2011

Characterisation of anchorless cell-surface proteins: novel candidates for a safe and universal group a streptococcal vaccine

Anna Henningham
University of Wollongong, annahen@uow.edu.au

Characterisation of Anchorless Cell-Surface Proteins: Novel Candidates for a Safe and Universal Group A Streptococcal Vaccine

A Thesis Submitted in Fulfilment of the Requirements for the Award of the Degree

Doctor of Philosophy (PhD)

from the

University of Wollongong

Wollongong, Australia

by

Anna Henningham

B Biotechnology (Honours)

School of Biological Sciences

University of Wollongong

2011

DECLARATION

This thesis is submitted under the regulations of the University of Wollongong in fulfilment of the degree of Doctor of Philosophy (PhD). It does not include any material published by another person except where due reference is made in the text. The experimental work described in this thesis is original work, performed by me, unless acknowledged below and has not been submitted for qualifications at any other academic institution.

I acknowledge the following individuals for their contribution to this work. Dr. Manfred Rohde, Helmholtz Zentrum für Infektionsforschung (HZI), Germany, captured images using transmission electron microscopy. Mr. Graham Magor and Dr. Michael Batzloff, Queensland Institute of Medical Research, Australia, performed opsonophagocytosis killing assays. Dr. Christine Gillen and Dr. Marcus Fülde, HZI, Germany, prepared the human heart extract and performed ELISA experiments using this extract.

_ _ _ _ _ _ _ _ _ _ _ _ _ _

Anna Henningham

January 10, 2011

ACKNOWLEDGEMENTS

ABSTRACT

Streptococcus pyogenes (group A streptococcus; GAS) causes ~700 million human infections each year, resulting in over 500,000 deaths. GAS can cause mild infections such as pharyngitis and impetigo, in addition to life threatening conditions including necrotising fasciitis, streptococcal toxic shock syndrome (STSS) and bacteremia. Repeated infection with GAS may result in the non-suppurative sequelae, acute rheumatic fever (ARF) and acute glomerulonephritis (APSGN). GAS remains sensitive to the antibiotic penicillin which can be administered as a means to treat infection or as prophylaxis. This strategy is utilised in regions with high GAS endemicity such as Indigenous populations living in northern Australia who suffer some of the highest rates of GAS auto-immune sequelae worldwide. However, issues with patient compliance and a growing concern over the possible emergence of resistant GAS strains may limit the usefulness of penicillin in the future. A vaccine capable of preventing GAS infection may be the only effective way to control and eliminate GAS infection and disease.

The development of a commercial GAS vaccine is hampered by the occurrence of many unique GAS serotypes, antigenic variation within the same serotype, differences in geographical distribution of serotypes and the production of antibodies cross-reactive with human tissue that may lead to auto-immune disease. Several independent studies have documented a number of GAS cell wall-associated or secreted metabolic enzymes which contain neither N-terminal leader sequences nor C-terminal cell wall anchors. A proteomic analysis of serotype M1T1 GAS cell wall extracts was undertaken for the purpose of vaccine development. This approach catalogued 13 novel anchorless proteins and following a series of characterisation experiments identified two protective vaccine candidates, arginine deiminase (ADI) and trigger factor (TF). ADI and TF conferred protective efficacy in two murine models of GAS infection; systemic and subcutaneous. These surface-exposed antigens are expressed across multiple GAS serotypes exhibiting ≥ 99% amino acid sequence identity. Vaccine safety concerns are alleviated by the observation that these vaccine candidates lack human homologs, while sera from human populations suffering repeated GAS infections and high levels of auto-immune complications do not recognise these enzymes. In addition, anti-sera raised against ADI and TF does not react with human heart extract. Both ADI and TF warrant further investigation as GAS vaccine candidates for the prevention of GAS disease, which despite many decades of research trying to find a suitable vaccine, remains a major cause of morbidity and mortality worldwide.

TABLE OF CONTENTS   Page

LIST OF FIGURES   Page

LIST OF TABLES

Page

ABBREVIATIONS

2D	two dimensional
3D	three dimensional
ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
ADI	arginine deaminase
AK	adenylate kinase
ANGIS	Australian National Genomic Information Service
ANOVA	analysis of variance
Ap	ampicillin
AP	animal passaged
APSGN	acute post-streptococcal glomerulonephritis
ARF	acute rheumatic fever
BCA	bicinchoninic acid
BCAT	branched chain amino-acid aminotransferase
BLAST	basic local alignment search tool
bp	base pairs
BSA	bovine serum albumin
ºC	degrees Celcius
CBS	citrate-buffered saline
CD	cluster of differentiation
CDS	colour developing solution
CFA	Freund's Complete Adjuvant
cfu	colony forming units
CHO	group A carbohydrate
CK	carbamate kinase
cm	centimetre
Cov	control of virulence
Cpa	collagen-binding protein
CRS	closely related to SIC
CTB	cholera toxin B
DAB	3,3'-diaminobenzidine
dH$_2$O	sterile glass distilled water
DMF	dimethylformamide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DRS	distantly related to SIC
DTT	dithiothreitol
E64	N-[N-(L-3-transcarboxyirane-2-carbonyl)-L-Leucyl]agmatine
E. coli	*Escherichia coli*
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EF-Tu	elongation factor Tu
ELISA	enzyme-linked immunoabsorbant assay
EM	electron microscopy
Enn	M related protein
FBA	fructose-bisphosphate aldolase
FbaA	fibronectin-binding protein A
FbaB	fibronectin-binding protein B
FBP54	fibronectin binding protein 54
fg	femtogram
Fg	fibrinogen
FITC	fluorescein isothiocyanate
Fn	fibronectin
g	9.8 ms^{-2}

g	gram
G	guage
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GAS	group A streptococcus
GRAB	protein G-related α2-macroglobulin-binding protein
h	hour
HDL	high-density lipoprotein
Hep-2	human epidermoid carcinoma cells
His-tag	hexahistidine tag
HRP	horse radish peroxidase
HZI	Helmholtz Zentrum für Infektionsforschung
IdeS/Mac-1	IgG-degrading enzyme of *Streptococcus pyogenes*
IFA	Freund's Incomplete Adjuvant
Ig	immunoglobulin
IPTG	isopropyl-β-D-thiogalactosidase
k	kilo
kb	kilo-bases
K_D	dissociation constant
kDa	kilo-Daltons
KLH	keyhole limpet hemocyanin
KPR	ketopantoate reductase
L	litre
L. lactis	*Lactococcus lactis*
LB	Luria Bertani
Lbp/Lmb/Lsp	laminin-binding protein
LPXTG	Gram-positive protein cell wall anchor motif
LTA	lipoteichoic acid
M	molar
m	milli
M. hominis	*Mycoplasma hominis*
mAbs	monoclonal antibodies
Mac-2	variant of IdeS/Mac-1
MALDI-TOF	matrix-assisted laser desorption ionisation time-of-flight
MALP-2	macrophage activating lipopeptide 2
MBP	maltose-binding protein
MBS	membrane blocking solution
MHC	major histocompatibility complex
min	minutes
mm	millimetre
Mrp	M related protein
MS	mass spectrometry
μ	micro
μF	microfarad
n	nano
NADP-GAPDH	NADP-dependent glyceraldehyde-3-phosphate dehydrogenase
NCBI	National Centre for Biotechnology Information
ng	nanograms
NHS	N-hydroxysuccinimide
Ni-NTA	nickel nitrilotriacetic acid
nm	nanometre
NMS	normal mouse sera
NPBP	nephritis plasmin binding protein
OD	optical density
OPD	*O*-phenylenediamine dihydrochloride
ORF	open reading frame

OTCase	orthinine carbamoyltransferase
p	pico
PAGE	polyacrylamide gel electrophoresis
PAM	plasminogen-binding group A streptococcal M-like protein
PBS	phosphate buffered saline
PBST	phosphate buffered saline with Tween-20
PCR	polymerase chain reaction
PEG	polyethylene glycol
PFBP	*S. pyogenes* fibronectin-binding protein
PFK	6-phosphofructokinase
PGK	phosphoglycerate kinase
Plg	plasminogen
Plr	plasmin receptor
PMF	peptide mass fingerprinting
PMN	polymorphonuclear leukocytes
PMSF	phenylmethylsulphonyl fluoride
Prp	PAM-related protein
PrtF2	protein F2
PVDF	polyvinylidene fluoride
®	registered
r	resistant
RHD	rheumatic heart disease
RRF	ribosome recycling factor
S. cristatus	*Streptococcus cristatus*
S. equi	*Streptococcus equi*
S. gordonii	*Streptococcus gordonii*
S. pneumoniae	*Streptococcus pneumoniae*
S. pyogenes	*Streptococcus pyogenes*
S. sanguis	*Streptococcus sanguis*
S. suis	*Streptococcus suis*
SA	streptavidin
SagP	streptococcal acid glycoprotein
SclA/SclB	streptococcal collagen-like protein
SCPAw	mutant form of C5a peptidase
ScpC	a serine protease
SD	standard deviation
SDH	streptococcal surface dehydrogenase
SDS	sodium dodecyl sulphate
sec	seconds
SEM	standard error of the mean
SEN	streptococcal surface enolase
SfbI	streptococcal fibronectin binding protein I
SFFBP-12	streptococcal fibronectin-fibrinogen binding protein
SfbX	streptococcal fibro fibronectin binding protein X
SibA	secreted immunoglobulin binding protein from GAS
Sib35	streptococcal immunoglobulin binding protein 35
SIC	streptococcal inhibitor of complement
SLO	streptolysin O
SLS	streptolysin S
SMEZ	streptococcal mitogenic exotoxin Z
SOD	superoxide dismutase
SOF/SfbII	serum opacity factor
Spa	streptococcal protective antigen
Spe	streptococcal pyrogenic exotoxins
SpyCEP/Spy0416	*S. pyogenes* cell envelope proteinase

SSA	streptococcal superantigen
Sse	streptococcal surface esterase
Stains-All	l-ethyl-2-[3-(l-ethyl-naphtho[l,M]thiazolin-2-ylidene)-2-methyl-propenyl]naphtho[l,2d]thiazolium bromide
STSS	Streptococcal toxic shock syndrome
TAFI	thrombin-activatable fibrinolysis inhibitor
TBS	tris-buffered saline
TBST	tris-buffered saline with Tween-20
TCA	trichloroacetic acid
TEM	transmission electron microscopy
TF	trigger factor
THBY	Todd-Hewitt Broth supplemented with 1% (w/v) yeast
TIM	triosephosphate isomerase
™	trade mark
tPa	tissue plasminogen activator
Tris-HCl	tris(hydroxymethyl)aminomethane hydrochloride
Tween-20	polyoxyethylenesorbitan monolaurate
U	units
uPAR	urokinase plasminogen activator receptor
UPTOP	unhindered presentation on tips of pili
URT	upper respiratory tract
UV	ultraviolet
V	volt
v/v	volume/volume
WCL	whole cell lysate
w/v	weight/volume

PUBLICATIONS

Henningham A, Cole JN, Gillen CM, Rohde M, Fulde M, Ramachandran V, Cork AJ, Hartas J, Magor G, Djordjevic SP, Cordwell SJ, Sriprakash KS, Nizet V, Chhatwal GS, Batzloff MR, Walker MJ. (2010) Anchorless cell-surface proteins: Novel candidates for a safe and universal group A streptococcal vaccine. (*Submitted to Journal of Experimental Medicine, invited to revise*)

Cole JN, Henningham A, Gillen CM, Ramachandran V & Walker MJ. (2008) Human pathogenic streptococcal proteomics and vaccine development. *Proteomics Clinical Applications* **2**: 387–410.

Cole JN, Aquilina JA, Hains PG, Henningham A, Sriprakash KS, Caparon MG, Nizet V, Kotb M, Cordwell SJ, Djordjevic SP & Walker MJ. (2007) Role of group A Streptococcus HtrA in the maturation of SpeB protease. *Proteomics* **7**, 4488-4498.

Walker MJ, Hollands A, Sanderson-Smith ML, Cole JN, Kirk JK, Henningham A, McArthur JD, Dinkla K, Aziz RK, Kansal RG, Simpson AJ, Buchanan JT, Chhatwal GS, Kotb M & Nizet V. (2007) DNase Sda1 provides selection pressure for a switch to invasive group A streptococcal infection. *Nature Medicine* **13**, 981-985.

Cole JN, Sanderson-Smith ML, Cork AJ, Henningham A, Conlan F, Ranson M, McArthur JD, & Walker MJ. (2007) Gene expression and tagging of streptococcal proteins In *Molecular Biology of Streptococci.* Edited by R. Hakenbeck & G. S. Chhatwal. Hethersett, UK: Horizon Scientific Press.

PATENTS

2008 *"Group A Streptococcus Pharmaceutical Compositions and Methods Thereof"*
Inventors: **Henningham A, Walker MJ, Cole JN**
PCT Application No.: PCT/AU2007/001236

Provisional Patents

2008 "*Gram positive cocci pharmaceutical compositions and methods thereof*"
Inventors: **Henningham A, Cole J, Gillen CM, Ogunniyi AD, Nizet V, Paton JC, Walker MJ**
2008903129 Australian Provisional Patent (filed 19th June 2008)

2007 "*Group A streptococcal pharmaceutical compositions and methods thereof*"
Inventors: **Henningham A, Walker MJ, Cole JN**
2007904592 Australian Provisional Patent (filed 24th August 2007)

CONFERENCE PRESENTATIONS

<u>Henningham A</u>, Batzloff MR, Cole JN, Gillen CM, Cork AJ, Williams LA, Ramachandran V, Rohde M, Fulde M, Sriprakash KS, Chhatwal GS, Walker MJ. Development of a safe group A streptococcal vaccine. *Bacterial Pathogenesis 10*, Sept 20-23, Barossa Valley, South Australia, Australia, 2009 (Oral Presentation).

<u>Henningham, A</u>, Batzloff MR, Cole JN, Gillen CM, Cork AJ, Ramachandran V, Rohde M, Fulde M, Sriprakash KS, Chhatwal GS, Walker MJ. Development of a safe group A streptococcal vaccine. *Microbial Pathogenesis & Host Response*, Sept 8-12, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 2009 (Poster Presentation).

<u>Henningham A</u>, Batzloff MR, Cole JN, Gillen CM, Hartas J, Sriprakash KS, Walker MJ. Protection against lethal *Streptococcal pyogenes* challenge following vaccination with anchorless cell wall-associated proteins. *XVII Lancefield International Symposium on Streptococci and Streptococcal Diseases*, June 22-26, Porto Heli, Greece, 2008 (Poster Presentation).

Walker MJ, <u>Henningham A</u>, Cork AJ, Cole JN, Ramachandran V, Gillen CM, Rohde M, Batzloff MR, Sriprakash KS, Nizet V, Chhatwal GS. Group A Streptococcal Anchorless Surface Proteins. *XVII Lancefield International Symposium on Streptococci and Streptococcal Diseases,* June 22-26, Porto Heli, Greece, 2008 (Oral Presentation).

Cole JN, Aquilina JA, Hains PG, <u>Henningham A</u>, Sriprakash KS, Caparon MG, Nizet V, Kotb M, Cordwell SJ, Djordjevic SP, Walker MJ. Role of group A streptococcal HtrA in the maturation of SpeB protease. *XVII Lancefield International Symposium on Streptococci and Streptococcal Diseases*, June 22-26, Porto Heli, Greece, 2008 (Poster Presentation).

Ramachandran V, <u>Henningham A</u>, Cork AJ, Aquillina JA and Walker MJ. Examination of Group A Streptococcal surface protein-protein interactions. *XVII Lancefield International Symposium on Streptococci and Streptococcal Diseases*, June 22-26, Porto Heli, Greece, 2008 (Poster Presentation).

Walker MJ, Cole JN, <u>Henningham A</u>, Cork A, Ramachandran V, Sanderson-Smith ML, Gillen CM, Hollands A, Rohde M, Aziz RK, Batzloff MR, Sriprakash KS, Aquilina JA, Nizet V, Kotb M, Chhatwal GS. Surface Proteins and Structures of Group A Streptococci. *Australian Society for Microbiology*, July 9-13, Adelaide, Australia, 2007 (Oral Presentation)

Ramachandran V, <u>Henningham A</u>, Cork AJ, Aquillina JA, Walker MJ. 2007. Examination of streptococcal surface protein-protein interactions. *Bacterial Pathogenesis 9*, Sept 23-26, Lorne, Victoria, Australia, 2007 (Poster Presentation).

1. LITERATURE REVIEW

1.1 Overview

Streptococcus pyogenes (Group A Streptococcus; GAS) is an important Gram-positive human pathogen capable of causing life-threatening conditions including STSS (streptococcal toxic shock syndrome) and necrotising fasciitis, in addition to relatively mild conditions such as pharyngitis and impetigo (Cunningham, 2000). The non-suppurative immune sequelae ARF (acute rheumatic fever) and APSGN (acute post-streptococcal glomerulonephritis) can occur following repeated GAS infection, and are characterised by high morbidity and mortality rates. Although GAS can survive in many human tissues, the most common sites of colonisation are the upper respiratory tract and the skin (Efstratiou, 2000). GAS is a highly evolved pathogen possessing several mechanisms to avoid innate and adaptive immune responses in order to colonise its human host. Infection by GAS is thought to be initiated via the adhesion of the bacterium to epithelial tissue in the host. Adhesion is mediated by various bacterial adhesin molecules, surface proteins and virulence factors; and is followed by colonisation and invasion of deeper tissue, resulting in invasive disease.

During the 20th century, GAS infections generally had a decreased severity than had been previously recorded, and since the advent of penicillin it was, at one stage, believed that GAS had almost been eliminated as a human pathogen. However, since the late 1980s there has been a resurgence of post-streptococcal immune sequelae and invasive GAS disease, particularly STSS, necrotising fasciitis and ARF, in both industrialised and developing countries (Stevens, 1995, Kaplan, 1991, Cunningham, 2000, Efstratiou, 2000, Davies *et al.*, 1996). The reasons for this increase in invasive infection are not entirely understood, but may include: changes in the characteristics of the bacterium (Carapetis *et al.*, 1995b), a re-emergence of highly virulent GAS strains (Nowak, 1994, Aziz *et al.*, 2008), a widespread prevalence of particular serotypes (Rogers *et al.*, 2007), or other host-related factors such as genetic susceptibility to disease. It is highly likely that this increase in invasive disease burden is due to a combination of both bacterial and host factors (Basma *et al.*, 1999).

Currently, GAS infection is endemic in Aboriginal communities in northern Australia. Several researchers have noted unusually high rates of the post-streptococcal sequelae ARF and APSGN amongst these indigenous communities, compared to rates

in urban Australian populations and in other industrialised countries (Martin *et al.*, 1996, Gardiner *et al.*, 1996, Carapetis *et al.*, 1996). In certain remote Aboriginal communities in the Top End of the Northern Territory, as many as 650 cases of ARF occur per 100,000 people per year (McDonald *et al.*, 2004). Current treatment of infection in these populations involves the prophylactic injection of penicillin; unfortunately, this strategy has had limited success due to low levels of patient compliance (Brandt *et al.*, 2000). GAS infection and immune sequelae are a major public health concern in these regions as there is no commercial vaccine available.

At present, the exact mechanism(s) by which GAS infect the human host and cause disease have not yet been fully elucidated. Consequently, further research on GAS, its adhesins, surface proteins and virulence factors is necessary in order to determine the mechanism(s) of GAS infection, and the precise roles that these molecules play in pathogenesis. This knowledge may facilitate the development of an effective and protective vaccine against GAS.

1.2   Group A Streptococcal Infection and Sequelae

GAS can cause a wide variety of human diseases of differing severity and it is the recent increase in invasive disease that has elicited widespread research into possible mechanisms of GAS infection. The diseases caused by GAS are outlined in Table 1.1, categorised by severity of disease.

Table 1.1 Four levels of disease caused by the human pathogen GAS. Adapted from Efstratiou (2000).

Category of disease	Disease types
Superficial diseases	pharyngitis, pyoderma, impetigo, erysipelas, vaginitis, post-partum infections
Deep infections	bacteremia, necrotising fasciitis, deep soft tissue infections, cellulitis, myositis, puerperal sepsis, pericarditis, meningitis, pneumonia, septic arthritis, septicemia
Toxin-mediated	scarlet fever, streptococcal toxic shock syndrome
Immunologically mediated	rheumatic fever, post-streptococcal glomerulonephritis, reactive arthritis, auto-immune neuropsychiatric disorders

GAS serotypes M1 and M3 are amongst the most common GAS strains associated with invasive disease in industrialised nations (Stevens, 1995). However, the capacity to initiate invasive disease is neither serotype limited nor specific (Cleary *et al.*, 1992). Invasive GAS infections such as STSS and necrotising fasciitis are characterised by the failure of several different organ systems and, in the case of

necrotising fasciitis, progressive soft tissue destruction (Breiman *et al.*, 1993). As many as 30 - 70% of patients with severe invasive GAS infection die despite medical treatment (Stevens, 1995). The clinical manifestations of STSS include a severe drop in blood pressure (hypotension), high fever and pain, multiple organ shock, and in certain cases, destruction of deep soft tissues (Basma *et al.*, 1999). Necrotising fasciitis is characterised by high fever, swelling and distinct tenderness to a muscle group, most often in a limb (Holm, 1996). During necrotising fasciitis, the sheath that surrounds muscle tissue is destroyed, resulting in localised swelling and bullae filled with clear yellow fluid. Without intervention, cutaneous gangrene can develop and systemic shock and organ failure can occur (Bisno *et al.*, 1996).

ARF is a non-suppurative immune sequelae that can occur following GAS infection. ARF can appear as an inflammation of the joints (arthritis), heart (carditis), central nervous system (chorea), skin (erythema marginatum) or subcutaneous nodules (Cunningham, 2003). Some patients do not exhibit any clinical symptoms preceding the onset of ARF, making diagnosis and treatment difficult (Veasy *et al.*, 1994). Rheumatic heart disease (RHD), a condition in which there is long term damage to the heart valves, can occur following repeated cases of ARF (Carapetis *et al.*, 2005a). Although, for the most part, ARF is no longer a major health problem in industrialised countries, it still continues to be a serious health problem in indigenous populations and developing nations and it is the most common cause of paediatric heart disease worldwide (Stollerman, 1997a). Until recently it was thought that ARF always occurred following a throat infection (Efstratiou, 2000). However, in the Northern Territory of Australia where ARF occurs at endemic levels, GAS throat colonisation and pharyngitis are rare. Rather, the skin is the primary tissue reservoir of GAS and pyoderma is the main form of GAS infection in these populations (McDonald *et al.*, 2004). ARF is triggered when antibodies generated against M protein on the GAS cell surface immunologically cross-react with proteins in human tissue, such as cardiac myosin, resulting in auto-immunity (Cunningham *et al.*, 1989). M protein and myosin are both alpha-helical coiled-coil molecules, and recently it was found that structural coiled-coil irregularities present in the M protein also occur in human myosin and tropomysin, further demonstrating the molecular basis of such cross-reactivity (McNamara *et al.*, 2008). Additionally, there is evidence that α-M protein antibodies may also cross react with laminin (Shikhman *et al.*, 1993) and that antibodies against N-acetyl glucosamine, a component of the group

A carbohydrate, may play a role in the development of ARF (Guilherme *et al.*, 2006b).

APSGN is another non-suppurative immune sequelae caused by GAS. Generally, nephrogenic GAS strains are those which cause skin infections such as impetigo and pyoderma, although, not all cases of impetigo result in glomerulonephritis, and pharyngeal strains can also contribute to APSGN (Stollerman, 1997b). APSGN mainly occurs in children and teenagers, with predominately more males affected than females. APSGN is characterised by symptoms such as oedema, hypertension (high blood pressure), urinary sediment abnormalities and decreased levels of complement components in serum (Vincent *et al.*, 2004). Currently, the exact mechanism(s) of APSGN pathogenesis are unknown, although, it is thought that immune complex deposition, direct activation of complement due to the presence of streptococcal products in the glomeruli, and the production of cross-reactive antibodies reactive with both streptococcal antigens and glomerular tissue may play a role in the pathogenesis of APSGN (Cunningham, 2000). It is hoped that a vaccine that effectively prevents the initial colonisation of GAS would also eliminate the occurrence of ARF and APSGN.

1.3   Identification of GAS

1.3.1   Lancefield Classification

In the early 20th century Rebecca Lancefield developed a technique to classify different groups of streptococci based on antigenic differences in cell wall polysaccharides (group A, B, C, F and G) or lipoteichoic acid (in the case of group D) (Cunningham, 2000). There are 20 serologically defined groups of β-haemolytic streptococci, A through to H and K through to V (Ahmed *et al.*, 1998). The unique carbohydrate antigen of GAS consists of *N*-acetyl-β-$_D$-glucosamine linked to a polymeric rhamnose backbone.

1.3.2   M Typing

Serological M typing is a method for categorising individual strains of GAS. Developed by Lancefield in 1962, this method is based on the antigenic specificity of M protein found on the surface of GAS. Extracted M protein is reacted in precipitation reactions with a number of typing sera, each exclusive to a particular serotype. The typing serum recognises the unique hypervariable region of the protein located at the N-terminus (Cunningham, 2000).

The characterisation and identification of clinical isolates of GAS by M typing relies on the availability of type-specific polyclonal anti-sera. Serologic M typing can be problematic as there are estimated to be over 120 different M types and M typing anti-sera are not readily available commercially and can be difficult to prepare (Vitali *et al.*, 2002). Additionally, some isolates are non-typeable due to either low expression levels of M protein, expression of a new M protein, expression of an M protein variant, or collection of isolates from different geographical regions to reference strains (Gardiner *et al.*, 1995). Subsequently, molecular methods such as *emm* sequence typing and *vir* typing have been developed.

1.3.3   *emm* Sequence Typing

Each M protein is encoded by unique *emm* genes. The 5' regions of the *emm* genes are highly variable and are hence distinct for each GAS M type (Facklam *et al.*, 1999). Currently, 124 *emm* types are recognised (Johnson *et al.*, 2006). During *emm* sequence typing the unique 5' end of the *emm* gene is amplified and sequenced using polymerase chain reaction (PCR) with primers universal for all *emm* genes (Vitali *et al.*, 2002, Beall *et al.*, 1996). The resultant sequences are used to interrogate genomic databases available at the Centres for Disease Control and Prevention website (www.cdc.gov). *emm* sequence typing is routinely performed to identify clinical isolates of GAS, enabling the association of certain GAS serotypes with specific diseases and immune sequelae.

1.3.4   T Typing

T typing is another epidemiological tool used to identify different GAS strains, either as an alternative or supplement to M typing (Krause, 2002). T proteins are GAS surface antigens exhibiting high levels of antigenic diversity (Bessen *et al.*, 2002). The T typing procedure involves antibody-antigen agglutination tests for T proteins, which use a similar methodology to that used during serological M typing. The T protein is much more stable than M protein, and for this reason, in certain cases, T typing can be preferential to serological M typing (Stollerman, 1997a). In recent times, PCR and sequencing reactions based on the genes encoding T proteins have also been developed.

1.3.5   *Vir* Typing

Vir typing is a molecular typing method which is also useful for the identification of isolates that cannot be typed by serologic based M typing procedures

(Hartas *et al.*, 1998). *Vir* typing involves amplification of the entire 5-7 kb variable *vir* regulon of GAS by long PCR. The *vir* regulon includes the *emm* genes encoding M protein in addition to other virulence factor genes (Gardiner *et al.*, 1998). The resultant PCR product is cleaved by the restriction enzyme *Hae*III and the fragments are resolved using agarose gel electrophoresis. Different DNA banding patterns occur for different M types of GAS permitting identification of unique strains.

1.4   Adhesion: Entry Into Host

The attachment of bacteria to mucosal surfaces and host cells is the first step in the pathogenesis of infectious disease (Beachey, 1981, Vercolloti *et al.*, 1985). Such attachment and subsequent colonisation is essential for GAS to infect the host. The common routes of entry for GAS are the nasopharynx, surgical wounds and the skin (Holm, 1996). The adherence of GAS to host cells is a complex procedure involving several different adhesins (Table 1.2) which are required for adhesion to various receptors on host cells and tissues (Jenkinson *et al.*, 1997). Successful attachment of GAS to host cells often results in up-regulation of virulence genes encoding proteins which mediate subsequent invasion of host tissues. A two-step adhesion model has been proposed for the adhesion of GAS to host cells. Initially, lipoteichoic acid (LTA) mediates a reversible weak hydrophobic interaction with host cells by overcoming electrostatic repulsions between the negatively charged bacterial surface and negative charges on the host cell surface (Hasty *et al.*, 1992). This is followed by a second specific interaction between an adhesin expressed on the surface of GAS and receptors on host cells (Hasty *et al.*, 1992, Hasty *et al.*, 1996, Courtney *et al.*, 1999a).

There are several possible approaches to prevent bacterial adhesion (and thus prevent the development of infectious disease) including: the introduction of purified adhesins or receptors to act as competitive inhibitors of bacterial adherence factors, the administration of sublethal doses of antibiotics to inhibit expression of adhesins and the development of vaccines based on adhesins or other bacterial surface proteins involved in pathogenesis (Ofek *et al.*, 2003, Beachey, 1981). The development of vaccines is the most widely explored approach, and there are several GAS adhesins, surface proteins and virulence factors under investigation as potential GAS vaccine candidates. The interaction of GAS virulence factors with proteins of the host is reviewed elsewhere (Nitsche-Schmitz *et al.*, 2007, Tart *et al.*, 2007, Musser *et al.*, 2009, Olsen *et al.*, 2009).

1.4.1 Fibronectin and Fibrinogen Binding Proteins

Although the exact molecular mechanism(s) of GAS adherence to human epithelial cells is not fully elucidated, the binding of fibronectin (Fn) via several GAS surface-associated proteins has been associated with this process (Rocha *et al.*, 1999). Fn is a 450 kilo Dalton (kDa) glycoprotein found in plasma, other body fluids, extracellular matrix (ECM) and basement membranes, which serves as a substrate for mammalian cell adhesion (Goodfellow *et al.*, 2000, Joh *et al.*, 1998). It is hypothesised that binding of Fn, in addition to other ECM molecules such as laminin, collagen and vitronectin, is important in GAS pathogenesis and the development of infection. Several GAS adhesins have also been observed to bind the plasma protein fibrinogen (Fg) (Courtney *et al.*, 2002b), a 340 kDa protein that plays an important role in blood clotting in the host.

Table 1.2 Putative GAS adhesins and host receptors. Table adapted from Courtney *et al.* (2002b) and Cunningham (2000).

Adhesin	Abbreviation	Host Receptor
Lipoteichoic acid	LTA	Fn
M protein	N/A	Fg, CD46, Fn, laminin, Plg
M-related proteins	Mrp and Enn	IgG, IgA, Fg
Protein F1	SfbI	Fn, integrins
Protein F2	PrtF2	Fn
S. pyogenes Fn-binding protein	PFBP	Fn
Fn-binding protein 54	FBP54	Fn, Fg
28 kDa protein	N/A	Fn
Streptococcal Fn-Fg binding protein	SFFBP-12	Fn? Fg?
Streptococcal surface dehydrogenase	SDH/GAPDH	Fn, Fg, Plg, lysozyme, myosin, actin
Vitronectin-binding protein	N/A	Vitronectin
Hyaluronic acid	N/A	CD44
Serum opacity factor	SOF/SfbII	Fn, Fg
Collagen-binding protein	Cpa	Collagen
Streptococcal collagen-like protein	SclA, SclB	Unknown
Streptococcal pyrogenic exotoxin B	SpeB	Integrin, laminin
Streptococcal Fn-binding protein X	SfbX	Fn
Laminin-binding protein	Lbp	Laminin
Fn-binding protein A	FbaA	Fn
Fn-binding protein B	FbaB	Fn

1.4.1.1 M Protein

M protein is a major surface-associated virulence factor expressed by GAS. A total of 124 distinct serotypes of GAS expressing unique forms of M protein have been identified by serological and/or genotyping methods (Johnson *et al.*, 2006). M proteins are comprised of four distinct repeat regions labelled A through to D (Figure 1.1). Region A is nearest the N-terminus and is highly variable, conferring serotype specificity. Region B can also vary amongst strains, whilst the C-repeat region is highly conserved amongst all M serotypes (Fischetti, 1991). Some M proteins also contain a D repeat region adjacent to the cell wall anchor motif (not shown in Figure 1.1).
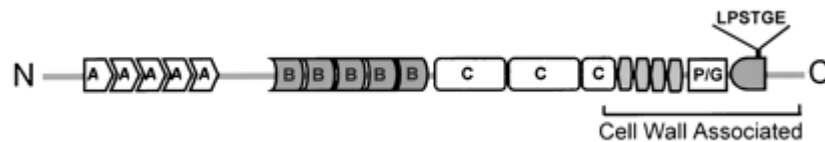
Figure 1.1 Schematic diagram of the streptococcal M6 protein. N represents the amino terminus whilst C represents the carboxyl terminus. The C-terminal cell wall associated region of the M protein is shown, as is the LPXTG membrane anchor region. Figure adapted from Biswas *et al.* (2001).

M proteins are known to function as adhesins mediating binding to human epithelial cells (Courtney *et al.*, 1994a, Courtney *et al.*, 1997, Wang *et al.*, 1994, Okada *et al.*, 1994, Perez-Casal *et al.*, 1995, Eyal *et al.*, 2003) via binding to a variety of human ECM and plasma proteins including Fg (Carlsson *et al.*, 2005), plasminogen (Plg) (Berge *et al.*, 1993, Ringdahl *et al.*, 2000, Sanderson-Smith *et al.*, 2008), CD46 (Okada *et al.*, 1995), Fn (Cue *et al.*, 2001), factor H (Perez-Casal *et al.*, 1995) and collagen (Dinkla *et al.*, 2003).

1.4.1.2 M-related Protein (Mrp and Enn)

In addition to surface expression of M protein, several GAS strains are known to express M-related proteins, designated Mrp and Enn. These M-related proteins possess structural similarity to the M protein and are known to bind to IgG and IgA (Podbielski *et al.*, 1996). Mrp has also been observed to bind Fg (Stenberg *et al.*, 1992) at two distinct binding sites and this interaction prevents the deposition of complement on the GAS cell surface via the classical pathway, facilitating resistance to phagocytosis (Courtney *et al.*, 2006a, Podbielski *et al.*, 1996, Thern *et al.*, 1998).

1.4.1.3 Protein F1/SfbI

Hanski and Caparon (1992) initially described Protein F1, a GAS surface protein observed to bind Fn with high affinity. The gene encoding protein F1, *prtf1*, is located within the FCT region of GAS (Bessen *et al.*, 2002). The FCT region is a variable genetic region in the GAS chromosome encoding the genes for several Fn-binding proteins, a collagen-binding protein and the surface T antigen. In a study of GAS adherence to respiratory epithelial cells, Protein F1-deficient mutants showed significantly less adherence than the wild-type strain, suggesting Protein F1 plays an important role in GAS adhesion to host cells (Hanski *et al.*, 1992, Okada *et al.*, 1994). The N-terminus of Protein F1 also binds Fg (Hanski *et al.*, 1992, Katerov *et al.*, 1998). This multiple binding affinity may endow Protein F1-positive strains with enhanced virulence.

SfbI, Streptococcal fibronectin binding protein I, is an allelic variant of Protein F1 (Talay *et al.*, 1992). Like Protein F1, the binding of Fn by SfbI was found to mediate GAS adherence to respiratory epithelial cells (Molinari *et al.*, 1997), subsequently triggering the internalisation of GAS by these nonphagocytic cells (Molinari *et al.*, 1997, Molinari *et al.*, 1999c). It is thought that GAS may invade epithelial cells as a means of avoiding the effects of antibiotic treatment and accessing deeper tissue and the bloodstream (LaPenta *et al.*, 1994).

1.4.1.4 Protein F2 (PrtF2), Fn-Binding Protein (PFBP) and Fn-Binding Protein B (FbaB)

Protein F2 (PrtF2), *S. pyogenes* Fn-binding protein (PFBP) and Fn-binding protein B (FbaB) are closely related Fn-binding members of the *prtF2* gene family (Ramachandran *et al.*, 2004). Protein F2 is encoded by the *prtF2* gene located in the FCT region (Bessen *et al.*, 2002). Mutagenesis of the *prtF2* gene in a M49 GAS isolate abolished the Fn-binding ability of the isolate (Jaffe *et al.*, 1996, Kreikemeyer *et al.*, 2004). Protein F2 possesses two Fn-binding domains, both located in the C-terminal portion of the protein (Jaffe *et al.*, 1996). PFBP is encoded by the *pfbp* gene and is 127.4 kDa in size (Rocha *et al.*, 1999). PFBP contains a LPXTG motif which is characteristic of cell wall-associated proteins in Gram-positive bacteria. In a study of the Fn-binding ability of PFBP, the C-terminal repeat region of PFBP was fused to the anchor region of M protein, and subsequently expressed on the cell surface of the

commensal species *Streptococcus gordonii*. The resultant PFBP fusion protein was observed to bind both soluble and immobolised fibronectin in bacterial dot blot assays, whilst the parental *S. gordonii* strain did not exhibit any binding (Rocha *et al.*, 1999). FbaB has a LPXTG cell wall anchor motif and Fn-binding repeats located at the C-terminus (Terao *et al.*, 2002b). FbaB has been found in GAS serotypes M3 and M18, which are commonly associated with STSS (Terao *et al.*, 2002b). In a study of the adhesion of GAS to human epidermoid carcinoma (HEp-2) cells, a FbaB-deficient mutant GAS strain had significantly lower levels of adhesion than the wild-type, suggesting that FbaB plays a role in GAS adherence to host cells (Terao *et al.*, 2002b).

1.4.1.5 Fibronectin-Binding Protein 54 (FBP54)

F̲ibronectin-b̲inding protein 5̲4 (FBP54) is a 54 kDa Fn and Fg binding protein of GAS encoded by the *fbp*54 gene (Courtney *et al.*, 1994). The Fn-binding domain and Fg-binding sites of FBP54 reside in the first 89 N-terminal amino acid residues (Hasty *et al.*, 1996, Courtney *et al.*, 1994). FBP54 was identified as a GAS surface protein following incubation of whole GAS cells with α-FBP54 sera (Courtney *et al.*, 1994, Courtney *et al.*, 1996). Despite this, FBP54 does not contain the LPXTG cell wall anchor motif, common in Gram-positive surface proteins. Anti-FBP54 antibodies have been recovered from the sera of ARF and APSGN patients, and thus, is thought that FBP54 may play a role in the development of these non-suppurative streptococcal immune sequelae (Courtney *et al.*, 1996).

1.4.1.6 Streptococcal Fibronectin-Fibrinogen Binding Protein-12 (SFFBP-12)

A study screening a M12 genomic library identified a novel protein designated S̲treptococcal F̲ibronectin-F̲ibrinogen B̲inding P̲rotein-12 (SFFBP-12) (Rocha *et al.*, 1997). SFFBP-12 was found to contain the LPXTG motif typical of Gram-positive surface proteins and to share sequence homologies (ranging from 21% to 75%) with Fn and Fg binding proteins from *Streptococcus dysgalactiae* and *Staphylococcus aureus* (Rocha *et al.*, 1997). The actual Fn and Fg binding capacities of SFFBP-12 are yet to be determined, but it is likely, given the sequence homology with other Fn and Fg binding proteins, that SFFBP-12 may also mediate adhesion of GAS to host cells via these receptors.

1.4.1.7 Streptococcal Surface Dehydrogenase (SDH/GAPDH)

S̲treptococcal s̲urface d̲ehydrogenase (SDH) is a 35.8 kDa GAS surface protein

possessing glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity, and binding affinity for host proteins Fn, lysozyme, plasmin and cytoskeletal proteins myosin and actin (Pancholi *et al.*, 1992). Additionally, SDH/GAPDH mediates adherence to human pharyngeal cells via a binding interaction with uPAR/CD37 (Jin *et al.*, 2005). Such interactions may mediate intracellular signalling pathways in these host cells. Although traditionally characterised as a glycolytic enzyme within the cytosol, SDH/GAPDH was shown to be cell wall-associated when western blots of GAS mutanolysin-derived cell wall extracts were probed with affinity purified α-SDH/GAPDH antibodies (Pancholi *et al.*, 1992). However, like FBP54, SDH/GAPDH does not contain the Gram-positive LPXTG anchor motif. It is thought that the combination of GAPDH enzyme activity and multiple binding abilities of SDH/GAPDH may enable SDH/GAPDH to play a role in GAS colonisation and infection of the host (Pancholi *et al.*, 1992).

1.4.1.8 Serum Opacity Factor (SOF/SfbII) and Streptococcal Fn-Binding Protein X (SfbX)

Serum opacity factor (SOF) is a cell-surface associated Fn-binding protein of GAS possessing enzymatic activity capable of opacifying mammalian serum (Rakonjac *et al.*, 1995). Previously SOF was thought to cleave apolipoprotein A1 of high-density lipoprotein (HDL) found in mammalian serum, resulting in an aggregation of HDL giving the serum an opaque appearance (Gillen *et al.*, 2002). Instead the binding of SOF to HDL displaces the lipid molecules of the HDL which then aggregate resulting in an opaque serum appearance (Courtney *et al.*, 2006). It is thought that the disruption of host HDL by SOF may diminish its anti-inflammatory functions, thereby contributing to the pathogenesis of GAS (Courtney *et al.*, 2006). SOF contains a C-terminal Fn-binding domain and has also been documented to possess a C-terminally located Fg-binding domain (Courtney *et al.*, 2002a). The ability of a M75 SOF-positive GAS strain to adhere to HEp-2 cells has been shown to occur independently of its OF activity, following the production of site-directed mutant forms of SOF and the use of these in latex bead cell adherence experiments (Gillen *et al.*, 2008). Recently, SOF was determined to serve as a receptor for the ECM and plasma protein, fibulin-1, via a binding site distinct to that utilised to bind Fn (Courtney *et al.*, 2009). In addition, SOF has been shown to contribute to the virulence of GAS *in vivo* in mouse infection experiments. In challenge studies, 80% of mice challenged intraperitoneally with a SOF-negative GAS mutant strain survived, whilst all mice challenged with the wild-

type SOF-positive M2 GAS strain died (Courtney *et al.*, 1999b). These findings indicate that SOF is an important virulence factor of GAS, playing a role in pathogenesis *in vivo*.

Streptococcal Fn-binding protein X (SfbX) is a Fn-binding protein containing four putative Fn-binding repeats with a high sequence homology to the Fn-binding domain found in SOF (Jeng *et al.*, 2003). The gene encoding SfbX, *sfbx*, is known to exist in a two gene operon with the *sof* gene (Jeng *et al.*, 2003), however, it is not known whether these two proteins function synergistically, and the precise role that SfbX plays in GAS virulence is not yet fully understood.

1.4.1.9 Fibronectin-Binding Protein A (FbaA)

Fn-binding protein A (FbaA) is another GAS Fn-binding protein which is thought to contribute to GAS virulence. In a murine model of GAS skin infection, mice challenged with a FbaA-deficient GAS mutant had lower mortality rates than mice challenged with the FbaA-positive wild-type (Terao *et al.*, 2001). These results suggest that FbaA is necessary for the development of GAS skin infection. FbaA has also been shown to bind host regulators of complement, Factor H and Factor H-like protein 1, thereby promoting resistance to phagocytosis (Pandiripally *et al.*, 2002).

1.4.2   Collagen Binding Protein (Cpa)

Collagen is a widely distributed protein in human tissues, playing a structural role in biological processes such as host cell attachment to the ECM, cell proliferation and differentiation during organogenesis and hematopoiesis (Visai *et al.*, 1995). The gene encoding GAS Collagen Binding Protein (*cpa*) is located within the FCT region of the GAS genome (Bessen *et al.*, 2002). Cpa is a 57 kDa protein that binds human collagen with a high affinity and it is thought that the binding of GAS to collagen Type IV may be associated with APSGN (Visai *et al.*, 1995). A GAS mutant deficient in Cpa was observed to have a significantly lower rate of internalisation by HEp-2 cells in comparison to the wild-type (Kreikemeyer *et al.*, 2005). Recently, Cpa has been identified as a minor pilin protein of GAS and is thought to covalently link to the pilin backbone and to the T antigen exclusively at the pilin tip (Quigley *et al.*, 2009). For more information on GAS pilus, see section 1.4.6.

1.4.3   The Role of Vitronectin

Vitronectin, also known as complement S protein and serum-spreading factor, is

another host ECM component recruited by GAS during adhesion to host cells (Liang *et al.*, 1997). It is known that α-vitronectin antibodies block the binding of GAS to cultured endothelial cells *in vitro* (Valentin-Weigand *et al.*, 1988), suggesting GAS surface protein(s) and/or adhesin(s) may bind to host cell surfaces via attachment to vitronectin. The precise role that vitronectin plays in GAS pathogenesis is not yet elucidated.

1.4.4 Laminin Binding Proteins

GAS has been reported to bind laminin with an apparent K_D of 4 x 10^{-8} M in an interaction that is functionally irreversible (Switalski *et al.*, 1984). Laminin is a 900 kDa glycoprotein composed of various carbohydrate moieties and three α-helical coiled-coil chains, designated A, B1 and B2 (Beck *et al.*, 1990). In mature tissue, laminin is a ubiquitous component of the basement membrane, playing an important role in cell adhesion and extravasation (Mota *et al.*, 1988). Two laminin binding proteins of GAS have been identified to date: Laminin-binding protein (Lbp/Lmp/Lsp) (Terao *et al.*, 2002a) and Streptococcal pyrogenic exotoxin B (SpeB) (Hytönen *et al.*, 2001).

Lbp of GAS is encoded by the *lbp* gene and has been observed to bind human laminin (Terao *et al.*, 2002a). Using Southern hybridisation, the *lbp* gene was detected in all ten GAS strains screened by Terao *et al.* (2002a), demonstrating that the presence of Lbp is not limited to a single serotype. A Lbp-deficient mutant strain was observed to have significantly less adherence to HEp-2 cells *in vitro* compared to the wild-type (Terao *et al.*, 2002a). Recombinant Lbp was found to bind laminin and to block adherence of GAS to laminin *in vitro* (Elsner *et al.*, 2002, Wahid *et al.*, 2005). Lbp is thought to hasten infections caused by GAS by binding laminin in underlying deep tissues following the initial adhesion of GAS to host cells (Terao *et al.*, 2002a). Following the resolution of a Lbp crystal structure (Linke *et al.*, 2008), a metal-binding site was identified containing a zinc ion which may facilitate the interaction of Lbp with host laminin (Linke *et al.*, 2009).

Streptococcal pyrogenic exotoxin B (SpeB) is a broad spectrum cysteine proteinase located on the surface of GAS and secreted into the culture supernatant. SpeB degrades human ECM components such as Fn and vitronectin in addition to IgG, IgA, IgD, IgE and IgM (Von Pawel-Rammingen *et al.*, 2003). SpeB can also bind to laminin in host ECM (Hytönen *et al.*, 2001). A SpeB variant, mSpeB2, expressed by

M1 and other widely disseminated invasive serotypes of GAS, has also been determined to bind to host integrins (Stockbauer *et al.*, 1999).

1.4.5 Other Molecules Playing a Role in Adhesion

1.4.5.1 Hyaluronic Acid Capsule

Hyaluronic acid is a linear polymer composed of glucuronic acid and *N*-acetylglucosamine repeating units. The genes involved in hyaluronic acid biosynthesis are encoded by the highly conserved *has* gene operon (Moses *et al.*, 1997), which contains three genes, *hasA*, *hasB* and *hasC*. Polysaccharide capsules such as the hyaluronic acid capsule of GAS obstruct antibody access to epitopes on the bacterium's surface, complement (Moses *et al.*, 1997) and other phagocyte-mediated host immune defence mechanisms permitting the survival of GAS. Hyaluronic acid capsule is thought to serve as a universal adhesin mediating attachment of GAS cells to keratinocytes, a major cell type found in pharyngeal epithelium and external skin tissue (Schrager *et al.*, 1998). One study found acapsular GAS mutants (created by transposon mutagenesis) were sensitive to phagocytic killing *in vitro*, in contrast to wild-type strains (Wessels *et al.*, 1991). Acapsular mutants exhibited reduced virulence compared to wild-type strains following intraperitoneal infection of CD1 mice and intranasal infecition of BALB/c mice (Ashbaugh *et al.*, 1998, Wessels *et al.*, 1994, Wessels *et al.*, 1991). In other studies, mice infected intramuscularly with wild-type GAS appeared to recover from the acute signs of infection within the first few days (Iida *et al.*, 2006). Death started to occur three weeks post GAS infection, a phenomenon researchers termed 'delayed death'. GAS recovered from the deceased mice had a notably thickened capsule (Iida *et al.*, 2006). When an isogenic *hasA* mutant was tested in the same intramuscular infection model 'delayed death' was not observed (Iida *et al.*, 2006). These results suggest that capsule is a critical factor in the occurrence of delayed death, a phenomenon in which GAS establish a persistant infection. An upregulation of capsule has also been observed to hinder the recognition of polyclonal antibodies specific for the surface protein G-related α2-macroglobulin-binding protein (GRAB), rendering these antibodies inefficient in the initiation of *in vitro* opsonophagocytosis (Dinkla *et al.*, 2007). Capsular masking of GAS surface proteins, such as GRAB, may enable GAS to avoid coating by antigen-specific antibodies in the host (Dinkla *et al.*, 2007). Recently, a mutation within the control of virulence regulatory sensor kinase (*covRS*) two-component regulon was determined to be associated with the upregulation of

hyaluronic acid capsule, resulting in hypervirulence of M1T1 GAS (Walker *et al.*, 2007). Hence, hyaluronic acid capsule appears to protect GAS from phagocytosis and enhance virulence in the host (Wessels *et al.*, 1994, Wessels *et al.*, 1991, Ashbaugh *et al.*, 1998).

1.4.5.2 Protein G-related α₂-macroglobulin-binding protein (GRAB)

Protein G̲-related α̲2-macroglobulin-b̲inding protein (GRAB) is a GAS cell-surface protein (possessing a LPXTG cell-wall anchor motif) that binds α_2-macroglobulin, a prominent protease inhibitor in human plasma via a high affinity binding region located at its N-terminus (Rasmussen *et al.*, 1999, Godehardt *et al.*, 2004). α_2-macroglobulin bound to the surface of GAS via GRAB can trap and inhibit host proteases, thereby protecting GAS surface virulence determinants from proteolytic degradation (Rasmussen *et al.*, 1999). A GRAB-deficient mutant strain was observed to have attenuated virulence compared to the wild-type following intraperitoneal infection of NMRI mice (Rasmussen *et al.*, 1999). The mice infected with the GRAB-deficient mutant strain survived longer, had smaller skin lesions and lower levels of GAS dissemination than mice infected with the wild-type (Toppel *et al.*, 2003). Such results suggest that GRAB plays an important role in initiating GAS infection in the host.

1.4.5.3 Streptococcal Collagen-like Proteins (SclA/Scl1, SclB/Scl2)

S̲treptococcal c̲ollagen-l̲ike protein SclA/Scl1 is a surface protein of GAS that possesses a collagen-like region, a hypervariable N-terminal region, a LPXTG cell wall anchor and signal sequence typical of Gram-positive bacterial surface proteins (Rasmussen *et al.*, 2000). The contribution of SclA/Scl1 protein to GAS host cell adherence was investigated by Lukomski *et al.* (2000) using an isogenic SclA/Scl1 protein-deficient mutant. Significantly less Δ*sclA/scl1* mutant bacteria were observed to adhere to human A549 epithelial cells grown *in vitro* compared to the wild-type strain (Lukomski *et al.*, 2000), a trait which was restored following genetic complementation (Caswell *et al.*, 2007). A motif has been identified in SclA/Scl1 protein which facilitates the binding of this collagen-like protein to the human collagen receptor integrin $\alpha_2\beta_1$ (Caswell *et al.*, 2008a, Humtsoe *et al.*, 2005). This interaction is thought to facilitate both the adherence and internalisation of GAS into host cells. SclA/Scl1 protein has also been observed to bind apolipoprotein B, one of the major constituents of low-density lipoprotein (Han *et al.*, 2006), plasma and HDL, most likely via a hydrophobic

interaction (Gao *et al.*, 2010). Recently, the SclA/Scl1 protein of M6 GAS was determined to bind factor H, a regulatory protein of the human complement system (Caswell *et al.*, 2008b). This binding interaction may facilitate GAS evasion of complement and subsequent opsonophagocytosis in the host (Caswell *et al.*, 2008b).

SclB/Scl2 protein is another collagen-like protein of GAS, closely related to SclA/Scl1 protein. SclB/Scl2 protein also contains a hypervariable N-terminus, the LPXTG cell wall anchor motif and a signal sequence (Rasmussen *et al.*, 2001). A SclB/Scl2 protein-deficient mutant strain demonstrated a 75% reduction in adherence to human foreskin fibroblasts compared to the wild-type (Rasmussen *et al.*, 2001), suggesting that SclB/Scl2 also contributes to the adhesion of GAS to host cells.

Additionally, both SclA/Scl1 protein and SclB/Scl2 protein have been recognised as a link between GAS and thrombin-activatable fibrinolysis inhibitor (TAFI) of the host. TAFI has anti-fibrinolytic properties, is capable of inactivating the anaphylatoxins C3a and C5a, and exhibits pro-inflammatory properties via the conversion of bradykinin (Påhlman *et al.*, 2007). The ability of GAS to bind TAFI via SclA/Scl1 protein and SclB/Scl2 protein (Påhlman *et al.*, 2007), and the possibility of subsequent activation of TAFI allow the bacteria a powerful mechanism of host inflammatory modulation.

1.4.6   Group A Streptococcal Pilus

Whilst pili have long been known to exist and perform important functional roles in many Gram-negative bacterial species, the discovery of pili and pilin proteins is a recent phenomenon in many Gram-positive species (reviewed in Ton-That & Schneewind, 2004). The pili of GAS is an area of increasing interest, stemming from reports in 2005 that GAS possess pilus-like structures extending from the cell surface (Mora *et al.*, 2005). Pilus structures in GAS may mediate adherence to host cells. GAS pili, like a number of other Gram-positive pili, appear to be formed via sortase mediated covalent polymerisation of LPXTG-containing proteins to form the pilus backbone and embedding of minor pilin proteins into the backbone (Mora *et al.*, 2005). The genes encoding GAS pili components can vary between different serotypes, but in all cases are located within the FCT locus. Pilin proteins include the serotype specific T antigen (backbone, major pilin protein) and Cpa (the minor pilin protein). In addition, a number of sortase proteins are required for the assembly of the pilus on the cell surface (Mora *et*

16

al., 2005). Of these sortase enzymes, the transpeptidase (SrtB) required for polymerisation of the backbone proteins is encoded on the FCT locus, whilst the housekeeping sortase (SrtA), essential for the covalent attachment of the complete pilus to the cell wall peptidoglycan, is encoded elsewhere on the chromosome (Scott *et al.*, 2006). Other possible accessory pilin proteins may include Spy0130 (in M1 strain SF370), FctX (in M3 strain 10394), and it has recently been found that there are 57 distinct pilus islands belonging to 7 different FCT regions (Falugi *et al.*, 2008). It appears that the entire 11 kb FCT locus, previously characterised as a pathogenicity island of GAS (Bessen *et al.*, 2002), is capable of *en bloc* horizontal gene transfer permitting the transfer of pilin genes between GAS isolates. GAS pili have been shown to contribute to the formation of biofilms and to mediate adherence to skin and pharyngeal cells *in vitro* (Manetti *et al.*, 2007, Abbot *et al.*, 2007). Investigation of the structure of the pilus components through X-ray crystallography (Kang *et al.*, 2007, Solovyova *et al.*, 2009) has revealed insights into the assembly of the pilus on the cell surface. For further reviews on GAS and Gram-positive pili, please refer to Scott & Zähner (2006), Telford *et al.* (2006), Mandlik *et al.* (2007) and Proft & Baker (2009).

1.5 Colonisation, Invasion and Host Response

GAS have evolved several mechanisms to interfere with the complement system including: binding of complement regulatory components such as factor H or factor H-like protein to the cell surface by M protein and SclA (Fischetti, 1991, Caswell *et al.*, 2008b), proteolytic degradation of chemotactic substances such as C5a by the streptococcal expressed C5a peptidase (Ji *et al.*, 1997), inhibition of the membrane attack complex formation by the streptococcal inhibitor of complement protein (SIC) (Åkesson *et al.*, 1996) and the inhibition of antibacterial proteins and peptides such as secretory leukocyte proteinase inhibitor, human neutrophil α-defensin and LL-37 (Fernie-King *et al.*, 2002, Frick *et al.*, 2003).

Although GAS are generally considered to be an extracellular pathogen, they also possess the ability to invade host cells. The mechanism(s) of GAS invasion of host cells are currently under investigation, and a number of surface proteins and adhesins have been implicated in this process including M protein, Protein F1/SfbI, PrtF2, SpeB, ADI, SclA/Scl1 and streptolysin O (SLO) (Molinari *et al.*, 1999a). It is thought that the survival of GAS within host cells is an important strategy employed by GAS in order to

avoid host immune response and defences. In addition, the internalisation of GAS into tonsil and respiratory epithelial cells permits the survival of the bacterium even during antibiotic treatment, allowing the potential for recurrent infection (Osterlund *et al.*, 1997a, Osterlund *et al.*, 1997b, Kaplan *et al.*, 2006). One study isolated viable GAS cells from mouse phagocytes during *in vivo* infection and found that these intracellular GAS cells were able to transfer infection when intravenously injected into naïve mice (Medina *et al.*, 2003). Another study considered the cellular location of GAS in human tissue biopsies collected from 17 patients suffering soft tissue infection including necrotising fasciitis, myositis and cellulitis. GAS were detected both extracellularly and intracellularly within phagocytic cells such as macrophages (Thulin *et al.*, 2006). Internalised GAS were mostly found in more recently infected tissue (as characterised by a lower bacterial load and less inflammation), and intracellular GAS were identified in tissue from patients who had undergone prolonged intravenous antibiotic treatment (Thulin *et al.*, 2006), Thus, invasion of GAS into host cells not only allows evasion of the host immune defence, but also evasion from antibiotic therapies and may enable the spread of GAS from primary sites of infection.

Colonisation of the host is a complex process affected by host factors including (but not exclusive to) the specificity and concentration of immunoglobulins in saliva, the types of mucins present and the presence or absence of normal flora on mucosal surfaces (Courtney *et al.*, 2002b). Although it is known that adhesion is the first step in the colonisation process, and the mechanism(s) involved in bacterial adhesion are currently being intensively investigated, the mechanism(s) of subsequent colonisation and the interactions between GAS and host cell surfaces are less well understood (Courtney *et al.*, 2002b). Several virulence factors and toxins of GAS are thought to be involved in colonisation of the host following adhesion to host tissues.

1.5.1 Invasins

1.5.1.1 M Proteins

M protein has evolved several different mechanisms to evade host immune defences including: the presence of a net negative charge on the N-terminus which results in electrostatic repulsion from host cells such as phagocytes, antigenic variation resulting in M proteins of different size and sequence, the ability to bind the regulatory protein of the complement system, factor H (Fischetti, 1991) and evasion of

phagocytosis by blocking the deposition of C3b (an opsonin) onto its surface (Fischetti, 1989). Such evasion of the host immune defence allows GAS to persist in infected tissues and potentially invade deep tissue or host cells. M protein from multiple GAS serotypes has been directly implicated in the process of host cell internalisation of GAS. M6 protein-specific serum IgG has been found to inhibit internalisation of M6 GAS by cultured human pharyngeal cells (Fluckiger *et al.*, 1998). These findings complement those of other studies in which M6- and M3-deficient GAS mutant strains were observed to have a decreased rate of internalisation by HEp-2 cells as compared to the respective wild-type strains (Jadoun *et al.*, 1998, Eyal *et al.*, 2003). Furthermore, M protein and M-like proteins from an M1 strain permitted survival of GAS within neutrophils (Staali *et al.*, 2003). Together, these findings suggest that different types of M protein may play a role in the internalisation of GAS by host cells which may facilitate cases of recurrent GAS infection.

1.5.1.2 Protein F1/SfbI and Protein F2 (PrtF2)

Presumably via a binding interaction with Fn, Protein F1 can promote the internalisation of GAS into host cells. In one study, the expression of Protein F1 in a naturally Protein F1 deficient *emm*1 strain (via the introduction of a plasmid carrying the *prtF1* gene), resulted in a rate of internalisation of GAS by HEp-2 cells 15 times greater than that of the control strain harbouring the plasmid vector only (Jadoun *et al.*, 1998). In the same study, a Protein F1-deficient isogenic mutant was internalised at a significantly lower rate than the wild-type (Jadoun *et al.*, 1998), suggesting that the expression of Protein F1 promotes the internalisation of GAS by host epithelial cells. Similarly, the allelic variant of Protein F1, SfbI, also promotes the internalisation of GAS by HEp-2 cells via the interaction of SfbI with host Fn (Molinari *et al.*, 1997). The attachment and internalisation of GAS by HEp-2 cells was blocked when the HEp-2 cells were pre-treated with exogenous purified SfbI protein, and also when GAS were treated with antibodies against full length SfbI or the Fn-binding domain of SfbI (Molinari *et al.*, 1997). Additionally, inert latex beads coated with purified recombinant SfbI were able to invade HEp-2 cells, showing that SfbI *per se* was sufficient to trigger internalisation (Molinari *et al.*, 1997). In a recent study, Protein F1/SfbI contributed to GAS resistance of phagocytosis (when expressed in an M1 background) and the expression of Protein 1/SfbI was also observed to partially inhibit the deposition of C3b on the surface of GAS cells (Hyland *et al.*, 2007). Thus, Protein F1/SfbI is a

multifunctional protein of GAS with the ability to mediate adherence and invasion of host cells which also exhibits anti-phagocytic properties.

In a similar manner to Protein F1/SfbI, PrtF2 plays a role in the internalisation of GAS by host cells. The ability of a PrtF2-deficient isogenic mutant (in a GAS strain also lacking the *sfbI* gene) to adhere to and invade HEp-2 cells in antibiotic protection assays and double immunofluoresence staining experiments was significantly reduced in comparison to the wild-type (Kreikemeyer *et al.*, 2004). In a study of clinical isolates collected from patients living in northern Australia, isolates containing *prtF2* (and lacking *prtF1*) were observed to invade HEp-2 cells with a high efficiency (Gorton *et al.*, 2005).

1.5.1.3 Streptococcal pyrogenic exotoxin B (SpeB)

The role of SpeB as a GAS virulence factor has been widely investigated, and SpeB has been observed to play a role in the invasion of A-549 human respiratory epithelial cells by GAS (Tsai *et al.*, 1998). An isogenic SpeB-deficient mutant strain was observed to have a two- to three-fold decrease in cellular invasion compared to the wild-type A20 strain (Tsai *et al.*, 1998). However, the findings of Tsai *et al.* (1998) contrast with those of Burns *et al.* (1998) and Jadoun *et al.* (2002). In a study utilising heterologous mutants, Burns *et al.* (1998) observed a significant increase in the internalisation of SpeB mutants by both human umbilical vein endothelial cells and A-549 cells in comparison to wild-type strains (M2 and M3) (Burns *et al.*, 1998). Using a SpeB-deficient M3 GAS mutant, Jadoun *et al.* (2002) also observed a significant increase in the internalisation of the SpeB mutant into HEp-2 cells in comparison to the wild-type. It is possible the differential rates of GAS internalisation in these studies may be due to the selection of different host cells, which may contain different surface receptors available for GAS attachment and subsequent invasion.

1.5.1.4 Arginine Deiminase (ADI/SagP)

ADI or streptococcal acid glycoprotein (SagP) is a virulence factor of GAS capable of inhibiting the proliferation of human T cells *in vitro* (Degnan *et al.*, 1998). An isogenic *sagP* mutant constructed in the M5 Manfredo GAS isolate was found to invade eukaryotic HEp-2 and A549 cells at a rate 3-5 times lower than the wild-type (Degnan *et al.*, 2000). In contrast, a *sagP* isogenic M3 GAS mutant was observed to have a 5-fold increase of internalisation by HEp-2 cells in comparison to the wild-type

(Marouni *et al.*, 2003). In addition, Marouni *et al.* (2003) also observed a down-regulation of SpeB transcription and translation in the *sagP* mutant strain, along with a partial decrease in the transcription of *hasA*, an essential gene of capsule synthesis. It is thought that ADI may play an indirect regulatory role in GAS, influencing the expression of GAS virulence factors and the consequent internalisation of GAS by host cells (Marouni *et al.*, 2003).

1.5.1.5 Streptococcal Collagen-like Protein A (SclA/Scl1)

Recently, the collagen-like protein SclA/Scl1 has also been implicated in the internalisation of GAS by host cells via interactions between SclA/Scl1 and the human collagen $\alpha_2\beta_1$ integrin, laminin and Fn (Caswell *et al.*, 2007, Caswell *et al.*, 2010). An isogenic *scl1* mutant was observed to have a significant decrease in both adherence to and internalisation by HEp-2 cells compared to the wild-type M41 isolate (Caswell *et al.*, 2007). Furthermore, internalised GAS were observed to re-emerge intact into the extracellular environment (Caswell *et al.*, 2007).

1.5.1.6 Lipoprotein of Streptococcus pyogenes (Lsp)

<u>L</u>ipoprotein of <u>S</u>treptococcus <u>p</u>yogenes (Lsp), a Lra1-lipoprotein family member, has also been observed to function in invasion of host cells by GAS. Following the construction of a *lsp*-deficient GAS mutant strain, adherence and internalisation of GAS by A-549 epithelial cells was reduced compared to the M49 wild-type M49 (Elsner *et al.*, 2002). Mutation of the *lsp* gene led to a decrease in the transcription of *prtF2*, *sof*, *speB*, and three important global regulatory operons, <u>c</u>ontrol <u>of v</u>irulence <u>r</u>egulatory <u>s</u>ystem (*covRS*), *mga* and *nra* (Elsner *et al.*, 2002). The *covRS* (Jadoun *et al.*, 2000) and *nra* genes (Molinari *et al.*, 2001) have been previously implicated in the interaction of GAS with host cells and the decreased transcription of these genes in the *lsp* mutant strain may influence the rate of host cell internalisation *in vitro*.

1.5.1.7 C5a Peptidase

Streptococcal C5a peptidase is a surface expressed endopeptidase which cleaves the C5a component of complement, inactivating C5a so it can no longer act as a chemo-attractant (Koroleva *et al.*, 2002). This results in a delayed infiltration of phagocytes to sites of GAS infection, which slows the clearance of GAS from host mucosal surfaces (Ji *et al.*, 1997) allowing GAS to further colonise the host (Stafslien *et al.*, 2000). C5a

peptidase has also been reported to be an invasin of GAS, as pre-incubation of M1 GAS with α-C5a peptidase serum resulted in 54% less invasion into HEp-2 cells than incubation without serum (Purushothaman *et al.*, 2004). In addition, when pre-incubated with HEp-2 cells, a recombinant form of C5a peptidase was observed to block the internalisation of whole GAS into HEp-2 cells (Purushothaman *et al.*, 2004).

1.5.1.8 Streptococcal Inhibitor of Complement (SIC)

Streptococcal inhibitor of complement (SIC) is encoded by the *sic* gene, located in the *mga* regulon of the GAS chromosome, and is abundantly expressed in highly virulent strains of GAS such as the M1 serotype (Frick *et al.*, 2003). Protein SIC inhibits complement-mediated lysis of GAS cells by blocking the membrane insertion site on the C5b67 complex during the activation of complement (Åkesson *et al.*, 1996). Other roles have been established for SIC including the inhibition of antibacterial agents such as lysozyme (Fernie-King *et al.*, 2002), human neutrophil α-defensin (Fernie-King *et al.*, 2002, Frick *et al.*, 2003), LL-37 (Frick *et al.*, 2003), β-defensins (Fernie-King *et al.*, 2004) and secretory leukocyte proteinase inhibitor (Fernie-King *et al.*, 2007, Fernie-King *et al.*, 2002). In addition to binding and promoting the inactivation of these antibacterial agents, SIC is also capable of binding to the cytoskeleton of host cells, which may in turn trigger cytoskeletal re-arrangement (Binks *et al.*, 2005). Two variants of SIC, known as CRS (closely related to SIC) and DRS (distantly related to SIC) have been identified in certain GAS strains (Hartas *et al.*, 1999). Like SIC, CRS and DRS disrupt complement activation by binding C6 and C7 (Binks *et al.*, 2003, Binks *et al.*, 2004). CRS is expressed by M57 strains whilst DRS is expressed by M12 and M55 GAS. Given GAS serotypes M1, M12, M55 and M57 are associated with APSGN, it is possible that SIC, CRS and DRS play a role in the pathogenesis of APSGN (Hartas *et al.*, 1999). A recent study has also shown SIC contributes to whole blood and serum resistance phenotypes exhibited by M1T1 GAS (Pence *et al.*, 2010).

1.5.1.9 IgG-Degrading Enzyme of Streptococcus pyogenes (IdeS/Mac-1)

IgG-degrading enzyme of *Streptococcus pyogenes* (IdeS), also know as Mac-1, is a 35 kDa protein with a single specificity for cleavage of human IgG (Von Pawel-Rammingen *et al.*, 2003). Through the cleavage of IgG antibodies, IdeS protects GAS from opsonisation and subsequent phagocytosis, allowing avoidance of the host immune response. Furthermore, the half length Fc IgG fragments generated by IdeS were found

to act as priming agents for polymorphonuclear leukocytes (PMNs) (Söderberg *et al.*, 2008b). In the host, during the inital stages of natural infection it is possible that PMNs could be located remotely to the site of infection, and this mechanism may contribute to a further inflammatory response in the host without harming the bacteria (Söderberg *et al.*, 2008b). GAS strains which are associated with the development of APSGN (M12 and M55) were observed to produce active IdeS *in vitro* (Von Pawel-Rammingen *et al.*, 2003), thus, it is possible IdeS may contribute to the pathogenesis associated with APSGN. GAS has also evolved the ability to recruit cystatin C, a host produced protease inhibitor, to act as a co-factor for IdeS (Vincents *et al.*, 2008) enhancing the virulent effects of IdeS. An allelic variant of IdeS/Mac-1 has been designated Mac-2, which is also capable of weak IgG endopeptidase activity (Söderberg *et al.*, 2008a, Agniswamy *et al.*, 2004).

1.5.1.10 Immunoglobulin (Ig)-Binding Proteins

Ig-binding proteins expressed by GAS have been associated with isolates involved in invasive disease. Although the precise role(s) of Ig-binding proteins in the pathogenesis of GAS are not yet fully elucidated, it is thought that Ig-binding proteins may help the pathogen evade the host immune defence systems (Fagan *et al.*, 2001). Secreted immunoglobulin binding protein from group A streptococcus (SibA) is an Ig-binding protein expressed by GAS. SibA is encoded by the *sibA* gene, is highly conserved and binds IgG, IgA and IgM (Fagan *et al.*, 2001). Currently, it is unknown what role SibA plays in the pathogenesis of GAS. Another IgG-binding protein, streptococcal immunoglobulin-binding protein 35 (Sib35) has a similar binding specificity to SibA, as it is also capable of binding IgG, IgA and IgM (Kawabata *et al.*, 2002). It is thought Sib35 is secreted into the culture supernatant and it is postulated to be surface-localised, although, it does not contain the typical Gram-positive LPXTG cell-wall anchor motif (Kawabata *et al.*, 2002). In a recent study, Sib35 was observed to activate mouse B cells and promote the proliferation of these B lymphocyte cells *in vitro* (Okamoto *et al.*, 2008).

