasciitis." Annu Rev Med **51**: 271-88.

Stevens, D. L., M. H. Tanner, et al. (1989). "Severe group A streptococcal infections associated with a toxic shock-like syndrome and scarlet fever toxin A." N Engl J Med **321**(1): 1-7.

Stollerman, G. H. (1991). "Rheumatogenic streptococci and autoimmunity." Clin Immunol Immunopathol **61**(2 Pt 1): 131-42.

Stollerman, G. H. (1997). "Rheumatic fever." Lancet **349**(9056): 935-42.

Sumby, P., K. D. Barbian, et al. (2005). "Extracellular deoxyribonuclease made by group A Streptococcus assists pathogenesis by enhancing evasion of the innate immune response." Proc Natl Acad Sci U S A **102**(5): 1679-84.

Sumby, P., S. F. Porcella, et al. (2005). "Evolutionary Origin and Emergence of a Highly Successful Clone of Serotype M1 Group A Streptococcus Involved Multiple Horizontal Gene Transfer Events." J Infect Dis **192**(5): 771-782.

Sumby, P., S. F. Porcella, et al. (2005). "Evolutionary origin and emergence of a highly successful clone of serotype M1 group a Streptococcus involved multiple horizontal gene transfer events." J Infect Dis **192**(5): 771-82.

Sumby, P., A. R. Whitney, et al. (2006). "Genome-wide analysis of group a streptococci reveals a mutation that modulates global phenotype and disease specificity." PLoS Pathog **2**(1): e5.

Summers, W. C. (2001). "Bacteriophage therapy." Annu Rev Microbiol **55**: 437-51.

Sung, C. K., H. Li, et al. (2001). "An rpsL cassette, janus, for gene replacement through negative selection in Streptococcus pneumoniae." Appl Environ Microbiol **67**(11): 5190-6.

Svensson, M. D., D. A. Scaramuzzino, et al. (2000). "Role for a secreted cysteine proteinase in the establishment of host tissue tropism by group A streptococci." Mol Microbiol **38**(2): 242-53.

Svensson, N., S. Oberg, et al. (2000). "Invasive group A streptococcal infections in Sweden in 1994 and 1995: epidemiology and clinical spectrum." Scand J Infect Dis **32**(6): 609-14.

Swift, H. F., A. T. Wilson, et al. (1943). "Typing Group a Hemolytic Streptococci by M Precipitin Reactions in Capillary Pipettes." J Exp Med **78**(2): 127-133.

Talkington, D. F., B. Schwartz, et al. (1993). "Association of phenotypic and genotypic characteristics of invasive Streptococcus pyogenes isolates with clinical components of streptococcal toxic shock syndrome." Infect Immun **61**(8): 3369-74.

Tao, J. H., C. S. Fan, et al. (2006). "Depression of biofilm formation and antibiotic resistance by sarA disruption in Staphylococcus epidermidis." World J Gastroenterol. **12**(25): 4009-4013.

Tenover, F. C., R. D. Arbeit, et al. (1995). "Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing." J Clin Microbiol **33**(9): 2233-9.

Terao, Y., Y. Mori, et al. (2008). "Group A streptococcal cysteine protease degrades C3 (C3b) and contributes to evasion of innate immunity." J Biol Chem **283**(10): 6253-60.

Thern, A., L. Stenberg, et al. (1995). "Ig-binding surface proteins of Streptococcus pyogenes also bind human C4b-binding protein (C4BP), a

regulatory component of the complement system." J Immunol **154**(1): 375-86.

Thern, A., M. Wastfelt, et al. (1998). "Expression of two different antiphagocytic M proteins by Streptococcus pyogenes of the OF+ lineage." J Immunol **160**(2): 860-9.

Thompson, J. D., T. J. Gibson, et al. (1997). "The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools." Nucleic Acids Res **25**(24): 4876-82.

Tiesler, E. and U. Beck (1976). "[Distribution of the isoenzymes of nucleases in group A Streptococci (author's transl)]." Zentralbl Bakteriol Orig A **234**(4): 462-72.

