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Analysis of the Streptococcal Hemoprotein Receptor: A Role in Virulence and Host Defense

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Citation	Huang, Ya-Shu. 2012. "Analysis of the Streptococcal Hemoprotein Receptor: A Role in Virulence and Host Defense." Georgia State University. https://doi.org/10.57709/2758528
DOI	https://doi.org/10.57709/2758528
Download date	2026-04-13 02:35:55
Link to Item	https://hdl.handle.net/20.500.14694/1908

ANALYSIS OF THE STREPTOCOCCAL HEMOPROTEIN RECEPTOR: A ROLE IN
VIRULENCE AND HOST DEFENSE

by

YA-SHU HUANG

Under the Direction of Zehava Eichenbaum

ABSTRACT

Group A streptococcus (GAS) is an important pathogen that produces a wide spectrum of suppurative infections and autoimmune sequelae in humans, ranging from less complex pharyngitis, impetigo to more severe manifestations such as necrotizing fasciitis, toxic shock syndrome, rheumatic fever and glomerulonephritis. The worldwide burden of GAS infections and sequelae is considerable, but an immunization program that defends against the hyper-variable GAS is missing. The streptococcal hemoprotein receptor (Shr), is an iron-regulated

protein involved in heme acquisition. An unspecified region in the amino terminus of Shr mediates the interactions with hemoglobin and two protein modules named NEAT1 and NEAT2 bind heme. In this study, we analyzed the molecular structure and function of Shr, investigated its antigenic properties and role in GAS disease production. We demonstrated that Shr is a new type of GAS adhesin that contributes to the pathogen interactions with extracellular matrix (ECM) proteins. Shr enabled bacterial adherence to host cells and was important for GAS virulence *in vivo*. Immunizations with Shr protein by intraperitoneal or intranasal administration conferred resistance to systemic GAS challenge in mice. Shr antiserum allowed bacterial opsonization and defended against GAS diseases in a murine model for passive vaccination. Studies with isolated Shr domains localized ECM-binding to the NEAT domains and showed that most of the protein is exposed on the bacterial surface. In addition, Shr N-terminal region and both of the NEAT modules elicited strong antibody response in rabbits. In conclusion, Shr is a protective antigen that contributes to GAS pathogenesis by facilitating both heme uptake and bacterial adherence. Since Shr is conserved among GAS strains and other pyogenic streptococci, this study demonstrates that Shr may be used to develop a vaccine against GAS strains and related pathogens.

INDEX WORDS: *Streptococcus pyogenes*, *Sia* operon, Adherence, Adhesin, Fibronectin, MSCRAMM, NEAT domain, Virulence, Immunization, Vaccine

ANALYSIS OF THE STREPTOCOCCAL HEMOPROTEIN RECEPTOR: A ROLE IN
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YA-SHU HUANG

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

in the College of Arts and Sciences

Georgia State University

2012

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Ya-Shu Huang
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May 2012

ACKNOWLEDGEMENTS

“When you want something, all the universe conspires in helping you to achieve it.” (Paulo Coelho, *The Alchemist*, 1988)

This dissertation would not have been possible without the help and support of several important people. I am truly thankful to my adviser, Dr. Zehava Eichenbaum. It has been a great pleasure to be mentored by her valuable guidance, complete support and incomparable kindness. I really enjoy her good influence, which is like a life-giving spring breeze, during my Ph.D. years. I want to also thank Dr. Morly Fisher and Dr. Li for their instructions on many experimental knowledge and skills. I thank my dissertation committee: Dr. Chung-Dar Lu, Dr. Parjit Kaur and Dr. Susanna Greer for contributing their ideas and suggestions to my work.

I am deeply grateful to my parents and my sister for always being there with me through good times and bad times. Their encouragement, support, trust and love are the most precious treasure that I own in the whole world. Being an international student, friends become my second family. My dear lab members: Chadia, Mahamoudou and Elizabeth. Thank you for being my sisters and brothers in the lab. Your laughter, caring and help will not be forgotten in my future life. I want to show my special thanks to my best friend in US, Yingju, for taking care of me in many aspects and for loving me with a generous sisterhood.

I would also like to thank the financial support and academic resources provided by Molecular Basis of Disease Program at Georgia State University.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS.....	vi
LIST OF TABLES.....	ix
LIST OF FIGURES	x
GENERAL INTRODUCTION.....	1
Bacterial adherence and pathogenesis.....	2
Host extracellular matrix (ECM) molecules.....	3
Adhesins in Gram-positive bacteria.....	6
Adhesins in Group A Streptococcus.....	10
Fibronectin-binding adhesins in GAS.....	12
<i>Streptococcus pyogenes</i> : Pathogenesis and epidemiology.....	15
Development of recent vaccines against streptococcal diseases	15
The streptococcal hemoprotein receptor (Shr).....	16
CHAPTER I: SHR IS A BROAD-SPECTRUM SURFACE RECEPTOR THAT CONTRIBUTES TO ADHERENCE AND VIRULENCE IN GAS.....	18
INTRODUCTION.....	18
MATERIALS AND METHODS.....	21
RESULTS.....	30
DISCUSSION.....	50
CHAPTER II: DEFENSE FROM THE GROUP A STREPTOCOCCUS BY ACTIVE PASSIVE VACCINATION WITH THE STREPTOCOCCAL HEMOPROTEIN RECEPTOR.....	56
INTRODUCTION.....	56

MATERIALS AND METHODS.....	59
RESULTS.....	62
DISCUSSION.....	73
CHAPTER III: ANALYSIS OF SHR DOMAINS.....	76
INTRODUCTION.....	76
MATERIALS AND METHODS.....	84
RESULTS.....	98
DISCUSSION.....	110
GENERAL DISCUSSION.....	120
REFERENCES	129
APPENDICES	140
Appendix A	141
Appendix B	142

LIST OF TABLES

Table 1. Analysis of Shr expression on the surface of GAS clinical isolates.....	44
Table 2. Oligonucleotide primers used in Chapter III.....	96
Table 3. Plasmids used in Chapter III.....	97

LIST OF FIGURES

Figure 1. An illustration of some of proteins and complex in the ECM network.....	4
Figure 2. The schematic illustration of fibronectin-binding repeats of SfbI.....	14
Figure 3. MtsR antiserum specifically recognizes MtsR protein.....	24
Figure 4. Shr protein domains and cellular location.....	31
Figure 5. Successful inactivation of <i>shr</i> in strain ZE4912 and mutant complementation in strain ZE4924.....	33
Figure 6. Shr is exposed on the surface of GAS.....	35
Figure 7. Shr binds <i>in vitro</i> to ECM components.....	37
Figure 8. Inhibition of Shr binding to immobilized ECM ligands.....	38
Figure 9. Shr expressed on <i>L. lactis</i> cell surface promotes bacterial binding to fibronectin.....	41
Figure 10. Adherence of GAS to cultured epithelial cells.....	43
Figure 11. Shr antibody levels in mice following recovery from GAS infections.....	46
Figure 12. Zebrafish survival following intramuscular injection.....	49
Figure 13. Anti-Shr response in serum after intraperitoneal vaccination.....	64
Figure 14. Protection of mice from systemic GAS challenge after intraperitoneal vaccination with Shr.....	65
Figure 15. Shr intranasal vaccination and protective immune response.....	68
Figure 16. Protection of mice from systemic GAS infection with passive immunization with anti- Shr.....	71
Figure 17. Serum mediation of phagocytosis and killing of bacteria with anti-Shr.....	72
Figure 18. Shr proteins in pyogenic streptococci and <i>Clostridium novyi</i>	79
Figure 19. The extended tandem β -zipper model.....	81

Figure 20. Gateway [®] Recombination reactions.....	86
Figure 21. Plasmid map of pYSH5.....	87
Figure 22. Plasmid map of pYSH7.....	88
Figure 23. Plasmid map of pYSH6.....	89
Figure 24. Plasmid map of pYSH8.....	90
Figure 25. Plasmid map of pYSH9.....	91
Figure 26. Binding of extracellular matrix proteins by Shr.....	99
Figure 27. Specificity of Shr domain antibodies.....	102
Figure 28. Surface presentation of Shr domains by whole cells.....	105
Figure 29. Passive immunization by Shr domain-specific antibody.....	107
Figure 30. Opsonization of bacteria with Shr domain-specific antibodies.....	109
Figure 31. Crystal structure of heme-bound IsdA NEAT domain from <i>Staphylococcus aureus</i>	113
Figure 32. Predicted structure of Shr NEAT1 domain.....	114
Figure 33. Adherence of GAS to cultured lung cells NCIH292.....	141
Figure 34. Surface presentation of Shr mutants in <i>L. lactis</i>	142

GENERAL INTRODUCTION

Bacterial adherence to the host mucosal and cutaneous surfaces is a critical step in the establishment of most infections. Successful colonization requires from pathogens to efficiently compete with the resident microbial populations for resources and space and to resist clearance by the host defense mechanisms. The digestive tract for example, is protected by the washing actions of the saliva and the bowel movements, which exclude bacteria with mechanic forces. The ciliated cells in the upper respiratory tract repulse microbial invaders by electrostatic forces and propel those that are trapped to the mucus away from the epithelial surfaces (Cunningham 2000). Surface adhesins with high affinity to molecules that are found on the host cells or extracellular matrix (ECM) are used by pathogenic bacteria to firmly attach and persist in desirable sites. In addition to attachment, some bacterial adhesins play additional roles in virulence and may also contribute to immune escape or nutrient acquisition, for example. The focus of this study is Group A Streptococcus (GAS), also known as *Streptococcus pyogenes*. GAS is an important bacterial pathogen that imposes a significant toll on human health worldwide. We hypothesized that an exported protein in GAS, named Shr (for Streptococcal Hemoprotein Receptor), is involved in adherence in addition to its previously described role in iron uptake. We inferred that the pleiotropic Shr protein has a significant impact on GAS virulence and is a likely candidate for vaccine development. This dissertation describes the analysis of Shr structure/function, its role in GAS disease process and illustrates its promise as a vaccine target. In this introduction, we provide an overview of bacterial adhesins with emphasis on the mechanisms used by Gram-positive pathogens, offer background information as for the pathology and epidemiology of streptococcal diseases and summarize the current status of vaccine development against GAS.

Bacterial adherence and pathogenesis

Bacterial pathogens express surface molecules that mediate binding to the host and thus facilitate colonization and the formation of microbial communities. The structure and location of bacterial adhesins are impacted by the configuration of the cell envelope, which is different between Gram-positive and Gram-negative organisms. In Gram-negative bacteria, adhesins are often anchored to the outer membrane, which serves as the cell exterior (enclosing on a thin peptidoglycan layer in the periplasmic space). In Gram-positive organisms, adhesins are often covalently bound to the thick cell wall, which is exposed to the extracellular environment (Abbot, Smith et al. 2007). Other Gram-positive adhesins span the peptidoglycan layer and have one end outside of the cell wall and the other embedded in the inner membrane via a lipid moiety or by a transmembrane segment. Because of their surface exposure, bacterial molecules that mediate adherence are also available for other interactions with the host. For example, adhesins may communicate with components of the immune system and shape antibody and cytokine productions by the host.

Several independent adhesins can work synergistically to enhance the attachment of a single bacterium. In fact, the simultaneous production of a different adhesin combination at separate disease stages enables the bacterium to colonize the variety of niches that it encounters during the infection process. The role of a particular adhesin, however, may be difficult to demonstrate experimentally because of this redundancy. In summary, while not all of the adhesins produced by an individual pathogen are essential for causing diseases, together, these molecules act to increase the effectiveness of a single pathogen and enhance its ability to colonize the host (Nobbs, Lamont et al. 2009).

Host extracellular matrix (ECM) molecules

Cells in the mammalian host produce and release various macromolecules that together form a complex network named the extracellular matrix (ECM). Cells bind to the ECM via specific receptors such as integrins and benefit from the structural support it provides (Fig. 1). In addition to offering strength, the ECM maintains tissue elasticity. The proteins that make up the ECM are typically large and multifunctional. Most of them are constructed in a modular fashion and contain multiple binding surfaces that facilitate multimerization and interactions with a wide range of macromolecules (Campbell 2003). Collagen, the most plentiful structural protein found in the host, is the major structural component of the ECM. Collagen forms a variety of interstitial (collagen types I, II, III) and basement (collagen type V) networks found in different tissue types.

Several glycoproteins, proteoglycans, hyaluronan and other components associate with the basic collagen networks to form the massive ECM (Cossart, Boquet et al. 2004). Gram-positive pathogens often choose to interact with the host ECM for adherence purpose (Kreikemeyer, Klenk et al. 2004; Hauck and Ohlsen 2006). In addition to specific interactions with immobilized components of the ECM, some bacterial adhesins target soluble components of the matrix. These soluble proteins then serve as an intermediate bridge between the bacterium and cognate receptors on the host cell surfaces. In some cases, bacterial binding to a host surface receptor (directly or via a soluble ECM component) initiates a signaling cascade in the host cell that leads to bacterial uptake. Similar mechanism allows bacteria to invade phagocytic cells while avoiding the phagosome and the phagolysosome (Ozeri, Rosenshine et al. 1998; Hauck and Ohlsen 2006; Caswell, Lukomska et al. 2007).

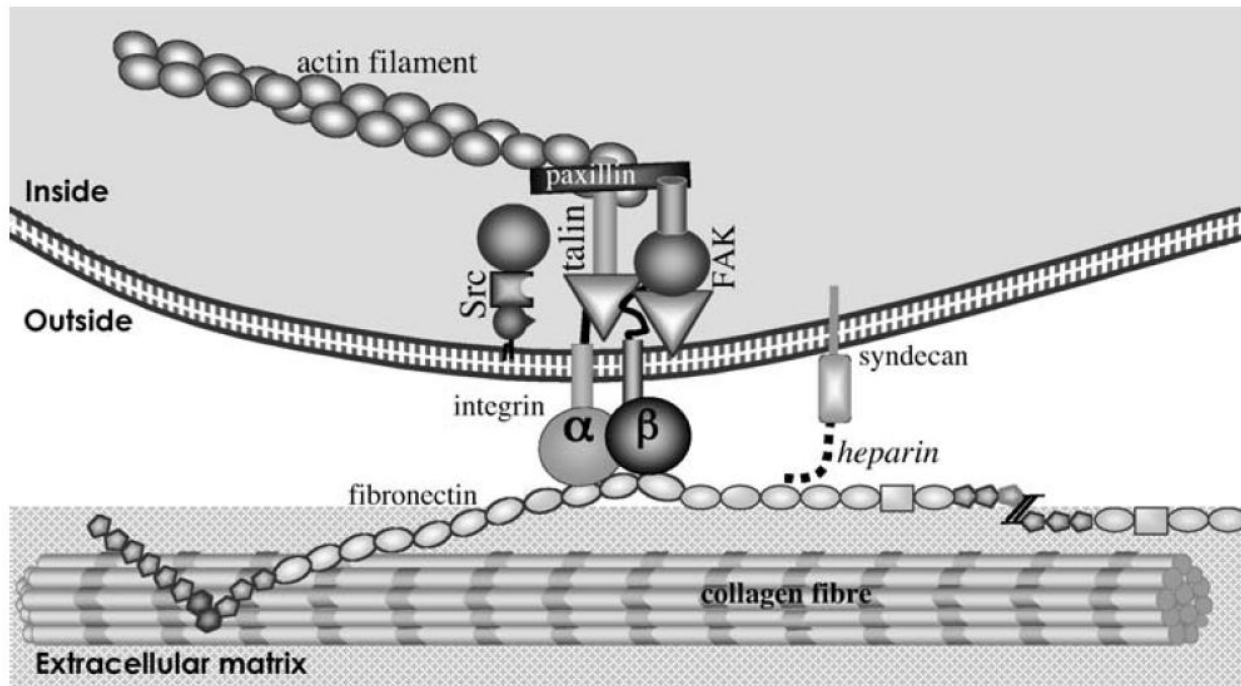


Figure 1. An illustration of some of the proteins and complex in the ECM network.

In the extracellular spaces, collagen is the main structural component. Several glycoproteins, proteoglycans, hyaluronan and other components associate with the basic collagen networks to form the massive ECM network. Inside the cell, actin filaments are the major structural component. The linking of intra- and extra- cellular networks is accomplished in diverse ways. For example, the ECM protein fibronectin binds both to collagen and to extracellular receptors such as integrins and syndecan, as illustrated above. (Figure adopted from Campbell, 2003)

The ECM molecules that serve as targets for bacterial adhesion include collagen, fibronectin, fibrinogen, vitronectin, laminin, elastin and glycosaminoglycans. Fibronectin is a large glycoprotein, which exists in a soluble form in the plasma and as a fibrillar polymer that is bound to the ECM. Fibronectin forms dimers in which the 250-kD subunits are linked by a pair of disulfide bonds. The ECM-fixed fibronectin is more effective in binding to integrins than the soluble form. The ECM bound molecule is proposed to exist in an extended form that exposes its integrin binding regions (Hynes 1999). Fibronectin can form a molecular bridge between the bacteria and the $\alpha 5\beta 1$ integrin molecules on the host cell (Ozeri, Rosenshine et al. 1998; Joh, Wann et al. 1999; Cue, Southern et al. 2000). Integrins are heterodimeric receptors that mediate cell adhesion, migration and tissue organization. They regulate the direct association of cells with each other and with the ECM. Further, by binding to ligands, integrins provide a transmembrane link for extracellular signals across the plasma membrane. This function is used by several bacterial pathogens to, directly or via fibronectin, contact integrins and trigger a set of conformational changes in the cell membrane that allows their internalization. Fibrinogen is a 340-kD plasma glycoprotein that forms a dimer. It plays an important role in the vascular system by mediating platelet adherence and aggregation at injury sites. Fibrinogen can also interact with a variety of ECM proteins and thus serves as a key factor in the matrix organization. Vitronectin, a 75-kD glycoprotein, is present in both the blood and the ECM. Vitronectin also controls the degradation of the ECM by stabilizing the plasminogen activator inhibitor-1. In addition, vitronectin binds to some complement factors, heparin and to the thrombin-antithrombin III complex. Thereby, it also participates in the regulation of clot formation. Laminin, is a massive 900-kD glycoprotein that serves as the major component of the basement membrane. Laminin is involved in several important processes including cell attachment and proliferation, chemotaxis

and angiogenesis. Elastin is highly flexible and therefore serves as the main constituent of the ECM in organs that are required to expand and contract such as the lung, skin and large arteries. Elastin consists of 36 domains with interlacing hydrophobic and cross-linking characteristics. The rubber-like mechanical properties of elastin are derived from several repetitive hydrophobic domains. These protein modules display an unstructured organization when elastin is in the relaxed state and a structured organization when it is in the extended state. Finally, glycosaminoglycans are a group of proteoglycans that consist of polysaccharide chains with a protein core. They are present in the ECM of connective tissues as well as on the surface of eukaryotic cells. Glycosaminoglycans function as coreceptors of cytokines and chemokines, regulators of enzymatic activity and signaling molecules in response to injury or infection. Thus, glycosaminoglycans are considered important for many cellular responses in development, homeostasis and diseases (Trowbridge and Gallo 2002).

Adhesins in Gram-positive bacteria

Most Gram-positive pathogens encode multiple adhesins, which are typically expressed according to different environmental cues representing the conditions that the bacterium may confront during infection. The adhesins described below are categorized according to their structure and host targets.

None-protein adhesins:

While most of the adhesins are proteins, both teichoic acid (TA) and polysaccharide capsules mediate attachment in some Gram-positive organisms. TA, a main component of the cell envelope in Gram-positive bacteria, is a glycerol phosphate polymer that can be bound to the cell

wall or embedded in the outer leaflet of the cytoplasmic membrane. The negatively charged TA associates with host cells through electrostatic attractions and weak hydrophobic interactions, and therefore it allows bacteria to overcome the electrostatic repulsions found between bacteria and mammalian cells. Some receptors of the innate immune system detect the presence of bacteria by interacting with TA. The scavenger receptor A and CD36 on macrophages bind to TA from *Staphylococcus aureus* (Greenberg, Fischer et al. 1996; Hoebe, Georgel et al. 2005). In addition, the TA of *S. aureus*, *Streptococcus agalactiae* and *Streptococcus pneumonia* interacts with the Toll-like receptor 2 of human macrophages and activate an innate immune response (Schroder, Morath et al. 2003; Henneke, Morath et al. 2005).

An antiphagocytic capsule is produced by most of the pathogens that are capable of producing systemic infections. The majority of capsules consist of polysaccharide polymers, although the specific molecular components vary from organism to organism. Studies in multiple pathogens have shown that the major role of the capsule is to enhance bacterial survival in the blood. The capsule facilitates the escape from phagocytosis and reduces the complement activation on the bacterial surface, which are both important defense mechanisms of the innate immune system. For example, the sialic acid-modified capsule of *S. agalactiae* blocks C3b deposition on the streptococcal surface and thereby prevents bacterial uptake by the phagocytic cells. A decrease in capsule synthesis correlates with an increase in C3b deposition in *S. agalactiae* (Marques, Kasper et al. 1992). The capsule in *S. aureus* contributes to abscess formation in rat model in a mechanism that is independent of the capsule-mediated immune evasion (Tzianabos, Wang et al. 2001).

Protein adhesins:

Protein adhesins are categorized into pili (otherwise known as fimbriae) and afimbrial-type based on their structures. Pili are protein polymers made of a repeating subunit (major pilin) that forms the backbone and one or more minor subunits that are typically located at the tip and facilitate adherence (Scott and Zähler 2006). In some pili, however, adherence is mediated by the shaft rather than the tip. Pili appear as filamentous structures, which typically extend 1 to 3 μm from the bacterial surface. Pilus synthesis is markedly different in Gram-negative and positive organisms. In Gram-negatives, the pilus subunits are held together only by hydrogen bonds, while in Gram-positive bacteria the pilus subunits are covalently linked to each other and the pilus is bound to peptidoglycan. Biosynthesis begins with the export of the pilus subunits by the general secretion pathway. At the surface, the subunits are then connected to each other by a dedicated enzyme with a sortase-like function. The pilus grows from the tip, starting with the minor subunits followed by the major subunits that create the shaft. Finally, each complete pilus is anchored to the peptidoglycan by a sortase enzyme. This mechanism for pilus biosynthesis substantially increases the strength of the pili structure in Gram-positive bacteria and makes it difficult to dissociate from the surface. Pili are important for virulence of several Gram-positive pathogens. In *S. pneumoniae* for example, the genes required for pilus production are located in the *rlr* pathogenicity islet, and the pili mediate pneumococcal adherence to lung epithelial cells (Barocchi, Ries et al. 2006).

In addition to pili, Gram-positive bacteria also produce afimbrial adhesins, which are held at the cell surface so that they can be available for interactions with the host. Afimbrial adhesins can be covalently bound to the cell wall, embedded in the cell membrane, or noncovalently associated

with other surface components. In general, cell wall-linked adhesins share the following characteristics: an N-terminal signal peptide, a sortase recognition motif at the C-terminus (LPX [T, S, A] G), followed by a hydrophobic transmembrane domain and a short positively-charged tail. The signal peptide allows the protein export by the general secretion apparatus. The hydrophobic segment and the positively-charged tail tether the C-terminus of the peptide in the membrane, presenting it in the correct configuration to the sortase enzyme, which cleaves it at the LPX site and links it to the peptidoglycan.

An important subfamily of afimbrial adhesins is called MSCRAMMs (microbial surface components recognizing adhesive matrix molecules). These proteins target ECM components, such as fibrinogen, fibronectin and collagen for adherence. Multiple MSCRAMMs have been identified and characterized in important Gram-positive pathogens including *S. aureus* and GAS. In addition to the cell-wall sorting signals described above, MSCRAMMs typically have a ligand binding domain in their N-terminus. Several MSCRAMMs are pleiotropic and exhibit affinity to other ligands and/or a catalytic activity in addition to ECM binding. ECM interactions are often mediated by one or more repetitive sequences in the protein (Patti, Allen et al. 1994). A single bacterium can express different MSCRAMMs that bind to the same ligand, while many MSCRAMMs are capable of binding to more than just one ECM component. For example, both of the *S. aureus* receptors, FnbpA and FnbpB, bind soluble and immobilized fibronectin. Fibronectin then connects the bacterium to the host integrin $\alpha 5\beta 1$, which in turn triggers the uptake of *S. aureus* into the host epithelial and endothelial cells (Wann, Gurusiddappa et al. 2000; Schwarz-Linek, Hook et al. 2006). A third adhesin in staphylococcus, Map (or Eap), recognizes a broad variety of ECM components, including fibrinogen, fibronectin,

thrombospondin, vitronectin, bone sialoprotein, osteopontin and collagen. This protein that is expressed in both a secreted and a cell-anchored form, mediates the attachment and internalization of *S. aureus* into several types of eukaryotic cells. Map serves as an anti-inflammatory protein as well as an adhesin. It binds directly to the host receptor ICAM-1 and leads to attenuation of leukocyte recruitment (Chavakis, Hussain et al. 2002).

Afimbrial adhesins can target other host molecules in addition to the ECM components. For example, the Staphylococcal adhesin Bbp, binds to the sialoprotein in osteoid (an organic portion of the bone matrix). These interactions are important to the development of staphylococcal osteomyelitis (Tung, Guss et al. 2000). *Listeria monocytogenes* is a Gram-positive food-borne pathogen that can produce invasive infections. The major adhesins of *L. monocytogenes*, internalin A and B, recognize the human E-cadherin and the hematocyte receptor, Met, respectively. E-cadherin, a cell-cell cohesion molecule, is the suggested target of *L. monocytogenes* at the gut epithelium, while Met is targeted by the pathogen during the invasive stage of the disease (Lecuit, Ohayon et al. 1997).

Adhesins in Group A Streptococcus

GAS is a Gram-positive human pathogen. It commonly causes a variety of mild diseases, including pharyngitis, impetigo, cellulitis and scarlet fever. Some episodes of GAS infections lead to severe complications such as rheumatic fever, rheumatic heart disease and acute glomerulonephritis. GAS can also produce systemic infections that involve the lungs, blood or muscle. Acute GAS infections are rare (~1:100,000) but life-threatening, these include necrotizing fasciitis (NF), myositis and streptococcal toxic shock syndrome (STSS)

(Cunningham 2000; Cunningham 2008). To ensure a successful colonization to a variety of sites in the human body, GAS uses a spectrum of adhesins including TA, capsule, pili and a number of MSCRAMMs. Hasty *et al* proposed a two-step model for the interaction of GAS with human cells (Hasty, Ofek et al. 1992). The TA first mediates weak and reversible interactions with the host, while other adhesins then direct the bacterial binding to other host ligands with high affinity. The hyaluronic acid (HA) capsule of GAS allows binding to collagen fibers and to the glycoprotein CD44 of human keratinocytes *in vitro* (Schrager, Alberti et al. 1998). However, the *in vivo* role of the capsule in adherence is controversial. The capsule was found to mediate GAS attachment in a study performed in a mouse skin infection model (Dinkla, Rohde et al. 2003), while another study suggested that the GAS capsule masks important surface adhesins and thus impairs adherence to human epithelial cells (Bartelt and Duncan 1978).

In 1933 Rebecca Lancefield established the classification of streptococci according to the antigenic properties of the M protein and T antigen. Contemporary investigations, however, established that both proteins partake in adherence; the M protein is afimbrial adhesin and the T antigen is part of the pili. The M protein is the major virulence factor in GAS and it forms α -helical coiled coil dimers, which have an extended shape and appear as hair-like projection on the cell surface. The M protein is an antiphagocytic protein that is also involved in adherence. The N-terminus of M protein is hypervariable and there are more than 200 M-protein based serotypes and genotypes in GAS (Cole, Barnett et al. 2011). Different M proteins exhibit binding to a combination of host plasma proteins, including the Fc portion of IgA and IgG, factor H and fibrinogen. Binding to these host proteins protects GAS from opsonization. Some M serotypes mediate GAS adherence to keratinocytes by binding to the membrane cofactor protein,

CD46 (Okada, Liszewski et al. 1995; Giannakis, Jokiranta et al. 2002; Sandin, Carlsson et al. 2006). T antigen, also called protein T, is a trypsin-resistant surface protein that serves as the major pilus subunit in GAS (Mora, Bensi et al. 2005). Studies done with the M1 strain SF370 demonstrated that bacterial adherence to human tonsil and primary skin cells is facilitated by the pili (Abbot, Smith et al. 2007). In a separate investigation, the pili were found to bind the salivary component gp340, leading to bacterial aggregation in saliva, which may facilitate bacterial clearance during pharyngeal infection (Edwards, Manetti et al. 2008).

GAS expresses several adhesins that bind directly to host cells. These include the collagen-like protein SclA, which carries a conserved collagen-like region and a hypervariable N-terminal portion (Rasmussen, Eden et al. 2000). The collagen-like region mimics the function of human collagens. It interacts with integrin $\alpha 2\beta 1$ and activates intracellular signaling pathways in the host cells (Humtsoe, Kim et al. 2005). Similar proteins with collagen-like sequences are found in a wide range of bacteria, including *S. pneumonia*, *Clostridium perfringens* and *Haemophilus* species. In addition, GAS produces multiple MSCRAMMs that bind to the ECM. Although several adhesins that bind to collagen and laminin were found (Terao, Kawabata et al. 2002; Kreikemeyer, Nakata et al. 2005), most of GAS MSCRAMMs bind to fibronectin (Talay 2005).

Fibronectin-binding adhesins in GAS

Fibronectin-binding adhesins are expressed by all streptococci. However, these receptors exhibit significant variety in their binding characteristics, such as ligand affinity and preferences for the soluble and/or the immobilized form of fibronectin. The typical fibronectin-binding receptors are associated with the peptidoglycan, although some appear to lack a cell wall sorting motif. There

are at least 11 fibronectin-binding proteins in GAS, including SfbI (aka PrtF1), protein F2 (PrtF2, aka FbaB and PFBP), SfbII (aka serum opacity factor, SOF) and FbaA (Kreikemeyer, Klenk et al. 2004). Most of the MSCRAMMs that bind fibronectin have similar structural organization and binding mechanism (Schwarz-Linek, Hook et al. 2006). The major fibronectin-binding activity is located to a tandem repeat (35-40 amino acid residues) region that is found at the C-terminal half of the receptor (Fig. 2). The number of the repeats varies in different adhesins, but the repeating units share significant homology with that found in various adhesins of GAS and other Gram-positive bacteria. Some proteins also contain an upstream fibronectin-binding domain (UFBD). Several of the fibronectin receptors in GAS have additional functions. For example, SOF and ScpA (aka C5a peptidase) have a lipoproteinase or a peptidase activity in addition to fibronectin binding (Courtney, Hasty et al. 2003; Purushothaman, Park et al. 2004). ScpA specifically inactivates the host complement factor, C5a, which inhibits the recruitment of the polymorphonuclear neutrophils (Brown, Gu et al. 2005). Notably, the ScpA binds fibronectin with high affinity through a unique mechanism that does not involve the common protein repeats.

***Streptococcus pyogenes*: Pathogenesis and epidemiology**

Each year, over 10 million of noninvasive GAS diseases, primarily throat and superficial skin infections occur worldwide. In addition, GAS causes an array of invasive diseases, due to which a massive cost is spent every year. The severe GAS diseases appear in the U.S. at a rate of about 3.6/100,000 in population, producing significant morbidity. In 2009 for example, 11,000 invasive infection cases were reported, leading to 1,300 deaths. (Centers for Disease Control and Prevention. 2010. Active Bacterial Core Surveillance Report, Emerging Infections Program Network, group A *Streptococcus*, 2009). Therapies with penicillin and clindamycin are the most effective ways to treat noninvasive streptococcal infections. However, while treatment with antibiotics may prevent the development of invasive GAS diseases, antibiotics are not an effective treatment at the systemic stage (Young, Aronoff et al. 2005; Cole, Barnett et al. 2011). For patients with necrotizing fasciitis for example, early surgery is required to remove damaged tissues and to prevent the spread of bacteria within the body. The mortality rate of this disease is usually higher than 50% because of the multiple organ failure.

