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Initiation of invasive disease in M1T1  
group A streptococcus

Andrew Hollands  
University of Wollongong

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# **Initiation of Invasive Disease in M1T1 Group A Streptococcus**

*A Thesis Submitted in Fulfilment of the Requirements*

*For the Award of the Degree*

*Doctor of Philosophy (PhD)*

*From the*

*University of Wollongong*

*By*

**Andrew Hollands**

School of Biological Sciences

2009

## DECLARATION

I, Andrew Hollands, declare that this thesis, submitted in fulfilment of the requirements for the award of Doctor of Philosophy (PhD), in the School of Biological Sciences, University of Wollongong, is wholly my own work unless otherwise referenced or acknowledged. The document has not been submitted for qualifications at any other academic institution.

A handwritten signature in black ink, appearing to read 'A Hollands', with a stylized, cursive script.

Andrew Hollands

30 November, 2009

## ABSTRACT

*Streptococcus pyogenes* (group A streptococcus; GAS) is an important human pathogen that colonizes epithelial and mucosal surfaces. Group A streptococcal disease can be relatively minor, such as streptococcal pharyngitis, or severe and life-threatening, such as necrotizing fasciitis. There has been a resurgence of severe infection with GAS since the mid-1980s that has been paralleled by the emergence of a globally disseminated clone, M1T1. The M1T1 clone of GAS presents as the most common cause of streptococcal pharyngitis in developed countries and are also overrepresented in cases of severe infection.

Most invasive bacterial infections are caused by species that more commonly colonize the human host with minimal or no symptoms. Although phenotypic or genetic correlates underlying a bacterium's shift to enhanced virulence potential have been studied, the *in vivo* selection pressures governing such shifts are poorly understood. The globally disseminated M1T1 clone of GAS is linked with the rare but life-threatening syndromes of necrotizing fasciitis and toxic shock syndrome. Mutations in the group A streptococcal control of virulence regulatory sensor kinase (*covR/S*) operon are associated with severe invasive disease, abolishing expression of a broad spectrum cysteine protease (SpeB) and allowing the recruitment and activation of host plasminogen on the bacterial surface. This study describes how a bacteriophage-encoded group A streptococcal DNase (Sda1), which facilitates the pathogen's escape from neutrophil extracellular traps (NETs), can serve as a selective force for *covR/S* mutation. The results provide a paradigm whereby horizontal gene transfer and natural selection exerted by the innate immune system

generate hypervirulent bacterial variants with increased risk of systemic dissemination.

This study sought to investigate if there was a cost of fitness associated with *covR/S* mutation that counterbalances the dramatic increase in virulence. It was found that *covR/S* mutant bacteria had reduced capacity to bind fibronectin and collagen, both components of the extracellular matrix bound by streptococcal adhesins. The *covR/S* mutant strain examined in this study also showed reduced capacity to bind to epithelial cell layers as a consequence of increased capsule expression. This mutant strain displayed reduced capacity to form biofilms. An animal model of skin colonization was used to show that the *covR/S* mutant strain has a colonization defect. This reduced capacity to colonize presents an explanation as to why hypervirulent *covR/S* mutant M1T1 group A streptococci are not rapidly spread amongst the community.

The role of SpeB in the course of infection is still unclear. This study utilized a SpeB-negative M1T1 clinical isolate, 5628, with a naturally occurring mutation in the gene encoding the regulator RopB, to elucidate the role of RopB and SpeB in systemic virulence. Allelic exchange mutagenesis was used to replace the mutated *ropB* allele in 5628 with the intact allele from the well characterized isolate 5448. The inverse allelic exchange was also performed to replace the intact *ropB* in 5448 with the mutated allele from 5628. An intact *ropB* was found to be essential for SpeB expression. While the *ropB* mutation was shown to have no effect on haemolysis of RBCs, extracellular DNase activity or survival in the presence of neutrophils, strains with the mutated *ropB* allele were less virulent in murine systemic models of

infection. An isogenic SpeB knockout strain containing an intact RopB showed similarly reduced virulence. Microarray analysis found genes of the SpeB operon to be the primary target of RopB regulation. These data show that an intact RopB and efficient SpeB production are necessary for systemic infection with GAS.



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## ABBREVIATIONS

°C	degrees Celsius
aa	amino acid
Ab	antibody
ANOVA	analysis of variance
APSGN	acute post-streptococcal glomerulonephritis
ARF	acute rheumatic fever
BLAST	basic local alignment search tool
bp	base pair
CCD	charge-coupled device
cDNA	complementary DNA
CFU	colony forming units
Cm	chloramphenicol
Co	collagen
CovR/S	control of virulence regulator/sensor
DNA	deoxyribonucleic acid
DTT	dithiothreitol
<i>E. coli</i>	<i>Escherichia coli</i>
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
Erm	erythromycin
FBP	fibronectin binding protein
FCT	fibronectin-binding, collagen-binding, T-antigen
Fn	fibronectin
<i>g</i>	acceleration due to gravity ( $9.8 \text{ ms}^{-2}$ )
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GAS	group A streptococcus
GEO	Gene Expression Omnibus
h	hours
HRP	horseradish peroxidase
IgA	immunoglobulin A
IgG	immunoglobulin G
kDa	kilodaltons
LA	Luria-Bertani agar
LB	Luria-Bertani broth
LTA	lipoteichoic acid
M	molar
MBC	minimum bactericidal concentration
MF	mitogenic factor
MHC	major histocompatibility complex
MIAME	minimum information about a microarray experiment
MIC	minimum inhibitory concentration
min	minutes
ml	millilitres
mM	millimolar
mm	millimetres
Mrp	M-related protein

NCBI	National Center for Biotechnology Information
NET	neutrophil extracellular trap
ng	nanogram
nm	nanometres
nt	nucleotide
OD	optical density
ORF	open reading frame
PAI	plasminogen activator inhibitor
PAM	plasminogen-binding group A streptococcal M-like protein
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Prp	PAM-related protein
RBC	red blood cell
RGD	arginine-glycine-aspartic acid
RPMI	Roswell Park Memorial Institute
<i>S. pyogenes</i>	<i>Streptococcus pyogenes</i>
SCP	streptococcal C5a peptidase
SD	standard deviation
Sda1	streptodornase 1
SDH	streptococcal surface dehydrogenase
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEN	streptococcal surface enolase
SIC	streptococcal inhibitor of complement-mediated lysis
Ska	streptokinase
SLO	streptolysin O
SLS	streptolysin S
SmeZ	streptococcal mitogenic exotoxin Z
SOF	serum opacity factor
Spe	streptococcal pyrogenic exotoxin
SpyCEP	<i>Streptococcus pyogenes</i> cell envelope protease
SSA	streptococcal superantigen
STSS	streptococcal toxic shock syndrome
TCA	trichloroacetic acid
TCF	tissue chamber fluid
THA	Todd-Hewitt agar
THB	Todd-Hewitt broth
THY	Todd-Hewitt broth supplemented with 1% (w/v) yeast extract
TNF	tumor necrosis factor
USA	United States of America
V	volts
v/v	volume/volume
w/v	weight/volume
WT	wildtype
μl	microlitres
μm	micrometres
μM	micromolar

## PUBLICATIONS

Walker, M. J., **Hollands, A.**, Sanderson-Smith, M. L., Cole, J. N., Kirk, J. K., Henningham, A., McArthur, J. D., Dinkla, K., Aziz, R. K., Kansal, R. G., Simpson, A. J., Buchanan, J. T., Chhatwal, G. S., Kotb, M. and Nizet, V. (2007). DNase Sda1 provides selection pressure for a switch to invasive group A streptococcal infection. *Nature Medicine* **13**(8): 981-5.

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## CONFERENCE PRESENTATIONS

**Hollands, A.,** Aziz, R. K., Kansal, R., Kotb, M., Nizet, V., Walker, M. J. (2008)  
Mutation in *ropB* suppresses *speB* expression and reduces group A streptococcal virulence. XVII Lancefield International Symposium on Streptococci and Streptococcal Diseases. June 22-26 Porto Heli, Greece.

## 1. INTRODUCTION

### 1.1 Overview

*Streptococcus pyogenes* (group A streptococcus; GAS) is an important Gram-positive pathogen responsible for a wide range of infections including relatively minor superficial diseases such as streptococcal pharyngitis through to severe life-threatening invasive diseases such as necrotising fasciitis (Cunningham, 2000). GAS colonises epithelial and mucosal surfaces and it has been estimated that approximately 15% of school age children each year suffer streptococcal pharyngitis (Carapetis *et al.*, 2005).

The incidence of severe group A streptococcal infections has been on the rise in developed countries since the mid-1980s (Kaplan, 1991; Efstratiou, 2000; Carapetis *et al.*, 2005). This resurgence has been paralleled by the emergence of a globally disseminated M1T1 clone that is distinguished by the acquisition of phages encoding virulence factors Sda1 and SpeA (Chatellier *et al.*, 2000; Aziz *et al.*, 2005; Aziz and Kotb, 2008). Studies of M1T1 clinical isolates from invasive disease cases have revealed an inverse relationship between expression of the extracellular cysteine protease SpeB and clinical severity (Kansal *et al.*, 2000). The existence of a SpeB-negative invasive phenotype has been hypothesized that results from mutations in the regulator *covR/S* (Sumby *et al.*, 2005).

In this chapter, a review is presented of literature relevant to the classification of GAS, epidemiology, disease types, group A streptococcal virulence factors and the role of the CovR/S regulatory system.

## **1.2 The Genus *Streptococcus***

The genus *Streptococcus* consists of Gram-positive, chain forming cocci that are commonly found in the normal oral and bowel flora of warm-blooded animals (Norton, 1986). Streptococci are a very diverse group of bacteria, with some species causing very specific infections while other species can infect a wide range of host organisms and tissue sites (Fischetti and Ferretti, 2000). The genus consists of 49 species and 8 subspecies, of which the 3 that most commonly cause infections in humans are *Streptococcus pyogenes* (group A streptococcus, GAS), *Streptococcus agalactiae* (group B streptococcus) and *Streptococcus pneumoniae* (pneumococcus) (Reichardt, 2001).

Haemolysis, the ability to disrupt red blood cells (RBCs) is an important trait used for identifying streptococci, with  $\beta$ -haemolysis of blood-agar being used as a laboratory diagnostic test (Norton, 1986). Streptococci are classified by the Lancefield scheme into approximately 20 groups based on immunological differences in cell surface antigens (Lancefield, 1933; Norton, 1986). Group A, B, C, F and G are identified by cell wall polysaccharides and group D by lipoteichoic acid (Cunningham, 2000). The antigen that is used for serotyping GAS is comprised of N-acetyl- $\beta$ -D-glucosamine linked to a polymeric rhamnose backbone (Cunningham, 2000).



### 1.3 Group A Streptococcus (*Streptococcus pyogenes*)

*S. pyogenes* was first isolated in 1874 by Billroth and is a human-specific pathogen (Weiss and Laverdiere, 1997; Krause, 2002). GAS colonises the throat and skin causing a wide range of infections that include relatively minor infections such as streptococcal pharyngitis through to severe invasive diseases such as necrotising fasciitis (Cunningham, 2000). Group A streptococcal attachment to and colonisation of host tissues is facilitated through the use of multiple adhesins (Hasty *et al.*, 1992). The most prominent of these adhesins is the M-protein, which is highly variable, having over 80 different types able to be distinguished by serological methods (Weiss and Laverdiere, 1997). GAS also produces a number of secreted virulence proteins such as streptokinase and pyrogenic exotoxins that play an important role in disease pathogenesis (Cunningham, 2000; Reichardt, 2001).

A number of *S. pyogenes* adhesins bind to fibronectin, solely or in addition to other ligands, suggesting that fibronectin binding plays an important role in attachment and colonisation. Nonetheless, it has been shown that fibronectin binding is not the only determinant of adherence to eukaryotic cells (Edwards *et al.*, 2004). Group A streptococci have historically been considered an extracellular pathogen; however, the bacterium has been shown to persist within phagocytic cells as well as being internalised by human epithelial cells (LaPenta *et al.*, 1994; Medina *et al.*, 2003).

## 1.4 Classification of GAS

As described above, GAS is distinguished from other streptococci by immunological differences in cell surface antigens (Lancefield classification). *S. pyogenes* causes a wide range of infections, thus a method for discriminating between disease isolates is required. A range of serological and genetic typing methods exist for classification of group A streptococcal strains (Cunningham, 2000). Procedures for serotyping group A streptococcal strains include M-typing and T-typing (Cunningham, 2000) as well as Class-I/Class-II serotyping (Bessen and Fischetti, 1990). *S. pyogenes* may also be classified by genotyping methods such as *emm*-typing (Beall *et al.*, 1996; Facklam *et al.*, 1999) and vir-typing (Gardiner *et al.*, 1995; Gardiner *et al.*, 1998).

M-typing classifies group A streptococcal strains based on the M-protein, which is expressed on the surface of GAS (Cunningham, 2000). The M-protein is a fibrillar coiled-coil protein that contains highly conserved C-repeats near the C-terminus and a hypervariable region close to the N-terminus (Figure 1.1) (Fischetti, 1989; Fischetti, 1991). It is this hypervariable region that forms the basis for classification into one of over eighty 80 distinct serotypes (Fischetti, 1989).

T-antigen typing allows for further classification of GAS (Cunningham, 2000). Correlation has been shown between certain M-types and specific T-types (Beall *et al.*, 1997; Beall *et al.*, 1998). The T-antigen is encoded by the *tee* gene in a highly variable locus known as the fibronectin-binding, collagen-binding, T-antigen (FCT) region (Bessen and Kalia, 2002). While the T-antigen was first identified and used

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**Figure 1.1** Schematic diagram of the fibrillar coiled-coil streptococcal M-protein indicating the conserved region and the variable region of the protein including the hypervariable region used for serological M-typing. The cell membrane, peptidoglycan cell wall and the group A streptococcal carbohydrate are shown. Figure from Fischetti *et al.* (1989).

long ago by Rebecca Lancefield and colleagues for classification purposes (Lancefield, 1928; Lancefield and Dole, 1946), it has only recently been identified as the major component of group A streptococcal pilli (Mora *et al.*, 2005).

M-proteins have been divided into two groups based on their reaction with antibodies against the conserved C-terminal region of the protein (Bessen *et al.*, 1989; Bessen and Fischetti, 1990). Class I M-proteins contain an epitope that reacts with the class I/II typing antibodies, whereas Class II M-proteins do not contain this epitope and hence do not react with these antibodies (Bessen *et al.*, 1989; Bessen and Fischetti, 1990). There is a strong positive correlation between strains expressing class I M-

proteins and rheumatic fever, suggesting that this epitope may play a role in pathogenesis (Bessen *et al.*, 1989; Bessen and Fischetti, 1990; Bessen *et al.*, 1995).

*Emm*-typing identifies isolates based on the nucleotide sequence of the *emm*-gene, which codes for the M-protein (Beall *et al.*, 1996; Facklam *et al.*, 1999). Sequencing of the variable 5'-end of the *emm* gene is conducted using primers based on common regions of the gene (Beall *et al.*, 1996). More than 150 *emm*-types have been identified, with differences in the *emm* gene corresponding to serologically distinct M-types (Facklam *et al.*, 1999).

As a classification method, M-protein serotyping presents a number of difficulties. These include problems in preparation of M-typing antisera, identifying strains with low-level expression of the M-protein or strains that express a novel M-protein type (Cunningham, 2000). Many group A streptococcal strains exist for which the M-type has not been defined and antisera are not available (Martin and Sriprakash, 1997). Up to 60% of isolates from some Aboriginal communities in Northern Australia are unable to be identified by M-typing (Gardiner *et al.*, 1997). Although *emm*-typing enables the identification of a larger number of distinct strains than M-typing, the presence of virulence determinants is not always consistent with the *emm*-type, as virulence genes may be obtained via processes such as phage acquisition which is independent of the evolution of the *emm*-type (Beres *et al.*, 2004). Despite their shortcomings, both M-typing and *emm*-typing remain two of the most common methods of classification of GAS and have been extensively used for epidemiological studies of group A streptococcal infections (Abolnik and Sexton, 1994; Carapetis *et al.*, 1999).

Vir-typing is another genotyping method that involves PCR amplification of a 5-7 kb variable region of the genome termed the *vir* regulon (Gardiner *et al.*, 1995). The PCR product is digested using the restriction enzyme, *Hae*III, and the restriction fragments visualised. The discriminatory power of Vir-typing restriction fragment length polymorphism is similar to that of M-typing and Vir-types have been shown to correspond to specific M-types (Gardiner *et al.*, 1995; Gardiner *et al.*, 1997).

### 1.5 Epidemiology of GAS

Group A streptococcal infections were in steady decline in developed countries following the Second World War. This decline predates the introduction of penicillin treatment, so it has been attributed to a combination of factors that are not clearly defined (Bisno, 1991). Since the mid-1980s, however, there has been a dramatic resurgence in severe group A streptococcal infections throughout the world, including developed countries such as the USA, Canada and Sweden (Bisno, 1991; Kaplan, 1996; Bessen *et al.*, 2000). This resurgence has not been clearly explained although it may be due to the emergence of more virulent strains, such as M1T1, or the acquisition of new virulence factors (Bronze and Dale, 1996; Efstratiou, 2000; Aziz and Kotb, 2008). This shift in *S. pyogenes* virulence is compounded by the lack of specific immunity in populations where group A streptococcal infections are not prevalent (Abolnik and Sexton, 1994; Bronze and Dale, 1996).

In developed countries, particular strains are predominantly associated with severe group A streptococcal infections. In countries such as USA, Canada and Europe, the M1 serotype of GAS, and to a lesser extent, M3 have been associated with severe

invasive disease and there remains a high mortality rate amongst patients with these infections, despite medical intervention (Stromberg *et al.*, 1991; Stevens, 1995; Struelens, 1995). In particular, the M1T1 clone has persisted as the most common cause of invasive infections in developed countries (Cleary *et al.*, 1998; Murono *et al.*, 1999; Chatellier *et al.*, 2000).

Group A streptococcal infections are, however, most prevalent in developing countries and in indigenous populations of industrialised countries. In such populations, the incidence of rheumatic fever in school age children is 100 to 200 per 100,000 compared with 0.5 per 100,000 in developed countries (Carapetis *et al.*, 2000; Olivier, 2000). *S. pyogenes* infections have remained endemic in indigenous populations and in developing countries. However, the epidemiology of group A streptococcal strains within these populations may differ significantly from that of the populations of developed countries, exhibiting much higher strain diversity (Carapetis *et al.*, 1999).

In India, group A streptococcal infections are common and have a significant effect on public health and on the national economy (Menon *et al.*, 2004; Shet and Kaplan, 2004). In contrast to western countries where a very small number of strains predominate, a wide range of *emm*-types are common in communities in India (Sagar *et al.*, 2004). Many of the strains commonly circulating in Indian communities also differ from those found in other countries, such as the USA (Sagar *et al.*, 2004).

Group A streptococcal infections are endemic in Aboriginal communities in northern Australia and occur at very high levels. Unlike the USA and Canada, where there are

a few dominant strains, there are many group A streptococcal strains commonly circulating in these communities that cause invasive disease (Delvecchio *et al.*, 2002). As a result, the division between invasive and non-invasive strains is not as clearly defined. Identifying the strains responsible for invasive infections is complicated by the presence of individuals with multiple infections by different group A streptococcal strains or multiple strains being present in the one infection site (Carapetis *et al.*, 1995).

The incidence of acute rheumatic fever (ARF) is higher in Aboriginal communities in northern Australia than that reported anywhere else in the world (Carapetis *et al.*, 1996). Children in Aboriginal communities in northern Australia also suffer from streptococcal skin infections at rates of up to 50% (Currie and Carapetis, 2000). The specific factors responsible for these high levels of infection are not clear, although environmental factors including household crowding and personal hygiene most likely play a role (Taplin *et al.*, 1973; Munoz *et al.*, 1992). Although the precise factors that contribute to the prevalence of group A streptococcal infections are not clearly understood, further elucidation of the mechanisms of colonisation and the virulence factors involved may lead to the development of more adequate treatment and prevention of group A streptococcal disease.

## **1.6 Group A Streptococcal Infection and Disease**

Group A streptococci colonise the throat or skin of the host and can result in a range of diseases and complications that include superficial, invasive, toxin-mediated and immune-mediated disease (Efstratiou, 2000). Human skin and mucosal surfaces are

the major reservoirs for group A streptococcal infections and it has been recognised that particular strains colonise throat or skin tissue preferentially (Bessen *et al.*, 2000; Cue *et al.*, 2000). *S. pyogenes* is transmitted from carrier to host by direct contact or aerosols of respiratory secretions (Norton, 1986).

### ***1.6.1 Superficial group A streptococcal disease***

Superficial Group A streptococcal infections include pharyngitis, pyoderma, erysipelas and vaginitis (Efstratiou, 2000). Streptococcal pyoderma, or impetigo, is a non-invasive disease of the skin and, along with non-invasive infection of the throat (streptococcal pharyngitis), constitutes the majority of streptococcal infections (Bisno, 1991; Bisno and Stevens, 1996). More extensive infection of the skin and cutaneous lymphatics may occur when group A streptococci gain entry into the body through local trauma or lesions (Bisno, 1991).

### ***1.6.2 Toxin-mediated disease***

Toxin-mediated group A streptococcal diseases include streptococcal toxic-shock like syndrome (STSS) and scarlet fever (Efstratiou, 2000; Reichardt, 2001). STSS is quite often associated with necrotising fasciitis and is most likely mediated by streptococcal superantigens (Stevens, 1995; Stevens, 2000). These superantigens interact with major histocompatibility complex (MHC) class II molecules resulting in the release of a large quantity of inflammatory cytokines through the non-specific activation of T-lymphocytes (Struelens, 1995; Cunningham, 2000). The result of



these processes is hypotension, fever and multiple organ failure (Stevens, 1997; Stevens, 2000).

### ***1.6.3 Immune-mediated sequelae***

Two immune-mediated complications that can arise from streptococcal infections are acute rheumatic fever (ARF) and acute post-streptococcal glomerulonephritis (APSGN) (Cunningham, 2000). These two conditions arise from cross-reactivity between an immune response mounted against a streptococcal infection and human tissues (Stollerman, 1997; Guzman-Cottrill *et al.*, 2004).

ARF can result in inflammation of the heart, joints, central nervous system or skin and usually follows within three weeks of a streptococcal infection (Guzman-Cottrill *et al.*, 2004). ARF has been considered to only be associated with throat infections (Efstratiou, 2000). However, this data is based on epidemiological studies in temperate regions and recent studies in Aboriginal communities in tropical northern Australia suggest that this may not necessarily be the case, with very high rates of ARF within populations where streptococcal throat infection is uncommon (McDonald *et al.*, 2004). In developing countries and indigenous populations, ARF and rheumatic heart disease remain the highest cause of heart disease in children and young adults (Carapetis *et al.*, 1996; Stollerman, 1997).

APSGN may follow streptococcal infections of the throat or skin and is an immune mediated condition that affects the kidneys (Cunningham, 2000). The exact mechanism of APSGN is not well defined, although research has shown raised levels

of specific immunoglobulins in nephritic patients indicating that an autoimmune response due to group A streptococcal infection may be involved (Koshi *et al.*, 1983). A recent study suggested that anti-SpeB antibodies may be cross-reactive with kidney endothelial cells and result in glomerular damage (Luo *et al.*, 2007). In addition, some studies have suggested that glomerulonephritis may result from immune complex formation with streptococcal antigens, such as SpeB or glyceraldehyde-3-phosphate dehydrogenase (GAPDH), followed by glomerular deposition, induction of cytokines and complement activation (Yoshizawa *et al.*, 2004; Batsford *et al.*, 2005; Viera *et al.*, 2007).