1.5.1.11 Serine Protease ScpC

Recently, ScpC, a novel group A streptococcal serine protease was discovered which is capable of directly inactivating key components of the host innate immune reponse, degrading the CXC chemokines, IL-8, KC and MIP-2 (Hidalgo-Grass *et al.*,

2006). Via the construction of *scpC*-deficient mutant strains in a M14 background, ScpC was observed to be essential for GAS virulence in a murine model of necrotising fasciitis (Hidalgo-Grass *et al.*, 2006).

1.5.2 Toxins

Toxins produced by pathogenic bacteria exert harmful effects on the host. GAS secrete several toxins, including streptolysin S, streptolysin O, superantigens, streptokinase, DNase, hyaluronidase and NADase (Courtney *et al.*, 1999a).

1.5.2.1 Streptolysin S (SLS) and Streptolysin O (SLO)

Streptolysin S (SLS) is a haemolytic toxin produced by almost all GAS strains. SLS can lyse a wide range of host cells, including erythrocytes, lymphocytes, PMNs, keratinocytes, macrophages and platelets (Betschel *et al.*, 1998, Goldmann *et al.*, 2009, Miyoshi-Akiyama *et al.*, 2005, Sierig *et al.*, 2003). SLS is encoded by a nine-gene operon known as *sag* (SLS-associated gene) which is comprised of the genes *sagA* to *sagI* (Nizet *et al.*, 2000). One study reports that the *sagA* gene is required for the complete expression and correct surface localisation of M protein (Biswas *et al.*, 2001). In several studies, SLS-deficient GAS mutants have been observed to be less virulent than wild-type strains in murine models of GAS infection (Betschel *et al.*, 1998, Datta *et al.*, 2005, Sierig *et al.*, 2003), and it is hypothesised that SLS contributes to early stages of GAS infection and the development of necrosis in a mouse model (Fontaine *et al.*, 2003). Thus, SLS plays both a direct role in the virulence of GAS through lysis of host cells, and an indirect role by influencing the expression of the M protein.

SLO is an oxygen-sensitive exotoxin which interacts with cholesterol in target cell membranes (Goldmann *et al.*, 2009) to form membrane pores which lead to cell destruction (Sierig *et al.*, 2003) in a wide range of eukaryotic cells including erythrocytes, leukocytes, macrophages and platelets. Recently it has been found that GAS is capable of triggering dose-dependent apoptosis of macrophages and neutrophils from both primary and cultured sources (Timmer *et al.*, 2009) presumably due to the activity of SLO. A *slo*-deficient GAS mutant promoted lower rates of macrophage apoptosis both *in vitro* and *in vivo*, and also had reduced virulence in a mouse model of systemic GAS infection in comparison to M1T1 wild-type GAS (Timmer *et al.*, 2009). The perforation of PMNs by SLO leads to activation of PMNs and the consequent release of heparin-binding protein from host cells, a potent inducer of vascular leakage

(Nilsson *et al.*, 2006). This hyper-inflammatory response may facilitate further pathogenesis of GAS in the host. SLO is encoded by the highly conserved *slo* gene and is expressed in almost all strains of GAS (Limbago *et al.*, 2000). In a murine model of invasive GAS disease (Limbago *et al.*, 2000) and also in a model of bacterial sepsis (Sierig *et al.*, 2003), mice infected with a *slo*-deficient GAS mutant were shown to have decreased mortality compared to mice infected with the wild-type. SLO has also been observed to contribute to the development of necrotic lesions in a murine subcutaneous infection model (Fontaine *et al.*, 2003). Thus, SLO plays a crucial role during the induction of host cell apoptosis, contributing to GAS virulence and evasion of the host immune response. Interestingly, SLO also shows promise as a novel anti-cancer therapeutic. Following the injection of SLO-expressing adenovirus into cervical cancer tumours in nude mice, the average size of SLO-treated tumours was determined to be only 30% of the size of the control group (Yang *et al.*, 2006).

1.5.2.2 Streptococcal Pyrogenic Exotoxins (Spes) and Superantigens

Superantigens are a class of proteins expressed by GAS which potently and non-specifically activate T-cells (Weiss *et al.*, 1997) by binding to regions on major histocompatibilitity complex (MHC) class II molecules and concurrently bind to the variable region of the β-chain of the T cell receptor (Alouf *et al.*, 2003). This results in expansion of these cells which stimulates an immune response, in particular the release of cytokines and other immune effectors, which can lead to tissue damage and organ failure typically seen in invasive GAS infection (Broudy *et al.*, 2002, Holm, 1996, Bisno *et al.*, 1996). The symptoms that occur during STSS result, in part, from an excess of superantigen-induced cytokine production (Stevens, 2000). Superantigens expressed by GAS include the streptococcal pyrogenic exotoxins (Spes), of which there is SpeA (Cleary *et al.*, 1992, Kazmi *et al.*, 2001, Müller-Alouf *et al.*, 2001, Musser *et al.*, 1991), SpeB (refer to section 1.5.14.3.1), SpeC (Tripp *et al.*, 2003), SpeG (Proft *et al.*, 2000, Proft *et al.*, 1999), SpeH (Proft *et al.*, 2000, Proft *et al.*, 1999), SpeI (Brouillard *et al.*, 2007), SpeJ (Baker *et al.*, 2004, McCormick *et al.*, 2001, Proft *et al.*, 1999), SpeL and SpeM (Proft *et al.*, 2004, Smoot *et al.*, 2002), streptococcal superantigen (SSA) (Mollick *et al.*, 1993), streptococcal mitogenic exotoxin Z (SMEZ) (Kamezawa *et al.*, 1997, Proft *et al.*, 2000, Yang *et al.*, 2005, Nooh *et al.*, 2006) and SMEZ-2 (Proft *et al.*, 1999). Each of these superantigens exhibit a plethora of toxic effects. The number of GAS superantigens is too extensive to review in detail here,

please refer to aforementioned references for specific detail on these toxins.

1.5.2.2.1 Streptococcal Pyrogenic Exotoxin (SpeB)

All GAS strains carry *speB*, the gene encoding SpeB, and the majority of strains are thought to express this highly conserved, secreted broad spectrum cysteine protease. SpeB is produced as a 40 kDa inactive zymogen, which is subsequently cleaved via autocatalysis, resulting in the 28 kDa active form of the protease (Musser *et al.*, 1996). It is thought that SpeB is involved in the development of the GAS-mediated auto-immune complication APSGN (Luo *et al.*, 2007). In a study of SpeB production by non-severe and severe (STSS, necrotising fasciitis) disease causing GAS isolates, there was a significantly greater amount of SpeB production and subsequent protease activity in the non-severe isolates compared to severe STSS isolates (Kansal *et al.*, 2000). These findings suggest an inverse correlation between severity of disease and SpeB activity. Initially, it was hypothesised that an on/off and/or post-translational regulatory mechanism(s) may regulate SpeB production. In such a system, isolates in which SpeB is switched 'off' have a greater likelihood of intact M protein and other virulence factors and are more likely to be associated with severe rather than non-severe disease (Kansal *et al.*, 2000). Successive studies have confirmed the propensity of GAS to undergo *in vivo* switching (Aziz *et al.*, 2004, Cole *et al.*, 2006), in which bacterial production of the DNase Sda1 enables evasion of neutrophil extracellular traps. This induces a selective pressure towards mutation(s) in the GAS *covRS*, resulting in SpeB-negative isolates, permitting deep tissue invasion and the transition from localised to systemic infection of the host (Walker *et al.*, 2007).

SpeB-mediated cleavage of host proteins and tissues has long been thought to facilitate the pathogenesis of GAS. SpeB is capable of cleaving host Fn and vitronectin (Kapur *et al.*, 1993) and Ig (Von Pawel-Rammingen *et al.*, 2003). Furthermore, SpeB has been observed to cleave the anti-bacterial peptide LL-37 (Schmidtchen *et al.*, 2002), properdin (a positive complement activation regulator found in serum) (Tsao *et al.*, 2006) and complement component C3 (Hsu *et al.*, 2008, Kuo *et al.*, 2008), resulting in the inhibition of several complement activation pathways. SpeB has also been observed to bind to integrin (Stockbauer *et al.*, 1999) and laminin (Hytönen *et al.*, 2001) of the host. Binding of such host proteins may facilitate an accumulation of SpeB on the host cell surface, enabling localised degradation of host tissue (Stockbauer *et al.*, 1999). In a

recent study, following treatment of host serum with SpeB, the classical, lectin and alternative complement pathways did not function properly (Kuo *et al.*, 2008). Additionally, SpeB was observed to cleave C3 in the same study (Kuo *et al.*, 2008), demonstrating that SpeB has multiple means of preventing opsonophagocyosis and complement-mediated clearance. Furthermore, a degraded form of the opsonin C3b has been found in serum isolated from patients suffering STSS and it has been experimentally shown that GAS and recombinant SpeB are capable of degrading C3b *in vitro* (Terao *et al.*, 2008).

The direct effect of SpeB on PMN-mediated phagocytosis has been investigated using isogenic SpeB mutants. In whole blood, there was a five-fold increase in phagocytosis of a SpeB-deficient mutant GAS strain as compared to the wild-type, NZ131 (M49); and following treatment with recombinant SpeB, the mitochondria of the PMN cells exhibited a loss of membrane potential and a decrease in dehydrogenase activity (Chiang-Ni *et al.*, 2006). Moreover, a *speB*-deficient mutant was observed to have a lower survival rate *in vivo* in infected mice and human blood, in comparison to the M1 wild-type (Terao *et al.*, 2008). Such results imply that SpeB facilitates GAS resistance to phagocytosis at the infection site, permitting further dissemination into the host. Following the generation of α-SpeB monoclonal antibodies (mAbs), one antibody (designated 10G) was determined to elicit cross-reactivity against kidney endothelial cells *in vitro* and was also capable of producing kidney injuries *in vivo* in mice (Luo *et al.*, 2007). Thus, in addition to proteolytic and other toxic effects, SpeB may also play a direct role in auto-immune disease and immune sequelae such as APSGN.

1.5.2.3 M1 Protein

M1 serotype GAS are often associated with cases of invasive GAS infection including STSS (Aziz *et al.*, 2008). In recent times, the multifunctional M1 protein has been observed to also function as a superantigen, capable of activating neutrophils (Herwald *et al.*, 2004) and monocytes (Påhlman *et al.*, 2006), potently inducing the proliferation of T cells and subsequent release of Th1 cytokines (Påhlman *et al.*, 2008). In addition to a direct activation of neutrophils and monocytes, M1 protein can also activate blood cells in an indirect manner, via the interaction of M1 protein with Fg. M1/Fg complexes bind to Fg receptors on platelets (Shannon *et al.*, 2007). If host IgG molecules against M1 protein bind to the M1/Fg complex, these IgG molecules will

interact with the Fc receptor on platelets leading to activation and a pro-inflammatory response, typical of severe invasive GAS infection (Shannon *et al.*, 2007).

1.6 The Role of Plasminogen (Plg) in GAS Infection

Plg is a glycoprotein found in plasma and extracellular fluid. Plg is converted to the active enzyme plasmin by several different serine proteases including tissue-type plasminogen activator (tPa) and urokinase plasminogen activator, both of which are host activators, and streptokinase (McArthur *et al.*, 2008), a potent Plg activator secreted by GAS. GAS express several surface proteins that bind Plg, and once surface-bound Plg is converted to plasmin, GAS can subsequently use this plasmin to degrade host tissues, basement membranes and ECM components. Thus, the acquisition of plasmin(ogen) by GAS is thought to contribute to pathogenesis and tissue invasion. For a review of the role Plg plays in GAS virulence, refer to Walker *et al.* (2005).

Five GAS Plg-binding receptors have been identified to date: streptococcal surface enolase (SEN) (Pancholi *et al.*, 1998), streptococcal surface dehydrogenase (SDH/GAPDH) (Pancholi *et al.*, 1993, Pancholi *et al.*, 1992) also designated as plasmin receptor (Plr) (Broder *et al.*, 1991), M proteins that bind Plg directly with high affinity such as Plg-binding group A streptococcal M-like protein (PAM) (Berge *et al.*, 1993) and PAM-related protein (Prp) (Sanderson-Smith *et al.*, 2007).

Additionally, GAS can acquire plasmin enzymatic activity via an interaction with Plg, Fg and streptokinase (Wang *et al.*, 1995). Several independent studies have reported this interaction and once GAS has acquired surface-associated plasmin enzyme activity, it is maintained even in the presence of potent physiological Plg regulators such as α_2-antiplasmin and α_2-macroglobulin (Wang *et al.*, 1995, Svensson *et al.*, 2002, D'Costa *et al.*, 1998). This unregulated enzyme activity provides the bacterium with invasive potential through tissue barriers. Mice expressing the human Plg transgene were found to have a greater susceptibility to GAS infection compared to wild-type mice (Sun *et al.*, 2004). This susceptibility was dependent on the expression of streptokinase, further illustrating the role streptokinase plays in the virulence and pathogenesis of GAS (Sun *et al.*, 2004). Another study assessed the contribution of both streptokinase and human Plg to virulence in a mouse model of GAS infection. Researchers infected mice with wild-type M1 GAS and a streptokinase-deficient mutant in the presence and absence of exogenous human Plg (Khil *et al.*, 2003). When

administered with the wild-type GAS, exogenous human Plg significantly increased mean lesion size and the percent survival of mice (Khil *et al.*, 2003), highlighting the synergy between streptokinase and host Plg.

1.6.1 Streptococcal Surface Enolase (SEN) and Streptococcal Surface Dehydrogenase (SDH/GAPDH)

Streptococcal surface enolase (SEN) is a 45 kDa protein located on the surface of GAS possessing the ability to bind human Plg (Pancholi *et al.*, 1998). SEN possesses a dual functionality as it is also a key glycolytic enzyme located in the cytoplasm of the cell where it catalyses the conversion of 2-phosphoglycerate to phosphoenolpyruvate. SEN binds Plg via exposed lysine residue(s) located at its C-terminus. Once SEN has bound plasmin, it has the ability to maintain proteolytic activity, even in the presence of regulators such as α_2-macroglobulin (Pancholi *et al.*, 1998). A recent study has shown that lysine residues in positions 252, 255, 434 and 435 play a key role in the aquisition of Plg by SEN (Cork *et al.*, 2009). Through binding to host Plg, SEN may play an important role in virulence during the establishment of GAS infection. Anti-SEN antibodies have been observed to react with human α-enolase, suggesting that enolase is a cross-reactive antigen that may play a role in the development of GAS auto-immune disease such as ARF (Fontán *et al.*, 2000).

SDH/GAPDH also exhibits binding affinities for several other human proteins, including Fn, lysozyme, myosin and actin as discussed in section 1.4.1.10. Furthermore, SDH/GAPDH also has ADP-ribosylating activity, a process involved in signal transduction events (Pancholi *et al.*, 1993). It is thought that the ADP-ribosylating activity of SDH/GAPDH may allow cellular communication between GAS and host cells during infection (Pancholi *et al.*, 1993). SDH/GAPDH has been observed to inhibit chemotaxis and hydrogen peroxide production activated by C5a, presumably via the capture of C5a (Terao *et al.*, 2006). An insertion mutatgenesis strategy was adopted to produce an enzymatically functional recombinant mutant of SDH/GAPDH, in which a 12-mer hydrophobic tail was added to diminish the functionality of the protein (Boel *et al.*, 2005). Unlike wild-type SDH/GAPDH protein, the mutant protein was not secreted into the medium, was observed to bind significantly less human Plg, showed poor adherence to human pharyngeal cells and lost innate anti-phagocytic activity (Boel *et al.*, 2005). Such results highlight the necessity of SDH export from the cell and

emphasise the role of SDH in GAS virulence.

Plasmin receptor (Plr) is a 41 kDa protein located on the surface of GAS observed to bind plasminogen with a high affinity (Broder *et al.*, 1991). Plr has also been found to possess GAPDH activity, and it is now accepted that Plr and SDH are the same GAS surface protein, initially thought to be distinct proteins as they were found on different strains of GAS (Winram *et al.*, 1996, Pancholi *et al.*, 2003, Yoshizawa *et al.*, 2004). Like SEN, a C-terminal lysine residue of Plr has been found to be crucial for plasmin binding (Winram *et al.*, 1998). In one study, serum from APSGN patients reacted with Plr, and following antibody staining of tissue samples, Plr was identified in glomeruli of all 22 patients assessed who were suffering early phase APSGN (Yamakami *et al.*, 2000). Findings such as these suggest Plr/SDH may contribute to the development of APSGN.

Although both SDH/GAPDH and SEN have been found on the GAS cell surface (Pancholi *et al.*, 1992), neither of these proteins contain the LPXTG cell wall anchor motif typically found in surface proteins of Gram-positive species. Currently, the mechanism(s) of transport of these proteins to the cell-surface is unknown. In one study utilising *emm* and *mrp* (encoding M protein and M-related protein respectively) isogenic mutants, a binding association between SDH/GAPDH and M protein was revealed, suggesting that M and M-related proteins may be involved in anchoring SDH/GAPDH to the bacterial surface (D'Costa *et al.*, 2000).

1.6.2 Plasminogen-binding Group A Streptococcal M-like Protein (PAM)

PAM is a 42 kDa M protein expressed on the surface of a number of group A streptococcal serotypes. PAM binds both Plg and plasmin with high affinity (Berge & Sjobring, 1993). The binding of PAM to Plg kringle two is mediated by two internal repeat sequences, known as the a1a2 repeats, located in the N-terminus of PAM (Wistedt *et al.*, 1998, Ringdahl *et al.*, 1998, Sanderson-Smith *et al.*, 2006b). Recently, a similar Plg-binding protein, Prp has been identified. In both PAM and Prp, the binding of Plg has been found to be mediated by highly conserved arginine and histidine residues in the a1a2 repeat (Sanderson-Smith *et al.*, 2007). The mutagenesis of four critical Plg-binding amino acids in Prp resulted in significantly less Plg binding and plasmin acquisition when the bacteria were grown *in vitro* in human plasma

(Sanderson-Smith *et al.*, 2008). Furthermore, this Prp mutant strain was observed to be significantly attenuated in virulence compared to wild-type (*emm*98.1) and complemented strains (Sanderson-Smith *et al.*, 2008).

1.7 Treatments for GAS Infection

Penicillin has been used for the successful treatment of GAS infection for many years and GAS still exhibits sensitivity to penicillin. Despite no known resistance to penicillin, some studies have reported a failure of penicillin in the treatment of GAS pharyngitis, with the percent failure estimated to be as high as 20-40% (Pichichero *et al.*, 2007). Several reviews outline a number of different factors which have been proposed as possible explanations for the failure of penicillin in the treatment of GAS infections including *in vivo* co-pathogenicity with other bacterial species, *in vivo* eradication of normal throat microflora, carrier state, internalisation of GAS into host cells, GAS tolerance of penicillin, poor penetration of penicillin into host tissues, lack of patient compliance or reoccurring exposure to GAS (Brook, 2007, Pichichero *et al.*, 2007).

Prior to the use of penicillin to treat streptococcal infections (the use of penicillin commenced in 1945), sulphonamides were the treatment of choice (Krause, 2002). In some cases, clindamycin is also used to treat patients with invasive infections as it rapidly halts the metabolic activity of GAS and consequently prevents further production of toxins, facilitating phagocytosis and subsequent clearance of GAS in the host (Holm, 1996). Macrolides are another alternative treatment to penicillin; however, concern exists regarding the expansion and spread of macrolide-resistant isolates (Michos *et al.*, 2009, Richter *et al.*, 2005). One study found that macrolide-sensitive GAS isolates may use biofilm formation as another mechanism to escape antibiotic treatment and persist in the host (Baldassarri *et al.*, 2006). Thus, macrolides are best avoided in the majority of cases, and the use of macrolides should be reserved for patients who are allergic to penicillin (Brook, 2007). Daptomycin is another novel antimicrobial agent proposed for the treatment of infections caused by Gram-positive bacteria (LaPlante *et al.*, 2004). The antibiotic cefuroxime axetil was observed to exhibit an efficacy comparable to penicillin in the treatment of GAS pharyngitis (Adam *et al.*, 2000). However, at the current time, specific reports of the use of daptomycin and cefuroxime axetil in the treatment of GAS infections and disease are limited.

Although there have been high success rates and reductions in the severity of GAS infections using these antibiotic based treatments, the development of new treatment methods, and more importantly new methods which prevent infection is essential as there is still a high morbidity and mortality rate associated with severe invasive GAS infections. Furthermore, attempts at antibiotic prophylaxis as a means of controlling the immune sequelae ARF and RHD have not eliminated these sequelae in developing countries or indigenous populations (Carapetis *et al.*, 2005b). Fluoride has been shown to decrease the adherence of GAS to buccal cells, collagen, Fn and laminin (Cao *et al.*, 2002). In another study, fluoride exposure was observed to attenuate the expression of GAS virulence factors, including M protein and SDH/GAPDH (Thongboonkerd *et al.*, 2002). Whilst the mechanism(s) of action mediated by fluoride on GAS adherence to host cells and tissues requires more thorough investigation, fluoride could possibly be used as a treatment or prophylactic agent for GAS infection (Cao *et al.*, 2002). Although, in most cases, the existing treatment of choice, penicillin, remains effective and other emerging treatment methods show promise in fighting GAS infection, the costs of antibiotic treatments and time off school or work represent an additional financial burden. A vaccine which enables the prevention of GAS infection may be the only effective way to control and eliminate GAS infection and disease.

1.8 GAS Vaccine Strategies

Traditionally, vaccines designed to prevent bacterial infection have been based on strategies including whole-cell vaccines containing heat-killed pathogens, live attenuated vaccines constructed via inactivation of bacterial genes responsible for essential metabolic functions or pathogenicity, toxoid-based preparations, polysaccharides conjugated to suitable carrier proteins and subunit vaccines containing either whole proteins (purified or recombinant), minimal peptide epitopes, cell wall or capsule antigens identified using biochemical, serological, microbiological or genetic techniques. The development of vaccines using such approaches is time-consuming, often taking many years or decades. However, a new era of vaccine development technology has emerged, utilising reverse vaccinology, aided by proteomics, whole genome sequencing, bioinformatics and DNA microarrays. Such technologies have allowed rapid discovery and identification of putative virulence factors, surface-associated proteins and possible vaccine candidates of many bacterial species including GAS. Examples of these technologies are described in further detail in section 1.8.15.

Despite the advent of such high throughput techniques, the development of GAS vaccines is challenging, facing obstacles such as the occurrence of many unique serotypes (of which there are greater than 120 M types), antigenic variation within the same serotype, differences in geographical distribution of serotypes, and the production of antibodies cross-reactive with human tissue which can lead to host auto-immune disease. GAS infection and post-infective immune sequelae are a continuing worldwide problem, occurring in both developed and developing regions. Thus, the imperative for efficacious vaccines to prevent streptococcal disease is high. Furthermore, whilst GAS remains sensitive to penicillin and antibiotics continue to be used in attempts to control streptococcal infection, there is increasing concern that such use, and particularly over-use, may lead to the emergence of resistant strains or promote the internalisation of GAS by host cells as a means of antibiotic avoidance.

In the case of GAS vaccines, blocking the attachment of GAS to the host cell surface and its receptors (and hence preventing subsequent colonisation) is likely to be an effective strategy in the prevention of infection (Wizemann *et al.*, 1999). As attachment is usually mediated via bacterial adhesins, it follows that major adhesins are key targets in vaccine development. Prophylactic vaccination with adhesin-containing vaccines has successfully blocked GAS infection in mice, although, such approaches have associated problems and shortcomings (outlined in Table 1.3). Another strategy in GAS vaccinology is the elucidation of the surfactome. It is possible that proteins located on, or associated with, the cell surface may be exposed to the host immune system during infection potentially resulting in cellular and humoral immune responses. Since GAS is known to colonise mucosal tissues in the host, the upper respiratory tract (URT) and the skin, a vaccine promoting both systemic (serum IgG) and mucosal immunity (mucosal IgA) is likely to offer long lasting protection against a broad range of GAS infections. GAS vaccinology has primarily focused on the major virulence factor, the M protein. Whilst one M protein based GAS vaccine has reached human clinical trials (Kotloff *et al.*, 2004, McNeil *et al.*, 2005), there are no licensed GAS vaccines commercially available and several factors have hampered the development of M protein based vaccines. In many cases, non-M protein vaccine candidates do not possess a conformation similar to host proteins and tissues (McMillan *et al.*, 2005). For this reason, such vaccine candidates are not likely to generate an auto-immune response in the host.

Table 1.3 Characteristics of existing GAS vaccine candidates tested in animal models.

Characteristics	M protein	C5a peptidase	FbaA	FBP54	CHO	GRAB	LTA	R28 protein	Pili	Protein F1	Sib35	SOF	Spa	SpeB	SpyCEP	Sse
Highly conserved across serotypes	-	+	-	+	+	+	+	ND	-	-	ND	-	ND	+	+	-
Ubiquitous expression	+	+	-	-	+	-	+	ND	-	-	ND	-	ND	-	ND	ND
Opsonic/Bactericidal antibodies	+	-	ND	ND	+	-	ND	ND	ND	-	+	+	+	ND	ND	-
Lack of possible toxicity and/or proteolytic activity	+	-	+	+	+	+	+	+	ND	+	+	-	+	-	-	-
Lack of auto-immune reactivity	-	ND	ND	ND	-	ND	ND	ND	ND	+	ND	ND	ND	-	ND	ND
Systemic protection	+	ND	+	+	+	ND	ND	+	ND	ND	+	+	+	+	ND	ND
Mucosal protection	+	+	ND	ND	+	ND	ND	ND	+	+	ND	-	ND	ND	+	ND
Subcutaneous protection	+	ND	ND	ND	ND	-	ND	ND	ND	-	ND	ND	ND	ND	ND	+

Abbreviations: CHO, group A carbohydrate; FbaA, fibronectin binding protein A; FBP54, fibronectin binding protein 54; GRAB, protein G-related α2-macroglobulin-binding protein; LTA, lipoteichoic acid; SfbI, streptococcal fibronectin binding protein I; Sib35, streptococcal immunoglobulin-binding protein 35; SOF, serum opacity factor; SpeB, streptococcal pyrogenic exotoxin B; Spa, streptococcal protective antigen; SpeB, streptococcal pyrogenic exotoxin B; SpyCEP, *Streptococcus pyogenes* cell envelope proteinase; Sse, streptococcal surface esterase; ND, not determined.

1.8.1   M protein

M proteins are an obvious target for GAS vaccine development, as they are known to be a major virulence factor of GAS and have elicited protective immunity in several studies. Human clinical trials of heat killed GAS based vaccines commenced in 1923, and M protein based GAS vaccinology has been an active topic of investigation since that time. Early studies involved the vaccination of human volunteers with crude M protein preparations followed by the administration of live GAS to the pharynx. This strategy effectively protected against GAS pharyngeal colonisation (D'Alessandri *et al.*, 1978, Fox *et al.*, 1973, Polly *et al.*, 1975), but an increased incidence of ARF in some of the vaccinated children compared to the unvaccinated control children was observed (Massell *et al.*, 1969). This significantly hampered the development of whole M protein based vaccines. Since then, a number of vaccine development studies have targeted the C-repeat region of the M protein, conserved amongst all serotypes of GAS (Bessen *et al.*, 1990, Medaglini *et al.*, 1995, Brandt *et al.*, 2000); while other studies have focused on peptides derived from the serotype specific N-terminal A-repeats of the M protein (Beachey *et al.*, 1986, Beachey *et al.*, 1981, Dale *et al.*, 1993, Dale *et al.*, 1999, Dale *et al.*, 1983, Dale *et al.*, 1996, Kotloff *et al.*, 2004). Type-specific antibodies against M protein have been found to neutralise the antiphagocytic effect of M protein, allowing prompt removal of GAS of the same serotype (Beachey *et al.*, 1981). Fischetti (1991) found that exposure to GAS in childhood can lead to the development of antibodies raised against the conserved region of M protein, which may offer protection against streptococcal pharyngitis in later life. If an M protein based vaccine can be produced which acts against the conserved regions of M protein shared by all GAS serotypes, the need to generate serotype-specific antibodies for protection against GAS infection would be eliminated.

1.8.1.1 Conserved C-repeat based M protein vaccines

To date there have been five different approaches for producing GAS vaccines based on the conserved, non-type specific C-repeats of the M protein. Production of a vaccine based on this conserved region eliminates limitations associated with serotype specific antibody responses. Whilst a vaccine based on the C-repeat region could theoretically offer protection against all serotypes, antibodies directed against this non-type specific conserved region of the M protein have been observed to be non-opsonic (Jones *et al.*, 1988). This indicates that epitopes contained within the N-terminal

serotype specific regions of M protein are responsible for the generation of opsonic α-M protein antibodies (Rothbard, 1945).

One research group produced four overlapping synthetic peptides encompassing the complete C-repeat region of the M6 protein. When Swiss CD1 mice were immunised intranasally with peptide-cholera toxin B (CTB) conjugates followed by an intranasal challenge with either a homologous M6 GAS strain or a heterologous M14 strain, a significant reduction in pharyngeal colonisation was observed (Bessen *et al.*, 1988, Bessen *et al.*, 1990).

A second approach synthesised two peptides comprising regions within the C-repeat region of the M5 protein (Bronze *et al.*, 1992). The peptides were conjugated to keyhole limpet hemocyanin (KLH) and used to immunise rabbits. The resultant anti-serum was pre-incubated with a heterologous M24 GAS isolate and was subsequently administered to BALB/c mice via the intranasal route in passive immunisation experiments. Mice administered the GAS pre-incubated with the anti-peptide serum had a significantly higher rate of survival than the control mice (which were administered GAS pre-incubated with normal rabbit serum) (Bronze *et al.*, 1992), indicating that serum IgG antibodies raised against the peptides were capable of eliciting passive protection. In active immunisation studies, BALB/c mice intranasally immunised with M5 protein-based peptide-CTB conjugates were also significantly protected against heterologous intranasal challenge with M24 GAS (Bronze *et al.*, 1992). Whilst an elevated level of serum IgG was detected against both the peptide and CTB, like previous findings, the IgG antibodies raised against the C-repeat region peptides were unable to induce opsonophagocytosis *in vitro* (Bronze *et al.*, 1992).

A third approach identified B- and T-cell epitopes within the C-repeat region of the M5 protein using human blood samples to screen overlapping peptides comprising the 99 amino acid C-repeat region (Guilherme *et al.*, 2006a). The identification of immunogenic epitopes led to the construction of a 55-amino acid protein, designated StreptInCor, encompassing the reactive epitopes. Following subcutaneous immunisation of BALB/c mice, StreptInCor was found to induce high titre serum IgG antibodies (Guilherme *et al.*, 2009). When StreptInCor was co-administered with the mucosal adjuvant, AFCo1 via the intranasal route, a mucosal IgA and systemic IgG response resulted (Guilherme *et al.*, 2009). At this stage it is not known whether StreptInCor is

capable of eliciting opsonic antibodies or protective immunity in mouse models of GAS infection.

A fourth approach utilises synthetic peptides based on minimal B- and T-cell epitopes (producing smaller peptides than StreptInCor). Following the identification of B-cell epitopes within the C-repeat region of the M protein (Pruksakorn *et al.*, 1992, Pruksakorn *et al.*, 1994), the laboratory of Good *et al.* constructed a 20 amino acid synthetic peptide designated p145 (Pruksakorn *et al.*, 1994). Two daughter epitopes, designated J8 and J14 have since been designed, which represent the minimal B-cell epitopes contained within the p145 sequence (Hayman *et al.*, 1997). Good and collegues have investigated the murine serum IgG response (Brandt *et al.*, 2000) and muscosal IgA response (Brandt *et al.*, 1999, Batzloff *et al.*, 2006) against these antigens, studied the opsonic potential of antibodies directed against these peptides (Pruksakorn *et al.*, 1994, Brandt *et al.*, 1996, Brandt *et al.*, 1997, Brandt *et al.*, 1999, Brandt *et al.*, 2000, Hayman *et al.*, 2002, Olive *et al.*, 2002a, Olive *et al.*, 2003, Batzloff *et al.*, 2003, Olive *et al.*, 2004, Olive *et al.*, 2005, Batzloff *et al.*, 2005, Olive *et al.*, 2006) and have examined the protective efficacy of these peptide antigens in mouse models with a number of different adjuvants including tetanus toxin (Brandt *et al.*, 2000), diphtheria toxin (Olive *et al.*, 2002b, Batzloff *et al.*, 2003, Pandey *et al.*, 2009), proteosomes (Batzloff *et al.*, 2005), lipid core technology (Hayman *et al.*, 2002, Olive *et al.*, 2002a, Olive *et al.*, 2003, Olive *et al.*, 2005, Olive *et al.*, 2006, Batzloff *et al.*, 2006, Moyle *et al.*, 2006a, Moyle *et al.*, 2006b, Moyle *et al.*, 2006c, Abdel-Aal *et al.*, 2008, Zaman *et al.*, 2010), liposaccharides (Simerska *et al.*, 2008b, Simerska *et al.*, 2008a, Fujita *et al.*, 2008) and H12 (Georgousakis *et al.*, 2009b), a protective segment of Protein F1 (Schulze *et al.*, 2003c, Schulze *et al.*, 2003a).

A final strategy of GAS vaccine development based on the C-repeat region of the M protein utilises live vaccine delivery vectors and is discussed further in section 1.8.14.

1.8.1.2 N-terminal based M protein vaccines

The other major branch of M protein vaccinology focuses on the highly variable N-terminal serotype specific portion of the M protein. One research group has developed a hexavalent vaccine containing protective N-terminal M protein fragments from six serotypes; M1, M3, M5, M6, M19 and M24 (Dale, 1999). The included

serotypes were selected due to a frequent association with pharyngitis and ARF in the United States (Kotloff *et al.*, 2004, Dale, 1999). Administration of this hybrid vaccine generated high titre opsonising antibodies in rabbits (Dale, 1999) and protected BALB/c mice against mucosal challenge (Hall *et al.*, 2004). In 2004, the vaccine reached phase I clinical trials and was found to result in a statistically significant increase in antibody titre for all six M protein-based fragments, with five of the six targeted GAS serotypes being opsonised by the resulting anti-sera (Kotloff *et al.*, 2004). Additionally, there was no evidence the antibodies were cross-reactive with human tissue (Kotloff *et al.*, 2004). Although this vaccine was successful in phase I clinical trials, one major shortcoming of this hexavalent vaccine preparation is that it only offers protection against six of at least 120 GAS M types. In an attempt to broaden the protection, a multivalent vaccine containing fragments based on the variable amino terminal of 26 different M proteins was produced using recombinant techniques (Hu *et al.*, 2002). Following immunisation of rabbits, type-specific serum IgG antibodies were generated which recognised 25 of the 26 M protein-based fragments in the vaccine (Hu *et al.*, 2002). In addition, none of the antibodies cross-reacted with host tissue (Hu *et al.*, 2002). *In vitro* opsonophagocytosis assays were performed and 18 of the 26 targeted vaccine strains had rates of $\geq 30\%$ opsonisation following incubation with specific polyclonal rabbit serum (Hu *et al.*, 2002). Additionally, this vaccine preparation was observed to be safe and immunogenic in phase I human clinical trials (McNeil *et al.*, 2005). In a study of 5,400 invasive GAS cases in the US from 2000-2004, the *emm* types in the 26-valent GAS vaccine covered 79% of serotypes isolated from patients (O'Loughlin *et al.*, 2007). However, these findings were disparate from those of a study conducted 2001-2005 in the US, in which the coverage of the vaccine serotypes was predicted to be approximately 60% (Nir-Paz *et al.*, 2010). In a study of isolates associated with severe GAS infection in Japan from 2001-2005, 81% of isolates were amongst those which comprise the 26-valent vaccine (Ikebe *et al.*, 2007). In a study of GAS isolates recovered from healthy school children in Ethiopia, 46% of serotypes responsible for infection in this region were not represented in the multivalent vaccine (Abdissa *et al.*, 2006), and the vaccine serotype coverage within Africa, Asia, Latin America, the middle east and the Pacific region is estimated to be only 39%, 60.5%, 72%, 63% and 34% respectively (Steer *et al.*, 2009b). Whilst the vaccine coverage of strains in the United States and Japan may be considered promising, this 26-valent GAS vaccine cannot provide 100% coverage in any region. Thus, it is obvious another strategy

encompassing a broader, more diverse collection of GAS serotypes is required for the design of a vaccine efficacious in all geographical regions.

1.8.1.3 Plasminogen-binding Group A Streptococcal M-like Protein

The protective efficacy of PAM has also been recently investigated. Following subcutaneous immunisation of Quackenbush mice with a PAM-derived peptide (a1) conjugated to KLH, a1-KLH was observed to generate a systemic IgG antibody response capable of inducing opsonophagocytosis of GAS *in vitro* and protecting mice against systemic homologous GAS challenge *in vivo* (Sanderson-Smith *et al.*, 2006a). However, given that the a1 peptide is derived from the highly variable, serotype specific A-repeat region of the M protein, it is likely the protection afforded by this peptide would be limited to PAM-expressing GAS strains and the capacity of PAM to protect against heterologous GAS challenge is yet to be investigated.

1.8.2   Heat killed GAS

Another strategy in GAS vaccinology has utilised heat killed GAS as a component in GAS based vaccine preparations. In one study, the administration of heat killed M24 GAS or pep M24 extracts (a pepsin derived extract containing surface antigens) in Swiss white mice via the intranasal route was, in both cases, observed to protect against lethal intranasal challenge with the homologous M24 strain and also a heterologous M6 strain (Bronze *et al.*, 1988). In attempts to bolster the systemic IgG antibody response, during the course of intranasal immunisations, an additional booster immunisation was administered via the intraperitoneal route. Serum IgG antibodies reactive against whole M24 GAS cells and pep M24 extracts were detected, both of which were found to be opsonic against the M24 strain, but not the heterologous M6 strain (Bronze *et al.*, 1988). In another study, whole heat killed M50, M55 or M12 GAS were intranasally adminisered to NMRI mice (Stjernquist-Desatnik *et al.*, 1990). In each case, researchers observed protection against intranasal challenge with an M50 GAS strain (Stjernquist-Desatnik *et al.*, 1990). The serum IgG response and opsonic antibody production was not investigated in this study. Whilst such studies present evidence indicating whole heat killed GAS vaccine preparations can protect against infection mediated by both homologous and heterologous strains, the safety concerns of administering whole GAS (albeit heat killed) to individuals is likely to preclude further development of such preparations. For example, whole M protein and CHO located on

the surface of heat killed GAS may mediate auto-immune sequelae in the immunised host.

1.8.3   C5a Peptidase

C5a peptidase is another virulence factor currently under investigation as a GAS vaccine antigen. In CD1 mice, following intranasal immunisation with recombinant C5a peptidase, significant levels of anti-C5a peptidase IgA salivary antibodies and serum IgG were detected (Ji *et al.*, 1997). These antibodies caused a reduction in the ability of homologous and heterologous GAS serotypes to colonise pharyngeal epithelia (Ji *et al.*, 1997, Park *et al.*, 2005). The protective potential of an inactive form of C5a peptidase was investigated in a murine disease model established in CD1 mice. The inactive C5a peptidase (designated SCPAw), administered via the subcutaneous route, was highly immunogenic, resulting in significantly elevated serum IgG compared to control mice which were instead administered tetanus toxoid (Cleary *et al.*, 2004). Following homologous and heterologous challenge, GAS were cleared more rapidly from the oral/nasal mucosa of immunised mice, compared to control mice (Cleary *et al.*, 2004). Antibodies reactive against C5a peptidase have also been detected in the sera of children with GAS-associated pharyngitis, indicating that C5a peptidase is expressed *in vivo* during the course of infection and is immunogenic in humans (Shet *et al.*, 2003, Shet *et al.*, 2004). Although these studies show promise for the development of a detoxified C5a peptidase-based vaccine, only approximately 40 GAS serotypes are known to express C5a peptidase (Dale, 1998). Thus, a vaccine containing C5a peptidase could not offer broad protection against all GAS serotypes. Furthermore, it is not known if C5a peptidase cross-reacts with host tissues, which could potentially result in auto-immunity.

1.8.4   Protein F1/SfbI

Protein F1/SfbI is another potential GAS vaccine candidate. CD1 mice intranasally immunised with either protein F1 alone, or protein F1 coupled to CTB, produced a specific humoral IgG response and a lung mucosal IgA immune response against protein F1 (Guzmán *et al.*, 1999). When protein F1-immunised mice were subjected to intranasal challenge with a homologous GAS strain, 80% survival was observed (Guzmán *et al.*, 1999). Likewise, when immunised CD1 or BALB/c mice were challenged with a heterologous GAS strain, in both cases, 90% survival ensued

(Guzmán *et al.*, 1999). However, intranasal immunisation of BALB/c mice with protein F1 coupled to CTB did not protect mice against subcutaneous GAS challenge with a homologous strain (McArthur *et al.*, 2004a). McArthur *et al.* (2004) found that sera from mice immunised with protein F1/CTB did not result in opsonophagocytosis. Protein F1, specifically the H12 region responsible for the Fn-binding, has also being investigated for use as a mucosal adjuvant as it has self-adjuvanting properties (Schulze *et al.*, 2003a, Schulze *et al.*, 2003c) and the ability to enhance the immune response against co-administered antigens, including the M protein based peptide J14 (Georgousakis *et al.*, 2009b).

1.8.5 Serum Opacity Factor (SOF/SfbII)

SOF has also been examined as a potential GAS vaccine component. SOF was found capable of generating opsonic antibodes in humans, rabbits and mice (Courtney *et al.*, 2003). Following a primary subcutaneous immunisation of SOF, followed by an intramuscular booster immunisation, Swiss NIH mice were protected against systemic challenge with homologous M2 GAS (Courtney *et al.*, 2003). Likewise, BALB/c mice immunised subcutaneously and boosted intramuscularly with a SOF protein lacking the signal sequence and Fn-binding repeat region (designated SOFΔFn) were protected against lethal intraperitoneal challenge with heterologous M49 GAS (Gillen *et al.*, 2008). Such results indicate that a SOF-containing vaccine may elicit protective effects against infection by SOF-positive GAS strains (Courtney *et al.*, 2003). The administration of SOF coupled to CTB via the intranasal route resulted in elevated mucosal IgA and IgG antibody responses (Schulze *et al.*, 2006a). However, BALB/c mice were not protected against lethal intranasal challenge with a heterologous strain in this model (Schulze *et al.*, 2006a).

Not all GAS serotypes express SOF, and SOF expression varies depending on geographical location, with approximately only 32-60% of previously surveyed strains determined to be SOF-positive (Beall *et al.*, 2000, Goodfellow *et al.*, 2000, Kreikemeyer *et al.*, 2002, Prakash *et al.*, 1991). Thus, a SOF-based GAS vaccine would not offer protection against non-SOF containing strains and could not induce broad protection against the many circulating GAS serotypes. Furthermore, given the ability of SOF to opacify serum via the disruption of HDL, SOF, at least administered in an active form, would presumably not be safe as a vaccine intended for human use. In

preliminary studies utilising inactive mutant forms of SOF recombinant protein, in which the OF activity was abrogated, immunisation with two SOF mutant proteins failed to protect against lethal heterologous systemic GAS challenge (Gillen *et al.*, 2008). It may be that mutating regions conferring OF activity reduced the accessibility of the protective epitopes contained within this region, or that the site-directed mutagenesis disrupted the structure of protective conformational epitopes (Gillen *et al.*, 2008). In any case, whilst SOF has been observed to produce opsonic antibodies and to elicit protection against homologous and heterologous GAS strains following systemic challenge, the inability of SOF to protect against mucosal challenge and the limited serotype distribution of SOF, coupled with its inherent OF activity, precludes the inclusion of SOF in GAS based vaccines.

1.8.6   Streptococcal Pyrogenic Exotoxin B (SpeB)

SpeB is another possible choice as a GAS vaccine candidate given its highly conserved nature and expression across many GAS serotypes. However, the use of SpeB as a vaccine candidate in its native form raises safety concerns given its proteolytic activity. One group tested the ability of SpeB to elicit a protective immune response following systemic challenge in CD1 mice by active subcutaneous immunisation with SpeB and also by passive immunisation with rabbit α-SpeB IgG antibodies administered via the intraperitoneal route (Kapur *et al.*, 1994). Both immunisation strategies were found to enhance the survival of the mice following intraperitoneal GAS challenge (Kapur *et al.*, 1994). More recently, a chimeric protein comprised of a mutant form of SpeB (in which the active site was inactivated via mutagenesis) and the receptor binding surface of SpeA, was intramuscularly administered to HLA-DQ8/human CD4+ transgenic mice and to BALB/c mice. This was observed to elicit a protective response in both mouse strains following intravenous challenge (Ulrich, 2008). The ability of SpeB to induce opsonic antibodies was not assessed by Ulrich (2008). The use of SpeB as a vaccine candidate, at least in the active form, is questionable given the inherent proteolytic activity of SpeB. In addition, the observation that SpeB may be associated with the induction of APSGN (Luo *et al.*, 2007) also raises questions to the suitability of this secreted GAS protein as a vaccine candidate.

1.8.7 Fibronectin-Binding protein 54 (FBP54)

FBP54 has shown potential as a GAS vaccine component. A statistically significant increase in the titre of α-FBP54 IgG antibodies was observed in the sera of GAS-infected patients, as compared to sera obtained from healthy individuals (Kawabata *et al.*, 2001) indicating FBP54 is expressed during natural infection. Following subcutaneous, oral and intranasal immunisation of BALB/c mice with FBP54, an elevated FBP54-specific IgG response was monitored for all three routes, with an increase in salivary IgA following oral and intranasal immunisation (Kawabata *et al.*, 2001). Whilst administration of FBP54 produced an immune response against GAS in mice, the ability of FBP54 to produce opsonic serum antibodies or to protect against GAS infection remains to be determined.

1.8.8 Fibronectin-Binding Protein A (FbaA)

When the protective immunity of FbaA was explored in a murine intraperitoneal immunisation and intraperitoneal GAS challenge model utilising BALB/c mice, immunisation with FbaA was observed to produce an elevated humoral IgG response similar to the M protein and to confer a level of protection on par with M protein (Ma *et al.*, 2009). Such findings demonstrate the promise of FbaA as a viable GAS vaccine candidate. The investigations conducted by Ma *et al.* (2009) did not examine whether experimental immunisation with FbaA induces opsonic antibodies nor the distribution of FbaA amongst GAS serotypes.

1.8.9 N-acetyl Glucosamine, the Group A Carbohydrate (CHO)

N-acetyl glucosamine, the group A carbohydrate, is present on the surface of all GAS serotypes irrespective of M type, and for this reason it may make a suitable vaccine candidate. In one study, antibodies reactive against CHO were found in the sera of children, with an increase in antibody titre correlating to an increase in age (Salvadori *et al.*, 1995). Furthermore, affinity purified α-CHO antibodies were observed to result in the opsonisation of three different GAS serotypes (Salvadori *et al.*, 1995). Mice immunised with CHO were protected against both intraperitoneal and intranasal GAS challenge (Sabharwal *et al.*, 2006). However, immunological cross-reactivity between CHO and host heart valve proteins (Goldstein *et al.*, 1967) and cytoskeletal proteins such as actin, keratin, myosin and vimentin (Shikhman *et al.*, 1993) preclude the use of full length CHO as a GAS vaccine antigen.

1.8.10 Streptococcal Secreted Esterase (Sse)

Recently, Streptococal secreted esterase (Sse) was identified as a new protective antigen of GAS. Following subcutaneous immunisation of Sse utilising Alum as an adjuvant, CD1 mice were protected against lethal subcutaneous challenge by homologous M1 and heterologous M3 GAS strains (Liu *et al.*, 2007). Furthermore, passive immunisation of CD1 mice with α-Sse serum (of mouse origin) also resulted in protection against virulent homologous GAS challenge (Liu *et al.*, 2007). Anti-Sse antibodes were detected in convalescent serum from pharyngitis patients, however, experimentally derived α-Sse serum was not opsonic in an *in vitro* assay (Liu *et al.*, 2007). Liu *et al.* (2007) identified two alternate varients of Sse within 10 serotypes of GAS. However, it is not clear if these two Sse variants are expressed by all serotypes of GAS, evoking questions about the capability of Sse to provide broad serotype protection.

1.8.11 Lipoteichoic Acid (LTA)

LTA is a cell surface component of GAS which mediates the adherence of GAS to host cells (Ofek *et al.*, 1975). Intranasal co-administration of LTA with CTB to BALB/c mice elicited an increase in serum IgG antibody and pharyngeal IgA antibody responses (Yokoyama *et al.*, 2002). When tested in an *in vitro* adherence assay, the rate of adherence of GAS to pharyngeal epithelial cells was significantly reduced following pre-treatment with the pharyngeal washings obtained from LTA-CTB immunised mice (Yokoyama *et al.*, 2002). This study, however, neglected to test the protective efficacy of LTA following GAS challenge, or the opsonic potential of α-LTA antibodies, and thus, at this time, the suitability of LTA as a GAS vaccine candidate is uncertain.

1.8.12 *Streptococcus pyogenes* Cell Envelope Proteinase (SpyCEP)

A bioinformatic and proteomic study, discussed in greater detail in section 1.8.15, identified a number of GAS surface proteins, one of which Spy0416, elicited a protective immune response against homologous mucosal infection in CD1 mice with an M23 GAS isolate (Rodreiguez-Ortega *et al.*, 2006). Spy0416 has been identified as *Streptococcus pyogenes* cell envelope proteinase (SpyCEP), a protein known to cleave and inactivate interleukin-8 (Edwards *et al.*, 2005) and play a key role in systemic bacterial dissemination (Kurupati *et al.*, 2010). SpyCEP is a highly conserved, cell wall anchored protein that also exists in a secreted form (Turner *et al.*, 2009). A recent study

assessed the protective efficacy of SpyCEP following intramuscular immunisation of BALB/c mice. Upon intramuscular and intranasal infection with GAS, a decrease in bacterial disemination to both the liver and the spleen was observed in both cases (Turner *et al.*, 2009). Furthermore, immunisation of BALB/c mice with SpyCEP also reduced organ disemination of the equine pathogen *Streptococcus equi* (Turner *et al.*, 2009). As such, SpyCEP shows potential as a cross-species streptococcal vaccine candidate, although, the protective efficacy of SpyCEP against lethal GAS challenge and the ability of α-SpyCEP antibodies to promote opsonophagocytosis is yet to be investigated.

1.8.13 Streptococcal Immunoglobulin-Binding Protein 35 (Sib35)

Sib35 is an Ig-binding protein of GAS which elicits protection against lethal systemic GAS challenge. Subcutaneous immunisation of BALB/c, C3H/HeN, C57/BL6 and CD1 mice with Sib35 resulted in significant protection of BALB/c, C3H/HeN and C57/BL6 mice strains (but not CD1 mice) compared to sham immunisation with phosphate buffered saline (PBS) (Okamoto *et al.*, 2005). In addition, polyclonal rabbit anti-serum raised against Sib35 was opsonic *in vitro* (Okamoto *et al.*, 2005). Thus, Sib35 shows promise as a GAS vaccine candidate.

1.8.14 Streptococcal Protective Antigen (Spa)

Streptococcal protective antigen (Spa) is a GAS vaccine antigen identified in a M protein-negative mutant GAS strain which was resistant to phagocytosis and virulent in mice despite the absence of M protein. Intraperitoneal administration of α-Spa (of rabbit origin) to Swiss mice mediated passive protection against subsequent homologous intraperitoneal GAS challenge (Dale *et al.*, 1999). In addition, anti-Spa antibodies were found to opsonise M3 and M28 GAS (Dale *et al.*, 1999). Whilst such experimental findings show Spa is promising as a GAS vaccine antigen, active immunisation studies are yet to be performed.

1.8.15 R28 Protein

R28 protein of GAS is a surface protein with sequence similarity to the α, β and Rib proteins of Group B streptococcus. Administration of C3H/NeH mice with α-R28 sera (of rabbit origin) via passive intraperitoneal immunisation resulted in protection of mice against lethal intraperitoneal challenge (Stalhammar-Carlemalm *et al.*, 1999). In addition, both passive (intraperitoneal) and active (subcutaneous) immunisation of

C3H/NeH mice with R28 or Rib resulted in protection against Group B streptococcus and GAS respectively (Stalhammar-Carlemalm *et al.*, 2000). Thus, R28 protein shows promise as a cross-species streptococcal vaccine antigen.

1.8.16 Superoxide Dismutase (SOD)

There have been preliminary investigations into the immune response directed against s̲up̲e̲r̲o̲x̲ide d̲i̲s̲mutase (SOD) and the opsonic activity mediated by SOD. Although strong antibody responses were directed against SOD following subcutaneous immunisation of Quakenbush mice, the resultant anti-serum promoted only a moderate level of opsonisation (in comparison to M1 protein) and mice immunised with SOD were not protected against homologous lethal intraperitoneal challenge with an M1 GAS strain (McMillan *et al.*, 2004b, McMillan *et al.*, 2004a). Although SOD was observed to be associated with the GAS surface and has opsonic potential, the lack of immune protection elicited by SOD in a mouse model impedes the use of SOD as a GAS vaccine.

1.8.17 Live Vaccine Delivery Vectors

Given the safety concerns regarding use of a heat-killed or de-toxified GAS vaccine preparation, live vaccine delivery systems utilising attenuated or commensal species seem a logical avenue of exploration in the quest for effective GAS vaccines. Live vaccine vectors may offer economic advantages over parenterally administered preparations as administration is simple, self-administration is a possibility, and there is low to no pain associated with vaccine delivery. All of these factors should assist in increasing patient compliance (Georgousakis *et al.*, 2009a). To date, several attenuated and commensal species have been utilised as vehicles for delivery of GAS antigens including *aroA*-attenuated *Salmonella*, vaccinia virus, *Lactococcus lactis* and *S. gordonii*. Initial experiments assessed the immunogenicity and protective efficacy of an *aroA*-attenuated strain of *Salmonella typhimurium* genetically engineered to express the GAS surface M5 protein (Poirier *et al.*, 1988). Oral immunisation with this M protein expressing *S. typhi* was observed to protect BALB/c mice against systemic and mucosal homogolous GAS challenge (Poirier *et al.*, 1988). In addition, the resultant α-M protein serum was opsonic against M5 GAS, but not M24 GAS (Poirier *et al.*, 1988). Vaccinia virus has also been utilised for the delivery of M protein based GAS vaccine preparations. CD1 mice were intranasally immunised with a vaccinia virus expressing

the C-repeat region of the M6 protein (Fischetti *et al.*, 1989). Following homologous (M6) and heterologous (M14) intranasal challenge, a significant reduction in pharyngeal colonisation of GAS was observed in mice administered the engineered vaccinia virus (Fischetti *et al.*, 1989).

An alternate approach utilised *Lactococcus lactis*, an intestinal commensal flora, as a live vaccine vector harbouring the genes encoding the C-repeat region from the M protein of M6 GAS. CD1 mice were intranasally immunised with the live vaccine vector which elicited a salivary IgA and serum IgG response (Mannam *et al.*, 2004). Mice were also subcutaneously immunised with the *L. lactis* strain which elicited circulating serum IgG (Mannam *et al.*, 2004). Following heterologous challenge with an M14 strain, the mice immunised via the intranasal route and the subcutaneous route were significantly protected against pharyngeal infection (Mannam *et al.*, 2004). The capacity of the C-repeat region expressing *L. lactis* strain to produce opsonic antibodies directed against M protein was not investigated in this study.

In recent times, another research group has generated *L. lactis* mutants which express the T3 GAS pilus (Quigley *et al.*, 2010). It is possible to engineer GAS vaccine antigens into the tip protein of the pilus. Utilising maltose-binding protein (MBP) from *Escherichia coli* as a proof of principle, Quigley *et al.* (2010) found that mucosal immunisation of CD1 mice with the *L. lactis* strain expressing the pilus-linked MBP resulted in both systemic (IgG) and mucosal (IgA) immune responses against MBP. This mucosal vaccine delivery system, designated UPTOP (unhindered presentation on tips of pili), could be utilised during vaccine design against a diverse range of bacterial pathogens. Although, the UPTOP system is yet to be used for the assessment of the immune response and protective efficacy of GAS antigens.

S. gordonii, a human commensal of the upper respiratory tract (URT), has also been explored as a live mucosal vaccine vector for GAS vaccine antigens. Initially, an *S. gordonii* strain was engineered to express the M6 protein via the introduction of the *emm*6 gene by chromosomal integration and transcriptional fusion (Pozzi *et al.*, 1992b). Subsequently, a fusion protein consisting of the M protein C-repeat region fused to the E7 protein of the human papillomavirus type 16 was produced (Pozzi *et al.*, 1992a). Once integrated into the *S. gordonii* chromosome, this recombinant protein was found to be immunogenic, generating serum IgG antibodies against the C-repeat region in

BALB/c mice following subcutaneous immunisation (Pozzi *et al.*, 1992a). The capacity of this fusion protein to induce opsonic serum antibodies or to elicit protection against lethal GAS infection in mouse models has not been investigated. Several strategies have been adopted to optimise the expression and immunogenicity of M protein on the surface of *S. gordonii*, including shortening of the N-terminus, inserting a 94 amino acid alpha-helical spacer region and increasing the number of C-repeat regions (Bolken *et al.*, 2002). The incorporation of the spacer region and duplication of C-repeat epitopes was observed to enhance the immunogenicity of the M protein when expressed on the surface of this commensal. In 2005, the response of 150 volunteers to intranasal or oral immunisation with a non-vaccine bearing wild-type strain of *S. gordonii* was investigated, and the preparation was generally well tolerated (Kotloff *et al.*, 2005). To date, the protective efficacy of M protein expressing *S. gordonii* strains has not been tested in mouse models of GAS infection.

1.8.18 Other Vaccine Development Strategies

In recent times, several new technologies including bioinformatics and proteomics based techniques have been utilised in the search for potential bacterial surface proteins, including those which may represent potential vaccine candidates. In the case of GAS, one such approach used bioinformatics to identify 30 open reading frames (ORF) in M1 GAS which were thought to encode putative extracellular lipoproteins (Lei *et al.*, 2004). Anti-sera obtained from mice infected with whole GAS and from patients suffering GAS pharyngitis and invasive infection was used to probe recombinant forms of the lipoproteins in western blots. Seven of the recombinant proteins were observed to react with each of the sera (Lei *et al.*, 2004), suggesting they are expressed during natural infection. Recombinant forms of 16 of the proteins were used to individually immunise CD1 mice via the subcutaneous route. Following immunisation the anti-sera raised against five of the proteins (Spy0385, Spy1245, Spy1274, Spy1390, Spy1558) was observed to inhibit the growth of GAS in an *in vitro* bactericidal assay compared to sham immunised sera (Lei *et al.*, 2004). Whilst experimental immunisation with these five proteins resulted in opsonic serum antibodies, further characterisation of these antigens is necessary, including testing if the proteins are capable of inducing a protective immune response in murine models of GAS disease.

Another research group utilised two alternate techniques in an attempt to identify potential GAS vaccine candidates. Firstly, mutanolysin extracted surface proteins were resolved using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and separated by whole gel elution. A pool of proteins 29 kDa in size were used to immunise Quackenbush mice, resulting in antibodies that were opsonic against M1 GAS (McMillan *et al.*, 2004a). Amino acid sequencing revealed the major antigen in this pool as SOD, a known virulence factor of several pathogenic bacteria (Wilks *et al.*, 1998, Yesilkaya *et al.*, 2000, Lynch *et al.*, 2000). A focus on the characterisation of SOD as a potential GAS vaccine candidate is outlined in section 1.8.13. Secondly, in the same study, a bioinformatics approach was used to search the M1 genome for proteins containing a LPXTG Gram-positive cell wall anchor motif and an export signal sequence, typical of cell surface proteins. Six such proteins were selected and the presumed antigenically exposed N-terminal regions of each were synthesised into peptides (McMillan *et al.*, 2004a). Each peptide was conjugated to KLH and used to immunise Quackenbush mice. Each of the six peptides was observed to be immunogenic, resulting in a serum IgG response (McMillan *et al.*, 2004a). Although the protective efficacy of the selected antigens was not investigated, this study illustrates the application of both proteomic and bioinformatic based screening techniques in identifying potential vaccine antigens.

A different research group utilised a combination of biochemical, bioinformatic and proteomic techniques to identify surface exposed proteins of an M1 GAS isolate, SF370. Whole GAS cells were treated with trypsin or proteinase K, or a combination of both, in order to 'shave' the surface exposed proteins (Rodreiguez-Ortega *et al.*, 2006). The resultant peptide fragments were identified using nano-LC-MS/MS followed by interrogation of the SF370 genomic database. A total of 68 proteins were identified, 12 of which contained a LPXTG cell-wall anchor motif, 11 lipoproteins, 37 transmembrane proteins and eight secreted proteins (Rodreiguez-Ortega *et al.*, 2006). Only four of the proteins identified were predicted to be cytosolic in nature (*spy0611*; putative elongation factor Tu [EF-Tu], *spy0717*; 50S ribosomal protein L31, *spy0792*; conserved hypothetical protein, and *spy1073*; 50S ribosomal protein L7/L12) (Rodreiguez-Ortega *et al.*, 2006). Of the 14 proteins which were recombinantly expressed and used to immunise CD1 mice intraperitoneally, one protein Spy0416 (SpyCEP), a putative cell envelope proteinase, elicited protection against mucosal infection with GAS

(Rodreiguez-Ortega *et al.*, 2006). A more detailed description of the protective efficacy of SpyCEP is outlined in section 1.8.12.

Another group of researchers also utilised proteomics based techniques, specifically, trypsin digestion of the cell surface and multidimensional nano-LS-MS/MS to enable identification of SF370-surface exposed proteins (Severin *et al.*, 2007). Severin *et al.* (2007) found a total of 79 proteins, 33 of which have not previously been associated with the cell surface of GAS. Amongst the proteins identified were four proteins containing the LPXTG cell-wall anchor motif, 12 lipoproteins, nine secreted proteins, 22 membrane-associated proteins, one bacteriophage-associated protein and 21 proteins traditionally known to exist in the cytoplasm (Severin *et al.*, 2007). Sixteen of the proteomically identified proteins were selected to individually immunise Swiss Webster mice via the subcutaneous route. Whilst this study confirmed the surface localisation of these 16 proteins using the resultant specific polyclonal mouse anti-serum in a whole cell enzyme-linked immunoabsorbant assay (ELISA) (Severin *et al.*, 2007), the ability of these putative surface antigens to protect against lethal GAS challenge is yet to be examined.

Recently, a study used genomic surface display libraries to identify novel GAS surface proteins. In short, a GAS genomic library was produced in which sheared genomic DNA was ligated into appropriate plasmids for bacterial surface display screens using human serum (Fritzer *et al.*, 2010). Biotinylated human serum was incubated with the *E. coli* cells expressing the genomic library and surface exposed antigenic fragments that bound the serum were detected using streptavidin (SA)-conjugated microbeads followed by MS (Fritzer *et al.*, 2010). This resulted in the identification of a number of antigens which conferred protection against GAS challenge in mouse models. Six antigens (Spy0269, Spy0292, SpyCEP, Spy0872, Spy0895 and Spy1666) were observed to protect against heterologous intranasal challenge, whilst Spy0292, SpyCEP and Spy0872 also elicited protective immunity against intravenous GAS challenge (Fritzer *et al.*, 2010). In both challenge models, antigens were administered using subcutaneous immunisation (Fritzer *et al.*, 2010). Each of the nine protective antigens identified in this study were highly conserved with greater than 97% sequence identity amongst the 13 sequenced GAS genomes.

A proteomic study conducted in our laboratory initially identified the major cell wall-associated proteins of GAS (Cole *et al.*, 2005). A mutanolysin extraction was performed on GAS strains NS931 (isolated from a patient suffering necrotising fasciitis), NS13 (isolated from a patient with bacteremia) and S43 (isolated from a case of bronchopneumonia) in an attempt to identify cell-wall associated proteins. The cell wall extracts were arrayed by two dimensional (2D) SDS-PAGE and excised protein spots identified by matrix-assisted laser desorption ionisation time-of-flight (MALDI-TOF) MS. From the protein spots examined, 74 separate cell-wall associated proteins were identified. Of these 74 proteins, 8 proteins have been previously reported as cell wall-associated in GAS, whilst 35 of the proteins have previously been identified in either cellular or extracellular proteomes of GAS (Cole *et al.*, 2005). During immunoblot analysis with pooled human anti-sera from individuals living in regions where GAS infection is endemic, 33 of the proteins identified in this study were found to be immuno-reactive. Additionally, 2D western blot experiments were performed, in which the cell-surface of intact whole GAS cells was biotinylated prior to mutanolysin extraction. A total of 23 biotinylated proteins were detected on the blot, and thus these 23 proteins were thought to be not only cell wall-associated but also surface exposed (Cole *et al.*, 2005). A more detailed description of this study follows in Chapter 3.

1.9   Conclusions

The human pathogen GAS can cause mild diseases such as pharyngitis and impetigo, invasive and potentially fatal diseases including necrotising fasciitis and STSS, and the post-infective sequelae ARF and APSGN. GAS infection is currently a significant public health concern, as there has been an increase in invasive GAS disease since the 1980s. GAS adheres to host cells via adhesin molecules and surface proteins, and once GAS has adhered to cells it can invade and colonise the human host, leading to infection of deeper tissue. To determine the mechanism(s) of GAS infection and consequent disease and sequelae, the roles that adhesins, surface proteins and virulence factors play in GAS pathogenesis must be fully elucidated. Current prophylactic treatment of GAS infection involves penicillin injection; however, this strategy has had limited success. Thus, the development of new prevention and treatment strategies is vital. An effective and protective vaccine against GAS may be the only way to eradicate GAS in the human population. GAS vaccine candidates under investigation (current and previous) include M protein, C5a peptidase, FbaA, FBP54, protein F1, SOF, FBP54,

CHO, R28, Sib35, Spa, SpeB, Sse, LTA, SpyCEP and the putative cell wall-associated proteins of this study.

1.10 Objectives

A number of putative cell wall-associated proteins identified in the proteomic study of Cole *et al.* (2005) were selected for further analysis and characterisation. Given the proteins were found in the cell-wall fraction and a number determined to be surface exposed, it was thought that these proteins may be presented to the immune system during the course of human infection. For this reason, it was hypothesised that these cell-wall associated proteins may be suitable GAS vaccine candidates.

The principal objectives of this project are as follows:

i)   to clone selected GAS cell-wall associated proteins into *E. coli* expression vectors and purify the recombinant proteins

ii)   to determine the protective efficacy of the recombinant vaccine antigens in two mouse models of GAS infection (a systemic infection model and a subcutaneous infection model)

iii)   to use antigen-specific polyclonal serum to confirm the surface localisation of the antigens on GAS using flow cytometry, confocal microscopy and immuno-electron microscopy

iv)   to determine the cross-reactivity of the candidate antigens with serum from infected patients living in two different geographical regions suffering endemic infection

v)   to assess the ability of protective antigens to promote opsonophagocytic killing

vi)   to test the reaction of protective antigens with human heart extract

2.   MATERIALS AND METHODS

Unless otherwise stated all media, solutions and buffers (Appendix I) were made with glass distilled water using analytical grade reagents.

2.1   Bacterial Strains, Plasmids, Media and Culture

Bacterial strains and plasmids used in this study are listed in Table 2.1 and Table 2.2 respectively. *E. coli* strains were cultured at 37ºC in Luria-Bertani (LB) broth (Sambrook *et al.*, 1989) with shaking at 200 rpm in a BioLine Shaking Incubator (Edwards Instrument Co., Australia) or on LB agar supplemented with 100 µg ampicillin (Ap) mL^{-1} (Appendix I). GAS strains were routinely grown at 37ºC on either commercially prepared Columbia agar plates supplemented with 5% (v/v) defibrinated horse blood (BioMérieux, Australia), or on Difco™ Todd-Hewitt Broth (Becton, Dickinson and Company, USA), supplemented with 1% (w/v) yeast extract (THBY) (Sigma, USA) agar supplemented with 2% (v/v) defibrinated horse blood (Equicell, Australia). Liquid GAS cultures were cultivated at 37ºC in stationary flasks in THBY. GAS isolates were kindly provided from the following collaborators: 5448 (an M1T1 clonal isolate), the animal passaged 5448AP variant, the isogenic 5448Δ*speB* mutant and 20174 (M3) from Prof. Malak Kotb (Department of Molecular Genetics, Biochemistry and Microbiology, University of Cincinnati College of Medicine, USA), HCS5 (M5) a throat isolate associated with ARF (Lyon *et al.*, 2004) from Prof. Michael Caparon (Washington University School of Medicine, Washington, USA), pM1 (M1) and 2036 (M6) from Dr. Michael Batzloff (Queensland Institute of Medical Research, Brisbane, Australia), NS88.2 (M98.1) and NS192 (M106) were isolated from patients in the Northern Territory of Australia (Delvecchio *et al.*, 2002, McKay *et al.*, 2004, Ramachandran *et al.*, 2004) and were provided by Prof. Bart Currie from the Menzies School of Health Research (Darwin, Australia).

2.2   Bioinformatic Analyses

2.2.1   BLAST Searching Streptococcal Genomes

One crucial criterion for the suitability of a vaccine candidate is the ubiquity of the genes encoding the vaccine antigens in all serotypes of GAS. At present, there are 13 complete sequenced GAS genomes which are publicly available on the internet. These genomes were selected for sequencing due to a high association with infection and disease. To determine the percent identity and level of conservation of the genes

Table 2.1 Bacterial strains used in this study.

Strain	Relevant Characteristics	Source or Reference
Group A Streptococcus		
5448	*emm*1, *tee*1	Aziz *et al.*, 2004
5448Δ*speB*	Isogenic *speB* mutant	Aziz *et al.*, 2004
5448AP	5448 passaged through a mouse	Aziz *et al.*, 2004
NS192	*emm*106, Vir type 3.2, *pfbp*$^+$, *sfbI*$^+$, *sof*$^+$, *sfbX*$^+$, *fbp54*$^+$, *fbaA*$^+$	McKay *et al.*, 2004
NS88.2	*emm*98.1, Vir type 17.4, *fbaB*$^+$, *sfbI*$^-$, *sof*$^+$, *sfbX*$^+$, *fbp54*$^+$, *fbaA*$^+$	McKay *et al.*, 2004
pM1	*emm*1, *sof*$^-$, 2031 strain passsaged in mice 12 times, Sp^r (200μg.mL^{-1})	Batzloff *et al.*, 2005
2036	*emm*6, *sof*$^-$	Brandt *et al.*, 2000
HSC5	*emm*5	Lyon & Caparon, 2004
20174	*emm*3, *speB*$^+$	UC
Escherichia coli		
One Shot® TOP10	Chemically competent cells, Plasmid storage strain	Invitrogen, USA
BL21 Star™(DE3) One Shot®	Chemically competent cells, protein expression strain	Invitrogen, USA

Abbreviations: *emm*, emm sequence type; *fbaA*, fibronectin-binding protein A gene; *fbp54*, fibronectin-binding protein 54 gene; *pfbp*, *S. pyogenes* fibronectin-binding protein gene; r, resistance; *sfbI*, SfbI gene; *sfbX*, streptococcal fibronectin-binding protein X gene; *speB*, streptococcal pyrogenic exotoxin B gene; *sof*, serum opacity factor gene; Sp, streptomycin; *tee*, T type; UC; University of Cincinnati.

encoding the vaccine antigens of this study, genomic BLAST interrogation of the sequenced GAS genomes was performed at National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi).

2.2.2   BLAST Searching the Human Proteome

Following the observation that immunisation of humans with M3 protein resulted in three cases of ARF (Massell *et al.*, 1969), there has been an increased concern about the cross-reactivity of GAS vaccine candidate antigens with human proteins. As a preliminary means of addressing this issue, the amino acid sequence of each vaccine antigen of this study was used to interrogate the human proteome utilising the BLASTP search engine available at the NCBI website (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi). The resultant percentage amino acid identity of any human homologues are reported.