Development of recent vaccines against streptococcal diseases

There is still no method available to prevent GAS infections and to effectively treat invasive GAS episodes (Steer, Batzloff et al. 2009). These challenges underscore the needs for a GAS vaccine. Multiple vaccine candidates have been investigated in the past fifty years, including M-protein and other antigens. Immunization with M protein provides protection from clinical strains. However, M1 protein, the most prevalent serotype in invasive cases, is a potent inducer of T-cell proliferation and therefore leads to strong release of Th1-type cytokines. Thus, vaccination with M1 and possibly with other M serotypes suffers from the serious concern that it

may lead to excessive T-cell activation and harmful inflammatory responses (Påhlman, Olin et al. 2008). Moreover, some M serotypes, such as M18 are implicated in the production of the post streptococcal sequelae, acute rheumatic fever, which can lead to a rheumatic heart disease (Smoot, Barbian et al. 2002). It is suggested that rheumatic M types share antigenic epitope with host components and as a result trigger autoimmune response with severe inflammatory disease. Streptococcal autoimmune complications can lead to lasting damage to the joints and the heart and are suggested to be the cause of several neurological disorders (Snider and Swedo 2003). A 26-valent vaccine that is based on the variable N-terminus of M was recently developed. This vaccine that excludes previously identified cross reactive epitopes was found protective and safe in phase I and II clinical trials (Shulman, Tanz et al. 2009). However, a significant concern raised by M-based vaccines is the wide variety (over 200) of the different M serotypes, since the spectrum and prevalence of the M types are likely to change over time, especially in response to the pressure imposed by immunization with selected serotypes. A vaccine candidate that is based on an antigen that is conserved among GAS strains would be more attractive. Several GAS surface components and virulence factors have been tested for their ability to elicit immunity. These include the ScpA, GAS surface carbohydrates, several fibronectin-binding proteins, exotoxins and pili. While some antigens were protective against one or more GAS strains, none of them has reached clinical trials.

The streptococcal hemoprotein receptor (Shr)

The Shr protein is encoded by the first gene of streptococcal iron acquisition (*sia*) operon and its expression is regulated by iron via the metal-dependent repressor, MtsR (Bates, Toukoki et al. 2005; Toukoki, Gold et al. 2010). Shr is able to acquire heme from the environment and various

host hemoproteins. Shr ligands include hemoglobin, myoglobin, heme-albumin and hemoglobin-haptoglobin (Bates, Montanez et al. 2003; Ouattara, Bentley Cunha et al. 2010).

Shr harbors a leader peptide, two DUF1533 domains (domain of unknown function) two heme-binding NEAT (NEAr Transporter) domains that are separated by a leucine rich repeat (LRR) region. A short hydrophobic segment is located at the C-terminus of Shr. The DUF1533 domain is 60 amino acids long and it is typically found in several copies in bacterial proteins from several species of *Clostridium*, *Streptococcus* and a few related bacteria. The InterPro protein domain database suggests that many of the proteins that harbor one or more DUF1533 domains also contain a LRR region. This suggests that these domains cooperate for some functions. The NEAT domain is about 125 residues and is often found in multiple copies in secreted or surface exposed proteins in Gram-positive organisms. All of the characterized NEAT domains bind heme and/or hemoproteins. Most of the bacterial species producing NEAT proteins are important human pathogens, these include *Bacillus anthracis*, *L. monocytogenes*, *Clostridium perfringens*, *S. aureus* and GAS (Andrade, Ciccarelli et al. 2002). LRR regions typically contain repeats of 20-29 amino acid segments that are rich with leucine. They are mostly found in eukaryotic proteins with diverse functions and cellular locations. This LRR domain is suggested to mediate protein-protein interactions (Kobe and Kajava 2001). For instance the LRR domain of Internalin A interacts with E-cadherin and is sufficient for internalization of *L. monocytogenes* into epithelial cells (Lecuit, Ohayon et al. 1997; Schubert and Heinz 2003).

In this dissertation, we investigate the importance of Shr for GAS pathogenesis and study its promise as a vaccine target for GAS and related pathogens.

CHAPTER I

SHR IS A BROAD-SPECTRUM SURFACE RECEPTOR THAT CONTRIBUTES TO ADHERENCE AND VIRULENCE IN GAS

INTRODUCTION

The group A Streptococcus (GAS), also known as *Streptococcus pyogenes*, is a versatile human pathogen that colonizes the mucosal surfaces of the upper respiratory pathways and the epidermis (Cunningham 2000). GAS usually produces self-limited infections such as pharyngitis, impetigo and erysipelas. Infrequently, GAS also causes severe invasive infections in a variety of body sites, including bacteremia, necrotizing fasciitis and myositis. In addition to the immediate damage caused by primary GAS infections, some episodes lead to the development of Streptococcal Toxic Shock Syndrome (STSS) or to autoimmune complications such as glomerulonephritis and acute rheumatic fever, resulting in renal injury or inflammation of connective tissue respectively. Acute rheumatic fever can trigger additional manifestations in the form of rheumatic heart disease and the neurological syndrome Sydenham's Chorea (Snider and Swedo 2003). The wide spectrum of GAS-related illnesses makes it an intriguing pathogen and underscores the complexity of GAS interactions with the human tissues and immune response.

The initial, non-specific interaction of GAS with the host tissues is provided by lipoteichoic acid (LTA). This amphipathic surface molecule is proposed to counteract the electrostatic repulsion between the bacteria and the host surfaces. The subsequent step in adherence involves high-

affinity binding to host components and is mediated by a large number of adhesins (Bisno, Brito et al. 2003; Kreikemeyer, Klenk et al. 2004; Talay 2005). These include M proteins from several serotypes, F and F-like proteins, hyaluronic acid (HA) capsule and the recently described pili (Abbot, Smith et al. 2007; Manetti, Zingaretti et al. 2007). Some of the adhesins in GAS bind directly to molecules on the host cell surface. For example, M6 and the HA capsule bind to the keratinocyte receptors CD46 and CD44 respectively (Okada, Liszewski et al. 1995; Schrage, Alberti et al. 1998; Giannakis, Jokiranta et al. 2002), and the GAS collagen-like protein, Sc11, interacts with the $\alpha_2\beta_1$ integrin expressed by human epithelial cells (Caswell, Lukomska et al. 2007). More often, GAS adhesins attach the bacterium to the extracellular matrix (ECM) or to plasma components that in turn function as a bridge between the bacterium and receptors on the host cells. This mechanism of bacterial attachment is very common among pathogenic Gram-positive cocci and the bacterial proteins involved are called MSCRAMMs (Microbial Surface Components Recognizing Adhesive Matrix Molecules) (Patti, Allen et al. 1994). GAS expresses several surface molecules that bind collagen or laminin (Elsner, Kreikemeyer et al. 2002; Terao, Kawabata et al. 2002; Dinkla, Rohde et al. 2003; Kreikemeyer, Nakata et al. 2005). However, many more GAS adhesins target the ECM component fibronectin (Kreikemeyer, Klenk et al. 2004; Talay 2005). At least 11 fibronectin-binding proteins were identified in GAS thus far, including F1 (SfbI), F2 (PFBP), Fbp54, SOF, and several M proteins. While a few of these GAS proteins (such as Fbp54) are ubiquitous and encoded by most serotypes, others are found only in some or even in a single serotype. Moreover, most of the fibronectin-binding proteins in GAS are individually regulated and are expressed under unique growth conditions or in response to certain environmental stimuli (Kreikemeyer, McIver et al. 2003; Kreikemeyer, Klenk et al. 2004).

The production of infection by GAS and its survival in the host environment is dependent on its ability to acquire essential nutrients such as iron. GAS growth *in vitro* in iron-restricted medium is supported by whole blood, serum, myoglobin, hemoglobin, and hemoglobin-haptoglobin complex, but not by the ferric-carrier proteins transferrin and lactoferrin (Francis, Booth et al. 1985; Eichenbaum, Muller et al. 1996; Podbielski, Woischnik et al. 1999). Hence, heme, the most abundant iron form in mammals, serves as a major source of iron for this hemolytic pathogen. The *sia* is an iron-regulated operon in GAS involved in heme acquisition and transport. In addition to five genes with unknown function, the *sia* codes for *shr*, *shp*, and the *siaABC* (*htsABC*) genes (Lei, Smoot et al. 2002; Bates, Montanez et al. 2003). The SiaABC proteins are the components of an ABC type heme-transporter. Shp is a surface protein that was shown to acquire heme from hemoglobin and transfer it to apoSiaA (HtsA) while forming a stable complex with it (Liu and Lei 2005). Shr, encoded by the first gene in the *sia* operon, is a 145 kDa protein which binds myoglobin, hemoglobin, and hemoglobin-haptoglobin complexes (Bates, Montanez et al. 2003). Two NEAT (NEAr Transporter) domains are found in Shr (Fig. 2A) (Andrade, Ciccarelli et al. 2002). While their functional role is not well understood, NEAT domains appear to share a common immunoglobulin-like fold, and some of them were found to bind heme (Mazmanian, Skaar et al. 2003; Newton, Klebba et al. 2005; Maresso, Chapa et al. 2006; Grigg, Vermeiren et al. 2007) and/or heme-containing proteins (Mazmanian, Skaar et al. 2003; Clarke, Wiltshire et al. 2004; Torres, Pishchany et al. 2006; Dryla, Hoffmann et al. 2007). In this study, we explored Shr's function and investigated its contribution to GAS pathogenesis. We demonstrate here that in addition to its likely role in heme acquisition, Shr is a MSCRAMM that specifically binds to fibronectin and laminin, and mediates bacterial attachment. We report that Shr is expressed *in vivo* and is important for GAS virulence in a zebrafish infection model.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *E. coli* strains DH5 α and Top10 (Invitrogen) were used for cloning and gene expression. The clinical GAS (*S. pyogenes*) isolates used in this study were obtained from the Georgia Emerging Infections Program (GEIP) and are listed in Table 1. The other GAS strains used in this study were MGAS5005, an M type 1 strain (Sumbly, Porcella et al. 2005); JRS6, an M type 6 strain (Scott, Guenther et al. 1986); NZ131, an M type 49 strain (Simon and Ferretti 1991); ZE4912, a *shr* mutant constructed in NZ131 (Provided by Dr. Bernard Beall, Centers for Disease Control and Prevention, Respiratory Diseases Branch, Atlanta, GA. Fig 3). The *shr* mutation in ZE4912 is a deletion-insertion mutation made by replacing an internal 0.3-kb *Bgl*III fragment with the spectinomycin resistance gene, *aad9* (personal communication, Dr. Bernard Beall). We confirmed the structure of the *shr* mutation in ZE4912 genome, by sequence analysis of a DNA fragment carrying the mutant *shr* allele amplified from ZE4912 chromosome, with ZE245 (5'-GTGCCACAAAACCAAGGCACAC-3') and ZE246 (5'-CAGTCGATGAGTATCGGCGAG-3') primers. The *Lactococcus lactis* strain MG1363, was used as a heterologous host for the expression of the native Shr protein from plasmid pXL14. *E. coli* cells were grown in Luria-Bertani broth with agitation. GAS was grown statically in Todd-Hewitt broth with 0.2% w/v yeast extract (THY, Difco Laboratories). *L. lactis* was grown statically at 30°C, in M17 (Difco Laboratories) supplemented with 0.5% w/v glucose. When necessary, spectinomycin at 100 μ g/ml or kanamycin at 70 μ g/ml was added to the medium.

Nucleic acid methods. Chromosomal and plasmid DNA extraction, DNA manipulations including restriction digests, cloning, and DNA transformation into *E. coli*, GAS, or *L. lactis* were done according to the manufacturer's recommendations and with standard protocols as previously described (Sambrook 1989; Eichenbaum, Federle et al. 1998). For RNA extraction and analysis, GAS cells were harvested at the logarithmic growth phase and total RNA was prepared using the RiboPure-Bacteria Kit (Ambion). RNA was quantified spectrophotometrically, and its integrity was examined by agarose gel electrophoresis. The absence of DNA contamination was verified by PCR. cDNA was produced by Superscript III reverse transcriptase (Invitrogen) according to the manufacturer's specification. One microgram of RNA was used in the reverse transcription step, and 1/20 of the reaction served as a template for 25 cycles of PCR. The primers SRAR (5'-CTGATGCTACTGCCATAGCAG-3') and SRAL (5'-GCGTTCAGGAGGTCTAGCTC-3') were used in the analysis of the *recA* gene and the primers 204A-Fwd (5'-GCTATGATGCTGTTAAGCGTGTGG-3') and 204A-Rev (5'-TCTGGAATGGCATGAGCTGTTC-3') were used in the analysis of *siaA* transcription.

Construction of the *shr* complemented strain ZE4924. To complement the *shr* mutation in ZE4912 we cloned the native *shr* gene into the temperature sensitive plasmid pJRS700 (Montanez, Neely et al. 2005). A 4.5-kb fragment amplified from NZ131 chromosome with the primers ZE170 (5'-TTTTTTATCGATTTAGCTCTTGCTGACTAG-3') and ZE174 (5'-TTTTTTATCGATTATGCAGTAGTGACATCTC-3') was cloned into the *Cla*I site of pJRS700 generating plasmid pXL14. Since ZE4912 cells harboring pXL14 did not grow well at 30°C (possibly due to the high gene dosage of *shr* carried by pXL14), we constructed a merodiploid strain (ZE4924), which contains both the mutant and the wild type alleles of *shr* in the

chromosome. The strain ZE4924 was made in ZE4912 by Campbell insertion. Plasmid pXL14 was introduced into ZE4912 cells. Clones in which pXL14 was integrated into the chromosome (by homologous recombination between the mutant *shr* allele in the chromosome and the wild type copy on pXL14) were selected on kanamycin plates at 37°C (the non-permissive temperature). The resulting chromosomal structure was verified by PCR analysis.

Antibodies. Rabbit polyclonal antibodies against Shr and rabbit anti-SiaA serum have been previously described (Bates, Montanez et al. 2003). Rabbit polyclonal antibodies against M protein type 49 (a killed whole-cell vaccine that was absorbed extensively to produce M49 specific antiserum) were provided by Dr. Bernard Beall (Centers for Disease Control and Prevention, Respiratory Diseases Branch, Atlanta, GA). Rabbit IgG against GAS type-specific carbohydrates was purchased from Abcam, Inc. Rabbit antiserum for the metal dependent repressor MtsR (Bates, Toukoki et al. 2005) was raised against purified MtsR using the same method employed for the production the Shr antiserum (Bates, Montanez et al. 2003). Western blot analysis determined that the MtsR antiserum specifically recognize purified MtsR protein and reacted with protein band at the correct size in protein extract from the wild type NZ131 strain but not from the *mtsR* mutant ZE491 (Fig. 3). Mouse antiserum against *Lactococcus lactis* surface components was raised by intraperitoneal injection with 10^8 CFU of MG1363 cells. A booster injection was given 10 days after the first injection. Antiserum was collected 10 days after the booster.



Figure 3. MtsR antiserum specifically recognizes MtsR protein.

Equal amount of total cell lysate of GAS NZ131 (wild type), ZE4912 (shr^-) and ZE491($mtsR^-$) were analyzed by SDS-PAGE, followed by Western blot with rabbit anti-MtsR. Purified MtsR protein is the positive control.

Preparation of GAS cell fractions. GAS cell fractionation was done as previously described with minor modifications (Pancholi and Fischetti 1992). Essentially, GAS was grown overnight in THY broth containing 20 mM glycine. Cells from 14 ml cultures at $O.D_{600} = 1.0$ were harvested, washed with phosphate buffered saline (PBS), and resuspended in 0.5 ml of either TEA (10 mM Tris-HCl buffer pH 8.0, 1 mM EDTA, and 0.75 $\mu\text{g}/\mu\text{l}$ of the protease inhibitor AEBSF, Roche) for the preparation of total cellular proteins, or in TEAR (TEA with 30% raffinose) for cellular fractionation. The cell suspensions were treated with the muralytic enzyme mutanolysin (500U) for 1 h at 37°C to dissolve the cell wall. For total protein extract, the mutanolysin treated cells were boiled in sample buffer for 10 min. For cell fractionation, the protoplasts were separated from the cell surface fraction by centrifugation, resuspended in TEA, and lysed by successive cycles of freezing (-80°C) and thawing (37°C). Membrane proteins were separated from the soluble intracellular proteins by centrifugation and were dissolved by boiling in sample buffer. The protein extracts were standardized with respect to the cell number in the corresponding culture, and were separated on a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Shr, M protein type 49 (M49), or SiaA were detected by Western blot as described previously (Bates, Montanez et al. 2003).

Purification of rShr (His₆-Xpress-Shr). The recombinant Shr was purified from the *E. coli* cells harboring plasmid pCB1 as previously described (Bates, Montanez et al. 2003) with the following modifications: the cells were resuspended in 20 mM Tris pH 8.0, 100 mM NaCl, 0.1% Triton X-100, 0.5 mg/ml lysozyme, and a protease inhibitor cocktail (Complete Mini, EDTA-free, Roche), and incubated on ice for 30 min. The bacteria were then lysed by sonication, and the rShr was purified over a nickel column (HisTrap HP, GE Healthcare) and subsequently

desalted by HiTrap Desalting column (GE Healthcare). The purified protein was examined by SDS-PAGE and Western blot analysis with anti-Shr antibodies, and quantified by the Bradford assay (Bio-Rad).

Enzyme-linked Immunosorbant assays (ELISA). Three types of ELISA assay were used in the binding experiments. *ELISA with immobilized bacteria.* This assay was preformed to study the availability of Shr on the bacterial cell surface. GAS or *L. lactis* cells from overnight cultures were harvested, washed with phosphate buffered saline (PBS), and used to coat 96-well EIA/RIA microplates (Costar, Corning Inc.) overnight at 4°C. The supernatant was then aspirated and the plates were washed with PBST (PBS containing 0.05% Tween 20). After blocking with PBST and 5% skim milk for 1 h at 37°C, the wells were washed with PBST and incubated with the primary antibody for 1h at 37°C. The microplates were next washed with PBST and incubated with secondary antibodies conjugated to alkaline phosphatase (AP) for 1h at 37°C. The reaction was developed using *p*-nitrophenyl phosphate system (KPN) and the absorbance was read at 405 nm. *Elisa with immobilized ligands and purified Shr protein.* This assay was used to investigate binding of Shr to host proteins. The microplates were coated with the ligands (25µg/ml), washed, and blocked as described above. Purified Shr (in PBST-1% BSA) was added to the coated wells in increasing concentrations. After 1 h incubation at 37 °C, Shr antiserum (diluted in PBST-1% BSA) was added to the wells, and the plates were incubated for 1 h at 37 °C. The wells were then washed with PBST, and binding was detected with AP-conjugated secondary antibody as described above.

Analysis of *L. lactis* cell binding to immobilized ligands by ELISA. The microplates were coated with the ligands (25µg/ml), washed, and blocked as described above. Samples of *L. lactis* cultures were harvested, washed with equal volume of PBS, diluted into the desired concentrations in PBST-1% BSA, and applied to the wells. Following 1 h incubation at 37 °C, the plates were washed with PBST, and reacted with the *L. lactis* antiserum. Binding was detected with secondary AP-conjugated antibody as described above.

Detection of anti-Shr antibodies in convalescent mice. Mice were injected subcutaneously with 2×10^7 CFU of the wild-type strain MGAS5005 as previously described (Schrager, Rheinwald et al. 1996) and were monitored for 27 days. The infected mice developed subcutaneous lesions, which healed over time in the surviving animals (~80% of the injected mice). On the 28th day post inoculation, the surviving mice were bled by cardiac punctation and the serum was obtained by centrifugation. ELISA assay was used to determine the presence and the titer of anti Shr antibodies in the mice sera. 96-well EIA/RIA microplates (Costar, Corning Inc.) were coated with 1µg/ml purified Shr. Following overnight coating at 4°C, the plates were washed with PBST (PBS containing 0.05% v/v Tween 20), and blocked with 1% w/v BSA in PBST for 1 h at 37°C. The plates were then washed with PBST and incubated for 1h at 37°C with mouse sera at different dilutions. Following incubation and washing, AP-conjugated anti-mouse antibodies diluted 1:1000 in blocking buffer, was added to the plates and incubated for 1h at 37°C. Bound antibodies were detected by using *p*-nitrophenyl phosphate system (KPN). Plate contents were incubated at room temperature for 30 min and read at 405 nm.

HEp-2 cell culture and adherence assay. HEp-2 cells were cultured in 24-well tissue culture plates with nutrient mixture medium (RPMI) supplemented with 10% fetal bovine serum at 37°C in an atmosphere with 5% CO₂. For adherence assays, cells grown to a semi confluent state (~10⁵ cells/well) with antibiotics (2% Penicillin-Streptomycin, Thermo Scientific Hyclon Antibiotics) were washed with PBS and inoculated with GAS cells (10⁷ CFU/well) harvested at the mid-log phase (OD₆₀₀ = 0.3-0.4), washed with PBS, and resuspended in RPMI medium. The bacteria were incubated with the tissue cultures for 2 h at 37 °C; at that time, non-adherent bacteria were removed by successive washes with PBS. To determine the number of adherent bacteria, the HEp-2 cells were detached with 100µl of 0.05% trypsin for 5 min at 37 °C and lysed with 400 µl per well of 0.025% Triton X-100. The bacterial number in each sample was determined by plating on THY agar plates. Each adherence experiment was performed in triplicate or quadruplicate.

Zebrafish care and virulence assays. Care and feeding of zebrafish (*Danio rerio*) followed published methods (Westerfield 1995; Neely, Pfeifer et al. 2002). Streptococci were cultured overnight in THY with 2% (w/v) peptone (THYP) at 37 °C, diluted 1:100 in THYP the next day, and incubated at 37°C. The cells were harvested at OD₆₀₀ = 0.3, washed once with THYP, and diluted to an appropriate concentration in fresh THYP. Streptococcal cells (10µl) were aseptically injected into groups of six anaesthetized male breeder zebrafish (Scientific Hatcheries). Following intramuscular (i.m.) infection, the fish were allowed to recover in 225 ml sterilized ddH₂O supplemented with aquarium salts (Instant Ocean; Aquarium Systems) at a concentration of 60 mg/l in a 25 °C incubator. Infected fish were monitored for 72 h and death was recorded in intervals of 12 h. The 50% lethal dose (LD₅₀) for infection was determined by

Reed-Muench method as previously described (Neely, Pfeifer et al. 2002) where zebrafish were challenged over a range of 10^3 - 10^7 CFU of each of the streptococcal strains.

Competitive assay in zebrafish model. The competitive index was defined as the change in the population ratio of two strains after growing in zebrafish muscle. NZ131 (wild type) and the mutant strain (ZE4912) were cultured separately as described above. Cells from each strain were mixed in a 1:1 ratio to a final concentration 10^8 CFU/ml. Zebrafish were infected i.m. with $10\mu\text{l}$ of this mixture, resulting in an infectious dose of 10^6 CFU. After 24 h, muscle lesions were collected from euthanized fish and homogenized in PBS. The homogenates were plated on THY and THY-spectinomycin plates, and the ratio of mutant to wild type strain in the lesion was determined.

Statistical analysis. Student's *t*-test was used to compare data sets derived from two groups to each other; a *P*-value ≤ 0.05 was considered significant. ANOVA analysis followed by the Tukey's HSD post hoc test were used for multigroup comparisons; a *P*-value ≤ 0.05 was considered significant. Zebrafish survival data were analyzed by the method of Reed and Muench for the calculation of the LD_{50} . Kaplan-Meier plots of zebrafish survival were used to compare infection by the wild type, mutant and complemented GAS strains. The statistical significance was evaluated by the log rank test (Glantz 2005).

RESULTS

Shr is attached to the GAS cell membrane and is exposed to the extracellular environment.

Shr is a large (1,275 amino acids) protein found in both the culture supernatant and in association with whole cells (Bates, Montanez et al. 2003). It has a functional leader peptide, and at its carboxy terminus it has a putative transmembrane domain followed by a positively charged tail (Fig. 4A). However, unlike proteins anchored to the surface of Gram-positive bacteria, Shr lacks a cell wall anchoring motif at its C terminus (Scott and Barnett 2006) suggesting that after it is exported to the cell surface, Shr may be bound to the cell membrane and not anchored to the cell wall. To determine the cellular location of Shr, GAS strain NZ131 (M type 49) was fractionated following digestion with the muralytic enzyme mutanolysin and examined by Western blot analysis. The Shr protein (at 145 kD) was not released by this process but was found instead in the membrane fraction (Fig. 4B). As a control, the cell wall protein M49, represented by the monomer at 37-40 kD (as predicted from the genome sequence) and the typical range of slower-migrating bands containing cell wall fragments (Fischetti, Jones et al. 1985) was found exclusively in the cell wall fraction, as expected (Fig. 4C). Therefore, Shr appears to remain associated with the cell membrane, probably by the transmembrane domain in its carboxy terminus.

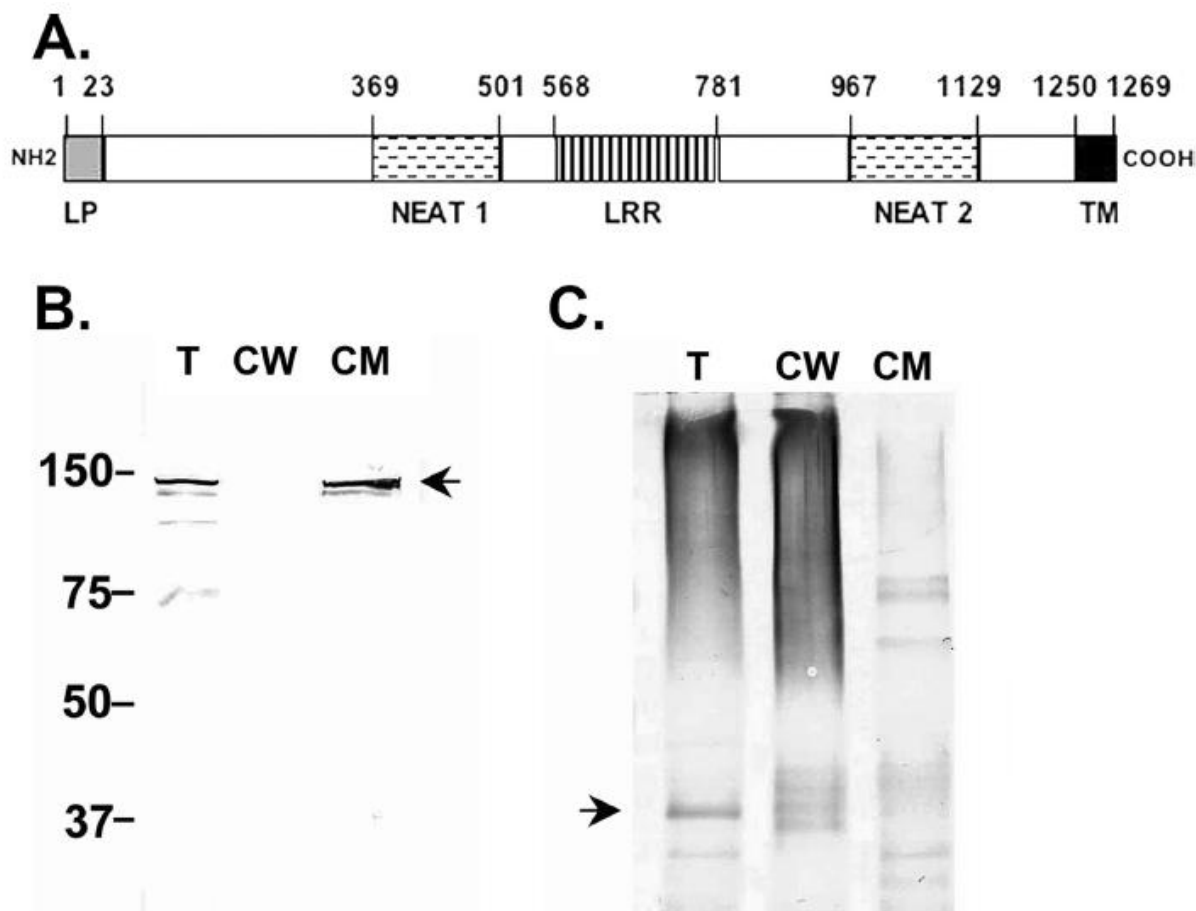


Figure 4. Shr protein domains and cellular location.

(A) Schematic representation of Shr protein. The SMART algorithm (<http://smart.embl-heidelberg.de>) was used for structural analysis of Shr. The location of protein domains (expressed as amino acid number) is shown. LP, leader peptide; NEAT 1, NEAT domain 1; LRR, Leucine rich repeats; NEAT 2, NEAT domain 2; TM, transmembrane domain. (B and C) Proteins prepared from NZ131 cells grown in THYB were analyzed by Western blotting using anti-Shr antibodies (B) or anti-M49 antibodies (C). T, total protein; CW, cell wall fraction; CM, cell membrane fraction. Full length Shr and M49 are indicated by the arrows.

The cellular location of Shr was further studied in an *shr* deletion-insertion mutant (ZE4912, Fig. 5A) and the mutant strain complemented with *shr* expressed from its own promoter in the chromosome (ZE4924, Materials and Methods). To examine the effect of *shr* mutation and of its complementation on the expression from the *sia* operon, we performed RT-PCR and Western blot analysis on RNA and proteins extracted from the isogenic strains NZ131, ZE4912, and ZE4924. As can be seen in Fig. 5, the transcription of *siaA* gene (Fig. 5B) and the production of this protein (Fig. 5C) were not significantly altered in either the mutant or in the complementation strain in comparison to the parent strain. Therefore, the mutation in *shr* does not seem to be polar on the downstream genes in the *sia* operon. On the other hand, cell fractionation and Western blot analysis showed that the *shr* mutation in ZE4912 resulted in the loss of Shr from the membrane fraction (Fig. 5D, panel 2) and in the formation of a truncated Shr fragment (25-kDa) that was secreted to the culture supernatant (data not shown). Complementation of *shr* in ZE4924 strain restored the presence of Shr protein in the cell membrane fraction (Fig. 5D, panel 3). These observations are consistent with the suggestion that the carboxy terminus of Shr is required for Shr association with the cell membrane, and with our previous observation that Shr has a leader peptide that can target its secretion.

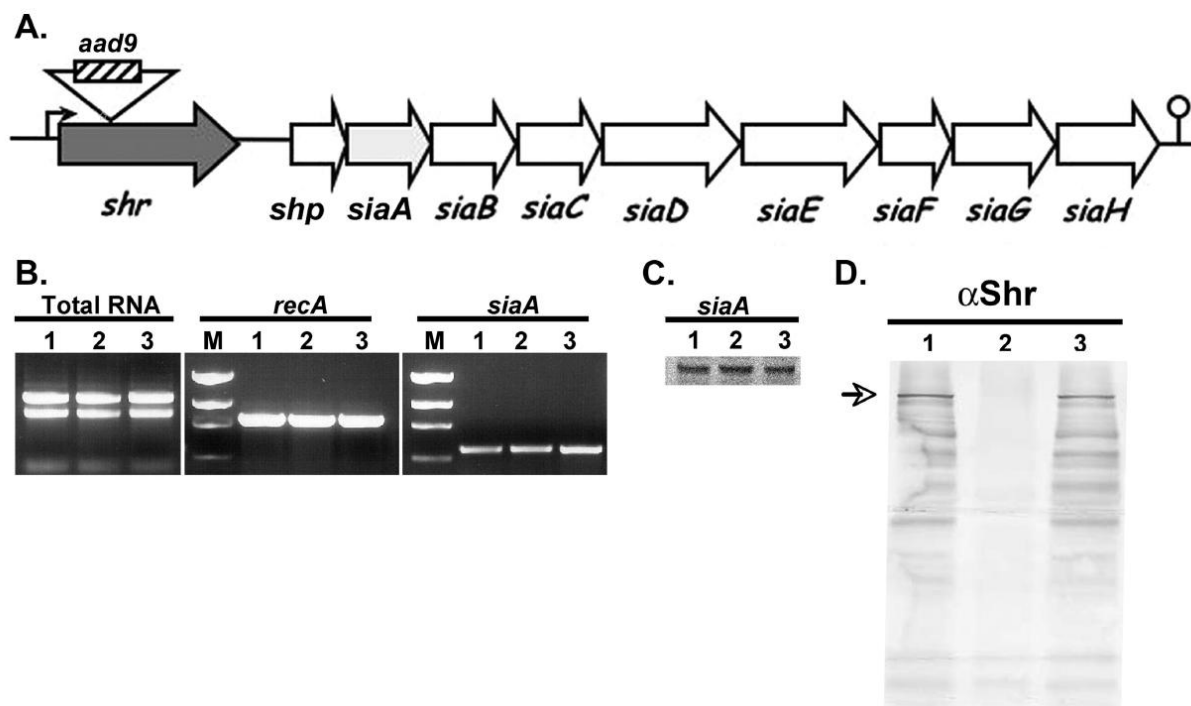


Figure 5. Successful inactivation of *shr* in strain ZE4912 and mutant complementation in strain ZE4924.

