Identification and characterisation of components expressed by Gram-positive bacterial pathogens during human infection

A thesis submitted for the degree of Doctor of Philosophy

By

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Abstract

Gram-positive pathogens are responsible for a wide range of global diseases, including nosocomial infections. The increasing incidence of antibiotic-resistant strains warrants the development of novel therapeutic strategies to combat these organisms. Novel potential targets for vaccine development are antigens, which are expressed during infection. Identification of *in vivo*-expressed proteins which are conserved amongst several Gram-positive pathogens provides the possibility of producing immunotherapeutics to target a range of diseases. Using a direct screening technique, 48 *in vivo*-expressed antigen-encoding loci were identified in *Streptococcus pyogenes*. Analysis of these loci along with those identified in screens of *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus agalactiae* and *Streptococcus pneumoniae* resulted in the identification of 13 families of conserved domains. Three highly conserved *S. aureus* protein domains were selected for analysis, IsdA-c (amino acids 215-310), SdrD-n (amino acids 30-200) and ScaB-c (amino acids 156-265). Antibodies against these domains were opsonic during infection by *S. aureus* and *S. agalactiae*, and IsdA C-terminus polypeptide showed promise in protecting against infection by these pathogens in a mouse model of septic arthritis. Thus these protein domains may represent cross-protective targets for vaccine development.

Also identified during the immunoscreening was a family of ten surface proteins in *S. aureus* and *S. epidermidis*, named the Staphylococcal conserved antigens (Sca). These proteins contain a conserved C-terminal ~110 amino acid CHAP (cysteine, histidine-dependent amidohydrolases/peptidases) domain. Analysis of the Sca proteins in *S. aureus* identified the whole family as peptidoglycan hydrolases with ligand binding capabilities. Functional analysis of ScaD revealed that this enzyme may have a key role in cell separation. ScaD was expressed early during exponential growth and overproduction of the protein in *S. aureus* complemented the essentiality of the two-component regulator YycG/YycF. Overproduction of ScaD in wild-type *S. aureus* resulted in defective growth, increased resistance to lysis and a disrupted autolytic profile, indicating that unregulated expression of this putatively important enzyme may be hazardous to the cell.

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Abbreviations

aa	Amino acids
Amp	Ampicillin
AP	Alkaline phosphatase
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BHI	Brain heart infusion
bp	base pairs
BSA	Bovine serum albumin
CFU	Colony forming units
°C	Degrees Celcius
CIAP	Calf intestinal alkaline phosphatase
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
ECM	extracellular matrix components
Ery	Erythromycin
g	grams
GAS	group A streptococcus
HPLC	high performance liquid chromatography
hr	hours
Kan	Kanamycin
kb	kilobase pairs
kDa	kilodaltons
L	litres
LB	Luria-Bertani medium
Lin	Lincomycin
M	Molar
Mb	Megabase pairs
mbar	millibar
MCS	Multiple cloning site
MIC	minimum inhibitory concentration
mM	millimolar
mg	milligrams
min	minutes
ml	millilitres
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSCRAMM	Microbial surface components recognising adhesive matrix molecules
MSSA	Methicicllin-sensitive *Staphylococcus aureus*
NBT	Nitroblue tetrazolium
Neo	Neomycin
OD	Optical density
ORF	Open reading frame
PCR	Polymerase chain reaction
PBS	Phosphate buffered saline
pfu	Plaque forming units

PMN	polymorphonuclear leukocyte
PVDF	Polyvinylidene difluoride
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute
RT	Room temperature
s	seconds
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SERAM	Secretable expanded repertoire adhesive molecules
t	time
TAE	Tris-acetate EDTA (buffer)
TBS	Tris buffered saline
TCS	two-component system
TEMED	N,N,N'N'-tetramethyl-ethylenediamine
Tet	Tetracycline
TIGR	The Institure of Genomic Research
Tris	Tris (hydroxymethyl) aminomethane
TSB	Tryptic soy broth
µF	microfarad
µg	microgram
µl	microlitre
µM	micromolar
VISA	vancomycin-intermediate *Staphylococcus aureus*
VRE	vancomycin-resistant enterococci
VRSA	vancomycin-resistant *Staphylococcus aureus*
v/v	volume for volume
WT	Wild-type
w/v	weight for volume
x	times
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
~	Approximately

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Chapter 1

Introduction

1.1 The Staphylococci

Consisting of 32 different species, the staphylococci are spherical bacteria approximately 0.5 – 1.5 μm in diameter, predominantly associated with skin, skin glands and mucous membranes. The name *Staphylococcus* was chosen by the Scottish surgeon Sir Alexander Ogdson, and is derived from the Greek words *staphyle*, meaning bunch of grapes, and *kokkos*, meaning grain, due to the cellular arrangement of the bacteria. Staphylococcal cell division is in more than one plane, hence cells are arranged in grape-like clusters (Wilkinson, 1997). This is a distinguishing feature from the closely related streptococci, which are slightly ovoid cells dividing in only one plane, resulting in chain formation.

Staphylococci are facultatively anaerobic, capable of generating energy by respiratory or fermentative pathways. They are chemo-organotrophs and are catalase-positive but oxidase-negative. Staphylococci are commonly halo-tolerant, with most species able to withstand concentrations of up to 1.7M (10% w/v) NaCl (Wilkinson, 1997). The staphylococcal genome consists of a single circular chromosome, along with prophages, plasmids, transposons and insertion sequences. They are classified within the Bacillus-Lactobacillus-Streptococcus cluster, defining Gram-positive bacteria with a low G+C content of DNA, ranging from 30-39%. The staphylococci are divided into two major groups, the coagulase-positive and coagulase-negative (CoNS) staphylococci, based on their ability to clot blood plasma. The most prominent pathogen of the *Staphylococcus* species, and indeed the most studied, is *Staphylococcus aureus*.

1.2 *Staphylococcus aureus*

S. aureus colonies can be distinguished from those of other staphylococci, e.g. *S. epidermidis* by the golden yellow colour, which is a result of carotenoid production,

specifically staphyloxanthin, in stationary phase cells. This differs considerably from the pale, translucent, white colonies of *S. epidermidis*. A further distinguishing trait is the ability of *S. aureus* to produce coagulase (Wilkinson, 1997).

The genome of *S. aureus* is approximately 2.8 Mb and has a low G+C content. The whole genome of *S. aureus* was originally sequenced from the Japanese-derived methicillin-resistant strain N315 and the vancomycin-resistant strain Mu50 (Kuroda *et al.* 2001). There are now several sequenced and annotated strains of *S. aureus*, including *S. aureus* COL at The Institute for Genomic Research (TIGR) database (www.tigr.org/tdb) and the widely used laboratory strain *S. aureus* NCTC8325 at Oklahoma University (www.genome.ou.edu/staph.html).

1.2.1. *S. aureus* diseases and infections

S. aureus is a virulent pathogen and is currently the most common cause of infections in hospitalised patients (Archer, 1998). It is a common commensal of humans, inhabiting the squamous epithelium of the anterior nares. Approximately 20% of the population are colonised with *S. aureus*, 60% are intermittent carriers and 20% are non-carriers (Foster, 2004). The organism can be carried asymptomatically on mucous membranes but is only transiently carried on the skin. Colonisation provides the pathogen with the opportunity to infect skin sites, with the potential to cause serious diseases and conditions. Approximately half of all skin infections are caused by *S. aureus*, including folliculitis, mastitis, pyodermas and impetigo. However, most commonly, skin infections result in absesses or furuncles (boils) (Tenover and Gaynes, 2000).

Five stages of infection have been identified in the pathogenesis of *S. aureus*: colonisation, local infection, systematic dissemination and/or sepsis, metastatic infection, and toxinosis (Archer, 1998). Infections spread locally (e.g. carbuncle, cellulitis, impetigo bullosa, wound infection) or can gain access to the blood. Once in the blood, the bacteria are able to spread widely to distant infection sites, causing diseases such as endocarditis, osteomyelitis, renal carbuncle and septic arthritis. The success of *S. aureus* as a pathogen

and its ability to cause a wide spectrum of diseases can be attributed to an extensive array of virulence factors (Chapter 1.3).

In the UK, 20,000 cases of *S. aureus* bacteraemia are reported each year and *S. aureus* is the leading potentially lethal cause of hospital-acquired infections (García-Lara *et al.* 2005; Archer, 1998). The presence of an indwelling medical device, such as an intravascular or urinary catheter, represents a significant risk factor for staphylococcal bacteraemia and a high proportion of these infections progress to the more serious endocarditis. *S. aureus* is also a common cause of nosocomial pneumonia, most often following the onset of influenza, and it is the most common cause of empyema (infection of the pleural space) (Tenover and Gaynes, 2000). Furthermore, *S. aureus* is the most common cause of surgical site infections, largely due to the presence of the bacteria on the skin and mucous membranes.

Staphylococcal osteomyelitis can be either acute, most often occurring in children, or chronic, which is more common in adults. Acute osteomyelitis affects the long bones of the lower extremity and is treatable with antimicrobial therapy (Tenover and Gaynes, 2000). In addition, *S. aureus* is responsible for a range of toxin-mediated diseases, including impetigo, food poisoning, toxic shock syndrome (TSS) and scalded skin syndrome (SSS).

1.2.2. Antibiotic resistance in *S. aureus*

The prevalence of strains of *S. aureus* resistant to multiple antibiotics has dramatically increased over recent years. Occurrence of resistance first began in the 1940s, soon after the introduction of penicillin (Wilson and Cockcroft, 1952). This was due to the production of β-lactamase (penicillinase), a serine protease that hydrolyses β-lactam rings rendering the antibiotic inactive. In 1960, a semi-synthetic penicillinase-resistant β-lactam was developed, methicillin. However, the first clinical isolate of methicillin-resistant *Staphylococcus aureus* (MRSA) was reported in 1961, just a year after the introduction of the antibiotic (Jevons, 1961). The mechanism of methicillin resistance

involves the acquisition of the *mecA* gene, which encodes a unique penicillin-binding protein PBP2a. PBPs are transpeptidases which catalyse the formation of cross-bridges in bacterial cell wall peptidoglycan (Hiramatsu *et al.* 2001). PBP2a has a low affinity for β-lactam antibiotics, allowing the staphylococci to survive at high concentrations (de Lencastre *et al.* 2007). PBP2a is intrinsically insensitive not only to methicillin, but to all β-lactams developed, including isoxazoyl penicillins (e.g. oxacillin), cephalosporins, cefamycins and carbapenems (Foster, 2004). *mecA* is acquired by *S. aureus* via incorporation of a large genetic element known as the staphylococcal cassette chromosome *mec* (SCC*mec*), which integrates into the *S. aureus* chromosome at a specific location (*attBscc*), located near the origin of replication (Hiramatsu *et al.* 2001).

One of the last available drugs to which *S. aureus* remained sensitive was the glycopeptide vancomycin. However, the first clinical isolate with decreased susceptibility to this antibiotic (MIC = 8 μg/ml) was isolated in Japan in 1997 (Hiramatsu *et al.* 1997). Several strains with increased vancomycin resistance were also identified in other countries, and these strains were designated vancomycin-intermediate *S. aureus* (VISA), with MICs ranging from 8-16 μg/ml. Analysis of 48 VISA isolates revealed a common thickening of the cell wall and it was proposed that this resulted in the trapping of vancomycin molecules, preventing them from reaching the cytoplasmic membrane where the functional targets of the antibiotic are located (Cui *et al.* 2003). In June 2002, a high-level vancomycin-resistant *S. aureus* (VRSA) strain was isolated, with an MIC of 1024 μg/ml (Chang *et al.* 2003). Analysis of this isolate identified an *in vivo*, interspecies transfer of the *vanA* operon from vancomycin-resistant *E. faecalis* (VRE) to a multi-resistance conjugative plasmid in an MRSA, resulting in the formation of cell wall precursors to which vancomycin cannot bind (Weigel *et al.* 2003).

MRSA strains were primarily associated with hospital-acquired infections, however, the emergence of highly invasive staphylococcal strains occurred in the USA in 1999 when four fatal community-based MRSA infections in children were reported (Lowy, 2007). Further outbreaks of community-associated MRSA (CA-MRSA) were seen in patients without any of the usual hospital-associated risk factors. Initially, these CA-MRSA

strains showed diverse genetic backgrounds, however, unique clonal lineages emerged, often carrying the genetic determinant of the PVL toxin (Chapter 1.4.2), which may make effective chemotherapy more complex (de Lencastre *et al.* 2007).

1.3 Virulence factors of *S. aureus*

The success of *S. aureus* as a pathogen and its ability to cause a wide range of diseases can be attributed to its extensive array of virulence determinants, some of which are summarised in Figure 1.1 and Table 1.1. These include surface proteins, toxins and exoenzymes, which are coordinately regulated to be expressed when needed during infection. These virulence factors promote the key stages of infection: 1) attachment of bacteria to host cells or extracellular matrices; 2) evasion of host defences; 3) invasion and spread of bacteria to new sites. A hypothetical model of the coordinated expression of virulence factors is shown in Figure 1.2. Surface proteins are primarily produced during the exponential phase of growth, allowing the attachment of infecting bacteria during the initial stages of infection. Extracellular toxins and exoenzymes are produced during the post-exponential (PXP) phase due to crowding, allowing bacteria to spread to new sites during stationary (STA) phase to repeat the cycle (Projan and Novick, 1997).

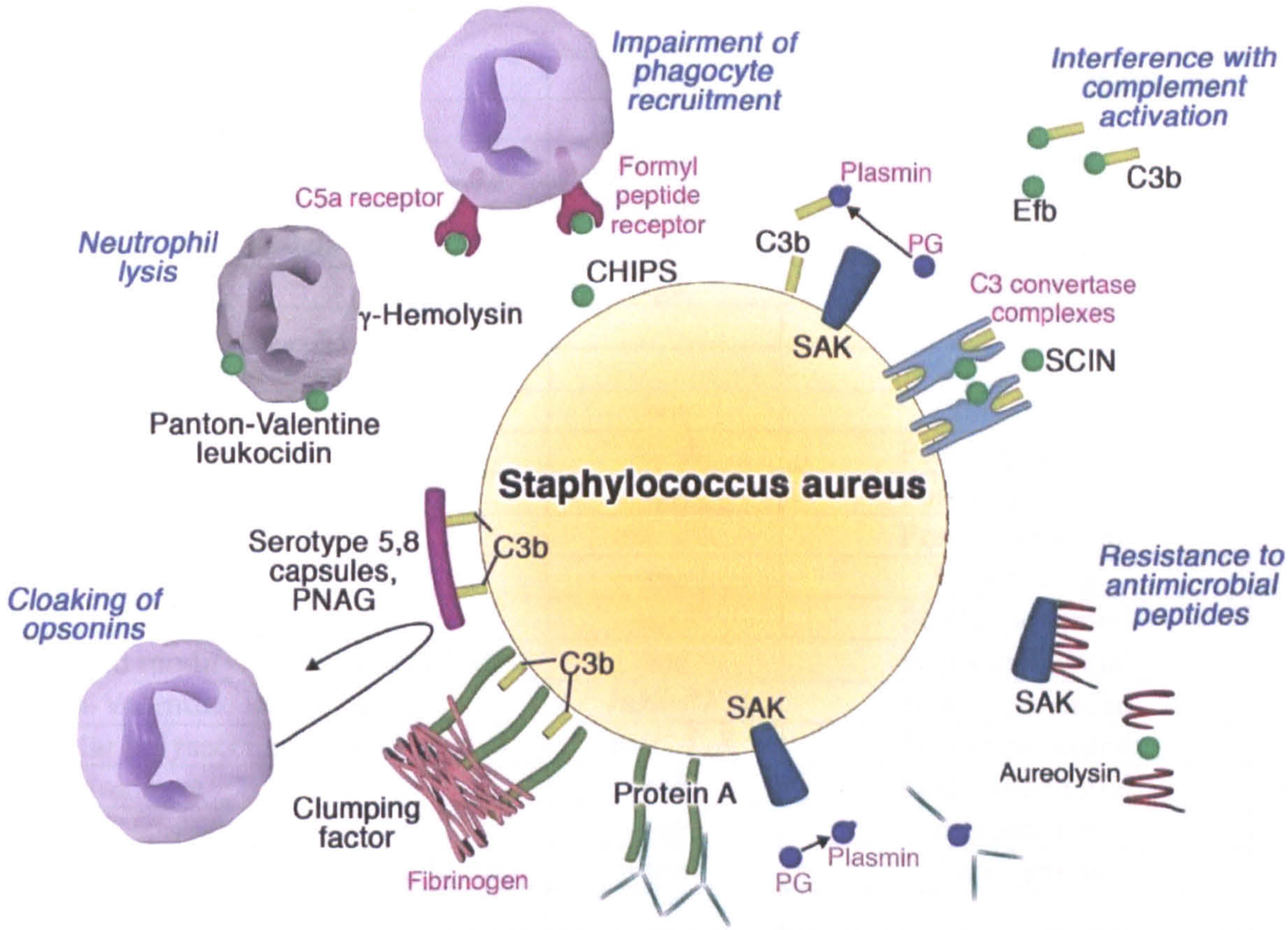


Figure 1.1: Virulence factors of *S. aureus*. Virulence factors are labelled in black, host components are labelled in pink. Adapted from Nizet (personal communication).

Virulence Factor	Gene	Growth phase of expression
Attachment		
Clumping factors A, B	*clfA, clfB*	Exponential
Fibronectin-binding proteins A, B	*fnbA, fnbB*	Exponential
Collagen-binding protein	*can*	Post-exponential
Coagulase	*cga*	Exponential
Host defence evasion		
Enterotoxin A	*sea*	Constitutive
Enterotoxins A, B, C1-3, D, E, H	*seb-h*	Post-exponential
Toxic shock syndrome toxin-1	*tst*	Post-exponential
Exfoliative toxins A, B	*eta, etb*	Post-exponential
Protein A	*spa*	Exponential
V8 protease	*sspA*	Post-exponential
Fatty acid modifying enzyme (FAME)	*fme*	Post-exponential
Panton-Valentine leukocidin	*lukS-PV*	Post-exponential
Capsular polysaccharide types 1, 5, 8	*cap1, 5, 8*	Post-exponential
Staphylokinase	*sak*	Post-exponential
CHIPS	*chp*	Exponential
SCIN	*scn*	Exponential
Invasion / tissue penetration		
α-toxin	*hla*	Post-exponential
β -haemolysin	*hlb*	Post-exponential
γ-haemolysin	*hlgA-C*	Post-exponential
δ-haemolysin	*hld*	Exponential
Aureolysin (metalloprotease)	*aur*	Post-exponential
Cysteine protease	*sspB*	Post-exponential
Staphopain	*scpA*	Post-exponential

Table 1.1: Virulence factors of *S. aureus*. Adapted from Projan and Novick (1997).

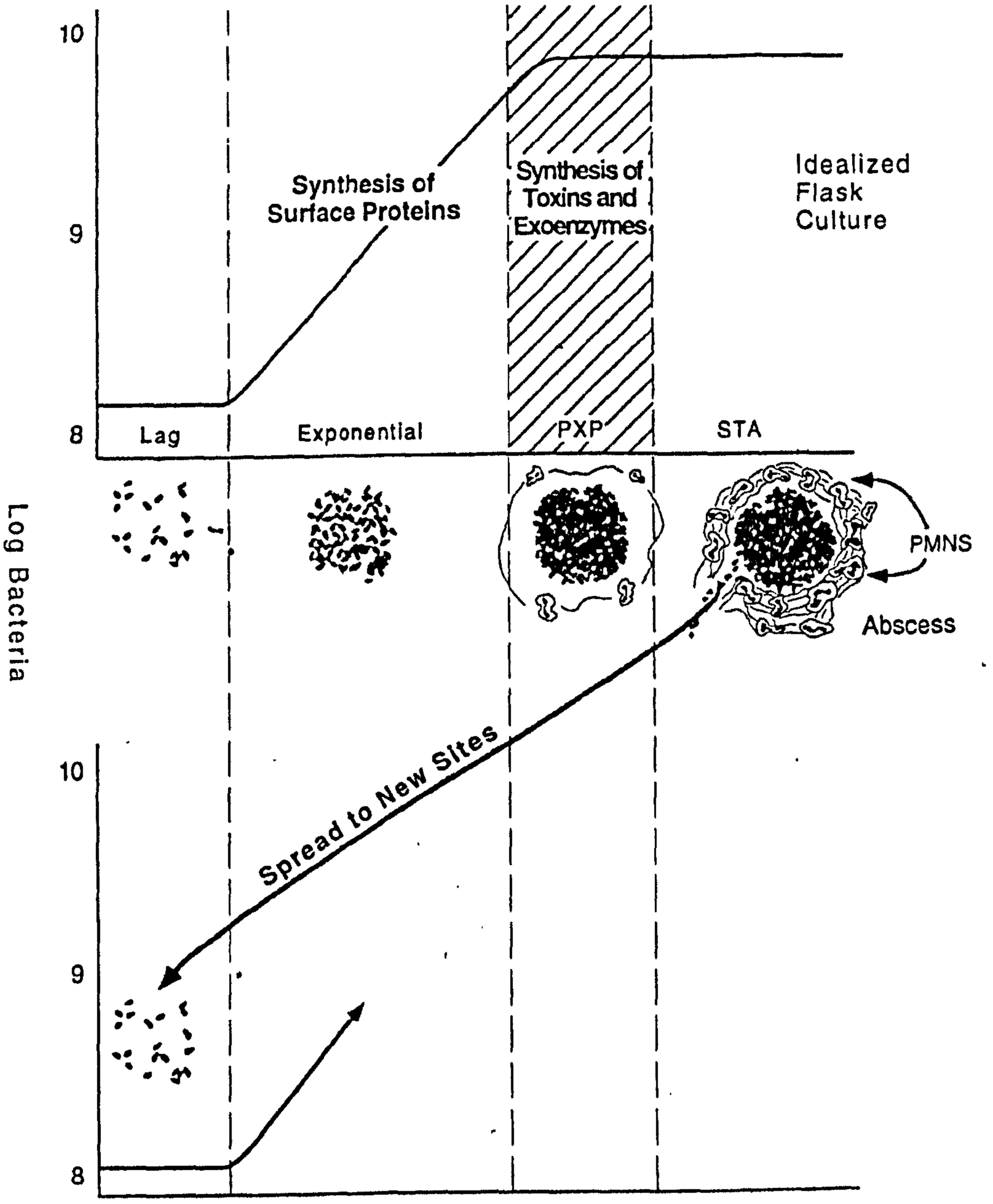


Figure 1.2: Synthesis of staphylococcal virulence factors in relation to growth. Surface proteins are produced during the exponential phase. Extracellular toxins and exoenzymes are produced during the post-exponential (PXP) phase due to crowding, allowing bacteria to spread to new sites during stationary (STA) phase to repeat the cycle (reproduced from Projan and Novick, 1997).

1.3.1. Adhesins

Attachment of staphylococci to host cells is essential for colonisation and subsequent dissemination. *S. aureus* expresses an array of adhesins which promote the attachment of bacteria to extracellular matrix components (ECM) of host epithelial and endothelial surfaces. Several adhesins are anchored covalently to the bacterial cell wall and are termed MSCRAMMs (microbial surface components recognising adhesive matrix molecules). These proteins are composed of an N-terminal signal sequence, which is cleaved during Sec-dependent secretion across the cytoplasmic membrane, and a conserved C-terminal cell wall sorting signal comprising an LPXTG motif followed by a hydrophobic stretch of amino acids and a positively charged tail located on the cytoplasmic side of the membrane (Roche *et al.* 2003; Marraffini *et al.* 2004). The enzyme sortase A (SrtA), an extracellular transpeptidase, recognises these surface proteins and cleaves them between the threonine and glycine residues of the LPXTG motif. This liberates the carboxyl of the threonine to form an amide bond with the amino group of the pentaglycine crossbridge, hence the C-terminal end is tethered to the cell wall peptidoglycan of the staphylococci (Ton-That *et al.* 1999). In *S. aureus*, the surface protein becomes linked to lipid II, and this product of the sorting reaction is incorporated into the cell wall envelope via the transglycosylation and transpeptidation reactions of cell wall biosynthesis (Marraffini *et al.* 2004). The product can only be released from the cell by cleavage by a murolytic enzyme, such as lysostaphin in *S. aureus*.

Protein A (Spa) is present in over 90% of *S. aureus* strains and can interact with several host components, including immunoglobulins G, A and E, platelets and von Willebrand factor (Fournier and Klier, 2004). The 42 kDa surface protein binds IgG molecules by their Fc portion or, alternatively, it can bind the Fab domains (Jansson *et al.* 1998). Protein A is suggested to have a role as a virulence factor and studies in animal models have shown that *spa* mutants caused less severe infections than wild-type strains (Palmqvist *et al.* 2002). It is predicted that the binding of protein A to IgG antibodies is involved in the evasion of the immune response, possibly by preventing opsonisation (Projan and Novick, 1997), and protein A competes with phagocytic cells for IgG-Fc

sites, hence reducing IgG-mediated opsonisation (Hartleib *et al.* 2000). In addition, the binding of the Fab domain induces the activation of B cells, conferring superantigenicity to protein A.

S. aureus expresses two closely-related fibronectin (Fn)-binding proteins, FnBPA and FnBPB (Foster and Höök, 1998), encoded by the genes *fnbA* and *fnbB*. It has been suggested that these two genes, separated by 682 bp, probably arose by gene duplication (Greene *et al.* 1995). Fibronectin plays an essential role in *S. aureus* infections of both implanted devices and human infections and the FnBP proteins were identified as sufficient for binding and invasion of host cells by *S. aureus* (Sinha *et al.* 2000). Furthermore, interactions between fibronectin and Fn-binding proteins have been implicated in the invasion of non-professional phagocytes (Joh *et al.* 1999). Studies with sera from convalescent patients has shown that antibodies against the FnBPs do not block fibronectin binding (Casolini *et al.* 1998), suggesting that the immune response against these virulence factors in *S. aureus* only produces antibodies that recognise FnBPs after they have bound to their ligand, hence, bacterial adhesion is not blocked.

The Sdr family of proteins comprises six proteins characterised by the presence of a domain containing Ser-Asp dipeptide repeats (Josefsson *et al.* 1998; Clarke *et al.* 2006). ClfA and ClfB, two structurally similar fibrinogen-binding proteins, are both associated with virulence. ClfA is an important virulence factor in infective endocarditis and staphylococcal arthritis (O'Brien *et al.* 2002) and has been demonstrated to inhibit phagocytosis (Higgins *et al.* 2006). ClfB has been suggested to contribute to the pathogenicity of biomaterial-related infections by promoting adherence to immobilised fibrinogen (Ní Eidhin *et al.* 1998) and, along with ClfA, mediates the binding and activation of platelets (O'Brien *et al.* 2002). SdrC, SdrD and SdrE have a similar structural organisation to ClfA and ClfB. *sdrC* is present in all strains of *S. aureus* and SdrD is associated with bone infections, osteomyelitis and methicillin resistance (Labandeira-Rey *et al.* 2007).

Collagenous proteins constitute a major component of the ECM and adherence to these ligands is mediated by the collagen-binding adhesin Cna. Although Cna has been shown to be a virulence factor in an experimental model of septic arthritis (Xu *et al.* 2004), vaccination of mice with a DNA vaccine encoding Cna did not protect against intra-peritoneal infection by *S. aureus*, despite inducing a strong antibody response (Therrien *et al.* 2007).

The iron-regulated surface determinant (Isd) proteins are a family of proteins in *S. aureus* which are involved in iron transport. IsdA is able to bind transferrin and is regulated by exogenous iron levels via the Fur protein, only being expressed under iron-limited conditions (Clarke *et al.* 2004). In addition to binding transferrin, IsdA is a broad spectrum adhesin able to bind both fibrinogen and fibronectin via a NEAT domain. IsdA contributes significantly to virulence of *S. aureus* and recently, it has been shown that IsdA binds lactoferrin, an anti-staphylococcal polypeptide found in human nasal secretions, hence increasing resistance of *S. aureus* to its killing (Clarke and Foster, 2008). IsdA is also required for nasal colonisation and confers resistance to antimicrobial peptides found on the skin (Clarke *et al.* 2006; 2007).

In addition to the covalently anchored adhesins, *S. aureus* also expresses several adhesins which are ionically associated with the cell wall, known as SERAMs (secretable expanded repertoire adhesive molecules) (Chavakis *et al.* 2005). Emp (extracellular matrix protein-binding protein) is expressed during stationary growth and binds to several host extracellular matrix molecules, including fibronectin, fibrinogen, collagen and vitronectin (Hussain *et al.* 2001). Eap (extracellular adherence protein) can bind several plasma proteins, including fibronectin, fibrinogen and prothrombin. This adhesin causes agglutination of *S. aureus* cells and significantly enhances bacterial adherence and internalisation into eukaryotic cells (Haggar *et al.* 2003). Ebh (ECM-binding protein homologue) binds fibronectin and may be involved in adhesion to endothelial cells (Sinha and Herrmann, 2005). Notably, the presence of anti-Ebh antibodies in the serum of *S. aureus*-infected patients indicates that this protein is expressed during infection (Clarke *et al.* 2002). Efb (extracellular-fibrinogen binding protein) was shown to be important for

pathogenesis in a rat wound model and antibodies against this adhesin protected against infection in a mouse mastitis model (Palma *et al.* 2001).

S. aureus capsular polysaccharides, representing 11 serologically distinct capsule types, have been identified in more than 90% of clinical isolates, and serotype 5 and 8 strains are the most predominant (Thakker *et al.* 1998; Watts *et al.* 2005). Although the role of these polysaccharides remains unclear, it has been demonstrated that optimal expression of capsule enhances bacterial virulence by increasing resistance to phagocytic killing by leukocytes (Thakker *et al.* 1998; Luong and Lee, 2002; Kampen *et al.* 2005).

The surface polysaccharide PNAG (poly-(1,6)-*N*-acetylglucosamine), also known as polysaccharide-adhesin (PS/A) and polysaccharide intercellular adhesin (PIA), promotes adhesion of bacteria and is implicated in biofilm formation (Projan and Novick, 1997; Maira-Litrán *et al.* 2002). Deletion of the intercellular adhesin (*ica*) locus, which encodes the biosynthetic enzymes for PNAG production, resulted in strains with decreased virulence in murine models and increased susceptibility to antibody-independent opsonic killing (Kropec *et al.* 2005).

1.3.2. Toxins and invasins

In order to allow dissemination of infection, a pathogen must be able to invade host tissues and virulence factors involved in this process in *S. aureus* include cytolytic toxins, exfoliative toxins, TSST-1 and staphylococcal enterotoxins.

Haemolysins are cytotoxic agents which have a role in the invasion of bacteria during infection. One of the most well-studied members of this group is α-toxin (α-haemolysin), a pore-forming toxin encoded by the gene *hla* (Bohach and Foster, 2000). This 293 residue monomer is secreted extracellularly by *S. aureus* and binds to the host membrane, oligomerising to form a ring-shaped pore. The toxin can lyse red blood cells in this manner, as well as mononuclear immune cells, epithelial and endothelial cells, and platelets. Death of these cells is attributed to disrupted osmoregulation, the influx and

efflux of cations and cell apoptosis. Several studies have shown that α-toxin acts as a virulence factor, as *hla*⁻ strains were significantly less virulent in experimental infections of keratitis, mastitis and peritonitis (Projan and Novick, 1997; Bayer *et al.* 1997). However, Bayer *et al.* (1997) also found that hyperproduction of α-toxin by *S. aureus*, as well as non-production of the toxin, resulted in reduced virulence in a model of endocarditis.

β-haemolysin is an enzyme that acts as a sphingomyelinase, encoded by the gene *hlb*. The action of β-haemolysin is antagonistic to that of α-toxin because β-haemolysin disrupts the membranes of erythrocytes, hence α-toxin cannot attach and multimerise (Projan and Novick, 1997). δ-haemolysin, a 26 residue peptide, has surfactant-like properties and is lytic towards many types of membranes, forming channels. Therefore, this toxin may be cytotoxic to tissues and may have an adverse effect on leukocytes (Bohach and Foster, 2000). δ-haemolysin has been observed to enhance the action of β-haemolysin, increasing its haemolytic properties (Projan and Novick, 1997). However, the specific role of this toxin in virulence is yet to be established.

S. aureus can produce four antigenically distinct exfoliative toxins, known as ETA, ETB, ETC and ETD (Ladhani, 2003). These toxins are responsible for a range of diseases, including localised blisters, extensive exfoliation and staphylococcal scalded skin syndrome (SSSS). ETA and ETB, predominant in Europe/USA and Japan respectively, are responsible for most human cases of SSSS. They are able to target and destroy specific proteins of the host epidermis. It has been suggested that ETD may play a wider role in staphylococcal infections as it is not strongly associated with SSSS. This protein may be important in allowing the organism to spread and invade local tissue (Ladhani, 2003). The role of exfoliative toxins as superantigens remains controversial. A study by Plano *et al.* (2000) concluded that the proteins did not exhibit superantigenic activity, but this conclusion is disputed elsewhere (Ladhani, 2003). Furthermore, exfoliative toxins have been shown to possess serine protease activity and share 25% homology with staphylococcal V8 protease (Chapter 1.3.3).

S. aureus produces several extracellular proteins with superantigenic properties, including enterotoxins and toxic shock syndrome toxin 1 (TSST-1). These are known as the pyrogenic toxin superantigens (PTSAgs). These superantigens directly cross-link certain T-cell receptor domains on major histocompatibility complex class II (MHC II) proteins, activating specific subsets of T cells (Holtfreter *et al.* 2004). This results in the proliferation of T cells and the release of cytokines and lymphokines, for example, tumour necrosis factor (TNF), interleukins and interferon-γ (Projan and Novick, 1997). This large systemic release of cytokines can lead to symptoms of toxic shock syndrome.

Seventeen staphylococcal enterotoxins (SEs) have been identified, encoded on mobile genetic elements. These toxins are known as SEs A-E and G-Q, and are recognised to cause staphylococcal food poisoning syndrome (Akineden *et al.* 2001). Loss of superantigen activity, for example due to a genetic mutation, also results in loss of enterotoxic activity, confirming the correlation of these two factors (Balaban and Rasooly, 2000). Nearly all clinical isolates of *S. aureus* contain enterotoxin genes, with extensive variation occurring between strains (Holtfreter *et al.* 2004). In general, *S. aureus* isolates have several of these genes, most often five. Furthermore, a correlation exists between the presence of enterotoxin genes in *S. aureus* and the ability of the organism to elicit T cell proliferation, substantiating the superantigenicity of these proteins and their roles as virulence factors involved in evading the host immune response. However, it has been proposed that the amounts and spectrum of superantigens secreted may differ between *S. aureus* carriage and *S. aureus* infection. Importantly, antibodies which neutralise these superantigens are highly specific and this lack of cross-inhibition is an important consideration in the design of superantigen vaccines (Holtfreter *et al.* 2004).

S. aureus expresses a bi-component pore-forming exotoxin that specifically acts on polymorphonuclear leukocytes, disrupting phagocytosis, an important defence against bacterial infection (Bohach and Foster, 2000). This toxin is known as Panton-Valentine leukocidin (PVL) and is encoded by the *luk-PV* locus, requiring assembly of two polypeptdies, LukS-PV and LukF-PV, into a heterooligomeric pore (Labandeira-Rey *et*

al. 2007). Presence of PVL has been strongly associated with strains of community-associated methicillin-resistant *S. aureus* (CA-MRSA), although it was established that the toxin was not the major determinant of disease caused by these strains (Voyich *et al.* 2006). However, expression of the *luk-PV* operon results in altered regulation of cell wall-anchored and secreted protein production, including upregulation of both SdrD and protein A (Spa) (Chapter 1.4.1), which may enhance tissue adherence and colonisation, thereby contributing to the virulence of PVL-positive strains (Labandeira-Rey *et al.* 2007).

1.3.3. Other extracellular enzymes

S. aureus expresses several extracellular proteases, which are predicted to act as virulence factors by several mechanisms, e.g. inactivation of antimicrobial peptides, cleavage of human IgG molecules and allowing dissemination of bacteria by tissue destruction (Reed *et al.* 2001). The serine protease V8 protease, also known as SspA, can cleave and inactivate IgG antibodies and it has been proposed that it functions, along with other proteases, to block the action of these antibodies (Projan and Novick, 1997). *sspA* is transcribed in an operon along with *sspB*, a cysteine protease, and *sspC*, which encodes staphostatin B (Rice *et al.* 2001; Shaw *et al.* 2005). *S. aureus* also expresses a cysteine protease called staphopain (ScpA) and a metalloprotease called aureolysin (Aur) (Arvidson, 2000). SspA is required for proteolytic maturation of SspB and is itself activated by Aur (Shaw *et al.* 2004). The temporal coordination of expression of these proteases through the quorum-sensing system *agr* is believed to enable *S. aureus* to switch from the expression of adhesive molecules to the expression of extracellular toxins and enzymes that damage host tissues and the immune system, allowing dissemination of infection (Shaw *et al.* 2005). This change from an adhesive to invasive phenotype requires the degradation of adhesins post-exponentially, which is contributed to by these proteases (McGavin *et al.* 1997; Shaw *et al.* 2004). Indeed, a *S. aureus* strain lacking SspA was severely attenuated in virulence in mouse abscess, bacteraemia and wound infection models, highlighting the contribution of this serine protease to bacterial pathogenesis (Coulter *et al.* 1998).

Staphylokinase (Sak) is a 136 amino acid serine protease-like molecule produced by *S. aureus*. It is a plasminogen activator, converting the precursor plasminogen to the fibrinolytic protease plasmin, which degrades proteins of the extracellular matrix. It has been demonstrated that staphylokinase interacts with α-defensins, almost completely inhibiting their bactericidal effects (Jin *et al.* 2004). Staphylokinase induces the secretion of these defensins from polymorphonuclear cells and complex binds them, neutralising their effects. This represents a mechanism by which *S. aureus* evades killing by the host innate immune system. The majority of staphylococci isolated from the nasal mucosa produce staphylokinase, further supporting the suggestion that the production of this surface protein increases bacterial survival in the host. In addition, it has been suggested that staphylokinase may enable *S. aureus* to bind host plasminogen via cell surface receptors, hence, promoting bacterial invasion (Jin *et al.* 2004).

S. aureus, along with most other strains of staphylococci of human origin, produce lipases. In response to bacterial infection, the host produces a variety of fatty acids and lipid molecules, which act as surfactants to disrupt the bacterial membrane. The lipases produced by *S. aureus* have been shown to have a negative effect on the immune system (Projan and Novick, 1997). Furthermore, an enzyme produced by *S. aureus*, fatty acid metabolising enzyme (FAME), is proposed to detoxify bactericidal fatty acids at the site of an infection.

Chemotaxis inhibitory protein of staphylococci (CHIPS) and Staphylococcal complement inhibitor (SCIN) are small, excreted molecules that play a crucial role in the defence against the innate immune system (Rooijakkers *et al.* 2006). CHIPS is a 14 kDa protein produced by 65% of clinical *S. aureus* strains which blocks neutrophil chemotaxis by binding neutrophil receptors (De Haas *et al.* 2004). SCIN is a 10 kDa protein that inhibits the central complement convertases, reducing opsonisation and subsequent phagocytosis (Rooijakkers *et al.* 2005; 2007). SCIN and CHIPS are produced early during exponential growth, allowing efficient modulation of the early immune response to infection.

1.4 The Streptococci

Streptococci are round/ovoid cocci, 0.6 – 1.0 μm in diameter. These bacteria divide in one plane, hence occur in pairs or chains. Streptococci are fermentative and, similar to the staphylococci, are facultative anaerobes. They are catalase-negative and oxidase positive. The streptococci are classified according to the Lancefield serological grouping system, based on immunological differences in cell wall polysaccharides (A, B, C, F, G) and lipoteichoic acids (D) (Cunningham, 2000).

1.5 *Streptococcus pyogenes*

Streptococcus pyogenes is a group A streptococcus (GAS) and is a frequent pathogen of humans. The major reservoir of *S. pyogenes* is the human oral-nasal mucosa and it has been estimated that 5-15% of individuals carry the bacterium as part of the normal flora of the respiratory tract with no symptoms of disease (Cue *et al.* 2000). The genome of *S. pyogenes* is approximately 1.89 Mb and over 150 M-protein (Chapter 1.6.1) genotypes exist (Park and Cleary, 2005).

1.5.1. *S. pyogenes* diseases and infections

Acute *S. pyogenes* diseases primarily occur in the respiratory tract or on the skin, and generally, isolates from the former do not infect the latter. The most common disease caused by *S. pyogenes* infection is pharyngitis, however, there has been a recent increase in systemic *S. pyogenes* disease worldwide (Cue *et al.* 2000). Streptococcal pharyngitis, commonly known as "strep throat", is treatable using β-lactam antibiotics. However, if left untreated this condition can lead to rheumatic fever and possible heart disease.

As mentioned in Chapter 1.2.1, impetigo, a localised infection of the skin, can be caused by infection with *S. aureus*. However, this condition is also a consequence of *S. pyogenes* infection. In its severe form, this can lead to the acute invasive disease, necrotising fasciitis, leading to the labelling of *S. pyogenes* as "flesh-eating bacteria". Other invasive,

toxigenic infections caused by this pathogen are myositis and streptococcal toxic shock syndrome (TSS). Streptococcal TSS involves bacteraemia, shock and, in approximately 30% of cases, death (Fischetti, 2000b).

Puerperal fever, sepsis occurring after childbirth, and scarlet fever, a disease of childhood, were formerly diseases of *S. pyogenes* which claimed many lives. Now, due to antibiotic therapy, these conditions are rare. Studies have shown that *S. pyogenes* infections are significantly more common in children compared to adults, suggesting multiple infections during childhood results in the development of protective antibodies (Cunningham, 2000).

1.5.2. Antibiotic resistance in *S. pyogenes*

Penicillin and related β-lactam antibiotics are the primary choice for the treatment of infections by *S. pyogenes* and currently, no resistance to penicillin has developed despite several years of exposure to this antibiotic (Albrich *et al.* 2004; Robinson *et al.* 2006). Patients with hypersensitivity to β-lactam antibiotics are generally treated with macrolides, including erythromycin and clindamycin, and macrolide-resistant GAS (MRGAS) is increasingly being recognised as a problem in many parts of the world (Robinson *et al.* 2006). Macrolides such as erythromycin inhibit protein synthesis by binding to 23S rRNA of the 50S bacterial ribsome subunit (Richter *et al.* 2008) and most of the resistance to these antibiotics occurs via one of two mechanisms. Macrolide efflux (M phenotype) by MefA results in resistance to 14- and 15-membered ring macrolides but not 16-membered ring macrolides, lincosamides and streptogramin B (MLS_B). Resistance may also be due to methylation of the ribosomal drug binding site by methylases encoded by *ermA* or *ermB*, resulting in resistance to all MLS_B antibiotics (Desjardins *et al.* 2004; Richter *et al.* 2008). These three major resistance genes (R genes) found in *S. pyogenes* are associated with mobile genetic elements and were acquired via horizontal gene transfer (Robinson *et al.* 2006). A study by Desjardins *et al.* (2004) identified the efflux (*mefA*) as the more common mechanism of resistance, particularly in pediatric GAS isolates (72% of isolates).

An almost linear association was identified between use of macrolide antibiotics and resistance in *S. pneumoniae*, and although weaker, a similar significant correlation was observed for macrolide-resistant *S. pyogenes* (Albrich *et al.* 2004). Erythromycin resistance was identified in 6% of adult GAS isolates and 10% of pediatric GAS isolates in 2004 (Desjardins *et al.* 2004). In a study in 2007, 16% of GAS isolates studied were erthromycin-resistant and 27% were resistant to tetracycline (Lloyd *et al.* 2007). It was reported that rates of resistance to macrolides, tetracycline and quinolones were rising, most likely due to extensive use of these antibiotics. Indeed, it was suggested that macrolides have replaced penicillin in the treatment of *S. pyogenes* infections due to favourable pharmacokinetics, despite maintained susceptibility of streptococci to penicillin, amoxycillin, cephalosporins and linezolid.

Emergence of resistance to macrolides led to the development of ketolides, a new class of antimicrobials derived from erythromycin, which have a 10-fold greater ribosomal binding affinity. However, the first available antibiotic, telithromycin, has variable activity against macrolide-resistant *S. pyogenes* and resistance to this antibiotic amongst European strains of *S. pyogenes* significantly increased from 2002/03 to 2004/05 (Richter *et al.* 2008).

1.6 Virulence factors of *S. pyogenes*

Similar to *S. aureus* (Chapter 1.3), S. *pyogenes* expresses an array of virulence determinants enabling the pathogen to infect and cause disease. A summary of these virulence factors is shown in Figure 1.3 and Table 1.2.

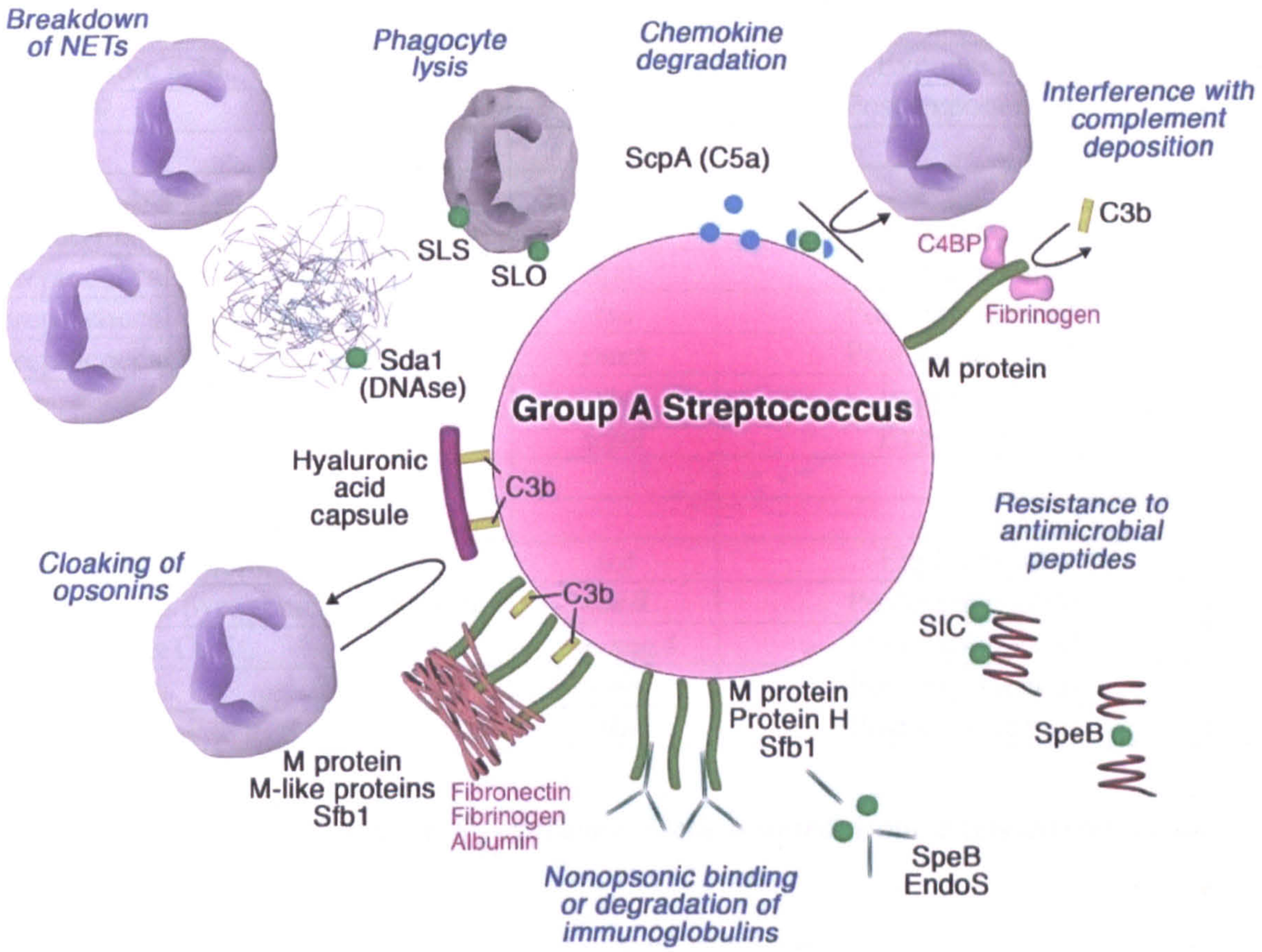


Figure 1.3: Virulence factors of Group A *Streptococcus*. Virulence factors are labelled in black, host components are labelled in pink. Adapted from Nizet (personal communication).

Virulence Factor	Gene	Growth phase of expression
Attachment		
Lipoteichoic acid	*lta*	Exponential
Protein F	*sfbI*	Post-exponential
Host defence evasion		
M proteins	*emm*	Exponential
Streptococcal pyrogenic exotoxins	*spe*	Post-exponential
Streptococcal superantigen	*ssa*	Post-exponential
Streptococcal mitogenic exotoxin Z	*smez*	Post-exponential
Sic	*sic*	Exponential
C5a peptidase	*scpA*	Exponential
Invasion / tissue penetration		
Serum opacity factor	*sof*	Exponential
Streptococcal pyrogenic exotoxin B	*speB*	Post-exponential
Streptolysins O, S	*slo*, *sagA*	Post-exponential
Enolase	*eno*	Post-exponential
Streptokinase	*ska*	Post-exponential

Table 1.2: Virulence factors of *S. pyogenes*. Data adapted from Kreikemeyer *et al.* (2003).

1.6.1. Adhesins

The cell surface proteins of *S. pyogenes* are similar in structure to those of other Gram-positive pathogens, including *S. aureus* (Chapter 1.3.1). *S. pyogenes* expresses MSCRAMMs, constituting a charged C-terminus, followed by a hydrophobic domain and a conserved LPXTG motif, which allows covalent linkage to the bacterial peptidoglycan via sortase activity. The M proteins of *S. pyogenes* are a family of cell wall-anchored proteins and with more than 100 variants these surface proteins are expressed by all isolates of *S. pyogenes* (Rivera *et al.* 2007). The proteins, encoded by *emm* genes, extend from the surface as alpha-helical coiled-coil dimers, appearing as fibrils on the bacterial surface (Cunningham, 2000). The M proteins represent key virulence factors associated with both colonisation and resistance to phagocytosis. They have important roles in evading the immune response by conferring resistance to phagocytosis by human neutrophils (Fischetti, 1989). This antiphagocytic effect is due to the binding of the M protein to factor H and fibrinogen. Factor H is a regulatory component of the complement pathway, which inhibits the deposition of soluble C3b, hence reducing opsonisation of the bacteria. Binding fibrinogen to the streptococcal surface also blocks activation of complement via the alternate pathway, hence the amount of C3b which can bind to *S. pyogenes* cells is greatly reduced. This therefore reduces phagocytosis by polymorphonuclear leukocytes (Cunningham, 2000). The M protein family also contains proteins that can bind IgG and IgA molecules, including Arp, protein H and Sir, and it is likely that these proteins facilitate the evasion of the host defence mechanisms (Stenberg *et al.* 1994).

Lipoteichoic acid (LTA) is an amphipathic molecule which can bind to a variety of host cell types and receptor molecules, including both fibrinogen and fibronectin. The negatively charged polyglycerol phosphate backbone of LTA allows it to react with the positively charged residues of streptococcal surface proteins (Cunningham, 2000). It has been proposed that LTA plays a critical role in the initial, reversible, interactions between streptococci and epithelial cells, facilitating the interaction of a second adhesin to result

in a high-affinity, host-cell specific attachment (Courtney *et al.* 1994). This second adhesin varies according to the type of host cell or tissue involved in the attachment process.

Protein F (SfbI) binds fibronectin at its amino terminal and is expressed by 50-70% of clinical isolates of *S. pyogenes* (Kreikemeyer *et al.* 2004). It is involved in binding to the dermis and Langerhans cells, and it is regulated by a superoxide signal (Cunningham, 2000). As well as being an adhesin, protein F mediates internalisation of *S. pyogenes* into non-phagocytic cells (Jadoun *et al.* 1998).

FBP54 can bind both fibronectin and fibrinogen and is present in all clinical strains tested (Courtney *et al.* 1994; Kawabata *et al.* 2001). Furthermore, FBP54 was demonstrated to be associated with infection as a significantly higher IgG titre against this adhesin was observed in patients with *S. pyogenes* infections compared to healthy volunteers. Immunisation with FBP54 elicited protection in mice against lethal *S. pyogenes* infection, indicating a putative role as a vaccine candidate for this streptococcal adhesin.

SOF (serum opacity factor) is a 112 kDa bi-functional protein which binds fibronectin at its C-terminus and contains an N-terminal domain responsible for turning mammalian sera opaque by cleaving a serum apolipoprotein (Rivera *et al.* 2007). The *sof* gene is co-transcribed with the gene *sfbX*, which encodes a second fibronectin-binding protein (Oehmcke *et al.* 2004). SOF promotes adherence to and invasion of human HEp-2 pharyngeal epithelial cells independently of SfbX, and it is the N-terminal domain of SOF that is responsible for this function (Timmer *et al.* 2006). Furthermore, SOF has been demonstrated to significantly contribute to virulence of *S. pyogenes* in a murine model of necrotising skin infection.

SpeB (streptococcal exotoxin B), a 40 kDa protein, mediates binding to laminin, a glycoprotein expressed on host cells (Hytönen *et al.* 2001). SpeB does not contain the Gram positive peptidoglycan anchoring motif LPXTG, it is suggested that the protein is anchored to the bacterial surface via its propeptide moiety. SpeB is controlled by the

multiple gene regulator Mga and all strains of *S. pyogenes* contain the *speB* gene, although expression levels vary.

1.6.2. Toxins and invasins

The family of proteins known as the pyrogenic toxin superantigens (PTSAgs) include the streptococcal pyrogenic exotoxins (SPEs), streptococcal superantigen (SSA) and streptococcal mitogenic exotoxin Z (SMEZ) (Earhart *et al.* 2000; Proft *et al.* 2004). These proteins have a highly conserved structure and, similar to the staphylococcal enterotoxins (Chapter 1.3.2), the PTSAgs have superantigenic properties, eliciting T cell proliferation. The PTSAgs induce fever in the host during infection (pyrogenicity) and SPEs enhance the susceptibility of the host to lethal endotoxin shock. SpeB (streptococcal pyrogenic exotoxin B) is a 28 kDa cysteine protease expressed by all strains of *S. pyogenes* in varying levels. The adhesive function of this protein has been mentioned previously (Chapter 1.6.1), however, SpeB also has an enzymatic role, converting the interleukin-1β precursor into its active form, an inflammatory cytokine (Cunningham, 2000). Moreover, it is thought that SpeB enhances internalisation of *S. pyogenes* by epithelial and endothelial cells, hence may have a pathogenic role in bacterial invasion. As well as promoting dissemination of infecting bacteria, it has also been suggested that this cysteine protease promotes phagocytic resistance and hence may also play a role in evasion of the host response (Voyich *et al.* 2004). Immunisation of mice with SpeB has been shown to confer protection against infection by *S. pyogenes* and inactivation of the protein significantly decreases lethality (Hytönen *et al.* 2001).

S. pyogenes can express two cytolysins, streptolysin O (SLO) and streptolysin S (SLS). SLO is a thiol-activated, cholesterol-binding protein and can act as a leukocidin, forming pores in the cell membranes of phagocytes, effectively destroying this immune response (Billington *et al.* 2000). Streptolysin O is also capable of interfering with other aspects of the host immune reaction, for example, immune cell chemotaxis, respiratory burst and lymphocytes. This interference, particularly during the early stages of infection, can prevent clearance of the invading bacteria, allowing them to invade and proliferate.

Furthermore, it has been suggested that streptolysin O may play a role in the regulation of bacterial invasion (Coye and Collins, 2004). This protein can induce the expression of cytokines, which may promote bacterial infection.

Streptolysin S (SLS) is a β-haemolysin, able to lyse erythrocytes. A study by Nizet *et al.* (2000) identified a nine-gene operon responsible for the production of SLS and found sequence homologies with the bacteriocin class of antimicrobial peptides. Streptolysin S can damage cell membranes of lymphocytes, neutrophils and platelets, and has been demonstrated to be a virulence determinant of *S. pyogenes* in an animal model of necrotic skin infection.

C5a peptidase is a proteolytic enzyme, encoded by the gene *scpA*, which is anchored to the surface of *S. pyogenes*. This 130 kDa endopeptidase contributes to streptococcal invasion by inhibiting the recruitment of polymorphonuclear leukocytes at the infection site. C5a peptidase cleaves the complement-derived chemotaxin C5a, which enhances the chemotaxis of phagocytes (Voyich *et al.* 2004). Therefore, phagocytosis is significantly inhibited.

S. pyogenes expresses several DNases, including Sda1. This contributes to virulence by allowing the organism to escape killing by neutrophil extracellular traps (NETs), which are composed of DNA and contain chromatin and neutrophil granule components, and act to capture and kill surrounding bacteria independent of phagocytic uptake (Sumby *et al.* 2005; Buchanan *et al.* 2006). Sda1 has been shown to promote virulence in a murine model of GAS nectrotising fasciitis and to be necessary and sufficient for effective degradation of NETs (Buchanan *et al.* 2006).

The 31 kDa protein Sic is secreted by most serotype M1 strains of *S. pyogenes*. This protein disrupts phagocytosis by altering normal polymorphonuclear leukocyte (PMN) cytoskeleton function (Voyich *et al.* 2004). Furthermore, Sic inactivates antibacterial peptides, including lysozyme and defensins.

S. pyogenes contains a capsule composed of a hyaluronic acid polymer, made up of repeating units of glucuronic acid and *N*-acetylglucosamine (Cunningham, 2000). This capsule plays a role in evading opsonisation and hence phagocytosis. The mechanism of resistance to phagocytosis may be due to the barrier created by the capsule, preventing phagocytes from accessing opsonic complement proteins bound to the streptococcal surface.

S. pyogenes can bind plasmin(ogen) to cell surface receptor proteins and this is thought to enhance bacterial invasion by activating extracellular matrix metalloproteases or collagenases (Cunningham, 2000). These plasminogen-binding proteins include glyceraldehyde-3-phosphate dehydrogenase (Plr), enolase and streptokinase.

1.7 Regulation of bacterial virulence

Most virulence factors are accessory genes, i.e. they are not essential for growth under all conditions. These accessory genes are often coordinately regulated so that they are only expressed when needed. Global regulators allow the control of expression of unlinked genes by a single regulatory determinant, enabling a pathogen to rapidly adapt to changing environments encountered during infection in order to maximise its virulence.

1.7.1. Regulation of *S. aureus* virulence determinants

The expression of virulence factors in *S. aureus* is regulated by several interacting regulators. The Agr (accessory gene regulator) two-component signal transduction system is composed of four structural genes, *agrB*, *agrD*, *agrC* and *agrA*, but is composed of two divergent transcripts, RNAII and RNAIII, which are under the control of promoters P2 and P3, respectively (Figure 1.4). *agrB* encodes a membrane-associated protease which modifies the prepropeptide of AgrD and generates small signal molecules. These signal molecules are recognised by the sensor kinase AgrC which subsequently activates the response regulator AgrA. AgrA upregulates production of the effector molecule RNAIII, which alters expression of a range of virulence factors in *S. aureus*,

both at the transcriptional and translational levels (Bronner *et al.* 2004). The Agr two-component system upregulates transcription of toxin genes whilst repressing synthesis of cell wall proteins, postexponentially. Transcription profiling demonstrated that mutation of *agr* lead to the altered expression of 138 genes in *S. aureus*, 104 of which were upregulated and 34 of which were downregulated (Dunman *et al.* 2001). Examples of these genes are shown in Table 1.3.

SarA (staphylococcal accessory regulator) is a 14.5 kDa DNA-binding protein encoded by three overlapping transcripts, driven by three distinct promoters, P1, P3 and P2 (Cheung *et al.* 2008). SarA promotes synthesis of fibronectin and fibrinogen-binding proteins for adhesion and toxins for tissue spread, whilst repressing expression of protein A and proteases. Upregulation of α, β and δ-toxin expression also occurs indirectly via upregulation of *agr* by *sarA*. Transcription profiling demonstrated that mutation of *sarA* lead to the altered expression of around 120 genes in *S. aureus*, 76 of which were upregulated and 44 of which were downregulated (Dunman *et al.* 2001). Examples of these genes are shown in Table 1.4.

SarA is a member of a family of regulatory proteins involved in the control of virulence genes, including the homologues SarR, SarS, SarT, SarU and Rot. It is suggested that all members of the SarA family bind target DNA by a similar mechanism, that is with the helix-turn-helix binding to the major groove of the DNA and the winged region interacting with the minor groove (Liu *et al.* 2006). SarR is involved in the regulation of *sarA* expression, and is expressed maximally during the post-exponential growth phase, leading to downregulation of *sarA* during late exponential to the stationary phases of growth. SarS promotes transcription of protein A and represses expression of α-toxin, and expression of *sarS* is repressed by both *sarA* and *agr* (Bronner *et al.* 2004). SarT represses α-haemolysin expression as well as expression of *sarU*, which is involved in regulation of RNAII and RNAIII from the *agr* locus and *agr*-target genes. Down-regulation of SarU by SarT results in repression of RNAIII, which in turn is also able to repress expression of *sarT*. This therefore constitutes a feedback loop, which may represent an alternative pathway for virulence gene regulation *in vivo* to that of *agr*

(Bronner *et al.* 2004). Originally named "repressor of toxin", the regulatory protein Rot in fact positively regulates 86 genes and negatively regulates 60 genes (Saïd-Salim *et al.* 2003). Rot generally has opposite effects on gene expression to *agr*, for example, Rot upregulates expression of protein A and the serine proteases SspB and SspC (down-regulated by *agr*) and down-regulates α-toxin and γ-haemolysin, which are upregulated by *agr*.

In addition to Agr and SarA, *S. aureus* contains several other regulatory systems. The two-component system SaeRS (*S. aureus* exoprotein expression) activates the expression of several virulence factors, including serine protease SspA, coagulase, α-haemolysin, β-haemolysin, Eap, Emp, protein A and FnbA (Rogasch *et al.* 2006). The *sae* locus consists of *saeR* which encodes the response regulator and *saeS* which encodes the sensor histidine kinase and SaeRS regulates gene expression primarily at the transcriptional level (Giraudo *et al.* 1997). SaeRS plays an important role in virulence during infection, inactivation of this regulatory system attenuated virulence in a murine infection model, as well as eliminating adhesion and internalisation of bacteria into lung epithelial cells (Liang *et al.* 2006).

Other two-component systems also regulate virulence factor expression, such as *arlRS* (autolysis-related locus) which is transcribed from exponential to post-exponential growth phase and consists of the response regulator ArlR and sensor protein ArlS (Bronner *et al.* 2004). In addition to the control of the potentially destructive autolysins, ArlRS also regulates expression of Ser-Asp-rich bone sialoprotein-binding proteins, cysteine protease, serine protease, protein A, β-haemolysin and leukotoxins (Liang *et al.* 2005). The *srrAB* (staphylococcal respiratory response) system is involved in adaptation to anaerobic growth of *S. aureus*, a common feature during infection, and is involved in the regulation of toxic shock syndrome toxin 1 (TSST-1) and protein A (Yarwood *et al.* 2001).

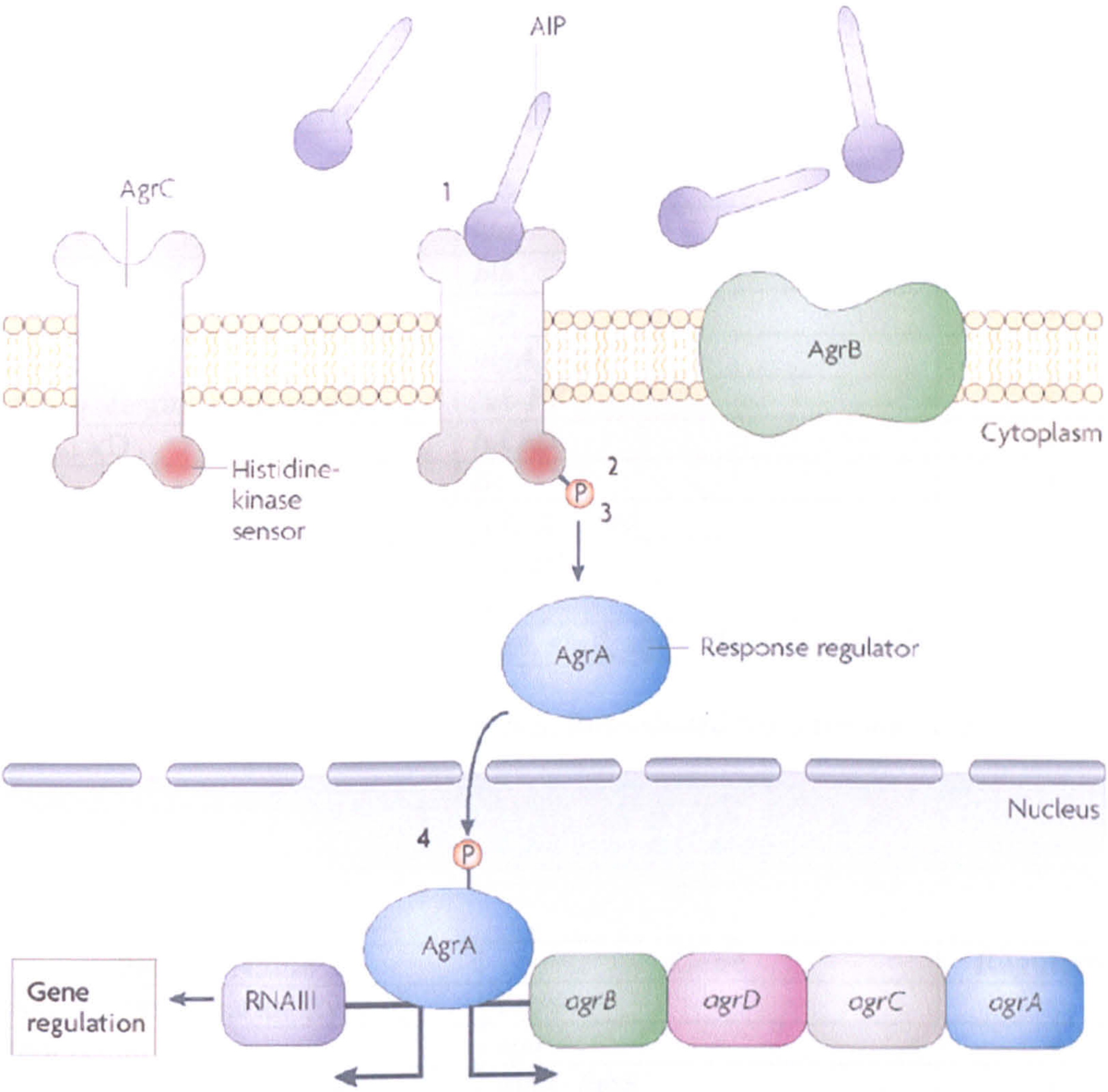


Figure 1.4: The *agr* two-component system in *S. aureus*. Autoinducing peptide (AIP) accumulates extracellularly and activates the *agr* system. The histidine kinase (AgrC) [1] recognises the signal, followed by histidine phosphorylation [2] and phosphotransfer to the response regulator (AgrA) [3], which then binds to the RNAIII transcript that encodes a small RNA that functions to modulate gene expression [4]. Reproduced from Cegelski *et al.* (2008).

Protein Name	Gene	Regulation
Capsular polysaccharide (type 5)	*cap5*	+
Capsular polysaccharide (type 8)	*cap8*	+
Protein A	*spa*	–
Fibronectin binding proteins	*fnbA, fnbB*	–
α-toxin	*hla*	+
β-toxin	*hlb*	+
δ-toxin	*hld*	+
γ-toxin	*hlgA, hlgCB*	+
Panton-Valentine Leukocidin	*luk-PV*	+
LukE-LukD	*lukED*	+
TSST-1	*tst*	+
Enterotoxins B, C, D	*seb, sec, sed*	+
Exfoliatin A and B	*eta, etb*	+
V8 serine protease	*sspA*	+
Proteases SlpA, B, C, D, E, F	*slpA, slpB, slpC, slpD, slpE, slpF*	+

Table 1.3: Examples of *agr*-regulated genes. Reproduced from Bronner *et al.* (2004).

Protein Name	Gene	Regulation
Capsular polysaccharide (type 8)	*cap8*	+
Protein A	*spa*	–
Fibronectin-binding proteins	*fnbA, fnbB*	+
Collagen adhesin	*cna*	–
AgrA, B, C, D	*agrA, agrB, agrC, agrD*	+
RNAIII		+
α-toxin	*hla*	+
δ-toxin	*hld*	+
γ-toxin	*hlgA, hlgCB*	+
Panton-Valentine Leukocidin	*lpv*	+
LukE-LukD	*lukED*	+
TSST-1	*tst*	+
Enterotoxin B	*seb*	+
V8 serine protease	*sspA*	–

Table 1.4: Examples of *sarA*-regulated genes. Reproduced from Bronner *et al.* (2004).

In addition to the two-component systems and SarA homologues, *S. aureus* also possesses an alternative sigma factor, σ^B, a regulator of a wide range of virulence genes, including *coa*, *sarA*, *fnbA*, *clfA*, *sspA*, *hla* and *seb* (Shaw *et al.* 2006). The regulation of σ^B activity is modulated by the gene products of *rsbU*, *rsbV* and *rsbW*. RsbW acts as an anti-sigma factor by binding σ^B to form a RsbW-σ^B complex, however, RsbV competes with σ^B to bind RsbW, releasing active σ^B (Bronner *et al.* 2004). Many laboratory strains of *S. aureus*, including the 8325-4 lineage, contain deletions in the *rbsU* gene, restoration of which results in a dramatic decrease in the production of exotoxins, including α-haemolysin and the major proteases (Horsburgh *et al.* 2002b).

1.7.2. Regulation of *S. pyogenes* virulence determinants

Throughout the exponential phase of growth of *S. pyogenes*, the multiple gene regulator Mga activates expression of virulence factors associated with adherence of bacteria to the extracellular matrix and epithelial cells (e.g. M protein, SOF), as well as molecules involved in the subversion of the immune response (e.g. M protein, C5a peptidase, Sic), thereby enhancing the early colonisation of the pathogen in the host (Kreikemeyer *et al.* 2003). When nutrients become exhausted and conditions more unfavourable for growth, bacteria enter the stationary phase of growth and the Mga-controlled pathways are repressed by the upregulation of the RofA-like protein (RALP) family of response regulators. The RALP family consists of four homologous members, RofA, Nra, RALP-3 and RALP-4, which together control bacterial-host cell interactions and avoidance of host cell damage (Kreikemeyer *et al.* 2003). Genes regulated by this family include SfbI, streptolysin S, SpeB, SpeA and Mga. Upregulation of genes by RALP and downregulation of the exponential phase regulator Mga allows the transition from colonisation to persistance and spread of invading *S. pyogenes* throughout the host. During late stationary phase, the transcriptional regulator Rgg (also known as RopB), whilst maintaining the downregulation of Mga, upregulates expression of the cysteine protease SpeB, resulting in complete loss of adhesins/invasins and lysis of host cells to allow spread to other tissue sites (Kreikemeyer *et al.* 2003).

In addition to the "stand-alone" virulence regulators, *S. pyogenes* also expresses several two component systems (TCS), including Ihk/Irr, FasBCAX and CsrRS (CovRS). Ihk/Irr is homologous to the *S. aureus* TCS ArlSR and is involved in the evasion of PMN-mediated killing (Federle *et al.* 1999; Voyich *et al.* 2003). FasBCAX consists of two sensor histidine kinases and one response regulator and its activity leads to the downregulation of adhesins and the upregulation of secreted aggressins at the end of exponential growth (Kreikemeyer *et al.* 2001). The most characterised of the streptococcal TCSs is CsrRS (CovRS). CsrRS regulates expression of streptolysin S, streptokinase and SpeB, in addition to the genes of the hyaluronic capsule, a crucial virulence determinant during adhesion (Kreikemeyer *et al.* 2003).

1.8 Identification of bacterial virulence factors

Gram positive pathogens such as staphylococci and streptococci cause significant morbidity and mortality world-wide. Infections by these organisms result in high numbers of hospitalised patients and the threat of highly antibiotic resistant strains, particularly associated with *S. aureus*, warrants the development of preventative measures to combat these infections. In particular, specific protection of high risk individuals, such as health care workers, patients facing surgery, implant recipients and immunocompromised patients, is necessary to reduce the morbidity associated with these infections. Identification of bacterial virulence factors and putative antigenic targets is fundamental for the development of effective vaccines. In particular, the identification of antigens expressed *in vivo* is key to finding an appropriate target, as many vital genes would be missed using *in vitro* methods, which do not represent the complex environment encountered by a pathogen during infection.

1.8.1. Proteomics

Proteomics is the study of the function, regulation and expression of proteins under different conditions, and can be used to elucidate virulence determinants of bacteria, which may represent potential novel targets for drug design. Strains exhibiting altered

modes of pathogenicity can be compared via protein differential display using two-dimensional (2-D) gel electrophoresis in association with mass spectrometry to enable the identification and characterisation of proteins (Cordwell, 2006).