2.3   Genomic DNA Extraction

Chromosomal DNA was extracted from GAS isolate 5448 using the protocol provided in the QIAGEN DNeasy Blood and Tissue Kit (USA) for use as a template to

Table 2.2 Plasmids used in this study.

Plasmid	Relevant Characteristics	Reference
pET160/GW/D-TOPO®	Ap^r	Invitrogen, USA
pET160-ADI	Ap^r, *sagP* of 5448 in pET160	This study
pET160-AK	Ap^r, *adk* of 5448 in pET160	This study
pET160-BCAT	Ap^r, *bcaT* of 5448 in pET160	This study
pET160-EF-Tu	Ap^r, *tufA* of 5448 in pET160	This study
pET160-FBA	Ap^r, *fba* of 5448 in pET160	This study
pET160-KPR	Ap^r, *apbA* of 5448 in pET160	This study
pET160-NADP-GAPDH	Ap^r, *gapN* of 5448 in pET160	This study
pET160-OTCase	Ap^r, *arcB* of 5448 in pET160	This study
pET160-PFK	Ap^r, *pfkA* of 5448 in pET160	This study
pET160-PGK	Ap^r, *pgk* of 5448 in pET160	This study
pET160-RRF	Ap^r, *rrf* of 5448 in pET160	This study
pET160-TF	Ap^r, *ropA* of 5448 in pET160	This study
pET160-TIM	Ap^r, *tpi* of 5448 in pET160	This study

Abbreviations: *adk*, adenylate kinase gene; Ap, ampicillin; *apbA*, ketopantoate reductase gene; *arcB*, ornithine carbamoyltransferase gene; *bcaT*, branched-chain-amino-acid aminotransferase gene; *fba*, fructose-bisphosphate aldolase gene; *gapN*, NADP-dependent glyceraldehyde-3-phosphate dehydrogenase gene; *pfkA*, 6-phosphofructokinase gene; *pgk*, phosphoglycerate kinase gene; r, resistance; *ropA*, trigger factor gene; *rrf*, ribosome recycling factor gene; *sagP*, arginine deiminase gene; *tpi*, triosephosphate isomerase gene; *tufA*, elongation factor Tu gene.

amplify selected genes during PCR. DNA was also extracted from 5448*ΔSpeB*, 5448AP, NS192, NS88.2, HCS5 and 20174 GAS isolates for use in PCR screening experiments. GAS isolates were cultured in 2 mL of THBY at 37ºC overnight. Cells were harvested by centrifugation at 5,000 x *g* for 10 min in an Eppendorf 5415C microcentrifuge (Eppendorf, Germany) at room temperature and resuspended in 180 μL of enzymatic lysis buffer (Appendix I). Resuspended cells were lysed for 30 min at 37ºC by adding 25 μL of proteinase K solution and 200 μL of buffer AL. A 200 μL volume of 100% ethanol was added to the cells and mixed thoroughly to precipitate the DNA. The mixture was applied to a DNeasy® Mini spin column, 500 μL of buffer AW1 was added and the spin column spun at 6,000 x *g* for 1 min to wash the bound DNA. The resultant flow-through was discarded and 500 μL of buffer AW2 was applied to the spin column. The column was centrifuged at 20,000 x *g* for 3 min. The spin column was placed in a clean 1.5 mL microcentrifuge tube, 200 μL of buffer AE directly applied onto the membrane and incubated for 1 min at room temperature. The membrane centrifuged at 6,000 x *g* for 1 min to elute the bound chromosomal DNA. The elution was repeated the extracted chromosomal DNA was resolved on an agarose gel. Extracted DNA was stored at -20ºC until required.

2.4   Extraction of Plasmid DNA

The Wizard® *Plus* SV Minipreps DNA Purification System (Promega, USA) was used to extract plasmid DNA. *E. coli* cultures were grown in 10 mL of LB broth containing 100 µg.mL^{-1} Ap overnight at 37ºC with shaking (200 rpm). Cells were harvested by centrifugation at 10,000 x *g* for 5 min and resuspended in 250 µL of Cell Resuspension Solution. The cells were lysed with 250 µL of Cell Lysis Solution, immediately mixed by inversion and incubated at room temperature for 5 min. A 10 µL volume of Alkaline Protease Solution was added to inactivate endonucleases released during lysis and the suspension was immediately mixed by inversion and incubated at room temperature for 5 min. Following incubation, 350 µL of Neutralisation Solution was added and the cell lysate was centrifuged at 14,000 x *g* in a 1.5 mL microcentrifuge tube for 10 min at room temperature. The cleared lysate was transferred to a Spin Column and centrifuged at 14,000 x *g* for 1 min. The flow-through was discarded and 750 µL of Column Wash Solution was applied to the column prior to centrifugation at 14,000 x *g* for 1 min. Following the disposal of the flow-through the wash procedure was repeated using 250 µL of Column Wash Solution, and the column centrifuged at 14,000 x *g* for 2 min. Following washes, the Spin Column was transferred to a clean 1.5 mL microcentrifuge tube and 100 µL of Nuclease-Free Water applied to the membrane. After incubating for 10 min at room temperature the plasmid DNA was eluted by centrifugation at 14,000 x *g* for 1 min. Plasmid DNA was resolved on an agarose gel and stored at -20ºC until required for use.

2.5   Amplification and Cloning of GAS Vaccine Antigens

2.5.1   Polymerase Chain Reaction

The genes encoding the vaccine antigens were amplified from the 5448 GAS isolate using PfuUltra™ High-Fidelity DNA Polymerase from *Pyrococcus furosius* (Stratagene, USA). *PfuUltra™* DNA Polymerase was selected because this polymerase produces blunt-ended PCR products and has 3' → 5' proofreading ability. None of the vaccine antigens contained traditional Gram-positive LPXTG cell wall anchor motifs, nor signal peptide signals (searched for using the SignalP program available at http://au.expasy.org/). Consequently, the oligonucleotide primers (Sigma Genosys, Australia) (listed in Table 2.3) and designed to amplify the entire genes encoding the vaccine antigens (gene sequences obtained from Genbank, http://www.ncbi.nlm.nih.gov/). The bases CACC were added to the 5' end of the

forward primer to enable correct directional cloning into the pET160/GW/D-TOPO®
vector (Invitrogen, USA). All PCR's were performed in a 50 μL reaction volume using
a Palm-Cycler Version 2.2 (Corbett Research, Australia). The components of a typical
PCR reaction are listed in Table 2.4. All solutions were kept on ice and thawed before
use. The temperature cycling of PCR reactions is outlined in Table 2.5. Annealing
temperatures were optimised for the amplification of each vaccine antigen gene
depending on the melting temperatures of the amplification primers. A negative control
was included in each PCR containing all components of the PCR reaction except
template DNA. PCR products were used immediately in pET160/GW/D-TOPO®
cloning reactions.

Table 2.3 Sequence of primers used to amplify vaccine antigen genes from 5448 GAS. The bases CACC were incorporated into all forward (5') primers to allow directional cloning into the pET160/GW/D-TOPO® vector. Forward primers listed in black, reverse primers listed in blue.

Protein antigen	Gene to be amplified	Primer sequence (5'→ 3') Forward/Reverse	Melting temperature (ºC)
ADI	*sagP*	CACCATGACTGCTCAAACACCA TTAAATATCTTCACGTTCAAATGG	68.3 60.6
AK	*adk*	CACCATGAATCTTTTAATCATG TTATTTGAGTTCTAGCAACGC	58.4 58.6
BCAT	*bcaT*	CACCATGATGACAATAGCAATTG TTAATCTACTTTTACAATCCAACC	63.8 57.0
EF-Tu	*tufA*	CACCATGGCAAAAGAAAAATAC TTAAGCTTCGATTTCTGAAAC	61.9 57.7
FBA	*fba*	CACCATGGCAATCGTTTCAGCA TTATGCTTTGTTAGCTGATCCGAAAAC	72.0 67.4
KPR	*apbA*	CACCATGTTAGTTTATATTGCTGGC TTATTGGATGTTCAACACTGC	64.0 60.0
NADP-GAPDH	*gapN*	CACCGTGAACGGTGAATGGAAA TTACTGGATATCAAATACAACAGA	70.8 56.9
OTCase	*arcB*	CACCATGACACAAGTATTTCAA TTACACTTTTGGAATAAAGAGGTT	59.9 58.7
PFK	*pfkA*	CACCATGAAACGTATTGCTGTT TTATTGTGACAATGAACGGTT	63.5 59.3
PGK	*pgk*	CACCATGGCTAAATTGACTGTT TTATTTTTCAGTCAATGCTGCCAAACC	62.6 69.8
RRF	*rrf*	CACCATGGCAAATGCAATTATTG TTACCCTGAGAGCAATTCTTT	67.6 59.3
TF	*ropA*	CACCATGTCTACATCATTTGAA TTACTTAACGCTTGCTGTGCT	59.9 61.5
TIM	*tpi*	CACCATGTCACGTAAACCAATTATT TTAGTTAAGGAAATCAAGCAAAGC	64.2 61.4

2.5.2   Invitrogen pET160/GW/D-TOPO® Cloning

A Champion™ pET160 Directional TOPO® Expression Kit (Invitrogen, USA)

was used to clone PCR products. The PCR products contained CACC at their 5' end, which anneals with a GTGG overhang in the TOPO cloning site of the vector (illustrated in Figure 2.1) to allow successful directional cloning. A representative pET160 clone is shown in Figure 2.1. The cloning reaction was performed in a 0.2 mL microcentrifuge tube. The pET160/GW/D-TOPO® cloning reaction contained the following components: ~10 ng of fresh PCR product (0.5 – 4 μL), 1 μL salt solution, 1 μL TOPO® vector; made to a total volume of 6 μL with sterile water. The components were mixed gently and incubated for 5 min at room temperature. Following the incubation, 3 μL of the reaction was added to a vial of One Shot® TOP10 chemically competent *E. coli* (Invitrogen, USA). The cells were gently mixed and incubated on ice for 30 min. Cells were then heat-shocked at 42ºC for 30 sec (without shaking) and immediately transferred to ice. Following the addition of 250 μL of SOC medium (Appendix I), the transformed cells were incubated at 37ºC for 1 h with shaking (200 rpm) and plated onto LB agar supplemented with Ap (100 μg.mL^{-1}) and grown overnight at 37ºC. The following day, eight single colonies were isolated for each vaccine antigen cloning and re-streaked onto LB agar with Ap 100 μg.mL^{-1} and grown overnight at 37ºC. Each potential transformant was inoculated into 1.5 mL LB broth supplemented with Ap 100 μg.mL^{-1} and grown overnight at 37ºC with shaking (200 rpm). The following day, plasmid extractions were performed (section 2.4) to isolate potential transformant DNA ready for screening.

Table 2.4 Final concentration of PCR reagents using *PfuUltra™* High-Fidelity DNA Polymerase.

Reagent	Volume (μL)	Final Concentration
PCR buffer (10X)	5	1X
dNTPs (2.5 mM each)	4	0.2 mM of each dNTP
DNA template	0.5 – 1	2 ng
DNA polymerase	1	1.25 U
Forward primer (5') (25 μM)	1	0.5 μM
Reverse primer (3') (25 μM)	1	0.5 μM
Sterile dH$_2$O	38 – 38.5	To final volume (50 μL)

Table 2.5 Temperature cycles used for PCR using *PfuUltra™* HF DNA Polymerase. The annealing temperature for each vaccine antigen gene depended on the melting temperature of the primers.

Step	Temperature	Time	Cycles
1.1 Denaturing	95ºC	2 min	1
2.1 Denaturing	95ºC	30 sec	
2.2 Annealing	51-61ºC	30 sec	30
2.3 Extension	72ºC	2 min 30 sec	
3.1 Extension	72ºC	10 min	1
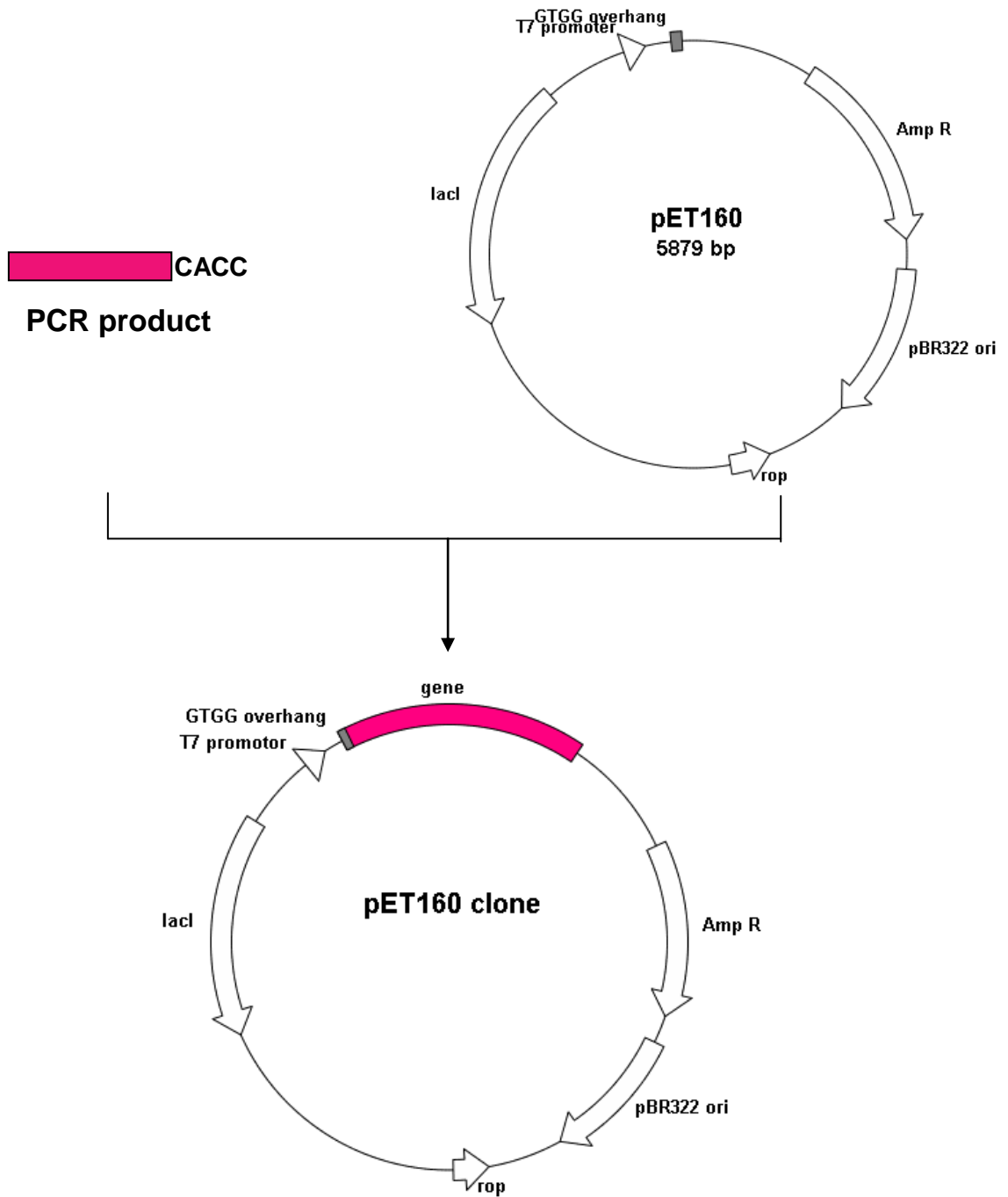
Figure 2.1 Schematic diagram illustrating cloning of the GAS vaccine antigen genes into the pET160/GW/D-TOPO® vector. The PCR product is engineered to contain a CACC 5' overhang which anneals to a GTGG overhang in the vector sequence allowing directional cloning. Abbreviations and definitions; Amp R, ampicillin resistance gene; ori, origin; rop, interacts with the pBR322 origin to facilitate low-copy replication in *E. coli*; lacI, encodes lac repressor.

2.6 DNA Manipulations

2.6.1 Agarose Gel Electrophoresis

Bio-Rad Mini-Sub™ or a Bio-Rad Wide Mini-Sub™ electrophoresis chambers (Bio-Rad, USA) were used for all agarose gel electrophoresis. Agarose gels were prepared in 1X TAE (Appendix I) buffer using 1% (w/v) electrophoresis grade agarose (Promega,USA). The sizes of the DNA fragments were estimated by comparison to a standard curve based on the separation of 5 μL of Hyperladder I molecular markers (Bioline, UK). Samples, routinely 10 μL, were mixed with 2 μL of bromophenol blue DNA loading dye (Appendix I) prior to being loaded in the gel. Gels were routinely electrophoresed in 1X TAE buffer and run at a constant voltage of 80 V for approximately 1.5 h using a Bio-Rad Power Pack 300 (Bio-Rad, USA). Gels were stained with ethidium bromide staining solution (Appendix I) for 30 min, destained in dH$_2$O for 10 min and viewed under ultra-violet (UV) light and photographed using the Nova Line Gel Documentation System (Novex, Australia).

2.6.2 Restriction Enzyme Digestion

Plasmid DNA (14 μL) was added to a 1.5 mL microcentrifuge tube containing 2 μL dH$_2$O. Ideally, restriction enzymes were selected for each clone that cut once within the vector and once within the insert, yielding two fragments. Restriction enzymes selected for each pET160 clone are listed in Table 2.6. Following the addition of 2 μL

Table 2.6 Restriction enzymes used to digest potential transformants for each of the GAS vaccine antigen expression vectors. Expected DNA fragment sizes indicated on the right. Each set of restriction enzymes cuts the potential clone twice, once within the vector sequence and once within the insert, resulting in two DNA fragments. pET160-NADP-GAPDH, pET160-RRF and pET160-TF however, are cut twice within the vector.

Clone	Restriction Enzyme/s	Expected Fragments (bp)
pET160-ADI	*Pst*I	2,153 & 4,923
pET160-AK	*Pst*I & *Hind*III	1,233 & 5,245
pET160-BCAT	*Hind*III & *Nhe*I	1,209 & 5,656
pET160-EF-Tu	*Pst*I & *Hind*III	1,150 & 5,886
pET160-FBA	*Pst*I	1,667 & 5,054
pET160-KPR	*Bst*EII	1,189 & 5,574
pET160-NADP-GAPDH	*Nco*I & *Nhe*I	1,870 & 5,373
pET160-OTCase	*Bst*EII	2,131 & 4,722
pET160-PFK	*Pst*I	1,251 & 5,602
pET160-PGK	*Nru*I	2,909 & 4,129
pET160-RRF	*Nco*I & *Nhe*I	1,024 & 5,373
pET160-TF	*Hind*III & *Pst*I	2,207 & 4,916
pET160-TIM	*Bst*EII	1,284 & 5,314

of the appropriate 10X Buffer (Fermentas, Australia), 1 µL (10 U) of each restriction enzyme (Fermentas, Australia) was added and the contents of the tube mixed gently by pipetting up and down. The reaction tube was centrifuged for 5 sec and incubated at 37ºC for a minimum of 2 h. Samples were resolved by agarose gel electrophoresis.

2.6.3 DNA Sequence Analysis

2.6.3.1 Cycle Sequence Reactions

Primers for DNA sequencing (Table 2.7) were purchased from Sigma-Genosys (Australia) and designed to sequence the 5' and 3' junctions of the multiple cloning site in the pET160/D-TOPO® vector. Due to the large size of the genes encoding NADP-GAPDH and TF (approximately 1.4 and 1.3 kb respectively), additional primers were designed to enable sequencing of the internal parts of these genes.

Table 2.7 Primers used for sequencing the multiple cloning site in the pET160/GW/D-TOPO® vector.

Primer	Sequence (5' – 3')	Melting Temperature
pET Forward (5')	TCTCGATCCCGCGAAATTAATACG	70.0ºC
pET Reverse (3')	TAGTTATTGCTCAGCGTGGC	66.1ºC
pET160-NADP-GAPDH Internal Forward (5')	GTGAAGTTATTCGTACCGCTG	61.1ºC
pET160-NADP-GAPDH Internal Reverse (3')	CGTCTTCAGGCATTCCGACAC	69.0ºC
pET160-TF Internal Forward (5')	GAAGTTTCAGATGGAAGACGTG	58.9ºC
pET160-TF Internal Reverse (3')	CTCATCATCAAGCTCTGGTAC	58.9ºC

All cycle sequencing reactions were performed in a 0.2 mL microcentrifuge tube. Each reaction was made up of 0.5 µL of BigDye™ Terminator Ready Reaction Mix (Perkin-Elmer Applied Biosystems, USA), 50-100 ng template DNA, 4 pmole of either the forward or the reverse oligonucleotide primer and sterile Milli-Q water to a final volume of 10 µL. The temperature cycling regime used is shown in Table 2.8 and was performed using a Palm-Cycler Version 2.2 (Corbett Research, Australia).

Table 2.8 Temperature cycling parameters used for DNA sequencing reactions.

Step	Temperature	Time	Cycles
DNA Denaturation	96ºC	10 sec	
Primer Annealing	50ºC	5 sec	25
Extension	60ºC	4 min	

2.6.3.2 Ethanol Precipitation

Once the temperature cycling reaction was completed, ethanol precipitation was performed to remove excess unincorporated dye terminators. The entire 10 μL PCR reaction, 2 μL of 3 M sodium acetate (pH 4.6) and 50 μL of ice cold 95% (v/v) ethanol were added to a 1.5 mL microcentrifuge tube. The contents of the tube were vortexed and placed on ice for 10 min to encourage precipitation. Following incubation, the plasmid DNA was pelleted by centrifugation at 16,000 x *g* for 20 min. The DNA pellet was washed with 250 μL of ice cold 70% (v/v) ethanol and centrifuged at 16,000 x *g* for 10 min. The ethanol was carefully aspirated from the tube and the pellet air-dried for approximately 1 h.

2.6.3.3 Electrophoresis of Sequencing Samples

Sequencing samples were routinely electrophoresed through 3130 POP7 polymer HiDi Formamide using a 3130XL sequencer (both supplied by Applied Biosystems, USA). Electrophoretic separation was conducted by Marie Turner (Technical Officer, School of Biological Sciences, University of Wollongong). The resultant DNA sequences were analysed using AutoAssembler Software (Perkin-Elmer Applied Biosystems, USA) and the BioManager program available at the Australian National Genomic Information Service (ANGIS) (http://www.angis.org.au). The ANGIS website is no longer available.

2.7   Bacterial Transformation

2.7.1   Preparation of Electrocompetent Cells

Electrocompetent BL21 Star™(DE3) One Shot® *E. coli* were selected for use as an expression strain for the recombinant proteins. A single colony from a freshly streaked LB agar plate was used to inoculate 50 mL of LB broth. This culture was grown overnight at 37ºC with shaking (200 rpm). The overnight culture was then used to inoculate 450 mL LB broth which was grown at 37ºC with shaking (200 rpm) until an OD_{600} of 1 was obtained. Cells were harvested by centrifugation in a Beckman JA-10 rotor (Beckman, USA) at 4,000 x *g* for 15 min at 4ºC and the supernatant discarded. For successful electroporation, a cell suspension of very low conductivity is necessary. To achieve this, the cells were washed several times to reduce the ionic strength of the suspension. Wash steps were as follows; 200 mL of ice cold dH_2O, 100 mL of ice cold dH_2O, 4 mL ice cold 10% (v/v) glycerol; the cells were re-centrifuged between each

wash and the supernatant discarded. Finally, the cells were resuspended in 0.6 mL of 10% (v/v) glycerol and stored as 50 μL aliquots at -80ºC.

2.7.2 Transformation of Electrocompetent Cells

Due to the possible instability of expression plasmids in BL21 Star™(DE3), a fresh aliquot of cells were transformed each time for recombinant protein expression. *E. coli* cells, plasmid DNA and electro-cuvettes were placed on ice. Plasmid DNA (1 μL, approximately 100 ng) was added to 50 μL of BL21 Star™ *E. coli* cells and mixed gently with a pipette tip. *E. coli* cells were transferred to the bottom of a 0.2 cm electro-cuvette and pulsed once using a Gene Pulser® (Bio-Red, USA) set to 25 μF capacitance and 2.5 kV. Following the pulse, the cells were transferred to a 5 mL tube and 350 μL LB broth added followed by incubation at 37ºC for 1 h with horizontal shaking (200 rpm). The transformed cells were used to inoculate 100 mL of LB supplemented with Ap (100μg.mL^{-1}) and incubated at 37ºC overnight.

2.8 Protein Expression & Purification from the pET160 vector

Recombinant proteins expressed from the pET160/GW/D-TOPO® vector contain a N-terminal hexahistidine tag (His-tag). Via affinity binding to a Nickel-nitrilotriacetic acid (Ni-NTA) chromatography column, the His-tag permits purification of the recombinant proteins from *E. coli* whole cell lysate preparations.

2.8.1 His-tag Affinity Chromatography of Recombinant Proteins

2.8.1.1 E. coli Culture Growth for Purification

A 100 mL culture of LB supplemented with 100 μg.mL^{-1} Ap was inoculated with a freshly transformed bacterial suspension. The culture was incubated overnight at 37ºC with shaking (200 rpm). This overnight culture was used to inoculate 900 mL of LB Ap 100 μg.mL^{-1}, which was grown at 37ºC with shaking (200 rpm) until an OD$_{600nm}$ of 0.5 – 0.8 was reached. At this stage, recombinant protein expression was induced with isopropyl-β-D-thiogalactosidase (IPTG) at a final concentration of 1 mM. Following induction, the culture was incubated at 37ºC with shaking (200 rpm) for a further 4 h. Cells were harvested by centrifugation at 5,000 x *g* for 10 min at 4ºC and the supernatant discarded. The cell pellet was stored overnight at -20ºC before proceeding with recombinant protein purification.

2.8.1.2 Denaturing Batch Purification of Recombinant Proteins

The bacterial pellet was thawed at room temperature for 10 min, resuspended in 20 mL of QIAGEN® lysis Buffer A (Appendix I) and stirred on a rotating wheel for 1 h at room temperature. The lysate was centrifuged at 10,000 x *g* for 25 min at 4°C and the supernatant collected. The resultant cleared lysate was filtered using a 0.22 μm Millex syringe driven filter device (Millipore, Australia), subsequently mixed with a 5 mL volume of 50% (v/v) Ni-NTA slurry (QIAGEN, Australia) and stirred on a rotating wheel for 1 h at room temperature. The lysate-slurry mixture was applied to a 10 mm chromatography econo-column (Bio-Rad, USA), and the column allowed to empty by gravity flow. A 5 mL volume of 50% (v/v) Ni-NTA slurry generates a 2.5 mL resin bed. The lysate flow-through was saved and stored at 4°C. The column was washed with 16 column volumes (40 mL) of QIAGEN® Buffer C (Appendix I). Typically, recombinant proteins were eluted with 5 x 2 mL fractions of QIAGEN® elution Buffer D (Appendix I) and 5 x 2 mL fractions of QIAGEN® elution Buffer E (Appendix I). Elution fractions were resolved using SDS-PAGE to determine which fractions contained recombinant protein. Fractions containing recombinant protein were pooled and prepared for dialysis.

Following SDS-PAGE of eluted fractions, those containing quantities of protein detectable using Coomassie blue stain (Appendix I) were pooled. To ensure removal of the recombinant proteins from the denaturing elution buffer, the pooled elution fractions were dialysed at 4°C against PBS with a decreasing concentration of urea, starting with 4 M urea/PBS and ending with 0.5 M urea/PBS. A decreasing urea gradient was used in the dialysis procedure to minimise the precipitation of recombinant protein.

2.9 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

SDS-PAGE was carried out using a Bio-Rad Mini Protean® 3 Cell (Bio-Rad, USA) electrophoresis unit and previously established methods (Laemmli, 1970). Resolving gels contained 12% bis-acrylamide (35:0.8 acrylamide:bis-acrylamide; Bio-Rad, USA) with a 4% stacking gel. Appropriate volumes of protein samples were mixed with either 2X or 5X Reducing Sample Buffer (Appendix I) and boiled for 10 min prior to loading onto the gel. Unstained Protein Molecular Weight Markers (Fermentas, USA) were resolved on each gel, and a standard curve constructed to estimate the molecular mass of protein samples. SDS-PAGE gels were run at a constant voltage of 120 V in 1X

SDS-PAGE running buffer (Appendix I) for approximately 2 h. Proteins were stained with Coomassie Blue stain (Appendix I) and destained with rapid destaining solution (Appendix I); by heating for 1 min at maximum power in a microwave and then gently shaking for 60 min on an orbital shaker. Gels were scanned with a GS-800™ calibrated densitometer (Bio-Rad, USA).

2.10 Protein Expression and Purification from the pGEX2T vector

Dr. Martina Sanderson-Smith previously cloned the *emm*1 gene from the NS696 GAS isolate into pGEX2T, creating the pGEX2T-M1 expression vector used in this study. The details for the expression and purificatiaon of recombinant M1 protein from this vector are described in Sanderson-Smith *et al.* (2006).

2.11 Bicinchoninic Acid (BCA) Concentration Assay

The bicinchoninic acid (BCA) assay was used to determine the final concentration of recombinant proteins following purification and dialysis. The BCA kit (Sigma, USA) consists of two Reagents, Reagent A, a BCA solution and Reagent B which contains copper (II) sulfate pentahydrate solution. The Working Reagent is prepared by adding 8 parts of Reagent A to 1 part of Reagent B. The amino acids cysteine, tryptophan, tyrosine, and the peptide bond are able to reduce Cu^{2+} to Cu^{1+} which then forms a purple-blue complex with BCA. This complex is measured at 560 nm and the absorption is proportional to the amount of protein in the solution. BCA assays were routinely performed in 96-well microtitre plates (Greiner Bioone, Germany), with BSA standards in the concentration range $100 - 1000$ $\mu g.mL^{-1}$ prepared in the same buffer as the dialysed protein (either 0.5 M urea/PBS or PBS). A 25 μL volume of samples and standards were added to 200 μL of Working Reagent in triplicate, incubated for 30 min at 37ºC and the absorbance read at 560 nm. A standard curve based on the absorbance and concentration of BSA standards was constructed and the concentration of protein samples was estimated by interpolation.

2.12 Western Blot Analysis
2.12.1 Transfer onto Nitrocellulose Membrane

Western transfer was carried out using a Mini Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad, USA). Pre-stained molecular size markers were resolved on SDS-PAGE gels prior to transfer to indicate the approximate mass of proteins and also the success of transfer of the proteins to the membrane. Following resolution of proteins

in the SDS-PAGE gel, the gel was equilibrated for 15 min in chilled transfer buffer (Appendix I) to remove residual salts associated with the gel. A 6 x 9 cm piece of 0.45 µm nitrocellulose membrane (PALL, USA), two fibre pads and two filter paper squares (both supplied by Bio-Rad, USA) were also pre-soaked in chilled transfer buffer for 15 min. The nitrocellulose membrane and gel were assembled between the fibre pads and filter paper in the gel holder cassette (Bio-Rad, USA) as shown in Figure 2.2. The gel holder cassette was inserted into the modular electrode assembly (Bio-Rad, USA) and placed into the buffer tank with a Bio-Ice cooling unit (Bio-Rad, USA). The tank was filled with chilled transfer buffer (Appendix I) and continually stirred during transfer at 100 V for 60 min at room temperature using a PowerPac 300 power supply (Bio-Rad, USA).
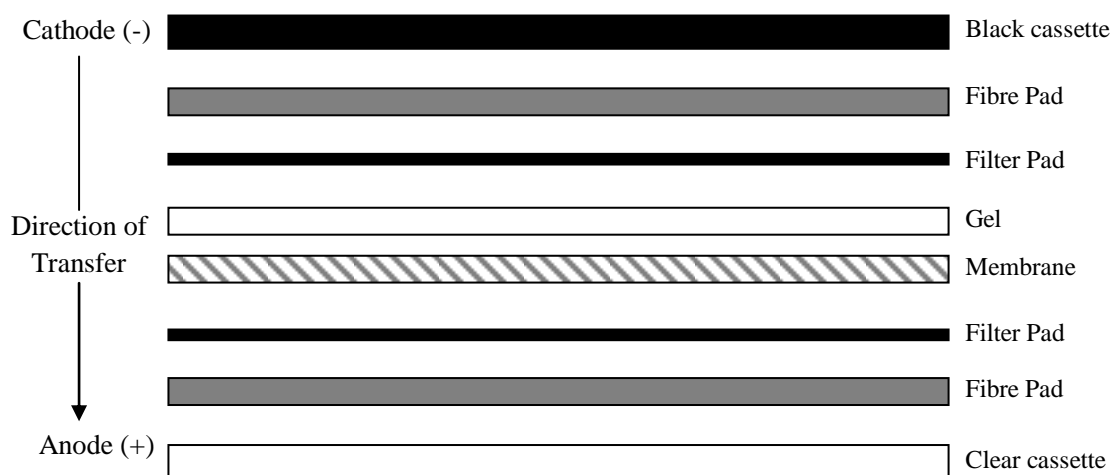
Figure 2.2 Assembly of the gel sandwich for western transfer using the Mini Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad, USA).

2.12.2 Western Blot Development

Following western transfer, membranes were blocked in 50 mL of 5 % (w/v) skim milk (Difco Laboratories, USA) in PBS overnight at 4ºC. The membrane was washed for 5 min in 50 mL of PBST (Appendix I). This wash step was repeated twice giving a total of three washes. The wash solution was discarded and the membrane was incubated at room temperature for 90 min on an orbital shaker in a volume of 5 mL of primary antibody (in a heat sealed bag) suitably diluted in 0.5% (w/v) skim milk in PBS. Polyclonal mouse anti-sera raised against each of the vaccine antigens was routinely used at a dilution of 1:2,000, unless stated otherwise. Human serum obtained from Australian Aboriginal and Indian individuals suffering endemic GAS infection was used at a dilution of 1:1,000. Following primary antibody incubation, three 5 min

PBST washes were performed. The secondary antibody, either goat anti-mouse IgG-HRP (Chemicon), used for the detection of murine primary anti-sera, or goat anti-human IgG-HRP (Bio-Rad, USA), used for the detection of human primary anti-sera, was diluted 1:2,000 in 0.5% (w/v) skim milk in PBS. Membranes were incubated in 30 mL of secondary antibody at room temperature for 90 min on an orbital shaker. Following secondary antibody incubation, three 5 min PBST wash steps were performed. In preparation for development, the membranes were equilibrated in 100 mM Tris-HCl (pH 7.6) for 5 min. A 50 mL volume of 3,3'-diaminobenzidine (DAB) Colour Development Solution (Appendix I) was applied and membranes were subjected to gentle rocking until sufficiently developed (up to a maximum time of 20 min). The membranes were thoroughly washed with dH$_2$O and scanned using a GS-800[™] calibrated densitometer (Bio-Rad, USA).

2.13   Mutanolysin Extraction of GAS Cell Wall Proteins

Mutanolysin is a N-acetylmuramidase similar to lysozyme, it cleaves the β-N-acetylmuramyl-(1,4)-N-acetylglucosamine linkage of the bacterial cell wall polymer peptidoglycan-polysaccharide. It has been used previously for the preparation of streptococcal cell wall extracts (Ji *et al.*, 1998, Wang *et al.*, 1994) and the method of Ji *et al.* (1998) was utilised in this study. A single GAS colony was used to inoculate 2 mL of THBY (Appendix I) and grown at 37ºC overnight without shaking. This overnight culture was added to 100 mL of THBY and the culture grown at 37ºC to late stationary phase (approximately 16 h growth) without shaking. GAS cells were harvested via centrifugation in a JA-10 rotor (Beckman, USA) at 7,560 x *g* for 20 min at 4ºC. Cells were washed twice in 5 mL of wash buffer containing the protease inhibitor PMSF (Appendix I) and re-centrifuged. Following washes, the bacterial cells were resuspended in 1.15 mL of mutanolysin mix (Appendix I) and incubated for 2 h at 37ºC with shaking (200 rpm). The sample was centrifuged at 16,000 x *g* in an Eppendorf 5415C microcentrifuge for 5 min (Eppendorf, Germany). The supernatant, containing the cell wall-associated proteins, was carefully aspirated and frozen at -20ºC until required for use.

2.14   Production of Polyclonal Anti-serum in Rabbits

Polyclonal rabbit anti-serum specific for ADI, FBA, KPR, M1 and TF was individually generated for use in electron microscopy experiments using routine

immunisation and bleed techniques (Harlow *et al.*, 1988). All procedures described herein complied with the Guidelines for the Care and Use of Animals for Scientific Purposes (National Health and Medical Research Council, Australia) and were approved by the University of Wollongong ethics committee.

Individual eight week old female New Zealand white rabbits (Merungora, NSW, Australia) were each immunised with single recombinant vaccine antigen on Days 0, 21, 42 and 77. For the primary injection on Day 0, 150 µg of recombinant protein was prepared in a 500 µL volume and this was emulsified with 500 µL of Freund's Complete Adjuvant (CFA) (Sigma, USA). A 500 µL volume of this emulsification (containing approximately 75 µg of recombinant protein) was administered intramuscularly to the hind leg thigh muscle using a 23 G needle. Booster immunisations were prepared and delivered in the same manner using Freund's Incomplete Adjuvant (IFA) (Sigma, USA) rather than CFA. Test bleeds, in which 5 to 10 mL of blood were collected were performed on Days -1, 28 and 52. The bleed on Day -1 allowed the collection of pre-immune serum for use as control serum; subsequent test bleeds permitted monitoring of the serum-specific IgG antibody response following each immunisation. A small patch on the ear was shaved and swabbed with 70% (v/v) ethanol. The ear was warmed under a lamp for 1 – 2 min to increase blood flow and the marginal ear vein nicked with a sterile scalpel. Blood drops were collected in sterile 15 mL tubes. Antibody levels were monitored by ELISA. Exsanguination via terminal cardiac puncture was performed on Day 85 by Dr. Tracy Maddocks (University of Wollongong, Australia) and the blood collected in 50 mL sterile tubes. Following collection, rabbit blood was left to clot for 1 h at room temperature. The clot was detached from the edges of the tube using a glass Pasteur pipette and the blood sample was stored at 4ºC overnight to allow the clot to contract. The serum was separated from the clot, red blood cells and other cellular debris by centrifugation at 3,000 x *g* for 15 min at 4ºC using a Heraeus® Megafuge® 1.0 R centrifuge (Kendro, Germany). The supernatant (serum fraction) was carefully aspirated and aliquots were stored at -80ºC.

2.15 Murine Subcutaneous Immunisation & Intraperitoneal GAS Challenge Model

A systemic challenge experiment was selected as an *en bloc* approach to initially screen the immunogenicity and protective efficacy of the GAS vaccine antigens. BALB/c mice were immunised thrice with each individual vaccine antigen and subsequently challenged with a lethal dose of GAS. The timeline of the mouse procedures is illustrated in Figure 2.3. Following challenge, mice were monitored for a period of ten days, deaths were recorded and Kaplan-Meier survival curves generated using GraphPad™ Prism 4.2 (GraphPad Software Inc., USA). The survival of mice immunised with GAS vaccine antigens was compared to the survival of mice sham immunised with PBS using the log-rank test in which $P < 0.05$ was considered a statistically significant difference. Immunisation and challenge experiments were conducted using 10 mice for each vaccine antigen unless otherwise stated. To further confirm the protective efficacy exhibited by ADI, KPR and TF, a second independent group of 10 mice were immunised and challenged. Logistically not all challenge experiments could be performed at the same time, thus, there are three groups of 10 mice for the positive (full length M1 protein) and negative (PBS) controls. This immunisation and systemic challenge model has been used in previous GAS challenge studies using both inbred BALB/c mice and outbred Quackenbush mice (Sanderson-Smith *et al.*, 2006a, Batzloff *et al.*, 2004). All animal experiments described in this thesis complied with Guidelines for the Care and Use of Animals for Scientific Purposes (National Health and Medical Research Council, Australia) and were approved by institutional ethics committees at the University of Wollongong and the Queensland Institute of Medical Research.
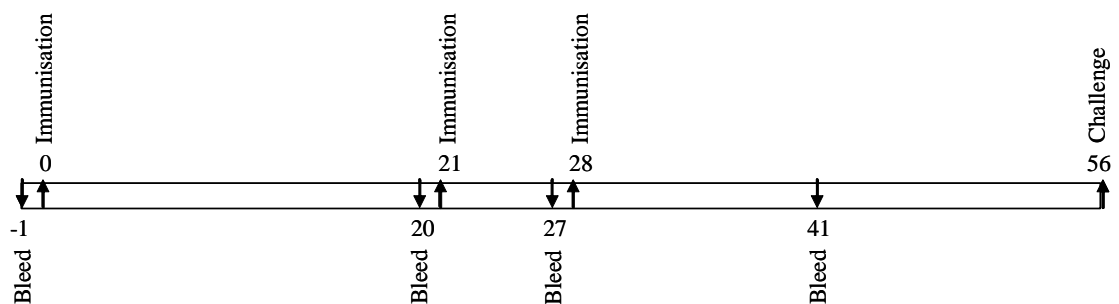
Figure 2.3 Timeline of mouse immunisation, bleed and challenge procedures. Day numbers indicated represent the following procedures; -1, pre-immune bleed; 0, primary immunisation; 20, bleed; 21, booster immunisation; 27, bleed; 28, booster immunisation; 41, final bleed; 56, challenge.

2.15.1 Subcutaneous Immunisation

Groups of 10 BALB/c mice (Animal Resource Centre, Perth, Australia) were individually immunised on Day 0 with each of the thirteen vaccine antigens; ADI, AK, BCAT, EF-Tu, FBA, KPR, NADP-GAPDH, OTCase, PFK, PGK, RRF, TF, TIM, recombinant M1 protein (positive control) or PBS (negative control). In this model, the widely characterised protective M protein (D'Alessandri *et al.*, 1978, Fox *et al.*, 1973, Polly *et al.*, 1975) was selected as a positive control antigen. Per mouse, 10 µg recombinant protein in a 25 µL volume of PBS (Appendix I) was emulsified with 25 µL CFA (Sigma, USA). To prepare the emulsification for immunisation, the protein and adjuvant were mixed in a 1.5 mL microcentrifuge tube by vortexing for 1 min and subsequently passed up and down a 1 mL syringe using an 18 G blunt ended needle (Becton Dickinson, USA). To account for volume loss during sample preparation, double the volume of required sample was prepared. Per mouse, a 50 µL volume of the preparation (containing a 1:1 ratio of protein to adjuvant) was injected subcutaneously at the base of the tail. Mice were administered booster immunisations on Day 21 and 28 consisting of 10 µg of recombinant protein in a 50 µL volume of PBS (without CFA) injected subcutaneously.

2.15.2 Bleeds

Mice were bled on Day -1 (prior to commencing immunisation), 20, 27 and 41. The first three bleeds were performed by snipping 1 mm from the end of the tail and obtaining 10 µL blood which was immediately mixed with 90 µL PBS (resulting in a 1:10 dilution). The final bleed, on Day 41, was via a scalpel cut to the tail vein. The tail was heated under a lamp for 10 sec to increase blood flow. A small nick was made with a sterile scalpel and a maximum volume of 200 µL of whole blood was collected in a 1.5 mL microcentrifuge tube. The blood was left to clot for 1 h at room temperature and the clot removed using a sterile toothpick. To obtain a serum fraction free of red blood cells and other cellular debris, following clotting and removal of the clot, all blood samples were centrifuged at 3,000 x *g* in an Eppendorf 5415C microcentrifuge (Eppendorf, Germany) for 10 min at room temperature. The supernatant (serum) was carefully aspirated and stored at -20ºC.

2.15.3 Enzyme-Linked Immunosorbent Assay (ELISA)

Enzyme-linked Immunosorbent Assay (ELISA) was performed to determine the serum-specific IgG titre against the GAS vaccine antigens following immunisation. Serum obtained from mice immunised with PBS and adjuvant only was tested in parallel with each specific test serum for background reactivity against each vaccine antigen. Additionally, corresponding pre-immune serum was tested against each vaccine antigen to ensure no pre-disposed serum reactivity. Firstly, 96-well microtitre plates (Greiner Bioone, Germany) were coated with 5 μg of recombinant protein in 100 μL of carbonate coating buffer (Appendix I) per well and incubated for 90 min at 37ºC. Following coating, the antigens were flicked out of the wells and the non-specific binding sites blocked with 5% (w/v) skim milk in PBS (200 μL per well) incubated overnight at 4ºC. Following blocking, the wells were washed twice with PBST and the test serum (primary antibody) generated in ten individual mice for each vaccine antigen was added singly to the top well in row 1 through to 10 respectively at a 1:100 dilution. A pool of serum from mice immunised with PBS and adjuvant only was added to the top wells in rows 11 and 12 at a 1:100 dilution (Figure 2.4). Primary sera was titrated down the plate in a 1:2 dilution (in 0.5% (w/v) skim milk/PBS), resulting in a final volume of 100 μL per well. Final bleed serum was titrated down two plates. Test sera was incubated for 90 min at 37ºC. Following incubation, the test serum was discarded and the wells were washed four times with PBST. After washing, the secondary antibody, goat-anti mouse IgG-HRP (Bio-Rad, USA) (100 μL) was added to all wells at a dilution of 1:3,000 (in 0.5% (w/v) skim milk/PBS) and incubated for 90 min at 37ºC. Following incubation, the secondary antibody was discarded and the wells were washed four times with PBST. A 100 μL volume of *O*-phenylenediamine dihydrochloride (OPD) solution was added per well and the microtitre plates incubated for 10 min in darkness. The absorbance was subsequently measured at 450 nm using a SpectraMax spectrophotometer (Molecular Dynamics, USA). The titre of each serum sample was defined as the lowest test serum dilution that had a greater absorbance than 0.2 at 450 nm.

Dilution	1	2	3	4	5	6	7	8	9	10	11	12
100	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	Pooled PBS serum	
200												
400												
800												
1600												
3200												
6400												
12800	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓

Figure 2.4 Typical 96-well microtitre plate layout for ELISA. Serum dilution factor indicated on the left, serum was diluted two-fold down either one or two plates. The row numbers are indicated along the top. M1 represents the serum from mouse 1 in that antigen group; M2 represents serum from mouse 2 and so on. Pooled PBS serum is a pool of the serum obtained from all ten mice immunised with PBS and adjuvant.

2.15.4 Intraperitoneal GAS Challenge

Consequent to subcutaneous immunisation, BALB/c mice were challenged with a passaged M1 strain (pM1) in the stationary phase administered via the intraperitoneal route on Day 56. The M1 strain, 2031, had previously been passaged 12 times through mice to increase the virulence of the strain and to ensure lethality in mice. Prior to GAS challenge, pM1 glycerol stocks were prepared as follows. A single colony of pM1 was inoculated into 5 mL THBY and grown overnight at 37ºC. The following day, the bacterial culture was centrifuged in an a Heraeus® Megafuge® 1.0 R centrifuge (Kendro, Germany) at 3,000 x g for 10 min at 4ºC. The supernatant was discarded, the bacterial pellet resuspended in THBY containing 15% (w/v) glycerol and 100 μL aliquots stored at -20ºC.

Ten tubes containing 5 mL of THBY were each inoculated with individual 100 μL glycerol stocks of pM1 and grown overnight at 37ºC without shaking. The following day, the 5 mL cultures were pooled and centrifuged in a Heraeus® Megafuge® 1.0 R centrifuge (Kendro, Germany) at 3,000 x g for 10 min at 4ºC. The supernatant was discarded, the pellets pooled into one tube, washed twice with 30 mL of chilled THBY and centrifuged under the same conditions. Following washing, the supernatant was discarded and the pellet resuspended in 5 mL THBY (neat solution). This neat solution was serially diluted in ten-fold dilutions. A 50 μL volume of the 10^{-4} to 10^{-8} dilutions was plated in triplicate using the pour plate method with THBY agar supplemented with 2% (v/v) horse blood (Equicell, Australia). For pour plating, 50 μL of serial dilution was pipetted onto the base of empty plastic Petri dishes. Approximately 15 mL of

THBY agar supplemented with 2% (v/v) horse blood was subsequently poured into each petri dish (already containing the serial dilution) and swirled ten times to the right, ten times to the left and ten times up and down to ensure uniform distribution of GAS throughout the agar. The plates were incubated overnight at 37ºC, whilst the neat solution was stored at 4ºC overnight. The following day, the colonies within each of the dilutions were counted and the cfu/mL of the neat solution was determined. This solution was appropriately diluted into chilled THBY to result in a dosage of approximately 2×10^7 cfu/mL (actual doses ranged from $6 \times 10^6 - 2.6 \times 10^7$ cfu/mL). This inoculum was administered to each mouse in a 400 μL volume intraperitoneally on Day 56.

2.16 Murine Intraperitoneal Immunisation & Subcutaneous GAS Challenge Model

Following the initial screening of the candidate antigens in the systemic challenge model, a murine GAS skin infection model was developed for further characterisation of specific antigens. Variations of this model have been previously used (McArthur *et al.*, 2004a, Medina *et al.*, 2003) but, to our knowledge, this study was the first time this murine GAS skin infection model was preceeded by intraperitoneal immunisation for testing protective efficacy of GAS vaccine candidates. The M1T1 clonally disseminated 5448 GAS isolate was selected for challenge in this model due to its high association with human infection and disease (Stevens, 1992, Johnson *et al.*, 2002). Prior to challenge, this isolate was passaged through mice, generating the 5448AP isolate, which was used in subsequent GAS subcutaneous infection studies. The schedule and timing of procedures mirrored those of the previous systemic challenge study described in section 2.15 and shown in Figure 2.3. The candidate antigens tested in this model were ADI, FBA, KPR, TF, M1 (positive control), and PBS (negative control). A second experiment was performed in which groups of two antigens which showed partial protection in the initial experiment were co-administered in a cocktail vaccine preparation. Immunisation and challenge experiments were performed in duplicate for each individual vaccine antigen and paired antigen combination and the data presented represent two independent groups of 10 mice unless otherwise stated.

2.16.1 Intraperitoneal Immunisation

Two groups of 10 C57BL/J6 mice (Animal Resources Centre, Perth, Australia) were immunised on Day 0. Per mouse, 10 μg recombinant protein in a 50 μL volume of PBS (Appendix I) was emulsified with 50 μL CFA (Sigma, USA) using the emulsification procedure described in section 2.15.1. Per mouse, a 100 μL volume of the preparation was injected intraperitoneally. Mice were administered intraperitoneal booster immunisations on Day 21 and 28 consisting of 10 μg of recombinant protein in a 100 μL volume of PBS.

2.16.2 Bleeds, Serum Preparation and ELISA

Bleeds were performed on Day -1, 20, 27 and 41 as per the method outlined in Sections 2.15.2. ELISA was utilised to determine the serum-specific IgG antibody titre against the vaccine antigens at each bleed, as previously described in Section 2.15.3.

2.16.3 Subcutaneous GAS Challenge

Mice were subcutaneously challenged on Day 56 with log phase 5448AP. The day before challenge (Day 55), a smooth patch of skin was prepared for infection with GAS by removing fur from a small patch on the back of the mouse using clippers followed by the application of Nair hair removal cream. A single 5448AP colony was used to inoculate 4 mL THBY which was grown for approximately 10 h at 37ºC without shaking. This 4 mL culture was subsequently sub-inoculated into a total volume of 40 mL THBY and grown overnight at 37ºC. On the day of challenge, this 40 mL overnight culture was used to inoculate 200 mL THBY which was grown at 37ºC until an OD_{600nm} of 0.6 was reached, at which point the bacterial culture was centrifuged in a Heraeus® Megafuge® 1.0 R centrifuge (Kendro, Germany) for 20 min at 10,000 x g. Following centrifugation, the supernatant was discarded and the pellet washed twice in 35 mL of 0.7% (w/v) saline and centrifuged as previously stated. Based on previous bacterial counts at this growth phase, an inoculum in 0.7% (w/v) saline containing 1×10^9 cfu/mL of 5448AP was prepared. A 100 μL volume of inoculum was subcutaneously administered to a shaved region on the back of the mouse. Following infection, a serial dilution of the inoculum was prepared in sterile 96-well microtitre plates (Greiner Bioone, Germany), plated in triplicate on HBA agar plates (BioMérieux, Australia) and grown overnight at 37ºC to determine the dose of GAS administered. Following infection, mice were monitored for a period of ten days and deaths recorded. Kaplan-

Meier survival curves were generated in GraphPad™ Prism 4.2 (GraphPad Software Inc., USA) and log-rank tests carried out to determine which antigens conferred significant levels of protection ($P < 0.05$) in comparison to PBS sham immunised mice.

2.17   Donor Screen for Opsonophagocytosis Killing Assay

The GAS isolate 5448 was grown to stationary phase and serially diluted 10-fold in PBS. Normal mouse sera (NMS) was heat inactivated at 60ºC for 25 min. A 50 µL volume of 1×10^{-4} and 1×10^{-5} dilutions of 5448 GAS was pre-incubated with 50 µL of heat inactivated NMS in duplicate for 20 min at room temperature. A 400 µL volume of heparinised human blood from prospective donors was added and the mixture was incubated end on end for 3 h at 37ºC. Following the incubation, a 50 µL volume of the mixture was plated with Columbia agar supplemented with 2% (v/v) horse blood using the pour plate technique. The agar plates were incubated at 37ºC overnight and GAS colonies were counted the following day. A donor was determined to support the growth of the GAS strain if the number of GAS surviving in their blood following the 3 hour incubation was equal to or greater than 32 times the number of CFU/mL in the original inoculum.

2.18   Opsonophagocytosis Killing Assay

The GAS isolate 5448 was grown to stationary phase and a 5×10^{-5} dilution was prepared in PBS. Based on the donor screen, this dilution was the most suitable for counting. The opsonophagocytosis killing assay was essentially performed as described in section 2.17 using a 5×10^{-5} dilution of 5448 with specific polyclonal mouse anti-sera raised against test antigens, α-M1 serum as the positive control and serum from mice sham immunised with PBS as the negative control; and heparinised human blood obtained from a single donor previously determined to support the growth of 5448 GAS. The survival of GAS incubated with the test sera was presented as a percent in comparison to the survival of GAS incubated with sera from mice sham immunised with PBS. Two biological replicates were undertaken each of which were plated in quadruplicate. This experiment was performed by Mr. Graham Magor and Dr. Michael Batzloff, Queensland Institute of Medical Research, Australia.

2.19   Hyaluronic Acid Capsule Determination

A 4 mL volume of THBY was inoculated with a single colony of GAS (5448 and 5448AP strains) and grown overnight at 37ºC without shaking. A 2 mL volume of

the overnight culture was diluted into 23 mL of pre-warmed THBY and grown to the early logarithmic growth phase (OD_{600nm} 0.5 − 0.6). To enable determination of cfu/mL a serial dilution (10-fold) was prepared in sterile 0.7 % (w/v) saline and each dilution, 10^{-1} to 10^{-8}, was plated in triplicate on HBA plates and grown overnight at 37ºC. In the meantime, 10 mL of overnight culture was centrifuged at 5,000 x g for 10 min at room temperature using the Hereaus® Megafuge® 1.0 R centrifuge (Kendro, Germany). The supernatant was discarded and the cell pellet resuspended in 500 μL of sterile Milli-Q water and the hyaluronic acid capsule released during shaking with 1 mL chloroform for 30 sec. The hyaluronic acid capsule (aqueous layer) was obtained following centrifugation at 16,000 x g for 5 min at room temperature using an Eppendorf 5415C microcentrifuge (Eppendorf, Germany). The amount of hyaluronic acid capsule extracted was quantified using previously established methods (Schrager et $al.$, 1996). In short, a 75 μL aliquot of capsule sample or hyaluronic acid standard, hyaluronic acid sodium salt from $Streptococcus$ $equi$ (0.5 mg/mL in sterile milli-Q water, Sigma, USA), were added to a microtitre plate in triplicate and diluted two-fold in milli-Q water. Following the addition of 100 μL of l-ethyl-2-[3-(l-ethyl-naphtho[l,M]thiazolin-2-ylidene)-2-methylpropenyl]naphtho[l,2d] thiazolium bromide (Stains-All Solution) (Appendix I) to all wells, the absorbance at 640 nm was immediately read using a SpectraMax® 250 microtitre plate reader (Molecular Devices, USA). The amount of hyaluronic acid extracted from each strain was determined following interpolation from the standard curve (using SOFTMax® Pro Version 1.1 software; Molecular Devices, USA) and fg capsule per cfu was calculated. All hyaluronic acid capsule assays were performed in triplicate and the data presented is the average and SEM of the three replicates.

2.20   SpeB Characterisation of Strains

2.20.1 Columbia Skim Milk Agar Plate Assays

To visually examine the SpeB activity of GAS strains, the Columbia skim milk agar plate-based assay was utilised (Ashbaugh et $al.$, 1998). Four individual 5448 and 5448AP colonies were transferred with sterile toothpicks to Columbia Skim Milk Agar plates, in the presence and absence of N-[N-(L-3-transcarboxyirane-2-carbonyl)-L-Leucyl]agmatine (E64) at a concentration of 28 μM (Appendix I) and incubated for 48 h at 37ºC. SpeB-positive colonies were observed to have a ring of precipitate around the site of inoculation. The results presented are representative of the four individual colonies assessed.

2.20.2 Collection of GAS Culture Supernatants

A 2 mL volume of THBY was inoculated with a single colony of GAS (5448 or 5448AP) and grown overnight at 37ºC. The overnight culture was sub-inoculated into 100 mL THBY and grown for 24 h at 37ºC under stationary conditions. The culture was spun in a Beckman JA-10 rotor (Beckman, USA) at 12,000 x g for 20 min at 4ºC. The supernatant was filtered using a 0.22 μm Millex syringe driven filter device (Millipore, Australia), 85 mL of ice cold 10% (v/v) TCA was added and the tube mixed by inversion and incubated on ice for 1 h. The suspension was centrifuged in a Beckman JA-10 rotor (Beckman, USA) at 10,000 rpm for 15 min at 4ºC, the supernatant discarded and the pellet air dried in a laminar flow cabinet for 1 h. The pellet was resuspended in 1 mL of 100 mM Tris (pH 7.6) and 1 mL 10% (v/v) TCA. The tube was vortexed and incubated on ice for 20 min. The sample was centrifuged at 16,000 x g for 15 min at room temperature using an Eppendorf 5415C microcentrifuge (Eppendorf, Germany) and the supernatant discarded. The pellet was washed with 500 μL of ice cold 100% (v/v) ethanol and centrifuged as above for 5 min. The supernatant was discarded and the pellet air dried in the flow hood for 30 min and resuspended in 100 μL of 100 mM Tris (pH 7.6).

2.20.3 SpeB Western Blots

Following the collection of culture supernatants, the supernatants were mixed with 5X Cracking Buffer (Appendix I) and appropriate volumes loaded onto 12% SDS-PAGE reducing gels. To detect the presence of SpeB in the culture supernatants, western blotting was performed using a 1:1,000 dilution of affinity-purified rabbit α-SpeB IgG (Toxin Technology Inc., USA). Western blotting was undertaken as described in section 2.12.

2.21 Confocal Microscopy

A 5 mL volume of THBY was inoculated with a single colony of GAS isolate 5448 and grown overnight at 37ºC without shaking. The following day, the cultures were centrifuged for 5 min at 3,000 x g in an Eppendorf 5810R Swing-bucket rotor centrifuge at 4ºC (Eppendorf, Germany) and the supernatant discarded. The pellet was washed twice using 5 mL sterile PBS, and centrifugation performed as previously stated. Following the washes, the pellet was resuspended in PBS to a final OD_{600nm} of 0.4. A wax circle with a diameter of approximately 1.2 cm was drawn on a Polysine

slide (Erie Scientific, USA) with an ImmEdge pen (Vector Laboratories, USA). Ten microlitres of GAS suspension was spread within the wax circle using a yellow pipette tip and the bacteria air dried onto the surface of the slide. The bacteria were fixed onto the slide by incubation in 3% (w/v) paraformaldehyde/PBS (10 min) in a Wheaton slide staining dish, followed by washing with PBS for 10 min. Each wax circle was blocked with 150 μL of 3 % (w/v) bovine serum albumin (BSA)/PBST at 37ºC for 30 min and then washed twice with PBS as previously indicated. Next, non-specific human IgG was used to block general Ig binding sites on the surface of the bacteria, a 1:200 dilution of human IgG was prepared in 0.3% (w/v) BSA/PBS and 150 μL was added within the wax circles on each slide and incubated for 1 h at 37ºC. Unless otherwise stated, all antibodies were diluted in 0.3% (w/v) BSA/PBS. Following two PBS washes, a 150 μL volume of specific pooled murine test anti-serum ($n = 10$) diluted 1:200 was applied to each wax circle and incubated overnight at 4ºC. Pre-immune serum (collected from mice prior to immunisation) and final bleed serum (collected from mice following the completed immunisation regime) specific for individual vaccine antigen was used. PBS washes were performed in triplicate and secondary antibody was applied to the slide. Goat anti-mouse IgG-FITC (Zymed, USA) was diluted 1:200 and a 150 μL volume was applied within each wax circle and incubated for 2 h at room temperature. Slides were washed thrice with PBS and 10 μL of Vectashield (Vector Laboratories, USA) was applied to each slide to mount glass coverslips which were subsequently adhered to the slide using clear nail polish. A Leica TCS SP confocal microscope mounted on a Leica DM IRBE inverted microscope (Leica Microsystems, Germany) configured for the detection of FITC (excitation: 488 nm; emission: 520 nm) was used to view stained cells. Using a 100X/1.0 FLUOTAR PL oil immersion objective lens, fluorescent and transmission images were obtained.

2.22 Electron Microscopic Analyses

2.22.1 Protein A Purification of Polyclonal Rabbit Anti-serum

A 2 mL volume of specific polyclonal rabbit serum was applied to a 2 mL protein A sepharose column (Sigma Aldrich, USA) previously equilibrated with PBS. The UV absorbance of the column elutions and flow-through was monitored during the purification process. Following the application of serum, PBS was applied to the column to wash away excess serum until the absorbance of the column flow-through had returned to the baseline level. To elute the antibody, a 1.2 mL volume of 0.1 M

glycine (pH 3.0) was used, eluate was collected in 2 mL microcentrifuge tubes containing 100 μL of 1M phosphate buffer. The elution fractions containing the most protein (as observed from the UV absorbance) were pooled and aliquoted into 200 μL volumes and stored at − 20ºC.

2.22.2 Transmission Immunogold Electron Microscopy (TEM)

Immunogold electron microscopy analyses were expertly performed by Dr. Manfred Rohde at the Department of Microbial Pathogenesis, HZI, Braunschweig, Germany. A 10 mL volume of THBY was inoculated with a single colony of 5448 GAS of interest and grown overnight at 37ºC. Following overnight culture, 100 μL of the settled bacteria was transferred to a 1.5 mL microcentrifuge tube and centrifuged at 5,500 x *g* for 2 min in an Eppendorf microcentrifuge (Eppendorf, Germany). The supernatant was discarded and the pellet resuspended in 100 μL of a 1:50 dilution of protein A purified test antibody (in PBS) and incubated for 1 h at 30ºC. Pellets were washed three times with a 1 mL volume of PBS and centrifuged under the same conditions. Following washes, the pellet was resuspended in 100 μL of PBS containing 0.5 mg/mL PEG 20,000, 5 μL protein A gold particles (15 nm in diameter) was added and the solution incubated for 30 min at 30ºC. Samples were washed thrice with PBS and fixed with 2% glutaraldehyde and 3% formaldehyde for 15 min at room temperature and then washed once with PBS. A 2 μL volume of resuspended sample was incubated a plastic coated copper grid for 5 min, washed with water and air dried. Samples were directly viewed using a transmission electron microscope (Zeiss EM 910 at an acceleration voltage of 80 kV). Images were recorded with a Slow Scan CCD camera.

2.23 Flow Cytometry

Flow cytometry was utilised to confirm surface localisation of vaccine antigens on 5448 GAS via fluorescent staining of specific mouse anti-sera. All solutions for flow cytometry were sterile filtered through a 0.22 μm Millex syringe driven filter (Millipore, Australia) to ensure the removal of any particulate matter. A 10 mL volume of THBY was inoculated with a single colony of 5448 GAS and the culture grown overnight at 37ºC without shaking. The following day, the 10 mL overnight culture was sub-inoculated into 30 mL of pre-warmed THBY which was grown at 37ºC to an OD_{600} of 0.6. GAS cells were centrifuged in a Heraeus® Megafuge® 1.0 R centrifuge (Kendro,

Germany) at 8,000 x *g* for 10 min. Following each resuspension step, bacteria were thoroughly vortexed to promote breakage of streptococcal chains. The supernatant was discarded, the pellet was resuspended in 50 mL of ice-cold PBS, re-centrifuged and the supernatant discarded. Bacteria were resuspended in 25 mL ice cold PBS and hence concentrated two-fold. Triplicate 1 mL aliquots of GAS were pipetted into 1.5 mL microcentrifuge tubes for each test serum, α-M1 serum (positive control), serum from mice immunised with PBS (negative control) and samples containing no primary antibody (control for background). GAS aliquots were centrifuged at 7,500 x *g* for 10 min in an Eppendorf 5415C microcentrifuge (Eppendorf, Germany) at room temperature. The supernatant was discarded and each pellet resuspended 200 μL of 3% (w/v) BSA/PBST (Appendix I) and incubated for 1 h on ice for blocking. GAS cells were re-centrifuged, the supernatant discarded, the cell pellet resuspended in 100 μL of non-specific human IgG diluted 1:150 in 0.3% (w/v) BSA/PBST and incubated on ice for 1 h. The human IgG was utilised to block IgG-binding proteins on the surface of the GAS prior to subsequent incubations with test serum and secondary antibody. The GAS cells were then re-centrifuged, the supernatant discarded and the cells washed by resuspending the cell pellets in 500 μL of ice cold PBS (PBS was step). The cells were re-centrifuged, the supernatant discarded, the cell pellet resuspended in 100 μL of specific mouse anti-serum diluted 1:50 in 0.3% BSA/PBST and incubated overnight at 4ºC. Following the incubation with primary serum, the cells were subjected to two PBS washes as previously described. Following washing, the supernatant was discarded, the cells were resuspended in a 100 μL volume of goat anti-mouse FITC conjugated IgG (Zymed, USA) diluted 1:150 in 0.3% (w/v) BSA/PBST and incubated on ice for 1 h. GAS cells were then washed twice with PBS. Following the wash, the supernatant was discarded, the cells were resuspended in 100 μL of ice cold PBS and 100 μL of 4% (w/v) paraformaldehyde/PBS was subsequently added. The cells were stored at 4ºC overnight to fix the GAS. Prior to cell counting using a Becton Dickson FACSort flow cytometer (Becton Dickson, USA) using CellQuest software (version 3.1f), 800 μL of PBS was added to each sample and the samples transferred to 5 mL round-bottom polystyrene flow cytometry tubes (Becton Dickson, USA). The FACSort was set as follows: FSC E00, 9.000 log scale; SSC 443, 1.00, log scale; and FL1, 999, 1.00, log scale. Data from 10,000 events were collected for each sample and triplicate tubes of each anti-serum were counted. Cell-surface-associated FITC-fluorescence (excitation: 488 nm; emission: 520 nm) was determined using a uniform population of gated cells

which excluded non-cellular debris. FlowJo software (version 7.2.5) was used to prepare histograms displaying FITC-fluorescence and to determine the geometric mean under the curve. Specific antibody binding was calculated by subtracting the average geometric mean of the no primary antibody control from the test sample. The histograms representing the surface labelling with the test serum were compared to the histogram of the serum from mice sham immunised with PBS using an unpaired t-test with a confidence interval of 95%.

2.24   Analysis of Peptide Array Membranes

Overlapping peptide spot-membranes of ADI, KPR and TF were constructed from an acid-stable AC-S01 type amino-PEGylated cellulose membrane (AIMS-Scientific-Products GmbH, Germany) (Beutling *et al.*, 2008). A series of 15-mer synthetic peptides were assembled at defined intervals on each membrane. The first peptide spot, positioned at the top left of the membrane corresponded to the first 15 amino acids at the N-terminus of the vaccine antigen. The second spot contained the last 12 amino acids contained in 'spot one' with the next three amino acids downstream. This pattern was repeated along the membrane until the C-terminus of each antigen was reached. The amino acid composition of each peptide spot on each of the membranes is given in Appendix II. The membranes were rehydrated with 100% (v/v) ethanol, washed with Tris-buffered saline (TBS) (Appendix I) for 10 min and blocked overnight at 4ºC with membrane blocking solution (MBS) (Appendix I). Membranes were washed for 10 min with TBS with 0.05% Tween-20 (TBST) (Appendix I) and probed with a pool of mouse anti-serum specific for each vaccine antigen diluted 1:100 in MBS for 3 h with gentle rocking at room temperature. Murine pre-immune and final bleed serum was tested for the subcutaneous immunisation regime described in section 2.15.1. Following three TBST washes, the membranes were incubated with a 1:2,000 dilution of goat anti-mouse IgG alkaline phosphatase conjugate (Sigma, USA) for 1.5 h at room temperature. Two TBST washes were carried out, followed by two 10 min washes with citrate buffered saline (CBS) (Appendix I). The membranes were developed with colour developing solution (CDS) (Appendix I) for 10 min and the reaction terminated by washing with PBS. The developed membrane arrays were scanned using a GS-800™ calibrated densitometer (Bio-Rad, USA). Following reaction of the first test serum, the membranes were stripped ready for reaction with control sera. In short, the membranes were washed twice with water (10 min) and incubated in an excess of

dimethylformamide (DMF) until the blue colour of spot signals dissolved. All wash steps were 10 min unless otherwise stated. The DMF step was repeated, followed by three water washes. Membranes were then washed three times with Stripping Mix A (Appendix I) in a sonication bath at 40ºC, followed by washing three times with Stripping Mix B (Appendix I). Finally, the membranes were subjected to three washes with 100% (v/v) alcohol.

2.25 Cross-Reactivity of Vaccine Antigens with Human Sera Obtained from Populations Suffering Endemic GAS Infection

The reactivity of vaccine antigens with human disease serum was tested using serum from two geographically distinct populations suffering endemic GAS infection. The vaccine antigens were analysed using both western blot and ELISA with a pool of serum obtained from an Australian Aboriginal population. Collection of sera complied with institutional human ethics. Pooled serum from Aboriginal adults ($n = 7$) and from school-aged Aboriginal children ($n = 30$) living in remote communities in northern Australia was obtained from the Menzies School of Health Research, Darwin, Northern Territory, Australia. GAS infections are known to be endemic in these populations and an incidence of 651 cases of acute rheumatic fever are estimated to occur per 100,000 individuals (Carapetis *et al.*, 1996). The serum from both Aboriginal adults and children diluted 1:1,000 in 0.5% skim milk/PBS was incubated with 2μg of each recombinant vaccine antigens immobilised on western blots (as described in section 2.12). Reactivity between the pool of serum from Aboriginal children ($n = 30$) and each of the candidate antigens was subsequently tested in ELISA. The pooled serum was added to the top row of a microtitre plate at a 1:100 dilution (diluted in 0.5% (w/v) skim milk/PBS). This test serum was diluted two-fold down the plate, with a final dilution of 1:12,800 (1:3,276,800 in the case of M1 protein which was diluted over several microtitre plates). Recombinant M1 protein incubated with a 1:200 dilution of the Aboriginal test serum was used to normalise the data between different microtitre plates. The serum-specific IgG antibody titre was defined as the lowest dilution of test serum with an absorbance equal to or greater than 0.2. A one-way ANOVA (Dunnett's post test; $P < 0.05$) was performed using GraphPad™ Prism 4.2 (GraphPad Software Inc., USA) to compare the serum-specific IgG titre of the candidate antigens to the widely characterised immunoreactive M1 protein.