(A) Schematic representation of the *sia* operon and the *shr* mutation in ZE4912. The *shr* mutation in strain ZE4912, consists of a small deletion and an insertion of the spectinomycin resistance gene *aad9*. ZE4924 strain is a merodiploid, which carries both the mutant and the wild type alleles of *shr* in the chromosome (see Materials and Methods). (B to D) RNA and proteins from the wild type NZ131 (1); the *shr* mutant ZE4912 (2); and the *shr* complemented strain ZE4924 (3) were prepared and analyzed. (B) Total RNA was extracted, loaded (1 μ g/well) on agarose gel, and examined for integrity. Reverse transcription-PCR products obtained with *recA* specific primers (used to control for equal amounts of RNA template) or with *siaA*-specific primers were analyzed by agarose gel electrophoresis. (C) Total proteins were prepared, and analysed by Western blot with anti-SiaA serum. (D) Proteins from the membrane fraction were analyzed by Western blotting with anti-Shr serum. Full length Shr is indicated by the arrow. (work (B) and (C) were performed by X. Li (Fisher, Huang et al. 2008)).

To test whether the membrane-bound Shr is exposed on the cell exterior or is buried within the peptidoglycan layer, we conducted whole-cell ELISA using Shr antibodies. GAS cells grown to stationary phase were used to coat ELISA plates and reacted with anti-Shr serum. An antiserum against the repressor protein MtsR (Bates, Toukoki et al. 2005) was used as a control for intracellular proteins, and normal rabbit serum (NRS) was used as a control for any non-specific reaction. As shown in Fig. 6, the Shr antiserum (black bars) reacted with the wild type (NZ131) and with the complementation strain (ZE4924). The specificity of the antiserum for Shr was demonstrated by its lack of reaction with *shr* mutant strain (ZE4912). In all tested strains, no significant reactivity was obtained with the MtsR antiserum or the NRS (stripes and white bars respectively). As expected, the positive control antibodies, which specifically recognize GAS type-specific carbohydrates, reacted with all of the GAS strains examined (gray bars). Similarly to its interaction with NZ131 whole cells, Shr antiserum specifically recognized cells of JRS4 (M type 6) and MGAS5005 (M type 1) strains (data not shown). These observations suggest that Shr is exposed on the surface and accessible for interactions with the host extracellular environment.

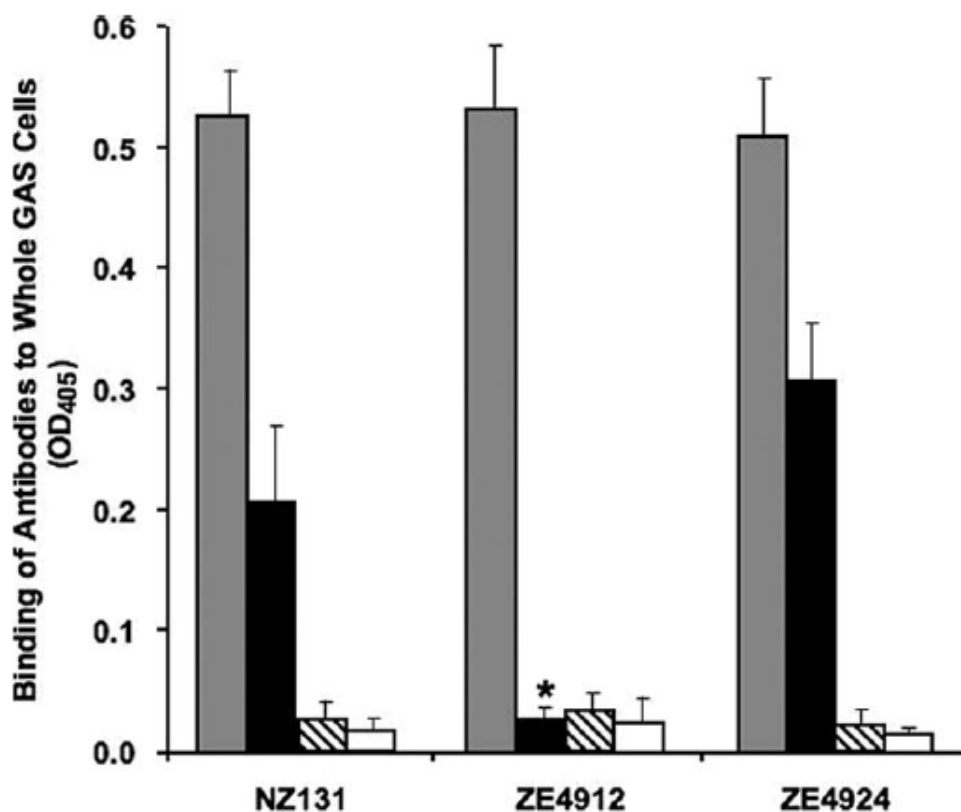


Figure 6. Shr is exposed on the surface of GAS.

Data are from ELISA with GAS cells immobilized onto microtiter plates. Wells coated with cells from the isogenic series NZ131, ZE4912, and ZE4924, were reacted with antibodies recognizing GAS type-specific carbohydrates (gray bar), Shr antiserum (black bar), or control antibodies MtsR antiserum (strips bar) or normal rabbit serum (white bar) as described in Materials and Methods. Antibody binding to GAS surface antigens was detected by anti-rabbit AP-conjugated secondary antibodies. The reaction was developed and measured at 405 nm. Each bar represents the average of at least three repeats, and the standard deviation (SD) is represented by the error bars. The significance of the difference in binding of anti-Shr serum to the three strains was examined by ANOVA ($P = 0.001$), followed by the Tukey's HSD test for each strain pair. The asterisk indicates that the difference between the strains is significant ($P < 0.01$ for each strain pair).

Shr is an MSCRAMM. The surface localization and exposure of Shr raised the possibility that it may function in bacterial adherence in addition to iron uptake. We therefore examined the interactions of Shr with ECM components, using ELISA assays. Different ligands immobilized onto microtiter plates were reacted with increasing concentrations of purified Shr. Shr binding was detected using Shr antiserum (Fig. 7). As control for non-specific interactions, wells coated with transferrin, fibronectin, laminin, or goat IgG, were incubated with the Shr antiserum directly. Only low background binding ($OD_{405} = 0.1 \pm 0.01$, Fig. 7, and data not shown) of the Shr antiserum to the control wells was observed demonstrating the specificity of the Shr antibodies. Although Shr did not bind to transferrin (Fig. 7, diamonds) and goat IgG (data not shown), Shr bound to immobilized fibronectin (Fig. 7, triangles) and laminin (Fig. 7, squares) in a dose-dependent and saturable manner.

Inhibition experiments demonstrated the specificity of binding of Shr to the ECM ligands fibronectin and laminin. When Shr was allowed to interact with immobilized fibronectin in the presence of increasing concentrations of soluble fibronectin, binding of Shr to the plates was inhibited in a dose dependent manner (Fig. 8A, triangles). On the other hand, transferrin, which was not recognized by Shr *in vitro*, could not compete with immobilized fibronectin for binding to Shr (Fig.8A diamonds). Similarly, soluble laminin inhibited binding of Shr to wells coated with laminin (Fig. 8B). Laminin also competed with fibronectin for Shr binding (up to 40% inhibition, Fig. 8A, squares). However, at similar molar concentrations, laminin was less effective at competition than was soluble fibronectin (80% inhibition). This demonstrates that binding of Shr is specific to a subset of ECM components.

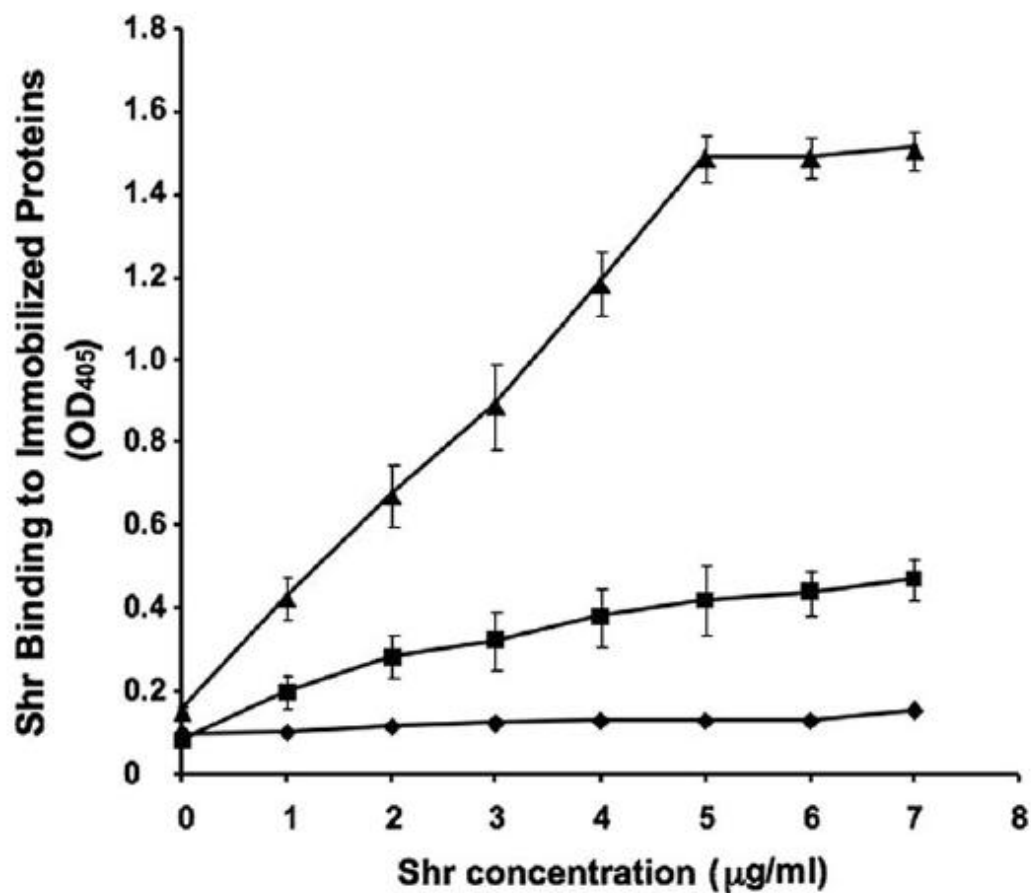


Figure 7. Shr binds *in vitro* to ECM components.

The Shr protein was added in increasing concentrations to microtiter plate wells coated with fibronectin (triangles), laminin (squares), or transferrin (diamonds) and incubated for 1 hour at 37°C. Bound Shr was detected by anti-Shr antibodies and anti-rabbit AP-conjugated secondary antibodies. Each datum point stands for the mean \pm SD (represented by the error bar) from four independent experiments done in duplicates.

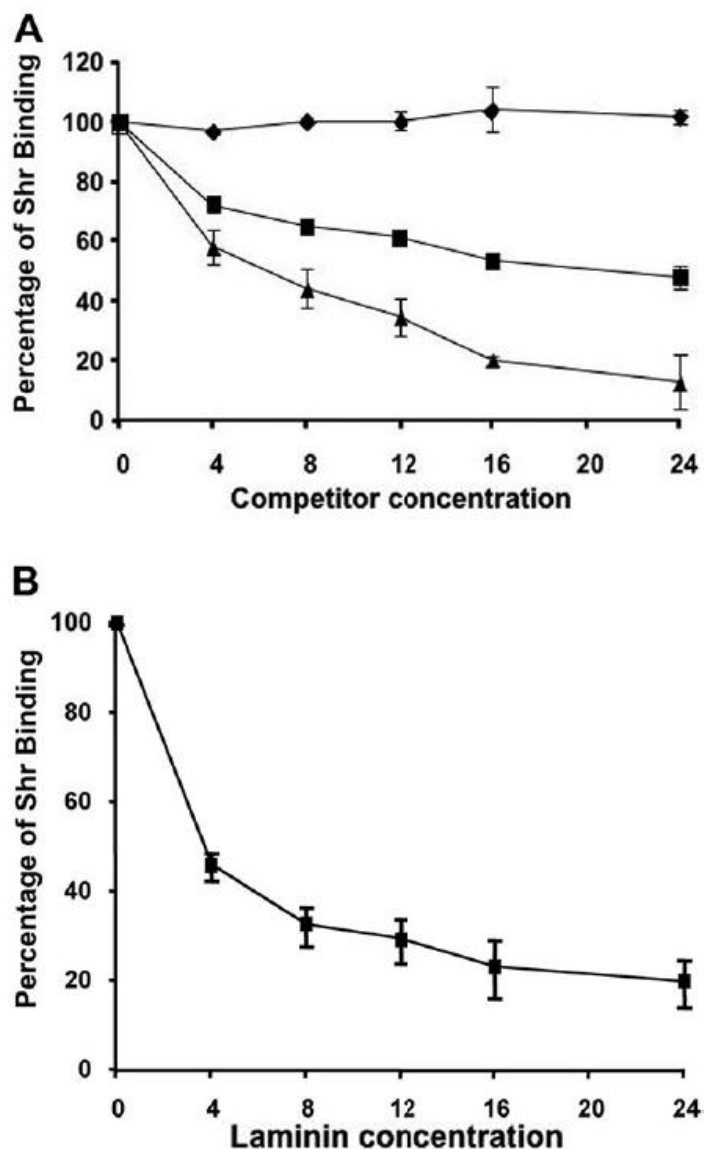


Figure 8. Inhibition of Shr binding to immobilized ECM ligands.

(A) Binding of purified Shr to immobilized fibronectin as a function of the competitor concentration. Shr (3.5 nM) was pre-incubated for 1.5 h at room temperature with increasing concentrations of fibronectin (triangles), laminin, (squares), or transferrin (diamonds) prior to its addition to fibronectin-coated wells. Bound Shr was detected with anti-Shr and anti-rabbit AP-conjugated secondary antibodies. (B) Binding of Shr to immobilized laminin as a function of soluble laminin concentration. Data are the same as in A except that 3.5 nM of Shr was pre-incubated with increasing concentrations of laminin before incubation in the laminin-coated wells. In both A and B, each datum point represents the mean of six independent experiments done in duplicate, and the standard deviation is represented by the error bars. The competitor amount is expressed as a multiple of the molar concentration Shr.

GAS expresses multiple fibronectin binding proteins on its surface (Kreikemeyer, Klenk et al. 2004; Talay 2005). To test the ability of Shr to mediate bacterial binding to fibronectin in the absence of the other GAS fibronectin-binding proteins, we expressed Shr in a heterologous host. Plasmid pXL14, which carries the native *shr* gene, was introduced into *L. lactis* MG1363 strain. The production of Shr protein in *Lactococcus* was confirmed by Western blot analysis (data not shown), and the presentation of Shr on the cell surface was tested by ELISA. Microtiter plates were coated with cells of the native *L. lactis* strain MG1363 or the recombinant strain that harbors pXL14 (MG1363/pXL14). The coated wells were reacted with Shr antiserum (Fig. 9A, black bars), *L. lactis* antibodies (Fig. 9A, gray bars), or NRS (Fig. 9A, white bars). The Shr antiserum reacted significantly ($P = 0.0037$) only with the bacteria that harbor the *shr* plasmid (pXL14), demonstrating specificity for Shr. As expected, the *L. lactis* antibodies interacted strongly and similarly with both MG1363 and MG1363/pXL14 strains, and only weak binding of the NRS to both strains was observed. Therefore, like in its native host, Shr protein produced in *L. lactis* is exposed on the bacterial cell surface.

Binding of the recombinant *L. lactis* cells expressing Shr to fibronectin and to transferrin was investigated. The two ligands were immobilized onto microtiter plates and the coated wells were incubated with bacteria. Binding of *Lactococcus* to the immobilized ligands was detected using the *L. lactis* antiserum. Only weak binding of MG1363 strain to fibronectin (Fig. 9B, black bars), transferrin (Fig. 9B, white bars), or uncoated wells (data not shown) was observed, demonstrating that *L. lactis* does not interact strongly with fibronectin or transferrin. In contrast, MG1363 cells harboring plasmid pXL14 demonstrated significant ($P = 0.001$) binding to immobilized fibronectin (Fig. 9B, black bars); these cells, however, did not bind to transferrin

(Fig. 9B, white bars). Therefore, *L. lactis* cells expressing Shr gained the ability to specifically bind to fibronectin. This observation demonstrates that Shr can mediate cell binding to the ECM and supports its possible role as bacterial adhesin.

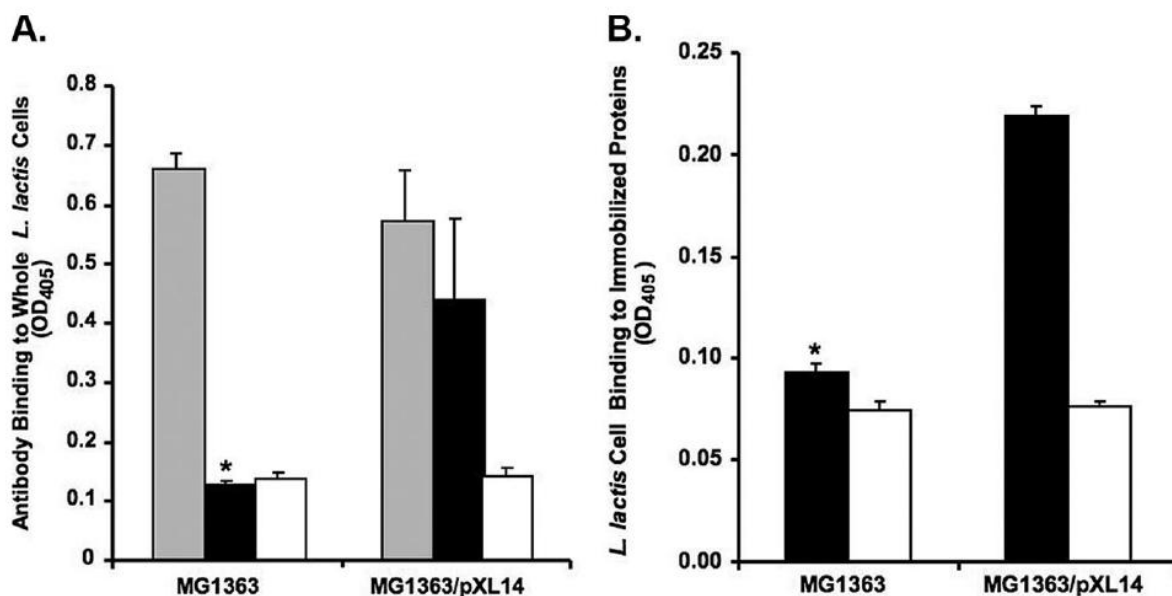


Figure 9. Shr expressed on *L. lactis* cell surface promotes bacterial binding to fibronectin.

Cells of the wild type *Lactococcal* strain MG1363 and of a recombinant MG1363 strain harboring plasmid pXL14 (MG1363/pXL14) were tested for Shr surface presentation and cell binding to different ligands. **(A)** ELISA assay with immobilized *L. lactis* cells and anti-Shr serum. Wells coated with *L. lactis* cells were reacted with anti-*L. lactis* serum (gray bar), anti-Shr serum (black bar), or with normal rabbit serum (white bar) as described in Materials and Methods. **(B)** Analysis of *L. lactis* cell binding to immobilized fibronectin. *L. lactis* cells were reacted with wells coated with fibronectin (black bar) or transferrin (white bar) as described in Materials and Methods. Bound bacteria were detected with anti-*L. lactis* serum. Antibody binding was detected with secondary AP-conjugated antibodies. Each bar represents the average of at least four repeats, and the SD is represented by the error bars. The asterisks indicate statistical significance for the differences between MG1363 and MG1363/pXL14 in Shr-antiserum binding (A, $P = 0.0037$) and in binding to immobilized fibronectin (B, $P = 0.001$).

To test the function of Shr in adherence of whole GAS cells directly, the *shr* mutant (ZE4912) was compared to its wild type parent (NZ131) as well as to the mutant complemented with the *shr* gene (ZE4924). Binding of GAS to HEp-2 cells was assayed following incubation at 37°C for 2 h. In comparison to the wild type strain, the *shr* mutant demonstrated about 40% reduction in adherence (Fig. 10). This difference is statistically significant ($P < 0.05$). Although the complemented strain bound to the HEp-2 cells better than the mutant, it only bound about 75% as well as the wild type. The difference in binding between the complemented strain and either the wild type or the *shr* mutant was not statistically significant. Thus, although complementation was not complete, Shr seems to act as an adhesin in GAS attachment to HEp-2 cells.

Shr production and surface presentation is conserved among clinical isolates. A collection of 17 clinical isolates representing seven different M serotypes was examined for Shr expression using whole cell ELISA. As summarized in Table 1, Shr antiserum reacted with most of the isolates (14 out of 17) and produced a signal that was at least 2 fold higher than the background signal produced with the control serum NRS. A significant variation in the strength of the produced signal was found among the different clinical isolates, however. This range of reaction may result from differences in the amount of Shr protein produced by each strain or may be due to antigenic differences in Shr and subsequently weaker interaction with the Shr antiserum. Shr sequence is very conserved among GAS genomes available in the database, however the sequence variability may increase among clinical isolates. In summary, Shr protein was present on the surface in 17 out of the 20 strains tested in this study (including NZ131, MGS5005 and JRS4), suggesting that this protein is important for the GAS infection process.

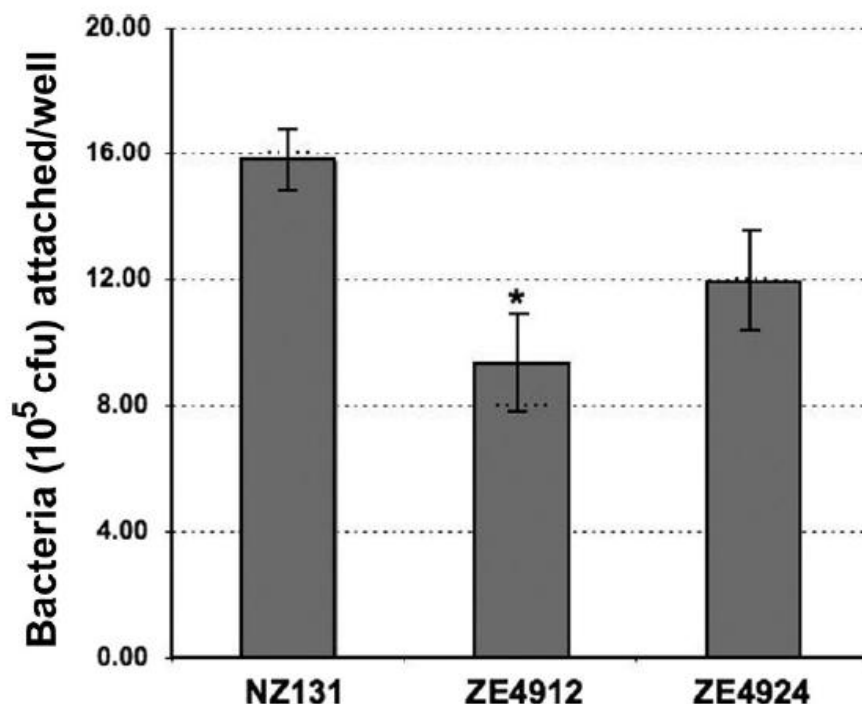


Figure 10. Adherence of GAS to cultured epithelial cells.

Mid-log phase cultures of wild-type strain (NZ131), *shr* mutant (ZE4912), and the complemented strain (ZE4924) were incubated with confluent HEp-2 cell monolayers for 2 h at 37 °C under 5% CO₂. Unattached bacteria were removed by washing, cells were detached and lysed, and the number of bound bacteria per well was determined by plating. Each bar represents the mean number of GAS cells attached as obtained from at least two independent experiments performed in triplicate. The standard error of the mean (SEM) is expressed by the error bar. The significance of the difference in adherence between the three strains was examined by ANOVA ($P = 0.025$) followed by the Tukey's HSD test for each strain pair. The asterisk indicates that the difference between NZ131 and ZE4912 is statistically significant ($P < 0.05$).

Table 1. Analysis of Shr expression on the surface of GAS clinical isolates.

Strain ^a	<i>emm</i> type ^b	Mean OD ₄₀₅ ± SD ^c		Shr/NRS ratio
		NRS	Shr	
GA04425 (M1)	01	0.03 ± 0.001	0.08 ± 0.025	2.7
GA03420 (M3)	NT	0.09 ± 0.001	0.18 ± 0.014	2.0
GA05099 (M43)	NT	0.13 ± 0.004	0.20 ± 0.001	1.5
GA06439	114	0.04 ± 0.001	0.05 ± 0.005	1.2
GA02581 (M1)	01	0.03 ± 0.006	0.08 ± 0.002	2.7
GA10156	75	0.02 ± 0.006	0.63 ± 0.008	31.5
GA05982	01	0.03 ± 0.004	0.05 ± 0.001	1.7
GA03198	11	0.07 ± 0.012	0.32 ± 0.001	4.6
GA10158 (M3)	03	0.03 ± 0.010	2.09 ± 0.071	69.7
GA10878	18	0.06 ± 0.008	0.88 ± 0.003	14.7
GA08067	12	0.08 ± 0.006	0.42 ± 0.015	5.2
GA10149	01	0.02 ± 0.001	0.10 ± 0.008	5.0
GA03971	59	0.01 ± 0.001	1.08 ± 0.016	108
GA03877	82	0.05 ± 0.001	0.16 ± 0.007	3.2
GA07775	04	0.14 ± 0.010	0.30 ± 0.013	2.1
GA0734	12	0.04 ± 0.008	0.87 ± 0.007	21.7
GA07346	22	0.05 ± 0.006	0.12 ± 0.003	2.4

^a Clinical strains obtained from the Georgia Emerging Infections Program. The M type (if available) is indicated in parentheses.

^b NT, not typed.

^c Immobilized GAS cells were reacted with NRS or with anti-Shr serum; antibody binding was detected with AP-conjugated secondary antibody.

(work was performed by M. Fisher (Fisher, Huang et al. 2008)).

Shr is expressed by GAS *in vivo* and elicits an immune response in mice. In cells growing *in vitro* in the laboratory, *shr* expression is regulated by the availability of metal in the medium (Bates, Toukoki et al. 2005). To determine if the conditions *in vivo* during GAS infection in a murine model allow the expression of Shr, we asked if a challenge with GAS leads to the production of Shr antibodies in mice. Mice were injected subcutaneously with strain MGAS5005 (M type 1) and sera collected 28 days after inoculation were assayed by ELISA for Shr antibody titer. Purified Shr was used to coat microtiter plates, which were then reacted with the mouse sera (Fig. 11). Sera from mice injected with GAS reacted strongly with purified Shr in most of the tested animals (9 out of 14), whereas none of the sera obtained from naïve mice were reactive against Shr. The conversion of most mice to Shr positive demonstrates that Shr is immunogenic, and that in most of the mice, it was produced during infection in sufficient amounts and duration to elicit an immune response.

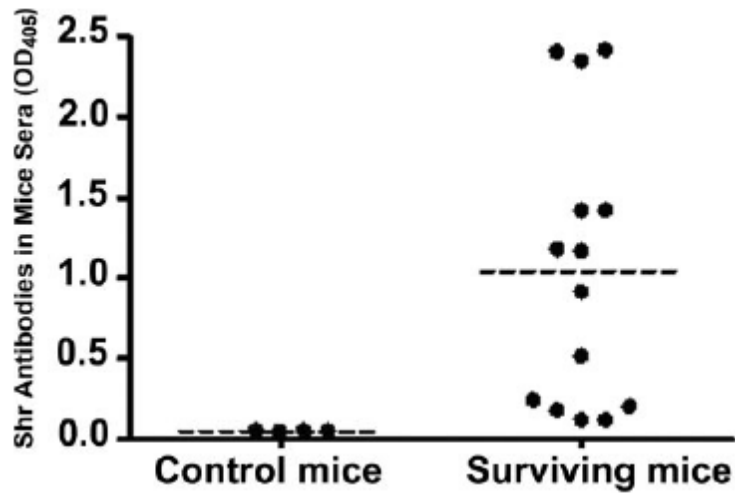


Figure 11. Shr antibody levels in mice following recovery from GAS infections.

Mice were inoculated subcutaneously with a sublethal dose of MGAS5005 (M1). Serum was collected from each mouse 28 days following challenge, diluted 1:1000, and tested for reactivity with purified Shr by ELISA as described in the Methods. Each datum point represents the titer obtained from an individual mouse. (work was performed by M. Fisher (Fisher, Huang et al. 2008)).

Shr is important for GAS pathogenesis in the zebrafish infection model. The use of zebrafish as an infection model for pathogenic streptococci was previously established. Intramuscular injection (i.m.) of zebrafish with the native fish pathogen, *S. iniae* causes a systemic disease, while injection with GAS causes mostly local muscle lesions and necrosis (Neely, Pfeifer et al. 2002). The effect of *shr* inactivation on virulence was investigated via the i.m. infection route, as previously described (Neely, Pfeifer et al. 2002; Montanez, Neely et al. 2005). Groups of six zebrafish were challenged with 10^6 CFU per fish of the wild type NZ131, the *shr* mutant (ZE4912), or the mutant complemented with *shr* (ZE4924) cells (Fig. 12). Injection with the wild type strain resulted in rapid fish death, with fish survival beginning to decline as early as 12 h post infection, reaching a final survival rate of 22% at 48 h post infection (filled squares). On the other hand, the *shr* mutant strain was attenuated for virulence; fish injected with the mutant strain (open squares) exhibited a slower decline in survival and the final survival rate was significantly higher than that of fish infected with the wild type strain (62% survival at 48 h post infection, $P < 0.0012$). The complemented strain (filled triangles) demonstrated intermediate virulence; fish infected with this strain died at a slower rate than those infected with the wild type strain (100% survival at 12 h and 24 h post infection). However, the final survival rate eventually declined to a level similar to that of fish infected with the wild type strain (25% 48 h post infection). Determination of LD_{50} was done by injecting each fish with a range of 10^4 - 10^7 CFU of each strain. Consistent with the attenuated phenotype observed for the *shr* mutant, the wild type LD_{50} value was significantly lower (5×10^4 CFU per fish) than that of the mutant (2.5×10^6 CFU per fish), and an intermediate LD_{50} value (5.6×10^5 CFU) was observed with the complemented strain, demonstrating partial complementation of virulence.