Tillett, W. S., S. Sherry, et al. (1948). "Streptococcal desoxyribonuclease; significance in lysis of purulent exudates and production by strains of hemolytic streptococci." Proc Soc Exp Biol Med **68**(1): 184-8.

Timmer, A. M., S. A. Kristian, et al. (2006). "Serum opacity factor promotes group A streptococcal epithelial cell invasion and virulence." Mol Microbiol **62**(1): 15-25.

Trotonda, M. P., Y. Q. Xiong, et al. (2009). "Role of mgrA and sarA in methicillin-resistant Staphylococcus aureus autolysis and resistance to cell wall-active antibiotics." J Infect.Dis. **199**(2): 209-218.

Tsiodras, S., H. S. Gold, et al. (2001). "Linezolid resistance in a clinical isolate of Staphylococcus aureus." Lancet **358**(9277): 207-208.

Udo, E. E. and E. Sarkhoo (2009). "Genetic Analysis of High-Level Mupirocin Resistance in ST80 clone of Community-Associated Methicillin-Resistant Staphylococcus aureus." J Med.Microbiol.

Unnikrishnan, M., D. M. Altmann, et al. (2002). "The bacterial superantigen streptococcal mitogenic exotoxin Z is the major immunoactive agent of Streptococcus pyogenes." J Immunol **169**(5): 2561-9.

Veasy, L. G., L. Y. Tani, et al. (1994). "Persistence of acute rheumatic fever in the intermountain area of the United States." J Pediatr **124**(1): 9-16.

Veasy, L. G., S. E. Wiedmeier, et al. (1987). "Resurgence of acute rheumatic fever in the intermountain area of the United States." N Engl J Med **316**(8): 421-7.

Verheust, C., N. Fornelos, et al. (2005). "GIL16, a new gram-positive tectiviral phage related to the Bacillus thuringiensis GIL01 and the Bacillus cereus pBClin15 elements." J Bacteriol **187**(6): 1966-73.

Viola, M., D. Quaratino, et al. (2005). "Allergic reactions to antibiotics, mainly betalactams: facts and controversies." Allerg Immunol (Paris) **37**(6): 223-9.

Vlaminckx, B. J., F. H. Schuren, et al. (2007). "Dynamics in prophage content of invasive and noninvasive M1 and M28 Streptococcus pyogenes isolates in The Netherlands from 1959 to 1996." Infect Immun **75**(7): 3673-9.

Voyich, J. M., D. E. Sturdevant, et al. (2003). "Genome-wide protective response used by group A Streptococcus to evade destruction by human

polymorphonuclear leukocytes." Proc Natl Acad Sci U S A **100**(4): 1996-2001.

Wagner, P. L. and M. K. Waldor (2002). "Bacteriophage control of bacterial virulence." Infect Immun **70**(8): 3985-93.

Waldor, M. K., D. I. Friedman, et al. (2005). Phages : their role in bacterial pathogenesis and biotechnology. Washington, D.C., ASM Press.

Walker, M. J., A. Hollands, et al. (2007). "DNase Sda1 provides selection pressure for a switch to invasive group A streptococcal infection." Nat Med **13**(8): 981-5.

Wannamaker, L. W. (1958). "The differentiation of three distinct desoxyrlbonucleases of group A Streptococci." J Exp Med **107**(6): 797-812.

Wannamaker, L. W. (1970). "Serum antibodies to streptococci in rheumatic fever, glomerulonephritis and chorea." Zentralbl Bakteriol Orig **214**(3): 331-8.

Wannamaker, L. W., B. Hayes, et al. (1967). "Streptococcal nucleases. II. Characterization of DNAse D." J Exp Med **126**(3): 497-508.

Wannamaker, L. W. and W. Yasmineh (1967). "Streptococcal nucleases. I. Further studies on the A, B, and C enzymes." J Exp Med **126**(3): 475-96.