The second group of human disease serum was collected from a population suffering endemic infection in Chandrigarh, India. The serum was pooled from teenagers in the following clinical disease groupings; healthy individuals ($n = 10$), pharyngitis ($n = 8$), ARF patients ($n = 9$) and rheumatic heart disease (RHD) patients ($n = 10$). Each of these four pooled sera was tested in ELISA against each of the vaccine antigens to determine the serum-specific IgG antibody titre against each antigen. ELISA technique essentially as described in section 2.15.3, each candidate antigen was tested in quadruplicate across microtitre 96-well plates. Both recombinant M1 protein incubated with a 1:1,600 dilution of the RHD serum in duplicate and the plate blank (duplicate wells incubated with all reagents except the test serum) were used to normalise the data between different microtitre plates. Reactions in wells were developed using 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) tablets (Roche, USA) incubated at 37 ºC for 30 min. The absorbance of each well was subsequently measured at 405 nm and was recorded. The titre was defined as the lowest dilution of test serum with an absorbance equal to or greater than 0.2. A one-way ANOVA (Dunnett's post test, $P < 0.05$) was performed in GraphPad™ Prism 4.2 (GraphPad Software Inc., USA) to compare the serum-specific IgG titre of the candidate antigens to the widely characterised immunoreactive M1 protein for each of the disease serum groups.

2.26   Cross-reactivity of Antigen Sera with Human Heart Extract

Human heart tissue (5 g) was provided by Prof. Maisch and Dr. Pankuweit from the University Hospital Marburg. The tissue was homogenised in 5 mL of PBS containing complete protease inhibitor cocktail (Roche, Germany) using a PolyTron PT2100. Debris was removed by centrifugation at 12,000 x g for 30 min at 4°C. The reactivity between human heart proteins and test serum from rabbits immunised with individual recombinant antigens was tested by ELISA. 96-well plates were coated with 20 µg/mL of the human heart extract in 0.1 M carbonate coating buffer (pH 9.6) and blocked with 1% (w/v) gelatin in PBS. The test serum was diluted two-fold starting with a 1:10 dilution in 1% (w/v) gelatin. Naive rabbit serum was utilised as a negative control. The secondary antibody, goat anti-rabbit IgG-HRP (Dianova), was diluted 1:3,000 in 1% (w/v) gelatine. Two biological replicates were undertaken using duplicate samples. This experiment was performed by Dr. Christine Gillen and Dr. Marcus Fülde, HZI, Germany. Collection and handling of human tissue complied with institutional human ethics.

3. SELECTION, EXPRESSION AND PURIFICATION OF GAS VACCINE ANTIGENS

3.1 Introduction

Currently, there is no commercial vaccine formulation available to prevent GAS infection. One GAS vaccine based on small fragments of the highly variable serotype specific portion of the M protein from 26 GAS serotypes has progressed to testing in human clinical trials (McNeil *et al.*, 2005). However, it is probable that this formulation may only provide protection against the included 26 GAS serotypes and will not elicit broad spectrum protection against the $\geq$ 120 known serotypes of GAS. To achieve global eradication of GAS infection, there is an obvious need for a vaccine capable of eliciting protection independent of serotype. It is possible that highly conserved surface antigens may provide one such mechanism of serotype independent protection.

A recent proteomic analysis of the GAS cell wall fraction identified 66 novel putative cell wall-associated proteins amongst three different strains of GAS: NS931 (*emm69)*, NS13 (*emm*53) and S43 (*emm*6) (Cole *et al.*, 2005). In this study the major cell wall-associated proteins of GAS were identified using a 2D SDS-PAGE proteomic analysis of mutanolysin cell wall extracts prepared using the method of Ji *et al.* (1998). In this technique, mutanolysin cleaves the β-1,4 linkage of the highly conserved *N*-acetylmuramyl-*N*-acetylglucosamine in the peptidoglycan-polysaccharide cell wall polymer resulting in the separation of protoplasts and cell wall extracts (Siegel *et al.*, 1981). Initially, protein spots were excised from the Coomassie blue stained 2D PAGE gel, digested with trypsin, and identified using standard peptide mass fingerprinting (PMF) techniques and MALDI-TOF MS (Cole *et al.*, 2005). Secondly, to determine which of the newly identified cell wall-associated proteins were also surface exposed, the surface of intact GAS cells was biotinylated prior to the preparation of mutanolysin extracts, which were then resolved by 2D SDS-PAGE and detected using western blot analysis (Cole *et al.*, 2005). A total of 23 proteins were detected on the western blot using a streptavidin-HRP conjugate and were consequently assumed to be exposed on the bacterial surface.

A similar proteomic analysis assessed cell wall extracts obtained from the M1T1 GAS isolate, 5448 (Cole, 2006) with the intention of identifying proteins which may be novel GAS vaccine candidates. Using a method analogous to that adopted by Cole *et al.*

(2005), this study identified cell wall-associated proteins and determined which of these proteins were surface exposed on the 5448 GAS isolate (Cole, 2006). A representative 2D SDS-PAGE gel of a mutanolysin extract of the 5448 GAS isolate grown in the presence and absence of E64 (a cysteine protease inhibitor) is depicted in Figure 3.1. The GAS cysteine protease, SpeB, has been reported to degrade many of the proteins in the M1T1 secreted proteome (Aziz *et al.*, 2004), thus, in an attempt to identify all cell wall-associated proteins of the SpeB positive GAS isolate 5448, E64 was used to inhibit the proteolytic actions of SpeB. A total of 30 individual protein spots were excised from the PAGE gel (Figure 3.1) and identified by Cole (2006) (Table 3.1). It is thought that cell wall-associated, surface exposed proteins identified in the proteomic studies performed by Cole *et al.* (2005) and Cole (2006), highlighted in Table 3.1, may be viable GAS vaccine antigens. Thus, immunisation with such proteins may protect against subsequent GAS infection. Additionally, details of proteins identified in the heterologous GAS isolates NS931, NS13 and S43 analysed in Cole *et al.* (2005) and the selection criteria applied when considering possible candidate vaccine antigens is summarised in Table 3.1. Ideally, proteins that were observed to be cell wall-associated in each of the four GAS strains analysed by Cole *et al.* (2005) and Cole (2006) (NS931, NS13, S43, 5448), were selected for further characterisation in the work described in this thesis. Furthermore, exposure on the cell surface was also a desirable trait for putative vaccine antigens. Finally, the amino acid identity with the human proteome was also investigated. The proteins most suitable for inclusion in this project as vaccine candidates have zero or a low percent identity (≤ 51 %) with individual proteins within the human proteome. A number of the selected candidate antigens, ADI, FBA, KPR and TF were determined to have no homology in the human proteome or possess a human homolog which does not share the same functionality as the GAS antigen.

The candidate GAS vaccine antigens selected for inclusion in this study are arginine deiminase (ADI), adenylate kinase (AK), branched chain amino-acid aminotransferase (BCAT), elongation factor Tu (EF-Tu), fructose-bisphosphate aldolase (FBA), ketopantoate reductase (KPR), NADP-dependent glyceraldehyde-3-phosphate dehydrogenase (NADP-GAPDH), orthinine carbamoyltransferase (OTCase), 6-phosphofructokinase (PFK), phosphoglycerate kinase (PGK), ribosome recycling factor (RRF), trigger factor (TF), and triosephosphate isomerase (TIM). It is interesting to note that many of these vaccine antigens also possess enzymatic functions, with several of the

Figure 3.1 Two-dimensional gel electrophoresis profiles of mutanolysin cell wall extracts from the 5448 GAS isolate grown in the presence and absence of cysteine protease inhibitor, E64. The extracts were harvested after growth to late stationary phase (37°C for 16 h) in THBY without shaking. The protein extracts (170 μg) were isoelectric focused over a linear pH gradient of 4 to 7 and resolved with a 12.5% SDS-PAGE gel. The gels were stained with colloidal Coomassie blue and destained in 1% (v/v) acetic acid. Protein spots were identified by peptide mass fingerprinting are indicated with numbered arrows. Molecular mass markers are given in kilodaltons (kDa). Proteins that were selected for further characterisation in this project are highlighted in grey in Table 3.1. Two-dimensional gel electrophoresis performed by Dr. Jason Cole, PhD thesis, University of Wollongong, 2006.
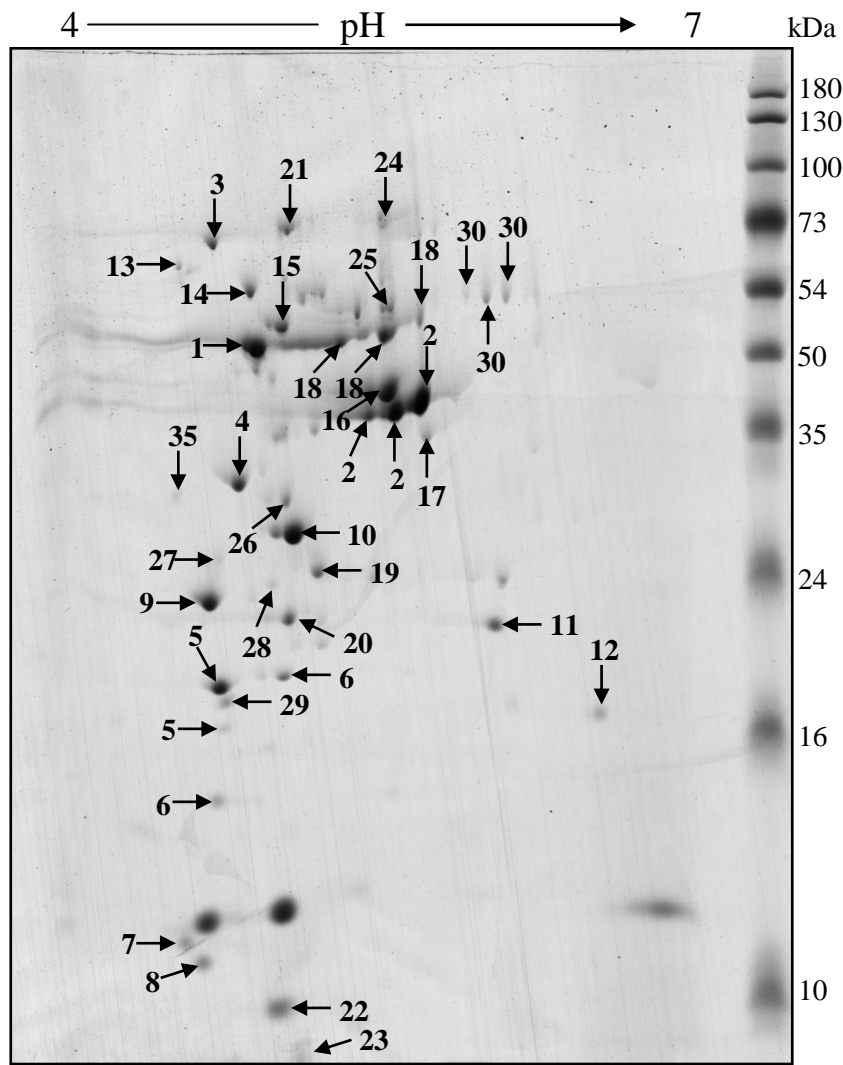
5448 + E64
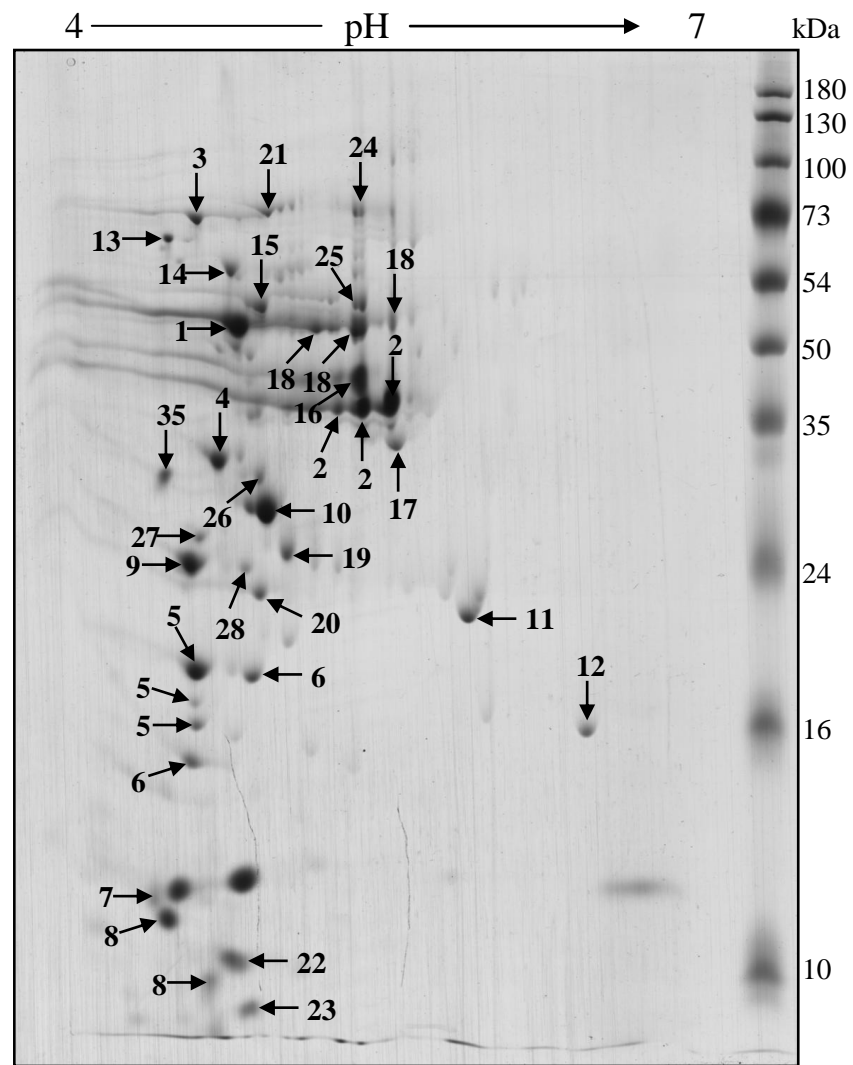
5448 - E64

Table 3.1 Selected cell wall-associated proteins identified in mutanolysin extracts of GAS strains NS931, NS13, S43 and 5448 by MALDI-TOF peptide mass fingerprinting analysis. Proteomic analysis performed by Dr. Jason Cole, PhD thesis, University of Wollongong, 2006. + indicates the presence of the protein; - indicates an absence of the protein. Shading indicates the proteins selected for characterisation in this thesis.

Protein	Arrow Number [a]	Cell wall-associated [b]				Cell surface-associated [c]				Human homolog [d]	
		NS931	NS13	S43	5448	NS931	NS13	S43	5448	Accession no. [f, g]	Identity (%) [h]
Enolase (SEN)	1	+	+	+	+	+	+	+	+	P09104	49
GAPDH	2	+	+	+	+	+	+	+	+	P04406	44
Chaperone protein DnaK (HSP70)	3	+	+	+	+	+	+	+	+	P38646	51
Putative carbamate kinase	4	+	+	+	+	+	+	+	+	N/A	0
Putative alkyl hydroperoxidase	5	+	+	+	+	+	+	-	+	P32119$^{\Omega}$	39
General stress protein, Gls24 family	6	-	-	-	+	-	-	-	+	N/A	0
Putative thioredoxin	7	-	-	-	+	-	-	-	-	B4DZQ3$^{\Omega}$	41
LSU ribosomal protein L12P	8	-	-	-	+	-	-	-	-	N/A	0
Triosephosphate isomerase (TIM)	9	+	+	+	+	+	+	+	+	P60174	40
Fructose-bisphosphate aldolase (FBA)	10	+	+	+	+	+	+	+	+	N/A	0
Ribosome recycling factor (RRF)	11	+	+	+	+	-	-	-	-	Q96E11	28
Putative phosphotransferase system (PTS), enzyme II component B	12	-	-	-	+	-	-	-	-	N/A	0
Trigger factor (TF)	13	+	+	-	+	-	-	-	+	Q02790[†]	23
60 kDa chaperonin (GroEL)	14	+	+	+	+	+	+	+	+	P10809	47
Putative dipeptidase	15	+	+	+	+	+	-	+	+	Q96KP4$^{\Omega}$	43
Orthinine carbamoyltransferase	16	+	+	+	+	+	+	+	+	P00480	40
6-phosphofructokinase (PFK)	17	+	+	+	+	-	-	+	-	Q01813	37
Arginine deaminase (ADI)	18	+	+	+	+	+	-	+	+	N/A	0
Purine nucleoside phosphorylase	19	+	+	+	+	-	-	+	+	P00491$^{\Omega}$	45
Superoxide dismutase (Mn)	20	+	+	+	+	+	-	-	-	P04179	50
Putative transketolase	21	+	+	+	+	+	+	+	+	P29401$^{\Omega}$	33
10 kDa chaperonin (GroES)	22	-	-	-	+	-	-	-	-	P61604	32
30S ribosomal protein S6	23	-	-	-	+	-	-	-	-	B9ZVW4$^{\Omega}$	25
Phosphoenolpyruvate-protein phosphotransferase	24	-	-	-	+	-	-	-	-	N/A	0

Table 3.1 Selected cell wall-associated proteins identified in mutanolysin extracts of GAS strains NS931, NS13, S43 and 5448 by MALDI-TOF peptide mass fingerprinting analysis. Proteomic analysis performed by Dr. Jason Cole, PhD thesis, University of Wollongong, 2006. + indicates the presence of the protein; - indicates an absence of the protein. Shading indicates the proteins selected for characterisation in this thesis.

Protein	Arrow Number [a]	Cell wall-associated [b]				Cell surface-associated [c]				Human homolog [d]	
		NS931	NS13	S43	5448	NS931	NS13	S43	5448	Accession no. [f, g]	Identity (%) [h]
NADP-dependent GAPDH	25	+	+	+	+	+	+	-	+	P47895[Ω]	32
Ketopantoate reductase (2-dehydropantoate 2-reductase) (KPR)	26	+	-	+	+	-	-	-	-	N/A	0
Protein phosphatase 2C	27	-	-	-	+	-	-	-	+	Q8N819	23
Adenylate kinase (AK)	28	+	-	+	+	-	-	-	-	P54819	39
Transcription elongation factor GreA	29	-	-	+	+	-	-	-	-	N/A	0
M protein	30	+	+	+	+	-	-	-	-	Q15149[†]	21
Branched chain amino-acid aminotransferase (BCAT)	NA [e]	+	-	+	-	-	-	-	-	P54687	32
Elongation factor Tu (EF-Tu)	NA	+	-	+	-	+	-	+	-	P49411	51
Phosphoglycerate kinase (PGK)	NA	+	+	+	-	-	+	+	-	P00558	43

a Numerical designation of the protein spot in the representative gel (Figure 3.1).

b Proteins identified by PMF in mutanolysin extracts.

c Proteins identified as surface exposed following biotinylation of whole intact GAS cells.

d Top match identified by BLAST-P searches of the *Homo sapiens* database (using an E threshold of 1) at the ExPASy/SIB BLAST network service (http://au.expasy.org/tools/blast/).

e Protein not identified in the representative 2D gel of the 5448 isolate (Figure 3.1), but identified in either NS931, NS13 or S43 GAS isolates shown in Cole *et al*. (2005).

f Swiss-Prot or TrEMBL accession number.

g N/A (not applicable) indicates a human homolog was not identified.

h GAS proteins without a known human homolog have zero percent identity.

† Human homolog identified using BLAST-P search does not have the same function as GAS protein.

Ω GAS or human protein has unknown function.

prospective antigens performing a key role in metabolism and other crucial housekeeping roles.

In order to determine the protective efficacy, confirm the surface localisation and examine the cross-reactivity of these putative surface proteins with human serum obtained from patients suffering endemic GAS infection, a total of 13 GAS vaccine antigens were expressed as hexahistidyl-tagged recombinant proteins. This chapter outlines the strategies adopted for the cloning, expression and purification of the vaccine antigens and the surveillance of gene presence and conservation amongst sequenced GAS genomes and representative GAS isolates.

3.2   Results

3.2.1   Gene Sequences Encoding GAS Vaccine Antigens

To design primers to amplify the genes encoding the 13 GAS vaccine antigens the entire gene sequence for each was obtained from Genbank (http://www.ncbi.nlm.nih.gov/). The full protein names (and alternatives), gene designation and Genbank accession numbers are listed in Table 3.2. None of the 13 vaccine antigens contain the traditional LPXTG cell wall anchor motif commonly found in Gram-positive bacterial surface proteins. Additionally, the SignalP protein server which predicts signal peptide cleavage sites (available at http://au.expasy.org/), did not

Table 3.2 Genbank accession numbers, ORF numbers and gene name designations for the genes encoding the 13 GAS vaccine antigens.

Protein Name	Gene Name	Accession Number	ORF Number
Arginine deiminase	*sagP*	AF468045	NA
Adenylate kinase	*adk*	AE014137	SpyM3_0061
Branched chain amino-acid aminotransferase	*bcaT*	AE014149	SpyM3_0626
Elongation factor Tu	*tufa*	AE014145	SpyM3_0432
Fructose-bisphosphate aldolase	*fba*	AE006614	SPy1889
Ketopantoate reductase (2-dehydropantoate 2-reductase)	*apbA*	AE010020	spyM18_0911
NADP-dependent glyceraldehyde-3-phosphate dehydrogenase	*gapN*	AE014157	SpyM3_1045
Orthinine carbamoyltransferase	*arcB*	AE014159	SpyM3_1194
6-phosphofructokinase	*pfk*	AE010047	spyM18_1231
Phosphoglycerate kinase	*pgk*	AE014167	SpyM3_1624
Ribosome recycling factor	*rrf*	AE009989	spyM18_0506
Trigger factor	*ropA*	AE014167	SpyM3_1634
Triosephosphate isomerase	*tpi*	AE006516	SPy0613

recognise signal peptide sequences within any of the genes encoding the 13 vaccine antigens. Thus, primers were designed to amplify the full gene sequence with no truncations.

3.2.2 Sequence Identity Between GAS Vaccine Antigens & Human Proteins

The amino acid identity shared between the 13 GAS vaccine antigens and the human proteome (indicated in Table 3.1) is a simplistic indication of possible shared epitopes between the vaccine antigens and host proteins. Shared epitopes may result in the production of auto-antibodies which react with vaccine antigens and also host proteins and tissue. If there is no significant amino acid identity, it is likely there are no shared epitopes. Conversely, the higher the amino acid identity the greater the likelihood of shared epitopes. ADI, FBA, KPR and TF were determined to have either no significant amino acid identity with the proteins of the human proteome or to possess a human homolog which has a different function (Table 3.1). It is likely that human homologues with differing function possess a different structure to the GAS antigens, therefore, in such cases sequence identity is unlikely to equate to shared epitopes. Whilst the remaining nine GAS vaccine antigens of this study share some sequence identity with the human proteome, none of the selected candidate antigens possess greater than 51% amino acid identity (Table 3.1).

3.2.3 Percent Conservation of GAS Vaccine Antigens Amongst Sequenced GAS Genomes

A high level of conservation of the genes encoding antigens is crucially important when considering putative GAS vaccine candidates. Conservation of vaccine antigens at a genetic level is likely to enable heterologous broad spectrum protection against GAS infection. At the time of analysis, there were 12 complete sequenced GAS genomes and one partially sequenced genome at NCBI, http://www.ncbi.nlm.nih.gov/. GAS isolates which have been sequenced generally have a high rate of association with human infection. Thus, a high level of conservation of vaccine antigen genes among GAS serotypes would be necessary if serotype independent protection is to be achieved. The nucleotide identity between the genes encoding each of the GAS vaccine antigens of this study amongst the 13 sequenced GAS genomes is shown in Table 3.3. A very high level of gene conservation is observed amongst the different GAS serotypes for each of the 13 vaccine antigens with a minimum 98% identity (Table 3.3). Thus, the

genes encoding the GAS vaccine antigens are ubiquitous and highly conserved, at least amongst currently sequenced GAS serotypes.

3.2.4 PCR Screening of Representative GAS Isolates for the Genes Encoding GAS Vaccine Antigens

In addition to determining the representation of the vaccine antigen genes in sequenced GAS genomes, the presence of the vaccine antigens genes in heterologous strains of both clinical and epidemiological significance was assessed. A protein which is ubiquitous among GAS strains and highly conserved has the potential to confer protection against many or all circulating GAS strains irrespective of serotype. To investigate the frequency of the genes encoding the GAS vaccine antigens, a number of representative and clinical GAS isolates were screened using PCR (Table 3.4). Each of the 13 vaccine antigens were detected in all of the nine strains assessed using PCR (Table 3.4) as well as in all of the 13 publically available GAS genomes (Table 3.3). This data suggests that the genes encoding the GAS vaccine antigens are likely to be present amongst many or possibly all GAS strains.

3.2.5 Cloning Vaccine Antigen Genes into the pET160 Vector

The globally disseminated M1T1 GAS isolate 5448 was selected as the template DNA for the amplification of the genes encoding the 13 vaccine antigens, due to its high association with human infection (Stevens, 1992, Johnson *et al.*, 2002). Amplification of each vaccine antigen gene resulted in PCR products of approximate expected molecular size; ADI 1.2 kb, AK 0.6 kb, BCAT 1 kb, EF-Tu 1.2 kb, FBA 0.9 kb, KPR 0.9 kb, NADP-GAPDH 1.4 kb, OTCase 1 kb, PFK 1 kb, PGK 1.2 kb, RRF 0.6 kb, TF 1.3 kb, and TIM 0.8 kb (Figure 3.2). A single amplicon was visible for all 13 vaccine antigens, indicating that only one discrete target sequence was amplified in all cases.

The strategy employed to clone the individual amplicons into the expression vector pET160 is summarised in section 2.4.2 and a representative plasmid map is shown in Figure 2.1. Following analysis of potential clones by restriction enzyme digestion (data not shown), DNA sequencing of expression vectors confirmed that each of the genes encoding the GAS vaccine antigens were successfully cloned into the expression vector in the correct frame for recombinant protein expression and without point mutations (data not shown).

Table 3.3 Percent amino acid identities of the 13 vaccine antigens amongst sequenced GAS genomes as determined using BLAST searches. GAS strain designations listed with serotype indicated in brackets.

	ADI	AK	BCAT	EF-Tu	FBA	KPR	NADP-GAPDH	OTCase	PFK	PGK	RRF	TF	TIM
M1 (M1)	99%	99%	99%	99%	100%	99%	99%	99%	99%	99%	100%	99%	100%
M49 591* (M49)	ND	99%	ND	98%	ND	ND	ND	99%	ND	98%	ND	100%	100%
MGAS10394 (M6)	99%	99%	99%	100%	99%	99%	99%	100%	100%	99%	100%	100%	99%
MGAS315 (M3)	100%	100%	100%	100%	99%	99%	99%	100%	99%	100%	100%	100%	100%
MGAS8232 (M18)	99%	99%	99%	98%	100%	99%	99%	100%	100%	99%	100%	99%	99%
SSI-1 (M3)	100%	100%	100%	100%	99%	98%	100%	100%	99%	100%	100%	100%	100%
MGAS10270 (M2)	99%	99%	99%	100%	100%	100%	99%	99%	99%	99%	99%	99%	99%
MGAS10750 (M4)	99%	100%	99%	100%	100%	99%	99%	99%	99%	99%	99%	99%	99%
MGAS2096 (M12)	99%	99%	99%	100%	99%	99%	99%	99%	99%	99%	99%	99%	100%
MGAS5005 (M1)	99%	99%	99%	99%	99%	99%	99%	99%	99%	99%	99%	99%	100%
MGAS6180 (M28)	99%	99%	99%	100%	100%	100%	99%	99%	99%	99%	99%	99%	99%
Manfredo (M5)	99%	99%	99%	99%	99%	99%	99%	99%	99%	98%	99%	99%	99%
MGAS9429 (M12)	99%	99%	99%	100%	99%	99%	99%	99%	99%	99%	99%	99%	99%

* This genome is only partially sequenced. ND, not determined due to partial genome availability.

Table 3.4 Presence of the genes encoding the 13 GAS vaccine antigens amongst nine clinical GAS isolates. Gene presence determined using PCR amplification.

Strain	Sequence Type	ADI	AK	BCAT	EF-Tu	FBA	KPR	NADP-GAPDH	OTCase	PFK	PGK	RRF	TF	TIM
5448	*emm*1	+	+	+	+	+	+	+	+	+	+	+	+	+
NS88.2	*emm*98.1	+	+	+	+	+	+	+	+	+	+	+	+	+
pM1	*emm*1	+	+	+	+	+	+	+	+	+	+	+	+	+
A20	*emm*23	+	+	+	+	+	+	+	+	+	+	+	+	+
HSC5	*emm*5	+	+	+	+	+	+	+	+	+	+	+	+	+
ALAB49	*emm*53	+	+	+	+	+	+	+	+	+	+	+	+	+
NS192	*emm*100	+	+	+	+	+	+	+	+	+	+	+	+	+
20174	*emm*3	+	+	+	+	+	+	+	+	+	+	+	+	+
2036	*emm*6	+	+	+	+	+	+	+	+	+	+	+	+	+

+, indicates gene detected using PCR; -, indicates gene not detected using PCR.
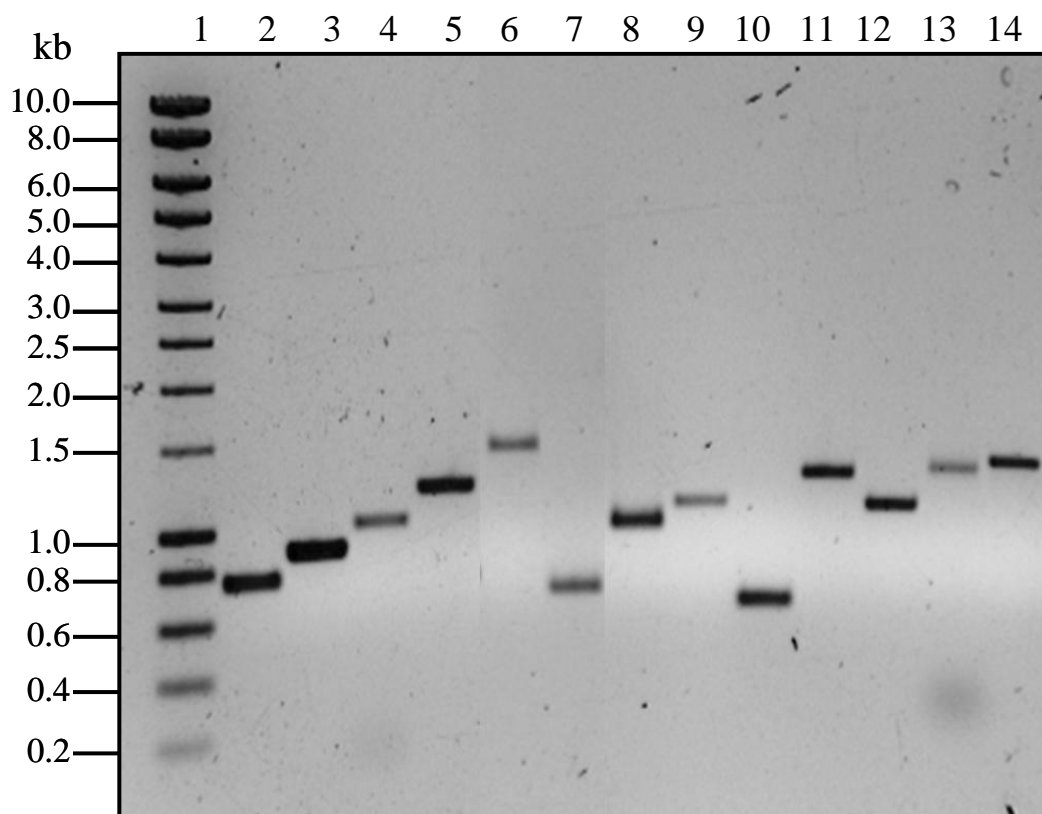
Figure 3.2 PCR amplification of the genes encoding the 13 GAS vaccine antigens from 5448 chromosomal DNA using the proof-reading Pfu Ultra High-Fidelity DNA polymerase. PCR products were resolved on a 1 % (w/v) TAE agarose gel stained with ethidium bromide. Lanes are as follows; 1, Bioline Hyperladder molecular size markers (sizes shown in kb); 2, *tpi*, encodes TIM; 3, *fba*, encodes FBA; 4, *pfkA*, encodes PFK; 5, *pgk*, encodes PGK; 6, *gapN*, encodes NADP-GAPDH; 7, *adk*, encodes AK; 8, *apbA*, encodes KPR; 9, *bcaT*, encodes BCAT; 10, *rrf*, encodes RRF; 11, *tufA*, encodes EF-Tu; 12, *arcB*, encodes OTCase; 13, *sagP*, encodes ADI; 14, *ropA*, encodes TF.

3.2.6 Expression and Purification of Recombinant Forms of GAS Vaccine Antigens

For recombinant protein expression successful clones were transformed into BL21 Star™ *E. coli* cells. When functionally expressing clones were established for each of the 13 GAS vaccine antigens, large scale expression cultures were prepared for recombinant protein purification. Whole cell lysates (WCL) of BL21 Star™ *E. coli* expressing each of the recombinant antigens are shown in Figure 3.3. The predicted size of each recombinant antigen is as follows; ADI 50.7 kDa, AK 28.3 kDa, BCAT 41.8 kDa, EF-Tu 48.4 kDa, FBA 35.8 kDa, KPR 38.4 kDa, NADP-GAPDH 54.0 kDa, OTCase 42.5 kDa, PFK 40.3kDa, PGK 46.7 kDa, RRF 25.1 kDa, TF 51.6 kDa and TIM 31.2 kDa. The expected sizes of the recombinant proteins was determined by adding the molecular mass of the vector encoded His-tag to the expected molecular mass of each vaccine antigen. In most

cases the molecular size of the recombinant antigens was as predicted, however EF-Tu and TIM were larger than expected, whilst PFK was smaller than expected (Figure 3.3). However, DNA sequencing (data not shown) confirmed the correct DNA sequence was cloned into the vector for all 13 GAS vaccine antigens. Whilst SDS coats proteins in a net negative charge during denaturing SDS-PAGE (resulting in a linear relationship between size and migration), it is also known that the hydrodynamic shape and charge-to-mass ratio of some proteins can also influence their migration during SDS-PAGE irrespective of the addition of SDS (Tung *et al.*, 1972). The pET160 vector used to create the 13 expression constructs encodes an N-terminal hexahistidyl tag (Figure 2.1) for the purification of the recombinant proteins using Ni-NTA affinity chromatography. Following expression, the BL21 Star™ *E. coli* cells were lysed and recombinant proteins purified using Ni-NTA affinity purification under denaturing conditions. Elution fractions were visualised on SDS-PAGE gels (data not shown) and those containing purified recombinant protein were pooled and dialysed into 0.5 M urea/PBS for use in subsequent vaccine characterisation experiments. Purified dialysed recombinant antigen is shown in lane 4 in Figure 3.3.

3.3   Discussion

Based on the proteomic studies previously performed in our laboratory (Cole, 2006, Cole *et al.*, 2005), 13 cell-wall associated proteins were selected as putative GAS vaccine candidates for further consideration and characterisation in this study. The antigens were selected based on a number of criteria including surface exposure, the percent amino acid identity shared with the human proteome and presence and conservation amongst GAS serotypes with clinical and epidemiological significance.

During early immunisation studies of humans with GAS vaccine preparations, the administration of partially purified M3 protein was observed to result in three cases of ARF (Massell *et al.*, 1969). Furthermore, it is well established that the host immune response to M protein can produce antibodies that react with host tissue such as cardiac myosin (Cunningham *et al.*, 1992, Dale *et al.*, 1985a, Dale *et al.*, 1986). As a consequence, there has been a heightened concern that antibodies generated against GAS vaccine antigens may cross-react with human proteins and tissues potentially leading to auto-immune disease.
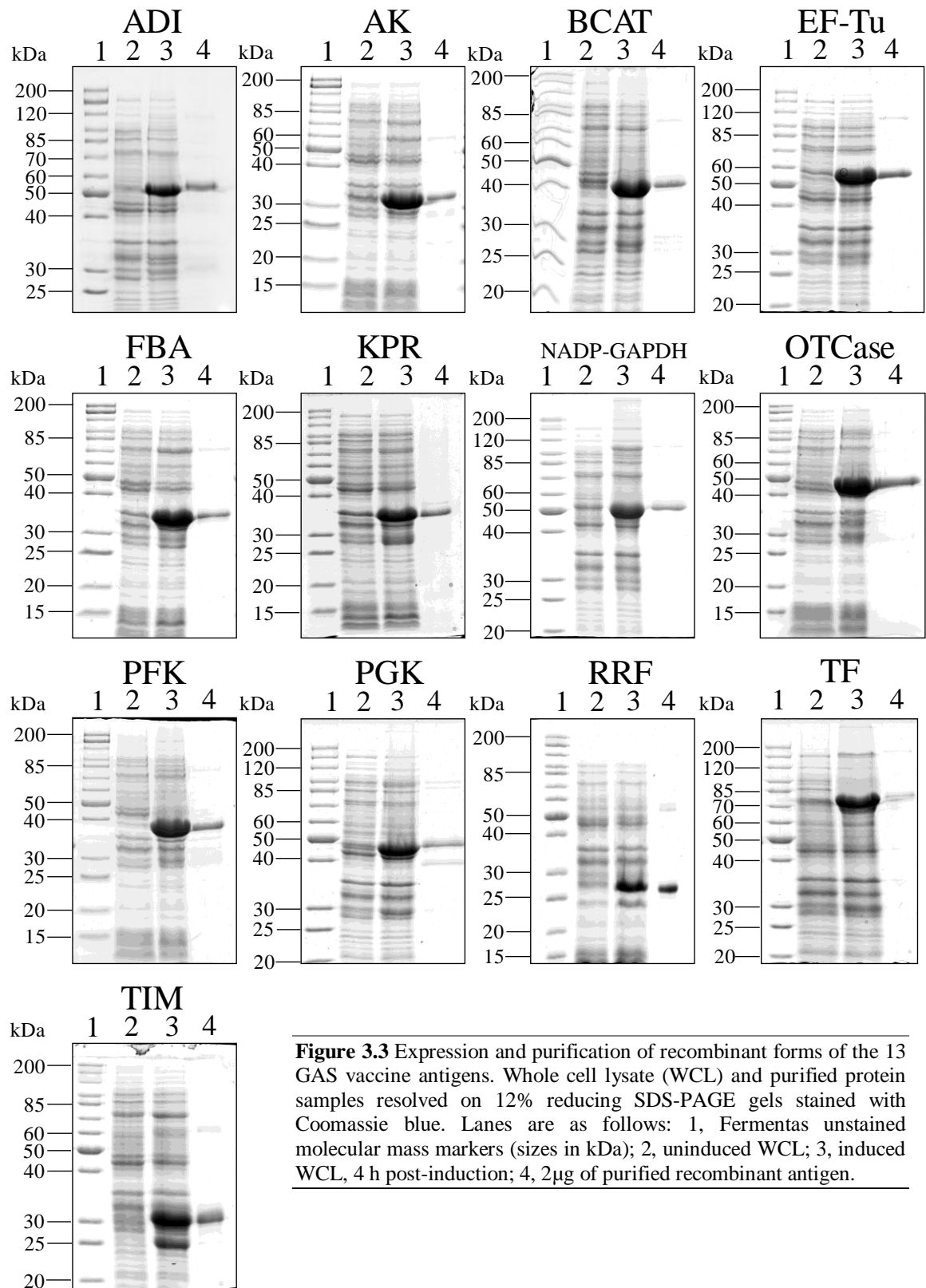
Figure 3.3 Expression and purification of recombinant forms of the 13 GAS vaccine antigens. Whole cell lysate (WCL) and purified protein samples resolved on 12% reducing SDS-PAGE gels stained with Coomassie blue. Lanes are as follows: 1, Fermentas unstained molecular mass markers (sizes in kDa); 2, uninduced WCL; 3, induced WCL, 4 h post-induction; 4, 2µg of purified recombinant antigen.

The amino acid identity the GAS vaccine antigens share with the human proteome is a simplistic consideration of the presence of possible cross-reactive epitopes, and is by no means a conclusive determination of antigenic cross-reactivity. An alternate approach could involve immunoinformatics (Korber *et al.*, 2006), a wing of bioinformatics focussing on immune-related databases, to investigate possible cross-reactive epitopes within vaccine antigens. Other studies have also tested the cross-reactivity of vaccine antigens with human tissues, in particular proteins known to be abundant in cardiac muscle and tissue. One group investigated the cross-reactivity of polyclonal rabbit anti-serum raised against the Fn-binding repeat of the Protein F1 of GAS with porcine heart myosin, porcine muscle myosin and bovine muscle myosin using ELISA (Valentin-Weigand *et al.*, 1994). The use of animal forms of myosin rather than human forms and testing only a single dilution of sera in ELISA limited the interpretation of this data. Other studies have also explored the cross-reactivity of α-streptococcal murine and human MAbs with myosin (Dale *et al.*, 1985a, Dale *et al.*, 1985b), laminin (Antone *et al.*, 1997, Galvin *et al.*, 2000), tropomyosin (Fenderson 1989) and vimentin (Kraus *et al.*, 1989). Whilst experiments such as these give an indication if vaccine antigens share cross-reactivity with the selected human proteins tested, this approach is limited to determining immunological cross-reactivity with only a single test protein at a time (eg. myosin) and cannot indicate a lack of cross-reactivity with other proteins of the human proteome. Perhaps a more encompassing approach would be to test the cross reactivity of specific polyclonal sera or MAbs with whole tissue extracts derived from human organs and tissues (eg. heart, kidney) rather than testing cross-reactivity with individual host proteins. Another limitation to some of these experimental studies is the use of basement membrane and extracellular proteins purified from animals, rather than from a human source. Whilst it is likely the animal homologues share a high sequence similarity with the human protein, it is unlikely the two forms would be 100% homologous. Furthermore, the use of murine monoclonal antibodies may not yield the same results as human MAbs, as it has previously been observed that MAbs from different origin can have differential recognition of host/pathogen molecules. As Cunningham (2000) points out, human α-myosin/α-streptococcal MAbs all react with *N*-acetylglucosamine (an important component of the group A carbohydrate) whilst murine MAbs do not. The bioinformatic interrogation of the human proteome using the BlastP search engine utilised

in this study was a basic approach to enable the elimination of antigens not suitable for this study due to high sequence similarity with human proteins. However, a combinatorial approach analysing the entire human proteome using immunoinformatics and experimentally testing for cross-reactive and immunogenic epitopes would be the next logical step in a thorough investigation into the presence of cross-reactive epitopes. The cross-reactivity of polyclonal anti-sera raised against protective antigens of this study with human heart extract is explored in Chapter 6.

The genes encoding each of the 13 GAS vaccine antigens were ubiquitous amongst sequenced GAS genomes and clinical GAS isolates screened by PCR and were found to have a high sequence identity between GAS serotypes. Thus, it is possible the candidate antigens of this study may provide heterologous GAS protection. Drawbacks of certain existing GAS vaccine candidates, M protein, SfbI, SOF and FbaA, is that they are not conserved between GAS serotypes nor necessarily present amongst all serotypes of GAS. However, it must be noted that the presence of the genes encoding the vaccine antigens does not necessarily mean that the proteins are expressed, or expressed under all conditions. In order to determine if the ubiquitous genes encoding the GAS vaccine antigens were expressed by a range of heterologous GAS isolates, further analysis has been conducted in the form of western blotting experiments screening for native forms of the candidate vaccine antigens in mutanolysin cell wall extracts. This data is presented in Chapter 4.

Given all of the 13 GAS vaccine antigens lack the LPXTG cell wall anchor motif commonly found in Gram-positive surface proteins and that they also lack apparent signal sequences, it is not known how these proteins are associated with the cell wall. It is possible the proteins may be released during autolysis and re-attach to the surface of adjacent cells or that a yet to be elucidated alternative secretory pathway may exist (Cole *et al.*, 2005). One author has suggested that enzymatic degradation of the bacterial cell wall using a combination of mutanolysin and lysozyme, as utilized in the proteomic studies conducted by Cole *et al.* (2005) and Cole (2006), may produce cell lysis and result in the contamination of cell wall samples with cytoplasmic proteins (Rodreiguez-Ortega *et al.*, 2006). However, an alternative proteomic study which utilised trypsin to 'shave' cell wall-associated proteins also identified 21 proteins commonly identified as cytoplasmic (Severin

et al., 2007). Whilst it is possible that the detection of these traditional cytoplasmic proteins associated with the cell surface may be artefactual and a result of chemical lysis or autolysis during sample processing, the existence of anchorless GAS cell surface-associated proteins of cytoplasmic origin is by no means a new phenomenon. The glycolytic surface exposed Plg binding virulence factors of GAS, SEN (Pancholi *et al.*, 1998) and SDH/GAPDH (Pancholi *et al.*, 1992) identified in the proteomic studies performed by Cole *et al.* (2005) and Cole (2006) (Table 3.1) are established examples of two traditionally cytoplasmic proteins lacking a LPXTG motif located on the surface of GAS possessing a dual functionality.

Several other recent investigations have utilised proteomics, genomics and reverse vaccinology approaches to facilitate the discovery of streptococcal surface proteins and potential protective antigens of GAS. ADI, EF-Tu, FBA, NADP-GAPDH, OTCase, PGK, TF and TIM (in addition to SEN and SDH/GAPDH) were identified as surface-associated proteins of GAS following proteolytic digestion of the GAS cell surface, trypsinisation of the fragments and multidimensional nano-LC MS/MS identification of the resultant peptides (Severin *et al.*, 2007). ADI, KPR, NADP-GAPDH, OTCase, PFK, PGK, TF and TIM (as well as SEN and SDH/GAPDH) were also found in GAS culture supernatants assessed by 2D electrophoresis and amino-terminal amino acid sequencing (Lei *et al.*, 2000). This data further supports the hypothesis that these proteins are somehow secreted from the cell.

The association of the vaccine antigens under investigation in this study with the cell surface does not appear to be unique to GAS, ADI and OTCase have been identified as surface-associated in *Streptococcus suis* (Winterhoff *et al.*, 2002a) and *Streptococcus sanguis* (Floderus *et al.*, 1990b). Following PMF identification of cell wall proteins extracted from *Streptococcus pneumoniae* using mutanolysin, FBA and SDH/GAPDH were identified and found to protect against lethal mucosal challenge with WU2 and 9VR *S. pneumoniae* isolates (Ling *et al.*, 2004). Extraction of mutanolysin derived cell wall fractions and subsequent PMF analysis has also been performed for Group B streptococcus, in which OTCase and PGK were determined to be associated with the cell wall (Hughes *et al.*, 2002). In an animal model of Group B streptococcus infection, Hughes *et al.* (2002)

observed OTCase and PGK offer a degree of protection against lethal intraperitoneal challenge with the M732 isolate of Group B streptococcus. Furthermore, PGK, SEN, SDH/GAPDH, FBA, TIM, AK, EF-Tu and RRF were amongst cell surface-associated proteins identified on *S. oralis* (Wilkins *et al.*, 2003). The immunogenicity and protective efficacy of the 13 GAS vaccine antigens will be tested in an intraperitoneal murine model of GAS infection. In an attempt to further confirm the surface localisation of the 13 vaccine antigens in this study, immunofluorescence microscopy and analysis of the distribution of the vaccine antigens in heterologous cell wall extracts has been performed. The results of these investigations are described in Chapter 4.

4. INITIAL SCREENING AND CHARACTERISATION OF PUTATIVE GAS VACCINE ANTIGENS

4.1 Introduction

The development of a human vaccine that protects against GAS infection has been a significant field of scientific investigation for many years, with the first human trials testing M protein dating back to the 1920s (Steer *et al.*, 2009a). However, as yet, no commercial GAS vaccine preparation is available. Whilst a number of GAS vaccine candidates have been identified which elicit protection in animal models of disease, a vaccine preparation which is efficacious against all serotypes of GAS and safe for use in humans remains elusive. Table 1.3 outlines the characteristics and possible shortcomings of proposed GAS vaccine candidates. Each GAS vaccine candidate listed in Table 1.3 has been observed to protect against at least one route of experimental GAS infection. However, none of the existing candidates have been reported to confer protection against all three commonly investigated experimental routes of GAS infection; systemic, intranasal and subcutaneous. Although M protein and CHO are ubiquitously expressed on all serotypes of GAS, the cross-reactivity of sera generated against these proteins and potential involvement of such proteins with auto-immune diseases (Dale *et al.*, 1985a, Dale *et al.*, 1985b, Dale *et al.*, 1986, Shikhman *et al.*, 1993) precludes the inclusion of full length M protein or CHO in vaccine preparations. Furthermore, although M protein (Lancefield, 1962), FbaA (Terao *et al.*, 2001), Sse (Liu *et al.*, 2007) Protein F1 and SOF (Goodfellow *et al.*, 2000, Delvecchio *et al.*, 2002) are present on the surface of GAS, these antigens do not exhibit sequence conservation among of the estimated 120 GAS serotypes. Thus, it is likely that protection afforded by vaccine preparations based on the M protein, FbaA, SfbI, SOF or Sse would be limited to a subset of GAS serotypes.

GAS vaccinology has primarily focused on the major virulence factor, the M protein. However, several factors have hampered the development of M protein based vaccines such as the large number of unique M serotypes, the potential antigenic variation within a serotype due to the continual evolution of M protein (Fischetti, 1989), and the presence of cross-reacting epitopes, which may trigger post-infective immune sequelae (Cunningham, 2003). Furthermore, circulating GAS strains can rapidly be replaced by a

new set of strains (Dale *et al.*, 2002, Kaplan *et al.*, 2001). Therefore, multivalent vaccine preparations based on the variable N-terminus of the M protein, such as the 26-valent formulation developed by McNeil *et al.* (2005), may need to be continuously reformulated in order to protect against vaccine driven changes in circulating strains. Additionally, multivalent vaccine formulations must be dynamic to ensure protection in geographical regions where GAS strains are diverse. Several existing GAS vaccine candidates including C5a peptidase, FBP54, CHO, GRAB and SpeB are conserved amongst GAS serotypes and the use of conserved antigens in GAS vaccine formulations eliminates concerns of serotype dependent protection.

Although there is no vaccine available to prevent GAS infection, GAS remains sensitive to penicillin. Penicillin is used in prophylactic and post-infection treatment strategies. However, it is possible that GAS may develop resistance to penicillin, and thus, the administration of penicillin may not necessarily be a feasible option in the future. In areas of high GAS endemicity prophylactic injection of penicillin is routinely performed. Unfortunately, this strategy has had limited success due to low levels of patient compliance (Brandt *et al.*, 2000). It has been reported that penicillin fails to eradicate GAS in as many as 35% of pharyngotonsillitis cases (Sela *et al.*, 1999). It is possible this treatment failure may be due to the co-presence of other bacterial species capable of degrading penicillin or poor patient compliance, however, it is also hypothesised that following penicillin treatment GAS may invade host cells, successfully evading the effects of antibiotics (Sela *et al.*, 1999). The internalisation of GAS by epithelial cells mediated by Protein F1 has been extensively studied (Molinari *et al.*, 1997, Molinari *et al.*, 1999b, Talay *et al.*, 2000, Ozeri *et al.*, 1998). In addition, other GAS antigens and surface proteins such as M protein (Cue *et al.*, 1998, Cue *et al.*, 2001, Dombek *et al.*, 1999), SOF (Timmer *et al.*, 2006, Gillen *et al.*, 2008), PrtF2 (Kreikemeyer *et al.*, 2004), FbaA (Terao *et al.*, 2001) and SpeB (Burns *et al.*, 1998, Tsai *et al.*, 1998) have also been shown to mediate internalisation of GAS by host cells. The need for new GAS vaccine candidates ubiquitiously expressed amongst serotypes which are highly conserved, non-toxic, do not exhibit proteolytic effects and which do not trigger auto-immune reactions is paramount.

This chapter investigates the distribution of GAS vaccine antigens in representative heterologous GAS cell wall extracts and the localisation of the antigens on the surface of an M1 GAS isolate using immunofluorescence microscopy. In addition, the immunogenicity of the antigens following administration to mice and the subsequent protective efficacy of the antigens exhibit against lethal systemic GAS challenge is evaluated. As humans are known to be the exclusive reservoir of GAS, the establishment of functional animal models of GAS infection and disease can be problematic as many GAS strains display low pathogenicity in mice. Nonetheless, a murine disease model utilising a mouse passaged M1 strain (pM1) was optimised for use in this study. This murine disease model incorporates a subcutaneous immunisation and an intraperitoneal challenge, representative of systemic infection, and has previously been utilised by other researchers to determine the level of protection against GAS challenge conferred by M protein and PAM (Batzloff *et al.*, 2004, Sanderson-Smith *et al.*, 2006a).

4.2   Results

4.2.1   Detection of Vaccine Antigens in GAS Cell Wall Extracts

The genes encoding the 13 putative GAS vaccine antigens were found to have a ubiquitous presence in sequenced GAS genomes (Table 3.3) and in selected representative clinical and epidemiological isolates screened by PCR (Table 3.4). To test the expression of the GAS vaccine antigens *in vitro*, mutanolysin derived cell wall extracts from nine representative GAS isolates were probed with polyclonal mouse anti-serum specific for each of the vaccine antigens in western blotting experiments. ADI, FBA, KPR, PFK, RRF, TF and TIM were detected in the cell wall extracts of each of the nine GAS strains tested (Table 4.1). The remaining vaccine antigens, AK, BCAT, EF-Tu, NADP-GAPDH, OTCase and PGK were detected in the majority of GAS cell wall extracts tested (Table 4.1). Whilst AK and PGK were not observed in the 5448 cell wall extract, they were detected in 5448Δ*speB* (an isogenic mutant containing a deletion of the speB gene), suggesting that AK and PGK may be degraded by the cysteine protease SpeB in wild-type 5448.

Table 4.1 Screening cell wall extracts from clinical GAS isolates ($n = 9$) for the presence of GAS vaccine antigens. GAS vaccine antigens were detected by western blotting mutanolysin cell wall extracts with specific polyclonal mouse anti-serum obtained after individual subcutaneous immunisation regime with each of the vaccine antigens.

Strain	Sequence Type	ADI	AK	BCAT	EF-Tu	FBA	KPR	NADP-GAPDH	OTCase	PFK	PGK	RRF	TF	TIM
5448	emm1	+	-	+	+	+	+	+	+	+	-	+	+	+
5448ΔspeB	emm1	+	+	+	+	+	+	+	+	+	+	+	+	+
NS88.2	emm98.1	+	-	+	-	+	+	+	+	+	+	+	+	+
pM1	emm1	+	+	+	+	+	+	-	+	+	+	+	+	+
A20	emm23	+	+	+	+	+	+	+	-	+	+	+	+	+
HSC5	emm14	+	+	-	+	+	+	-	+	+	+	+	+	+
NS192	emm53	+	+	+	+	+	+	+	+	+	+	+	+	+
20174	emm100	+	+	+	+	+	+	+	+	+	+	+	+	+
2036	emm6	+	+	+	+	+	+	-	+	+	+	+	+	+

+, indicates antigen detected; -, indicates antigen not detected in western blotting. ADI, arginine deiminase; AK, adenylate kinase; BCAT, branched chain amino-acid aminotransferase; EF-Tu, elongation factor Tu; FBA, fructose bisphosphate aldolase; KPR, ketopantoate reductase; NADP-GAPDH, NADP-dependent glyceraldehyde-3-phosphate dehydrogenase; OTCase, orthinine carbamoyltransferase; PFK, 6-phosphofructokinase; PGK, phosphoglycerate kinase; RRF, ribosome recycling factor; TF, trigger factor; TIM, triosephosphate isomerase.

4.2.2 Detection of Vaccine Antigens on the Surface of GAS Cells Using Immunofluorescence Microscopy

In order to investigate the presentation of the 13 vaccine antigens on the GAS cell surface, immunofluorescence microscopy was undertaken utilising antigen specific polyclonal mouse serum. Pooled mouse anti-serum raised against M1 protein ($n = 10$) and anti-serum obtained from mice sham immunised with PBS ($n = 10$) were used as positive and negative control sera, respectively. Mouse matched pre-immune serum ($n = 10$) was also employed as a negative control. Polyclonal anti-serum specific for ADI, full-length M1 protein, EF-Tu and OTCase were bound to the GAS cell surface resulting in a distinct surface fluorescence (Figure 4.1). Anti-serum raised against BCAT, FBA, KPR, NADP-GAPDH, PGK, TF and TIM also bound to the surface of GAS, resulting in lower but still a clearly visible quantity of fluorescence at the GAS cell surface (Figure 4.1). Serum from mice sham immunised with PBS, in addition to α-AK, α-PFK and α-RRF serum (Figure 4.1) did not react with the GAS surface, there was no visible fluorescence in these samples. In all cases, the mouse matched pre-immune serum did not react with the surface of the cell (Figure 4.1).

4.2.3   Determination of the Protective Efficacy of Vaccine Antigens in an Intraperitoneal Murine GAS Challenge Model

In order to assess the protective efficacy of the vaccine antigens against lethal GAS challenge, recombinant ADI, AK, BCAT, EF-Tu, FBA, KPR, NADP-GAPDH, OTCase, PFK, PGK, RRF, TF and TIM were individually used to immunise BALB/c mice via the subcutaneous route prior to lethal challenge.

4.2.3.1 Specific High Titre Serum IgG Antibodies Against Recombinant Vaccine Antigens in BALB/c Mice Following Subcutaneous Immunisation

BALB/c mice ($n = 10$) were immunised as described in section 4.2.3. Following each immunisation, the titre of serum IgG antibodies raised against each recombinant antigen was determined using ELISA. The confirmation of high titre serum IgG antibodies reactive against each GAS vaccine antigen was crucial prior to challenge with GAS. Mice were bled prior to immunisation (Day -1; pre-immune serum) and following each immunisation on Days 20, 27 and 41 respectively. The titre was defined as the lowest
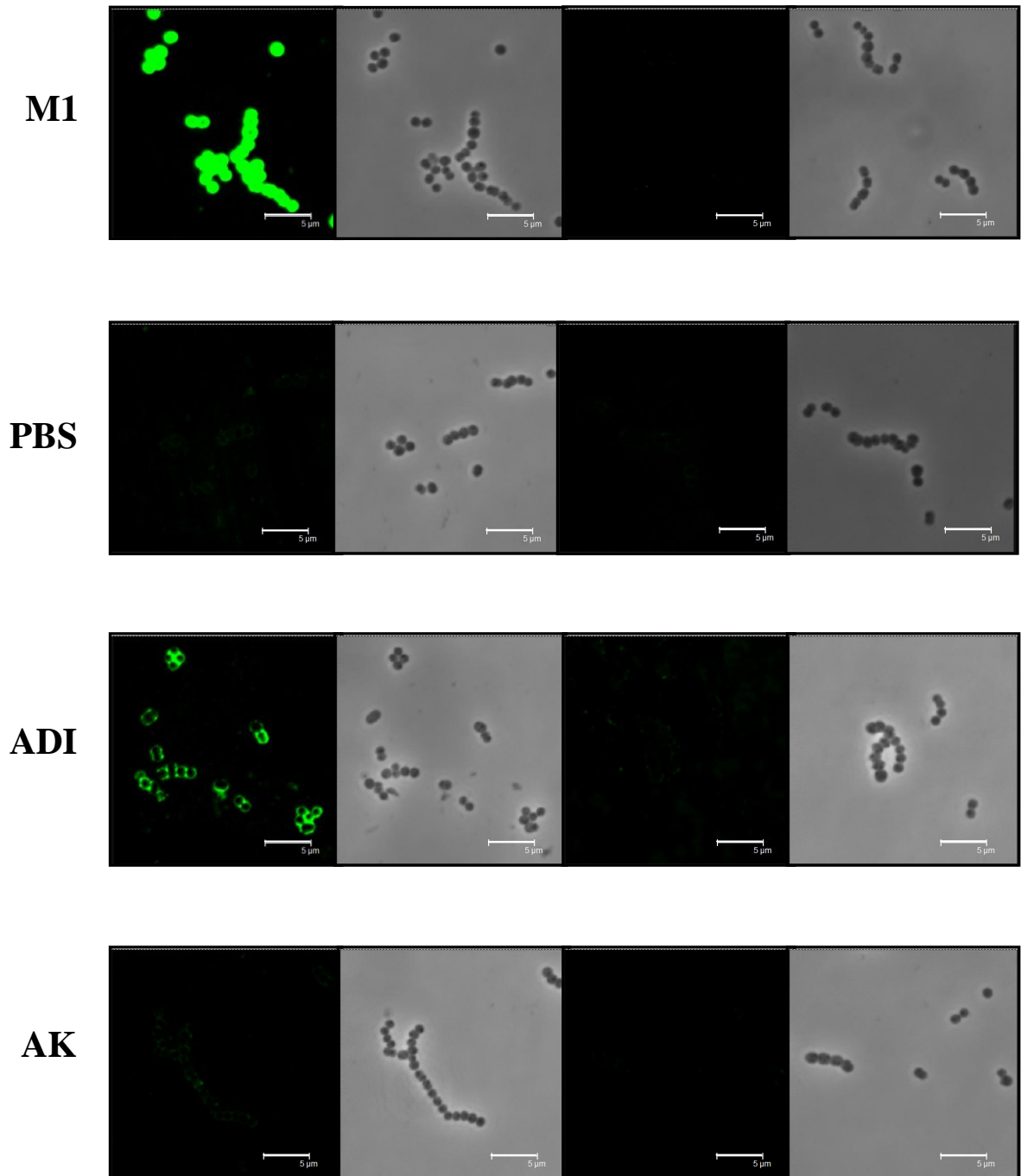
Figure 4.1 Detection of GAS vaccine antigens via immunofluorescence on the surface of GAS strain pM1 (*emm*1) using pools of specific polyclonal anti-serum from BALB/c mice immunised with the individual antigens (*n* = 10). Serum collected 15 days prior to challenge (final bleed). Serum collected prior to immunisation (pre-immune) was used as a negative control. Antibodies bound to the surface of the GAS were visualised with goat-anti mouse IgG-FITC (Zymed). From left to right; final bleed fluorescence image, final bleed transmission image, pre-immune fluorescence image, pre-immune transmission image. Scale bars, in white, indicate 5 μm.

BCAT
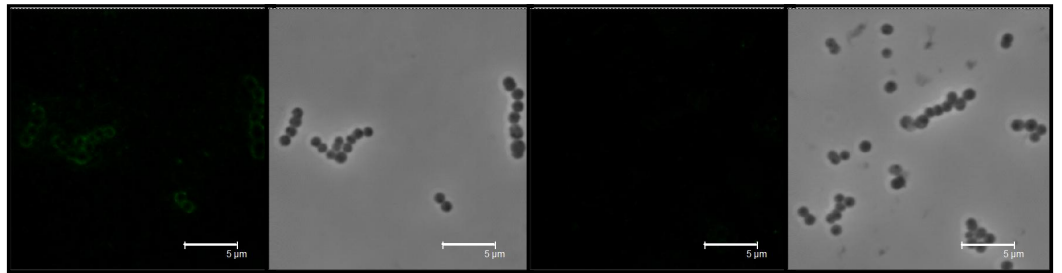
EF-Tu

FBA

KPR

Figure 4.1 *Cont.*

NADP-GAPDH

OTCase

PFK

PGK
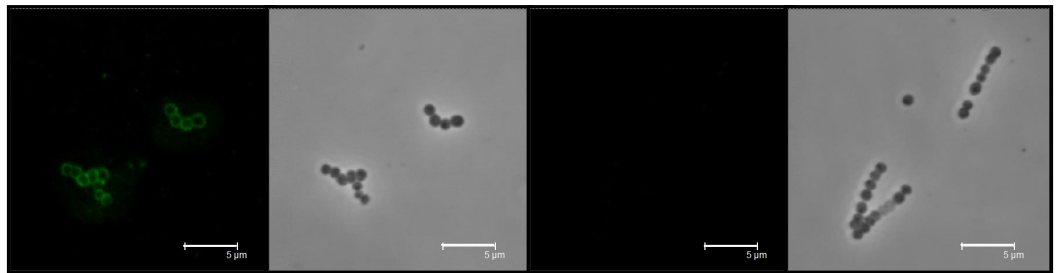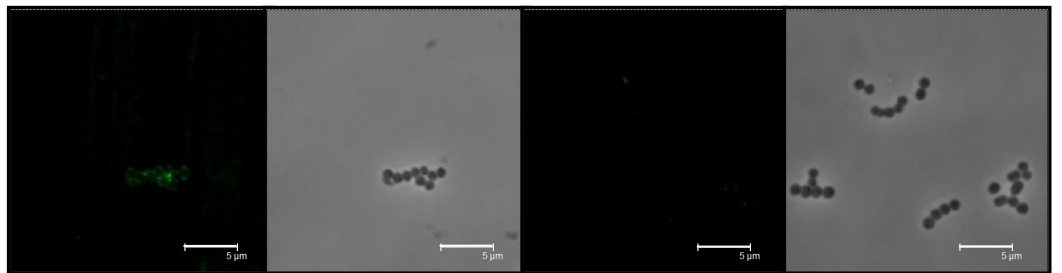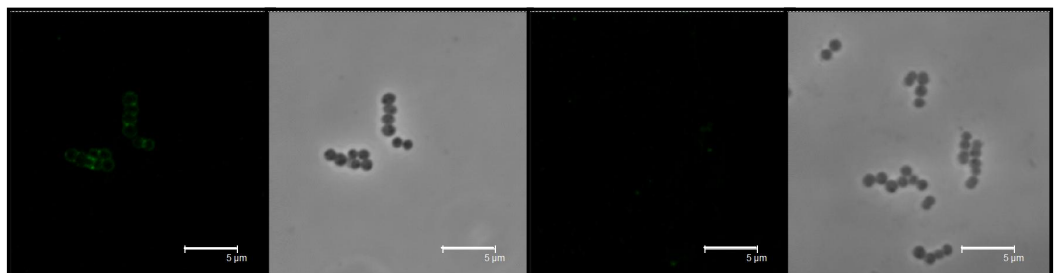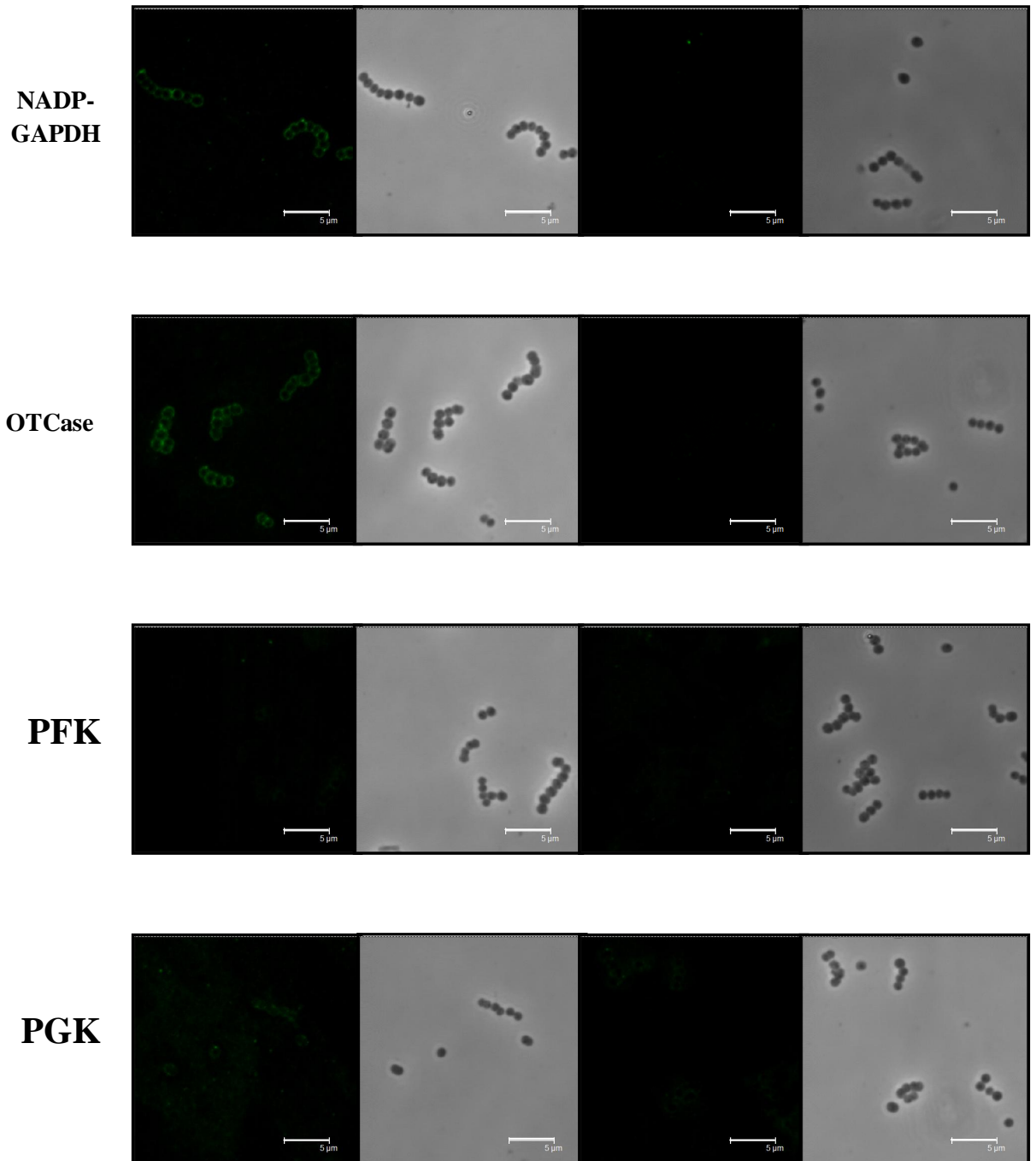
Figure 4.1 *Cont.*
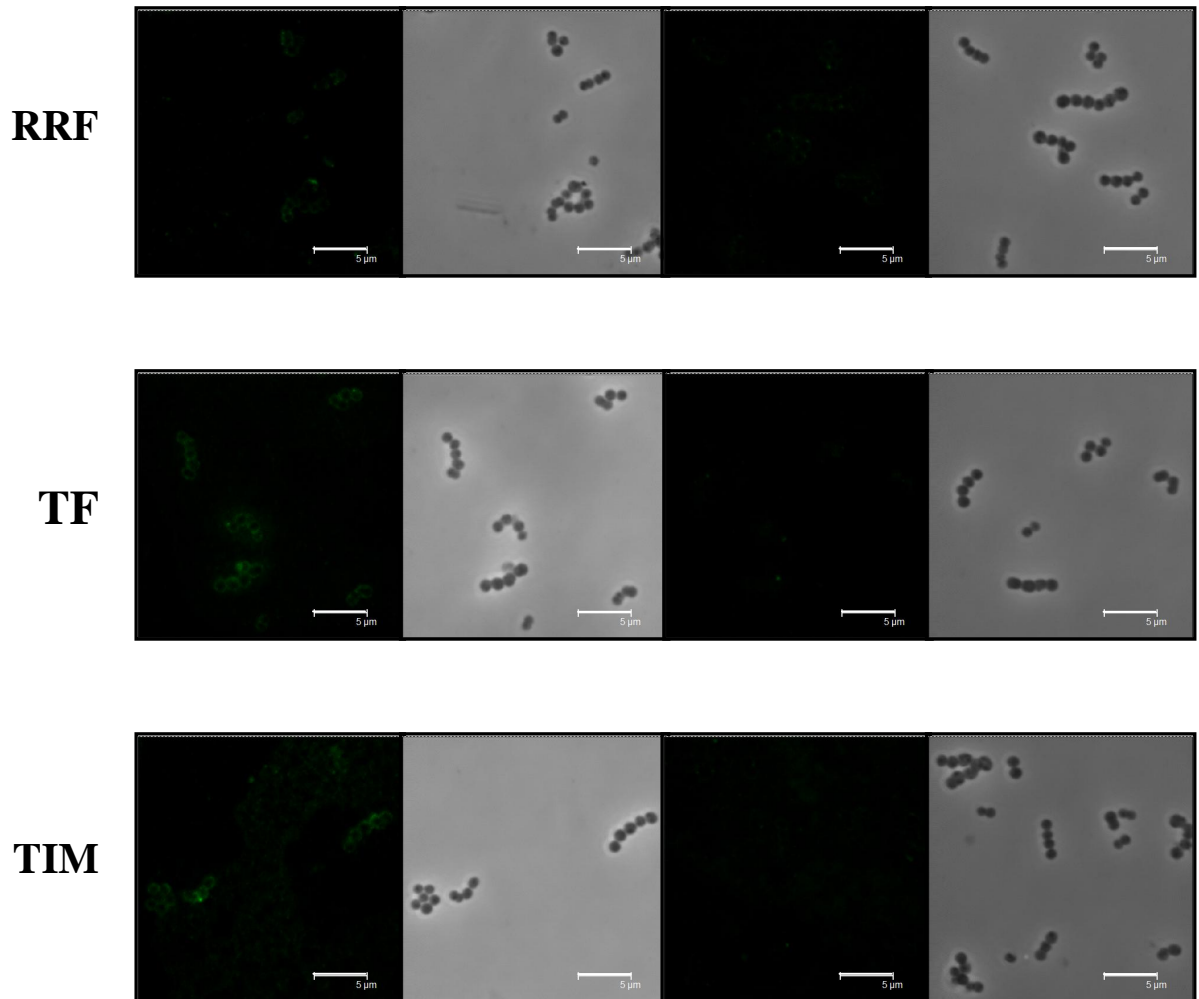
Figure 4.1 *Cont.*

serum dilution that had an absorbance greater than 0.2 at 450 nm. Immunisation of mice with each of the GAS vaccine antigens resulted in high titre serum IgG antibodies (Figure 4.2), with titres ranging from 1.75×10^5 for NADP-GAPDH to 1.58×10^8 for ADI in serum taken on day 41 (15 days before challenge). The mouse matched pre-immune sera did not react with any of the vaccine antigens (data not shown). Thus, each of the 13 GAS vaccine antigens was highly immunogenic in BALB/c mice, generating a strong systemic antibody response following subcutaneous immunisation.