The impact of Shr on the infection process in GAS was also evaluated by testing the ability of the *shr* mutant to compete with the wild type strain for growth in the muscle tissue during co-infection. Zebrafish were inoculated by i.m. injection with 10^6 CFU of a mixed culture comprised of a 1:1 wild type to mutant ratio. The lesion area was excised 24 hours post infection, and the number of viable bacteria of each strain in the tissue was determined by comparing the bacterial recovery on plates with spectinomycin (the mutant's marker) to that on antibiotic-free plates. The competitive index (CI) is defined as the output ratio of mutant to wild-type bacteria divided by the input ratio of mutant to wild type bacteria. The CI of the *shr* mutant was calculated to be 0.011 ± 0.002 . The growth rate of the *shr* mutant *in vitro* in THYB was identical to that of the wild type strain. In addition, the chromosomal mutation in *shr* was stable during growth in THYB in the absence of spectinomycin, as was evident from the plating efficiency (100%) of this strain on selective plates after overnight growth in the absence of antibiotics. Therefore, the low competition index suggests that inactivation of *shr* dramatically reduced the *in vivo* fitness of GAS in comparison to the wild type strain in zebrafish tissue. Since Shr is found both on the cell surface and in the culture supernatant, the fact that the presence of the wild type strain during infection could not rescue the attenuated phenotype of the mutant suggests that the surface associated form of Shr is important for GAS fitness during infection.

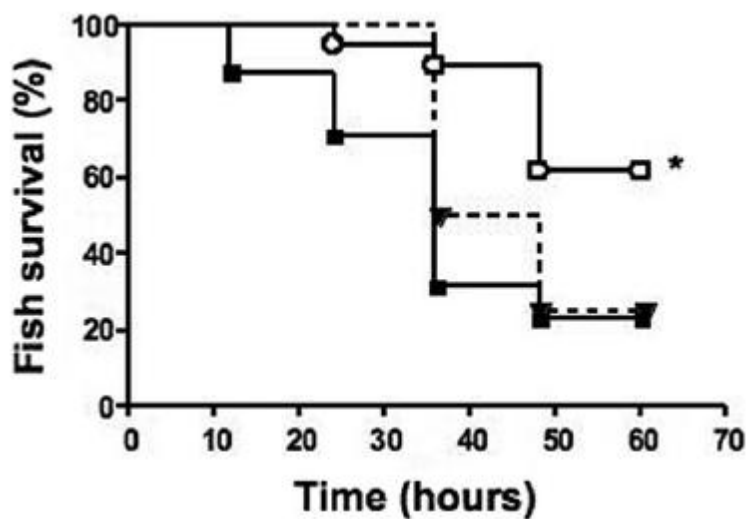


Figure 12. Zebrafish survival following intramuscular injection.

Kaplan-Meier plots showing survival of zebrafish following intramuscular challenge with 10^6 CFU of GAS wild type (NZ131, filled squares), *shr* mutant (ZE4912, open squares), and the complemented (ZE4924, filled triangles) strains. The asterisk represent statistical significance difference between ZE4912 (n = 18) and NZ131 (n = 56) ($P = 0.0012$) and between ZE4912 (n = 18) and ZE4924 (n = 12) ($P = 0.03$) by the log rank test.

DISCUSSION

The human pathogen GAS exports numerous proteins to its surface where they carry out the many tasks that mediate colonization and disease. Examples of these tasks include escape and subversion of the immune response, adherence, and nutrient scavenging (Bisno, Brito et al. 2003). Shr is a large, complex, and highly conserved surface protein in GAS (Fig. 4A) that does not share extensive sequence homology with other proteins in the database (Bates, Montanez et al. 2003). Although the functional role of Shr has not yet been deciphered, this protein has been shown to bind hemoproteins and is also suggested to mediate heme acquisition and transport in conjunction with Shp and the SiaABC heme-transporter (Bates, Montanez et al. 2003; Nygaard, Blouin et al. 2006). The major objectives of this study were to explore in depth the function of Shr and its role in GAS pathogenesis. The results show that the contribution of Shr to the GAS infection process extends beyond iron uptake to include interaction with ECM and adherence to epithelial cells. Therefore, Shr represents a new MSCRAMM in GAS necessary for virulence in the zebrafish model for necrotizing fasciitis.

When GAS is grown in culture under laboratory conditions, Shr is found in the supernatant as well as in association with whole GAS cells. Even though Shr contains a leader peptide that can mark proteins for secretion (Bates, Montanez et al. 2003), the mechanism that facilitates the association of Shr with GAS surface remains unclear. The majority of surface proteins in Gram-positive bacteria are covalently attached to the peptidoglycan by a sortase enzyme, which recognizes a C-terminus cell wall sorting motifs followed by a hydrophobic segment and a charged tail (Marraffini, Dedent et al. 2006; Scott and Barnett 2006). The LPXTG sequence serves as the sorting signal for the housekeeping sortase in GAS. Shr does not have an LPXTG

motif in its C-terminus or the QVPTG signal that is recognized by *srtC2*, an accessory sortase that is also produced by the GAS NZ131 strain (Barnett, Patel et al. 2004). Using cell fractionation and immunoblotting, this study demonstrated that Shr is not bound to the cell wall; rather, Shr is found in the membrane fraction (Fig. 4B). The absence of recognizable cell wall sorting signals in Shr sequence is consistent with these findings. It seems likely that Shr remains associated with the cytoplasmic membrane after its export to the surface due to the putative trans-membrane segment and the charged tail found in its C-terminus (Fig. 4A), as was found to be the case for ActA of *Listeria monocytogenes* (Kocks, Gouin et al. 1992). Shr is not present in the membrane of the mutant GAS strain ZE4912, since the mutation is located in the 5' coding region of the *shr* gene and therefore results in the formation of a transacted protein secreted to the culture supernatant (Fig. 5 and data not shown). The secretion of the abridged Shr fragment supports the hypothesis that the C-terminus in Shr is required for its membrane anchoring and therefore for its association with GAS cells.

The ability of anti-Shr serum to recognize Shr on the surface of whole GAS cells (Fig. 6, black bars, NZ131 and ZE4924 strains) shows that the wild-type Shr protein spans the cell wall in GAS and is exposed to the extracellular environment. Therefore, Shr may be able to interact with large extracellular molecules, in addition to small ligands capable of diffusing the cell wall. Since Shr appears to be anchored to the cytoplasmic membrane through its C-terminus, it is possible that it can deliver heme from the extracellular compartment directly to the transport components found in the cell membrane. This heme transport scheme is different from the one proposed by Maresso *et al.* for heme uptake by the Isd proteins in *S. aureus*, where heme is relayed in a cascade fashion from surface-exposed NEAT-proteins, such as IsdA, IsdB, or IsdH,

to an IsdC protein that is found deeper in the peptidoglycan; heme is then transferred by IsdC to the IsdDEF membrane transporter for uptake (Maresso and Schneewind 2006).

Using solid phase binding assays, we were able to demonstrate that Shr also binds the ECM components fibronectin and laminin (Fig. 7). The competition studies performed with soluble and immobilized fibronectin and laminin demonstrate that Shr interactions with these adhesive molecules are specific (Fig. 8). Therefore, Shr appears to have a broader spectrum of ligands than previously suggested. Shr availability on the GAS surface combined with its affinity for ECM components qualifies this receptor as a new MSCRAMM in GAS. The report also states that recombinant *L. lactis* cells that express Shr on the surface binds specifically to immobilized fibronectin (Fig. 9, MG1363/pXL14), which is an activity not found in the native *L. lactis* cells (Fig. 9, MG1363). This observation shows that Shr is able to mediate bacterial attachment to the ECM and further supports its role as a MSCRAMM. Interestingly, the Shr amino acid sequence does not contain the typical fibronectin-binding repeats found in other fibronectin-MSCRAMMs (Kreikemeyer, Klenk et al. 2004; Schwarz-Linek, Hook et al. 2006). Thus, it is not clear which part of this large protein mediates the binding to fibronectin or to laminin. It was reported that the NEAT-protein IsdA in *S. aureus* binds to several non-heme host proteins, including the ferric carrier transferrin (Taylor and Heinrichs 2002), several matrix and plasma proteins, and hemoglobin (Clarke, Wiltshire et al. 2004). The results presented here demonstrate that Shr does not bind to transferrin, as it has a similar but not identical binding pattern as IsdA. The ligand binding in IsdA is attributed to its single NEAT domain, but the residues involved in binding to the serum and ECM proteins were not determined. The sequence homology between IsdA and Shr is limited to the NEAT regions and is quite low even within these domains. Moreover, Shr is

a significantly larger and more complex protein than IsdA (145 and 38 kDa respectively), and has a central LRR segment, a unique amino terminal region, and two NEAT domains. Additional analysis is required to determine the domains and the residues involved in the recognition of various Shr ligands.

The data presented shows that Shr helps facilitate GAS attachment to HEp-2 epithelial cells, where a reduction in binding of about 40% was observed in the *shr* mutant. This decrease in adherence is statistically significant and can be partially restored in the complementation strain (Fig. 10). Since GAS expresses several adhesins that mediate binding to HEp-2 cells, it is not surprising that only a small reduction in adherence is observed in the *shr* mutant. As far as we can determine, Shr is the only adhesin reported to be induced in response to the iron restriction likely encountered during infection. Therefore, Shr's contribution to adherence may be more significant under such disease-specific conditions. Shr-dependent adherence may take place through fibronectin binding or additional serum-bridging molecules as found for other GAS adhesins (Kreikemeyer, Klenk et al. 2004; Talay 2005). Alternatively, Shr may interact directly with a host cell receptor via a mechanism that may be assisted by its LRR domain (Fig. 4A). LRR are suggested to provide a scaffold for the formation of protein-protein interactions (Kobe and Kajava 2001), and the LRR domain in Internalin was shown to be necessary and sufficient for binding to E-cadherin in *L. monocytogenes* (Lecuit, Ohayon et al. 1997).

It seems likely that *shr* complementation was not able to restore adherence to the level observed in the wild type due to the nature of the mutation and the method used for complementation. The *shr* mutation in ZE4912 results in the production of a truncated Shr fragment that is secreted into

the extracellular medium. This Shr fragment, which is also produced by the complementation strain, may serve as a competitor interfering with the Shr-mediated attachment to epithelial cells and impairing adherence in the complementation strain. In addition, the complementation strain ZE4924 is a merodiploid strain created by Campbell insertion. Since this type of mutation is unstable, it may excise and reduce the efficiency of complementation in the binding assays.

This study presents several experiments that suggest Shr is important for GAS virulence. A single infection event was found to be sufficient to trigger a significant antibody response to Shr in convalescent mice, indicating that Shr is expressed *in vivo* in adequate amount and duration to elicit a host response (Fig. 11). While the majority of the injected mice (9 out of 14) developed high Shr titer, the anti-Shr antibody level in the remaining five mice was low. The observed variations in the antibody response following GAS challenge may result from differences in the time it took individual mice to clear the bacteria, or from variation in the efficiency of their immune response, or both. Analysis of Shr production in different GAS strains, including a collection of 17 clinical isolates (Table 1), revealed that Shr could be detected on the surface of most of the examined strains. Inactivation of *shr* resulted in attenuation of virulence in a zebrafish model (Fig. 12, ZE4912) with the LD₅₀ of the mutant about 50 times higher than that of the wild-type strain. As in the adherence assay, only partial restoration of virulence was observed with the complemented strain. Both the kinetics of survival in zebrafish post infection and the LD₅₀ value of ZE4924 complementation strain demonstrated an intermediate level of virulence in comparison to the wild type NZ131 and the *shr* mutant ZE4912. This suggests that either instability of the complemented strain or the production of both the full length and a truncated fragment of Shr by ZE4924 prevented complete recovery of virulence. The recovery

of the mutant strain from the lesion tissue was much less than that of the wild-type strain after co-infection (CI of 0.01). This observation demonstrates that inactivation of *shr* results in a mutant strain that is significantly less fit than the wild type parent strain. Shr is found both in the culture supernatant and on the cell surface. The inability of the wild-type strain to complement the *in vivo* growth defect of the mutant strain during co-infection of the zebrafish muscle strongly suggests that it is the surface-anchored form of Shr that is important for GAS ability to persist in the host during infection.

In summary, given that Shr is a broad-spectrum surface receptor contributing to iron acquisition (Bates, Montanez et al. 2003) (and manuscript in preparation), ECM binding, and adherence, its contribution to the disease process in GAS appears complex,. Ongoing research is on the way to further investigate this important multi-functional surface protein and to better understand its role in the disease process.

CHAPTER II

DEFENSE FROM THE GROUP A STREPTOCOCCUS BY ACTIVE PASSIVE VACCINATION WITH THE STREPTOCOCCAL HEMOPROTEIN RECEPTOR

INTRODUCTION

Streptococcus pyogenes or Group A Streptococcus (GAS) is a versatile pathogen capable of producing a spectrum of illness ranging from mild infections such as pharyngitis and impetigo to invasive diseases including myositis and necrotizing fasciitis (Cunningham 2000). GAS infections can also trigger a number of disabling sequelae. For example, pharyngitis, an ordinary childhood disease of which there are hundreds of millions of cases per year worldwide, can lead to acute rheumatic fever (RF) in about 3% of the untreated patients (Steer, Danchin et al. 2007). RF is thought to result from cross-reaction of antibodies and T cell receptors with tissues of the heart, synovium, and/or neurons of the basal ganglia in the brain (Guilherme, Fae et al. 2005; Guilherme, Ramasawmy et al. 2007). In addition to the induction of heart disease, the damage inflicted by autoimmune reactions is hypothesized to produce a number of neuropsychiatric disorders including Sydenhym's chorea and obsessive-compulsive disorder (Snider and Swedo 2003; Snider and Swedo 2004). All together, GAS costs billions of dollars in the US alone and more than 500,000 deaths per year globally. An effective GAS vaccine is therefore highly desirable, especially for the developing parts of the world where RF and rheumatic heart disease are leading causes of disability and mortality in children (Carapetis, Steer et al. 2005; Steer, Danchin et al. 2007).

GAS M protein has been extensively studied as a vaccine candidate since early observations that it elicits lasting immunity (Cohen-Poradosu and Kasper 2007; Dale 2008; Steer, Batzloff et al. 2009). However, the antiphagocytic M protein is a highly variable antigen (Beall, Facklam et al. 1996; Facklam, Martin et al. 2002). The N-terminal domain, the molecule's most outward facing and least conserved region, evokes a M type specific antibody (Lancefield 1962). More than 150 of M types are known, and the number and identity of the prevalent strains varies significantly in different parts of the globe (Kaplan, Wotton et al. 2001; Shulman, Tanz et al. 2009; Steer, Jenney et al. 2009). In addition to the complications arising from extensive antigenic variation, M-based vaccination programs suffer from safety concerns. Several M serotypes were implicated in RF development, and cross reactivity has been found between some M epitopes and human tissues.

Recent studies suggest that a safe and effective M-base vaccine for GAS may be in reach. Two vaccines based on peptides derived from the N-terminal domain of the M protein were found to be protective in clinical trials without adverse outcome (Kotloff, Corretti et al. 2004; McNeil, Halperin et al. 2005). In its current formulation, M vaccine provides protection against 26 serotypes, covering approximately 85% of GAS strains in the US (Shulman, Tanz et al. 2009). Nevertheless, this vaccine is expected to have fairly limited coverage in the developing countries, and there are concerns that it may trigger a shift in serotype prevalence (Steer, Magor et al. 2009). Therefore, there is a significant interest in identifying additional protective antigens that may facilitate broad immunization programs. A number of GAS components have been investigated, including antigens derived from virulence factors or surface components, such as the C5a peptidase (Ji, Carlson et al. 1997), the conserved C-terminal region of the M protein (Pandey, Batzloff et al. 2009), group A carbohydrates (Sabharwal, Michon et al. 2006),

lipoteichoic acid (Dale, Baird et al. 1994; Yokoyama and Harabuchi 2002) and several fibronectin binding proteins (Kawabata, Kunitomo et al. 2001; Courtney, Hasty et al. 2003; McArthur, Medina et al. 2004). Antigens that were identified *in silico* and/or by proteomics or genomics methods have been also studied (Rodriguez-Ortega, Norais et al. 2006; Turner, Kurupati et al. 2009). Although a protective response was observed with some antigens in one or more models, difficulties arising from limited expression among GAS isolates or the high concentration required for effective antibody response were reported. Other than the N-terminus peptides of the M protein, no additional GAS antigens have reached human trials since the 1970s.

The streptococcal hemoprotein receptor (Shr) is highly conserved in GAS genomes. It binds to several hemoproteins and ECM components and is implicated in iron acquisition and adherence (Bates, Montanez et al. 2003; Fisher, Huang et al. 2008). Shr is available on the bacterial surface for antibody binding, and recent analysis confirmed the expression of Shr in 14 out of 17 tested clinical isolates representing 12 M types (Fisher, Huang et al. 2008). Here, we investigate the ability of Shr to elicit protective immunity. We show that Shr is highly immunogenic and that vaccination with Shr in both active and passive models protected mice from systemic GAS challenge.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* DH5 α harboring plasmid pCB1, which expresses a recombinant Shr protein (His6-Xpress-Shr, rShr (Bates, Montanez et al. 2003)), were grown in Luria-Bertani broth at 37°C. The GAS strains MGAS5005 (M type 1 (Sumbly, Porcella et al. 2005)), MGAS315 (M type 3 (Beres, Sylva et al. 2002)) and ZE491 (M type 49, *mtsR* (Bates, Toukoki et al. 2005)) were grown at 37°C in Todd-Hewitt broth (Difco Laboratories) with 0.2% w/v yeast extract (THY). The *Lacococcus lactis* MG1363 and MG1363 harboring plasmid pXL14 (expressing the native Shr protein (Fisher, Huang et al. 2008)) were cultivated in M17 (Difco Laboratories) supplemented with 0.5% w/v glucose (GM17) at 30°C. When necessary, ampicillin (100 μ g/ml) or kanamycin (70 μ g/ml) was added to the medium.

Mouse vaccinations. CD-1 female mice were used (weight, 20 to 22 grams; Charles River Laboratories). For *systemic immunization*, recombinant Shr was purified as previously described (Fisher, Huang et al. 2008) and was quantified using the Modified Lowry Protein Assay Kit (Thermo Scientific). Purified rShr protein (40 μ g) was administered intraperitoneally (i.p.) on days 0 (emulsified in Complete Freund's adjuvant, CFA), 14, 28, and 42 (emulsified in Incomplete Freund's adjuvant, IFA). For mock vaccination, phosphate-buffered saline (PBS) mixed with CFA or IFA was administrated. Serum samples were collected on days 0, 7, 21, 35 and 49, and the anti-Shr immunoglobulin (Ig) G response in individual animals was determined. *Mucosal immunization* was done as previously described (Quigley, Hatkoff et al. 2010). *L. lactis* cells (MG1363/pXL14 or MG1363) were briefly washed and resuspended in saline to give 1×10^8 CFU/ μ l. Mice were vaccinated intranasally (i.n.) by four administrations of 2×10^9 CFU (20

μl) into the nostril, every 10 days for three consecutive days (i.e. on days 1, 2, 3, 14, 15, 16, 27, 28, 29, and on days 40, 41 and 42). The anti-Shr IgG titer in serum samples collected on day 50 was determined. Lung lavage samples were collected on day 52 as previously described (Quigley, Hatkoff et al. 2010), and the anti-Shr IgA level was determined. For *passive immunization*, 100 μl of rabbit rShr-antiserum (Bates, Montanez et al. 2003) or serum samples from naive animals (normal rabbit serum, NRS) were mixed with 400 μl PBS and administrated intraperitoneally. All of the vaccination and mouse challenge experiments were repeated at least twice.

GAS infection model. GAS cells at the mid-logarithmic phase ($OD_{600} = 0.7$) were harvested, washed and resuspended in saline. Cell concentration was determined by microscopic counts and verified by plating. Mice were infected with 0.1 ml of cells suspension (LD_{80}) and were observed four times a day after challenge. Animals exhibiting signs of severe distress were euthanized and counted as dead. All experiments involving animals were conducted according to protocols approved by the Georgia State University Institutional Animal Care and Use Committee.

Shr-antibody detection. The antibody response was measured by enzyme-linked immunosorbent assay (ELISA) (Quigley, Hatkoff et al. 2010). Briefly, microtiter plates (Costar, Corning Inc.) were coated with rShr (50 ng per well), washed and blocked with 1% w/v BSA in PBS-Tween. Serum or lung fluid samples were allowed to react with the coated plates for 1 h at 37°C. Antibody production was detected using anti-mouse IgG or anti-mouse IgA secondary antibodies (Sigma). The absorbance at 405 nm was measured after a 30-min incubation.

Antibody titers were defined as the dilution producing the same OD₄₀₅ at 2 times of the background level (the reading obtained with serum or lung fluid samples of nonimmunized mice).

Opsonophagocytic killing assay. ZE491 GAS cells (OD₆₀₀ = ~0.7) were harvested and washed. A total of 0.1 ml of diluted (1: 200,000) cell samples were added into 0.5 ml of fresh rabbit whole blood, together with 0.1 ml of rabbit Shr antiserum (Bates, Montanez et al. 2003) or NRS (as control), and incubated with shaking at 37°C for 3 h. Surviving bacteria were enumerated by plating for viable counts. Opsonization expressed as the percent reduction in mean CFU was calculated as $[1 - (\text{CFU in the presence of Shr antiserum}) / (\text{CFU in the presence of NRS})] \times 100$ (Olive, Sun et al. 2006).

Statistical analysis. Data presented are average or representative of data from experiments repeated at least two times. The Student's *t* test was used for testing significance when comparing two groups. Kaplan-Meier plots of survival and log-rank tests were used for comparison of protection by immunization.

RESULTS

Intraperitoneal vaccination with Shr elicits a robust humoral response and protection.

Previous studies demonstrated that Shr is expressed *in vivo* and suggested that it is required for GAS disease process (Fisher, Huang et al. 2008), raising the possibility that the multifunctional Shr may serve as a vaccine target. To examine Shr antigenic potential, a recombinant protein (rShr, (Bates, Montanez et al. 2003)) was expressed, purified and administered intraperitoneally to mice. The time course development of the anti-Shr response was monitored in serum samples from four representative mice (Fig. 13A). All four animals developed a significant serum reaction to Shr after a single antigen administration (IgG titer range, 10^3 - 10^4). The anti-Shr IgG level continued to ascend after the second immunization and appeared to be saturated after the third immunization, demonstrating an anti-Shr titer range of $(1.25 - 6.25) \times 10^6$. End point titer analysis in the rest of the cohorts' mice revealed a robust anti-Shr response in the immunized animals, with a mean titer of 5.6×10^5 (Fig. 13B). In contrast to the significant anti-Shr response found in the experimental group (ie, Shr-immunized mice), Shr-specific antibody was not seen in serum samples obtained from the control group (ie, the mock vaccination group).

To test for protective immunity, vaccinated mice were challenged *i.p.* with a lethal dose of MGAS5005 (M type 1) on day 49 of the vaccination experiment, and animal survival was monitored for 5 days. A rapidly progressing fatal infection developed in most of the mice in the control group (mock vaccination), resulting in 21% survival at the end point. Similarly, an aggressive disease was previously reported in a murine model for systemic GAS infection with MGAS5005 (Sumby, Porcella et al. 2005). A less severe disease developed after infection of

mice immunized with rShr, and the end point survival rate of this group was 78%, 3.7-fold higher than that of the mock-vaccinated group ($P = 0.002$) (Fig. 14). Therefore, systemic immunization of mice with rShr provides protection from systemic GAS challenge.

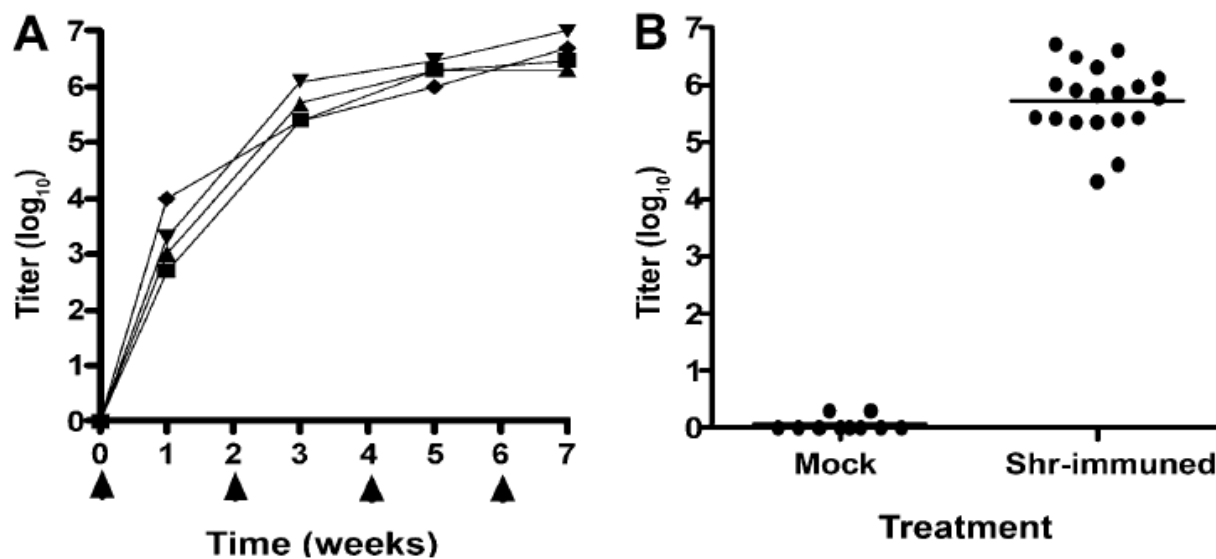


Figure 13. Anti-Shr response in serum after intraperitoneal vaccination.

Mice were administrated with rShr (arrow heads), and the resulting serum IgG titers were determined by ELISA performed in quadruplicates. Each datum point represents the average response in individual animals. **(A)** Shr antibody development. Shr-specific IgG levels were determined in serum samples collected from 4 representative mice on days 0, 7, 21, 35 and 49. **(B)** End point anti-Shr IgG response. The anti-Shr IgG titers found in serum samples collected on day 49 from mice administrated with rShr (Shr-immuned; n=19) or with PBS (mock; n=10) are shown. The horizontal bar indicates the mean titer response.

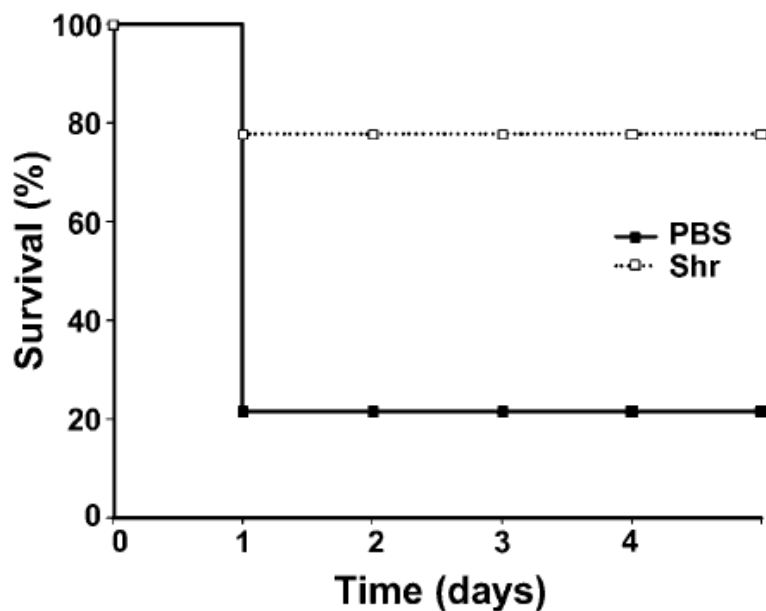


Figure 14. Protection of mice from systemic GAS challenge after intraperitoneal vaccination with Shr.

Immunized mice were infected intraperitoneally with 5×10^8 CFU. Kaplan-Meier survival curves of antigen-immunized mice (shr; n=18) and mock-vaccinated mice (PBS; n=14) are shown. The statistical significance ($P = 0.002$) was determined by the log-rank test. Two independent experiments producing similar results were conducted; the data shown are from a representative experiment.

Intranasal vaccination with Shr results in strong antibody response and enhanced defense.

The ability of Shr to elicit immune response by mucosal vaccination was examined using the probiotic *L. lactis* as a delivery vector. Mice intranasally received the recombinant bacteria expressing Shr on the surface (MG1363/pXL14, (Fisher, Huang et al.)) or the native host cells (MG1363). The presence of anti-Shr IgA in undiluted lung lavage samples collected 10 days after the last antigen administration (day 52) was determined by ELISA. A measurable Shr-IgA activity was exhibited by all of the mice that were immunized with Shr-expressing bacteria (Fig. 15A); 9 out of 13 mice developed a Shr-specific IgA level that was at least three times higher than that seen in untreated animals (with an end point IgA titer (the titer is expressed as a dilution ratio) that varied from 1:4 to 1:64; data not shown). Mice that were vaccinated with unmodified *L. lactis* cells showed only the background IgA level seen with untreated animals ($P = 0.01$). The induction of anti-Shr IgG in the serum after the course of mucosal vaccination (day 50) was tested as well (Fig. 15B). All of the mice administered with the recombinant *L. lactis* (MG1363/pXL14) developed a significant IgG response to Shr, demonstrating a mean endpoint titer (\pm standard deviation) of $5.5 \times 10^4 \pm 2 \times 10^4$, whereas only background response was found in serum samples from pre-immune mice or mice vaccinated with native *L. lactis* (MG1363, $P < 0.0001$). Therefore, mucosal administration with *L. lactis* expressing Shr resulted in a measurable immune response in secretions and a strong reaction in the serum.

We next asked whether the anti-Shr immune response triggered by the mucosal immunization was sufficient to protect against GAS infection. Vaccinated mice were infected intraperitoneally (on day 52) with MGAS5005. Aggressive infections were seen after the challenge in mice that were immunized with the native *L. lactis* strain (MG1363), demonstrating only a 25% survival

rate on the fifth day after infection. In contrast, most of the mice that were administrated with Shr-producing lactococci (MG1363/pXL14) were able to recover and demonstrated an 87% survival rate, 3.48-fold higher than that of the control group ($P = 0.014$) (Fig. 15C). Thus, mucosal immunization with Shr-producing bacteria resulted in protective immunity against systemic GAS challenge.