Proteomics techniques have successfully been utilised to identify 25 proteins differing in expression levels between the virulent *Mycobacterium tuberculosis* H37Rv and the non-virulent *M. bovis* BCG (Jungblut *et al.* 1999), and to identify several proteins of *Helicobacter pylori* which were reactive with pooled sera from infected patients, including urease B subunit, enolase and catalase (McAtee *et al.* 1998).

Proteome analysis has also been used in the identification of potential *S. aureus* vaccine candidate antigens (Vytvytska *et al.* 2002). Sera from healthy individuals and patients suffering from *S. aureus* infections were screened for antibodies against staphylococcal lysates and recombinant proteins representing surface antigens. 2-DE immunoblotting using *S. aureus* cell wall protein preparations and the human sera revealed a number of highly immunogenic staphylococcal proteins, which were isolated and analysed by mass spectrometry. This led to the identification of 15 proteins including known and novel vaccine candidates, demonstrating that serological proteome analysis is a powerful tool for the identification of novel staphylococcal antigens (Vytvytska *et al.* 2002).

1.8.2. Identification of *in vivo* expressed antigens

Techniques using human immunogenicity as a primary means of selection on a genome-wide basis are particularly valuable for the selection of putative antigenic targets. Indeed, antibodies that are induced in the host provide molecular proof of the *in vivo* expression of the corresponding antigenic proteins (Meinke *et al.* 2004). These antigenome techniques have been utilised in several studies to identify antigenic components of human pathogens. For example, several *in vivo*-expressed antigens in an *S. aureus* MRSA strain were identified by Etz *et al.* (2002). *S. aureus* peptide libraries were displayed on the surface of *E. coli* via fusion to one of two outer membrane proteins and probed with sera selected for high antibody titre and opsonic activity. This method identified 60

antigenic proteins, a considerable fraction of which were surface and secreted proteins, including the fibronectin binding proteins FnbpA and FnbpB (Etz *et al.* 2002). Similar immunoscreening using sera from patients with septicaemia due to an epidemic strain of MRSA (EMRSA-15) to probe an EMRSA-15 genomic library resulted in the identification of an immunodominant ABC transporter protein (Burnie *et al.* 2000). An antibody preparation against this protein is currently being evaluated as an immunotherapeutic to be used in conjunction with the antibiotic vancomycin (NeuTec Pharma).

Xu *et al.* (1997) constructed genomic libraries of two *Enterococcus faecalis* strains and screened these with serum from a rabbit immunised with surface proteins of an *E. faecalis* endocarditis isolate and sera from four patients with enterococcal endocarditis. This identified a number of antigens, including bacterial virulence factors, transporters, two-component regulators, metabolic enzymes and cell surface proteins (Xu *et al.* 1997). A similar method was utilised by Lang *et al.* (2000) to identify antigens of *Staphylococcus epidermidis*. A genomic DNA library, constructed using a Lambda Zap Express cloning vector, was screened using serum from a patient with *S. epidermidis* infective endocarditis. This led to the identification of a novel antigen, named SsaA, thought to have a role in virulence (Lang *et al.* 2000).

More recently, antigenome technology was used to identify the range of antigens of both *Staphylococcus* and *Streptococcus* species (Meinke *et al.* 2005). Comprehensive peptide libraries were constructed and displayed on the surface of *E. coli*, which were then probed with high-titre sera from patients either infected with or asymptomatically carrying the appropriate pathogen. Reactive clones were selected, sequenced and immunogenicity confirmed by Western blot analysis. This resulted in the identification of approximately 800 antigens of the five staphylococcal and streptococcal species screened, with a bias towards proteins involved in cell envelope and cellular processes/pathogenesis, as expected for these predominantly extracellular pathogens. This included previously identified protective antigens, such as PspA, M1 protein, Sip and ClfA (Meinke *et al.* 2005).

In two similar studies, *in vivo*-expressed antigens of *S. aureus* and *S. epidermidis* were identified by probing bacteriophage expression libraries with serum samples from infected and uninfected individuals (Clarke *et al.* 2006; Pourmand *et al.* 2006). In *S. aureus*, eleven putative antigens were selected for further analysis and recombinant proteins were produced in order to determine the specific antibody titres in a large collection of human serum samples. Seven of these antigens demonstrated significantly increased concentrations of reactive IgG in serum from diseased patients compared to those in healthy individuals. Furthermore, significantly higher concentrations of reactive IgG to four antigens, including IsdA and IsdH (Chapter 1.3.1), were found in serum samples from non-nasal-carriers of *S. aureus*, compared with those in healthy carriers. Subsequent work revealed that vaccination of cotton rats with IsdA protected against nasal carriage and that this antigen is required for nasal colonisation, making IsdA a promising vaccine candidate (Clarke *et al.* 2006). In *S. epidermidis*, 53 antigenic loci were identified, including members of a novel family of proteins which have homologues in *S. aureus*. Analysis of three selected antigens, AtlE, ScaB and GehD, by screening human serum samples, revealed that there were significant increases in the amounts of reactive IgG in infected individuals compared to healthy individuals. Furthermore, vaccination of mice with recombinant antigens stimulated an immune response which, *in vitro*, opsonised *S. epidermidis* (Pourmand *et al.* 2006). This demonstrates that these antigens may have important functions in disease and may constitute potential targets for immunotherapy.

1.9 Therapeutic antibody and vaccine development

Several approaches against *S. aureus* are being developed. StaphVAX™ is a bivalent polysaccharide and protein-conjugated vaccine, directed against the two most prevalent capsular polysaccharides, types 5 and 8, which are associated with 80 – 90% of *S. aureus* clinical infections (Jones, 2002). During Phase III clinical studies in haemodialysis patients, a single immunisation of StaphVAX™ was demonstrated to prevent *S. aureus* bacteraemia for up to ten months. The vaccine induced high levels of capsular

polysaccharide-specific antibodies within 10-14 days, suggesting the potential to prevent *S. aureus* infections in patients with short-term risk, such as those facing surgery (Fattom *et al.* 2004). However, the vaccine failed to provide significant protection against *S. aureus* bacteraemia after one year, the primary end point of the phase III study.

More recently, a prophylaxis has been developed against *S. aureus* consisting of a humanised monoclonal antibody (MAb) that recognises the surface MSCRAMM ClfA (Chapter 1.3.1). Passive immunisation with this MAb, named Aurexis® (tefibazumab), was demonstrated to significantly protect against intravenous MRSA challenge in murine septicaemia and rabbit infective endocarditis (IE) models (Patti, 2004; Domanski *et al.* 2005), and was reported to be well-tolerated and effective in human trials (Weems *et al.* 2006).

Approaches to vaccination against streptococcal species primarily focused on the surface M protein, a major virulence factor present throughout this genus. Antibodies to the amino terminus of the M protein have been demonstrated to be opsonic and provide protection against infection by streptococci of the same M protein type. However, these antibodies are generally type-specific as antibodies directed against the amino terminus of one M-type do not protect against infection from other M-types (McMillan *et al.* 2004a). Vaccines based on M proteins are hindered by the highly antigenically variable amino terminus of the M protein, with over 100 *emm*-types reported (McMillan *et al.* 2004b). Furthermore, some M protein epitopes are immunologically cross-reactive with human heart proteins and have been implicated in pathology of rheumatic heart disease. The carboxyl terminal of the M protein has also been the focus of vaccination studies as this region is more conserved between strains, however, the carboxyl terminus is less immunogenic and less opsonic than the amino terminus (McMillan *et al.* 2004a).

In attempt to combat these difficulties, a vaccine against *S. pyogenes* was developed which contained amino-terminal M protein fragments from 26 different serotypes (Hu *et al.* 2002). These included the majority of serotypes which cause streptococcal pharyngitis, those frequently isolated from cases of invasive disease, and those associated

with acute rheumatic fever (Dale, 2008). This had the aim of developing a vaccine to have a significant impact on the total disease burden caused by *S. pyogenes* in North America and Western Europe. The 26-valent vaccine was shown to be immunogenic in rabbits and evoke broadly opsonic antibodies against the majority of serotypes. More recently, the vaccine was demonstrated to be well-tolerated and immunogenic in adult volunteers, and is currently being considered for pediatric trials, the primary target group for the vaccine (Dale, 2008).

Several studies have focused on the identification of *S. pyogenes* vaccine candidates other than the M protein. These include the adhesin SfbI, which demonstrated 80% and 90% protection against challenge with homologous and heterologous strains of *S. pyogenes*, respectively, after intranasal immunisation of mice with the protein (Guzmán *et al.* 1999). Serum opacity factor (SOF) was shown to evoke bactericidal antibodies, which can opsonise both homologous and heterologous SOF-positive streptococci (approximately 45% of strains of *S. pyogenes*) in human blood and protect mice against streptococcal infection (Courtney *et al.* 2003). The fibronectin-binding protein FBP54, which is present in all strains of *S. pyogenes*, was used to immunise mice both orally and subcutaneously, resulting in significantly longer survival after challenge with *S. pyogenes* (Kawabata *et al.* 2001).

Vaccines against *S. pneumoniae* are currently in use. A 23-valent polysaccharide vaccine (PPV) has been available since 1983 and is effective in reducing invasive pneumococcal disease among adults and the elderly. However, the PPV vaccine is ineffective in children under 2 years old and in immunocompromised patients (McGee, 2007). A 7-valent conjugative vaccine (PCV-7) was introduced in the USA in 2000 and in Europe in 2001. This vaccine includes the most common serotypes in children, accounting for approximately 80% of strains of *S. pneumoniae*. Following the introduction of PCV-7 in the routine immunisation of US children, a signficant decrease was seen in the incidence of invasive pneumococcal diseases, acute otitis media, community-acquired respiratory infections and nasopharyngeal colonisation (Leibovitz, 2008). However, introduction of this vaccine also resulted in an increase in carriage of replacement serotypes of *S.*

pneumoniae, some of which are antibiotic-resistant, which may concerningly lead to replacement disease (McGee, 2007; Leibovitz, 2008).

1.10 Aims of the project

The primary aim of this project was to identify antigens expressed by *S. pyogenes* which were cross-reactive with *S. aureus*-infected patient sera. Results from this were then combined with results of four previous screens using *S. aureus*, *S. epidermidis*, *S. agalactiae* and *S. pneumoniae*. Comparative analyses allowed the identification of putative conserved and cross-reactive antigens amongst these Gram-positive pathogens. These conserved protein fragments were further characterised to assess their roles as vaccine targets, including both opsonisation and protection studies.

The secondary aim of the project was to further characterise conserved proteins which were identified during the immunoscreening process. This included overexpression of recombinant proteins to determine their activities and mutant analysis of one specific member of a large conserved family in order to elucidate its role.

Chapter 2

Materials and Methods

2.1 Media

2.1.1. 2YT

Tryptone	16 g l^{-1}
Yeast	10 g l^{-1}
NaCl	5 g l^{-1}

Oxoid Agar No. 1 (1% (w/v)) was added for 2YT agar.

0.7% (w/v) agarose was added for 2YT top agarose.

2.1.2. B2

Casein acid hydrolysate	10 g l^{-1}
Yeast extract	25 g l^{-1}
K_2HPO_4	1 g l^{-1}
NaCl	25 g l^{-1}

The pH was adjusted to 7.5. Once autoclaved and cooled, sterile glucose was added to a final concentration of 1 mM.

2.1.3. Brain heart infusion (BHI)

Brain heart infusion (BHI)	37 g l^{-1}

Oxoid Agar No. 1 (1.5% (w/v)) was added for BHI agar.

2.1.4. Luria-Bertani (LB) (Sambrook *et al.* 1989)

Tryptone	10 g l^{-1}
Yeast extract	5 g l^{-1}
NaCl	10 g l^{-1}

The pH was adjusted to 7.2 using NaOH. Oxoid Agar No. 1 (1.5 % (w/v)) was added for LB agar.

2.1.5. LK (modified LB with potassium)

Tryptone	10 g l^{-1}
Yeast extract	5 g l^{-1}
KCl	7 g l^{-1}

Oxoid Agar No. 1 (1.0 % (w/v)) was added for LK bottom agar. Oxoid Agar No. 1 (0.7 % (w/v)) was added for LK top agar.

2.1.6. Phage agar

Casamino acids	3 g l^{-1}
Yeast extract	3 g l^{-1}
NaCl	5.9 g l^{-1}

Oxoid Agar No. 1 (1.0 % (w/v)) was added for phage bottom agar. Oxoid Agar No. 1 (0.33 % (w/v)) was added for phage top agar.

2.1.7. Super optimal broth (SOB)

Tryptone	2% (w/v)
Yeast extract	0.5% (w/v)
NaCl	10 mM
KCl	2.5 mM

Once autoclaved and cooled, filter-sterilsed supplements of $MgCl_2$ and $MgSO_4$ were added, each to 10 mM (final concentration).

2.1.7.1. Super optimal broth with catabolite repression (SOC)

SOC was prepared by the addition of sterile glucose (to 20 mM) to SOB.

2.1.8. Terrific broth (TB)

Tryptone	12 g l^{-1}
Yeast	24 g l^{-1}
Glycerol	0.4 % (v/v)

The media was autoclaved and cooled, then 0.17 M KH_2PO_4 / 0.72 M K_2HPO_4 was added.

2.1.9. Todd-Hewitt Broth (THY)

Todd-Hewitt broth	36.4 g l^{-1}
Yeast extract	2 g l^{-1}

The pH was adjusted to 7.4 using 0.1 M Tris-Cl (pH 10.0).

2.1.10. Tryptic Soy Broth (TSB)

Tryptic soy broth (TSB)	30 g l^{-1}

Oxoid Agar No. 1 (1.5% (w/v)) was added for TSB agar.

2.1.10.1. Iron-limited TSB

For iron-limited TSB, 10g Chelex-100 was added to 1 l TSB and stirred overnight at 4°C. The Chelex-100 was decanted by filtration and the media sterilised by autoclaving. 20 µM Dipyridyl and 400 µl 1 M $MgSO_4$ were then added to the media.

2.2 Antibiotics

All antibiotics used in this study are listed in Table 2.1. Stock solutions were filter-sterilised using 0.2 µm pore size and stored at -20°C. Concentrations used are also listed in Table 2.1.

2.3 Buffers and stock solutions

2.3.1. Phosphate buffered saline (PBS)

NaCl	8 g l^{-1}
Na_2HPO_4	1.4 g l^{-1}
KCl	0.2 g l^{-1}
KH_2PO_4	0.2 g l^{-1}

The pH was adjusted to 7.4 using HCl.

2.3.2. PBS-T

PBS + Tween-20	0.1% (v/v)

Antibiotic	Stock Concentration (mg ml^{-1})	Working Concentration in *E. coli* (µg ml^{-1})	Working Concentration in *S. aureus* (µg ml^{-1})
Ampicillin (Amp)	50[a]	100	-
Erythromycin (Ery)	5[b] or 75[b]	300	5
Kanamycin (Kan)	50[a]	50	50
Lincomycin (Lin)	25[c]	-	25
Neomycin (Neo)	50[a]	-	50
Spectinomycin (Spc)	100[a]	100	-
Tetracycline (Tet)	5[c]	12.5	5

Table 2.1: Antibiotics used in this study. Stock concentrations of antibiotics were dissolved in dH_20[a], 95% (v/v) ethanol[b], or 50% (v/v) ethanol[c], filter-sterilised and stored at -20 °C. The inducing concentration of Ery was 0.15 µg ml^{-1}.

2.3.3. Tris buffered saline (TBS)

Tris	2.4 g l^{-1}
NaCl	29.2 g l^{-1}

The pH was adjusted to 7.5 using HCl.

2.3.4. TBS-T

TBS + Tween-20	0.1% (v/v)

2.3.5. Phage buffer

$MgSO_4$	1 mM
$CaCl_2$	4 mM
Tris-Cl (pH7.8)	50 mM
NaCl	5.9 g l^{-1}
Gelatin	1 g l^{-1}

2.3.6. SM buffer

NaCl	5.8 g l^{-1}
$MgSO_4.7H_2O$	4 g l^{-1}
Tris-Cl (pH7.5)	50 mM

2.3.7. HiTrap™ buffers

2.3.7.1. START buffer

$NaPO_4$ (pH 7.4)	0.02 M
NaCl	0.5 M
+/- Urea	8 M

2.3.7.2. Elution buffer

$NaPO_4$ (pH 7.4)	0.02 M
NaCl	0.5 M
Imidazole	0.5 M
+/- Urea	8 M

2.3.8. QIAGEN buffers

The composition of QIAGEN buffers is detailed in the manufacturer's instructions provided with the kits.

2.3.9. SDS-PAGE buffers

2.3.9.1. Sample prep buffer

Tris / HCl pH 6.8	0.125 M
Sucrose	10% (w/v)
SDS	4% (w/v)
Bromophenol blue	1 g l^{-1}

β-mercaptoethanol (7% (v/v)) was added just before use.

2.3.9.2. SDS-PAGE gel formulations and construction of gel

The following components were added together in a sterile 30 ml universal:

17.5% (w/v) Resolving gel:

30% (w/v) Acrylamide/Bis (37.5:1) (BioRad)	3.275 ml
3M Tris-Cl (pH8.3)	0.95 ml
dH_2O	3.2 ml
10% (w/v) Ammonium persulphate	100 µl
10% (w/v) SDS	75 µl
TEMED	10 µl

The components were gently mixed and pipetted into prepared gel-casting apparatus. A layer of isopropanol was pipetted onto the top of the gel to isolate it from the air. Once set, the isopropanol was removed and the gel washed with dH_2O. The following components were then added together in a sterile 30 ml universal:

Stacking gel:

30% (w/v) Acrylamide/Bis (37.5:1) (BioRad)	0.83 ml
3M Tris-Cl (pH8.3)	1.25 ml
dH_2O	2.88 ml
10% (w/v) Ammonium persulphate	100 µl
10% (w/v) SDS	50 µl
TEMED	10 µl

The components were gently mixed and pipetted on top of the resolving gel. A plastic comb was inserted into the stacking gel to create wells and to separate the gel from the air. Once the gel had solidified, the comb was removed and the gel was transferred to a gel-running tank, submerged in SDS-PAGE electrophoresis buffer.

2.3.9.3. SDS-PAGE electrophoresis buffer

Tris	3 g l^{-1}
Glycine	14.4 g l^{-1}
SDS	1 g l^{-1}

2.3.9.4. Coomassie blue staining solution

Coomassie B blue (R250)	1.15 g l^{-1}
Methanol	40% (v/v)
Acetic acid	10% (v/v)

2.3.9.5. Destain solution

Acetic acid	10% (v/v)
Methanol	5% (v/v)

2.3.9.6. Renaturing gel incubation buffer

Triton X-100	1 ml
1M $MgCl_2$	10 ml
1M sodium citrate (pH5)	25 ml
dH_2O	to 1 l

2.3.9.7. 10X Renaturing gel stain

Methylene blue	2 g
2 M KOH	1.79 ml
dH_2O	to 200 ml

10X renaturing stain was diluted to 1X using dH_2O prior to use.

2.3.10. Western blotting solutions

2.3.10.1. Transfer buffer for N-terminal sequencing

100 mM CAPS buffer (pH11)	10% (v/v)
Methanol	10% (v/v)

2.3.10.2. Transfer buffer for antibody detection

Tris	3 g l^{-1}
Glycine	14.4 g l^{-1}
Methanol	20% (v/v)

2.3.10.3. Blocking solution

Western blocking solution consisted of TBS-T containing 6% (w/v) dried skimmed milk powder (Marvel).

2.3.10.4. Primary antibody solution

The primary antibody was diluted 1:1000 in blocking solution for anti-sera and stored at -20°C.

2.3.10.5. Secondary antibody solution

The appropriate secondary antibody was diluted 1:30,000 in TBS-T.

2.3.10.6. Alkaline phosphatase (AP) buffer

Tris / HCl pH 9.5	0.1 M
NaCl	5.8 g l^{-1}
$MgCl_2.6H_2O$	10.2 g l^{-1}

2.3.10.7. Colour substrate solution

160 µl NBT/BCIP stock solution (Roche) was diluted in 25 ml AP buffer.

2.3.11. Southern blotting solutions

2.3.11.1. 50X TAE

Trisma base	242 g l^{-1}
Glacial acetic acid	57.1 ml l^{-1}
Na_2EDTA (0.5 M pH 8.0)	100 ml l^{-1}

Before use the buffer was diluted 1:50 to produce TAE.

2.3.11.2. Depurination solution

HCl	250 mM

2.3.11.3. Denaturation solution

NaOH	0.5 M
NaCl	1.5 M

2.3.11.4. Neutralisation solution

Tris-Cl	0.5 M
NaCl	3 M

The pH was adjusted to 7.5.

2.3.11.5. 20X SSC

NaCl	3 M
Sodium citrate	300 mM

The pH was adjusted to 7.0 with 1M NaOH. 20X SSC was diluted with dH_2O to make 10X, 5X and 0.5X SSC.

2.3.11.6. Standard prehybridisation buffer

SSC	5X
N-lauroylsarcosine	0.1% (w/v)
SDS	0.02% (w/v)
Blocking reagent	1% (w/v)

2.3.11.7. Hybridisation solution

The DNA probe was diluted in prehybridisation buffer to a concentration of 5-25 ng/ml.

2.3.11.8. 2X wash solution

SDS	0.1% (w/v)
SSC	2X

2.3.11.9. 0.5X wash solution

SDS	0.1% (w/v)
SSC	0.5X

2.3.11.10. Maleic acid buffer

Maleic acid	0.1 M
NaCl	0.15 M

The pH was adjusted to 7.5 and the buffer autoclaved prior to use.

2.3.11.11. Washing buffer

0.3% (v/v) Tween was dissolved in maleic acid buffer.

2.3.11.12. Blocking solution

Maleic acid buffer containing 1 % (w/v) blocking reagent (Roche).

Blocking reagent was dissolved in maleic acid buffer by microwaving and was stored at -20°C.

2.3.11.13. Antibody solution

Blocking solution containing 0.2 µl ml^{-1} Anti-digoxigenin-AP conjugate (Roche).

2.3.11.14. Detection buffer

Tris-HCl (pH 9.5)	100 mM
NaCl	100 mM
$MgCl_2 \cdot 6H_2O$	50 mM

The pH was adjusted to 7.5 using 1 M NaOH.

2.3.11.15. TE buffer

Tris-HCl	1 mM
EDTA	0.1 mM

The pH was adjusted to 7.5 using 1 M HCl.

2.4 Bacterial Strains and Plasmids

2.4.1. Bacterial maintenance, culture and storage conditions

Bacterial strains used in this study are listed in Tables 2.2 and 2.3.

2.4.1.1. *Streptococcus* and *Staphylococcus* strains

Streptococcus and *Staphylococcus* strains (Table 2.2) were taken from glycerol stocks and grown on BHI agar plates containing antibiotics where appropriate to maintain selection of resistance markers. Plate cultures were stored for up to two weeks at 4°C, before being re-streaked from glycerol stocks. For long-term storage, a single colony

was spread onto a BHI agar plate containing relevant antibiotics and grown overnight at 37°C. A loopful of cells was resuspended in 1 ml BHI containing 15 % (v/v) glycerol in a sterile 1.5 ml microfuge tube. These glycerol stocks were then snap-frozen in liquid nitrogen and stored at -70°C. Liquid cultures were normally prepared by inoculation of culture medium with a single isolated colony. Unless otherwise stated, cultures were grown overnight in 5 ml medium in a sterile plastic 25 ml universal, and were aerated on a rotary shaker at 250 rpm. These cultures were then used to inoculate 250 ml conical flasks (culture : flask volume ratio 1:2.5), and were aerated on a rotary shaker at 250 rpm. All *S. aureus* plates or liquid medium cultures were grown at 37°C unless otherwise stated. These conditions are referred to as standard conditions.

2.4.1.2. *Escherichia coli* strains

Escherichia coli strains (Table 2.3) were cultured, at 37°C, using LB broth or LB agar containing antibiotics where necessary to ensure selection of plasmids. Plate cultures were stored at 4°C for up to two weeks before re-streaking from glycerol stocks. For long-term storage, glycerol stocks of *E. coli* strains were prepared as for *S. aureus* (Chapter 2.4.1.1), using LB medium rather than BHI.

2.4.2. Plasmids

Plasmids used in this study are listed in Table 2.4. Plasmid DNA was purified using QIAGEN plasmid kits according to the manufacturer's protocol. Purified plasmid DNA was stored in EB buffer at -20°C.

2.4.3. Bacteriophage

Bacteriophage ϕ11 (Mani *et al.* 1993) and ϕ85 were used for phage transduction of *S. aureus* (Chapter 2.X). These phage are *S. aureus*-specific, temperate, transducing phage of serological group B, and require Ca^{2+} ions for maintenance of infection in bacterial cells. The genome size of ϕ11 is approximately 45 kb (Novick, 1990).

Strain	Relevant Genotype / Markers	Source / Reference
S. pyogenes HSC-5	M5 serotype group A streptococcus	Lab stock
S. agalactiae 6313	Serotype III group B streptococcus	Lab stock
S. pneumoniae	Serotype III clinical isolate	Lab stock
S. aureus 8325-4	8325 cured of known prophages	Novick (1963)
S. aureus SH1000	Functional *rbsU*$^+$ derivative of 8325-4	Horsburgh *et al.* (2002b)
S. aureus spa	*spa::tet* Tc^r in SH1000 background	Harris *et al.* (2002)
S. aureus RN4220	Restriction deficient transformation recipient	Kreiswirth *et al.* (1983)
S. aureus MS014	RN4220 P_{spac}-*yycFG*	Dubrac and Msadek (2004)

Table 2.2: *Streptococcus* and *Staphylococcus* strains used in this study.

Strain	Relevant Genotype / Markers	Source / Reference
BL21 (DE3)	*F*$^-$ *ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm* (DE3)	Novagen
EL250	*F*$^-$ *mcrA* Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80 *lacZ* ΔM15 Δ*lac*X74 *rec*A1 *deo*R *rec*A1 *end*A1 *ara*D139 Δ(*ara-leu*)7649 *gal*U *gal*K *rsp*L *nup*G	Lab stock
Top10	*F*$^-$ *mcrA* Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80 *lacZ* ΔM15 Δ*lac*X74 *rec*A1 *deo*R *ara*D139 Δ(*ara-leu*)7697 *gal*K *rsp*L (Str^r) *end*A1 *nup*G	Invitrogen
XLOLR	*Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 thi-1 recA1 gyrA96 relA1 lac* [F′ *proAB lacI*qZΔ*M15* Tn*10* (Tetr)] Su- (nonsuppressing) λr (lambda resistant)	Stratagene
XL1-Blue	MRF′ strain Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1recA1 gyrA96 relA1 lac* [F′ *proAB lacI*qZΔ*M15* Tn*10* (Tetr)]	Stratagene
GroD	BL21 containing pREP4groESL and pETDa	This Study
GroDN	BL21 containing pREP4groESL and pETDna	This Study
GroDC	BL21 containing pREP4groESL and pETDca	This Study
ELD2	EL250 containing pTOPD2	This Study

Table 2.3: *E. coli* strains used in this study.

Plasmid Name	Relevant Genotype	Marker	Source / Reference
pBK-CMV	Phagemid vector containing MCS flanked by T3 and T7 promoters	Kan^R	Novagen
pET-24d	6x His overexpression vector	Kan^R	Novagen
pET-21d	6x His overexpression vector	Amp^R	Novagen
pETC	pET-24d containing *scaC* without the signal peptide	Kan^R	This Study
pETD	pET-24d containing *scaD* without the signal peptide	Kan^R	This Study
pETE	pET-24d containing *scaE* without the signal peptide	Kan^R	This Study
pETF	pET-24d containing *scaF* without the signal peptide	Kan^R	This Study
pETG	pET-24d containing *scaG* without the signal peptide	Kan^R	This Study
pETJ	pET-24d containing *scaJ* without the signal peptide	Kan^R	This Study
pETDa	pET-21d containing *scaD* without the signal peptide	Amp^R	This Study
pETDn	pET-24d containing *scaD* N-terminal 130 amino acids without the signal peptide	Kan^R	This Study
pETDc	pET-24d containing *scaD* C-terminal 110 amino acids	Kan^R	This Study
pREP4groESL	Overexpression plasmid for chaperones GroES and GroEL	Kan^R	Amrein *et al.* (1995)
pETDna	pET-21d containing *scaD* N-terminal 130 amino acids without the signal peptide (393 bp)	Amp^R	This Study
pETDca	pET-21d containing *scaD* C-terminal 110 amino acids (327 bp)	Amp^R	This Study
pOB	pGEM3Zf(+) *erm* containing cloning vector	Amp^R / Ery^R	Horsburgh *et al.* (2002a)
pCR®2.1-TOPO®	Cloning vector allowing insertions via *Taq*-mediated TOPO® Cloning reaction	Amp^R / Ery^R	Invitrogen
pTOPD2	pCR®2.1-TOPO® containing 2.5 kb *scaD* region	Amp^R / Kan^R	This Study
pGL400	Vector containing P*spac* promoter and *tet* resistance gene	Amp^R / Ery^R / Tet^R	García-Lara, unpublished
pGL456	Cloning vector containing *amp* and *ery*	Amp^R / Ery^R	García-Lara, unpublished

pGL439	Vector containing P*spac* promoter and *tet* resistance gene, flanked by *Asc*I and *Fse*I restriction sites	Kan^R / Amp^R / Tet^R	García-Lara, unpublished
pDPs1	pGL456 containing1.3 kb *scaD* region with insertion of P_{spac} promoter and *tet* resistance marker	Amp^R / Ery^R / Tet^R	This Study
pMAD	Temperature-sensitive cloning vector with *bgaB* for colorimetric selection	Amp^R / Ery^R	Arnaud *et al.* (2004)
pGL433b	Cloning vector containing *kan* resistance gene cassette	Kan^R / Amp^R	García-Lara, unpublished
pDPs2	pMAD containing1.3 kb *scaD* region with insertion of P_{spac} promoter and *tet* resistance marker	Amp^R / Ery^R / Tet^R	This Study
pDMut1	pMAD containing inactivated *scaD* gene region with *kan* insertion	Amp^R / Ery^R / Kan^R	This Study
pMJ8426	Cloning vector containing constitutive P*pcn*	Spc^R / Tet^R	Jana *et al.* (2000)
pDComp	pMJ8426 containing *scaD* under control of P*pcn*	Spc^R / Tet^R	This Study

Table 2.4: Plasmids used in this study.

2.5 Centrifugation

A number of different centrifuges were used for the harvesting of cells and precipitated material. These were:

1) Eppendorf microfuge 5415D; max. volume 2 ml, max. speed 3200 rpm (10,000 x g).

2) Centaur 2 centrifuge (Sanyo); max. volume 50 ml, max. speed 10,000 rpm.

3) Avanti™ J251 (Beckman); max. volumes and speeds dependent on the rotor used:

JA-20: max. volume 50 ml, max. speed 20,000 rpm (48,384 x g).

JA-14: max. volume 250 ml, max. speed 14,000 rpm (30,074 x g).

JA-10.5: max. volume 500 ml, max. speed 10,000 rpm (18,480 x g).

Unless otherwise stated, centrifugation was carried out at room temperature.

2.6 Determination of bacterial cell density

2.6.1. Spectrophotometric measurement

To quantify the bacterial yield of a culture, spectrophotometric measurements at 600 nm (OD_{600nm}), were performed. These measurements were taken using a Jenway 6100 spectrophotometer. Where necessary, the culture samples were diluted 1:10 in unused sterile culture medium to give a reading below 0.8 on this type of spectrophotometer.

2.6.2. Viable cell counts (CFU)

An alternative method for the quantification of cell numbers involved viable cell counts. Bacterial samples were serially diluted 1:10 in PBS in duplicate. 100 µl samples of each dilution were spread using sterile glass beads onto duplicate BHI agar plates containing antibiotics where necessary. After overnight incubation at 37°C, the number of colony forming units (CFU) were determined.

2.7 Growth Experiments

Strains were streaked from glycerol stocks onto BHI plates containing suitable antibiotics (Chapter 2.4.1.1). 5 ml BHI in a sterile 30 ml universal tube was inoculated using a single colony and grown overnight at 37°C, with aeration on a rotary shaker at 250 rpm. The overnight pre-culture was used to inoculate 100 ml pre-warmed BHI in a 250 ml conical flask to an OD_{600nm} 0.01. The 100 ml culture was grown at 37°C, 250 rpm and the culture OD_{600nm} was determined in duplicated over the desired time period.

2.8 Cell autolysis

S. aureus was grown to mid-exponential phase (OD_{600nm} 0.5-1.0) in BHI. Cells were harvested by centrifugation (4000 rpm, 4°C, 10 min), washed in 50 mM Tris-Cl (pH 7.5) and resuspended in lysis buffer (50 mM Tris-Cl (pH 7.5) containing 0.05% (v/v) Triton-X 100). Generalised cell lysis of cells was followed spectrophotometrically while incubation at 37°C with shaking at 250 rpm.

2.9 Microscopy

2.9.1. Phase contrast microscopy

1 ml samples of cells were harvested at specific points during growth in BHI, placed in a microcentrifuge and immediately chilled on ice for 5 min. The cell samples were recovered by centrifugation for 3 min (10,000 , RT) and resuspended in 1 ml dH_2O. 100 µl was spread on a slide and allowed to air dry. Once dry, and after heat fixation, a dilute methylene blue solution (0.001 % (w/v) in dH_2O) was spread over the mounted cells and washed with dH_2O. A coverslip was placed on top immediately and the stained cells were viewed under the microscope.

2.9.2. Transmission electron microscopy

Cell pellets were fixed in Karnovsky's fixative in 100 mM sodium cacodylate buffer for 3h at 4°C. The specimens were then washed in 100 mM sodium cacodylate buffer containing 10% (w/v) sucrose 3 times with 30 min intervals at 4°C. Secondary fixation was carried out in 2 % (w/v) osmium tetroxide for 1 h at room temperature (Hayat, 1981). Dehydration was through a graded series of ethanol (75% (v/v), 15 min; 95% (v/v), 15 min; 100% (v/v), 15 min; 100% (v/v), 15 min; 100% (v/v) dried over anhydrous copper sulphate for 15 min) at room temperature. The specimens were then placed in an intermediate solvent, propylene oxide, for 2 changes of 15 min. Infiltration was accomplished by placing the specimens on propylene oxide-Spurr resin (50:50) overnight at room temperature. The specimens were then left in Spurr resin for 6-8 h at room temperature after which they were embedded in fresh Spurr resin for 8 h at 70°C (Glauert, 1974). Ultrathin sections (~70-90nm) were cut on a Reichert Ultracut E ultramicrotome and stained for 15 min with 3% (w/v) uranyl acetate-50 % (v/v) ethanol followed by staining with Reynold's lead citrate for 2 min (Watson, 1958; Reynolds, 1963). The sections were examined using a Philips CM10 Transmission Electron Microscope at an accelerating voltage of 80 kV. Electron micrographs were recorded on Kodak 4489 Electron Microscope Film.

2.10 Protein overexpression

2.10.1. Growth and induction of expression

E. coli BL21 containing the pET24-d or pET21-d overexpression plasmid with appropriate insert was grown overnight in 5 ml LB and 50 µg/ml kanamycin or 100 µg/ml ampicillin, as appropriate. 2.5 ml culture was used to inoculate 250 ml TB containing antibiotic, and grown at 37°C, 250 rpm until it reached an OD_{600nm} of 0.6-1.0. Protein overexpression was then induced using 1 mM IPTG and the culture grown for a further 4-6 h. Cells were harvested by centrifugation (6000 x g, 15 min, 4°C) and the pellet was stored at -20°C.

2.10.2. Analysis of recombinant protein solubility

Cells prepared in Chapter 2.10.1 were resuspended in 0.5 ml START buffer and 1 mg/ml lysozyme was added. The suspension was left at room temperature for 1 h before being sonicated (Sanyo Soniprep 150) three times for 10s. The suspension was then centrifuged (13,000 rpm, 10 min) and the supernatant (soluble fraction) separated from the pellet (insoluble fraction). The insoluble pellet was resuspended in 0.5 ml START buffer containing 8M urea. Both the soluble and insoluble fractions were analysed by SDS-PAGE.

2.10.3. Protein purification using HiTrap™ column

Harvested cells were resuspended in 5 ml START buffer and freeze-thawed three times (10 min at -80°C). The suspension was sonicated three times for 10s (Sanyo Soniprep 150) and centrifuged (18,000 rpm, 25 min, 4°C). The supernatant (soluble fraction) was removed and the pellet, containing the insoluble protein, was resuspended in 50 ml START buffer with 8M urea. The protein suspension was then filtered through 0.45 µm filters.

A 5 ml HiTrap™ column (Amersham Biosciences) was washed with 15 ml dH_2O, then charged with 20 ml 50mM $NiSO_4.6H_2O$. Excess $NiSO_4.6H_2O$ was washed out with 15 ml dH_2O. The Bio-Rad Econo Gradient pump and Fraction collector tubing was flushed with START buffer containing 8M urea. The charged HiTrap column was attached to the pump and equilibrated with the START buffer and 8M urea. The filtered His-tagged protein was then applied to the column and eluted using an increasing gradient of elution buffer. The eluted fractions were analysed by SDS-PAGE and purified protein was dialysed into PBS and 8M urea using 12-14 kDa cut-off dialysis membrane (Medicell International Ltd). The HiTrap™ column was washed with 10 ml 0.1M EDTA followed by 10 ml dH_2O. The column was then flushed with 10 ml 20% (v/v) ethanol and stored at 4°C.

2.10.4. N-terminal sequencing

Proteins under investigation were separated by 17.5% (w/v) SDS-PAGE gel (Chapter 2.12) and blotted onto a PVDF membrane (Chapter 2.13) as previously described but using CAPS buffer (Chapter 2.3.10.1). After the transfer procedure the membrane was soaked in dH_20 for 5 min, followed by 5 min in methanol at room temperature. The membrane was then stained with Coomassie blue staining solution for 1 min and then destained for 3 x 5 min in destain solution (50% (v/v) methanol). The blot was then dried, protein side up, on blotting paper for 1h at room temperature. The protein under investigation was then excised from the membrane and sequenced by Dr Arthur Moir (Department of Molecular Biology and Biotechnology, University of Sheffield) using an ABI 476A sequencer.

2.11 Preparation of cell walls

2.11.1. Preparation of purified cell walls

A mid-exponential phase culture (OD_{600nm} 1.0) of the required strain was harvested (11,000 g, 10 min, 4°C) and the cell pellet resuspended in ice-cold 50 mM Tris-HCl (pH 7.5). Bacterial cells were then autoclaved (15 p.s.i. for 5 min) before being disrupted. *S. subtilis* cells were disrupted by passing the cell suspension twice through a French pressure cell (Aminco, 180 MPa, 4°C). *S. luteus*, *S. aureus* and *S. epidermidis* cells were disrupted using the Braun homogeniser (Foster, 1992). Insoluble material was recovered by centrifugation (27,000 g, 10 min, 4°C) and the pellet resuspended in 30 ml 50 mM Tris-HCl (pH 7.5) plus 2% (w/v) SDS. After incubation at 50°C for 10 min and centrifugation as before, the pellet was resuspended in 30 ml of 50 mM Tris-HCl (pH 7.5) plus 3 % (w/v) SDS, 50 mM dithiothreitol (DTT) and 1mM EDTA. The suspension was boiled for 20 min, insoluble material recovered as before and the pellet resuspended and boiled once more in the same buffer. These walls were then collected by centrifugation as before and washed five times in dH_2O by centrifugation and resuspension. The concentration of the cell suspension was determined by pipetting 200

µl onto a pre-weighed weighing boat, evaporating off the liquid in a 60°C oven and weighing the resulting dried boat. The final cell wall suspension was stored at -20°C.

2.11.2. Preparation of cell wall-associated proteins

2.11.2.1. FastPrep method of cell wall preparation

Cell walls were prepared as described by Clarke *et al.* (2002). Bacteria were grown in 1 l TSB to an OD_{600nm} 1.0. Cells were harvested by centrifugation (8000 g, 10 min, 4°C). The pellet was then resuspend in 15 ml cold TBS. The cells were recovered by centrifugation (8000 g, 5 min, 4°C), and the pellet resuspended in 2 ml ice cold buffer solution (50 mM Tris-HCl (pH 7.5), 0.1M NaCl, 0.5 mM PMSF). 0.5 ml of the bacterial suspension was then transferred to a FastPrep tube (Anachem). The tubes were inserted in the FastPrep instrument (Anachem), the speed set to 6 and time to 40 s. Disruption was repeated 10 times to ensure bacteria cells were broken, with cooling on ice in between. The tubes were cooled on ice and breakage of the cells verified by light microscopy. The FastPrep beads were allowed to settle, and the supernatant / suspension removed into a clean tube. 1 ml cold 50 mM Tris-HCl and 0.1 M NaCl was added to the suspension, before centrifuging at 4000 g for 10 min at 4°C. The supernatant was retained and 5 ml cold 50 mM Tris-HCl and 0.1 M NaCl added to the pellet. After mixing, the insoluble material was recovered by centrifugation (15,000 g, 10 min, 4°C). The pellets were resuspend in 1 ml ice cold 50 mM Tris-HCl (pH 7.5), and recovered by centrifugation (as above) to give native cell walls.

2.11.2.2. Ionically bound proteins

The native cell wall material was resuspended in 20 µl SDS sample buffer with 5.6% (v/v) BME (added just before use). The suspension was boiled for 3 min, and cooled at RT for 5 min. Insoluble material was removed by centrifugation (13,000 g, 5 min, RT) and the supernatant retained.

2.12 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

2.12.1. Mini Protean SDS-PAGE

Discontinuous SDS-PAGE was performed according to Laemmli (1970) using BioRad Mini-Protean II gel slabs (70 mm (L) x 80 mm (W) x 0.75 mm thickness), following the manufacturer's instructions (BioRad). Reagents for SDS-PAGE were prepared as described in Chapter 2.3.9. Samples were diluted at least 1:4 with sample buffer and heated at 100°C for 10 min. Electrophoresis was performed at room temperature with constant voltage of 200 V for 1 h.

2.12.2. Coomassie blue staining

After electrophoresis, protein gels were placed in approximately 30 ml Coomassie blue stain (Chapter 2.3.9.4) for 30-60 min. They were then destained in two volumes of approximately 30 ml destain solution (Chapter 2.3.9.5) overnight until the background was clear. Gels were dried using Gel-Dry™ Drying Solution and DryEase Mini Cellophane (Invitrogen). Molecular masses were determined by comparison to the Prestained SDS-PAGE Standards, Low Range (BioRad) (Table 2.5). The gel was dried by placing between two DryEase™ minicellophane (Invitrogen) sheets which had been pre-soaked in Gel-Dry™ gel drying solution for 5 min. The sandwich was then fastened into a gel drying frame (Novex) and left to stand on the bench overnight to dry. For a permanent electronic record, protein gels were scanned using an EPSON Perfection 3170 scanner.

2.12.3. Renaturing SDS-PAGE analysis of peptidoglycan hydrolases

Peptidoglycan hydrolase activity was detected by renaturing gel electrophoresis as described by Foster (1992) using purified *S. aureus*, *B. subtilis* or *M. luteus* vegetative cell walls as the substrate incorporated into the acrylamide gel to a concentration of 0.1% (w/v). A Protean II (BioRad) gel apparatus was used for electrophoresis (Chapter 2.12.1).

Electrophoresed gels were rinsed in dH_2O to remove SDS and then placed in 200 ml of renaturing solution (Chapter 2.3.9.6) at RT for 30 min, followed by incubation overnight at 37°C in another 200 ml of renaturing solution. The gels were then rinsed in dH_2O and incubated in 1X renaturing stain (Chapter 2.3.9.7) for 3 h at RT. After destaining in dH_2O, peptidoglycan hydrolase activity was visualised as zones of clearing in the blue opaque background. The gel was dried by placing between two DryEase™ minicellophane (Invitrogen) sheets which had been pre-soaked in 10% (v/v) glycerol for 5 min. The sandwich was then fastened into a gel drying frame (Novex) and left to stand on the bench overnight to dry. For a permanent electronic record, protein gels were scanned using an EPSON Perfection 3170 scanner.

Protein	Approximate molecular weight (Da)
Phosphorylase B	104,365
Bovine serum albumin	97,273
Ovalbumin	50,419
Carbonic anhydrase	37,191
Soybean trypsin inhibitor	29,184
Lysozyme	20,163

Table 2.5: Prestained SDS-PAGE Standards, Low Range (BioRad).

2.13 Western blotting

2.13.1. Electrophoresis and blotting

Protein samples were separated by 17.5% (w/v) SDS-PAGE (Chapter 2.12) and the gel was equilibrated in transfer buffer (Chapter 2.3.10.2) for 15 min. PVDF membrane was placed in methanol for a few seconds, before the BioRad Transfer Apparatus (BioRad) was assembled, as follows:

Top electrode BLACK sandwich plate (cathode)
White sponge (soaked in transfer buffer)
Filter paper (soaked in transfer buffer)
Gel
PVDF membrane (wetted with transfer buffer)
Filter paper (soaked in transfer buffer)
White sponge (soaked in transfer buffer)
Bottom electrode WHITE sandwich plate (anode)

The electroblotting apparatus was connected to a power pack and transfer of the polypeptides onto the PVDF membrane of pore size 0.2 µm (BioRad) was done in appropriate transfer buffer at 60 V for 1h.

2.13.2. Western blotting involving detection with alkaline phosphatase-conjugated secondary antibodies

The PVDF membrane containing the transferred protein was soaked overnight at 4°C in blocking buffer (TBS-T containing 6% (w/v) dried skimmed milk powder). The membrane was then washed briefly with TBS-T (Chapter 2.3.4) before adding 30 ml primary antibody solution (Chapter 2.3.10.4) for 90 min with agitation. The primary antibody was removed and the membrane washed for 3 x 10 min with TBS-T. The membrane was then placed in 30 ml specific secondary antibody solution (Chapter

2.3.10.5) for 60 min with agitation. The secondary antibody was raised against antibodies from the animal in which the primary originated and was also conjugated with AP to allow colourmetric detection. The secondary antibody was then discarded and the membrane washed again for 3 x 10 min in TBS-T. The membrane was pre-equilibrated with AP buffer, before being replaced with 25 ml colour substrate solution. The blot was then placed in the dark to allow the membrane to develop. Proteins bound with primary antibodies were visualized by the development of purple bands. After sufficient time the membrane was washed with 25 ml TE for 5 min to stop the reaction. The membrane was then air-dried and stored in the dark. For a permanent electronic record, Western blots were scanned using an EPSON Perfection 3170 scanner.

2.13.3. Ligand affinity blotting

Recombinant polypeptides were separated by 17.5% (w/v) SDS-PAGE (Chapter 2.12) and transferred to PVDF membranes by electroblotting (Chapter 2.13). Membranes were blocked overnight at 4°C in PBS-T (Chapter 2.3.2) containing 3% (w/v) BSA (Sigma). The membranes were incubated with the appropriate ligand labelled with biotin using a Biotin Labelling Kit (Roche), to probe the membrane at a final concentration of ~2 µg/ml. Bound ligands were detected using alkaline phosphatase-conjugated avidin (BioRad) and NBT-BCIP solution (Roche).

2.14 Immunoscreening the genomic DNA library with antisera

Plaques of *E. coli* XL1 Blue infected with the λ phage containing *S. pyogenes* genomic DNA library were transferred to nitrocellulose membranes pre-treated with 10 mM IPTG (Stratagene). The membranes were washed in TBS-T, blocked (TBS-T + 6% (w/v) skimmed milk) and probed with human sera (diluted 1:1000). Detection was carried out using alkaline phosphatase-conjugated anti-human IgG (FC specific) (1:30,000 in TBS-T) and visualised with NBT-BCIP (160 µl in 25 ml AP buffer).

2.15 ELISA analysis of specific antibody titre

An enzyme-linked immunosorbent assay (ELISA) was used to determine the titre of specific antibody against recombinant antigens. Briefly, 100 µl of appropriate recombinant polypeptides (10 µg/ml) or BSA in PBS (5 µg/ml), was added to wells of a 96-well microtiter plate (Nunc) overnight at 4°C. Plates were washed three times with PBS-T, and the remaining protein-binding sites were blocked with 5% (w/v) BSA in PBS for 2 h at room temperature. The plates were again washed three times in PBS-T, and different concentrations of human sera (1:100 and 1:1000) diluted in PBS-T were added. The plates were incubated for a further 1 h before being washed three times, and alkaline phosphatase-conjugated monoclonal antibody against human IgG (diluted 1:50,000 in PBS-T) was added, followed by 1 h of incubation. The plates were again washed three times in PBS-T and finally, bound antibodies were detected by using the Sigma Fast *p*-nitrophenyl phosphate system (Sigma). Plates were read at 405 nm in a Victor microtiter plate reader (Wallac).

2.16 DNA purification techniques

2.16.1. QIAquick Gel Extraction

DNA was purified from an agarose gel using a QIAquick gel extraction kit (QIAGEN). The DNA excised from the gel using a sharp scalpel blade. The gel slice was weighed and 3X the volume of Buffer QG added. The suspension was vortexed and incubated at room temperature until the gel had completely dissolved. 1X gel volume of isopropanol was added to the mixture before transferring it to a QIAquick spin column and centrifuging at 13,000 rpm for 1 min. The flow-through was discarded and 0.75 ml Buffer PE added to the column. This was then centrifuged as before, the flow-through discarded and centrifuged again. DNA was eluted with 50 µl Buffer EB, and the recovered DNA was stored at -20°C.

2.16.2. QIAquick PCR Purification

PCR reactions were purified using a QIAquick PCR purification kit (QIAGEN). Briefly, 5X volume of Buffer PB was added to the PCR reaction. The mixture was applied to a QIAquick column and centrifuged at 13,000 rpm for 1 min. The flow-through was discarded and the column washed with 0.75 ml Buffer PE by centrifuging as before. The flow-through was again discarded and the centrifugation repeated. DNA was eluted with 50 µl Buffer EB, and the recovered DNA was stored at -20°C.

2.16.3. Purification of dye-terminator sequencing reactions using DyeEX spin columns

Unincorporated nucleotides and primers were removed from dye-terminator sequencing reactions using a DyeEX spin kit (QIAGEN), according to the manufacturer's instructions.

2.16.4. Genomic DNA preparation

Genomic DNA was purified from *S. aureus* using a DNeasy kit (QIAGEN), based on the manufacturer's instructions. A colony was grown overnight in 5 ml BHI and cells recovered by centrifugation (13,000 rpm, 1 min). Pellets were resuspended in 1 ml dH_2O and centrifugation repeated. Pellets were then resuspended in 180 µl dH_2O. 5 µl lysostaphin (5 mg ml^{-1}) and 5 µl RNase (0.5 mg ml^{-1}) were added and samples incubated at 37°C for 30 min. Once cells had lysed, 25 µl proteinase K and 200 µl Buffer AL were added, mixed by vortexing and incubated at 70°C for 30 min. 200 µl ethanol was added and samples pipetted into DNeasy mini spin columns in 2 ml collection tubes. Columns were centrifuged at 8000 rpm for 1 min and the flow-through discarded. 500 µl Buffer AW1 was then added, centrifugation repeated and the flow-through discarded again along with the collection tube. 500 µl Buffer AW2 was added and columns centrifuged at 13,000 rpm for 3 min. Flow-through was discarded and DNA eluted into clean eppendorf tubes using 200 µl Buffer AE (8000 rpm, 1 min).

2.16.5. Small scale plasmid preparation from *E. coli*

A single colony of the *E. coli* strain containing the plasmid was used to inoculate 5 ml LB containing appropriate antibiotics. The culture was incubated overnight at 37°C, 250 rpm, unless otherwise stated. Cells were harvested at 13,000 rpm for 1 min. Plasmid DNA was isolated and purified using QIAprep Spin Miniprep kit DNA purification system (QIAGEN), according to the manufacturer's instructions. Plasmid DNA was eluted using 50 µl Buffer EB and stored at -20°C.

2.16.6. Large scale plasmid preparation from *E. coli* and *S. aureus*

Large amounts of plasmid (up to 500 µg) were isolated from *E. coli* and *S. aureus* using a Plasmid Midi kit DNA purification system (QIAGEN). A single colony of the bacteria was used to inoculate 100 ml LB (for *E. coli*) or BHI (for *S. aureus*), containing appropriate antibiotics. The culture was grown overnight at 37°C, 250 rpm, unless otherwise stated. Cells were harvested by centrifugation (6000 rpm for 10 min) and resuspended in buffer P1. For *S. aureus* cells, 50 µl lysostaphin (5 mg ml^{-1}) was also added. The protocol was then continued according to the manufacturer's instructions. Washed DNA pellets were air-dried overnight, resuspended in 200 µl dH_2O and incubated at 37°C for 10 min to allow the DNA to redissolve. Samples were then centrifuged at 5500 rpm for 3 min to ensure resuspension of all DNA. Purified plasmid DNA was stored at -20°C.

2.16.7. Precipitation of DNA using glycogen

Samples were prepared as follows:

DNA	100 µl
3M sodium acetate	10 µl
Glycogen (20 mg ml^{-1})	5 µl
Ethanol	300 µl

Samples were incubated at -20°C for 15-60 min before being centrifuged at 13,000 rpm, 10 min, RT. Pellets were then washed five times with 70% (v/v) ethanol, air-dried and redissolved to the required volume.

2.17 *In vitro* DNA manipulation techniques

2.17.1. Polymerase chain reaction (PCR)

2.17.1.1. Primer design

The primers used for PCR amplification were short synthetic oligonucleotides (19 - 74 bp) that were based on DNA sequences from published studies or obtained from cloning vector information and *S. aureus* databases (http://www.ncbi.nlm.nih.gov/ and http://www.genome.ou.edu/staph.html). Suitable restriction sites were introduced where necessary at the 5' ends of primers to enable subsequent cloning. The primers used in this study are shown in Table 2.6. Oligonucleotide primers for PCR and sequencing reactions were all synthesised by eurofins MWG GmbH (http://www.eurofinsdna.com/).

Primer	Sequence (5'→ 3')	Source
T3	AATTAACCCTCACTAAAGGG	Stratagene
T7 forward	GTAATACGACTCACTATAGGG	Stratagene
T7 reverse	GCTAGTTATTGCTCAGCGG	Stratagene
pETC_F	TTTTTTTCCATGGCAGAACAAAATAACAATGG	This study
pETC_R	TTTTTTCTCGAGATGAATGAAATTATATGAAC	This study
pETD_F	TTTTTTTCCATGGCATCTGAGCAAGATAACTACG	This study
pETD_R	TTTTTTCTCGAGGTGAATGAAGTTATAACCAG	This study
pETE_F	TTTTTTTCCATGGCACAACAACATGGCACACAA	This study
pETE_R	TTTTTTCTCGAGGTGGATGTAATTATATTTTCCT	This study
pETF_F	TTTTTTTCCATGGCATATACGAATGATAGCAAAACA	This study
pETF_R	TTTTTTCTCGAGATGGATGTAATTATATGATGAA	This study
pETG_F	TTTTTTTCCATGGCAGTTAATTATTATAGTAAAAA	This study
pETG_R	TTTTTTCTCGAGGTAAATATAGTTAAAGTTACGT	This study
pETI_F	TTTTTTTCCATGGCACCTGATTTAAATAAAGGTAAA	This study
pETI_R	TTTTTTCTCGAGCTCCTTATCATGAATAAAAC	This study
pETJ_F	TTTTTTTCCATGGCTGAAAATTATACAAATTACA	This study
pETJ_R	TTTTTTCTCGAGAATATGGATGTAGTTGTAGTT	This study
pETD_F2	TTTTTTTCCATGGCATATACTTCAGGACGTAACTT	This study
pETD_R2	TTTTTTCTCGAGACCACTTGAGATTGAACGG	This study
D1F	TTTTTTGGATCCTGGTTCCAGATGATATATTA	This study
D1R	TTTTTTGGATCCTGATATTCCAATTCAAGTATT	This study
D2F	TTTTTTGGATCCTGTTAATATCTTGCCACGCA	This study
D2R	TTTTTTGGATCCGTAACGATATTTTGCGGATAT	This study
PS_RedF	TAACATTAATTTTGTCACACGGACATTACGAAAATATTTTAG ATTTAAAATTAGAAATCCCTTTGAGAATGTTT	This study
PS_RedR	ATAGTAGCTGTAGCGATTTTCTTCATTTTAAAAATATCCTCC TAAAAATTGTGAGCGGCTCACAATTCCAC	This study
InvD1	TTTTTTGTCGACTTAAAATTTAGATTTTATAAAAG	This study
InvD2	TTTTTTGTCGACAAATCCTCCTATAAAAATTTT	This study
D3	TTTTTTGGCCGGCCAATTTTAAATCTAAAATATTTTCGT	This study
D4	TTTTTTGGCGCGCCTTTAGGAGGATATTTTTAAAATG	This study
PsF1	TCTGTCATCTTACATCCTCA	This study
PsF2	AGCCAACTAATGACAATGATT	This study
PsR	GAAGTTATAACCAGCAGCTT	This study
Mut1	TTTTTTGGATCCCAATTAAGCAATGCTCCAG	This study
Mut2	TTTTTTGTCGACTATATGATGTTGGGTCGTTT	This study
Mut3	TTTTTTCTCGAGCAATCTCAAGTGGTTATACT	This study
Mut4	TTTTTTGAATTCCACGTTTAACTTAATGTGAG	This study
KanF	TTTTTTGTCGACCAGCGAACCATTTGAGG	This study
KanR	TTTTTTCTCGAGATTCCTCGTAGGCGCTCGG	This study
CompF	TTTTTTGGATCCCACAAGGACGCTAATTTCT	This study
CompR	TTTTTTGAATTCGGCGTAAACATAGCCATC	This study

Table 2.6: Synthetic oligonucleotides used as primers for PCR amplification of DNA fragments in this study. Relevant restriction sites are underlined.

2.17.1.2. DNA amplification

Where accurate DNA amplification was not required, PCR reactions were carried out using 1.1x ReddyMix™ PCR Master Mix (ABgene). The following components were added, on ice, to a 0.5 ml thin-walled PCR tube:

PCR Master Mix	45 µl
Forward primer	300 nM
Reverse primer	300 nM
Template DNA	100 – 500 ng
dH_2O	to 50 µl

PCR amplification was performed using an Eppendorf 5330 Mastercycler. The lid was heated to 105°C for the duration of the PCR amplification, and the block was pre-heated to 94°C. The following thermal cycling programme of 30 cycles of steps 2 - 4 was used:

1) Denature	95°C	5 min
2) Denature	95°C	30 s
3) Anneal	50-60°C	30 s
4) Extension	72°C	t min (t=1 min kb^{-1} + 10 %)
5) Extension	72°C	10 min

2.17.1.3. Hi-fidelity DNA amplification

DNA amplifications that required 3'-5' proof-reading activity were performed with Extensor Hi-Fidelity PCR Master Mix (ABgene). The following components were added, on ice, to a 0.5 ml thin-walled PCR tube:

Extensor Master Mix	12.5 µl
Forward primer	200 nM
Reverse primer	200 nM

Template DNA	100-250 ng
dH_2O	to 25 µl

PCR amplification was performed using an Eppendorf 5330 Mastercycler. The lid was heated to 105°C for the duration of the PCR amplification, and the block was pre-heated to 94°C. Once the tubes were added, the DNA was denatured at 94°C for 2 min, followed by 10 cycles of programme A, then 20 cycles of programme B:

Programme A:

Denaturation	94°C	10 s
Annealing	50-68°C	30 s
Extension	68°C	t min (t = 45 sec kb^{-1})

Programme B:

Denaturation	94°C	10 s
Annealing	50-68°C	30 s
Extension	68°C	t min (t = 45 sec kb^{-1} + 10s / cycle)

Once all the cycles were complete a final step of 68°C for 7 min was added to allow complete extension of the primers. The PCR products were stored at -20 °C.

2.17.2. Restriction digestion of DNA

DNA restriction digests were performed according to the manufacturer's instructions, in volumes from 20 – 100 µl. The reactions were prepared as follows:

Restriction enzyme buffer	10% (v/v)
Restriction enzyme(s)	5 units
DNA solution	To final volume

Where appropriate the reaction volume was made up with dH_2O. The restriction digests were incubated at 37°C for 2.5 h, unless otherwise stated. Before digested DNA was used for further manipulation, it was purified using QIAGEN PCR purification kit (Chapter 2.15.2) or separated by 1% (w/v) agarose gel electrophoresis (Chapter 2.16.5) and purified using a QIAquick gel extraction kit (Chapter 2.15.1).

2.17.3. Alkaline phosphatase treatment

The 5' ends of plasmids digested with only one restriction enzyme were dephosphorylated using Calf Intestinal Alkaline Phosphatase (CIAP) (Roche) to reduce the re-ligation of the vector during subsequent cloning steps. The reactions were prepared as follows:

Restriction digest reaction	20 – 50 µl
CIAP buffer (10x)	8 µl
CIAP	1 µl
dH_2O	to 80 µl

Reactions were incubated at 37°C for 15 min. The DNA was then immediately separated by 1% (w/v) agarose gel electrophoresis (Chapter 2.17.5) and purified by gel extraction (Chapter 2.16.1).

2.17.4. DNA ligation

The following were added on ice:

Digested plasmid DNA	100 ng
Digested DNA insert	x ng*
10X DNA ligase buffer (Promega)	2 µl
T4 DNA ligase (Promega)	1 µl

*the amount of insert added was calculated using the following equation to allow a 1:1 and 1:3 molar ratio of vector:insert to be used:

$$\frac{\text{100 ng of plasmid} \times \text{kb size of insert}}{\text{kb size of plasmid}} \times \text{molar ratio of } \frac{\text{insert}}{\text{plasmid}} = \text{ng of insert}$$

The reaction was made up to 20 µl with dH_2O and incubated overnight at 15°C using an Eppendorf 5330 Mastercycler. The completed ligation mix was then purified using glycogen (Chapter 2.16.7) used to transform *E. coli* (Chapter 2.20.1).

2.17.5. Agarose gel electrophoresis

DNA fragments were separated by horizontal gel electrophoresis using various size electrophoresis tanks (Life Technologies). An appropriate volume of agarose gel (1% (w/v)) was dissolved in TAE by microwaving and poured into the gel tray. Once set, the gel was submerged in a suitable volume of TAE electrophoresis buffer (Chapter 2.3.11.1). For visualisation of the DNA, 10 µl ethidium bromide (10 mg/ml, BioRad) was added to the TAE running buffer. DNA loading dye (6X, MBI Fermentas) was added to a 1X final concentration with the samples and loaded into the gel wells. To estimate the sizes of DNA fragments, 1 kb DNA size markers (Abgene) were also loaded onto the gel. If the concentration of DNA was also to be determined, Hyperladder I (Bioline) DNA markers were used (Table 2.7). The gel was run at 100 V for 1h and visualised using a UV transilluminator at 260 nm.Gels were photographed using BioDoc-It system (UVP) and printed by Sony Video Graphic Printer using Thermal Print Media (UPP-110HA). Photographs were then scanned using an Epson Perfection 3170 scanner.

1 kb DNA Size Marker (Abgene)	Hyperladder I (Bioline)	
Band Size (bp)	**Band Size (bp)**	**ng / Band**
10,000	10,000	100
8,000	8,000	80
6,000	6,000	60
5,000	5,000	50
4,000	4,000	40
3,000	3,000	30
2,500	2,500	25
2,000	2,000	20
1,500	1,500	15
1,000	1,000	100
750	800	80
500	600	60
250	400	40
	200	20

Table 2.7: Size of DNA bands used as size markers for 1% (w/v) TAE agarose gel electrophoresis.

2.18 DNA hybridization techniques

2.18.1. Labelling of DNA probes with digoxigenin

Gel purified DNA fragments were labelled by a random priming method using a commercially available digoxigenin (DIG) DNA labelling and detection kit (Roche). DNA to be labelled (up to 3 µg in a maximum of 15 µl EB in a microfuge tube) was denatured at 100°C for 10 min, then immediately chilled on ice for 10 min. The following components were added on ice:

Random hexanucleotide mixture	2 µl
dNTP labelling mixture (containing DIG-dUTP)	2 µl
Klenow enzyme (2 units)	1 µl

The volume of the reaction was made up to 20 µl with dH_2O. The reaction was incubated overnight at 37°C, then gel purified (Chapter 2.16.1). The purified probe was then quantified (Chapter 2.18.2) and, if not used immediately, stored at -20°C. Prior to use the probe was denatured by boiling for 10 min, followed by immediate chilling on ice for 10 min.

2.18.2. Quantification of DIG-labelled DNA probes

The amount of DIG-labelled material in a volume of labelled DNA was determined by comparison to labelled control DNA of known concentration supplied in the DIG DNA labelling and detection kit (Roche). The sample DNA and the control DNA were diluted according to the manufacturer's instructions, using a single pre-dilution step to obtain an estimated concentration of 1 ng μl^{-1}, followed by five serial 10-fold dilutions. 1 µl of each 10-fold dilution was spotted onto Hybond-N+ Extra (positively charged) nylon membrane (Amersham Life Sciences). The DNA was permanently bound to the membrane using a UV crosslinker (Chapter 2.18.4) and DIG-labelled DNA was then detected immunologically, using AP-linked anti-dioxygenin antibody (Chapter

2.3.11.13). The spot intensities of the control and probe dilutions were compared visually to estimate the concentration of the probe.

2.18.3. Southern blotting

Digested genomic DNA was separated by 1% (w/v) agarose gel electrophoresis (Chapter 2.17.5). DIG-labelled DNA molecular weight marker of size range 0.12-21.2 kb (Roche) was loaded onto the gel to estimate the size of DNA fragments following development of the blot using AP-conjugated anti-DIG antibody. The gel was soaked in depurination solution (Chapter 2.3.11.2) for 10 min and washed in dH_2O. This acts to nick large DNA molecules, aiding their transfer onto the membrane during the blotting procedure. The gel was then soaked in denaturation buffer (Chapter 2.3.11.3) twice for 15 min and rinsed in dH_2O. The gel was neutralised by soaking in neutralisation buffer (Chapter 2.3.11.4) twice for 15 mins. The DNA was transferred from the gel to a Hybond-N+ Extra membrane by vacuum blotting at 70 mbar for 90 mins, using 10X SSC as the transfer buffer.

2.18.4. Fixing the DNA to the membrane

DNA was fixed to the Hybond-N+ Extra (positively charged) nylon membrane with the use of a UV crosslinker (Amersham Life Sciences RPN 2500) at 70 mJ / cm^2 for 15 sec.

2.18.5. Prehybridization and hybridization

All prehybridisation and hybridisation procedures were done in Techne Hybridiser HB-1D. Membranes to be probed with the DIG-labelled DNA were prehybridised for 2 h at 68°C in pre-hybridisation solution (Chapter 2.3.11.6) (20 ml per 100 cm^2 of membrane). Just prior to use, the labelled probe (Chapter 2.18.1) was denatured in a microfuge tube by placing in a boiling water bath for 10 min. The labelled probe was immediately chilled on ice for 10 min and added to pre-heated hybridisation solution to give a final probe concentration of 5-25 ng ml^{-1}. The membrane was then hybridised with the

labelled probe overnight at 68°C. After hybridisation the solution was retained for future use and stored at -20°C. Unbound probe was removed by washing the membrane twice in 2X wash solution (Chapter 2.3.11.8) for 5 min at room temperature. The membrane was then washed twice in 0.5X wash solution (Chapter 2.3.11.9) for 5 min at 68 °C.

2.18.6. Colorimetric detection of DIG-labelled DNA

The hybridised and washed membranes were equilibrated with washing buffer (Chapter 2.3.11.11) for 1 min and then blocked for 30 min with gentle rocking in blocking solution (Chapter 2.3.11.12). The membrane was then transferred to antibody solution containing a 1:5000 dilution of stock anti-DIG-AP antibody (Chapter 2.3.11.13) (Roche). After 30 min incubation with gentle rocking, the membrane was washed twice for 15 min with washing buffer. The membrane was equilibrated for 2 min with detection buffer (Chapter 2.3.11.14) before 10 ml colour substrate solution (Chapter 2.3.10.7) was applied to the membrane. This was then incubated in the dark to allow the membrane to develop. The presence of anti-DIG-AP bound to DIG-labelled DNA was visualised by the appearance of purple bands. After the colour had developed sufficiently, the membrane was washed in 1X TE (Chapter 2.3.11.15) for 5 min to stop the reaction. The membrane was then air-dried, scanned using an EPSON Perfection 3170 scanner and stored in the dark.

2.19 Phage techniques

2.19.1. Preparation of phage lysates

The donor strain was grown in broth with appropriate antibiotics at 37°C until in log phase. 1-3 ml of cultures were centrifuged (5000 rpm, 10 min, 4°C). The pellet was resuspended in 5 ml BHI in a 30 ml universal and 5 ml phage buffer was added. 30 µl stock lysate (phi 11, phi 85) was added and the mixture left at room temperature for 10 min. The lysate was then incubated on a slow shaker (50 rpm) at 30°C for 2-4 h until

clear. If lysis took longer, the tube was left stationary overnight at 25°C. Once clear, the lysate was filter-sterilised (0.2 µm filter) and stored at 4°C.

2.19.2. Determination of phage concentration

S. aureus SH1000 was grown in BHI until log phase (OD_{600nm} 0.6-1.0). Phage lysate was diluted up to 10^{-7} and 100 µl diluted phage mixed with 400 µl SH1000 culture. 50 µl 1M $CaCl_2$ was added and the tube was left for 10 min. 5 ml phage top agar (cooled to 50°C) was added to the phage mixture and used to overlay a phage bottom agar plate. Once set, plates were incubated at 37°C overnight. Plaques were counted and pfu/ml calculated (no. plaques x 10^8).

2.19.3. Phage transduction

The recipient bacteria were grown overnight in 20 ml LK. The cells were recovered by centrifugation at 5000 rpm for 10 min and the pellet resuspended in 1.5 ml LK. 500 µl and 100 µl phage lysate was added to 500 µl cells (control = no phage lysate) and the volume made up to 2 ml with LK + 10 mM $CaCl_2$. Tubes were incubated statically at 37°C for 25 min, followed by 15 min at 37°C with gentle shaking. 1 ml ice-cold 0.02 M sodium citrate was added on ice and left for 5 min before centrifuging at 5000 rpm for 10 min. The supernatant was removed and the pellet resuspended in 1 ml ice-cold 0.02 M sodium citrate. Mixtures were left on ice for 45-90 min, before 100 µl and 200 µl aliquots were spread onto LK-citrate plates (0.05% (w/v) sodium citrate). Plates were incubated at 37°C for 90 min, then overlayed with 5 ml LK top agar containing 6 times the normal concentration of selective antibiotic. When set, plates were left at 37°C for 24-48 h until colonies developed.

2.20 Transformation techniques

2.20.1. Transformation of *E. coli* by electroporation

2.20.1.1. Preparation of electrocompetent *E. coli* cells

E. coli Top 10 was grown overnight in LB and 2.5 ml was used to inoculate 250 ml LB. The culture was grown at 37°C, 250 rpm, until log phase (OD_{600} 0.5-0.6). The culture was chilled on ice for 30 min before cells were harvested at 5000 rpm, 15 min, 4°C, in 50 ml tubes. Pellets were washed twice with 25 ml ice-cold dH_2O and then twice with 2 ml ice-cold 10% (v/v) glycerol. The final pellet was resuspended in 1.5 ml 10% glycerol, snap frozen in liquid nitrogen and stored at -80°C.

2.20.1.2. Electroporation of *E. coli* competent cells

50 µl electrocompetent *E. coli* Top 10 cells were defrosted on ice and 4 µl plasmid was added (1 µl control plasmid, e.g. pUC18). Cells were transferred to a pre-chilled 0.1 cm cuvette and electroporated at 2500 V, 25 µF, 200 Ω. 1 ml SOC was added immediately and cells recovered at 37°C, 250 rpm for 90 min. Various sized aliquots were plated onto LB containing appropriate antibiotics and incubated at 37°C overnight.

2.20.2. Transformation of *S. aureus* by electroporation

2.20.2.1. Preparation of *S. aureus* electrocompetent cells

S. aureus RN4220 was grown overnight at 37°C, 250 rpm in 10 ml B2 medium. 2 ml of culture was used to inoculate 1l B2. The culture was grown at 37°C, 250 rpm, until log phase (OD_{600nm} 0.5-1.0). Cells were harvested by centrifugation for 10 min at 5000 rpm, 4°C. The pellet was washed three times with 300 ml sterile dH_2O at room temperature, then three times with decreasing volumes of 10% (v/v) glycerol (i.e. 100 ml, 50 ml, 25

ml). Finally, cells were resuspended in 1.25 ml 10% (v/v) glycerol before being snap-frozen in liquid nitrogen and stored at -80°C.

2.20.2.2. Electroporation of *S. aureus* competent cells

50 µl electrocompetent *S. aureus* RN4220 cells were defrosted on ice and 5 – 20 µg plasmid DNA was added. The plasmid/cell mixture was then transferred to a 0.1 cm gap cuvette (BioRad) and electroporated at room temperature at 2300 V, 25 µF, 100 Ω using a BioRad Gene Pulser. Cells were recovered by adding 1 ml pre-warmed B2 (containing an inducing concentration of Ery (i.e. 0.15 µg ml^{-1}), or no addition for Tet or Kan/Neo, where appropriate). Cells were recovered at 37°C, 250 rpm for 90 min before being plated out on BHI agar containing appropriate antibiotics and incubated overnight at 37°C.