#### ***1.6.4 Group A streptococcal invasive disease***

While superficial diseases represent the majority of streptococcal infections, a proportion of these lead to more serious invasive diseases such as bacteraemia, cellulitis, puerperal sepsis and necrotising fasciitis (Stevens, 2001). Invasive disease results from skin or mucous membrane infection that is closely followed by deep infection of the connective tissue (Reichardt, 2001). Severe invasive group A streptococcal infections such as necrotising fasciitis account for approximately 30% of the approximately 500,000 deaths from GAS each year (Carapetis *et al.*, 2005). The incidence of such acute conditions has been on the rise since the mid-1980s (Kaplan, 1991). This resurgence has been paralleled by the emergence of a globally disseminated M1T1 clone of GAS (Chatellier *et al.*, 2000; Walker *et al.*, 2005; Tart *et al.*, 2007). While M1T1 GAS has become the most common cause of streptococcal pharyngitis in North America, this clone is also overrepresented in cases of severe invasive disease (Cleary *et al.*, 1992; Demers *et al.*, 1993).

## 1.7 Virulence Factors

GAS has many proven and putative virulence factors that may contribute to invasive disease. This review will briefly describe many of the known virulence determinants, and focus on those that are relevant to the research presented in this thesis.

### 1.7.1 *M-protein*

M-protein is an abundant surface protein in GAS and is the basis for serological M-typing (Cunningham, 2000). It has been described as the major virulence factor for GAS due to its anti-phagocytic capacity (Fischetti, 1989). M-protein has an alpha-helical coiled-coil structure with destabilizing insertions that resembles mammalian myosin and tropomyosin (McNamara *et al.*, 2008). This similarity causes the M-protein immune response to cross-react with human myosin and tropomyosin and this may be the cause of post-infection immune sequelae such as acute rheumatic fever (Cunningham, 2000). As well as the molecular mimicry displayed, M-protein is also able to bind host proteins such as fibrinogen to block deposition of opsonic antibodies and complement, preventing phagocytic uptake and elimination (Ringdahl *et al.*, 2000; Sandin *et al.*, 2006). M-protein has also been shown to mediate adherence to host tissues (Okada *et al.*, 1995).

Numerous proteins have been found in GAS with similarities to M-protein, such as M-related protein (Mrp) (Podbielski *et al.*, 1996), Enn protein (Podbielski *et al.*, 1994), plasminogen-binding M-like protein (PAM) (Berge and Sjobring, 1993) and the PAM-related protein (Prp) (Sanderson-Smith *et al.*, 2007). These may have

developed as a result of intergenomic recombination (Podbielski *et al.*, 1994). The Mrp and Enn proteins are able to bind IgG and IgA and can also contribute to the antiphagocytic ability of GAS (Podbielski *et al.*, 1996). PAM and Prp bind plasminogen and may be involved in the invasive spread of GAS (Berge and Sjobring, 1993; Sanderson-Smith *et al.*, 2007)

### ***1.7.2 Hyaluronic acid capsule***

The group A streptococcal capsule is composed of hyaluronic acid, a high-molecular weight glycosaminoglycan polymer, which is synthesized by the *has* operon (*hasA*, *hasB* and *hasC*) (Crater and van de Rijn, 1995). The hyaluronic acid that composes the group A streptococcal capsule is identical to that in human connective tissue and is composed of alternating residues of N-acetylglucosamine and glucuronic acid (Crater and van de Rijn, 1995; Bisno *et al.*, 2003).

While the classical role of the hyaluronic acid capsule is resistance to phagocytosis (Wessels *et al.*, 1991; Dale *et al.*, 1996), capsule has also been shown to be a low-affinity adhesin acting through the CD44 receptor (Schrager *et al.*, 1998). Acapsular mutants have been shown to be susceptible to phagocytosis and to be less virulent *in vivo* (Wessels *et al.*, 1991; Wessels and Bronze, 1994; Ashbaugh *et al.*, 1998).

### ***1.7.3 C5a peptidase***

Streptococcal C5a peptidase (SCP) is encoded by the gene *scpA* and interferes with the host complement pathway by cleaving C5a near the C-terminus and inhibiting its

chemoattractant ability (Wexler *et al.*, 1985; Cleary *et al.*, 1992). This delays the recruitment of neutrophils and results in increased virulence *in vivo* (O'Connor and Cleary, 1987; Ji *et al.*, 1996). C5a peptidase also shows potential as a vaccine candidate as it is highly conserved between strains and immunisation with recombinant SCP results in increased clearance of group A streptococci from intranasally infected mice (Cleary *et al.*, 2004).

#### ***1.7.4 Extracellular matrix binding proteins and lipoteichoic acid***

GAS colonises regions of the body that have strong barrier defence mechanisms, such as the throat and skin. Therefore without adherence to host tissue, group A streptococci would be removed by exfoliation and mucous flow (Cunningham, 2000). *S. pyogenes* utilises multiple adhesins in the crucial initial phase of attachment to host tissue (Hasty *et al.*, 1992; Courtney *et al.*, 2002; Kreikemeyer *et al.*, 2004). These adhesins bind to a range of host proteins including fibronectin, fibrinogen, collagen, vitronectin and the CD46 receptor (Cunningham, 2000). Some of the major group A streptococcal adhesins and their host targets are listed in Table 1.1. Lipoteichoic acid (LTA), has been shown to be a vital component of group A streptococcal attachment (Simpson and Beachey, 1983) and it has been suggested that LTA is essential in the first step of a two step model of adhesion (Figure 1.2) (Hasty *et al.*, 1992).

In the two-step model of adhesion proposed by Hasty *et al.* (1992), a relatively weak and reversible interaction between streptococcal LTA and ligands on the host cell

**Table 1.1** Group A Streptococcal adhesins and their host target molecules.

Adhesin	Ligands Bound	Reference
28kDa fibronectin binding protein	Fibronectin	(Courtney <i>et al.</i> , 1992)
Collagen binding protein	Collagen	(Visai <i>et al.</i> , 1995)
FbaA	Fibronectin	(Terao <i>et al.</i> , 2001)
FbaB	Fibronectin	(Terao <i>et al.</i> , 2002)
FBP54	fibronectin, fibrinogen	(Courtney <i>et al.</i> , 1994)
Laminin binding protein	Laminin	(Terao <i>et al.</i> , 2002)
Lipoteichoic acid (LTA)	Fibronectin	(Simpson and Beachey, 1983)
M-Protein	Fibronectin, fibrinogen, CD46, galactose, laminin, sialic acid containing receptors, plasminogen	(Ringdahl and Sjobring, 2000; 2001; 2004)
PFBP	Fibronectin	(Rocha and Fischetti, 1999)
Serum opacity factor (SOF)	Fibronectin, fibrinogen, ApoA1	(Courtney <i>et al.</i> , 2002)
SfbI/PrtFI	Fibronectin, IgG	(Medina <i>et al.</i> , 2000; 2000)
SfbX	Fibronectin	(Jeng <i>et al.</i> , 2003)
Streptococcal surface dehydrogenase (SDH)	Fibronectin, lysozyme, myosin, actin, plasmin	(Pancholi and Fischetti, 1992; 1996)
Vitronectin binding protein	Vitronectin	(Valentin-Weigand <i>et al.</i> , 1988)

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**Figure 1.2** Hypothetical two-step model of adhesion of group A streptococcus with host tissue. The first step occurs through interaction of hydrophobic components on the bacterial surface, such as LTA, with hydrophobic domains of the host cell molecules. The first step is necessary before the second step, involving more specific interactions, such as those between M protein and an M protein receptor or fibronectin binding proteins and fibronectin. Successful completion of the second step would make the interaction essentially irreversible. Figure from Hasty *et al.* (1992)

surface is considered to be the first stage of attachment. The second stage of attachment involves a much stronger, high affinity interaction between group A streptococcal adhesins and specific host cell surface receptors. The interaction between LTA and receptors such as fibronectin on the host cell surface is a non-specific, hydrophobic interaction and may enable the bacteria to adhere to a wide range of cell types (Hasty *et al.*, 1992; Courtney *et al.*, 1997). The second stage of adhesion is essentially irreversible and the specific interactions of adhesins expressed on group A streptococcal cell surface with host tissue receptors may confer tissue specificity (Okada *et al.*, 1994; Wang and Stinson, 1994). This two-step process enables GAS to overcome electrostatic repulsion of the negatively charged group A streptococcal surface by hydrophobic interactions in order to allow the strong specific binding of adhesins to occur (Hasty *et al.*, 1992).

In addition to being an extracellular pathogen, GAS is able to be internalised and persist within human cells (LaPenta *et al.*, 1994). GAS has been shown to be internalised by both human epithelial (LaPenta *et al.*, 1994) and phagocytic cells (Medina *et al.*, 2003). While it is clear that internalisation of GAS takes place, it is not clear what function it serves. Internalisation may represent successful containment of the infection by host epithelial cells (Schrager *et al.*, 1996) or may be involved in antibiotic evasion and persistence (Neeman *et al.*, 1998). Internalisation and survival within phagocytic cells suggests an additional virulence mechanism that exploits the innate immune system (Medina *et al.*, 2003). Group A streptococci have been shown to undergo phenotypic switching in response to environmental factors, which may result in increased virulence that enables the bacteria to escape phagosomes and survive within phagocytic cells (Medina *et al.*, 2003). The ability of

*S. pyogenes* to evade immune defence and antibiotic treatment may be an important factor in the persistence of disease, particularly in endemic regions (Molinari *et al.*, 1997).

A number of *S. pyogenes* adhesins have been shown to be involved in the process of internalisation as well as attachment, but the exact mechanisms involved are not clearly defined (Courtney *et al.*, 2002; Kreikemeyer *et al.*, 2004). The interaction between high affinity fibronectin binding proteins (FBPs), such as SfbI, and fibronectin appears to be an important element in internalisation of GAS (Molinari *et al.*, 1997; Ozeri *et al.*, 1998; Talay *et al.*, 2000). Laminin, a common component of basement membranes, will bind to M1 GAS and promote internalisation (Switalski *et al.*, 1984). Peptides containing an Arg-Gly-Asp (RGD) motif can activate internalisation of GAS (Cue *et al.*, 1998; Cue *et al.*, 2000). This tripeptide is present in the cell binding sites of most endogenous extracellular proteins that bind to integrins including fibronectin and laminin (Karp, 2002). The RGD sequence is a common integrin binding motif present in a number of streptococcal proteins, such as FbaB and the integrin binding variant of the streptococcal cysteine protease SpeB2 (Stockbauer *et al.*, 1999; Terao *et al.*, 2002).

### ***1.7.5 Hyaluronidase***

While hyaluronic acid capsule is important for survival of GAS in the host, it has long been known that GAS also expresses hyaluronidase, capable of degrading hyaluronic acid (Douglas, 1941; Nuala, 1944). At least three hyaluronidases are present in the group A streptococcal genome including *hylA* (Hynes *et al.*, 1995;



Hynes *et al.*, 2000). The contribution of hyaluronidase to group A streptococcal virulence is not currently well understood; however, recently group A streptococcal hyaluronidase was shown to facilitate subcutaneous spread of the bacteria (Starr and Engleberg, 2006)

### ***1.7.6 Streptokinase***

The secreted group A streptococcal plasminogen activator, streptokinase, is able to activate plasminogen via two distinct pathways and is not inhibited by PAI-1 or PAI-2 or host plasmin inhibitors  $\alpha$ 2-antiplasmin or  $\alpha$ 2-macroglobulin (Coleman and Benach, 1999). Plasminogen binding and activation may occur indirectly, through binding of a trimolecular complex (streptokinase, plasminogen and fibrinogen) to cell surface fibrinogen or plasminogen receptors, or directly via lysine dependent cell surface plasminogen receptors (Sanderson-Smith *et al.*, 2004). This indicates that GAS is capable of plasminogen activation unregulated by host plasminogen activators and inhibitors. The capacity of GAS to bind and activate plasminogen has been demonstrated to vary between strains and this may relate to differing capacities to invade through host tissue barriers (Svensson *et al.*, 1999; McKay *et al.*, 2004).

### ***1.7.7 Extracellular DNases***

*S. pyogenes* possesses multiple DNases, including mitogenic factor (MF) (Sriskandan *et al.*, 2000), MF-2 and MF-3 (Hasegawa *et al.*, 2002), SdaD (Podbielski *et al.*, 1996), Spd1 (Broudy *et al.*, 2002) and the phage-acquired Sda1 found in M1T1 GAS (Aziz *et al.*, 2004; Sumby *et al.*, 2005). The presence and relative importance of the

various streptococcal DNases varies between strains (Sriskandan *et al.*, 2000; Hasegawa *et al.*, 2002; Sumby *et al.*, 2005). The presence of the phages that encode the virulence genes *sda1* and *speA* differentiates M1T1 GAS from other M1 isolates (Aziz *et al.*, 2005; Aziz and Kotb, 2008). The potent extracellular DNase, Sda1, allows M1T1 GAS to escape killing by neutrophils extracellular traps (NETs) and has been shown to be the major DNase that contributes to virulence (Sumby *et al.*, 2005; Buchanan *et al.*, 2006). NETs are composed of DNA and histones and are secreted by neutrophils to entrap and kill invading bacteria (Brinkmann *et al.*, 2004).

#### ***1.7.8 Streptococcal inhibitor of complement***

The streptococcal inhibitor of complement-mediated lysis (SIC) protein is encoded by the highly polymorphic gene *sic* and is present in only a few group A streptococcal M-types, including the highly virulent M1T1 (Akesson *et al.*, 1996; Stockbauer *et al.*, 1998; Fernie-King *et al.*, 2001). SIC is highly immunogenic and displays a high level of sequence divergence, even amongst isolates of the same M-type suggesting that it may be under strong immune pressure (Stockbauer *et al.*, 1998; Hoe *et al.*, 2000). SIC acts by binding C6 and C7, preventing their incorporation into the membrane attack complex (Akesson *et al.*, 1996; Lukomski *et al.*, 2000). SIC has also been shown to bind and inhibit the action of secretory leukocyte proteinase inhibitor, lysozyme and the antimicrobial peptides  $\alpha$ -defensin and LL-37 (Fernie-King *et al.*, 2002; Frick *et al.*, 2003). The ability of SIC to combat numerous innate immune defences may result in the reduced virulence of a *sic* knockout in a murine intranasal infection model (Lukomski *et al.*, 2000).

### **1.7.9 IL-8 protease**

*Streptococcus pyogenes* cell envelope protease (SpyCEP, also known as ScpC) is a cell surface protease capable of cleaving the human CXC chemokine IL-8 (Edwards *et al.*, 2005; Hidalgo-Grass *et al.*, 2006). IL-8 is a multifunctional chemokine with a major role in recruitment and activation of neutrophils (Kunkel *et al.*, 1991; Middleton *et al.*, 1997). SpyCEP has been shown to promote resistance to killing by neutrophils (Zinkernagel *et al.*, 2008). In a murine model of infection a SpyCEP/ScpA double mutant was shown to result in reduced mortality and lesion size compared to an isogenic ScpA knockout, suggesting that SpyCEP is important for full virulence (Hidalgo-Grass *et al.*, 2006). In a separate study, a SpyCEP knockout was shown to result in larger lesions than its isogenic wildtype; however, this increase in lesion size was attributed to increased neutrophil infiltration and tissue damage due to cytotoxic molecules produced by the activated neutrophils at the site of infection (Sumby *et al.*, 2008).

### **1.7.10 Streptolysin O and streptolysin S**

GAS possesses two main cytolytins, Streptolysin S (SLS) and Streptolysin O (SLO) (Kehoe *et al.*, 1987; Nizet *et al.*, 2000). The SLS biosynthesis pathway is encoded by a 9 gene operon (*sagA – sagI*) that results in the production of a potent oxygen-stable cytolytin responsible for the  $\beta$ -haemolysis used to characterize GAS (Nizet *et al.*, 2000; Datta *et al.*, 2005). SLS has been shown to have a cytolytic effect on a broad range of cells, including lymphocytes, neutrophils and platelets (Keiser *et al.*, 1964; Taketo and Taketo, 1966; Ginsburg, 1972; Hryniewicz and Pryjma, 1977). The

cytolytic effect of SLS is similar to that of the complement system, forming transmembrane pores resulting in osmotic cell lysis (Ginsburg, 1999; Carr *et al.*, 2001). SLS has been shown to contribute significantly to virulence in a number of animal models (Betschel *et al.*, 1998; Humar *et al.*, 2002; Fontaine *et al.*, 2003; Sierig *et al.*, 2003; Engleberg *et al.*, 2004).

In contrast to SLS, SLO is an oxygen-labile, cholesterol-dependent cytolysin expressed from a single gene, *slo* (Weller *et al.*, 1996; Palmer, 2001). While SLO does not contribute significantly to group A streptococcal  $\beta$ -haemolysis, it has been shown to induce apoptosis in various cell types including macrophages, neutrophils and epithelial cells (Timmer *et al.*, 2009). It has also been shown to contribute significantly to group A streptococcal virulence in murine models of infection (Limbago *et al.*, 2000; Timmer *et al.*, 2009).

#### ***1.7.11 Streptococcal superantigens***

GAS expresses a number of potent superantigens, including the streptococcal pyrogenic exotoxins SpeA, SpeC and SpeF, streptococcal superantigen (SSA) and streptococcal mitogenic exotoxin (SmeZ) (Abe *et al.*, 1991; Tomai *et al.*, 1992; Mollick *et al.*, 1993; Norrby-Teglund *et al.*, 1994; Unnikrishnan *et al.*, 2002). Superantigens are able to simultaneously bind human MHC class II and T cell receptors resulting in excessive unregulated T-cell activation (Marrack and Kappler, 1990; Manders, 1998). This unregulated T-cell activation results in the production of large numbers of cytokines including TNF- $\alpha$ , interleukin 1 $\beta$  and interferon- $\gamma$ . Overwhelming production of these immunomodulators can result in STSS,

characterized by symptoms such as hypotension and multiple organ failure due to activation of the complement, coagulation and fibrinolytic pathways (Manders, 1998; Bisno *et al.*, 2003).

#### **1.7.12 *SpeB***

SpeB is a secreted cysteine protease initially expressed as 40 kDa zymogen which is then converted to the 28 kDa active form by autocatalytic processing (Musser *et al.*, 1996). SpeB is known to cleave numerous host proteins including components of the extracellular matrix, cytokine precursors, immunoglobulins, and antimicrobial peptides (Cunningham, 2000; Hynes, 2004; Nyberg *et al.*, 2004), which could interfere with host immune functions. However, SpeB has also been shown to cleave a range of group A streptococcal proteins such as the fibrinogen-binding M1 protein (Raeder *et al.*, 1998; Ringdahl *et al.*, 2000), various superantigens (Kansal *et al.*, 2003; Aziz *et al.*, 2004), the secreted plasminogen activator streptokinase (Rezcallah *et al.*, 2004) as well as the DNase Sda1 (Aziz *et al.*, 2004), and thus possibly interferes with the proven virulence functions of these bacterial factors. The precise role(s) of SpeB throughout the course of infection are undoubtedly complex, and not surprisingly, different studies using different *in vivo* animal models have produced seemingly contradictory results (Lukomski *et al.*, 1998; Svensson *et al.*, 2000; Ashbaugh and Wessels, 2001). Recently, the loss of SpeB activity through mutations in *covR/S* has been linked to the switch to an invasive phenotype in M1T1 GAS (Sumby *et al.*, 2006).

## 1.8 The CovR/S Regulatory System

CovR/S (Control of Virulence Response/Sensor, also known as CsrR/S) is a two-component regulatory system consisting of a sensory component, CovS, and a response component, CovR (Levin and Wessels, 1998). CovR/S has been shown to respond to environmental  $Mg^{2+}$  and conditions of mild stress, such as low pH, high osmolarity or high temperature (Gryllos *et al.*, 2003; Dalton and Scott, 2004). CovR/S was originally found to regulate capsule production as *covR/S* mutants were found to have a mucoid phenotype (Levin and Wessels, 1998). CovR/S has since been found to regulate up to 15% of the group A streptococcal genome (Graham *et al.*, 2002; Sumby *et al.*, 2006). Many of the genes regulated by CovR/S are virulence determinants as described above, including SpeB, streptodornase, streptokinase, streptolysin O, streptococcal inhibitor of complement and the hyaluronic acid capsule synthesis operon (Sumby *et al.*, 2006).

The response element, CovR, has been shown to act mainly as a repressor, with the vast majority of differentially regulated genes being expressed at lower levels in a wildtype strain of GAS compared to its isogenic *covR* deletion mutant (Federle *et al.*, 1999; Graham *et al.*, 2002). Similarly, *covS* mutant bacteria have been found to up-regulate numerous virulence related genes, with the notable exception of *speB*, which is up-regulated in *covR* deletion mutants but down-regulated in *covS* inactivation mutant strains (Heath *et al.*, 1999; Graham *et al.*, 2002; Sumby *et al.*, 2006).

Mucoid strains of GAS have long been associated with outbreaks of severe disease (Veasy *et al.*, 1987; Westlake *et al.*, 1990). Wessels *et al.* (1991) showed

dramatically increased virulence of such mucoid group A streptococci due to the up-regulation of hyaluronic acid capsule. Later, Levin and Wessels (1998) identified *CovR/S* as a regulator responsible for the mucoid phenotype and dramatically increased virulence following inactivation mutation.

Hyaluronic acid capsule has been shown to be a low level adhesin via the CD44 receptor and to facilitate M-protein mediated adhesion to keratinocytes; however, highly encapsulated strains exhibit reduced entry into keratinocytes despite increased virulence of these strains in a subcutaneous model of infection (Schrager *et al.*, 1996; Schrager *et al.*, 1998). Recently, an inverse correlation was shown between ability of isolates to adhere to host cells and their virulence in a mouse model of infection (Miyoshi-Akiyama *et al.*, 2009). While the authors did not find a significant correlation between hyaluronic acid production and virulence in this study, it is worth noting that the isolate expressing the highest level of hyaluronic acid was a mucoid M1 isolate that was also the most virulent (Miyoshi-Akiyama *et al.*, 2009).

Mouse passage of group A streptococci has been shown to produce SpeB-negative, highly encapsulated variants with increased virulence (Raeder *et al.*, 2000). Ravins *et al.* (2000) observed inactivation of *covR/S* in a highly-encapsulated, mouse-passaged M6 isolate. Engleberg *et al.* (2001) also noted spontaneous mutations in *covR/S* in M1 group A streptococci recovered from murine subcutaneous infections associated with larger more necrotic lesions.

Cole *et al.* (2006) observed that upon subcutaneous infection of mice, wildtype SpeB-positive M1T1 group A streptococci routinely switched to a SpeB-negative phenotype with increased plasminogen accumulation on the group A streptococcal

surface and systemic dissemination of the bacteria. While this switch was not identified as being linked to mutations in *covR/S*, the loss of SpeB expression was shown to be a crucial factor. These authors proposed a model of invasive disease initiation, whereby SpeB is required for M1T1 survival at the site of local infection; however, SpeB also disrupts the plasminogen activation system through degradation of numerous self and host proteins as previously described. The switch to a SpeB negative phenotype allows accumulation of surface plasmin activity enabling systemic spread of the bacteria (Figure 1.3). An inverse relationship has been shown between SpeB expression and disease severity in M1T1 isolates, supporting the hypothesis that SpeB loss is associated with severe invasive disease in humans (Kansal *et al.*, 2000).

Please see print copy for image



**Figure 1.3** Proposed model for group A streptococcal systemic disease initiation. (A) *S. pyogenes* M1T1 (blue) gain entry through the skin and a host innate immune response is initiated. (B) During the initial stages of infection, *S. pyogenes* M1T1 express SpeB to combat the host response. (C) Loss of SpeB activity in a subpopulation of group A streptococci (green) leads to the accumulation of surface plasmin activity. (D) Transition of *S. pyogenes* M1T1 is facilitated by surface plasmin activity, resulting in systemic infection. From Cole *et al.* (2006)



Recently, Sumby *et al.* (2006) also documented the switch to a mucoid, SpeB-negative phenotype upon subcutaneous infection of mice and utilised whole-genome sequencing to connect this switch to a single mutation in the *covR/S* locus, thereby complementing the model of infection proposed by Cole *et al.* (2006) with a mechanism for the switch to a SpeB-negative phenotype and the initiation of invasive disease. In addition, Sumby *et al.* (2006) investigated the transcriptome of invasive and pharyngeal isolates and found two distinct transcriptomes that were termed an invasive transcriptome profile and pharyngeal transcriptome profile. The invasive transcriptome profile encompassed *covS* mutant group A streptococci with up-regulation of multiple virulence factors and down-regulation of *speB*. The observation of *covR/S* mutant invasive clinical isolates coupled with spontaneous *covS* mutation during murine infection suggests that *covR/S* mutation may play a pivotal role in the initiation of human invasive disease caused by M1T1 GAS.