4.2.3.2 Protective Efficacy of GAS Vaccine Antigens in Systemic Murine GAS Challenge Model

Following subcutaneous immunisation, mice were challenged with a lethal dose of approximately 2 x 10^7 cfu/mL of GAS strain pM1 on Day 56 (actual doses ranged from 2 x 10^7 – 2.9 x 10^7 cfu/mL). For logistical reasons the protective efficacy of the 13 candidate antigens was tested in two separate cohort experiments with 10 mice per vaccine antigen group. For each individual experiment, in addition to the test antigens, one negative control group ($n = 10$) comprised of mice sham immunised with PBS and one positive control group ($n = 10$) comprised of mice immunised with M1 protein were included. Cohort one consisted of ten groups; AK, BCAT, RF-Tu, FBA, KPR, RRF, TF, TIM, M1 protein and PBS sham group. Cohort two consisted of seven groups; ADI, NADP-GAPDH, OTCase, PFK, PGK, M1 protein and PBS sham group. The survival curves presented display the combined results for the control groups for both experimental cohorts. After lethal challenge, mice immunised with M1 protein, ADI, AK, BCAT, KPR, NADP-GAPDH, OTCase, PFK, PGK, RRF and TF were observed to have significantly increased survival ($P < 0.05$), compared to mice sham immunised with PBS only (Figure 4.3). In this study, 100% of mice immunised with ADI and KPR survived challenge with a lethal dose of GAS. This 100% survival rate is on par with the survival rate of the widely characterised protective M1 protein (Cole *et al.*, 2008), which was used as a positive control in this experiment. Experimental immunisation with EF-Tu, FBA and TIM failed to protect BALB/c mice from lethal intraperitoneal infection ($P > 0.05$; Figure 4.3). Therefore, ten of the 13 antigens tested; ADI, AK, BCAT, KPR, NADP-GAPDH, OTCase, PFK, PGK, RRF and TF, show promise as GAS vaccine candidates in this systemic murine model of infection.
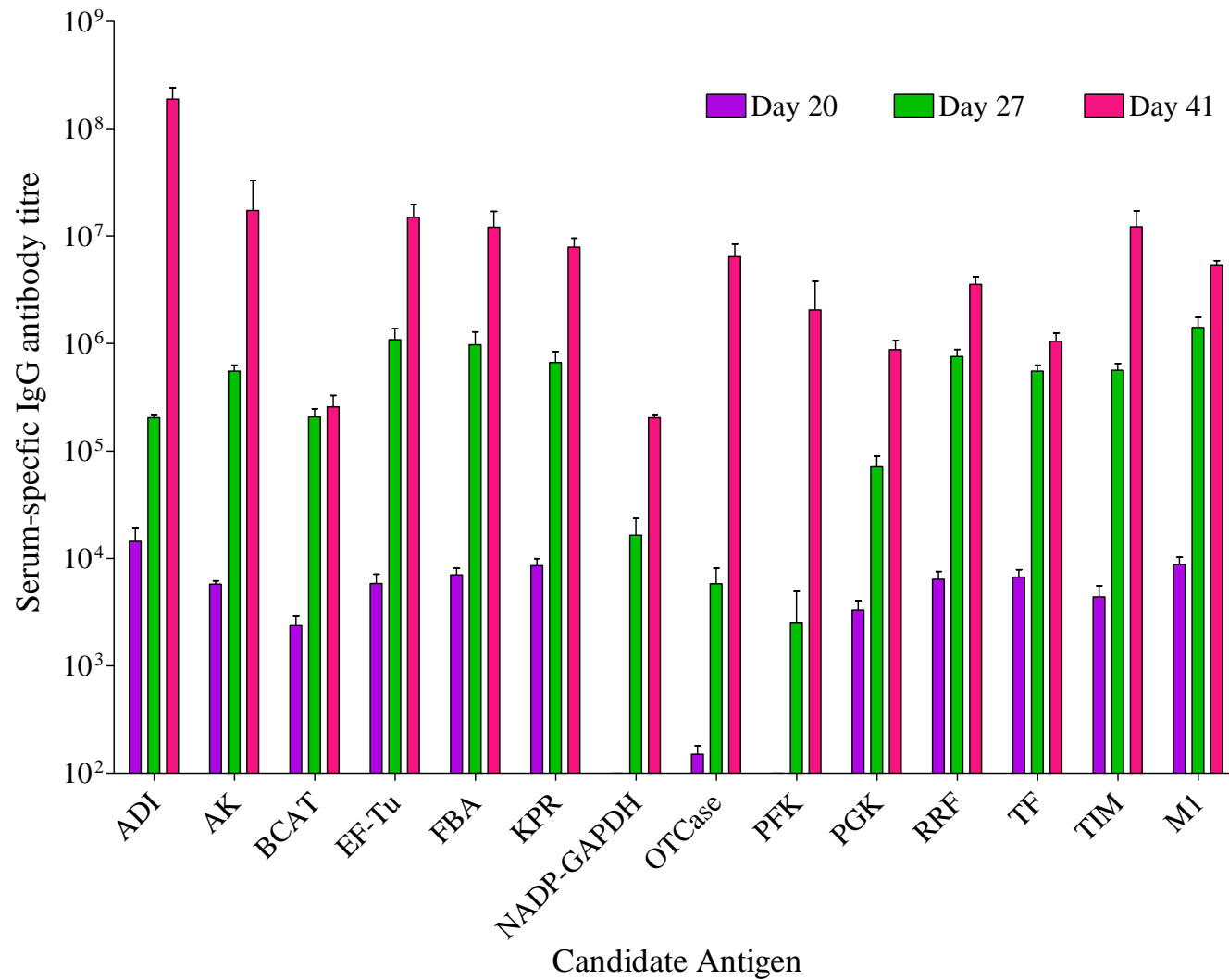
Figure 4.2 Specific serum IgG antibody titres in BALB/c mice (*n* = 10) as determined by ELISA following subcutaneous immunisation with individual GAS vaccine antigens. Primary immunisation with 10 µg antigen in Freund's Complete Adjuvant on Day 0; two booster immunisations on Days 21 and 28 contained 10 µg of antigen in PBS. Mice were bled on Day -1 (no reactivity of serum with antigens, data not shown), 20, 27 and 41. Titres presented as mean ± SEM. Titre defined as the dilution with an absorbance ≥ 0.2 at 450 nm.

Figure 4.3 Protective efficacy exhibited by GAS vaccine antigens against lethal intraperitoneal challenge with the GAS isolate pM1 (*emm*1) following subcutaneous immunisation of BALB/c mice. Primary immunisation performed on Day 0, 10 µg antigen in CFA; booster immunisations, administered on Days 21 and 28 contained 10 µg of antigen in PBS. Intraperitoneal challenge with pM1 GAS on Day 56 (28 days following final immunisation). Two experimental cohorts were challenged, with doses ranging from $2 - 2.9$ x 10^7 cfu/mL. Kaplan-Meier survival curves display survival of 10 mice for each GAS vaccine antigen and 20 mice for M1 protein (positive control group) and mice sham immunised with PBS (negative control group). * indicates a significant difference, $P < 0.05$, as determined using the log-rank test comparing survival of mice immunised with GAS vaccine antigens to the survival of mice sham immunised with PBS (no antigen). A) M1 protein, B) AK, C) ADI, D) BCAT, E) EF-Tu, F) FBA, G) KPR, H) NADP-GAPDH, I) OTCase, J) PFK, K) PGK, L) RRF, M) TF, N) TIM.

A M protein

P < 0.0001*

– ■ – PBS – ◆ – M1

B AK

P = 0.0042*

– ■ – PBS – ◆ – AK

C ADI

P = 0.0013*

– ■ – PBS – ◆ – ADI

D BCAT

P = 0.0078*

– ■ – PBS – ◆ – BCAT

E EF-Tu

P = 0.0653

– ■ – PBS – ◆ – EF-Tu

F FBA

P = 0.8935

– ■ – PBS – ◆ – FBA

G KPR

P = 0.0013*

– ■ – PBS – ◆ – KPR

H NADP-GAPDH

P = 0.0447*

– ■ – PBS – ◆ – NADP-GAPDH

114

I OTCase

J PFK

K PGK

L RRF

M TF

N TIM

Figure 4.3 *Cont.*

115
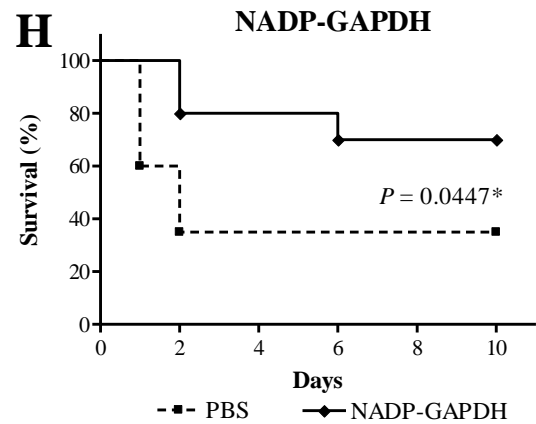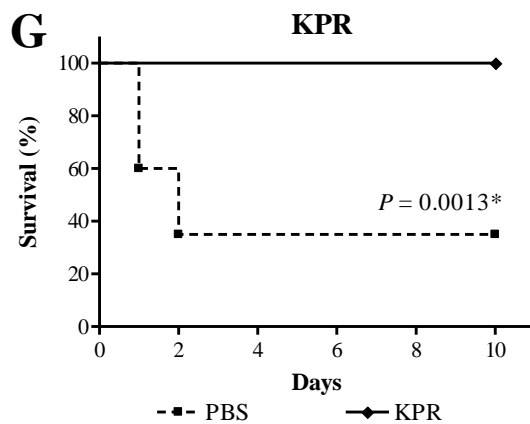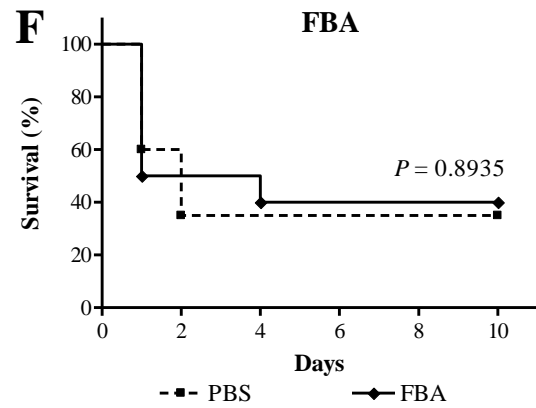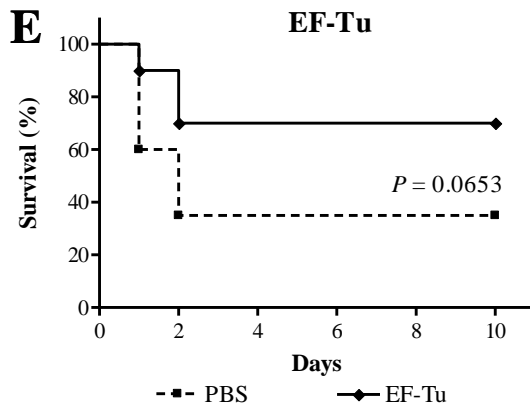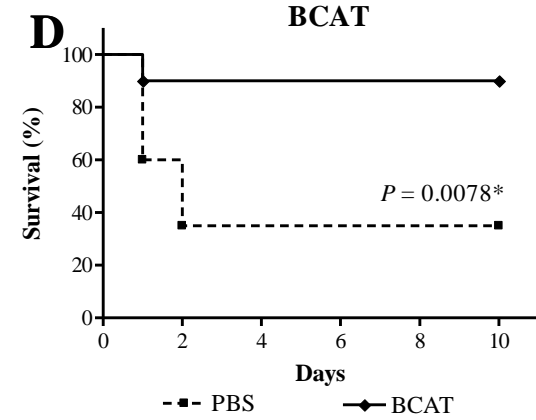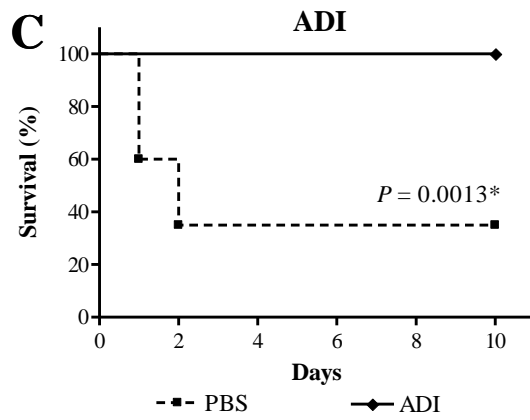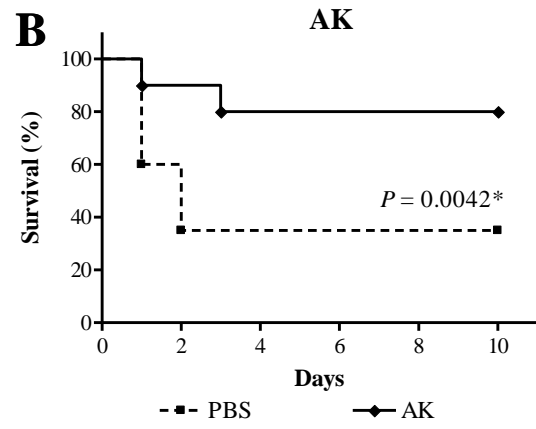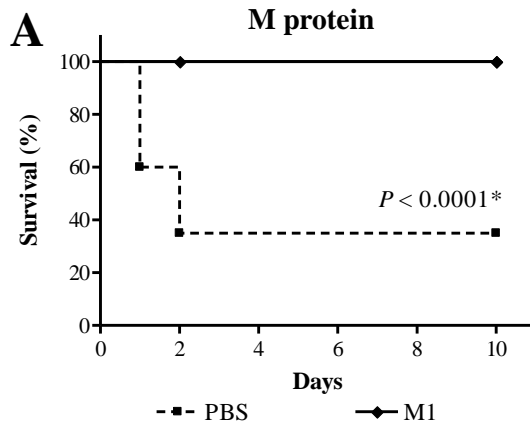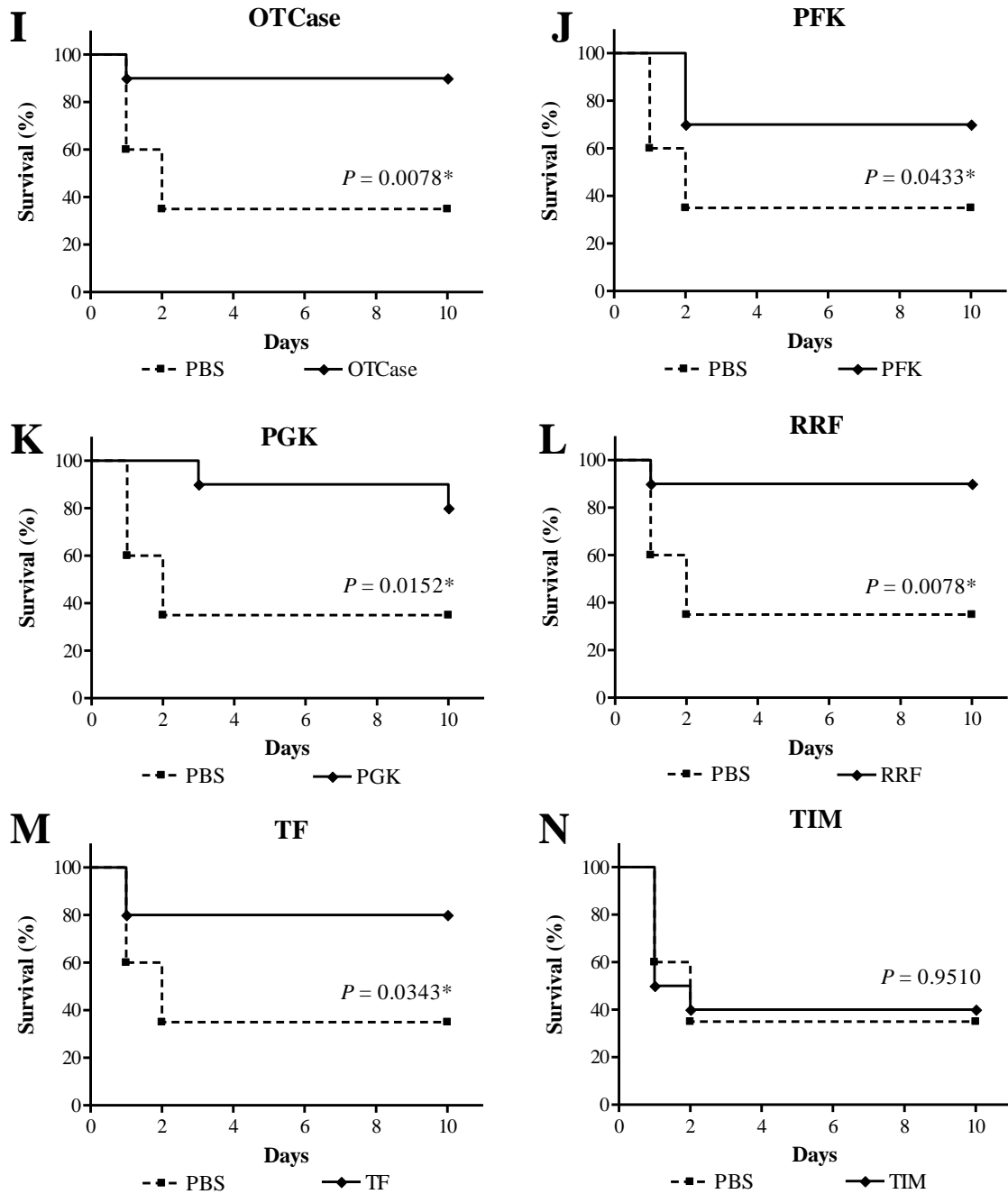
4.3 Discussion

The protective efficacy of 13 GAS vaccine antigens was tested in a systemic (intraperitoneal) GAS challenge model. Many previous studies testing the protective efficacy of GAS vaccine candidates have utilised systemic GAS challenge models, including those examining FbaA (Terao *et al.*, 2005), FBP54 (Kawabata *et al.*, 2001), CHO (Sabharwal *et al.*, 2006), R28 (Stalhammar-Carlemalm *et al.*, 1999), SOF (Courtney *et al.*, 2003), SpeB (Kapur *et al.*, 1994), Sib35 (Okamoto *et al.*, 2005) and Spa (Dale *et al.*, 1999). Although it is not approved for use in humans, CFA was selected as the adjuvant for this initial screening experiment as it generates high titre anti-sera with avid specificity. CFA is also capable of inducing both humoral and cell-mediated (Th1) based immune responses (Stills, 2005). In this study, ten of the candidate antigens; ADI, AK, BCAT, KPR, NADP-GAPDH, OTCase, PFK, PGK, RRF and TF were observed to offer significant protection ($P < 0.05$) against lethal systemic challenge with pM1 GAS (Figure 4.3). Furthermore, subcutaneous immunisation of BALB/c mice with each of the individual thirteen GAS vaccine antigens resulted in high titre serum IgG antibodies (Figure 4.2). Western blotting experiments of mutanolysin derived cell wall extracts revealed ADI, FBA, KPR, PFK, RRF, TF and TIM were present in the cell wall fraction of all nine GAS isolates tested (Table 4.1). The presence of these antigens in all nine GAS isolates indicates that the expression of these antigens is not serotype specific and it is possible the antigens may be ubiquitous in nature. Incubation of whole GAS cells with specific polyclonal anti-sera raised against ADI, BCAT, EF-Tu, FBA, KPR, NADP-GAPDH, OTCase, PGK, TF and TIM resulted in distinct visible fluorescence at the GAS cell surface in immunofluorescence experiments (Figure 4.1). Of the 13 antigens assessed in this study, ADI, KPR and TF show the most promise as potential GAS vaccine antigens as they were found to be surface localised and to confer protection against lethal GAS infection.

For a GAS vaccine candidate to be considered suitable for use in humans it is essential that it provides protection against infection, that it is exposed to the host immune system and thus presumably localised on the cell surface and that it does not induce auto-immune disease. This chapter investigated the protective efficacy and exposure of the antigens to the human immune system. Chapter 6 will address concerns pertaining to the issue of antigen safety via investigation of the reactivity of the 13 GAS vaccine antigens

with human serum obtained from two geographically distinct populations in which GAS infection and disease is endemic.

Of the candidate antigens observed to protect BALB/c mice against systemic challenge in this study, several have been previously reported to play a role in the virulence of, or to elicit protection against infection in GAS or other bacterial species. In this study, ADI was observed to be surface localised on GAS following immunofluorescence microscopy (Figure 4.1), expressed by all analysed GAS isolates (Table 4.1) and to protect BALB/c mice against lethal intraperitoneal challenge ($P < 0.05$; Figure 4.3). ADI has been previously identified as a cell wall-associated protein in *S. suis* (Winterhoff *et al.*, 2002b) and *S. sanguis* (Floderus *et al.*, 1990a). GAS ADI, also known as streptococcal acid glycoprotein (SagP), in addition to OTCase and carbamate kinase (CK) is part of the arginine deiminase system in which L-arginine is converted to citrulline with concomitant production of ATP and ammonia (Zúñiga *et al.*, 2002). The resultant ammonia can act to raise the pH of the cytoplasm, and thus ADI, in concert with OTCase and CK, may protect GAS from the low internal pH of host cells (Curran *et al.*, 1995, Degnan *et al.*, 2000). ADI has been observed to play a role in shielding streptococcal cells from acidic environments on the respiratory surfaces or within phagolysosomes during cellular invasion, via the production of ammonia (Curran *et al.*, 1995, Curran *et al.*, 1998, Degnan *et al.*, 2000). In addition, in *Listeria monocytogenes*, ADI has been found to promote extracellular acid tolerance (Ryan *et al.*, 2009). ADI has previously been reported to influence GAS virulence factor expression, including the expression of hyaluronic acid capsule and the pyrogenic exotoxin SpeB (Marouni *et al.*, 2003). Through the modulation of the expression of such virulence factors, ADI is also thought to influence the internalisation of GAS into epithelial cells (Marouni *et al.*, 2003). ADI expressed by GAS is thought to play a role in the inhibition of human peripheral blood mononuclear cell proliferation (Degnan *et al.*, 2000, Degnan *et al.*, 1998) which may allow GAS the ability to down-regulate or modulate the host immune response to infection. ADI of *Streptococcus cristatus* has been found to act as a signal molecule responsible for cell–cell communication between *S. cristatus* and *Porphyromonas gingivalis*, two oral bacterial species (Xie *et al.*, 2007). ADI has also been observed to inhibit the proliferation of murine tumour lines both *in vivo* and *in vitro* (Yoshida *et al.*, 1998).

Trigger factor (TF; also known as RopA) was found to be a protective antigen in this study (Figure 4.3). Previous reports on TF indicate that it is a ribosome-associated chaperone and peptidyl–prolyl cis–trans isomerase implicated in SpeB protease maturation in GAS (Lyon *et al.*, 2003, Lyon *et al.*, 1998). OTCase, another protective antigen from this study, has been identified as a cell-wall protein of Group B streptococcus (Hughes *et al.*, 2002), *S. sanguis* (Floderus *et al.*, 1990a) and *S. suis* (Winterhoff *et al.*, 2002b) and as a Fn-binding protein of *Staphylococcus epidermis* (Hussain *et al.*, 1999). Likewise, the protective antigen PGK has been identified as a cell-surface protein of *S. oralis* (Wilkins *et al.*, 2003), *S. pneumoniae* (Ling *et al.*, 2004) and Group B streptococcus (Hughes *et al.*, 2002). OTCase and PGK were observed to offer a degree of protection, although non-significant, against lethal intraperitoneal Group B streptococcus infection following passive immunisation (Hughes *et al.*, 2002). FBA is an anchorless glycolytic protein of GAS that was not protective against systemic challenge in this study. FBA has previously been detected in a cell-wall fraction of *S. pneumoniae* and was subsequently observed to partially protect mice against respiratory infection with pneumococci (Ling *et al.*, 2004). The study conducted by Ling *et al.* (2004) utilised intraperitoneal immunisation followed by mucosal infection with pneumococcus. In contrast, this study of the protective efficacy of GAS antigens utilised intraperitoneal infection with GAS (Figure 4.3). The difference in challenge route may have influenced the lack of protection observed in mice immunised with FBA following intraperitoneal GAS challenge.

In addition to the ability to protect against lethal challenge, another consideration when characterising putative vaccine candidates is the extent of localisation of the antigen on the surface of GAS. All of the existing GAS vaccine antigens reported in the literature (outlined in Table 1.3) have been found associated with the cell surface, however, not all of these antigens are reported to contain the traditional LPXTG cell wall anchor motif. Typically, Gram-positive surface proteins destined for transport across the cytoplasmic membrane contain a N-terminal signal sequence and/or the highly conserved hydrophobic C-terminal membrane anchor sequence, LPXTG. M protein (Lancefield, 1962), C5a peptidase (O'Connor *et al.*, 1986, Chen *et al.*, 1990), FbaA (Terao *et al.*, 2001), Protein F1 (Talay *et al.*, 1992), R28 protein (Stalhammar-Carlemalm *et al.*, 1999), SOF (Rakonjac *et al.*, 1995) and Spa (Dale *et al.*, 1999) each contain C-terminal LPXTG cell wall anchor

motifs. SpeB (Kapur *et al.*, 1993), Sib35 (Kawabata *et al.*, 2002) and Sse (Liu *et al.*, 2007) harbour putative signal peptide sequences, presumably leading to the secretion of these antigens from the cell. It is possible these antigens subsequently re-associate with the cell surface. However, very few of the proteins identified in GAS cell wall extracts by Cole *et al.* (2005) contained either of these motifs, with only seven (of the 74 proteins identified) possessing N-terminal signal sequences and three containing the LPXTG motif. Some previously characterised surface-associated proteins of GAS such as FBP54 (Courtney *et al.*, 1996), SEN (Pancholi *et al.*, 1998) and SDH/GAPDH (Pancholi *et al.*, 1992) are known to lack any of the known signal sequence or anchor motifs. It is speculated that such proteins may be localised on the bacterial surface via re-association following passive autolysis or via secretion by an unknown mechanism (Chhatwal, 2002). *S. pneumoniae* α-enolase has been observed to re-associate with the cell surface by interacting with receptors on both encapsulated and unencapsulated pneumococci (Bergmann *et al.*, 2001). A similar type of re-association mechanism may be utilised by the anchorless proteins of this study, but further investigation is required. The enolase and GAPDH of *Lactobacillus crispatus* bind to LTA on the bacterial surface in a pH-dependent manner, demonstrating one possible mechanism via which these glycolytic enzymes can associate with the bacterial cell wall (Antikainen *et al.*, 2007). Alternatively, a microdomain known as the ExPortal has been identified in the cytoplasmic membrane of GAS, which is involved in the export of proteins, such as SpeB, from the cell (Rosch *et al.*, 2004). It is not known if any of the cell-wall associated proteins of this study are exported through this ExPortal, but such a scenario is possible. Other potential alternatives for the secretion and export of these cell wall-associated proteins include internal signal sequences (which may not yet have been identified or defined in the literature), post-translational acylation, association with another secreted protein or the general secretory (Sec) pathway (Cole *et al.*, 2008).

Each of the 13 GAS vaccine antigens of this study detected in cell wall extracts (Table 4.1) are anchorless. Despite an as yet undetermined mechanism of export, ADI, BCAT, EF-Tu, FBA, KPR, NADP-GAPDH, OTCase, PGK, TF and TIM were observed to be localised on the surface of intact M1 GAS cells in immunofluorescence microscopy (Figure 4.1). The visualisation of whole intact GAS cells stained with specific polyclonal anti-sera raised against the aforementioned antigens further supports the notion that these antigens are bona fide GAS surface proteins. Thus, it is likely that the vaccine antigens of this study are accessible to the host immune system following GAS infection. Three of the

candidate antigens, ADI, KPR and TF, were selected for further characterisation of their suitability for use as GAS vaccine antigens as they were observed to be protective in a systemic model of murine GAS infection, were observed to be associated with the GAS cell-surface and found to have no significant identity with the proteins of the human proteome. The non-protective protein, FBA, was selected as a negative control antigen in subsequent characterisation studies.

5. FURTHER CHARACTERISATION OF PROTECTIVE GAS VACCINE ANTIGENS ADI, KPR AND TF

5.1 Introduction

The work described in the previous chapter detailed the surface exposure and protective efficacy that each of the 13 GAS vaccine candidates conferred against lethal systemic GAS infection in mice following a single round of screening. The genes encoding ADI, KPR and TF were determined to be present and highly conserved amongst representative GAS isolates and sequenced genomes. ADI, KPR and TF were shown to be associated with the GAS cell-surface in immunofluorescence microscopy and had no significant amino acid identity with the proteins of the human proteome. The suitability of ADI, KPR and TF as GAS vaccine candidates will be further investigated in this chapter. The non-protective protein, FBA, was utilised as a negative antigen for comparison purposes in the characterisation studies outlined in this chapter.

When tested in a systemic (intraperitoneal) GAS challenge model, experimental immunisation with the individual candidate antigens ADI, KPR or TF conferred significant protection against lethal pM1 challenge as compared to mice sham immunised with PBS ($P < 0.05$; Figure 4.2). The data presented in this chapter confirms the ability of ADI, KPR and TF to protect against lethal GAS infection, by confirming the protective efficacy of the antigens against intraperitoneal GAS challenge and utilising an alternative murine GAS infection model, subcutaneous challenge. In this infection model C57BL/J6 mice were immunised intraperitoneally and subsequently challenged via the subcutaneous route using the hyper-virulent 5448AP GAS isolate (Aziz *et al.*, 2004). This subcutaneous GAS challenge model has previously been utilised to determine the virulence of wild-type GAS strains and corresponding animal passaged or knockout mutant GAS strains in humanised Plg transgenic mice (Walker *et al.*, 2007, Cole *et al.*, 2006, Sanderson-Smith *et al.*, 2008). Subcutaneous GAS challenge has been utilised during other GAS vaccine studies testing the ability of Sse (Liu *et al.*, 2007) and Protein F1 (McArthur *et al.*, 2004a) to protect mice against subcutaneous GAS infection with M1 or M3 isolates (Liu *et al.*, 2007) and an M23 isolate (McArthur *et al.*, 2004a) respectively. However, this is the first study in which a subcutaneous GAS challenge has been preceded by an intraperitoneal immunisation regime

to determine the protective efficacy of GAS vaccine antigens.

This chapter further examines the surface localisation of ADI, KPR and TF. As discussed in the previous chapter, most of the existing GAS vaccine candidates in the literature have been reported to be surface localised proteins either containing LPXTG cell wall anchor motifs or secreted proteins containing signal peptide sequences. Exposure of antigens on the cell surface enables presentation of the antigen to the host immune system. Surface exposure of antigens also allows ready access for antibody recognition and antibody binding. The cell surface exposure, localisation and abundance of ADI, KPR and TF on the surface of GAS is investigated using both immuno-electron microscopy (immuno-EM) and flow cytometry. Several existing GAS vaccine candidates including M protein (Beachey *et al.*, 1981), CHO (Salvadori *et al.*, 1995), SOF (Courtney *et al.*, 2003), Sib35 (Okamoto *et al.*, 2005) and Spa (Dale *et al.*, 1999) have been found to neutralise GAS via the production of serum opsonic antibodies. An investigation of the ability of polyclonal anti-sera raised against ADI, KPR or TF to promote opsonophagocytosis of GAS is presented. To determine linear B-cell epitopes within ADI, KPR and TF, peptide membrane arrays were synthesised produced using SPOT-peptide array technology (Frank, 2002) in which overlapping peptides comprising 15 amino acids each were positioned adjacently with a consecutive offset of 3 amino acids towards the C-terminus of the antigen. Specific polyclonal mouse anti-sera was used to probe the membranes and identify potential epitopes.

5.2   Results

5.2.1   Confirmation of the Protective Efficacy of ADI, KPR and TF in a Murine Intraperitoneal GAS Challenge Model

In the previous chapter, individual experimental immunisation with recombinant ADI, KPR or TF conferred protection against lethal systemic challenge with a M1 GAS isolate, pM1 ($P < 0.05$; Figure 4.3). Experimental immunisation with FBA, however, did not protect BALB/c mice against GAS challenge ($P > 0.05$; Figure 4.3). In order to confirm the protective efficacy of ADI, KPR and TF, the subcutaneous immunisation followed by intraperitoneal challenge experiments were repeated using an additional 10 BALB/c mice per antigen. The survival data from the previous experiment (presented in Chapter 4) and

this repeat experiment were pooled. The total pooled data represents 20 mice immunised with KPR, TF and FBA, 18 mice immunised with ADI (two mice died of incidental non-related causes prior to challenge) and 30 mice immunised with recombinant M1 protein (positive control group) and PBS (negative control group) respectively. As per the findings in Chapter 4, subcutaneous immunisation with the individual antigens ADI, KPR or TF, as well as immunisation with the positive control antigen, M1 protein, elicited a high titre of serum IgG antibodies (data not shown) and protected mice against lethal intraperitoneal challenge with pM1 GAS ($P < 0.05$; Figure 5.1). Experimental immunisation of mice with recombinant FBA protein did not protect mice against lethal intraperitoneal challenge with pM1 GAS ($P > 0.05$; Figure 5.1). These findings confirm the protective efficacy of ADI, KPR and TF against lethal systemic GAS challenge.

5.2.2 Opsonophagocytosis Mediated by α-ADI, α-KPR and α-TF Sera

Following the confirmation that ADI, KPR and TF elicit protection against systemic GAS infection with an M1 GAS isolate (Figure 5.1), the ability of polyclonal sera raised against ADI or TF to promote opsonophagocytosis of M1 GAS was investigated *in vitro*. Specific mouse anti-sera raised against the GAS vaccine antigens or M1 protein following subcutaneous immunisation of BALB/c mice was incubated with GAS cells and whole human blood. Treatment of GAS with α-ADI and α-M1 protein sera significantly reduced the survival of GAS in whole human blood in comparison to GAS incubated with sera from PBS sham immunised mice ($P < 0.05$; Figure 5.2). As α-ADI and α-M1 protein sera resulted in the killing of GAS in whole human blood, it is possible that *in vivo* ADI and M1 protein protect infected mice via an antibody-mediated mechanism. Opsonophagocytic killing of GAS is hypothesised to play a role, at least in part, in the efficacy of protective GAS antigens by facilitating bacterial clearance in the infected host.

5.2.3 Protective Efficacy of ADI, KPR and TF in a Murine Subcutaneous GAS Challenge Model

In addition to determining the protective efficacy of GAS vaccine antigens in a murine systemic GAS challenge model, the protective efficacy of ADI, TF and KPR was also investigated in a mouse model of subcutaneous GAS infection. The ability of antigens to protect against one route of GAS infection does not guarantee that the same antigen will
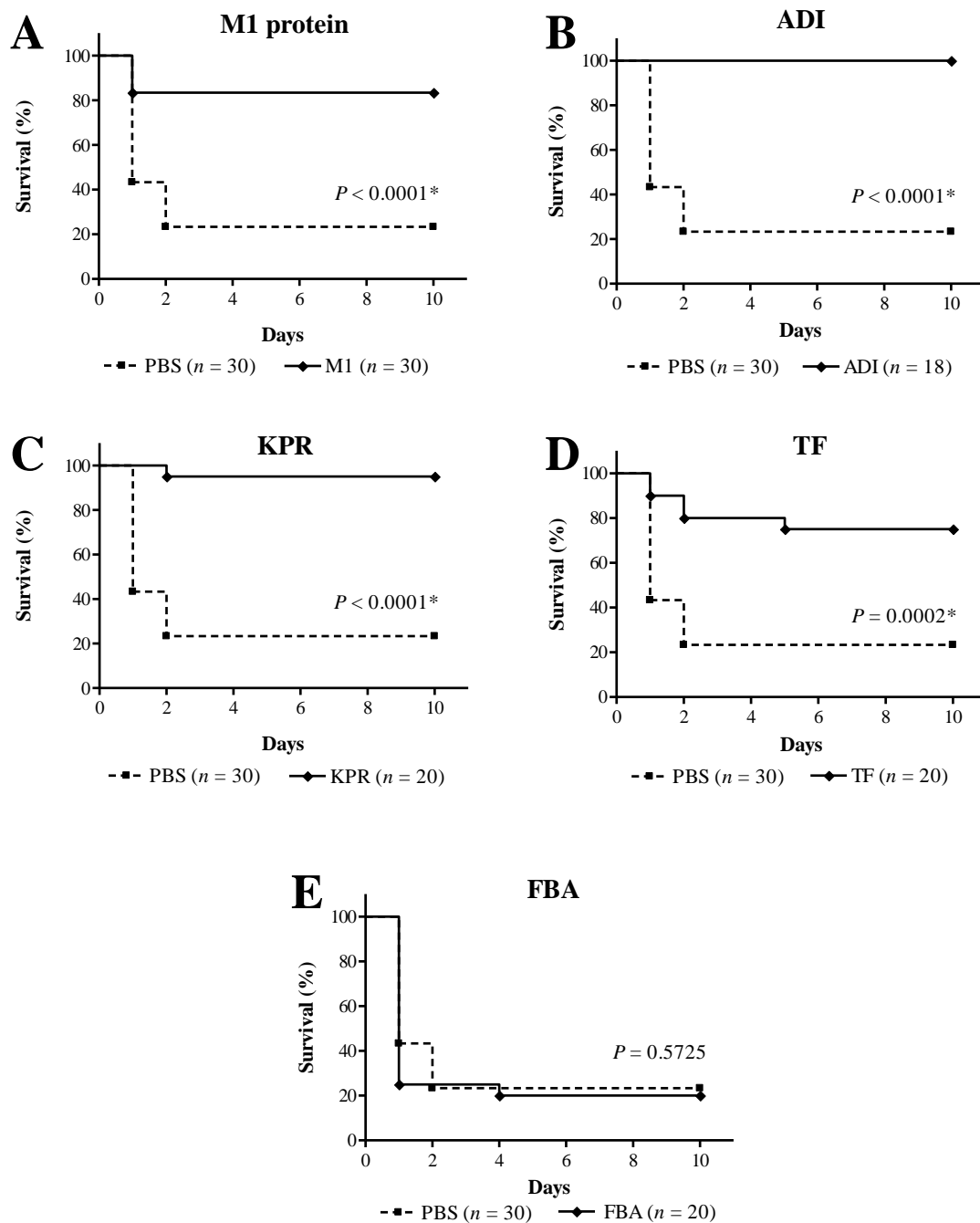
Figure 5.1 Confirmation of protective efficacy exhibited by GAS vaccine antigens against lethal intraperitoneal challenge with GAS isolate pM1 (*emm*1) following subcutaneous immunisation of BALB/c mice. Primary immunisation performed on Day 0, 10 μg antigen in CFA; booster immunisations, administered on Days 21 and 28 contained 10 μg of antigen in PBS. Intraperitoneal challenge with pM1 GAS on Day 56 (28 days following final immunisation). Mice immunised with M1 protein were used as a positive control group and PBS sham immunised mice served as the negative control group. Survival data presented for two independent replicates, in which the intraperitoneal doses of GAS ranged from $2 - 2.9 \times 10^7$ cfu/mL. * indicates a significant difference in the survival of mice post GAS infection ($P < 0.05$) as determined using the log-rank test comparing the survival of mice immunised with individual GAS vaccine antigens to the survival of mice sham immunised with PBS. A) M1 protein, B) ADI, C) KPR, D) TF, E) FBA.
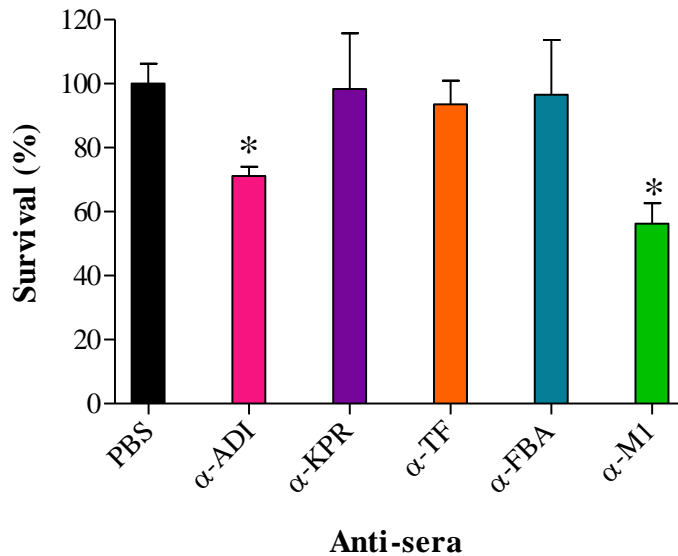
Figure 5.2 Percent survival of the serotype M1 GAS isolate 5448 following opsonophagocytosis killing assay. GAS were pre-incubated with specific polyclonal mouse anti-sera raised against ADI, KPR, TF or M1 protein (serum from mice sham immunised with PBS was the negative control) and were subsequently incubated with normal heparinised human blood. Blood from a single human donor previously determined to support the growth of the GAS isolate 5448 was used. Quadruplicate data from two independent repeat experiments is presented. The percent survival of GAS when incubated with specific test anti-sera was calculated in reference to the number of GAS (cfu/mL) surviving in the negative control (PBS sham serum). * indicates a significant difference ($P < 0.05$) between the test anti-sera and the PBS sham control serum.

exhibit protection against other routes of GAS infection. For instance, SOF has been shown to protect against lethal systemic GAS challenge (Courtney *et al.*, 2003), but when mice were intranasally immunised with SOF they were not protected against intranasal GAS challenge (Schulze *et al.*, 2006a). Similarly, Protein F1 is widely known to protect mice against intranasal GAS infection (Guzmán *et al.*, 1999, McArthur *et al.*, 2004b, Schulze *et al.*, 2003c), however, immunisation with recombinant Protein F1 did not elicit protection against subcutaneous GAS challenge (McArthur *et al.*, 2004a). It is hypothesised that if the GAS vaccine antigens of this study elicit protection against GAS administered to mice via a number of alternate routes, these antigens may also engender, in humans, protection against multiple types of GAS infection.

5.2.3.1 High Titre Serum IgG Antibodies Specific for Recombinant ADI, KPR and TF in C57BL/J6 Mice Following Intraperitoneal Immunisation

The intraperitoneal immunisation regime for the subcutaneous GAS challenge model followed the same schedule as was employed for the previous immunisation and challenge experiments described in Section 4.2.l. Immunisation of mice with each of the

individual candidate antigens resulted in high titre specific serum IgG antibodies (Figure 5.3), with titres of 1.7×10^5 for ADI, 1.49×10^5 for KPR, 1.24×10^5 for TF and 2.5×10^5 for FBA in serum taken on day 41 (final bleed serum; taken 15 days before challenge). Mouse matched pre-immune sera did not react with the candidate antigens in ELISA (data not shown). These results indicate each of the antigens are immunogenic when individually administered via the intraperitoneal route. These findings, along with those presented in section 4.2.1.1, suggest that when adjuvanted with CFA; ADI, KPR, TF and FBA are highly immunogenic in mice irrespective of immunisation route.

5.2.3.2 SpeB and Hyaluronic Acid Capsule Expression of the Subcutaneous GAS Challenge Isolate 5448AP

The challenge strain used in the subcutaneous GAS challenge model is the hyper-invasive isolate 5448AP, an animal passaged derivative of the globally disseminated M1T1 5448 isolate. The 5448 GAS isolate has been associated with invasive infection in humans including necrotising fasciitis and STSS (Aziz *et al.*, 2004). 5448AP shows higher levels of virulence in comparison to wild-type 5448 in a murine subcutaneous GAS challenge model (Walker *et al.*, 2007). The cause of this hyper-virulence is a result of a mutation in the *covRS* gene (Sumby *et al.*, 2006). Mutations in this regulon alter gene expression in up to 15% of the GAS genome (Sumby *et al.*, 2006). Of the changes in gene expression outlined by Sumby *et al.* (2006) it is known that the expression of SpeB protease is down-regulated and hyaluronic acid capsule expression is up-regulated in GAS strains harbouring mutation(s) in *covS*.

Prior to the challenge of C57BL/J6 mice, the SpeB expression profile of the challenge strain 5448AP was confirmed. SpeB expression in wild-type 5448 and the challenge strain 5448AP was determined by growing each strain on Columbia skim milk agar. SpeB activity is indicated by a dark halo of precipitate around the site of inoculation (Ashbaugh *et al.*, 1998). SpeB activity was observed to be absent in the 5448AP isolate (Figure 5.4) in contrast to wild-type 5448. The SpeB activity of the wild-type was diminished in the presence of E64, a broad spectrum cysteine protease inhibitor (Figure 5.4). Moreover, western blot analysis of GAS culture supernatants indicates that the supernatant from 5448AP was deficient in SpeB, whilst the culture supernatant from the
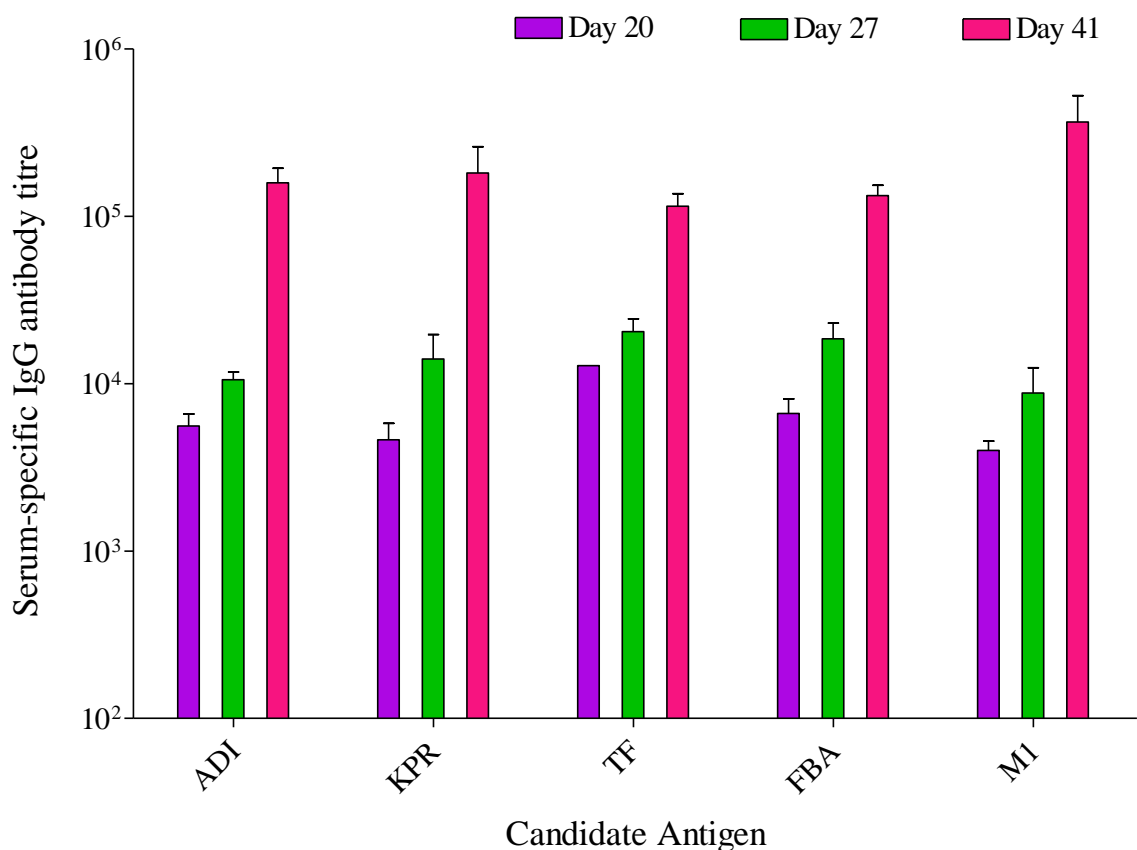
Figure 5.3 Serum-specific IgG antibody titres in C57BL/J6 mice ($n = 10$) as determined by ELISA following intraperitoneal immunisation with GAS vaccine antigens. Primary immunisation with 10 μg of antigen in Freund's Complete Adjuvant on Day 0; booster immunisations administered on Days 21 and 28 contained 10 μg of antigen in PBS. Mice were bled on Days -1 (pre-immune bleed; no reactivity of serum with antigens, data not shown), 20, 27 and 41. Titres presented as mean ± SEM. Titre defined as the dilution with an absorbance ≥ 0.2 at 450 nm.

wild-type 5448 strain was observed to contain SpeB (Figure 5.4F).

Following the observation that SpeB was not expressed in the 5448AP challenge strain, the relative quantity of extracted hyaluronic acid capsule were compared between 5448AP and 5448 wild-type GAS using l-ethyl-2-[3-(l-ethyl-naphtho[l,M]thiazolin-2-ylidene)-2-methyl-propenyl]naphtho[l,2d]thiazolium bromide (Stains-All) as described by Benchetrit *et al.* (1977). This technique exploits a shift in absorbance at 600 nm following binding of Stains-All to mucopolysaccharides contained within hyaluronic acid (Benchetrit *et al.*, 1977). The 5448AP isolate expressed 104.4 fg capsule/cfu, which was significantly higher than the 30.4 fg/cfu of capsule expressed by the wild-type ($P < 0.05$; Figure 5.5). These results demonstrate the hyper-encapsulation of the 5448AP isolate in comparison to
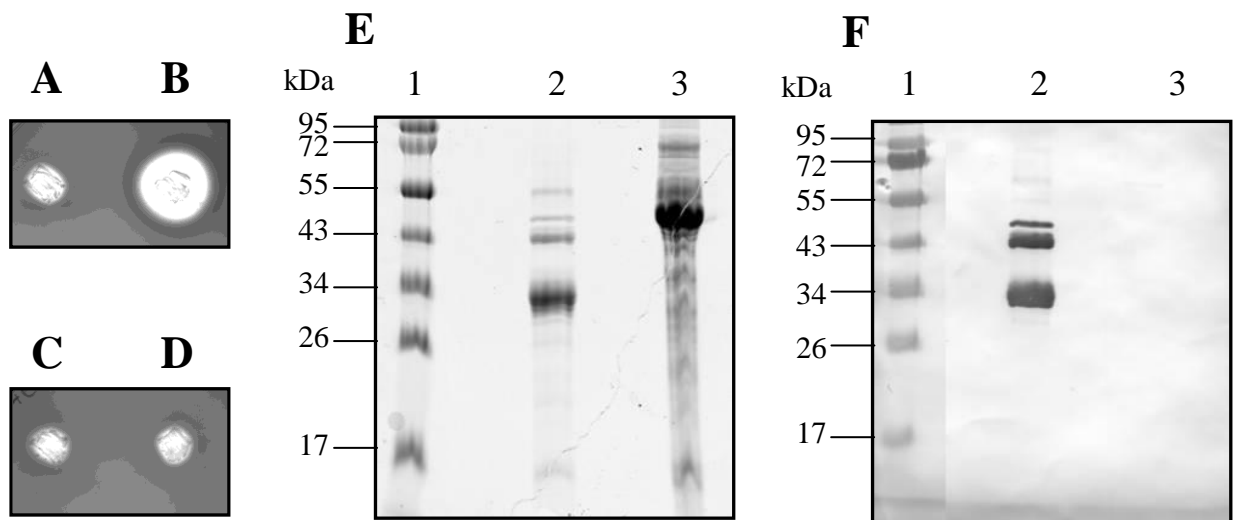
Figure 5.4 SpeB activity and expression profiles of 5448 and 5448 AP GAS isolates. (A-D) Columbia skim milk agar plate assays. Single colonies were patched onto plates in the presence and absence of the cysteine protease inhibitor E64 (28 μM) and incubated at 37 °C for 48 hours. SpeB protease activity is indicated by a darkened ring of precipitate around the site of inoculation. (A) 5448AP; (B) 5448; (C) 5448AP +E64, (D) 5448 +E64. (E) Coomassie blue stained 12% SDS-PAGE gel; lane 1) Fermentas pre-stained molecular mass markers (kDa), 2) 5448 stationary phase supernatant, 3) 5448AP stationary phase supernatant. (F) The supernatants were transferred to a PVDF membrane and probed with commercially purchased rabbit α-SpeB antibody.

wild-type 5448. It is hypothesised that the increased capsule expression exhibited by the GAS 5448AP isolate may, in part, limit the exposure of GAS surface antigens to the host immune system during infection studies using this isolate. Given that mice are not a natural host for GAS infection, the hyper-virulence in mice exhibited by the GAS isolate 5448AP render it suitable for use in the subcutaneous GAS challenge model despite the upregulation in capsule production.

5.2.3.3 Protective Efficacy of ADI, KPR and TF in a Murine Subcutaneous GAS Challenge Model

The protective efficacy of GAS vaccine antigens was further assessed using subcutaneous GAS challenge following individual intraperitoneal immunisation with ADI, KPR, TF, FBA or full length M1 protein. For logistical reasons a number of separate experimental cohorts were established at staggered time points to test the protective efficacy of vaccine antigens. Each cohort contained groups of 10 mice for each antigen under investigation in addition to one negative control group (consisting of 10 mice sham
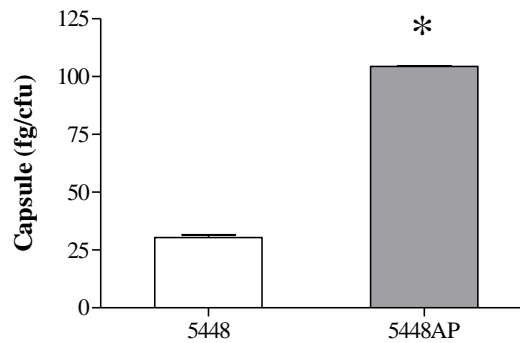
Figure 5.5 Hyaluronic acid capsule production of 5448 and 5448AP GAS isolates. GAS was grown to mid-log phase (OD of 0.6 at 600 nm) in THBY at 37°C under stationary conditions. The hyaluronic acid capsule was extracted using chloroform and quantified using the Stains-All technique previously described by Benchetrit *et al.* (1977). The data presented represent the mean ± SEM of three replicates. * indicates a significant difference in capsule production ($P < 0.05$).

immunised with PBS) and one positive control group (containing 10 mice immunised with recombinant M1 protein). All data generated in individual experimental cohorts was combined and is presented herein. One mouse sham immunised with PBS and two mice immunised with KPR died of incidental causes during the course of the experiment prior to GAS challenge. Subcutaneous doses of 5448AP GAS for the independent cohorts ranged from $8.5 \times 10^8 – 3.5 \times 10^9$ cfu/mL. After lethal subcutaneous GAS challenge with 5448AP, mice immunised with M1 protein were observed to have significantly increased survival ($P < 0.05$; Figure 5.6) compared to mice sham immunised with PBS. Experimental immunisation with ADI, KPR, TF or FBA failed to elicit a significant increase in mouse survival compared to mice sham immunised with PBS ($P > 0.05$; Figure 5.6). The effect of ADI, KPR and TF when administered in paired antigen combinations was subsequently investigated. It was hypothesised that these antigens may act synergistically when co-administered and antigen cocktails may elicit significant protection against lethal subcutaneous GAS challenge.

Two independent groups of 10 mice were immunised via the intraperitoneal route with a combination of ADI and KPR or a combination of ADI and TF as per the schedule described in Section 4.2.2.1. An additional mouse was immunised with the ADI and TF cocktail resulting in a total of 21 mice. Experimental immunisation with the ADI and TF combination was observed to produce a significant elevation in survival post 5448AP GAS

Figure 5.6 Protective efficacy exhibited by GAS vaccine antigens against lethal subcutaneous challenge with GAS isolate 5448AP following intraperitoneal immunisation of C57BL/J6 mice. Primary immunisation performed on Day 0, 10 μg of antigen in CFA; booster immunisations administered on Days 21 and 28 contained 10 μg of antigen in PBS. Subcutaneous challenge with 5448AP GAS was performed on Day 56 (28 days following final immunisation). A number of individual experimental cohorts of mice were subcutaneously challenged with 8.5 x 10^8 – 3.5 x 10^9 cfu/mL 5448AP GAS. Kaplan-Meier survival curves display the combined data for all cohorts. Mice immunised with M1 protein were the positive control group and mice sham immunised with PBS were the negative control group. * indicates a significant difference, $P < 0.05$, as determined using the log-rank test comparing the survival of mice immunised with individual vaccine antigens to the survival of mice sham immunised with PBS. A) ADI, B) FBA, C) KPR, D) TF, E) ADI + TF, F) ADI + KPR, G) M1 protein.

A ADI

Survival (%)

(*P* = 0.0747)

Days

-■- PBS (*n* = 79) ◆ ADI (*n* = 40)

B FBA

Survival (%)

(*P* = 0.6430)

Days

-■- PBS (*n* = 79) ◆ FBA (*n* = 20)

C KPR

Survival (%)

(*P* = 0.0813)

Days

-■- PBS (*n* = 79) ◆ KPR (*n* = 38)

D TF

Survival (%)

(*P* = 0.3483)

Days

-■- PBS (*n* = 79) ◆ TF (*n* = 40)

E ADI+TF

Survival (%)

(*P* = 0.0075)*

Days

-■- PBS (*n* = 79) ◆ ADI + TF (*n* = 21)

F ADI+KPR

Survival (%)

(*P* = 0.1219)

Days

-■- PBS (*n* = 79) ◆ ADI + KPR (*n* = 20)

G M1 protein

Survival (%)

(*P* < 0.0001)*

Days

-■- PBS (*n* = 79) ◆ M1 (*n* = 80)

131
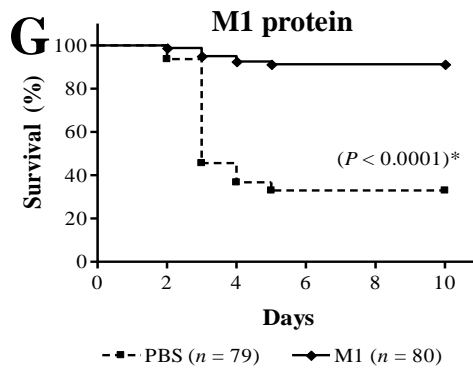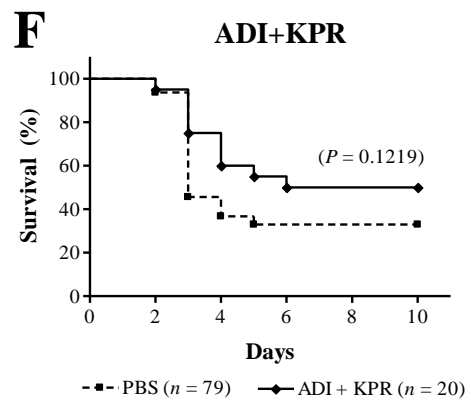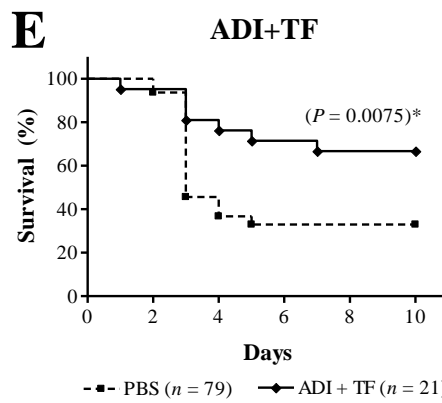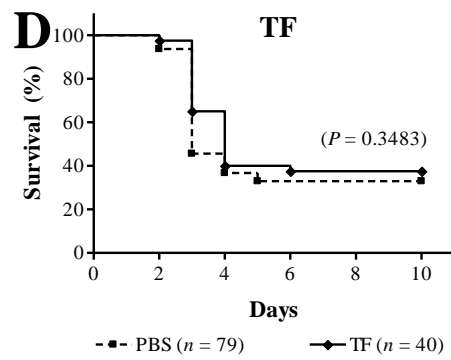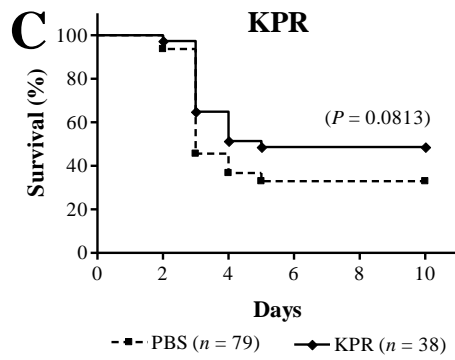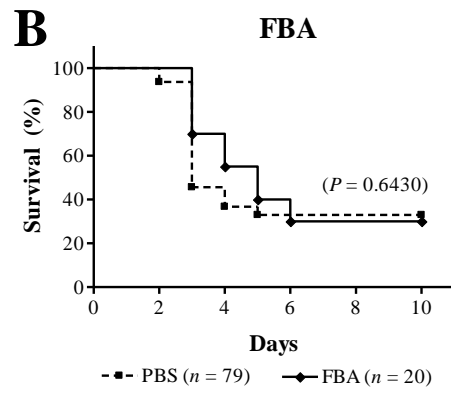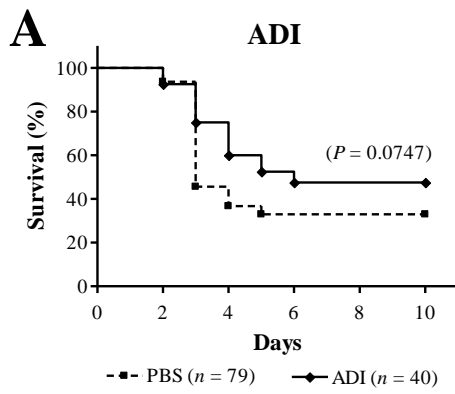
subcutaneous infection compared to mice sham immunised with PBS ($P < 0.05$; Figure 5.6). Experimental immunisation with a combination of ADI and KPR did not protect against lethal 5448AP subcutaneous challenge ($P > 0.05$; Figure 5.6). Given ADI and TF exhibit protection in a paired combination prior to subcutaneous GAS infection, further investigation of the suitability of ADI and TF as GAS vaccine antigens is justified.

5.2.4 Surface Localisation and Abundance of ADI, KPR and TF

5.2.4.1 Detection of ADI, KPR and TF on the Cell Surface of GAS Using Transmission Electron Microscopy

The distribution of ADI, KPR and TF on the surface of GAS was further investigated using IgG antibodies purified from specific rabbit anti-serum by protein A affinity chromatography. Whole GAS cells were incubated with the purified antibodies and antibodies bound to the surface of the bacterial cell were detected using gold particles conjugated to Protein A. Antibodies raised against M1 protein were utilised as a positive control and as expected pre-incubation with α-M1 IgG resulted in an accumulation of protein A conjugated gold particles on the GAS cell surface (Figure 5.7). Pre-incubation of GAS with α-ADI, α-KPR, α-TF, and α-FBA IgG antibodies also led to an accumulation of protein A gold particles on the surface of GAS. This data further confirms that each of the GAS candidate antigens ADI, FBA, KPR and TF are associated with the cell surface. In contrast, protein A conjugated gold particles did not accumulate on the surface of GAS pre-incubated with rabbit matched pre-immune IgG (Figure 5.7).

5.2.4.2 Quantification of ADI, KPR & TF on the GAS Cell Surface Using Flow Cytometry

Flow cytometry was employed to quantify cell surface-associated fluorescence following incubation of GAS cells with specific mouse polyclonal anti-sera raised against either ADI, KPR or TF. Antibodies bound to the surface of GAS were detected with a FITC-conjugated secondary antibody. Incubation of GAS with anti-sera raised against M1 protein, ADI, TF and KPR produced a significant shift in cell surface associated fluorescence in comparison to GAS incubated with PBS sham sera ($P < 0.05$; Figure 5.8). These results support the hypothesis that ADI, KPR and TF are localised and/or associated with the cell surface of GAS. In contrast, incubation with anti-serum raised against FBA
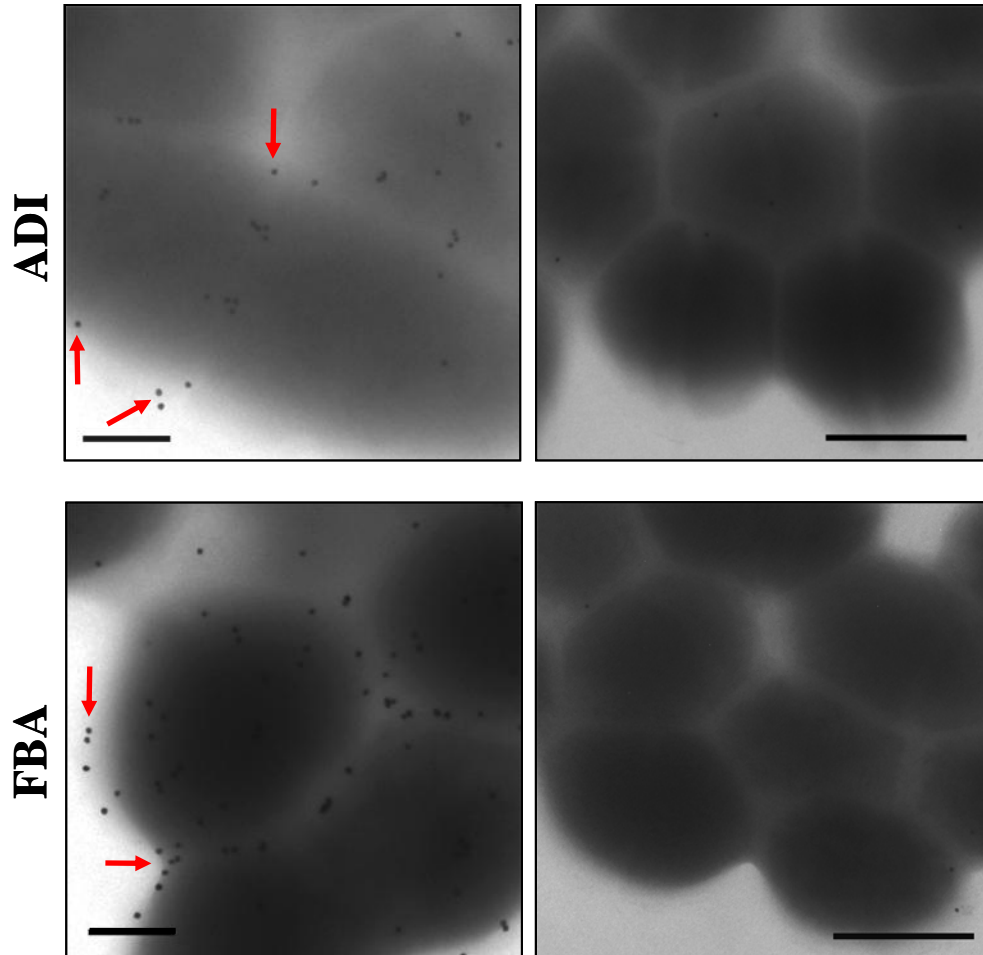
Figure 5.7 Detection of vaccine antigens on the surface of GAS isolate 5448 using transmission immuno-electron microscopy. Each antigen was administered to an individual New Zealand white rabbit. The resultant polyclonal sera (final bleed sera) was subsequently purified by protein A affinity chromatography. The isolated IgG antibodies were used to probe the GAS cell surface. Serum collected prior to immunisation (pre-immune sera) which was subsequently protein A purified, was used a negative control. Antibodies bound to the surface of GAS were visualised using protein A conjugated gold particles (indicated by red arrows). Final bleed serum shown in the left panel, scale bar represents 200nm; the corresponding pre-immune serum shown in the right panel, scale bar represents 500nm.