2.21 Lambda Red cloning

A single colony of *E. coli* EL250 containing an appropriate plasmid was grown overnight at 30°C, 250 rpm in LB containing appropriate antibiotic. 1 ml overnight culture was used to inoculate 50 ml fresh LB with antibiotic and cells were grown at 30°C to exponential phase (OD_{600nm} 0.4-0.5). Cells were then heat-shocked for 15 min by swirling at 42°C, transferred to a slurry ice-water bath for 10 min with swirling to induce the lambda red system. Cells were harvested by centrifugation at 5500 rpm, 10 min, 4°C. The supernatant was removed and cells resuspended in 25 ml sterile ice-cold water. The centrifugation process was repeated and cells were washed with ice-cold water twice more. The supernatant was removed after the last wash and the cell pellet was resuspended in 500 µl with ice-cold sterilised water. The freshly prepared competent cells were kept once for 1 h before they were used for electroporation. Electroporation cuvettes (BioRad) were cooled in an ice-water bath for at least 10 min prior to use. A 50 µl sample of electrocompetent cells was mixed with 1-5 µl of DNA samples containing 0.08-0.4 µg of purified DNA fragments, transferred to the electroporation cuvette, and incubated on ice for 1 min. The cells were transformed by electroporation at 1.75 kV, 25 µF, 200 Ω.

Following electroporation, cells were recovered by adding 1 ml LB, transferring the cell suspension into a sterile eppendorf tube and incubating at 30°C, 250 rpm, for 90 min. 50-100 µl aliquots were then plated on LB plates containing appropriate antibiotic and incubated for 2 days at 30°C.

2.22 Opsonisation

In order to determine the opsonophagocytic activity of specific antisera, assays were carried out using both *S. aureus* SH1000 and *S. agalactiae* 6313 in the presence of human neutrophils. Neutrophils were isolated from freshly obtained human blood (kindly provided by Dr. Lynne Prince and Dr. Kathryn Vaughan, University of Sheffield Medical School). Baby rabbit serum (Cederlane Laboratories Ltd.) was used as a source of complement and antibodies against *S. aureus* or *S. agalactiae* were removed prior to use. Bacteria were grown to OD_{600nm} 0.5, washed once in PBS and diltued to 10^7 cells/ml in PBS. 100 µl washed cells (either *S. aureus* or *S. agalactiae*) were added to 500 µl baby rabbit serum and incubated at 4°C for 30 min on a rotating wheel. The baby rabbit serum was filter sterilised before use. Bacteria were grown to log phase (OD_{600nm} 0.5) in either BHI (*S. aureus*) or THY (*S. agalactiae*), and resuspended in PBS. To allow expression of iron-regulated genes, such as *isdA* in *S. aureus*, both species were also grown in iron-limited TSB.

For the assay, 1 x 10^5 cfu bacteria were incubated with 2.5 x 10^5 neutrophils, along with the specific antiserum (1:10 dilution) and complement source (1:10 dilution). Samples were made up to 500 µl using RPMI medium (Sigma). Appropriate controls were carried out by replacing the antiserum with either pre-immune serum or no serum (RPMI). In addition, controls were carried out with no neutrophils and with no complement source, each being replaced by RPMI.

Immediately after mixing, a sample was removed and vortexed, and the number of bacteria counted by diluting in TSB containing 0.25% (v/v) Tween-20 and plating onto BHI agar plates. The bacteria/neutrophil samples were incubated at 37°C on a slow

shaker and samples were removed after 1.5 h (and 3 h), and the number of bacteria counted again as above. The plates were incubated overnight at 37°C and the percentage of initial bacteria calculated at each time point.

Chapter 3

Identification of *Streptococcus pyogenes* components recognised by human sera

3.1 Introduction

Successful vaccine development requires identification of suitable targets. Developments in whole-genome sequencing of bacteria have enabled comprehensive analysis for vaccine candidates by bioinformatics-based screens. This approach has been utilised to identify putative antigenic targets in a number of bacterial pathogens, including *Streptococcus pneumoniae* (Wizemann *et al.* 2001) and *Neisseria meningitidis* (Pizza *et al.* 2000). Surface or secreted proteins are the most promising candidates for immunogenic targets as they are involved in processes such as cell adhesion, intracellular entry and immune system evasion. These proteins are at the interface between pathogen and host immune defences.

The success of genomics-based approaches greatly relies on the proper identification and annotation of genes and is difficult to apply to those of no known function, which comprise approximately 40% of sequenced genomes (Weichhart *et al.* 2003). Furthermore, bioinformatic analysis does not reveal those proteins which are expressed *in vivo* during infection and are able to induce an immune response, an important factor for the development of effective targets for vaccines or therapeutic antibodies.

To overcome the limitations of whole-genome analysis approaches, alternative methods have been developed to identify immunogenic proteins produced *in vivo*. For example, Weichhart *et al.* (2003) identified novel antigens of *Staphylococcus aureus* using *in vitro* protein selection by ribosome display. Proteins expressed *in vivo* during infection which are able to induce an immune response were identified using human antibodies from *S. aureus*-infected patients, identifying 14 possible candidate vaccine peptides.

Comparative proteome analysis has also led to the identification of putative vaccine candidates. Using two-dimensional electrophoresis, Jungblut *et al.* (1999) identified 25 proteins differing in expression levels between the virulent *Mycobacterium tuberculosis* H37Rv and the non-virulent *M. bovis* BCG. Similarly, McAtee *et al.* (1998) identified several vaccine candidates of *Helicobacter pylori* by Western blotting for proteins reactive with pooled sera from infected patients and identification by N-terminal sequencing. Proteins identified include urease B subunit, enolase and catalase.

Another promising strategy for antigen identification involves screening bacterial genomic DNA expression libraries with human sera to identify proteins expressed *in vivo* which stimulate an immune response within the host. This technique was successfully used to identify novel putative virulence determinants of *Enterococcus faecalis*, an opportunistic pathogen (Xu *et al.* 1997). The same method was employed by Lang *et al.* (2000) to identify antigens of *Staphylococcus epidermidis*, resulting in the identification of a novel protein, SsaA, thought to have a role in virulence. Immunoscreening has been used to identify putative antigens of *Staphylococcus aureus* in several studies, including an immunodominant ABC transporter protein in MRSA, homologous to the *B. subtilis* ABC transporter YkpA (Burnie *et al.* 2000), as well as Empbp, IsaA and Coagulase (Etz *et al.* 2002).

Recently, immunoscreening has identified antigenic components of both *S. aureus* (Clarke *et al.* 2006) and *S. epidermidis* (Pourmand *et al.* 2006), including a conserved family of proteins named the Sca family (See Section 3.3.1.1). Further to this, antigenic components of both *Streptococcus agalactiae* and *Streptococcus pneumoniae* were identified by similar methods (Stapleton *et al.* unpublished). In this study, a genomic expression library of *Streptococcus pyogenes* was screened for putative cross-reactive antigens using sera from *S. aureus*-infected patients. Bioinformatic analysis of the output from all five screens enabled the identification of conserved polypeptide domains, which may represent putative antigenic targets for prophylaxis against a range of Gram positive pathogens.

3.2 Results

3.2.1. λZAP expression library preparation

In order to identify antigens expressed by *Streptococcus pyogenes* which were cross-reactive with human sera, a lambda ZAP expression library was screened in *E. coli* by plaque hybridisation. The lambda ZAP Express (Stratagene) library was previously constructed by Dr S. Clarke as follows: *S. pyogenes* HSC-5 genomic DNA was partially cut with *Sau*3A and the resulting fragments (2-10 kb in length) ligated into the *Bam*HI site of Lambda ZAP Express, according to the manufacturer's instructions. The library was shown to have > 30% phage-containing inserts.

3.2.2. Library Titration

The phage titre of the library was determined by plaque enumeration using *E. coli* XL1-blue as the host strain. The phage titre was determined to be 7.3×10^7 pfu/ml.

3.2.3. Human sera samples

Nineteen sera were used to screen the *S. pyogenes* genomic expression library. These included sera from 11 patients infected with *S. aureus*, sera from 4 nasal carriers of *S. aureus* and sera from 4 non-carriers (Table 3.1) (all kindly provided by Dr R. Read, University of Sheffield).

3.2.4. Immunoscreening of the expression library with human sera

A primary screen of the *S. pyogenes* library using human sera was carried out by infection of phage into *E. coli* XL1-blue, resulting in plaque formation. Clones cross-reactive with human sera were identified after plaque lift onto nitrocellulose membranes. A summary of the screening protocol is shown in Figure 3.1.

In order to achieve good overall coverage of the *S. pyogenes* genome library, approximately 3.6×10^4 pfu were screened per agar plate, and 10 plates were screened per serum. Therefore, at least 10.8×10^4 insert-containing plaques were screened for each serum. The genome size of *S. pyogenes* is approximately 1.89 Mb (www.tigr.org); if the insert size for each clone were 2 kb, 216 Mb of inserts would be screened for each serum, giving more than 100 times coverage of the genome.

Serum antibodies bound to antigens present on the nitrocellulose membranes were detected using alkaline phosphatase-conjugated anti-human antibody (Chapter 2.14). Membranes were developed and positive primary clones were identified as purple spots against a pink background (Figure 3.2). The number of positive clones varied between sera, up to 10 putative positive clones were picked per plate for each serum.

Positive clones from the primary screen were then subjected to a second round of screening at approximately 200 pfu per plate. Up to 20 well-separated clones were picked for each serum in the secondary screen. To ensure these clones were both pure and antigenic, a tertiary screen was carried out. The positive clones were spotted directly onto membranes (approx. 20 pfu) and probed with the original sera to confirm their reactivity.

These pure clones were then excised to yield pBK-CMV-based phagemids (Figure 3.3) in *E. coli* XL1-blue. A total of 114 phagemids were excised from 14 sera. To confirm the presence of insert DNA within the phagemid, restriction digests were carried out using *Eco*RI and *Pst*I (Figure 3.4). The pBK-CMV backbone is seen as a 4.5 kb fragment in each clone, with *S. pyogenes* inserts represented by the various other fragments on the gel. In order to accurately identify and map the clones within the *S. pyogenes* genome, sequencing reactions were carried out. T3 and T7 primers (Table 2.6) were used for sequencing as they flank the insertion site in the vector, allowing the *S. pyogenes* inserts to be identified. DNA sequences corresponding to the ends of the cloned fragments were compared to the *S. pyogenes* MGAS8232 genome by BLAST analysis at TIGR (http://tigrblast.tigr.org/cmr-blast/), allowing the entire insert sequence to be identified.

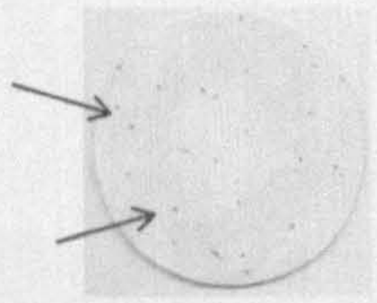
Serum Number	Serum Type	Positive Clones Identified	Total No. of Clones
D	*S. aureus*-infected patient sera	C	1
G		A	1
F		D	1
55		-	0
44		-	0
36		A, B, G, M, P	5
31		D, E, F, I, L, M, N, Q	8
30		B, C	2
28		A, B, C, D, H, I, L, M, O, P, R	11
24		B, D, H, I, K, O, P, Q, S, T	10
21		-	0
		Total	**39**
D62	Nasal carriers	C, E, F, G, H, I, J, M, N, P, Q, R, S	13
D74		A, B, C, G, H	5
D75		B, C, D, E, G, H, I	7
D78		A, C, D, E, F	5
		Total	**30**
D59	Non-carriers	-	0
D60		A, B, E, F, H, I	6
D63		-	0
D65		B, C, E, F, H, I	6
		Total	**12**
		Total Clones Isolated	**81**

Table 3.1: Positive clones identified from immunoscreening of the *S. pyogenes* library with human sera.

Bacteriophage lambda ZAP library of *S. pyogenes* was constructed, containing 2-10 kb DNA fragments.

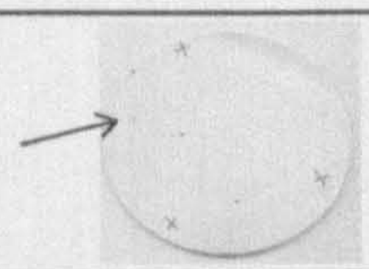
↓

Primary Screen: Phage library infected into *E. coli* XL1-blue. Plaque lift onto nitrocellulose membranes. *S. aureus*-infected patient, hospital worker and community sera used to identify antigens.



↓

Secondary Screen: A further round of screening was carried out to isolate pure clones.

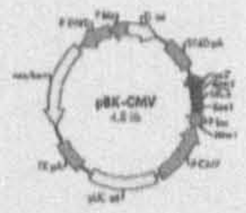


↓

Tertiary Screen: A final screen was carried out to ensure clones were both pure and antigenic.

↓

Positive clones were excised as kanamycin-resistant phagemids



↓

Phagemid DNA was purified and inserts sequenced.

↓

Database analysis (http://tigrblast.tigr.org/cmr-blast/) was used to identify antigen-encoding genes of *S. pyogenes*.
81 clones identified – 48 loci

Figure 3.1: Flow diagram illustrating the method for screening the λZAP expression library to identify *S. pyogenes* antigens.

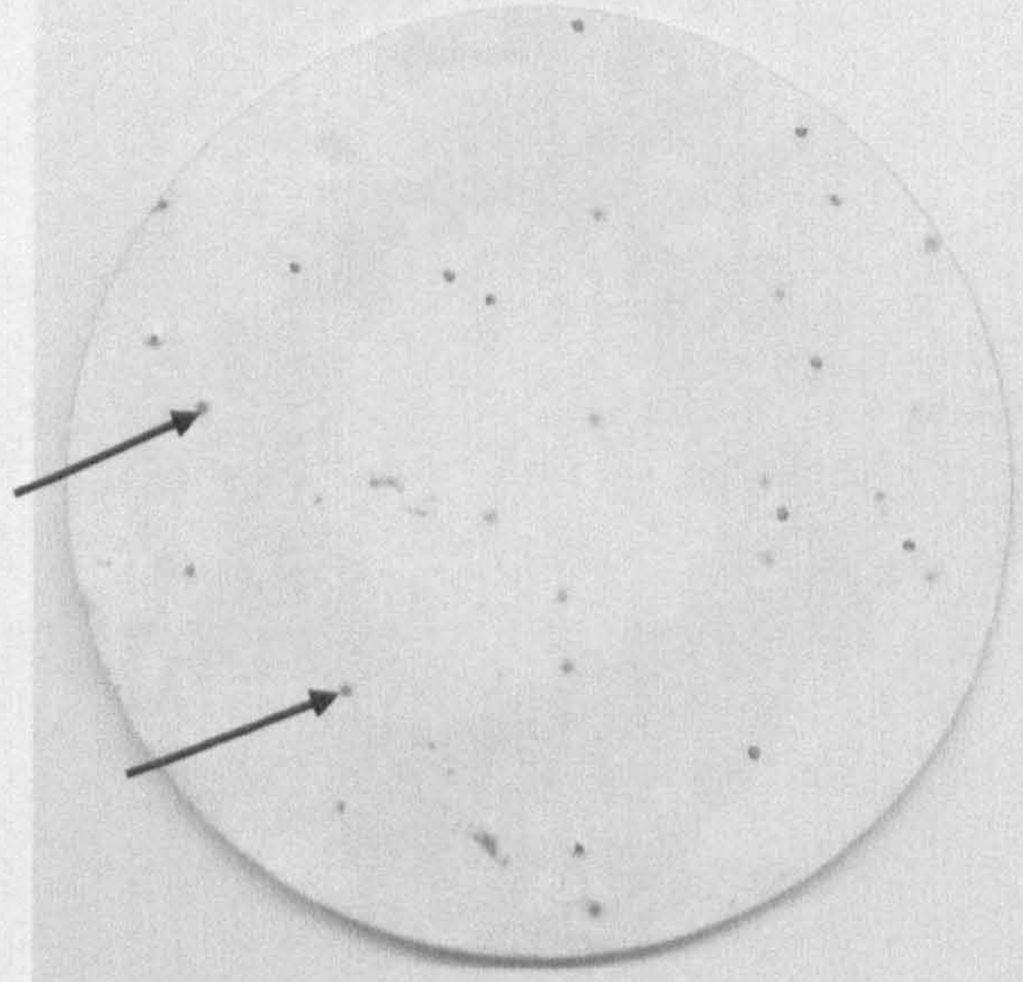
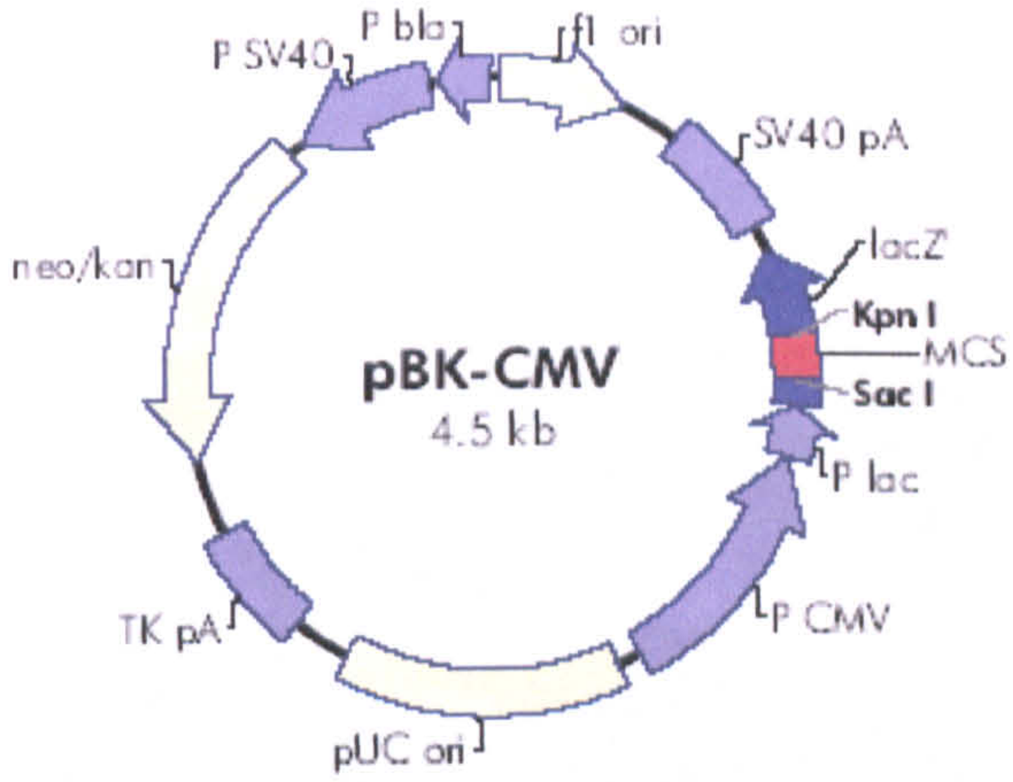


Figure 3.2: Screening of the genomic library of *S. pyogenes*. Purple spots (identified by arrows) correspond to positive clones on the nitrocellulose membrane.



pBK-CMV Multiple Cloning Site Region
(sequence shown 952–1196)

T7 Promoter

AACGACGGCCAGTGAATT**GTAATACGACTCACTATAGGGC**GAATTGGGTACACTTACCTGGTACCCCACCCGGGTGGAAA...

T7 primer binding site

...ATGGGCCCGCGGCCGCTCTAGAAGTACTCTCGAGAAGCTTTTTGAATTCTTTGGATCCACTAGTGTCGACCTGCAG...

*Bam*H1

T3 Promoter

β-gal α -fragment

...GCGCGCGAGCTCCAGCTTTTGTT**CCCTTTAGTGAGGGTTAATTT**CGAGCTTGGCGTAATCAAGGTCATAGCTGTTTCCTGT

T3 primer binding site

Figure 3.3: The pBK-CMV phagemid vector and polylinker sequence containing the multiple cloning site (MCS) flanked by T3 and T7 promoters (Novagen) (highlighted in red). The *Bam*HI site into which inserts were cloned is underlined.

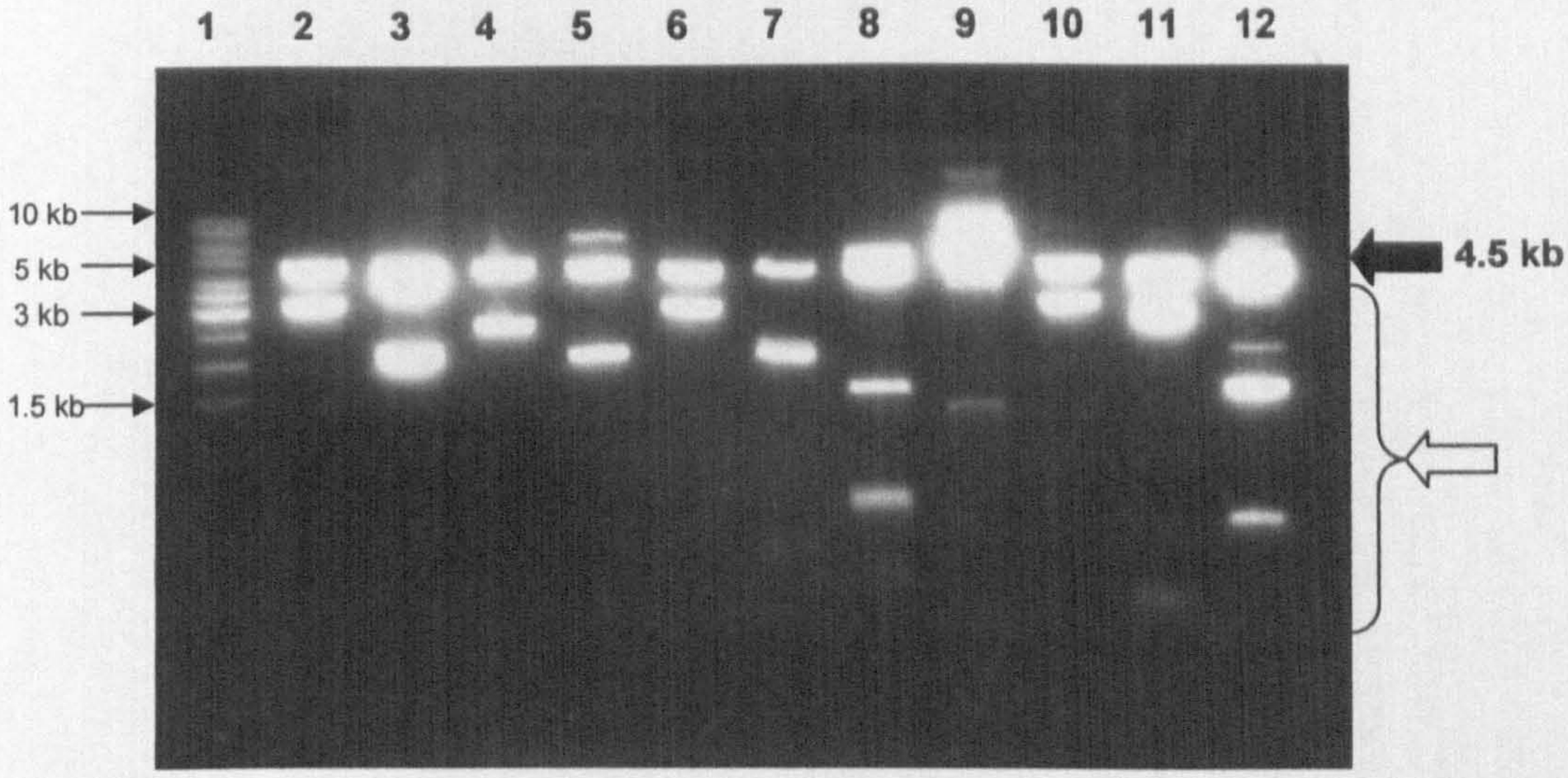


Figure 3.4: 1% (w/v) agarose gel electrophoresis of restriction digests (*Eco*RI and *Pst*I) of representative *S. pyogenes* genomic library phagemid clones. The pBK-CMV backbone can be seen by the filled arrow at 4.5 kb. Inserts are shown by the open arrow. The gel shows examples of clones obtained from screening the library with *S. aureus*-infected patient sera 28. Lane 1, molecular size marker; 2, clone 28A; 3, 28B; 4, 28C; 5, 28D; 6, 28H; 7, 28I; 8, 28L; 9, 28M; 10, 28O; 11, 28P; 12, 28R.

3.2.5. Bioinformatic analysis of cloned antigens

The location of the sequenced clones on the *Streptococcus pyogenes* MGAS8232 genome was determined using BLAST analysis, using the following website: http://tigrblast.tigr.org/cmr-blast/. In total, 81 clones were identified during the immunoscreen, corresponding to 48 loci in the *S. pyogenes* genome (Table 3.2). 172 full and partial open reading frames were identified within these loci and the cellular location of each of the proteins was predicted using SOSUIsignal (http://bp.nuap.nagoya-u.ac.jp/sosui/sosuisignal/sosuisignal_submit.html). Proteins were designated as secreted if they possessed a signal sequence and membrane proteins were assigned according to the presence of membrane spanning regions.

Locus	Locus coordinates	Clone coordinates (Clone occurrence)	Genes within locus (cloned region amino acids)	Designation, putative function or homology	Cellular location
1	37324 – 45562	41633 – 45562 (1) 37324 – 41408 (1)	NT03SP0027 (*182-1257)	PurL, putative phosphoribosyl-formylglycinamide synthase	C
			NT03SP0028 (1-503)	PurF, amidophosphoribosyltransferase	C
			NT03SP0029 (1-340)	PurM, phosphoribosyl-formylglycinamidine cyclo-ligase	C
			NT03SP0030 (1-184)	PurN, phosphoribosylglycinamide-formyltransferase	C
			NT03SP0031 (1-454*)	PurH, phosphoribosyl-aminoimidazolecarboxamide formyltransferase/IMP cyclohydrolase	C
2	48201 – 49201	48201 – 49201 (1)	NT03SP0033 (*366-421)	PurD, phosphoribosylamine glycine ligase	C
			NT03SP0034 (1-203)	PurE, phosphoribosylaminoimidazole carboxylase, catalytic subunit	C
			NT03SP0035 (1-5*)	PurK, phosphoribosylaminoimidazole carboxylase, ATPase subunit	C
3	94720 – 96213	94720 – 96213 (1)	NT03SP0087 (*160-655*)	RpoB, DNA-directed RNA polymerase, β-subunit	C
4	98613 – 100784	98613 – 100784 (1) 98613 – 100368 (1)	NT03SP0088 (*239-961*)	RpoC, DNA-dependent RNA polymerase, B subunit	C
5	137624 – 139810	137624 – 139810 (1)	NT03SP0132 (*374-666)	NtpI, V-type sodium ATP synthase subunit I	M
			NT03SP0133 (1-159)	NtpK, V-type sodium ATP synthase, subunit K	M
			NT03SP0134 (1-194)	NtpE, putative V-type sodium-ATPase, subunit E	C
			NT03SP0135 (1-50*)	ATP synthase (C/AC39) subunit	C
6	152987 – 156015	153672 – 155740 (1)	NT03SP0149 (*2-118)	conserved hypothetical protein	C
		153000 – 155247 (1)	NT03SP0150 (1-571)	Slo, streptolysin O precursor	S
		152987 – 156015 (1)	NT03SP0151 (1-80)	conserved hypothetical protein	M
		154105 – 154988 (1)	NT03SP0152 (*10-55)	conserved hypothetical protein	M

		153672 – 156015 (1)			
7	180256 – 182603	180256 – 182603 (1)	NT03SP0176 (*65-259) NT03SP0177 (1-159) NT03SP0178 (*203-406)	putative AtsA-ElaC family protein cytidine/deoxycytidylate deaminase family protein putative lipoprotein	C C S
8	212312 – 215406	212312 – 215406 (1)	NT03SP0214 (*131-295) NT03SP0215 (1-276) NT03SP0216 (1-151) NT03SP0217 (1-220) NT03SP0218 (1-185*)	sugar ABC transporter sugar ABC transporter conserved hypothetical protein conserved hypothetical protein NanH, N-acetylneuraminate lyase	M M C M C
9	243678 – 247256	243678 – 247256 (1)	NT03SP0247 (*197-420) NT03SP0248 (1-349) NT03SP0249 (1-159) NT03SP0250 (1-395*)	uncharacterised UPF0051 family protein putative aminotransferase NifU family protein conserved hypothetical intein-containing protein	C C C C
10	323813 – 325818	323813 – 325818 (1)	NT03SP0341 (*90-683) NT03SP0342 (1-73*)	Unknown hylP3, phage-associated hyaluronidase	C C
11	327252 – 328956	327252 – 328956 (1)	NT03SP0343 (*215-634) NT03SP0344 (1-142)	hypothetical phage protein hypothetical phage protein	C C
12	511889 – 513957	511889 – 513957 (1) 512754 – 513956 (1)	NT03SP0579 (*178-620) NT03SP0580 (1-238*)	PTS system, β-glucosides-specific IIABC component BglA, 6-phospho-β-glucosidase	M C
13	585054 – 588139	585054 – 588139 (1)	NT03SP0663 (*26-68) NT03SP0664 (1-110) NT03SP0665 (1-269) NT03SP0666 (1-66) NT03SP0667 (1-265) NT03SP0668 (1-113) NT03SP0669 (1-24*)	hypothetical phage protein hypothetical phage protein RecT, prophage pi1 protein 11, recombinase hypothetical phage protein hypothetical phage protein conserved hypothetical protein conserved hypothetical phage protein	C C C C C C M
14	608681 – 612244	608681 – 612244 (1)	NT03SP0704 (*37-247) NT03SP0705 (1-658)	hypothetical phage protein conserved hypothetical protein	C C

			NT03SP0706 (1-307*)	hylP2, phage-associated hyaluronidase	C
15	612964 – 614784	612964 – 614784 (1)	NT03SP0707 (*208-628)	putative pathogenicity protein	C
			NT03SP0708 (1-142)	conserved hypothetical protein	C
			NT03SP0709 (1-36*)	hypothetical phage protein	C
16	690793 – 693691	690793 – 693034 (1)	NT03SP0790 (*327-581)	RgpFc, polysaccharide biosynthesis protein	C
		690793 – 693691 (1)			
			NT03SP0791 (1-710*)	putative membrane protein	M
17	778815 – 782706	778815 – 782706 (1)	NT03SP0882 (*202-227)	RpiA, ribose 5-phosphate isomerase	C
		780477 – 782358 (1)	NT03SP0883 (1-403)	DeoB, phosphopentomutase	C
			NT03SP0884 (1-146)	ArsC, arsenate reductase	C
			NT03SP0885 (1-269)	DeoD, purine nucleoside phosphorylase, family 2	C
			NT03SP0886 (1-40)	hypothetical protein	C
			NT03SP0887 (1-201*)	DeoD, purine nucleoside phosphorylase	C
18	786173 – 789854	786173 – 789186 (1)	NT03SP0891 (*188-230)	PyrF, orotidine 5'-phosphate decarboxylase	C
		786665 – 789186 (1)			
		786665 – 789854 (1)	NT03SP0892 (1-209)	PyrE, orotate phosphoribosyltransferase	C
			NT03SP0893 (1-484)	AmiC, 6-aminohexanoate-cyclic-dimer hydrolase	C
			NT03SP0894 (1-287)	putative amino acid ABC transporter	S
			NT03SP0895 (1-66*)	amino acid ABC transporter	M
19	808341 – 810784	808341 – 810784 (1)	NT03SP0913 (*90-309)	metallo-β-lactamase superfamily protein	C
			NT03SP0914 (1-254)	oxidoreductase, short-chain dehydrogenase/reductase family	C
			NT03SP0915 (1-338*)	RecJ, single-stranded-DNA exonuclease	C
20	985219 – 988955	985219 – 988955 (1)	NT03SP1096 (*429-572)	Pgm, phosphoglucomutase	C
			NT03SP1097 (1-318)	sugar ABC transporter	M
			NT03SP1098 (*272-354)	sugar ABC transporter	M
21	1039380 – 1041995	1039380 – 1041995 (1)	NT03SP1152 (*63-261)	ABC-transporter	M
			NT03SP1153 (1-204)	conserved hypothetical protein	M
			NT03SP1154 (1-43)	hypothetical protein	S
			NT03SP1155 (1-73)	hypothetical phage protein	C

			NT03SP1156 (*137-262)	SpeL, pyrogenic toxin	M
22	1043956 – 1045813	1043956 – 1045795 (1) 1043956 – 1045813 (1)	NT03SP1158 (1-204*) NT03SP1159 (1-174) NT03SP1160 (*19-214)	hypothetical phage protein hypothetical phage protein cell wall hydrolase	M C C
23	1048585 – 1050136	1048585 – 1050136 (1)	NT03SP1165 (1-38*) NT03SP1166 (1-53) NT03SP1167 (1-211) NT03SP1168 (*215-410)	hypothetical phage protein hypothetical phage protein hypothetical phage protein conserved hypothetical protein	C C C C
24	1051571 – 1060562	1051571 – 1053571 (1) 1053568 – 1058282 (1) 1057456 – 1060562 (2)	NT03SP1169 (1-310*) NT03SP1170 (1-683) NT03SP1171 (1-259) NT03SP1172 (1-760) NT03SP1173 (1-495) NT03SP1174 (1-109) NT03SP1175 (1-119) NT03SP1176 (1-201) NT03SP1177 (1-129) NT03SP1178 (1-121) NT03SP1179 (*55-102)	hypothetical phage protein hypothetical phage protein minor phage-associated tail protein minor phage-associated tail protein hypothetical phage protein hypothetical phage protein gp168, structural phage protein hypothetical phage protein hypothetical phage protein hypothetical phage protein hypothetical phage protein	C C C M C C C C C C C
25	1077004 – 1079885	1077004 – 1079885 (1)	NT03SP1206 (1-134*) NT03SP1207 (1-34) NT03SP1208 (1-454) NT03SP1209 (1-227) NT03SP1210 (1-94)	putative bacteriophage protein hypothetical protein helicase-related phage protein putative bacteriophage protein hypothetical phage protein	C S C C C
26	1098055 – 1099278	1098055 – 1099278 (1)	NT03SP1237 (1-81) NT03SP1238 (1-182) NT03SP1239 (1-74)	putative IS1239 transposase transposase, IS30 family hypothetical protein	C C C
27	1210692 – 1212408	1210692 – 1212408 (1)	NT03SP1359 (1-36*) NT03SP1360 (1-143) NT03SP1361 (*209-594)	hypothetical phage protein hypothetical phage protein conserved hypothetical phage protein	C C C
28	1226452 – 1228702	1226452 – 1228702 (1)	NT03SP1384 (1-69*) NT03SP1385 (1-497) NT03SP1386 (*240-422)	hypothetical phage protein conserved hypothetical phage protein putative structural phage protein	C C C

29	1233350 – 1236360	1233350 – 1236360 (1)	NT03SP1395 (1-24*) NT03SP1396 (1-113) NT03SP1397 (1-55) NT03SP1398 (1-265) NT03SP1399 (1-309) NT03SP1400 (1-109) NT03SP1401 (*26-68)	conserved hypothetical phage protein conserved hypothetical protein hypothetical phage protein hypothetical phage protein RecT, prophage pi1 protein 11, recombinase hypothetical phage protein hypothetical phage protein	C C C C C C C
30	1290707 – 1293060	1290707 – 1293060 (1)	NT03SP1463 (1-337) NT03SP1464 (1-142) NT03SP1465 (*154-411)	ArgF, ornithine carbamoyl transferase acetyltransferase, GNAT family ArcA, arginine deiminase	C C C
31	1323988 – 1326555	1323988 – 1326267 (1) 1324309 – 1326555 (1)	NT03SP1494 (1-444*) NT03SP1495 (1-308) NT03SP1496 (*245-259)	ABC transporter ABC-transporter MalF, ABC-transporter	S M M
32	1456633 – 1458454	1456633 – 1458454 (1)	NT03SP1632 (1-38*) NT03SP1633 (1-142) NT03SP1634 (*252-671)	hypothetical phage protein hypothetical phage protein hypothetical phage protein	C C C
33	1459302 – 1463090	1459302 – 1463090 (1)	NT03SP1635 (1-341*) NT03SP1636 (1-659) NT03SP1637 (1-52) NT03SP1638 (*33-238)	hylP3, hyaluronidase conserved hypothetical protein putative conserved domain protein hypothetical phage protein	C C C C
34	1541198 – 1543384	1541198 – 1543384 (1)	NT03SP1743 (1-230*) NT03SP1744 (*85-580)	iron compound ABC transporter iron compound ABC transporter	M M
35	1544830 – 1547236	1544830 – 1547236 (1) 1544935 – 1547236 (1) 1544935 – 1546996 (1)	NT03SP1745 (*108-907*)	leucine-rich repeat domain protein	M
36	1555527 – 1559783	1556817 – 1558985 (1) 1556817 – 1557963 (1) 1556418 – 1559783 (1) 1555527 – 1559783 (1) 1556817 – 1558779 (1)	NT03SP1753 (1-172*) NT03SP1754 (1-294) NT03SP1755 (1-31) NT03SP1756 (*145-975)	mannose-6-phosphate isomerase, class I ScrK, fructokinase hypothetical protein secreted endoglycosidase	C C S S
37	1569578 – 1570909	1569578 – 1570909 (1)	NT03SP1765 (*88-529*)	UvrA, excinuclease ABC, A subunit	C

38	1595563 – 1598153	1595563 – 1598153 (1)	NT03SP1792 (1-398) NT03SP1793 (1-239) NT03SP1794 (1-181*)	Transporter transcriptional regulator, CRP family PepX, X-pro dipeptidyl-peptidase	M C C
39	1604401 – 1606870	1604401 – 1606870 (2) 1604808 – 1606870 (1)	NT03SP1802 (*272-374) NT03SP1803 (1-223) NT03SP1804 (1-400) NT03SP1805 (*215-259)	conserved hypothetical protein DeoC, deoxyribose-phosphate aldolase NupC, putative nucleoside transporter Udp-1, uridine phosphorylase	C C M C
40	1655413 – 1657728	1655413 – 1657728 (1) 1655695 – 1657723 (1)	NT03SP1845 (1-275*) NT03SP1846 (*96-561)	LacG, 6-phospho-β-galactosidase LacE, PTS system, lactose-specific IIBC components	C M
41	1662420 – 1665210	1662420 – 1665210 (1)	NT03SP1853 (*228-257) NT03SP1854 (1-95) NT03SP1855 (1-111) NT03SP1856 (1-156) NT03SP1857 (1-49) NT03SP1858 (*79-130)	LacR, lactose phosphotransferase system repressor conserved hypothetical protein conserved hypothetical protein putative phage family integrase putative integrase RpsI, ribosomal protein S9	C C C C C C
42	1741557 – 1744675	1741557 – 1744675 (6) 1743154 – 1744675 (9)	NT03SP1935 (1-264*) NT03SP1936 (1-83) NT03SP1937 (*142-538)	Mga, regulatory protein conserved hypothetical protein Isp, autolysin	C M S
43	1786418 – 1790263	1786418 – 1790263 (1)	NT03SP1987 (*110-510) NT03SP1988 (1-421) NT03SP1989 (1-31) NT03SP1990 (1-335*)	AhpF, alkyl hydroperoxide reductase HutI, imidazolone-propionase hypothetical protein HutU, urocanate hydratase	C C S C
44	1792999 – 1795500	1792999 – 1795500 (1) 1794048 – 1795500 (1)	NT03SP1993 (*31-557) NT03SP1994 (1-198) NT03SP1995 (1-25*)	Fhs, formate-tetrahydrofolate ligase conserved hypothetical protein putative amino acid permease	C C M
45	1803098 – 1805513	1803098 – 1805513 (1)	NT03SP1999 (*68-255) NT03SP2000 (1-346) NT03SP2001 (*462-631)	RpsB, ribosomal protein S2 Tsf, translation elongation factor Ts PepO, endopeptidase O	C C C
46	1839526 – 1841207	1839526 – 1841207 (1)	NT03SP2035 (1-209*) NT03SP2036 (*191-426)	AspS, aspartyl-tRNA synthetase HisS, histidyl-tRNA synthetase	C C
47	1864737 –	1864737 – 1867258 (1)	NT03SP2066 (1-63*)	TrmU, tRNA methyltransferase	C

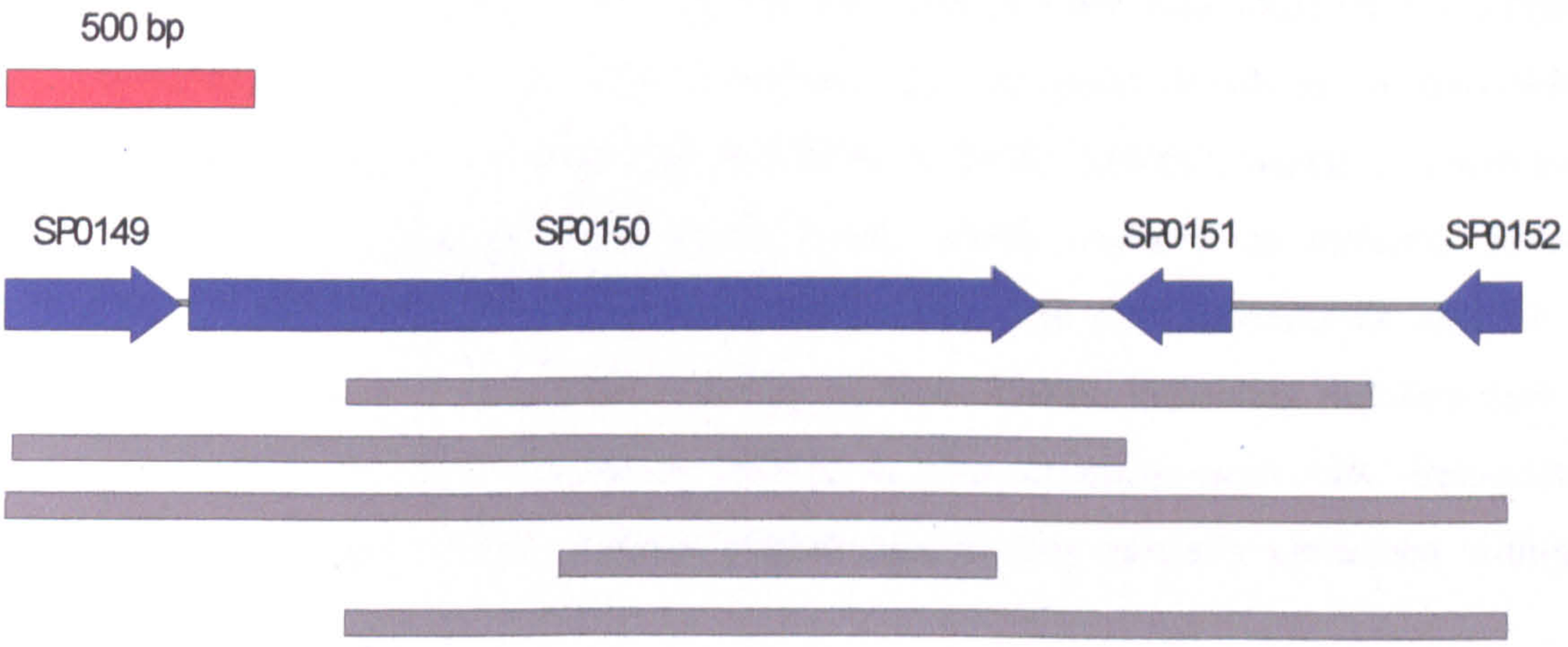
	1869288	1867255 – 1869288 (1)	NT03SP2067 (1-41) NT03SP2068 (1-223) NT03SP2069 (1-290) NT03SP2070 (1-204) NT03SP2071 (1-266) NT03SP2072 (*176-280)	hypothetical protein SdhB, L-serine dehydratase β subunit SdhA, L-serine dehydratase α subunit IsaA, immunodominant antigen A ABC transporter, permease ABC transporter	S S M S M M
48	1891068 – 1891842	1891068 – 1891842 (1)	NT03SP2093 (1-177) NT03SP2094 (1-87)	IS30 transposase putative IS1239 transposase	C C

Table 3.2: Identification of *S. pyogenes* loci encoding antigens recognised by human sera. The genomic expression library was screened with sera from 11 patients with confirmed *S. aureus* bacteremia, 4 nasal carriers and 4 non-carriers. Locus, clone and gene co-ordinates correspond to the *S. pyogenes* MGAS8232 genome (http://cmr.tigr.org), and function is assigned according to the TIGR4 genome database. Asterisks indicate partially cloned genes. Predicted subcellular location is shown according to SOSUIsignal (http://bp.nuap.nagoya-u.ac.jp/sosui/sosuisignal/sosuisignal_submit.html): C, cytoplasmic; M, membrane; S, secreted / surface protein.

3.2.6. Analysis of putative antigenic loci

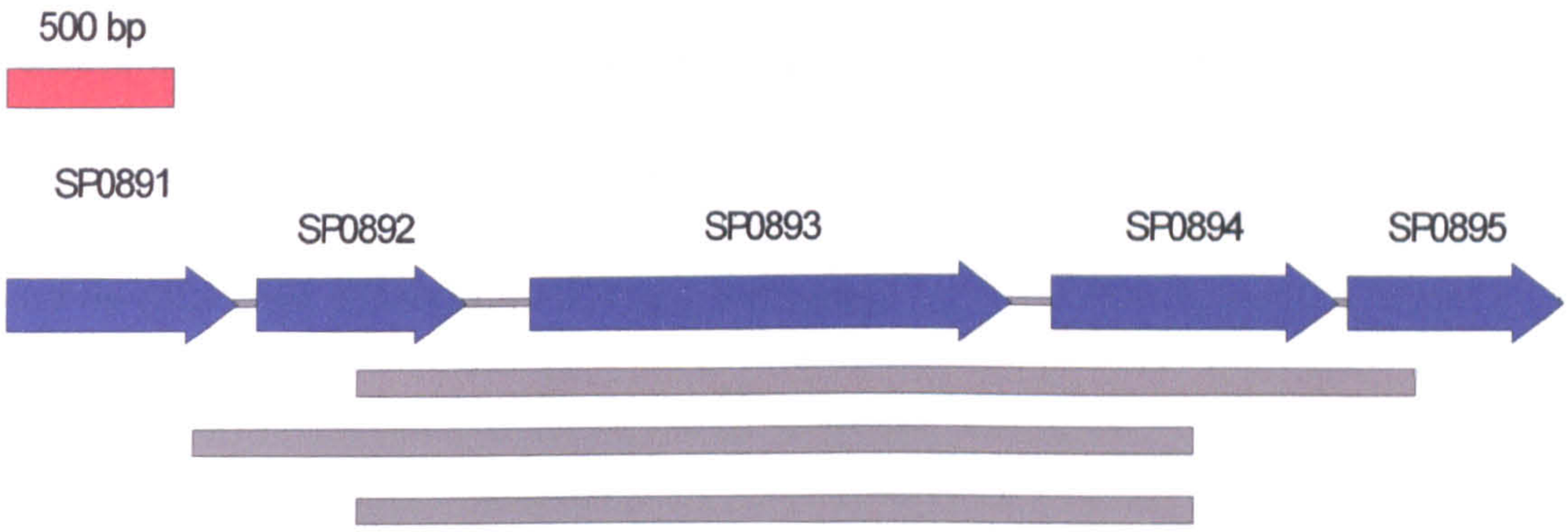
Loci identified during the immunoscreen of *S. pyogenes* (Table 3.2) which were of particular interest were selected for further analysis. These included loci containing putatively surface located proteins and loci which were identified by multiple clones during the screen. The loci are shown below, the grey bars represent clones which were identified during the screen.

Locus 6 (3029 bp; 152987 – 156015; 5 clones)



Analysis of locus 6 revealed the presence of four ORFs, represented by five individual clones. As SP0150 is represented by all five clones, this is most likely the antigenic protein in the locus. SP0150 encodes the toxin streptolysin O (Slo) and contains a signal peptide, suggesting that it is secreted or surface-located (Billington *et al.* 2000).

Locus 18 (3682 bp; 786173 – 789854; 3 clones)

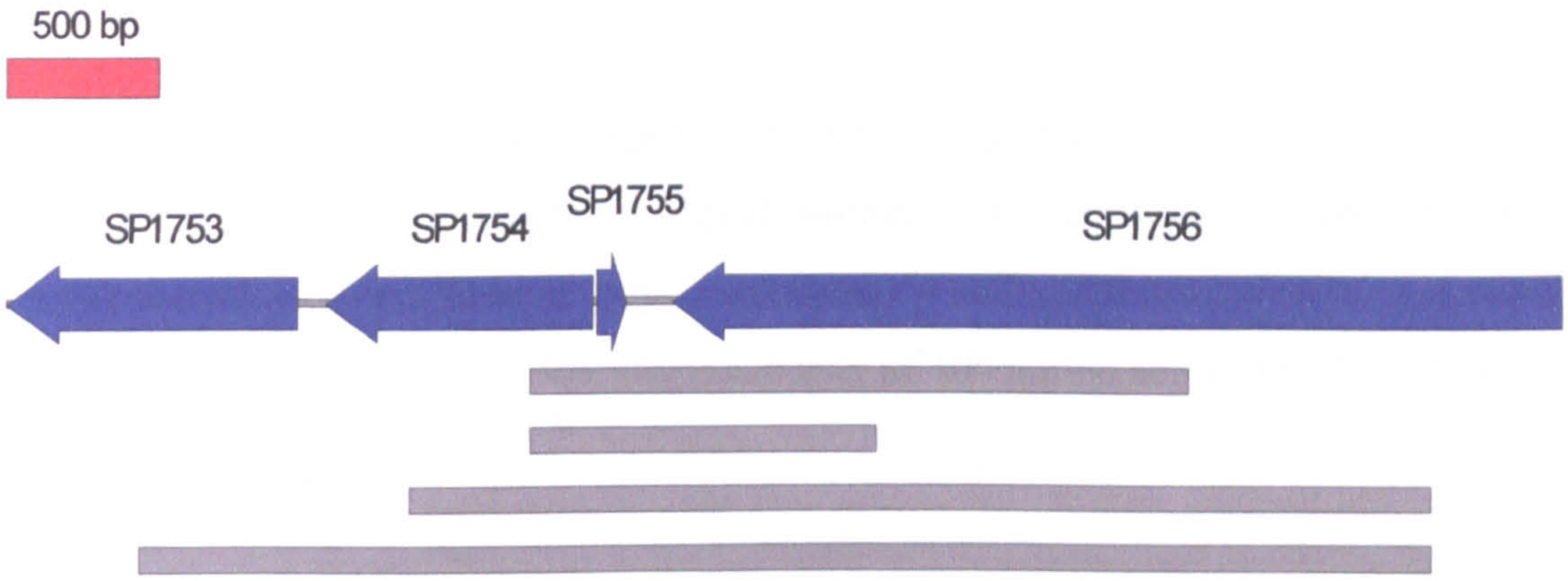


Analysis of locus 18 identified five ORFs, represented by three individual clones. SP0891 and SP0892 encode PyrF and PyrE, respectively, enzymes involved in pyrimidine ribonucleotide biosynthesis (Turnbough and Switzer, 2008). SP0893, which is represented by all three clones, encodes the enzyme AmiC which contains an amidase domain (PF01425). SP0894 is a secreted/surface-located amino acid ABC transporter, and the N-terminus of this protein is also represented by all three clones, indicating that this may be the antigenic protein within the locus. SP0895 is also an amino acid ABC transporter, although it is does not contain a signal peptide and is only partially contained within a single clone.

Locus 35 (2407 bp; 1544830 – 1547236; 3 clones)

Analysis of locus 35 identified a single ORF represented by three individual clones. SP1745 is a leucine-rich repeat protein of 1240 amino acids. Sequence analysis revealed the presence of two NEAT domains from amino acids 330-466 and 937-1094, suggesting a role for this protein in the transport of iron (Andrade *et al.* 2002).

Locus 36 (4257 bp; 1555527 – 1559783; 5 clones)

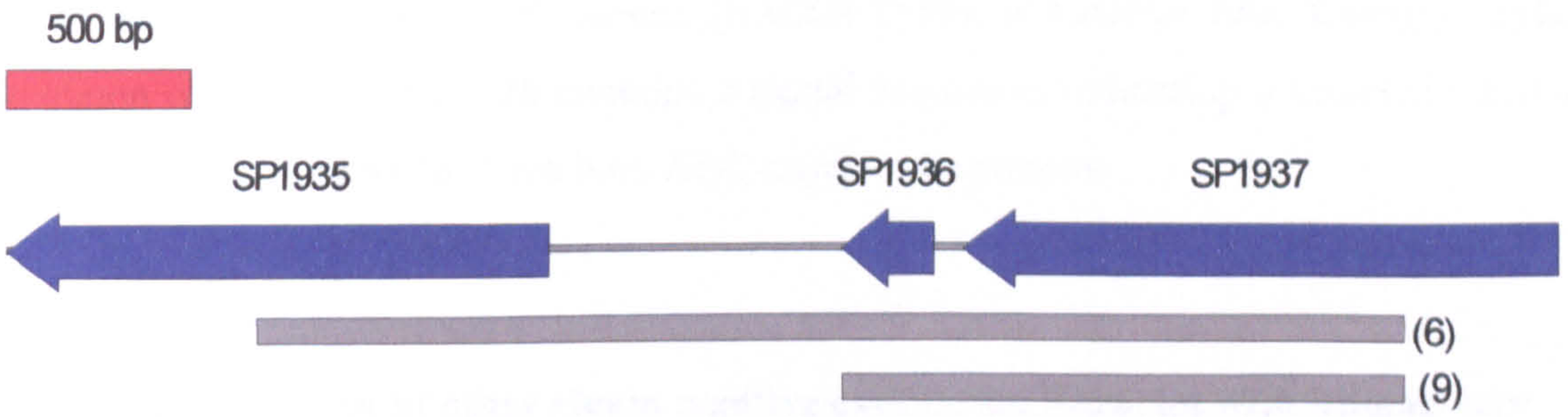


Locus 36 consists of four ORFs represented by five individual clones. SP1754 encodes a fructokinase of 294 amino acids, SP1755 encodes a hypothetical protein of 31 amino acids and SP1756 encodes a putative endoglycosidase of 975 amino acids. SP1756 contains a signal peptide, indicating a secreted / surface location and as the C-terminus of this protein is represented by all five clones, it can be suggested that this is the most likely antigen within this locus.

Locus 39 (2470 bp; 1604401 – 1606870; 3 clones)

Analysis of locus 39 identified four ORFs, represented by 3 individual clones in two groups. SP1802 is a conserved hypothetical protein of 374 amino acids, and SP1803 is a deoxyribose-phosphate aldolase, DeoC, involved in nucleotide metabolism (Han *et al.* 2004). SP1804 is a putative nucleoside transporter of 400 amino acids and SP1805 is a 259-residue uridine phosphorylase.

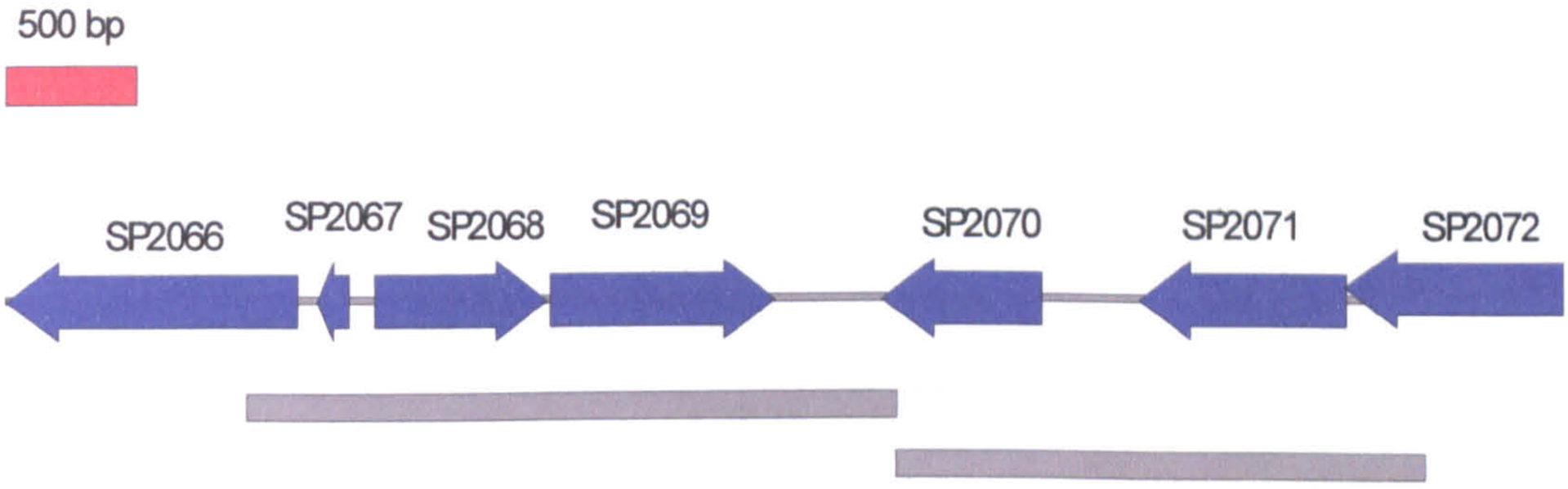
Locus 42 (3120 bp; 1741557 – 1744676; 15 clones)



Locus 42 consists of 3 ORFs, represented by 15 individual clones in two distinct groups. SP1937 is an immunogenic secreted protein (Isp) of 538 amino acids (McIver *et al.* 1996). The C-terminus of this protein is represented by all 15 clones isolated. Isp is a putative autolysin and contains a CHAP domain at the C-terminus, denoting an amidase function. A signal sequence is also present, indicating that Isp is a secreted / surface located protein.

SP1936, an 83-residue protein of unknown function, contains homologues throughout *S. pyogenes* (83% identity over the full length of the protein). These homologues have been named immunogenic proteins (http://www.tigr.org), suggesting the surface-located SP1936 may also be immunogenic.

Locus 47 (4552 bp; 1864737 – 1869288; 2 clones)



Locus 47 contains seven ORFs represented by two clones in the screen. SP2066 is a tRNA methyltransferase and SP2067 is a surface-located hypothetical protein. SP2068 and SP2069 are both L-serine dehydratases, enzymes likely involved in the gluconeogenesis pathway. SP2070 is an immunogenic secreted antigen (IsaA), which is 66% identical over 295 amino acids to IsaA in *S. aureus* (SACOL2584), a putative lytic transglycosylase (Stapleton *et al.* 2007). SP2070 contains a signal sequence, indicating a secreted / surface location. SP2071 and SP2072 are both ABC transporter proteins.

3.3 Immunoscreening of other Gram positive expression libraries with human sera

Previously, bacteriophage expression libraries of *S. aureus* LH607 and *S. epidermidis* 138 were probed with sera from patients with staphylococcal infection in order to identify antigenic and cross-reactive proteins (Clarke *et al.* 2006; Pourmand *et al.* 2006). In addition, libraries of *Streptococcus agalactiae* 6313 and *Streptococcus pneumoniae* (serotype III clinical isolate) were screened as above by Dr. Melanie Stapleton and Hilde Moseby, respectively. Results of these screens are shown in Table 3.3 and Table 3.4.

Locus	Locus co-ordinates	Clone coordinates (Clone occurrence)	Genes within locus (cloned region amino acids)	Designation, putative function or homology	Cellular location
1	?-30318	?-30318 (5)	GBS0016 (?-447)	PcsB, cell wall hydrolase	S
2	37239 – 40554	37239 – 40554 (1)	GBS0024 (*195-1241*)	phosphoribosylformylglycinamide synthase	C
3	58525 – 60012	58525 – 60012 (1)	GBS0042 (*213-420)	PurD, phosphoribosylamine-glycine ligase	C
			GBS0043 (1-163)	PurE, phosphoribosylaminoimidazole carboxylase	C
			GBS0044 (1-34*)	PurK, phosphoribosylaminoimidazole carboxylase	S
4	99888 – 102659	99888 – 102659 (3)	GBS0088 (*154-183)	unknown	C
			GBS0089 (1-139)	unknown	C
			GBS0090 (1-159)	ComXI, role unknown	C
			GBS0091 (1-230)	putative phosphoglycerate mutase	C
			GBS0092 (1-183*)	serine-type D-ala D-ala carboxypeptidase	M
5	275220 – 277811	275220 – 277811 (1)	GBS0257 (*110-180)	unknown	M
			GBS0258 (1-304)	GlyQ, glycyl-tRNA synthetase (alpha subunit)	C
			GBS0259 (1-213)	acyl carrier protein phosphodiesterase	C
			GBS0260 (1-168*)	GlyS, glycyl-tRNA synthetase (beta subunit)	C
6	320442 – 322053	320442 – 322053 (1)	GBS0297 (*80-246)	putative membrane protein	M
			GBS0298 (1-361)	histidine kinase	M
			GBS0299 (1-8*)	response regulator	C
7	666392 – 668629	666392 – 668629 (1)	GBS0651 (*154-667)	CylE, unknown	C
			GBS0652 (1-232*)	CylF, unknown	C
8	860546 – 863346	860546 – 863346 (1)	GBS0836 (1-266*)	ribonucleoside diphosphate reductase, β-subunit	C
			GBS0837 (*599-719)	ribonucleoside diphosphate reductase 2, α-subunit	C
9	1345584 – 1350543	1348424 – 1350368 (1)	GBS1301 (*36-253)	OrfB, transposase	C
			GBS1302 (1-96)	pXO1, transposase	C
		1345584 – 1350543 (1)	GBS1306 (1-822)	histidine triad protein	S
10	1351880 – 1354239	1351880 – 1354239 (3)	GBS1308 (*309-1094*)	ScpB, C5a peptidase	S
		1352247 – 1353873 (1)			
11	1375822 – 1377466	1375822 – 1377466 (1)	GBS1329 (1-225*)	LacG, 6-phospho-β-galactosidase	S
			GBS1330 (*276-568)	LacE, lactose-specific PTS	M

				system enzyme IIBC component	
12	1407262 – 1409398	1407262 – 1409398 (1)	GBS1356 (1-401*) GBS1357 (1-196) GBS1358 (1-133*)	ASP1, agglutinin receptor AbiGI, abortive infection protein AbiGII , abortive infection protein	M C C
13	1588571 – 1590843	1588571 – 1590843 (1)	GBS1529 (*555-1310)	cell wall surface anchor protein	C
14	1732604 – 1734906	1732604 – 1734906 (1)	GBS1674 (1-242*) GBS1675 (1-184) GBS1676 (1-237) GBS1677 (1-48*)	HtpX, heat shock protein membrane protein GidB, methyltransferase sodium-transport family protein	M M C M
15	1750256 – 1750411	1750256 – 1750411 (1)	GBS1691 (*64-113*)	unknown	C
16	1754356 – 1755833	1754356 – 1755833 (1)	GBS1696 (*48-124) GBS1697 (1-237) GBS1698 (*49-134)	DhaK3, dihydroxyacetone kinase glycerol transport protein unknown	C M C
17	1810660 – 1812243	1810660 – 1812243 (1)	GBS1752 (1-46*) GBS1753 (*20-499)	unknown putative multi-drug resistance protein	M M

Table 3.3: Identification of *S. agalactiae* 6313 loci encoding antigens recognised by human sera. The genomic expression library was screened with 11 patients with confirmed *S. aureus* bacteremia, 4 nasal carriers and 4 non-carriers. Locus, clone and gene co-ordinates correspond to the *S. agalactiae* NEM316 genome (http://cmr.tigr.org), and function is assigned according to this genome database. "?" refers to sequence which could not be mapped to the *S. agalactiae* NEM316 genome. Results obtained by Dr. Melanie Stapleton. Asterisks indicate partially cloned genes. Predicted subcellular location is shown according to SOSUIsignal (http://bp.nuap.nagoya-u.ac.jp/sosui/sosuisignal/sosuisignal_submit.html): C, cytoplasmic; M, membrane; S, secreted / surface protein.

Locus	Locus co-ordinates	No. of Clones	Genes within locus (cloned region amino acids)	Designation, putative function or homology	Cellular location
1	58164-61441	13	SP0055 (*62-75) SP0056 (1-432) SP0057 (*708-1312)	hypothetical protein PurB, adenylosuccinate lyase StrH, β-N-acetylhexosaminidase	S C S
2	177326-180252	15	SP0186 (*151-943) SP0187 (1-183*)	UvrA, excinuclease ABC subunit A peptidase M24 family protein	C C
3	249373-253330	1	SP0273 (*315-693) SP0274 (1-904*)	FusA, translation elongation factor G DNA polymerase III, α subunit	C C
4	559641-561854	22	SP0588 (*561-737) SP0589 (1-205) SP0590 (1-294) SP0591 (1-22*)	Pnp, polyribonucleotide nucleotidyltransferase CysE, serine acetyltransferase acetyltransferase, GNAT family CysS, cysteinyl-tRNA synthetase	C C C C
5	615142 - 621723	1	SP0648 (1-2093*)	BgaA, β-galactosidase	S
6	885552-887635	6	SP0932 (*88-420) SP0933 (1-265) SP0934 (1-32) SP0935 (1-55*)	ProA, γ -glutamyl phosphate reductase ProC, pyrroline-5-carboxylate reductase hypothetical protein Tmk, thymidylate kinase	C C C C
7	944840-945893	1	SP1003 (*193-543*)	conserved hypothetical protein	S
8	963764-966490	2	SP1020 (*105-359) SP1021 (1-279) SP1022 (1-199) SP1023 (1-143) SP1024 (1-13*)	PrfA, peptide chain release factor 1 HemK protein Sua5/YciO/YrdC family protein acetyltransferase, GNAT family GlyA, serine hydroxymethyltransferase	C C C C C
9	1106295-1113004	1	SP1171 (1-235*) SP1172 (1-39) SP1173 (1-124) SP1174 (1-819) SP1175 (*94-802)	hydrolase, haloacid dehalogenase-like family protein hypothetical protein conserved hypothetical protein conserved domain protein conserved domain protein	C C S S C
10	1216210-1218716	1	SP1282 (1-519) SP1283 (*39-299)	ABC transporter HtpX, heat shock protein	C M
11	1776017-1780064	1	SP1870 (*187-318) SP1871 (1-250) SP1872 (1-321) SP1873 (1-80) SP1874 (1-240) SP1875 (1-189)	iron-compound ABC transporter iron-compound ABC transporter iron-compound ABC transporter conserved hypothetical protein RluB, ribosomal large subunit pseudouridine synthase B ScpB, segregation and condensation protein B	M C S C C C

			SP1876 (*220-242)	ScpA, segregation and condensation protein A	C
12	1863087-1865606	1	SP1960 (*240-1078*)	RpoC, RNA polymerase, β subunit	C
13	2051178-2054073	2	SP2141 (1-424*) SP2142 (1-289) SP2143 (*663-886)	glycosyl hydrolase-related protein ROK family protein conserved hypothetical protein	C C C
14	2108143-2112376	1	SP2188 (*67-290) SP2189 (1-318) SP2190 (1-693) SP2191 (*110-175)	HslO, chaperonin putative TIM-barrel protein, NifR3 family CbpA, choline binding protein A conserved hypothetical protein	C C S M
15	2118276-2121133	1	SP2195 (*8-152) SP2196 (1-242) SP2197 (1-335) SP2198 (1-249) SP2199 (*25-96)	CtsR, transcriptional regulator ABC transporter putative ABC transporter ABC transporter conserved hypothetical protein	C C M M S
16	2131598-2137361	1	SP2211 (?) SP2212 (1-81) SP2213 (?) SP2214 (1-346) SP2215 (1-259) SP2216 (1-392) SP2217 (1-164) SP2218 (*165-272)	IS66 family element, Orf3, degenerate transposase family protein, truncation IS66 family element, Orf2, interruption Tsf, translation elongation factor Ts RpsB, ribosomal protein S2 Usp45, secreted protein MreD, putative rod shape-determining protein MreC, rod shape-determining protein	? C ? C C S M M

Table 3.4: Identification of *S. pneumoniae* loci encoding antigens recognised by human sera. The genomic expression library was screened with 11 patients with confirmed *S. aureus* bacteremia, 4 nasal carriers and 4 non-carriers. Locus, clone and gene co-ordinates correspond to the *S. pneumoniae* TIGR4 genome (http://cmr.tigr.org), and function is assigned according to this genome database. Results obtained by Hilde Moseby. Asterisks indicate partially cloned genes. "?" indicates insufficient protein sequence data available for analysis. Predicted subcellular location is shown according to SOSUIsignal (http://bp.nuap.nagoya-u.ac.jp/sosui/sosuisignal/sosuisignal_submit.html): C, cytoplasmic; M, membrane; S, secreted / surface protein.

3.3.1. Identification of potential antigenic protein families

Data from the screens of expression libraries of *S. aureus* (Clarke *et al.* 2006), *S. epidermidis* (Pourmand *et al.* 2006), *S. agalactiae*, *S. pneumoniae* and *S. pyogenes* (this study) were subjected to bioinformatic analysis in order to identify potential families of cross-reactive antigens. Open reading frames identified in the *S. aureus* screen which corresponded to known or potential surface located or antigenic proteins were subjected to BLAST analysis to identify homologous proteins from the screens of the four other species. These were then grouped into putative antigenic families based on the sequence homology. Proteins were considered to have homologous regions if they contained regions of ≥15 residues which were at least 25% identical and 40% similar. If the similarity was greater than 40%, then 21-24% identity was also considered homologous. The homologous regions from all strains are detailed in Table A.1 (see Appendix).

The bioinformatic analysis identified 13 families from nine *S. aureus* proteins which contained homologous regions of potentially cross-reactive proteins identified from at least four of the five expression libraries screened. These 13 families are summarised in Table 3.6 and Figure 3.5.

SdrD (SACOL0609) and ClfA (SACOL0856) are surface-located fibrinogen-binding proteins, anchored covalently to the cell wall via an LPXTG motif (Josefsson *et al.* 1998). These two proteins are members of a multigene family of proteins in *S. aureus* which contain serine-aspartate (SD) repeats, including SdrD, SdrC, SdrE, ClfA, ClfB and Pls (Clarke and Foster, 2006). SdrD has a direct homologue in *S. epidermidis*, which is 37% identical over 1118 amino acids to that of *S. aureus*. Similar cell wall anchor family proteins are present in the streptococci; these proteins also contain regions of repeating peptides. FnbpB is an LPXTG surface protein able to bind fibronectin, along with the related protein FnbpA. These two proteins are similar in structure, and this structure is also conserved within the fibronectin-binding proteins of streptococci (Joh *et al.* 1994).

SACOL2666 (ScaH) is an ionically bound surface protein, which contains both a C-terminal CHAP domain (amino acids 493-617) and a glucosaminidase domain (amino acids 327-468). ScaH is a member of a family of ten proteins in *S. aureus*, designated the staphylococcal conserved antigen (Sca) family, which contain a conserved C-terminal CHAP domain (Pourmand *et al.* 2006). This family has direct homologues in *S. epidermidis* and ScaH is 55% identical in the two species (http://www.ebi.ac.uk/emboss/align/). Related proteins are also found in streptococci, including Usp45 in *S. pneumoniae*, PcsB in *S. agalactiae* and Isp in *S. pyogenes*, which all contain a C-terminal CHAP domain homologous to that of ScaH.

FmtB, also known as SasB, is an LPXTG protein and contains 18 repeats of 71 amino acids. It is present throughout the staphylococci and shares low similarity with the cell wall surface anchor family proteins in the streptococci, predominantly over the repeat regions of these proteins (e.g. FmtB is 16% identical over 688 amino acids to SA1647 of *S. agalactiae*). SasC is 31% identical over 2076 amino acids to SasB and contains 17 repeats of 71 amino acids (Brummell, 2005).

Three members of a multigene family proposed to be involved in iron transport were identified to contain homologous fragments. IsdA (SasE), IsdB (SasJ) and IsdH (SasI) are surface proteins and are present throughout strains of *S. aureus*, as well as having homologues in *S. epidermidis* and *Bacillus* species. The proteins contain at least one NEAT domain, putatively associated with iron transport (Grigg *et al.* 2007). Although no direct homologues of Isd proteins have been identified in the streptococci, a NEAT domain was identified during the immunoscreen in a leucine-rich repeat domain protein (SP1745) of *S. pyogenes*. NEAT domains have also been identified in *Listeria* species and *Clostridium perfringens* (Andrade *et al.* 2002).

S. aureus protein	Common fragment (amino acids)
SACOL0609 (SdrD)	30 – 200
SACOL0856 (ClfA)	155 – 320
SACOL2509 (FnbpB)	36 – 170
SACOL2666 (ScaH)	345 – 465
	510 – 619
SACOL2150 (FmtB / SasB)	1220 – 1510
	1511 – 1780
SACOL1806 (SasC)	1095 – 1275
	1600 – 1760
SACOL1140 (IsdA / SasE)	215 – 310
SACOL1781 (IsdH / SasI)	275 – 405
	650 – 760
SACOL1138 (IsdB / SasJ)	490 – 620

Table 3.6: Domains of *S. aureus* antigens which represent common homologous fragments across a range of cross-reactive antigens from staphylococcal and streptococcal species.