## 1.9 Project Aims

The aim of this study was to further investigate the switch to a SpeB-negative phenotype and the initiation of invasive disease in group A streptococcus. The specific objectives were: 1) To investigate the role of the phage-acquired DNase, Sda1, in the switch to a SpeB negative phenotype through *covR/S* mutation, 2) To elucidate the fitness cost of *covR/S* mutation, 3) To investigate whether mutation of the regulator *ropB* represents an alternative method of switching to a hypervirulent, SpeB-negative phenotype.

## **2. DNASE-MEDIATED RESISTANCE TO NEUTROPHIL KILLING PROVIDES SELECTION PRESSURE FOR A GENETIC AND PHENOTYPIC SWITCH, PROMOTING INVASIVE GROUP A STREPTOCOCCAL INFECTION**

### **2.1 Introduction**

GAS is responsible for an estimated ~700 million infections each year worldwide (Carapetis *et al.*, 2005). Severe invasive group A streptococcal infections such as necrotising fasciitis account for approximately 30% of the estimated 500,000 deaths from GAS each year (Carapetis *et al.*, 2005). Epidemic invasive disease is associated with the emergence of the globally disseminated group A streptococcal M1T1 clone (Cunningham, 2000; Walker *et al.*, 2005), which is distinguished from related strains by acquisition of prophages encoding virulence genes such as superantigen SpeA and DNase Sda1 (Aziz *et al.*, 2005; Sumby *et al.*, 2005; Aziz and Kotb, 2008).

In the M1T1 group A streptococcal clone, the transition from local to systemic infection has been linked to mutations in the two-component regulatory system, *covR/S* (Cole *et al.*, 2006; Sumby *et al.*, 2006). Mutation of *covR/S* results in a global phenotypic change involving differential regulation of multiple virulence factors, including up-regulation of the potent extracellular DNase, Sda1, and marked down-regulation of the secreted cysteine protease, SpeB (Sumby *et al.*, 2006). The *covR/S* mutation and changes in gene expression are recapitulated upon subcutaneous challenge of mice (Sumby *et al.*, 2006).

Sda1 is a virulence factor that degrades DNA NETs and provides GAS with increased capacity to resist killing by neutrophils (Sumby *et al.*, 2005; Buchanan *et al.*, 2006). Loss of SpeB expression allows accumulation of active plasmin, a broad spectrum host protease, on the group A streptococcal bacterial surface (Cole *et al.*, 2006). An inverse correlation has been found between SpeB expression and disease severity in MIT1 GAS (Kansal *et al.*, 2000). This study sought to investigate the contribution of the bacteriophage-encoded Sda1 to the switch to a SpeB-negative phenotype.

## **2.2 Materials and Methods**

### ***2.2.1 Culture of group A streptococci***

*S. pyogenes* strains were routinely propagated at 37°C on horse blood agar (BioMérieux) or in static liquid cultures of Todd-Hewitt broth (Difco) supplemented with 1% (w/v) yeast extract (THY). Invasive group A streptococcal isolate 5448 (MIT1) and the isogenic animal-passaged SpeB-negative variant 5448AP have been described previously (Aziz *et al.*, 2004). The isogenic mutants 5448 $\Delta$ *sda1* (Buchanan *et al.*, 2006), 5448 $\Delta$ *speB* (Aziz *et al.*, 2004) and GAS strain SF370 (Ferretti *et al.*, 2001), have also been described previously.

### ***2.2.2 Construction of recombinant group A streptococcal strains***

Allelic exchange was used to precisely replace the deleted *sda1* chromosomal locus in 5448 $\Delta$ *sda1* with the wildtype (WT) *sda1* gene to construct strain 5448RC*sda1*<sup>+</sup>.

The technology employed to construct 5448RC*sdaI*<sup>+</sup> was similar to that used in the construction of 5448 $\Delta$ *sdaI* (Buchanan *et al.*, 2006). The PCR primers RCSdapHY304Fwd and RCSdapHY304Rev were employed for amplification of flanking DNA upstream and downstream of *sdaI* in the 5448 chromosome (Table 2.1). Following amplification, the *sdaI* gene was cloned by *PstI*/*EcoRI* digestion and T4 ligation into the temperature-sensitive plasmid pHY304. 5448 $\Delta$ *sdaI* was transformed with the resulting plasmid (pHY*sdaI*) by electroporation. Integration of pHY*sdaI* into the chromosome via single-crossover was achieved by culture at the permissive temperature for plasmid replication (30°C). Following subculture at 37°C, single-crossover chromosomal insertions were selected using chloramphenicol (*AsdaI*) and erythromycin (pHY304). Double-crossover was achieved by serial passage at 30°C, and double-crossover reverse-complemented mutants were identified following removal of antibiotic selection. The reverse-complemented strain 5448RC*sdaI*<sup>+</sup> was characterized as sensitive to both chloramphenicol and erythromycin; confirmed as *sdaI* PCR-positive using the forward primer SdaF2 and reverse primer SdaR4 (Table 2.1); and able to express Sda1 upon assaying for DNase activity (as described below).

The isogenic 5448 $\Delta$ *smeZ* mutant was constructed in a manner identical to 5448 $\Delta$ *sdaI*, as previously described (Buchanan *et al.*, 2006). A precise, in-frame allelic exchange replacement of the *smeZ* gene in GAS strain 5448 with a chloramphenicol acetyltransferase (*cat*) antibiotic resistance cassette was generated. The specific primer sets used for amplification of the flanking DNA upstream and downstream of *smeZ* in the 5448 chromosome are given in Table 2.1. The primers

SmeZ-UpR and SmeZ-DownF contain a 25 bp 5' extension corresponding to the 5' and 3' ends of the *cat* gene, respectively.

Integrational mutagenesis of *ska* and *emm1* was performed essentially as previously described (Nizet *et al.*, 2000). Internal fragments of the genes *ska* and *emm1* were PCR amplified from GAS strain 5448 using specific primer pairs (Table 2.1) and cloned by *Bam*HI/*Xba*I digestion and T4 ligation into the temperature-sensitive plasmid pVE600721. 5448AP was transformed with the resultant plasmids by electroporation and chloramphenicol resistant transformants were grown at the permissive temperature for plasmid replication (30°C). Single-crossover Campbell-type chromosomal insertions were selected by shifting to the non-permissive temperature (37°C), while maintaining chloramphenicol selection. Integrational knockouts were confirmed by PCR using the forward primer T7-For and reverse primer *emm1*out-R or M1skaout-R (Table 2.1). Confirmed integrational knockouts were designated 5448AP $\Delta$ *emm1* and 5448AP $\Delta$ *ska*.

**Table 2.1** Oligonucleotide primers used in this study for construction of recombinant group A streptococcal strains.

Primer	Direction	bp	Sequence
RCSdapHY304Fwd	Forward	29	GGGCTGCAGCTTAAACGTTGGTATTTTAA
RCSdapHY304Rev	Reverse	28	GGGGAATTCGGATAGCTTACAACACTAGTG
SdaF2	Forward	21	ATGTCTAAACATTGGAGACAT
SdaR4	Reverse	20	ATCAGATGATAAAGCAGACA
SmeZ-UpF	Forward	20	GGTGGTATATCCAGTGATTTTTTTCTCCATAA ATAGCCTCTTTTCAGGAGTTAT
SmeZ-UpR	Reverse	54	GGTGGTATATCCAGTGATTTTTTTCTCCATAA ATAGCCTCTTTTCAGGAGTTAT
SmeZ-DownF	Forward	54	TACTGCGATGAGTGGCAGGGCGGGGCGTAAT TCAATTTTTCAATATAACTTTTA
SmeZ-DownR	Reverse	20	AACGACACCTCTTTCAGCGA
Emm1-dis-F-BamHI	Forward	30	GCGGATCCTAGTCCTGACTCGCTTGGTCTA
Emm1-dis-R-XbaI	Reverse	30	GCATCTAGACTTGCAGCAAACAATCCCGCA
M1ska-dis-F-BamHI	Forward	30	GCATCTAGACTTGCAGCAAACAATCCCGCA
M1ska-dis-R-XbaI	Reverse		GCGTCTAGACGCGCACATGTCCCTTTAACAA
T7-For	Forward	21	GTAATACGACTCACTATAGGG
Emm1out-R	Reverse	24	GAGGTAAAGGCTAACGGTGATGGT
M1skaout-R	Reverse	24	TTGAGCCCTGGTCTGAAATCGTCA

### 2.2.3 DNA sequence analysis of the *covR/S* operon

To screen group A streptococcal strains for mutations in the *covR/S* locus, 12 primers for PCR and DNA sequence analysis were used (Table 2.2 and Figure 2.2A). Firstly, primers *p1* and *p12* were used to PCR amplify the intact *covR/S* locus from genomic DNA which was extracted by phenol-chloroform. Then, an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) was used to directly sequence the amplified PCR product with the 12 primers and the sequence assembled by the use of Sequencher version 4.5 (Gene Codes Corporation). Using BLAST N analysis, the assembled sequences were aligned against group A streptococcal genomes and a single adenine base insertion mutation was identified at position 877 in the 5448AP *covS* gene, using numbering relative to the ATG start codon of 5448 *covS*. Other *in vivo*-derived, SpeB-negative GAS strain 5448 derivatives were analysed for *covR/S* mutations in an identical manner.

**Table 2.2** Oligonucleotide primers used in this study for *covR/S* PCR and sequence analysis.

Primer	Direction	bp	Sequence
p1	Forward	19	GCTATTCCGGTACAGGTCT
p2	Forward	19	GTCAATGGTCGTGAAGGGT
p3	Forward	22	GATGTCTATATTCGTTATCTCC
p4	Forward	22	GATGATTTTTACCACAGATAAC
p5	Forward	20	GCATATTGGTCTCTTACAAC
p6	Forward	21	GCAAATTGTAGATGGGTATCA
p7	Reverse	20	GCGGAAAATAGCACGAATAC
p8	Reverse	20	AGGCAATCAGTGTAAGGCA
p9	Reverse	21	CTTGCGCCAAATAACTCAACA
p10	Reverse	21	ATCAAAAAGCCTGCTCAAATGA
p11	Reverse	21	CTTCATGTCATCCATCATTG
p12	Reverse	19	TTGCTCTCGTGTGCCATCT

#### **2.2.4 *SpeB* activity assays**

*SpeB*-positive and *SpeB*-negative isolates were routinely identified by the Columbia skim milk agar assay (Ashbaugh *et al.*, 1998). Quantitative *SpeB* assays were undertaken as previously described (Collin and Olsen, 2000). Group A streptococcal strains were grown overnight at 37°C. Cultures were then diluted 1:50 and grown for 18 h at 37°C. The cultures were centrifuged at 3200 x *g* and the supernatants sterile filtered using a 0.2 µm syringe driven filter (Whatman). 200 µl of filtered supernatant was mixed with 200 µl of activation buffer (1mM EDTA, 20 mM DTT in 0.1 M Sodium Acetate buffer, pH 5.0) and incubated for 30 min at 40°C. 400 µl of 2% (w/v) azocasein in activation buffer was then added and incubated for a further 1 h at 40°C. TCA was then added to a final concentration of 15% (v/v) and thoroughly mixed. The mixture was then centrifuged at 15,000 x *g* for 5 min and the OD<sub>366</sub> of the supernatant was then measured to indicate cleavage of the azocasein by *SpeB*.

#### **2.2.5 Western blot analysis**

Western blot identification of α-enolase, GAPDH, streptokinase and M1 protein, were conducted essentially as previously described (Cole *et al.*, 2006), with the exception that generic rabbit anti-M protein serum (anti-M53) was used to identify M1 protein. Following SDS-PAGE of cell wall extracts or culture supernatants, proteins were transferred to nitrocellulose membrane at 100 V for 1 h using the Mini Trans-Blot (Bio-Rad). The membranes were blocked in a solution of 5% (w/v) skim milk (Difco) in PBS for 1 h at room temperature. After a 10 min wash with PBS, the membranes were incubated for 2 h with primary Ab diluted 1:1,000 in PBS.

Following three washes for 10 min each with PBS, the membranes were incubated for 1 h with a 1:1,000 dilution of goat anti-rabbit IgG HRP conjugate (Bio-Rad) for SpeB, Ska, SEN, and GAPDH blots. Excess secondary Ab was removed by three PBS washes for 10 min each and all blots developed in a solution of 100 mM Tris-HCl (pH 7.6) containing 1.4 mM diaminobenzidine and 0.06% (v/v) hydrogen peroxide.

#### ***2.2.6 Plasminogen binding and cell surface plasmin activity***

GAS strain NS113324 was used as an internal control for bacterial surface plasmin acquisition assays undertaken by incubating bacteria overnight in human plasma. Plasminogen binding assays were conducted as previously described (McKay *et al.*, 2004). Approximately 70 ng of <sup>125</sup>I-plasminogen was added to 250 µl of cell suspension and incubated at room temperature for 45 min. The cells were harvested by centrifugation, the supernatant carefully aspirated and pellet-associated radioactivity measured using an automated Wallac gamma counter (Perkin Elmer, Jugesheim, Germany). The results were expressed as a percentage of input radioactivity. All measurements were determined in triplicate.

Plasmin acquisition in human plasma by *S. pyogenes* isolates was determined essentially as described previously (Wang *et al.*, 1994). Frozen plasma was purchased from the Red Cross Blood Bank (Sydney, NSW, Australia), defrosted on ice and pooled. Aliquots of pooled plasma were depleted of plasminogen by incubation at 4°C on ice with excess lysine-sepharose 4B for 1–2 h with gentle agitation. The extent of plasminogen depletion and the plasmin activity of plasma



was determined by chromogenic assay with Spectrozyme® PL (American Diagnostics Inc., Stamford, CT, USA), in the presence and absence of streptokinase, and by Western blot using a polyclonal rabbit anti-human plasminogen antibody (Calbiochem, Kilsyth, VIC, Australia). Group A streptococci were cultured overnight as stationary cultures in 25 ml THY inoculated with 1 colony. Group A streptococci were pelleted by centrifugation at  $800 \times g$ , washed in 50 ml of PBS, pH 7.4, prewarmed to  $37^{\circ}\text{C}$ , and resuspended to  $\text{OD}_{600} = 0.7$  (corresponding to log phase, in which streptokinase secretion is induced). A 2 ml aliquot of this suspension was pelleted as above and resuspended in an equal vol of 100% plasma or plasminogen depleted plasma at  $37^{\circ}\text{C}$ . Group A streptococci were incubated in plasma for 3 h at  $37^{\circ}\text{C}$ , pelleted by centrifugation and washed twice with 1 vol of ice-cold 0.01 M EDTA, 0.1% (w/v) gelatin in PBS, pH 7.4. Group A streptococci were resuspended in 0.1% (w/v) gelatin in PBS, pH 7.4 to  $\text{OD}_{600} = 0.75$ . Aliquots (100  $\mu\text{l}$ ) of this suspension were incubated in triplicate in the presence and absence of 20  $\mu\text{l}$  Spectrozyme PL, 2.5 mM at  $37^{\circ}\text{C}$  for 60 min in a 96-well plate. The reaction was quenched with 80  $\mu\text{l}$  of 1.75 M acetic acid, the plates centrifuged at  $800 \times g$ , and  $\text{OD}_{405}$  of supernatants determined. Plasmin activity was determined as the difference between  $\text{OD}_{405}$  in the presence and absence of substrate, thus accounting for differences in the sedimentation efficiency of group A streptococcal isolates. Each isolate was assayed in at least two independent experiments. Plasmin equivalents and the linear range of the assay ( $\text{OD}_{405} = 0\text{--}0.6$ ) were determined using a standard curve of purified plasmin (Roche Diagnostics, GmbH, Mannheim, Germany).

### ***2.2.7 Virulence of group A streptococci in a humanized plasminogen transgenic mouse model***

Transgenic humanized plasminogen *AlbPLGI* mice heterozygous for the human plasminogen transgene (Sun *et al.*, 2004) were backcrossed greater than  $n = 6$  with C57BL/J6 mice (Animal Resources Centre, Perth, Australia). The group A streptococcal strains 5448 and 5448AP were harvested at logarithmic phase ( $OD_{600} = 0.4$ ), washed twice with sterile 0.7% saline and diluted to the required dose. The number of viable bacteria was determined by counting colony forming units (CFU) after plating a dilution series onto blood agar. The SpeB expression status of 5448 and 5448AP was also determined as described above ( $n = 50$ ). The 5448AP inoculum was found to be 100% SpeB-negative, while the 5448 inoculum was 100% SpeB-positive. Groups of *AlbPLGI* mice ( $n = 10$ ) were subcutaneously infected with group A streptococci and mortality was monitored for 10 days. Alternatively, groups of *AlbPLGI* mice ( $n = 5$ ) were subcutaneously infected with either 5448 or 5448AP for 48 h and the lesion (site of infection), blood, spleen and liver harvested. Lesion, spleen and liver samples were homogenized in 2 ml of sterile 0.7% saline. The number of viable bacteria was determined by counting CFU after plating a dilution series onto blood agar.

### ***2.2.8 Tissue chamber implantation and fluid recovery***

This study utilized the subcutaneous teflon chamber model developed by Kazmi and coworkers (Kazmi *et al.*, 2001). Teflon chambers were inserted surgically under the skin of 6-week-old female BALB/C mice. Three weeks after surgery, tissue chamber fluid (TCF) was collected and tested for sterility. Mice that had contaminated TCF,

or those that had open surgical wounds, were excluded from further experimentation. To prepare inocula, bacteria were grown overnight in THY, checked for SpeB phenotype as detailed above, then subcultured for 18 h in THY. Bacterial pellets were washed twice in sterile phosphate buffered saline (PBS) and resuspended in sterile PBS to  $1 \times 10^9$  CFU/ml. 100  $\mu$ l of this bacterial suspension was injected into the subcutaneous chambers using sterile 25-gauge needles. At 24 h post-injection, sterile 25-gauge needles were used to collect the TCF to analyse bacterial content and SpeB status (Kazmi *et al.*, 2001; Aziz *et al.*, 2004).

### **2.2.9 DNase activity assays**

Supernatants were collected from mid-logarithmic ( $OD_{600} = 0.4$ ) or stationary phase cultures of group A streptococcal strains grown in THY or tissue-cage fluid. Calf thymus DNA (1.0  $\mu$ g/ $\mu$ l) was combined with bacterial supernatant (2.5  $\mu$ l) in final volume of 50  $\mu$ l buffer (300 mM Tris, 3 mM  $CaCl_2$ , 3 mM  $MgCl_2$ ) for 15 min at room temperature. To halt DNase activity, 12.5  $\mu$ l of 0.33 M EDTA was added to the reaction. Visualization of relative DNA degradation was undertaken by side-by-side comparison of DNA using 1% agarose gel electrophoresis.

### **2.2.10 Live cell imaging for visualization of NETs**

NETs were visualized as previously described (Buchanan *et al.*, 2006). Briefly, human neutrophils were isolated and purified from venous blood using the PolyMorphPrep kit (Axis-Shield) as per the manufacturer's instructions and seeded at  $2 \times 10^5$  per well in 96-well plates in RPMI without phenol red (Invitrogen). Group

A streptococci were added to the wells at a multiplicity of infection of 1:100 (GAS:neutrophils) and Sytox Orange (Molecular Probes) added to a final concentration of 0.1  $\mu$ M. Cells were visualized without fixation or washed using a Zeiss Axiovert 100 inverted microscope with appropriate fluorescent filters, and images captured with a CCD camera. For quantification, NETs were enumerated for each treatment by counting one transect after staining from 3 independent wells; a NET was defined as a discrete area of bright orange fluorescence larger in size than a neutrophil. Presented data are representative of experiments undertaken on three separate occasions.

#### ***2.2.11 Neutrophil killing assays***

Neutrophil killing assays were performed as previously described (Buchanan *et al.*, 2006). Briefly, human neutrophils were isolated and purified from venous blood using the PolyMorphPrep kit (Axis-Shield) as per the manufacturer's instructions and seeded into 96-well plates at  $2 \times 10^5$  cells/well. Logarithmic-phase bacteria grown in THY were diluted to the desired concentration in RPMI media + 2% heat inactivated autologous human plasma, then added to neutrophils at a multiplicity of infection of 1:10 (GAS:neutrophils). Plates were centrifuged at 500 x g for 10 min then incubated at 37°C in 5% CO<sub>2</sub>. Following incubation for 1 h, neutrophils were lysed with 0.02% Triton X-100 and the contents of the well serially diluted and plated on Todd-Hewitt agar for overnight incubation and enumeration of CFU. Internal control wells without neutrophils were used to determine baseline bacterial counts at the assay endpoints. Percent survival of group A streptococci was

calculated as ([CFU/ml experimental well] / [CFU/ml control well]) x 100%. All assays were performed in triplicate.

### ***2.2.12 Monitoring the in vivo phase-shift of group A streptococcal strains***

Separate cohorts of C57BL/J6 mice ( $n = 10$ ) were inoculated subcutaneously with a non-lethal dose of GAS to examine the *in vivo* phase-shift of SpeB during infection. The inocula used in these experiments were plated out onto blood agar plates then individual colonies tested for SpeB expression status as described above ( $n = 50$ ). The 5448, 5448 $\Delta$ *sda1*, 5448RC*sda1*<sup>+</sup> and 5448 $\Delta$ *smez* inocula were found to be 100% SpeB-positive. On day 3 post-infection, mice were sacrificed by CO<sub>2</sub> asphyxiation and representative bacteria isolated from skin lesions according to the method of Cole *et al.* (2006). The SpeB status of individual colonies ( $n = 50$ ) was determined as described above.

All animal experiments were conducted according to the Guidelines for the Care and Use of Laboratory Animals (National Health and Medical Research Council, Australia) and were approved by the University of Wollongong Animal Ethics Committee.

### ***2.2.13 Statistical analyses***

Statistical analysis of SpeB expression and status, plasminogen-binding, surface plasmin activity, human neutrophil killing assays, and NET quantification were performed using a one way ANOVA with a Dunnett's Multiple Comparison Test.

Differences were considered statistically significant at  $P < 0.05$ . Differences in survival of humanized plasminogen transgenic mice infected with the group A streptococcal strains 5448, 5448AP and 5448AP $\Delta ska$  were determined by the log-rank (Mantel-Cox) test for comparison of survival curves. Differences were considered significant at  $P < 0.05$ . All statistical tests were performed using GraphPad Prism version 4.02.

#### ***2.2.14 Ethics approvals***

Permission to obtain human blood and undertake animal experiments was obtained from University of California, San Diego and University of Wollongong ethics committees. Volunteers provided informed consent before blood samples were obtained.

#### ***2.2.15 Experimental acknowledgements***

Acknowledgement of experimental assistance provided for this section is outlined in Table 2.3.