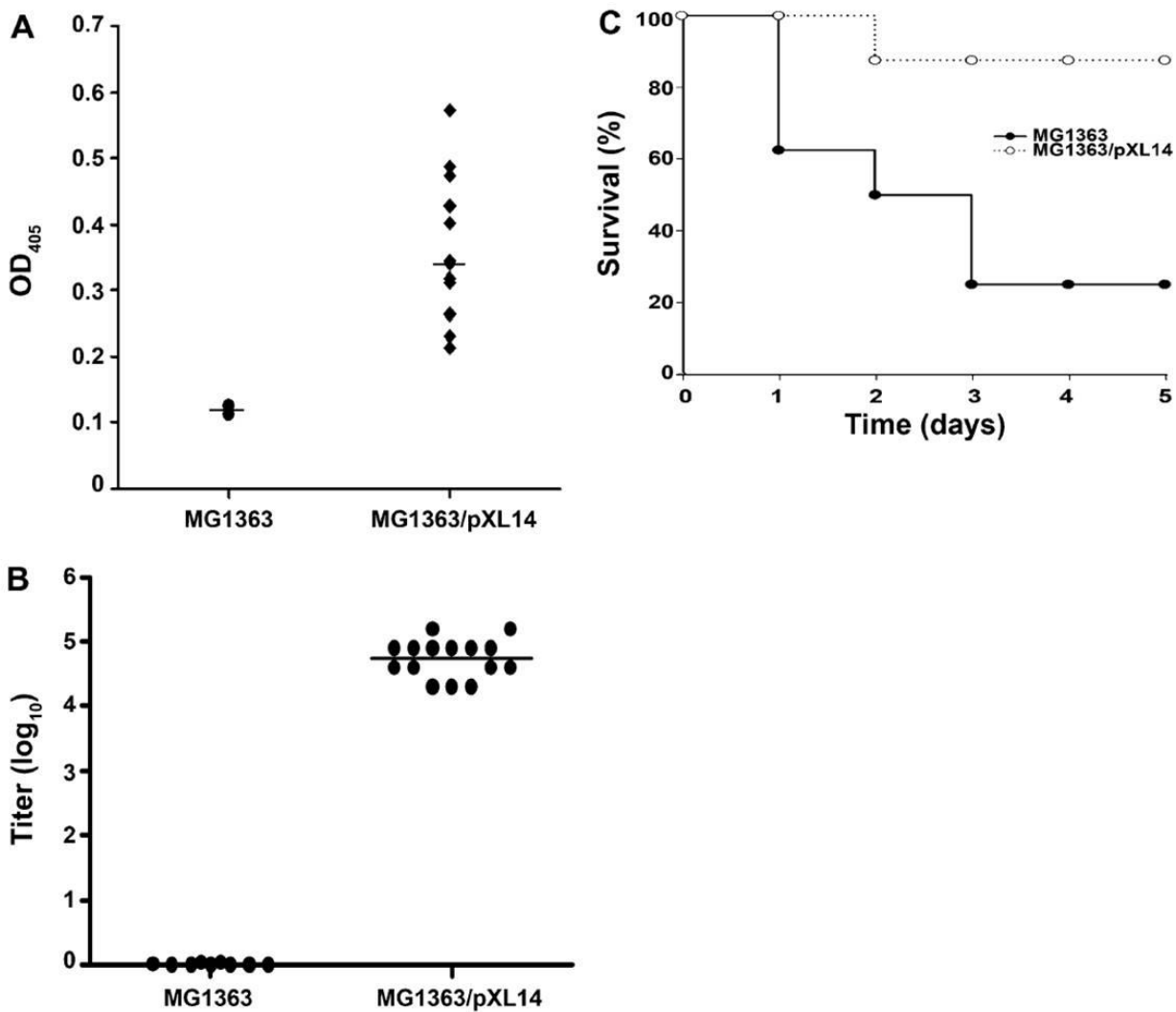


Figure 15. Shr intranasal vaccination and protective immune response.

Mice were immunized with 2×10^9 CFU of *L. lactis* (MG1363) or *L. lactis* expressing Shr (MG1363/pXL14). Endpoint antibody response (on day 50 and 52, respectively) was determined by ELISA performed in quadruplicate. **(A)** Shr-specific IgA level in undiluted lung lavage specimens from mice treated with MG1363 (n=2) or MG1363/pXL14 (n=13). Each datum point represents the mean response in individual animals. The statistical significance ($P = 0.01$) was determined by the Student *t* test. **(B)** Shr-specific IgG titer in serum from individual mice treated with MG1363 (n=15) or with MG1363/pXL14 (n=15). The statistical significance ($P < 0.0001$) was determined by the Student *t* test. **(C)** Kaplan-Meier survival curves of vaccinated mice after systemic GAS challenge. Mice immunized with MG1363 (n=8) or MG1363/pXL14 (n=8) were intraperitoneally received 1×10^8 CFU. The statistical significance ($P = 0.014$) was determined by the log-rank test. The data shown are pooled data from two independent experiments.

Passive immunity with rabbit Shr antiserum. The studies described above show that Shr vaccination administered via both the intraperitoneal and intranasal routes resulted in significant serum anti-Shr IgG. To examine whether the observed humoral response to Shr could account for the protection exhibited by the vaccinated animals, we tested the ability of rabbit Shr antiserum (Bates, Montanez et al. 2003) to protect naive mice from upcoming GAS infection. Pretreatment of mice with Shr antiserum (with a titer of 10^6) prior to intraperitoneal inoculation of MGAS5005 provided significant defense; the survival rate at the end point for mice administered with the Shr antiserum was 60%, whereas only a 20% survival rate was observed with the control mice that were treated with NRS prior to the challenge ($P = 0.0308$) (Fig. 16A). The same Shr antiserum also defended mice from infection with the invasive M type 3 strain MGAS315, demonstrating 53% and 20% survival rates in mice treated with anti-Shr or NRS, respectively ($P = 0.034$) (Fig. 16B). These observations indicate that Shr antibodies are protective against multiple GAS serotypes and suggest that the defense from GAS obtained by vaccination is mediated at least in part by serum antibody to Shr.

Rabbit Shr-antiserum is bactericidal. Because rabbit Shr-antiserum protects mice from GAS infection, we tested whether it contains opsonizing antibodies. The GAS metal-dependent repressor, MtsR, represses the expression of Shr in vitro (Bates, Toukoki et al. 2005). To ensure Shr production under the experimental conditions, we used the *mtsR* mutant, ZE491, in which Shr expression is deregulated (Bates, Toukoki et al. 2005). ZE491 cells were allowed to grow in rabbit whole blood in the presence of Shr-antiserum or NRS, and the change in the bacterial load after 3 hours of incubation was determined by viable counts. A significant reduction in the bacterial number (mean \pm standard deviation, $70\% \pm 17\%$) was observed in cultures treated with

Shr antiserum, comparison with those that were treated with NRS (Fig. 17), indicating that the anti-Shr serum facilitates phagocytosis and bacterial killing in whole blood.

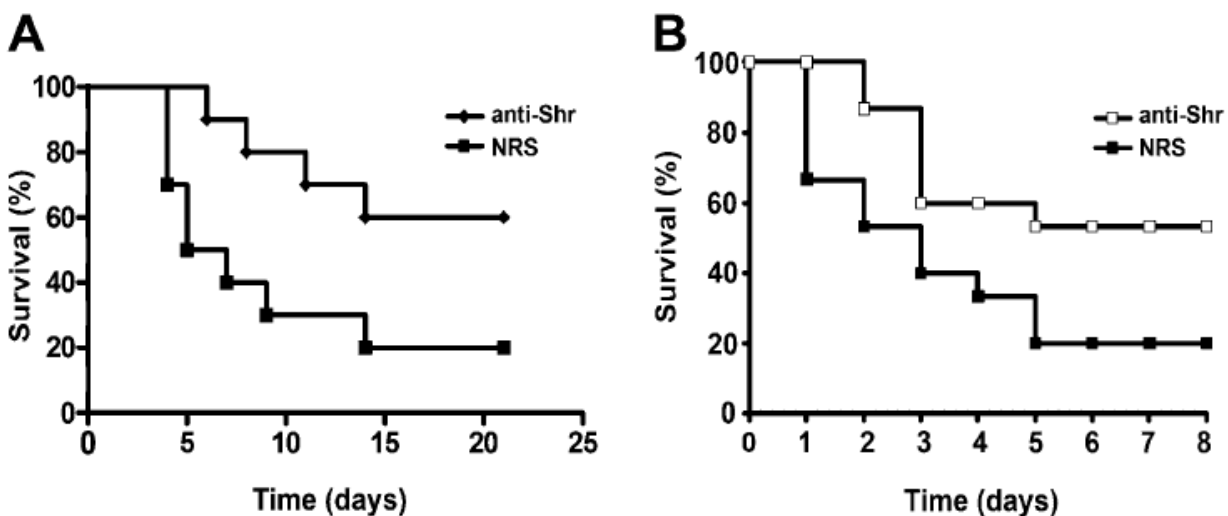


Figure 16. Protection of mice from systemic GAS infection with passive immunization with anti-Shr.

Kaplan-Meier survival curves are shown for mice challenged with 5×10^7 CFU 4 h after intraperitoneal administration of rabbit Shr antiserum (anti-Shr) or with normal rabbit serum (NRS). **(A)** MGAS5005 challenge. The results are representative of two independent experiments ($n=10$ for both groups; $P=0.0308$). **(B)** MGAS315 challenge. The data shown are pooled from three independent experiments ($n=15$ for both groups; $P=0.034$). The statistical significance was determined by the log-rank test.

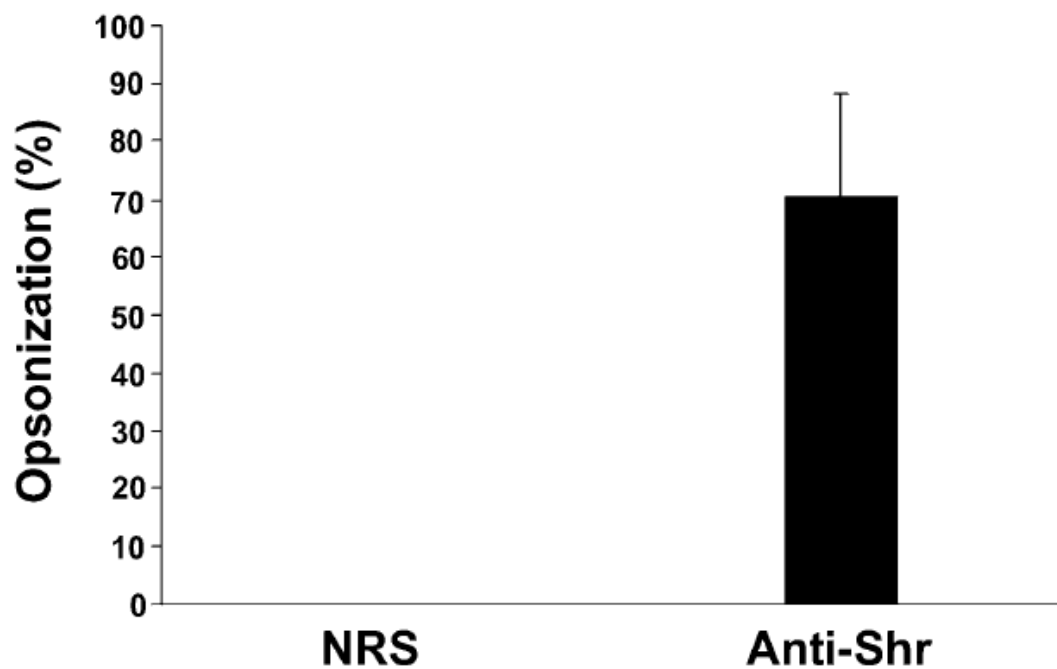


Figure 17. Serum mediation of phagocytosis and killing of bacteria with anti-Shr.

ZE491 cells were incubated in rabbit whole blood in the presence of Shr antiserum or normal rabbit serum (NRS). Bacterial growth after 3 h of incubation with Shr antiserum was compared with that of control NRS. The mean opsonization percentage (measured as the percentage reduction in CFU) derived from 4 independent experiments is shown; the standard deviation (SD) is represented by the error bar ($P < 0.001$).

DISCUSSION

An effective prevention program for GAS is greatly needed, especially in the developing world, where antibiotic therapy fails to control the occurrences of rheumatic fever and rheumatic heart disease as it does in developed countries (Guilherme, Ramasawmy et al. 2007; Steer, Danchin et al. 2007; Steer, Jenney et al. 2009). The increasing reports of drug-resistant GAS further underscore the need for GAS vaccine (Ardanuy, Domenech et al. 2010; Feng, Lin et al. 2010; Ikebe, Wada et al. 2010; Malli, Tatsidou et al. 2010). The hyper-variable M protein is the most prominent and promising GAS antigen (Kotloff, Corretti et al. 2004; McNeil, Halperin et al. 2005). However, M-based vaccination would likely require unique formulation for different geographic areas, and the efficacy of the vaccine may be hampered by rapid changes in GAS populations. Conserved antigens, which can be used alone or in combination with M-epitopes to increase coverage and combat population shifts, are therefore required. Here, we show that a conserved protein, Shr, is a promising antigen that can elicit robust humoral response and provide defense from systemic GAS infections in both active and passive immunization models.

We previously reported that GAS infection led to serum conversion in most of the surviving mice (Fisher, Huang et al. 2008). In this study, we measured the serum immune response triggered by Shr vaccination using CFA/IFA or *L. lactis* as adjuvants in systemic and mucosal application, respectively. The vigorous Shr-specific IgG response developed in mice after intraperitoneal vaccination (Fig. 13), and the high antibody levels observed in the intranasally immunized animals (Fig. 15B), together suggest that Shr is a strong immunogen. Moreover, both vaccination protocols resulted in enhanced resistance to systemic GAS challenge, showing that the immune reaction to Shr is protective (Fig. 14 and 15C). It is interesting to note,

however, that although the mucosal antigen administration produced a lower anti-Shr IgG titer ($(5.5 \times 10^4 \pm 2 \times 10^4)$) than did intraperitoneal vaccination with rShr (5.6×10^5), a similar level of mouse recovery from GAS infection was seen in both cases. Immunization with *L. lactis* typically leads to a T-helper 1 (Th1)-biased response (Repa, Grangette et al. 2003; Hanniffy, Carter et al. 2007; Quigley, Hatkoff et al. 2010). In addition to supporting IgG2a production over other immunoglobulin subtypes, Th1 adaptive response promotes cellular immunity, which could complement the protection provided by serum IgG.

Mucosal immunity is an important prevention and control measure of mucosal pathogens such as GAS. *Lactococcus* species was previously used successfully to elicit mucosal defense against *Streptococcus pneumoniae*, Group B Streptococcus, and Group A Streptococcus (Buccato, Maione et al. 2006; Hanniffy, Carter et al. 2007). Here, we show that in addition to the strong production of serum antibody, intranasal immunization with *L. lactis* expressing Shr triggered anti-Shr IgA formation in secretions (Fig. 15A). Shr-specific IgA was seen in the lung lavage specimens obtained from most mice, although the antibody titer was modest. Mucosal vaccination with M-derived peptides provided protection from intranasal challenge even in the absence of strong IgA response, suggesting a protective role for serum IgG (Olive, Clair et al. 2002; Olive, Sun et al. 2006). Nevertheless, IgA is important for GAS defense (D'Alessandri, Plotkin et al. 1978; Kawabata, Kunitomo et al. 2001; Batzloff, Pandey et al. 2006); thus, the mucosal delivery of Shr needs improvement. Investigations of alternative mucosal adjuvants, such as the lipid core peptide (Olive, Sun et al. 2006), proteosomes (Jones, Allard et al. 2003), or GAS pilus-based UPTOP (for unhindered presentation of polypeptides on tips of pili) system (Quigley, Hatkoff et al. 2010), are warranted.

Mice that were administrated with anti-Shr rabbit serum prior to infection demonstrated significantly higher survival rates after challenge with MGAS5005 or MGAS315 (60% and 53%, respectively) than did mice treated with NRS (20%) (Fig. 16). The observation that humoral response to Shr is protective suggests that the defense conferred by active vaccination with Shr is mediated, at least in part, by serum antibodies. Protection by serum antibodies is further supported by the findings that Shr antiserum is bacteriocidal (Fig. 17). In addition to facilitating GAS clearance by phagocytes, Shr serum antibodies are likely to interfere with Shr iron acquisition and adherence (Bates, Montanez et al. 2003; Fisher, Huang et al. 2008) and thus may act directly to limit GAS growth and spread. The observation that Shr confers passive immunity suggests that Shr antiserum may be useful in therapy of invasive diseases, such as streptococcal toxic shock syndrome (STSS). STSS is a rapidly progressing, superantigen-mediated disease that involves bacteremia, hypotension and multi-organ failure (Johansson, Thulin et al. 2010). The absence of protective antibodies against GAS M protein and superantigens is found to be associated with higher risk for STSS. Effective GAS antibodies may be useful in STSS treatment as they may act in reducing the bacterial load in the blood and thus superantigen production.

CHAPTER III

ANALYSIS OF SHR DOMAINS

INTRODUCTION

Shr is a conserved heme-acquiring protein that binds *in vitro* to hemoglobin, myoglobin and hemoglobin-haptoglobin complex (Bates, Montanez et al. 2003). We recently identified Shr as a new MASCRAMM that mediates GAS attachment to epithelial cells and contributes to the infection process (Fisher, Huang et al. 2008). Shr is exposed to the extracellular environment while the C-terminus of the receptor is tethered to the cell membrane. Purified Shr specifically interacts with the host ECM proteins fibronectin and laminin and its expression by *Lactococcus lactis* allows the recombinant bacteria to bind to immobilized fibronectin. These observations suggest that fibronectin binding is important for the role of Shr in adherence. *In vivo*, Shr contributes to virulence in an intramuscular zebrafish infection model and a high titer of Shr antibody was observed in sera from convalescent mice. Immunization studies in murine models revealed that intraperitoneal administration of Shr triggers a robust production of Shr-specific IgGs in the serum (Huang, Fisher et al. 2011). Intranasal immunization with recombinant *L. lactis* that expresses Shr elicits a specific IgA reaction in the lung lavage and a strong IgG reaction in the serum. Both immunization routes provide protection from systemic GAS challenge. In addition, rabbit Shr antiserum is bactericidal *in vitro* and defends from GAS disease when administrated to mice prior to challenge. Overall, these findings suggest that Shr is a promising vaccine candidate. We inferred that the contribution of the multifunctional Shr to GAS pathogenesis and its promise as a protective antigen merit more detailed studies of its structural domains.

Shr contains two NEAT (NEAr Transporter) domains. Andrade *et al* (2002) identified the NEAT domain (~125 amino acids) in genes from Gram-positive pathogens that are located near transporters of iron complexes. Up to five copies of the NEAT domain were found in a single protein. Although low sequence homology can be shared by separate NEAT domains, they are suggested to have similar secondary structure that consists mainly of β -strands. Our search for the NEAT structure in the PDB (Protein Data Bank, <http://www.rcsb.org/pdb/>) (Bernstein, Koetzle et al. 1977) showed that the NEAT domains are folded into an immunoglobulin-like β -sandwich structure.

Shr NEAT domains are separated by a leucine rich repeat (LRR) region. In addition, this complex protein contains a leader peptide and two copies of the DUF1533 motif (Fig. 4). The different regions in Shr are likely to facilitate its interactions with multiple host ligands. Shr protein modules and sequence motifs are found in different combinations in a variety of proteins from important Gram-positive pathogens. The NEAT domains, for example, are typically located in exported or secreted proteins. All of the NEAT-proteins characterized to date are parts of proteins that are involved in heme uptake. The Isd (iron-regulated surface determinants) system in *Staphylococcus aureus* includes the best characterized NEAT proteins. A few proteins in *Bacillus* species and *Clostridium novyi* contain a LRR region in addition to one or more NEAT modules. DUF1533 motif is found in variable copies with or without additional domains mostly in proteins that are predicted to be on the bacterial surface. Shr presents the only proteins type in the database, in which the NEAT, LRR and DUF1533 domains appear together. In addition to GAS, Shr orthologues are found in a few human and animal pathogens. These include the toxin-producing *C. novyi* and the pyogenic *Streptococcus dysgalactiae*, *Streptococcus equi* subsp.

zooepidemicus and *equi*. The *shr* gene in *S. equi* subsp. *equi* has a nonsense mutation that leads to the production of a truncated version of Shr, containing the N-terminal part of the proteins with two copies of DUF1533 and a partial NEAT domain (Meehan, Burke et al. 2010). Shr orthologues in Streptococcus share high amino acid homology (58-86% identity) (Fig. 18). The orthologue in *C. novyi* is more remote and exhibits limited sequence similarity (30%) (Meehan, Burke et al. 2010; Ouattara, Bentley Cunha et al. 2010). The presence of domains that are shared by several proteins from important pathogens raises the possibility that a broad-spectrum vaccine could be developed based on Shr protein modules.

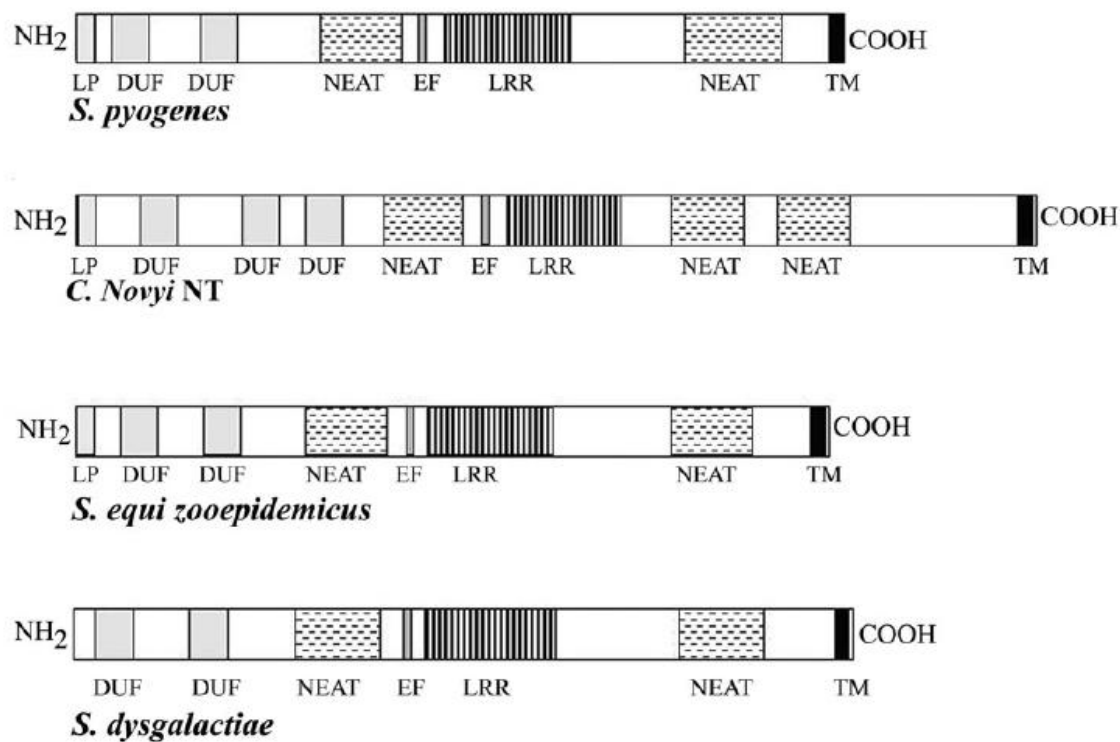


Figure 18. Shr proteins in pyogenic streptococci and *Clostridium novyi*.

LP: leader peptide; DUF: domain of unknown function 1533; NEAT: NEAr Transporter domain; EF: EF-hand motif; TM: transmembrane domain. (Figure adopted from Ouattara, Bentley Cunha et al. 2010)

Gram-positive bacteria express a wide variety of MSCRAMMs with fibronectin binding ability. These adhesins share overall structural organization and binding mechanism, while the protein lengths in each case vary greatly (Schwarz-Linek, Hook et al. 2006). The major fibronectin-binding activity in these molecules is typically localized to a region with tandem repeats of 35-40 amino acids, named FnBR (fibronectin binding repeat). FnBRs are typically found in the C-terminal half of the adhesin. The number of FnBRs varies among individual adhesins, but the repeating unit shares significant sequence homology (Fig. 19A). FnBR targets the N-terminal domain of fibronectin, which consists of five copies of the Fn-type 1 module (¹⁻⁵F1) (Fig. 2). The binding mechanism was deciphered by NMR study of a paired FnBR/Fn (¹F1²F1 in Fig. 19). This investigation revealed an anti-parallel orientation of the binding partners with an additional β -strand, and the binding mechanism was termed the tandem β -zipper model (Fig. 19B) (Schwarz-Linek, Werner et al. 2003). The structural information provided by the FnBR of *S. dysgalactiae* allowed the identification of the pattern and numbers of the FnBR repeats in the larger fibronectin-binding protein SfbI (GAS) and FnBPA (*S. aureus*). In SfbI, FnBPA and other fibronectin-binding proteins, an additional upstream fibronectin-binding domain (UFBD) is found (Fig. 19A). In FnBPA, the B1-B2 regions and repeats 4-5 are considered as the upstream fibronectin-binding domain. This region is known to use the same binding mechanism to the Fn-type 1 module as the FnBRs. The upstream fibronectin-binding domain (UR) of SfbI binds to an additional binding site, the gelatin-binding fragment in fibronectin. Therefore, the UR serves to enhance SfbI binding the ECM protein (Fig. 19A).

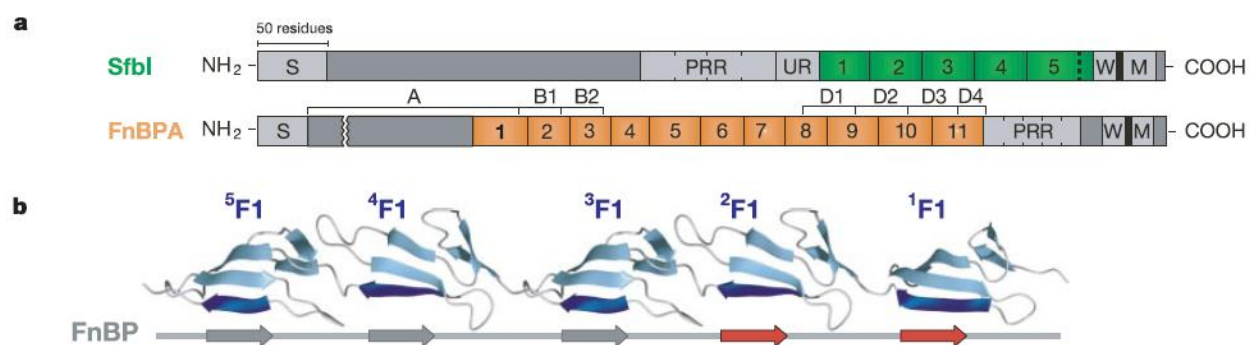


Figure 19. The extended tandem β -zipper model.

A, Schematic presentation of SfbI and FnBPA showing 1 - 5 F1-binding repeats (green and orange boxes). S, signal peptide. W, cell wall recognition motif. M, cell membrane spanning region. In SfbI, UR represents the upstream FN-binding domain. In Fn BPA, B1-B2 and box 4-5 represent the upstream FN-binding domain. B, The extended tandem **β -zipper model**. A 1 - 5 F1-binding segment is shown (grey line) with the binding β -strand (navy blue) of five sequential modules of consensus F1 fold (light blue). (Figure adopted from Schwarz-Linek, Werner et al. 2003)

The absence of the typical FnBPs and UFBD described above in Shr is intriguing and suggests that Shr binds to fibronectin using a different binding module(s) (Fig. 4A). NEAT domains have diverse ligand specificity, some interact only with heme while others bind heme and/or heme-containing proteins, such as hemoglobin and hemoglobin-haptoglobin complex (Grigg, Vermeiren et al. 2007). The single NEAT domain of IsdA, has an unusually wide spectrum of ligands. These include several serum proteins (fetuin, asialofetuin, transferrin and hemoglobin) and the ECM components, fibrinogen and fibronectin (Clarke, Wiltshire et al. 2004). This observation suggests the implication of Shr NEAT domain in ECM binding.

NEAT domains are distributed in a collection of proteins involved in iron scavenging in important Gram-positive pathogens. Many of them are negatively regulated by iron. The NEAT-receptors are typically expressed during infection since iron is highly limited in the human body. NEAT proteins have been drawing an increasing attention in the development of new strategies to prevent and treat staphylococcal diseases. Recently, an investigation of cell wall proteins in *S. aureus* identified two NEAT proteins, IsdA and IsdB, as potential vaccine candidates (Stranger-Jones, Bae et al. 2006). In addition, Kuklin *et al* reported that immunization with IsdB induced rapid antibody responses in rhesus macaques and increased the survival of infected animals in a murine sepsis model. The protection observed in this study correlates with the IgG titer found in the infected animals (Kuklin, Clark et al. 2006). The immunity elicited by IsdA and IsdB is suggested to be mediated by antibody interference with heme acquisition (Kim, DeDent et al. 2010). However, none of these studies has identified the protective epitopes in IsdA and IsdB. The structural homology of different NEAT domains may provide common protective epitopes

in NEAT proteins, including Shr. In this chapter, we analyze the functions and antigenic properties of Shr domains using isolated Shr fragments and antisera raised against them.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* DH5 α and TOP10 were used for cloning and maintenance of plasmids. *E. coli* XL1 Blue, XL10 Gold and BL21 StarTM (DE3) were used for recombinant protein expression. The GAS strains used in this chapter were NZ131 (wild type), ZE4912 (a *shr* deletion-insertion mutant made by replacing an internal fragment with the spectinomycin resistance gene *aad9*) (Fisher, Huang et al. 2008) and the following GAS mutants with in-frame deletion of various *shr* regions: ZE4925 (Δ NEAT1), ZE4926 (Δ NEAT2), ZE4929 (Δ NEAT1-2) (Ouattara, Bentley Cunha et al. 2010). In addition, we used the GAS strains ZE491 (an *mtsR* mutant) (Bates, Toukoki et al. 2005), MGAS5005 (M type 1) and MGAS315 (M type 3). The *Streptococcus dysgalactiae* subsp. *equisimilis* strain ATCC 12388 (Vandamme, Pot et al. 1996) was used to clone *shr*. *E. coli* cells were grown in Luria-Bertani broth at 37°C. *Streptococcus* cells were grown at 37°C in Todd-Hewitt broth (Difco Laboratories) with 0.2% w/v yeast extract (THY). When necessary, ampicillin at 100 μ g/ml or kanamycin at 70 μ g/ml was added to the growth medium.

DNA methods. Chromosomal DNA extraction was performed by MasterPureTM Gram Positive DNA Purification kit (Epicentre). Plasmid DNA was purified with Wizard[®] plus Minipreps (Promega). PCR for cloning was performed by using AccuTaq DNA Polymerase (Sigma-Aldrich). PCR products were purified with QIAquick PCR Purification kit (Qiagen). DNA manipulations including restriction digests, ligation and DNA transformation were done according to the manufacturer's recommendation and with standard protocols (Sambrook 1989). The oligonucleotide primers used are listed in Table 2. The plasmids used are listed and described in Table 3.

Construction of protein expression vectors. (i) Construction of the vectors expressing His₆-maltose-binding protein (MBP)-tagged NEAT1 (pYSH6) or NEAT2 (pYSH8). To generate the expression vectors for the His₆-MBP fusion proteins, we used the two-step cloning approach, Gateway[®] Technology with Clonase II system by Invitrogen (Hartley, Temple et al. 2000). The Gateway[®] Technology is based on the bacteriophage lambda site-specific recombination system, which facilitates the integration of lambda DNA into the *E. coli* chromosome. Naturally, lambda-site-specific recombination occurs between the *attB* site on the *E. coli* chromosome and the *attP* site on the phage genome. This reaction results in two recombinant *attL* and *attR* sites to the lambda genome (Landy 1989). The first cloning step involves a recombination reaction (BP) between an *attB*-flanked DNA fragment of choice and an *attP*-containing donor vector. This reaction produces the entry clone (Fig. 20A). The resulting clone is then used in a second recombination (LR) with a destination vector (pDEST-HisMBP), which contains a His₆-MBP tag (Nallamsetty, Austin et al. 2005). This reaction, which is taking place between the *attL* on the entry clone and the *attR* of the destination vector, generates the expression vector with His₆-MBP fusion protein. The entry clones in this study were generated using DNA fragment containing the NEAT coding sequences amplified from the NZ131 chromosome using primers with attached *attB* sites. The resulting fragments were then cloned into the pDONR[™] 221 by BP Clonase[™] II. The recombinant entry clones were selected on kanamycin (50 µg/ml). The NEAT1 (pYSH5) and NEAT2 (pYSH7) entry clones (Fig. 21 and Fig. 22, respectively) were reacted with the destination vector pDEST-HisMBP, using LR Clonase[™] II. These reactions generated the expression vector pYSH6 (His₆-MBP-NEAT1) and pYSH8 (His₆-MBP-NEAT2), which were selected on ampicillin (100 µg/ml) (Fig. 23 and Fig. 24). The identity and orientation of each insert were verified by restriction enzyme and DNA sequence analysis. **(ii) Construction**

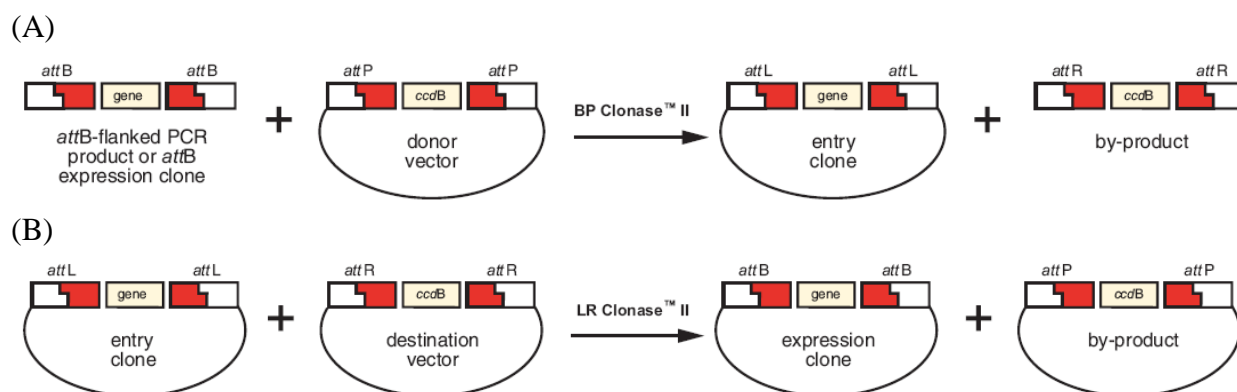


Figure 20. Gateway[®] Recombination reactions.