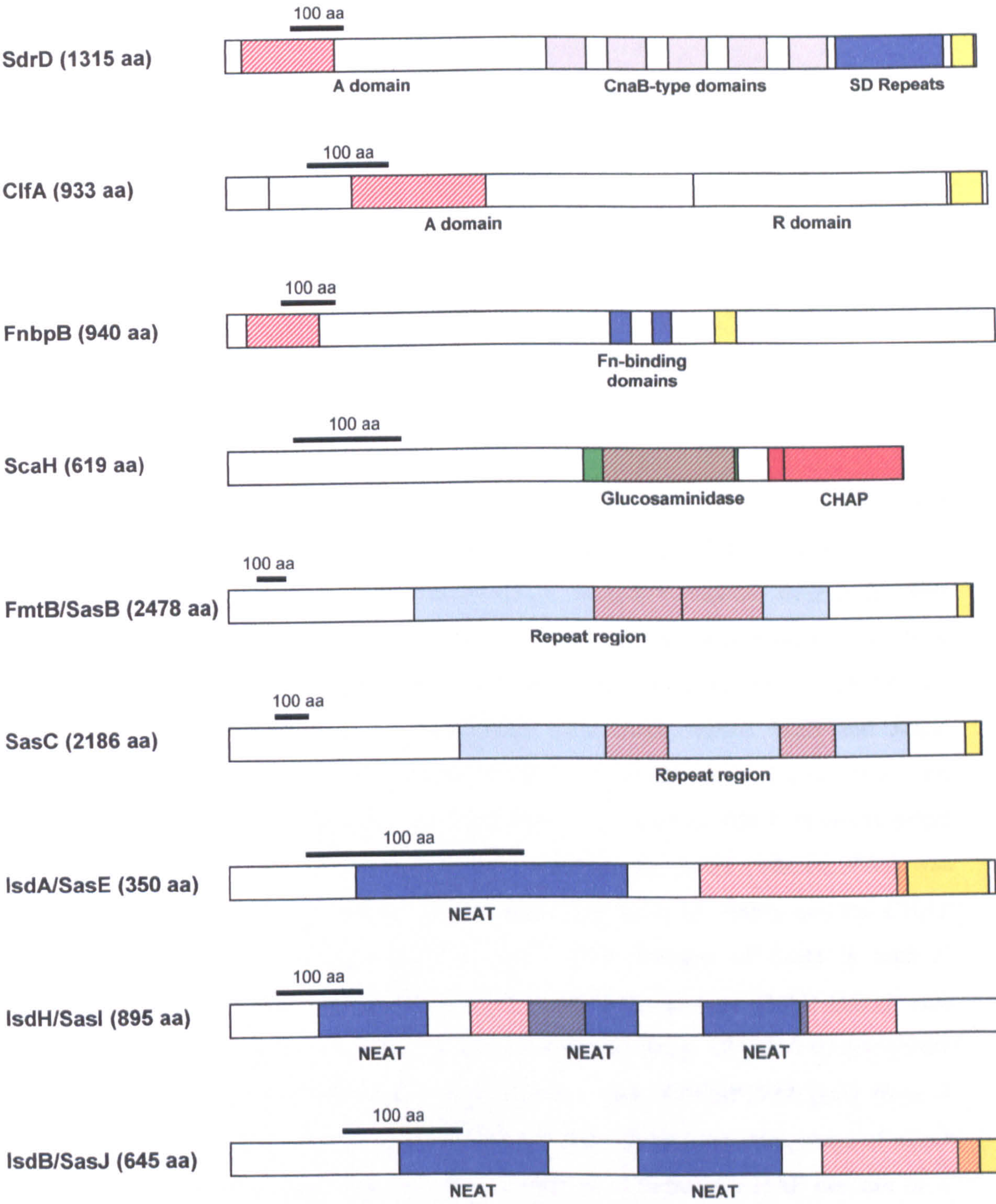


Figure 3.5: Proteins with conserved domains identified during the immunoscreening. Conserved regions are highlighted by the red stripes. Pfam domains were identified according to http://pfam.sanger.ac.uk. Gram-positive anchor domains are shown in yellow, all other Pfam domains are as labelled.

3.3.1.1. Alignment of protein families

For each of the 13 families, all of the homologous regions of proteins from *S. epidermidis*, *S. pneumoniae*, *S. agalactiae* and *S. pyogenes* were aligned with the *S. aureus* full-length protein to determine which families were particularly well-conserved. This analysis identified the N-terminus of SdrD (amino acids 30-200) (Figure 3.6) and the C-terminus of IsdA (215-310) (Figure 3.7) as the most conserved domains amongst these families, as they contained homologous regions from proteins of all species within the most conserved fragments. Homologous fragments within other families mapped to more divergent regions of the *S. aureus* proteins (not shown).

In addition to these two protein domains, one further domain was selected for subsequent analysis. Screening of the *S. epidermidis* expression library identified two novel proteins, SE0433 and SE2319 (Pourmand *et al.* 2006), which share 42% identity over their entire length and 60% identity in their C-terminal regions (www. ebi.ac.uk/emboss/align/). These were found to be members of the Sca family of proteins, with a conserved ~110 amino acid C-terminal CHAP domain. SE0433 and SE2319 were hence named ScaB and ScaA, respectively. The Sca family consists of 10 proteins in *S. epidermidis*, ScaA-J. Based on sequence homology, this family was also identified in *S. aureus*, which contains direct homologues of the ten *S. epidermidis* proteins. Furthermore, ScaH (SACOL2666) was identified in the *S. aureus* expression library screen (Clarke *et al.* 2006), and the CHAP domain of this protein is 36% identical to the CHAP domains of ScaB in both *S. epidermidis* (SE0433) and *S. aureus* (SACOL0723). Further members of this family were identified from screening of the streptococcal expression libraries: SP_2216 (Usp45) from *S. pneumoniae*, GBS0016 (PcsB) from *S. agalactiae*, and NT03SP1937 (Isp) from *S. pyogenes*. As the Sca family has members in several Gram positive species, some of which are potentially antigenic during infection, the conserved C-terminal CHAP domain of *S. aureus* ScaB (SACOL0723) was also chosen for further analysis.

Fragments from the alignments were mapped to the *S. aureus* proteins as shown in Figures 3.7-3.9. The conserved fragments, $SdrD_{30\text{-}200}$ (Figure 3.8), $IsdA_{215\text{-}310}$ (Figure 3.9) and

$ScaB_{146-265}$ (Figure 3.10), were subjected to BlastP analysis (http://tigrblast.tigr.org/cmr-blast/) and fragments identified during the alignments of the protein families were mapped to the *S. aureus* protein. Putative Pfam domains of each protein were identified using http://pfam.sanger.ac.uk.

This analysis demonstrated that the N-terminus was predominantly the homologous domain for each of the fragments identified by $SdrD_{30-200}$ (Figure 3.8). A direct homologue of SdrD was identified in *S. epidermidis*, SE0331, which contained a comparable SD repeat region, Gram-positive anchor domain and CnaB-type domains. Several amidases were also identified, four of which contained CHAP domains.

When domains homologous to $IsdA_{215-310}$ were analysed (Figure 3.9), it was apparent that the regions within each protein were distributed more diversely, unlike the relatively well-conserved N-terminus seen for SdrD. However, homologous fragments were generally present in the N-terminal region of each protein.

Five of the six proteins identified to be homologous to ScaB contained CHAP domains and the sequence homology occurred in this region in each case (Figure 3.10). The analysis also identified two direct homologues of ScaB in *S. epidermidis* which were identified in the screen, SE0433 (ScaB) and SE2319 (ScaA). ScaA was also identified by the SdrD domain, although the N-terminus was shown to be the homologous region in this case.

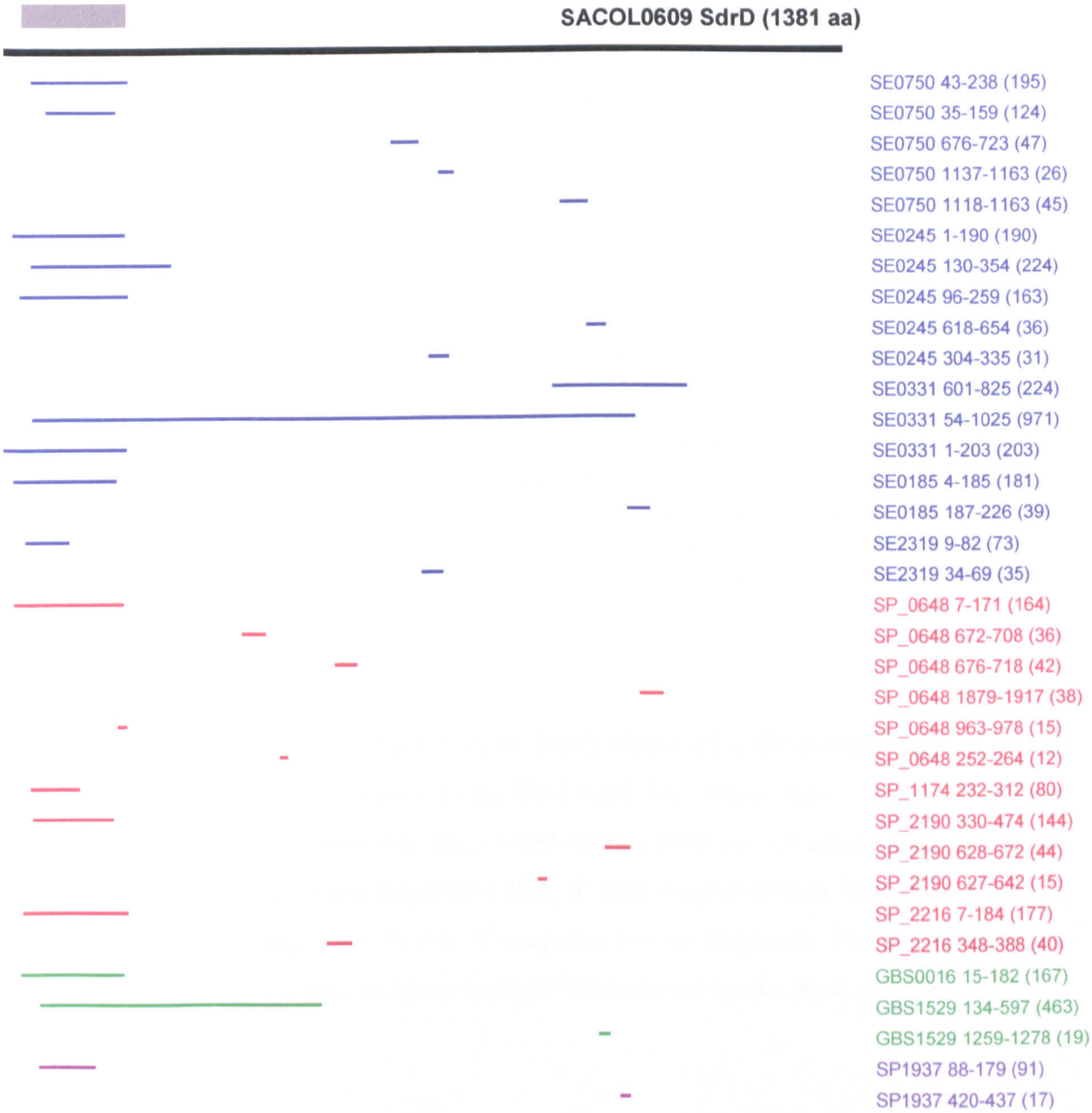


Figure 3.6: Alignment of the SdrD antigen family identified in the immunoscreen. The full-length *S. aureus* protein is shown by the bold black line. Homologous regions of proteins from other species are mapped to this, cloned regions (plus size) in amino acids are shown. Blue, *S. epidermidis* protein fragments; Red, *S. pneumoniae* protein fragments; Green, *S. agalactiae* protein fragments; Purple, *S. pyogenes* protein fragments. The conserved region chosen for further analysis (amino acids 30-200) is shown by the thick grey line.

Figure 3.7: Alignment of the IsdA antigen family identified in the immunoscreen. The full-length *S. aureus* protein is shown by the bold black line. Homologous regions of proteins from other species are mapped to this, cloned regions (plus size) in amino acids are shown. Blue, *S. epidermidis* protein fragments; Red, *S. pneumoniae* protein fragments; Green, *S. agalactiae* protein fragments; Purple, *S. pyogenes* protein fragments. The conserved region chosen for further analysis (amino acids 215-310) is shown by the thick grey line.

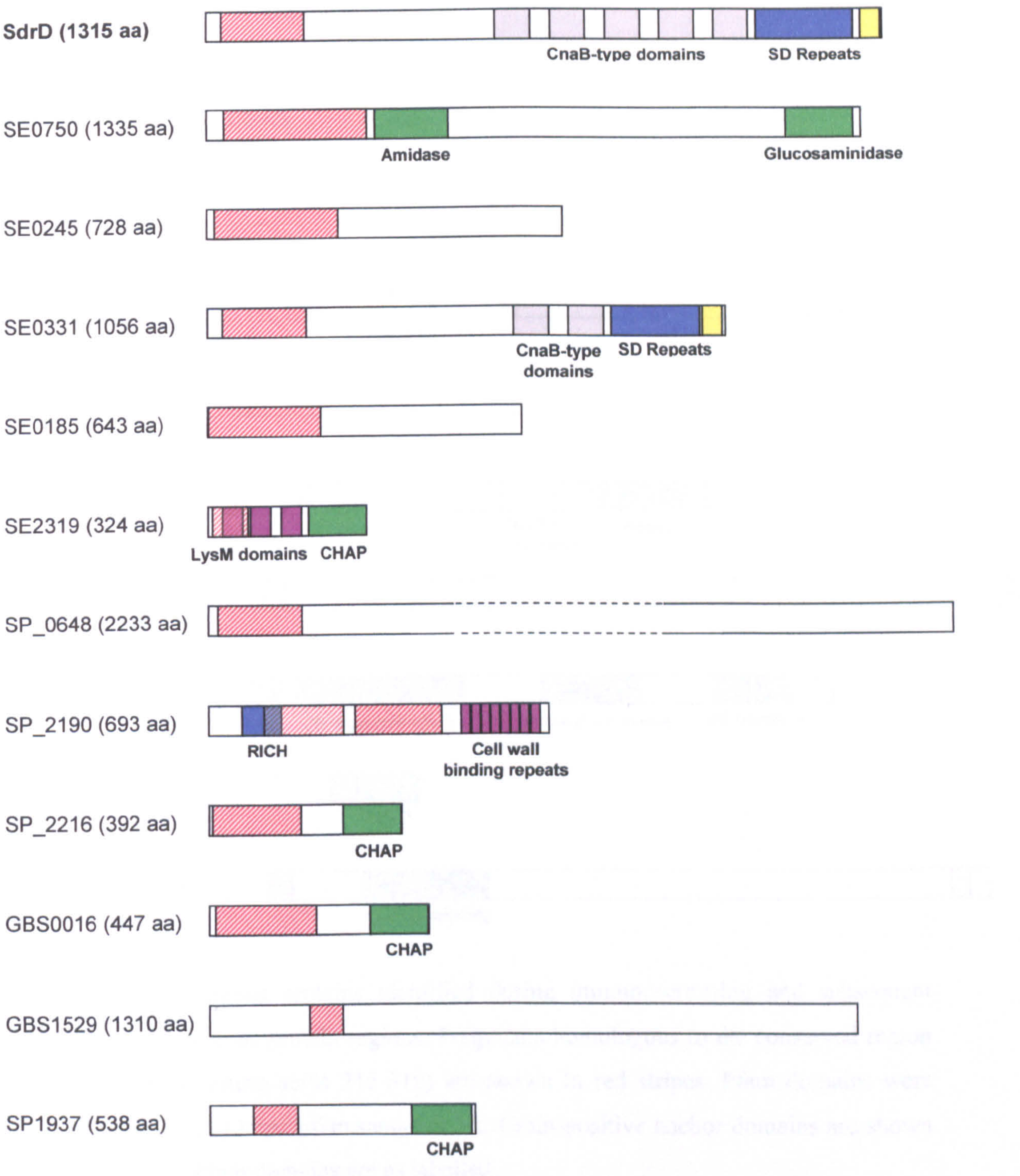


Figure 3.8: Homologous proteins identified during immunoscreening and subsequent alignment of homologous protein regions. Fragments homologous to the conserved region of SdrD identified (amino acids 30-200) are shown in red stripes. Pfam domains were identified according to http://pfam.sanger.ac.uk. Gram-positive anchor domains are shown in yellow, all other Pfam domains are as labelled.

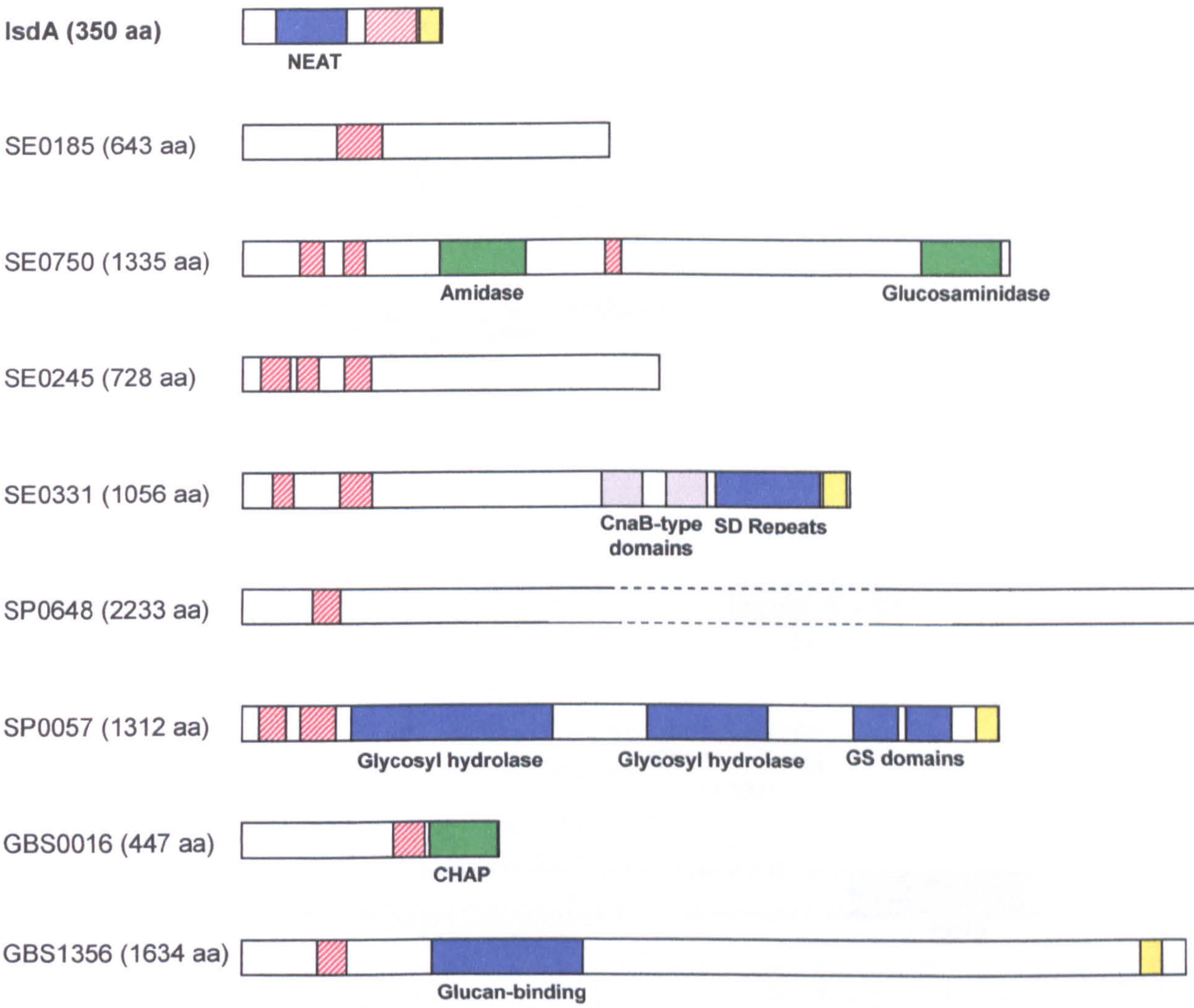


Figure 3.9: Homologous proteins identified during immunoscreening and subsequent alignment of homologous protein regions. Fragments homologous to the conserved region of IsdA identified (amino acids 215-310) are shown in red stripes. Pfam domains were identified according to http://pfam.sanger.ac.uk. Gram-positive anchor domains are shown in yellow, all other Pfam domains are as labelled.

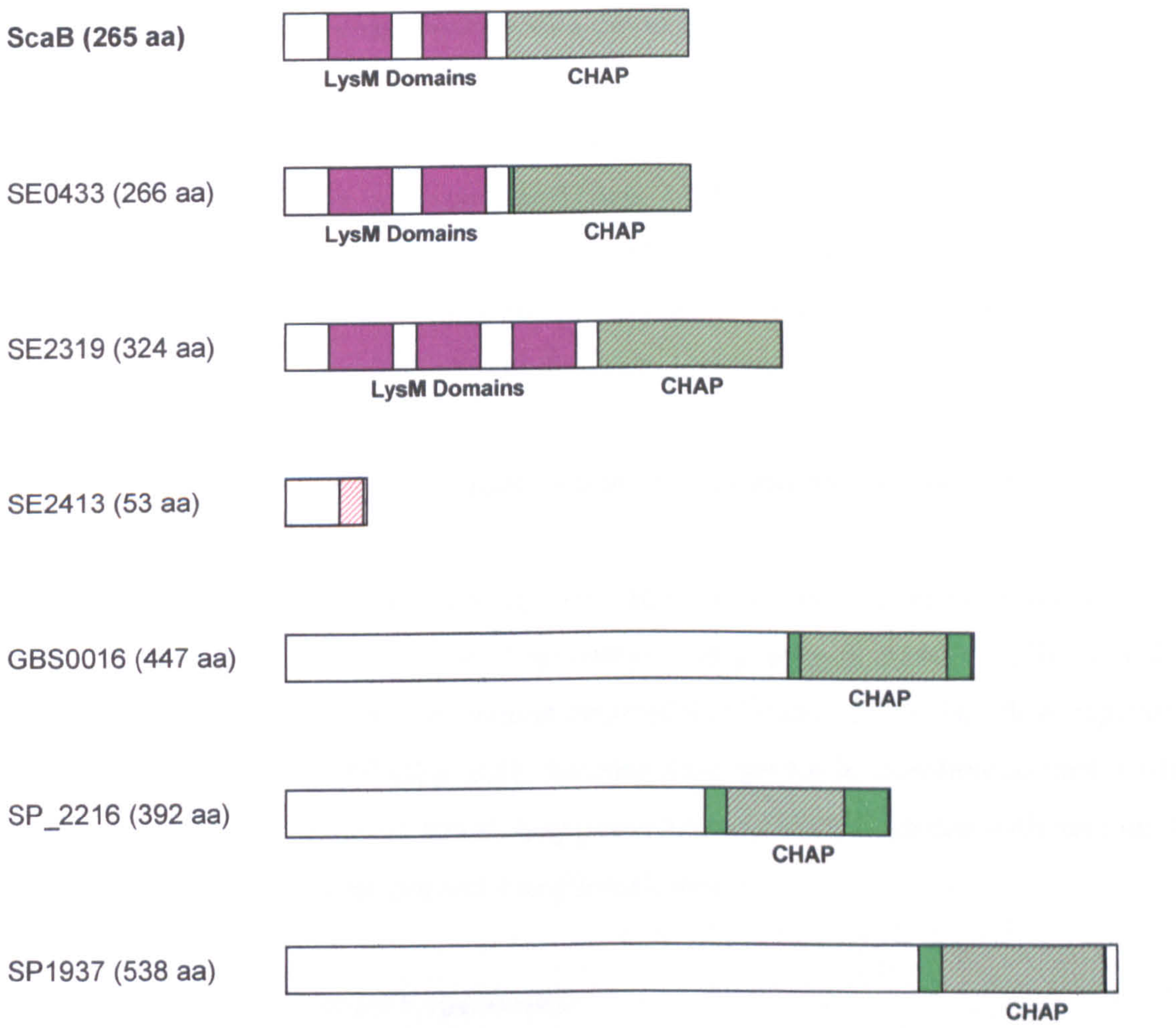


Figure 3.10: Homologous proteins identified during immunoscreening. Fragments homologous to the conserved CHAP domain of ScaB (amino acids 146-265) are shown in red stripes. Pfam domains were identified according to http://pfam.sanger.ac.uk and as labelled.

3.4 Use of conserved protein domains as vaccine candidates

3.4.1. Overexpression of conserved protein domains

In order to facilitate the analysis of the cross-genera identified domains for vaccination, the recombinant proteins were produced in *E. coli*. IsdA-c (amino acids 215-310), SdrD-n (amino acids 30-200) and ScaB-c (amino acids 156-265) were overexpressed by Dr. Melanie Stapleton (unpublished), and the purified recombinant polypeptides were used to raise polyclonal antisera in rabbits by the Antibody Resource Centre, Sheffield University.

3.4.2. Role of specific antisera in opsonisation of *S. aureus* and *S. agalactiae*

In order to determine the opsonophagocytic activity of specific antisera against the 3 conserved domains, sera were tested in assays using both *S. aureus* SH1000 and *S. agalactiae* 6313 in the presence of human neutrophils (Chapter 2.22). To allow expression of iron-regulated genes, including *isdA*, bacteria were grown in iron-limited media when the IsdA-c antiserum was being tested. Log phase bacteria were incubated with neutrophils, along with the specific antiserum and complement source.

3.4.2.1. Preliminary Control Experiments

Figure 3.11 demonstrates that killing of *S. aureus* is greatly increased in the presence of specific antisera. When no neutrophils are present, bacterial counts increase both with pre-immune serum (262% initial CFU after 3h) and no serum (657% initial CFU after 3h). With no complement, bacteria either increase (274% initial CFU after 3h with pre-immune serum) or decrease only slightly (73% initial CFU after 3h with no serum). When both neutrophils and complement are present, killing occurs with both pre-immune serum and no serum (48% and 40% initial CFU after 3h, respectively). However, the level of killing is highest when SdrD-n or ScaB-c antisera are present (1.6% and 2.3% initial CFU after 3h, respectively).

In iron-limited media (Figure 3.11 B), killing occurs in the no neutrophil and no complement controls in pre-immune serum (18% and 10% initial CFU after 3h, respectively), and the no neutrophil control with no serum (52% initial CFU after 3h). When both neutrophils and complement are present, killing occurs in both cases (pre-immune serum: 4% initial CFU after 3h; no serum: 18% initial CFU after 3h). With only neutrophils there is an increase of initial CFU (150% after 3h). Importantly, the level of killing is highest when the IsdA-c antiserum is present (0.3% initial CFU after 3h).

Figure 3.12 demonstrates an increased level of killing of *S. agalactiae* in the presence of specific antisera. Control experiments demonstrate that in the presence of pre-immune serum, bacterial numbers increase in all cases.

In THY medium (Figure 3.12 A), some killing occurs when no antiserum is present. With both neutrophils and complement included in the assay only 4% initial CFU is recovered after 3h. Similarly, when only complement is included only 9% initial CFU remains after 3h. When neutrophils alone are assessed, surviving bacteria increase (289% initial CFU after 3h), suggesting that neutrophils are not effective at opsonising *S. agalactiae* in the absence of a complement source. Importantly, bacteria are most effectively killed when SdrD-n and ScaB-c antisera are present (0.8% and 1.6% initial CFU after 3h, respectively).

Under iron-limited conditions (Figure 3.12 B), bacteria are not killed in the absence of antisera. When both neutrophils and complement were present 100% initial CFU remained after 3h and when complement was removed, 122% remained. As above, killing occurred in the absence of neutrophils (29% initial CFU after 3h). However, when these controls were compared to the sample including IsdA-c antiserum it was clear that the specific antibodies played a role in opsonising the bacterial cells as only 3.9% initial CFU remained after 3h.

3.4.2.2. Opsonisation Assays

In order to confirm the statistical significance of the opsonophagocytic killing, experiments were repeated in triplicate and data interpreted by *t*-test analysis (Figures 3.13, 3.14; Table 3.9).

Figure 3.13 and Table 3.9 show that incubation of *S. aureus* with the SdrD-n and ScaB-c antisera resulted in significant reductions in the number of bacteria recovered (3% and 5% initial CFU after 90 min, respectively) compared to pre-immune serum (37% initial CFU after 90 min; $P = 0.0168$ and 0.0185, respectively) and no serum (71% initial CFU after 90 min; $P = 0.0001$ and 0.0003, respectively). Incubation with IsdA-c antiserum also resulted in a significant reduction in the number of bacteria recovered (2.7% initial CFU after 90 min, compared to 66% for pre-immune and 53% for no serum; $P = 0.0192$ and 0.0005, respectively).

Analysis of the opsonisation of *S. agalactiae* (Figure 3.14; Table 3.9) showed that incubation of bacteria with SdrD-n and ScaB-c antisera increased the opsonic killing after 90 min (20% and 30% initial CFU after 90 min, respectively) compared to the no serum control (44% initial CFU after 90 min; $P = 0.0766$ and 0.2534, respectively), and was significantly different when compared to pre-immune serum (223% intial CFU after 90 min; $P = 0.0004$ and 0.0005, respectively). Incubation with IsdA-c antiserum resulted in a significant reduction in the number of bacteria recovered (28% initial CFU after 90 min, compared to 291% for pre-immune and 107% for no serum; $P = 0.0007$ and 0.0145, respectively).

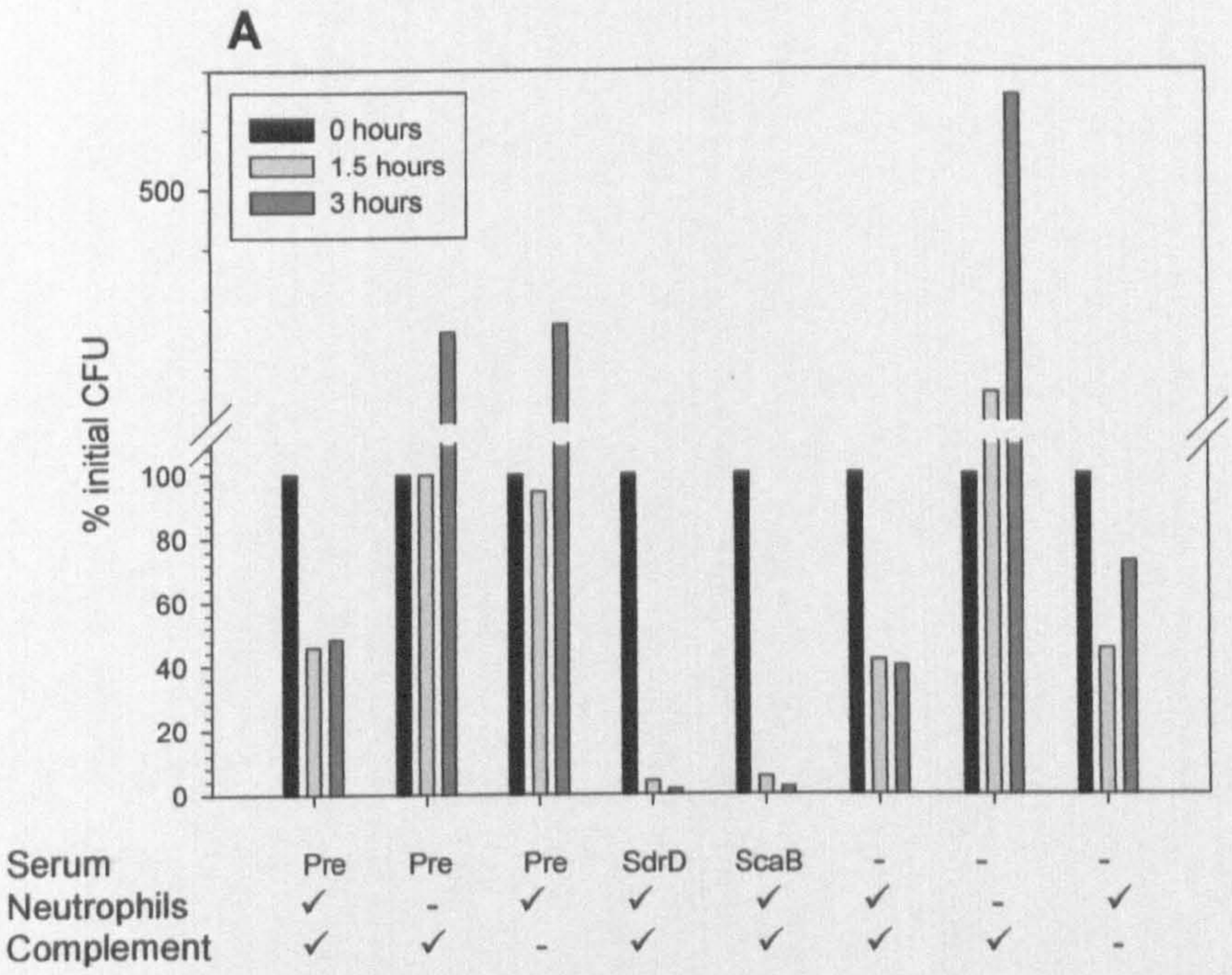
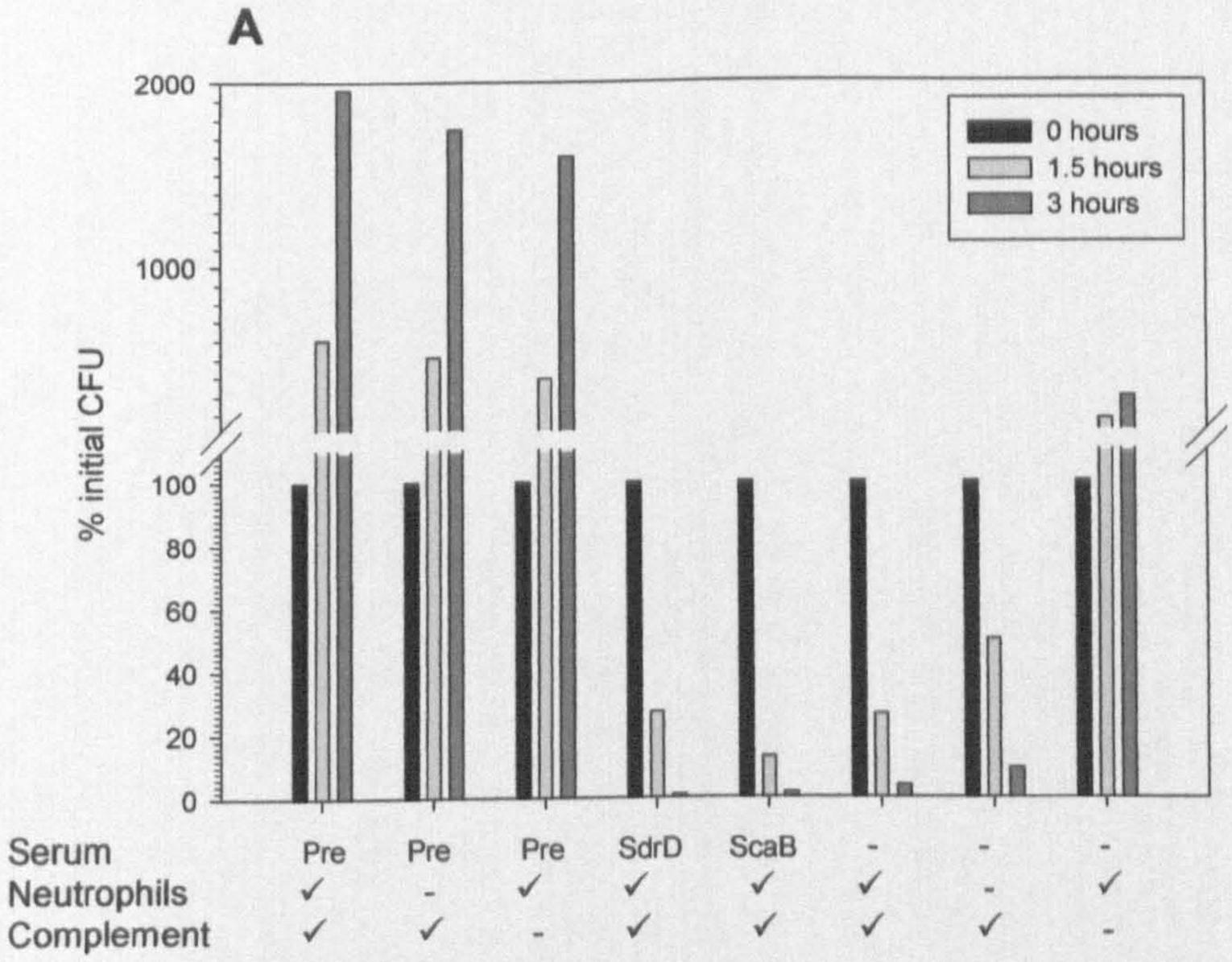


Figure 3.11: Opsonisation of *S. aureus* SH1000. Panel A, *S. aureus* grown in BHI; Panel B, *S. aureus* grown in iron-limited media (TSB). Components included in each assay are identified by the key.



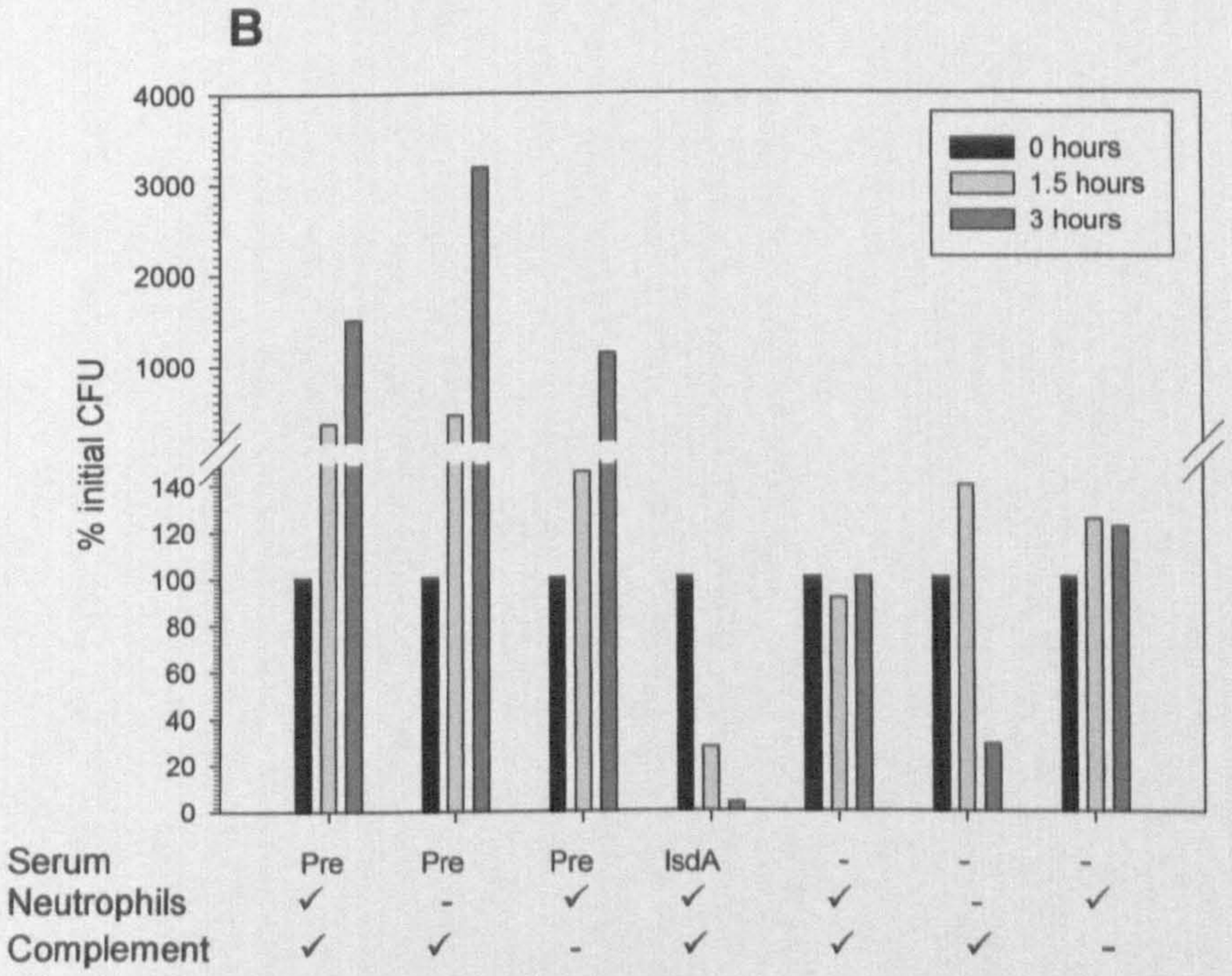


Figure 3.12: Opsonisation of *S. agalactiae* 6313. Panel A, *S. agalactiae* grown in THY; Panel B, *S. agalactiae* grown in iron-limited media (TSB). Components included in each assay are identified by the key.

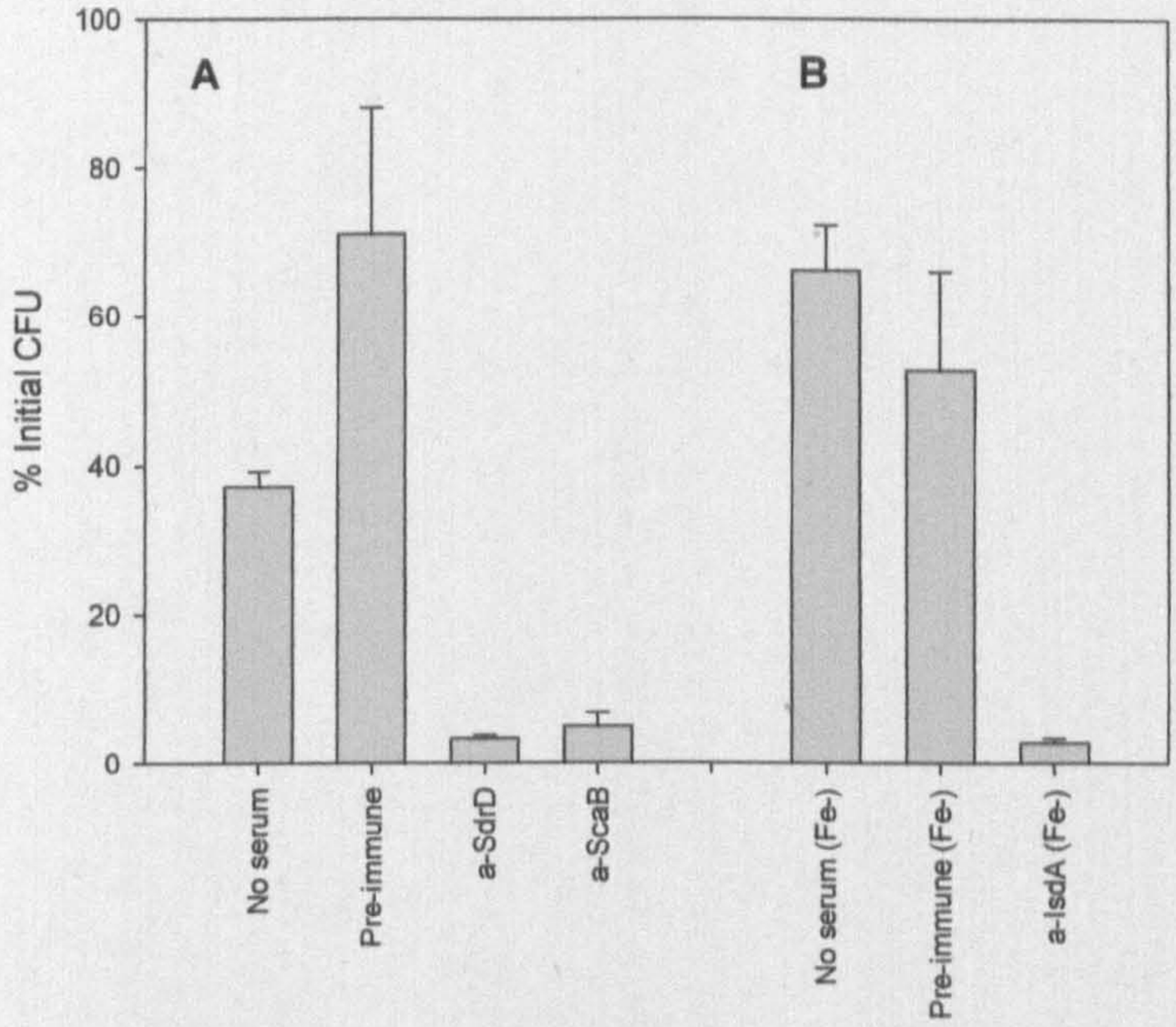


Figure 3.13: Percentage bacteria remaining after 1.5 h opsonisation of *S. aureus* SH1000 grown with (A) and without (B) iron. All samples contained both neutrophils and complement, results are from triplicate samples. Standard error bars are shown.

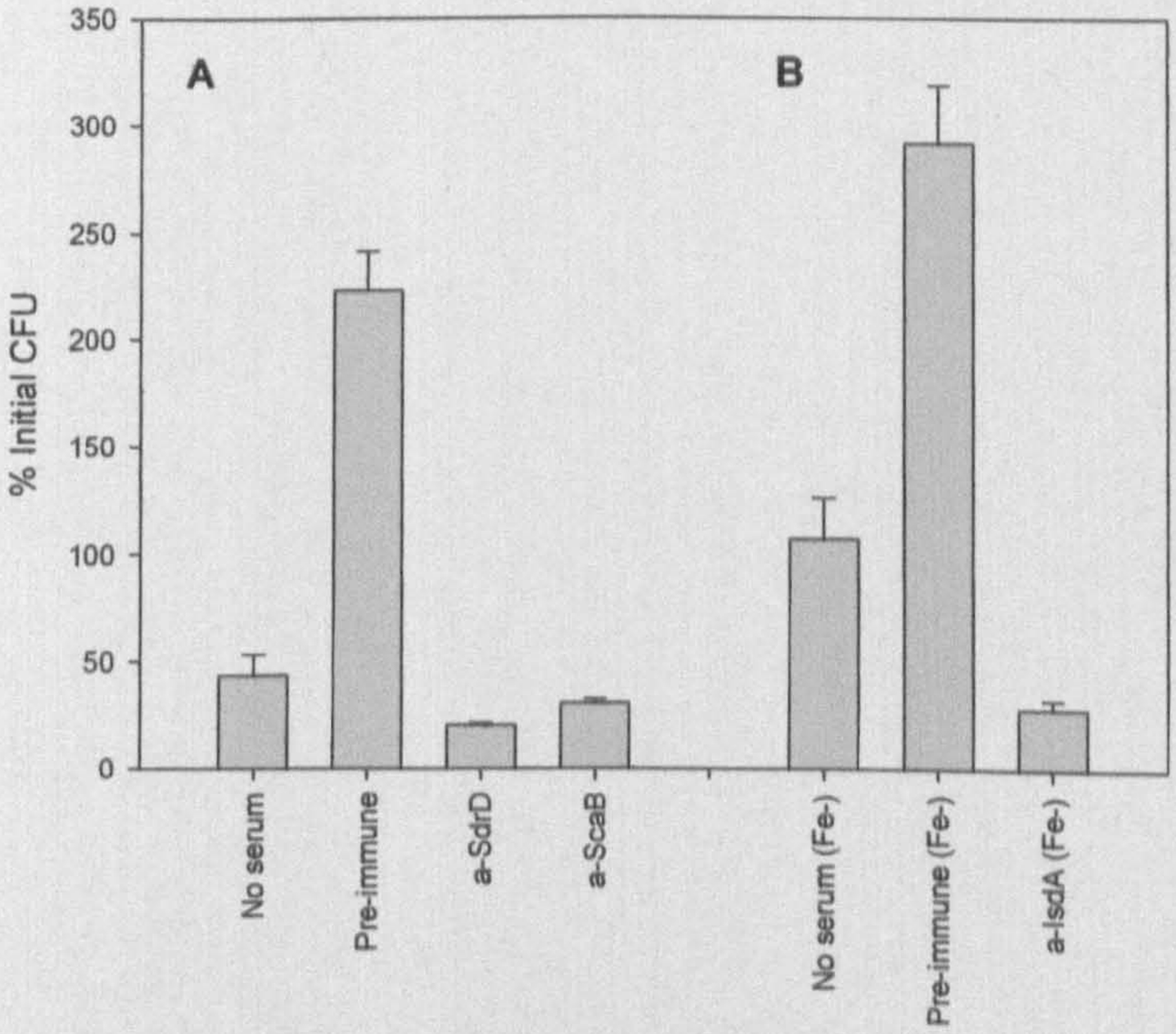


Figure 3.14: Percentage bacteria remaining after 1.5h opsonisation of *S. agalactiae* 6313 grown with (A) and without (B) iron. All samples contained both neutrophils and complement, results are from triplicate samples. Standard error bars are shown.

	Pre-immune serum	No serum
S. aureus		
a-SdrD	0.0001	0.0168
a-ScaB	0.0003	0.0185
a-IsdA	0.0005	0.0192
S. agalactiae		
a-SdrD	0.0766	0.0004
a-ScaB	0.2534	0.0005
a-IsdA	0.0145	0.0007

Table 3.6: *t*-test analysis of effect of immune sera on bacterial opsonisation as measured by killing. *P*-values were calculated by comparing results to both the pre-immune serum control and the no serum control.

3.4.3. Protection studies

IsdA-c and ScaB-c polypeptides were tested in mouse models of septic arthritis (in collaboration with Prof. Andrej Tarkowski and Dr. Margareta Verdrengh, Department of Rheumatology and Inflammation Research, University of Göteborg, Sweden). Mice were immunised with 50 µg protein and infected with either *S. aureus* or *S. agalactiae*. Control mice were injected with ovalbumin (OVA). After 20 (IsdA-c) or 14 (ScaB-c) days mice were assessed for prevalence of arthritis, mortality and weight loss.

Vaccination with IsdA-c (Table 3.7) displayed some efficacy in the case of staphylococcal infection, although differences were less evident with streptococcal infection. After infection with *S. aureus*, immunised mice had a lower prevalence of arthritis and mortality, although no differences were observed in the weight changes between groups. The protection against *S. agalactiae* infection was less apparent. No data was obtained for the percentage arthritis as induction of arthritis failed (for unknown reasons). There was no difference in the level of mortality between the immunised mice and the control mice, however, immunised mice demonstrated a reduction in weight loss.

Vaccination with ScaB-c (Table 3.8) resulted in a trend to higher survival and lesser arthritis in the staphylococcal group, although differences are relatively minor. Immunisation with ScaB-c did not, however, provide any protection against *S. agalactiae* infection.

	S. aureus		*S. agalactiae*	
	IsdA-c	**OVA**	**IsdA-c**	**OVA**
Arthritis	70%	100%	ND	ND
Mortality	0%	40%	40%	40%
Weight change	0%	0%	-0.9%	-10.0%

Table 3.7: Effect of vaccination with IsdA-c polypeptide in mouse septic arthritis model. Prevalence of arthritis, death and changes in weight were assessed. ND = no data.

	S. aureus		*S. agalactiae*	
	ScaB-c	**OVA**	**ScaB-c**	**OVA**
Arthritis	44%	57%	44%	11%
Mortality	10%	30%	10%	10%
Weight change	-10.0%	-4.6%	-6.2%	-3.4%

Table 3.8: Effect of vaccination with ScaB-c polypeptide in mouse septic arthritis model. Prevalence of arthritis, death and changes in weight were assessed.

3.5 Discussion

The primary aim of this study was to identify antigenic components of *S. pyogenes* which were cross-reactive with antibodies raised against *S. aureus* antigens expressed during infection. The immunoscreen allowed the detection of genes expressed *in vivo* rather than *in vitro* and the use of a genomic library of *S. pyogenes* meant that a large number of clones could be screened, decreasing the possibility of potential antigens being missed. Furthermore, expression using this method does not rely on the correct in-frame cloning of insert DNA and the use of phage lambda allows the production of insoluble proteins or those toxic to *E. coli*.

Despite these advantages, the immunoscreen was not completely comprehensive. For antigens to be identified they must be represented in the library and expressed from either their own or the *lac* promoter, hence, the partial *Sau*3A digest of the genomic library may disrupt some genes. In addition, many clones identified in the screen contained several ORFs, so the true immunotarget could not be conclusively determined.

In total, 81 clones were identified by the screen, representing 48 different loci in the *S. pyogenes* genome. Interestingly, the largest proportion of clones was identified from sera of *S. aureus*-infected patients, and that of nasal carriers. The fewest clones were isolated from immunoscreening with sera samples of non-carriers. The immunoscreen identified 128 putatively cytoplasmic proteins, 31 membrane proteins and 13 secreted or surface-located proteins. Surface / secreted proteins in particular represent promising vaccine targets as they have key roles in the interaction between pathogen and host cells. The high proportion of cytoplasmic proteins may be due to epitopes shared with staphylococcal proteins or proteins of other bacterial species to which we are exposed. Alternatively, these antigens may be released upon cell lysis.

The most commonly identified locus in the immunoscreen was locus 42, represented by 15 separate clones. Mapping of the clones to this region showed that Isp, a putative autolysin, was the most likely antigen. Isp (immunogenic secreted protein) is conserved amongst

strains of *S. pyogenes*, as well as containing homologous regions to proteins of other streptococcal species (McIver *et al.* 1996). Bioinformatic analysis revealed that Isp is in fact a member of the Sca family of proteins, along with Usp45 from *S. pneumoniae* and PcsB from *S. agalactiae*. Analysis of antibody titre in convalescent sera from a patient with GAS throat demonstrated that Isp is expressed during infection and generates an antibody response (McIver *et al.* 1996), therefore, this antigen may represent an appropriate immunotherapy target.

Another putative autolysin, IsaA, was identified by two clones during the immunoscreen (locus 47). NT03SP2070 is 66% identical over 295 amino acids to SACOL2584 (IsaA) in *S. aureus*. SACOL2584 is a putative lytic transglycosylase, which is related to SACOL2088 (SceD), a protein of similar function. When tested in a mouse septic arthritis model, a mutant of *isaA* was slightly attenuated for pathogenicity, and an *isaA sceD* double mutant was significantly attenuated (Stapleton *et al.* 2007). IsaA of *S. aureus* has also been previously identified to be expressed during *S. aureus* infection by immunoscreening (Etz *et al.* 2002), and a significantly higher titre of reactive IgG against IsaA was detected in serum from *S. aureus*-infected patients (Clarke *et al.* 2006).

A leucine-rich repeat domain protein (NT03SP1745) was identified by three clones in the *S. pyogenes* screen (locus 35). Leucine-rich repeat domains are present throughout Gram positives and have been demonstrated to be involved in virulence. LrrG, a leucine-rich repeat domain protein of GBS, is present in *S. agalactiae* and has a homologue in *S. pyogenes*. Vaccination with this protein was shown to protect mice against lethal infection by GBS (Seepersaud *et al.* 2005). Furthermore, disruption of the *elrA* gene, which encodes a leucine-rich repeat domain protein of *Enterococcus faecalis*, significantly attenuated bacterial virulence in a mouse peritonitis model (Brinster *et al.* 2007).

Analysis of locus 36 identified a secreted endoglycosidase (NT03SP1756) as a putative antigen, recognised by five clones. This *S. pyogenes* enzyme specifically hydrolyses human IgG by removing the glycan chains from the γ-chains of the IgG molecule (Collin and Olsén, 2001), a mechanism which blocks antibody-mediated phagocytosis and killing by

neutrophils (Voyich *et al.* 2004). A homologue of this endoglycosidase, EndoE, has also been identified in *E. faecalis* (Collin and Fischetti, 2004), suggesting this virulence-associated protein may also be conserved amongst several Gram positive pathogens.

Five clones from the screen identified the toxin streptolysin O (locus 6), a key virulence factor of *S. pyogenes* infection. This toxin can permeabilise mammalian cell membranes and is expressed during infection. It is associated with several streptococcal diseases, including acute rheumatic fever (Martins *et al.* 2008).

Several ABC transporters were identified during the screen as being cross-reactive antigens. These proteins are ubiquitous among bacteria, involved in the import and export of a wide variety of molecules. The protein complexes consist of two types of domain: an ATP-binding cassette (ABC), which is highly conserved, and a transmembrane domain that is less well-conserved (Linton and Higgins, 1998). Some ABC transporters have been demonstrated to be involved in virulence, for example, immunization with the iron uptake ABC transporter proteins PiaA and PiuA has been demonstrated to prevent respiratory infection by *S. pneumoniae* (Jomaa *et al.* 2006). An immunodominant ABC transporter was also identified in MRSA (Burnie *et al.* 2000). This ABC transporter is homologous to the *B. subtilis* protein YkpA, which is putatively involved in cell wall biosynthesis. It was therefore proposed that targeting of this antigen in MRSA by the immune response may disrupt synthesis of the cell wall, reducing the virulence of the bacteria.

Once *S. pyogenes* antigenic components had been identified in this study, the results were combined with results from other studies in order to identify putative cross-reactive antigens amongst five Gram positive species. To reduce the number of database searches to an appropriate and manageable number, the *S. aureus* proteins identified in the immunoscreen (Brummell, 2005; Clarke *et al.* 2006) were used as the basis to search the other genomes. A threshold limit of 25% identity and 40% similarity over at least 15 amino acids was used for the analysis (or 21-24% identity if the similarity was greater than 40%). This limit allowed for expected differences between species. Using a relatively low threshold level of homology allowed for cross-species variability so that antigenic groups

could be determined with the potential to protect across the range of Gram positive pathogens. The bioinformatic analysis of genome databases identified many homologous fragments which were not found during the original immunoscreen of that species. As discussed previously, many antigens may have been missed during the screening due to the technique utilised, therefore, additional screening may have given a more comprehensive complement of antigens but was not deemed worthwhile due to time constraints.

Primary bioinformatic analysis identified 13 families of proteins containing homologous fragments of proteins from at least four of the five screened species. SACOL2666, also named ScaH (Pourmand *et al.* 2006), has been shown to be a member of a multigene family in both *S. aureus* and *S. epidermidis*. The Sca family consists of 10 proteins, which have a conserved C-terminal ~110 amino acids. This C-terminus (amino acids 510-619 in ScaH) was identified during the screen as being cross-reactive and conserved between species (Table 3.5). The Sca family will be discussed further in Chapter 4. ScaH has also been shown to be part of a family of four putative N-acetylglucosaminidases, along with Atl (SACOL1062), SACOL1825 and SagA (SACOL2298) (Syed Mohamad, 2007). The glucosaminidase domain has been identified as being amino acids 338–466 in ScaH, corresponding approximately to that found to be conserved during the screening analysis.

ClfA is an LPXTG protein which binds fibrinogen (Fg) by an A domain of approximately 500 amino acids. ClfA is structurally related to another Fg-binding protein, ClfB, although the genes are not highly conserved, with only 27% identity in the A domain. ClfA is an important virulence factor in infective endocarditis and staphylococcal arthritis, mediating the binding and activation of platelets during infection (O'Brien *et al.* 2002). It has also been demonstrated that ClfA, along with another related protein, FnbpA, promotes early valve colonisation during infective endocarditis (Que *et al.* 2005). The analysis of the protective effect of immunisation with ClfA revealed a marked reduction in the severity of arthritis in a mouse model of infection, suggesting that ClfA is a crucial virulence determinant for this disease (Josefsson *et al.* 2001). Monoclonal antibodies generated against the ligand-binding domain of ClfA were shown to provide significant protection in mice against sepsis-associated death by *S. aureus* infection, as well as demonstrating broad

reactivity among clinical strains (Hall *et al.* 2003). Furthermore, ClfA has been demonstrated to inhibit phagocytosis, both in the presence and absence of fibrinogen, and the effect was proposed to be comparable to that of protein A (Higgins *et al.* 2006). Recent studies have evaluated the role of Aurexis® (tefibazumab), a humanised monoclonal antibody that recognises ClfA. This MAb was demonstrated to significantly protect against intravenous MRSA challenge in murine septicaemia and rabbit infective endocarditis (IE) models (Patti, 2004; Domanski *et al.* 2005), and was reported to be well-tolerated and effective in human trials (Weems *et al.* 2006). Aurexis® represents a novel approach using passive immunisation against a specific MSCRAMM and this MAb is currently under development as adjunctive therapy in combination with the antibiotic vancomycin.

Fibronectin-binding protein B (FnbpB) is 30.5% identical over 933 amino acids to ClfA (Brummell, 2005). In addition to mediating an interaction between bacterial and host cells during the initial stages of colonisation, FnbpB, along with its homologue FnbpA, can initiate internalisation into non-professional phagocytes (Clarke and Foster, 2006). The structures of FnbpB and FnbpA are similar to each other, and to equivalent fibronectin-binding proteins in streptococci, e.g. PFBP of *S. pyogenes* (Keane *et al.* 2007; Rocha and Fischetti, 1999). Despite their similarily, FnbpB and FnbpA have demonstrated different roles in infective endocarditis. Whereas FnbpA induced the aggregation of platelets when present on *S. carnosus* cells, FnbpB did not (Heilmann *et al.* 2004).

SasB has been named Mrp (Wu and De Lencastre, 1999) and FmtB (Komatsuzawa *et al.* 2000). SasB is an LPXTG family protein containing 18 repeats of 71 amino acids. The role of this protein remains to be fully determined, although mutation of *fmtB* has been shown to affect oxacillin resistance in MRSA (Komatsuzawa *et al.* 2000). ELISA analysis of SasB with serum samples from diseased and healthy individuals, and carriers and non-carriers of *S. aureus* demonstrated that this protein is positively associated with disease, and with nasal carriage (Clarke *et al.* 2006).

Another Sas protein was identified in the analysis, SasC. This protein contains 17 repeats of 71 amino acids and is 31% identical over 2076 amino acids to SasB. The N-terminal A

region has a similar predicted structure to the A regions of ClfA and SdrD (Roche *et al.* 2003). Despite this similarity, SasC is unable to bind many of the human ligands these proteins have been shown to adhere to, including fibronectin, fibrinogen and fetuin (Brummell, 2005). The role of SasC remains to be determined.

Two fragments of SasI were identified as being conserved during the analysis. SasI has also been named HarA (Dryla *et al.* 2003) and IsdH (Mazmanian *et al.* 2003). This protein is part of a family of proteins involved in the uptake of haem-iron across the cellular envelope, although it is encoded by a gene at a separate locus to the other Isd proteins (Mazmanian *et al.* 2003). The *isd* genes are transcriptionally controlled by the iron-responsive regulator Fur and are expressed under iron-limited conditions (Clarke and Foster, 2006; Mazmanian *et al.* 2003). SasI contains three NEAT domains, the active sites for ligand binding, and binds human haptoglobin, a protein that sequesters free haemoglobin in serum, in the transfer of hemin via the Isd proteins (Liu *et al.* 2008). SasI has been shown to be present in 96% of invasive strains of *S. aureus*, and a significantly elevated IgG titre was observed in convalescent patient sera, confirming its expression during infection (Roche *et al.* 2003; Clarke *et al.* 2006).

Another member of the Isd family of proteins, IsdB (SasJ), was identified to contain a conserved domain. IsdB contains two NEAT domains and can bind several iron-containing proteins (Mazmanian *et al.* 2003). It is proposed to be involved in the binding of haemoglobin and subsequent transfer of heme to IsdA during heme acquistion via the Isd proteins (Liu *et al.* 2008). IsdB is positively associated with infection, as a significantly elevated IgG titre was observed in convalescent patient sera (Roche *et al.* 2003). Vaccination with IsdB has provided significant protection in animal models, including rhesus macaques, in which a more than five-fold increase in antibody titres was observed after a single immunisation (Kuklin *et al.* 2006).

IsdA, also known as StbA (Taylor and Heinrichs, 2002), FrpA (Morrissey *et al.* 2002) and SasE (Roche *et al.* 2003) was first identified for its ability to bind transferrin. IsdA is an LPXTG cell wall protein regulated by exogenous iron levels via the Fur protein, only being

expressed under iron-limited conditions (Clarke *et al.* 2004). It is part of a family of iron-regulated surface determinant (Isd) proteins, some of which were also identified as containing conserved regions in this screen, e.g. SasC/IsdC and SasJ/IsdH. In addition to binding transferrin, IsdA is a broad spectrum adhesin able to bind both fibrinogen and fibronectin via a NEAT domain. Recently, it has been shown that IsdA also binds lactoferrin, an anti-staphylococcal polypeptide found in human nasal secretions, hence conferring resistance to its killing (Clarke and Foster, 2008). IsdA is required for nasal colonisation and mutation of *isdA* resulted in a reduction in the ability of bacteria to bind human nasal epithelial cells and to colonise the anterior nares of cotton rats (Clarke *et al.* 2006). Furthermore, significantly higher titres of reactive IgG against IsdA are detected in serum samples from individuals infected with *S. aureus*, indicating that IsdA is a marker of infection (Clarke *et al.* 2006). Alignment of all homologous fragments found during the screen with the IsdA protein resulted in a highly conserved C-terminal region (amino acids 215-310; Table 3.5; Figure 3.6). This conserved C-terminus has been shown to decrease the cellular hydrophobicity of *S. aureus*, conferring resistance to hydrophobic fatty acids, antimicrobial peptides of the host cell. This function therefore promotes the survival of the pathogen on human skin (Clarke *et al.* 2007). As IsdA has been proven to be a putative antigenic target expressed during infection, with virulence properties, this conserved C-terminus was chosen for further analysis to assess its ability to protect against infection by Gram positive pathogens.

Recombinant IsdA-c polypeptide was tested in mouse models of septic arthritis using both *S. aureus* and *S. agalactiae*. The protein offered some protection against *S. aureus* infection, reducing the incidence of both arthritis and mortality. Whilst there was no confirmed protection against *S. agalactiae*, a reduction in weight loss was observed. Specific antisera raised against IsdA-c was also shown to opsonise *S. aureus* and *S. agalactiae*. Significantly fewer bacteria survived in the presence of the IsdA-c antiserum when compared to either pre-immune or no serum controls, inferring an ability of the antiserum to opsonise these pathogens for phagocytic uptake and subsequent killing. Similar results were found by Stranger-Jones *et al.* (2006), who found that complement-

mediated opsonophagocytic killing of *S. aureus* Newman was increased in the presence of antibodies against full-length IsdA protein.

SdrD was identified as part of the serine-aspartate (SD) repeat protein family, which also includes ClfA and ClfB (Josefsson *et al.* 1998). Similar to ClfA and ClfB, it contains an N-terminal ligand binding domain (A domain) and an R domain composed of SD dipeptide repeats. However, unlike the Clf proteins, SdrD also contains five B repeat domains of unknown function. The N-terminus of SdrD (part of the A domain) was identified during the analysis as being conserved. The presence of the gene *sdrD* has been shown to be associated with bone infections, osteomyelitis and methicillin resistance, as well as having higher expression in strains expressing Panton-Valentine leukocidin (PVL), which cause staphylococcal necrotizing pneumonia (Labandeira-Rey *et al.* 2007). Whilst two *sdr* genes have been shown to be present in all strains, only *sdrC* is present in every case. *sdrD* is generally absent in MSSA strains (Sabat *et al.* 2006) and in total is only present in approximately 48% of invasive *S. aureus* strains (Peacock *et al.* 2002).

Although SdrD-n was not tested in protection studies with mice, it did produce promising results by opsonisation. Significantly fewer *S. aureus* were recovered after opsonisation with specific SdrD-n antibodies, compared to pre-immune serum and no serum controls. In a similar study, Stranger-Jones *et al.* (2006) found that antibodies against full-length SdrD mediated complement-dependent phagocytosis of *S. aureus*. However, results here also show that significantly fewer *S. agalactiae* survived opsonisation in the presence of the antiserum compared to pre-immune serum. These results suggest that the antiserum may be effective in providing cross-reactive protection against Gram positive pathogens, and that the N-terminus may be the antigenic domain of the protein. To fully determine the cross-protective ability of this antiserum, it could be tested in the mouse septic arthritis model, similarly to IsdA-c and ScaB-c.

In addition to the homologous regions of IsdA and SdrD, one additional fragment, the C-terminus of ScaB, was chosen for further analysis (Chapter 3.3.1.1.). ScaB was identified from screening the *S. epidermidis* expression library (Pourmand *et al.* 2006) and was found

to be a member of a multigene family with a conserved C-terminal CHAP domain. ScaH was also identified during the *S. aureus* screen (Clarke *et al.* 2006) and this C-terminus was found to represent one of the 13 families of conserved fragments among the Gram positives screened. The CHAP domain of ScaB is 41.8% identical to that of ScaH in *S. aureus*, and as this domain is part of a family of proteins present throughout the Gram positive pathogens, this fragment was further analysed.

ScaB-c protein was used to immunise mice which were subsequently infected with both *S. aureus* and *S. agalactiae*. The protein failed to provide any protection against streptococcal infection, although results showed a trend towards higher survival and lesser arthritis in the staphylococcal-infected group. Despite the low level of protection in mice, the specific antibodies against ScaB-c were effective at opsonising bacteria for phagocytic uptake by human neutrophils. Significantly fewer *S. aureus* cells survived incubation with neutrophils in the presence of ScaB-c antiserum compared to controls with both pre-immune and no serum. Fewer *S. agalactiae* were shown to survive phagocytosis, with bacterial counts being significantly lower in the presence of antiserum compared to pre-immune serum. Taken together, these results suggest that specific ScaB-c antisera may be cross-reactive and protective against Gram positive infections.

My work has highlighted protein domains potentially efficacious as novel vaccine components. Their development will require further study. Analysis of SdrD-n and ScaB-c antibodies have shown that both these antigens contribute to the killing of *S. aureus* and *S. agalactiae*. The absence of SdrD from all strains of *S. aureus* means that its effect may be limited as an individual vaccine target and must be assessed across a wider range of strains and species. IsdA-c has been shown to be an effective protector of infection by both *S. aureus* and *S. agalactiae*. Although several studies have identified proteins which increase opsonophagocytic uptake and killing of pathogens by human neutrophils, few have identified antigens capable opsonising numerous species. Identification of the protective fragment of the IsdA protein and confirmation of its ability to confer cross-protection across different Gram positive species makes this an attractive target for immunotherapy. Further work to elucidate its ability to protect a range of Gram positive species, using

mammalian models of infection, would help to evaluate the potential for this antigen as a vaccine candidate against Gram positive pathogens.

Chapter 3 – Summary

- Immunoscreening of a *S. pyogenes* genomic DNA expression library with human sera identified 48 putative cross-reactive antigenic loci in the *S. pyogenes* genome.
- Bioinformatic analysis of results of immunoscreens of five Gram positive pathogens identified 13 groups of homologous fragments which are potentially cross-reactive.
- Three conserved protein fragments were identified as being particularly highly conserved across species.
- Antibodies against the IsdA C-terminus, SdrD N-terminus and ScaB C-terminus were shown to be opsonic during infection by *S. aureus* and *S. agalactiae*.
- IsdA C-terminus polypeptide can protect against infection of *S. aureus* and *S. agalactiae* in mouse models of arthritis.

Chapter 4

Characterisation of the Sca family of proteins in *Staphylococcus aureus*

4.1 Introduction

The Gram-positive cell wall is a 15-30 nm thick layer, made up primarily of peptidoglycan (Figure 4.1). Peptidoglycan consists of glycan strands of repeating disaccharides of *N*-acetylmuramic acid-(β1-4)-*N*-acetylglucosamine (MurNAc-GlcNAc), which are cross-linked to form a murein sacculus surrounding the cell (Navarre and Schneewind, 1999). Attached to this are accessory molecules such as teichoic acids, teichuronic acids, polyphosphates and carbohydrates (Figure 4.1). Surface proteins are also anchored to the cell wall via a C-terminal LPXTG motif or by hydrophobic interactions (Fischetti, 2000a). The cell wall is vital for maintaining bacterial integrity and functions to provide a protective exoskeleton to resist mechanical and physical stresses, such as osmotic pressure in the cytoplasm and elevated hydrostatic turgor pressures (Koch, 2006). The peptidoglycan layer is a highly dynamic and tightly regulated macromolecule and is constantly remodelled to allow growth and division (Boneca, 2005). Furthermore, the cell wall represents the primary site of interaction with the environment. Peptidoglycan and attached surface proteins are at the interface between the bacteria and the host, therefore, these proteins represent key virulence factors during infection.

In order to allow cell growth and division, the bacterial cell wall peptidoglycan must be constantly remodelled, allowing the insertion of new material permitting the expansion of the cell. Remodelling requires the activity of peptidoglycan hydrolases. Those enzymes capable of hydrolysing their own peptidoglycan are known as autolysins.