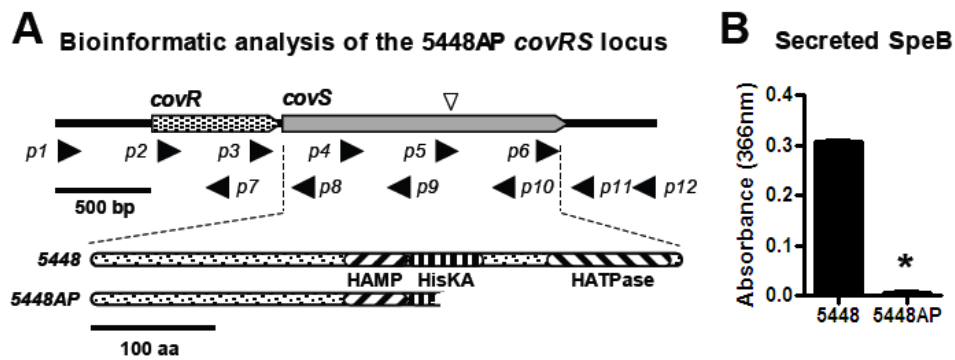
**Table 2.3** Acknowledgement of experimental assistance provided for this section

Experimental Section	Contributor	Contribution (%)
DNA sequence analysis	Mark Walker	33.3
	Josh Kirk	33.3
	Ramy Aziz	33.3
SpeB activity assay	Andrew Hollands	100
Western Blot analysis	Andrew Hollands	100
Plasminogen binding and plasmin acquisition assays	Jason Cole	100
Construction of 5448AP $\Delta$ ska and 5448AP $\Delta$ emm1	Andrew Hollands	100
Transgenic mouse breeding	Andrew Hollands	40
	Anna Henningham	30
	Jason MacArthur	30
Murine virulence studies	Andrew Hollands	40
	Jason Cole	30
	Martina Sanderson-Smith	30
Tissue cage implantation and in vivo DNase assays	Ramy Aziz	50
	Rita Kansal	50
In vitro DNase activity assays	Andrew Hollands	100
Neutrophil studies	Andrew Hollands	30
	Mark Walker	40
	Amelia Simpson	20
	John Buchanan	10
Construction of 5448 $\Delta$ SmeZ	Arthur Jeng	50
	Kalpna Chalasani	50
Construction of 5448RCSda1 <sup>+</sup>	Martina Sanderson-Smith	100
SpeB switching studies	Andrew Hollands	25
	Mark Walker	25
	Martina Sanderson-Smith	25
	Jason Cole	25

## 2.3 Results

### 2.3.1 *covS* mutation results in loss of *SpeB* expression

To elucidate the selection pressure for the rapid loss of *SpeB* expression *in vivo*, the human M1T1 group A streptococcal isolate 5448 was compared with its isogenic animal passaged *SpeB*-negative variant 5448AP (Aziz *et al.*, 2004). DNA sequence analysis shows 5448AP contains a single adenine base insertion at position 877 of the *covS* gene (Figure 2.1A) and lacks *SpeB* production (Figure 2.1B).



**Figure 2.1** DNA sequence analysis and *SpeB* expression of GAS strains 5448 and 5448AP. **(A)** DNA sequence comparison of GAS strains 5448 and 5448AP confirms the presence of a 1 base adenine addition at the 3' end of *covS* (nt position 877) encoded by 5448AP (unfilled arrowhead). Primers used for DNA sequence analysis are indicated by filled arrowheads (*p1* to *p12*). This insertion mutation results in the truncation of the *CovS* open reading frame at amino acid 300 from the *CovS* methionine start codon. Putative conserved *CovS* domains are indicated by striped regions in lower panel: HAMP: Histidine kinases/adenylyl cyclases/methyl-binding proteins/phosphatases; HisKA, Histidine kinase domain (phosphoacceptor); HATPase: Histidine kinase-like ATPase. Scales, in base pairs (bp; upper panel bar) and amino acids (aa; lower panel bar), are indicated. **(B)** In comparison to GAS strain 5448, secreted *SpeB* protease activity is abrogated in 5448AP. Asterisk indicates statistically significant difference from 5448, where  $P < 0.05$ .

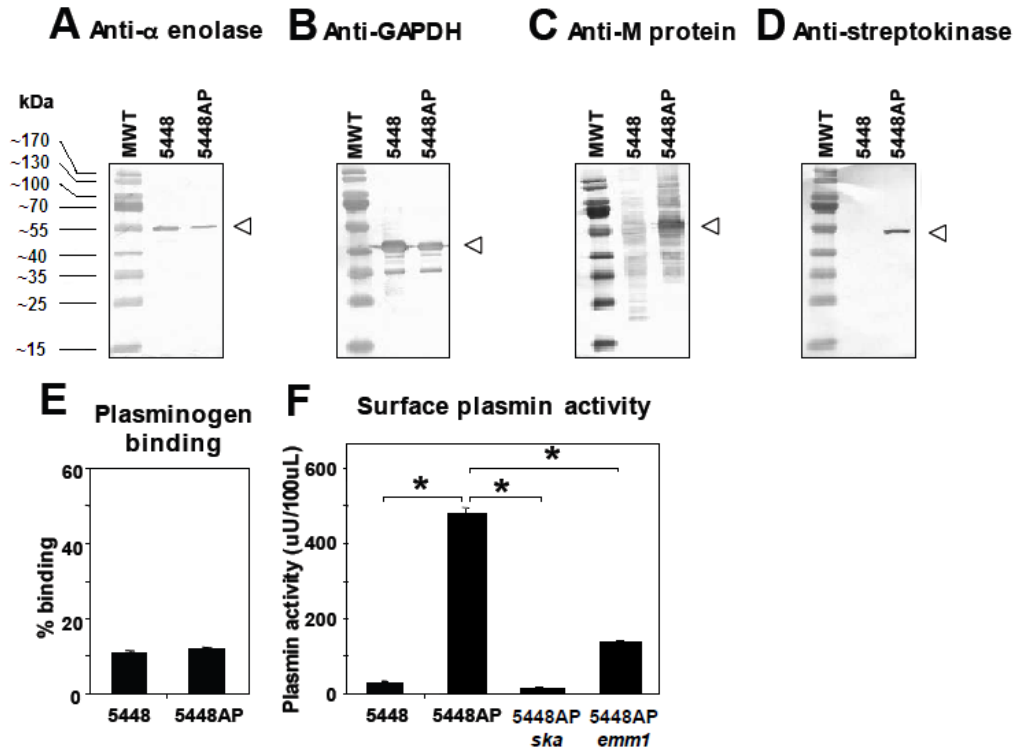


### ***2.3.2 covS mutant group A streptococci accumulate greater levels of surface plasmin***

Whilst equivalent to WT 5448 in expression of plasminogen receptors  $\alpha$ -enolase (Pancholi and Fischetti, 1998) (Figure 2.2A) and GAPDH (Pancholi and Fischetti, 1992) (Figure 2.2B1D), 5448AP exhibits higher levels of the fibrinogen-binding M1 protein (Ringdahl *et al.*, 2000; McArthur and Walker, 2006) (Figure 2.2C) and streptokinase (Figure 2.2D). Although washed 5448 and 5448AP cells bind identical levels of human plasminogen (Figure 2.2E), 5448AP accumulates significantly higher levels of surface plasmin activity following growth in human plasma (Figure 2.2F). The observed phenotypes of 5448AP parallel those seen upon allelic replacement of the *speB* gene in the parent strain (mutant 5448 $\Delta$ *speB*) (Cole *et al.*, 2006), indicating that surface plasmin acquisition by 5448AP reflects the loss of SpeB. Additionally, other gene expression changes, such as the increase in streptokinase or M-protein expression associated with *covR/S* mutation (Sumby *et al.*, 2006), may also contribute to surface plasmin acquisition by 5448AP. Both 5448AP $\Delta$ *ska* and 5448AP $\Delta$ *emm1* were found to accumulate significantly less surface plasmin activity than 5448AP (Figure 2.2F).

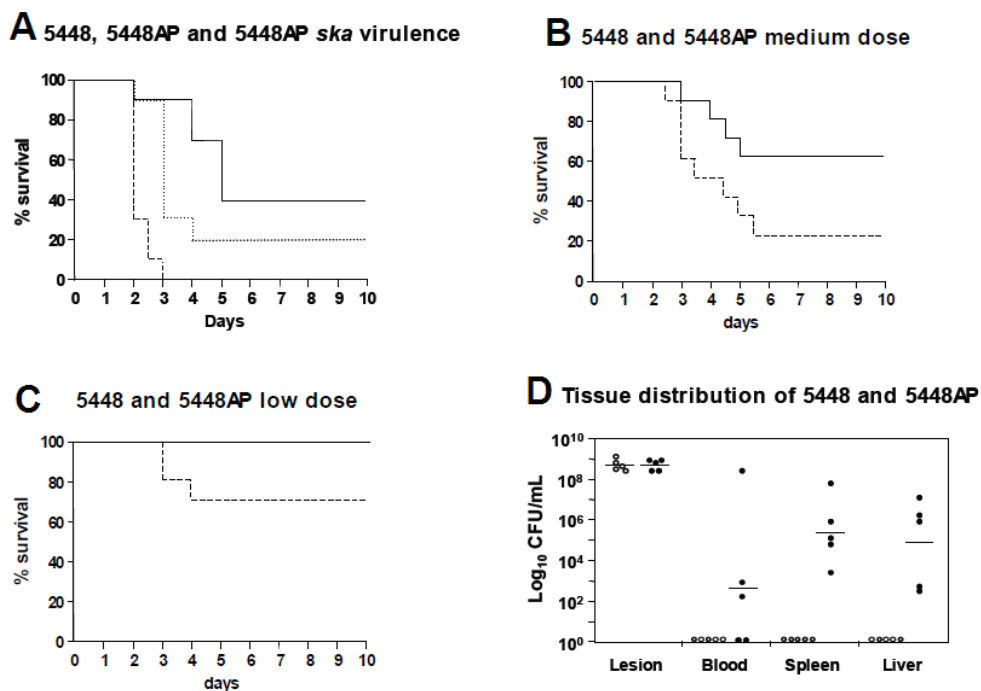
### ***2.3.3 covS mutation confers increased virulence and dissemination to organs***

Compared to WT, the 5448AP strain was found to be hypervirulent in a humanized plasminogen mouse subcutaneous infection model ( $P < 0.05$ ; Figure 2.3A-C). Isogenic mutagenesis of 5448AP was undertaken to construct a streptokinase-deficient strain (5448AP $\Delta$ *ska*), which showed reduced virulence in comparison to



**Figure 2.2** Molecular and phenotypic analyses of GAS strains 5448 and 5448AP. Western blot analysis of cell wall extracts indicates that equivalent amounts of (A)  $\alpha$ -enolase (unfilled arrowhead) and (B) GAPDH (unfilled arrowhead) are produced by strains 5448 and 5448AP. Western blot analysis of GAS indicates that a higher amount of (C) M1 protein (unfilled arrowhead) is produced in cell wall extracts and (D) streptokinase (unfilled arrowhead) is secreted into culture supernatants by strain 5448AP, in comparison to 5448. Molecular mass markers (MWT) are given in kilo-Daltons (kDa). (E) Washed 5448 and 5448AP cells bind equivalent amounts of human plasminogen. (F) Following overnight growth at 37°C in human plasma, 5448AP accumulates significantly higher levels of surface plasmin activity than 5448; 5448AP $\Delta$ *ska* and 5448AP $\Delta$ *emm1* accumulate significantly less plasmin activity than 5448AP. Asterisk indicates statistically significant difference from 5448 or 5448AP, where  $P < 0.05$ .

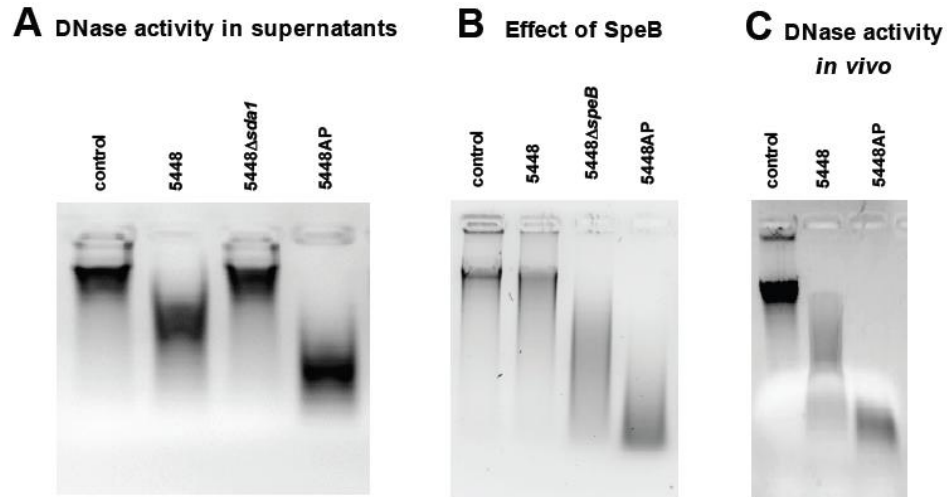
5448AP ( $P < 0.05$ ; Figure 2.3A). This observation is consistent with the reduced virulence of *ska*-deficient GAS previously reported (Sun *et al.*, 2004). Enumeration of bacterial counts in the site of infection, blood, spleen and liver of humanized plasminogen mice, suggest that the enhanced virulence of strain 5448AP is as a result of a widespread systemic infection following breakout from the site of local infection, immediately prior to the death of the mice (Figure 2.3D). These humanized animal model data reflect observations made in the clinical setting for group A streptococcal M1T1 strains, where mutations in *covR/S* correlate with human invasive disease severity (Kansal *et al.*, 2000; Sumby *et al.*, 2006).



**Figure 2.3** (A) Survival curves following subcutaneous infection of humanized plasminogen transgenic mice with GAS strain 5448 ( $3.2 \times 10^8$  colony forming units/dose; solid line), 5448AP ( $1.6 \times 10^8$  colony forming units/dose; dashed line) and 5448AP $\Delta ska$  ( $1.5 \times 10^8$  colony forming units/dose; dotted line). (B) Survival curves following subcutaneous infection of humanized plasminogen transgenic mice with GAS strain 5448 ( $6.6 \times 10^7$  CFU/dose; solid line) and 5448AP ( $2.9 \times 10^7$  CFU/dose; dashed line). (C) Survival curves following subcutaneous infection of humanized plasminogen transgenic mice with GAS strain 5448 ( $5.8 \times 10^6$  CFU/dose; solid line) and 5448AP ( $1.3 \times 10^7$  CFU/dose; dashed line). (D) Enumeration of bacterial counts in the site of infection, blood, spleen and liver of humanized plasminogen mice subcutaneously infected with GAS strain 5448 ( $2.6 \times 10^7$  colony forming units/dose; open circles) and 5448AP ( $4.9 \times 10^7$  colony forming units/dose; filled circles).

#### 2.3.4 *covS* mutation results in increased DNase activity in vitro and in vivo

DNA degradation by supernatants from 5448AP was increased compared to the 5448 parental strain (Figure 2.4A), consistent with both the up-regulation of *sda1* in *covS* mutant GAS (Sumby *et al.*, 2006) and the known ability of SpeB to degrade Sda1 (Aziz *et al.*, 2004). Removal of SpeB is shown to increase DNase activity but not to the same levels as 5448AP, illustrating the combined effects of up-regulation of Sda1 and down-regulation of SpeB (Figure 2.4B). In addition, increased DNA degradation by infection chamber fluid from 5448AP infected mice compared to 5448 was observed, demonstrating that this phenomenon also occurs *in vivo* (Figure 2.4C).



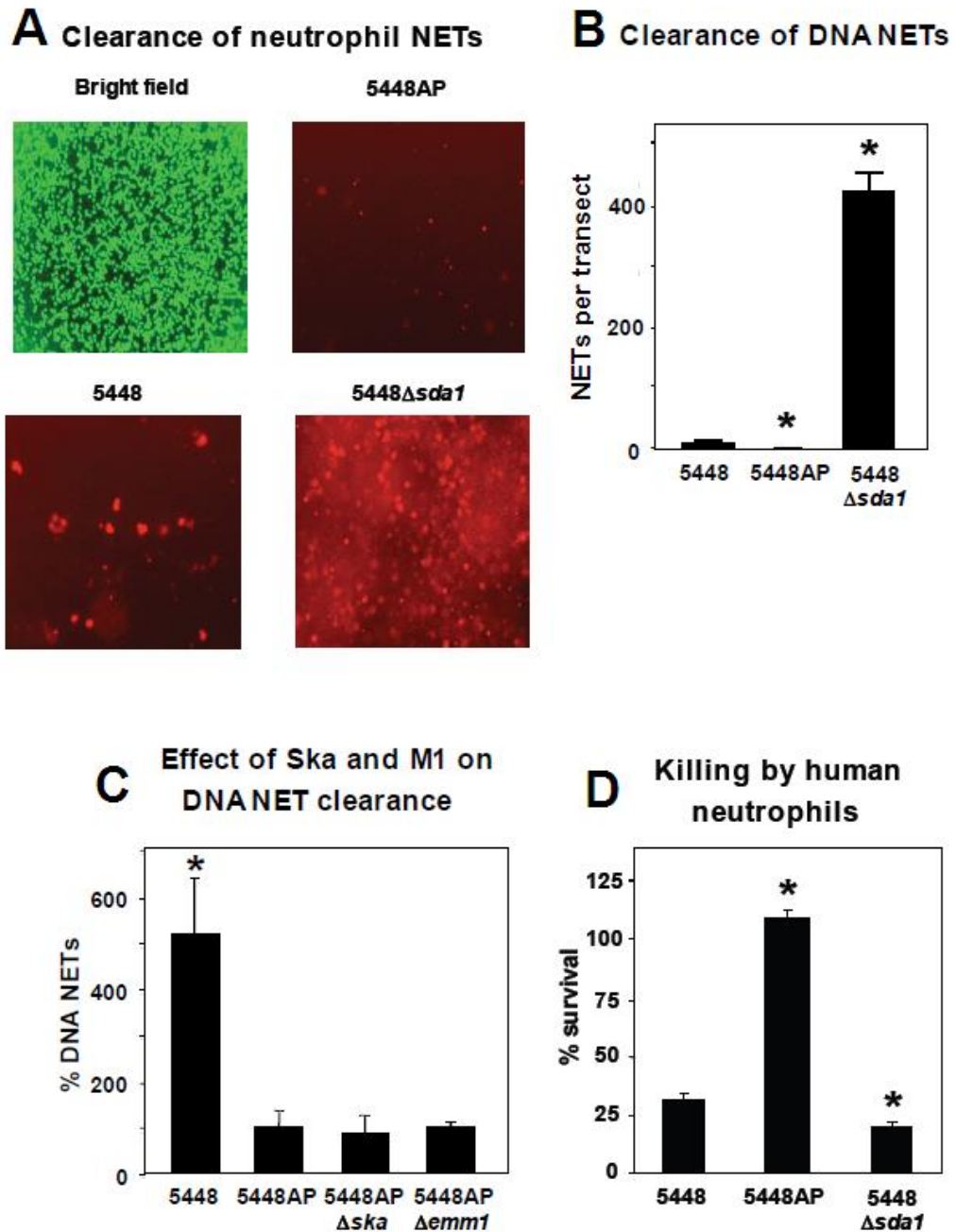
**Figure 2.4** DNase activity assays *in vitro* and *in vivo* (A) DNase expression in GAS mid-logarithmic phase culture supernatants as assessed by degradation of calf thymus DNA (control). (B) DNase expression in stationary-phase culture supernatants as assessed by degradation of calf thymus DNA (control). (C) DNase expression in tissue cage fluid 24 h following inoculation with 5448 or 5448AP. DNase activity of uninoculated tissue cage fluid was also determined (control).

### ***2.3.5 covS mutation results in increased clearance of NETs and increased resistance to killing by neutrophils***

Compared to the 5448 parent strain, the enhanced DNase activity of 5448AP was associated with clearance of NETs (Figure 2.5A and B) and increased resistance to neutrophil killing (Figure 2.5C). Neither streptokinase nor M1 protein contribute to NET clearance (Figure 2.5D).

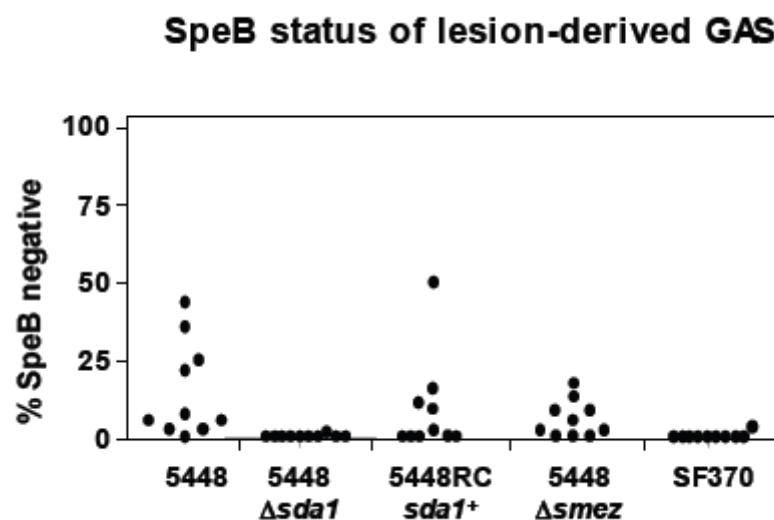
### ***2.3.6 sda1 is essential for the in vivo switch to a SpeB-negative phenotype.***

Neutrophils and NET-mediated extracellular killing play a pivotal role in antibacterial clearance at the initial site of infection (Brinkmann *et al.*, 2004; Buchanan *et al.*, 2006). It was hypothesized that acquisition of the potent bacteriophage-encoded DNase Sda1 by the M1T1 clone provides the selective force



**Figure 2.5** (A) Clearance of NETs by GAS. Neutrophils were visualized using bright field microscopy, whilst NETs were visualized using Sytox Orange staining. (B) Quantification of the clearance of NETs by GAS. NETs were visualized using Sytox Orange staining and enumerated for each treatment by counting a transect from 3 independent wells. Asterisk indicates statistically significant difference from 5448, where  $P < 0.05$ . (C) NETs clearance by GAS strain 5448 in comparison to 5448AP, 5448AP $\Delta$ ska and 5448AP $\Delta$ emm1. Asterisk indicates statistically significant difference from 5448AP (100%), where  $P < 0.05$ . (D) Killing of GAS by human neutrophils at a multiplicity of infection (GAS:neutrophils) = 1:10. Asterisk indicates statistically significant difference from 5448, where  $P < 0.05$ .

for loss of SpeB expression *in vivo*, since the cysteine protease is capable of degrading this important neutrophil survival factor. To examine this possibility, C57BL/J6 mice were subcutaneously challenged separately with 5448 and the isogenic 5448 $\Delta$ *sda1* mutant, predicting that absence of Sda1 would reduce the selective advantage for mutation to a SpeB-negative phenotype. Loss of SpeB expression *in vivo* during subcutaneous murine infection was abrogated in the isogenic 5448 $\Delta$ *sda1* mutant compared to WT 5448 (Figure 2.6; 1/500 SpeB-negative 5448 $\Delta$ *sda1* colony versus 76/500 SpeB-negative 5448 colonies;  $P < 0.05$ ). DNA sequence analysis of 10 selected SpeB-negative 5448 colonies suggests that mutations in *covR/S* have resulted in loss of SpeB expression (Table 2.4), as previously reported (Sumby *et al.*, 2006). Reverse complementation was used to replace the mutated chromosomal locus in 5448 $\Delta$ *sda1* with the WT allele to construct 5448RC*sda1*<sup>+</sup>. This complemented mutant regained the capacity to switch to the SpeB-negative phenotype (Figure 2.6). There was no significant difference observed between 5448 and 5448RC*sda1*<sup>+</sup> (45/500 SpeB-negative 5448RC*sda1*<sup>+</sup>



**Figure 2.6** The capacity of GAS strains 5448, 5448 $\Delta$ *sda1*, 5448RC*sda1*<sup>+</sup>, 5448 $\Delta$ *smez* and SF370 to phase-shift to a SpeB-negative phenotype was examined 3 days post-subcutaneous infection of mice.

colonies;  $P = 0.39$ ). The isogenic mutant 5448 $\Delta$ *smez*, derived in a manner identical to 5448 $\Delta$ *sda1*, was found to retain the capacity to phase-switch similar to the WT strain 5448 (Figure 2.5; 33/500 SpeB-negative 5448 $\Delta$ *smez* colonies;  $P = 0.11$ ). These observations suggest that the phase-switching phenotype is due to allelic replacement of the *sda1* gene and not due to the methodology used to construct an isogenic GAS mutant in strain 5448. The M1 serotype GAS strain SF370 is known not to encode Sda1 (Aziz *et al.*, 2005; Sumby *et al.*, 2005). SF370 was found to have minimal capacity to switch to the SpeB-negative phenotype compared to 5448 (Figure 2.5; 2/500 SpeB-negative SF370;  $P < 0.05$ ) consistent with the absence of Sda1 and thus lack of selective advantage for mutation to the SpeB-negative phenotype.

**Table 2.4** *CovR/S* DNA sequence analysis of selected GAS M1T1 strain 5448 SpeB-negative derivatives isolated 3 days following subcutaneous infection of C57BL/J6 mice.