KPR

TF

M1
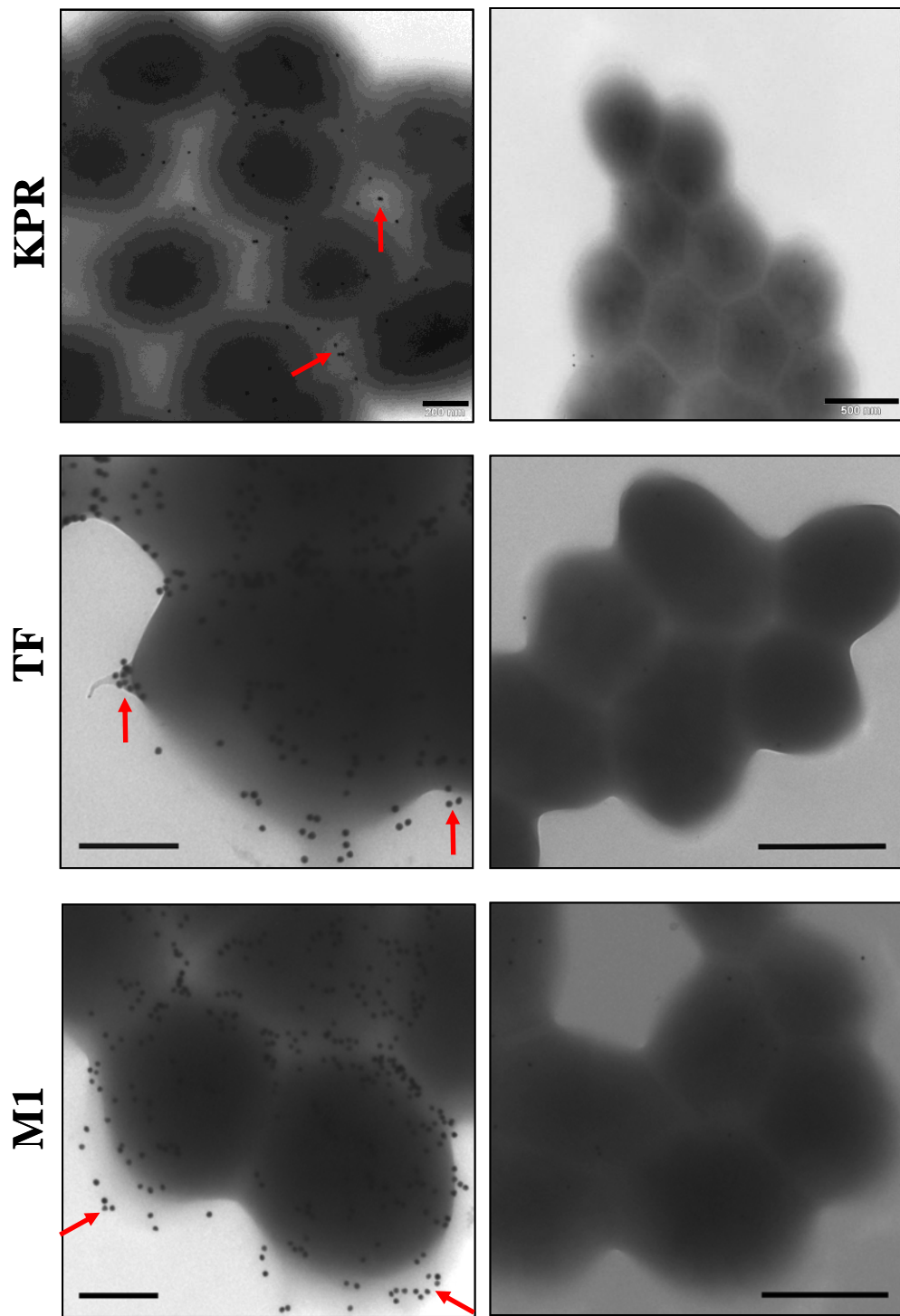
Figure 5.7 *Cont.*
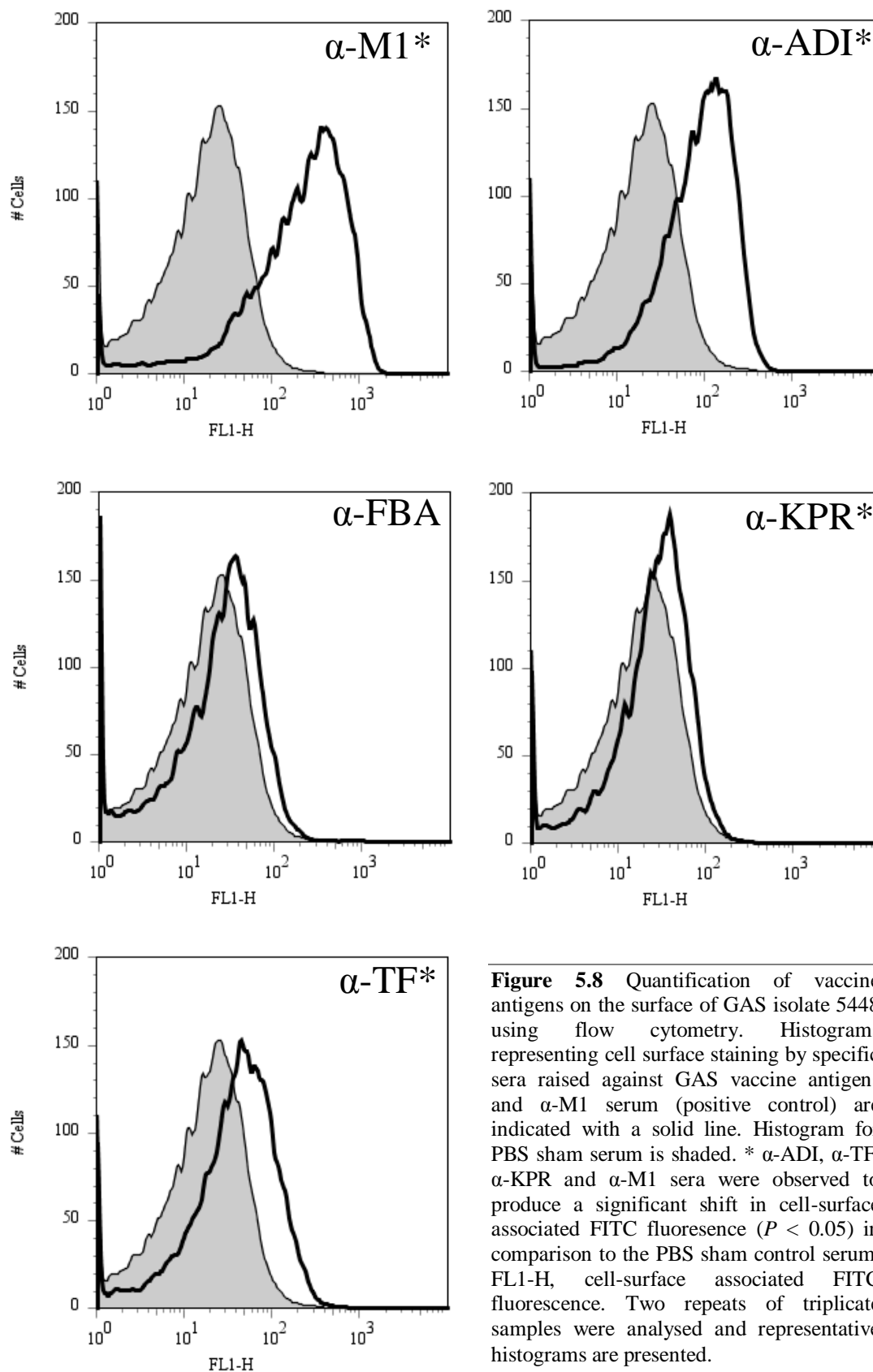
Figure 5.8 Quantification of vaccine antigens on the surface of GAS isolate 5448 using flow cytometry. Histograms representing cell surface staining by specific sera raised against GAS vaccine antigens and α-M1 serum (positive control) are indicated with a solid line. Histogram for PBS sham serum is shaded. * α-ADI, α-TF, α-KPR and α-M1 sera were observed to produce a significant shift in cell-surface associated FITC fluoresence ($P < 0.05$) in comparison to the PBS sham control serum. FL1-H, cell-surface associated FITC fluorescence. Two repeats of triplicate samples were analysed and representative histograms are presented.

did not result in a significant shift in cell surface-associated fluorescence in comparison to the PBS sham control serum ($P > 0.05$; Figure 5.8). The observed shift in cell surface fluorescence which occurred following staining with α-ADI and α-TF sera correlates with the protective efficacy exhibited by these antigens following lethal GAS challenge.

5.2.5   Mapping B-Cell Epitopes of ADI, KPR and TF

To conduct a preliminary characterisation of the discrete B-cell epitopes within ADI, KPR and TF, individual overlapping peptide array membranes were synthesised containing 15-mer peptides spanning the complete sequence of each of the antigens. Each consecutive peptide was offset by three amino acids towards the C-terminus of the protein. The membranes were probed with specific polyclonal mouse anti-serum and respective control sera (mouse matched pre-immune serum and serum from mice sham immunised with PBS). Incubation of the ADI and TF peptide arrays with either of the control sera resulted in a minimal colourmetric reaction (Figure 5.9B & C and 5.11B & C respectively). In the case of the KPR peptide array, a number of peptides reacted with both of the aforementioned control sera (Figure 5.10B and C). This reaction was consistent between the PBS sham sera and the mouse matched pre-immune serum.

Several discrete peptide spots were detected on each of the peptide arrays following probing with specific polyclonal anti-serum (Figures 5.9A, 5.10A and 5.11A respectively). There was a differing intensity of colourmetric development amongst developed peptide spots. Presumably, the lighter spots contained fewer reactive amino acids within the 15-mer peptide. Thus, for the interpretation in this study only the darker peptide spots were considered as reactive. Probing of the ADI and TF peptide arrays with specific anti-sera resulted the identification of 12 and 8 linear B-cell epitopes respectively (shaded in yellow in Figure 5.9D & 5.11D respectively). Probing of the KPR peptide membrane array with α-KPR sera resulted in the detection of 3 concise linear B-cell epitopes (shaded in yellow in Figure 5.10D), however, it also resulted in the development of one region containing 12 adjacent peptide spots. In this case concise specific linear epitope(s) could not be defined. Regions containing greater than four adjacent developed peptides possibly represent two or more individual contiguous immunogenic linear epitopes of which the distinct beginning and end of each could not be resolved. For this reason, the region on the KPR peptide
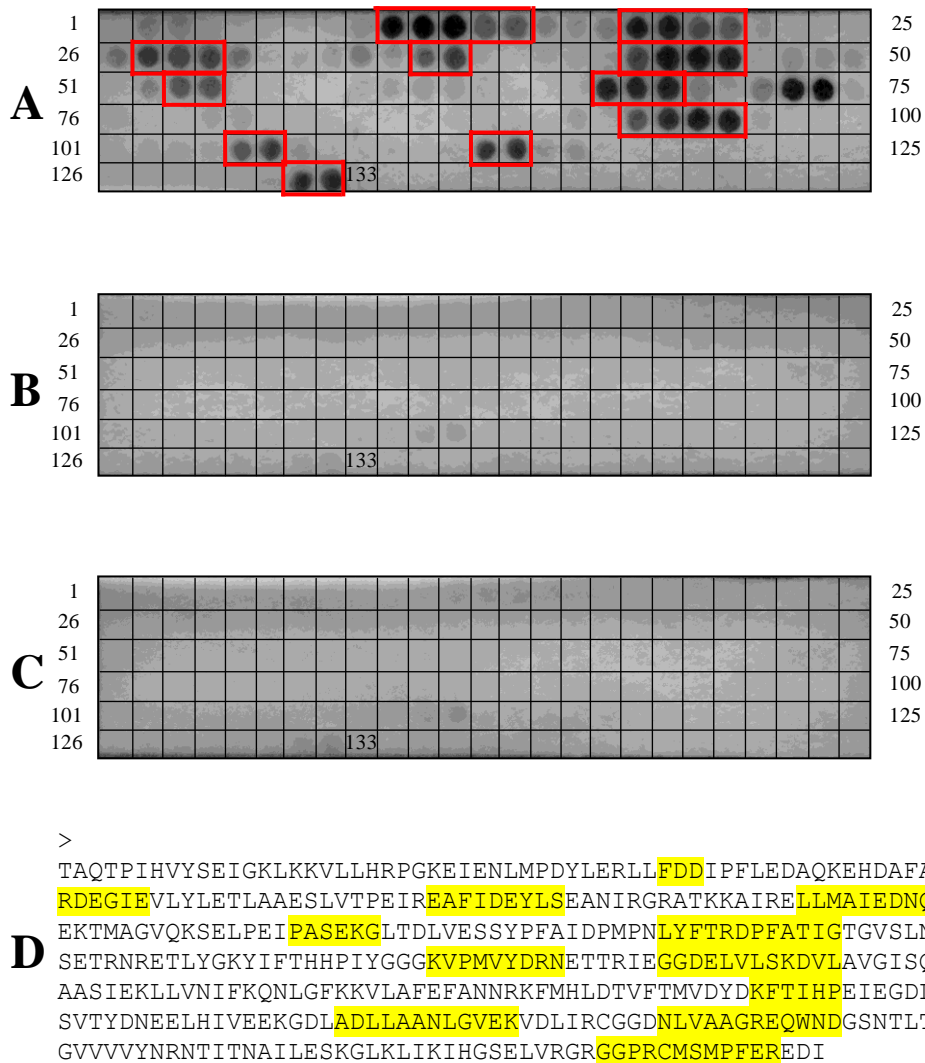
Figure 5.9 Determination of the linear B-cell epitopes of ADI. A membrane-bound array of 133 overlapping peptides (15 amino acids each, with a consecutive offset of 3 amino acids towards the C-terminus), covering the 410 amino acid sequence of ADI was probed with polyclonal mouse anti-serum. Reactive peptide spots were detected using alkaline phosphatase based colourmetric detection. The array was probed with (A) a pool of serum from BALB/c mice ($n = 10$) subcutaneously immunised with ADI, reactive peptides boxed in red; (B) corresponding mouse matched pooled pre-immune serum ($n = 10$); or (C) a pool of serum from BALB/c mice ($n = 10$) sham immunised with PBS via the subcutaneous route. (D) Linear B-cell epitopes identified within the ADI sequence highlighted in yellow.

membrane containing greater than four adjacent developed peptides was defined as an immunogenic region (indicated by gray shading in Figure 5.10D).
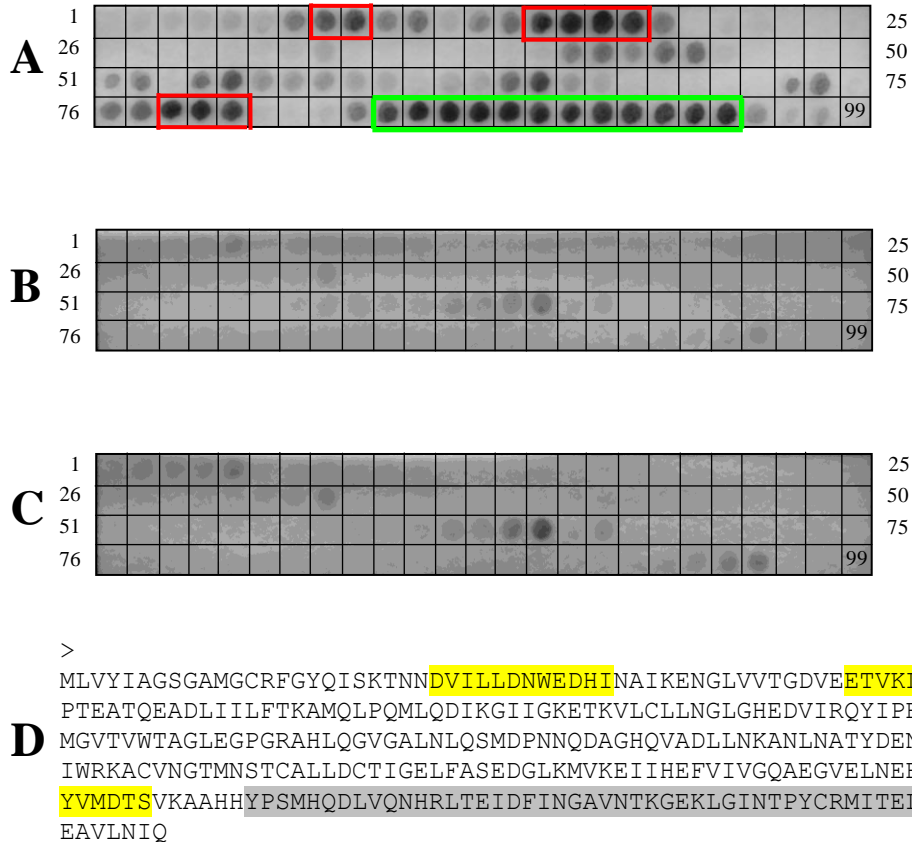
Figure 5.10 Determination of the linear B-cell epitopes of KPR. A membrane-bound array of 99 overlapping peptides (15 amino acids each, with a consecutive offset of 3 amino acids towards the C-terminus) covering the 307 amino acid sequence of KPR was probed with polyclonal mouse anti-serum. Reactive peptide spots were detected using alkaline phosphatase based colourmetric detection. The array was probed with (A) a pool of serum from BALB/c mice ($n = 10$) subcutaneously immunised with KPR, reactive peptides boxed in red and green; (B) corresponding mouse matched pooled pre-immune serum ($n = 10$); or (C) a pool of serum from BALB/c mice ($n = 10$) sham immunised with PBS via the subcutaneous route. (D) Linear B-cell epitopes identified within the KPR sequence highlighted in yellow; two or more indistinguishable adjacent epitopes, termed immunogenic regions, are highlighted in grey.

5.3   Discussion

When administered individually to mice via intraperitoneal immunisation none of the four candidate antigens; ADI, KPR, TF or FBA conferred protection against lethal subcutaneous GAS challenge ($P > 0.05$; Figure 5.6). However, the positive control, M1 protein, was protective in these experiments ($P < 0.05$; Figure 5.6). A mutation in *covS* is known to increase hyaluronic acid capsule expression in the 5448AP isolate (Sumby *et al.*, 2006). The increased production of capsule may result in steric hindrance and consequently reduce the accessibility of antigen localised on the GAS cell surface to circulating antibody. In one study, an upregulation of capsule hindered the recognition of polyclonal antibodies
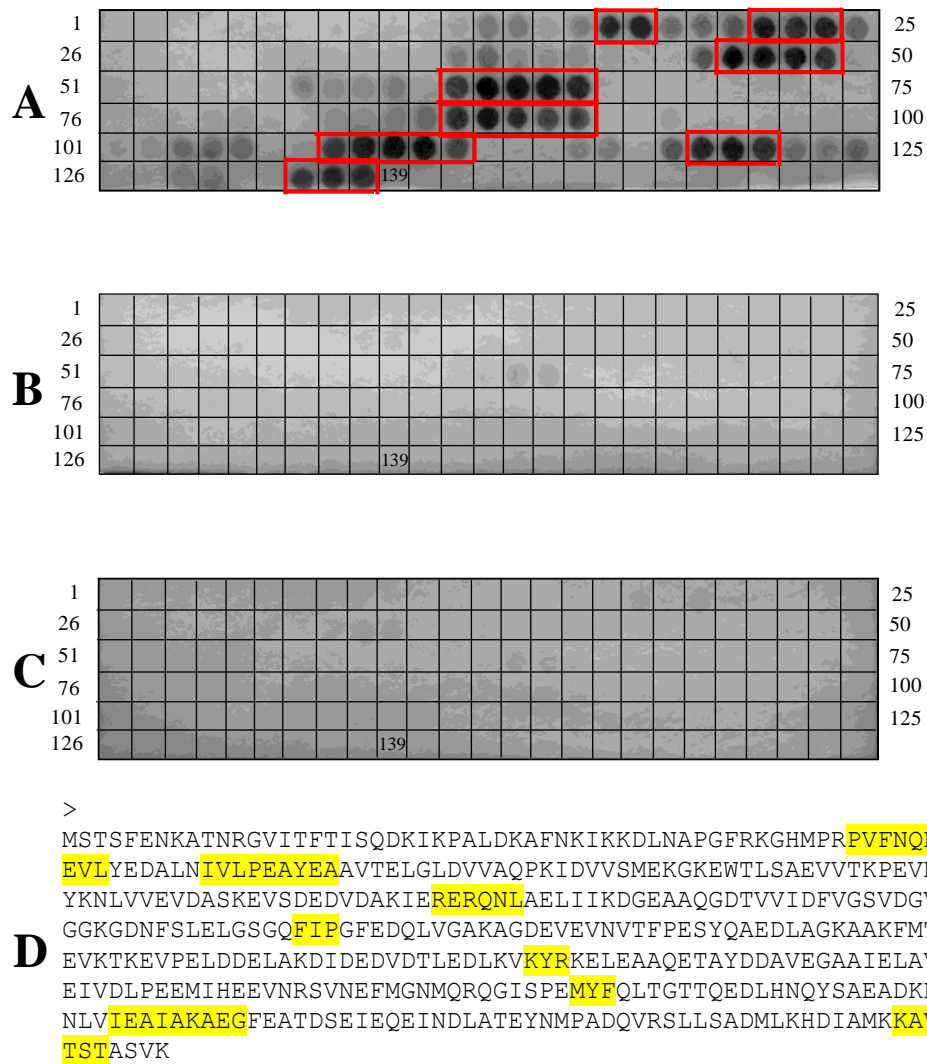
Figure 5.11 Determination of the linear B-cell epitopes of TF. A membrane-bound array of 139 overlapping peptides (15 amino acids each, with a consecutive offset of 3 amino acids towards the C-terminus) covering the 427 amino acid sequence of TF was probed with polyclonal mouse anti-serum. Reactive peptide spots were detected using alkaline phosphatase based colourmetric detection. The array was probed with (A) a pool of serum from BALB/c mice (*n* = 10) subcutaneouly immunised with TF, reactive epitopes are boxed in red; (B) corresponding mouse matched pooled pre-immune serum (*n* = 10); or (C) a pool of serum from BALB/c mice (*n* = 10) sham immunised with PBS via the subcutaneous route. (D) Linear B-cell epitopes identified within the TF sequence highlighted in yellow.

specific protein GRAB, rendering these antibodies inefficient in the initiation of *in vitro* opsonophagocytosis (Dinkla *et al.*, 2007). Although M1 protein was observed to elicit protection against infection with this capsule-abundant strain, M protein is known to be one of the most prominent surface proteins of GAS, existing as long hair-like extensions protruding approximately 500 Å into the extracellular milieu (Phillips *et al.*, 1981). Although each of the vaccine antigens of this study were detected following western blotting of cell wall extracts (Table 4.1), the relative expression levels and conformational

arrangement of ADI, KPR and TF on the cell surface of GAS remains unknown and is worthy of further investigation. The presence of the vaccine antigens on the cell surface of GAS was quantified *in vitro* using flow cytometry. Subsequent measurement of cell-surface associated fluoresence indicated a high level of α-M1 serum bound to the surface of 5448 GAS (Figure 5.7) confirming the abundance of this protein on the surface of GAS. The polyclonal serum used to detect ADI, KPR and TF, also produced a significant shift in cell surface associated fluorescence in comparison to the PBS sham serum ($P < 0.05$), further supporting the hypothesis that these antigens are localised on or are associated with the surface of GAS. Whilst the flow cytometry and immuno-EM analyses indicate the presence of ADI, KPR and TF on the cell surface of GAS, 3D configuration and arrangement of these antigens on the GAS cell surface remains unknown.

When administered in a two-antigen cocktail combination ADI and TF were observed to act synergistically, conferring significant protection against lethal subcutaneous 5448AP GAS challenge ($P < 0.05$; Figure 5.6). It is hypothesised that vaccine preparations containing a number of GAS antigens (or fragments thereof) may overcome issues of serotype dependent protection in cases where the expression of individual antigens is limited to a subset of serotypes. A number of GAS vaccine preparations described in the literature have contained two or more GAS-based antigenic constituents. For example, immunisation with a fusion protein consisting of fragments of SpeA and SpeB protected mice against lethal GAS infection (Ulrich, 2008). The 26-valent GAS vaccine preparation under investigation by Dale *et al.* contains N-terminal M protein fragments derived from 26 distinct GAS serotypes expressed as four recombinant fusion proteins in addition to a fragment from Spa (Hu *et al.*, 2002). This preparation has entered human clinical trials and has been well tolerated (Kotloff *et al.*, 2004, McNeil *et al.*, 2005). One of the C-repeat region based M protein vaccine preparations contains J8 coupled to an N-terminal segment of the M protein from the GAS isolate 88/30 (Olive *et al.*, 2003, Olive *et al.*, 2005). Subcutaneous administration of this conjugate to B10:Br mice resulted in protection against homologous GAS intraperitoneal challenge (Olive *et al.*, 2005, Olive *et al.*, 2003). Another M protein C-repeat region based epitope, JJo has been recombinantly produced as a fusion protein with H12 (a fragment of Protein F1) (Georgousakis *et al.*, 2009b). The administration of this fusion protein to BALB/c mice via the subcutaneous route resulted in

a high titre of specific serum IgG antibodies (Georgousakis *et al.*, 2009b), however, the protective efficacy of JJo-H12 is yet to be investigated.

Cocktail vaccine preparations are also under investigation in other pathogenic streptococcal species. A combination of PiuA and PiaA was observed to protect BALB/c mice against intraperitoneal infection with pneumococcus (Brown *et al.*, 2001). Furthermore, the survival was greater than that observed following individual immunisation with PiuA or PiaA (Brown *et al.*, 2001). The pneumococcal antigens PspA, PsaA and PdB have also been co-administered to BALB/c mice via the intraperitoneal route (Ogunniyi *et al.*, 2000). These antigens were observed to act in a synergistic manner and elicited heterologous protection against lethal pneumococcal systemic infection (Ogunniyi *et al.*, 2000). At this stage, the mechanisms providing elevated protection in the subcutaneous GAS infection model following the co-administration of ADI and TF are not yet elucidated, however, further investigation of the suitability of ADI and TF as GAS vaccine candidates administered individually or in combination is warranted.

A murine infection model utilising subcutaneous immunisation and subcutaneous challenge has been recently used to assess the protective efficacy of the streptococcal esterase, Sse, via both active and passive immunisation experiments (Liu *et al.*, 2007). Additionally, researchers have performed intranasal immunisation followed by subcutaneous GAS challenge to examine the protective efficacy of Protein F1 (McArthur *et al.*, 2004a). Experimental immunisation with Protein F1 did not protect BALB/c mice against lethal subcutaneous GAS challenge (McArthur *et al.*, 2004a). Whilst murine GAS skin infection models are not as commonly utilised as systemic or intranasal infection models in the literature, the subcutaneous infection model utilised in this study is of great significance in Australia as the skin is the major site of GAS infection for Aboriginal populations (Currie *et al.*, 2000) who have been widely reported to suffer endemic GAS infection (Carapetis *et al.*, 1999). In these populations the prevalence of pyoderma in children has been reported to be as high as 70% (Gardiner *et al.*, 1996). GAS skin infections can predispose invasive systemic infection and auto-immune sequelae and ARF and APSGN. To our knowledge, this is the first study in which a murine GAS subcutaneous challenge model has been preceeded by an intraperitoneal immunisation regime to

determine the immunogenicity and protective efficacy of GAS vaccine candidates. Thus, this newly developed intraperitoneal immunisation and subcutaneous GAS challenge model is an important tool for the study of putative GAS vaccine candidates.

Peptide array membranes containing 15-mer peptides comprising the sequence of ADI, KPR and TF respectively were probed with specific polyclonal anti-sera to determine linear B-cell epitopes contained within these antigens. Twelve, 3 and 8 linear B-cell epitopes were determined within the sequences of ADI, KPR and TF respectively (Figures 5.9 – 5.11 respectively). Antibodies raised against one or more of these epitopic regions may be contributing to the protective immune response in mice mediated following experimental immunisation. In this approach one region of the KPR peptide array contained 12 developed adjacent peptide spots (Figure 5.10A). Contiguous linear epitopes separated by fewer than 15 amino acids, such as this region on the KPR array could not be defined and was consequently termed an immunogenic region. As the peptides immobilised on the membrane arrays were offset by three amino acids, all epitopes of this study were defined in multiples of 3 amino acids. Perhaps the minimal reactive B-cell epitopes of each individual antigen could be determined via the synthesis and subsequent probing of a peptide array membrane with a consecutive overlap of only one amino acid. Once identified, the minimal reactive B-cell epitopes of ADI, KPR and TF could be synthesised and used to immunise mice in conjunction with a carrier molecule, possibly another previously investigated GAS vaccine candidate such as H12 (Schulze *et al.*, 2003c, Schulze *et al.*, 2003a) or a lipid based preparation for example. The contribution of individual epitopes to the protective efficacy of the conjugate could be subsequently evaluated. In regards to the linear immunogenic regions identified within ADI, KPR and TF in this study individual amino acids located within these epitopes could be mutagenised in recombinant forms of the antigens via site directed mutagenesis. These mutated antigens could then be used in immunisation and GAS challenge experiments to determine key residues involved in antibody recognition and binding. Moreover, such mutants may shed light on functionally essential amino acids of the catalytic site of each of these anchorless enzymes.

A study performed by Schulze *et al.* (2003) determined the reactive B-cell epitopes and a T-cell epitope contained within the Fn-binding repeat of Protein F1. A similar peptide

membrane technique as that employed in this study was used for the determination of B-cell epitopes (Schulze *et al.*, 2003b). To identify the T-cell epitope spleen cells isolated from mice immunised with a recombinant protein encompassing the Fn-binding region of Protein F1 were stimulated *in vitro* with 15 individual peptides (Schulze *et al.*, 2003b). T-cell proliferation in response to each of the peptides was determined by the extent of [^{3}H]thymidine incorporation into the newly generated T-cells (Schulze *et al.*, 2003b). The mapping and analysis of T-cell epitopes within ADI, KPR or TF could be explored using a similar method of T-cell stimulation.

Whilst the peptide array membranes for ADI, KPR and TF enabled the determination of linear B-cell epitopes, the presence and distribution of conformational epitopes of these antigens is yet to be explored. One method to determine conformational epitopes is epitope excision mass spectrometry (McLaurin *et al.*, 2002, Stefanescu *et al.*, 2007). In this technique specific polyclonal anti-serum raised against ADI, KPR or TF respectively would be bound to N-hydroxysuccinimide (NHS)-activated sepharose columns. Full length recombinant protein applied to the respective antibody conjugated sepharose column would be subsequently exposed to protease(s), resulting in the degradation of regions of the antigen not bound by the antibodies. Following sufficient washing of the column MALDI-MS could be used to determine the amino acid sequence of bound conformational epitopes. The knowledge of conformational epitopes of these antigens could facilitate the design of cocktail vaccine preparations containing combinations of conformational and linear epitopes from ADI, KPR or TF and/or other GAS vaccine candidates previously described in the literature.

Whilst ADI and TF elicited protective immune responses in both the systemic and subcutaneous GAS challenge models, further extensive characterisation of the suitability of these antigens as GAS vaccine candidates is considered a worthwhile endeavour. Future characterisation of ADI and TF could include the determination of the protective efficacy of these antigens in an intranasal immunisation and intranasal GAS challenge model or testing the immunogenicity and protective efficacy of the GAS vaccine antigens when co-administered with Alum, an adjuvant which is currently approved as safe for human use (Petrovsky *et al.*, 2004). Investigation of the protection these antigens confer against

infection with heterologous GAS strains and further exploration of vaccine cocktail formulations could also be examined. An important avenue of exploration is the safety of ADI and TF when administered to human hosts as GAS vaccine antigens. Chapter 6 will investigate the reactivity of antigens with human serum obtained from two geographically distinct populations in which GAS infection is endemic. In addition, the cross-reactivity of α-ADI and α-TF sera with human heart extract will be examined.

6.   PRELIMINARY INVESTIGATION INTO THE SAFETY OF GAS VACCINE ANTIGENS

6.1   Introduction

M protein has been widely characterised as a GAS vaccine component, however, the development of M protein based GAS vaccines has been significantly hindered by observed cross-reactivity of α-M protein antibodies with human heart proteins such as myosin, tropomyosin and vimentin (Dale *et al.*, 1982, Dale *et al.*, 1985a, Dale *et al.*, 1985b, Fenderson *et al.*, 1989, Kraus *et al.*, 1989). This molecular mimicry may lead to the development of the post-streptococcal immune sequelae ARF and RHD in the host. Recent investigations into the structure of the M1 protein unveiled a number of instabilities and irregularites throughout the coiled coil α-helical structure (McNamara *et al.*, 2008). Very similar structural irregularities were discovered in human myosin and tropomyosin and it is hypothesised that cross-reactivity of circulating α-M1 antibodies with these host proteins may initiate GAS auto-immune sequelae in the host (McNamara *et al.*, 2008). Since reports of an increased occurrence of ARF in individuals vaccinated with M protein based preparations compared to unvaccinated individuals (Massell *et al.*, 1969) researchers have approached putative GAS vaccine candidates and their potential to induce auto-immunity in the host with vigilance.

GAS infections and auto-immune sequelae have a high prevalence in developing countries and amongst indigenous populations of industrialised nations. The Aboriginal population of the Northern Territory of Australia has one of the highest reported rates of GAS disease and auto-immune sequelae worldwide (Currie *et al.*, 2000); with the incident rate of ARF as high as 650 per 100,000 and the prevalence of RHD in one rural Indigenous community measured at 24 cases per 1,000 (compared to 0.14 per 1,000, the rate of RHD of non-Aboriginal individuals living in the same region) (Carapetis *et al.*, 1996). GAS strains isolated from Indigenous Australians suffering GAS infection are diverse. One study conducted in the top end of the Northern Territory, Australia, in which Indigenous Australians comprised 73% of the patients surveyed, isolated GAS belonging to 75 distinct vir types from 37 cases of invasive disease (bacteremia and necrotising fasciitis) and 38 cases of non-invasive disease (pyoderma and pharyngitis) (Delvecchio *et al.*, 2002). In

addition, two different GAS strains (of distinct vir type) were isolated from two of the patients surveyed (Delvecchio *et al.*, 2002). Likewise, another study detected multiple GAS strains in 8 of 12 swabs obtained from cases of pyoderma in Aboriginal children and adults living in the same geographical region (Carapetis *et al.*, 1995a). Such results indicate that co-infection with two or more GAS strains is possible. A similar diversity is evident in the epidemiological profile of GAS infections in populations in northern India (Sagar *et al.*, 2008, Menon *et al.*, 2001, Dey *et al.*, 2005, Sagar *et al.*, 2004, Kumar *et al.*, 2009), in which no individual serotype(s) of GAS appear to be dominant in these populations. The diversity of GAS strains infecting individuals in these two geographical regions is in striking contrast to the clonality of strains which has been documented in industrialised populations, in which M1 and M3 isolates predominantly cause invasive GAS infection (Musser *et al.*, 1991, Upton *et al.*, 1995, Vlaminckx *et al.*, 2003, Talkington *et al.*, 1993, Schwartz *et al.*, 1990, Gaworzewska *et al.*, 1988, Aziz *et al.*, 2008). The selection of serotypes for the N-terminal M protein based 26-valent GAS vaccine under development by Dale *et al.* was based on the most frequent serotypes causing GAS infection in industrialised nations; M1.0, M1.2, M2, M3, M5, M6, M11, M12, M13, M14, M18, M19, M22, M24, M29, M33, M43, M59, M75, M76, M77, M89, M92, M101, M114 (Hu *et al.*, 2002). These 26 serotypes are infrequent or absent in infections in developing nations and Indigenous populations. In a recent review, Smeesters *et al.* (2009) theoretically predicted the level of coverage the 26-valent vaccine would provide in 16 different populations worldwide based on current epidemiological information. The theoretical percent coverage ranged from 86% in Mexico to as low as 21% in Nepal (Smeesters *et al.*, 2009). The percent coverage in Indigenous Australian populations and in India is predicted to be only 25% and 21% respectively, a stark contrast to the 84% coverage in the USA and Canada (Smeesters *et al.*, 2009). Presumably, the under-representation of GAS serotypes infecting these populations in this vaccine preparation will limit the usefulness of this vaccine in individuals living in these regions in which the rate of GAS infection and auto-immune sequelae is one of the highest worldwide. The use of ubiquitious, conserved surface antigens in GAS vaccine preparations, such as ADI, KPR and TF should result in broad protection in all populations and circumvent issues of serotype specificity.

The work in this chapter aimed to ascertain the human immune response to the GAS vaccine antigens and to identify any potential cross-reactivity between antigen specific antibodies and human tissues. Human serum was obtained from patients suffering endemic GAS infection living in the Northern Territory of Australia and in Chandigarh, India. The serum from Aboriginal individuals was collected from patients admitted to the Royal Darwin Hospital and from children during remote community surveys. The serum from Chandigarh, India, was from individuals ranging from 9 to 22 years old. Serum from both populations was screened for reactivity with the GAS vaccine antigens in ELISA. Human serum was obtained from these populations for these studies as the diversity of GAS strains infecting individuals in these regions is high and repeated cases of GAS infection in these populations results in some of the highest global rates of auto-immune sequelae. ELISA was also utilised to test the cross-reactivity of polyclonal anti-serum raised against ADI, KPR and TF with human heart extract.

6.2   Results

6.2.1   Cross-Reactivity of Human Serum with GAS Vaccine Antigens

6.2.1.1 Reactivity of Serum Obtained from Australian Aboriginals Suffering Endemic GAS Infection with GAS Vaccine Antigens

The Aboriginal population of the Northern Territory of Australia has one of the highest reported rates of GAS disease and auto-immune sequelae (Currie *et al.*, 2000). This experiment aimed to examine whether members of this population who suffer frequent GAS infections (Carapetis *et al.*, 1999) have developed antibodies reactive against the vaccine antigens of this study. It is hypothesised that an immune response will not be mounted against the vaccine antigens in individuals of this population who suffer repeated GAS infections as these antigens protected against GAS infection in mice and it is obvious that re-infection of individuals of this population is not resulting in protective immunity. The immune response was firstly investigated using two separate pools of serum obtained from Aboriginal children (*n* = 30) and Aboriginal adults (*n* = 7) respectively. The serum was used to probe recombinant forms of the antigens in western blots. M1 protein reacted with the pooled serum obtained from both Aboriginal children and adults (Figure 6.1), while none of the 13 vaccine antigens exhibited a band of expected molecular mass with either of the pools of serum (Figure 6.1). Whilst a band was developed in the lanes in which

RRF was loaded (Figure 6.1B & 6.1C), this band is not the same size as recombinant RRF (Figure 6.1A). It is possible the patient serum has reacted with a higher mass multimeric form of recombinant RRF in this lane. The reaction of the 13 GAS vaccine antigens with the pool of serum from Aboriginal children suffering endemic GAS infection was further explored using ELISA.

In comparison to M1 protein, the sera from Aboriginal children had a minimal serum IgG antibody response with the 13 putative vaccine antigens of this study ($P < 0.05$; Figure 6.2). Although the pooled sera from Aboriginal individuals (children and adults) suffering endemic GAS infection reacted with a band in the lane in which RRF was loaded in the western blots (Figure 6.1), recombinant RRF did not have a significant reaction with the serum from Aboriginal children when tested by ELISA (Figure 6.2).

6.2.1.2 Reactivity of Serum from an Indian Population Suffering Endemic GAS Infection with GAS Vaccine Antigens

The human immune response mounted against the GAS vaccine antigens was also investigated using four discrete pools of serum isolated from individuals living in Chandigarh, India. The patients were classified into the following clinical disease categories, control ($n = 10$), pharyngitis ($n = 8$), ARF ($n = 9$) and RHD ($n = 10$). For each of the four pools of sera, the serum IgG antibody response detected against the 13 vaccine antigens of this study was significantly less than the response mounted against M1 protein ($P < 0.05$; Figure 6.3).

6.2.2 Reactivity of α-ADI, α-KPR and α-TF Sera With Human Heart Extract

Following the observation that recombinant ADI, KPR and TF did not react with patient serum collected from two geographically distinct populations suffering endemic GAS infection, the cross-reactivity of anti-serum raised against ADI, KPR or TF with human heart extract was determined. Human heart extract was prepared by homogenising of a 5 g sample of human heart tissue resulting in the release of proteins. α-ADI, α-KPR and α-TF serum did not significantly react with the human heart extract ($P > 0.05$; Figure 6.4). Similarly, neither the negative control sera nor the naïve rabbit sera, exhibited a significant reaction with the extract ($P > 0.05$; Figure 6.4). In contrast, α-M1 serum had a significant reaction with the human heart extract ($P < 0.05$; Figure 6.4).
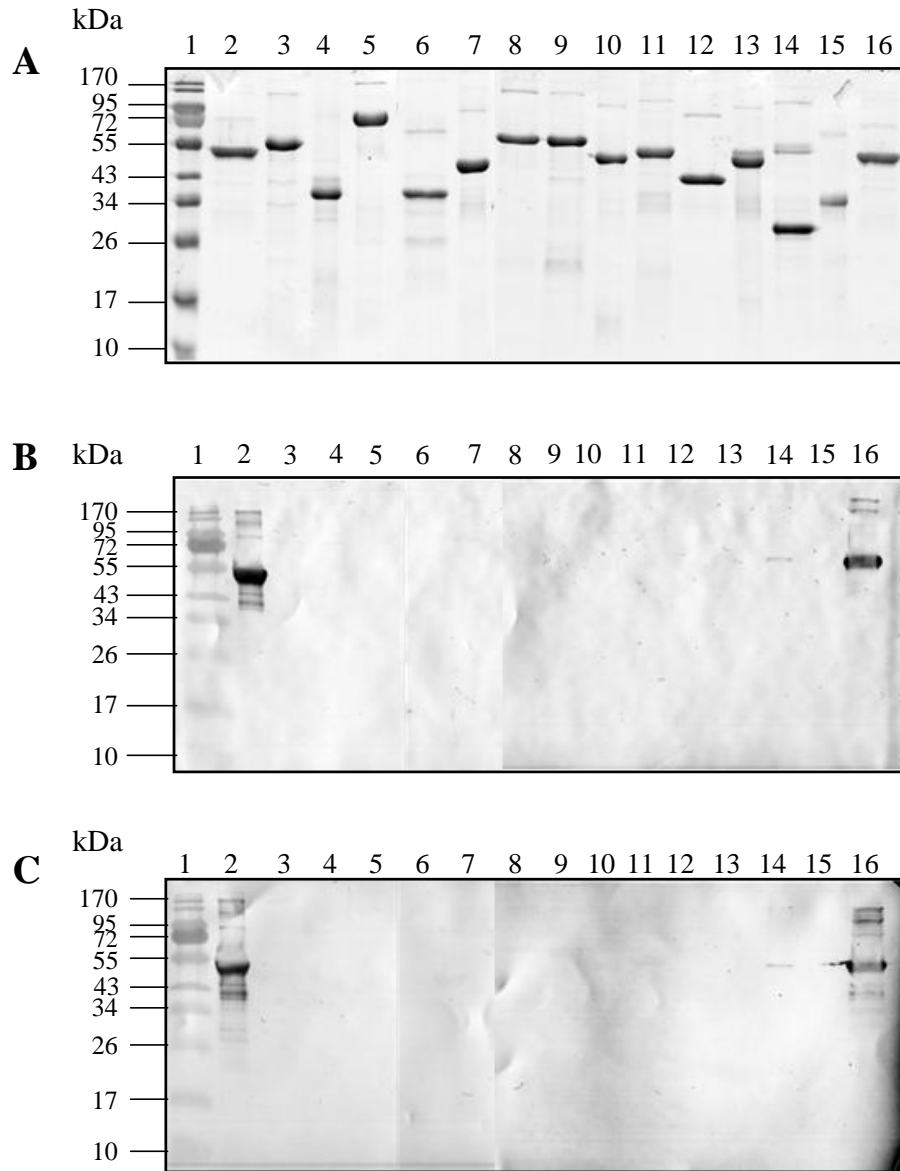
Figure 6.1 The immune response detected against GAS vaccine antigens in sera from Aboriginal individuals was investigated using western blotting. Vaccine antigens and control (M1 protein) were resolved on 12% SDS-PAGE reducing gels and either (A) stained with Coomassie brilliant blue, or transferred to PVDF membrane and (B) probed with a pool of adult Aboriginal serum (*n* = 7) diluted 1:1,000, or (C) probed with a pool of serum (*n* = 30) diluted 1:1,000 obtained from Aboriginal children living in remote communities of the Northern Territory (Australia) suffering endemic GAS infection. Lanes are as follows; 1, Fermentas prestained molecular mass marker (kDa); 2, M1 protein; 3, ADI; 4, KPR; 5, TF; 6, AK; 7, BCAT; 8, EF-Tu; 9, FBA; 10, OTCase; 11, NADP-GAPDH; 12, PFK; 13, PGK; 14, RRF; 15, TIM; 16, M1 protein.
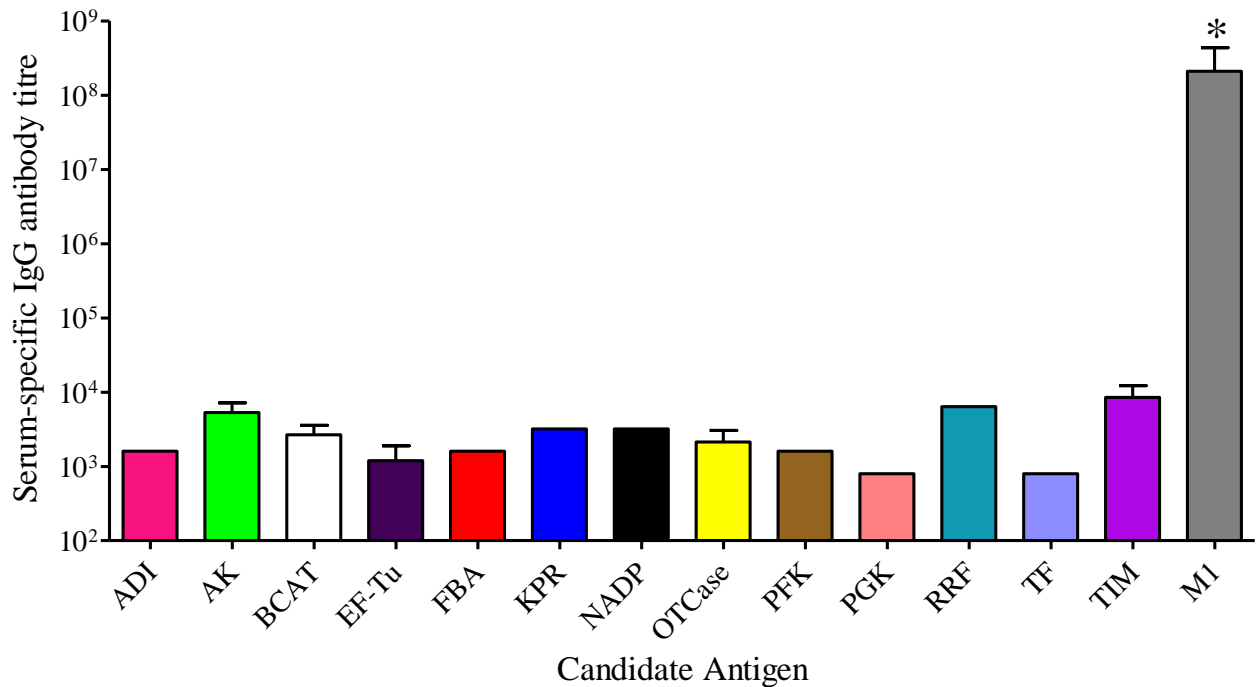
Figure 6.2 Serum obtained from Aboriginal children (n = 30) living in remote communities of the Northern Territory (Australia) suffering endemic GAS infection does not recognise GAS vaccine antigens in ELISA. * The response against M1 protein is significantly higher than the response with test antigens (P = 0.05) as determined using ANOVA one-way analysis of variance. The response against each vaccine antigen was tested in triplicate and the mean ± SD is presented.

6.3 Discussion

The potential cross-reactivity of GAS vaccine candidates with host proteins and tissue, specifically cardiac proteins including myosin, tropomysin, vimentin and laminin has been an obstacle for GAS vaccinology dating back to a report describing the development of ARF following the administration of M protein to humans in early vaccine trials (Massell *et al.*, 1969). In this study, a preliminary investigation of antigen safety was performed in which the reactivity of GAS vaccine antigens with patient serum obtained from two distinct geographical populations suffering endemic GAS infection was tested. The vaccine antigens of this study did not significantly react with any of the pools of infected patient serum in ELISA experiments (P < 0.05; Figure 6.2 and 6.3). This was in direct contrast to the reaction detected against M protein (P > 0.05; Figure 6.2 and 6.3). The Aboriginal population from which the tested sera was obtained has the highest incidence of ARF worldwide (Carapetis *et al.*, 1996). It has been previously hypothesised that the presence of circulating α-M protein antibodies (McNamara *et al.*, 2008) and antibodies against GAS CHO (Goldstein *et al.*, 1967) may lead to cases of auto-immune sequelae.

Thus, the lack of immune response mounted against the vaccine antigens of this study by individuals of these populations who suffer high rates of GAS auto-immune sequelae, suggests that these antigens are not involved in triggering auto-immune disease. The lack of immune response against these antigens, which have been shown in this study to be highly immunogenic in mice, suggests that GAS may have evolved a mechanism of shielding these antigens from the host immune response. Whilst α-M protein antibodies have been observed to protect against lethal GAS infection in this study and many others (outlined in Cole *et al.*, 2008), in populations suffering endemic GAS infection α-M protein antibodies are clearly not providing broad spectrum protection against repeated GAS infection. The high α-M protein titres in human serum observed in Figure 6.2 may reflect the diversity of M types continually infecting individuals in this population. Additionally, it is possible that M protein may be immune-dominant in natural infection and that the host immune response may preferentially recognise M protein over other GAS surface proteins due to its abundance on the surface of GAS.

A previous study examined the reaction between the proteins Protein F1 and SOF, and patient serum obtained from an Indigenous population in Australia suffering endemic GAS infection (Goodfellow *et al.*, 2000). High levels of α-Protein F1 and α-SOF serum IgG were detected in all pools of sera obtained from patients grouped in the following clinical disease categories, RHD, healthy control, non-RHD, ARF, bacteremia and APSGN (Goodfellow *et al.*, 2000). Yet, this population suffers recurrent GAS disease, thus the natural immune response in humans against Protein F1 and SOF does not appear to offer protection from GAS re-infection. However, experimental immunisation with both Protein F1 and SOF has resulted in protection against lethal GAS infection in mouse models (Guzmán *et al.*, 1999, Schulze *et al.*, 2006b, Schulze *et al.*, 2003c, Schulze *et al.*, 2003a, Gillen *et al.*, 2008). The lack of protection that antibodies reactive against Protein F1 and SOF provide in natural human infection may be a consequence of the variability within their sequence or differential expression of these proteins.

The potential for antigenic epitopes within putative GAS vaccine candidates to elicit circulating antibodies which are cross-reactive with human proteins is of significant concern, particularly M protein based preparations. Other studies have tested the cross-

Figure 6.3 Four discrete pools of serum from individuals living in Chandigarh, India suffering endemic GAS infection does not recognise GAS vaccine antigens in ELISA. Sera pooled according to the following clinical disease categories; A) control ($n = 10$), B) pharyngitis ($n = 8$), C) ARF ($n = 9$) and D) RHD ($n = 10$). * The response against M1 protein is significantly higher than the response with test antigens ($P < 0.05$) as determined using ANOVA one-way analysis of variance. The response against each antigen was tested in triplicate and the mean ± SD is presented.
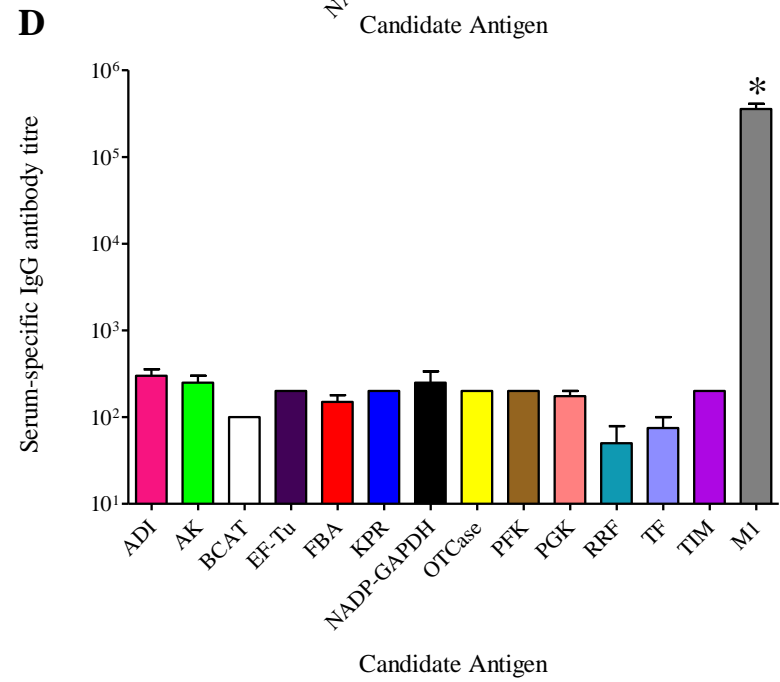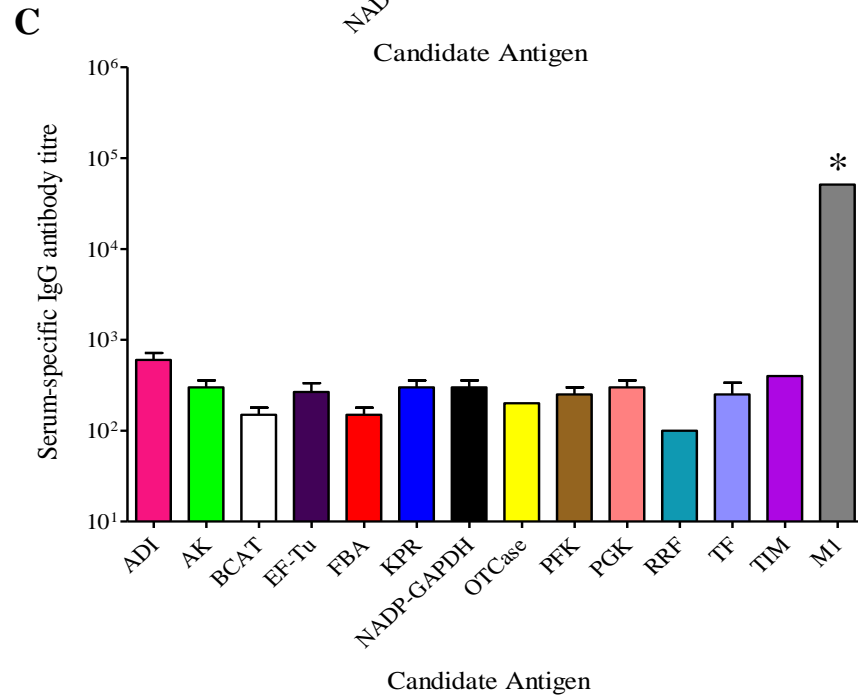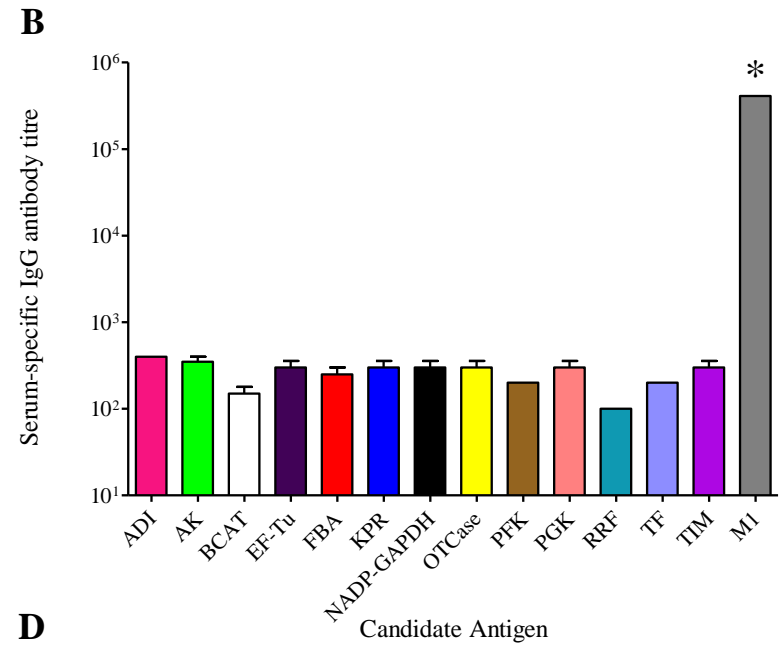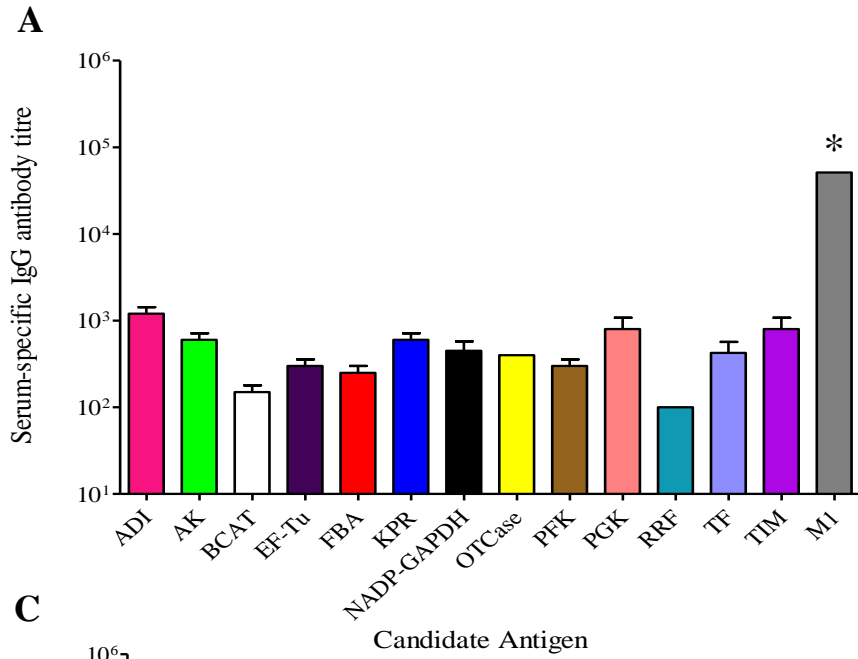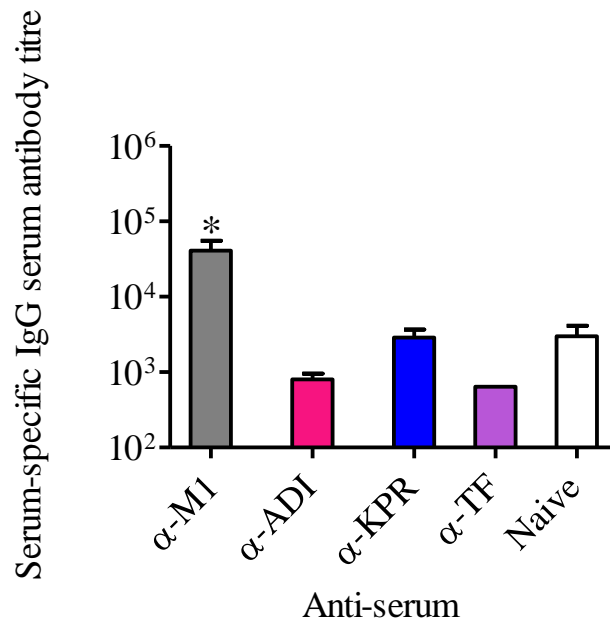
Figure 6.4 Reactivity of rabbit polyclonal anti-sera raised against ADI, KPR and TF with human heart extract tested by ELISA. Naïve rabbit sera was used as a negative control. * The response with α-M1 sera was significantly higher than that with the test sera ($P < 0.05$). Each sera was tested in triplicate and the mean ± SEM is presented.

reactivity of antibodies generated against GAS vaccine antigens with individual human cardiac proteins such as myosin (Dale *et al.*, 1985a, Dale *et al.*, 1986, Cunningham *et al.*, 1989, Cunningham *et al.*, 1992), tropomyosin (Fenderson *et al.*, 1989), laminin (Antone *et al.*, 1997, Galvin *et al.*, 2000), actin, keratin and vimentin (Shikhman *et al.*, 1993). In this study the capacity of polyclonal anti-serum raised against ADI, KPR or TF to react with human heart extract was evaluated. Whole heart extract rather than individual cardiac proteins was used as it was considered a more robust evaluation of the potential cross-reactivity of the vaccine antigens of this study. The α-ADI, α-KPR and α-TF serum did not significantly react with the human heart extract ($P > 0.05$; Figure 6.4), in contrast to α-M1 protein serum. These results suggest that unlike full length M protein, the B-cell epitopes contained within ADI, KPR and TF do not cross-react with human cardiac proteins. Thus, it is hypothesised that the administration of ADI, KPR or TF will not result in production of antibodies which cross-react with host heart proteins.

7.   CONCLUSIONS AND FUTURE DIRECTIONS

This investigation utilised a proteomic approach to screen for novel efficacious GAS vaccine candidates. Following a 2D SDS-PAGE analysis of GAS cell wall fractions, 13 putative GAS vaccine antigens were produced as recombinant proteins in *E. coli*. Individual experimental immunisation with ten of the vaccine antigens; ADI, AK, BCAT, KPR, NADP-GAPDH, OTCase, PFK, PGK, RRF and TF protected BALB/c mice against lethal systemic GAS challenge. Given ADI, KPR and TF share 0% amino acid identity with the proteins of the human proteome, the suitability of these three antigens as GAS vaccine candidates was further evaluated. ADI, KPR and TF were confirmed to be surface localised following staining of the GAS cell surface with α-ADI, α-KPR and α-TF sera in immunofluorescence microscopy, immuno-EM and flow cytometry. ADI, KPR and TF conferred synergistic protection against lethal subcutaneous GAS challenge in C57BL/6J mice and neither antigen reacted with patient serum collected from two geographically distinct populations suffering endemic GAS infection. Unlike α-M1 sera, serum raised against ADI, KPR or TF did not significantly react with human heart extract. In addition, antibodies raised against ADI were determined to be opsonic, and thus, the protection mediated by ADI may, at least in part, be due to opsonophagocytic killing of GAS. Hence, ADI, KPR and TF are GAS vaccine candidates worthy of further investigation.

Due to the number of antigens included in this study, for logistical reasons only the specific serum IgG response directed against individual antigens was evaluated following experimental immunisation. IgG subtypes, levels of serum IgM, serum IgA or salivary IgA directed against the antigens was not examined. Future immunisation studies utilising ADI, KPR and TF will more widely examine the host immune response triggered by these antigens. A more in depth knowledge of the host mucosal and serum-based immune responses against these antigens may assist in determining the optimal route and delivery system of these antigens to the human host. The B-cell epitopes of these antigens were determined using peptide arrays and subsequent studies could attempt to elucidate possible T-cell epitopes contained within these antigens using techniques such as those utilised in Schulze *et al.* (2003). An evaluation of the protective efficacy mediated by individual B-cell and T-cell epitopes of ADI, KPR and TF and the relative contribution of these epitopes

to host immunity against GAS could be investigated. For instance if no T-cell epitopes were identified in the antigens, a universal T-cell helper epitope could be introduced to the recombinant antigens such as P25, a T-cell help epitope recently co-administered with J14 and a lipid moiety (Abdel-Aal *et al.*, 2008). In this study there was a correlation between a high titre of specific serum α-ADI, α-KPR and α-TF IgG antibodies and the survival of mice following lethal GAS challenge. These results suggest that B-cells producing antibodies that are specific for ADI, KPR or TF play a role in mediating protection against GAS in murine infection models. While B-cells produce antibodies capable of inhibiting GAS adhesion to host cells and tissues or mediating opsonophagocytic killing, T-cells also play an important role in the host immune response. Helper T-cells activate B-cells which initiates production of specific Ig. Following immunisation of mice with ADI, KPR or TF the role that specific T-cells play in protection against GAS infection could be explored. To evaluate this, CD4$^+$ and CD8$^+$ T-cells could be depleted from BALB/c mice using rat α-CD4 and α-CD8 MAbs administered via the intraperitoneal route. The effect of T-cell depletion on the survival of mice post GAS challenge could then be evaluated. This technique was recently employed to explore the role T-cells play in J8-DT mediated protection (Pandey *et al.*, 2009). In the case of J8-DT, the depletion of CD4$^+$ cells (but not CD8$^+$ cells) significantly diminished mouse survival post GAS challenge, suggesting that CD4$^+$ T-cells are essential for active protection against GAS promoted by J8-DT (Pandey *et al.*, 2009).

A number of GAS vaccine candidates have been observed to offer protection against challenge with heterologous GAS strains including M protein based fragments (reviewed in section 1.8.1), C5a peptidase (Cleary *et al.*, 2004, Park *et al.*, 2005), FbaA (Terao *et al.*, 2005), FBP54 (Kawabata *et al.*, 2001), Protein F1 (Guzmán *et al.*, 1999, Schulze *et al.*, 2003c, Schulze *et al.*, 2006b), Sib35 (Okamoto *et al.*, 2005), SOF (Courtney *et al.*, 2003, Gillen *et al.*, 2008), SpyCEP (Rodreiguez-Ortega *et al.*, 2006, Turner *et al.*, 2009) and SSe (Liu *et al.*, 2007). In order to further determine the suitability of ADI, KPR and TF as broad spectrum GAS vaccine candidates, challenge experiments with heterologous GAS strains are required. Whilst ADI, KPR and TF elicited protection against lethal systemic and subcutaneous challenge with M1 GAS isolates, at this stage it is unknown if these novel antigens will provide protection against heterologous challenge. The high level of amino

acid conservation amongst sequenced GAS isolates suggests ADI, KPR and TF should evoke protection irrespective of GAS serotype, however, this requires experimental investigation. For the successful global eradication of GAS disease it is essential that a GAS vaccine can mediate protection against the 120 known circulating GAS serotypes. Although the 26-valent GAS vaccine preparation formulated by Dale *et al.* was observed to be immunogenic and well tolerated by individuals in clinical trials (Kotloff *et al.*, 2004, McNeil *et al.*, 2005), a major shortcoming of this preparation is the limited serotype coverage, as the preparation only contains epitopes specific for 26 GAS serotypes.

There are several different immune mechanisms vaccine preparations may trigger in order to elicit protective immunity. Active immunisation with antigens can result in the production of antibodies which bind to surface adhesins of GAS consequently blocking adhesion (and potential invasion). In addition, immunisation can lead to the production of serum antibodies which bind to surface antigens of GAS thereby promoting opsonophagocytic killing and subsequent clearance of GAS in the host. Vaccines may be designed to trigger systemic or mucosal immunity, or both. Given the variety of diseases caused by GAS and the ability of GAS to infect different sites of the human host (eg. respiratory mucosa, the skin, the blood), a vaccine capable of engendering both systemic and mucosal immunity may be the most effective against GAS infection. Other studies have utilised mucosal (intranasal) GAS infection models during the evaluation of a number of GAS vaccine candidates including heat-killed whole GAS (Bronze *et al.*, 1988, Stjernquist-Desatnik *et al.*, 1990), M protein (Polly *et al.*, 1975, D'Alessandri *et al.*, 1978, Bessen *et al.*, 1988, Fischetti *et al.*, 1989, Bessen *et al.*, 1990, Bronze *et al.*, 1992, Olive *et al.*, 2002b, Batzloff *et al.*, 2004, Hall *et al.*, 2004, Batzloff *et al.*, 2006, Olive *et al.*, 2006, Schulze *et al.*, 2006b, Olive *et al.*, 2007), C5a peptidase (Ji *et al.*, 1997, Cleary *et al.*, 2004, Park *et al.*, 2005), Protein F1 (Medina *et al.*, 1998, Guzmán *et al.*, 1999, Schulze *et al.*, 2003c, Schulze *et al.*, 2006b), CHO (Sabharwal *et al.*, 2006) and SpyCEP (Turner *et al.*, 2009). It would be useful to determine if ADI, KPR and TF can engender protection against mucosal GAS challenge. The testing of these antigens in an intranasal immunisation and intranasal GAS infection model would reveal if immunisation with ADI, KPR and TF can promote a mucosal IgA response in the host. Intranasal challenge experiments require the optimisation of a robust, reliable, reproducible intranasal GAS infection model, ideally one in which a

human safe adjuvant can be used. Currently there are several mucosal adjuvants under investigation in mouse models for use with GAS antigens including Ringers lactate buffer (Polly *et al.*, 1975, D'Alessandri *et al.*, 1978), CTB (Bessen *et al.*, 1988, Bessen *et al.*, 1990, Bronze *et al.*, 1992, Fischetti *et al.*, 1989, Olive *et al.*, 2002b, Guzmán *et al.*, 1999, Schulze *et al.*, 2003c, Schulze *et al.*, 2006a, Park *et al.*, 2005), lipid based adjuvant preparations (Hall *et al.*, 2004, Batzloff *et al.*, 2006, Olive *et al.*, 2007, Olive *et al.*, 2006) and MALP-2 (Schulze *et al.*, 2006b). However, none of these adjuvants are in use in human trials. Whilst the mucosal colonisation of GAS in mice following intranasal infection may mimic the colonisation that occurs during human pharyngitis, the main route of GAS infection of Australian Indigenous individuals suffering endemic GAS infection is by the skin (Currie *et al.*, 2000). It is hypothesised that long lasting broad spectrum protection against GAS infection would require stimulation of both humoral and mucosal host immune responses to negate the varied colonisation niches occupied by GAS in the host.

The investigation of the protection conferred by ADI, KPR or TF when adjuvanted with Alum, one of the only adjuvants currently approved as safe for human use (Petrovsky *et al.*, 2004), is suggested for future experiments. Previously in our studies ADI, KPR and TF have been adjuvanted with Alum and subcutaneously administered (data not shown). This resulted in high serum IgG titres comparable to the titres resulting following experimental immunisation of the antigens with CFA (described in section 4.2.3.1). However, the ability of ADI and TF to elicit a protective immune response against lethal GAS challenge when adjuvanted with Alum is yet to be investigated. Whilst this study has tested vaccination with ADI and TF both individually and together, there is the possibility for either or both of the antigens to be administered in a cocktail combination with any of the GAS vaccine candidate(s) listed in Table 1.1. Additionally, the antigens could be conjugated to M protein C-repeat region epitopes such as J8 or J14. Previous reports have coupled J8 to DT (Batzloff *et al.*, 2003), tetanus toxin (Brandt *et al.*, 2000) and a N-terminal fragment of M protein derived from the 88/30 GAS isolate (Olive *et al.*, 2005). Utilising protective GAS antigens, such as ADI, KPR or TF as the carrier molecule for J8, rather than non-GAS based proteins or molecules, may bolster the host immune response against GAS-specific epitopes. Another line of investigation is the inclusion of ADI, KPR, TF or epitopic segments thereof, into any of the many newly developing vaccine delivery

systems, be it live organism carrier or chemical/molecular based formulations. Novel adjuvants and vaccine delivery systems include biological macromolecules such as heat-shock proteins (Suto *et al.*, 1995), polysaccharide based adjuvants such as inulin (a storage polysaccharide of *Compositae*, a class of flowering plants) (Petrovsky, 2006) and particulate based systems (Liang *et al.*, 2006, Copland *et al.*, 2005, O'Hagan *et al.*, 2006) such as ISCOMs (Kersten *et al.*, 2003), liposomes (Kersten *et al.*, 2003) or virosomes (Westerfeld *et al.*, 2005). Alternatively, the heterologous expression of the GAS antigens ADI, KPR or TF on the surface of commensal organisms such as *S. gordonii* could be pursued. Such an approach has been utilised for the presentation of M6 protein (Pozzi *et al.*, 1992a, Pozzi *et al.*, 1992b) and JJoH12 (a M protein C-repeat region based recombinant antigen) (Georgousakis, 2008) to the host immune system. Methods which deliver the antigens in a formulation safe for human use whilst ensuring the structural integrity and immunogenicity of the antigens are a worthwhile research pursuit.

One concern regarding vaccination with ADI, KPR or TF is the possible side-effects of immunising hosts with antigens possessing enzyme activity. Some of the previously explored GAS vaccine candidates such as C5a peptidase, SpeB and SOF, at least in wild-type forms, were ultimately deemed unsuitable for use in GAS vaccine preparations due to their proteolytic and/or enzymatic nature. In an attempt to circumvent undesirable side-effects potentially mediated by the enzyme activity of these antigens researchers have produced inactivated forms of these antigens, generated via either site-directed mutagensis or truncation or a combination of both of these approaches. One research group constructed an inactivated form of C5a peptidase, designated SCPAw, in which the signal sequence and cell wall anchor were truncated and two essential catalytic residues of the active site were replaced with alanine to inactivate protease activity (Cleary *et al.*, 2004). Intranasal administration of SCPAw promoted rapid clearance of GAS from the oral/nasal mucosa of mice following mucosal GAS infection (Cleary *et al.*, 2004). Thus, abrogation of the enzyme activity of C5a peptidase did not appear to have a negative effect on C5a peptidase-mediated protection against GAS. Similarly, a mutant form of SpeB, designated C47S, was engineered via site directed mutagenesis (Ulrich, 2008). In this molecule a cysteine residue was mutated to a serine residue which consequently diminished the proteolytic activity of SpeB (Ulrich, 2008). The co-administration of SpeB C47S with a SpeA mutant protected

100% of mice tested following GAS challenge via the intravenous route (Ulrich, 2008). The protective efficacy of truncated forms of SOF lacking opacity factor activity has also been tested. A number of recombinant SOF-based proteins were generated and one SOF fragment lacking the signal sequence and Fn-binding repeat region, designated SOFΔFn, was observed to protect BALB/c mice against lethal intraperitoneal challenge with heterologous M49 GAS (Gillen *et al.*, 2008).