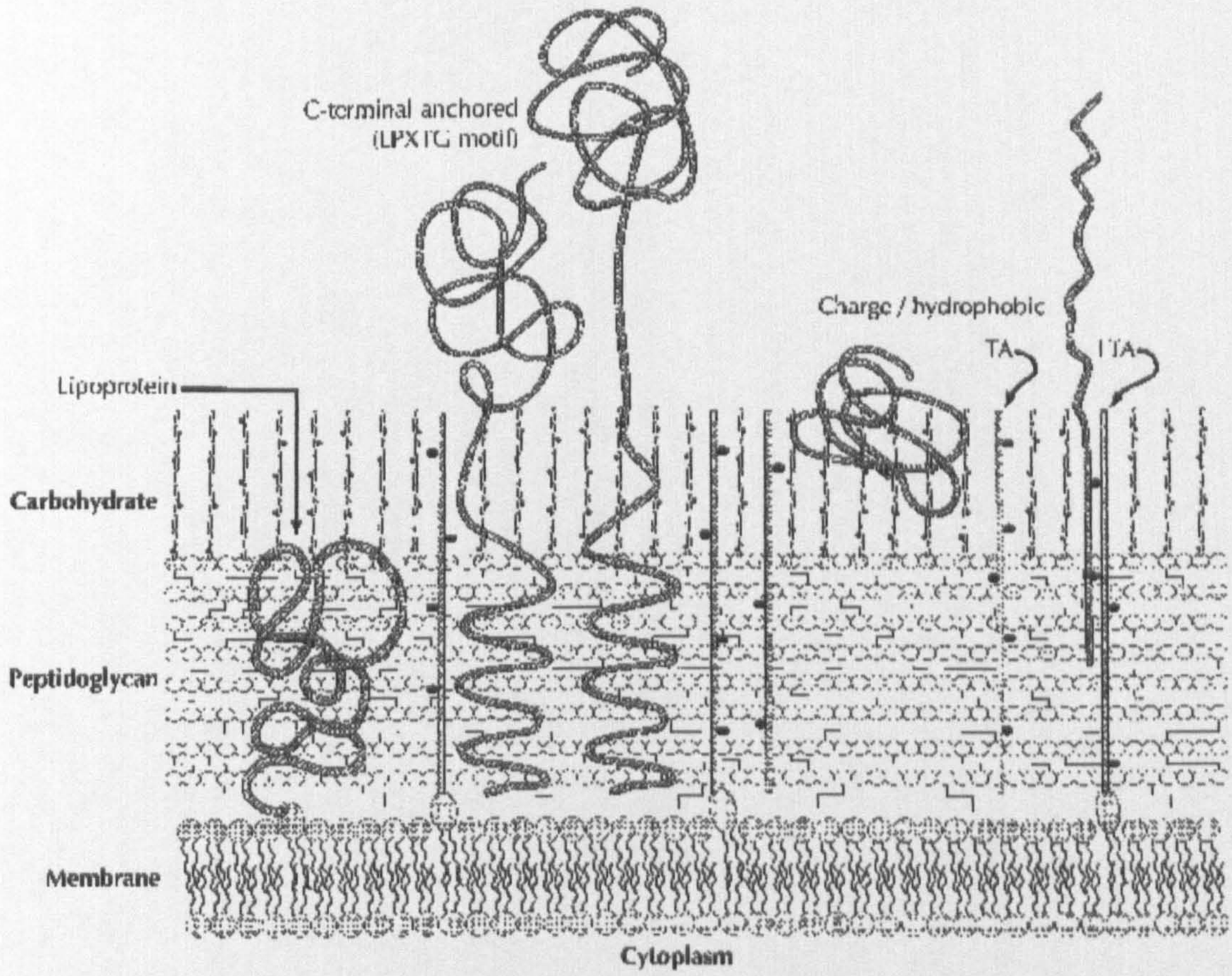


Figure 4.1: Major surface structures of the cell wall of Gram-positive bacteria. Linked to the surface of the peptidoglycan are surface proteins and polysaccharide structures, including teichoic acid (TA) and lipoteichoic acid (LTA). Reproduced from Fischetti (2000a).

As described in Chapter 3, immunoscreening of genomic libraries of five Gram positive pathogens resulted in the identification of a novel family of proteins, the staphylococcal conserved antigen (Sca) family. Studies have identified ScaA and ScaH in *S. aureus* as peptidoglycan hydrolases with the ability to bind various human ligands (Pourmand *et al.* 2006; Heilmann *et al.* 2005; Kajimura *et al.* 2005; Syed Mohamad 2007).

Several classes of peptidoglycan hydrolase exist, each able to hydrolyse different bonds within the peptidoglycan structure (Figure 4.2). *N*-acetylglucosaminidases cleave the β-1,4 glycosidic bond between the GlcNAc and MurNAc, *N*-acetylmuramoyl-L-alanine amidases hydrolyse the amide bond between the *N*-acetylmuramic acid and the peptide side chain, endopeptidases cleave the amide bonds between two amino acids, and muramidases and lytic transglycosylases cleave the β-1,4 glycosidic bond between MurNAc and GlcNAc (Vollmer *et al.* 2008).

Peptidoglycan hydrolases are multifunctional enzymes and have been implicated in numerous cellular processes, including cell growth, cell wall turnover, peptidoglycan maturation, cell division, separation, motility, chemotaxis, genetic competence, protein secretion and pathogenicity (Smith *et al.* 2000; Blackman *et al.* 1998). As bacteria produce several peptidoglycan hydrolases of each class, with overlapping functions, it is difficult to ascertain the specific role of an individual enzyme.

4.1.1. Cell separation

During cell division, peptidoglycan hydrolases are required to cleave the septum and allow separation of the daughter cells. In Gram-positive bacteria, the septum is formed and cleaved by these enzymes along a mid-line (Vollmer *et al.* 2008). Several examples of strains lacking various peptidoglycan hydrolases have resulted in defective cell separation. For example, two enzymes have been identified in *S. aureus* to be responsible for separation of daughter cells, Atl and Sle1 (Sugai *et al.* 1994; Kajimura *et al.* 2005). Atl is the most predominant peptidoglycan hydrolase in *S. aureus* and this bi-functional enzyme contains a C-terminal glucosaminidase domain and an N-terminal amidase

domain, a proform undergoing proteolytic processing to generate two extracellular cell wall hydrolases (Foster, 1995; Oshida *et al.* 1995). It has been predicted that the 62 kDa amidase and the 51 kDa glucosaminidase form a ring structure on the cell surface at the septal site and, once the septum has been formed, these autolysins digest the peptidoglycan to allow the daughter cells to separate (Figure 4.3) (Yamada *et al.* 1996). Inactivation of Atl in *S. aureus* results in growth in large cell clusters, indicating an inability of cells to separate correctly (Foster, 1995; Biswas *et al.* 2006). Furthermore, studies in *B. subtilis* involving the inactivation of peptidoglycan hydrolases have resulted in cells which grow in long chains with abnormal septa (Priyadarshini *et al.* 2007; Carbillido-López *et al.* 2006), indicating that these enzymes are vital for the correct separation of cells during cell division.

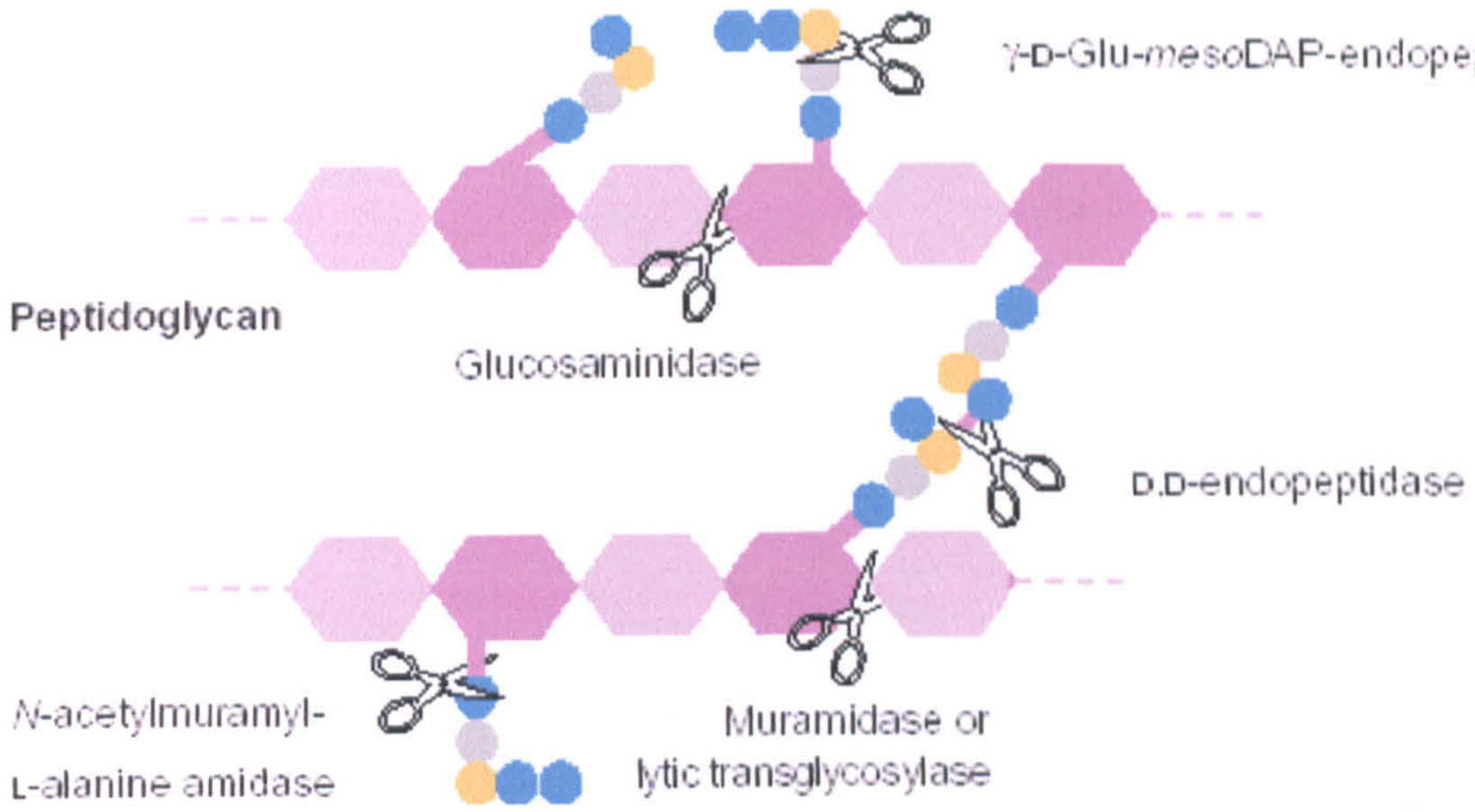


Figure 4.2: Bond specificities of peptidoglycan hydrolases on bacterial peptidoglycan. Adapted from Boneca *et al.* 2005.

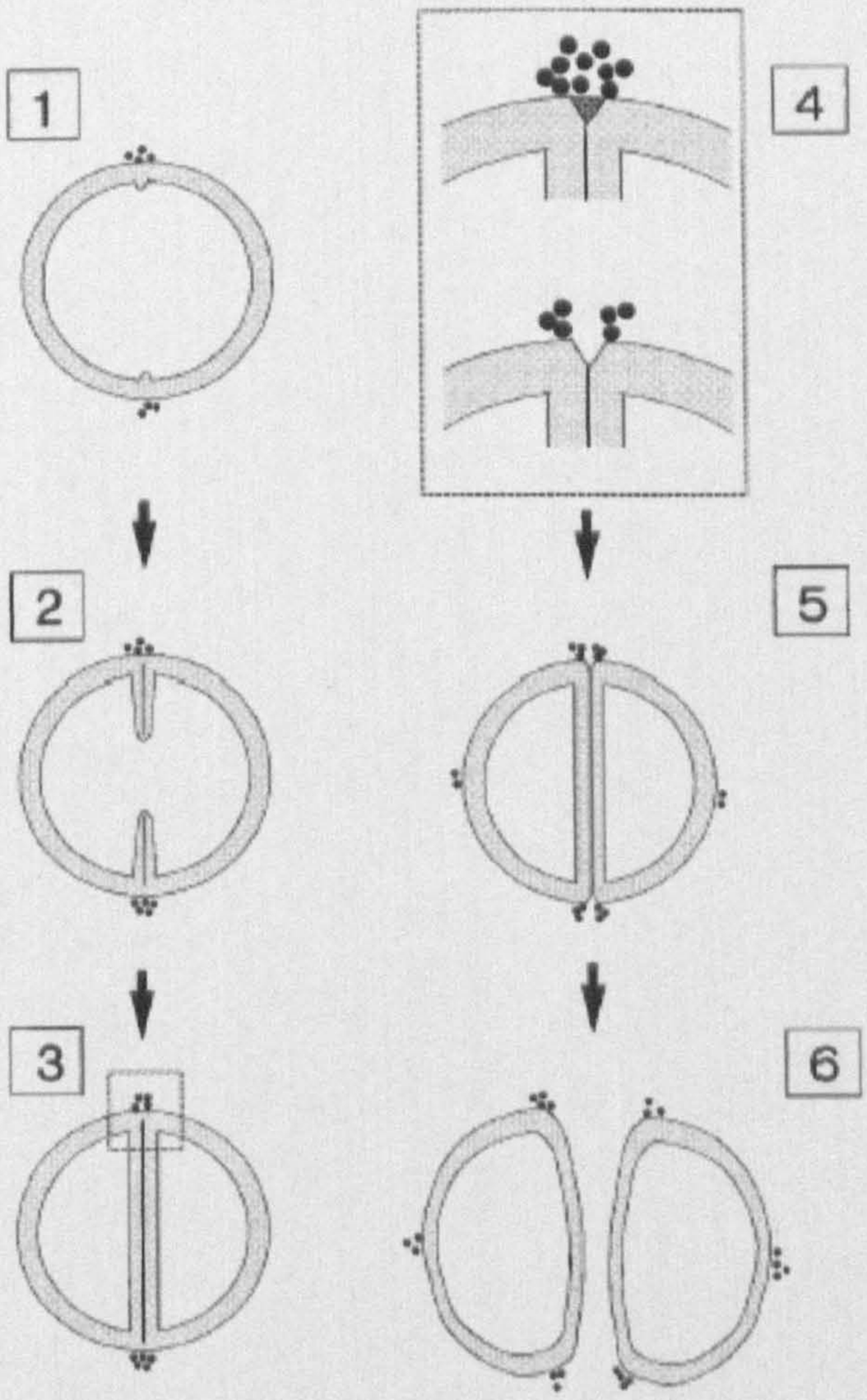


Figure 4.3: Model for the function of *atl* gene products in cell separation in *S. aureus*. The processed Atl hydrolase enzymes (black dots) form a ring at potential septal sites (1) and once the septum has been formed (2, 3), the peripheral peptidoglycan is hydrolysed (4, 5) to allow separation of the daughter cells (6). Reproduced from Yamada *et al.* (1996).

4.1.2. Cell wall turnover

Peptidoglycan hydrolases release fragments from the cell wall during growth, a process termed cell wall turnover. The inside-to-outside mechanism of cell wall growth involves the attachment of new peptidoglycan outside the cytoplasmic membrane, underneath the existing cell wall (Vollmer *et al.* 2008). The inner layer of the cell wall consists of newly synthesised, unstressed peptidoglycan, which passes outward as the cell elongates (in rod-shaped bacteria), becoming the middle, stress-bearing zone (Smith *et al.* 2000). The outer layer consisting of old peptidoglycan must be solubilised, a function carried out by peptidoglycan hydrolases. These autolysins cleave the outer layer of peptidoglycan, removing the oldest wall material from the outer surface and releasing it into the growth medium. This role in cell wall turnover has been demonstrated in *B. subtilis*, where inactivation of the major autolysin-encoding gene *lytC* led to cell wall and septal thickening and a large decrease in the rate of turnover (Blackman *et al.* 1998). In *S. aureus*, mutant cells lacking the major autolysin Atl displayed a rough outer surface, indicative of a lack of peptidoglycan release at the cell surface (Foster, 1995).

4.1.3. Peptidoglycan maturation

New peptidoglycan is synthesised at the outer surface of the cytoplasmic membrane from disaccharide pentapeptides which are transglycosylated to give glycan strands and cross-linked by transpeptidation, both carried out by penicillin-binding proteins (Sauvage *et al.* 2008). Endopeptidases and lytic transglycosylases are also involved in this process, degrading the original glycan strand to allow new peptidoglycan to be synthesised without disturbing the cell wall integrity in a "three-for-one" model (Höltje, 1998). In addition, autolysins have been shown to be involved in certain modifications to this peptidoglycan structure. For example, glucosaminidase activity in *S. aureus* is responsible for observed minor satellite peaks in glycan strand analysis (Boneca *et al.* 2000).

4.1.4. Antibiotic-induced lysis

It is long-established that inhibition of peptidoglycan synthesis by β-lactam antibiotics induces autolysis leading to bacterial death. Penicllin G-induced autolysis of *S. aureus* begins with localised perforation of the cell wall at initiated nascent cross walls, followed by general lysis (Sugai *et al.* 1997). It is suggested that peptidoglycan hydrolases are responsible for this effect. The major autolysin of *S. aureus*, Atl, has been implicated in the onset of penicillin-induced lysis, involved in the initial perforation of the cell wall during the autolytic process. When exposed to antibiotic, the cell wall localised at the presumptive site of cross wall formation is digested by *atl* gene products without forming a new cross wall, which causes a wall defect to form at the site. This leads to autolysis of the cell (Sugai *et al.* 1997).

4.1.5. Pathogenesis

Involvement of autolysins in pathogenesis has been demonstrated, for example, a *S. aureus* mutant lacking the two putative lytic transglycosylases IsaA and SceD was attentuated for virulence in a mouse septic arthritis model of infection (Stapleton *et al.* 2007). SceD is also required for nasal colonisation of *S. aureus*, suggesting that alteration of peptidoglycan by autolysins may allow bacteria to adapt to different host environments. In addition, Aaa of *S. aureus* and its homologue in *S. epidermidis* Aae, have been demonstrated to be adhesins, able to bind several human ligands, therefore promoting colonisation of host tissues (Heilmann *et al.* 2003; 2005).

In this chapter, the Sca family of proteins in *S. aureus* was further characterised. Recombinant proteins were overexpressed to determine their hydrolytic activities, ligand binding abilities and potential as antigens. In particular, analysis of ScaD was undertaken to characterise this novel autolysin.

4.2 Results

4.2.1. Bioinformatic analysis of the Sca family

The staphylococcal conserved antigen (Sca) family consists of ten proteins in *S. aureus*, named ScaA to ScaJ (Figure 4.4). Bioinformatic analysis of the protein sequences of the Sca family (http://pfam.sanger.ac.uk) demonstrated the presence of a conserved C-terminal CHAP domain (PF05257). The CHAP (cysteine, histidine-dependent amidohydrolases/peptidases) domain is a region between 110 and 140 amino acids in length, associated with amidase function (Bateman and Rawlings, 2003). Proteins which contain this domain are often involved in bacterial cell wall metabolism, functioning in peptidoglycan hydrolysis. The CHAP domain contains two invariant residues, a cysteine and a histidine, which form part of the putative active site of the protein. The CHAP domains of the Sca proteins vary in length from 114 to 125 amino acids and occur at the C-terminus of each protein. These regions contain a high level of homology between proteins, the percentage identity of each CHAP domain to that of ScaD is shown in Figure 4.4.

Three of the Sca proteins (ScaA, ScaB and ScaE) contain LysM (lysin motif) domains of 43 amino acids (PF01476). These domains were originally identified in bacterial lysins and are involved in bacterial cell wall degradation (Bateman and Bycroft, 2000). The LysM repeats are one of the most commonly occurring domains in bacterial surface proteins and putatively function in peptidoglycan binding. The LysM domains of the *E. faecalis* protein AtlA and the *L. lactis* protein AcmA are both required to maintain activity of these autolysins (Eckert *et al.* 2006; Steen *et al.* 2005). This suggests that peptidoglycan binding via these motifs is crucial, potentially allowing the correct positioning or conformation of the catalytic domain towards its substrate.

Analysis of the Sca proteins using SignalP 3.0 Server (www.cbs.dtu.dk/services/SignalP/) demonstrated the presence of a signal peptide in each case (Table 4.1), indicating that these proteins are secreted / surface-located. Analysis of the hydrophobicity

(http://bioweb.pasteur.fr/seqanal/interfaces/toppred.html) of Sca proteins revealed that the proteins are mainly hydrophilic, with the exception of a hydrophobic fragment at the N-terminus characteristic of the signal peptide. The isoelectric point (pI) of each Sca protein was predicted using ExPASy (www.expasy.ch/tools/pi_tool.html) and results are shown in Table 4.1.

ScaA was first identified as Aae in *S. epidermidis*, a surface-located autolysin with adhesive properties (Heilmann *et al.* 2003). The same protein was found during immunoscreening of *S. epidermidis* with human sera and bioinformatic analysis revealed it to be a member of a ten-protein family (Pourmand *et al.* 2006). These ten proteins contained a conserved C-terminal domain and were named the Staphylococcal conserved antigen family. The Sca proteins A-J have direct homologues in *S. aureus*. ScaA of *S. aureus* (SACOL0507) has been identified previously as Aaa (Heilmann *et al.* 2005) and Sle1 (Kajimura *et al.* 2005). Aaa was shown to act as an adhesin, binding the human ligands fibrinogen, fibronectin and vitronectin. Furthermore, the autolytic activity of the protein was confirmed using both *S. carnosus* and *S. aureus* cells. It was further demonstrated that the C-terminal portion of the protein was responsible for this observed activity and it was proposed that the protein acts as an *N*-acetylmuramyl-L-alanine amidase (Kajimura *et al.* 2005).

ScaB (SACOL0723) shares 39.1% identity and 53.4% similarity with ScaA (SACOL0507). The identity within the C-terminal CHAP domain is 56.7%. ScaB of *S. epidermidis* (SE0433) was identified by Pourmand *et al.* (2006) by immunoscreening with patient sera (Chapter 3). Further analysis revealed this protein to have adhesive properties, binding both fibronectin and lactoferrin (Pourmand, 2005).

ScaC (SACOL2581) is 255 amino acids in length and is highly homologous to the 267-residue ScaD (SACOL2291), sharing 76.7% identity and 81.9% similarity. The 117 amino acid C-terminal CHAP domains of these proteins are 88.9% identical. Analysis of the *S. epidermidis* ATCC 12228 genome demonstrated that the homologues of ScaC (SE1872) and ScaD (SE2124) are both 267 amino acids in length. These proteins are 100% identical at the amino acid level in this organism and the gene sequences are 99.6%

identical, differing by only 3 out of 774 base pairs. The DNA sequence of ScaD (SACOL2291) is the most homologous to these *S. epidermidis* proteins (74.4% identity to SE1872, 74.2% identity to SE2124), compared to SACOL2581 (74.1% identity to SE1872, 73.8% identity to SE2124). This high level of homology between ScaC and ScaD suggests that these paralogues may have arisen via gene duplication.

ScaC / ScaD has been identified previously in *S. epidermidis* by screening of a genomic library with patient serum and was named staphylococcal secretory antigen A, SsaA (Lang *et al.* 2000). The 267-residue protein identified was shown to be 75.2% identical to a 255-residue protein in *S. aureus* (ScaC), and was also 69.2% identical to the exoprotein SceB of *S. carnosus*. SsaA was demonstrated to be expressed during *S. epidermidis* sepsis, particularly in association with infective endocarditis, suggesting it may have a role in pathogenesis.

ScaD has also been named Ssa in *S. aureus* and was demonstrated to be required for the expression of high level resistance to macrolide-lincosamide-streptogramin B (MLS_B) antibiotics (Martin *et al.* 2002). This study also identified the C-terminus of Ssa to be highly similar to that of six additional proteins in *S. aureus*, now identified as ScaC, ScaJ, ScaF, ScaE, ScaA and ScaB. ScaD has been demonstrated to be under the control of the two-component regulator YycG/YycF (Dubrac and Msadek, 2004), and further work identified other Sca proteins under the control of this promoter, ScaB, ScaJ, ScaC and ScaE (Dubrac *et al.* 2007).

The N-terminus of both ScaD and ScaC share a degree of identity with the N-terminus of the autolysin LytM (SACOL0263). LytM is a glycyl-glycine endopeptidase belonging to the lysostaphin-type metallopeptidases (Odintsov *et al.* 2003). Alignment of the first 213 amino acids of LytM with the first 150 residues of ScaD and the first 138 residues of ScaC demonstrated that these domains were 28.9% and 27.5% identical to that of LytM, respectively.

ScaH is the largest member of the Sca family, with 619 amino acids. In addition to the conserved CHAP domain at the C-terminus, an additional Pfam domain was identified,

an endo-β-*N*-acetylglucosaminidase catalytic domain (PF01832). This domain is 142 residues in length in ScaH and further analysis demonstrated homology of this domain with three other glucosaminidases in *S. aureus*, SACOL1062 (Atl), SACOL1825 and SACOL2298 (SagA) (Syed Mohamad, 2007).

ScaI is 348 amino acids in length in *S. aureus*, although it is not present in all strains of the organism. Analysis of ten sequenced *S. aureus* genomes (www.tigr.org) revealed that ScaI is only found in three: *S. aureus* COL, *S. aureus* subsp. a*ureus* MRSA476 and *S. aureus* subsp. *aureus* USA300-FPR3757. These strains are all methicillin-resistant (MRSA), and the absence of the protein from all other sequenced methicillin-sensitive strains may suggest a possible link between the presence of ScaI and resistance to this antibiotic.

ScaJ is highly related to ScaC and ScaD, sharing 39.5% and 39.2% identity, respectively. Homology is particularly high within the CHAP domain, which is 62.7% identical to that of ScaD and 59.3% identical to that of ScaC. *scaJ* (SACOL2295) is located four genes downstream of *scaD* (SACOL2291) in *S. aureus*.

4.2.1.1. Homologues of ScaD in other species

BlastP analysis of SACOL2291 (ScaD) at http://tigrblast.tigr.org/cmr-blast/ identified several homologous proteins in other species (Table 4.2). Homology primarily occurred to the C-terminal region of ScaD, indicating that this CHAP domain is conserved throughout several Gram positive species (Figure 4.5). Several of the proteins identified by this analysis are also putative Sca family members, e.g. Isp, Usp45 and PcsB. PcsB of *S. pneumoniae* (SP_2216) is 34% identical to ScaD over 84 amino acids. This peptidoglycan hydrolase has been identified as being critical in cell separation (Ng *et al.* 2004). Interestingly, PcsB was also identified as the most immunogenic protein in a screen for conserved vaccine candidates (Giefing *et al.* 2008).

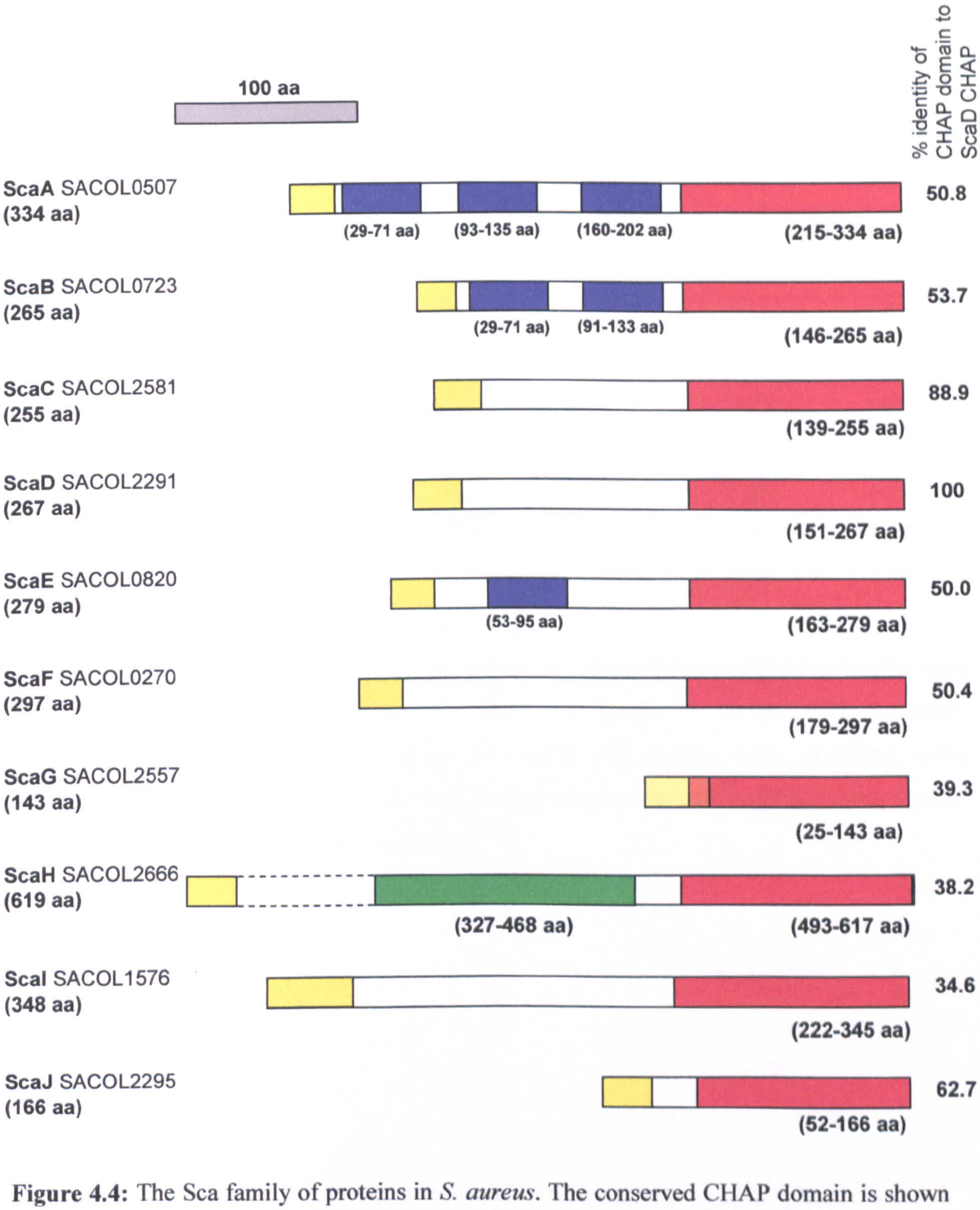


Figure 4.4: The Sca family of proteins in *S. aureus*. The conserved CHAP domain is shown in red, LysM domains are shown in blue, the glucosaminidase domain of ScaH is shown in green, predicted signal peptides (www.cbs.dtu.dk/services/SignalP/) are shown in yellow. The percentage identity of each CHAP domain to that of ScaD is also detailed.

Name	Locus	Gene length (bp)	Protein length (aa)	Molecular weight (kDa)	pI	Signal Sequence (aa)
ScaA	SACOL0507	1005	334	35.8	9.67	1-25
ScaB	SACOL0723	798	265	28.2	6.12	1-25
ScaC	SACOL2581	768	255	27.6	7.77	1-26
ScaD	SACOL2291	804	267	29.3	8.96	1-27
ScaE	SACOL0820	840	279	30.3	10.02	1-24
ScaF	SACOL0270	894	297	33.0	5.85	1-24
ScaG	SACOL2557	432	143	16.9	10.15	1-35
ScaH	SACOL2666	1860	619	69.3	5.96	1-27
ScaI	SACOL1576	1047	348	37.8	9.32	1-47
ScaJ	SACOL2295	501	166	17.4	5.59	1-27

Table 4.1: Summary of the Sca family of proteins in *S. aureus*. Gene and protein lengths were obtained from the TIGR CMR database (http://cmr.jcvi.org/tigr-scripts/CMR/CmrHomePage.cgi), pI scores and molecular weights were predicted using ExPASy (www.expasy.ch/tools/pi_tool.html) and signal sequences were predicted using SignalP 3.0 Server (www.cbs.dtu.dk/services/SignalP/).

Species	Locus	Protein name	Protein length (aa)	Identity	Similarity	Homologous region of protein (aa)	Homologous region of ScaD (aa)
S. pyogenes 8232	NT03SP1747	Immunogenic secreted protein 2 (Isp2)	505	34%	50%	388 – 499	158 – 266
	NT03SP0021	Secreted 45 kD protein (Usp45)	398	36%	44%	280 – 374	147 – 243
	NT03SP1937	Autolysin (Isp)	538	30%	49%	425 – 530	166 – 266
	NT03SP0032	Choline-binding protein D (cbpD)	374	32%	43%	59 – 170	164 – 267
	NT03SP1956	Low temperature requirement C protein	169	37%	57%	83 – 125	212 – 250
S. agalactiae NEM316	NT04SA1845	Immunogenic secreted protein precursor homologue	512	32%	51%	380 – 508	146 – 266
	NT04SA0050	PcsB	447	34%	47%	325 – 420	148 – 243
	NT04SA1923	Glucan binding protein B	169	37%	53%	60 – 139	164 – 243
	NT04SA1174	Immunogenic secreted protein precursor homologue	86	40%	59%	22 – 82	201 – 266

S. pneumoniae TIGR4	SP_2201	Choline-binding protein D (CbpD)	448	36%	50%	72 – 171	168 – 267
	SP_2216	PcsB, secreted 45 kD protein (Usp45)	392	34%	48%	280 – 363	159 – 243
E. faecalis V583	EF_0252	*N*-acetylmuramoyl-L-alanine amidase	503	37%	52%	377 – 498	148 – 266
L. lactis subsp. *lactis* IL1403	L159324	*N*-acetylmuramidase (AcmB)	475	35%	47%	360 – 472	162 – 266
	L96658	Usp45	456	32%	39%	327 – 448	150 – 258
B. subtilis 168	NT01BS5140	YybN	145	31%	44%	92 – 129	208 – 245
				34%	42%	24 – 71	21 – 66

Table 4.2: BlastP analysis (http://tigrblast.tigr.org/cmr-blast/) of homologues of ScaD (SACOL2291) in Gram-positive bacteria.

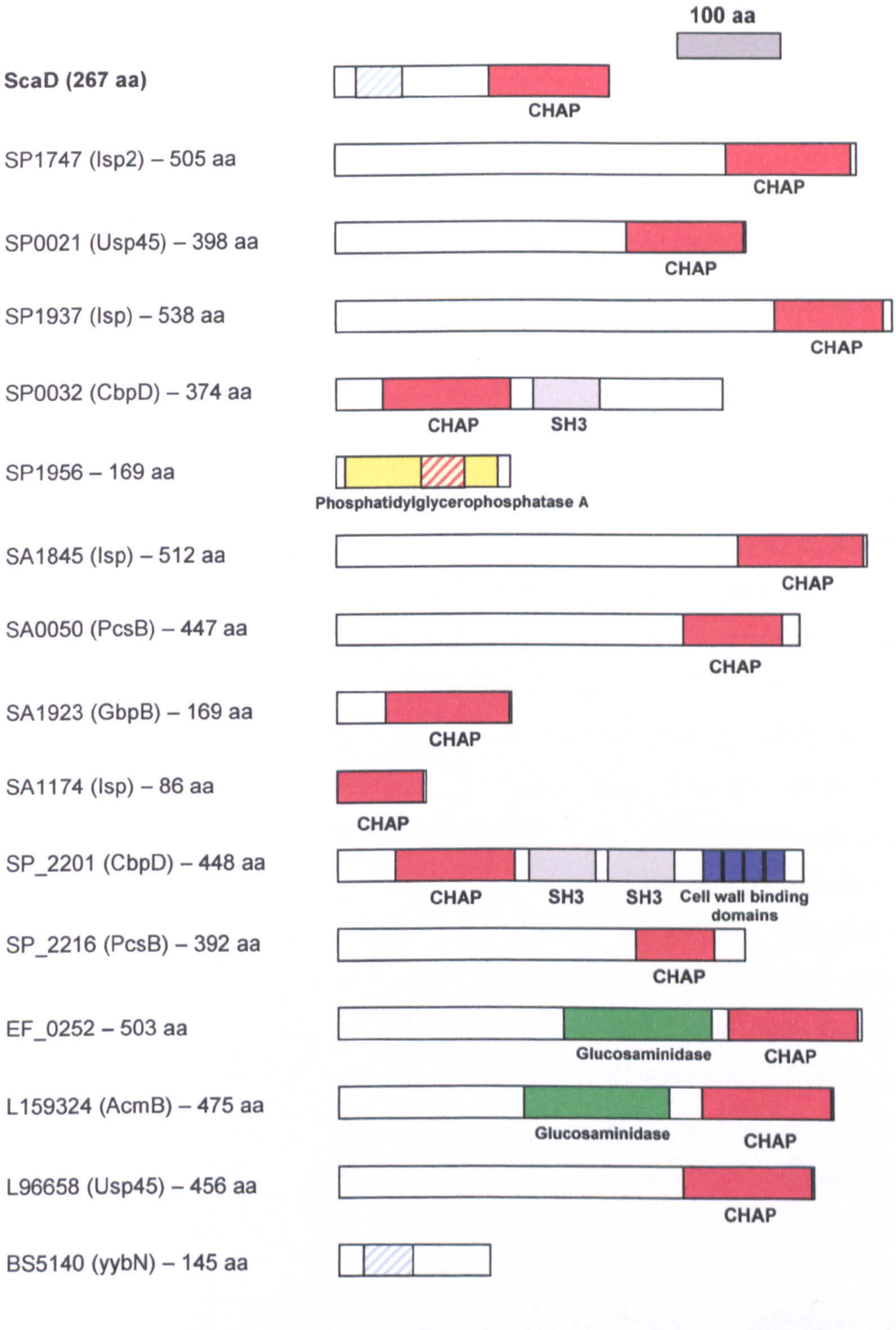


Figure 4.5: Homologues of ScaD. Only SP1956 and BS5140 did not contain CHAP domains. Homology occurred within CHAP domains in all cases except BS5140; the homologous region here is shown by blue stripes. Homology of SP1956 to the ScaD CHAP is shown by red stripes.

4.2.2. Characterisation of recombinant Sca proteins

4.2.2.1. pET overexpression system

The pET expression system was initially developed by Studier and Moffat (1986) using bacteriophage T7 RNA polymerase. The gene of interest is cloned into the pET vector downstream of a T7 promoter with expression of the gene controlled by this inducible promoter.

The pET-24d(+) vector was chosen to overexpress and purify the Sca proteins. The pET-24d(+) plasmid carries an N-terminal T7·Tag® sequence and a C-terminal His·Tag® sequence for convenient and economical purification of the protein of interest under different conditions. The pET-24a(+) plasmid can be seen in Figure 4.6. The maps of pET-24c(+) and pET-24d(+) are the same as pET-24a(+) except that pET-24d(+) is a 5308 bp plasmid; subtract 2 bp from each site beyond *Bam*HI at 198. pET-24d(+) is a 5307 bp plasmid; the *Bam*HI site is in the same reading frame as pET-24c(+). An *Nco*I site is substituted for the *Nde*I site with a net 1 bp deletion at position 238 of pET-24c(+). As a result, *Nco*I cuts pET-24d(+) at 234, and *Nhe*I cuts at 229. For the rest of the sites, subtract 3 bp from each site beyond position 239 in pET-24a(+). *Nde*I does not cut pET-24d(+) (Novagen pET system manual 10th edition, 2002-2003). pET-24d(+) has a multiple cloning site with a choice of eight restriction enzyme sites for cloning. The plasmid also carries the *lac* repressor gene, *lacI*, which limits basal expression of the gene of interest. A kanamycin resistance gene (*kan*^{R}) provides a selectable marker for *E. coli* clones. Another plasmid that is used in this study, pET-21d(+), has the same characteristics as the pET-24d(+) vector except that it contains an ampicillin resistance gene (*amp*^{R}) for selection.

The gene of interest is initially cloned into a host that does not contain the T7 promoter, such as *E. coli* Top10, eliminating the possibility of plasmid instability due to the production of proteins toxic to the host. Once established in a non-expressing host, the plasmid is transformed into an expression host strain, such as *E. coli* BL21, which

contains a chromosomal copy of the RNA polymerase gene under *lacUV5* control. *E. coli* BL21 is lysogenic for bacteriophage DE3, a lambda derivative that has the immunity region of phage *21* and carries a DNA fragment containing the *lacI* promoter and the gene for T7 RNA polymerase (Studier and Moffatt, 1986). Once a DE3 lysogen has been formed, the lac*UV5* promoter can be induced by IPTG causing the transcription of the T7 gene and the gene of interest in the plasmid. BL21 is commonly used as the host strain because it is deficient in the *lon ompT* protease, an enzyme which can degrade proteins during purification (Grodberg *et al.* 1988).

4.2.2.2. Construction of overexpression plasmids

Overexpression strains for ScaA, ScaB and ScaH have been created previously (Pourmand *et al.* 2006; Brummell, 2005). Constructs were created to overexpress the remaining 7 Sca proteins.

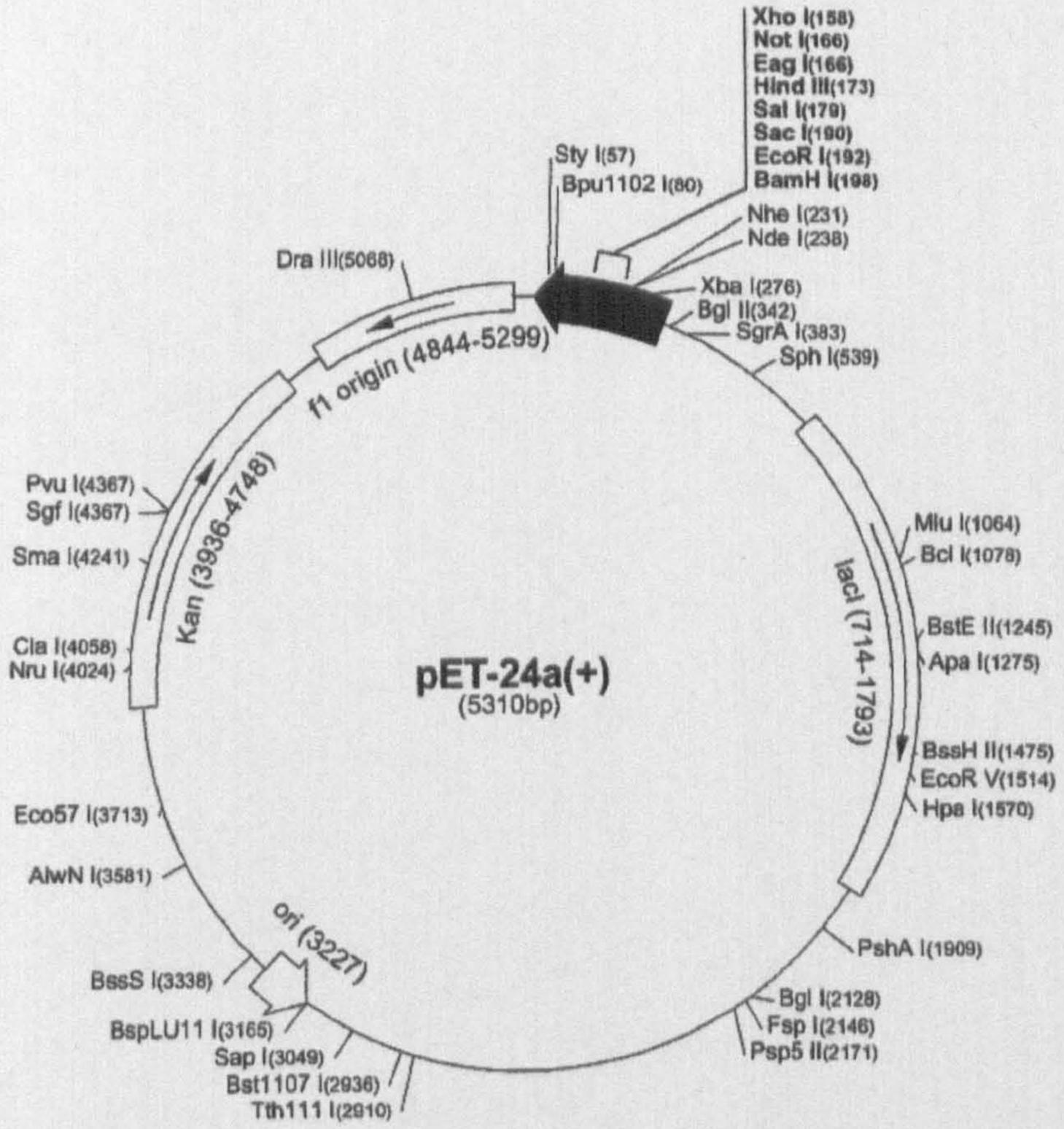
Sequence analysis revealed that *Nco*I and *Xho*I were suitable enzymes to clone each of the 7 *sca* genes. Primers were designed to amplify each of the genes without their signal sequences. Predicted signal sequences were identified using SignalP 3.0 Server (www.cbs.dtu.dk/services/SignalP/). Lengths of signal sequences and sizes of amplified gene products are shown in Table 4.3. Primers were designed to be in frame with the pET-24d vector once cloned.

PCR reactions (Chapter 2.17.1) were carried out using SH1000 genomic DNA and primer pairs pETC_F/pETC_R (*scaC*), pETD_F/pETD_R (*scaD*), pETE_F/pETE_R (*scaE*), pETF_F/pETF_R (*scaF*), pETG_F/pETG_R (*scaG*), pETI_F/pETI_R (*scaI*), pETJ_F/pETJ_R (*scaJ*) (Table 2.6). Amplified products were separated by 1% (w/v) TAE agarose gel (Chapter 2.17.5). The correct sized products were seen for *scaC*, *scaD*, *scaE*, *scaF*, *scaG* and *scaJ*, however, no product could be amplified for *scaI* (Figure 4.7). Further bioinformatic analysis revealed that ScaI is not present in all strains of *S. aureus*, therefore, could not be amplified using SH1000 genomic DNA as a template.

DNA from the 6 correct inserts was excised from the gel and purified (Chapter 2.16.1), before being digested with *Nco*I and *Xho*I. Digested inserts were ligated into the pET-24d vector (Chapter 2.17.4), which had been digested with the same enzymes. Ligations were then electroporated into competent *E. coli* Top10 cells (Chapter 2.20.1) and colonies containing the recombinant plasmids were isolated by selecting for kanamycin resistance. Plasmid DNA was purified from transformants using Qiagen mini-prep kits (Chapter 2.16.5) and recombinant plasmids were digested with *Nco*I and *Xho*I and separated by 1% (w/v) TAE agarose gel electrophoresis to confirm the presence of the insert (Figure 4.7). Inserts were also verified by PCR using recombinant plasmid DNA as a template with T7 forward and reverse primers, which flank the insert in the pET-24d vector (Figure 4.5). Positive clones were identified in all 6 cases. Recombinant plasmids were named pETC (*scaC*), pETD (*scaD*), pETE (*scaE*), pETF (*scaF*), pETG (*scaG*) and pETJ (*scaJ*). Inserts were sequenced to verify their fidelity and all 6 sequences matched that expected.

Recombinant plasmids were then transformed into the overexpression host strain *E. coli* BL21 (DE3) by electroporation (Chapter 2.20.1). Clones were verified by plasmid preparation, restriction digest and PCR, as above (results not shown).

A



B

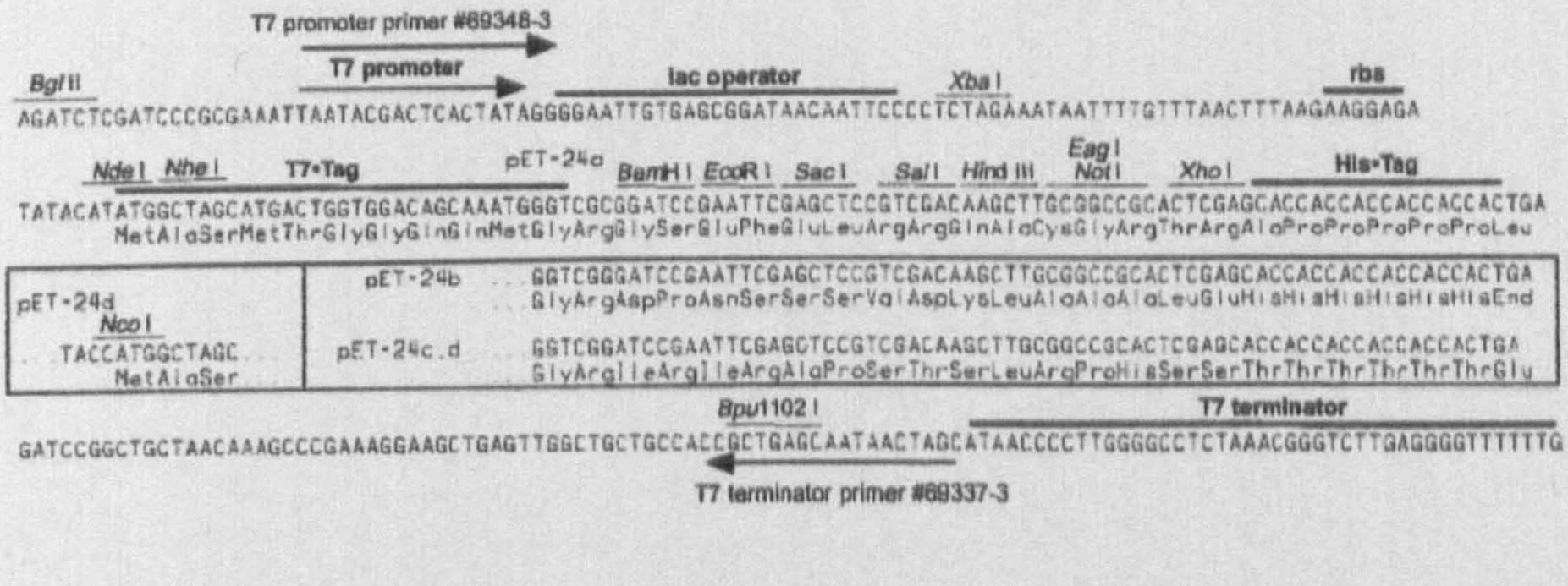


Figure 4.6: The pET-24 expression system (reproduced from the Novagen pET system manual 10th Edition, 2002-2003). A, map of pET-24a(+); B, pET-24a-d cloning/expression region.

Sca Protein	Predicted Signal Sequence (amino acids)	Expected Size of Amplified Insert (bp)
C	1-26	690
D	1-27	720
E	1-24	765
F	1-24	819
G	1-35	324
I	1-47	903
J	1-27	501

Table 4.3: Predicted signal sequences for 7 Sca proteins by SignalP 3.0 Server (www.cbs.dtu.dk/services/SignalP/). Expected sizes of genes amplified for overexpression without signal sequences are also shown.

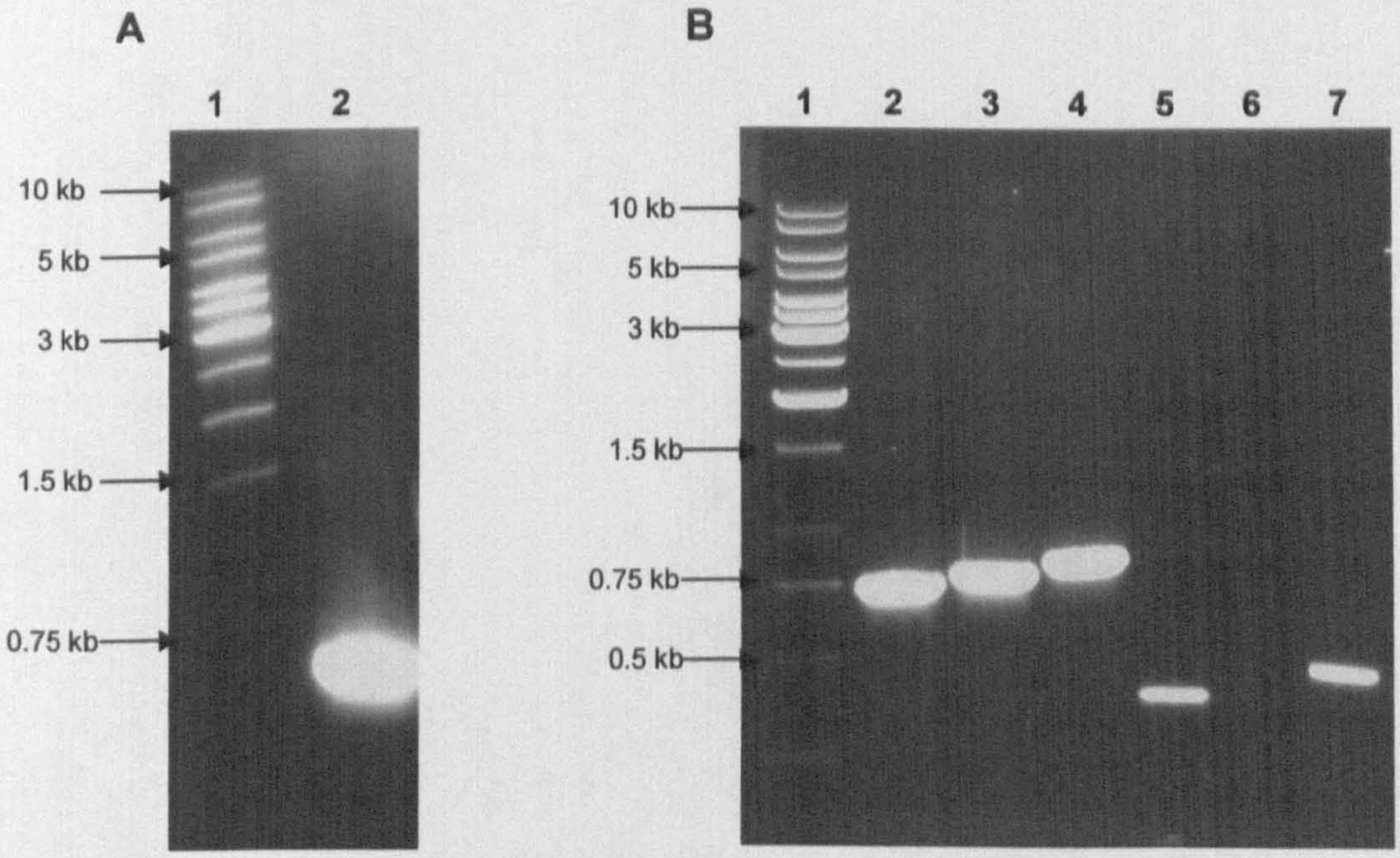


Figure 4.7: 1% (w/v) TAE agarose gels showing amplified overexpression inserts. Panel A: Lane 1, molecular size marker; 2, *scaC*. Panel B: Lane 1, molecular size marker; 2, *scaD*; 3, *scaE*; 4, *scaF*; 5, *scaG*; 6, *scaI* (no product); 7, *scaJ*. Expected sizes were seen for products of 6 *sca* genes. No product could be amplified for *scaI*.

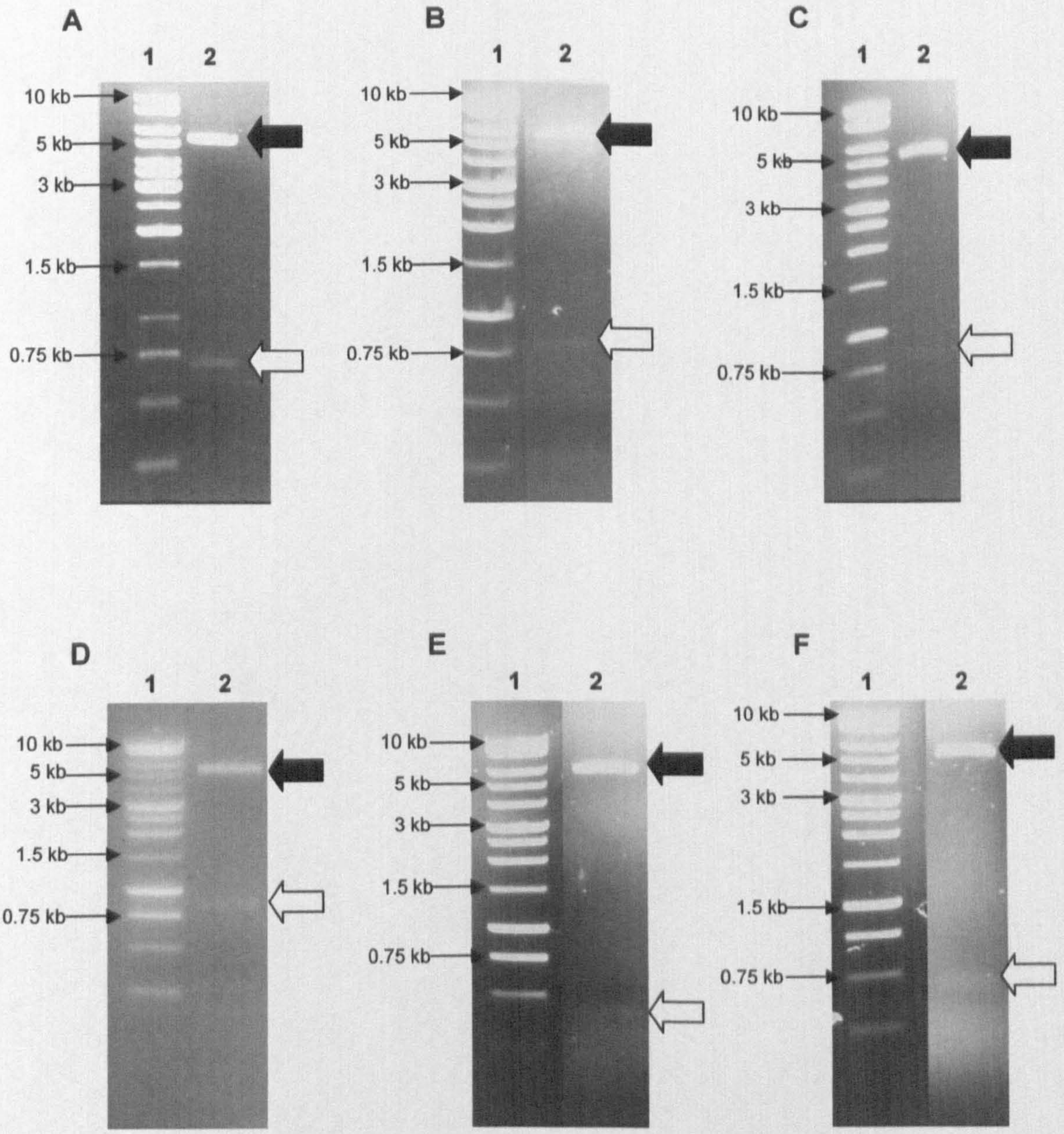


Figure 4.8: 1% (w/v) TAE agarose gels showing the restriction digests of overexpression constructs using *Nco*I and *Xho*I. Panel A, pETC; Panel B, pETD; Panel C, pETE; Panel D, pETF; Panel E, pETG; Panel F, pETJ. Lane 1, molecular size marker; 2, digested DNA. Filled arrows indicate the digested pET-24d vector (5.3 kb), unfilled arrows indicate the *sca* gene overexpression insert.

4.2.2.3. Overexpression of recombinant proteins

Expression of the Sca proteins was induced using 1 mM IPTG, as described in Chapter 2.10. To verify overexpression of the proteins, uninduced and induced total protein samples of *E. coli* BL21 were analysed by 17.5% (w/v) SDS-PAGE. Results of total cell protein analysis showed successful overexpression of the six Sca proteins (Figure 4.9).

4.2.2.4. Solubility of recombinant proteins

In order to allow purification, it was necessary to determine the solubility of the recombinant proteins. Induced culture samples were disrupted by sonication and separated by centrifugation (Chapter 2.10.2). Total proteins from supernatant and pellet were then analysed by 17.5% (w/v) SDS-PAGE. All six recombinant Sca proteins were present in the lysate pellet rather than the supernatant (Figure 4.10), indicating that the recombinant Sca proteins were insoluble.

4.2.2.5. Purification of recombinant proteins using a HiTrap affinity column

Cells harvested from the overexpression culture were prepared for purification as described in Chapter 2.10.3. Due to the insolubility of the recombinant Sca proteins, the protein lysate was treated with 8M urea in START buffer to solubilise the protein before purification by HiTrap™ affinity chromatography (Pharmacia Biotech). The HiTrap™ column used is pre-packed with Chelating Sepharose™ High Performance, which consists of highly cross-linked agarose beads to which iminodiacetic acid has been coupled by stable ether groups. Several amino acids, such as histidine, form complexes with many metal ions. The column matrix, charged with suitable metal ions, will selectively retain proteins if complex-forming amino acid residues are exposed on the surface of the protein.

The column was prepared and charged with nickel ions, which bind the six histidine residues of the His-tagged protein. The sample was applied to the column and washed

with START buffer to remove unbound proteins. START buffer containing a gradient of 0-0.5 M imidazole was used to elute the recombinant protein from the column. Eluate was collected into 50 fractions (1 ml/fraction) and eluted proteins were analysed by 17.5% (w/v) SDS-PAGE to determine when the purified protein had eluted from the column (Figure 4.11). Fractions containing purified recombinant protein were combined and dialysed overnight at 4°C into PBS + 8M urea. In order to solubilise the recombinant proteins, they were subsequently dialysed further into decreasing concentrations of urea (4M, 2M, 1M, no urea), however, when dialysed into 2-4 M urea, all six Sca proteins precipitated out of solution. The recombinant proteins were therefore stored in PBS + 8M urea.

Eight recombinant Sca proteins were successfully purified as described above. However, ScaJ could not be purified by this method, for unknown reasons. Unpurified protein was used in further analyses. The nine recombinant Sca proteins, including unpurified ScaJ, were analysed by 17.5% (w/v) SDS-PAGE (Figure 4.12). The correct sized fragments were seen for all recombinant proteins. As seen previously (Pourmand, 2005), ScaB was overexpressed in two forms, seen as two fragments on the gel. The lower fragment is a 114-residue N-terminally truncated protein, the higher fragment is full-length ScaB. Two fragments were also seen for rScaD although the identity of the two forms remains to be determined. ScaH was overexpressed by Syed Mohamad (2007) and produced several fragments on the gel due to hydrolysis of the full-length protein upon storage.

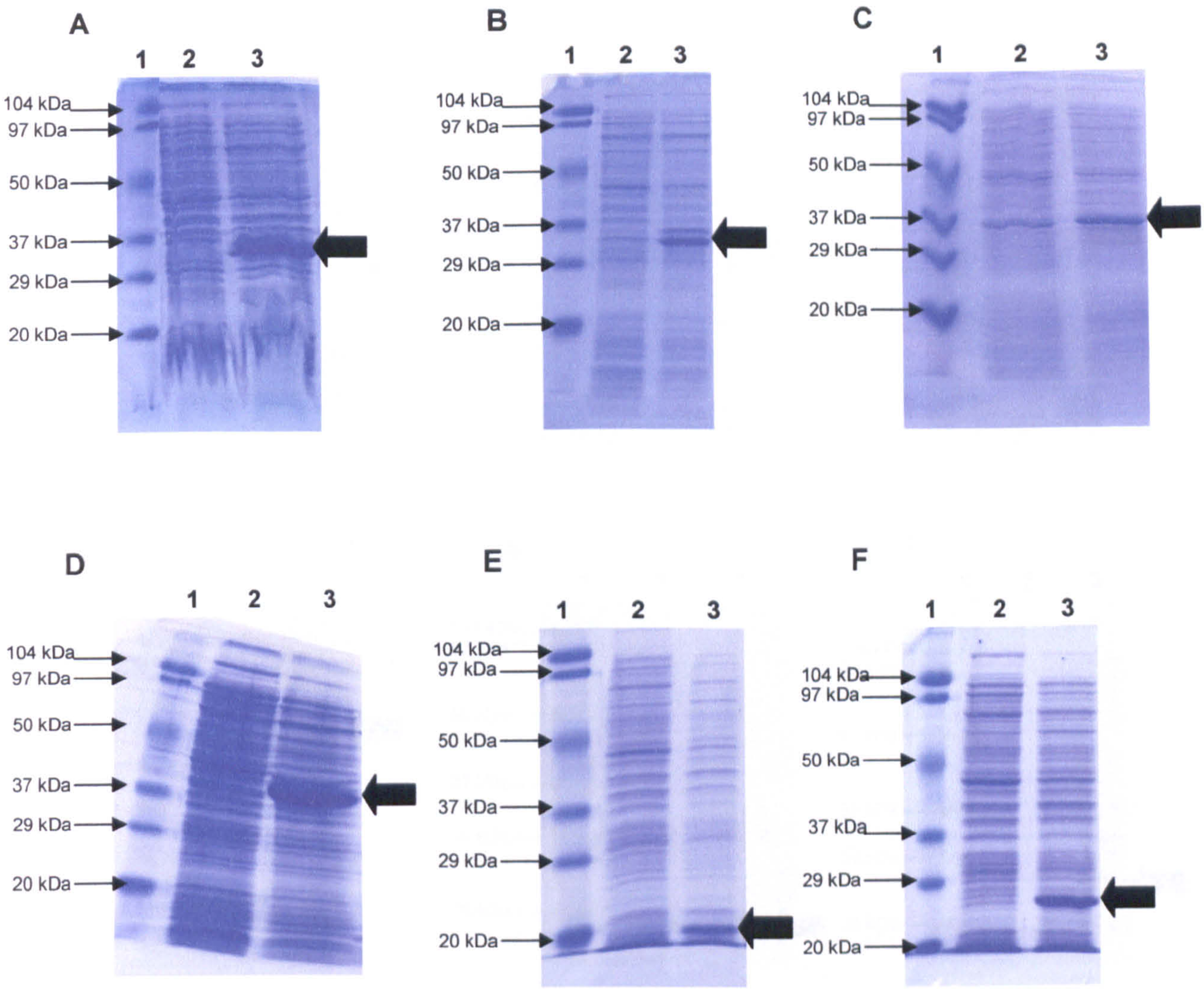


Figure 4.9: 17.5% (w/v) SDS-PAGE analysis of overexpression of six Sca proteins. Panel A, ScaC; Panel B, ScaD; Panel C, ScaE; Panel D, ScaF; Panel E, ScaG; Panel F, ScaJ. Lane 1, molecular mass marker; 2, uninduced *E. coli* BL21 protein fraction; 3, induced protein fraction containing the overexpressed Sca protein (identified by the filled arrows).

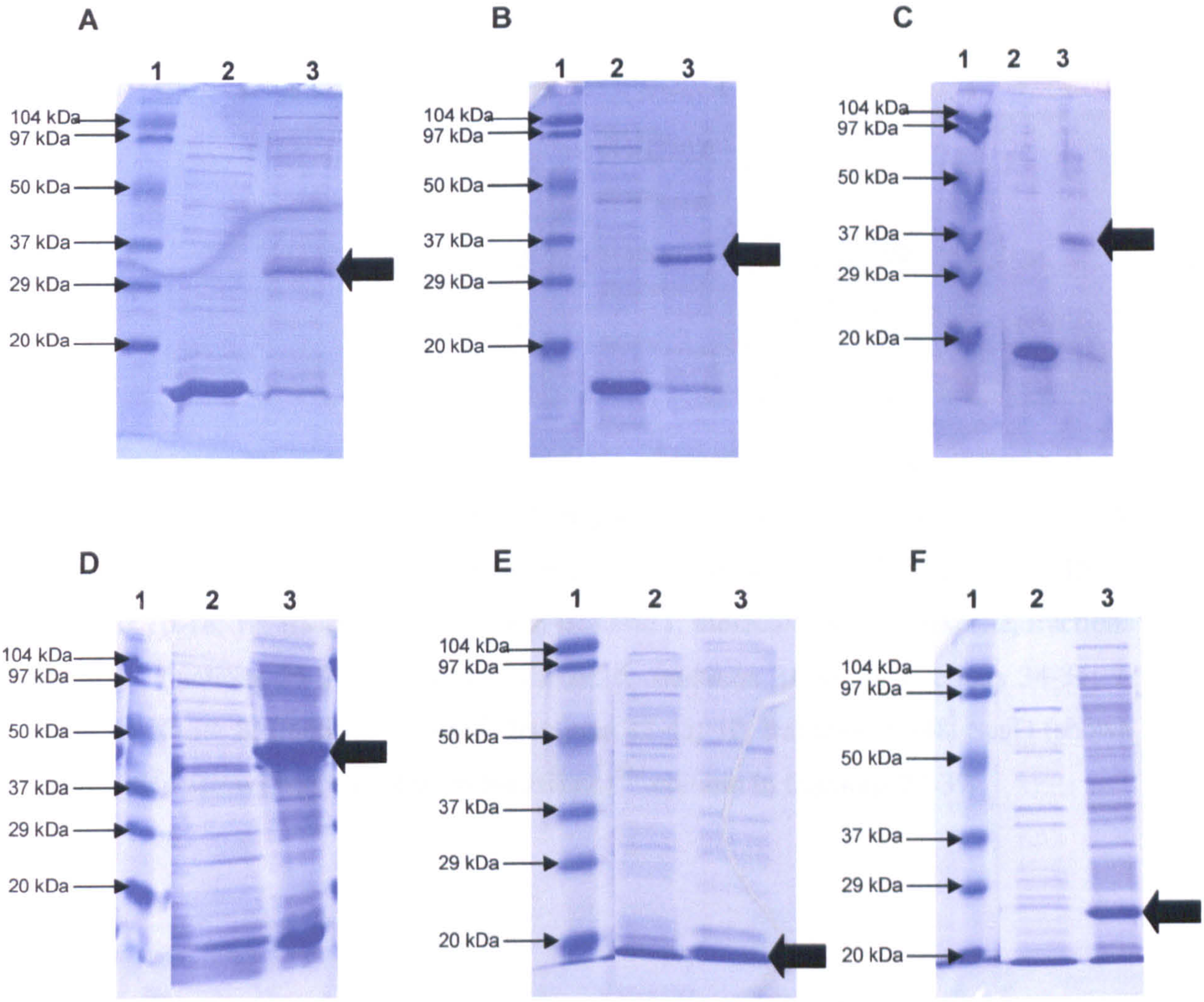


Figure 4.10: 17.5% (w/v) SDS-PAGE analysis of solubility of six Sca proteins. Panel A, ScaC; Panel B, ScaD; Panel C, ScaE; Panel D, ScaF; Panel E, ScaG; Panel F, ScaJ. Lane 1, molecular mass marker; 2, soluble *E. coli* BL21 protein fraction; 3, insoluble protein fraction. All six Sca proteins were insoluble (identified by filled arrows).

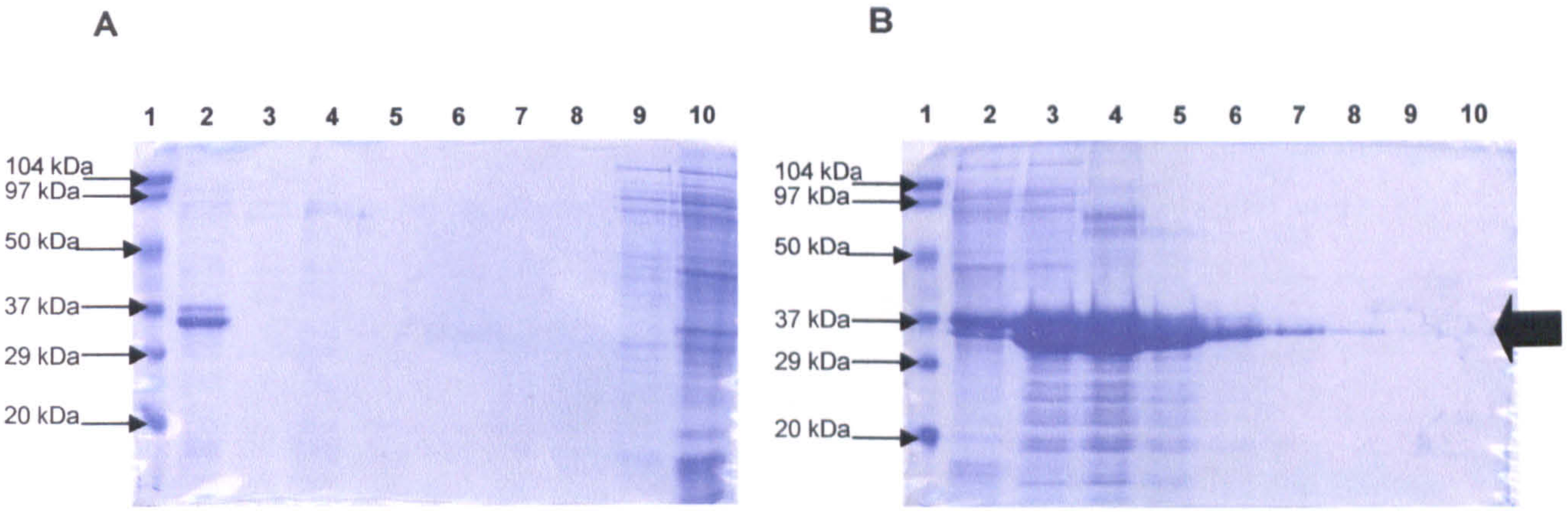


Figure 4.11: 17.5% (w/v) SDS-PAGE analysis of purification fractions of ScaD. Panel A: Lane 1, molecular mass marker; 2, unpurified extract; 3, purification waste; 4, fractions 1-3; 5, fractions 4-6; 6, fractions 7-9; 7, fractions 10-12; 8, fractions 13-15; 9, fractions 16-18; 10, fractions 19-21. Panel B: Lane 1, molecular mass marker; 2, fractions 22-24; 3, fractions 25-27; 4, fractions 28-30; 5, fractions 31-33; 6, fractions 34-36; 7, fractions 37-39; 8, fractions 40-42; 9, fractions 43-45; 10, fractions 46-48. ScaD (shown by the filled arrow) was eluted from the HiTrap^{TM} column in fractions 22-39.