GAS strain	Mouse ID	Tissue <sup>a</sup>	Mutation <sup>b</sup>	Consequence <sup>c</sup>
5448-APD1	OS41B	Blood	C to T nt 838 <i>covS</i>	H to Y aa 280 <i>covS</i>
5448-APD2	OS41B	Lesion	C to T nt 838 <i>covS</i>	H to Y aa 280 <i>covS</i>
5448-APD3	OS41B	Lesion	C to T nt 838 <i>covS</i>	H to Y aa 280 <i>covS</i>
5448-APD4	OS45R	Blood	C to T nt 838 <i>covS</i>	H to Y aa 280 <i>covS</i>
5448-APD5	OS45R	Blood	C to T nt 838 <i>covS</i>	H to Y aa 280 <i>covS</i>
5448-APD6	OS46L	Lesion	$\Delta$ nt 83 <i>covS</i>	Truncation in CovS
5448-APD7	OS46L	Lesion	$\Delta$ nt 83 <i>covS</i>	Truncation in CovS
5448-APD8	OS46L	Lesion	G to A nt 331 <i>covR</i>	A to T aa 111 <i>covR</i>
5448-APD9	OS322B	Lesion	$\Delta$ nt 406-1503 <i>covS</i>	Truncation in CovS
5448-APD10	OS492L	Lesion	C to T nt 838 <i>covS</i>	H to Y aa 280 <i>covS</i>

<sup>a</sup>Murine tissue from which GAS strain was isolated 3 days post-subcutaneous infection.

<sup>b</sup>Mutation positions are based upon nucleotide (nt) position in the *covR* or *covS* genes, relative to each ATG start codon.

<sup>c</sup>Substitutions in CovR and CovS are based upon amino acid (aa) position in each open reading frame, relative to each start codon.

## 2.4 Discussion

The resurgence of group A streptococcal disease in the mid-1980s was paralleled by the emergence of the globally disseminated M1T1 clone as a major cause of severe group A streptococcal invasive disease (Chatellier *et al.*, 2000; Walker *et al.*, 2005; Tart *et al.*, 2007). Recent genome-scale analyses have found that M1T1 differs from other M1 strains primarily in that the M1T1 clone has acquired two lysogenised bacteriophage genomes encoding Sda1 and SpeA, respectively (Aziz *et al.*, 2005; Sumby *et al.*, 2005; Aziz and Kotb, 2008). While the introduction of SpeA into the group A streptococcal population increases the propensity to cause streptococcal toxic shock, this study has shown that positive selection pressure *in vivo* is placed upon the bacteriophage-encoded secreted DNase Sda1.

The animal passaged strain 5448AP was found to have a mutation in *covS* that resulted in loss of SpeB expression. The *covS* mutant strain accumulated greater surface plasmin activity, despite binding equivalent amounts of plasminogen. Accumulation of active plasmin on the bacterial surface may result from increased expression of the plasminogen activator, streptokinase, and a loss of SpeB, which has been shown to degrade streptokinase (Rezcallah *et al.*, 2004). This accumulation of plasmin, a broad spectrum protease, may contribute to the invasive spread of GAS (Cole *et al.*, 2006).

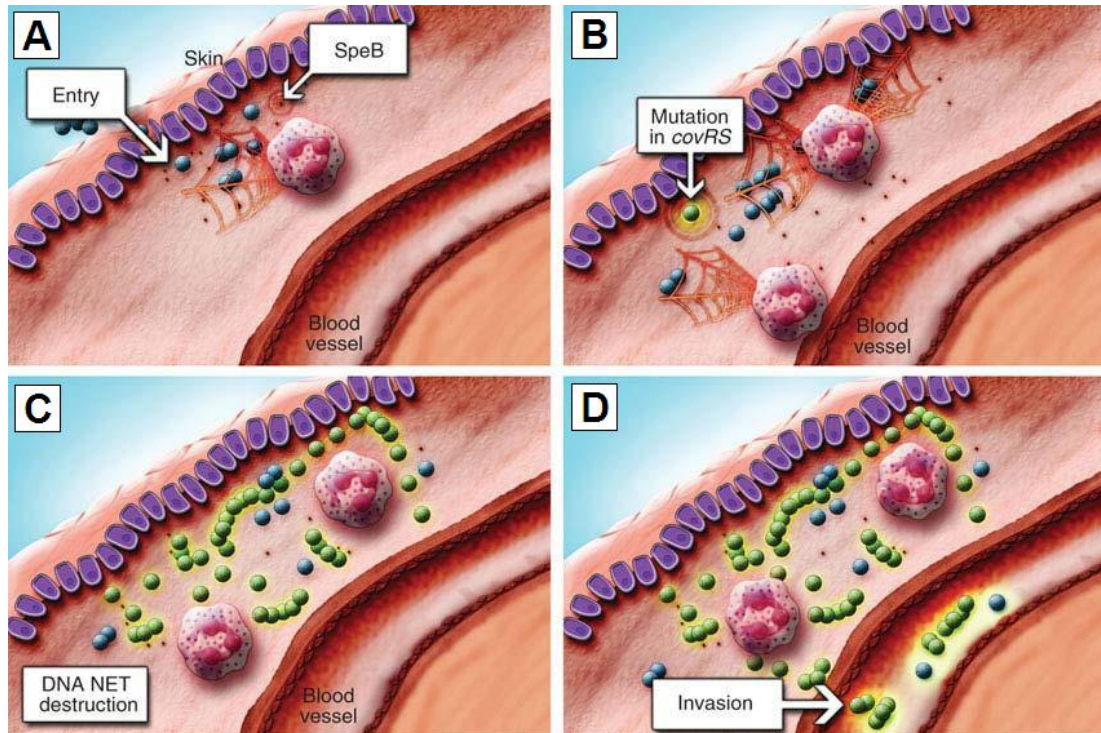
The *covS* mutant strain 5448AP displays significantly increased virulence and dissemination to organs in a murine model of infection. This increased virulence is partially abrogated by mutagenesis of the *ska* gene, suggesting that plasmin



activation and accumulation plays a significant role in this increased virulence, but that other gene regulation changes may contribute.

Loss of SpeB spares Sda1 from degradation and results in improved ability of GAS to degrade NETs and resistant killing by human neutrophils. This ability to resist killing by neutrophils may provide *covS* mutant bacteria with a survival advantage at the site of infection. *In vivo*, the ability to phase-switch to a SpeB-negative phenotype is abrogated by isogenic mutagenesis deleting the gene *sda1*. The genetic basis for loss of SpeB expression through *covR/S* mutation has been previously described (Sumby *et al.*, 2006). All colonies sequenced were found to contain mutations in the *covR/S* operon. Loss of SpeB has also been shown to result in increased invasive propensity of M1T1 by the accumulation of surface-bound plasmin activity (Cole *et al.*, 2006). Therefore, it was hypothesized that the bacteriophage-mediated acquisition of the *sda1* gene by the ancestral M1T1 has provided evolutionary selection pressure for increased neutrophil resistance via SpeB loss, which results in a hyperinvasive phenotype and can lead to severe invasive disease progression (Figure 2.6).

The evolution of bacterial pathogens principally occurs either through deletion events or horizontal gene transfer and acquisition (Ochman and Moran, 2001), which is exemplified by the bacteriophage-mediated acquisition of the *sda1* gene by M1T1. These data provide a paradigm for bacteriophage-mediated acquisition of virulence determinants and development of severe disease by otherwise benign human pathogens.



**Figure 2.6** Model for group A streptococcal invasive disease initiation and progression. (A) Following entry via the skin, group A streptococci (blue) are able to express SpeB (required during the early stages of the infection process; 1 black dots). An innate immune response is mounted by host neutrophils and entrapment of group A streptococci in NETs (orange) begins. (B) Within the group A streptococcal population, a mutation in *covR/S* occurs (green), resulting in loss of SpeB expression and improved resistance to killing by neutrophils. (C) Selection pressure by neutrophils results in an increase in the proportion of *covR/S* mutant phenotype group A streptococci within the bacterial population, improved NET clearance and neutrophil resistance. (D) Loss of SpeB expression allows the accumulation of surface plasmin activity leading to systemic infection.

### **3. THE COST OF EVOLUTION TO A HYPERVIRULENT PHENOTYPE BY M1T1 GROUP A STREPTOCOCCUS IS REDUCED CAPACITY TO COLONIZE.**

#### **3.1 Introduction**

An inverse relationship has been observed between SpeB expression and clinical severity in M1T1 clinical isolates (Chatellier *et al.*, 2000). Recently, a connection has been identified between loss of SpeB expression through mutation in the two-component regulatory system *covR/S* and development of invasive disease in the murine model (Sumby *et al.*, 2006; Walker *et al.*, 2007). SpeB is a secreted cysteine protease initially expressed as a 40 kDa zymogen which is then converted to the 28 kDa active form by autocatalytic processing (Musser *et al.*, 1996). SpeB has been shown to cleave multiple host proteins, including components of the extracellular matrix (ECM), cytokine precursors, immunoglobulins and antimicrobial peptides (Cunningham, 2000; Hynes, 2004; Nyberg *et al.*, 2004), which could impair host immune functions. However, SpeB also cleaves a broad range of self proteins, such as the fibrinogen-binding M1 protein (Raeder *et al.*, 1998; Ringdahl *et al.*, 2000; Cole *et al.*, 2007), various superantigens (Kansal *et al.*, 2003; Aziz *et al.*, 2004), the secreted plasminogen activator streptokinase (Rezcallah *et al.*, 2004), as well as the DNase Sda1 (Aziz *et al.*, 2004). Degradation of these bacterial effectors may alter group A streptococcal virulence. The exact role that SpeB plays during the course of infection remains unclear.

CovR/S is an important global gene regulator, responsible for regulating approximately 10% of the group A streptococcal genome (Sumby *et al.*, 2006). Specific point mutations in *covR/S* and truncation mutations of *covS* result in up-regulation of many genes encoding virulence factors such as streptodornase, streptokinase, streptolysin O, streptococcal inhibitor of complement and the hyaluronic acid capsule synthesis operon, whereas SpeB expression is significantly down-regulated (Sumby *et al.*, 2006; Walker *et al.*, 2007). It is hypothesized that it is the sparing of group A streptococcal virulence determinants from SpeB degradation, along with up-regulation of multiple virulence factors, that results in the proliferation and invasive spread of the *covR/S* mutant M1T1 group A streptococci (Cole *et al.*, 2006; Sumby *et al.*, 2006; Walker *et al.*, 2007). Furthermore, enhanced resistance to killing by neutrophils exerts a positive selection pressure on *covR/S* bacteria (Walker *et al.*, 2007). This evolution of M1T1 GAS *in vivo* results in the proliferation and invasive spread throughout the host of *covR/S* mutant group A streptococci (Cole *et al.*, 2006; Sumby *et al.*, 2006; Walker *et al.*, 2007).

Given the routine occurrence of *covR/S* mutation and the dramatically increased virulence of *covR/S* mutant M1T1 group A streptococci (Walker *et al.*, 2007), the question arises whether counter-balancing selection pressure acts to maintain the wildtype GAS M1T1 population in the presence of the hypervirulent neutrophil-resistant *covR/S* allele. This study sought to elucidate the cost of *covR/S* mutation in the GAS M1T1 population.

## 3.2 Materials and Methods

### 3.2.1 Bacterial strains, media and growth conditions

The well characterized M1T1 clinical isolate 5448 and its mouse-passaged *covS* mutant derivative 5448AP were used in this study (Walker *et al.*, 2007). Group A streptococcal strains were grown in Todd-Hewitt broth (THB), or on Todd-Hewitt agar plates (THA). *Escherichia coli* were grown in Luria-Bertani broth (LB) or on Luria-Bertani agar (LA). For antibiotic selection, erythromycin (Erm) was used at 5 µg/ml for GAS and 500 µg/ml for *E. coli*.

### 3.2.2 Allelic exchange mutagenesis

This technique was performed essentially as previously described (Nizet *et al.*, 2000). An intragenic fragment of *hasA* was amplified using primers hasAint-F-BamHI (5'-GCA GGA TCC TTG GAA CAT CAA CTG TAG G'3') and hasAint-R-XbaI (5'-GCA TCT AGA TTA ATT CAA ATG TCC TGT TGC AGC-3') and cloned by *Bam*HI/*Xba*I digestion and T4 ligation into the temperature sensitive vector pHY304. 5448 and 5448AP were transformed with the resultant plasmid by electroporation and Erm resistant transformants were grown at the permissive temperature for plasmid replication (30°C). Single-crossover Campbell-type chromosomal insertions were selected by shifting to the nonpermissive temperature (37°C) while maintaining Erm selection. Integrational knockouts were confirmed by PCR. Confirmed plasmid integrational knockouts were designated 5448Δ*hasA* and 5448APΔ*hasA*.

### 3.2.3 ECM binding assays

Fibronectin or collagen was bound to 96-well plates (Costar) as previously described (Jeng *et al.*, 2003). Bacteria were grown to mid-logarithmic phase, washed in sterile PBS and diluted to  $2 \times 10^6$  CFU/ml. The plates were washed three times with sterile PBS and 100  $\mu$ l ( $2 \times 10^5$  CFU) of bacterial suspension was added to each well. The plates were centrifuged at  $500 \times g$  for 10 min and then incubated at 37°C for 1 h. Following incubation, the plates were washed three times with sterile PBS to remove non-adherent bacteria. 100  $\mu$ l of 0.25% trypsin/1mM EDTA (Gibco) was added to each well and incubated for 10 min at 37°C to remove adherent bacteria. Bacteria were then serially diluted in PBS and plated onto THA for enumeration. Assays were performed in triplicate and significance determined by an unpaired t-test.

### 3.2.4 Epithelial cell adherence assays

Adherence assays were performed using Hep-2 (human pharyngeal epithelial cells) and HaCat (human keratinocyte cells) as previously described (Timmer *et al.*, 2006). Competition adherence assays were performed by adding an equal mix of 5448 and 5448AP to cell monolayers as described above. The assays were performed as above and plated on THA overnight. Fifty individual colonies from each condition were picked to inoculate cultures for microtitre SpeB assays. A microtitre assay for SpeB activity was used, based on previously described method by Collin *et al.* (2000) with volumes adjusted proportionally to a total volume of 200  $\mu$ l. Assays were performed in triplicate and statistical significance determined using an unpaired t-test.

### **3.2.5 Hyaluronic acid capsule assay**

Bacterial cultures were grown to mid-log phase in THB. 5 ml of OD<sub>600</sub>=0.4 culture was spun down and resuspended in 500 µl H<sub>2</sub>O. Serial dilutions of bacterial suspension were plated on THA to confirm equivalent cfu. 400 µl of the bacterial suspension was placed in a 2 ml screw cap tube with 1 ml chloroform. Tubes were shaken at full speed for 5 min in a mini-beadbeater-8 (Biospec Products). Tubes were then spun at ~13,000 x *g* for 10 min and hyaluronic acid content of the aqueous phase was determined using a Hyaluronic Acid Test Kit (Corgenix) as per the manufacturer's specifications.

### **3.2.6 Biofilm quantification assays**

Biofilm quantization was performed using a crystal violet stain assay as described previously (Tendolkar *et al.*, 2004). Bacterial cultures were grown to mid-logarithmic phase at 37°C in THB. 200 µl aliquots of the cultures were plated in 96-well microtitre plates. The plates were incubated at 37°C for 24 h. The cultures were then aspirated and the wells washed three times with sterile PBS and allowed to dry. 200 µl of 0.2% aqueous crystal violet solution was added to each well and allowed to stand for 15 min. The wells were then washed another three times with sterile PBS. Crystal violet bound to the biofilm was extracted with an 80:20 (v/v) mixture of ethanol and acetone. The absorbance of the extracted crystal violet was measured at 595 nm using an automated platereader. Assay was performed in triplicate and statistical significance determined using an unpaired t-test.

### **3.2.7 In vivo adherence assay**

Bacterial cultures were grown to mid-logarithmic phase ( $OD_{600}=0.4$ ) and washed with sterile PBS. Bacteria were diluted to  $2 \times 10^7$  CFU/ml of 5448 and  $2 \times 10^7$  CFU/ml of 5448AP. 10  $\mu$ l of bacterial suspension was spotted onto prewarmed THA plates. Once the droplets had dried, circles of agar containing the bacteria were excised using an 8mm biopsy punch. Shaved CD1 mice were anaesthetized with Ketamine/Xylazine and bacterial agar discs were affixed to the mouse with Tegaderm transparent wound dressing (3M). After 1 h, the mice were euthanized with isoflurane. The skin under the bacterial disks was excised and placed into 2 ml screw cap tubes containing 1ml PBS. The tubes were shaken in a mini-beadbeater-8 (Biospec Products) on mix setting for 2 min to remove non-adherent bacteria. The skin was then transferred to a fresh screw cap tube containing PBS and shaken for a further 2 min. The skin was then transferred to a fresh 2 ml tube containing 1 ml PBS and 1 mm silica/zirconia beads (Biospec Products). The tissue was then homogenized by shaking twice with the mini-beadbeater-8 at full for speed for 1 min, placing on ice in between. The homogenate was serially diluted in sterile PBS and plated on THA. Following overnight incubation, 50 colonies per skin sample were picked and screened for SpeB activity as previously described.

### **3.2.8 Growth Curves**

Overnight cultures of GAS bacteria were diluted into 10 ml fresh THB to the same  $OD_{600}$ . The  $OD_{600}$  was then measured every 30 min to measure growth curves for strains.



### ***3.2.9 Chain length assays***

Bacteria were grown overnight at 37°C in THB. Group A streptococci were then serially diluted in sterile PBS in a 96-well flat bottom plate and centrifuged for 5 min at 3,200 x g to create a monolayer. Group A streptococci were then viewed at a dilution of  $10^{-1}$  for visualization and  $10^{-2}$  for enumeration using a Zeiss Axiovert 100 inverted microscope and images captured with a CCD camera. Chain length was enumerated by counting chains in a random field of view and averaging. Statistical significance was determined using an unpaired t-test.

### ***3.2.10 Anti-microbial peptide resistance assays***

Bacteria were grown to mid-logarithmic phase and resuspended in PBS + 20% THB at  $1 \times 10^5$  CFU/ml. 90 µl of bacteria was then added to 10 µl of varying concentrations of LL-37 in a 96-well plate. At 24 hrs, 5 µl from each well was plated on THA and incubated overnight. The minimum inhibitory concentration (MIC) was defined as the lowest antimicrobial concentration yielding no detectable bacterial growth, and minimum bactericidal concentration (MBC) was defined as the lowest antimicrobial concentration yielding no surviving bacteria when the sample was plated on THA. Where there was variance in the MIC or MBC, the values are shown as a range.

### ***3.2.11 Ethics approvals***

Permission to obtain human blood and undertake animal experiments was obtained from University of California, San Diego and University of Wollongong ethics committees. Volunteers provided informed consent before blood samples were obtained.

### ***3.2.12 Experimental acknowledgements***

Acknowledgement of experimental assistance provided for this section is outlined in Table 3.1

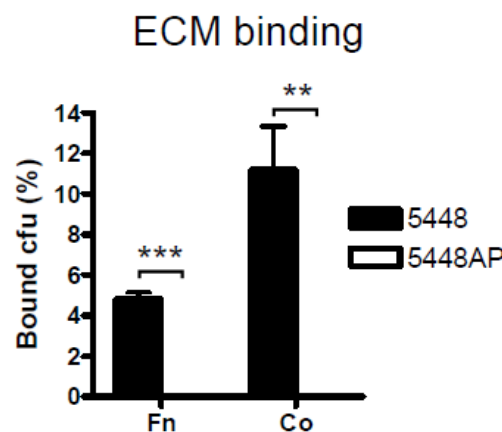
**Table 3.1** Acknowledgement of experimental assistance provided for this section.

<b>Experimental Section</b>	<b>Contributor</b>	<b>Contribution (%)</b>
Construction of mutant strains	Andrew Hollands	100
Epithelial cell adherence assays	Andrew Hollands	80
	Anjuli Timmer	20
Biofilm assays	Andrew Hollands	100
Growth Curves	Morgan Pence	100
Antimicrobial peptide assays	Morgan Pence	100
Chain length assays	Andrew Hollands	100
Skin Colonization	Andrew Hollands	100

### 3.3 Results

#### 3.3.1 *covR/S* mutant MIT1 group A streptococci have reduced capacity to bind ECM components

The well characterized MIT1 clinical isolate 5448 and the isogenic *covS* mutant derivative 5448AP were used in this comparative analysis. Binding to the ECM is an important initial step in the colonization process and is a property associated with the colonization of damaged tissues (Westerlund and Korhonen, 1993; Kreikemeyer *et al.*, 2004). The *covR/S* mutant strain 5448AP was found to have significantly reduced capacity to bind fibronectin ( $P < 0.001$ ) and collagen ( $P < 0.01$ ) compared to the wildtype parental strain 5448 (Figure 3.1). This reduction in capacity to bind ECM components may affect the ability of *covR/S* mutant group A streptococci to colonize the host.



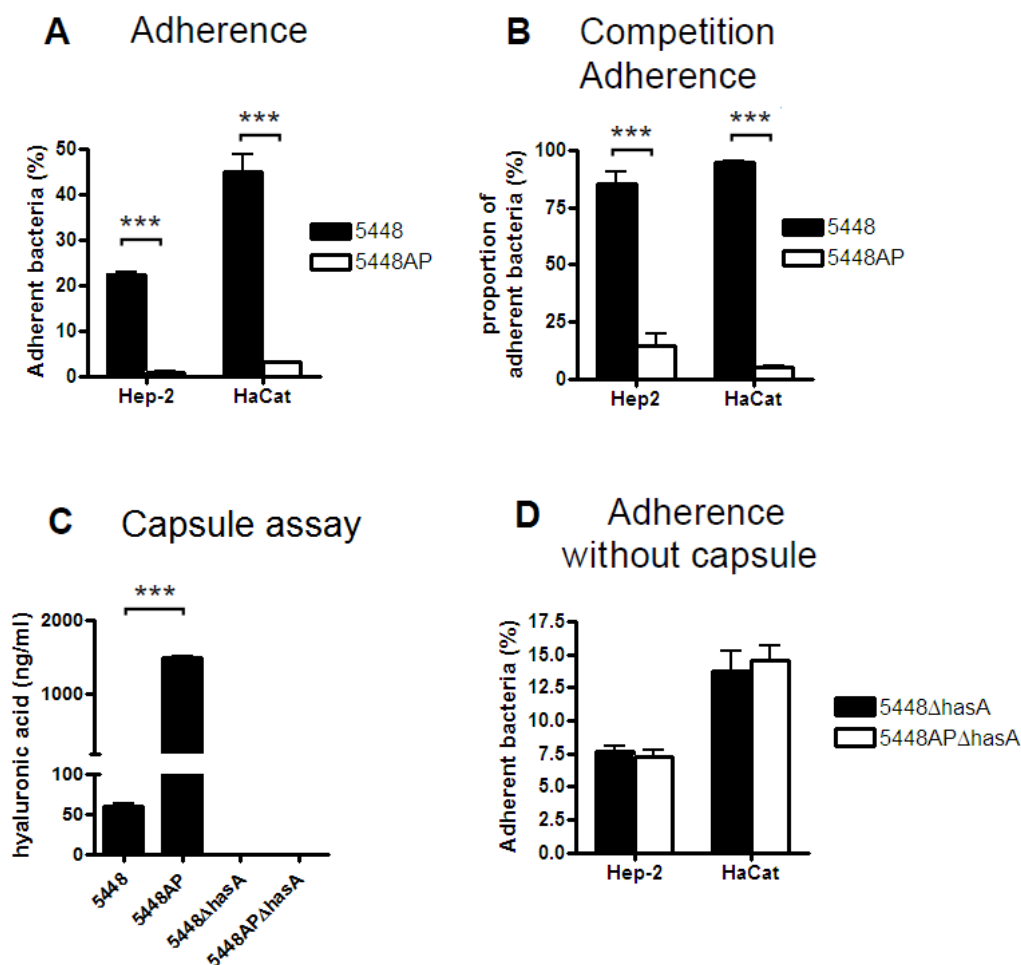
**Figure 3.1** *covR/S* mutation results in reduced binding to the extracellular matrix. Binding of 5448 and 5448AP to immobilized fibronectin (Fn) and collagen (Co).. Statistical significance is denoted by \*\* ( $P < 0.01$ ) or \*\*\* ( $P < 0.001$ )

### 3.3.2 Ability to adhere to epithelial cells is reduced in *covR/S* mutant *MIT1* group *A streptococci*

To investigate the ability of wildtype and *covS* mutant bacteria to adhere to epithelial cells, this study utilized two human cell lines: Hep-2 cells, a human laryngeal cell line, and HaCat cells, a human keratinocyte cell line, reflecting both throat and skin focal points of group A streptococcal infection. 5448AP had a marked decrease in adherence compared to wildtype 5448 ( $P < 0.001$ ) in both Hep-2 and HaCat cells (Figure 3.2A).