Watanabe, Y., Y. Todome, et al. (2002). "Cysteine protease activity and histamine release from the human mast cell line HMC-1 stimulated by recombinant streptococcal pyrogenic exotoxin B/streptococcal cysteine protease." Infect Immun **70**(7): 3944-7.

Weeks, C. R. and J. J. Ferretti (1986). "Nucleotide sequence of the type A streptococcal exotoxin (erythrogenic toxin) gene from Streptococcus pyogenes bacteriophage T12." Infect Immun **52**(1): 144-50.

Wessels, M. R. and M. S. Bronze (1994). "Critical role of the group A streptococcal capsule in pharyngeal colonization and infection in mice." Proc Natl Acad Sci U S A **91**(25): 12238-42.

Wessels, M. R., J. B. Goldberg, et al. (1994). "Effects on virulence of mutations in a locus essential for hyaluronic acid capsule expression in group A streptococci." Infect Immun **62**(2): 433-41.

Wessels, M. R., A. E. Moses, et al. (1991). "Hyaluronic acid capsule is a virulence factor for mucoid group A streptococci." Proc Natl Acad Sci U S A **88**(19): 8317-21.

Westberg, N. G., G. B. Naff, et al. (1971). "Glomerular deposition of properdin in acute and chronic glomerulonephritis with hypocomplementemia." J Clin Invest **50**(3): 642-9.

Wilcox, M. H., J. Hall, et al. (2003). "Use of perioperative mupirocin to prevent methicillin-resistant Staphylococcus aureus (MRSA) orthopaedic surgical site infections." J Hosp.Infect. **54**(3): 196-201.

Wilson, A. T. (1959). "The relative importance of the capsule and the M-antigen in determining colony form of group A streptococci." J Exp Med **109**(3): 257-70.

Wilson, G. G. and N. E. Murray (1991). "Restriction and modification systems." Annu Rev Genet **25**: 585-627.

Wilson, P., J. A. Andrews, et al. (2003). "Linezolid resistance in clinical isolates of Staphylococcus aureus." J Antimicrob.Chemother. **51**(1): 186-188.

Witzenrath, M., B. Schmeck, et al. (2009). "Systemic use of the endolysin Cpl-1 rescues mice with fatal pneumococcal pneumonia`." Critical Care Medicine **37**: 642-649.

World Health Organization (2001) "Rheumatic fever and rheumatic heart disese: report of a WHO expert consultation, Geneva." Who Tech Rep Ser **923**.

Yoong, P., D. Nelson, et al. (2004). "Identification of a broadly active phage lytic enzyme with lethal activity against antibiotic-resistant Enterococcus faecalis and Enterococcus faecium " Journal of Bacteriology **186**: 4808-4812.

Yoong, P., R. Schuch, et al. (2004). "Identification of a broadly active phage lytic enzyme with lethal activity against antibiotic-resistant Enterococcus faecalis and Enterococcus faecium." J Bacteriol **186**(14): 4808-12.

Yoshizawa, N. (2000). "Acute glomerulonephritis." Intern Med **39**(9): 687-94.

Young, R. (1992). "Bacteriophage lysis: mechanism and regulation." Microbiol Rev **56**(3): 430-81.

Yu, C. E. and J. J. Ferretti (1989). "Molecular epidemiologic analysis of the type A streptococcal exotoxin (erythrogenic toxin) gene (speA) in clinical Streptococcus pyogenes strains." Infect Immun **57**(12): 3715-9.

Yu, C. E. and J. J. Ferretti (1991). "Frequency of the erythrogenic toxin B and C genes (speB and speC) among clinical isolates of group A streptococci." Infect Immun **59**(1): 211-5.

Yutsudo, T., H. Murai, et al. (1992). "A new type of mitogenic factor produced by Streptococcus pyogenes." FEBS Lett **308**(1): 30-4.

Zabriskie, J. B. (1964). "The Role of Temperate Bacteriophage in the Production of Erythrogenic Toxin by Group a Streptococci." J Exp Med **119**: 761-80.