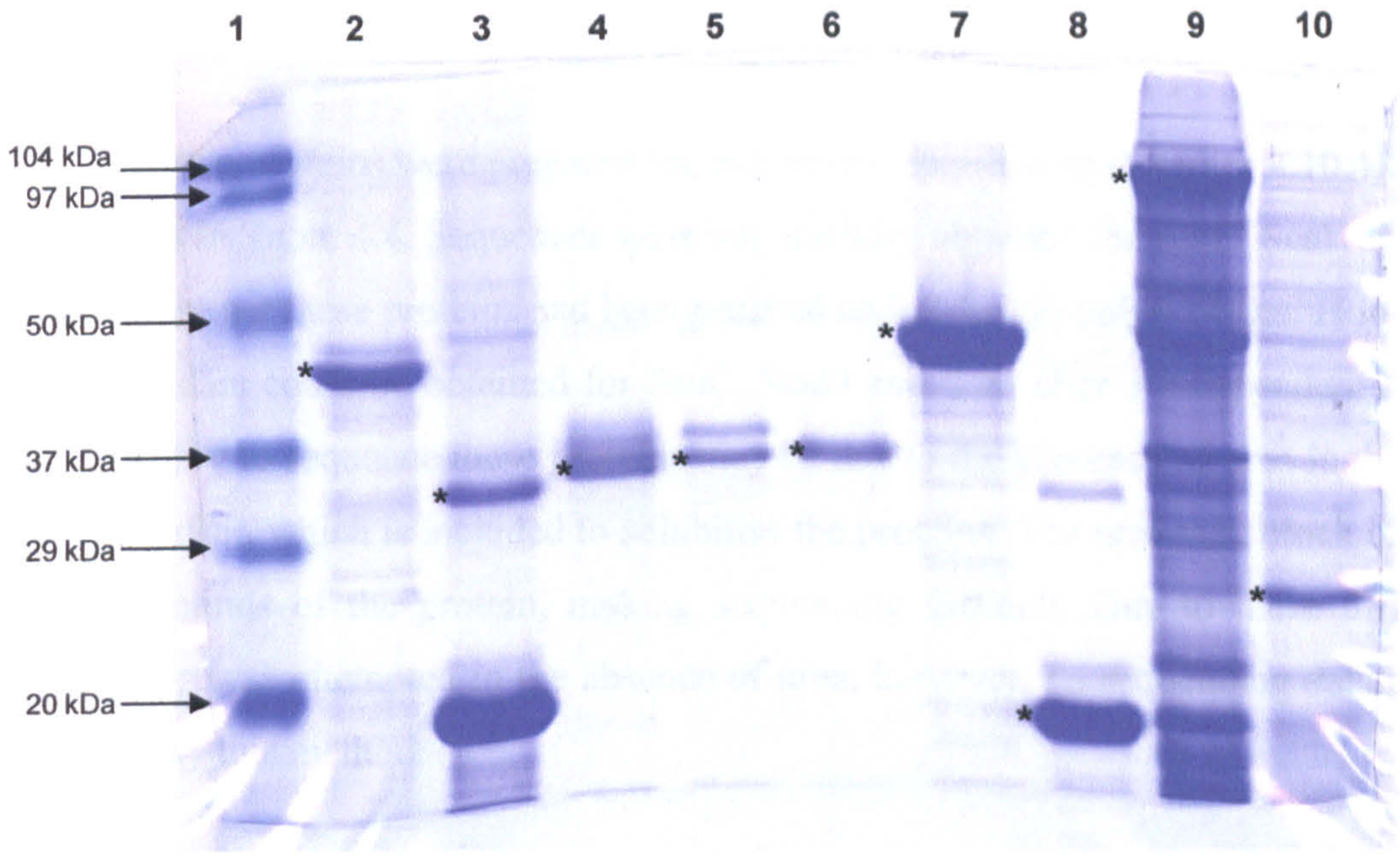


Figure 4.12: 17.5% (w/v) SDS-PAGE analysis of recombinant Sca proteins. Lane 1, molecular mass marker; 2, rScaA; 3, rScaB; 4, rScaC; 5, rScaD; 6, rScaE; 7, rScaF; 8, rScaG; 9, rScaH; 10, rScaJ. Sizes of full-length proteins are shown by asterisks. rScaB produced two fragments, the lower fragment is a 114-residue N-terminally truncated protein. rScaD also produced two fragments. ScaH produced many bands due to hydrolysis of the full-length protein upon storage.

4.2.2.6. N-terminal analysis of the recombinant proteins

Recombinant proteins were prepared for N-terminal sequencing (Chapter 2.10.4). Results are shown in Table 4.4. Sequences were successfully obtained for ScaE, ScaF and ScaG, confirming that these proteins had been purified as full-length polypeptides. However, no sequence data could be obtained for ScaC, ScaD and ScaJ after 3 independent attempts. The inability to sequence these proteins may be due to the presence of urea in the protein storage buffer, which is included to solubilise the proteins. The urea may block or modify the N-terminus of the protein, making sequencing difficult. Due to these difficulties, sequencing was attempted in the absence of urea, however, no sequencing data could be obtained by this method.

4.2.2.7. Analysis of recombinant proteins using mass spectrometry

As recombinant ScaC, ScaD and ScaJ could not be verified by N-terminal sequence analysis, these proteins were digested with trypsin and analysed by mass spectrometry using MASCOT (kindly carried out by Dr. Arthur Moir). All three recombinant proteins failed to be identified by this method, both after 4 h digests and overnight digests with trypsin. Undigested proteins were also assessed. Analysis of ScaC produced a peak at approximately 25.9 kDa, corresponding to the expected size of the full length overexpressed protein without the signal sequence (Figure 4.13). No data was obtained for ScaD and ScaJ.

4.2.3. Reaction of recombinant proteins with human serum

The ability of the recombinant Sca proteins to react with human sera was tested in Western blots. Three sera from the immunoscreen described in Chapter 3 were tested for antibodies that react with the nine recombinant Sca proteins. BSA was included as a negative control. Results are shown in Figure 4.14 and summarised in Table 4.5. rScaA, rScaB and rScaE bound antibodies in all three sera. rScaD and rScaF failed to bind any of the sera tested.

Sca Protein	N-Terminal Sequence	Identification
C	Unable to sequence	-
D	Unable to sequence	-
E	A Q Q H G T Q	SACOL0820 (ScaE)
F	A Y T N D S	SACOL0270 (ScaF)
G	A V N Y Y S K	SACOL2557 (ScaG)
J	Unable to sequence	-

Table 4.4: N-terminal sequence analysis of recombinant Sca proteins.

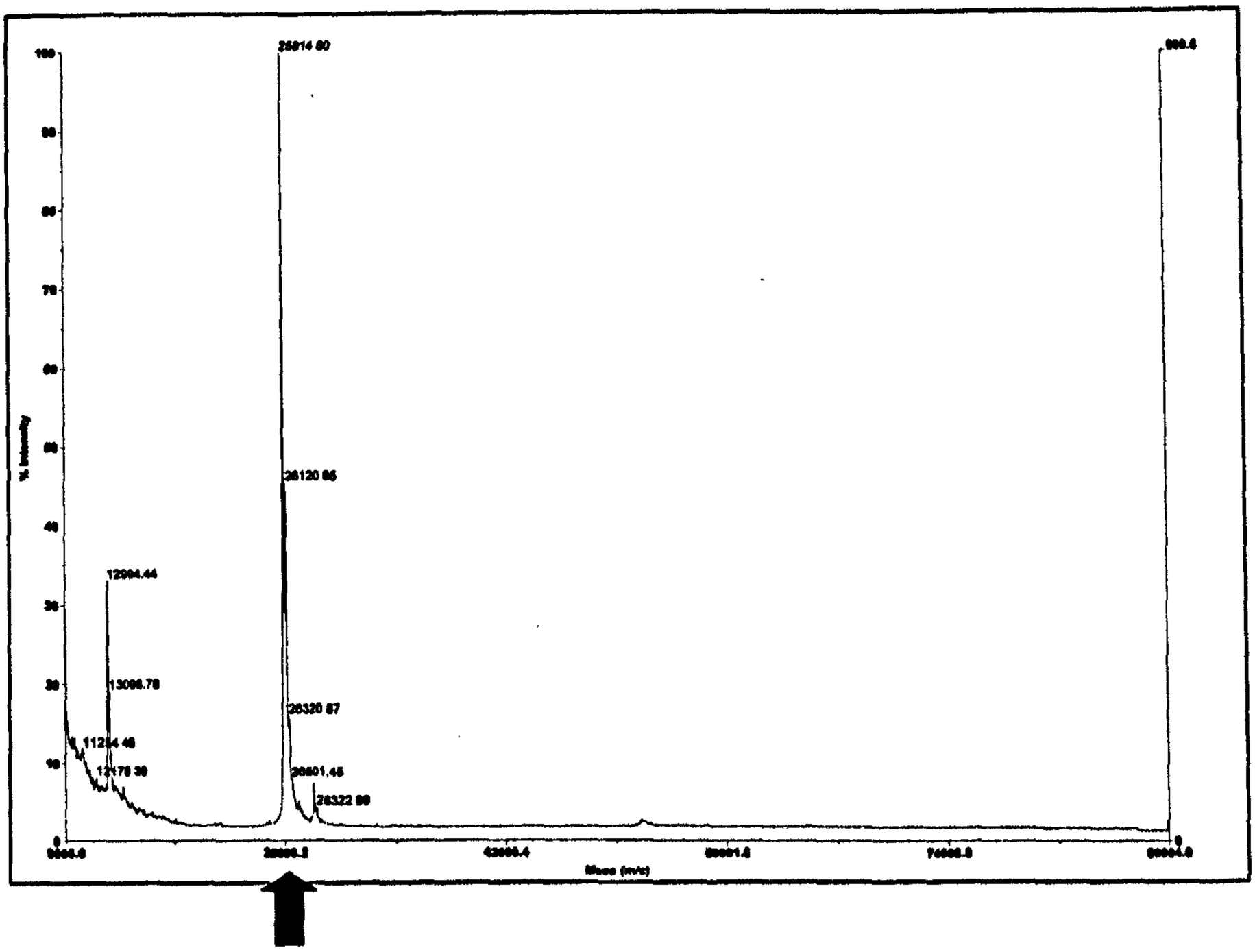
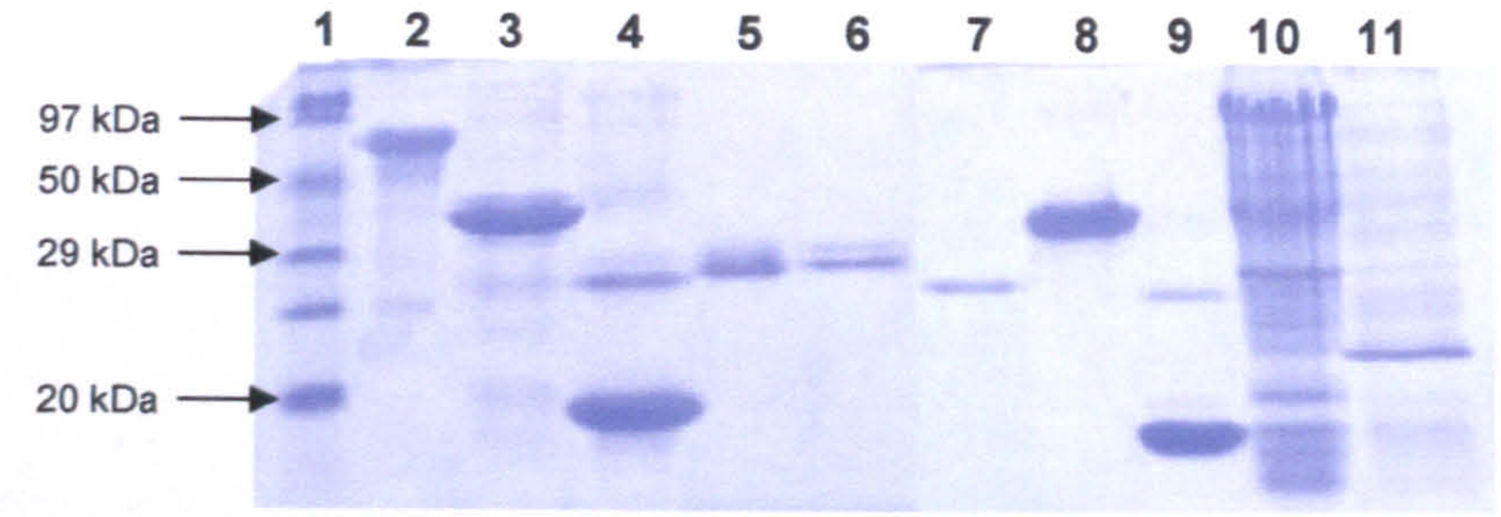
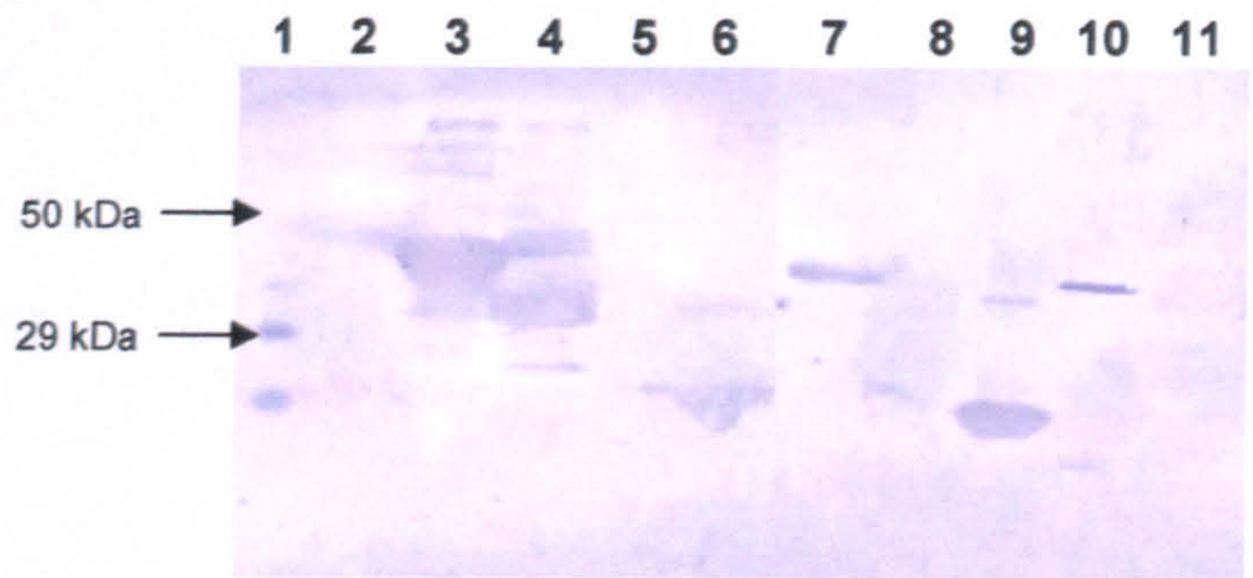
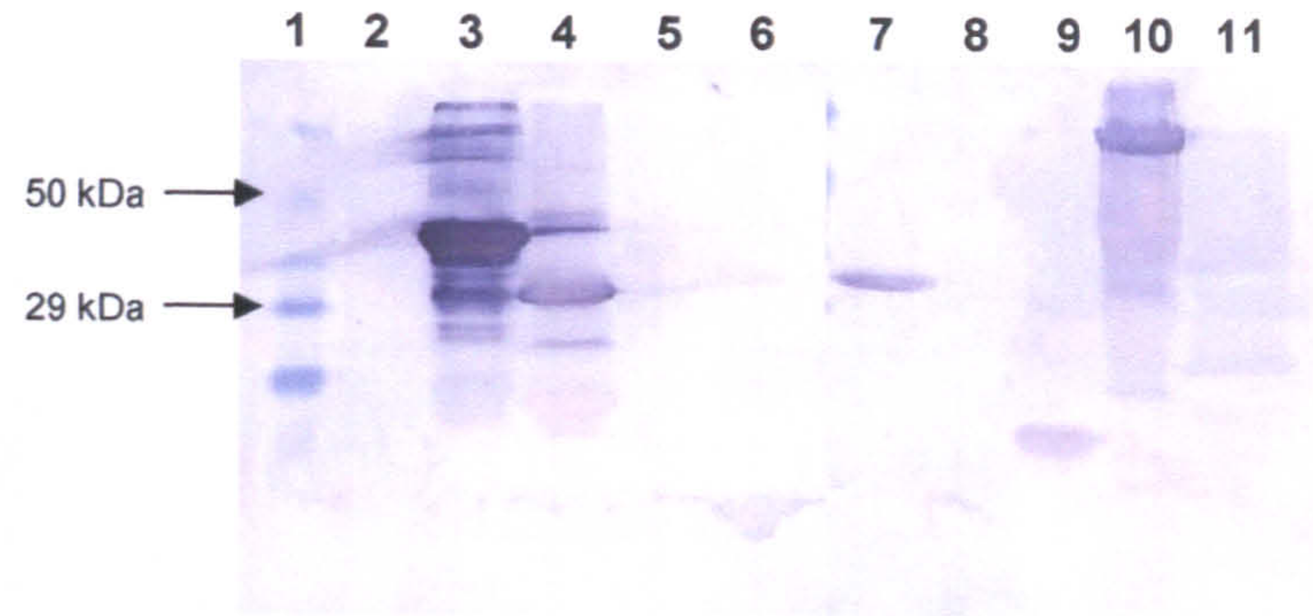


Figure 4.13: Mass spectrometry analysis of undigested ScaC. A peak at 25.9 kDa (shown by the filled arrow) corresponded to the expected size of the full-length recombinant protein without its signal peptide.







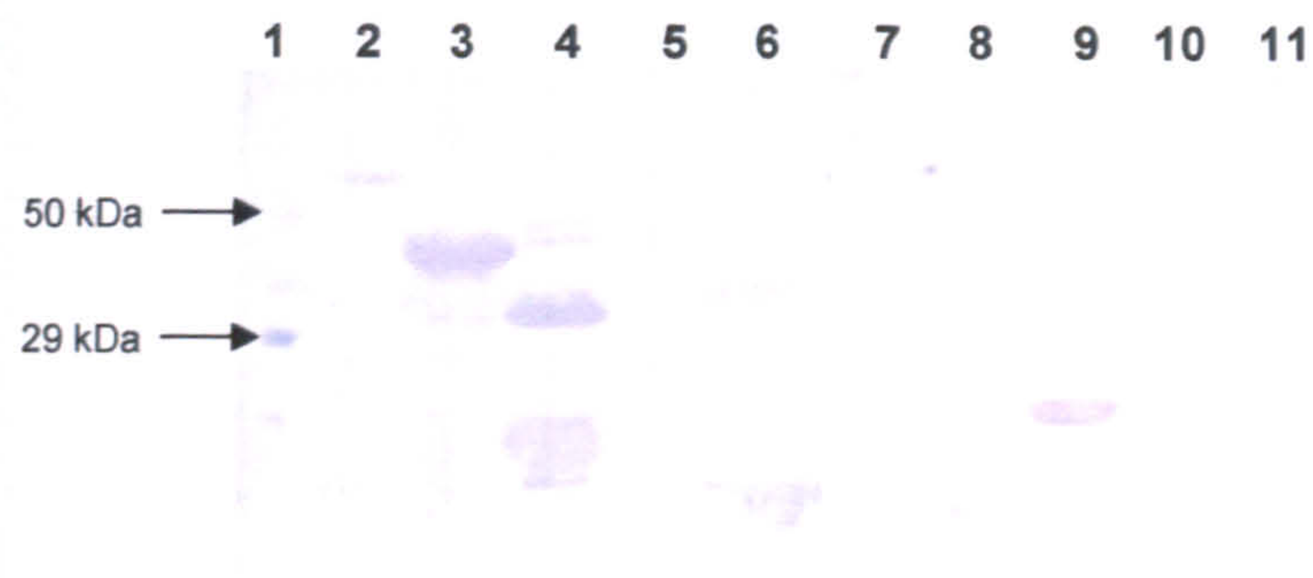


Figure 4.14: Western blot analysis demonstrating the reactivity of recombinant Sca proteins with human sera. Panel A) Coomassie-stained SDS-PAGE; Panel B) Human serum 28; Panel C) Human serum 24; Panel D) Human serum D62. Lane 1, molecular mass marker; 2, BSA; 3, rScaA; 4, rScaB; 5, rScaC; 6, rScaD; 7, rScaE; 8, rScaF; 9, rScaG; 10, rScaH; 11, rScaJ (unpurified).

Sca Protein	Human Serum		
	24	28	D62
ScaA	✓	✓	✓
ScaB	✓	✓	✓
ScaC	x	✓	x
ScaD	x	x	x
ScaE	✓	✓	✓
ScaF	x	x	x
ScaG	x	✓	x
ScaH	✓	✓	x
ScaJ	x	✓	x

Table 4.5: Summary of the reactivity of recombinant Sca proteins with human serum antibodies.

4.2.4. Peptidoglycan hydrolase activity of the recombinant Sca proteins

The peptidoglycan hydrolase activity of the recombinant Sca proteins was tested by zymogram analysis (Chapter 2.12.3). rScaA and rScaH have previously been demonstrated to lyse peptidoglycan from *S. aureus*, *S. epidermidis*, *Bacillus subtilis* and *Micrococcus luteus* (Pourmand, 2004; Syed Mohamad, 2007). The ability of nine Sca proteins to hydrolyse peptidoglycan from purified cell walls of three of these species, *S. aureus*, *B. subtilis* and *M. luteus*, was assessed. All recombinant Sca proteins were able to digest *S. aureus* peptidoglycan (Figure 4.15). Notably, both the full-length rScaB and the N-terminally truncated fragment demonstrated activity, hence the C-terminus is most likely the active domain. rScaA, rScaB, rScaE, rScaF, rScaG and rScaH were also able to hydrolase peptidoglycan from *B. subtilis* and *M. luteus* (results not shown). rScaC, rScaD and rScaJ did not produce lytic bands of clearing during zymograph analysis with *B. subtilis* and *M. luteus* cell walls as substrate. Whether this is due to lack of hydrolytic activity or insufficient protein on the gel remains to be clarified. The peptidoglycan hydrolase activity of the recombinant Sca proteins is summarised in Table 4.6.

4.2.5. Ligand binding ability of the Sca proteins

To determine the possible involvement of the recombinant Sca proteins in adhesion to human ligands, they were tested in binding assays with a range of plasma proteins (Chapter 2.13.3). Fibrinogen (Fg), fibronectin (Fn), fetuin (Ft), mucin (Mc), laminin (Lm), lactoferrin (Lf) and transferrin (Tf) were labelled with biotin and used to probe western blots of the nine recombinant Sca proteins. Western blots are shown in Figure 4.16 and results are summarised in Table 4.7.

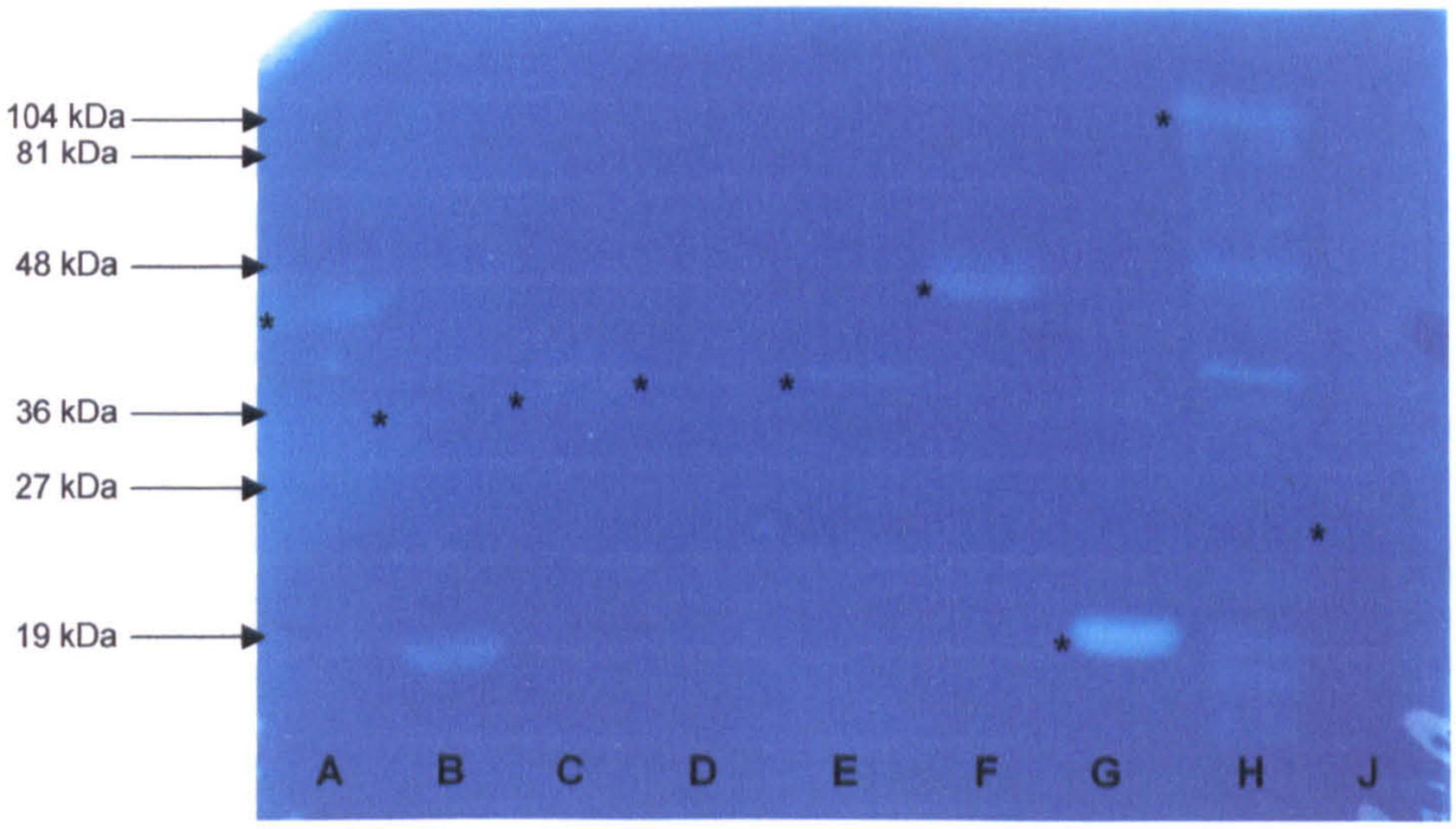
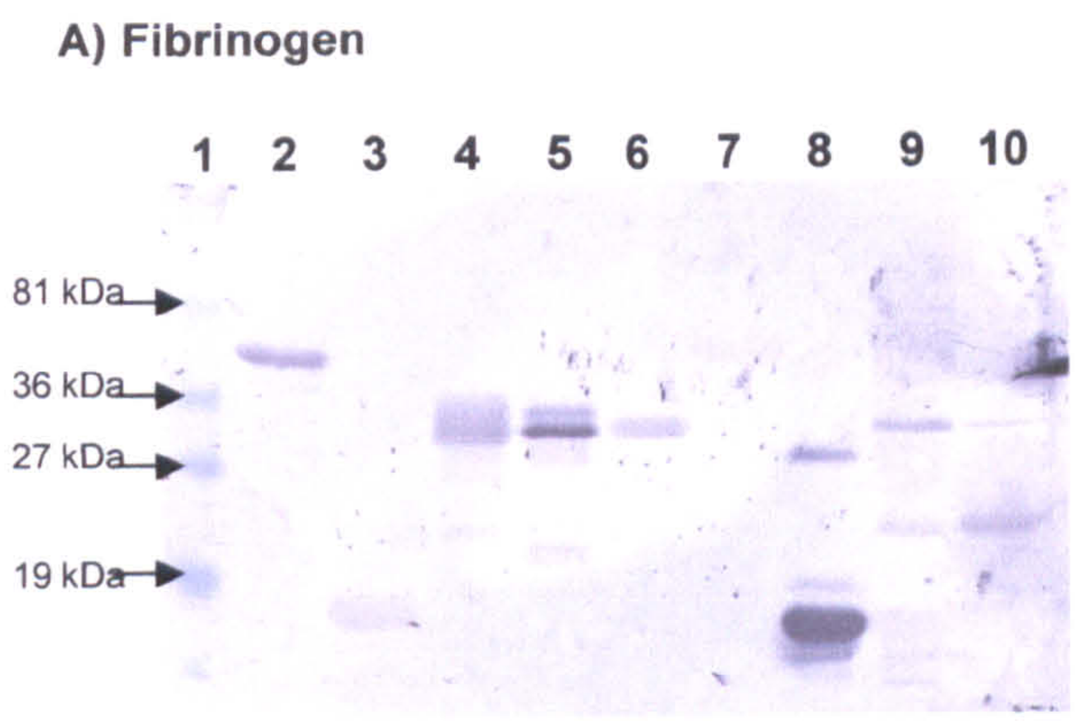
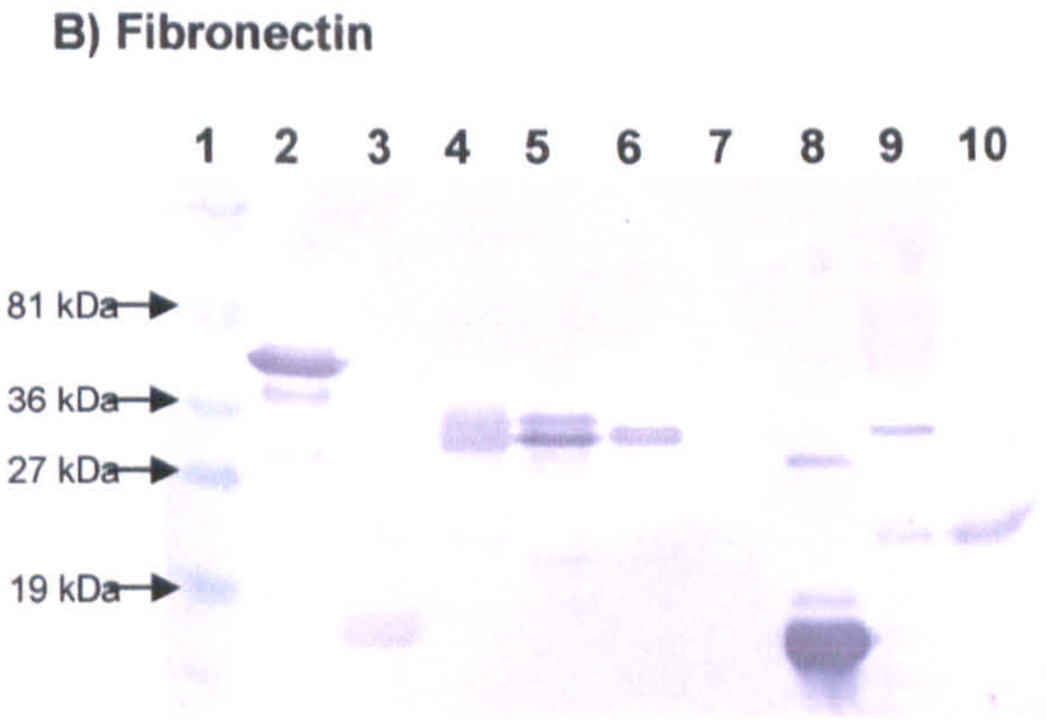


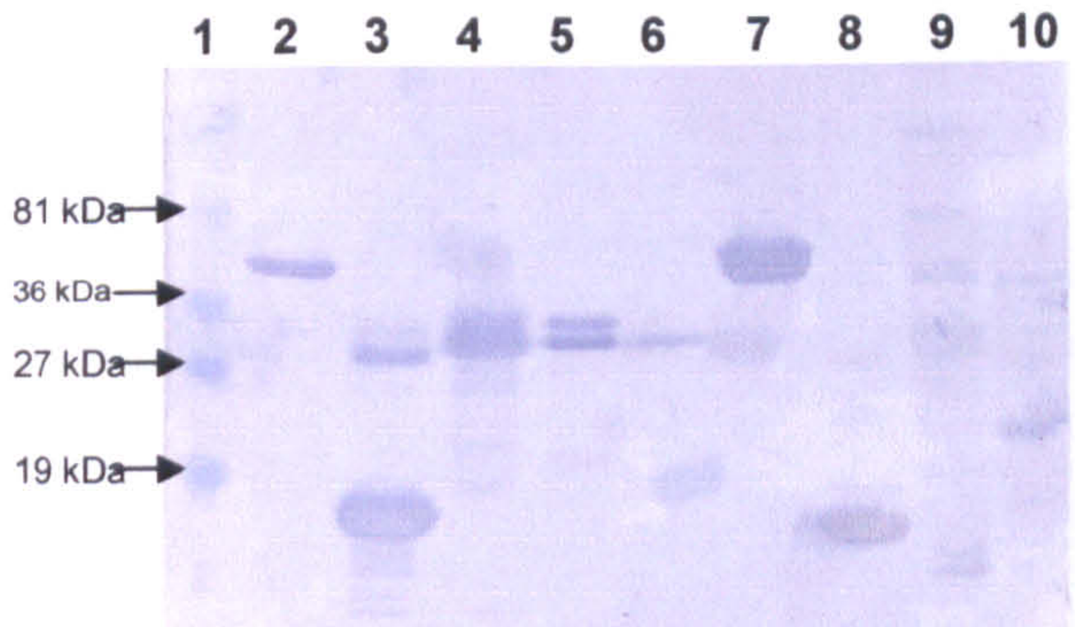
Figure 4.15: Zymogram analysis showing activity of the Sca proteins. Recombinant proteins were analysed by 17.5% (w/v) SDS-PAGE renaturing gel electrophoresis containing 0.1% (w/v) purified *S. aureus* cell walls as substrate. Lytic fragments demonstrating autolytic activity are seen as bands of clearing in the stained gel. Sizes of full-length proteins are marked with an asterisk.

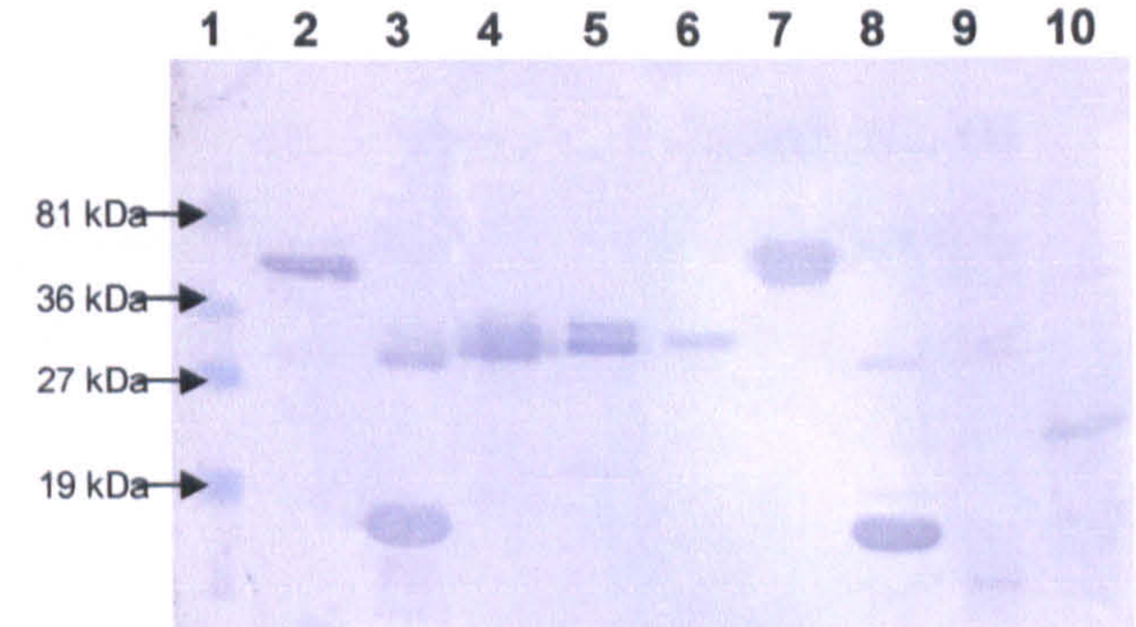
Substrate	rScaA	rScaB	rScaC	rScaD	rScaE	rScaF	rScaG	rScaH	rScaJ
S. aureus	✓	✓	✓	✓	✓	✓	✓	✓	✓
B. subtilis	✓	✓	U	U	✓	✓	✓	✓	U
M. luteus	✓	✓	U	U	✓	✓	✓	✓	U

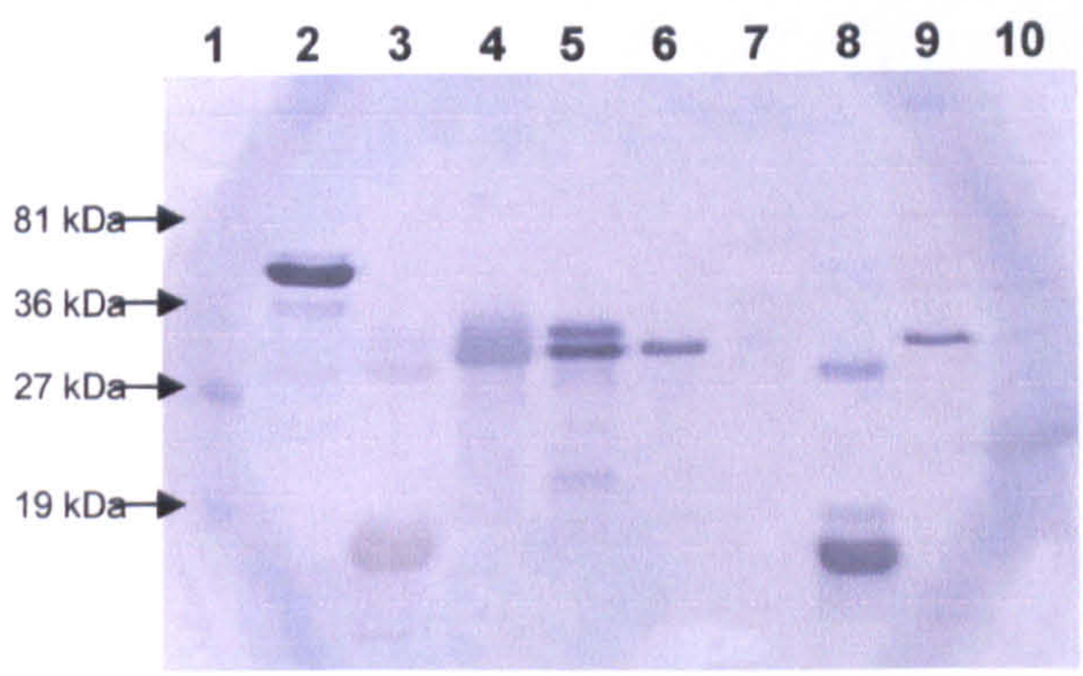
Table 4.6: Summary of peptidoglycan hydrolase activity of recombinant Sca proteins with three purified cell wall substrates. Confirmed peptidoglycan hydrolase activity is shown by a tick, unconfirmed activity is shown by "U".

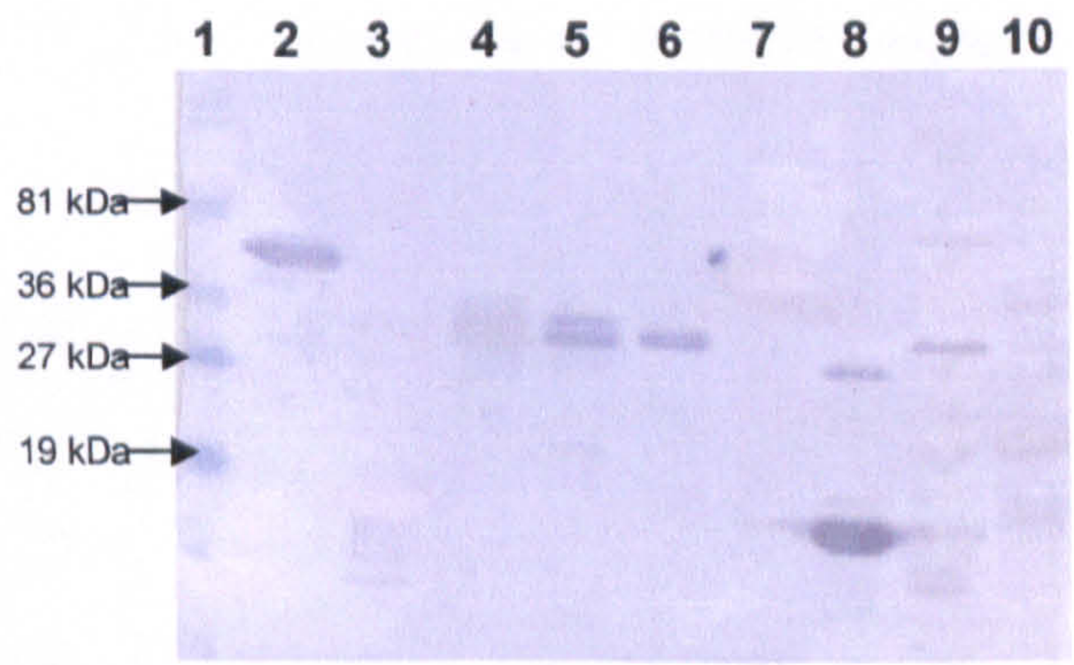

A) Fibrinogen
1 2 3 4 5 6 7 8 9 10
81 kDa
36 kDa
27 kDa
19 kDa


B) Fibronectin
1 2 3 4 5 6 7 8 9 10
81 kDa
36 kDa
27 kDa
19 kDa


C) Fetuin
1 2 3 4 5 6 7 8 9 10
81 kDa
36 kDa
27 kDa
19 kDa


D) Mucin
1 2 3 4 5 6 7 8 9 10
81 kDa
36 kDa
27 kDa
19 kDa


E) Laminin
1 2 3 4 5 6 7 8 9 10
81 kDa
36 kDa
27 kDa
19 kDa


F) Lactoferrin
1 2 3 4 5 6 7 8 9 10
81 kDa
36 kDa
27 kDa
19 kDa

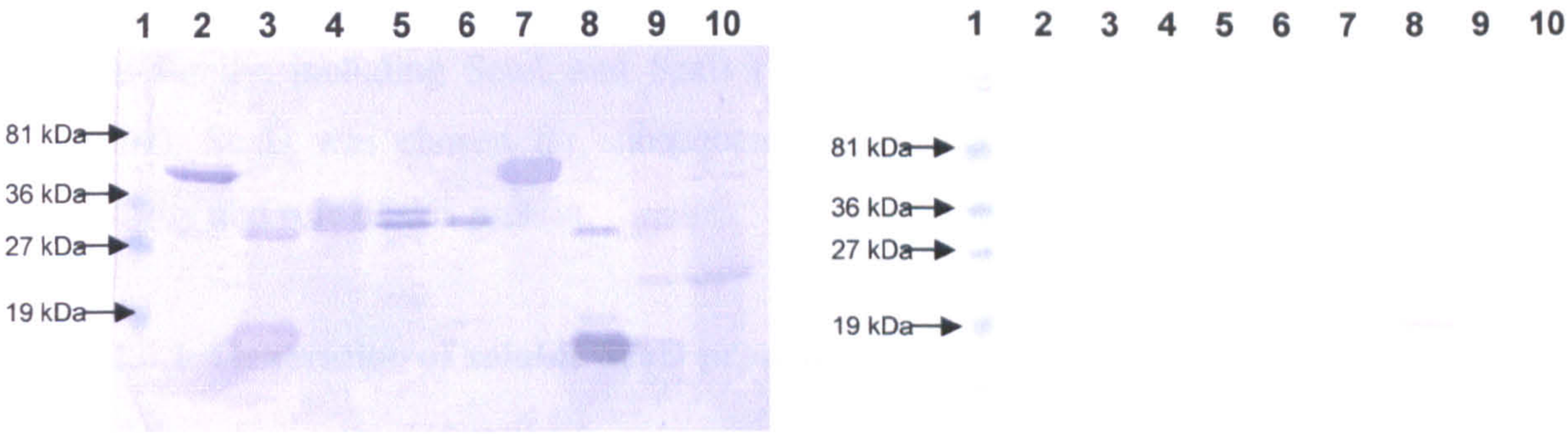


Figure 4.16: Western blots showing binding of recombinant Sca proteins to human ligands [A) fibrinogen, B) fibronectin, C) fetuin, D) mucin, E) laminin, F) lactoferrin, G) transferrin, H) no ligand control]. Lane 1, molecular mass marker; 2, rScaA; 3, rScaB; 4, rScaC; 5, rScaD; 6, rScaE; 7, rScaF; 8, rScaG; 9, rScaH; 10, rScaJ (unpurified).

Ligand	rScaA	rScaB	rScaC	rScaD	rScaE	rScaF	rScaG	rScaH	rScaJ
Fibrinogen	✓	✓	✓	✓	✓	x	✓	✓	✓
Fibronectin	✓	✓	✓	✓	✓	x	✓	✓	✓
Fetuin	✓	✓	✓	✓	✓	✓	✓	x	✓
Mucin	✓	✓	✓	✓	✓	✓	✓	x	✓
Laminin	✓	✓	✓	✓	✓	x	✓	✓	✓
Lactoferrin	✓	x	✓	✓	✓	x	✓	✓	✓
Transferrin	✓	✓	✓	✓	✓	✓	✓	x	✓

Table 4.7: Summary of the ability of recombinant Sca proteins to bind human ligands.

4.2.6. Characterisation of ScaD

Previous studies within the laboratory have further characterised several members of the Sca family, including ScaA and ScaB (Pourmand, 2004) and ScaH (Syed Mohamad, 2007). ScaD was chosen for subsequent analysis to attempt to further elucidate the activity and role of this protein.

4.2.6.1. Generation of soluble ScaD protein

As recombinant ScaD could not be solubilised by step-wise dialysis into PBS, an alternative method was sought. In a study by Amrein *et al.* (1995), production of soluble, active $p50^{csk}$ kinase was successfully achieved by co-overexpression of the protein in *E. coli* with the bacterial chaperones GroES and GroEL. It is proposed that these chaperones associate with nascent polypeptide chains, aiding their correct folding and hence increasing the solubility of the recombinant polypeptides. The two chaperones are overexpressed via the plasmid pREP4groESL, which carries a kanamycin resistance marker. Therefore, the ScaD overexpression plamsid pETD (Chapter 4.3.2), which is also kanamycin resistant could not be used to co-overexpress ScaD with these chaperones. pET-21d (Novagen) has the same characteristics as the pET-24d vector except that it contains an ampicillin resistance gene rather than a kanamycin gene (Chapter 4.3.1). This allowed the easy transfer of the *scaD* insert DNA from pETD into pET-21d.

4.2.6.1.1. Transfer of *scaD* overexpression insert into pET-21d

Plasmid pETD was digested with *Nco*I and *Xho*I and separated by 1% (w/v) TAE agarose gel electrophoresis. The 720 bp *scaD* insert was gel purified and ligated into pET-21d digested with the same enzymes. The ligation was transformed into competent *E. coli* Top10 and colonies containing the recombinant plasmid were isolated by selecting for ampicillin resistance. Plasmid DNA was purified from transformants using Qiagen mini-prep kits and presence of the insert-containing plasmid was verified by PCR using T7 forward and reverse primers (Table 2.6). Both PCR using plasmid template DNA and

colony PCR of *E. coli* Top10 cells were employed to verify the *scaD* insert in the pET-21d plasmid. The products of the PCR were analysed by 1% (w/v) TAE agarose gel electrophoresis and the correct sized bands were seen, confirming the presence of the *scaD* insert within the pET-21d vector (Figure 4.17). The recombinant plasmid was named pETDa. Recombinant pETDa was then transformed into the overexpression host strain *E. coli* BL21 (DE3) pREP4groESL by electroporation. Transformants were selected on both kanamycin and ampicillin and clones were verified by colony PCR, as above (results not shown). The resulting strain, containing two overexpression plasmids, was named GroD.

4.2.6.1.2. Co-overexpression of recombinant ScaD with GroES and GroEL

In order to produce soluble protein, different overexpression conditions were assessed. Strain GroD was grown in 100 ml TB and overexpression induced using increasing levels of IPTG (10 μM, 100 μM, 1 mM and 2 mM). Also, cultures were grown at 25°C, 30°C and 37°C, both before and after induction. Overexpression of the proteins was verified by analysis of uninduced and induced total protein samples of strain GroD by 17.5% (w/v) SDS-PAGE. The solubility of the proteins from cultures of each condition was also determined by analysis of the insoluble and soluble fractions of induced cultures by 17.5% (w/v) SDS-PAGE, as previous (Chapter 4.3.4). Results showed that the 29 kDa recombinant ScaD was successfully overexpressed along with both GroES (10 kDa) and GroEL (58 kDa), however, ScaD remained insoluble in every case (Figure 4.18).

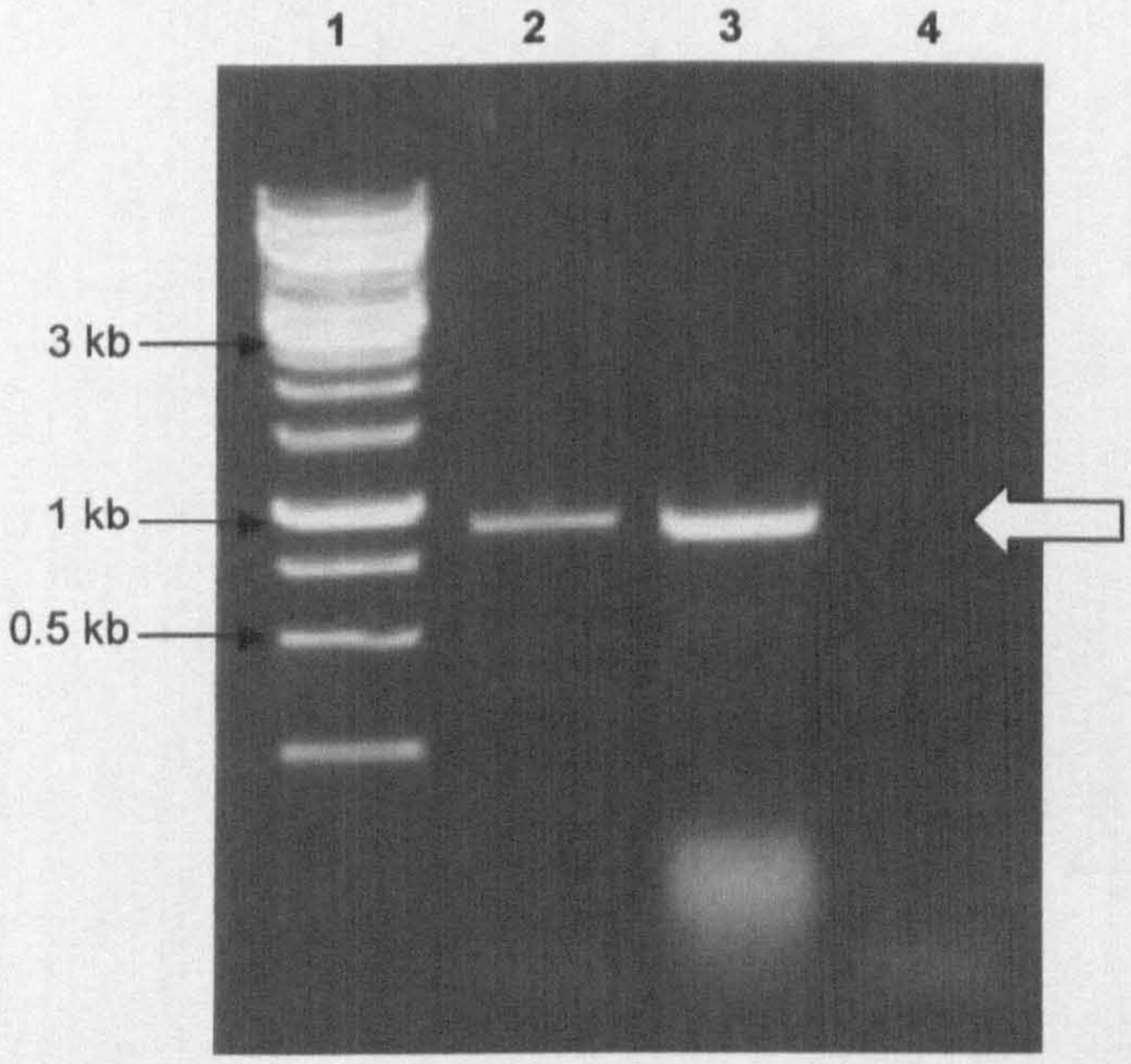
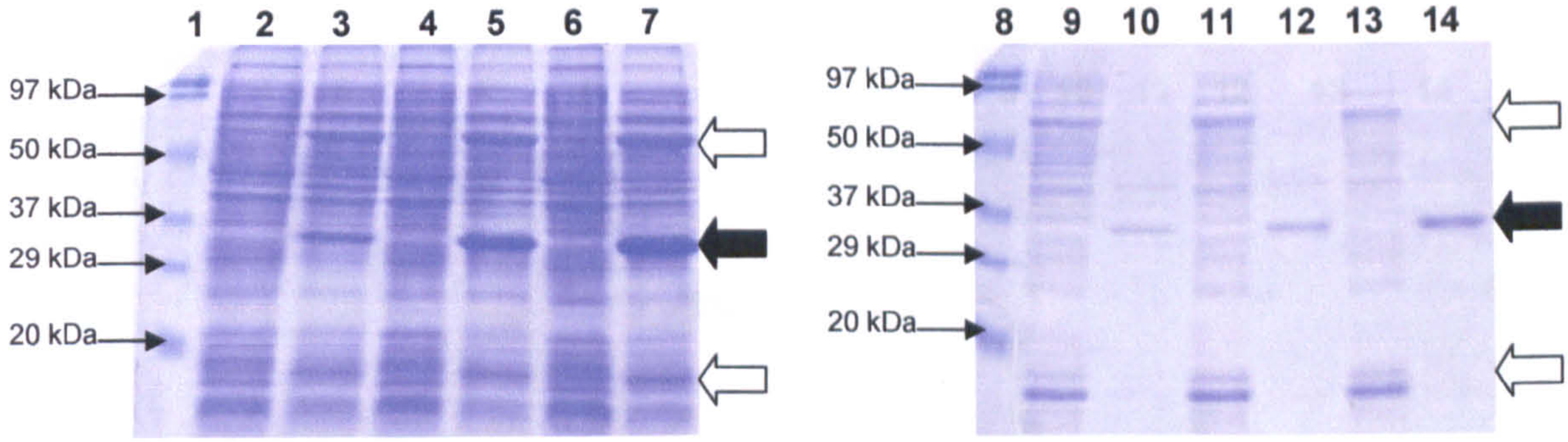
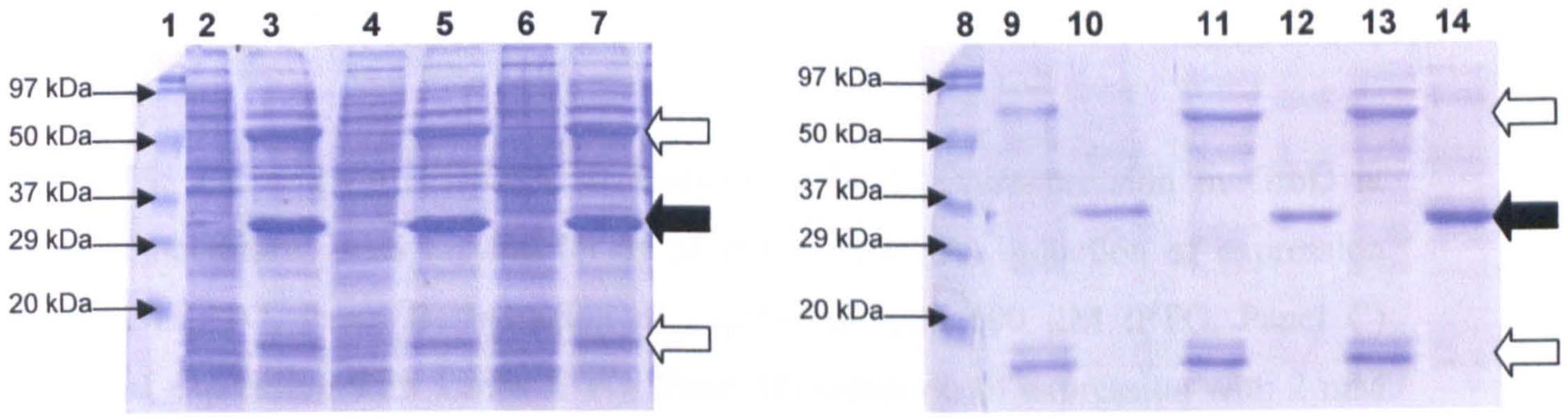


Figure 4.17: 1% (w/v) TAE agarose gel electrophoresis analysis of PCR using T7 forward and T7 reverse primers to confirm *scaD* insert in pETDa overexpression plasmid. Lane 1, molecular size marker; 2, PCR product using pETDa template DNA; 3, PCR product using colony of Top10 pETDa as template; 4, no template negative control. The correct sized insert was seen at approx. 720 bp, shown by the unfilled arrow.

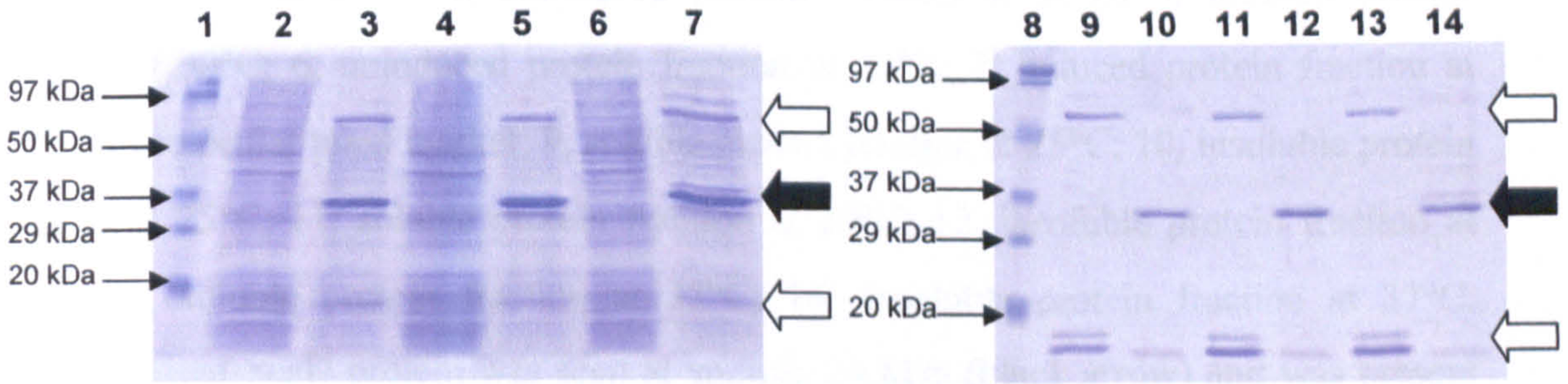
A) 10 µM IPTG



B) 100 µM IPTG



C) 1 mM IPTG



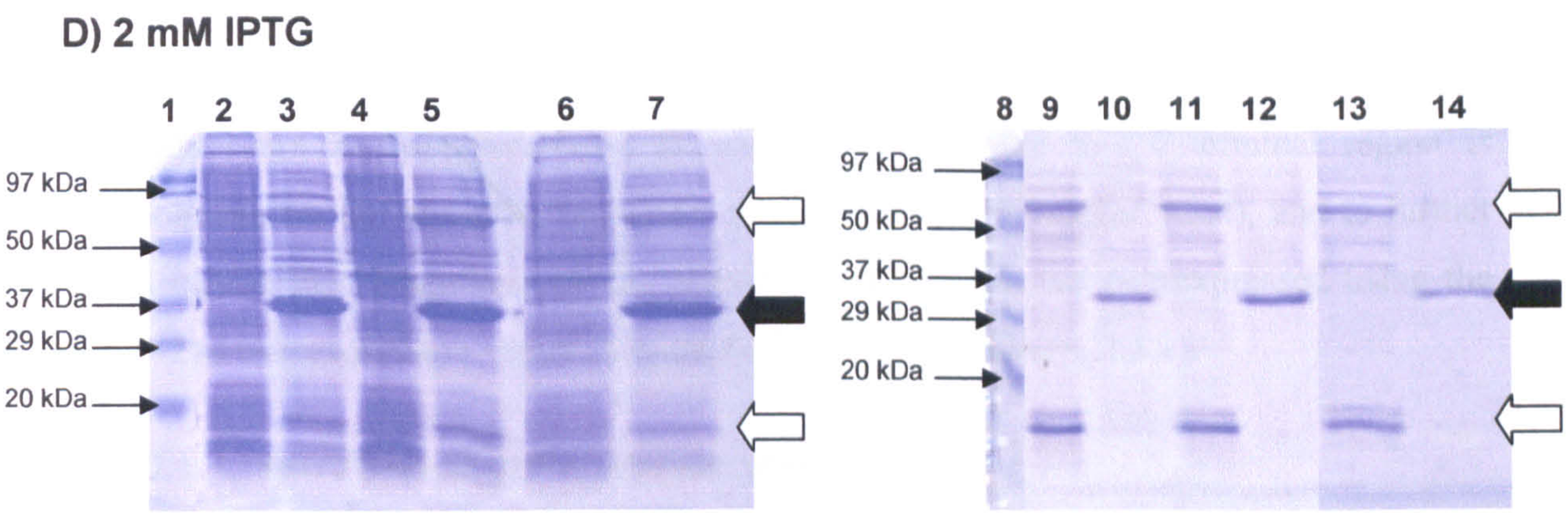


Figure 4.18: 17.5% (w/v) SDS-PAGE analysis of ScaD overexpression in GroD at different temperatures with varying levels of IPTG. Panel A) induction of expression with 10 μM IPTG, Panel B) induction of expression with 100 μM IPTG, Panel C) induction of expression with 1 mM IPTG, Panel D) induction of expression with 2 mM IPTG. Lane 1, molecular mass marker; 2, uninduced protein fraction at 25°C; 3, induced protein fraction at 25°C; 4, uninduced protein fraction at 30°C; 5, induced protein fraction at 30°C; 6, uninduced protein fraction at 37°C; 7, induced protein fraction at 37°C; 8, molecular mass marker; 9, soluble protein fraction at 25°C; 10, insoluble protein fraction at 25°C; 11, soluble protein fraction at 30°C; 12, insoluble protein fraction at 30°C; 13, soluble protein fraction at 37°C; 14, insoluble protein fraction at 37°C. Overexpressed ScaD protein was seen at approx. 29 kDa (black arrow) and was present in the insoluble protein fraction in all cases. Co-overexpressed chaperones GroEL and GroES were seen at 58 kDa and 10 kDa, respectively (white arrows).

4.2.7. Characterisation of the N-terminus and C-terminus of ScaD

The C-terminus of ScaD consists of a predicted CHAP domain, associated with peptidoglycan hydrolase activity. In order to verify that this C-terminal region is responsible for the observed hydrolytic activity of ScaD (Chapter 4.2.4), and to further characterise the N-terminus of this protein, each domain was overexpressed using the pET-24d overexpression vector previously described (Chapter 4.2.2.1).

4.2.7.1. Construction of overexpression plasmids

Overexpression plasmids were constructed as described in Chapter 4.2.2.2. Sequence analysis revealed that *Nco*I and *Xho*I were suitable enzymes to clone both the N- and C-terminus of *scaD* and primers were designed to amplify each fragment. The N-terminus was amplified without the signal peptide (Table 4.3). PCR reactions were carried out using SH1000 genomic DNA and primer pairs pETD_F/pETD_R2 (N-terminal 130 amino acids) and pETD_F2/pETD_R (C-terminal 110 amino acids) (Table 2.6). Amplified products were separated by 1% (w/v) TAE agarose gel and the correct sized products were seen for both the N- and C-terminus inserts (Figure 4.19).

DNA was excised from the gel and purified, before being digested with *Nco*I and *Xho*I. Digested inserts were ligated into the pET-24d vector, which had been digested with the same enzymes. Ligations were then electroporated into competent *E. coli* Top10 cells and colonies containing the recombinant plasmids were isolated by selecting for kanamycin resistance. Plasmid DNA was purified from transformants using Qiagen mini-prep kits and recombinant plasmids were digested with *Nco*I and *Xho*I and separated by 1% (w/v) TAE agarose gel electrophoresis to confirm the presence of the insert (Figure 4.20). Positive clones were identified in both cases. Recombinant plasmids were named pETDn (*scaD* N-terminus) and pETDc (*scaD* C-terminus). Recombinant plasmids were then transformed into *E. coli* BL21 (DE3) cells by electroporation and clones were verified by plasmid preparation and PCR using T7 forward and T7 reverse primers (Table 2.6) (results not shown).

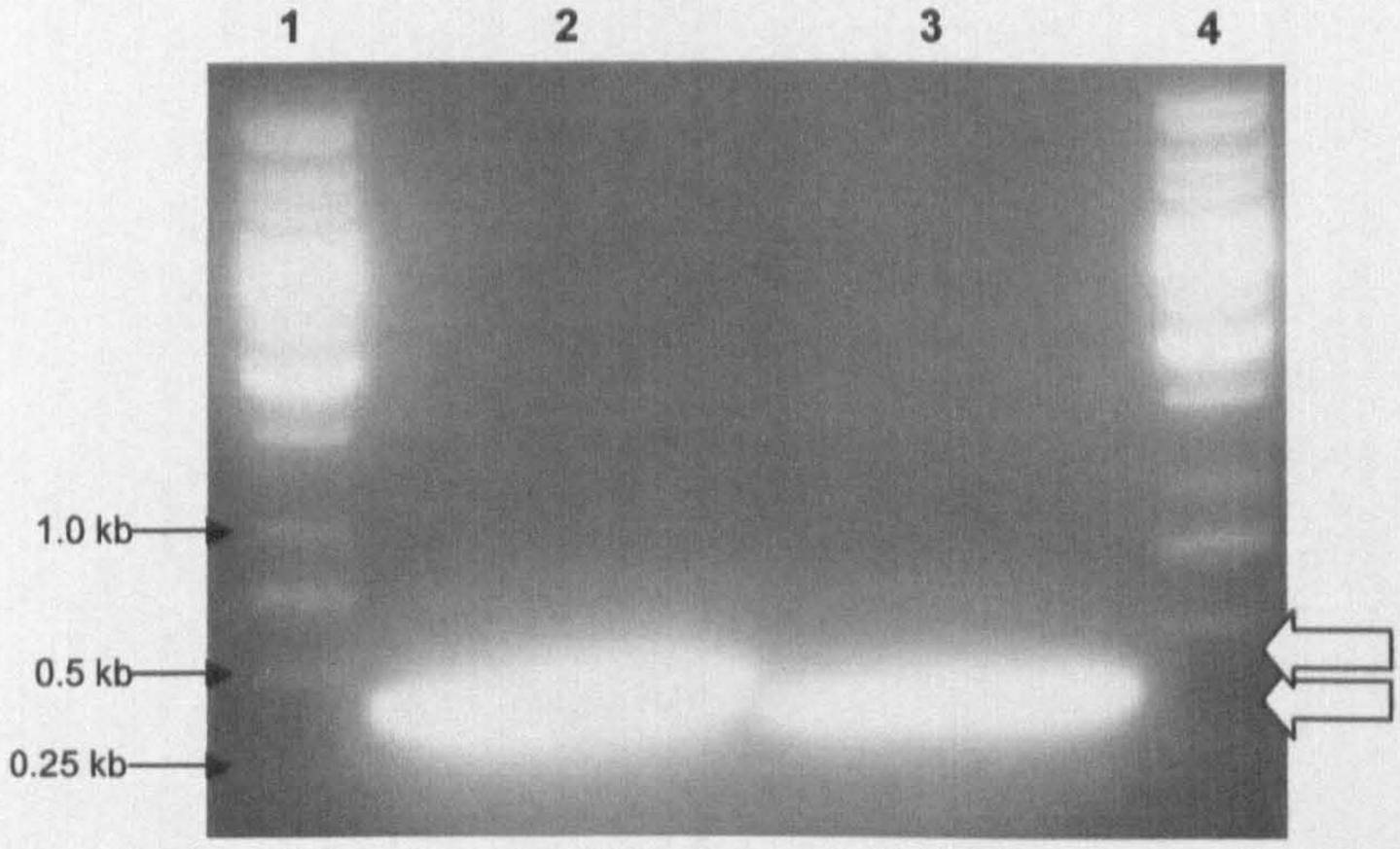


Figure 4.19: 1% (w/v) TAE agarose gel of PCR products of *scaD* N-terminus and *scaD* C-terminus overexpression inserts. Lane 1, molecular size marker; 2, amplified *scaD* N-terminal 393 bp; 3, amplified *scaD* C-terminal 327 bp; 4, molecular size marker. The correct sized products were seen for both overexpression inserts (unfilled arrows).

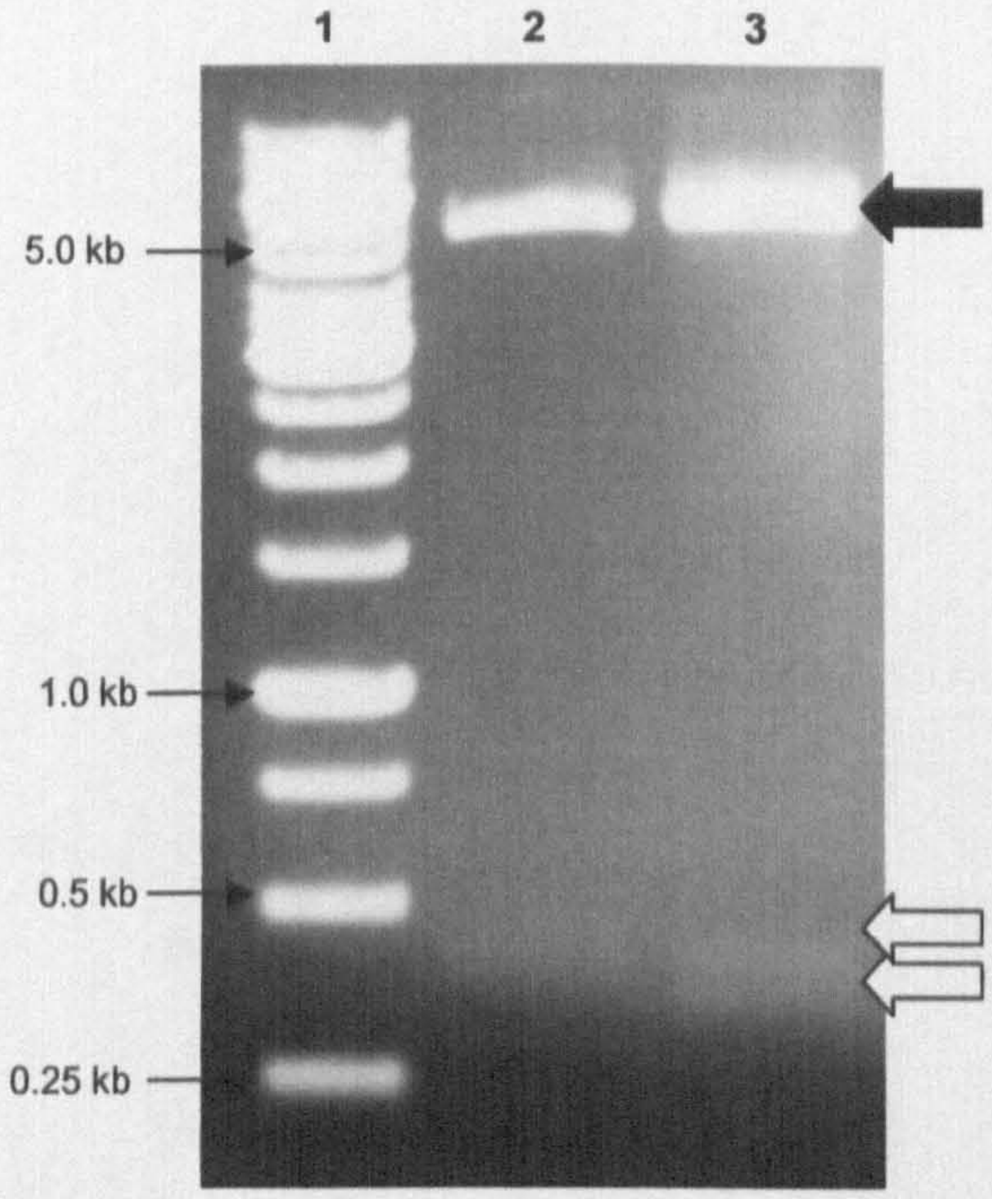


Figure 4.20: Verification of pETDn and pETDc by 1% (w/v) agarose gel analysis of restriction digests using *Nco*I and *Xho*I. Lane 1, molecular size marker; 2, digested pETDn; 3, digested pETDc. Filled arrows indicate the digested pET-24d vector (5.3 kb), unfilled arrows indicate the overexpression inserts at 393 bp (N-terminus) and 327 bp (C-terminus).