In a competition binding assay, 5448 outperformed 5448AP in adherence to both Hep-2 and HaCat cells (Figure 3.2B). The observation that the adherence of 5448AP was not rescued by co-infection with 5448 suggests that a cell surface factor and not a secreted factor, is responsible for this defect in epithelial cell binding.

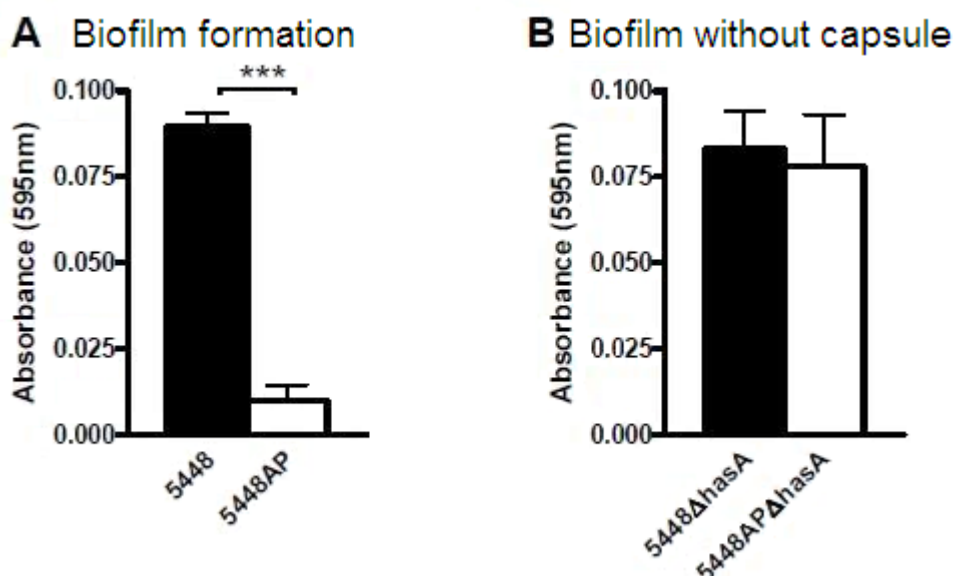
Up-regulation of capsule has previously been shown in *covS* mutant GAS (Sumby *et al.*, 2006). It was hypothesized that this up-regulation of capsule in the 5448AP was the cause of decreased epithelial cell binding. 5448AP was found to have significantly more hyaluronic acid capsule than 5448, while the mutant strains 5448 $\Delta$ *hasA* and 5448AP $\Delta$ *hasA* were found to have no capsule (Figure 3.2C). Comparison of wildtype and *covS* mutant group A streptococci, both with a genetically defined deletion in the capsule biosynthesis gene *hasA*, revealed there was no difference in adherence between 5448 $\Delta$ *hasA* and *covS* mutant 5448AP $\Delta$ *hasA* strains using either cell line (Figure 3.2D). These data suggest that up-regulation of capsule in 5448AP is the principal reason for reduced binding to epithelial cells.



**Figure 3.2** *CovR/S* mutation results in reduced capacity to adhere to epithelial cells. **(A)** Adherence to Hep-2 cells (human pharyngeal epithelial cells) and HaCat cells (human keratinocytes). **(B)** Competitive adherence to Hep-2 and HaCat cells. **(C)** Hyaluronic acid capsule levels. **(D)** Adherence to Hep-2 and HaCat cells using capsule deficient mutants 5448ΔhasA and 5448APΔhasA. Statistical significance is denoted by \*\*\* ( $P < 0.001$ ).

### 3.3.3 Biofilm formation is reduced in *covR/S* mutant MIT1 group A streptococci

Biofilm formation has been proposed to play a role in group A streptococcal colonization as well as being involved in persistence and recurrence of group A streptococcal infection (Lembke *et al.*, 2006; Manetti *et al.*, 2007). It was found that 5448AP exhibits significantly less biofilm formation than 5448 ( $P < 0.001$ ) (Figure 3.3A). Using mutant strains deficient in capsule, no difference was found in ability to form biofilms (Figure 3.3B). This reduction in biofilm formation may contribute to a reduced ability of *covR/S* mutant MIT1 group A streptococci to colonize a new host.

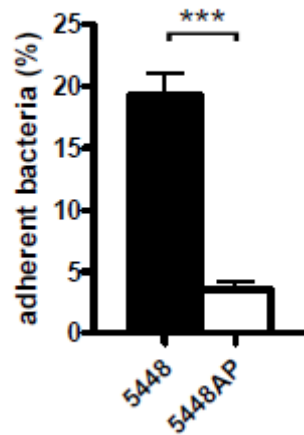


**Figure 3.3** *CovR/S* mutation reduces biofilm formation. (A) Biofilm formation after 24 h growth in 96-well polystyrene microtitre plates. (B) Biofilm formation using capsule deficient mutants 5448ΔhasA and 5448APΔhasA. Statistical significance is denoted by \*\*\* ( $P < 0.001$ ).

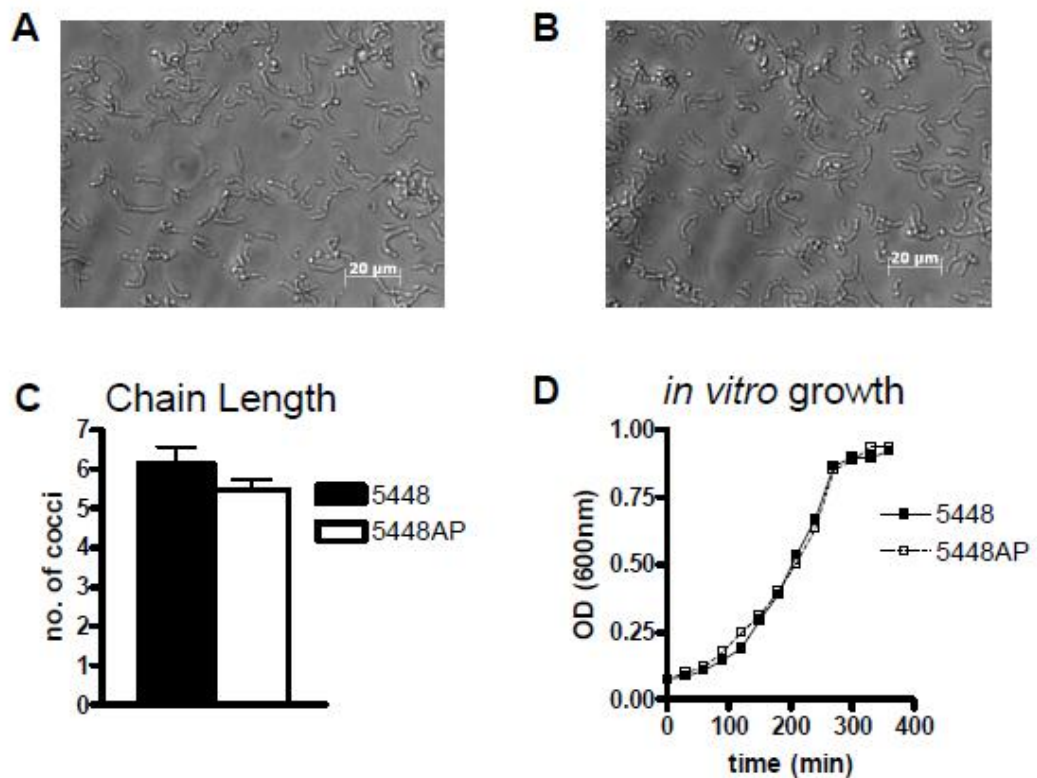
### 3.3.4 *covR/S* mutant M1T1 group A streptococci have reduced capacity to colonize

A mouse model of skin colonization was used to investigate the comparative capacity of 5448 and 5448AP to colonize the host at a relevant infection site. 5448AP was found to have significantly reduced ( $P < 0.001$ ) ability to adhere, and hence, colonize live mouse skin compared to 5448 (Figure 3.4). Reduced survival on live skin may relate to growth characteristics or sensitivity to antimicrobial peptides (Zasloff, 2002). However, no difference in growth rate or chain length between 5448 and 5448AP (Figure 3.5A-D), nor resistance to killing by the human cathelicidin antimicrobial peptide LL-37 was observed (Table 3.2). The similarity of growth characteristics and lack of difference in susceptibility to antimicrobial peptides suggests that the colonization defect is related to the reduced adherence and not to survival on the skin. These data suggest that the cost of *covR/S* mutation is reduced capacity to colonize the host and that this reduced colonization capacity is due to adherence defects in *covR/S* mutant M1T1 group A streptococci.

### Skin colonization



**Figure 3.4** *covR/S* mutation results in reduced colonization capacity. Adherence of bacteria to live mouse flank after 1 h incubation is shown. Statistical significance is denoted by \*\*\* ( $P < 0.001$ ).



**Figure 3.5** *covR/S* mutation does not alter growth characteristics. (A) Chain length of 5448. (B) Chain length of 5448AP. (C) Enumeration of chain length of 5448 and 5448AP. (D) Growth curves in Todd-Hewitt broth.

**Table 3.2** Resistance to killing by the human cathelicidin antimicrobial peptide LL-37.

Strain	Minimum Inhibitory Concentration	Minimum Bactericidal Concentration
5448	14 $\mu$ M	24 – 28 $\mu$ M
5448AP	16 $\mu$ M	28 $\mu$ M

### 3.4 Discussion

MIT1 GAS is the most common cause of streptococcal infections in several western countries (Cleary *et al.*, 1992; Demers *et al.*, 1993; Carapetis *et al.*, 2005). An inverse relationship between SpeB expression in MIT1 clinical isolates and disease severity led to the hypothesis that inactivation of SpeB through mutation in the two-component regulatory system *covR/S* facilitates invasive disease initiation (Chatellier *et al.*, 2000; Sumby *et al.*, 2006; Walker *et al.*, 2007). While *covR/S* mutation in MIT1 GAS results in improved neutrophils resistance and propensity for bacterial dissemination (Sumby *et al.*, 2006; Walker *et al.*, 2007), this study sought to investigate the counterbalancing fitness cost associated with *covR/S* mutation. Such a fitness cost would select for the maintenance of the wildtype phenotype in the population. In order to examine this evolutionary process, the MIT1 group A streptococcal isolate 5448 and its *covS* mutant derivative 5448AP were utilized (Walker *et al.*, 2007).

Binding to ECM components is an important initial step in the colonization process (Westerlund and Korhonen, 1993; Kreikemeyer *et al.*, 2004). GAS possesses several different fibronectin and collagen binding proteins, suggesting that attachment to these ECM components is important for bacterial colonization (Delvecchio *et al.*, 2002; Jeng *et al.*, 2003; Kreikemeyer *et al.*, 2004; Ramachandran *et al.*, 2004;



Kreikemeyer *et al.*, 2005). It was found that the *covR/S* mutant strain 5448AP showed reduced capacity to bind to both fibronectin and collagen. This binding defect may affect the capacity of *covR/S* mutant group A streptococci to colonize the host.

Utilizing two human epithelial cell lines, 5448AP was found to have significantly reduced capacity to adhere to either cell line compared to 5448. To confirm that the reduced adherence was due to a cell surface component, a competition adherence assay was used. With an equal mix of 5448 and 5448AP, mostly 5448 were able to bind to epithelial cells. Therefore, it was concluded that a cell surface component and not a secreted factor, was responsible for the decreased adherence in the *covR/S* mutant strain.

It was hypothesized that capsule, known to be up-regulated in *covR/S* mutant M1T1 group A streptococci (Sumby *et al.*, 2006) may mask prerequisite adhesins and result in the binding defect. Comparison of the capsule deficient isogenic mutants 5448 $\Delta$ hasA and 5448AP $\Delta$ hasA demonstrated that the two strains exhibited similar capacity to adhere to epithelial cells, suggesting that the up-regulated capsule in 5448AP is the principal cause of the reduction in binding ability. Bacterial adherence to epithelial cells is a critical step in the colonization process that may contribute to a colonization deficiency of *covR/S* mutant M1T1 group A streptococci (Beachey, 1981).

Biofilm formation has been proposed to play a role in group A streptococcal colonization as well as being involved in persistence and recurrence of group A

streptococcal infection (Lembke *et al.*, 2006; Manetti *et al.*, 2007). 5448AP had reduced capacity to form biofilms compared to 5448. However, capsule deficient mutants 5448 $\Delta$ hasA and 5448AP $\Delta$ hasA showed no difference in ability to form biofilms. This observation supports the contention that *covR/S* mutation reduces the colonization capacity of M1T1 GAS due to overproduction of capsule.

A murine model of colonization was utilised to examine these factors in an *in vivo* context. Significantly more 5448 than 5448AP were recovered from the mouse skin colonization model, demonstrating that *covR/S* mutation confers a colonization defect in M1T1 GAS, despite the dramatic increase in virulence at subsequent stages of infection. These data are supported by a recently published finding of an inverse correlation between ability to adhere to host cells and group A streptococcal virulence (Miyoshi-Akiyama *et al.*, 2009). The comparison of 5448 and 5448AP reveals that *covR/S* mutation is associated with increased virulence in this strain, but that this increase in virulence comes at the cost of the ability to adhere to host cells. Similarly, Miyoshi-Akiyama *et al.* (2009) found that high-virulence isolates of GAS were associated with a low capacity to adhere to epithelial cells. Although Miyoshi-Akiyama *et al.* (2009) did not find a significant correlation between hyaluronic acid production and virulence, it is worth noting that the isolate expressing the highest level of hyaluronic acid was a mucoid M1 isolate that was also the most virulent.

These findings highlight a duality in group A streptococcal infection, whereby it was hypothesized that an intact *covR/S* is needed for efficient colonization but a mutation in *covR/S* confers increased propensity for virulence following colonization of the host. *In vivo* evolution and selection, such as that described in this paper, has been

recently described in relation to persistent *Pseudomonas aeruginosa* infection in cystic fibrosis patients. Positive selection of mutations has been observed, allowing for genetic changes advantageous to life within the host (Smith *et al.*, 2006). Such research illustrates how *in vivo* selection and proliferation can affect genetic and phenotypic changes suited to bacterial survival in the host. Such mutation may result in a fitness cost in an alternate environmental niche, as described in this study.

In summary, it was demonstrated that *covR/S* mutation in M1T1 GAS results in defective ability to bind to components of the ECM and epithelial cells and reduced biofilm forming capacity. These combined defects result in reduced ability to colonize the host. These findings illustrate how *in vivo* evolution can select for variant bacterial populations subject to different selection pressures as the microorganism transits through various ecological niches associated with pathogenesis. In particular, reduced colonization capacity provides a potential explanation as to why the *covS* mutation conferring hypervirulence has not become fixed in the globally disseminated M1T1 clone of GAS, but rather may arise anew under innate immune selection in individual patients.

## **4. A NATURALLY OCCURRING MUTATION IN *RopB* SUPPRESSES *SpeB* EXPRESSION AND REDUCES M1T1 GROUP A STREPTOCOCCAL SYSTEMIC VIRULENCE.**

### **4.1 Introduction**

Studies of M1T1 clinical isolates from invasive disease cases have revealed an inverse relationship between expression of the extracellular cysteine protease *SpeB* and clinical severity (Kansal *et al.*, 2000). The existence of a *SpeB*-negative invasive phenotype has been hypothesized that results from mutations in the regulator *covR/S* (Sumby *et al.*, 2005). *SpeB* is a secreted cysteine protease initially expressed as 40 kDa zymogen which is then converted to the 28 kDa active form by autocatalytic processing (Musser *et al.*, 1996). *SpeB* is known to cleave numerous host proteins including components of the extracellular matrix, cytokine precursors, immunoglobulins and antimicrobial peptides (Cunningham, 2000; Hynes, 2004; Nyberg *et al.*, 2004), which could interfere with host immune functions. However, *SpeB* has also been shown to cleave a range of group A streptococcal proteins such as the fibrinogen-binding M1 protein (Raeder *et al.*, 1998; Ringdahl *et al.*, 2000), various superantigens (Kansal *et al.*, 2003; Aziz *et al.*, 2004), the secreted plasminogen activator streptokinase (Rezcallah *et al.*, 2004) as well as the DNase Sda1 (Aziz *et al.*, 2004), and thus possibly interfere with the proven virulence functions of these bacterial factors.

This chapter examined the effect of a natural mutation in the gene encoding the regulator RopB (also known as Rgg (Chaussee *et al.*, 2003)) identified in a SpeB-negative group A streptococcal clinical isolate. RopB is a group A streptococcal transcriptional regulator that has been shown to be essential for expression of SpeB and binds directly to the promoter region of *speB* (Chaussee *et al.*, 1999; Neely *et al.*, 2003). In studies performed in different group A streptococcal serotype strains, RopB has variably been suggested to be involved in the regulation of other group A streptococcal genes including those associated with metabolism of non-glucose carbohydrates and amino acids (Chaussee *et al.*, 2004; Dmitriev *et al.*, 2006), response to thermal and oxidative stress (Chaussee *et al.*, 2004; Pulliainen *et al.*, 2008) and the expression of virulence factors including DNases (MF-1 and MF-3) and haemolysins (streptolysin S and streptolysin O) (Chaussee *et al.*, 2002; Dmitriev *et al.*, 2006; Dmitriev *et al.*, 2008). Subsequent investigations into the effect of RopB on virulence have yielded differing results. A study utilizing a zebrafish intramuscular infection model with serotype M5 GAS showed that inactivation of RopB resulted in decreased virulence (Neely *et al.*, 2002), whereas a study utilizing a murine intraperitoneal infection model with serotype M49 GAS showed that inactivation of RopB resulted in increased virulence (Pulliainen *et al.*, 2008).

While such global differences in virulence effects could in part result from the differing animal models used, it may also reflect strain-specific variation in the RopB regulon. For example, separate studies have shown *ropB* mutation to have either no effect on haemolysis and DNase activity or, alternatively, to increase expression of haemolysin and DNase-encoding genes and the associated phenotypic activities (Chaussee *et al.*, 1999; Dmitriev *et al.*, 2006). This strain-specific variation is

highlighted in a recent work by Dmitriev *et al.* (2008) that shows inter- and intra-serotypic variation in the transcriptome of *ropB* mutant group A streptococci, with only members of the *SpeB* operon being commonly regulated in all strains tested.

It is in the light of the current uncertainty surrounding RopB and its role in virulence that this study sought to investigate the role of this transcriptional regulator in the serotype M1T1 group A streptococcal background that is the leading agent of severe human infection. This analysis begins with a naturally-occurring mutation in *ropB* identified in one such strain.

## **4.2 Materials and Methods**

### ***4.2.1 Bacterial strains, media and growth conditions***

The M1T1 group A streptococcal clinical isolates, 5448 and 5628, used in this study have been previously described (Kazmi *et al.*, 2001). Both 5448 and 5628 are *speA*-positive M1T1 strains that were isolated from patients with STSS that were recruited through an ongoing population-based surveillance for invasive Group A streptococcal infections in Ontario, Canada. Both strains were determined to be derived from the same clone as detailed elsewhere (Chatellier *et al.*, 2000). The group A streptococcal strain 5448 $\Delta$ *speB* has been previously described (Aziz *et al.*, 2004). Group A streptococcal strains were grown in Todd-Hewitt broth containing 1% yeast extract (THY), or on Todd-Hewitt agar plates (THA). *Escherichia coli* were grown in Luria-Bertani broth (LB) or on Luria-Bertani agar (LA). For antibiotic

selection, erythromycin (Erm) was used at 5 µg/ml for GAS and 500 µg/ml for *E. coli*.

#### **4.2.2 Sequencing**

Sequencing was performed using primers listed in Table 4.1. First the genes were amplified using primers RopB-F1 and RopB-R18 for *ropB*, CovRS-F1 and CovRS-R12 for *covR/S*, LuxS-F1 and LuxS-R7 for *luxS*, RofA-F1 and RofA-R10 for *rofA* and RopA-F1 and RopA-R9 for *ropA*. The genes were then sequenced using all internal primers and the sequences aligned using ContigExpress (Invitrogen).

#### **4.2.3 Allelic exchange mutagenesis**

Allelic exchange mutagenesis was performed essentially as previously described (Jeng *et al.*, 2003). The *ropB* allele plus upstream and downstream flanking regions was amplified from 5448 or 5628 using the primers RopB-F-BamHI (5'-CAG GAT CCC TCA TTT CAG TTG ACA AGA AAC-3') and RopB-R-XbaI (5'-CGC TCT AGA TAC CAA AAG GCT AGA CCT CTG-3'). The PCR products and temperature sensitive vector pHY304 were ligated using T4 ligase to create the plasmids pHY5448RopB and pHY5628RopB. GAS strain 5448 was transformed with the plasmid pHY5628RopB and GAS strain 5628 was transformed with the plasmid pHY5448RopB, and Erm resistant transformants were grown at the permissive temperature for plasmid replication (30°C). Single-crossover chromosomal insertions were selected by shifting to the nonpermissive temperature (37°C) while maintaining Erm selection. Single crossover colonies were then grown

**Table 4.1** Primers used for sequencing the *ropB* locus (including *speB*), the *covR/S* locus, *luxS*, *rofA* and *ropA*.

Primer name	Sequence (5'-3')
RopB-F1	GACTGTTCGTTAGAAAAGCCA
RopB-F2	CTCCTGATACGATGATAA
RopB-F3	AAAGTTTCTTTCAAGGC
RopB-F4	CTTTGATTTGTTTCGACAT
RopB-F5	TACCATGAATGGTAATAG
RopB-F6	TATCTCACTACCATTTTGC
RopB-F7	TGAGTTTCTCTTTATTAG
RopB-F8	GAACGGTGTGTGTGTCT
RopB-R9	AGTCACCCATTGATAAAG
RopB-R10	AGGCGGCTTCAACGGTTA
RopB-R11	AGACTACACTTACACACT
RopB-R12	ATCCAAAAATCAGCAGCTATC
RopB-R13	TTAACAAAATGAGAACGG
RopB-R14	TGATAGTCGCTTATGATA
RopB-R15	CATATTGACAAACATCCGAATCG
RopB-R16	GCTGTTGAGATAAACTAC
RopB-R17	CTAGACCTCTGCTCACTAG
CovRS-F1	GCTATTCGGTACAGGTGT
CovRS-F2	GTCAATGGTCGTGAAGGGT
CovRS-F3	GATGTCTATATTCGTTATCTCC
CovRS-F4	GATGATTTTTACCACAGATAAC
CovRS-F5	GCATATTGGTCTCTTACAAC
CovRS-F6	GCAAATTGTAGATGGGTATCA
CovRS-R7	GCGGAAAATAGCACGAATAC
CovRS-R8	AGGCAATCAGTGTAAGGCA
CovRS-R9	CTTGTGCCAAATAACTCAACA
CovRS-R10	ATCAAAAGCCTGCTCAAATGA
CovRS-R11	CTTTCATGTCATCCATCATTG
CovRS-R12	TTGCTCTCGTGTGCCATCT
LuxS-F1	GCAGCTCTATTGCACCTAT
LuxS-F2	AAGAAGTTATCGTCGAAA
LuxS-F3	AATCCTACTGACCTATTT
LuxS-R4	TAGTGGCAACACGGTGAA
LuxS-R5	TGAAAACCTGTTTCGACAG
LuxS-R6	ATAATGGCAATGGTTAC
LuxS-R7	GTACCTTACAATCAAGATGTT
RofA-F1	TCTTGAGCTAATGCAACCGT
RofA-F2	GAATCCGTTAGGAGATGA
RofA-F3	GTTTCGATAATATCATGG
RofA-F4	AAAGGATGTGTAAATTGG
RofA-F5	ACAAGGTTTCCAAATAAG
RofA-R6	AAGCAATTAACATAAGCG
RofA-R7	TCTGCAACATTTTATTCC
RofA-R8	GGCATTAAAGTTTATGAC
RofA-R9	TAGGAAGAGAGGTCCCTT
RofA-R10	GAACTTGAATCTGGATTTATTG
RopA-F1	TCTTGTCTGCAAAATACGTC
RopA-F2	TCTTCTTGAGTTGTACCA
RopA-F3	CTCAACACCATCAACTGA
RopA-F4	GATTTGTAGCTTTGTTTTT
RopA-R5	TTAAGGAACAAAACGTACAAG
RopA-R6	ATGTTGACACACTTGAAG
RopA-R7	TGTTGTGTCAATGGAAAA
RopA-R8	ATGCCATAGTCATCCGTT
RopA-R9	AATCCTTCTTTGATAGTTTATC



in the absence of antibiotic selection at 30°C and Erm sensitive colonies were then screened for the presence of the appropriate *ropB* allele using DNA sequence analysis. Confirmed allelic exchange mutants were designated 5448R- (5448 containing the mutant *ropB* allele from 5628) and 5628R+ (5628 containing the wildtype (WT) *ropB* allele from 5448).