Further studies of the suitability of ADI and TF as GAS vaccine antigens should focus on the structure of these novel antigens, including the determination and subsequent abrogation of essential active site residues such that enzymatic activity will be eliminated. Crystal structures have been resolved for ADI of *Pseudomonas aeruginosa* by two research groups to a resolution of 2.7 and 2.45 Å respectively (Oudjama *et al.*, 2002, Galkin *et al.*, 2004). Following the elucidation of these crystal structures researchers have targeted residues via site directed mutagenesis of the active site of ADI from *Mycoplasma hominis* (Wei et al., 2007). A number of single residue (D160E, E212D, H268F, H268Y and D270E) and double residue (D160E/D270E, D160E/E212D and E212D/D270E) mutant forms of ADI were constructed (Wei *et al.*, 2007). The introduced mutations had a minimal effect on the native structure of ADI (investigated using tryptophan emission fluorescence and circular dichroism) whilst marring substrate binding (Wei *et al.*, 2007). A logical next step in the investigation of the suitability of ADI as a GAS vaccine candidate would be the resolution of a crystal structure of GAS ADI. If the key residues were found to be conserved between GAS and *M. hominis*, the residues previously mutated in *M. hominis* could be targeted by site directed mutagenesis in GAS forms of ADI. Subsequently, the protective efficacy of the ADI mutant proteins could be assessed in murine GAS challenge models. Researchers have also resolved a crystal structure for TF to a resolution of 2.5 Å in *Vibrio cholerae* (Ludlam *et al.*, 2004). Ludlam *et al.* (2004) found that TF of *V. cholerae* exists as a dimer, with each monomer comprised of three functionally discrete domains. The effects of mutagenising key residues of the TF catalytic site has not yet been explored. This novel experiment could be investigated using GAS TF. Additionally, elucidating the structure of GAS ADI, KPR and TF could provide insight into how these anchorless antigens associate with the cell surface. Site directed mutagenesis of surface exposed residues may reveal if the antigens are interacting with other GAS surface protein/s as a

mechanism of localisation on the GAS cell surface or aid in the identification of novel anchoring motifs.

This study identified three novel anchorless GAS vaccine antigens, ADI, KPR and TF which protected against lethal infection in two murine GAS infection models. Furthermore, ADI, KPR and TF are highly conserved amongst representative GAS serotypes, localised on the GAS cell surface, lack significant amino acid sequence identity with proteins of the human proteome and do not react with sera obtained from patients suffering endemic GAS infection. Additionally, anti-sera raised against ADI, KPR or TF does not react significantly with human heart extract. The characterisation of these novel antigens conducted in this study indicates that ADI, KPR and TF warrant further investigation as GAS vaccine candidates for the prevention of GAS disease, which despite many decades of research trying to find a suitable vaccine, remains a major cause of morbidity and mortality worldwide.

REFERENCES

Abbot, E.L., Smith, W.D., Siou, G.P., Chiriboga, C., Smith, R.J., Wilson, J.A., *et al.* (2007). Pili mediate specific adhesion of *Streptococcus pyogenes* to human tonsil and skin. *Cellular Microbiology* **9,** 1822-1833.

Abdel-Aal, A.B., Batzloff, M.R., Fujita, Y., Barozzi, N., Faria, A., Simerska, P., *et al.* (2008). Structure-activity relationship of a series of synthetic lipopeptide self-adjuvanting group a streptococcal vaccine candidates. *Journal of Medicinal Chemistry* **51,** 167-172.

Abdissa, A., Asrat, D., Kronvall, G., Shittu, B., Achiko, D., Zeidan, M., *et al.* (2006). High diversity of group A streptococcal *emm* types among healthy schoolchildren in Ethiopia. *Clinical Infectious Diseases* **42,** 1362-1367.

Adam, D., Scholz, H. and Helmerking, M. (2000). Comparison of short-course (5 day) cefuroxime axetil with a standard 10 day oral penicillin V regimen in the treatment of tonsillopharyngitis. *Journal of Antimicrobial Chemotherapy* **45 Suppl,** 23-30.

Agniswamy, J., Lei, B., Musser, J.M. and Sun, P.D. (2004). Insight of host immune evasion mediated by two variants of group a Streptococcus Mac protein. *J Biol Chem* **279,** 52789-52796.

Ahmed, S. and Ayoub, E.M. (1998). Severe, invasive group A streptococcal disease and toxic shock. *Pediatric Annals* **27,** 287-292.

Åkesson, P., Sjöholm, A.G. and Björck, L. (1996). Protein SIC, a novel extracellular protein of *Streptococcus pyogenes* interfering with complement function. *The Journal of Biological Chemistry* **271,** 1081-1088.

Alouf, J.E. and Müller-Alouf, H. (2003). Staphylococcal and streptococcal superantigens: molecular, biological and clinical aspects. *International Journal of Medical Microbiology* **272,** 429-440.

Antikainen, J., Kuparinen, V., Lähteenmäki, K. and Korhonen, T.K. (2007). pH-dependent association of enolase and glyceraldehyde-3-phosphate dehydrogenase of *Lactobacillus crispatus* with the cell wall and lipoteichoic acids. *J Bacteriol* **189,** 4539-4543.

Antone, S.M., Adderson, E.E., Mertens, N.M.J. and Cunningham, M.W. (1997). Molecular analysis of V gene sequences encoding cytotoxic anti-streptococcal/anti-myosin monoclonal antibody 36.2.2 that recognises the heart cell surface protein laminin. *Journal of Immunology* **159,** 5422-5430.

Ashbaugh, C.D., Warren, H.B., Carey, V.J. and Wessels, M.R. (1998). Molecular analysis of the role of the group A streptococcal cysteine protease, hyaluronic capsule, and M protein in a murine model of human invasive soft-tissue infection. *Journal of Clinical Investigation* **102,** 550-560.

Aziz, R.K. and Kotb, M. (2008). Rise and persistence of global M1T1 clone of *Streptococcus pyogenes*. *Emerging Infectious Diseases* **14,** 1511-1517.

Aziz, R.K., Pabst, M.J., Jeng, A., Kansal, R., Low, D.E., Nizet, V. and Kotb, M. (2004). Invasive M1T1 group A Streptococcus undergoes a phase-shift in vivo to prevent proteolytic degradation of multiple virulence factors by SpeB. *Mol Microbiol* **51,** 123-134.

Baker, H.M., Proft, T., Webb, P.D., Arcus, V.L., Fraser, J.D. and Baker, E.N. (2004). Crystallographic and mutational data show that the streptococcal pyrogenic exotoxin J can use a common binding surface for T-cell receptor binding and dimerization. *J Biol Chem* **279,** 38571-38576.

Baldassarri, L., Creti, R., Recchia, S., Imperi, M., Facinelli, B., Giovanetti, E., *et al.* (2006). Therapeutic failures of antibiotics used to treat macrolide-susceptible *Streptococcus pyogenes* infections may be due to biofilm formation. *Journal of Clinical Microbiology* **44,** 2721-2727.

Basma, H., Norrby-Teglund, A., Guedez, Y., McGeer, A., Low, D.E., El-Ahmedy, O., *et al.* (1999). Risk factors in the pathogenesis of invasive group A streptococcal infections: role of protective humoral immunity. *Infection and Immunity* **67,** 1871-1877.

Batzloff, M.R., Hartas, J., Zeng, W., Jackson, D.C. and Good, M.F. (2006). Intranasal vaccination with a lipopeptide containing a conformationally constrained conserved minimal peptide, a universal T cell epitope, and a self-adjuvanting lipid protects mice from group a streptococcus challenge and reduces throat colonization. *J Infect Dis* **194,** 325-330.

Batzloff, M.R., Hayman, W.A., Davies, M.R., Zeng, M., Pruksakorn, S., Brandt, E.R. and Good, M.F. (2003). Protection against group A streptococcus by immunisation with J8-Diphtheria toxoid: Contribution of J8- and Diphtheria toxoid-specific antibodies to protection. *J Infect Dis* **187,** 1598-1608.

Batzloff, M.R., Yan, H., Davies, M., Hartas, J. and Good, M.F. (2004). Preclinical evaluation of a vaccine based on conserved region of M protein that prevents group A streptococcal infection. *Indian Journal of Medical Research* **119 Suppl,** 104-107.

Batzloff, M.R., Yan, H., Davies, M.R., Hartas, J., Lowell, G.H., White, G.*, et al.* (2005). Toward the development of an anti-disease, transmission-blocking intranasal vaccine for group A streptococcus. *J Infect Dis* **192,** 1450-1455.

Beachey, E.H. (1981). Bacterial Adherence: adhesin-receptor interactions mediating the attachment of bacteria to mucosal surfaces. *The Journal of Infectious Diseases* **143,** 325-345.

Beachey, E.H., Gras-Masse, H., Tarter, A., Jolivet, M., Audibert, F., Chedid, L. and Seyer, J.M. (1986). Opsonic antibodies evoked by hybrid peptide copies of types 5 and 24 streptococcal M proteins synthesized in tandem. *J Exp Med* **163,** 1451–1458.

Beachey, E.H., Seyer, J.M., Dale, J.B., Simpson, W.A. and Kang, A.H. (1981). Type-specific protective immunity evoked by synthetic peptide of *Streptococcus pyogenes* M protein. *Nature* **292,** 457-459.

Beall, B., Facklam, R. and Thompson, T. (1996). Sequencing *emm*-specific PCR products for routine and accurate typing of group A streptococci. *Journal of Clinical Microbiology* **34,** 953-958.

Beall, B., Gherardi, G., Lovgren, M., Facklam, R.R., Forwick, B.A. and Tyrrell, G.J. (2000). *emm* and *sof* gene sequence variation in relation to serological typing of opacity-factor-positive group A streptococci. *Microbiology* **146,** 1195-1209.

Beck, K., Hunter, I. and Engel, J. (1990). Structure and function of laminin: anatomy of a multidomain glycoprotein. *FASEB Journal* **4,** 148-160.

Benchetrit, L.C., Pahuja, S.L., Gray, E.D. and Edstrom, R.D. (1977). A sensitive method for the assay of hyaluronidase activity. *Analytical Biochemistry* **79,** 431-437.

Berge, A. and Sjöbring, U. (1993). PAM, a novel plasminogen-binding protein from *Streptococcus pyogenes*. *J Biol Chem* **268,** 25417-25424.

Bergmann, S., Rohde, M., Chhatwal, G.S. and Hammerschmidt, S. (2001). alpha-Enolase of *Streptococcus pneumoniae* is a plasmin(ogen)-binding protein displayed on the bacterial cell surface. *Mol Microbiol* **40,** 1273-1287.

Bessen, D. and Fischetti, V.A. (1988). Influence of intranasal immunization with synthetic peptides corresponding to conserved epitopes of M protein on mucosal colonization by group A streptococci. *Infect Immun* **56,** 2666-2672.

Bessen, D. and Fischetti, V.A. (1990). Synthetic peptide vaccine against mucosal colonization by group A streptococci. I. Protection against a heterologous M serotype with shared C repeat region epitopes. *Journal of Immunology* **145,** 1251–1256.

Bessen, D.E. and Kalia, A. (2002). Genomic localisation of a T serotype locus to a recombinatorial zone encoding extracellular matrix-binding proteins in *Streptococcus pyogenes*. *Infect Immun* **70,** 1159-1167.

Betschel, S.D., Borgia, S.M., Barg, N.L., Low, D.E. and De Azavedo, J.C.S. (1998). Reduced virulence of group A streptococcal Tn916 mutants that do not produce streptolysin S. *Infect Immun* **66,** 1671-1679.

Beutling, U., Dikmans, A. and Frank, R. (2008) Selection of peptide ligands from chemical synthetic repertoires using SPOT-peptide array technology. In, (ed.^(eds.). Helmholtz Centre for

Infectious Diseases, Braunschweig, Germany.

Binks, M., McMillan, D.J. and Sriprakash, K.S. (2003). Genomic location and variation of the gene for CRS, a complement binding protein in the M57 strains of Streptococcus pyogenes. *Infection and Immunity* **71,** 6701-6706.

Binks, M. and Sriprakash, K.S. (2004). Characterisation of a complement-binding protein, DRS, from strains of *Streptococcus pyogenes* containing the *emm*12 and *emm*55 genes. *Infect Immun* **72,** 3981-3986.

Binks, M.J., Fernie-King, B.A., Seilly, D.J., Lachmann, P.J. and Sriprakash, K.S. (2005). Attribution of the various inhibitory actions of the streptococcal inhibitor of complement (SIC) to regions within the molecule. *J Biol Chem* **280,** 20120-20125.

Bisno, A.L. and Stevens, D.L. (1996). Current concepts: streptococcal infections of skin and soft tissues. *The New England Journal of Medicine* **334,** 240-245.

Biswas, I., Germon, P., McDade, K. and Scott, J.R. (2001). Generation and surface localization of intact M protein in *Streptococcus pyogenes* are dependent on sagA. *Infect Immun* **69,** 7029–7038.

Boel, G., Jin, H. and Pancholi, V. (2005). Inhibition of cell surface export of group A streptococcal anchorless surface dehydrogenase affects bacterial adherence and antiphagocytic properties. *Infect Immun* **73,** 6237-6248.

Bolken, T.C., Franke, C.A., Jones, K.F., Bell, R.H., Swanson, R.M., King, D.S.*, et al.* (2002). Analysis of factors affecting surface expression and immunogenicity of recombinant proteins expressed by gram-positive commensal vectors. *Infect Immun* **70,** 2487-2491.

Brandt, E.R., Hayman, W.A., Currie, B., Carapetis, J., Jackson, D.C., Do, K.A. and Good, M.F. (1999). Functional analysis of IgA antibodies specific for a conserved epitope within the M protein of group A streptococci from Australian Aboriginal endemic communities. *International Immunology* **11,** 569-576.

Brandt, E.R., Hayman, W.A., Currie, B., Carapetis, J.R., Wood, Y., Jackson, D.C.*, et al.* (1996). Opsonic human antibodies from an endemic population specific for a conserved epitope on the M protein of group A streptococci. *Immunology* **89,** 331-337.

Brandt, E.R., Hayman, W.A., Currie, B., Pruksakorn, S. and Good, M.F. (1997). Human antibodies to the conserved region of the M protein: opsonization of heterologous strains of group A streptococci. *Vaccine* **15,** 1805-1812.

Brandt, E.R., Sriprakash, K.S., Hobb, R.I., Hayman, W.A., Zeng, W., Batzloff, M.R.*, et al.* (2000). New multi-determinant strategy for a group A streptococcal vaccine designed for the Australian Aboriginal population. *Nature Medicine* **6,** 455-459.

Breiman, R.F., Davis, J.P., Facklam, R.R. and Gray, B.M. (1993). Defining the group A streptococcal toxic shock syndrome. *JAMA* **269,** 390-391.

Broder, C.C., Lottenberg, R., von Mering, G.O., Johnston, K.H. and Boyle, M.D.P. (1991). Isolation of a procaryotic plasmin receptor: relationship to a plasminogen activator produced by the same micro-organism. *The Journal of Biological Chemistry* **266,** 4922-4928.

Bronze, M.S., Courtney, H.S. and Dale, J.B. (1992). Epitopes of group A streptococcal M protein that evoke cross-protective local immune responses. *Journal of Immunology* **148,** 888-893.

Bronze, M.S., McKinsey, D.S., Beachey, E.H. and Dale, J.B. (1988). Protective immunity evoked by locally administered group A streptococcal vaccines in mice. *Journal of Immunology* **141,** 2767-2770.

Brook, I. (2007). Overcoming penicillin failures in the treatment of Group A streptococcal pharyngo-tonsillitis. *International Journal of Pediatric Otorhinolaryngology* **71,** 1501-1508.

Broudy, T.B., Pancholi, V. and Fischetti, V.A. (2002). The in vitro interaction of *Streptococcus pyogenes* with human pharyngeal cells induces a phage-encoded extracellular DNase. *Infection and Immunity* **70,** 2805-2811.

Brouillard, J.N., Günther, S., Varma, A.K., Gryski, I., Herfst, C.A., Rahman, A.K.*, et al.* (2007). Crystal structure of the streptococcal superantigen SpeI and functional role of a novel loop

domain in T cell activation by group V superantigens. *Journal of Molecular Biology* **367,** 925-934.

Brown, J.S., Ogunniyi, A.D., Woodrow, M.C., Holden, D.W. and Paton, J.C. (2001). Immunization with components of two iron uptake ABC transporters protects mice against systemic *Streptococcus pneumoniae* infection. *Infect Immun* **69,** 6702-6706.

Burns, E.H., Lukomski, S., Rurangirwaa, J., Podbielski, A. and Musser, J.M. (1998). Genetic inactivation of the extracellular cysteine protease enhances *in vitro* internalization of group A streptococci by human epithelial and endothelial cells. *Microbial Pathogenesis* **24,** 333-339.

Cao, J. and Doyle, R.J. (2002). Fluoride modifies adhesion of Streptococcus pyogenes. *FEMS Immunology and Medical microbiology* **32,** 175-177.

Carapetis, J., Gardiner, D.L., Currie, B. and Mathews, J.D. (1995a). Multiple strains of *Streptococcus pyogenes* in skin sores of Aboriginal Australians. *Journal of Clinical Microbiology* **33,** 1471-1472.

Carapetis, J., Robins-Browne, R., Martin, D., Shelby-James, T. and Hogg, G. (1995b). Increasing severity of invasive group A streptococcal disease in Australia: clinical and molecular epidemiological features and identification of a new virulent M-nontypeable clone. *Clinical Infectious Diseases* **21,** 1220-1227.

Carapetis, J.R., McDonald, M. and Wilson, N.J. (2005a). Acute rheumatic fever. *The Lancet* **366,** 155-168.

Carapetis, J.R., Steer, A.C., Mulholland, E.K. and Weber, M. (2005b). The global burden of group A streptococcal diseases. *Lancet Infect Dis* **5,** 685-694.

Carapetis, J.R., Walker, A.M., Hibble, M., Sriprakash, K.S. and Currie, B.J. (1999). Clinical and epidemiological features of group A streptococcal bacteraemia in a region with hyperendemic superficial streptococcal infection. *Epidemiology and Infection* **122,** 59-65.

Carapetis, J.R., Wolff, D.R. and Currie, B.J. (1996). Acute rheumatic fever and rheumatic heart disease in the top end of Australia's Northern Territory. *Medical Journal of Australia* **164,** 146-149.

Carlsson, F., Sandin, C. and Lindahl, G. (2005). Human fibrinogen bound to *Streptococcus pyogenes* M protein inhibits complement deposition via the classical pathway. *Mol Microbiol* **56,** 28-39.

Caswell, C.C., Barczyk, M., Keene, D.R., Lukomska, E., Gullberg, D.E. and Lukomski, S. (2008a). Identification of the first prokaryotic collagen sequence motif that mediates binding to human collagen receptors, integrins alpha2beta1 and alpha11beta1. *J Biol Chem* **283,** 36168-36175.

Caswell, C.C., Han, R., Hovis, K.M., Ciborowski, P., Keene, D.R., Marconi, R.T. and Lukomski, S. (2008b). The Scl1 protein of M6-type group A Streptococcus binds the human complement regulatory protein, factor H, and inhibits the alternative pathway of complement. *Mol Microbiol* **67,** 584-596.

Caswell, C.C., Lukomska, E., Seo, N.S., Höök, M. and Lukomski, S. (2007). Scl1-dependent internalization of group A Streptococcus via direct interactions with the $\alpha_2\beta_1$ integrin enhances pathogen survival and re-emergence. *Mol Microbiol* **64,** 1319-1331.

Caswell, C.C., Oliver-Kozup, H., Han, R., Lukomska, E. and Lukomski, S. (2010). Scl1, the multifunctional adhesin of group A Streptococcus, selectively binds cellular fibronectin and laminin, and mediates pathogen internalization by human cells. *FEMS Microbiology Letters* **303,** 61-68.

Chen, C.C. and Cleary, P.P. (1990). Complete nucleotide sequence of the streptococcal C5a peptidase gene of *Streptococcus pyogenes*. *J Biol Chem* **265,** 3161-3167.

Chhatwal, G.S. (2002). Anchorless adhesins and invasins of Gram-positive bacteria: a new class of virulence factors. *TRENDS in Microbiology* **10,** 205-208.

Chiang-Ni, C., Wang, C.H., Tsai, P.J., Chuang, W.J., Lin, Y.S., Lin, M.T.*, et al.* (2006). Streptococcal pyrogenic exotoxin B causes mitochondria damage to polymorphonuclear cells preventing phagocytosis of group A streptococcus. *Medical Microbiology and Immunology* **195,**

55-63.

Cleary, P.P., Kaplan, E.L., Handley, J.P., Wlazlo, A., Kim, M.L., Hauser, A.R. and Schlievert, P.M. (1992). Clonal basis for resurgence of serious *Streptococcus pyogenes* disease in the 1980s. *The Lancet* **339,** 518-521.

Cleary, P.P., Matsuka, Y.V., Hunyh, T., Lam, H. and Olmsted, S.B. (2004). Immunisation with C5a peptidase from either group A or B streptococci enhances clearance of group A streptococci from intranasally infected mice. *Vaccine* **22,** 4332-4341.

Cole, J.N. (2006) Characterisation of cell wall proteins, virulence factor maturation and invasive disease trigger of Group A Streptococcus; PhD Thesis. In, (ed.^(eds.). Wollongong, University of Wollongong.

Cole, J.N., Henningham, A., Gillen, C.M., Ramachandran, V. and Walker, M.J. (2008). Human pathogenic streptococcal proteomics and vaccine development. *Proteomics Clinical Applications* **2,** 387–410.

Cole, J.N., McArthur, J., McKay, F.C., Sanderson-Smith, M., Cork, A.J., Ranson, M*., et al.* (2006). Trigger for group A streptococcal M1T1 invasive disease. *FASEB J* **20,** E1139-E1145.

Cole, J.N., Ramirez, R.D., Currie, B.J., Cordwell, S.J., Djordjevic, S.P. and Walker, M.J. (2005). Surface analysis and immune reactivity of major cell-wall associated proteins of group A streptococcus. *Infection and Immunity* **73,** 3137-3146.

Copland, M.J., Rades, T., Davies, N.M. and Baird, M.A. (2005). Lipid based particulate formulations for the delivery of antigen. *Immunology & Cell Biology* **83,** 97-105.

Cork, A.J., Jergic, S., Hammerschmidt, S., Kobe, B., Pancholi, V., Benesch, J.L*., et al.* (2009). Defining the structural basis of human plasminogen binding by streptococcal surface enolase. *J Biol Chem* **284,** 17129-17137.

Courtney, H.S., Dale, J.B. and Hasty, D.L. (1996). Differential effects of the streptococcal fibronectin-binding protein, FPB54, on adhesion of group A streptococci to human buccal cells and HEp-2 tissue culture cells. *Infect Immun* **64,** 2415-2419.

Courtney, H.S., Dale, J.B. and Hasty, D.L. (1999a) Strategies for prevention of group A streptococcal adhesion and infection. In *Handbook of bacterial adhesion: principles, methods, and applications.*, Y.H. An, R.J. Friedman (ed.^(eds.). Totowa, N.J., Humana Press, pp. 553–579.

Courtney, H.S., Dale, J.B. and Hasty, D.L. (2002a). Mapping the fibrinogen-binding domain of serum opacity factor of group A streptococci. *Current Microbiology* **44,** 236-240.

Courtney, H.S., Hasty, D.L. and Dale, J.B. (2002b). Molecular mechanisms of adhesion, colonisation, and invasion of group A streptococci. *Annals of Medicine* **34,** 77-87.

Courtney, H.S., Hasty, D.L. and Dale, J.B. (2003). Serum opacity factor (SOF) of Streptococcus pyogenes evokes antibodies that opsonize homologous and heterologous SOF-positive serotypes of group A streptococci. *Infect Immun* **71,** 5097-5103.

Courtney, H.S., Hasty, D.L., Li, Y., Chiang, H.C., Thacker, J.L. and Dale, J.B. (1999b). Serum opacity factor is a major fibronectin-binding protein and a virulence determinant of M type 2 Streptococcus pyogenes. *Molecular Microbiology* **32,** 89-98.

Courtney, H.S., Li, Y., Dale, J.B. and Hasty, D.L. (1994). Cloning, sequencing, and expression of a fibronectin/fibrinogen-binding protein from group A streptococci. *Infect Immun* **62,** 3937-3946.

Courtney, H.S., Li, Y., Twal, W.O. and Argraves, W.S. (2009). Serum opacity factor is a streptococcal receptor for the extracellular matrix protein fibulin-1. *J Biol Chem* **284,** 12966-12971.

Courtney, H.S., Zhang, Y.M., Frank, M.W. and Rock, C.O. (2006). Serum opacity factor, a streptococcal virulence factor that binds to apolipoproteins A-I and A-II and disrupts high density lipoprotein structure. *J Biol Chem* **281,** 5515-5521.

Cue, D., Dombek, P.E., Lam, H. and Cleary, P.P. (1998). *Streptococcus pyogenes* serotype M1 encodes multiple pathways for entry into human epithelial cells. *Infect Immun* **66,** 4593-4601.

Cue, D., Lam, H. and Cleary, P.P. (2001). Genetic dissection of the *Streptococcus pyogenes* M1

protein: regions involved in fibronectin binding and intracellular invasion. *Microbial Pathogenesis* **31,** 231-242.

Cunningham, M.W. (2000). Pathogenesis of group A streptococcal infections. *Clinical Microbiology Reviews* **13,** 470-511.

Cunningham, M.W. (2003). Autoimmunity and molecular mimicry in the pathogenesis of post-streptococcal heart disease. *Frontiers in Bioscience* **8,** s533-543.

Cunningham, M.W., Antone, S.M., Gulizia, J.M., McManus, B.M., Fischetti, V.A. and Gauntt, C.J. (1992). Cytotoxic and viral neutralizing antibodies crossreact with streptococcal M protein, enteroviruses, and human cardiac myosin. *Proceedings of the National Academy of Sciences of the United States of America* **89,** 1320-1324.

Cunningham, M.W., McCormack, J.M., Fenderson, P.G., Ho, M.K., Beachey, E.H. and Dale, J.B. (1989). Human and murine antibodies cross-reactive with streptococcal M protein and myosin recognize the sequence GLN-LYS-SER-LYS-GLN in M protein. *Journal of Immunology* **143,** 2677-2683.

Curran, T.M., Lieou, J. and Marquis, R.E. (1995). Arginine deiminase system and acid adaptation of oral streptococci. *Applied and Environmental Microbiology* **61,** 4494-4496.

Curran, T.M., Ma, Y., Rutherford, G.C. and Marquis, R.E. (1998). Turning on and turning off the arginine deiminase system in oral streptococci. *Canadian Journal of Microbiology* **44,** 1078-1085.

Currie, B.J. and Carapetis, J.R. (2000). Skin infections and infestations in Aboriginal communities in northern Australia. *Australas J Dermatol* **41,** 139-145.

D'Alessandri, R., Plotkin, G., Kluge, R.M., Wittner, M.K., Fox, E.N., Dorfman, A. and Waldman, R.H. (1978). Protective studies with group A streptococcal M protein vaccine. III. Challenge of volunteers after systemic or intranasal immunization with Type 3 or Type 12 group A Streptococcus. *J Infect Dis* **138,** 712-718.

D'Costa, S.S. and Boyle, M.D. (1998). Interaction of a group A Streptococcus within human plasma results in assembly of a surface plasminogen activator that contributes to occupancy of surface plasmin-binding structures. *Microbial Pathogenesis* **24,** 341-349.

D'Costa, S.S., Romer, T.G. and Boyle, M.D. (2000). Analysis of expression of a cytosolic enzyme on the surface of *Streptococcus pyogenes*. *Biochemical and Biophysical Research Communications* **278,** 826-832.

Dale, J.B. (1998). Group A streptococcal vaccines. *Pediatric Annals* **27,** 301-308.

Dale, J.B. (1999). Multivalent group A streptococcal vaccine designed to optomize the immunogenicity of six tandem M protein fragments. *Vaccine* **17,** 193-200.

Dale, J.B. and Beachey, E.H. (1982). Protective antigenic determinant of streptococcal M protein shared with sarcolemmal membrane protein of human heart. *J Exp Med* **156,** 1165-1176.

Dale, J.B. and Beachey, E.H. (1985a). Epitopes of streptococcal M proteins shared with cardiac myosin. *J Exp Med* **162,** 583-591.

Dale, J.B. and Beachey, E.H. (1985b). Multiple, heart-cross-reactive epitopes of streptococcal M proteins. *J Exp Med* **161,** 113-122.

Dale, J.B. and Beachey, E.H. (1986). Sequence of myosin-crossreactive epitopes of streptococcal M protein. *J Exp Med* **164,** 1785-1790.

Dale, J.B., Chiang, E.Y. and Lederer, J.W. (1993). Recombinant tetravalent group A streptococcal M protein vaccine. *Journal of Immunology* **151,** 2188-2194.

Dale, J.B., Chiang, E.Y., Liu, S., Courtney, H.S. and Hasty, D.L. (1999). New protective antigen of group A streptococci. *Journal of Clinical Investigation* **103,** 1261-1268.

Dale, J.B., Seyer, J.M. and Beachey, E.H. (1983). Type-specific immunogenicity of a chemically synthesized peptide fragment of type 5 streptococcal M protein. *J Exp Med* **158,** 1727–1732.

Dale, J.B. and Shulman, S.T. (2002). Dynamic epidemiology of group A streptococcal serotypes. *Lancet* **359,** 889.

Dale, J.B., Simmons, M., Chiang, E.C. and Chiang, E.Y. (1996). Recombinant, octavalent group A

streptococcal M protein vaccine. *Vaccine* **14,** 944–948.

Datta, V., Myskowski, S.M., Kwinn, L.A., Chiem, D.N., Varki, N., Kansal, R.G*., et al.* (2005). Mutational analysis of the group A streptococcal operon encoding streptolysin S and its virulence role in invasive infection. *Mol Microbiol* **56,** 681-695.

Davies, H.D., McGeer, A., Schwartz, B., Green, K., Cann, D., Simor, A.E. and Low, D.E. (1996). Invasive group A streptococcal infections in Ontario, Canada. *The New England Journal of Medicine* **335,** 547-554.

Degnan, B.A., Fontaine, M.C., Doebereiner, A.H., Lee, J.J., Mastroeni, P., Dougan, G*., et al.* (2000). Characterisation of an isogenic mutant of *Streptococcus pyogenes* Manfredo lacking the ability to make streptococcal acid glycoprotein. *Infect Immun* **68,** 2441-2448.

Degnan, B.A., Palmer, J.M., Robson, T., Jones, C.E.D., Fischer, M., Glanville, M*., et al.* (1998). Inhibition of human peripheral blood mononuclear cell proliferation by *Streptococcus pyogenes* cell extract is associated with arginine deiminase activity. *Infect Immun* **66,** 3050-3058.

Delvecchio, A., Currie, B.J., McArthur, J.D., Walker, M.J. and Sriprakash, K.S. (2002). *Streptococcus pyogenes prtFII*, but not *sfbI*, *sfbII*, or *fbp54*, is represented more frequently among invasive-disease isolates of tropical Australia. *Epidemiology and Infection* **128,** 391-396.

Dey, N., McMillan, D.J., Yarwood, P.J., Joshi, R.M., Kumar, R., Good, M.F*., et al.* (2005). High diversity of group A Streptococcal *emm* types in an Indian community: the need to tailor multivalent vaccines. *Clinical Infectious Diseases* **40,** 46-51.

Dinkla, K., Rohde, M., Jansen, W.T.M., Kaplan, E.L., Chhatwal, G.S. and Talay, S.R. (2003). Rheumatic fever-associated *Streptococcus pyogenes* isolates aggregate collagen. *Journal of Clinical Investigation* **11,** 1905-1912.

Dinkla, K., Sastalla, I., Godehardt, A.W., Janze, N., Chhatwal, G.S., Rohde, M. and Medina, E. (2007). Upregulation of capsule enables *Streptococcus pyogenes* to evade immune recognition by antigen-specific antibodies directed to the G-related alpha2-macroglobulin-binding protein GRAB located on the bacterial surface. *Microbes and Infection* **9,** 922-931.

Dombek, P.E., Cue, D., Sedgewick, J., Lam, H., Ruschkowski, S., Finlay, B.B. and Cleary, P.P. (1999). High-frequency intracellular invasion of epithelial cells by serotype M1 group A streptococci: M1 protein-mediated invasion and cytoskeletal rearrangements. *Mol Microbiol* **31,** 859-870.

Edwards, R.J., Taylor, G.W., Ferguson, M., Murray, S., Rendell, N., Wrigley, A*., et al.* (2005). Specific C-terminal cleavage and inactivation of interleukin-8 by invasive disease isolates of *Streptococcus pyogenes*. *J Infect Dis* **192,** 783-790.

Efstratiou, A. (2000). Group A streptococci in the 1990s. *Journal of Antimicrobial Chemotherapy* **45,** 3-12.

Elsner, A., Kreikemeyer, B., Braun-Kiewnick, A., Spellerberg, B., Buttaro, B.A. and Podbielski, A. (2002). Involvement of Lsp, a member of the LraI-Lipoprotein Family in *Streptococcus pyogenes*, in eukaryotic cell adhesion and internalisation. *Infect Immun* **70,** 4859-4869.

Eyal, O., Jadoun, J., Bitler, A., Skutelski, E. and Sela, S. (2003). Role of M3 protein in the adherence and internalization of an invasive *Streptococcus pyogenes* strain by epithelial cells. *FEMS Immunology and Medical Microbiology* **38,** 205-213.

Facklam, R., Beall, B., Efstratiou, A., Fischetti, V., Johnson, D., Kaplan, E*., et al.* (1999). *emm* typing and validation of provisional M types for group A streptococci. *Emerging Infectious Diseases* **5,** 247-253.

Fagan, P.K., Reinscheid, D., Gottschalk, B. and Chhatwal, G.S. (2001). Identification and characterisation of a novel secreted immunoglobulin binding protein from group A streptococcus. *Infection and Immunity* **69,** 4851-4857.

Falugi, F., Zingaretti, C., Pinto, V., Mariani, M., Amodeo, L., Manetti, A.G*., et al.* (2008). Sequence variation in group A Streptococcus pili and association of pilus backbone types with lancefield T serotypes. *J Infect Dis* **198,** 1834-1841.

Fenderson, P.G., Fischetti, V.A. and Cunningham, M.W. (1989). Tropomyosin shares immunologic

epitopes with group A streptococcal M proteins. *Journal of Immunology* **142,** 2475-2481.

Fernie-King, B.A., Seilly, D.J., Binks, M.J., Sriprakash, K.S. and Lachmann, P.J. (2007). Streptococcal DRS (distantly related to SIC) and SIC inhibit antimicrobial peptides, components of mucosal innate immunity: a comparison of their activities. *Microbes and Infection* **9,** 300-320--307.

Fernie-King, B.A., Seilly, D.J., Davies, A. and Lachmann, P.J. (2002). Streptococcal inhibitor of complement inhibits two additional components of the mucosal innate immune system: secretory leukocyte proteinase inhibitor and lysozyme. *Infect Immun* **70,** 4908-4916.

Fernie-King, B.A., Seilly, D.J. and Lachmann, P.J. (2004). The interaction of streptococcal inhibitor of complement (SIC) and its proteolytic fragments with the human beta defensins. *Immunology* **111,** 444-452.

Fischetti, V. (1989). Streptococcal M protein: molecular design and biological behaviour. *Clinical Microbiology Reviews* **2,** 285-314.

Fischetti, V.A. (1991). Streptococcal M protein. *Scientific American* **264,** 32-39.

Fischetti, V.A., Hodges, W.M. and Hruby, D.E. (1989). Protection against streptococcal pharyngeal colonization with a vaccinia: M protein recombinant. *Science* **244,** 1487-1490.

Floderus, E., Linder, L.E. and Sund, M.L. (1990a). Arginine catabolism by strains of oral streptococci. *APMIS* **11,** 1045-1052.

Floderus, E., Linder, L.E. and Sund, M.L. (1990b). Arginine catabolism by strains of oral streptococci. *Acta Pathologica Microbiologica et Immunologica Scandinavica* **11,** 1045-1052.

Fluckiger, U., Jones, K.F. and Fischetti, V. (1998). Immunoglobulins to group A streptococcal surface molecules decrease adherence to and invasion of human pharyngeal cells. *Infect Immun* **66,** 874-979.

Fontaine, M.C., Lee, J.J. and Kehoe, M.A. (2003). Combined contributions of streptolysin O and streptolysin S to virulence of serotype M5 *Streptococcus pyogenes* strain Manfredo. *Infect Immun* **71,** 3857-3865.

Fontán, P.A., Pancholi, V., Noclari, M.M. and Fischetti, V.A. (2000). Antibodies to streptococcal surface enolase react with human α-enolase: implications in postsrteptococcal sequelae. *The Journal of Infectious Diseases* **182,** 1712-1721.

Fox, E.N., Waldman, R.H., Wittner, M.K., Mauceri, A.A. and Dorfman, A. (1973). Protective study with a group A streptococcal M protein vaccine. Infectivity challenge of human volunteers. *Journal of Clinical Investigation* **52,** 1885-1892.

Frank, R. (2002). The SPOT-synthesis technique. Synthetic peptide arrays on membrane supports--principles and applications. *Journal of Immunological Methods* **267,** 13-26.

Frick, I.-M., Åkesson, P., Rasmussen, M., Schmidtchen, A. and Björck, L. (2003). SIC, a secreted protein of *Streptococcus pyogenes* that inactivates bacterial peptides. *J Biol Chem* **278,** 16561-16566.

Fritzer, A., Senn, B.M., Minh, D.B., Hanner, M., Gelbmann, D., Noiges, B.*, et al.* (2010). Novel conserved group A streptococcal proteins identified by the antigenome technology as vaccine candidates for a non-M protein-based vaccine. *Infect Immun* **78,** 4051-4067.

Fujita, Y., Abdel-Aal, A.B., Wimmer, N., Batzloff, M.R., Good, M.F. and I, T. (2008). Synthesis and immunological evaluation of self-adjuvanting glycolipopeptide vaccine candidates. *Bioorganic & Medicinal Chemistry* **16,** 8907-8913.

Galkin, A., Kulakova, L., Sarikaya, E., Lim, K., Howard, A. and Herzberg, O. (2004). Structural insight into arginine degradation by arginine deiminase, an antibacterial and parasite drug target. *J Biol Chem* **279,** 14001-14008.

Galvin, J.E., Hemric, M.E., Ward, K. and Cunningham, M.W. (2000). Cytotoxic mAb from rheumatic carditis recognises heart valves and laminin. *Journal of Clinical Investigation* **106,** 217-224.

Gao, Y., Liang, C., Zhao, R., Lukomski, S. and Han, R. (2010). The Scl1 of M41-type group A Streptococcus binds the high-density lipoprotein. *FEMS Microbiology Letters* **309,** 55-61.

Gardiner, D.L., Goodfellow, A.M., Martin, D.R. and Sriprakash, K.S. (1998). Group A streptococcal Vir Types are M-protein gene (*emm*) sequence type specific. *Journal of Clinical Microbiology* **36,** 902-907.

Gardiner, D.L., Hartas, J., Currie, B.J., Mathews, J.D., Kemp, D.J. and Sriprakash, K.S. (1995). *Vir* typing: a long-PCR typing method for group A streptococci. *PCR Methods and Applications* **4,** 288-293.

Gardiner, D.L. and Sriprakash, K.S. (1996). Molecular epidemiology of impetiginous group A streptococcal infections in Aboriginal communities of Northern Australia. *Journal of Clinical Microbiology* **34,** 1448-1452.

Gaworzewska, E. and Colman, G. (1988). Changes in the pattern of infection caused by *Streptococcus pyogenes*. *Epidemiology and Infection* **100,** 257-269.

Georgousakis, M., McMillan, D., Batzloff, M.R. and Sriprakash, K.S. (2009a). Moving forward: a mucosal vaccine against group A streptococcus. *Expert Review of Vaccines* **8,** 747-760.

Georgousakis, M.M. (2008) Towards a mucosal vaccine against group A streptococcus based on a live bacterial delivery system. In *School of Population Health*, (ed.^(eds.). University of Queensland.

Georgousakis, M.M., Hofmann, A., Batzloff, M.R., McMillan, D.J. and Sriprakash, K.S. (2009b). Structural optimisation of a conformational epitope improves antigenicity when expressed as a recombinant fusion protein. *Vaccine* **27,** 6799-6806.

Gillen, C.M., Courtney, H.S., Schulze, K., Rohde, M., Wilson, M.R., Timmer, A.M*., et al.* (2008). Opacity factor activity and epithelial cell binding by the serum opacity factor protein of *Streptococcus pyogenes* are functionally discrete. *J Biol Chem* **283,** 6359-6366.

Gillen, C.M., Towers, R.J., McMillan, D.J., Delvecchio, A., Sriprakash, K.S., Currie, B*., et al.* (2002). Immunological response mounted by Aboriginal Australians living in the Northern Territory of Australia against *Streptococcus pyogenes* serum opacity factor. *Microbiology* **148,** 169-178.

Goldmann, O., Sastalla, I., Wos-Oxley, M., Rohde, M. and Medina, E. (2009). *Streptococcus pyogenes* induces oncosis in macrophages through the activation of an inflammatory programmed cell death pathway. *Cellular Microbiology* **11,** 138-155.

Goldstein, I., Halpern, B. and Robert, L. (1967). Immunological relationship between streptococcus A polysaccharide and the structural glycoproteins of heart valve. *Nature* **213,** 44-47.

Goodfellow, A.M., Hibble, M., Talay, S.R., Kreikemeyer, B., Currie, B.J., Sriprakash, K.S. and Chhatwal, G.S. (2000). Distribution and antigenicity of fibronectin binding proteins (SfbI and SfbII) of *Streptococcus pyogenes* clinical isolates from the Northern Territory, Australia. *Journal of Clinical Microbiology* **38,** 389-392.

Gorton, D., Norton, R., Layton, R., Smith, H. and Ketheesan, N. (2005). Presence of fibronectin-binding protein gene prtF2 in invasive group A streptococci in tropical Australia is associated with increased internalisation efficiency. *Microbes and Infection* **7,** 421-426.

Guilherme, L., Faé, K.C., Higa, F., Chaves, L., Oshiro, S.E., Freschi de Barros, S*., et al.* (2006a). Towards a vaccine against rheumatic fever. *Clinical and Developmental Immunology* **13,** 125-132.

Guilherme, L., Kalil, J. and Cunningham, M. (2006b). Molecular mimicry in the autoimmune pathogenesis of rheumatic heart disease. *Autoimmunity* **39,** 31-39.

Guilherme, L., Postol, E., Freschi de Barros, S., Higa, F., Alencar, R., Lastre, M*., et al.* (2009). A vaccine against *S. pyogenes*: Design and experimental immune response. *Methods* **49,** 316-321.

Guzmán, C.A., Talay, S.R., Molinari, G., Medina, E. and Chhatwal, G.S. (1999). Protective immune response against *Streptococcus pyogenes* in mice after intranasal vaccination with fibronectin-binding protein SfbI. *J Infect Dis* **179,** 901-906.

Hall, M.A., Stroop, S.D., Hu, M.C., Walls, M.A., Reddish, M.A., Burt, D.S*., et al.* (2004). Intranasal immunization with multivalent group A streptococcal vaccines protects mice against intranasal challenge infections. *Infect Immun* **72,** 2507-2512.

Han, R., Caswell, C.C., Lukomska, E., Keene, D.R., Pawlowski, M., Bujnicki, J.M.*, et al.* (2006). Binding of the low-density lipoprotein by streptococcal collagen-like protein Scl1 of *Streptococcus pyogenes*. *Mol Microbiol* **61,** 351-367.

Hanski, E. and Caparon, M. (1992). Protein F, a fibronectin-binding protein is an adhesin of the group A streptococcus Streptococcus pyogenes. *Proceedings of the National Academy of Sciences of the United States of America* **89,** 6172-6176.

Harlow, E. and Lane, D. (1988) Antibodies, A Laboratory Manual New York, Cold Spring Harbour Press.

Hartas, J., Hibble, M. and Sriprakash, K.S. (1998). Simplification of a locus-specific DNA Typing method (Vir Typing) for Streptococcus pyogenes. *Journal of Clinical Microbiology* **36,** 1428-1429.

Hartas, J. and Sriprakash, K.S. (1999). *Streptococcus pyogenes* strains containing *emm*12 and *emm*55 possess a novel gene coding for distantly related SIC protein. *Microbial Pathogenesis* **26,** 25-33.

Hasty, D.L. and Courtney, H.S. (1996). Group A streptococcal adhesion: all of the theories are correct. *Advanced Experimental Medicine and Biology* **408,** 81-94.

Hasty, D.L., Ofek, I., Courtney, H.S. and Doyle, R.J. (1992). Multiple adhesins of Streptococci. *Infection and Immunity* **60,** 2147-2152.

Hayman, W.A., Brandt, E.R., Relf, W.A., Cooper, J., Saul, A. and Good, M.F. (1997). Mapping the minimal murine T cell and B cell epitopes within a peptide vaccine candidate from the conserved region of the M protein of group A streptococcus. *International Immunology* **9,** 1723-1733.

Hayman, W.A., Toth, I., Flinn, N., Scanlin, M. and Good, M.F. (2002). Enhancing the immunogenicity and modulating the fine epitope recognition of antisera to a helical group A streptococcal peptide vaccine candidate from the M protein using lipid-core peptide technology. *Immunology and Cell Biology* **80,** 178-187.

Herwald, H., Cramer, H., Mörgelin, M., Russell, W., Sollenberg, U., Norrby-Teglund, A.*, et al.* (2004). M protein, a classical bacterial virulence determinant, forms complexes with fibrinogen that induce vascular leakage. *Cell* **116,** 367-379.

Hidalgo-Grass, C., Mishalian, I., Dan-Goor, M., Belotserkovsky, I., Eran, Y., Nizet, V.*, et al.* (2006). A streptococcal protease that degrades CXC chemokines and impairs bacterial clearance from infected tissues. *EMBO Journal* **25,** 4628-4637.

Holm, S.E. (1996). Invasive group A streptococcal infections. *The New England Journal of Medicine* **335,** 590-591.

Hsu, J.F., Chuang, W.J., Shiesh, S.C., Lin, Y.S., Liu, C.C., Wang, C.C.*, et al.* (2008). Streptococcal pyrogenic exotoxin B cleaves human S-adenosylhomocysteine hydrolase and induces hypermethioninemia. *J Infect Dis* **198,** 367-374.

Hu, M.C., Walls, M.A., Stroop, S.D., Reddish, M.A., Beall, B. and Dale, J.B. (2002). Immunogenicity of a 26-valent group A streptococcal vaccine. *Infect Immun* **70,** 2171-2177.

Hughes, M.J.G., Moore, J.C., Lane, J.D., Wilson, R., Pribul, P.K., Younes, Z.N.*, et al.* (2002). Identification of major outer surface proteins of *Streptococcus agalactiae*. *Infect Immun* **70,** 1254-1259.

Humtsoe, J.O., Kim, J.K., Xu, Y., Keene, D.R., Höök, M., Lukomski, S. and Wary, K.K. (2005). A streptococcal collagen-like protein interacts with the alpha2beta1 integrin and induces intracellular signaling. *J Biol Chem* **280,** 13848-13857.

Hussain, M., Peters, G., Chhatwal, G.S. and Herrmann, M. (1999). A lithium chloride-extracted, broad-spectrum-adhesive 42-kilodalton protein of *Staphylococcus epidermis* is ornithine carbamoyltransferase. *Infect Immun* **67,** 6688-6690.

Hyland, K.A., Wang, B. and Cleary, P.P. (2007). Protein F1 and *Streptococcus pyogenes* resistance to phagocytosis. *Infect Immun* **75,** 3188-3191.

Hytönen, J., Haataja, S., Gerlach, D., Podbielski, A. and Finne, J. (2001). The SpeB virulence factor

of *Streptococcus pyogenes*, a multifunctional secreted and cell surface molecule with strepadhesin, laminin-binding and cysteine protease activity. *Mol Microbiol* **39,** 512-519.

Iida, K.-i., Seki, M., Saito, M., Kawamura, Y., Kajiwara, H. and Yoshida, S.-i. (2006). Capsule of *Streptococcus pyogenes* is essential for delayed death of mice in a model of streptococcal toxic shock syndrome. *Microbiology and Immunology* **50,** 127-130.

Ikebe, T., Hirasawa, K., Suzuki, R., Ohya, H., Isobe, J., Tanaka, D.*, et al.* (2007). Distribution of *emm* genotypes among group A streptococcus isolates from patients with severe invasive streptococcal infections in Japan, 2001-2005. *Epidemiology and Infection* **135,** 1227-1229.

Jadoun, J., Ozeri, V., Burstein, E., Skutelsky, E., Hanski, E. and Sela, S. (1998). Protein F1 is required for efficient entry of *Streptococcus pyogenes* into epithelial cells. *J Infect Dis* **178,** 147-158.

Jadoun, J. and Sela, S. (2000). Mutation in csrR global regulator reduces *Streptococcus pyogenes* internalization. *Microbial Pathogenesis* **29,** 311-317.

Jaffe, J., Natanson-Yaron, S., Caparon, M.G. and Hanski, E. (1996). Protein F2, a novel fibronectin-binding protein from *Streptococcus pyogenes*, possesses two binding domains. *Molecular Microbiology* **21,** 373-384.

Jeng, A., Sakota, V., Li, Z., Datta, V., Beall, B. and Nizet, V. (2003). Molecular genetic analysis of a group A Streptococcus operon encoding serum opacity factor and a novel fibronectin-binding protein, SfbX. *J Bacteriol* **185,** 1208-1217.

Jenkinson, H.F. and Lamont, R.J. (1997). Streptococcal adhesion and colonisation. *Critical Reviews in Oral Biology and Medicine* **8,** 175-200.

Ji, Y., Carlson, B., Kondagunta, A. and Cleary, P.P. (1997). Intranasal immunisation with C5a peptidase prevents nasopharyngeal colonisation of mice by the group A Streptococcus. *Infect Immun* **65,** 2080-2087.

Ji, Y., Schnitzler, N., DeMaster, E. and Cleary, P.P. (1998). Impact of M49, Mrp, Enn, and C5a Peptidase proteins on colonization of the mouse oral mucosa by *Streptococcus pyogenes*. *Infect Immun* **66,** 5399-5405.

Jin, H., Song, Y.P., Boel, G., Kochar, J. and Pancholi, V. (2005). Group A streptococcal surface GAPDH, SDH, recognizes uPAR/CD87 as its receptor on the human pharyngeal cell and mediates bacterial adherence to host cells. *Journal of Molecular Biology* **350,** 27-41.

Johnson, D.R., Kaplan, E.L., VanGheem, A., Facklam, R.R. and Beall, B. (2006). Characterization of group A streptococci (*Streptococcus pyogenes*): correlation of M-protein and *emm*-gene type with T-protein agglutination pattern and serum opacity factor. *J Med Microbiol* **55,** 157-164.

Johnson, D.R., Wotton, J., Shet, A. and Kaplan, E.L. (2002). A comparison of group A streptococci from invasive and uncomplicated infections: are virulent clones responsible for serious streptococcal infections? *J Infect Dis* **185,** 1586-1595.

Jones, K.F. and Fischetti, V.A. (1988). The importance of the location of antibody binding on the M6 protein for opsonisation and phagocytosis of group A M6 streptococci. *J Exp Med* **167,** 1114-1123.

Kamezawa, Y., Nakahara, T., Nakano, S., Abe, Y., Nozaki-Renard, J. and Isono, T. (1997). Streptococcal mitogenic exotoxin Z, a novel acidic superantigenic toxin produced by a T1 strain of *Streptococcus pyogenes*. *Infect Immun* **65,** 3828-3833.

Kang, H.J., Coulibaly, F., Clow, F., Proft, T. and Baker, E.N. (2007). Stabilizing isopeptide bonds revealed in gram-positive bacterial pilus structure. *Science* **318,** 1628-1628.

Kansal, R.G., McGeer, A., Low, D.E., Norrby-Teglund, A. and Kotb, M. (2000). Inverse relation between disease severity and expression of the streptococcal cysteine protease, SpeB, among clonal M1T1 isolates recovered from invasive group A streptococcal infection cases. *Infect Immun* **68,** 6362-6369.

Kaplan, E.L. (1991). The resurgence of group A streptococcal infections and their sequelae. *European Journal of Clinical Microbiology and Infectious Diseases* **10,** 55-57.

Kaplan, E.L., Chhatwal, G.S. and Rohde, M. (2006). Reduced ability of penicillin to eradicate

ingested group A streptococci from epithelial cells: clinical and pathogenetic implications. *Clinical Infectious Diseases* **43,** 1398-1406.

Kaplan, E.L., Wotton, J.T. and Johnson, D.R. (2001). Dynamic epidemiology of group A streptococcal serotypes associated with pharyngitis. *Lancet* **358,** 1334-1337.

Kapur, V., Maffei, J.T., Greer, R.S., Li, L.L., Adams, G.J. and Musser, J.M. (1994). Vaccination with streptococcal extracellular cysteine protease (interleukin-1 beta convertase) protects mice against challenge with heterologous group A streptococci. *Microbial Pathogenesis* **16,** 443-450.

Kapur, V., Topouzis, S., Majesky, M.W., Li, L.L., Hamrick, M.R., Hamill, R.J*., et al.* (1993). A conserved *Streptococcus pyogenes* extracellular cysteine protease cleaves human fibronectin and degrades vitronectin. *Microbial Pathogenesis* **15,** 327-346.

Katerov, V., Andreev, A., Schalén, C. and Totolian, A.A. (1998). Protein F, a fibronectin-binding protein of *Streptococcus pyogenes*, also binds human fibrinogen: isolation of the protein and mapping of the binding region. *Microbiology* **144,** 119-126.

Kawabata, S., Kunitomo, E., Terao, Y., Nakagawa, I., Kikuchi, H., Totsuka, K.-I. and Hamada, S. (2001). Systemic and mucosal immunisations with fibronectin-binding protein FBP54 induce protective immune response against *Streptococcus pyogenes* challenge in mice. *Infect Immun* **69,** 924-930.

Kawabata, S., Tamura, Y., Murakami, J., Terao, Y., Nakagawa, I. and Hamada, S. (2002). A novel, anchorless streptococcal surface protein that binds to human immunoglobulins. *Biochemical and Biophysical Research Communications* **296,** 1329-1333.

Kazmi, S.U., Kansal, R., Aziz, R.K., Hooshdaran, M., Norrby-Teglund, A., Low, D.E*., et al.* (2001). Reciprocal, temporal expression of SpeA and SpeB by invasive M1T1 group a streptococcal isolates *in vivo*. *Infect Immun* **69,** 4988-4995.

Kersten, G.F. and Crommelin, D.J. (2003). Liposomes and ISCOMs. *Vaccine* **21,** 915-920.

Khil, J., Im, M., Heath, A., Ringdahl, U., Mundada, L., Cary Engleberg, N. and Fay, W.P. (2003). Plasminogen enhances virulence of group A streptococci by streptokinase-dependent and streptokinase-independent mechanisms. *J Infect Dis* **188,** 497-505.

Korber, B., LaBute, M. and Yusim, K. (2006). Immunoinformatics comes of age. *PLOS Computational Biology* **2,** 0484-0492.

Koroleva, I.V., Efstratiou, A. and Suvorov, A.N. (2002). Structural heterogeneity of the streptococcal C5a peptidase gene in Streptococcus pyogenes. *Journal of Bacteriology* **184,** 6384-6386.

Kotloff, K.L., Corretti, M., Palmer, K., Campbell, J.D., Reddish, M.A., Hu, M.C*., et al.* (2004). Safety and immunogenicity of a recombinant multivalent group A streptococcal vaccine in healthy adults: Phase I trial. *Journal of the American Medical Association* **292,** 709-715.

Kotloff, K.L., Wasserman, S.S., Jones, K.F., Livio, S., Hruby, D.E., Franke, C.A. and Fischetti, V.A. (2005). Clinical and microbiological responses of volunteers to combined intranasal and oral inoculation with a *Streptococcus gordonii* carrier strain intended for future use as a group A streptococcus vaccine. *Infect Immun* **73,** 2360-2366.

Kraus, W., Seyer, J.M. and Beachey, E.H. (1989). Vimentin-cross-reactive epitope of type 12 streptococcal M protein. *Infect Immun* **57,** 2457-2461.

Krause, R.M. (2002). A half-century of streptococcal research: then & now. *Indian Journal of Medical Research* **115,** 215-241.

Kreikemeyer, B., Beckert, S., Braun-Kiewnick, A. and Podbielski, A. (2002). Group A streptococcal RofA-type global regulators exhibit a strain-specific genomic presence and regulation pattern. *Microbiology* **148,** 1501-1511.

Kreikemeyer, B., Nakata, M., Oehmcke, S., Gschwendtner, C., Normann, J. and Podbielski, A. (2005). *Streptococcus pyogenes* collagen type I-binding Cpa surface protein. Expression profile, binding characteristics, biological functions, and potential clinical impact. *J Biol Chem* **280,** 33228-33239.

Kreikemeyer, B., Oehmcke, S., Nakata, M., Hoffrogge, R. and Podbielski, A. (2004). *Streptococcus*

pyogenes fibronectin-binding protein F2: expression profile, binding characteristics, and impact on eucaryotic cell interactions. *J Biol Chem* **279,** 15850-15859.

Kumar, R., Vohra, H., Chakraborty, A., Sharma, Y.P., Bandhopadhya, S., Dhanda, V*., et al.* (2009). Epidemiology of group A streptococcal pharyngitis & impetigo: a cross-sectional & follow up study in a rural community of northern India. *Indian Journal of Medical Research* **130,** 765-771.

Kuo, C.F., Lin, Y.S., Chuang, W.J., Wu, J.J. and Tsao, N. (2008). Degradation of complement 3 by streptococcal pyrogenic exotoxin B inhibits complement activation and neutrophil opsonophagocytosis. *Infect Immun* **76,** 1163-1169.

Kurupati, P., Turner, C.E., I, T., Lawrenson, R.A., Alam, F.M., Nohadani, M.S., G W*, et al.* (2010). Chemokine-cleaving *Streptococcus pyogenes* protease SpyCEP is necessary and sufficient for bacterial dissemination within soft tissues and the respiratory tract. *Mol Microbiol* **76,** 1387-1197.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680-685.

Lancefield, R.C. (1962). Current knowledge of type-specific M antigens of group A streptococcus. *Journal of Immunology* **89,** 307-313.

LaPenta, D., Rubens, C., Chi, E. and Cleary, P.P. (1994). Group A streptococci efficiently invade human respiratory epithelial cells. *Proceedings of the National Academy of Sciences of the United States of America* **91,** 12115-12119.

LaPlante, K.L. and Rybak, M.J. (2004). Daptomycin - a novel antibiotic against Gram-positive pathogens. *Expert Opinion on Pharmacotherapy* **5,** 2321-2331.

Lei, B., Liu, M.-Y., Chesney, G.L. and Musser, J.M. (2004). Identification of new candidate vaccine antigens made by *Streptococcus pyogenes*: purification and characterisation of 16 putative extracellular lipoproteins. *J Infect Dis* **189,** 79-89.

Lei, B., Mackie, S., Lukomski, S. and Musser, J.M. (2000). Identification and immunogenicity of group A streptococcus culture supernatant proteins. *Infect Immun* **68,** 6807-6818.

Liang, M.T., Davies, N.M., Blanchfield, J.T. and Toth, I. (2006). Particulate systems as adjuvants and carriers for peptide and protein antigens. *Current Drug Delivery* **3,** 379-388.

Liang, O.D., Preissner, K.T. and Chhatwal, G.S. (1997). The hemopexin-type repeats of human vitronectin are recognised by Streptococcus pyogenes. *Biochemical and Biophysical Research Communications* **234,** 445-449.

Limbago, B., Penumalli, V., Weinrick, B. and Scott, J.R. (2000). Role of Streptolysin O in a mouse model of invasive group A streptococcal disease. *Infect Immun* **68,** 6384-6390.

Ling, E., Feldman, G., Portnoi, M., Dagan, R., Overweg, K., Mulholland, F*., et al.* (2004). Glycolytic enzymes associated with the cell surface of *Streptococcus pneumoniae* are antigenic in humans and elicit protective immune responses in the mouse. *Clin Exp Immunol* **138,** 290-298.

Linke, C., Caradoc-Davies, T.T., Proft, T. and Baker, E.N. (2008). Purification, crystallization and preliminary crystallographic analysis of *Streptococcus pyogenes* laminin-binding protein Lbp. *Acta Crystallographica, Section F, Structural Biology and Crystallization Communications* **64,** 141-143.

Linke, C., Caradoc-Davies, T.T., Young, P.G., Proft, T. and Baker, E.N. (2009). The laminin-binding protein Lbp from *Streptococcus pyogenes* is a zinc-receptor. *J Bacteriol* **191,** 5814-5823.

Liu, M., Zhu, H., Zhang, J. and Lei, B. (2007). Active and passive immunizations with the streptococcal esterase Sse protect mice against subcutaneous infection with group A streptococci. *Infect Immun* **75,** 3651-3657.

Ludlam, A.V., Moore, B.A. and Xu, Z. (2004). The crystal structure of ribosomal chaperone trigger factor from *Vibrio cholerae*. *Proceedings of the National Academy of Sciences of the United States of America* **101,** 13436-13441.

Lukomski, S., Nakashima, K., Abdi, I., Cipriano, V.J., Ireland, R.M., Reid, S.D*., et al.* (2000).

Identification and characterisation of the *scl* gene encoding a group A Streptococcus extracellular protein virulence factor with similarity to human collagen. *Infect Immun* **68,** 6542-6553.

Luo, Y.H., Kuo, C.F., Huang, K.J., Wu, J.J., Lei, H.Y., Lin, M.T*., et al.* (2007). Streptococcal pyrogenic exotoxin B antibodies in a mouse model of glomerulonephritis. *Kidney International* **72,** 716-724.

Lynch, M. and Kuramitsu, H. (2000). Expression and role of superoxide dismutases (SOD) in pathogenic bacteria. *Microbes and Infection* **2,** 1245-1255.

Lyon, W.R. and Caparon, M.G. (2003). Trigger Factor-mediated prolyl isomerization influences maturation of the *Streptococcus pyogenes* cysteine protease. *J Bacteriol* **185,** 3661-1667.

Lyon, W.R. and Caparon, M.G. (2004). Role for serine protease HtrA (DegP) of *Streptococcus pyogenes* in the biogenesis of virulence factors SpeB and the hemolysin Streptolysin S. *Infect Immun* **72,** 1618-1625.

Lyon, W.R., Gibson, C.M. and Caparon, M.G. (1998). A role for Trigger Factor and an Rgg-like regulator in the transcription, secretion and processing of the cysteine proteinase of *Streptococcus pyogenes*. *EMBO Journal* **17,** 6263-6275.

Ma, C.Q., Li, C.H., Wang, X.R., Zeng, R.H., Yin, X.L., Feng, H.D. and L, W. (2009). Similar ability of FbaA with M protein to elicit protective immunity against group A streptococcus challenge in mice. *Cellular & Molecular Immunology* **6,** 73-77.

Manetti, A.G., Zingaretti, C., Falugi, F., Capo, S., Bombaci, M., Bagnoli, F*., et al.* (2007). *Streptococcus pyogenes* pili promote pharyngeal cell adhesion and biofilm formation. *Mol Microbiol* **64,** 968-983.

Mannam, P., Jones, K.F. and Geller, B.L. (2004). Mucosal vaccine made from live, recombinant *Lactococcus lactis* protects mice against pharyngeal infection with *Streptococcus pyogenes*. *Infect Immun* **72,** 3444-3450.

Marouni, M.J., Ziomek, E. and Sela, S. (2003). Influence of group A streptococcal acid glycoprotein on expression of major virulence factors and internalisation by epithelial cells. *Microbial Pathogenesis* **35,** 63-72.

Martin, D.R. and Sriprakash, K.S. (1996). Epidemiology of group A streptococcal disease in Australia and New Zealand. *Recent Advances in Microbiology* **4,** 1-40.

Massell, B.F., Honikman, L.H. and Amezcua, J. (1969). Rheumatic fever following streptococcal vaccination. Report of three cases. *Journal of the American Medical Association* **207,** 1115-1119.

McArthur, J., Medina, E., Mueller, A., Chin, J., Currie, B.J., Sriprakash, K.S*., et al.* (2004a). Intranasal vaccination with streptococcal fibronectin binding protein Sfb1 fails to prevent growth and dissemination of *Streptococcus pyogenes* in a murine skin infection model. *Infect Immun* **72,** 7342-7345.

McArthur, J., Schulze, K., Chin, J., Currie, B.J., Sriprakash, K.S., Talay, S.R*., et al.* (2004b). Immune responses of a liposome/ISCOM vaccine adjuvant against streptococcal fibronectin binding protein 1 (Sfb1) in mice. *Indian Journal of Medical Research* **119,** 115-120.

McArthur, J.D., McKay, F.C., Ramachandran, V., Shyam, P., Cork, A.J., Sanderson-Smith, M.L*., et al.* (2008). Allelic variants of streptokinase from *Streptococcus pyogenes* display functional differences in plasminogen activation. *FASEB J* **22,** 3146-3153.

McCormick, J.K., Pragman, A.A., Stolpa, J.C., Leung, D.Y. and Schlievert, P.M. (2001). Functional characterization of streptococcal pyrogenic exotoxin J, a novel superantigen. *Infect Immun* **69,** 1381-1388.

McDonald, M., Currie, B.J. and Carapetis, J.R. (2004). Acute rheumatic fever: a link in the chain that links the heart to the throat? *The Lancet Infectious Diseases* **4,** 240-245.

McKay, F.C., McArthur, J.D., Sanderson-Smith, M.L., Gardam, S., Currie, B.J., Sriprakash, K.S*., et al.* (2004). Plasminogen binding by group A streptococcal isolates from a region of hyperendemicity for streptococcal skin infection and a high incidence of invasive infection.

Infect Immun **72,** 364-370.

McLaurin, J., Cecal, R., Kierstead, M.E., Tian, X., Phinney, A.L., Manea, M*., et al.* (2002). Therapeutically effective antibodies against amyloid-β peptide target amyloid-β residues 4–10 and inhibit cytotoxicity and fibrillogenesis. *Nature Medicine* **8,** 1263-1269.

McMillan, D.J. and Chhatwal, G.S. (2005). Prospects for a group A streptococcal vaccine. *Current Opinion in Molecular Therapeutics* **7,** 11-16.

McMillan, D.J., Davies, M.R., Browning, C.L., Good, M.F. and Sriprakash, K.S. (2004a). Prospecting for new group A streptococcal vaccine candidates. *Indian Journal of Medical Research* **119,** 121-125.

McMillan, D.J., Davies, M.R., Good, M.F. and Sriprakash, K.S. (2004b). Immune response to superoxide dismutase in group A streptococcal infection. *FEMS Immunology and Medical Microbiology* **40,** 249-256.

McNamara, C., Zinkernagel, A.S., Macheboeuf, P., Cunningham, M.W., Nizet, V. and P, G. (2008). Coiled-coil irregularities and instabilities in group A Streptococcus M1 are required for virulence. *Science* **319,** 1405-1408.

McNeil, S.A., Halperin, S.A., Langley, J.M., Smith, B., Warren, A., Sharratt, G.P*., et al.* (2005). Safety and immunogenicity of 26-valent group a streptococcus vaccine in healthy adult volunteers. *Clinical Infectious Diseases* **41,** 1114-1122.

Medaglini, D., Pozzi, G., King, T.P. and Fischetti, V.A. (1995). Mucosal and systemic immune responses to a recombinant protein expressed on the surface of the oral commensal bacterium *Streptococcus gordonii* after oral colonization. *Proceedings of the National Academy of Sciences of the United States of America* **92,** 6868–6872.

Medina, E., Goldmann, O., Toppel, A.W. and Chhatwal, G.S. (2003). Survival of *Streptococcus pyogenes* within host phagocytic cells: a pathogenic mechanism for persistence and systemic invasion. *J Infect Dis* **187,** 597-603.

Medina, E., Talay, S.R., Chhatwal, G.S. and Guzman, C.A. (1998). Fibronectin-binding protein I of *Streptococcus pyogenes* is a promising adjuvant for antigens delivered by mucosal route. *European Journal of Immunology* **28,** 1069-1077.

Menon, T., Whatmore, A.M., Srivani, S., Kumar, M.P., Anbumani, N. and Rajaji, S. (2001). *Emm* types of *Streptococcus pyogenes* in Chennai. *Indian Journal of Medical Microbiology* **19,** 161-162.

Michos, A.G., Bakoula, C.G., Braoudaki, M., Koutouzi, F.I., Roma, E.S., Pangalis, A*., et al.* (2009). Macrolide resistance in *Streptococcus pyogenes*: prevalence, resistance determinants, and emm types. *Diagnostic Microbiology and Infectious Disease* **64,** 295-299.

Miyoshi-Akiyama, T., Takamatsu, D., Koyanagi, M., Zhao, J., Imanishi, K. and Uchiyama, T. (2005). Cytocidal effect of *Streptococcus pyogenes* on mouse neutrophils in vivo and the critical role of streptolysin S. *J Infect Dis* **192,** 107-116.

Molinari, G. and Chhatwal, G.S. (1999a). Streptococcal invasion. *Current Opinion in Microbiology* **2,** 56-61.

Molinari, G., Chhatwal, G.S., Sela, S. and Jadoun, J. (1999b). Role played by the fibronectin-binding protein SfbI (Protein F1) of *Streptococcus pyogenes* in bacterial internalisation by epithelial cells. *J Infect Dis* **179,** 1049-1050.

Molinari, G., Chhatwal, G.S., Sela, S. and Jadoun, J. (1999c). Role played by the fibronectin-binding protein SfbI (Protein F1) of *Streptococcus pyogenes* in bacterial internalisation by epithelial cells. *The Journal of Infectious Diseases* **179,** 1049-1050.

Molinari, G., Rohde, M., Talay, S.R., Chhatwal, G.S., Beckert, S. and Podbielski, A. (2001). The role played by the group A streptococcal negative regulator Nra on bacterial interactions with epithelial cells. *Mol Microbiol* **40,** 99-114.

Molinari, G., Talay, S.R., Valentin-Weigand, P., Rohde, M. and Chhatwal, G.S. (1997). The fibronectin-binding protein of *Streptococcus pyogenes*, SfbI, is involved in the internalisation of group A streptococci by epithelial cells. *Infect Immun* **65,** 1357-1363.

Mollick, J.A., Miller, G.G., Musser, J.M., Cook, R.G., Grossman, D. and Rich, R.R. (1993). A novel superantigen isolated from pathogenic strains of *Streptococcus pyogenes* with aminoterminal homology to staphylococcal enterotoxins B and C. *Journal of Clinical Investigation* **92,** 710-719.