4.2.7.2. Overexpression of recombinant proteins

The N- and C-terminal fragments of ScaD were overexpressed as described in Chapter 2.10. Overexpression was induced with 1 mM IPTG and successful overexpression was verified by analysis of *E. coli* total protein before and after induction by 17.5% (w/v) SDS-PAGE. Analysis demonstrated overexpression of both polypeptides of the expected sizes (Figure 4.21). Similarly to the full-length ScaD, two recombinant polypeptides were overexpressed for the C-terminus. The identity of these fragments remains to be determined.

4.2.7.3. Solubility of recombinant proteins

Induced culture samples were disrupted by sonication and separated by centrifugation (Chapter 2.10.2). Total proteins from supernatant and pellet were then analysed by 17.5% (w/v) SDS-PAGE. Both fragments of ScaD were present in the lysate pellet rather than the supernatant, indicating that the recombinant polypeptides are insoluble (Figure 4.22).

4.2.7.4. Purification of recombinant proteins using a HiTrap affinity column

ScaD N- and C-terminus polypeptides were purified as described previously (Chapter 4.2.2.5; Chapter 2.10.3). The C-terminus fragment was successfully purified and eluted from the HiTrap™ column. Fractions of eluate were collected and analysed by 17.5% (w/v) SDS-PAGE, and fractions containing recombinant polypeptide were combined and dialysed overnight at 4°C into PBS containing 8M urea. The N-terminus fragment failed to be purified by this method. Insoluble recombinant protein was lost during filtration prior to purification, therefore, unfiltered extract was diluted ten-fold in START buffer and loaded onto the HiTrap™ column for purification. However, the column became blocked and no protein could be recovered from the purification despite repeated attempts. Analysis of ScaD N-terminus polypeptide was therefore carried out using unpurified extract.

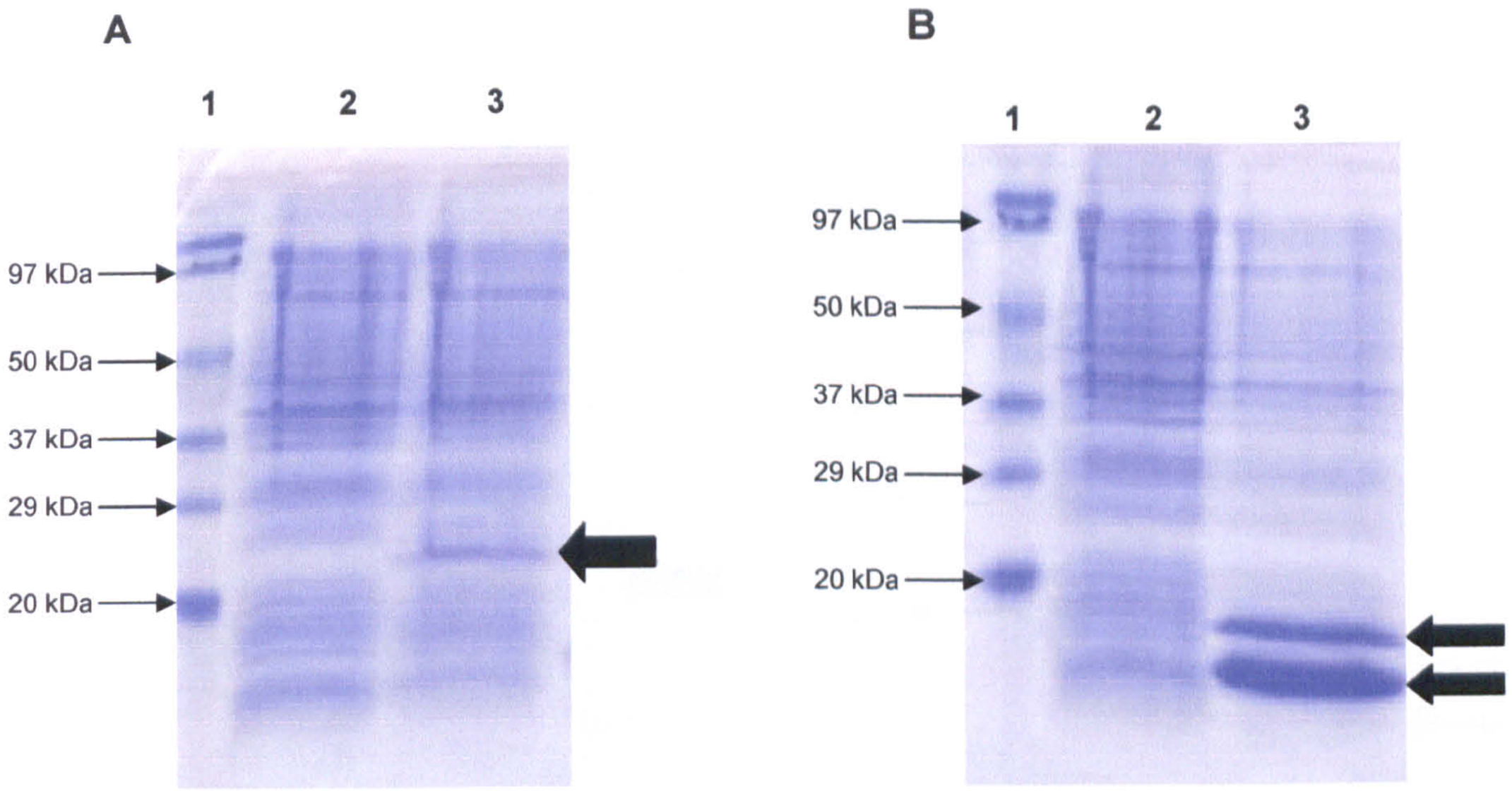


Figure 4.21: 17.5% (w/v) SDS-PAGE analysis of overexpression of ScaD N-terminus and C-terminus polypeptides. Panel A, N-terminus; Panel B, C-terminus. Lane 1, molecular mass marker; 2, uninduced *E. coli* BL21 protein fraction; 3, induced protein fraction containing the overexpressed fragment of ScaD (identified by the filled arrows). ScaD C-terminus produced two bands during overexpression.

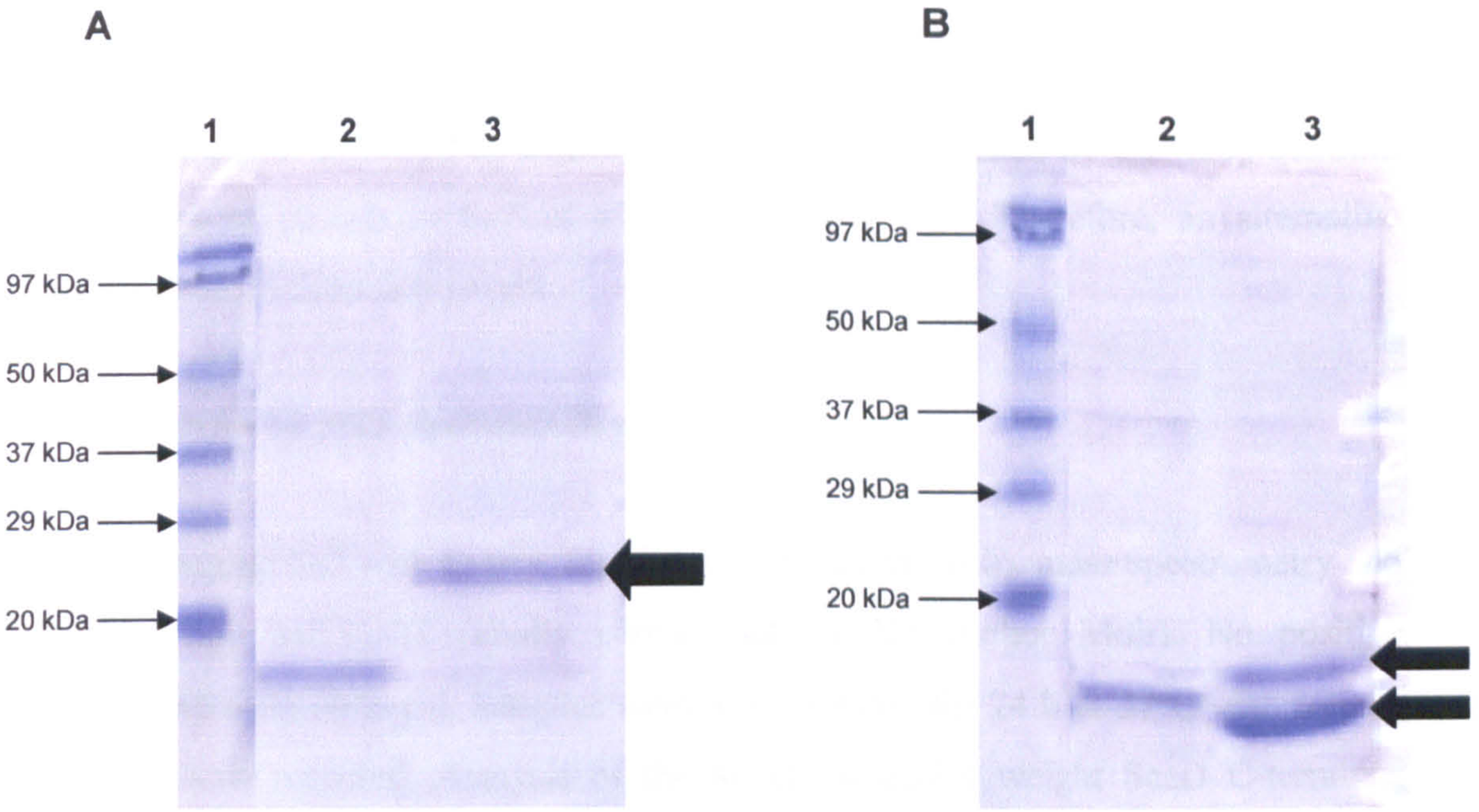


Figure 4.22: 17.5% (w/v) SDS-PAGE analysis of solubility of ScaD N-terminus and C-terminus polypeptides. Panel A, N-terminus; Panel B, C-terminus. Lane 1, molecular mass marker; 2, soluble *E. coli* BL21 protein fraction; 3, insoluble protein fraction. Both ScaD N-terminus and C-terminus were insoluble (identified by filled arrows).

4.2.7.5. N-terminal analysis of the recombinant proteins

In order to verify the identity of the recombinant polypeptides, the N-terminus was sequenced (Chapter 2.10.4). No sequence data could be obtained by this method, both from samples of protein in buffers with and without urea. Therefore, an alternative method of identification was sought.

4.2.7.6. Analysis by mass spectrometry

Proteins were digested with trypsin for 4 h at 37°C, analysed by mass spectrometry and identified using MASCOT (kindly carried out by Dr. Arthur Moir). No positive identifications were obtained. Samples were also digested for 24 h at 37°C with trypsin and the analysis repeated. Analysis of the lower molecular weight ScaD C-terminus fragment identified four digested fragments (Table 4.8). SsaA (ScaD) of *S. aureus* N315 was identified as being the most likely match for the polypeptide, although results were not significant. The region of the protein identified by this analysis is shown in Figure 4.19. As this corresponds to the expected identity of the protein, it was confirmed that the C-terminus had been successfully overexpressed. Verification of the identity of the N-terminus could not be made using this method.

4.2.7.7. Generation of soluble protein

As both the N- and C-terminus polypeptides were insoluble and due to the problems encountered during purification of the N-terminus, both protein fragments were co-overexpressed with the bacterial chaperones GroES and GroEL, as described in Chapter 4.2.6.2. pETDn and pETDc were digested with *Nco*I and *Xho*I, gel purified and ligated into pET-21d digested with the same enzymes. Ligations were transformed into competent *E. coli* Top10 cells and positive transformants identified by selecting for ampicillin resistance. Plasmid DNA was purified from cells using a Qiagen plasmid prep kit and presence of the insert was verified by PCR using primers T7 forward and T7

reverse (Table 2.6). The recombinant plasmids were named pETDna (*scaD* N-terminus) and pETDca (*scaD* C-terminus).

Recombinant pETDna and pETDca were transformed into electrocompetent *E. coli* BL21 containing pREP4groESL and positive transformants were identified by selecting for both ampicillin and kanamycin resistance. Presence of the pETDna and pETDca plasmids was verified by colony PCR using T7 forward and T7 reverse primers. The resulting strains were named GroDN (*scaD* N-terminus) and GroDC (*scaD* C-terminus).

4.2.7.7.1. Co-overexpression of recombinant ScaD with GroES and GroEL

In order to produce soluble protein, different overexpression conditions were assessed. Strains GroDN and GroDC were grown in 100 ml TB at 25°C, 30°C and 37°C, and overexpression was induced with 1 mM IPTG. Protein overexpression was verified by analysis of uninduced and induced total protein samples of strains GroDN and GroDC by 17.5% (w/v) SDS-PAGE. The solubility of the proteins from cultures of each condition was also determined by analysis of the insoluble and soluble fractions of induced cultures by 17.5% (w/v) SDS-PAGE, as previous. Results showed that the recombinant ScaD polypeptides were successfully overexpressed along with both GroES (10 kDa) and GroEL (58 kDa), however, ScaD N-terminus and C-terminus remained insoluble in each case (Figure 4.24).

Fragment of ScaD (aa)	Peptide Identified
183 – 199	K.IGSTWGNASNWANAAAR.A
200 – 209	R.AGYTVNNTPK.A
210 – 237	K.AGAIMQTTQGAYGHVAYVESVNSNGSVR.V
238 – 253	R.VSEMNYGYGPGVVTSR.T

Table 4.8: Fragments identified during mass spectrometry analysis of trypsin-digested ScaD C-terminus (lower molecular weight band). Four fragment corresponding to the C-terminus of the protein were identified.

```
1      MKKIATATIA TAGFATIAIA SGNQAHASEQ DNYGYNPNDP TSYSYTYTID
51     AQGNYHYTWK GNWHPSQLNQ DNGYYSYYYY NGYNNYNNYN NGYSYNNYSR
101    YNNYSNNNQS YNYNNYNSYN TNSYRTGGLG ASYSTSSNNV QVTTTMAPSS
151    NGRSISSGYT SGRNLYTSGQ CTYYVFDRVG GKIGSTWGNA SNWANAAARA
201    GYTVNNTPKA GAIMQTTQGA YGHVAYVESV NSNGSVRVSE MNYGYGPGVV
251    TSRTISASQA AGYNFIH
```

Figure 4.23: Sequence analysis of ScaD. The overexpressed C-terminal residues are underlined. The residues corresponding to fragments identified by mass spectrometry analysis of the lower molecular mass band of the recombinant C-terminus are shown in red, the first amino acid of each fragment is highlighted in blue.

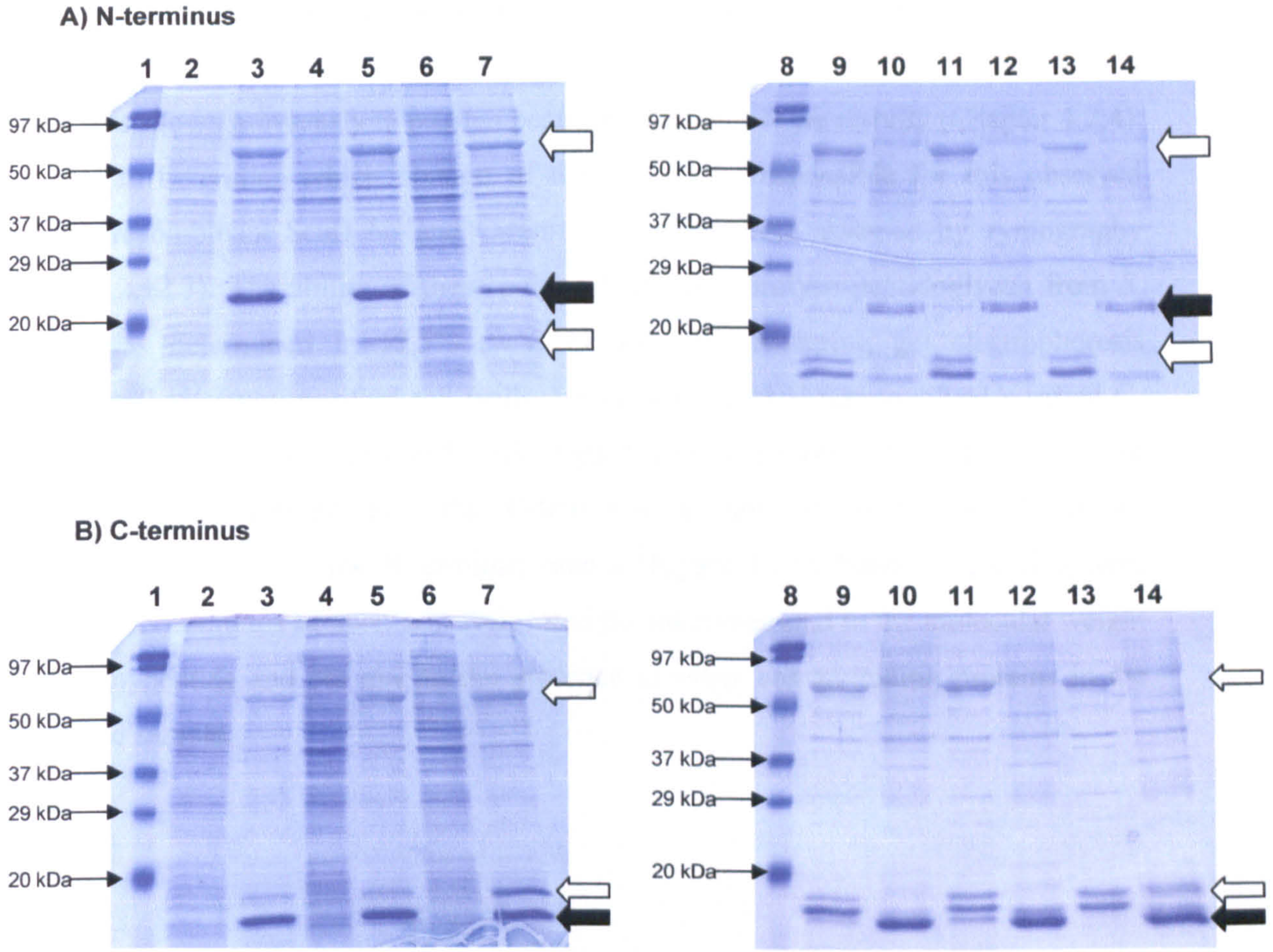


Figure 4.24: 17.5% (w/v) SDS-PAGE analysis of ScaD N-terminus (Panel A) and C-terminus (Panel B) overexpression in GroDN and GroDC at different temperatures. Lane 1, molecular mass marker; 2, uninduced protein fraction at 25°C; 3, induced protein fraction at 25°C; 4, uninduced protein fraction at 30°C; 5, induced protein fraction at 30°C; 6, uninduced protein fraction at 37°C; 7, induced protein fraction at 37°C; 8, molecular mass marker; 9, soluble protein fraction at 25°C; 10, insoluble protein fraction at 25°C; 11, soluble protein fraction at 30°C; 12, insoluble protein fraction at 30°C; 13, soluble protein fraction at 37°C; 14, insoluble protein fraction at 37°C. Overexpressed polypeptides were overexpressed at the expected sizes (filled arrows) and were both present in the insoluble protein fraction in all cases. Co-overexpressed chaperones GroEL and GroES were seen at 58 kDa and 10 kDa, respectively (unfilled arrows).

4.2.8. Peptidoglycan hydrolase activity of ScaD N-terminus and C-terminus

rScaD was shown previously to possess peptidoglycan hydrolase activity (Chapter 4.2.4). In order to determine which domain of the protein is responsible for this observed activity, recombinant ScaD N- and C-terminal regions were assessed by zymography (Chapter 2.12.3). The ability of these polypeptides to hydrolyse peptidoglycan from *S. aureus* was determined in 17.5% (w/v) SDS-PAGE renaturing gel electrophoresis containing 0.1% (w/v) purified cell walls. Unpurified ScaD N-terminus and purified C-terminus were assessed, along with full-length ScaD as a postive control. Analysis of zymograms demonstrated that the C-terminus is able to hydrolyse *S. aureus* peptidoglycan, however, the N-terminus cannot (Figure 4.25). Bands of clearing were seen in the N-terminus sample, however, these do not correspond to the molecular weight of the polypeptide and are due to the presence of other contaminating proteins in the unpurified sample.

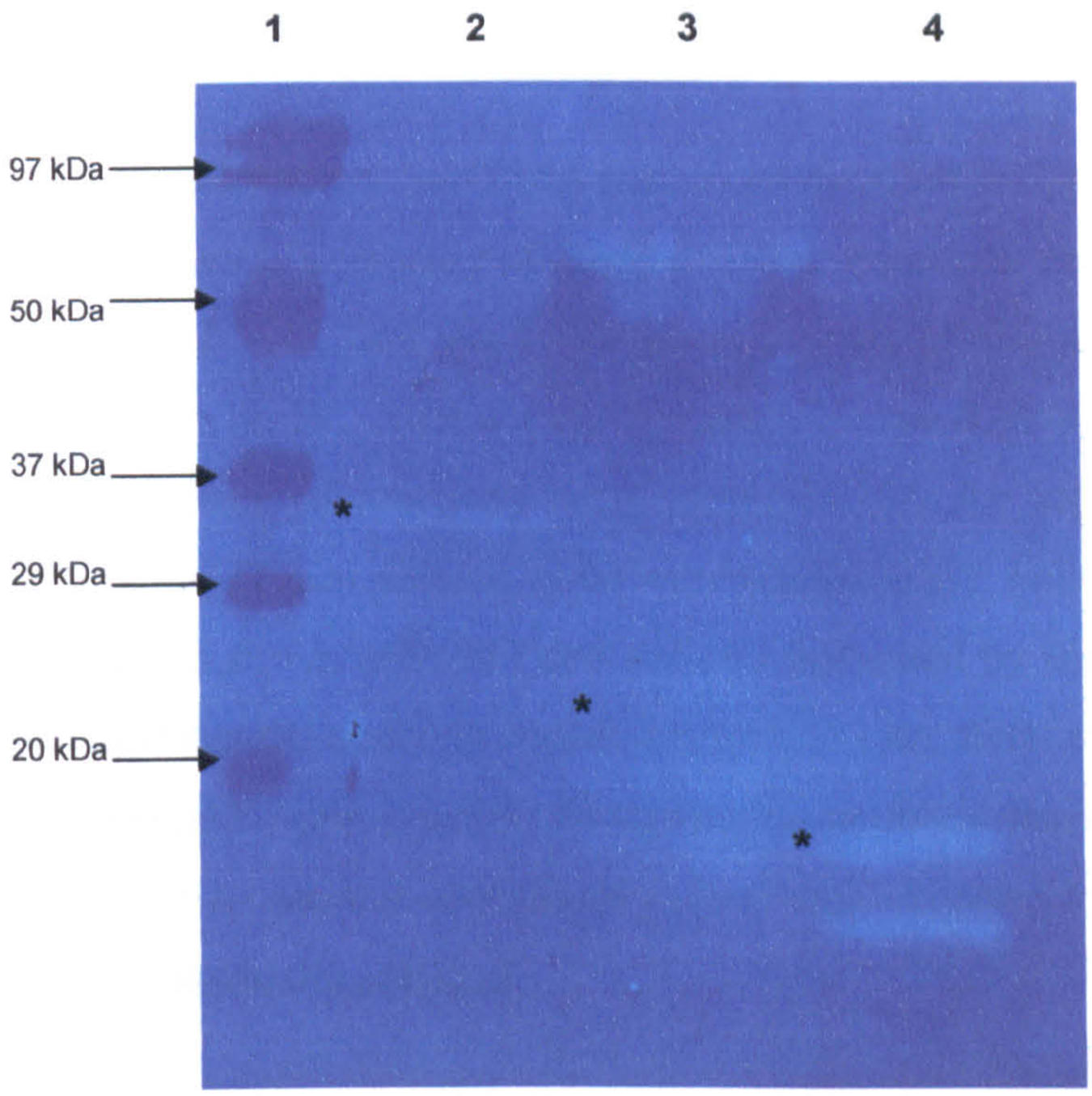


Figure 4.25: Zymogram analysis showing activity of ScaD. Full-length ScaD, the unpurified N-terminus and the purified C-terminus were analysed by 17.5% (w/v) SDS-PAGE renaturing gel electrophoresis containing 0.1% (w/v) purified *S. aureus* cell walls as substrate. Lane 1, molecular mass marker; 2, rScaD; 3, ScaD N-terminus; 4, ScaD C-terminus. Lytic bands demonstrating autolytic activity are seen as bands of clearing in the stained gel for rScaD and rScaD C-terminus. Sizes of proteins are marked with an asterisk.

4.2.9. Immunisation with ScaD

Approximately 1 mg of recombinant ScaD was used to produce antibodies in a rabbit by the Antibody Resource Centre, University of Sheffield. Five injections were given to the rabbit at two week intervals. The first bleed was taken after the third injection to assess the immune status of the rabbit. Analysis at this stage revealed poor immunogenic response to the protein (lack of reactivity seen during ELISA for antiserum diluted 1:200), hence further doses of recombinant ScaD were administered. Following the final injection of the rabbit, blood was collected and the sera harvested by centrifugation. A second analysis revealed that the antibody response remained low, therefore, the sera was affinity purified using rScaD. Approximately 80 µg specific antibody was recovered from the serum by this method, an amount considerably lower than expected levels as several milligrams of antibody are usually obtained.

4.2.9.1. Analysis of anti-rScaD

To confirm the reactivity of the anti-ScaD antibodies, they were assessed in Western blots using recombinant ScaD protein and whole cell lysate of *S. aureus* SH1000. SH1000 was grown to log phase (OD_{600nm} 0.5-1.0) in BHI and 1 OD unit of culture pellet was resuspended in sample prep buffer. The *S. aureus* lysate was separated by 17.5% (w/v) SDS-PAGE, along with rScaD and BSA (negative control). One protein gel was stained with Coomassie blue, another was blotted onto a PVDF membrane and used in a Western blot with the rScaD antibodies (Chapter 2.13). Results indicated that the antibodies reacted with rScaD, as well as with likely ScaD in the whole cell lysate. The antibodies also cross-reacted with other staphylococcal proteins, although they did not bind to BSA (Figure 4.26).

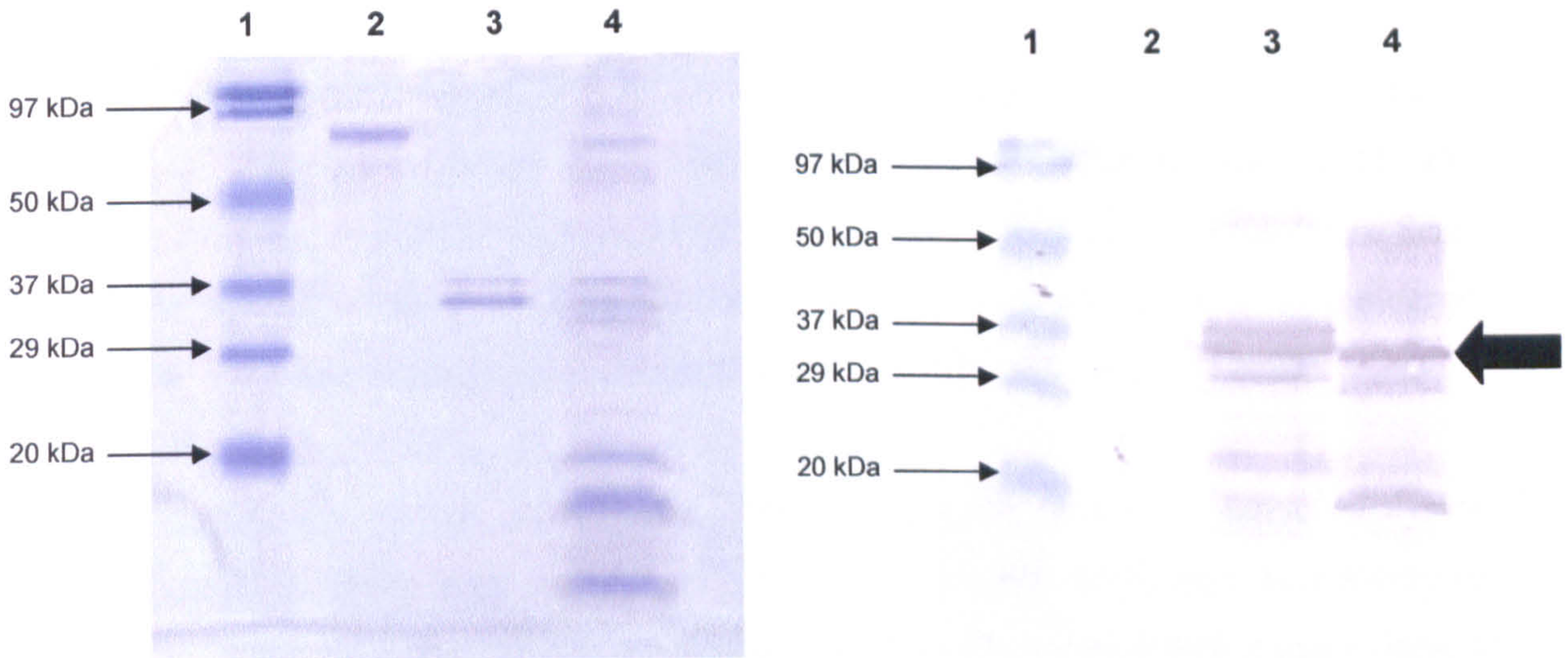


Figure 4.26: Western blot analysis of reactivity of anti-ScaD antiserum. Panel A, 17.5% SDS-PAGE analysis; Panel B, Western blot probed with anti-ScaD antiserum. Lane 1, molecular mass marker; 2, BSA; 3, rScaD; 4, SH1000 whole cell lysate. Antibodies bound to the rScaD, as well as ScaD in SH1000 (shown by the filled arrow).

4.3 Discussion

The staphylococcal conserved antigen (Sca) family was originally identified by immunoscreening of a *S. epidermidis* genomic library with patient sera. Subsequent analysis revealed direct homologues of these proteins in *S. aureus*. Bioinformatic analysis of the ten Sca proteins in *S. aureus* identified a highly homologous conserved CHAP domain with putative amidase function. This CHAP domain was also found to be conserved throughout several other Gram positive species. One protein, ScaH, also contains a glucosaminidase domain, indicating a role as a bi-functional autolysin. ScaH was also identified to be part of another, four-protein, family of glucosaminidases, including Atl, a novel protein (SACOL1825) and SagA (Syed Mohamad, 2007).

As the Sca proteins contain a C-terminal CHAP domain, their roles as putative autolysins were assessed. Zymography analysis of recombinant proteins confirmed their ability to hydrolyse peptidoglycan. Notably, overexpression of ScaB produced two polypeptides: a full-length protein and an N-terminally truncated fragment. The truncated polypeptide also demonstrated hydrolytic activity, indicating that the C-terminus containing the CHAP domain is the predicted active domain of this protein. Analysis of the overexpressed C-terminus and N-terminus fragments of ScaD further supported this, as only the C-terminus was shown to hydrolyse peptidoglycan. The CHAP domain is predicted to confer amidase function (Bateman and Rawlings, 2003; Rigden *et al.* 2003), therefore, it can be proposed that the Sca proteins are a family of amidases. Further analysis of these proteins to confirm the nature of the hydrolytic activity and the specific bonds cleaved by these enzymes is required, however, for such biochemical analysis it would be necessary to solubilise the recombinant proteins, which could not be achieved in this study.

Autolysins are fundamental enzymes possessed by all bacteria and in addition to their key roles in cellular physiology, are considered to be virulence factors, e.g. LytA, the major autolysin of *S. pneumoniae* (Berry and Paton, 2000), IsaA and SceD, two lytic

transglycosylases of *S. aureus* (Stapleton *et al.* 2007) and IspC of *L. monocytogenes* (Wang and Lin, 2006).

Bacteria possess several peptidoglycan hydrolases of each class, as demonstrated by the ten-protein Sca family of putative amidases. The presence of several enzymes which are able to carry out the same activity implies functional redundancy, the reason for which is not fully understood. Several studies in which single genes encoding autolysins have been mutated have resulted in only minor changes to the phenotype of the bacteria (Biswas *et al.* 2006; Huard *et al.* 2003) and this can be explained by the presence of multiple enzymes which can compensate for those lost. For example, two autolysin genes, *cwlC* and *lytC*, were demonstrated to have mutually compensatory roles in mother cell lysis during sporulation of *B. subtilis* (Smith and Foster, 1995). Two orders of functional redundancy exist: a process may be performed by several enzymes, and individual enzymes may be involved in numerous processes (Smith *et al.* 2000). For this reason, no single peptidoglycan hydrolase has been identified as being essential for viability. The CHAP domain protein PcsB was originally identified as essential (Ng *et al.* 2004), however, recently a gene deletion mutant has been created in *S. pneumoniae*, indicating that whilst this enzyme is crucial for correct cell separation and septum formation, and deletion drastically reduces growth, it is not in fact essential for viability (Giefing *et al.* 2008). In *B. subtilis*, endopeptidase-type autolysin activity, carried out by one or other of LytE and YvcE (CwlO), was found to be essential. Whilst neither gene is independently essential, no strains could be obtained in which both autolysins were inactivated, indicating the mutually compensatory, yet essential, nature of this class of autolysins (Bisicchia *et al.* 2007).

The occurrence of peptidoglycan hydrolases such as PcsB, which have an important role in bacterial physiology, highlights the importance of the bacterial cell wall in maintaining viability and cell division. This may also give a reason for the redundancy of autolysins found in most species. The role of these enzymes in maintaining proper structure and functioning of the cell wall means that cells may have back-up autolysins in the case of a non-functional enzyme of choice. Furthermore, alteration of cell wall structure by

peptidoglycan hydrolases may be vital for adaptation to different environmental niches (Stapleton *et al.* 2007). Similarly, modification of peptidoglycan may be necessary under conditions of stress and different enzymes may be employed by the bacteria under different conditions. It could therefore be proposed that the Sca family of putative autolysins may be expressed under different environmental conditions, explaining the existence of several highly related enzymes with similar activities. Autolysins such as these must be well-regulated in order to ensure that enzymes with the correct bond specificities target the appropriate peptidoglycan at specific times, which is particularly important due to their potential to compromise cell wall integrity (Koch, 2006; Antignac *et al.* 2007).

The role of ScaA (Sle1) as a peptidoglycan hydrolase was assessed by Kajimura *et al.* (2005). Mutants lacking this enzyme were shown to form clusters and exhibited irregular morphology with unseparated daughter cells, consistent with a role in splitting of the septum during cell division. However, this was not in agreement with a similar study, in which a ScaA (Aaa) mutant displayed growth characteristics comparable to that of wild-type *S. aureus*, although this may have been attributable to increased expression of the major autolysin Atl in the mutant (Heilmann *et al.* 2005).

Whilst the role of ScaA remains unclear, as an amidase it is likely involved in peptidoglycan structural dynamics. The peptidoglycan cell wall is essential to the survival and functioning of bacteria, enabling cells to resist osmotic and turgor pressures, and to grow and divide. Peptidoglycan must be continuously synthesised to maintain the integrity and viability of the cell whilst facilitating growth (Biswas *et al.* 2006; Koch, 2006). Autolysins are fundamentally involved in this process of cell wall turnover, cleaving the cross-linked covalent network to allow incorporation of newly synthesised material (Smith *et al.* 2000). For example, a *B. subtilis* mutant lacking the amidase LytC showed a marked reduction in the rate of cell wall turnover, and this was exacerbated in a mutant lacking both LytC and the glucosaminidase LytD, indicating that these autolysins were involved in the turnover of the peptidoglycan (Blackman *et al.* 1998). In *S. aureus*, partial inhibition of cell wall synthesis also resulted in repression of autolysin activity,

suggesting that cell wall synthetic and hydrolytic enzymes are closely regulated (Antignac *et al.* 2007). Cell wall turnover by peptidoglycan hydrolases is also important to allow the secretion of large proteins through the bacterial cell wall (Smith *et al.* 2000).

The *S. aureus* cell wall is subject to specific modifications in order to maintain its unique structure and such peptidoglycan maturation can involve peptidoglycan hydrolase activity. For example, analysis of *S. aureus* cell wall glycan strands by reverse-phase HPLC resulted in the presence of minor satellite peaks, identified to be the products of *N*-acetylglucosaminidase activity involved with modification of the glycan strand structure (Boneca *et al.* 2000). Furthermore, structural analysis of the peptidoglycan of the closely-related *B. subtilis* identified modifications resulting from the activities of glucosaminidases, endopeptidases and amidases (Atrih *et al.* 1999), and similar analysis of *Lactococcus lactis* cell wall muropeptides identified a novel carboxypeptidase enzyme involved in peptidoglycan maturation (Courtin *et al.* 2006). Cell walls from Gram-positive bacteria contain various secondary polymers, such as teichoic acids and anchored proteins, which are frequently involved in adhesion and invasion. Therefore, remodelling of these surface molecules may be necessary under different environmental conditions and enzymes including peptidoglycan hydrolases may be involved in this process (Boneca, 2005). Other cell wall modifications including O-acetylation of peptidoglycan have been proposed to serve as a level of control of autolysin activity. Indeed, *E. faecalis* produces autolysins with different specificities for O-acetylated peptidoglycan (Pfeffer *et al.* 2006). *N*-deacetylation of peptidoglycan in *Listeria monocytogenes* confers resistance to host lysozyme, preventing bacterial degradation and subsequent release of immunostimulants, hence providing a means of evading immune detection (Boneca *et al.* 2007).

Autolysins are also involved in cell separation and their effects are fairly well characterised. Mutants lacking various peptidoglycan hydrolases result in cells of irregular shapes and sizes growing in long chains with abnormal septa (Priyadarshini *et al.* 2007; Carbillido-López *et al.* 2006), indicating that these enzymes are vital for the correct separation of cells during cell division.

The Sca proteins contain signal peptides, denoting a secreted / surface location. They do not contain LPXTG motifs, indicating that they are ionically rather than covalently bound to the cell surface. Along with the covalently bound MSCRAMMs, these SERAMs (secretable expanded repertoire adhesive molecules) play key roles in pathogenesis, involved in the interaction between pathogen and host cells during colonisation and invasion (Chavakis *et al.* 2005). In particular, the ability to adhere to human extracellular matrix components and serum proteins promotes initial attachment and subsequent dissemination during infection (Clarke and Foster, 2006).

Several ligand-binding SERAMs have been identified which contribute to the pathogenicity of *S. aureus*. For example, Eap (extracellular adherence protein), a 70 kDa fibrinogen-binding protein, inhibits the recruitment of neutophils during bacterial peritonitis in mice, acting as an anti-inflammatory agent (Chavakis *et al.* 2002). Eap also enhances interactions between major histocompatibility complex molecules and human leukocytes, indicating a role in septic shock and fever (Scriba *et al.* 2008). Efb (extracellular fibrinogen-binding) protein shares significant homology with coagulase, another protein that binds fibrinogen (Rivera *et al.* 2007). Efb has been shown to inhibit the host immune response by blocking convertases during the complement cascade (Jongerius *et al.* 2007) and has demonstrated potential as a vaccine (Shannon *et al.* 2006). Coagulase is expressed at varying levels in almost all strains of *S. aureus* and is positively associated with infective endocarditis as approximately 40% of neonatal and adult endocarditis is caused by coagulase-positive strains (Rivera *et al.* 2007).

ScaA (Aaa) has been shown previously to be an adhesin, able to bind fibrinogen, fibronectin and vitronectin (Heilmann *et al.* 2005). Studies in this laboratory have also demonstrated the adhesive properties of ScaB (able to bind fibronectin and lactoferrin) and ScaH (able to bind seven human plasma proteins) (Pourmand, 2005; Syed Mohamad, 2007). The ligand binding ability of all the Sca proteins was determined during this study and results showed that all nine proteins tested in ligand affinity blots were able to bind a wide range of human ligands. In total, *S. aureus* contains many adhesins, several of

which can bind multiple ligands. The overlapping nature of the host components to which these proteins bind may imply an ability of bacteria to attach to host organisms in a range of environmental conditions (Clarke and Foster, 2006).

The Sca proteins are likely dual-function proteins, acting as both autolysins and adhesins. Homologues of Atl, the major autolysin of *S. aureus*, have also been demonstrated to possess both peptidoglycan hydrolase activity and adhesive properties. For example, AtlE of *S. epidermidis* is involved in the primary attachment to polystyrene and binds vitronectin (Heilmann *et al.* 1997), and Aas of *S. saprophyticus* and AtlC of *S. caprae* are both able to bind fibronectin (Hell *et al.* 1998; Allignet *et al.* 2001). All four of these proteins display the same overall organisation, containing both *N*-acetylmuramoyl-L-alanine amidase and endo-β-*N*-acetylglucosaminidase enzymatic domains.

Although these enzymes have been proven to have dual activities, the real contribution of this ligand-binding ability to bacterial virulence has not been established. Whilst proteins such as Aaa (ScaA) demonstrated high affinity for ligands such as fibrinogen (K_d = 12.3 nM) and fibronectin (K_d = 29.9 nM) (Heilmann *et al.* 2005), they may only be expressed in relatively small amounts in *S. aureus*. Therefore, it could be proposed that the ability to bind these ligands has little impact on virulence, or even that it is simply fortuitous. Indeed, the ligands tested in this study were chosen according to commercial availability but may not represent those which are of most importance *in vivo*. The fibrinogen-binding ability of ClfA, an important virulence factor in infective endocarditis and staphylococcal arthritis (Que *et al.* 2005; Josefsson *et al.* 2001), demonstrated to represent a successful vaccine candidate (Weems *et al.* 2006), has been proposed to be of minor importance for the development of septic arthritis (Palmqvist *et al.* 2004). Depletion of plasma fibrinogen in mice did not reduce the arthritogenicity of ClfA-expressing *S. aureus*, indicating that the adhesin activity of this protein may not be the major contributing factor to bacterial virulence.

The key roles of peptidoglycan hydrolases in the maintenance of cell wall structure and viability makes them interesting targets for prophylaxis. In particular, those enzymes

expressed during infection and recognised by the human immune system could represent putative vaccine candidates. Several Gram-positive autolysins have been identified which increase pathogenicity by interacting with the host immune response. For example, the autolysin p60 in *Listeria monocytogenes* indirectly suppresses the innate host immune response by enhancing the activation of natural killer (NK) cells which produce deleterious cytokines, allowing increased virulence during initial infection (Humann *et al.* 2007). Furthermore, the surface-associated autolysin Auto is required for entry of this organism into eukaryotic cells and for virulence in infection models of both mice and guinea pigs (Cabanes *et al.* 2004).

Several autolysins have been identified during screens for *in vivo*-expressed antigens in *S. aureus*. For example, Weichhart *et al.* (2003) identified the bi-functional autolysin Atl using *in vitro* protein selection by ribosome display, Clarke *et al.* (2006) identified Atl, the lytic transglycosylase IsaA and the bi-functional autolysin ScaH by immunoscreening using human sera, and Etz *et al.* (2002) identified IsaA, ScaC, ScaD, ScaB and ScaA, also by immunoscreening with sera. The nine recombinant Sca proteins were tested for their ability to react with patient sera in Western blots and results indicated that they may be expressed during infection, in agreement with these previous studies. However, both ScaD and ScaF failed to bind antibodies from any of the three serum samples tested.

In addition to the inability to bind human antibodies during Western blot analysis, ScaD displayed a relative lack of reactivity during rabbit immunisation. The recombinant protein failed to generate the expected immune response and only a low level of polyclonal antibodies was obtained from the immunised animal. Despite the lack of immmunogenicity seen here, ScaD homolgues have been identified as putative antigens previously. In *S. epidermidis*, screening of a genomic DNA library with *S. epidermidis*-infected patient serum identified a novel 257-residue antigen, named SsaA (Lang *et al.* 2000). SsaA is in fact ScaC/ScaD, which are identical at the amino acid level in *S. epidermidis*. SsaA is 74.2% identical to ScaC (255 amino acids) and 74.5% identical ScaD (267 amino acids) in *S. aureus*. Analysis of antibody titres in human sera demonstrated elevated levels of anti-SsaA IgG antibodies in *S. epidermidis*-infected sera,

with a particular association with infective endocarditis. This suggests that both ScaC and ScaD may have a role in pathogenesis in *S. epidermidis*, and due to the high homology of these proteins with their counterparts in *S. aureus*, it would be expected that ScaC and ScaD would be antigenic in this organism as well.

Furthermore, screening of *S. pneumoniae* for vaccine antigens using human antibodies identified two homologues of ScaD, CbpD (SP_2201) and PcsB (SP_2216) (Giefing *et al.* 2008), which share homology to ScaD in their CHAP domains (Table 4.2; Figure 4.5). In particular, PcsB was found to be the most immunogenic protein during this screen, hence the lack of reactivity seen for its homologue ScaD is surprising. Isp, a homologue of ScaD in *S. pyogenes*, has also been identified to be immunogenic (McIver *et al.* 1996). Further analysis using more human sera samples is required to fully assess the antigenic properties of ScaD in *S. aureus*.

Since several Sca proteins were shown to react with human serum antibodies and are conserved amongst Gram-positive pathogens, this family of autolysin/adhesins may provide interesting targets for immunotherapy. Further work to elucidate their association with virulence and roles in infection is required to determine their use as vaccine targets.

Chapter 4 – Summary

- Ten Sca proteins in *S. aureus* have been demonstrated to be peptidoglycan hydrolases.
- The C-terminus of the Sca proteins, containing the CHAP motif, is proposed to be the active domain of these proteins.
- The Sca proteins are able to bind a range of human ligands, hence may be involved in invasion and colonisation.
- Several Sca proteins react with human serum antibodies, therefore, are associated with staphylococcal infection.

Chapter 5

Analysis of the role of *scaD* in *Staphylococcus aureus*

5.1 Introduction

In Chapter 4, the Sca family of autolysins was characterised. One member, ScaD, was identified during a transposon mutagenesis screen as being putatively essential, as no mutants were isolated with transposon insertions in the gene encoding this enzyme (Foster, unpublished). Thus, ScaD may constitute an essential enzyme. Such a role would have important implications for vaccine development and cellular physiology, therefore, further work to analyse the role of *scaD* was undertaken.

Expression of *scaD* is controlled by a two-component system in *S. aureus*, YycG/YycF (Dubrac and Msadek, 2004). This regulatory system is specific to low-G+C Gram-positive bacteria and is highly conserved. It is reported to be essential for cell growth in *B. subtilis*, *S. aureus*, *S. pneumoniae*, *S. mutans*, *E. faecalis* and *L. monocytogenes* (Fabret and Hoch, 1998; Martin *et al.* 1999; Throup *et al.* 2000; Senadheera *et al.* 2005; Hancock and Perego, 2004; Kallipolitis and Ingmer, 2001). YycF is a response regulator which is highly conserved and essential in all species studied so far, with the exception of *L. lactis*. YycG is a histidine kinase and is less well conserved between species, suggesting variability in the signals detected in different bacteria (Bisicchia *et al.* 2007).

Two genomic organisations of the *yyc* operon have been observed: the *yycFGHIJ*-type (Class I) operon in *Bacillus*, *Staphylococcus* and *Listeria* species, and the *yycFGJ*-type (Class II) operon found amongst *Streptococcus* species (Szurmant *et al.* 2007; Bisicchia *et al.* 2007). In *B. subtilis*, YycH has been demonstrated to down-regulate YycF-dependent gene expression, most likely affecting phosphorylation levels of YycF by inhibiting YycG (Szurmant *et al.* 2005). It has been suggested that YycI and YycH interact to control the

activity of YycG, forming a ternary complex with the essential histidine kinase (Szurmant *et al.* 2007).

YycF recognises the promoter regions of several genes in *S. aureus*, including several putative autolysins. Indeed, Dubrac *et al.* (2007) reported that this two-component system activated the transcription of nine genes involved in cell wall degradation, including *lytM*, *atlA*, *isaA*, *sceD*, *ssaA* and four *ssaA*-related genes. Depletion of YycFG led to a significant decrease in peptidoglycan biosynthesis and turnover, and an increase in cell wall modifications such as peptidoglycan cross-linking and glycan chain length. YycG and YycF were therefore renamed as WalK (histidine kinase) and WalR (response regulator) to reflect their functions as regulators for cell wall metabolism.

In *S. pneumoniae*, the essentiality of YycG/YycF was originally attributed to its positive regulatory effect on *pcsB* expression, which encodes a peptidoglycan hydrolase (Ng *et al.* 2004). Severe depletion of PcsB lead to abnormal and uncontrolled cell wall synthesis, resulting in a rapid cessation of growth, a phenotype similar to that observed during depletion of YycFG. Constitutive expression of *pcsB* in strains depleted for YycFG reversed these cell morphology defects, as well as allowing inactivation of the essential *yycF* (Ng *et al.* 2003). However, more recently the gene encoding this autolysin, *pcsB*, was inactivated, which caused an extreme disruption of growth (Giefing *et al.* 2008). This demonstrated that whilst PcsB is crucial for correct cell wall synthesis and cell division, it is not in fact essential for viability.

In this chapter, the essentiality of *scaD* in *S. aureus* is investigated and the role of the peptidoglycan hydrolase further elucidated, including the effects of this protein on strains depleted for YycG/YycF.

5.2 Results

5.2.1. Analysis of the essentiality of *scaD*

To establish the essentiality of *scaD* in *S. aureus*, it was attempted to place the gene under the control of the IPTG-inducible promoter P*spac*.

5.2.1.1. Use of the Lambda Red system

The first attempt to place *scaD* under the control of the P*spac* promoter was done using the Lambda Red system (Yu *et al.* 2000). The cloning vector used was pOB (Figure 5.1) (Horsburgh *et al.* 2002a), which has an ampicillin (*amp*) and erythromycin (*ery*) resistance gene for selection in *E. coli* and *S. aureus*, respectively.

A fragment of approximately 4.4 kb containing the *scaD* gene and its spanning region was amplified by PCR using primers D1F and D1R (Table 2.6) and SH1000 genomic DNA as a template. Primers were designed to incorporate *Bam*HI restriction sites at the 5' and 3' ends of the DNA fragment to facilitate cloning. The amplified gene region was named D1. D1 and the vector pOB were each digested with *Bam*HI, the vector was treated with alkaline phosphatase to prevent religation, and products were resolved in a 1% (w/v) agarose gel before being gel purified. The insert was then ligated into the digested plasmid, ligation reactions purified with glycogen and used to transform electrocompetent *E. coli* Top10 cells. Transformants were selected on LB agar containing ampicillin (100 μg/ml). Despite 15 independent attempts, the fragment D1 could not be cloned into pOB in this way.

Due to the inability to clone insert D1 into pOB, primers were redesigned to amplify a smaller gene region in the hope that this would facilitate the cloning of the insert. Primers were designed to amplify *scaD* and the upstream and downstream regions to generate a product of 2.5 kb, and *Bam*HI restriction sites were incorporated into the primers to enable cloning. Primers D2F and D2R (Table 2.6) were used to amplify the fragment from SH1000 genomic DNA and the resulting fragment was named D2. As previously, D2 and

pOB were each digested with *Bam*HI, the vector was treated with alkaline phosphatase to prevent religation and products were resolved in a 1% (w/v) agarose gel before being gel purified. The insert was ligated into the digested plasmid, ligation reactions purified with glycogen and used to transform electrocompetent *E. coli* Top10 cells. Transformants were selected on LB agar containing ampicillin (100 μg/ml). However, after 10 independent attempts, the fragment D2 could not be cloned into pOB.

Due to the problems encountered with the ligation of fragments D1 and D2 into pOB, an alternative method was sought. Insert D2 was cloned into the vector pCR®2.1-TOPO® (Figure 5.2) using a TOPO TA Cloning® kit (Invitrogen). TOPO TA Cloning® allows the direct insertion of *Taq* polymerase-amplified PCR products into a plasmid vector via an overhanging 3' deoxythymidine (T) in the linearised vector, which allows the insertion of PCR products with a single deoxyadenosine (A) at the 3' end, added by the *Taq* polymerase (Invitrogen User Manual, Version U, 10th April 2006). PCR products are inserted into a *lacZα* gene, allowing selection of insert-containing clones by blue/white selection on X-gal. The cloning reaction was carried out according to the manufacturer's instructions and six white colonies were selected for analysis. Plasmid DNA was prepared and restriction digests using *Bam*HI carried out. Products were analysed by 1% (w/v) TAE agarose gel electrophoresis and results demonstrated the correct insertion of the 2.5 kb product in each case (Figure 5.3). The plasmid was named pTOPD2.

For Lambda Red cloning, plasmid pTOPD2 was transformed into electrocompetent *E. coli* EL250 cells. Transformants were selected on LB agar containing ampicillin (100 μg/ml) and clones were verified by digesting with *Bam*HI, as previously described. The resulting clone was named ELD2.

In order to insert the P*spac* promoter and *tet* resistance gene into pTOPD2 by Lambda Red recombination, primers PS_RedF and PS_RedR (Table 2.6) were designed to amplify the 2.2 kb P*spac* cassette from plasmid pGL400 (Figure 5.4) (García-Lara, unpublished). Primers were designed in such a way that they contained homologous sequences from both the P*spac* cassette and also from the cloned *scaD* gene fragment in pTOPD2. This allows

the insertion of the P*spac* cassette by recombination of the homologous sequences at each end with the gene region in the plasmid. The primers were designed such that the promoter cassette would be inserted upstream of the ribosome binding site of *scaD* (Figure 5.4).

The Lambda Red recombination system involves a two-step process: firstly the preparation of *E. coli* ELD2 cells and secondly, the recombination of the amplified P*spac* cassette into the plasmid. Strain ELD2 was grown to OD_{600nm} 0.4-0.5, before cells were shifted to 42°C for 15 min to induce the expression of the Lambda Red genes. Electrocompetent cells were then prepared and were transformed with the amplified P*spac* cassette. After 10 independent attempts, it was concluded that the P*spac* cassette could not be inserted into pTOPD2 using Lambda Red recombination technology.

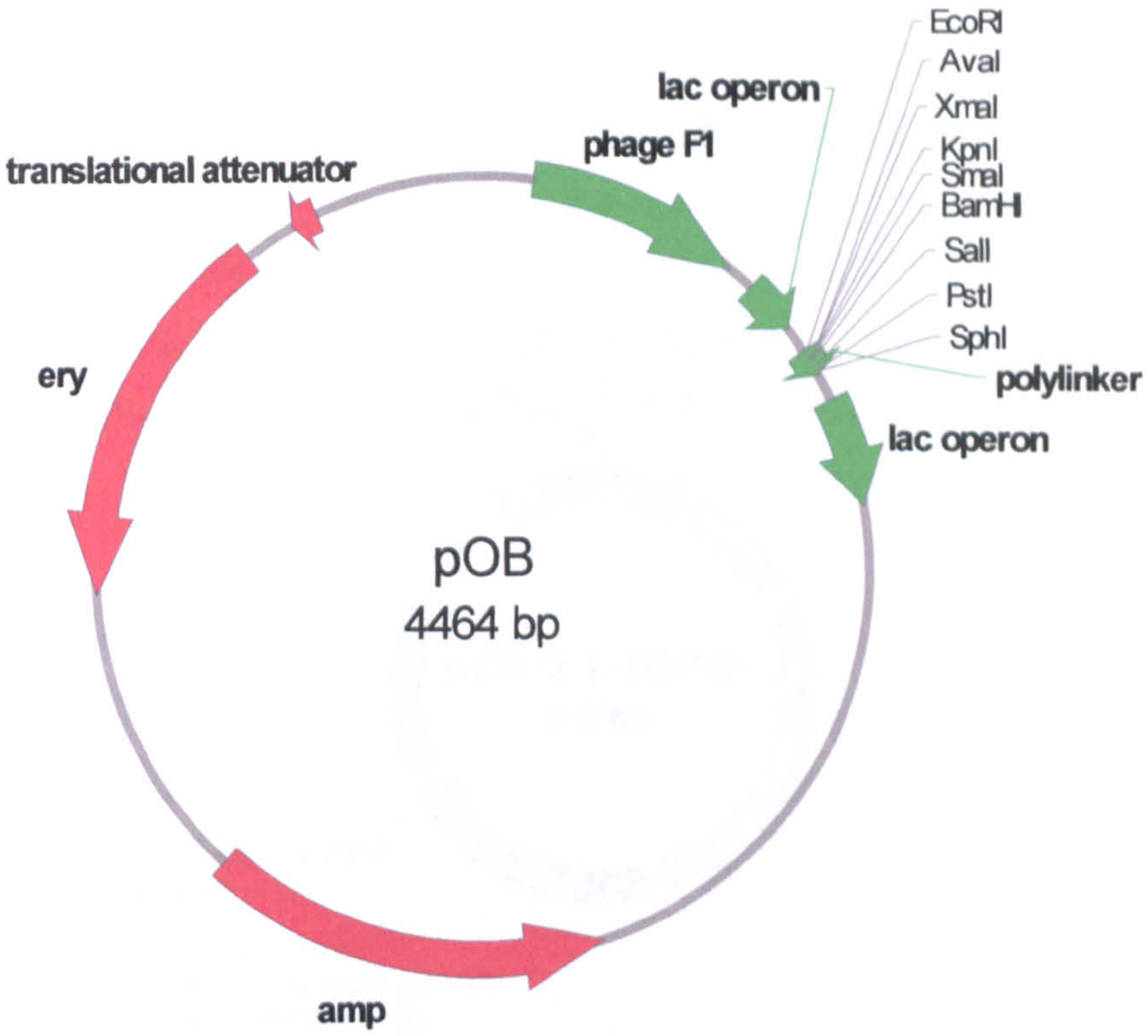
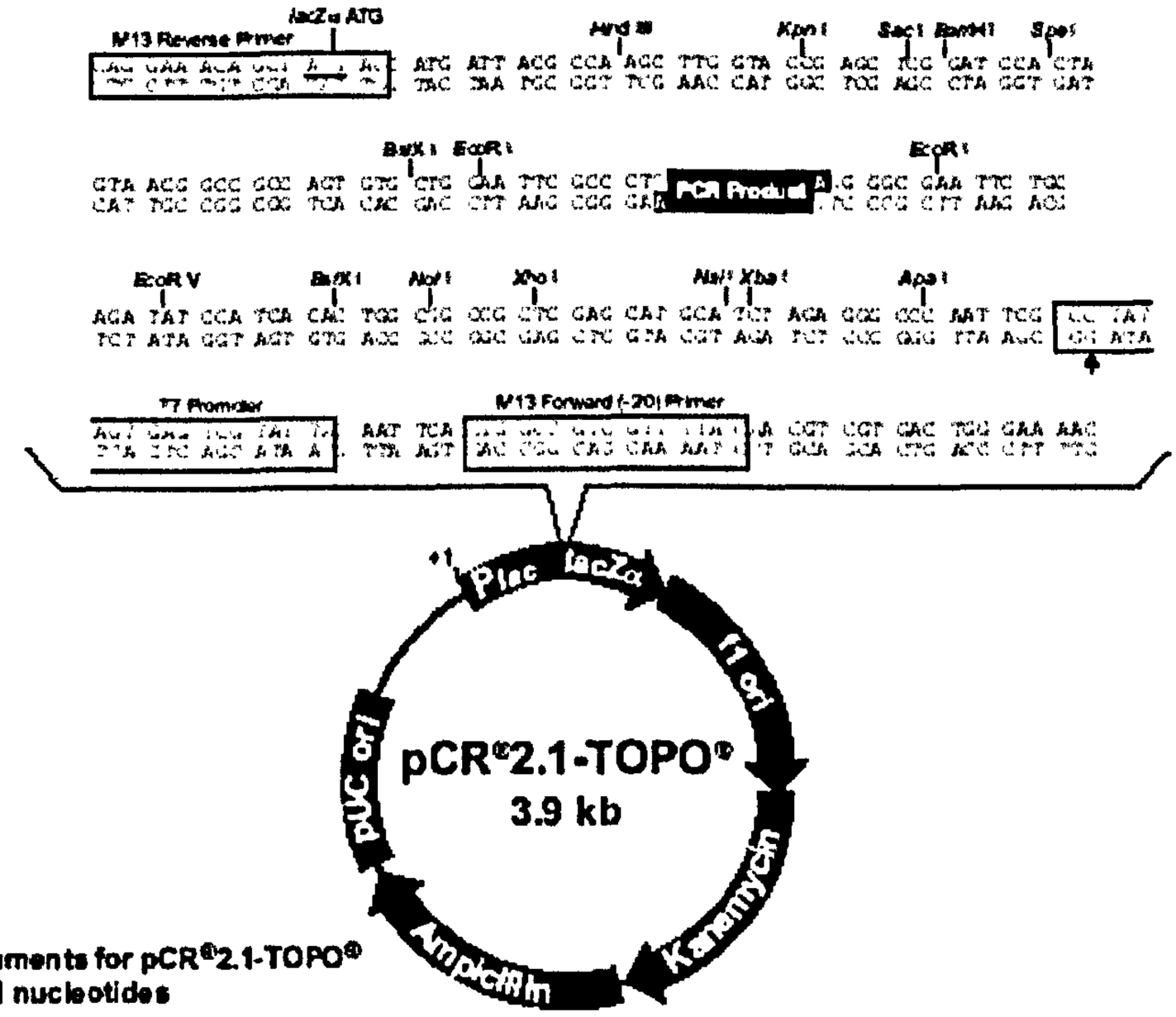


Figure 5.1: Physical map of pOB. The plasmid contains an ampicillin resistance gene (*amp*) for selection in *E. coli* and an erythromycin resistance marker (*ery*) for selection in *S. aureus* (Horsburgh *et al.* 2002a).



Comments for pCR®2.1-TOPO®
3931 nucleotides

LacZα fragment: bases 1-547
M13 reverse priming site: bases 205-221
Multiple cloning site: bases 234-357
T7 promoter/priming site: bases 364-383
M13 Forward (-20) priming site: bases 391-406
f1 origin: bases 548-985
Kanamycin resistance ORF: bases 1319-2113
Ampicillin resistance ORF: bases 2131-2991
pUC origin: bases 3136-3809

Figure 5.2: Map of pCR®2.1-TOPO® and sequence surrounding the TOPO® Cloning site (Invitrogen TOPO TA Cloning® User Manual, Version U, 10th April 2006).

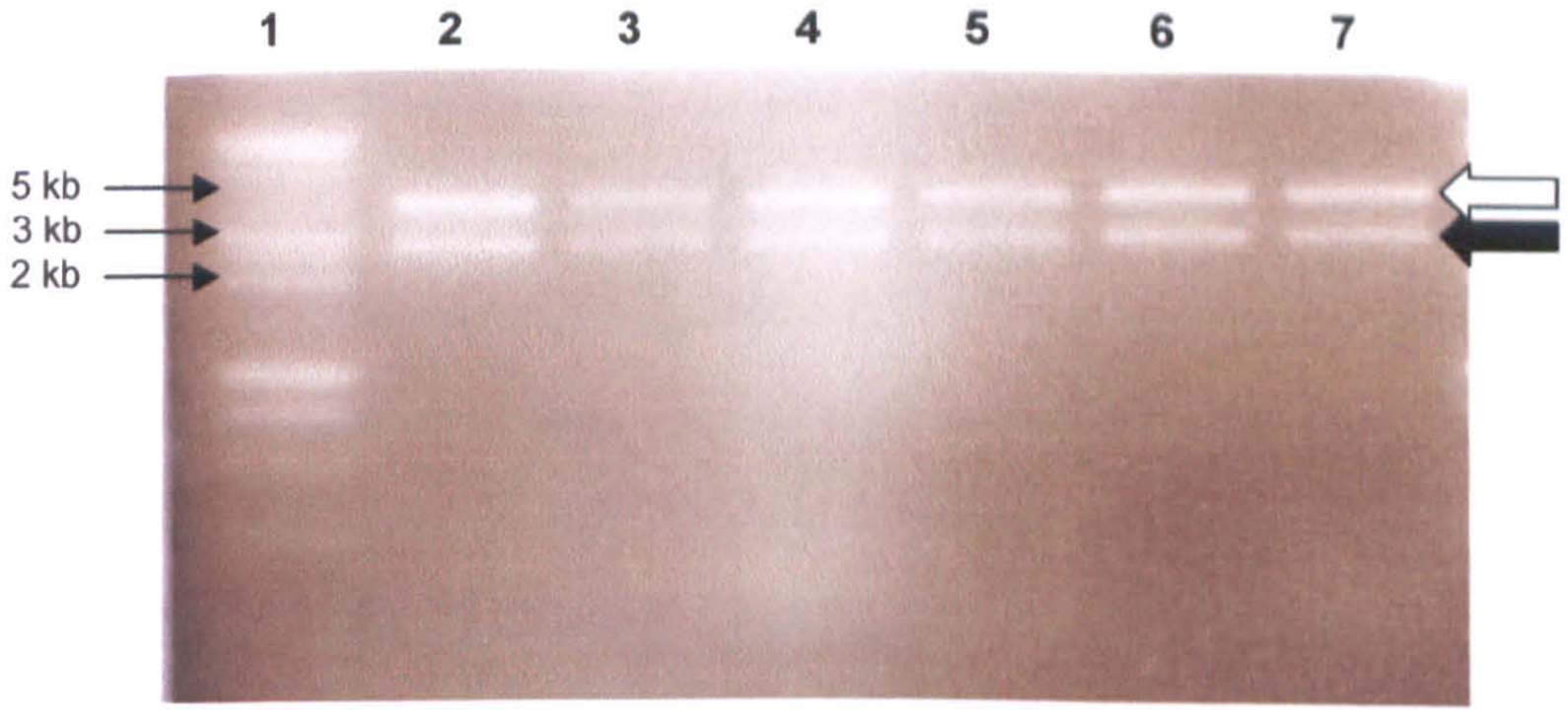


Figure 5.3: 1% (w/v) agarose gel analysis of restriction digests of pTOPD2 using *Bam*HI. Lane 1, molecular size marker; 2, clone A; 3, clone B; 4, clone C; 5, clone D; 6, clone E; 7, clone F. The vector was seen at the expected size of 3.9 kb (unfilled arrow) and the insert at 2.5 kb (filled arrow).

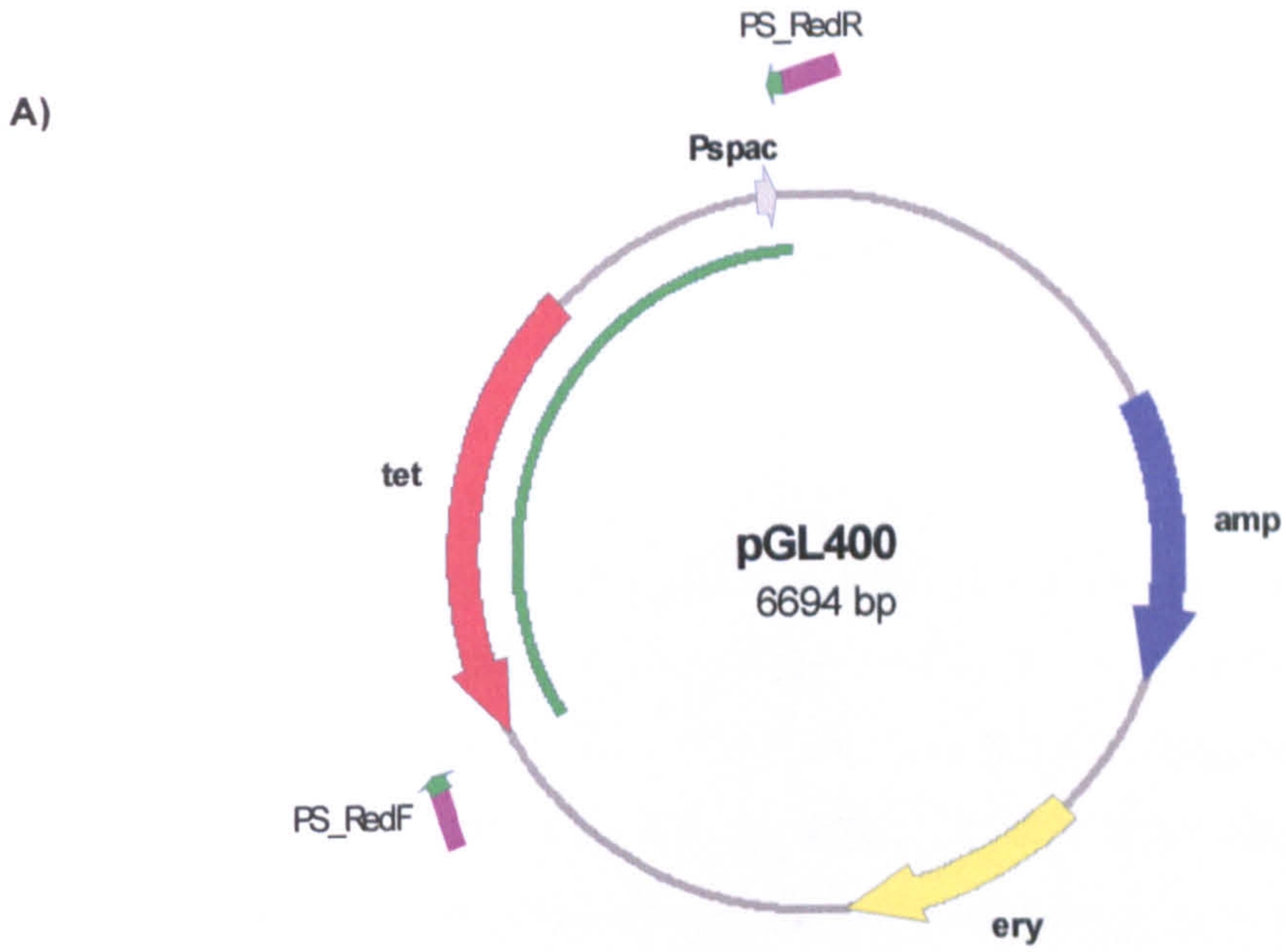


Figure 5.4: Insertion of P*spac* promoter and *tet* resistance gene into *scaD* gene region in pTOPD2 by Lambda Red recombination. Panel A) Primers PS_RedF and PS_RedR were designed to amplify the 2.2 kb P*spac* cassette from plasmid pGL400 (Green) (García-Lara, unpublished). Panel B) Primers also contained homologous sequences from the cloned *scaD* fragment in pTOPD2 (Purple). Panel C) This allowed insertion of the P*spac*/*tet* cassette by recombination of homologous sequences at each end with the *scaD* region.

5.2.1.2. Conventional cloning

As the P*spac* cassette could not be inserted into pTOPD2 by Lambda Red recombination technology, conventional cloning methods were attempted.

5.2.1.2.1. Inverse PCR

A restriction site was engineered upstream of *scaD* in pTOPD2 in order to insert the P*spac* cassette via conventional cloning. Primers were designed to amplify the plasmid in an inverse PCR reaction (Figure 5.5). They were designed to amplify from the chosen insertion site of the promoter cassette, upstream of the *scaD* ribosome binding site. Sequence analysis of pTOPD2 and the promoter cassette revealed that *Sal*I was a suitable enzyme to use for the cloning, and restriction sites were incorporated into the primers to generate the new enzyme site within the plasmid. The plasmid could then be religated, the P*spac* cassette amplified with the same restriction sites at the 3' and 5' ends, and the cassette ligated into the plasmid. Primers InvD1 and InvD2 were used to amplify the 6.4 kb product using pTOPD2 as template DNA. However, no products of the correct size could be obtained by this method, despite modifications to the PCR protocol.

5.2.1.2.2. Transfer of *scaD* to pGL456

Due to problems encountered previously, cloning was attempted with a new vector, pGL456 (Figure 5.6). This plasmid contains an ampicillin resistance marker (*bla*) for selection in *E. coli* and an erythromycin resistance gene (*erm*) for selection in *S. aureus*.

Insert D2 and pGL456 were both digested with *Bam*HI and the vector was subsequently treated with alkaline phosphatase to prevent religation. The insert was ligated into the plasmid, ligation reactions purified using glycogen and used to transform electrocompetent *E. coli* Top10. Transformants were selected on LB agar containing ampicillin (100 µg/ml) and verified by plasmid preparation, restriction digestion using *Bam*HI and analysis by 1%

(w/v) agarose gel electrophoresis. However, as was seen previously with pOB, the insert was unable to be cloned into pGL456.

5.2.1.2.3. Generation of *scaD*::P*spac* insert

As previous attempts to clone the *scaD*::P*spac* into the vector pGL456 failed, primers were designed to amplify the region containing *scaD* in two separate arms. Primer pairs D2F/D3 (Arm A) and D2R/D4 (Arm B) were used to amplify the region from SH1000 genomic DNA, generating products of 0.9 kb and 1.7 kb, respectively. The primers were designed to incorporate the restriction enzyme sites *Fse*I and *Asc*I into the 3' and 5' ends of arms A and B, respectively. In order to generate an insert containing the P*spac* promoter and tetracycline cassette, a three-way ligation was attempted. A 2.2 kb fragment containing the inducible promoter and antibiotic resistance cassette was digested from plasmid pGL439 (García-Lara, unpublished) using *Fse*I and *Asc*I (Figure 5.7). The fragment was gel purified and used in a ligation reaction with Arms A and B, digested with *Fse*I and *Asc*I, respectively. Ligations were then used as a template for a second PCR using primer pair D2F/D2R to generate a product of 4.8 kb. Amplification products were analysed by 1% (w/v) TAE agarose gel electrophoresis and the correct sized product was seen (Figure 5.8, Panel A). The insert was verified by restriction digests using *Bam*HI, *Fse*I and *Asc*I, and results demonstrated that the amplified insert was correct (Figure 5.8, Panel B). The insert was named DP1 (Figure 5.9).