#### **4.2.4 *SpeB* activity assays**

Cysteine protease activity assays were performed as described by Collin *et al.* (Collin and Olsen, 2000). Group A streptococcal strains were grown overnight at 37°C. Cultures were then diluted 1:50 and grown for 17 h at 37°C. The cultures were centrifuged at 3200 x g, and the supernatants sterile-filtered through 0.2 µm syringe-driven filters (Whatman). 200 µl of filtered supernatant was mixed with 200 µl of activation buffer (1 mM EDTA, 20 mM DTT in 0.1 M sodium acetate buffer, pH 5.0) and incubated for 30 min at 40°C. 400 µl of 2% (w/v) azocasein in activation buffer was then added and incubated for a further 1 h at 40°C. Trichloro-acetic acid was then added to a final concentration of 15% (w/v) and thoroughly mixed. The mixture was then centrifuged at 15,000 x g for 5 min and the OD<sub>366</sub> of the supernatant was then measured to indicate cleavage of the azocasein by *SpeB*.

#### **4.2.5 *SpeB* western blot**

For Western blot analysis, bacterial cultures were grown to late stationary phase (17 h) and pelleted by centrifugation at 3,200 x g for 10 min. The supernatants were sterile-filtered through 0.22 µm syringe-driven filters (Millipore). Supernatants were

diluted 1:8 and 10 µl of each sample was run on a 10% Bis-Tris Gel with MOPS running buffer (Invitrogen). Proteins were then transferred to a Nitrocellulose membrane (Invitrogen) by use of a Trans-Blot SD semi-dry transfer cell (BioRad) for 1 h at 20 V. The presence of SpeB in culture supernatants was detected using primary rabbit anti-SpeB diluted 1:1,000 for 1.5 h. Following subsequent washing, the membrane was incubated with a secondary goat anti-rabbit-HRP conjugate diluted 1:10,000 for 1 h. SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) was used to expose autoradiography film as per the manufacturer's instructions.

#### ***4.2.6 Haemolytic activity assay***

Fresh, heparinised human blood from healthy volunteers was washed twice with sterile PBS and resuspended to a final concentration of 2% (v/v) in PBS. Bacterial cultures were grown to mid-log phase ( $OD_{600}=0.4$ ), pelleted by centrifugation at 3,200 x g and the supernatants sterile-filtered through 0.22 µm syringe-driven filters (Millipore). 100 µl of blood was mixed with 100 µl of serially diluted supernatant. The plates were incubated for 1 h at 37°C, then 1 h at 4°C. Following centrifugation at 1,500 x g for 10 min, 100 µl was transferred into a fresh 96-well flat bottom plate and the absorbance at 405 nm was recorded.

#### ***4.2.7 DNase activity assays***

DNase assays were performed as previously described (Aziz *et al.*, 2004; Buchanan *et al.*, 2006). Briefly, group A streptococcal strains were grown to mid-log phase

(OD<sub>600</sub>=0.4), and the supernatants collected by centrifugation at 3200 x g. 2.5 µl of bacterial supernatant was added to 7.5 µl of calf thymus DNA (1 µg/ml) and 40 µl of DNase buffer (3 mM MgCl<sub>2</sub>, 3 mM CaCl<sub>2</sub>, 300 mM Tris; pH 7.4). The reaction was incubated for 5 min at 37°C and then stopped by the addition of 12.5 µl 0.33 M EDTA (pH 7.3). The relative DNA degradation was then compared by gel electrophoresis on a 1% agarose gel.

#### ***4.2.8 Neutrophil killing assays***

Human neutrophils were isolated from venous blood of healthy volunteers using the PolyMorphPrep kit as per the manufacturer's instructions (Axis-Shield, Norway). In 96-well plates,  $2 \times 10^5$  neutrophils were mixed with  $2 \times 10^4$  colony forming units (CFU) of logarithmic phase GAS in RPMI containing 2% heat-inactivated autologous plasma in a total volume of 200 µl. The plates were centrifuged at 500 x g for 5 min and incubated at 37°C in 5% CO<sub>2</sub> for 30 min. The neutrophils were then hypotonically lysed in H<sub>2</sub>O, serially diluted and plated on THA. The plates were incubated overnight at 37°C and CFU enumerated. Control wells containing bacteria but no neutrophils were used to determine survival. Percentage survival was calculated as [CFU/ml experimental well]/[CFU/ml control well] x 100%.

#### ***4.2.9 In vivo SpeB switching studies***

GAS strain 5628R+ was grown to mid-log phase (OD<sub>600</sub>=0.4), pelleted by centrifugation at 3,200 x g and washed twice with sterile PBS. Bacteria were then resuspended in PBS at a final concentration of  $1 \times 10^9$  CFU/ml. 100 µl of bacterial

suspension ( $1 \times 10^8$  CFU) was injected subcutaneously into the flank of 10 week old C57BL6/J mice. Three days post-infection, bacteria were recovered from the lesion and screened for SpeB status using the skim-milk agar method as previously described (Ashbaugh *et al.*, 1998).

#### ***4.2.10 Tissue cage implantation and in vivo bacterial growth***

To obtain *in vivo*-derived RNA for microarray analysis, a previously described subcutaneous murine teflon chamber model was used (Kazmi *et al.*, 2001). Approximately  $10^8$  CFU of bacteria were injected in sterile Teflon chambers that had been surgically inserted under the skin of age-matched female BALB/c mice. After 24 h, bacteria were collected, tested for purity and phenotypic homogeneity on blood agar plates and Columbia-casein agar plates, respectively. Only pure isolates with homogeneous protease activity (either positive or negative) were further selected for RNA extraction. Selected isolates recovered from teflon chambers were centrifuged, and their pellets were washed twice in PBS, sheared with beads (QBiogene), then processed for RNA extraction according to the RNEasey protocol (Qiagen).

#### ***4.2.11 Expression microarrays***

For transcriptome analysis, oligomer-based microarrays printed in the Molecular Resource Center at the University of Tennessee Health Science Center were used. Each array consists of duplicates of 2,328 oligomers (70-mers) that represent all ORFs in M1 GAS strain SF370 (GenBank accession # NC\_002737) in addition to oligomers representing ORFs from prophages in strains MGAS8232 (GenBank

accession# NC\_003485) and MGAS315 (GenBank accession # NC\_004070). The arrays also contained positive and negative controls of streptococcal ribosomal DNA and alien DNA (Stratagene), respectively.

#### ***4.2.12 cDNA preparation and microarray hybridization***

Bacterial RNA was treated with DNase Turbo (Ambion) for 1 h to remove any genomic DNA contamination, then converted it to dendrimer-labeled cDNA using the 3DNA Array 900TM kits (Genisphere, [http://www.genisphere.com/array\\_detection\\_900.html](http://www.genisphere.com/array_detection_900.html)) following the manufacturer's protocol. Equal amounts of dendrimer-labeled cDNA from different pairs of isolates were mixed, applied to the glass microarrays, and incubated for 16 h. After this first hybridization, the arrays were washed, labelled with a mixture of Alexa Fluor 546 and Alexa Fluor 647 (Genisphere), incubated for 3 h, washed again, then scanned using a GenePix 4000B scanner (Axon Instruments, Inc.). This study followed a cyclic design that allowed comparison of every condition with each other at least twice, and guaranteed dye swapping to eliminate effects of non-specific binding.

#### ***4.2.13 Analysis of microarray data***

To analyse the microarray data, GenePixPro 4.0 software (Axon Instruments, Inc.) was used for image processing, fluorescent normalization and spot finding, then GeneSpring GX 7.3.1 (Agilent Technologies) was used for normalization, statistical analysis, clustering analysis and gene-list generation. All primary microarray data

were submitted to the NCBI Gene Expression Omnibus (GEO) in accordance with MIAME standards (GEO accession # GSE13656).

#### ***4.2.14 Murine systemic infection models***

Group A streptococcal strains were grown to mid-log phase ( $OD_{600}=0.4$ ), pelleted by centrifugation at  $3,200 \times g$  and washed twice with sterile PBS. For intravenous challenge, bacteria were then resuspended in PBS at a final concentration of  $1 \times 10^9$  CFU/ml. 200  $\mu$ l of bacterial suspension ( $2 \times 10^8$  CFU) was injected into the lateral tail vein of 10 week old C57BL6/J mice ( $n=5$  for each group). For intraperitoneal infection, the bacteria were resuspended at a concentration of  $2.5 \times 10^8$  CFU/ml in PBS containing 5% (w/v) mucin. 200  $\mu$ l of bacterial suspension ( $5 \times 10^7$  CFU) was injected into the peritoneal cavity of C57BL6/J mice ( $n=5$  for each group). For both experiments, the mice were monitored for 10 days and deaths recorded every 24 h. Statistical significance was determined using the log-rank (Mantel-Cox) test for comparison of survival curves.

#### ***4.2.15 Experimental acknowledgements***

Acknowledgement of experimental assistance provided for this section is outlined in Table 4.2

**Table 4.2** Acknowledgement of experimental assistance provided for this section.

Experimental Section	Contributor	Contribution (%)
DNA sequence analysis	Andrew Hollands	100
Construction of mutant strains	Andrew Hollands	100
SpeB assays and Western Blot	Andrew Hollands	100
Growth Curves	Andrew Hollands	100
Haemolysis assays	Andrew Hollands	100
DNase assays	Andrew Hollands	100
Neutrophil assays	Andrew Hollands	100
Switching studies	Andrew Hollands	100
Tissue cage implantation	Andrew Hollands	60
	Rita Kansal	40
cDNA preparation, microarray hybridization and analysis	Andrew Hollands	70
	Ramy Aziz	30
Murine Virulence studies	Andrew Hollands	100

### 4.3 Results

#### ***4.3.1 Sequence analysis of clinical isolate 5628 reveals intact speB and covR/S but mutation in ropB***

M1T1 GAS clinical isolate 5628 was found on screening to lack SpeB activity by azocasein assay. DNA sequencing was performed using primers listed in Table 4.1. No mutations were found in the strain 5628 in the *speB* gene nor in the *covR/S* locus, which has been previously linked to loss of SpeB expression (Sumby *et al.*, 2006; Walker *et al.*, 2007). Further sequencing was performed on the previously described regulators *luxS*, *rofA*, *ropA* and *ropB* (Beckert *et al.*, 2001; Lyon and Caparon, 2003;

Marouni and Sela, 2003; Neely *et al.*, 2003). This analysis revealed a single base pair deletion of nucleotide 452 of 843 in *ropB*, leading to truncation of RopB at amino acid 170 of the 280 amino acid protein (Figure 4.1A). The truncation of RopB suggested that this may be the cause of lack of SpeB expression in this strain.

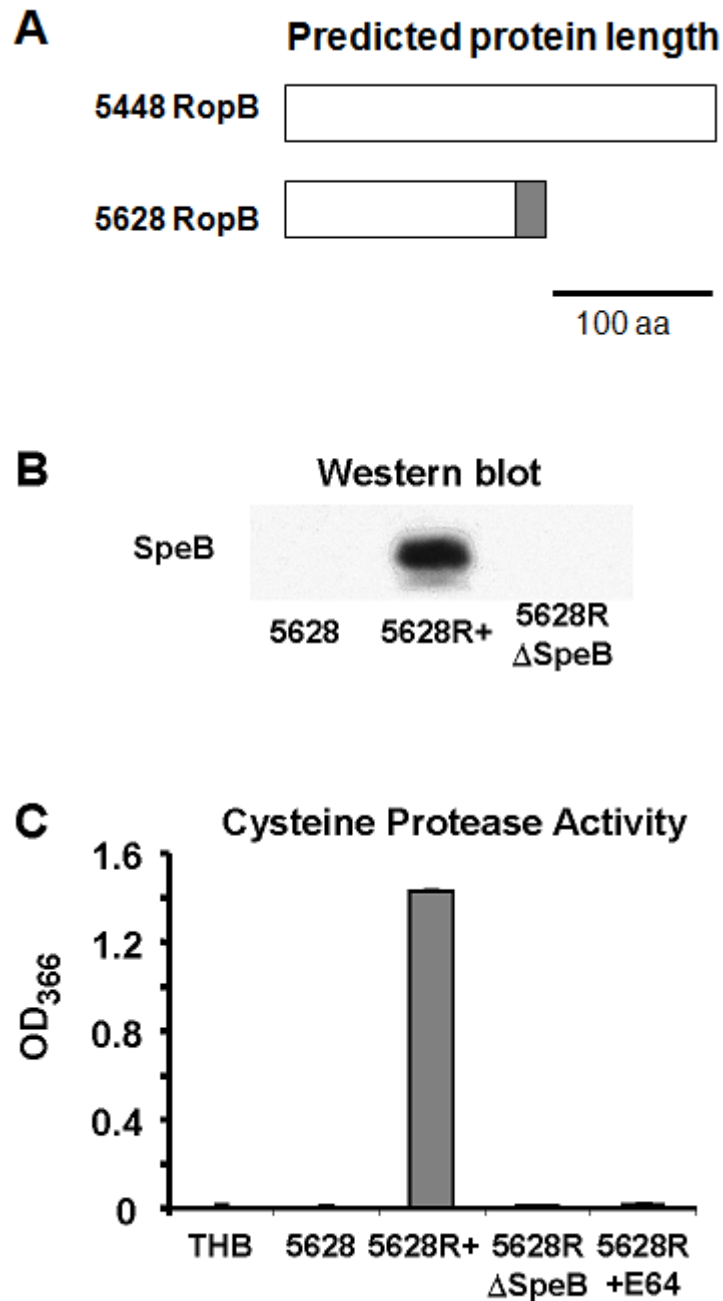
#### ***4.3.2 Repair of the 5628 ropB allele restores SpeB expression and activity***

Western blot detected SpeB in overnight culture supernatants of 5628R+, containing the intact *ropB* allele, but not in the clinical isolate 5628 nor the isogenic mutant 5628RΔSpeB, containing the intact *ropB* allele but lacking *speB* (Figure 4.1B). This expression correlated with a restoration of extracellular protease activity as detected by azocasein degradation assay (Figure 4.1C). Protease activity was abrogated by the addition of the cysteine protease inhibitor E64. These data demonstrated that the *ropB* point mutation was indeed responsible for the lack of SpeB expression in the clinical isolate 5628.

#### ***4.3.3 ropB mutation does not affect bacterial growth, haemolysis, DNase activity or resistance to neutrophil killing***

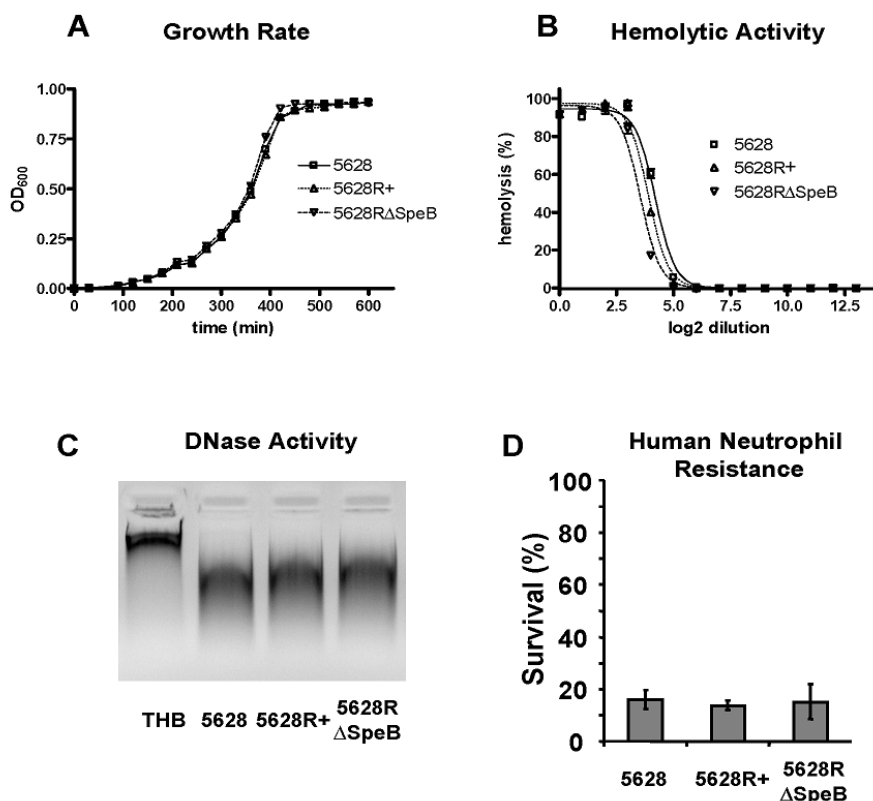
GAS strain 5628 and its isogenic mutants 5628R+ and 5628RΔSpeB were grown in THB, and the OD<sub>600</sub> was measured over time. No significant difference was found among the growth rates of the three bacterial strains (Figure 4.2A). Haemolysis assays were performed to determine the effect of *ropB* mutation on the cytolytic ability of GAS. No significant difference was seen in haemolytic activity among





**Figure 4.1** Mutation in *ropB* results in truncation of the RopB protein and abrogation of SpeB expression. (A) Schematic representation of the RopB protein expressed by GAS strains 5448 and 5628. In 5628, white region represent homology to 5448, shaded region indicates unique protein sequence in 5628. Scale bar is shown. (B) Western blot for SpeB using overnight culture supernatants. (C) SpeB activity assay using azocasein substrate.

strains containing the WT or mutated *ropB* allele (Figure 4.2B). Extracellular DNase activity of mid-log phase bacteria and group A streptococcal resistance to neutrophil killing were also unaffected by mutation in *ropB* (Figure 4.2C and D).



**Figure 4.2** *RopB* mutation does not affect *in vitro* growth, haemolytic activity, extracellular DNase activity or resistance to neutrophil killing. (A) OD<sub>600</sub> of *in vitro* grown bacterial cultures in THB. (B) Haemolysis of red blood cells by bacterial culture supernatants. (C) Degradation of calf thymus DNA by bacterial culture supernatants, run on a 1% agarose gel. (D) Bacterial survival after 30 min incubation with human neutrophils.

#### 4.3.4 *SpeB*-positive bacteria revert to a *SpeB*-negative phenotype on subcutaneous infection

C57BL/6/J mice were subcutaneously infected with the *SpeB*-positive strain 5628R+. Three days post-infection, bacteria were recovered from the lesion and screened for *SpeB* status using the skim-milk agar method. While the inoculum was 100% *SpeB*-positive, bacterial populations recovered from the lesions of individual mice were found to be 2%, 4%, 6%, 8%, and 24% *SpeB*-negative. Five representative colonies were picked, and the *covR/S* locus was sequenced in each. All colonies sequenced were found to have substitution mutations in *covR/S* resulting in truncation of CovS (Table 4.3). This finding reveals that when *SpeB*-negative colonies are selected for *in*

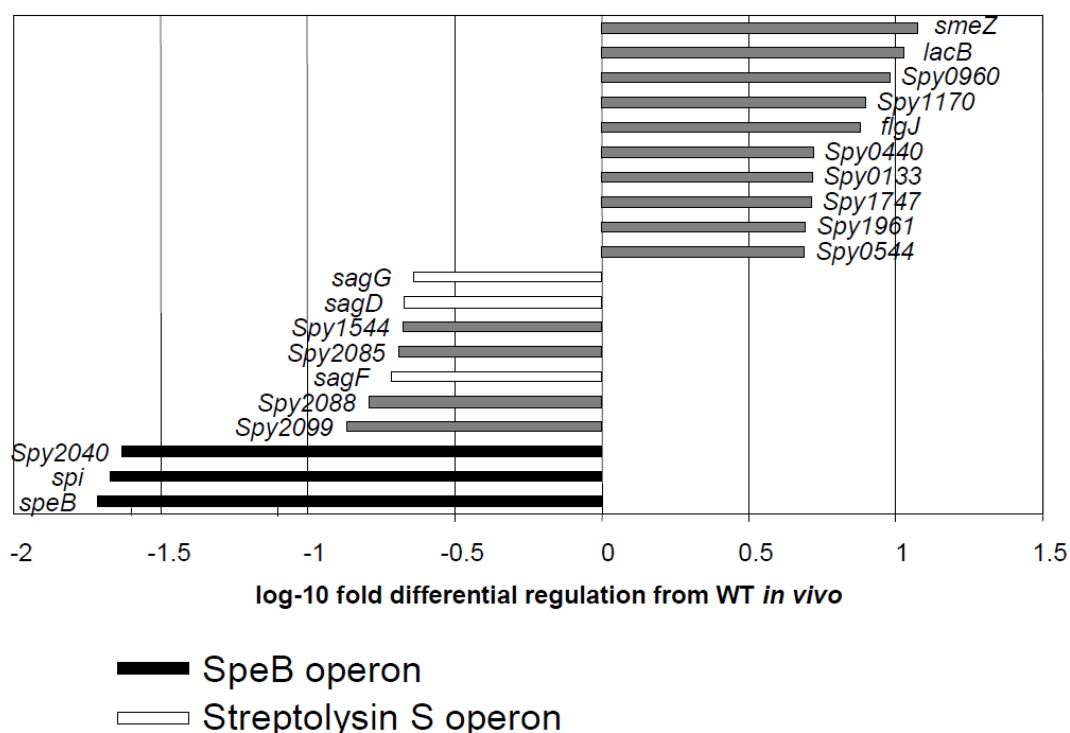
*vivo*, this selection is predominantly a phenomenon associated with CovR/S inactivation. The *ropB* mutation of strain 5628 was not recapitulated on passage in the mouse subcutaneous infection model.

**Table 4.3** *covR/S* DNA sequence analysis of selected GAS M1T1 strain 5628 SpeB-negative derivatives isolated 3 days following subcutaneous infection of C57BL6/J mice.