Two recombination reactions constitute the basis of the Gateway[®] Technology. (A) BP reaction facilitates recombination of an *attB* substrate (*attB*-PCR product) with an *attP* substrate (donor vector) to create an *attL*-containing entry clone. This reaction is catalyzed by BP Clonase[™] II enzyme mix. (B) LR reaction facilitates recombination of an *attL* substrate (entry clone) with an *attR* substrate (destination vector) to create an *attB*-containing expression. This reaction is catalyzed by LR Clonase[™] II enzyme mix. (Figure adopted from Invitrogen User Manual 25-0749)

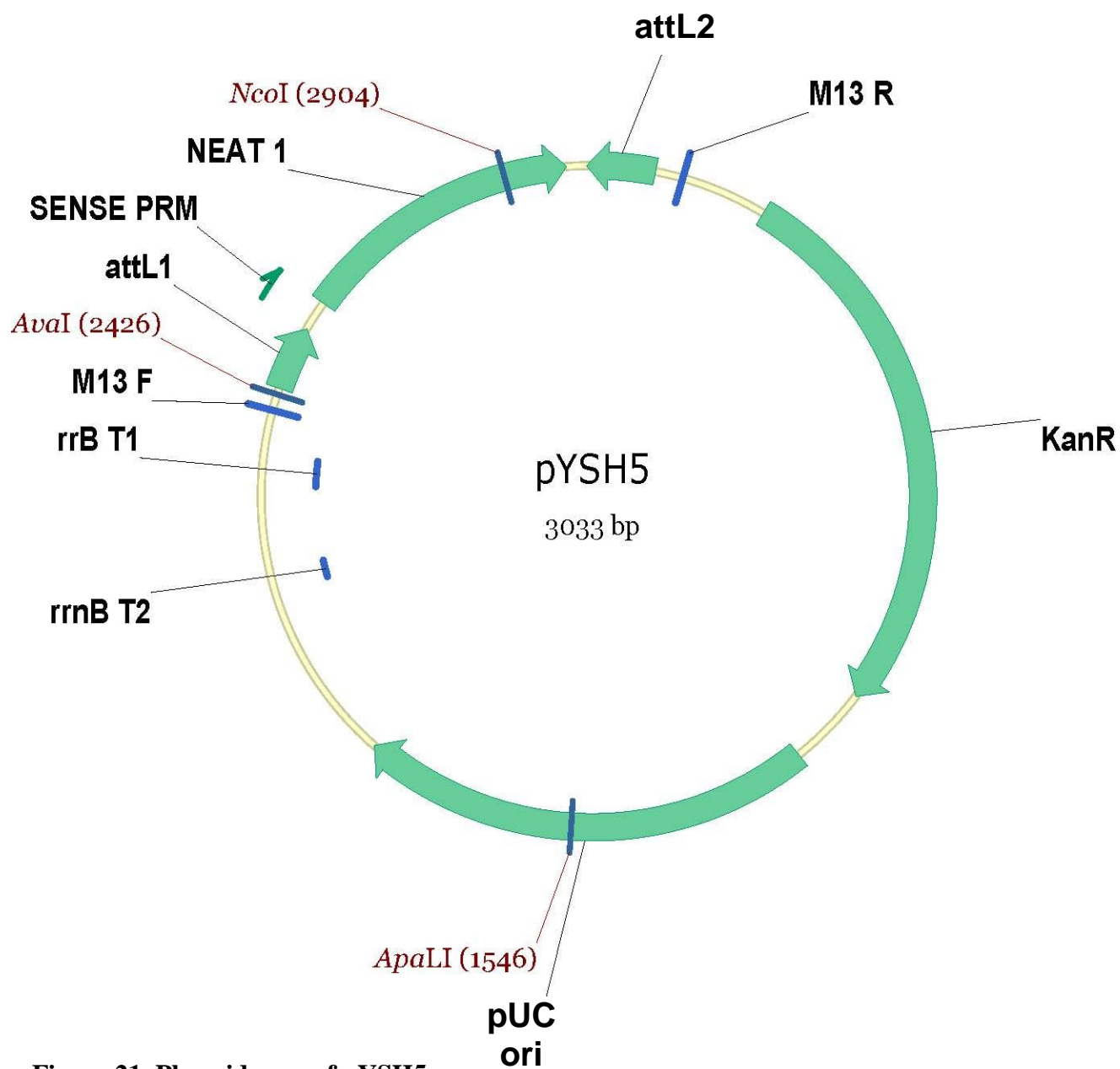


Figure 21. Plasmid map of pYSH5.

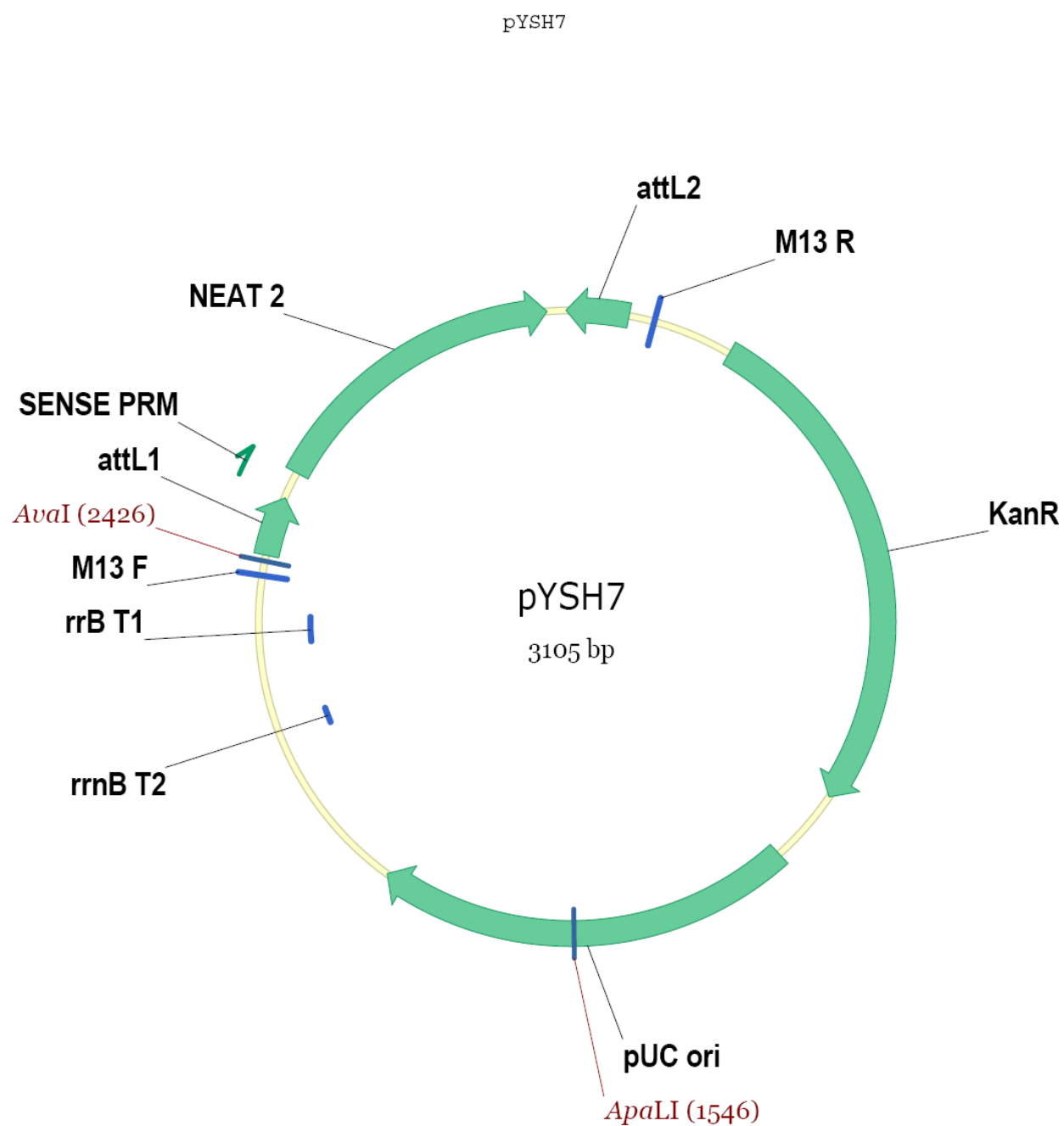


Figure 22. Plasmid map of pYSH7.

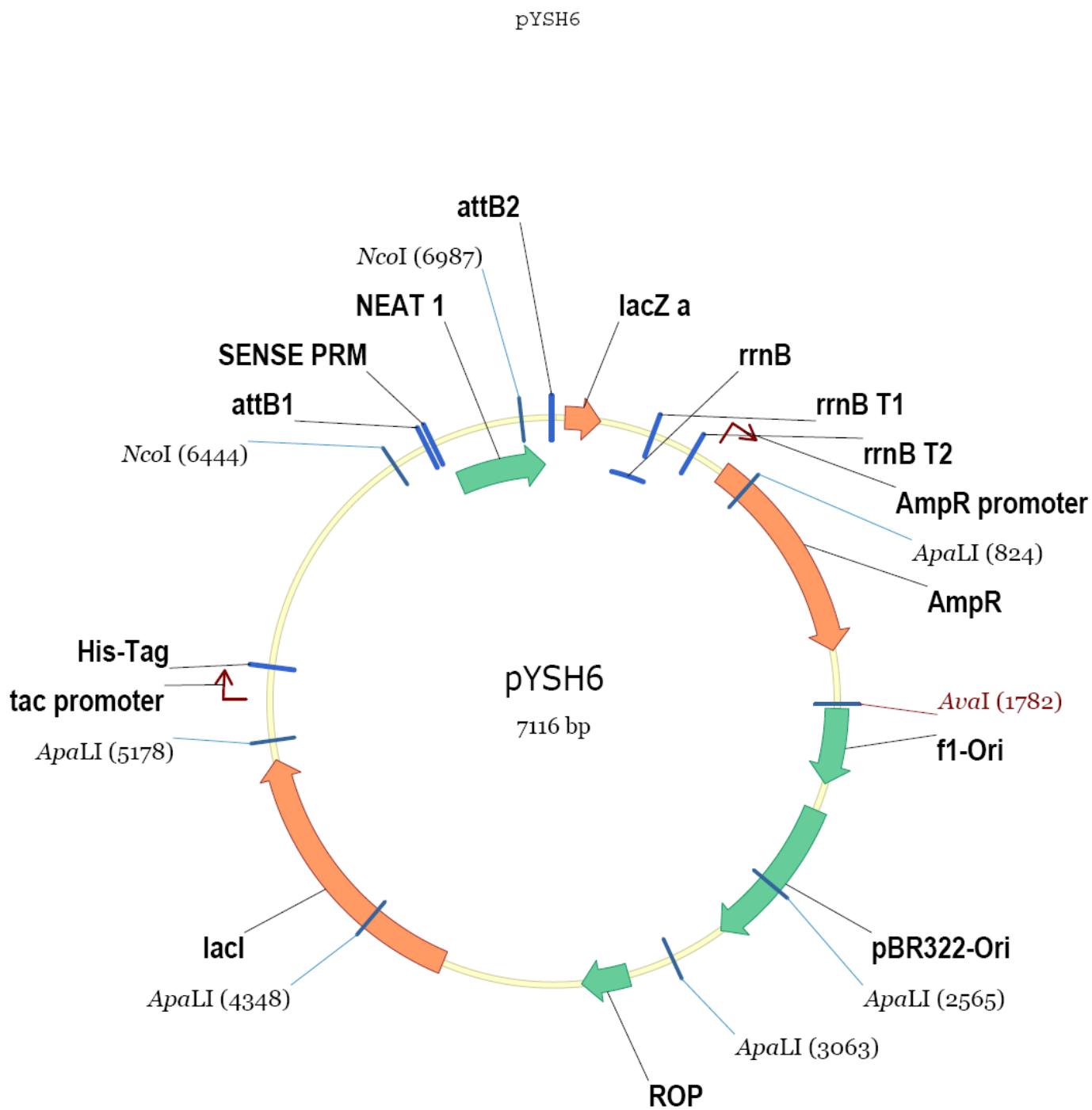


Figure 23. Plasmid map of pYSH6.

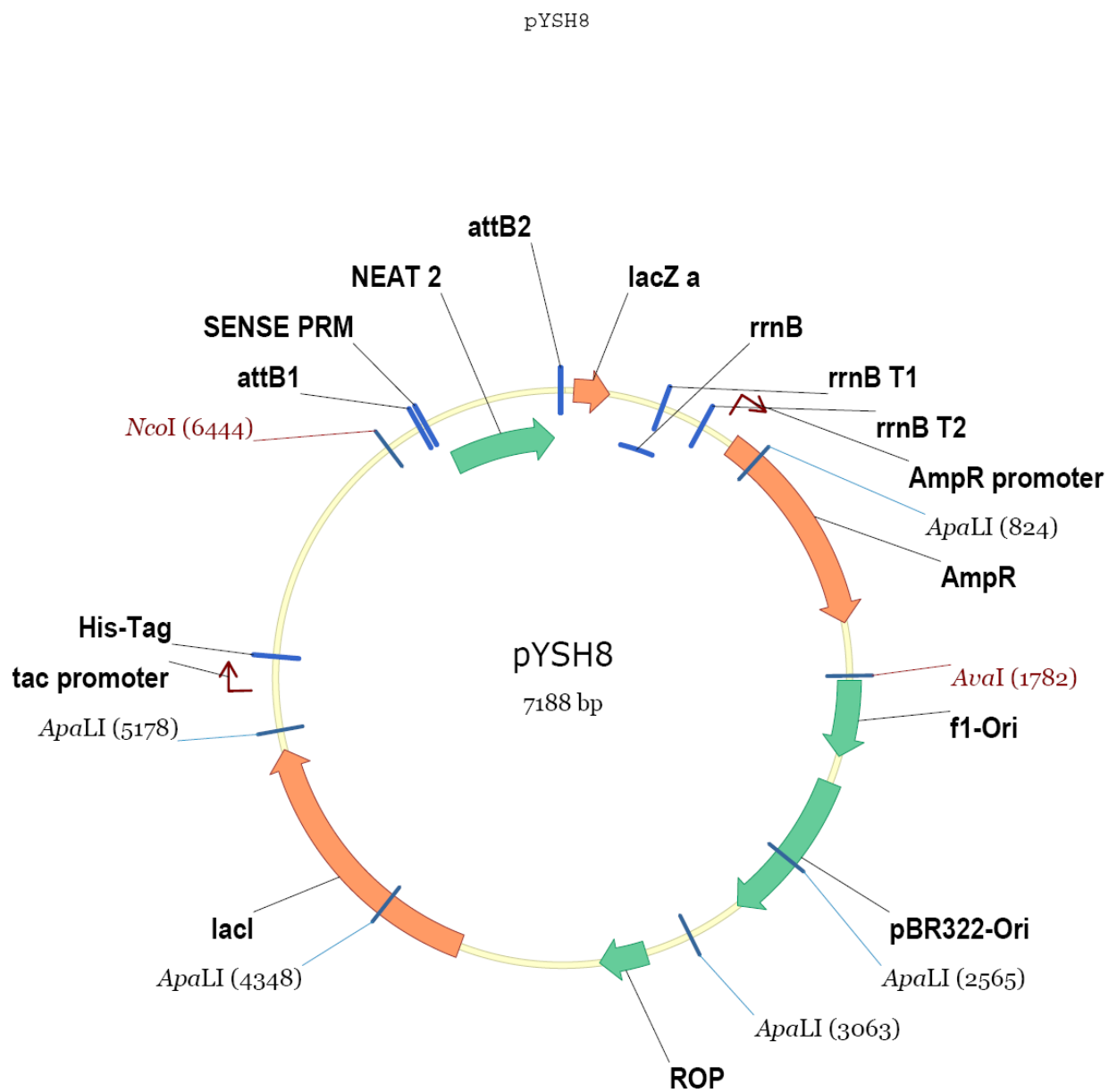


Figure 24. Plasmid map of pYSH8.

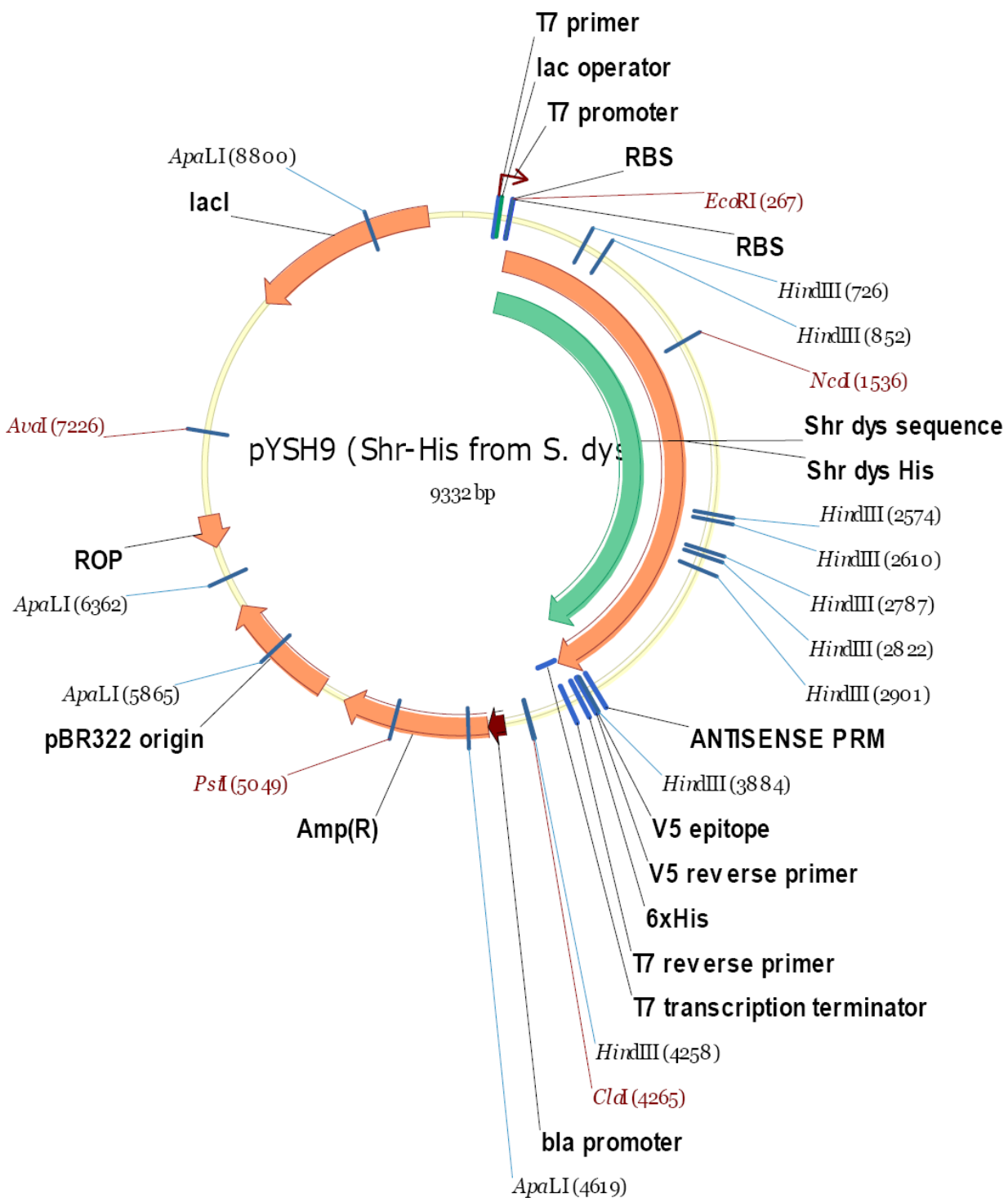


Figure 25. Plasmid map of pYSH9.

of the vector expressing His₆-tagged Shr protein from *S. dysgalactiae*. A 3.6-kb DNA fragment containing Shr coding sequence (except the N-terminal leader peptide and the C-terminal membrane anchoring segment) was amplified from the *S. dysgalactiae* chromosome and cloned into pET101/D-TOPO vector (Invitrogen). The recombinant clone was selected on ampicillin (100 µg/ml) and confirmed by restriction enzyme and PCR analysis. The resulting plasmid, pYSH9 (Fig. 25), expresses His₆-tagged Shr fusion protein under the control of T7 RNA polymerase promoter.

Overexpression and purification of Shr proteins. His₆-Shr (pCB1) (Bates, Montanez et al. 2003) was expressed and purified as described in Chapter I. The expression of His₆-MBP proteins from pYSH6 and pYSH8 and of Shr-His₆ from pYSH9 were induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and purified as previously described (Fisher, Huang et al. 2008). The expression of Strep-NTD (pEB10), Strep-NTD-NEAT1 (pEB11) or Strep-NEAT2 (pHSL2) (Ouattara, Bentley Cunha et al. 2010) were induced with 200 ng/ml anhydrotetracycline overnight at 28 °C. *E. coli* cells were harvested, resuspended in the lysis buffer (100 mM Tris-HCl pH 8, 500 mM sucrose, 1 mM EDTA) containing 0.5 mg/ml lysozyme, 0.5% β-D glucopyranoside and protease inhibitor cocktail (Complete, mini-EDTA-free, Roche) and lysed by gentle sonication. The cell debris was centrifuged and the cleared lysate was then applied to a Strep-Tactin Superflow column (IBA) with a 5 ml bed volume and purified using FPLC. A step gradient elution program was applied and the Strep-tag proteins were eluted with 5 column volumes of 100 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 2.5 mM desthiobiotin. The eluted proteins were analyzed by 10% SDS-PAGE and Western blot using antibody specific for Shr, His-tag, Strep-Tag or MBP as described previously (Bates, Montanez

et al. 2003). The purified proteins underwent dialysis in PBS (pH 7.4) in 1: 40,000 ratio to remove the imidazole from the protein solution. Protein concentrations were measured using the Modified Lowry Protein Assay kit (Pierce Biotechnology) and aliquots were stored at -20 °C for further study.

Antibodies. Rabbit polyclonal antibodies against GAS type-specific carbohydrates, fibronectin and laminin were purchased from Abcam, plc. Rabbit polyclonal antibodies against M protein type 49 were described in chapter I. Rabbit antisera were raised against purified His₆-MBP-NEAT1, His₆-MBP-NEAT2, Strep-NTD and the Shr-His₆ protein from *S. dysgalactiae*. Antisera for all proteins were obtained following previously described protocols (Bates, Montanez et al. 2003). Briefly, the purified proteins (~1 mg) were emulsified with Freund's complete adjuvant (Sigma) and injected each into female New Zealand rabbit. Each rabbit was subsequently immunized with two booster injections in Freund's incomplete adjuvant (Sigma), at two weeks intervals. Serum samples were collected one week after each booster to monitor titer development. The specificity of each antiserum was verified by Western Blot and ELISA and the titer was determined by ELISA. This protocol was approved by the Georgia State University Institutional Animal Care and Use Committee.

Enzyme-linked Immunoabsorbent Assays (ELISA). The following ELISAs with immobilized proteins or whole cells were performed. **(i) Ligand binding by immobilized Shr domains.** This assay was used to investigate binding between various host proteins and isolated Shr domains. 96-well EIA/RIA microplate wells (Costar, Corning) were coated overnight at 4 °C with 20 pmole of purified Shr proteins, washed and blocked with PBST-5% skim milk. Ligands in

increasing concentrations (in PBST-1% skim milk) were allowed to react with the coated wells for 1 h at 37 °C. A ligand-specific antibody (diluted in PBST-1% skim milk) was added to the wells and the plates were incubated for 1 h at 37 °C. The wells were then washed with PBST and binding was detected with alkaline phosphatase-conjugated secondary antibody (Sigma) diluted in PBST-1% skim milk. The chromogenic reaction was allowed to develop for 30 min following the addition of the pNPP substrate (KPN). Plates were then read at 405 nm using an automated reader. **(ii) Antiserum binding to immobilized bacteria.** This assay was used to study the surface accessibility of Shr proteins. GAS cells at $OD_{600} = 0.7$ were harvested, washed with PBS and immobilized overnight at 4 °C onto 96-well EIA/RIA microplate wells. Excess cells were aspirated and the plates were washed with PBST. After blocking with 5% skim milk in PBST for 1 h at 37°C, the wells were washed with PBST and incubated with primary antibodies for 1h at 37°C. The microplates were next washed with PBST and the antigen-antibody binding was detected with AP-conjugated secondary antibodies as described above.

Mouse passive immunization and infection.

For passive immunization, 100 µl of Shr specific antiserum or normal rabbit serum (NRS) was mixed with 400 µl PBS and administrated intraperitoneally. MGAS5005 cells at the mid-logarithmic phase ($OD_{600} = 0.7$) were harvested, washed and resuspended in saline. Cell concentration was determined by microscopic counts and verified by plating. Mice were infected with 0.1 ml of bacterial suspension (5×10^7 cfu) 4-5 hr after the antiserum injection and were checked four times a day after challenge. Animals exhibiting signs of severe distress were euthanized and counted as dead. All experiments were repeated three times. These experiments were conducted according to protocols approved by the Georgia State University Institutional Animal Care and Use Committee.

Opsonophagocytic Killing Assay

This assay was performed as described in Chapter II. Essentially, ZE491 GAS cells ($OD_{600} = 0.7$) were harvested and washed. A total of 0.1 ml of diluted (1: 200,000) cells were added into 0.5 ml of fresh rabbit blood, together with 0.1 ml of rabbit Shr specific antiserum or NRS (as control) and were incubated with gentle inverting at 37°C for 3 h. Surviving bacteria were enumerated by plating for viable counts. Opsonization expressed as the percentage of reduction in mean CFU was calculated as $[1 - (\text{CFU in the presence of Shr antiserum}) / (\text{CFU in the presence of NRS})] \times 100$ (Olive, Sun et al. 2006).

Table 2. Oligonucleotide primers used in Chapter III.

Name	Sequence (5'-3')	Location
ZE353	gagaacctgtacttccagtcttatcaagcaggtgaggttct	TEV-NEAT1
ZE354	ggggaccactttgtacaagaaagctgggtattaatcatcagtttctacctgataacc	<i>attB2</i> -NEAT1
ZE355	ggggacaagtttgtacaaaaagcaggctcggagaacctgtacttccag	3'MBP-TEV
ZE356	gagaacctgtacttccaggagaacggccttagagcattaaat	TEV-NEAT2
ZE357	ggggaccactttgtacaagaaagctgggtattattgtggattattagtttcttgac	<i>attB2</i> -NEAT2
ZE410	caccatgcaattagcgggaaaacaatac	Shr <i>(S. dysgalactiae)</i>
ZE411	gcgattagggctatctttaaatttgg	Shr <i>(S. dysgalactiae)</i>

Table 3. Plasmids used in Chapter III.

Plasmid	Description	Reference or source
pDONR™ 221	Gateway® donor vector for cloning entry vectors	Invitrogen
pYSH5	Entry vector carrying the NEAT1 domain from NZ131	This study
pYSH6	Entry vector carrying the NEAT2 domain from NZ131	This study
pDEST-HisMBP	Gateway® destination vector for cloning MBP fusion proteins	Addgene (Nallamsetty, Austin et al. 2005)
pYSH7	Expression vector for the His ₆ -MBP-NEAT1 fusion protein	This study
pYSH8	Expression vector for the His ₆ -MBP-NEAT2 fusion protein	This study
pET101/D-TOPO	Directional TOPO cloning vector for cloning C-terminal His ₆ tagged protein	Invitrogen
pYSH9	Expression vector for the Shr-His ₆ (<i>S. dysgalactiae</i>)	This study
pCB1	Expression vector for the His ₆ -Shr (<i>S. pyogenes</i>)	(Bates, Montanez et al. 2003)
pEB10	Expression vector for the Strep-NTD (<i>S. pyogenes</i>)	(Ouattara, Bentley Cunha et al. 2010)
pEB11	Expression vector for the Strep-NTD-NEAT1 (<i>S. pyogenes</i>)	(Ouattara, Bentley Cunha et al. 2010)
pHSL2	Expression vector of the Strep-NEAT2 (<i>S. pyogenes</i>)	(Ouattara, Bentley Cunha et al. 2010)

RESULTS

The NEAT 2 mediates most of Shr binding to ECM.

We have recently found that Shr functions as an adhesin and binds host fibronectin and laminin. To determine the domains contributing to the ability of Shr to bind protein components of ECM, immobilized Shr, NTD, NTD-NEAT1 and NEAT2 were allowed to react with the ECM components using ELISAs. Ligand binding was detected with antibodies specific for fibronectin or laminin. When fibronectin was added in the course of increasing concentrations to the immobilized proteins, Shr as well as the recombinant fragments NTD-NEAT1 and NEAT2 bound to it in a concentration-dependent then a saturated manner (Fig. 26A). The NEAT2 protein demonstrated the highest binding to fibronectin, while only low level binding was seen in NTD-NEAT1. No interactions with fibronectin were demonstrated by the immobilized NTD protein. These observations suggest that NEAT regions mediate the observed Shr binding to fibronectin. Similar observations were made with laminin. As shown in Fig. 26B, Shr and NEAT2 proteins bound to laminin, while no significant binding to laminin was observed by the NTD or NTD-NEAT1. In short, these observations indicate that while both Shr NEAT domains contribute to the interaction with ECM components, the NEAT2 domain plays a major role in the ECM-binding activity of Shr.