Mora, M., Bensi, G., Capo, S., Falugi, F., Zingaretti, C., Manetti, A.G*., et al.* (2005). Group A Streptococcus produce pilus-like structures containing protective antigens and Lancefield T antigens. *Proceedings of the National Academy of Sciences of the United States of America* **102,** 15641-15646.

Moses, A.E., Wessels, M.R., Zalcman, K., Albertí, S., Natanson-Yaron, S., Menes, T. and Hanski, E. (1997). Relative contributions of hyaluronic acid capsule and M protein to virulence in a mucoid strain of the group A Streptococcus. *Infection and Immunity* **65,** 64-71.

Mota, G.F.A., Carneiro, C.R.W., Gomes, L. and Lopes, J.D. (1988). Monoclonal antibodies to *Staphylococcus aureus* laminin-binding proteins cross react with mammalian cells. *Infection and Immunity* **56,** 1580-1584.

Moyle, P.M., Olive, C., Good, M.F. and Toth, I. (2006a). Method for the synthesis of highly pure vaccines using the lipid core peptide system. *Journal of Peptide Science* **12,** 800-807.

Moyle, P.M., Olive, C., Ho, M.F., Burgess, M., Karpati, L., Good, M.F. and Toth, I. (2006b). Method for the synthesis of multi-epitopic *Streptococcus pyogenes* lipopeptide vaccines using native chemical ligation. *Journal of Organic Chemistry* **71,** 6846-6850.

Moyle, P.M., Olive, C., Ho, M.F., Good, M.F. and Toth, I. (2006c). Synthesis of a highly pure lipid core peptide based self-adjuvanting triepitopic group A streptococcal vaccine, and subsequent immunological evaluation. *Journal of Medicinal Chemistry* **49,** 6364-6370.

Müller-Alouf, H., Proft, T., Zollner, T.M., Gerlach, D., Champagne, E., Desreumaux, P*., et al.* (2001). Pyrogenicity and cytokine-inducing properties of *Streptococcus pyogenes* superantigens: comparative study of streptococcal mitogenic exotoxin Z and pyrogenic exotoxin A. *Infect Immun* **69,** 4141-4145.

Musser, J.M., Hauser, A.R., Kim, M.L., Schlievert, P.M., Nelson, K. and Selander, R.K. (1991). *Streptococcus pyogenes* causing toxic-shock-like syndrome and other invasive diseases: clonal diversity and pyrogenic exotoxin expression. *Proceedings of the National Academy of Sciences of the United States of America* **88,** 2668-2672.

Musser, J.M. and Shelburne, S.A. (2009). A decade of molecular pathogenomic analysis of group A Streptococcus. *Journal of Clinical Investigation* **119,** 2455-2463.

Musser, J.M., Stockbauer, K., Kapur, V. and Rudgers, G.W. (1996). Substitution of cysteine 192 in a highly conserved *Streptococcus pyogenes* extracellular cysteine protease (interleukin 1b convertase) alters proteolytic activity and ablates zymogen processing. *Infect Immun* **64,** 1913-1917.

Nilsson, M., Sørensen, O.E., Mörgelin, M., Weineisen, M., Sjöbring, U. and Herwald, H. (2006). Activation of human polymorphonuclear neutrophils by streptolysin O from *Streptococcus pyogenes* leads to the release of proinflammatory mediators. *Thrombosis and Haemostasis* **95,** 982-990.

Nir-Paz, R., Korenman, Z., Ron, M., Michael-Gayego, A., Cohen-Poradosu, R., Valinsky, L*., et al.* (2010). *Streptococcus pyogenes emm* and T types within a decade, 1996-2005: implications for epidemiology and future vaccines. *Epidemiology and Infection* **138,** 53-60.

Nitsche-Schmitz, D.P., Rohde, M. and Chhatwal, G.S. (2007). Invasion mechanisms of Gram-positive pathogenic cocci. *Thrombosis and Haemostasis* **98,** 488-496.

Nizet, V., Beall, B., Bast, D.J., Datta, V., Kilburn, L., Low, D.E. and De Azavedo, J.C.S. (2000). Genetic locus for Streptolysin S production by group A streptococci. *Infection and Immunity* **68,** 4245-4254.

Nooh, M.M., Aziz, R.K., Kotb, M., Eroshkin, A., Chuang, W.J., Proft, T. and Kansal, R. (2006). Streptococcal mitogenic exotoxin, SmeZ, is the most susceptible M1T1 streptococcal superantigen to degradation by the streptococcal cysteine protease, SpeB. *J Biol Chem* **281,**

35281-35288.

Nowak, R. (1994). Flesh-eating bacteria: not new, but still worrisome. *Science* **364,** 1665.

O'Connor, S.P. and Cleary, P.P. (1986). Localization of the streptococcal C5a peptidase to the surface of group A streptococci. *Infect Immun* **53,** 432-434.

O'Hagan, D.T., Singh, M. and Ulmer, J.B. (2006). Microparticle-based technologies for vaccines. *Methods* **40,** 10-19.

O'Loughlin, R.E., Roberson, A., Cieslak, P.R., Lynfield, R., Gershman, K., Craig, A*., et al.* (2007). The epidemiology of invasive group A streptococcal infection and potential vaccine implications: United States, 2000-2004. *Clinical Infectious Diseases* **45,** 853-862.

Ofek, I., Beachey, E.H., Jefferson, W. and Campbell, G.L. (1975). Cell membrane-binding properties of group A streptococcal lipoteichoic acid. *J Exp Med* **141,** 990-1003.

Ofek, I., Hasty, D.L. and Sharon, N. (2003). Anti-adhesion therapy of bacterial diseases: prospects and problems. *FEMS Immunology and Medical Microbiology* **38,** 181-191.

Ogunniyi, A.D., Folland, R.L., Briles, D.E., Hollingshead, S.K. and Paton, J.C. (2000). Immunization of mice with combinations of pneumococcal virulence proteins elicits enhanced protection against challenge with *Streptococcus pneumoniae*. *Infect Immun* **68,** 3028-3033.

Okada, N., Liszewski, M.K., Atkinson, J.P. and Caparon, M. (1995). Membrane cofactor protein (CD46) is a keratinocyte receptor for the M protein of the group A streptococcus. *Proceedings of the National Academy of Sciences of the United States of America* **92,** 2489-2493.

Okada, N., Pentland, A.P., Falk, P. and Caparon, M.G. (1994). M protein and protein F act as important determinants of cell-specific tropism of *Streptococcus pyogenes* in skin tissue. *Journal of Clinical Investigation* **94,** 965-977.

Okamoto, S., Tamura, Y., Terao, Y., Hamada, S. and Kawabata, S. (2005). Systemic immunization with streptococcal immunoglobulin-binding protein Sib 35 induces protective immunity against group A *Streptococcus* challenge in mice. *Vaccine* **23,** 4852-4859.

Okamoto, S., Terao, Y., Tamura, Y., Hamada, S. and Kawabata, S. (2008). Streptococcal immunoglobulin-binding protein Sib35 exerts stimulatory and mitogenic effects toward mouse B lymphocytes. *FEMS Microbiology Letters* **281,** 73-80.

Olive, C., Batzloff, M., Horváth, A., Clair, T., Yarwood, P., Toth, I. and Good, M.F. (2003). Potential of lipid core peptide technology as a novel self-adjuvanting vaccine delivery system for multiple different synthetic peptide immunogens. *Infect Immun* **71,** 2373-2383.

Olive, C., Batzloff, M., Horváth, A., Clair, T., Yarwood, P., Toth, I. and Good, M.F. (2004). Group A streptococcal vaccine delivery by immunization with a self-adjuvanting M protein-based lipid core peptide construct. *Indian Journal of Medical Research* **119 Suppl,** 88-94.

Olive, C., Batzloff, M.R., Horváth, A., Wong, A., Clair, T., Yarwood, P*., et al.* (2002a). A lipid core peptide construct containing a conserved region determinant of the group A streptococcal M Protein elicits heterologous opsonic antibodies. *Infect Immun* **70,** 2734-2738.

Olive, C., Clair, T., Yarwood, P. and Good, M.F. (2002b). Protection of mice from group A streptococcal infection by intranasal immunisation with a peptide vaccine that contains a conserved M protein B cell epitope and lacks a T cell autoepitope. *Vaccine* **20,** 2816-2825.

Olive, C., Hsien, K., Horváth, A., Clair, T., Taywood, P., Toth, I. and Good, M.F. (2005). Protection against group A streptococcal infection by vaccination with self-adjuvanting lipid core M protein peptides. *Vaccine* **23,** 2298-2303.

Olive, C., Schulze, K., Sun, H.K., Ebensen, T., Horvath, A., Toth, I. and Guzman, C.A. (2007). Enhanced protection against *Streptococcus pyogenes* infection by intranasal vaccination with a dual antigen component M protein/SfbI lipid core peptide vaccine formulation. *Vaccine* **25,** 1789-1797.

Olive, C., Sun, H.K., Ho, M.F., Dyer, J., Horváth, A., Toth, I. and Good, M.F. (2006). Intranasal administration is an effective mucosal vaccine delivery route for self-adjuvanting lipid core peptides targeting the group A streptococcal M protein. *J Infect Dis* **194,** 316-324.

Olsen, R.J., Shelburne, S.A. and Musser, J.M. (2009). Molecular mechanisms underlying group A

streptococcal pathogenesis. *Cellular Microbiology* **11,** 1-12.

Osterlund, A. and Engstrand, L. (1997a). An intracellular sanctuary for *Streptococcus pyogenes* in human tonsillar epithelium--studies of asymptomatic carriers and in vitro cultured biopsies. *Acta Otolaryngologica* **117,** 883-888.

Osterlund, A., Popa, R., Nikkilä, T., Scheynius, A. and Engstrand, L. (1997b). Intracellular reservoir of *Streptococcus pyogenes* in vivo: a possible explanation for recurrent pharyngotonsillitis. *Laryngoscope* **107,** 640-647.

Oudjama, Y., Tricot, C., Stalon, V. and Wouters, J. (2002). Overexpression, purification, crystallization and preliminary X-ray crystallographic analysis of *Pseudomonas aeruginosa* L-arginine deiminase. *Acta Crystallographica Section D Biological Crystallography* **58,** 2150-2152.

Ozeri, V., Rosenshine, I., Mosher, D.F., Fässler, R. and Hanski, E. (1998). Roles of integrins and fibronectin in the entry of *Streptococcus pyogenes* into cells via protein F1. *Mol Microbiol* **30,** 625-637.

Påhlman, L.I., Marx, P.F., Mörgelin, M., Lukomski, S., Meijers, J.C. and Herwald, H. (2007). Thrombin-activatable fibrinolysis inhibitor binds to *Streptococcus pyogenes* by interacting with collagen-like proteins A and B. *J Biol Chem* **282,** 24873-24881.

Påhlman, L.I., Mörgelin, M., Eckert, J., Johansson, L., Russell, W., Riesbeck, K.*, et al.* (2006). Streptococcal M protein: a multipotent and powerful inducer of inflammation. *Journal of Immunology* **177,** 1221-1228.

Påhlman, L.I., Olin, A.I., Darenberg, J., Mörgelin, M., Kotb, M., Herwald, H. and Norrby-Teglund, A. (2008). Soluble M1 protein of *Streptococcus pyogenes* triggers potent T cell activation. *Cellular Microbiology* **10,** 404-414.

Pancholi, V. and Chhatwal, G.S. (2003). Housekeeping enzymes as virulence factors for pathogens. *International Journal of Medical Microbiology* **293,** 391-401.

Pancholi, V. and Fischetti, V.A. (1992). A major surface protein on group A streptococci is glyceraldehyde-3-phosphate dehydrogenase with multiple binding activity. *Journal of Experimental Medicine* **176,** 415-426.

Pancholi, V. and Fischetti, V.A. (1993). Glyceraldehyde-3-phosphate dehydrogenase on the surface of group A streptococci is also an ADP-ribosylating enzyme. *Proceedings of the National Academy of Sciences of the United States of America* **90,** 8154-8158.

Pancholi, V. and Fischetti, V.A. (1998). α-enolase, a novel strong plasmin(ogen) binding protein on the surface of pathogenic Streptococci. *The Journal of Biological Chemistry* **273,** 14503-14515.

Pandey, M., Batzloff, M. and Good, M.F. (2009). Mechanism of protection induced by group A Streptococcus vaccine candidate J8-DT: contribution of B and T-cells towards protection. *PLos One* **4,** e5147.

Pandiripally, V., Gregory, E. and Cue, D. (2002). Acquisition of regulators of complement activation by *Streptococcus pyogenes* serotype M1. *Infect Immun* **70,** 6206-6214.

Park, H.S. and Cleary, P.P. (2005). Active and passive intranasal immunizations with streptococcal surface protein C5a peptidase prevent infection of murine nasal mucosa-associated lymphoid tissue, a functional homologue of human tonsils. *Infect Immun* **73,** 7878-7886.

Pence, M.A., Rooijakkers, S.H., Cogen, A.L., Cole, J.N., Hollands, A., Gallo, R.L. and Nizet, V. (2010). Streptococcal inhibitor of complement (SIC) promotes innate immune resistance phenotypes of invasive M1T1 group A Streptococcus. *Journal of Innate Immunity* **2,** 587-595.

Perez-Casal, J., Okada, N., Caparon, M.G. and Scott, J.R. (1995). Role of the conserved C-repeat region of the M protein of *Streptococcus pyogenes*. *Mol Microbiol* **15,** 907-916.

Petrovsky, N. (2006). Novel human polysaccharide adjuvants with dual Th1 and Th2 potentiating activity. *Vaccine* **24 Suppl 2,** S2/26–S22/29.

Petrovsky, N. and Aguilar, J.C. (2004). Vaccine adjuvants: current state and future trends. *Immunology and Cell Biology* **82,** 488-496.

Phillips, G.N.J., Flicker, P.F., Cohen, C., Manjula, B.N. and Fischetti, V.A. (1981). Streptococcal M

protein: alpha-helical coiled-coil structure and arrangement on the cell surface. *Proceedings of the National Academy of Sciences of the United States of America* **78,** 4689-4693.

Pichichero, M.E. and Casey, J.R. (2007). Systematic review of factors contributing to penicillin treatment failure in *Streptococcus pyogenes* pharyngitis. *Otolaryngology–Head and Neck Surgery* **137,** 851-857.

Podbielski, A., Schnitzler, N., Beyhs, P. and Boyle, M.D. (1996). M-related protein (Mrp) contributes to group A streptococcal resistance to phagocytosis by human granulocytes. *Mol Microbiol* **19,** 429-441.

Poirier, T.P., Kehoe, M.A. and Beachey, E.H. (1988). Protective immunity evoked by oral administration of attenuated aroA *Salmonella typhimurium* expressing cloned streptococcal M protein. *J Exp Med* **168,** 25-32.

Polly, S.M., Waldman, R.H., High, P., Wittner, M.K. and Dorfman, A. (1975). Protective studies with a group A streptococcal M protein vaccine. II. Challenge of voulenteers after local immunization in the upper respiratory tract. *J Infect Dis* **131,** 217-224.

Pozzi, G., Contorni, M., Oggioni, M.R., Manganelli, R., Tommasino, M., Cavalieri, F. and Fischetti, V.A. (1992a). Delivery and expression of a heterologous antigen on the surface of streptococci. *Infect Immun* **60,** 1902-1907.

Pozzi, G.O., M R, Manganelli, R. and Fischetti, V.A. (1992b). Expression of M6 protein gene of *Streptococcus pyogenes* in *Streptococcus gordonii* after chromosomal integration and transcriptional fusion. *Research in Microbiology* **143,** 449-457.

Prakash, K. and Dutta, S. (1991). Application of serum opacity factor in subtyping of group A streptococci & identification of new M-types. *Indian Journal of Medical Research* **93,** 222-224.

Proft, T., Moffatt, S.L., Berkahn, C.J. and Fraser, J.D. (1999). Identification and characterisation of novel superantigens from *Streptococcus pyogenes*. *J Exp Med* **189,** 89-101.

Proft, T., Moffatt, S.L., Weller, K.D., Paterson, A., Martin, D. and Fraser, J.D. (2000). The streptococcal superantigen SMEZ exhibits wide allelic variation, mosaic structure, and significant antigenic variation. *J Exp Med* **191,** 1765-1776.

Proft, T., Webb, P.D., Handley, V. and Fraser, J.D. (2004). Identification & characterisation of the two novel streptococcal pyrogenic exotoxins SPE-L & SPE-M. *The Indian Journal of Medical Research* **119 Suppl,** 37-43.

Pruksakorn, S., Currie, B.J., Brandt, E.R., Martin, D., Galbraith, A., Phornphutkul, C.*, et al.* (1994). Towards a vaccine for rheumatic fever: identification of a conserved target epitope on M protein of group A streptococci. *The Lancet* **344,** 639-642.

Pruksakorn, S., Galbraith, A., Houghten, R.A. and Good, M.F. (1992). Conserved T and B cell epitopes on the M protein of group A streptococci. Induction of bactericidal antibodies. *Journal of Immunology* **149,** 2729-2735.

Purushothaman, S.S., Park, H. and Cleary, P.P. (2004). Promotion of fibronectin independent invasion by C5a peptidase into epithelial cells in group A Streptococcus. *The Indian Journal of Medical Research* **119 Suppl,** 44-47.

Quigley, B.R., Hatkoff, M., Thanassi, D.G., Ouattara, M., Eichenbaum, Z. and Scott, J.R. (2010). A foreign protein incorporated on the tip of T3 pili in *Lactococcus lactis* elicits systemic and mucosal immunity. *Infect Immun* **78,** 1294-1303.

Quigley, B.R., Zähner, D., Hatkoff, M., Thanassi, D.G. and Scott, J.R. (2009). Linkage of T3 and Cpa pilins in the *Streptococcus pyogenes* M3 pilus. *Mol Microbiol* **72,** 1379-1394.

Rakonjac, J.V., Robbins, J.C. and Fischetti, V.A. (1995). DNA sequence of the serum opacity factor of group A streptococci: identification of a fibronectin-binding repeat domain. *Infect Immun* **63,** 622-631.

Ramachandran, V., McArthur, J.D., Behm, C.E., Gutzeit, C., Dowton, M., Fagan, P.K.*, et al.* (2004). Two distinct genotypes of *prtF2*, encoding a fibronectin binding protein, and the evolution of the gene family in *Streptococcus pyogenes*. *J Bacteriol* **186,** 7601-7609.

Rasmussen, M. and Björck, L. (2001). Unique regulation of SclB - a novel collagen-like surface

protein of *Streptococcus pyogenes*. *Mol Microbiol* **40,** 1427-1438.

Rasmussen, M., Edén, A. and Björck, L. (2000). SclA, a novel collagen-like surface protein of Streptococcus pyogenes. *Infection and Immunity* **68,** 6370-6377.

Rasmussen, M., Muller, H.P. and Björck, L. (1999). Protein GRAB of *Streptococcus pyogenes* regulates proteolysis at the bacterial surface by binding a2-macroglobulin. *J Biol Chem* **274,** 15336–15344.

Richter, S.S., Heilmann, K.P., Beekmann, S.E., Miller, N.J., Miller, A.L., Rice, C.L*., et al.* (2005). Macrolide-resistant *Streptococcus pyogenes* in the United States, 2002-2003. *Clinical Infectious Diseases* **41,** 599-608.

Ringdahl, U., Svensson, M., Wisdedt, A.C., Renné, T., Kellner, R., Müller-Ester, W. and Sjöbring, U. (1998). Molecular co-operation between protein PAM and streptokinase for plasmin acquistion by *Streptococcus pyogenes*. *J Biol Chem* **173,** 6424-6430.

Rocha, C.L. and Fischetti, V.A. (1997). Identification and characterization of a new protein from *Streptococcus pyogenes* having homology with fibronectin and fibrinogen binding proteins. *Advances in Experimental Medicine and Biology* **418,** 737-739.

Rocha, C.L. and Fischetti, V.A. (1999). Identification and characterisation of a novel fibronectin-binding protein on the surface of group A streptococci. *Infection and Immunity* **67,** 2720-2728.

Rodreiguez-Ortega, M.J., Norais, N., Bensi, G., Liberatori, S., Capo, S., Mora, M*., et al.* (2006). Characterization and identification of vaccine candidate proteins through analysis of the group A Streptococcus surface proteome. *Nat Biotechnol* **24,** 191-197.

Rogers, S., Commons, R., Danchin, M.H., Selvaraj, G., Kelpie, L., Curtis, N*., et al.* (2007). Strain prevalence, rather than innate virulence potential, is the major factor responsible for an increase in serious group A streptococcus infections. *J Infect Dis* **195,** 1625-1633.

Rosch, J. and Caparon, M. (2004). A microdomain for protein secretion in Gram-positive bacteria. *Science* **304,** 1513-1515.

Rothbard, S. (1945). Bacteriostatic effect of human sera on group A streptococci: I. Type-specific antibodies in sera of patients convalesching from group A streptococcal pharyngitis. *J Exp Med* **82,** 93-106.

Ryan, S., Begley, M., Gahan, C.G. and Hill, C. (2009). Molecular characterization of the arginine deiminase system in *Listeria monocytogenes*: regulation and role in acid tolerance. *Environmental Microbiology* **11,** 432-445.

Sabharwal, H., Michon, F., Nelson, D., Wealing, D., Fuchs, K., Manjarrez, R.C*., et al.* (2006). Group A streptococcus (GAS) carbohydrate as an immunogen for protection against GAS infection. *J Infect Dis* **193,** 129-135.

Sagar, V., Bakshi, D.K., Nandi, S., Ganguly, N.K., Kumar, R. and Chakraborti, A. (2004). Molecular heterogeneity among north Indian isolates of Group A Streptococcus. *Letters in Applied Microbiology* **39,** 84-88.

Sagar, V., Kumar, R., Ganguly, N.K. and Chakraborti, A. (2008). Comparative analysis of *emm* type pattern of Group A Streptococcus throat and skin isolates from India and their association with closely related SIC, a streptococcal virulence factor. *BMC Microbiology* **8,** 150-158.

Salvadori, L.G., Blake, M.S., McCarty, M., Tai, J.Y. and Zabriskie, J.B. (1995). Group A streptococcus-liposome ELISA antibody titers to group A polysaccharide and opsonophagocytic capabilities of the antibodies. *J Infect Dis* **171,** 593-600.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecule Cloning - A Laboratory Manual New York, Cold Spring Harbor Laboratory Press.

Sanderson-Smith, M., Batzloff, M.R., Sriprakash, K.S., Dowton, M., Ranson, M. and Walker, M.J. (2006a). Divergence in the plasminogen-binding group a streptococcal M protein family: functional conservation of binding site and potential role for immune selection of variants. *J Biol Chem* **281,** 3217-3226.

Sanderson-Smith, M., Dinkla, K., Cole, J.N., Cork, A.J., Maamary, P.G., McArthur, J*., et al.* (2008). M protein-mediated plasminogen binding is essential for the virulence of an invasive

Streptococcus pyogenes isolate. *FASEB J* **22,** 2715-2722.

Sanderson-Smith, M., Dowton, M., Ranson, M. and Walker, M.J. (2007). The plasminogen-binding group A streptococcal M protein-related protein Prp binds plasminogen via arginine and histidine residues. *J Bacteriol* **189,** 1435-1440.

Sanderson-Smith, M.L., Walker, M.J. and Ranson, M. (2006b). The maintenance of high affinity plasminogen binding by group A streptococcal plasminogen-binding M-like protein is mediated by arginine and histidine residues within the a1 and a2 repeat domains. *J Biol Chem* **281,** 25965-25971.

Schmidtchen, A., Frick, I.M., Andersson, E., Tapper, H. and Björck, L. (2002). Proteinases of common pathogenic bacteria degrade and inactivate the antibacterial peptide LL-37. *Mol Microbiol* **46,** 157-168.

Schrager, H.M., Albertí, S., Cywes, C., Dougherty, G.J. and Wessels, M.R. (1998). Hyaluronic acid capsule modulates M protein-mediated adherence and acts as a ligand for attachment of group A *Streptococcus* to CD44 on human keratinocytes. *Journal of Clinical Investigation* **101,** 1708-1716.

Schrager, H.M., Rheinwald, J.G. and Wessels, M.R. (1996). Hyaluronic acid capsule and the role of streptococcal entry into keratinocytes in invasive skin infection. *Journal of Clinical Investigation* **98,** 1954-1958.

Schulze, K. and Guzman, C.A. (2003a). Identification of the domains of the fibronectin-binding protein I of *Streptococcus pyogenes* responsible for adjuvanticity. *FEMS Immunology and Medical Microbiology* **37,** 173-177.

Schulze, K., Medina, E., Chhatwal, G.S. and Guzman, C.A. (2003b). Identification of B- and T-cell epitopes within the fibronectin-binding domain of the SfbI protein of *Streptococcus pyogenes*. *Infect Immun* **71,** 7197-7201.

Schulze, K., Medina, E., Chhatwal, G.S. and Guzman, C.A. (2003c). Stimulation of long-lasting protection against *Streptococcus pyogenes* after intranasal vaccination with non adjuvanted fibronectin-binding domain of the SfbI protein. *Vaccine* **21,** 1958-1964.

Schulze, K., Medina, E. and Guzman, C.A. (2006a). Intranasal immunization with serum opacity factor (SOF) of *Streptococcus pyogenes* fails to protect mice against lethal mucosal challenge with a heterologous strain. *Vaccine* **24,** 1446-1450.

Schulze, K., Olive, C., Ebensen, T. and Guzman, C.A. (2006b). Intranasal vaccination with SfbI or M protein-derived peptides conjugated to diphtheria toxoid confers protective immunity against a lethal challenge with *Streptococcus pyogenes*. *Vaccine* **24,** 6088-6095.

Schwartz, B., Facklam, R.R. and Breiman, R.F. (1990). Changing epidemiology of group A streptococcal infection in the USA. *Lancet* **336,** 1167-1171.

Scott, J.R. and Zähner, D. (2006). Pili with strong attachments: Gram-positive bacteria do it differently. *Mol Microbiol* **62,** 320-330.

Sela, S. and Barzilai, A. (1999). Why do we fail with penicillin in the treatment of group A streptococcus infections? *Annals in Medicine* **31,** 303-307.

Severin, A., Nickbarg, E., Wooters, J., Quazi, S.A., Matsuka, Y.V., Murphy, E*., et al.* (2007). Proteomic analysis and identification of *Streptococcus pyogenes* surface-associated proteins. *J Bacteriol* **189,** 1514-1522.

Shannon, O., Hertzén, E., Norrby-Teglund, A., Mörgelin, M., Sjöbring, U. and Björck, L. (2007). Severe streptococcal infection is associated with M protein-induced platelet activation and thrombus formation. *Mol Microbiol* **65,** 1147-1157.

Shet, A., Kaplan, E., Johnson, D. and Cleary, P.P. (2004). Human immunogenicity studies on group A streptococcal C5a peptidase (SCPA) as a potential vaccine against group A streptococcal infections. *Indian Journal of Medical Research* **119 Suppl,** 95-98.

Shet, A., Kaplan, E.L., Johnson, D.R. and Cleary, P.P. (2003). Immune response to group A streptococcal C5a peptidase in children: implications for vaccine development. *J Infect Dis* **188,** 809-817.

Shikhman, A.R., Greenspan, N.S. and Cunningham, M.W. (1993). A subset of mouse monoclonal antibodies cross-reactive with cytoskeletal proteins and group A streptococcal M proteins recognizes N-acetyl-beta-D-glucosamine. *Journal of Immunology* **151,** 3902-3913.

Siegel, J.L., Hurst, S.F., Liberman, E.S., Coleman, S.E. and Bleiweis, A.S. (1981). Mutanolysin-induced spheroplasts of *Streptococcus mutants* are true protoplasts. *Infect Immun* **31,** 808-815.

Sierig, G., Cywes, C., Wessels, M.R. and Ashbaugh, C.D. (2003). Cytotoxic effects of Streptolysin O and Streptolysin S enhance the virulence of poorly encapsulated group A streptococci. *Infect Immun* **71,** 446-455.

Simerska, P., Abdel-Aal, A.B., Fujita, Y., Batzloff, M.R., Good, M.F. and Toth, I. (2008a). Synthesis and in vivo studies of carbohydrate-based vaccines against group A streptococcus. *Biopolymers* **90,** 911-616.

Simerska, P., Abdel-Aal, A.B., Fujita, Y., Moyle, P.M., McGeary, R.P., Batzloff, M.R.*, et al.* (2008b). Development of a liposaccharide-based delivery system and its application to the design of group A streptococcal vaccines. *Journal of Medicinal Chemistry* **51,** 1447-1452.

Smeesters, P.R., McMillan, D.J., Sriprakash, K.S. and Georgousakis, M.M. (2009). Differences among group A streptococcus epidemiological landscapes: consequences for M protein-based vaccines? *Expert Review of Vaccines* **8,** 1705-1720.

Smoot, J.C., Barbian, K.D., Van Gompel, J.J., Smoot, L.M., Chaussee, M.S., Sylva, G.L.*, et al.* (2002). Genome sequence and comparative microarray analysis of serotype M18 group A Streptococcus strains associated with acute rheumatic fever outbreaks. *Proceedings of the National Academy of Sciences of the United States of America* **99,** 4668-4673.

Söderberg, J.J., Engström, P. and von Pawel-Rammingen, U. (2008a). The intrinsic immunoglobulin g endopeptidase activity of streptococcal Mac-2 proteins implies a unique role for the enzymatically impaired Mac-2 protein of M28 serotype strains. *Infect Immun* **76,** 2183-2188.

Söderberg, J.J. and von Pawel-Rammingen, U. (2008b). The streptococcal protease IdeS modulates bacterial IgGFc binding and generates 1/2Fc fragments with the ability to prime polymorphonuclear leucocytes. *Molecular Immunology* **45,** 3347-3353.

Solovyova, A.S., Pointon, J.A., Race, P.R., Smith, W.D., Kehoe, M.A. and Banfield, M.J. (2009). Solution structure of the major (Spy0128) and minor (Spy0125 and Spy0130) pili subunits from *Streptococcus pyogenes. European Biophysics Journal* **39,** 469-480.

Staali, L., Mörgelin, M., Björck, L. and Tapper, H. (2003). *Streptococcus pyogenes* expressing M and M-like surface proteins are phagocytosed but survive inside human neutrophils. *Cellular Microbiology* **5,** 253-265.

Stafslien, D.K. and Cleary, P.P. (2000). Characterisation of the streptococcal C5a peptidase using a C5a-green fluorescent protein fusion protein substrate. *Journal of Bacteriology* **182,** 3254-3258.

Stalhammar-Carlemalm, M., Areschoug, T., Larsson, C. and Lindahl, G. (1999). The R28 protein of *Streptococcus pyogenes* is related to several group B streptococcal surface proteins, confers protective immunity and promotes binding to human epithelial cells. *Mol Microbiol* **33,** 208-219.

Stalhammar-Carlemalm, M., Areschoug, T., Larsson, C. and Lindahl, G. (2000). Cross-protection between group A and group B streptococci due to cross-reacting surface proteins. *J Infect Dis* **182,** 142-149.

Steer, A.C., Batzloff, M.R., Mulholland, K. and Carapetis, J.R. (2009a). Group A streptococcal vaccines: facts versus fantasy. *Current Opinion in Infectious Diseases* **22,** 544-552.

Steer, A.C., Law, I., Matatolu, L., Beall, B.W. and Carapetis, J.R. (2009b). Global *emm* type distribution of group A streptococci: systematic review and implications for vaccine development. *Lancet Infect Dis* **9,** 611-616.

Stefanescu, R., Iacob, R.E., Damoc, E.N., Marquardt, A., Amstalden, E., Manea, M.*, et al.* (2007). Mass spectrometric approaches for elucidation of antigen-antibody recognition structures in molecular immunology. *European Journal of Mass Spectrometry* **13,** 69-75.

Stenberg, L., O'Toole, P. and Lindahl, G. (1992). Many group A streptococcal strains express two

different immunoglobulin-binding proteins, encoded by closely linked genes: characterization of the proteins expressed by four strains of different M-type. *Mol Microbiol* **6,** 1185-1194.

Stevens, D.L. (1992). Invasive group A streptococcus infections. *Clinical Infectious Diseases* **14,** 2-11.

Stevens, D.L. (1995). Streptococcal toxic-shock syndrome: spectrum of disease, pathogenesis, and new concepts in treatment. *Emerging Infectious Diseases* **1,** 69-78.

Stevens, D.L. (2000). Streptococcal toxic shock syndrome associated with necrotising fasciitis. *Annual Review of Medicine* **51,** 271-288.

Stills, H.F. (2005). Adjuvants and antibody production: Dispelling the myths associated with Fruend's Complete and other adjuvants. *Institute of Laboratory Animal Resources Journal* **46,** 280-293.

Stjernquist-Desatnik, A., Kurl, D.N., Schalén, C. and Christensen, P. (1990). Protective effect of heterologous gram-positive vaccine against lethal upper respiratory tract infection with type M50 group A streptococci in mice. *Vaccine* **8,** 150-152.

Stockbauer, K.E., Magoun, L., Liu, M., Burns, E.H.J., Gubba, S., Renish, S.*, et al.* (1999). A natural variant of the cysteine protease virulence factor of group A Streptococcus with an arginine-glycine-aspartic acid (RGD) motif preferentially binds human integrins $\alpha_V\beta_3$ and $\alpha_{IIb}\beta_3$. *Proceedings of the National Academy of Sciences of the United States of America* **96,** 242-247.

Stollerman, G.H. (1997a). Changing streptococci and prospects for the global eradication of rheumatic fever. *Perspectives in Biology and Medicine* **40,** 165-189.

Stollerman, G.H. (1997b). Rheumatic fever. *The Lancet* **349,** 935-942.

Sumby, P., Whitney, A.R., Graviss, E.A., DeLeo, F.R. and Musser, J.M. (2006). Genome-wide analysis of group a streptococci reveals a mutation that modulates global phenotype and disease specificity. *PLoS Pathogens* **2,** 41-49.

Sun, H., Ringdahl, U., Homeister, J.W., Fay, W.P., Engelberg, N.C., Yang, A.Y.*, et al.* (2004). Plasminogen is a critical host pathogenicity factor for Group A Streptococcal infection. *Science* **305,** 1283-1286.

Suto, R. and Srivastava, P.K. (1995). A mechanism for the specific immunogenicity of heat shock protein-chaperoned peptides. *Science* **269,** 1585-1588.

Svensson, M.D., Sjöbring, U., Luo, F. and Bessen, D.E. (2002). Roles of the plasminogen activator streptokinase and the plasminogen-associated M protein in an experimental model for streptococcal impetigo. *Microbiology* **148,** 3933-3945.

Switalski, L.M., Speziale, P., Höök, M., Wadström, T. and Timpl, R. (1984). Binding of *Streptococcus pyogenes* to laminin. *The Journal of Biological Chemistry* **259,** 3734-3738.

Talay, S.R., Valentin-Weigand, P., Jerlstrom, P.G., Timmis, K.N. and Chhatwal, G.S. (1992). Fibronectin-binding protein of *Streptococcus pyogenes*: sequence of the binding domain involved in adherence of streptococci to epithelial cells. *Infect Immun* **60,** 3837-3844.

Talay, S.R., Zock, A., Rohde, M., Molinari, G., Oggioni, M., Pozzi, G.*, et al.* (2000). Co-operative binding of human fibronectin to SfbI protein triggers streptococcal invasion into respiratory epithelial cells. *Cellular Microbiology* **2,** 521-535.

Talkington, D.F., Schwartz, B., Black, C.M., Todd, J.K., Elliott, J., Breiman, R.F. and Facklam, R.R. (1993). Association of phenotypic and genotypic characteristics of invasive *Streptococcus pyogenes* isolates with clinical components of streptococcal toxic shock syndrome. *Infect Immun* **61,** 3369-3374.

Tart, A.H., Walker, M.J. and Musser, J.M. (2007). New understanding of the group A Streptococcus pathogenesis cycle. *Trends Microbiol* **15,** 318-325.

Terao, Y., Kawabata, S., Kunitomo, E., Murakami, J., Nakagawa, I. and Hamada, S. (2001). Fba, a novel fibronectin-binding protein from *Streptococcus pyogenes*, promotes bacterial entry into epithelial cells, and the fba gene is positively transcribed under the Mga regulator. *Mol Microbiol* **42,** 75-86.

Terao, Y., Kawabata, S., Kunitomo, E., Nakagawa, I. and Hamada, S. (2002a). Novel laminin-

binding protein of *Streptococcus pyogenes*, Lbp, is involved in adhesion to epithelial cells. *Infect Immun* **70,** 993-997.

Terao, Y., Kawabata, S., Nakata, M., Nakagawa, I. and Hamada, S. (2002b). Molecular characterisation of a novel fibronectin-binding protein of *Streptococcus pyogenes* strains isolated from toxic shock-like syndrome patients. *The Journal of Biological Chemistry* **277,** 47428-47435.

Terao, Y., Mori, Y., Yamaguchi, M., Shimizu, Y., Ooe, K., Hamada, S. and Kawabata, S. (2008). Group A streptococcal cysteine protease degrades C3 (C3b) and contributes to evasion of innate immunity. *J Biol Chem* **283,** 6253-6260.

Terao, Y., Okamoto, S., Kataoka, K., Hamada, S. and Kawabata, S. (2005). Protective immunity against *Streptococcus pyogenes* challenge in mice after immunization with fibronectin-binding protein. *J Infect Dis* **192,** 2081-2091.

Terao, Y., Yamaguchi, M., Hamada, S. and Kawabata, S. (2006). Multifunctional Glyceraldehyde-3-phosphate Dehydrogenase of *Streptococcus pyogenes* Is Essential for Evasion from Neutrophils. *J Biol Chem* **281,** 14215-14223.

Thongboonkerd, V., Leungpailin, J., Cao, J., Pierce, W.M., Cai, J., Klein, J.B. and Doyle, R.J. (2002). Fluoride exposure attenuates expression of *Streptococcus pyogenes* virulence factors. *J Biol Chem* **277,** 16599-16605.

Thulin, P., Johansson, L., Low, D.E., Gan, B.S., Kotb, M., McGeer, A. and Norrby-Teglund, A. (2006). Viable group A streptococci in macrophages during acute soft tissue infection. *PLoS Medicine* **3,** e53.

Timmer, A.M., Kristian, S.A., Datta, V., Jeng, A., Gillen, C.M., Walker, M.J*., et al.* (2006). Serum opacity factor promotes group A streptococcal epithelial cell invasion and virulence. *Mol Microbiol* **62,** 15-25.

Timmer, A.M., Timmer, J.C., Pence, M.A., Hsu, L.C., Ghochani, M., Frey, T.G*., et al.* (2009). Streptolysin O promotes group A Streptococcus immune evasion by accelerated macrophage apoptosis. *J Biol Chem* **284,** 862-871.

Toppel, A.W., Rasmussen, M., Rohde, M., Medina, E. and Chhatwal, G.S. (2003). Contribution of Protein G-related α_2-macroglobulin-binding protein to bacterial virulence in a mouse skin model of group A streptococcal infection. *J Infect Dis* **187,** 1694-1703.

Tripp, T.J., McCormick, J.K., Webb, J.M. and Schlievert, P.M. (2003). The zinc-dependent major histocompatibility complex class II binding site of streptococcal pyrogenic exotoxin C is critical for maximal superantigen function and toxic activity. *Infect Immun* **71,** 1548-1550.

Tsai, P.J., Kuo, C.F., Lin, K.Y., Lin, Y.S., Lei, H.Y., Chen, F.F*., et al.* (1998). Effect of group A streptococcal cysteine protease on invasion of epithelial cells. *Infect Immun* **66,** 1460–1466.

Tsao, N., Tsai, W.H., Lin, Y.S., Chuang, W.J., Wang, C.H. and Kuo, C.F. (2006). Streptococcal pyrogenic exotoxin B cleaves properdin and inhibits complement-mediated opsonophagocytosis. *Biochemical and Biophysical Research Communications* **339,** 779-784.

Tung, J.S. and Knight, C.A. (1972). Relative importance of some factors affecting the electrophoretic migration of proteins in sodium dodecyl sulfate-polyacrylamide gels. *Analytical Biochemistry* **48,** 153-163.

Turner, C.E., Kurupatia, P., Wilesa, S., Edwards, R.J. and Sriskandan, S. (2009). Impact of immunization against SpyCEP during invasive disease with two streptococcal species: *Streptococcus pyogenes* and *Streptococcus equi*. *Vaccine* **27,** 4923–4929.

Ulrich, R.G. (2008). Vaccine based on a ubiquitous cysteinyl protease and streptococcal pyrogenic exotoxin A protects against *Streptococcus pyogenes* sepsis and toxic shock. *Journal of Immune Based Therapies and Vaccines* **6,** 8.

Upton, M., Carter, P.E., Morgan, M., Edwards, G.F. and Pennington, T.H. (1995). Clonal structure of invasive *Streptococcus pyogenes* in Northern Scotland. *Epidemiology and Infection* **115,** 231-141.

Valentin-Weigand, P., Grulich-Henn, J., Chhatwal, G.S., Muller-Berghaus, G., Blobel, H. and

Preissner, K.T. (1988). Mediation of adherence of streptococci to human endothelial cells by complement S protein (vitronectin). *Infection and Immunity* **56,** 2851-2855.

Valentin-Weigand, P., Talay, S.R., Kaufhold, A., Timmis, K.N. and Chhatwal, G.S. (1994). The fibronectin binding domain of the Sfb protein adhesin of *Streptococcus pyogenes* occurs in many group A streptococci and does not cross-react with heart myosin. *Microbial Pathogenesis* **17,** 111-120.

Veasy, L.G., Tani, L.Y. and Hill, H.R. (1994). Persistence of acute rheumatic fever in the intermountain area of the United States. *The Journal of Pediatrics* **124,** 9-16.

Vercolloti, G.M., McCarthy, J.B., Lindholm, P. and Peterson, P.R. (1985). Extracellular matrix proteins (fibronectin, laminin and type IV collagen) bind and aggregate bacteria. *American Journal of Pathology* **120,** 13-21.

Vincent, M.T., Celestin, N. and Hussain, A.N. (2004). Pharyngitis. *American Family Physician* **69,** 1465-1471.

Vincents, B., Vindebro, R., Abrahamson, M. and von Pawel-Rammingen, U. (2008). The human protease inhibitor cystatin C is an activating cofactor for the streptococcal cysteine protease IdeS. *Chemistry & Biology* **15,** 960-968.

Visai, L., Bozzini, S., Raucci, G., Toniolo, A. and Speziale, P. (1995). Isolation and characterisation of a novel collagen-binding protein from *Streptococcus pyogenes* strain 6414. *The Journal of Biological Chemistry* **270,** 347-353.

Vitali, L.A., Zampaloni, C., Prenna, M. and Ripa, S. (2002). PCR M Typing: a new method for rapid typing of group A streptococci. *Journal of Clinical Microbiology* **40,** 679-681.

Vlaminckx, B.J., Mascini, E.M., Schellekens, J., Schouls, L.M., Paauw, A., Fluit, A.C.*, et al.* (2003). Site-specific manifestations of invasive group a streptococcal disease: type distribution and corresponding patterns of virulence determinants. *Journal of Clinical Microbiology* **41,** 4941-4999.

Von Pawel-Rammingen, U. and Björck, L. (2003). IdeS and SpeB: immunoglobulin-degrading cysteine proteinases of *Streptococcus pyogenes*. *Current Opinion in Microbiology* **6,** 50-55.

Walker, M.J., Hollands, A., Sanderson-Smith, M.L., Cole, J.N., Kirk, J.K., Henningham, A.*, et al.* (2007). DNase Sda1 provides selection pressure for a switch to invasive group A streptococcal infection. *Nature Medicine* **13,** 981-985.

Wang, H., Lottenberg, R. and Boyle, M.D.P. (1995). A role for fibrinogen in the streptokinase-dependent acquisition of plasmin(ogen) by group A streptococci. *The Journal of Infectious Diseases* **171,** 85-92.

Wang, J.R. and Stinson, M.W. (1994). M protein mediates streptococcal adhesion to HEp-2 cells. *Infect Immun* **62,** 442-448.

Wei, Y., Zhou, H., Sun, Y., He, Y. and Luo, Y. (2007). Insight into the catalytic mechanism of arginine deiminase: functional studies on the crucial sites. *Proteins* **66,** 740-750.

Weiss, K.A. and Laverdiére, M. (1997). Group A *Streptococcus* invasive infections: a review. *Canadian Journal of Surgery* **40,** 18-25.

Wessels, M.R., Moses, A.E., Goldberg, J.B. and DiCesare, T.J. (1991). Hyaluronic acid capsule is a virulence factor for mucoid group A streptococci. *Proceedings of the National Academy of Sciences of the United States of America* **88,** 8317-8321.

Westerfeld, N. and Zurbriggen, R. (2005). Peptides delivered by immunostimulating reconstituted influenza virosomes. *Journal of Peptide Science* **11,** 707-712.

Wilkins, J.C., Beighton, D. and Homer, K.A. (2003). Effect of acidic pH on expression of surface-associated proteins of *Streptococcus oralis*. *Applied and Environmental Microbiology* **69,** 5290-5296.

Wilks, K.E., Dunn, K.L., Farrant, J.L., Reddin, K.M., Gorringe, A.R., Langford, P.R. and Kroll, J.S. (1998). Periplasmic superoxide dismutase in meningococcal pathogenicity. *Infect Immun* **66,** 213-217.

Winram, S.B. and Lottenberg, R. (1996). The plasmin-binding protein Plr of group A streptococci is

identified as glyceraldehyde-3-phosphate dehydrogenase. *Microbiology* **142,** 2311-2320.

Winram, S.B. and Lottenberg, R. (1998). Site-directed mutagenesis of streptococcal plasmin receptor protein (Plr) identifies the C-terminal Lys334 as essential for plasmin binding, but mutation of the plr gene does not reduce plasmin binding to group A streptococci. *Microbiology* **144,** 2025-2035.

Winterhoff, N., Goethe, R., Gruening, P., Rohde, M., Kalisz, H., Smith, H.E. and Valentin-Weigand, P. (2002a). Identification and characterisation of two temperature-induced surface-associated proteins of *Streptococcus suis* with high homologies to members of the arginine deiminase system of *Streptococcus pyogenes*. *J Bacteriol* **184,** 6768-6776.

Winterhoff, N., Goethe, R., Gruening, P., Rohde, M., Kalisz, H., Smith, H.E. and Valentin-Weigand, P. (2002b). Identification and characterisation of two temperature-induced surface-associated proteins of Streptococcus suis with high homologies to members of the arginine deiminase system of Streptococcus pyogenes. *Journal of Bacteriology* **184,** 6768-6776.

Wistedt, A.C., Kotarsky, H., Marti, D., Ringdahl, U., Castellino, F.J., Schaller, J. and Sjöbring, U. (1998). Kringle 2 mediates high affinity binding of plasminogen to an internal sequence in streptococcal surface protein PAM. *J Biol Chem* **273,** 24420-24424.

Xie, H., Lin, X., Wang, B., Wu, J. and Lamont, R.J. (2007). Identification of a signalling molecule involved in bacterial intergeneric communication. *Microbiology* **153,** 3228-3234.

Yamakami, K., Yoshizawa, N., Wakabayashi, K., Takeuchi, A., Tadakuma, T. and Boyle, M.D. (2000). The potential role for nephritis-associated plasmin receptor in acute poststreptococcal glomerulonephritis. *Methods* **21,** 185-197.

Yang, L., Thomas, M., Woodhouse, A., Martin, D., Fraser, J.D. and Proft, T. (2005). Involvement of streptococcal mitogenic exotoxin Z in streptococcal toxic shock syndrome. *Journal of Clinical Microbiology* **43,** 3570-3573.

Yang, W.S., Park, S.O., Yoon, A.R., Yoo, J.Y., Kim, M.K., Yun, C.O. and Kim, C.W. (2006). Suicide cancer gene therapy using pore-forming toxin, streptolysin O. *Molecular Cancer Therapeutics* **5,** 1610-1619.

Yesilkaya, H., Kadioglu, A., Gingles, N., Alexander, J.E., Mitchell, T.J. and Andrew, P.W. (2000). Role of manganese-containing superoxide dismutase in oxidative stress and virulence of *Streptococcus pneumoniae*. *Infect Immun* **68,** 2819-2826.

Yokoyama, Y. and Harabuchi, T. (2002). Intranasal immunization with lipoteichoic acid and cholera toxin evokes specific pharyngeal IgA and systemic IgG responses and inhibits streptococcal adherence to pharyngeal epithelial cells in mice. *International Journal of Pediatric Otorhinolaryngology* **63,** 235-241.

Yoshida, J., Takamura, S. and Nishio, M. (1998). Characterisastion of a streptococcal antitumour glycoprotein (SagP). *Life Sciences* **62,** 1043-1053.

Yoshizawa, N., Yamakami, K., Fujino, M., Oda, T., Tamura, K., Matsumoto, K., *et al.* (2004). Nephritis-associated plasmin receptor and acute poststreptococcal glomerulonephritis: characterization of the antigen and associated immune response. *Journal of the American Society of Nephrology* **15,** 1785-1793.

Zaman, M., Abdel-Aal, A.B., Phillipps, K.S., Fujita, Y., Good, M.F. and Toth, I. (2010). Structure-activity relationship of lipopeptide Group A streptococcus (GAS) vaccine candidates on toll-like receptor 2. *Vaccine* **28,** 2243-2248.

Zúñiga, M., Pérez, G. and González-Candelas, F. (2002). Evolution of arginine deiminase (ADI) pathway genes. *Molecular Phylogenetics and Evolution* **25,** 429-444.

APPENDIX I

Growth and Nutrient Media

<u>Luria Bertani (LB) Broth</u>

Bactotryptone	10 g/L
Yeast extract	5 g/L
NaCl	10 g/L

<u>LB Agar Medium</u>

LB Broth	1 L
Agar	15 g/L

Following autoclaving, agar medium cooled prior to the addition of antibiotics.

<u>SOC Medium</u>

Bactotryptone	20 g/L
Yeast extract	5 g/L
1 M NaCl	1 % (w/v)
1 M KCl	0.25 % (w/v)
2 M MgSO4 (filter sterilised, added after autoclaving)	1 % (w/v)
1 M Glucose (filter sterilised, added after autoclaving)	2 % (w/v)

<u>Todd-Hewitt Broth (THBY)</u>

Todd-Hewitt Broth powder (Difco)	30 g/L
Yeast extract	10 g/L

<u>THBY Agar Medium</u>

THBY	1 L
Agar	15 g/L

QIAGEN DNeasy® Blood & Tissue Kit buffers

<u>Enzymatic Lysis Buffer</u>

Tris-HCl (pH 8)	20 mM
EDTA	2 mM
Triton X-100	1.2 % (v/v)
Lysozyme (added just before use)	20 mg/mL

Agarose Gel Electrophoresis Reagents

<u>Agarose gels</u>

Electrophoresis grade agarose	1 % (w/v)
1 X TAE	100 mL

<u>10X TAE (Running Buffer)</u>

Tris base	48.4 g/L
Glacial Acetic acid	11.43 mL
EDTA	3.72 g/L

<u>Bromophenol Blue DNA Loading Dye</u>

Bromophenol blue	5 mg/10 mL
Glycerol	7.5 mL
TE buffer	2.5 mL

Ethidium Bromide Staining Solution
Ethidium bromide   1 µg/mL
Distilled water

Qiagen® Expressionist Protein Purification Buffers
Buffer A
NaH2PO4   100 mM
Tris-HCl   10 mM
Guanidine HCl   6 M
Imidazole   20 mM
pH 8.0

Buffer C, D and E
NaH2PO4   100 mM
Tris-HCl   10 mM
Urea   8 M
Imidazole   20 mM
Buffer C = pH 6.3, Buffer D = pH 5.9, Buffer E = pH 4.5

SDS-PAGE Solutions
2X Cracking Buffer (Store at room temperature. Add DTT immediately prior to use.)
Tris-HCl (pH 6.8)   90 mM
Bromophenol blue   0.02 % (w/v)
Glycerol   20 % (v/v)
SDS   2 % (w/v)
DTT   100 mM

5X Cracking Buffer (Store at room temperature. Add DTT immediately prior to use.)
Tris-HCl (pH 6.8)   225 mM
Bromophenol blue   0.05 % (w/v)
Glycerol   50 % (v/v)
SDS   5 % (w/v)
DTT   250 mM

10X Running Buffer
Tris-Base   30.3 g/L
Glycine   144 g/L
SDS   10 g/L
pH 8.3, do not adjust. Store at 4 °C

Resolving Gel (12%)
40% (w/v) Bis Acrylamide   1.55 mL
1.5 M Tris-HCl (pH 8.8)   1.25 mL
10% (w/v) SDS   50 µL
dH20   2.25 mL
TEMED   25 µL
10% (w/v) APS   25 µL

Stacking Gel (4%)
40% (w/v) Bis Acrylamide   225 µL
0.5 M Tris-HCl (pH 6.8)   600 µL

10% (w/v) SDS	25 μL
dH20	1.6 mL
TEMED	12.5 μL
10% (w/v) APS	12.5 μL

Coomassie Blue Stain

Coomassie Brilliant Blue R-250	0.25 % (w/v)
Methanol	40 % (v/v)
Acetic acid	10 % (v/v)

Rapid Destain

Methanol	40 % (v/v)
Acetic acid	10 % (v/v)

Western Transfer Solutions

Western Transfer Buffer

Tris-HCl	3.03 g/L
Glycine	14.4 g/L
Methanol	20 % (v/v)

Chill to 4 ºC before use.

10X Phosphate-buffered Saline (PBS)

NaCl	80 g/L
KCl	2 g/L
NaH_2PO_4	14.4 g/L
KH_2PO_4	2.4 g/L

pH 7.4 ± 0.2. Do not adjust. Dilute to 1X for use.

PBST

1X PBS	1 L
Tween-20	0.05 % (v/v)

DAB Colour Development Solution

DAB (3,3′-Diaminobenzidine)	0.5g/L
Hydrogen Peroxide	0.06 % (v/v)

Mutanolysin Extraction Buffers

TE Buffer

Tris-HCl	100 mM
EDTA	25 mM

pH 8.0

Wash Buffer

PMSF (phenylmethylsulphonyl fluoride)	1 mM
Isopropanol	10 % (v/v)
TE buffer	90 % (v/v)

Prepare immediately before use by firstly dissolving PMSF in a small volume of ice cold isopropanol.

TE-Sucrose (TES) Buffer

Tris-HCl	50 mM

EDTA	1 mM
Sucrose	20 % (w/v)

pH 8.0. Autoclave. Chill on ice before use.

Lysozyme Solution

Lysozyme from egg white	100 mg/mL

Made fresh in chilled TES buffer and stored on ice before use.

Mutanolysin Solution

Mutanolysin (Sigma, USA)	5,000 U/mL

Made up in chilled, filter sterilised K2HPO4 and stored at -20 ℃.

Mutanolysin Mix (Prepare immediately before use and store on ice.)

TES Buffer	1 mL
Lysozyme Solution	100 μL
Mutanolysin Solution	50 μL

ELISA Solutions

Carbonate Coating Buffer (pH 9.6)

Sodium carbonate	1.93 g/L
Sodium hydrogen carbonate	3.81 g/L

Peptide Membrane Array Solutions

Tris-buffered Saline (TBS)

NaCl	8 g/L
KCl	0.2 g/L
Tris base	6.1 g/L

pH 7.0

TBST

TBS with 0.05% (v/v) Tween-20

Membrane Blocking Solution (MBS)

Skim milk	2 % (w/v)
Tween 20	0.2 % (v/v)

Made up in TBS.

Citrate-buffered Saline (CBS)

NaCl	8 g/L
KCl	0.2 g/L
Citric acid	10.51 g/L

pH 7.0

Colour Developing Solution (CDS)

Dissolve 50 mg 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) in 1 mL of 70% (v/v) DMF in water; store at -20 ℃. Dissolve 60 mg 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt (BCIP) in 1 mL DMF; store at -20 ℃.

Always prepare CDS fresh: To 10 mL CBS add 50 μL 1 M magnesium chloride, 40 μL BCIP and 60 μL MTT.

Stripping Mixture A

Urea	8 M
SDS	1 % (w/v)
2-mercaptoethanol (added prior to use)	0.5 % (v/v)

Made up in PBS. Store at room temperature
pH adjusted to 7.0 with acetic acid.

Stripping Mixture B

Acetic acid	10 % (v/v)
Ethanol	50 % (v/v)
Water	40 % (v/v)

Store at room temperature.

Hyaluronic Acid Capsule Assays

Stains-All Solution (Store protected from light at 4 ℃.)

Stains-All (Sigma, USA)	20 mg
Acetic acid glacial	60 μL
50% (v/v) formamide	100 mL

SpeB Plate Activity Assays

Columbia Skim Milk Agar

Solution 1

Columbia broth	8.75 g
Agar technical (Agar No. 3)	5 g
dH2O	250 mL

Solution 2

Skim milk powder	7.5 g
dH2O	250 mL

Autoclave Solution 1 and Solution 2. Allow to cool to 50 ℃ and combine. Store at 4 ℃.

For plates containing E64 (N-[N-(L-3-transcarboxyirane-2-carbonyl)-L-Leucyl]-agmatine), E64 was added evenly to the surface of the plate with a final concentration of 28 μM.

APPENDIX II

Table A. 1 Individual 15-mer peptide spot sequences for ADI, KPR and TF peptide array membranes.

Peptide Spot	ADI	KPR	TF
1	TAQTPIHVYSEIGKL	MLVYIAGSGAMGCRF	MSTSFENKATNRGVI
2	TPIHVYSEIGKLKKV	YIAGSGAMGCRFGYQ	SFENKATNRGVITFT
3	HVYSEIGKLKKVLLH	GSGAMGCRFGYQISK	NKATNRGVITFTISQ
4	SEIGKLKKVLLHRPG	AMGCRFGYQISKTNN	TNRGVITFTISQDKI
5	GKLKKVLLHRPGKEI	CRFGYQISKTNNDVI	GVITFTISQDKIKPA
6	KKVLLHRPGKEIENL	GYQISKTNNDVILLD	TFTISQDKIKPALDK
7	LLHRPGKEIENLMPD	ISKTNNDVILLDNWE	ISQDKIKPALDKAFN
8	RPGKEIENLMPDYLE	TNNDVILLDNWEDHI	DKIKPALDKAFNKIK
9	KEIENLMPDYLERLL	DVILLDNWEDHINAI	KPALDKAFNKIKKDL
10	ENLMPDYLERLLFDD	LLDNWEDHINAIKEN	LDKAFNKIKKDLNAP
11	MPDYLERLLFDDIPF	NWEDHINAIKENGLV	AFNKIKKDLNAPGFR
12	YLERLLFDDIPFLED	DHINAIKENGLWTG	KIKKDLNAPGFRKGH
13	RLLFDDIPFLEDAQK	NAIKENGLWTGDVE	KDLNAPGFRKGHMPR
14	FDDIPFLEDAQKEHD	KENGLWTGDVEETV	NAPGFRKGHMPRPVF
15	IPFLEDAQKEHDAFA	GLWTGDVEETVKLP	GFRKGHMPRPVFNQK
16	LEDAQKEHDAFAQAL	VTGDVEETVKLPIMK	KGHMPRPVFNQKFGE
17	AQKEHDAFAQALRDE	DVEETVKLPIMKPTE	MPRPVFNQKFGEEVL
18	EHDAFAQALRDEGIE	ETVKLPIMKPTEATQ	PVFNQKFGEEVLYED
19	AFAQALRDEGIEVLY	KLPIMKPTEATQEAD	NQKFGEEVLYEDALN
20	QALRDEGIEVLYLET	IMKPTEATQEADLII	FGEEVLYEDALNIVL
21	RDEGIEVLYLETLAA	PTEATQEADLIILFT	EVLYEDALNIVLPEA
22	GIEVLYLETLAAESL	ATQEADLIILFTKAM	YEDALNIVLPEAYEA
23	VLYLETLAAESLVTP	EADLIILFTKAMQLP	ALNIVLPEAYEAAVT
24	LETLAAESLVTPEIR	LIILFTKAMQLPQML	IVLPEAYEAAVTELG
25	LAAESLVTPEIREAF	LFTKAMQLPQMLQDI	PEAYEAAVTELGLDV
26	ESLVTPEIREAFIDE	KAMQLPQMLQDIKGI	YEAAVTELGLDWAQ
27	VTPEIREAFIDEYLS	QLPQMLQDIKGIIGK	AVTELGLDWAQPKI
28	EIREAFIDEYLSEAN	QMLQDIKGIIGKETK	ELGLDWAQPKIDW
29	EAFIDEYLSEANIRG	QDIKGIIGKETKVLC	LDWAQPKIDWSME
30	IDEYLSEANIRGRAT	KGIIGKETKVLCLLN	VAQPKIDWSMEKGK
31	YLSEANIRGRATKKA	IGKETKVLCLLNGLG	PKIDWSMEKGKEWT
32	EANIRGRATKKAIRE	ETKVLCLLNGLGHED	DWSMEKGKEWTLSA
33	IRGRATKKAIRELLM	VLCLLNGLGHEDVIR	SMEKGKEWTLSAEW
34	RATKKAIRELLMAIE	LLNGLGHEDVIRQYI	KGKEWTLSAEWTKP
35	KKAIRELLMAIEDNQ	GLGHEDVIRQYIPEH	EWTLSAEWTKPEVK
36	IRELLMAIEDNQELI	HEDVIRQYIPEHNIL	LSAEWTKPEVKLGD
37	LLMAIEDNQELIEKT	VIRQYIPEHNILMGV	EWTKPEVKLGDYKN
38	AIEDNQELIEKTMAG	QYIPEHNILMGVTVW	TKPEVKLGDYKNLW
39	DNQELIEKTMAGVQK	PEHNILMGVTVWTAG	EVKLGDYKNLWEVD
40	ELIEKTMAGVQKSEL	NILMGVTVWTAGLEG	LGDYKNLWEVDASK
41	EKTMAGVQKSELPEI	MGVTVWTAGLEGPGR	YKNLWEVDASKEVS
42	MAGVQKSELPEIPAS	TVWTAGLEGPGRAHL	LWEVDASKEVSDED
43	VQKSELPEIPASEKG	TAGLEGPGRAHLQGV	EVDASKEVSDEDVDA
44	SELPEIPASEKGLTD	LEGPGRAHLQGVGAL	ASKEVSDEDVDAKIE
45	PEIPASEKGLTDLVE	PGRAHLQGVGALNLQ	EVSDEDVDAKIERER

46	PASEKGLTDLVESSY	AHLQGVGALNLQSMD	DEDVDAKIERERQNL
47	EKGLTDLVESSYPFA	QGVGALNLQSMDPNN	VDAKIERERQNLAEL
48	LTDLVESSYPFAIDP	GALNLQSMDPNNQDA	KIERERQNLAELIIK
49	LVESSYPFAIDPMPN	NLQSMDPNNQDAGHQ	RERQNLAELIIKDGE
50	SSYPFAIDPMPNLYF	SMDPNNQDAGHQVAD	QNLAELIIKDGEAAQ
51	PFAIDPMPNLYFTRD	PNNQDAGHQVADLLN	AELIIKDGEAAQGDT
52	IDPMPNLYFTRDPFA	QDAGHQVADLLNKAN	IIKDGEAAQGDTWI
53	MPNLYFTRDPFATIG	GHQVADLLNKANLNA	DGEAAQGDTWIDFV
54	LYFTRDPFATIGTGV	VADLLNKANLNATYD	AAQGDTWIDFVGSV
55	TRDPFATIGTGVSLN	LLNKANLNATYDENV	GDTWIDFVGSVDGV
56	PFATIGTGVSLNHMF	KANLNATYDENWPN	WIDFVGSVDGVEFD
57	TIGTGVSLNHMFSET	LNATYDENWPNIWR	DFVGSVDGVEFDGGK
58	TGVSLNHMFSETRNR	TYDENWPNIWRKAC	GSVDGVEFDGGKGDN
59	SLNHMFSETRNRETL	ENWPNIWRKACVNG	DGVEFDGGKGDNFSL
60	HMFSETRNRETLYGK	VPNIWRKACVNGTMN	EFDGGKGDNFSLELG
61	SETRNRETLYGKYIF	IWRKACVNGTMNSTC	GGKGDNFSLELGSGQ
62	RNRETLYGKYIFTHH	KACVNGTMNSTCALL	GDNFSLELGSGQFIP
63	ETLYGKYIFTHHPIY	VNGTMNSTCALLDCT	FSLELGSGQFIPGFE
64	YGKYIFTHHPIYGGG	TMNSTCALLDCTIGE	ELGSGQFIPGFEDQL
65	YIFTHHPIYGGGKVP	STCALLDCTIGELFA	SGQFIPGFEDQLVGA
66	THHPIYGGGKVPMVY	ALLDCTIGELFASED	FIPGFEDQLVGAKAG
67	PIYGGGKVPMVYDRN	DCTIGELFASEDGLK	GFEDQLVGAKAGDEV
68	GGGKVPMVYDRNETT	IGELFASEDGLKMVK	DQLVGAKAGDEVEVN
69	KVPMVYDRNETTRIE	LFASEDGLKMVKEII	VGAKAGDEVEVNVTF
70	MVYDRNETTRIEGGD	SEDGLKMVKEIIHEF	KAGDEVEVNVTFPES
71	DRNETTRIEGGDELV	GLKMVKEIIHEFVIV	DEVEVNVTFPESYQA
72	ETTRIEGGDELVLSK	MVKEIIHEFVIVGQA	EVNVTFPESYQAEDL
73	RIEGGDELVLSKDVL	EIIHEFVIVGQAEGV	VTFPESYQAEDLAGK
74	GGDELVLSKDVLAVG	HEFVIVGQAEGVELN	PESYQAEDLAGKAAK
75	ELVLSKDVLAVGISQ	VIVGQAEGVELNEEE	YQAEDLAGKAAKFMT
76	LSKDVLAVGISQRTD	GQAEGVELNEEEITQ	EDLAGKAAKFMTTIH
77	DVLAVGISQRTDAAS	EGVELNEEEITQYVM	AGKAAKFMTTIHEVK
78	AVGISQRTDAASIEK	ELNEEEITQYVMDTS	AAKFMTTIHEVKTKE
79	ISQRTDAASIEKLLV	EEEITQYVMDTSVKA	FMTTIHEVKTKEVPE
80	RTDAASIEKLLVNIF	ITQYVMDTSVKAAHH	TIHEVKTKEVPELDD
81	AASIEKLLVNIFKQN	YVMDTSVKAAHHYPS	EVKTKEVPELDDELA
82	IEKLLVNIFKQNLGF	DTSVKAAHHYPSMHQ	TKEVPELDDELAKDI
83	LLVNIFKQNLGFKKV	VKAAHHYPSMHQDLV	VPELDDELAKDIDED
84	NIFKQNLGFKKVLAF	AHHYPSMHQDLVQNH	LDDELAKDIDEDVDT
85	KQNLGFKKVLAFEFA	YPSMHQDLVQNHRLT	ELAKDIDEDVDTLED
86	LGFKKVLAFEFANNR	MHQDLVQNHRLTEID	KDIDEDVDTLEDLKV
87	KKVLAFEFANNRKFM	DLVQNHRLTEIDFIN	DEDVDTLEDLKVYR
88	LAFEFANNRKFMHLD	QNHRLTEIDFINGAV	VDTLEDLKVKYRKEL
89	EFANNRKFMHLDTVF	RLTEIDFINGAVNTK	LEDLKVKYRKELEAA
90	NNRKFMHLDTVFTMV	EIDFINGAVNTKGEK	LKVKYRKELEAAQET
91	KFMHLDTVFTMVDYD	FINGAVNTKGEKLGI	KYRKELEAAQETAYD
92	HLDTVFTMVDYDKFT	GAVNTKGEKLGINTP	KELEAAQETAYDDAV
93	TVFTMVDYDKFTIHP	NTKGEKLGINTPYCR	EAAQETAYDDAVEGA
94	TMVDYDKFTIHPEIE	GEKLGINTPYCRMIT	QETAYDDAVEGAAIE
95	DYDKFTIHPEIEGDL	LGINTPYCRMITELV	AYDDAVEGAAIELAV
96	KFTIHPEIEGDLRVY	NTPYCRMITELVHAK	DAVEGAAIELAVANA

97	IHPEIEGDLRVYSVT	YCRMITELVHAKEAV	EGAAIELAVANAEIV
98	EIEGDLRVYSVTYDN	MITELVHAKEAVLNI	AIELAVANAEIVDLP
99	GDLRVYSVTYDNEEL	ITELVHAKEAVLNIQ	LAVANAEIVDLPEEM
100	RVYSVTYDNEELHIV	-	ANAEIVDLPEEMIHE
101	SVTYDNEELHIVEEK	-	EIVDLPEEMIHEEVN
102	YDNEELHIVEEKGDL	-	DLPEEMIHEEVNRSV
103	EELHIVEEKGDLADL	-	EEMIHEEVNRSVNEF
104	HIVEEKGDLADLLAA	-	IHEEVNRSVNEFMGN
105	EEKGDLADLLAANLG	-	EVNRSVNEFMGNMQR
106	GDLADLLAANLGVEK	-	RSVNEFMGNMQRQGI
107	ADLLAANLGVEKVDL	-	NEFMGNMQRQGISPE
108	LAANLGVEKVDLIRC	-	MGNMQRQGISPEMYF
109	NLGVEKVDLIRCGGD	-	MQRQGISPEMYFQLT
110	VEKVDLIRCGGDNLV	-	QGISPEMYFQLTGTT
111	VDLIRCGGDNLVAAG	-	SPEMYFQLTGTTQED
112	IRCGGDNLVAAGREQ	-	MYFQLTGTTQEDLHN
113	GGDNLVAAGREQWND	-	QLTGTTQEDLHNQYS
114	NLVAAGREQWNDGSN	-	GTTQEDLHNQYSAEA
115	AAGREQWNDGSNTLT	-	QEDLHNQYSAEADKR
116	REQWNDGSNTLTIAP	-	LHNQYSAEADKRVKT
117	WNDGSNTLTIAPGW	-	QYSAEADKRVKTNLV
118	GSNTLTIAPGWWY	-	AEADKRVKTNLVIEA
119	TLTIAPGVWVYNRN	-	DKRVKTNLVIEAIAK
120	IAPGWWYNRNTIT	-	VKTNLVIEAIAKAEG
121	GWWYNRNTITNAI	-	NLVIEAIAKAEGFEA
122	WYNRNTITNAILES	-	IEAIAKAEGFEATDS
123	NRNTITNAILESKGL	-	IAKAEGFEATDSEIE
124	TITNAILESKGLKLI	-	AEGFEATDSEIEQEI
125	NAILESKGLKLIKIH	-	FEATDSEIEQEINDL
126	LESKGLKLIKIHGSE	-	TDSEIEQEINDLATE
127	KGLKLIKIHGSELVR	-	EIEQEINDLATEYNM
128	KLIKIHGSELVRGRG	-	QEINDLATEYNMPAD
129	KIHGSELVRGRGGPR	-	NDLATEYNMPADQVR
130	GSELVRGRGGPRCMS	-	ATEYNMPADQVRSLL
131	LVRGRGGPRCMSMPF	-	YNMPADQVRSLLSAD
132	GRGGPRCMSMPFERE	-	PADQVRSLLSADMLK
133	GGPRCMSMPFEREDI	-	QVRSLLSADMLKHDI
134	-	-	SLLSADMLKHDIAMK
135	-	-	SADMLKHDIAMKKAV
136	-	-	MLKHDIAMKKAVEVI
137	-	-	HDIAMKKAVEVITST
138	-	-	AMKKAVEVITSTASV
139	-	-	MKKAVEVITSTASVK