Mouse	Mutation	Consequence
1	C to T nt 340 <i>covS</i>	Truncation of CovS at aa 113
2	C to T nt 340 <i>covS</i>	Truncation of CovS at aa 113
3	C to T nt 340 <i>covS</i>	Truncation of CovS at aa 113
4	C to T nt 154 <i>covS</i>	Truncation of CovS at aa 51
5	C to T nt 340 <i>covS</i>	Truncation of CovS at aa 113

#### 4.3.5 *SpeB* is the principal target of *RopB* regulation in vivo

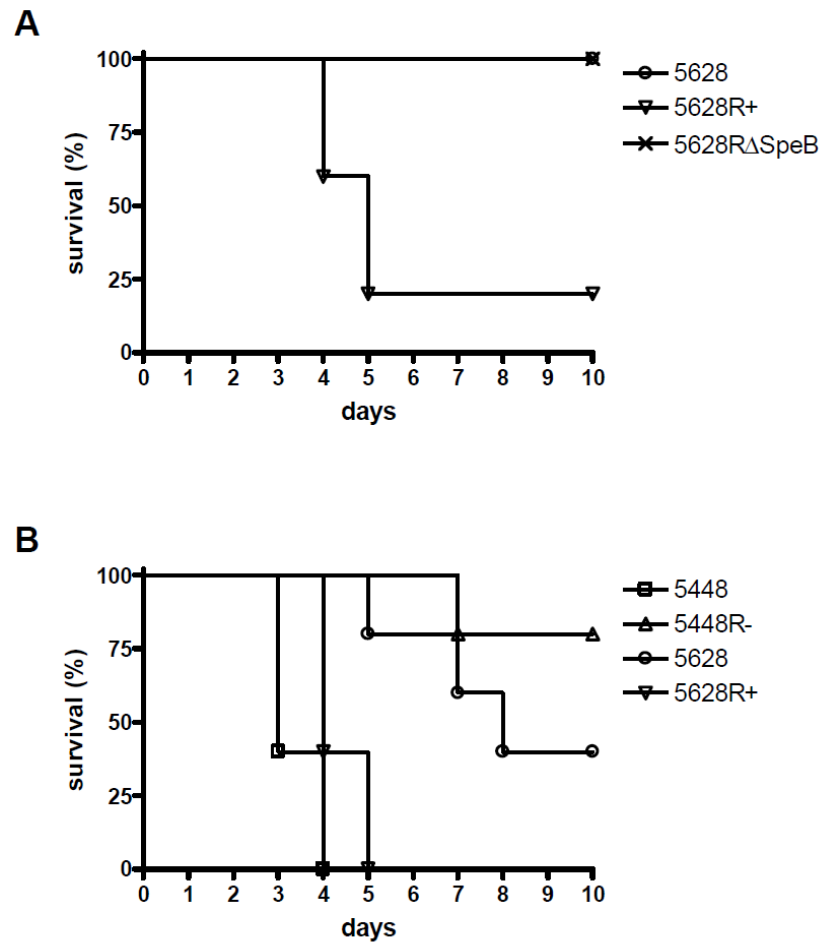
Microarray analysis was performed on *in vivo*-derived RNA from the well characterized GAS strain 5448 and its derivative 5448R-, containing the mutated *ropB* allele from 5628. Forty-seven genes were found to be down-regulated and 52 genes were found to be up-regulated in 5448R- greater than 2-fold ( $P < 0.05$ ). The most strongly down-regulated genes in 5448R- are members of the *SpeB* operon, as would be expected from the lack of *SpeB* expression and activity in 5628 (Figure 4.3). In addition to the *SpeB* operon, genes of the streptolysin S operon were also found to be strongly down-regulated in the *ropB* mutant strain. Apart from the gene encoding the superantigen SmeZ, there was an absence of virulence-related genes found to be strongly up-regulated in the strain 5448R-, which expressed the truncated RopB.



**Figure 4.3** Microarray analysis emphasizes the down-regulation of the SpeB operon. The figure shows the top 10 genes up- and down-regulated in the *ropB* mutant bacteria compared to WT when the bacteria are inoculated *in vivo* ( $P < 0.05$ ). The values plotted represent log ratios (log mutant/WT), and genes co-clustered in operons are highlighted (black bars, SpeB operon; white bars, SLS operon).

#### 4.3.6 *RopB* is required for virulence in systemic infection

To determine the effect of *ropB* mutation on virulence, C57BL6/J mice were subjected to intraperitoneal infection with mid-logarithmic phase bacteria. GAS strain 5628R+ expressing a full length RopB showed increased virulence compared to the strain 5628 with a truncated RopB (Figure 4.4A). Strain 5628RΔSpeB containing a full-length RopB but lacking SpeB also showed reduced virulence, suggesting that the reduced virulence of 5628 may primarily result from the lack of SpeB expression in this strain. Virulence was also examined in an intravenous model of systemic infection. The well characterized strain 5448 and its isogenic mutant 5448R-, containing the *ropB* allele from 5628, were further included to examine the



**Figure 4.4** RopB and SpeB-negative bacteria show reduced virulence in systemic infection models. (A) Intraperitoneal infection of C57BL6/J mice with  $5 \times 10^7$  CFU of group A streptococcal strains with 5% mucin (n=5 per group). (B) Intravenous challenge of C57BL6/J mice with  $2 \times 10^8$  CFU of group A streptococcal strains (n=5 per group).

effect of this allelic variation in a well-characterized virulent strain of GAS. The two strains containing a truncated RopB (5628 and 5448R-) demonstrated similarly reduced virulence compared to the two strains containing a full-length RopB (5628R+ and 5448) (Figure 4.4B), confirming that inactivation of RopB results in decreased virulence during systemic infection.

### 4.3 Discussion

The cumulative contribution of the secreted cysteine protease SpeB to the pathogenesis of invasive group A streptococcal infection is at present unclear, and studies in various group A streptococcal serotypes and animal models have produced varying results (Lukomski *et al.*, 1998; Svensson *et al.*, 2000; Ashbaugh and Wessels, 2001). In the globally-disseminated clonal M1T1 serotype associated with epidemic invasive group A streptococcal infection, an inverse relationship has been found between SpeB activity and clinical disease severity (Kansal *et al.*, 2000). Recently, a connection was found between inactivation of SpeB through mutation in the two-component regulatory system *covR/S* and development of invasive disease in the murine model (Sumby *et al.*, 2006; Walker *et al.*, 2007). This study investigated a second mechanism of inactivation of SpeB, namely through truncation of the regulator RopB. It was shown that in M1T1 GAS, when the *covR/S* locus is intact and DNase Sda1 activity is unaffected, *ropB* point mutation results in reduced virulence *in vivo*, despite inactivation of SpeB activity.

The SpeB-negative, serotype M1T1 clinical isolate 5628 was used to investigate the role of RopB in virulence. RopB has previously been shown to be necessary for SpeB production (Chaussee *et al.*, 1999; Neely *et al.*, 2003) as well as regulation of other group A streptococcal virulence factors (Chaussee *et al.*, 2002; Chaussee *et al.*, 2004; Dmitriev *et al.*, 2006; Dmitriev *et al.*, 2008; Pulliainen *et al.*, 2008). The M1T1 strain 5628 contains a point mutation in the *ropB* gene that results in a truncation of the last 110 amino acids of the 280 amino acid protein. Restoration of full-length RopB in 5628R+ resulted in gaining SpeB expression, but did not result in changes

in haemolysis, DNase activity or resistance to killing by neutrophils. The *ropB* mutation in this particular strain does not result in the kind of global phenotypic change found with *covR/S* mutation where differential expression was observed of multiple genes encoding virulence determinants, including *sic*, *ska*, *slo*, *speA*, *speJ*, *scpC* and the hyaluronic acid synthesis operon (Sumbly *et al.*, 2006) which were all unaffected in the *ropB* mutant examined in this work.

Recently, it was demonstrated that WT, SpeB-positive bacteria undergo a phase-shift to a SpeB-negative phenotype after subcutaneous infection of mice (Aziz *et al.*, 2004). This phase switch was observed to be the result of mutations in the two-component regulatory system *covR/S* (Walker *et al.*, 2007). CovR/S is a global regulator, whose inactivation is linked to an invasive phenotype and involves up-regulation of many genes encoding virulence factors such as streptodornase, streptokinase, streptolysin O, streptococcal inhibitor of complement and the hyaluronic acid capsule synthesis operon (Sumbly *et al.*, 2006). Of the down-regulated genes in *covR/S* mutant strains, SpeB would appear to be one of the most important due to its ability to degrade many host and self-proteins. It is the loss of SpeB expression in these mutant bacteria that is hypothesized to allow the invasive spread of GAS by sparing from SpeB degradation self-proteins involved in plasminogen accumulation and activation (Cole *et al.*, 2006). In this chapter it was found that SpeB-positive bacteria with a restored RopB reverted to a SpeB-negative phenotype on subcutaneous infection. Furthermore, the switch to a SpeB-negative phenotype appeared to occur exclusively through mutations in *covR/S*. This result suggests that *covR/S* mutations are the predominant method of phenotypic switching in GAS and that *ropB* mutation is not readily selected for *in vivo*. This leads to the

conclusion that *ropB* mutation in 5628 does not represent a similar mechanism to *covR/S* mutation and may (a) be an incidental occurrence or (b) arise secondary to different selection pressures than those associated with the shift to invasive infection.

Microarray analysis was conducted using *in vivo*-derived RNA, since the transcriptome of bacteria grown *in vitro* may differ greatly from the transcriptome found during the course of infection. Implantation of tissue cages allows for bacteria to be grown in an *in vivo* environment for 24 hours prior to recovery and RNA extraction. The microarray data showed members of the *SpeB* operon to be the most strongly down-regulated genes following inactivation of *RopB*. This finding supported the *in vitro* and *in vivo* data that suggested *SpeB* is the main target of *RopB* regulation in this strain.

Virulence studies utilizing systemic models of infection showed that a full-length *RopB* was required for virulence. The clinical isolate 5628 expressing a truncated *RopB*, as well as the allelic exchange mutant 5448R- also expressing the truncated *RopB* from 5628, showed reduced virulence compared to strains containing a full-length *RopB*. These data illustrate the necessity for an intact *RopB* for full virulence *in vivo*. In addition, the strain 5628RΔ*SpeB* expressing an intact *RopB* but lacking *SpeB* showed similarly reduced virulence to the strain with a truncated *RopB*. While the down-regulation of the streptolysin S operon may contribute to the reduced virulence, this finding implies that the loss of *SpeB* in this strain is the main cause of the lack of virulence exhibited. This finding leads to the conclusion that inactivation of *SpeB* alone is not sufficient to initiate invasive disease. Moreover, these data suggest that *SpeB* loss, only in the context of mutation of the *covR/S* regulatory



circuit, promotes invasion. Of note, the additional virulence factors streptokinase and M1 protein, are both up-regulated in *covR/S* mutant M1T1 strains (Sumby *et al.*, 2006), but not in the *ropB* mutant under investigation in this study. Streptokinase and M1 protein are believed to play a role in the accumulation of host plasmin at the group A streptococcal cell surface, a process which is thought to accentuate invasive disease (Walker *et al.*, 2005; Cole *et al.*, 2006; McArthur *et al.*, 2008).

Together these data provide evidence of RopB's role in virulence. In M1T1 GAS, an intact RopB and efficient SpeB production are necessary for systemic infection.

## 5. CONCLUSIONS

The M1T1 clone has arisen as the predominant cause of severe group A streptococcal disease in developed countries (Cleary *et al.*, 1998; Murono *et al.*, 1999; Chatellier *et al.*, 2000). M1T1 GAS is distinguished by the presence of the phages that encode the virulence genes *sdal* and *speA* (Aziz *et al.*, 2005; Aziz and Kotb, 2008). This study sought to investigate the contribution of the bacteriophage-encoded Sda1 to the switch to a SpeB negative phenotype. It was demonstrated that the presence of Sda1 is necessary to facilitate the switch to a SpeB-negative phenotype through mutation of *covR/S*. Loss of SpeB spares Sda1 from degradation and results in greater clearance of DNA NETs and enhanced resistance to killing by neutrophils. Loss of SpeB also results in greater accumulation of plasmin on the group A streptococcal surface, facilitating invasive spread of the bacteria. It was proposed that the acquisition of *sdal* by M1T1 GAS has provided evolutionary selection pressure for increased resistance to neutrophils by SpeB loss, resulting in a hyperinvasive phenotype. These data demonstrate how acquisition of new genes by horizontal gene transfer of bacteriophage-mediated acquisition may allow for new selection pressures on the bacterium, allowing increased invasive disease potential.

In M1T1 isolates, an inverse relationship has been found between SpeB expression and disease severity (Kansal *et al.*, 2000). It was demonstrated how *in vivo* selection pressure facilitates the switch to a hyperinvasive, SpeB-negative phenotype through *covR/S* mutation. While *covR/S* mutation in M1T1 GAS results in increased resistance to killing by neutrophils and invasive disease potential (Sumby *et al.*, 2006; Walker *et al.*, 2007), this study sought to investigate the counterbalancing

fitness cost associated with *covR/S* mutation. Such a fitness cost would select for the maintenance of the wildtype phenotype in the population. In order to examine this evolutionary process, this study utilized the M1T1 group A streptococcal isolate 5448 and its *covS* mutant derivative 5448AP (Walker *et al.*, 2007). It was found that *covS* mutant M1T1 group A streptococci have reduced capacity to bind to components of the ECM, reduced ability to form biofilms and reduced ability to bind to epithelial cells. These defects were found to be primarily related to the hyperencapsulation of *covS* mutant group A streptococci, as capsule deficient strains showed equal propensity for biofilm formation and adherence to epithelial cells. Significantly more 5448 were recovered from the murine skin colonization model in comparison with 5448AP, demonstrating that *covS* mutation confers a colonization defect in M1T1 group A streptococci, despite the dramatic increase in virulence at subsequent stages of infection. These findings demonstrate how genetic and phenotypic changes suited to bacterial survival in the host may result in a fitness cost in an alternate environmental niche.

It was demonstrated how loss of SpeB expression in M1T1 group A streptococci through mutations in *covR/S* leads to a hyperinvasive phenotype and the initiation of invasive disease. This study also investigated a second mechanism of loss of SpeB expression through mutation in the gene encoding the standalone regulator RopB. It was found that in M1T1 GAS, *ropB* mutation results in decreased virulence *in vivo*, despite the loss of SpeB expression. This strain did not display the kind of global phenotypic change that occurs with *covR/S* influencing virulence encoding genes including *sic*, *ska*, *slo*, *speA*, *speJ*, *scpC* and the hyaluronic acid synthesis operon (Sumby *et al.*, 2006), which were all unaffected in the *ropB* mutant examined in this

work. Reduced virulence of isogenic *speB* mutant strains of GAS has previously been shown (Lukowski *et al.*, 1998; Cole *et al.*, 2006). These previous findings, together with the reduced virulence of *ropB* mutant, suggest that SpeB plays an important role in virulence that is counteracted by the global phenotypic change that occurs with *covR/S* mutation. In addition, it was found that SpeB-positive bacteria with a restored RopB reverted to a SpeB negative phenotype on subcutaneous infection through mutations in *covR/S*. These data suggest that *covR/S* mutations are the predominant method of phenotypic switching in GAS and that *ropB* mutation is not readily selected for *in vivo*.

Together, these findings reveal how acquisition of the bacteriophage-encoded DNase *sdal* facilitates *in vivo* selection pressure for the switch to a SpeB-negative, hyperinvasive phenotype through mutations in *covR/S* resulting in the initiation of invasive disease. While *covR/S* mutation results in dramatically increased virulence, it also confers a colonisation defect that prevents these mutations from being fixed in the genome by spread of hypervirulent *covR/S* mutant M1T1 group A streptococci. Furthermore, this switch is specific for *covR/S* mutation as loss of SpeB through mutation of *ropB* does not result in the same kind of global phenotypic change and is not sufficient to facilitate the initiation of invasive disease.

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## 7. Appendix

### Appendix 1: Gene Expression Changes Due to *ropB* Mutation

**Table 7.1** Genes differentially regulated greater than 2-fold ( $P < 0.05$ ) from microarray analysis of GAS strains 5448 and 5448R-

Array Spot ID	Description	Fold change (5448/5448R-)
NT01SP1804_5J22	SPy2039_pyrogenic exotoxin B; streptopain precursor	51.87
NT01SP1803_5J21	SPy2038_inhibitor of SpeB; co-transcribed	47.20
NT01SP1805_5J23	SPy2040_hypothetical protein	42.72
NT01SP1853_5L23	SPy2099_similar to transcriptional regulator (GntR family), putative	7.33
NT01SP1844_5L14	SPy2088_amino acid permease, putative	6.18
NT01SP0648_2K14	SPy0743_sagF	5.19
NT01SP1842_5L12	SPy2085_formate--tetrahydrofolate ligase	4.87
NT01SP1379_4I10	SPy1544_ornithine carbamoyltransferase	4.71
NT01SP0646_2K12	SPy0741_sagD	4.69
NT01SP0649_2K15	SPy0744_sagG	4.36
NT01SP1420_4K2	SPy1595_pot. starch degradation products transport system permease protein amyD	4.34
NT01SP0696_2M14	SPy0796_hypothetical protein	4.26
NT01SP0700_2M18	SPy0800_pore forming protein ebsa, putative	3.93
NT01SP1845_5L15	SPy2089_histidine ammonia-lyase	3.70
NT01SP0160_1G14	SPy0175_SgaT protein, putative	3.55
NT01SP1416_4J22	SPy1589_conserved hypothetical protein	3.53
NT01SP0652_2K18	SPy0747_LPXTG-motif cell wall anchor domain protein	3.45
NT01SP0162_1G16	SPy0177_hexulose-6-phosphate synthase SgbH, putative	3.37
NT01SP0043_1B18	_alcohol dehydrogenase/acetaldehyde dehydrogenase	3.24
NT01SP0115_1E17	SPy0128_LPXTG-motif cell wall anchor domain protein	3.24
NT01SP1415_4J21	SPy1588_histidine kinase; spt10S, hk07, YesNM	3.06
NT01SP0107_1E9	SPy0121_similar to Lactobacillus acidophilus dA/dG-kinase	3.05
NT01SP1495_4N5	SPy1682_glycerol uptake facilitator protein	2.96
NT01SP1847_5L17	SPy2091_hypothetical protein	2.87
NT01SP0643_2K9	SPy0738_streptolysin S associated protein SAGA-related protein	2.87
NT01SP1908_5O6	SPy2156_aspartyl-tRNA synthetase	2.86
NT01SP1412_4J18	SPy1584_shikimate 5-dehydrogenase	2.77
NT01SP0453_2C11	SPy0519_conserved hypothetical protein	2.67
NT01SP0892_3E17	SPy1010_7,8-dihydro-8-oxoguanine-triphosphatase	2.61
NT01SP0116_1E18	SPy0129_conserved hypothetical protein	2.57
NT01SP1690_5F4	SPy1906_type I restriction-modification system, M subunit	2.44
NT01SP1442_4K24	SPy1618_cysteine synthase A	2.38
NT01SP1397_4J4	SPy1566_conserved hypothetical protein	2.34
NT01SP1338_4G17	SPy1502_methylenetetrahydrofolate dehydrogenase/methenyltetrahydrofolate cyclohydrolase	2.30
NT01SP1275_4E12	SPy1434_cation-transporting ATPase, E1-E2 family	2.25
NT01SP0789_3A11	SPy0895_hypothetical protein	2.22

NT01SP1799_5J17	SPy2034_conserved hypothetical protein	2.22
NT01SP1597_5B11	SPy1795_similar to ferrichrome ABC transporter (binding protein), putative	2.20
NT01SP0984_3I13	SPy1113_acid phosphatase	2.14
NT01SP1826_5K20	SPy2063_ribosomal large subunit pseudouridine synthase D	2.10
NT01SP1731_5G21	_hypothetical protein	2.10
NT01SP0448_2C6	SPy0514_catabolite control protein A	2.09
NT01SP1590_5B4	SPy1787_ABC transporter, ATP-binding protein, putative	2.08
NT01SP1611_5B23	SPy1815_PTS system, sucrose-specific IIBC component	2.07
NT01SP0130_1F8	SPy0144_conserved hypothetical protein	2.06
NT01SP1724_5G14	SPy1941_cysteinyl-tRNA synthetase	2.03
NT01SP1051_3L8	SPy1179_transcriptional regulator, GntR family, putative	2.00
NT01SP0841_3C14	SPy0952_hypothetical protein (Prophage 370.2)	0.49
NT01SP0419_2B1	SPy0480_hypothetical protein	0.48
NT01SP0037_1B12	SPy0037_Helix-turn-helix domain protein	0.46
NT01SP1243_4D4	SPy1398_ribosomal small subunit pseudouridine synthase A	0.44
NT01SP0032_1B7	SPy0032_phosphoribosylamine--glycine ligase	0.42
NT01SP1304_4F14	SPy1465_Structural protein (Prophage 370.3)	0.42
NT01SP1897_5N19	SPy2147_hypothetical protein (Prophage 370.4)	0.41
NT01SP1066_3L23	SPy1198_Helix-turn-helix domain protein	0.40
NT01SP0472_2D6	SPy0540_Glycosyl transferases domain protein	0.39
NT01SP1343_4G22	SPy1507_arginine transport system permease protein	0.38
NT01SP1244_4D5	SPy1399_glucosamine-6-phosphate isomerase	0.38
NT01SP0756_2P2	_exotoxin type c precursor	0.37
NT01SP1525_4O11	SPy1717_CopY	0.37
NT01SP1941_5P13	SPy2193_Cobalt transport protein superfamily	0.37
NT01SP0468_2D2	SPy0536_nitroreductase family protein	0.35
NT01SP1587_5B1	SPy1784_cdd4-like protein	0.34
NT01SP1445_4L3	SPy1621_response regulator, rr03 S.pneumo; yvqC	0.31
NT01SP0868_3D17	SPy0980_BRO family, N-terminus family (Prophage 370.2)	0.31
NT01SP1297_4F7	SPy1457_Structural protein (Prophage 370.3)	0.29
NT01SP0873_3D22	SPy0986_gp113 (Prophage 370.2)	0.29
NT01SP0605_2I19	SPy0694_major tail shaft protein (Prophage 370.1)	0.28
NT01SP1250_4D11	SPy1405_putative lipoprotein	0.28
NT01SP0546_2G8	SPy0631_mannose-specific phosphotransferase system component IIAB, putative	0.27
NT01SP0898_3E23	SPy1017_hypothetical protein	0.27
NT01SP0570_2H8	SPy0656_conserved hypothetical protein (Prophage 370.1)	0.27
NT01SP0474_2D8	SPy0542_UDP-glucose 6-dehydrogenase	0.26
NT01SP1759_5I1	SPy1983_collagen-like protein Scl1, sclA	0.26
NT01SP0152_1G6	SPy0168_hypothetical protein	0.25
NT01SP1055_3L12	SPy1183_oxaloacetate decarboxylase, alpha subunit	0.25
NT01SP0617_2J7	SPy0710_N-acetylmuramoyl-L-alanine amidase domain protein (Prophage 370.1)	0.25
NT01SP1953_6A1	SPy2205_putative multiple membrane domain protein	0.25
NT01SP0475_2D9	SPy0543_macrolide-efflux protein	0.24
NT01SP1044_3L1	SPy1172_hypothetical protein	0.24
NT01SP0937_3G14	SPy1062_response regulator, yesNM, rr09	0.23
NT01SP1873_5M19	SPy2121_DNA mismatch repair protein MutL, putative	0.23
NT01SP0834_3C8	SPy0945_tec protein (Prophage 370.2)	0.23

NT01SP0952_3H5	SPy1077_methyl transferase	0.23
NT01SP0148_1G2	SPy0164_transcription antitermination protein NusG	0.22
NT01SP0143_1F21	SPy0158_surface-located membrane protein 1 (Imp1), putative	0.22
NT01SP0431_2B13	SPy0496_MutR, putative	0.22
NT01SP0141_1F19	SPy0155_ATP synthase archaeal, B subunit	0.21
NT01SP0611_2J1	SPy0701_hyaluronidase (Prophage 370.1)	0.21
NT01SP0476_2D10	SPy0544_repressor protein, putative	0.21
NT01SP1742_5H8	SPy1961_dna polymerase iii, alpha chain polc-type (ec 2.7.7.7) (poliii)	0.20
NT01SP1555_4P17	SPy1747_acetyl-CoA carboxylase, biotin carboxyl carrier protein	0.19
NT01SP0120_1E22	SPy0133_conserved hypothetical protein	0.19
NT01SP0385_1P15	SPy0440_3-oxoacyl-(acyl carrier protein) reductase, putative	0.19
NT03SP1160_6M10	spyM18_1242_flgJ	0.13
NT01SP1042_3K23	SPy1170_D-lactate dehydrogenase, putative	0.13
NT01SP0849_3C22	SPy0960_hypothetical	0.11
NT01SP1705_5F19	SPy1922_galactose-6-phosphate isomerase, LacB subunit	0.09
SpyM3_1716_6H7	SpyM3_1716_smeZ	0.08