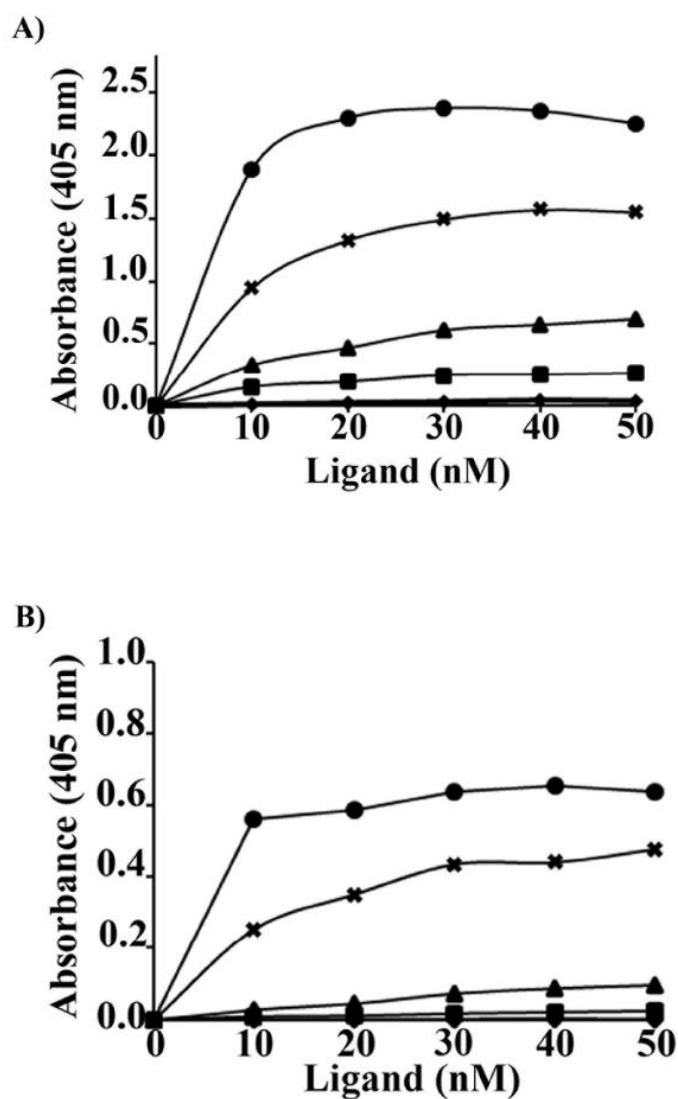


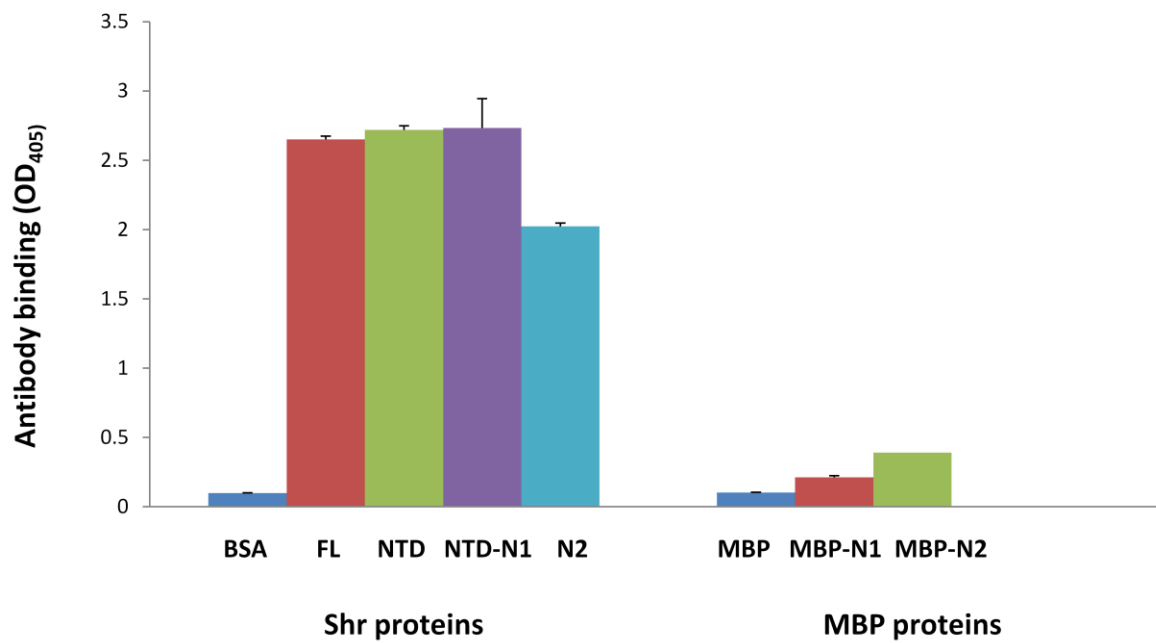
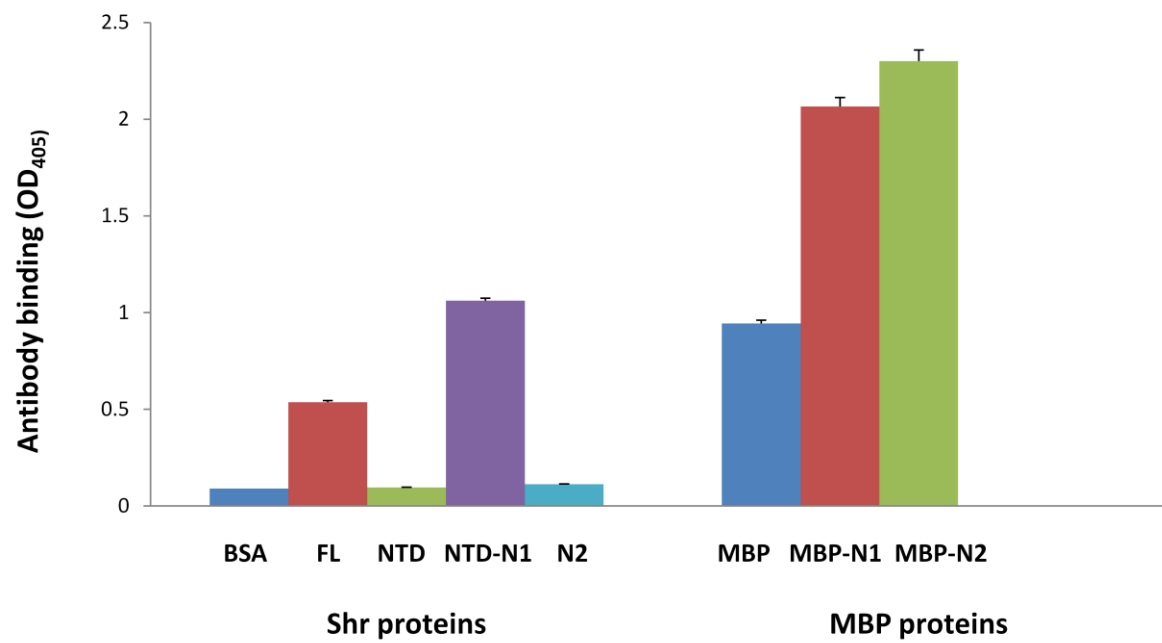
Figure 26. Binding of extracellular matrix proteins by Shr.

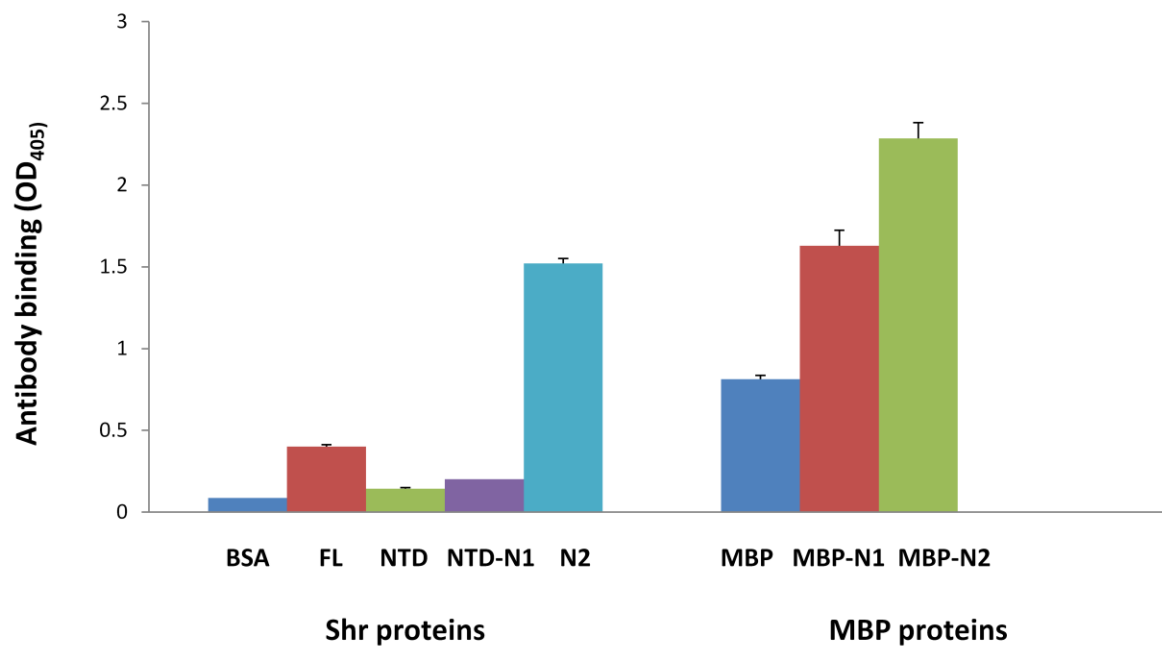
ELISA assays show fibronectin (A) and laminin (B) binding by Shr (crosses) and NEAT2 (dots). NTD-NEAT1 (triangles) slightly bound fibronectin but did not bind to laminin. In contrast, NTD (squares) or BSA (diamonds) did not bind to fibronectin or laminin. The plates were coated with rShr or Shr fragments and subsequently reacted with increasing concentrations of fibronectin or laminin as described in Materials and Methods. Each data point represents the average of triplicates of at least 3 independent repeats.

Production of antisera to Shr domains with high titers

Separate Shr domains mediate the protein binding to different ligands: the NTD binds to hemoglobin while the NEAT domains bind to heme and host ECM protein components (Ouattara, Bentley Cunha et al. 2010). To further investigate the contribution of each domain to Shr function and role as a protective immunogen, antibodies specific for separated Shr regions were generated. We previously found that the NEAT domains are insoluble and accumulate in inclusion bodies when they are overexpressed as separate protein fragments. To increase solubility, we cloned NEAT1 and NEAT2 as fusion proteins to MBP under T7 RNA polymerase promoter system (pYSH6 and pYSH8 respectively). A previously described Strep-NTD protein (Ouattara, Bentley Cunha et al. 2010) and the two NEAT fusion proteins were overexpressed and purified by affinity chromatography. The purified proteins were used to immunize rabbits. Serum samples were collected a week after each antigen administration and titer development was monitored by ELISA. Antibody titers were defined as the serum dilution producing the same OD₄₀₅ at 2 times of the background level (i.e. the reading obtained with serum collected from the same rabbit before immunization). The titer of anti His₆-MBP-NEAT1 and anti-NTD are 6.25×10^6 while the titer of anti His₆-MBP-NEAT2 is 1.25×10^6 . Antigen recognition and specificity of each antiserum were analyzed by Western blot assay and ELISA. BSA was used as a negative control. To exclude the interactions with the MBP portion of the His₆-MBP-NEAT1 and His₆-MBP-NEAT2 fusion proteins, the antisera were reacted with Strep-NTD, Strep-NTD-NEAT1 and Strep-NEAT2 in addition to the antigen used in vaccination and the full-length Shr protein (Fig. 27).

The NTD antiserum reacted strongly (~26-fold over the background) and equally with the full-length Shr and the Strep-NTD, suggesting that this domain is well accessible to antibody even when it is presented as part of the entire Shr protein. Surprisingly, the NTD antiserum crossreacted with both of the NEAT domains. Its reaction with the NEAT2 fragment was the strongest (4-fold above the background) (Fig. 27A). His₆-MBP-NEAT1 antiserum reacted with both NEAT1 and MBP, as expected. This antiserum was specific for the NEAT1 domain and did not demonstrate significant interaction with the isolated NTD or NEAT2 proteins (Fig. 27B). Anti His₆-MBP-NEAT1 also interacted with the full-length Shr, however, this reaction was modest (5-fold above the background) comparing to the reaction of the NTD antiserum. Therefore, NEAT1 is at least partially available for antibody in the intact Shr protein. His₆-MBP-NEAT2 antiserum reacted with both NEAT2 and MBP (Fig. 27C). Like the anti His₆-MBP-NEAT1, the antiserum for NEAT2 appeared specific and did not show significant interaction with NEAT1 and NTD. The Anti His₆-MBP-NEAT2 recognized the full-length Shr, producing a signal that is 4-fold over the background, and thus demonstrated that this domain is also available on the surface of the full-length Shr.

(A) Anti-NTD**(B) Anti His₆-MBP-NEAT1**

(C) Anti His₆-MBP-NEAT2**Figure 27. Specificity of Shr domain antibodies.**

The Shr protein or MBP-fusion proteins were immobilized in equal molarity onto microtiter plates and allowed to react with antisera raised with isolated Shr domains using ELISA. Antisera to the following proteins were used: strep-NTD (A), His₆-MBP-NEAT1 (B) and His₆-MBP-NEAT2 (C). BSA and MBP serve as controls for background signal. FL, full-length Shr; NTD, N-terminal domain; NTD-N1, NTD-NEAT1 fragment; N2, NEAT2 domain; MBP-N1, His₆-MBP-NEAT1 protein; MBP-N2, His₆-MBP-NEAT2 protein.

Surface presentation of Shr domains

The antibodies raised for isolated Shr domains were used to determine the availability of separate Shr regions on GAS surface by ELISA with immobilized GAS cells (Fig. 28). Commercially available antibodies for GAS surface carbohydrates and NRS were used as positive and negative controls respectively. The antiserum reaction with cells of the wild type strain (NZ131) was compared to that with the *mtsR* mutant (ZE491), in which Shr expression is upregulated. As expected, the carbohydrate-specific antibody produced a clear signal (~ 3-fold above the background) in both of the tested strains. The antiserum raised against the full-length Shr generated a reaction that was 2- and 4-fold above the background with NZ131 and ZE491 respectively. This demonstrates that, as we reported in Chapter II, Shr is readily available on GAS surface. However, in the wild type strain, only the NTD antibody reacted with the immobilized cells, and no significant reaction was observed with either NEAT1 or NEAT2 antiserum. Repetition of the analysis with cells that overproduced Shr (ZE491) allowed the detection of the receptor by all of the tested antisera. While the strongest reaction was observed with anti-NTD (OD₄₀₅ = 1.35), both NEAT1 and NEAT2 antisera reacted effectively with the immobilized cells (OD₄₀₅ = 0.3 and 0.33 respectively). Therefore, all 3 domains appear to, at least in part, transverse the thick cell wall and are available on the surface. The reduced efficiency of NEAT1 and NEAT2 antisera when compared to anti-NTD or anti-Shr could result from the lower expression of Shr in the wild type strain. Alternatively, a surface protein that is missing in the *mtsR* mutant might mask NEAT1 and NEAT2 domains in the wild type strain.

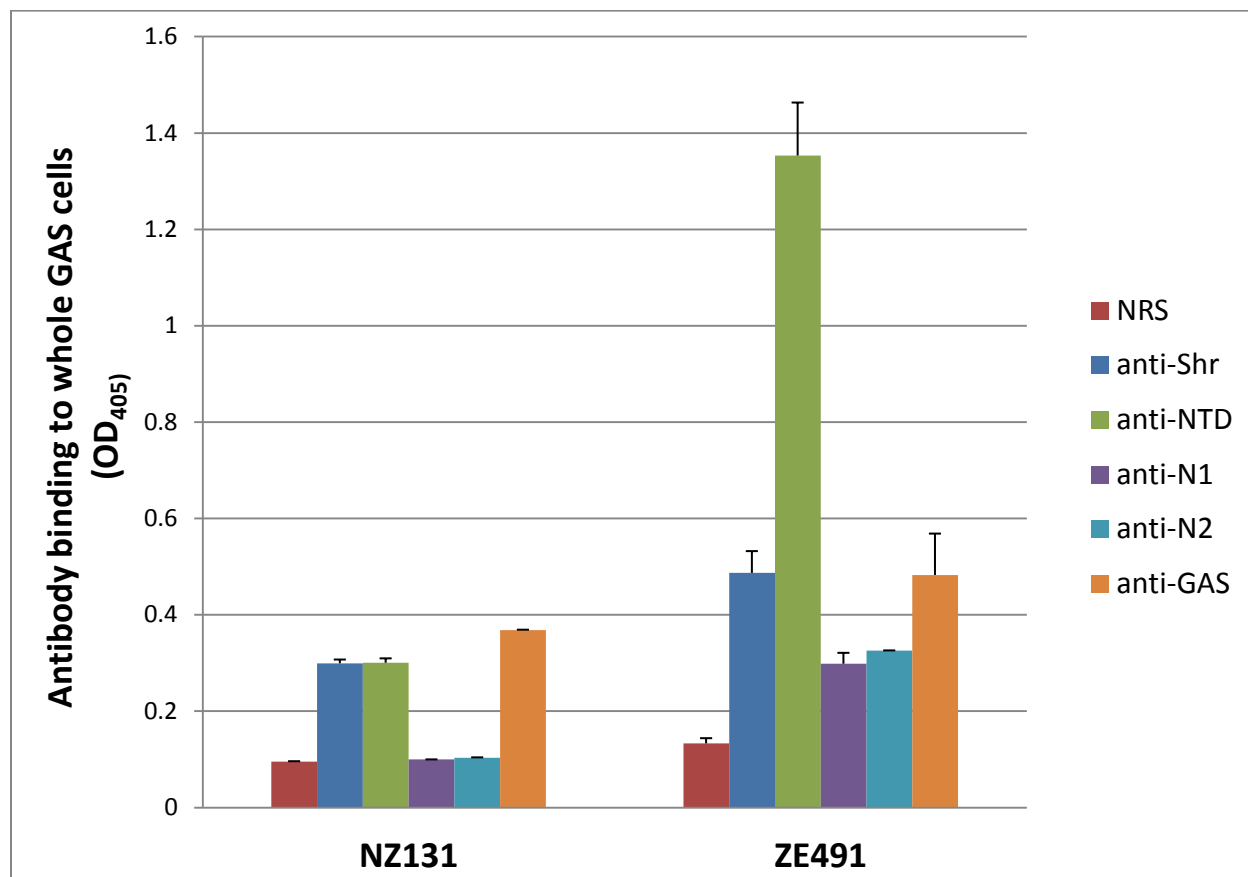


Figure 28. Surface presentation of Shr domains by whole cells.

Cells of the GAS wild type strain NZ131 and the *mtsR* mutant ZE491 were allowed to react with Shr antisera using ELISA with immobilized bacteria. Normal rabbit serum (NRS) served as the negative control. Antisera raised with the full-length Shr (anti-Shr), strep-NTD (anti-NTD), His₆-MBP-NEAT1 (anti-N1), His₆-MBP-NEAT2 (anti-N2) and GAS carbohydrate antibody (anti-GAS) were used.

Passive immunity with antisera raised against isolated Shr domains

Our previous studies showed that rabbit antiserum raised against the full-length Shr protects naive mice from GAS infection. Pretreatment of mice with Shr hyper immune serum (with a titer of 10^6) prior to intraperitoneal infection of MGAS5005 (M type 1) or MGAS315 (M type 3) increased mice survival 2-3 folds when compared to mice administrated with NRS. Some of the Shr domains are shared by proteins from important Gram-positive pathogens. This raises the possibility of developing broad protection based on Shr fragments. We therefore asked which of Shr regions is protective in a passive immunization model. These experiments were performed as described in chapter II, except for that the Shr antibodies were replaced by domain antisera. Mice were treated with NRS, NTD, NEAT1 or NEAT2 antiserum 4-5 hours prior to intraperitoneal infection with 5×10^7 cfu of MGAS5005 strain and monitored for 5 days. The end point survival rate of mice administrated with the NTD, NEAT1 or NEAT2 antisera were 60%, 27% and 27%, respectively, whereas 40% survival rate was observed with the control mice that were treated with NRS (Fig. 29). Therefore, unlike the protection observed with antiserum raised against the full-length Shr, none of the tested antibodies significantly defended mice from GAS. The survival curve of mice treated with anti-NTD was not statistically different from the mock vaccinated mice. Surprisingly, mice administrated with NEAT1 and NEAT2 antisera showed lower survival rate (27% and 27%, respectively) than the control group (40% survival rate) and the group treated with anti-NTD (60%). Therefore, it appears that the antisera specific for the NEAT domain sensitize the mice for GAS infection.

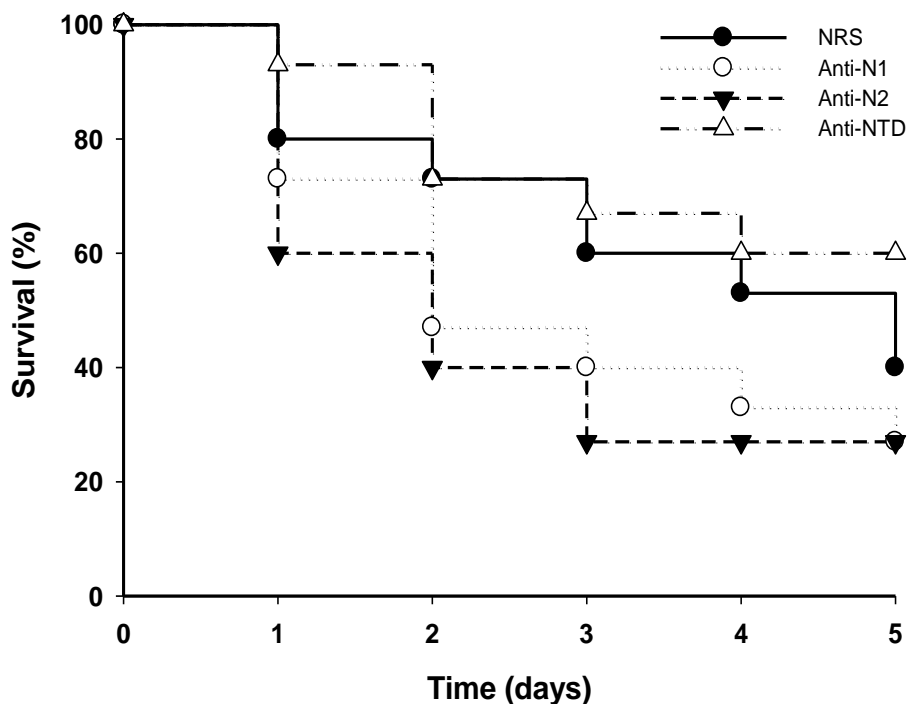


Figure 29. Passive immunization by Shr domain-specific antibody.

Kaplan-Meier survival curves are shown for mice challenged with 5×10^7 CFU of MGAS5005 strain 4 h after intraperitoneal administration of rabbit Shr antisera. Antiserum for His₆-MBP-NEAT1 (anti-N1), His₆-MBP-NEAT2 (anti-N2), strep-NTD (anti-NTD) or normal rabbit serum (NRS) were used. The data shown are pooled from three independent experiments (total n=15 for all groups). The statistical significance was determined by the log-rank test.

Opsonophagocytosis by antisera raised against isolated Shr domains

The ability of the NEAT1 and NEAT2 antisera to opsonize bacteria in whole blood was compared to that of the serum raised against the full-length Shr. ZE491 cells were incubated in whole blood in the presence of different Shr antisera or NRS. GAS viable counts were determined following 3 hours of incubation in blood. As reported before, Shr antiserum resulted in a significant bacterial opsonization ($70\% \pm 17\%$) in comparison to NRS (Fig. 30). A similar reduction in bacterial survival was found in cultures that were treated with NEAT1 ($69\% \pm 29\%$) or NEAT2 ($85\% \pm 2\%$) antiserum, respectively.

Production of anti-Shr from *S. dysgalactiae*

S. dysgalactiae codes for an Shr orthologue that shares the same molecular architecture with GAS Shr (See Fig. 18). To begin investigating the role of this protein in pathogenesis, an antibody against this protein was therefore generated. A His-tagged recombinant protein (132 kD) was constructed, over-expressed, purified and administered to rabbits. The development of titers was monitored from the rabbit serum collected a week after each injection or boost. One week after the final immunization, the final IgG titers were determined as 1×10^6 by ELISA.

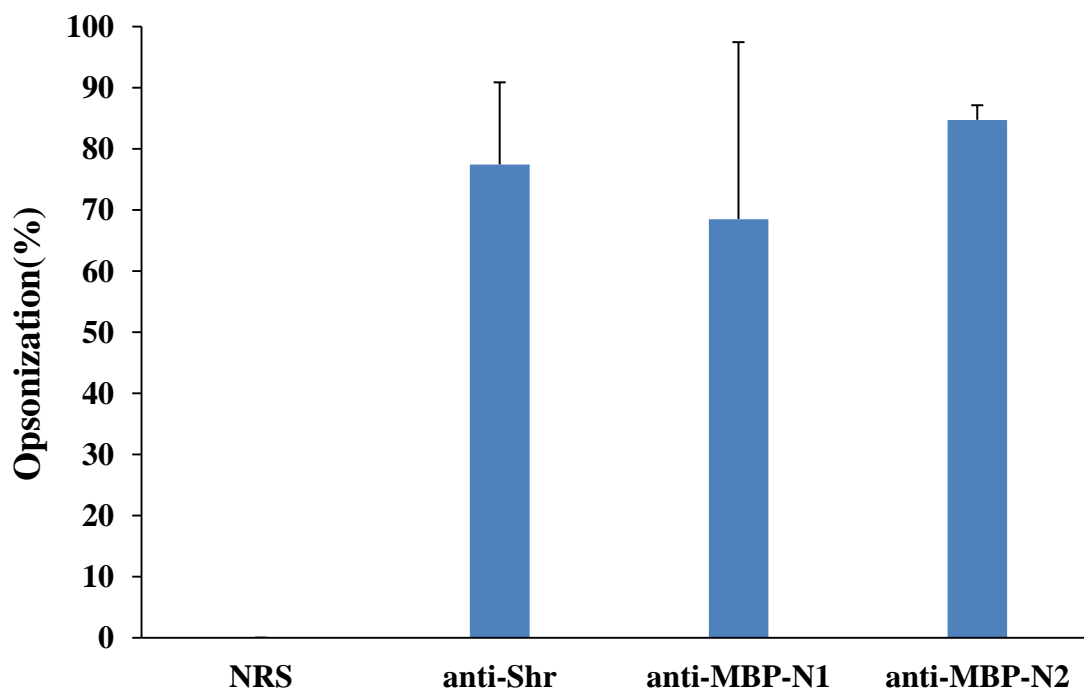


Figure 30. Opsonization of bacteria with Shr domain-specific antibodies.

ZE491 cells were incubated in rabbit whole blood in the presence of rabbit antiserum for Shr, His₆-MBP-NEAT1, His₆-MBP-NEAT2 or normal rabbit serum (NRS). Bacterial growth after 3 h of incubation with Shr antiserum was compared with that in the presence of the control NRS. The mean opsonization percentage (measured as the percentage reduction in CFU) derived from 4 independent experiments is shown; the standard deviation (SD) is represented by the error bar. (The data of NRS and anti-Shr were previously described in chapter II.)

DISCUSSION

The protein domains of Shr in GAS or its orthologues in other Streptococci have been studied by our laboratory and other investigators. These studies have largely focused on Shr binding to hemoglobin and heme (Zhu, Liu et al. 2008; Meehan, Burke et al. 2010; Ouattara, Bentley Cunha et al. 2010). In Chapter I, we determined that in addition to its role in iron acquisition, Shr binds to fibronectin and laminin and functions in adherence. However, the mechanisms of Shr binding to ECM remained unknown. In chapter II, we demonstrated that Shr is a protective antigen in GAS, but the involvement of the separate domains in the induced immunity was not illustrated. In this chapter, we investigated Shr functional domains focusing on the NTD, NEAT1 and NEAT2 regions. We localized the binding to fibronectin and laminin to the NEAT domains of Shr and showed that it is mostly mediated by NEAT2. We raised antisera for separate regions of Shr and used them to show that the NTD, NEAT1 and NEAT2 domains transverse GAS peptidoglycan layer and are exposed on the surface. In addition, we tested the ability of the domain-antibodies to defend mice and to opsonize bacteria. The Shr antibodies were bactericidal in whole blood. However, none of the antisera were able to protect mice effectively from infections. Surprisingly, both NEAT1 and NEAT2 antibodies appeared to increase mice sensitivity to GAS infections.

NEAT2 strongly interacts with both fibronectin and laminin molecules, while NEAT1 binds only to fibronectin and with a weaker affinity (Fig. 26). Therefore, NEAT2 appears to play more significant role in Shr interactions with the ECM. This is the first time that NEAT domains are found to be part of MSCRAMMs in GAS. Ligand interactions with the common fibronectin binding proteins in GAS had been extensively studied (Talay, Valentin-Weigand et al. 1994;

Jaffe, Natanson-Yaron et al. 1996) . For example, SfbI and F2 bind fibronectin through a tandem β -zipper binding mechanism using fibronectin-binding repeats (Schwarz-Linek, Werner et al. 2003). These repeats are 35-40 residues long and are located at the C-terminal half of the adhesin. A second region found immediately upstream to the repeats acts to enhance SfbI affinity to fibronectin (Fig. 19). The number of the fibronectin-binding repeats in SfbI ranges from 2 to 7 in different strains, suggesting that the protein evolution involves domain deletion and duplication events (Towers, Fagan et al. 2003). Protein F2 is only functionally related to SfbI and is expressed in a small subset of GAS strains that do not express SfbI. Protein F2 has two fibronectin binding domains in its C-terminus, which are ~100 aa residues apart. One of these regions is composed of three consecutive repeats of 21-37 residues and the other is made of ~100 non-repeated residues. Shr lacks all the binding repeats described for GAS or other bacteria and thus appears to use a new protein pattern and mechanism to bind fibronectin.

In addition to the interactions with ECM components, both of the NEAT domains in Shr bind heme, and therefore exhibit an unusual ligand binding property. The only example of a NEAT domain that binds ligand other than heme and heme-containing proteins is the single NEAT domain of the staphylococcal IsdA protein. IsdA is an iron-regulated adhesin that interacts with a broad spectrum of ligands, including heme, several heme proteins, fibronectin and fibrinogen (Clarke, Wiltshire et al. 2004). IsdA binds these ligands with a single NEAT domain. Therefore, some NEAT domains may have a binding motif for some ECM proteins. The crystal structure of several NEAT domains including that of IsdA (PDB entry 2ITF) has been solved (Pilpa, Fadeev et al. 2006; Grigg, Vermeiren et al. 2007; Sharp, Schneider et al. 2007). These studies revealed

an immunoglobulin-like β -sandwich fold with a large hydrophobic heme-binding pocket (Fig. 31). The Ig-like fold contains eight β -strands, forming two β -sheets in the sandwich.

To learn more about the possible fold of Shr NEAT domains, we looked for homologous targets in the Swissprot database using BLAST (Basic Local Alignment Search Tool). No matches with significant similarity in primary sequence were found. The two NEAT domains in Shr share 30.9% identity encompassing 55 residues and only ~20% identity with other NEAT domains in the database. We performed structure alignment of Shr NEAT1 and NEAT2 by BLAST analysis with PDB (Protein Domain Bank). While NEAT1 matched two domain models with 27% sequence similarity, NEAT2 failed to match any modeled structure. The two models that matched NEAT1 are that of IsdC protein from *S. aureus*. One is a crystal structure with heme bound that was solved by X-ray fractionation (PDB entry 2O6P) and the other is in solution with zinc protoporphyrin, which was predicted by NMR (PDB entry 2k78). IsdC is the central heme shuttle protein in the Isd system. It receives heme from IsdA, IsdB and IsdH and delivers it to the cognate ABC transporter. Based on the information of 2O6P given by PDB, we used two structure modeling servers to predict the structure of a heme-bound NEAT1 (Fig. 32). This analysis predicts that NEAT1 shares notable structural similarity with that of IsdA and suggests that NEAT1 has an immunoglobulin-like β -sandwich fold composed of eight β -strands that form the heme-binding pocket.

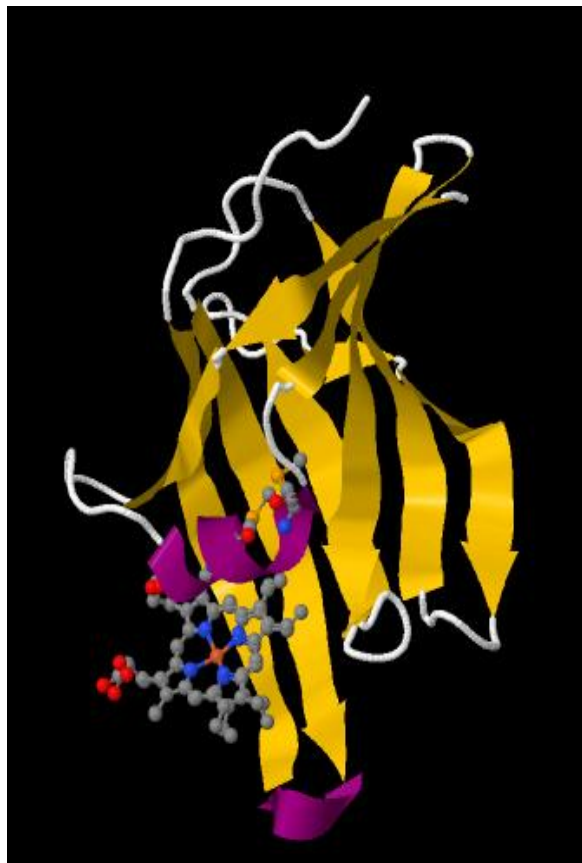


Figure 31. Crystal structure of heme-bound IsdA NEAT domain from *Staphylococcus aureus*.

Secondary structure of the IsdA NEAT domain bound with the heme (PDB entry 2ITF). Yellow ribbons represent β -sheets, pink ribbons represent α -helix. Heme is shown in sticks within the binding pocket (Figure adopted from Grigg, Vermeiren et al. 2007 and viewed by Jmol program).

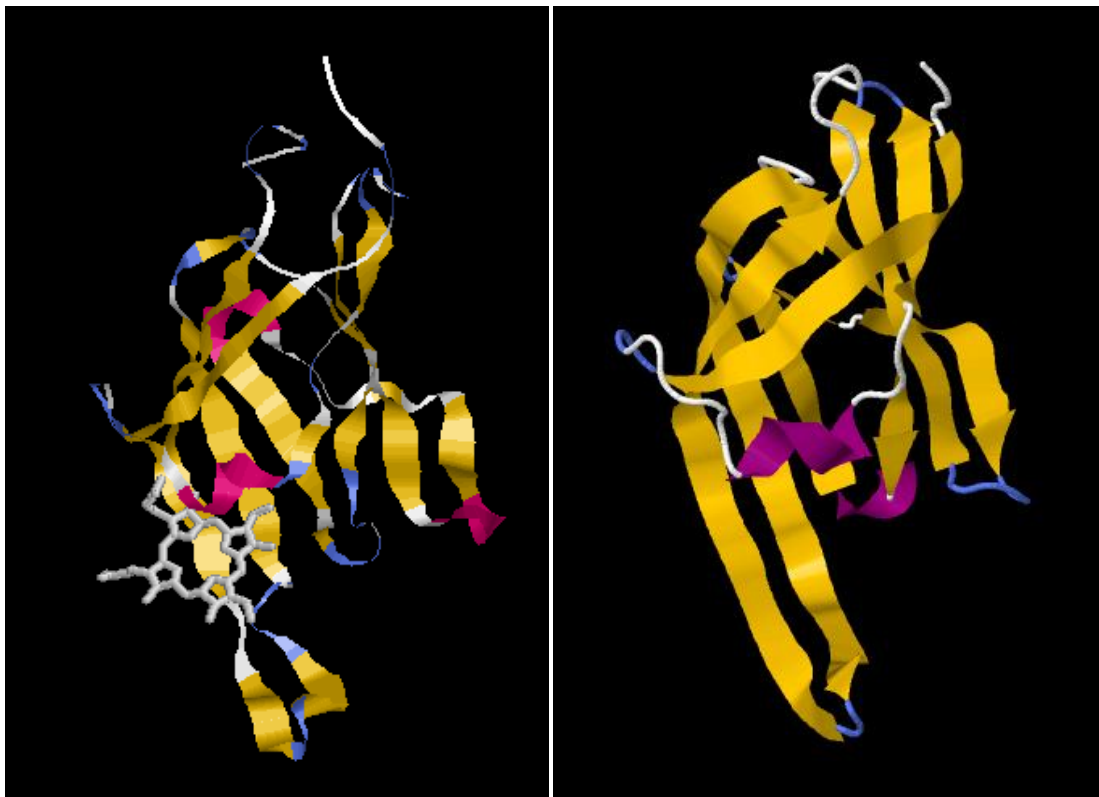


Figure 32. Predicted structure of Shr NEAT1 domain.

(Left) The structure of NEAT1 domain with heme bound is predicted based on the template, IsdC NEAT domain from *S. aureus* (PDB entry 2O6P), by Structure and Alignment Server (<http://bmcc3.cs.gsu.edu/>) and viewed by RasMol program. (Right) The structure of NEAT1 domain is predicted based on the same template by Protein Homology/analogy Recognition Engine v.2 (Phyre2) (Kelley and Sternberg 2009) (right) and viewed by Jmol program. Yellow ribbons represent β -sheets, pink ribbons represent α -helix and blue color represents turns. The gray stick represents the structure of heme (left).

As in all NEAT domain characterized to date, the suggested structure of Shr NEAT1 is rich with β -strands. β -sheets are found in the typical fibronectin binding region of several bacterial receptors and are a structural feature that is important for ligand interaction (Schwarz-Linek, Werner et al. 2003). Fibronectin binding by bacterial receptors involves a β -strand that lines up in an antiparallel arrangement to the triple-strand of the F1 segment in the fibronectin, forming an extended tandem β -zipper (Fig. 19). It is thus conceivable that some NEAT domains also bind fibronectin with one of the eight β -strands at either side of the β -sandwich (Fig. 32). Out of the two NEAT domains in Shr, NEAT2 is the key binder to the ECM. It seems possible that the Shr evolved via duplication of an ancestor NEAT domain followed by divergent evolution that led to domains with separate ligand activity. Domain duplication may facilitate an increased sensitivity to ligands that are present at low concentration such as heme, or it may enhance bacterial interactions with host surface (Andrade, Ciccarelli et al. 2002).

Our analysis demonstrated that the antisera raised with isolated NEAT1 or NEAT2 were quite specific and did not interact significantly with the other NEAT domain (Fig. 27). These observations are surprising as both protein modules are expected to share similar fold. Therefore, distinctive structural characteristics are found in each domain, which lead to unique antigenic determinants. Surprisingly, immunization with Shr NTD produced an antiserum which cross-reacted with both NEAT1 and NEAT2 regions (Fig. 27). Shr NTD does not have significant sequence homology with non-Shr proteins in the database. However, it contains two copies of domain with unknown function (DUF1533) that is found in various copies in mostly hypothetical proteins from the firmicutes phylum. It seems possible that the NEAT domain, which is widely

spread in the Firmicutes, and the DUF1533 share a common ancestor, or that one had evolved from the other. Additional studies are needed to map the epitopes that are shared by Shr NTD and the NEAT domains and to further explore this hypothesis.

We examined the surface architecture of Shr domains in GAS NZ131 (wild type) and ZE491 (*mtsR* mutant) using the antibodies generated with separate Shr regions (Fig. 28). Only the NTD antibody interacted with NZ131 cells, while no reaction with the NEAT1 or NEAT2 antisera was observed. However, a significant reaction between both NEAT antibodies and GAS cells was observed when ZE491 cells were used. Since both antisera are specific and recognize only the NEAT domain used to produce them, this observation indicates that both NEAT domains are exposed at least in part, outside of GAS cell wall. Due to inactivation of *mtsR*, which represses the *shr* gene, the expression of Shr in ZE491 is 3-4 folds higher than that in NZ131. The failure of the NEAT antisera to detect Shr in NZ131 may result from the lower expression of Shr in this strain. It is also possible that Shr NEAT domains are covered by a separate surface protein that is missing in the *mtsR* mutant strain. The NTD antibody produces a very strong signal with both the wild type and *mtsR* mutant strains. This observation suggests that the NTD of Shr is well exposed on the bacterial surface. However, cross reactions with other Shr regions may have also contributed to the interactions between the NTD antiserum and GAS cells.

Because of their surface presentation and contribution to bacterial colonization, NEAT-containing proteins provide a collection of candidates for vaccine development against several Gram-positive pathogens. The protective potential of NEAT proteins from *S. aureus* has been

examined in several vaccine models. For example, a multivalent vaccine that involves IsdA and IsdB protects mice from kidney infections by staphylococcus in both active and passive immunization protocols (Stranger-Jones, Bae et al. 2006). Vaccination with IsdB induced rapid antibody responses in rhesus macaques and defended mice from *S. aureus* in a sepsis infection model (Kuklin, Clark et al. 2006). Immunization with IsdA and IsdB antibodies protected mice from staphylococcal abscess formation and lethal challenge (Kim, DeDent et al. 2010). In addition, mucosal immunization with IsdA-chimera protein induces antigen-specific humoral response in mice (Arlian and Tinker 2011). In Chapter II, we reported that Shr elicit immunity from GAS in mouse infection model. In this chapter, we tested the antigenic and protective potential of separate Shr domains. Hyperimmune sera were obtained for each of the tested antigens, indicating that they are all immunogenic. However, none of these sera were able to effectively defend mice from GAS infection in passive vaccination experiments. Administration of the NTD antiserum resulted in 60% survival, which is higher but not statistically significant than the survival rate of the control group (40%) (Fig. 29). The NTD mediates Shr binding to hemoglobin (Ouattara, Bentley Cunha et al. 2010), which is the first step of heme uptake from the host. Therefore, anti-NTD may prevent hemoglobin binding and thus interferes with the iron uptake process during infections. The NTD contains two copies of DUF1533, which is found in several repeats in putative proteins from *Clostridia* and in two species of *Paenibacillus* (Fig. 18). Hence, the NTD domain may allow the development of broad-spectrum protection. More experiments are needed to examine the effectiveness of NTD based vaccine.

Mice treated with NEAT1 or NEAT2 antiserum showed only 27% survival rate at the end point. This survival rate is lower than that of the control group that was treated with NRS. Therefore, it

seems that these antisera augmented the severity of GAS infections. The lack of protection might be the result of steric hindrance. The weak interactions of the NEAT antisera with wild type GAS, suggest that the domains may be only partially accessible. It is also possible that the protein fold of isolated fragments is different from that in the full length Shr. Therefore, the resulting antibodies may be less effective in interacting with Shr. The increased mortality in the mice treated with the NEAT antisera is surprising and the mechanism involved is not clear. It is possible that these antibodies react with host components and thus impair the host immune response. A structural modeling using the Protein Homology/analogy Recognition Engine v.2 (Phyre2) (Kelley and Sternberg 2009) suggests that a region in Shr that contains the LRR and NEAT2 shares the same fold as toll-like receptors (TLR). It thus seems possible that the NEAT antisera facilitate an autoimmune reaction with TLRs and trigger the production of harmful inflammatory cytokines.

A significant reduction in the number of viable bacteria in whole blood was found in the presence of antiserum against Shr, NEAT1 and NEAT2 in comparison to NRS (77%, 69% and 85%, Fig. 30). Therefore, all the tested antisera were similarly effective in opsonizing GAS. This observation is intriguing, considering that the NEAT antisera reaction with whole GAS cells is weaker than that of anti-Shr antibody (Fig. 28). It is possible that Shr is upregulated in GAS while growing in the blood and that this overexpression facilitates stronger interaction with the antisera. The observed opsonization by the NEAT antibodies also supports the proposal that an autoimmune response rather than inability to interact with GAS cells is responsible for the observed failure to protect mice from GAS infections. Additional work, however, is required to

characterize GAS expression profile in different infection sites (such as blood, intraperitoneal cavity etc.) and to illustrate the interaction of Shr antibodies with GAS and the host components.

In conclusion, this chapter dissects Shr at the submolecular level and illustrates the receptor interactions with host components and antigenic potential. We have mapped the ECM binding region in Shr and suggested a possible binding mechanism for the interactions with fibronectin. We tested availability of Shr domains on GAS surface and initiated the identification of protective regions in Shr.

GENERAL DISCUSSION

GAS is a dangerous human pathogen that produces a number of simple disease as well as severe infections and immune complications. The antigenic variability of key surface proteins in GAS allows this pathogen to escape the host adaptive immune system. As a result, individuals can experience repeating episodes of GAS infections throughout their life span. The long term goal of this dissertation is to gain a better understanding of the molecular mechanisms of GAS virulence, so that new and efficient strategies for prevention and therapy could be developed. The focus of this work is a conserved surface protein named Shr. Shr is a complex protein that contains a unique N-terminal region and two NEAT domains with LRR in between. This dissertation summarizes our investigations of Shr structure and function, its contributions to GAS interactions with the human host and its antigenic properties. In the sections below, we discuss our findings and their importance to the field of GAS pathogenesis and vaccine development.

Shr and adherence

Adherence is an early and critical step in the infection process. GAS can colonize multiple sites in the human body including the epidermis, various niches at the upper respiratory tract and many otherwise sterile tissues. This versatility is reflected in the complex array of adhesins used by GAS. In addition to TA and pili, GAS employs over a dozen of afimbrial adhesins, most of which are MSCRAMMs that target fibronectin and other ECM components (Patti, Allen et al. 1994). One of the main contributions of this dissertation is the finding that Shr is a part of GAS

adhesins family. This receptor specifically binds to fibronectin and laminin and contributes to GAS interactions with epithelial cells (Fig. 7, 8, and 10).

Shr inactivation had only a moderate impact (2 fold) on GAS adherence to the human epithelial Ep-2 cell line (Fig. 10). This cell line was originally thought to be derived from an epidermoid carcinoma of the larynx, but was subsequently traced to HeLa cell contamination. Differently from our studies with Ep-2, significantly larger contribution of Shr to attachment (15 fold) was found when the NCIH292 cell line was tested (Appendix A, Fig. 33). NCIH292 cells are derived from human lung cells and therefore may represent a better adherence model for a mucosal pathogen such as GAS. Shr affinity for specific tissues indicates that *in vivo* the receptor would likely contribute to GAS interactions with host surface only in certain infection sites.

Shr is the first adhesin in GAS that was found to be expressed in response to iron limitation. Other adhesins, such as the M protein, SfbI or SOF are regulated by other environmental cues such as atmospheric compositions, temperature and/or nutrient availability. Therefore, while GAS codes for multiple adhesins, only a subset is likely to be expressed at certain niches in the body. Since tissue models are able to reproduce only some of the conditions that bacteria experience during infection, a better evaluation of Shr contribution to adherence could be achieved using animal models. Indeed, an *shr* mutant is significantly disadvantaged in comparison to the wild type strain in an intramuscular zebrafish infection model (Fig. 12). However, this infection model does not differentiate the relative contribution to iron uptake from that to adherence by the pleiotropic Shr.

Fibronectin binding by Shr

In vitro, Shr binds directly and specifically to fibronectin and laminin, but not to collagen or fibrinogen. Thus, Shr is selective and interacts with only a subset of ECM components (Fig. 7 and 8). Furthermore, *L. lactis* cells expressing Shr gained the ability to exclusively interact with fibronectin (Fig. 9B). These observations demonstrate that Shr mediates whole-cell binding to the ECM and supports its role as a GAS MSCRAMM. Analysis of Shr domains showed that both of the Shr NEAT domains contribute to its interactions with ECM components; however, NEAT2 plays a more prominent role in this process (Fig. 26). The single NEAT domain of IsdA from *S. aureus* also binds fibrinogen and fibronectin in addition to heme (Clarke, Wiltshire et al. 2004). Therefore, like the staphylococcal IsdA, Shr uses the NEAT modules for adherence in addition to heme scavenging. It is interesting that the NEAT in IsdA binds hemoglobin and hemoglobin-haptoglobin complexes. However, this activity in Shr is localized to a separate region in the N-terminal part of the receptor (Ouattara, Bentley Cunha et al. 2010).

Our work provided only the second example for ECM binding by NEAT domains. The mechanisms by which fibronectin and laminin bind to NEAT are still not known. Fibronectin binding by other MSCRAMMs such as SfbI is mediated by a tandem β -zipper mechanism (Schwarz-Linek, Werner et al. 2003). This model involves antiparallel deposition of β -strands of the bacterial receptor to β -strands from the F1 region of fibronectin. Characterized NEAT domains and the predicted fold of Shr NEATs contain several β -strands (Fig 31 and 32). It seems feasible that like in other bacterial receptors, one or more of the β -strands in NEAT participate in fibronectin binding. It is important to note, however, that only a few NEAT domains bind fibronectin and that significant differences in the affinity to fibronectin are found between Shr

NEAT1 and NEAT2. Therefore, additional work is needed to determine exactly how the NEAT module allows binding of non-heme ligands and to identify the specific properties that are required for fibronectin binding by a subset of NEAT domains.

Shr Topology

Typical Gram-positive MSCRAMMs have an N-terminal leader peptide, one or more repetitive motifs that mediate ligand binding and a C-terminal transmembrane domain with the cell wall anchoring motif LPX[T,S,A]G (Patti, Allen et al. 1994). We localized Shr, which lacks the sortase anchoring motif, to the cell membrane fraction in GAS (Fig. 4). We suggest that the hydrophobic segment found in the protein carboxy terminus anchors Shr to the membrane. Nevertheless, Shr is still exposed on the cell surface in both GAS (Fig. 6) and heterologous Gram-positive hosts such as *L. lactis* (Fig. 9A). Since the cell membrane resides underneath the peptidoglycan layers, the extruding portion of Shr protein must pass through the cell wall to extend to the exterior. Using antibodies raised against separate regions of Shr, we showed that epitopes from the NTD, NEAT1 and NEAT2 are available for interactions outside of bacterial surface (Fig. 28). These findings are consistent with the predicted topology of Shr (Mobylye@Pasteur, <http://mobylye.pasteur.fr/cgi-bin/portal.py?#forms::toppred>). This *in silico* analysis suggests that Shr has only two hydrophobic helices; one is located at the N-terminal leading peptide which is cleaved during export and the other is found at the C-terminus and is followed by a few charged residues. Together, our observations and analysis suggest that Shr protrudes through cell wall; the N-terminus of the mature protein and the two NEAT domains are localized at least in part, at the extracellular environment, while the C-terminus is embedded in the cell membrane with the charged tail in the cytoplasm. The availability of NEAT2 domain for

antibody outside of the cell wall is also consistent with the adherence function that we propose for NEAT2.

The multiple fibronectin-binding proteins expressed by GAS complicate the analysis of Shr interactions with this ligand *in vivo*. Therefore, we constructed a number of in-frame deletions in Shr and expressed the mutants in *L. lactis* (Appendix B, Fig. 34). Unfortunately, we found that unlike the full length protein, these *shr* mutants were not stable in *L. lactis*. Thus, new approaches, such as Shr-coated latex beads, are needed to investigate the net effect of Shr on GAS adherence and invasion (Ozeri, Rosenshine et al. 1998).

Antigenic studies of Shr

A key contribution of this work to the field of GAS pathogenesis is the finding that Shr is a protective antigen. *In silico* analysis shows that the *shr* gene is highly conserved in the available GAS genomes. This observation together with our finding that Shr protein was expressed on the surface of many clinical isolates (Table 1) suggest that a vaccine based on Shr may provide a broad protection against streptococcal diseases. Therefore, Shr vaccine could avert the problems of M protein-derived vaccines, such as protection against limited serotypes and cross reaction with host epitopes.

Shr is highly immunogenic. Systemic vaccination with purified full length Shr protein resulted in robust antibody response in serum (Fig. 13). Also, Shr triggered strong serum response in mice administrated intranasally with recombinant *L. lactis* expressing Shr (Fig. 15). Shr conservation and strong antigenicity are somehow hard to reconcile with the fact that an individual fails to

mount protective response to GAS and thus may experience multiple infections. It is possible that while the purified Shr is a strong immunogen, the host response to GAS may be dominated by other antigens such as the plentiful M protein. Therefore, GAS may use the hypervariable M protein as a “decoy” immunogen that leads to an immune response that is essentially ineffective against the wide spectrum of M serotypes. While a measurable IgA response was observed in mice treated intranasally with Shr-producing *L. lactis*, the resulting IgA titers were quite weak. The mucosal immune response to Shr may be improved by using purified protein and alternative adjuvant.

Both systemic and mucosal vaccination with Shr elicited protection from systemic GAS challenge by a highly virulent GAS strain (Fig. 14 and 15). These observations are very promising and indicate that additional studies exploring Shr as vaccine candidate are justified. They also indicate that Shr vaccine could be administered by more than one route. Most of GAS infections are initiated at the upper respiratory tract and the skin. Therefore, testing the ability of Shr vaccine to protect from mucosal and cutaneous challenges are the logical next steps. Murine model can serve as a suitable tool for skin infection. Unfortunately, there is no good murine model for the upper respiratory tract infections produced by GAS. Therefore, experiments for mucosal protection may require *in vivo* model in higher animals such as rhesus macaques. The staphylococcal NEAT protein, IsdB, has been tested in rhesus macaque model and was found to elicit rapid antibody responses when administered intramuscularly (Kuklin, Clark et al. 2006).

Shr antibody defenses against GAS

The efficacy of an antibody can be examined by passive immunization experiments (Dale, Baird et al. 1994; Sabharwal, Michon et al. 2006). Shr rabbit antiserum effectively defended mice from systemic challenge by two GAS serotypes (Fig. 16). These findings are impressive, especially considering that rabbit antiserum was used in these experiments rather than isolated IgGs or affinity purified Shr antibody. In addition to Shr, two NEAT proteins in *S. aureus* were identified as protective antigens. Purified antibodies for IsdA or IsdB defended mice from renal abscess formation and a lethal intravenous challenge (Kim, DeDent et al. 2010). Unlike Shr antiserum, IsdA and IsdB antibody do not promote opsonophagocytic killing (Kim, DeDent et al. 2010). The opsonizing ability of Shr antiserum explains at least in part its protective action (Fig. 17). Shr antibody appears to be a key component of the host defense. It is most likely to work by neutralizing Shr on the bacterial surface. The establishment of lethal infection by the i.p. route used in our study is not likely to require adherence. Thus, the potential anti-adherence properties of the Shr antiserum may not seem important to GAS defense in this model. Therefore, Shr antibody may protect mice by interfering with Shr ability to obtain iron. *In vitro* model for adherence and growth is needed to further characterize the protective properties of Shr antiserum.

The survival rate of mice passively immunized with anti-Shr is lower than that of actively immunized mice (Fig. 14, 15, and 16). The reduced protection by passive vaccination may result from the absence of T-cell mediated immunity. CD4⁺ cells are the major pathway for the development of long-lasting immunity. The CD4⁺ cells bind to the epitopes presented by antigen-presenting cells and coordinate the induced immune response. Another limitation of

passive immunization is that the protection is limited by the amount of antibody and application time. Additional work is needed to optimize the protocols for passive immunization, which may in turn enhance the observed protection.

The antisera of NEAT domains are opsonizing and their ability to facilitate bacterial killing in blood is similar to that of anti-Shr (Fig. 30). However, these antisera failed to protect mice in the passive immunization. Mice treated with NTD antiserum had a higher survival rate than the control group, but the difference was small (20%) and not statistically significant (Fig. 29). It is not clear why the antiserum generated with the full length protein is more protective than the domain antisera. It seems conceivable that protective epitopes that are found in the full length Shr may be missing in the isolated domains. In addition, some of the antibody generated against an isolated domain may not interact with the full length proteins, since the domain may fold differently and some epitopes are not accessible in the complete receptors. Finally, an effective defense may require the simultaneous neutralization of several Shr functions and the antibodies recognizing multiple sites in the protein.

A significant concern is raised from the observation that the antisera for NEAT1 and NEAT2 sensitize mice to GAS infections. We propose that the NEAT domains which have immunoglobulin-like fold, share epitopes with host proteins. Thus, the NEAT antisera may induce autoimmune complications. A structural modeling program (Phyre2) suggests that the LRR-NEAT2 region in Shr folds similarly to the ligand-binding site of toll-like receptors (TLRs). TLRs are essential components of the innate immune response and are therefore one of the key factors involved in recognizing and defending against invading pathogens. NEAT

antibodies may interact with the ligand binding site of TLRs and thus block their activity, which in turn will attenuate the innate immune system. Alternatively, these interactions may overactivate the receptor and therefore lead to excessive and harmful cytokine responses. The effect of NEAT antiserum on host needs to be tested in mice without GAS challenge or with non-Shr-expressing bacteria in order to begin addressing these concerns.

The efforts for GAS vaccine development has focused mostly on the variable N-terminus of M protein. However, recent studies examined several conserved epitopes in GAS, these include the ScpA, cell wall carbohydrates and a few fibronectin-binding proteins (Ji, Carlson et al. 1997; Kawabata, Kunitomo et al. 2001; Sabharwal, Michon et al. 2006). Several studies have used modern proteomics and genomics methods and identified new immunogenic determinant in GAS (Rodriguez-Ortega, Norais et al. 2006; Dale 2008; Steer, Batzloff et al. 2009). In this work, we demonstrate that Shr is a MSCRAMM that contributes to the progress of GAS pathogenesis beyond iron acquisition. The conservation of Shr in multiple streptococci and the domains it shares with other Gram-positive pathogens suggest that Shr may provide broad protection against GAS and related pathogens. We initiate the investigation of Shr antigenic properties and provide strong support to the promise of Shr-based vaccine.

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APPENDICES

APPENDIX A

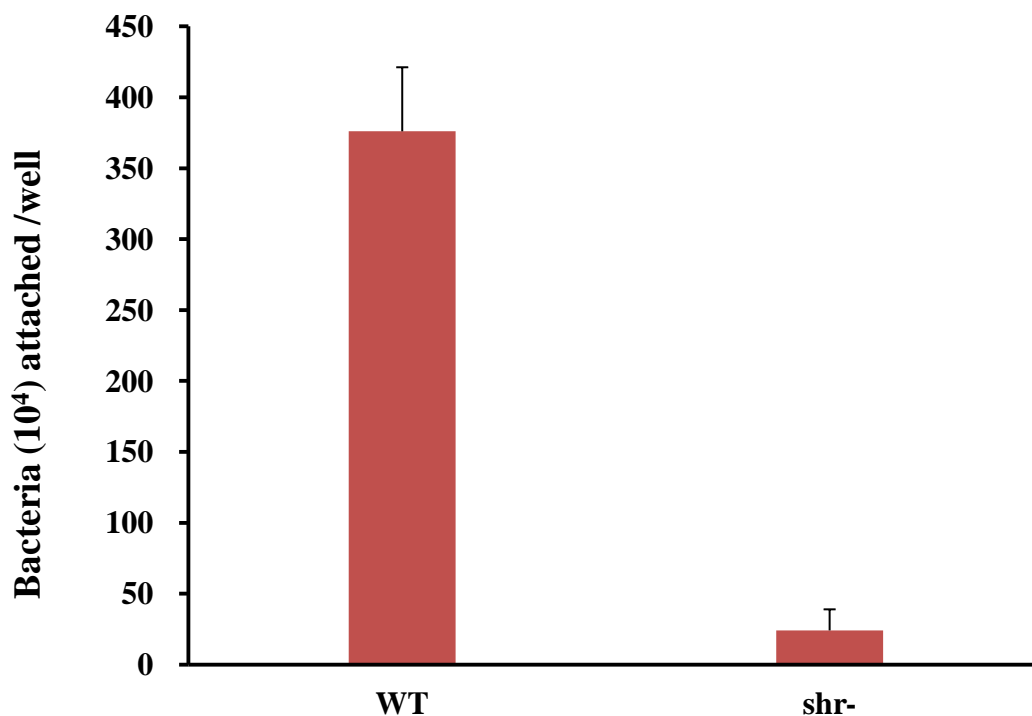


Figure 33. Adherence of GAS to cultured lung cells NCIH292.

Mid-log phase cultures of wild-type strain (NZ131) and *shr* mutant (ZE4912) were incubated with confluent NCIH292 cell monolayers for 2 h at 37 °C under 5% CO₂. Unattached bacteria were removed by washing, cells were detached and lysed, and the number of bound bacteria per well was determined by plating. Each bar represents the mean number of GAS cells attached as obtained from at least two independent experiments performed in triplicate. The standard deviation is expressed by the error bar. The difference between NZ131 and ZE4912 is significant ($P < 0.005$).

APPENDIX B

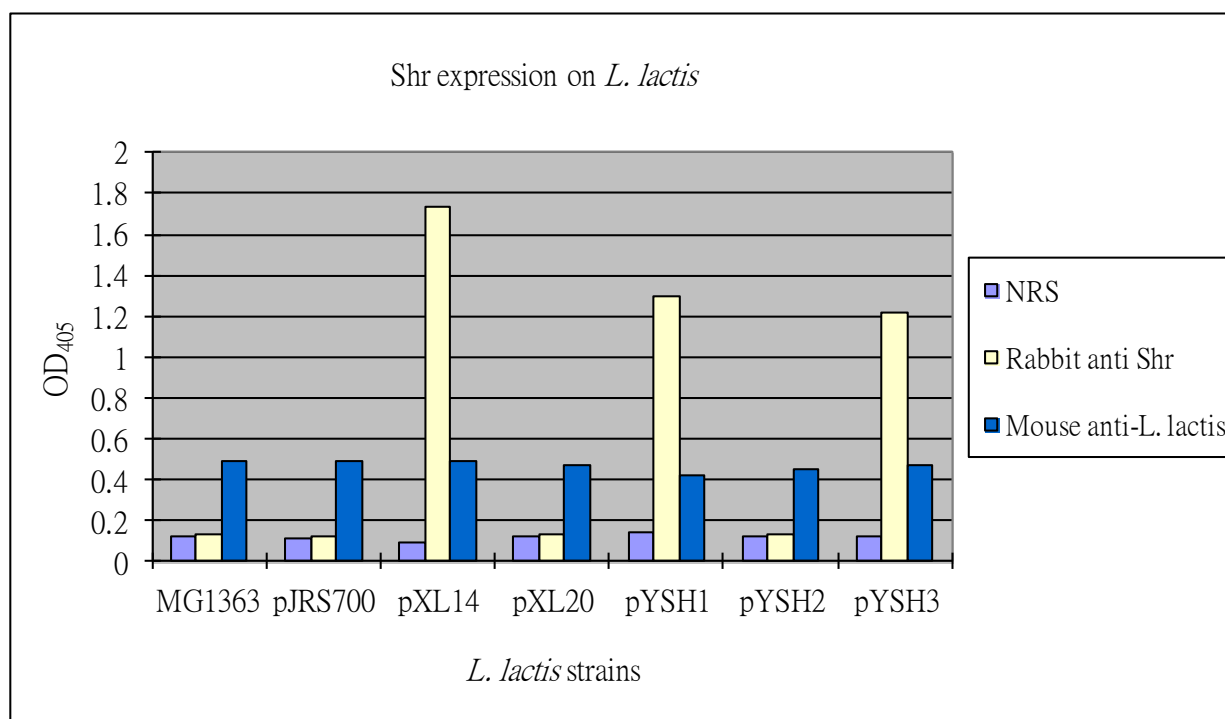


Figure 34. Surface presentation of Shr mutants in *L. lactis*.

L. lactis strains expressing Shr mutants were immobilized in the wells of microtiter plate and then detected with anti-Shr and anti-*L. lactis* antibodies in ELISA. Normal rabbit serum (NRS) serves as negative control, showing the background signal of rabbit serum. MG1363 is the naïve *L. lactis*. pJRS700 is the *L. lactis* strain with the empty vector without inserted shr gene. pXL14 is the *L. lactis* strain that expresses full-length Shr protein on the surface. pXL20 is the *L. lactis* strain expressing a NTD-NEAT1 domain deletion mutant. pYSH1 is the *L. lactis* strain expressing a NEAT1 domain deletion mutant. pYSH2 is the *L. lactis* strain that expresses a partial NEAT2 deletion mutant. pYSH3 is the *L. lactis* strain expressing a NEAT2 deletion mutant. Each bar represents the detection by anti-Shr as from two independent experiments.