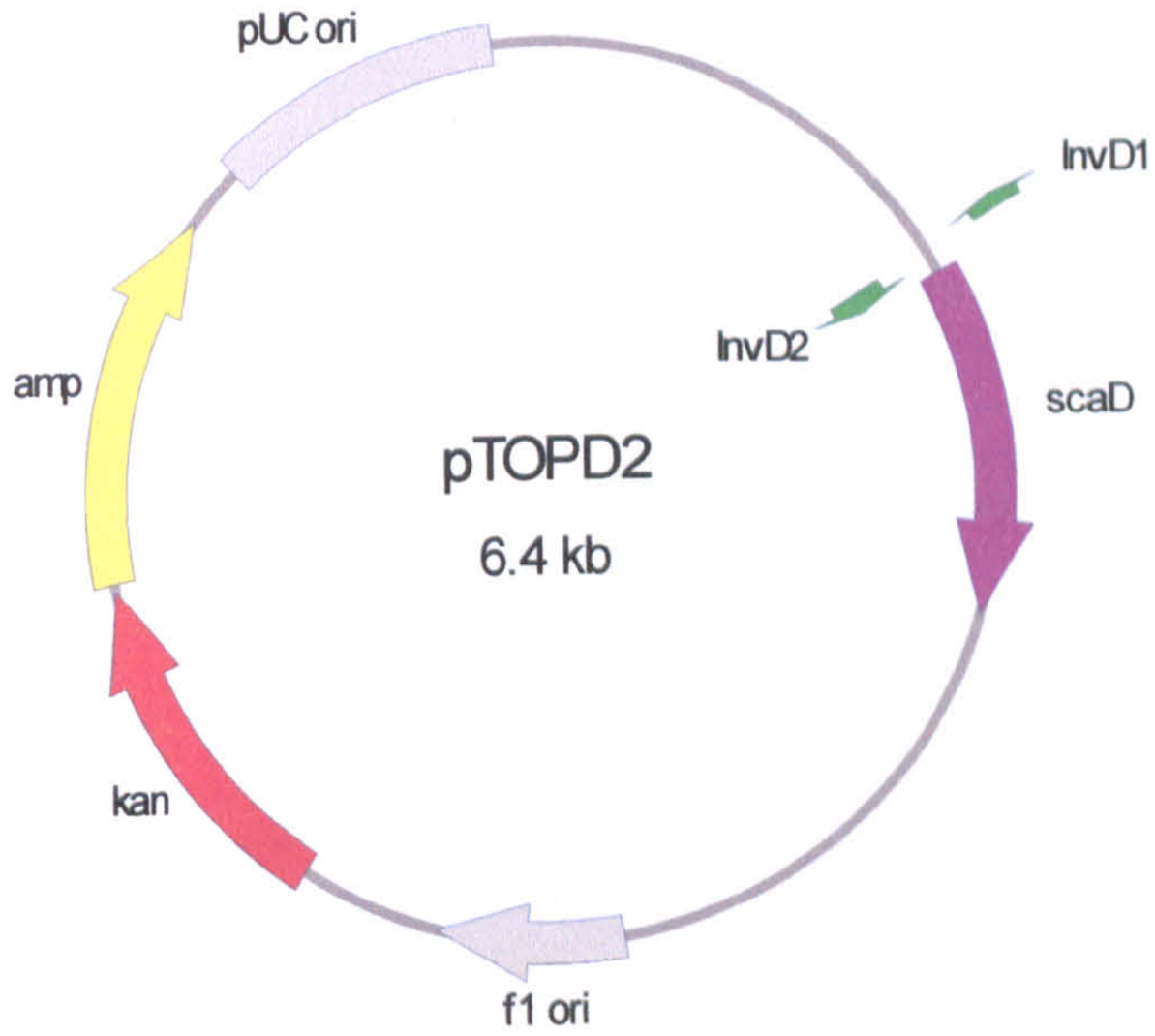


Figure 5.5: Physical map of pTOPD2 demonstrating positions of primers InvD1 and InvD2 for inverse PCR to create *Sal*I restriction site upstream of *scaD*.

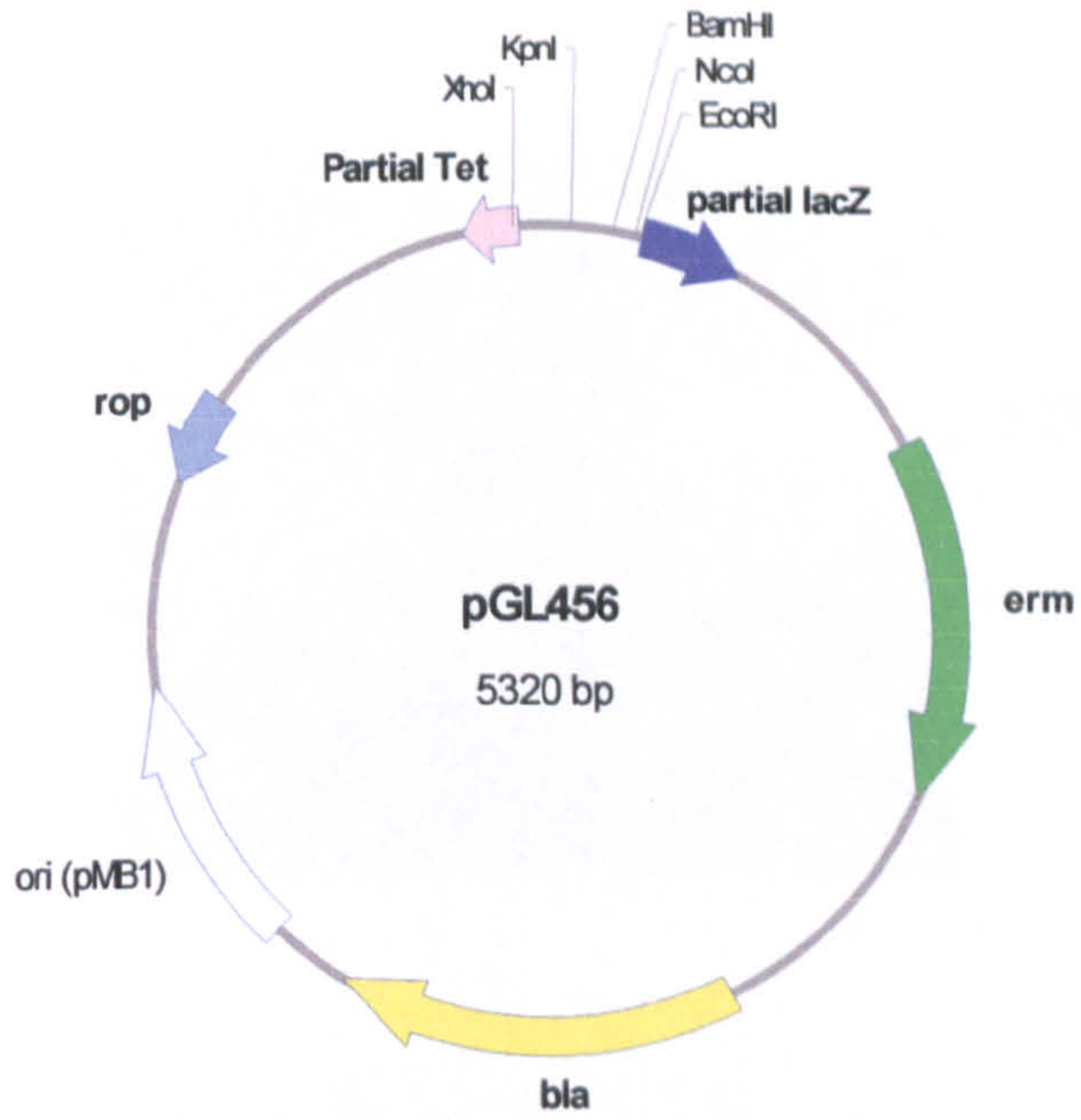


Figure 5.6: Physical map of pGL456 (García-Lara, unpublished). This plasmid contains an ampicillin resistance gene (*bla*) for selection in *E. coli* and an erythromycin resistance gene (*erm*) for selection in *S. aureus*.

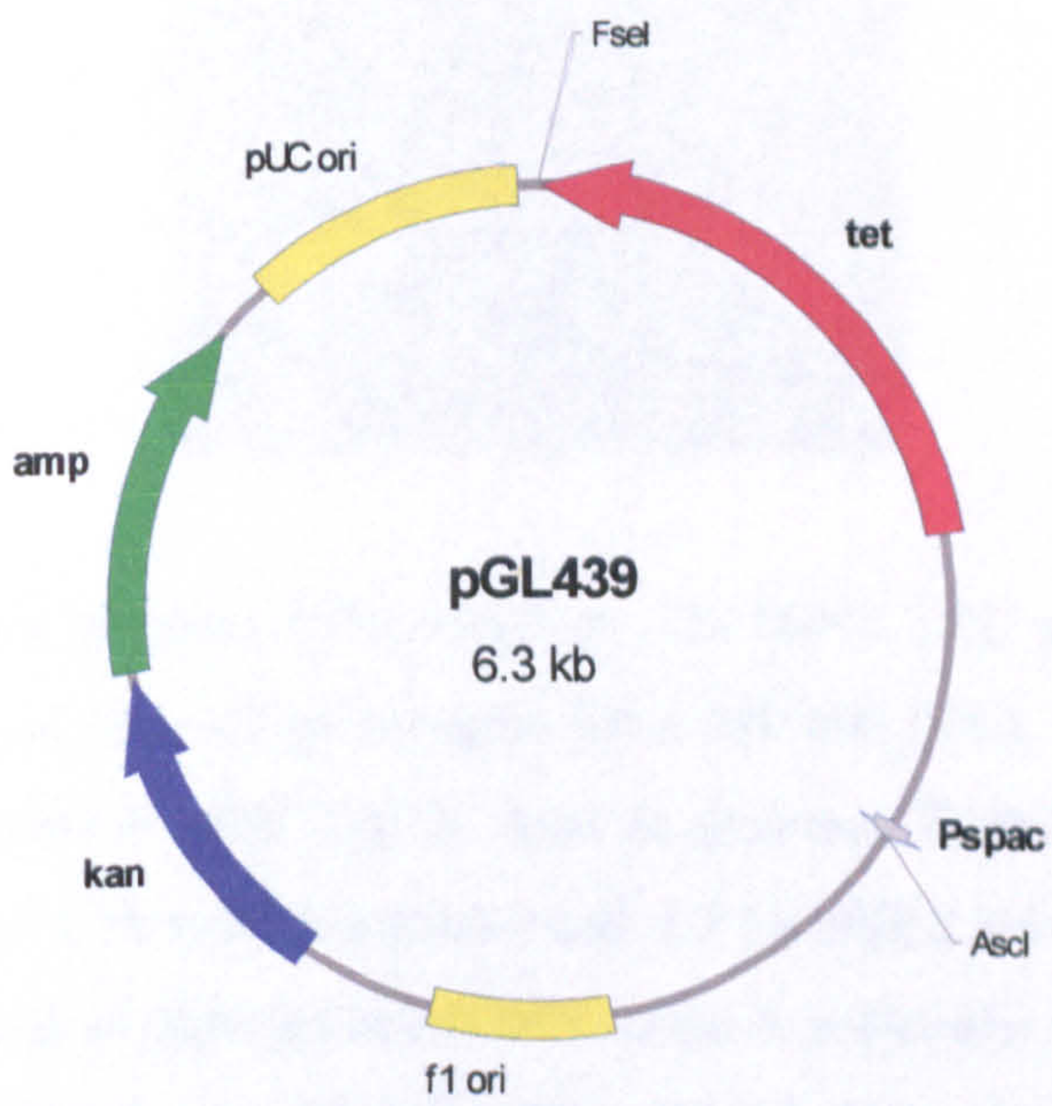


Figure 5.7: Physical map of pGL439 (García-Lara, unpublished). This plasmid contains a tetracycline resistance gene (*tet*) and P*spac* promoter flanked by the restriction sites *Fse*I and *Asc*I.

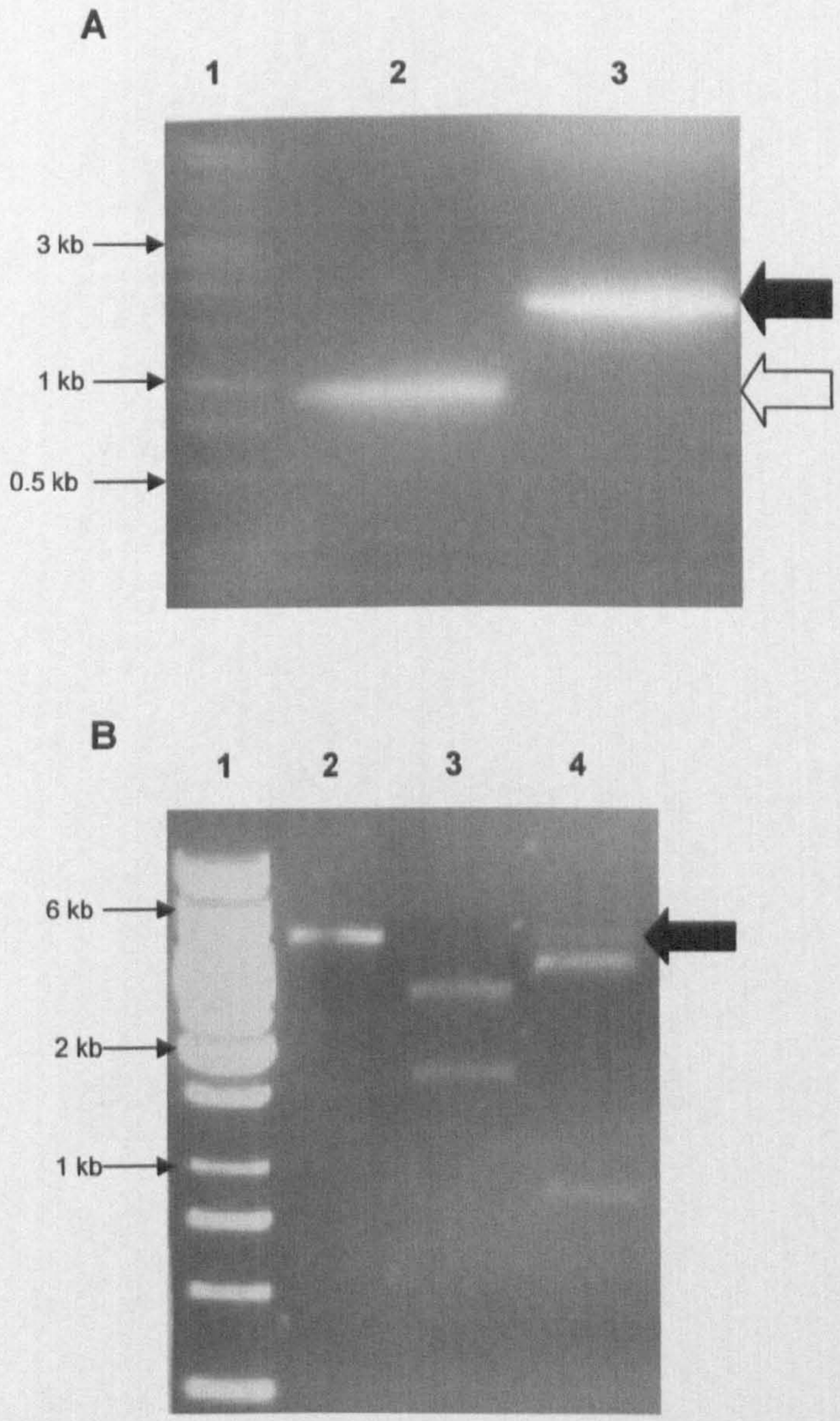


Figure 5.8: Generation of insert DP1. Panel A, 1% (w/v) TAE agarose gel analysis of products of amplification of *scaD* gene region from SH1000 DNA. Lane 1, molecular size marker; 2, Arm A (primers D2F/D3); 3, Arm B (primers D2R/D4). The correct sized products were seen at 0.9 kb (unfilled arrow) and 1.7 kb (filled arrow). Panel B, 1% (w/v) TAE agarose gel analysis of digested insert DP1. Lane 1, molecular size marker; 2, *Bam*HI-digested; 3, *Asc*I-digested; 4, *Fse*I-digested (incomplete digest, undigested DNA highlighted by the filled arrow). Bands were seen at the correct sizes to verify the DP1 insert.

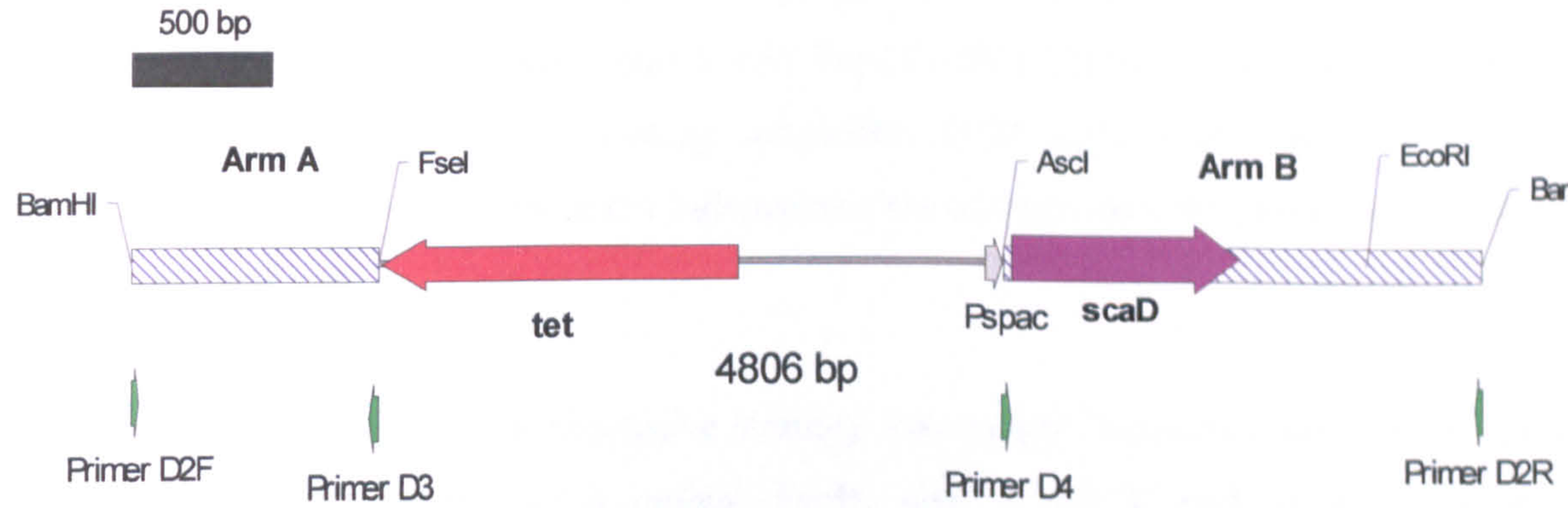


Figure 5.9: Map of insert DP1 containing *scaD*::P*spac* and a tetracycline antibiotic marker. Arms A and B were amplified from SH1000 DNA using primer pairs D2F/D3 and D2R/D4, respectively. The insert was constructed by ligation of the 2.2 kb P*spac* cassette with the two gene region arms followed by amplification using primers D2F/D2R.

5.2.1.2.4. Cloning of *scaD*::P*spac* into pGL456

The DP1 insert was digested with *Bam*HI and gel purified, before being ligated into pGL456 digested with the same enzyme. Ligations were then precipitated with glycogen and used to transform electrocompetent *E. coli* Top10 cells (Chapter 2.20.1). Transformants were selected on LB agar containing ampicillin (100 µg/ml) and agar containing tetracycline (12.5 µg/ml). Despite ten independent transformations, the insert could not be cloned into pGL456.

As cloning attempts failed, an alternative strategy was sought. Sequence analysis of insert DP1 revealed the presence of a unique *Eco*RI site at the 3' end of the fragment, approximately 500 bp upstream from the 3' *Bam*HI site (Figure 5.9). As pGL456 contained both *Bam*HI and *Eco*RI within the multiple cloning site, these restriction enzymes were used for cloning. Fragment DP1 was digested with *Bam*HI/*Eco*RI, resulting in the formation of two products of 4.3 kb and 0.5 kb. The 4.3 kb fragment was gel purified and used in a ligation reaction with pGL456 digested with the same enzymes. Ligations were purified using glycogen and used to transform electrocompetent *E. coli* Top10 cells (Chapter 2.20.1). Transformants were selected on LB agar containing tetracycline (12.5 µg/ml), resulting in one putative clone. Plasmid preparation and restriction digestion using enzymes *Bam*HI/*Eco*RI and *Asc*I resulted in bands of the expected sizes (Figure 5.10). The resulting plasmid was named pDPs1 (Figure 5.11).

5.2.1.2.5. Transformation of pDPs1 into RN4220

pGL456 is unable to replicate in *S. aureus*, therefore, transfer requires recombination of the homologous regions of the insert into the bacterial genome. pDPs1 was used to transform electrocompetent *S. aureus* RN4220 (Chapter 2.20.2) and colonies were selected on BHI agar containing tetracycline (5 µg/ml). However, even after ten independent attempts no colonies were obtained.

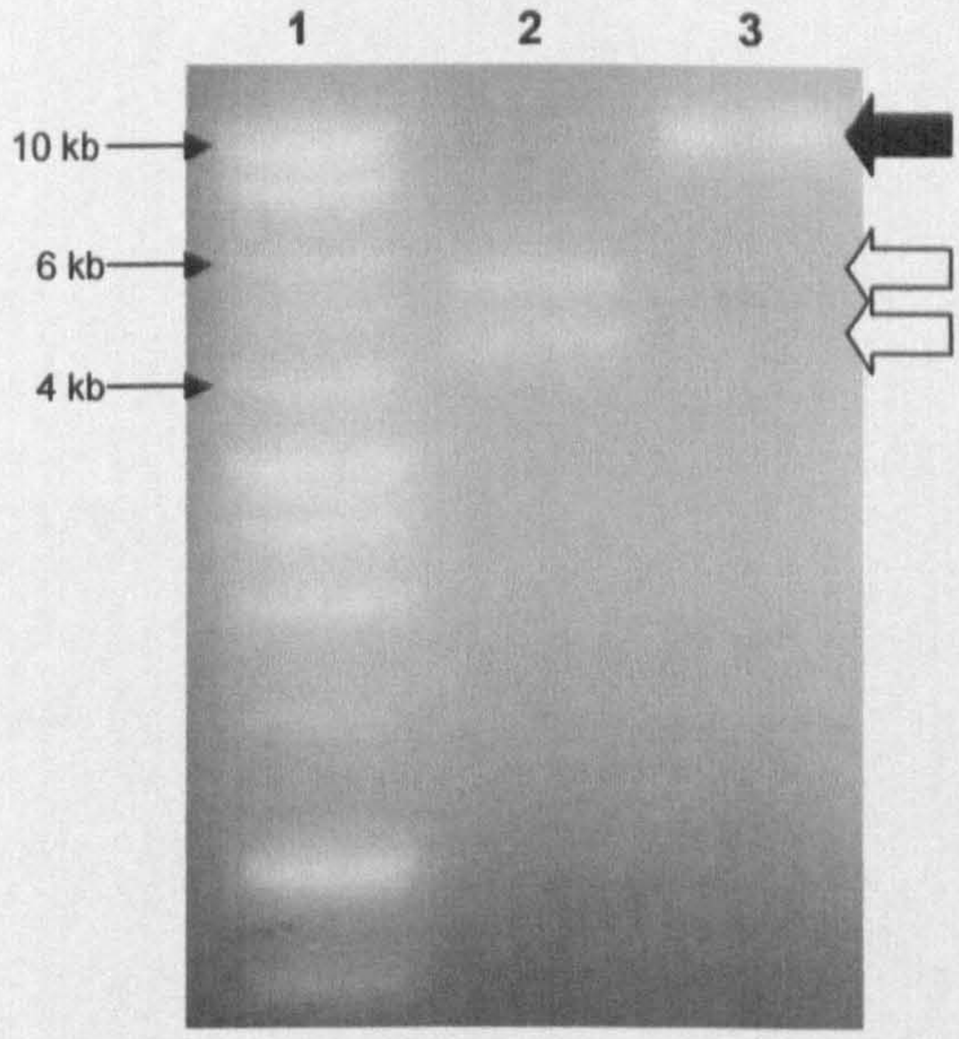


Figure 5.10: 1% (w/v) TAE agarose gel analysis of restriction digests of pDPs1. Lane 1, molecular size marker; 2, *Bam*HI/*Eco*RI digest of pDPs1; 3, *Asc*I digest of pDPs1. The correct sized products at 5.4 kb and 4.3 kb are shown by the unfilled arrows, and the linearised plasmid is shown at 9.7 kb by the filled arrow.

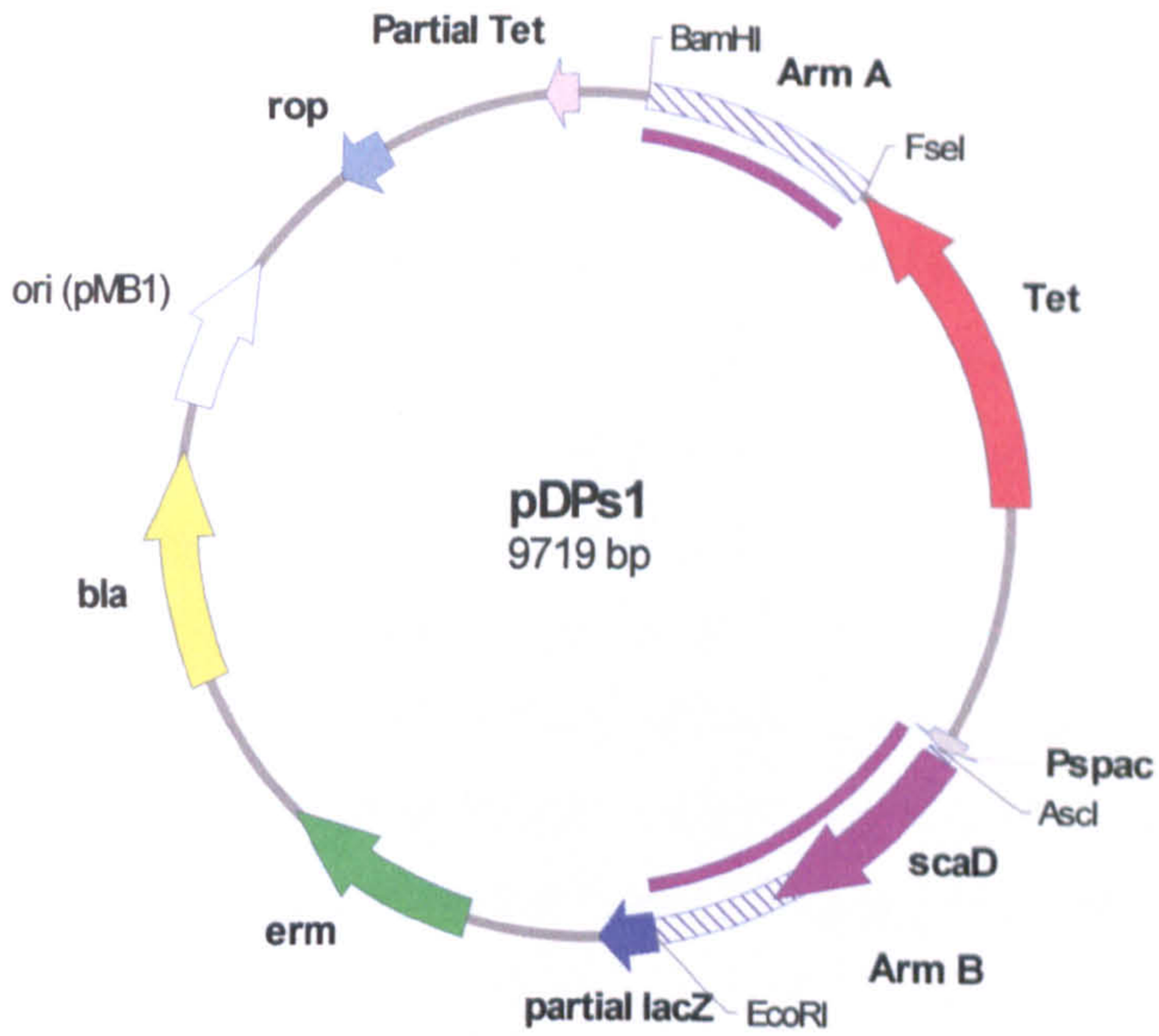


Figure 5.11: Physical map of pDPs1. The insert containing P*spac*::*scaD* and a tetracycline resistance marker was cloned into pGL456 using *Bam*HI and *Eco*RI. *S. aureus* sequence is highlighted by the inner purple lines.

5.2.1.2.6. Transfer of P*spac*::*scaD* into pMAD

To facilitate the recombination of the P*spac*::*scaD* into the *S. aureus* chromosome, the insert was transferred into the temperature sensitive plasmid pMAD (Arnaud *et al.* 2004). pMAD (Figure 5.12) is a temperature-sensitive plasmid, replicating at 30°C in *S. aureus*. Temperatures above 37°C lead to greatly impaired replication of the plasmid, permitting homologous recombination of target genes during mutagenesis. The plasmid contains an ampicillin resistance gene (*bla*) for selection in *E. coli* and an erythromycin resistance gene (*ermC*) for selection in *S. aureus*. In addition, a *bgaB* gene is present, which encodes a thermostable β-galactosidase. When plated onto X-gal, this allows colorimetric blue/white selection of bacteria that have lost the plasmid, facilitating the identification of clones during recombination.

The *scaD*::P*spac* insert was digested from pDPs1 (Chapter 5.2.1.2.4) using restriction enzymes *Bam*HI and *Eco*RI. The digested fragment was gel purified and ligated into pMAD, which had been digested with the same enzymes. Ligations were purified using glycogen and used to transform competent *E. coli* Top10 cells (Chapter 2.20.1). Transformants were selected on LB agar containing tetracycline (12.5 μg/ml) at 30°C. Three positive transformants were selected and purified plasmid was digested with *Bam*HI/*Eco*RI and *Asc*I, to produce the expected fragments of 9.7 kb and 4.3 kb, and 14 kb, respectively (Figure 5.13). The plasmid construct was named pDPs2 (Figure 5.14).

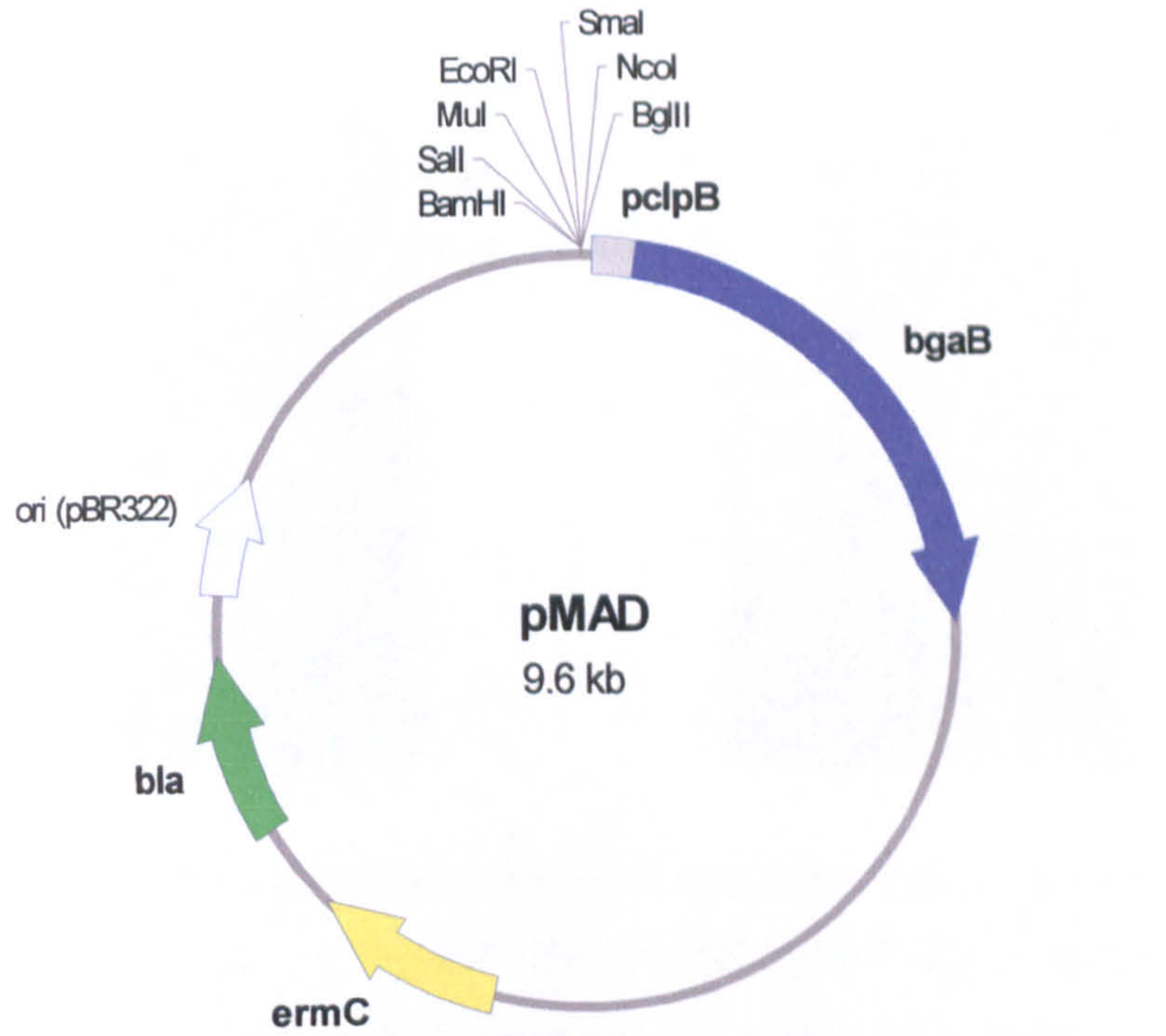


Figure 5.12: Physical map of pMAD (Arnaud *et al.* 2004). This plasmid contains an ampicillin resistance gene (*bla*) for selection in *E. coli* and an erythromycin resistance gene (*ermC*) for selection in *S. aureus*. There is a replication sequence (*ori*) for replication of plasmid at low temperature. The plasmid also contains a *bgaB* gene, encoding a thermostable β-galactosidase, which allows colorimetric blue/white selection of bacteria when plated onto X-gal.

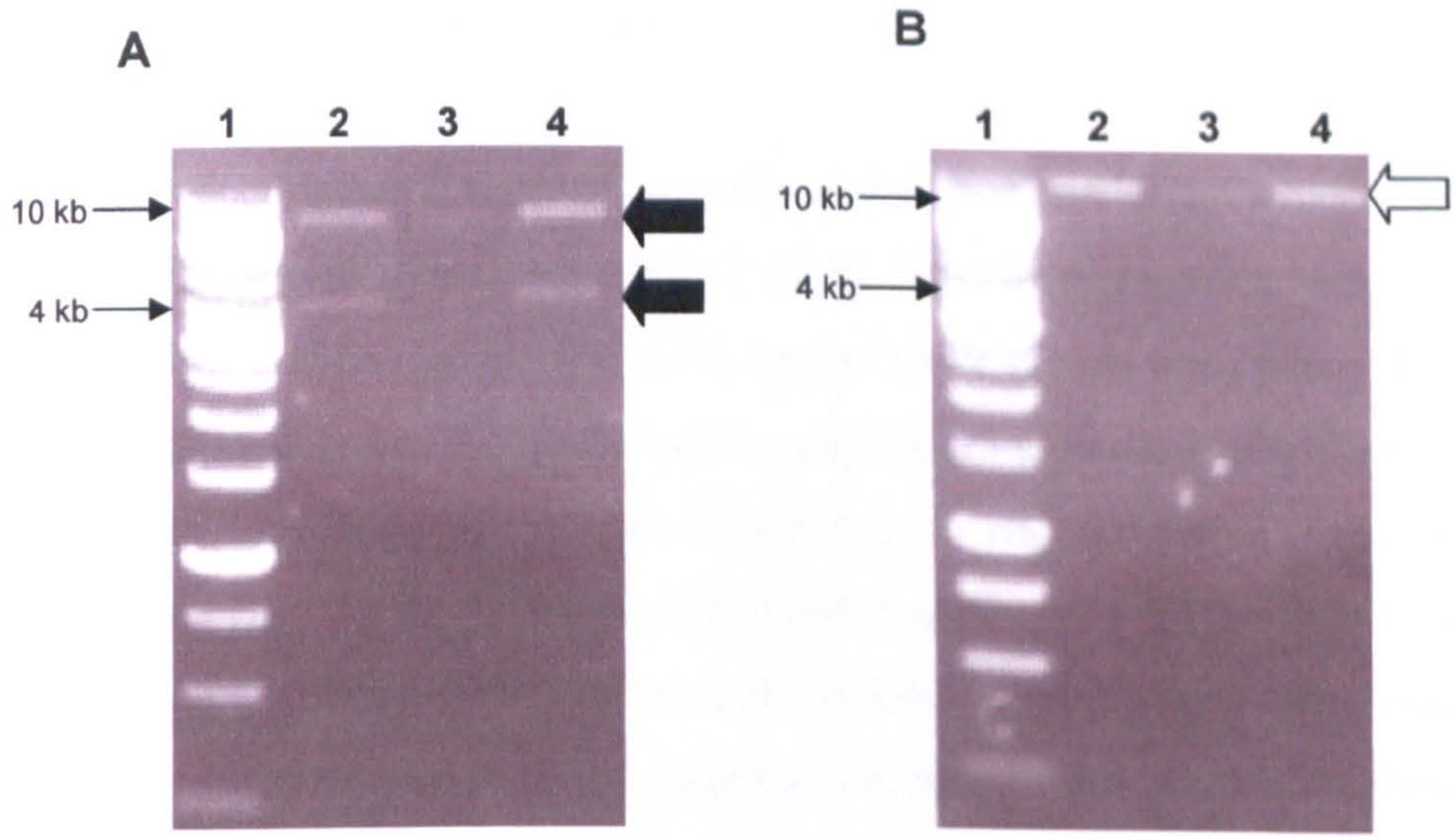


Figure 5.13: 1% (w/v) TAE agarose gel analysis of restriction digests of pDPs2 from *E. coli* Top10 transformants. Panel A, digestion with *Bam*HI and *Eco*RI; Panel B, digestion with *Asc*I. Lane 1, molecular size marker; 2, clone 1; 3, clone 2; 4, clone 3. The correct sized fragments at 9.7 kb and 4.3 kb were seen for *Bam*HI/*Eco*RI (filled arrows) and at 14 kb for *Asc*I (unfilled arrow).

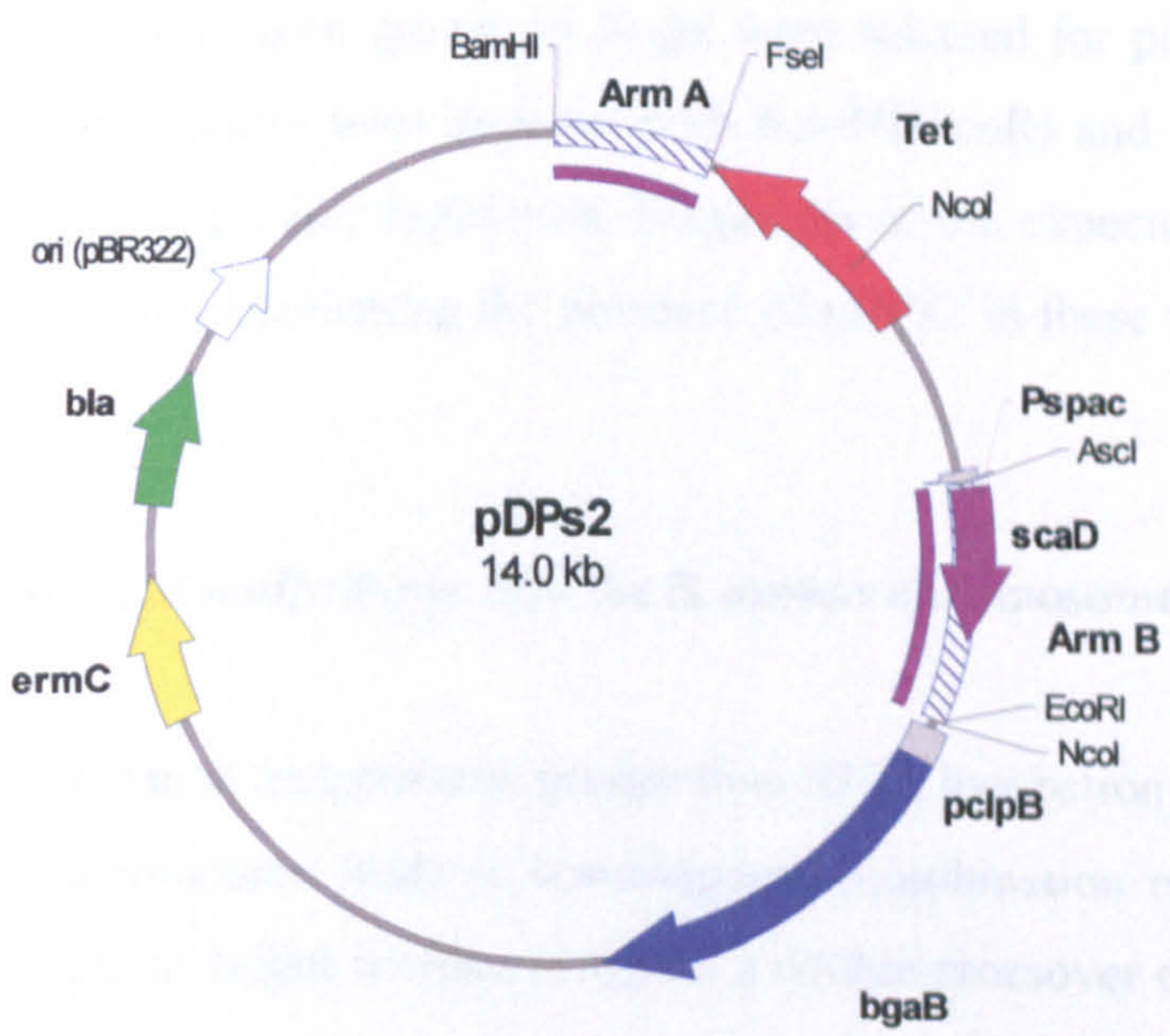


Figure 5.14: Physical map of pDPs2. The insert containing P*spac*::*scaD* and tetracycline resistance marker was cloned into pMAD using *Bam*HI and *Eco*RI. *S. aureus* sequence is highlighted by the inner purple lines.

5.2.1.3. Transfer of pDPs2 into *S. aureus*

A midi preparation (Chapter 2.16.6) of plasmid pDPs2 was made and used to transform electrocompetent *S. aureus* RN4220 (Chapter 2.20.2). Transformants were selected on BHI agar containing tetracycline (5 μg/ml) at 30°C and positive clones were patched onto BHI agar containing erythromycin (5 μg/ml) and lincomycin (25 μg/ml). Three clones resistant to both antibiotics were selected for plasmid preparation and restriction digestion. Upon digestion with *Bam*HI and *Eco*RI, the correct sized fragments of 9.7 kb and 4.3 kb were seen for clones 1 and 2, and digestion with *Nco*I resulted in the expected fragments of 11.5 kb and 2.5 kb for clones 1 and 2, confirming the presence of the plasmid in these strains (results not shown).

Plasmid pDPs2 was transferred into *S. aureus* SH1000 by phage transduction (Chapter 2.19). Transductants were selected on LK top agar containing tetracycline (5 μg/ml) at 30°C and subsequently patched onto BHI agar containing erythromycin (5 μg/ml), lincomycin (25 μg/ml) and X-gal (80 μg/ml). Four transductants resistant to both antibiotics which were blue when grown on X-gal were selected for plasmid preparation and restriction digestion. Clones were digested with *Bam*HI/*Eco*RI and *Asc*I and analysed by 1% (w/v) TAE agarose gel electrophoresis. Fragments of the expected sizes were seen for 3 of the 4 transductants, confirming the presence of pDPs2 in these strains (results not shown).

5.2.1.4. Recombination of *scaD*::P*spac* into the *S. aureus* chromosome

As pMAD cannot replicate at temperatures greater than 37°C, incubation of strains carrying the plasmid at high temperature leads to homologous recombination of the cloned gene region. Several attempts to isolate a clone in which a double crossover event had occurred were carried out.

SH1000 pDPs2 was grown at 30°C overnight in 5 ml BHI containing tetracycline (5 μg/ml). This culture was used to inoculate a fresh 5 ml of BHI without antibiotic to

OD_{600nm} 0.01 and cultures were incubated at 30°C, 250 rpm for 2 h, followed by 6 h at 42°C. Cultures were serially diluted and plated onto BHI agar containing tetracycline (5 µg/ml) and X-gal (80 µg/ml), before being incubated overnight at 42°C. Clones having undergone a single crossover event produced blue colonies due to integration of the vector. However, clones in which a double crossover event had occurred, resulting in loss of the pMAD vector, were seen as white colonies (Arnaud *et al.* 2004). Despite repeated attempts, no correct colonies were obtained.

The next attempt involved passaging of strains at 42°C until white colonies were isolated. A single colony of SH1000 pDPs2 was streaked 20 times onto BHI agar containing tetracycline (5 µg/ml) and X-gal (80 µg/ml) and incubated at 42°C for 48 h. A single colony was then picked from each strain and streaked out again onto the same medium. Plates were incubated at 42°C for a further 48 h and the process repeated up to ten times. After several attempts, no white colonies were isolated by this method.

As passaging of strains on medium containing tetracycline had failed, the method was repeated on BHI agar containing no antibiotics. After 5-6 passages white colonies were observed. However, when these strains were plated back onto medium containing tetracycline, they were unable to grow. Analysis of these colonies by PCR using primer pairs PsF1/PsR and PsF2/PsR (Table 2.6) did not result in bands of the expected sizes (3.7 kb and 3 kb, respectively). Instead, bands were seen corresponding to that of wild-type DNA (1.5 kb and no product, respectively), indicating that these strains had lost the plasmid without undergoing homologous recombination.

LB medium was also used in the hope that a less-rich medium may increase the chances of recombination and plasmid loss. However, no white colonies were isolated after up to ten passages at 42°C. Other attempts involved variation of temperatures and levels of antibiotic, however, no correct clones could be obtained.

5.2.1.5. Identification by Southern hybridisation

No white colonies were identified from attempts to isolate strains in which the plasmid had resolved due to homologous recombination. To determine the identity of the strains, three clones (A, B and C) were analysed by Southern hybridisation (Chapter 2.18). Genomic DNA from *S. aureus* SH1000 and clones A, B and C was digested with *Eco*RI and separated by 1% (w/v) agarose gel electrophoresis, blotted onto a nylon membrane and probed with a digoxygenin labelled DNA fragment of the 0.8 kb Arm A (Figures 5.9, 5.14). The possible restriction patterns are shown in Figure 5.15. Homologous recombination could occur in two locations, (1) and (2). Two outcomes for single crossover events are possible (1 and 2), yielding fragments of 3.6 kb/14 kb and 5.8 kb/11.8 kb. A double crossover event would result in a single band at 5.8 kb (3) and SH1000 genomic DNA would result in a band at 3.6 kb.

Analysis of the three clones by Southern hydridisation (Figure 5.16) demonstrated that clones A and B (Lanes 2 and 3) represented strains which had undergone a single crossover event, with hybridising bands at approximately 3.6 kb and 14 kb. Clone C demonstrated the same banding pattern as SH1000 wild-type DNA (Lanes 1 and 4), although the band is faint so cannot be fully verified.

5.2.1.6. Transductions

Transductional outcross to resolve the single crossover insertions by recombination was attempted using phage transduction with the strains that had undergone a single crossover event (Chapter 5.2.1.5). Lysates were made of strains A and B and were used to transduce SH1000 (Chapter 2.19). Transductants were selected on LK top agar containing tetracycline and resulting colonies were patched onto BHI agar plates containing tetracycline (5 μg/ml) and plates containing erythromycin (5 μg/ml) and lincomycin (25 μg/ml). Colonies resistant to Tet but unable to grow on Ery/Lin represented putative clones with resolved plasmids. However, all 585 patched transductants demonstrated resistance to both antibiotics, indicating that homologous recombination via double crossover had not occurred.

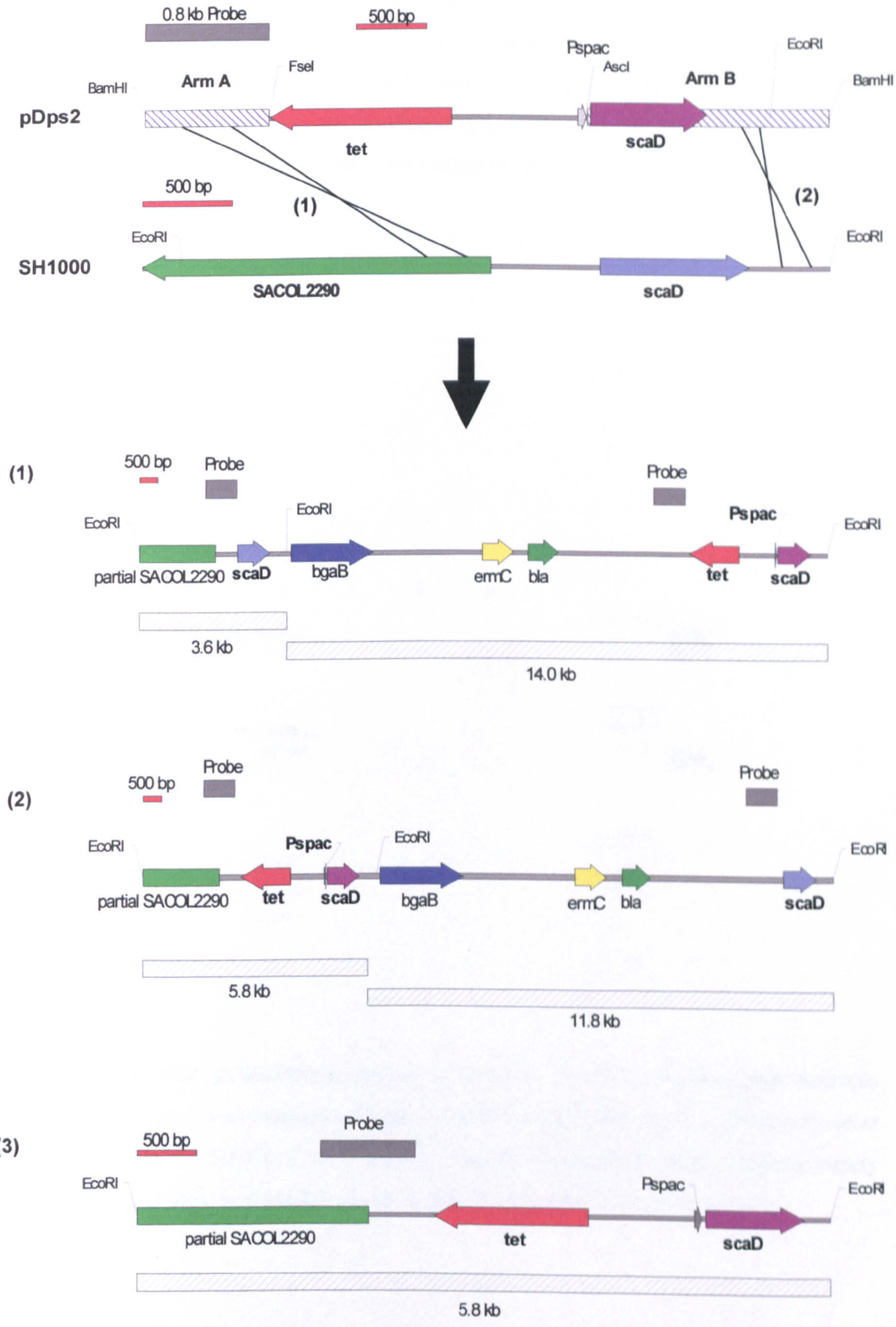

0.8 kb Probe
500 bp
Pspac
EcoRI
FseI
AscI
BamHI
Arm A
Arm B
BamHI
pDps2
tet
scaD
500 bp
(1)
(2)
EcoRI
EcoRI
SH1000
SACOL2290
scaD
(1)
500 bp
Probe
Probe
EcoRI
EcoRI
Pspac
EcoRI
partial SACOL2290
scaD
bgaB
ermC
bla
tet
scaD
3.6 kb
14.0 kb
(2)
500 bp
Probe
Probe
EcoRI
Pspac
EcoRI
EcoRI
partial SACOL2290
tet
scaD
bgaB
ermC
bla
scaD
5.8 kb
11.8 kb
(3)
500 bp
Probe
EcoRI
Pspac
EcoRI
partial SACOL2290
tet
scaD
5.8 kb

Figure 5.15 (previous page): Homologous recombination of *scaD* region from pDPs2 into SH1000. Recombination could occur in two possible locations, (1) and (2). Single crossover events could lead to two possible outcomes, (1) and (2); double crossover would lead to the outcome in (3). Relevant *Eco*RI restriction sites and expected size of fragments hybridising to the probe during Southern blot analysis are shown.

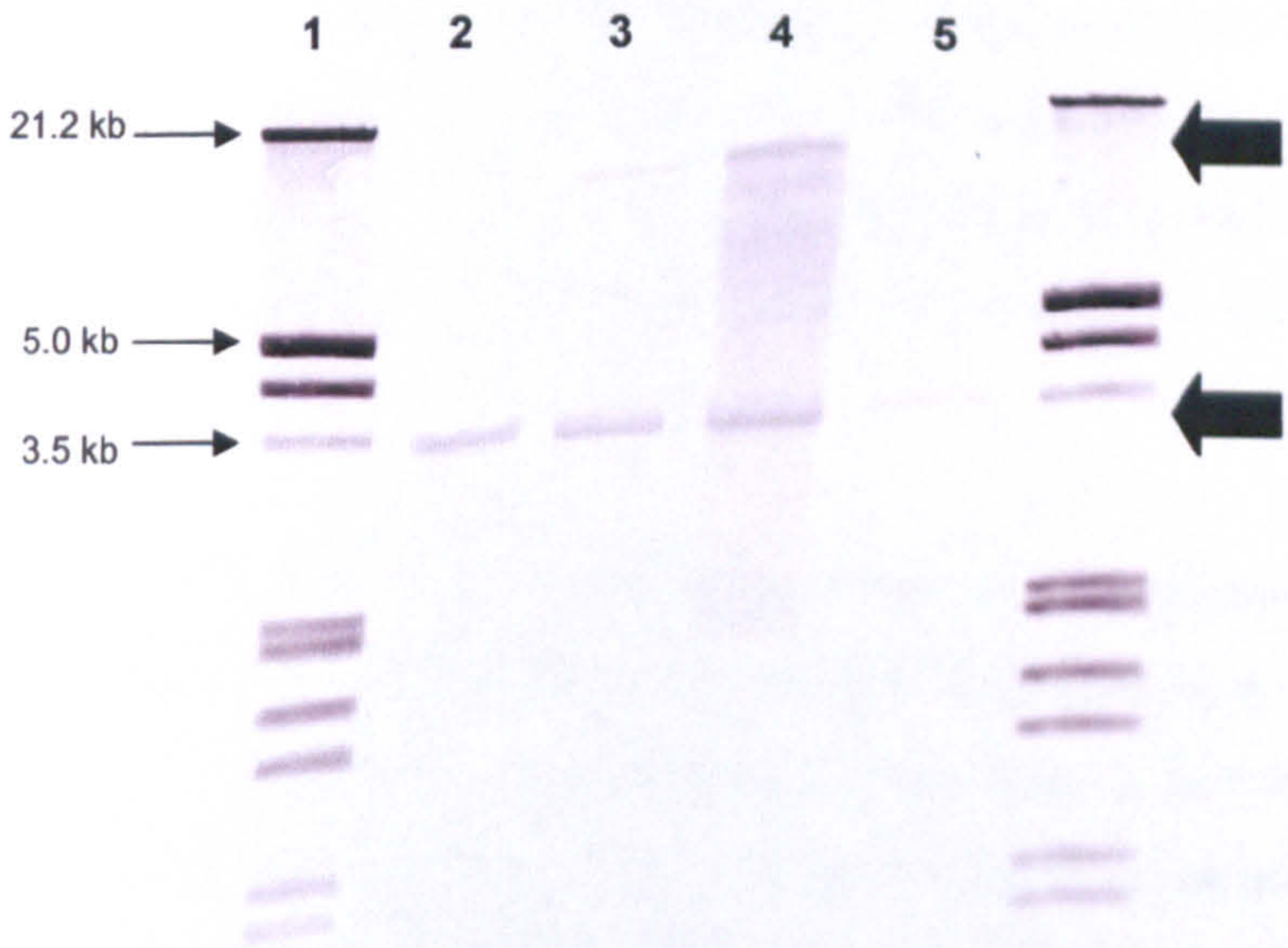


Figure 5.16: Southern hybridisation analysis of clones A, B and C, resulting from attempts to induce homologous recombination of P*spac*::*scaD* into SH1000. Lane 1, DNA molecular weight marker III; 2, SH1000; 3, A; 4, B; 5, C. Results demonstrate bands at approximately 3.6 kb and 14 kb (filled arrows) for clones A and B, indicating a single crossover.

5.2.2. Analysis of the role of ScaD

The inability to place *scaD* under the control of the inducible P*spac* promoter was not fully understood. Insertional inactivation of *scaD* was also attempted to determine if a mutation could be made and the gene was therefore not essential.

5.2.2.1. Insertional inactivation of *scaD* gene using a kanamycin cassette

Construction of *scaD* mutation was carried out by amplifying two fragments of the *scaD* locus using SH1000 genomic DNA as the template. Primers were designed to amplify a fragment of 1.3 kb (A) consisting of 130 bp of the 5' end of *scaD* and 1.2 kb of upstream sequence. A second fragment of 0.8 kb (B) consisted of 340 bp of the 3' end of *scaD* and 440 bp downstream sequence. This resulted in the deletion of 330 bp of *scaD*. Fragment A was amplified using primer pair Mut1/Mut2 (Table 2.6) and incorporated the restriction sites *Bam*HI and *Sal*I. Fragment B was amplified using primer pair Mut3/Mut4 (Table 2.6), which incorporated the restriction sites *Xho*I and *Eco*RI, respectively (Figure 5.17). The kanamycin cassette (1.5 kb) was amplified from pGL433b (García-Lara, unpublished) using primer pair KanF/KanR, which incorporated the restriction sites *Sal*I and *Xho*I (Figure 5.18).

The PCR products of fragments A, B and the Kan cassette were digested with *Sal*I, *Xho*I and *SalI*/*Xho*I, respectively, before being gel purified and used in a series of ligation reactions. Three ligation reactions were carried out: 1, A + Kan; 2, B + Kan; 3, A + Kan + B. The ligation reactions were precipitated with glycogen before being used as the template DNA in PCRs using primers Mut1/KanR, KanF/Mut4, Mut1/Mut4, respectively (Figure 5.17). Products were analysed by 1% (w/v) TAE agarose gel electrophoresis and results demonstrated the successful amplification of the two shorter fragments, but failure to produce the full insert containing arms A, B and the Kan cassette (Figure 5.19). Fragments were named AK (Arm A + Kan) and BK (Arm B + Kan).

Fragments AK and BK were gel purified and digested with *Sal*I and *Xho*I, respectively. These fragments were then ligated to Arm B and Arm A, digested with the same enzymes. Ligations were precipitated with glycogen and used as the template for a second PCR using primers Mut1 and Mut4. Analysis of PCR products by 1% (w/v) TAE agarose gel electrophoresis demonstrated a fragment of approximately 3.6 kb, indicating that the full insert had been successfully amplified in both cases. Fragments were gel purified and digested with both *Sal*I and *Xho*I to verify their identity (Figure 5.20). The resulting fragment was named ABK.

Fragment ABK was digested with *Bam*HI/*Eco*RI and ligated into pMAD, which had been digested with the same enzymes. Ligation reactions were precipitated with glycogen before being used to transform electrocompetent *E. coli* Top10 cells. Transformants were selected on LB agar containing ampicillin (100 µg/ml) at 30°C and patched onto LB agar containing kanamycin (50 µg/ml). 135 transformants were patched onto kanamycin and of these, 8 displayed resistance to both antibiotics. Plasmid DNA was prepared from these strains and digested with *Bam*HI/*Eco*RI. All 8 strains contained the correct plasmid with bands at 9.7 kb and 3.6 kb (Figure 5.21). The resulting recombinant plasmid was named pDMut1.

5.2.2.2. Transfer of pDMut1 into *S. aureus*

A midi preparation (Chapter 2.16.6) of plasmid pDMut1 was made and used to transform electrocompetent *S. aureus* RN4220 (Chapter 2.20.2) Transformants were selected on BHI agar containing kanamycin (50 µg/ml) at 30°C, resulting in four colonies. These putative clones were then patched onto BHI agar containing erythromycin (5 µg/ml) and lincomycin (25 µg/ml), resulting in one transformant which was resistant to both antibiotics. A plasmid preparation from this clone was digested using *Bam*HI/*Eco*RI and *Sall*/*Xho*I, and analysed by 1% (w/v) TAE agarose gel electrophoresis. The correct sized fragments of 9.7 kb / 3.6 kb, and 11.8 kb / 1.5 kb were seen for the clone, confirming the presence of the plasmid in this strain (results not shown).

Plasmid pDMut1 was transferred into *S. aureus* SH1000 by phage transduction (Chapter 2.19). Transductants were selected on LK top agar containing kanamycin (50 µg/ml) at 30°C and subsequently patched onto BHI agar containing erythromycin (5 µg/ml), lincomycin (25 µg/ml), and X-gal (80 µg/ml). Four transductants resistant to both antibiotics which were blue when grown on X-gal were selected for plasmid preparation and restriction digestion. Clones were digested with *Bam*HI/*eco*RI and *Sal*I/*Xho*I, and analysed by 1% (w/v) agarose gel electrophoresis. Fragments of the expected sizes were seen for all four transductants, confirming the presence of pDMut1 in these strains (results not shown).

5.2.2.3. Recombination of inactivated *scaD* into the *S. aureus* chromosome

In order to isolate a mutant which had undergone homologous recombination of the inactivated *scaD* into *S. aureus*, SH1000 pDMut1 was passaged at 42°C on BHI containing kanamycin (50 µg/ml) and X-gal (80 µg/ml), as described previously (Chapter 5.2.1.4). However, similarly to the plasmid pDPs2, after several attempts no colonies could be isolated in which a double crossover event had occurred. The *scaD* gene could therefore not be inactivated in *S. aureus* in this study.

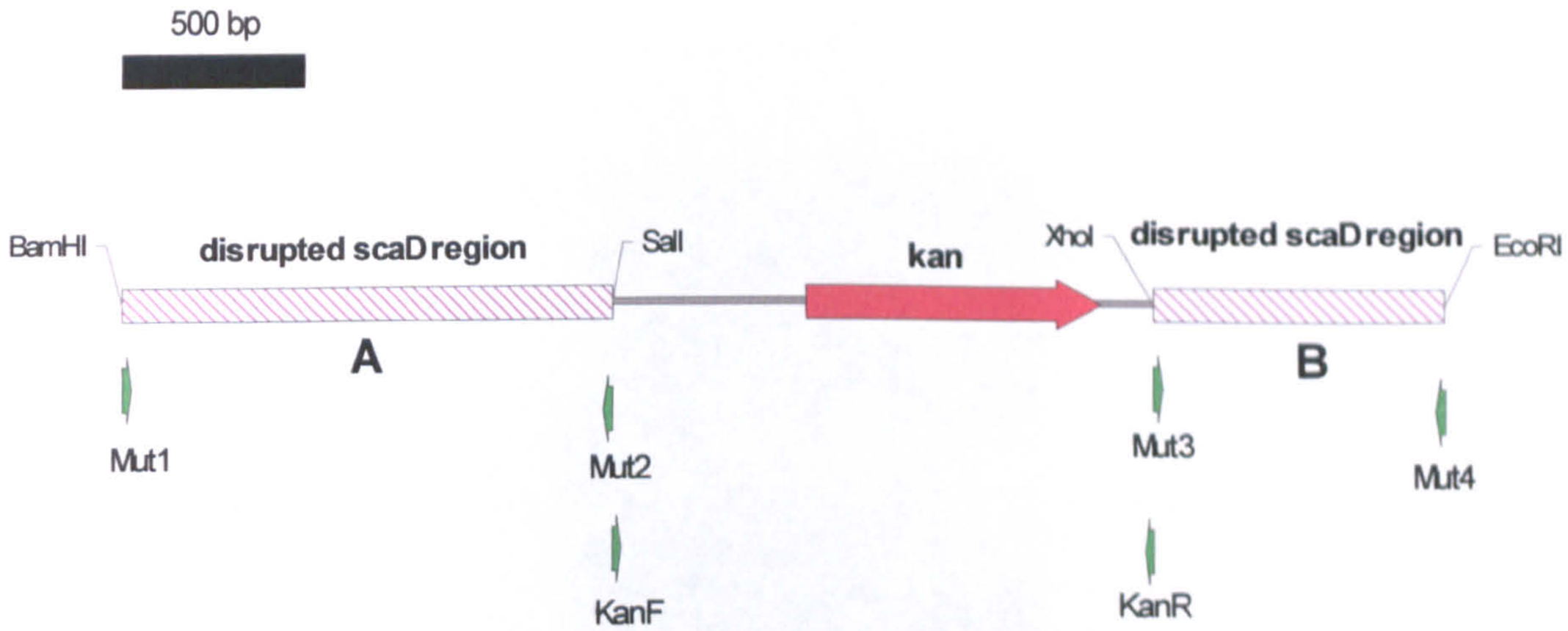


Figure 5.17: Physical map of the disrupted *scaD* gene region and kanamycin cassette for insertional inactivation of *scaD*. Relevant primers are shown.

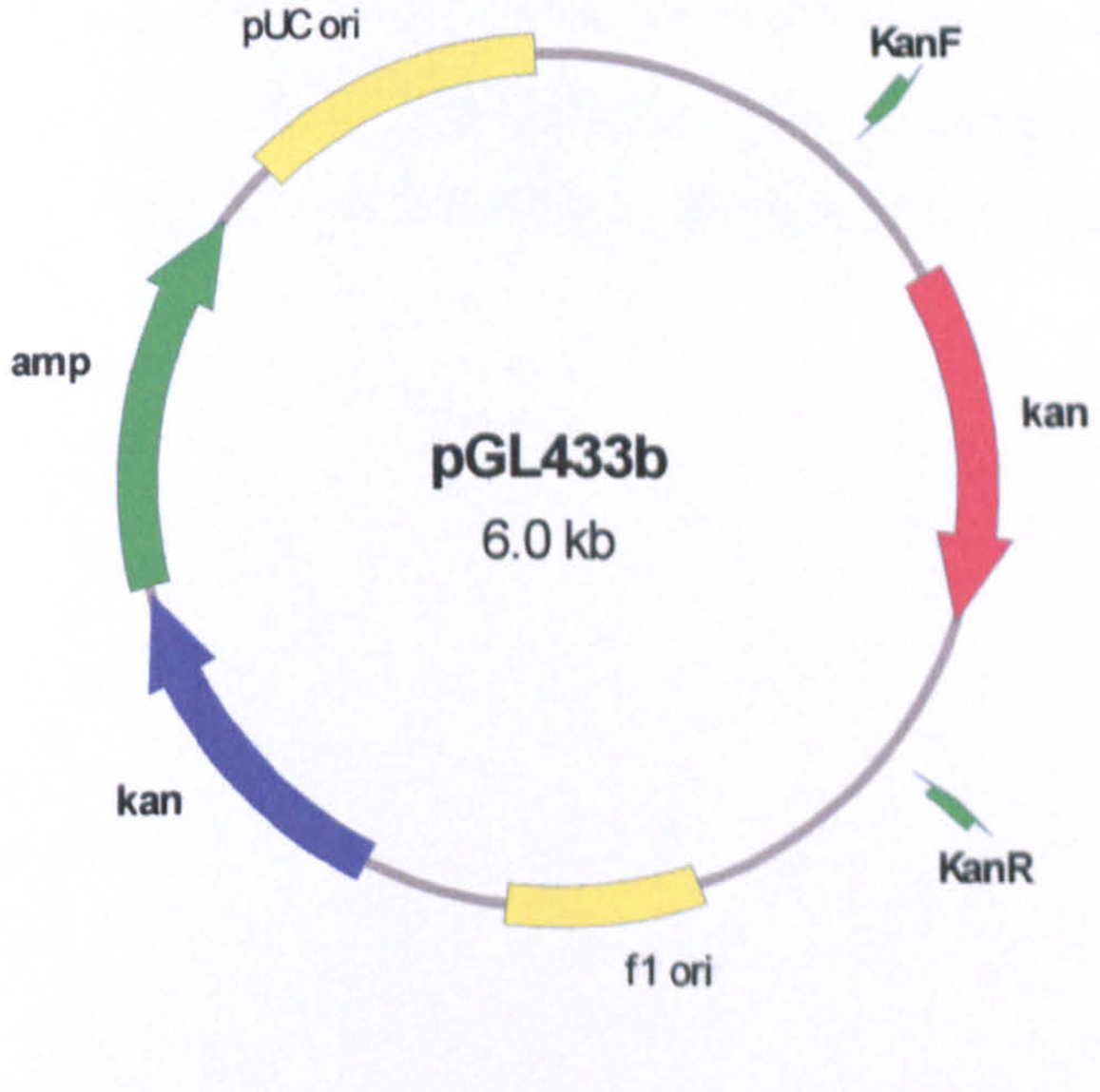


Figure 5.18: Physical map of pGL433b (García-Lara, unpublished). A 1.5 kb region containing a kanamycin resistance gene (*kan*) was amplified from the plasmid using primers KanF and KanR (Table 2.6).

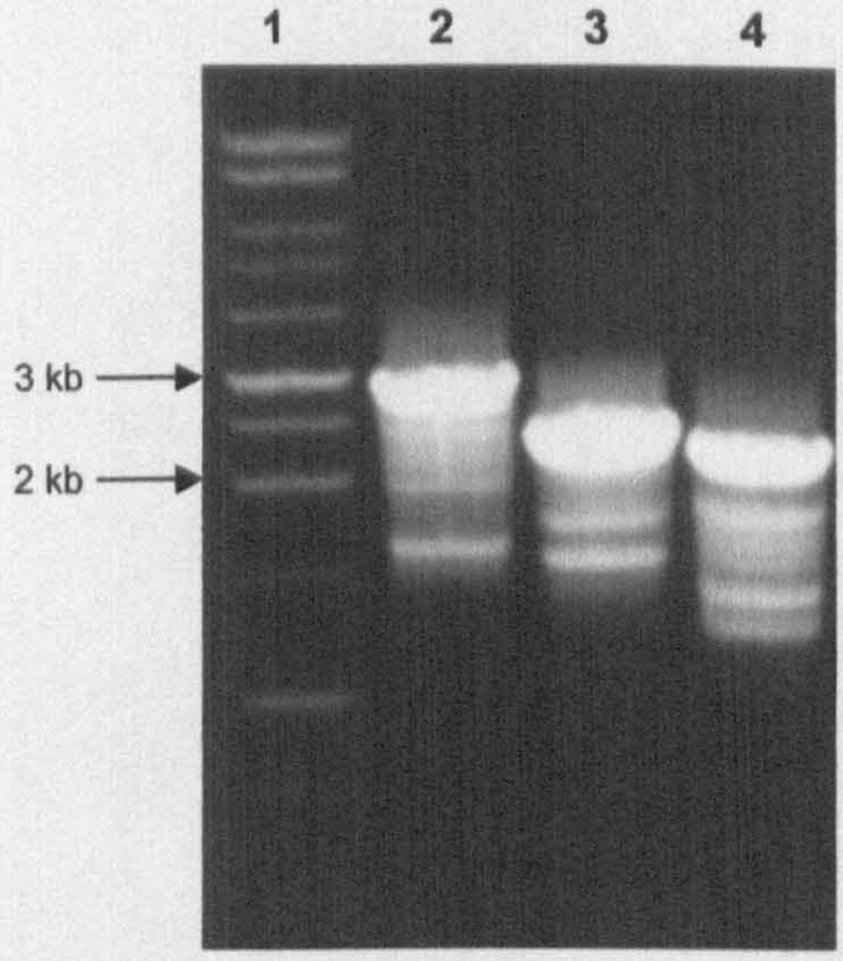


Figure 5.19: 1% (w/v) TAE agarose gel analysis of PCR products from amplification of *scaD* ligations. Lane 1, molecular size marker; 2, Arm A + Kan (Primers Mut1/KanR); 3, Arm B + Kan (Primers KanF/Mut4); 4, Arms A + B + Kan (Primers Mut1/Mut4). Results indicate the successful amplification of Arms A and B each with the Kan cassette (bands at 2.8 kb and 2.3 kb, respectively). The 3-way ligation (lane 4) failed to produce a product at the expected size of 3.6 kb. Correct fragments were named AK (Arm A + Kan) and BK (Arm B + Kan).

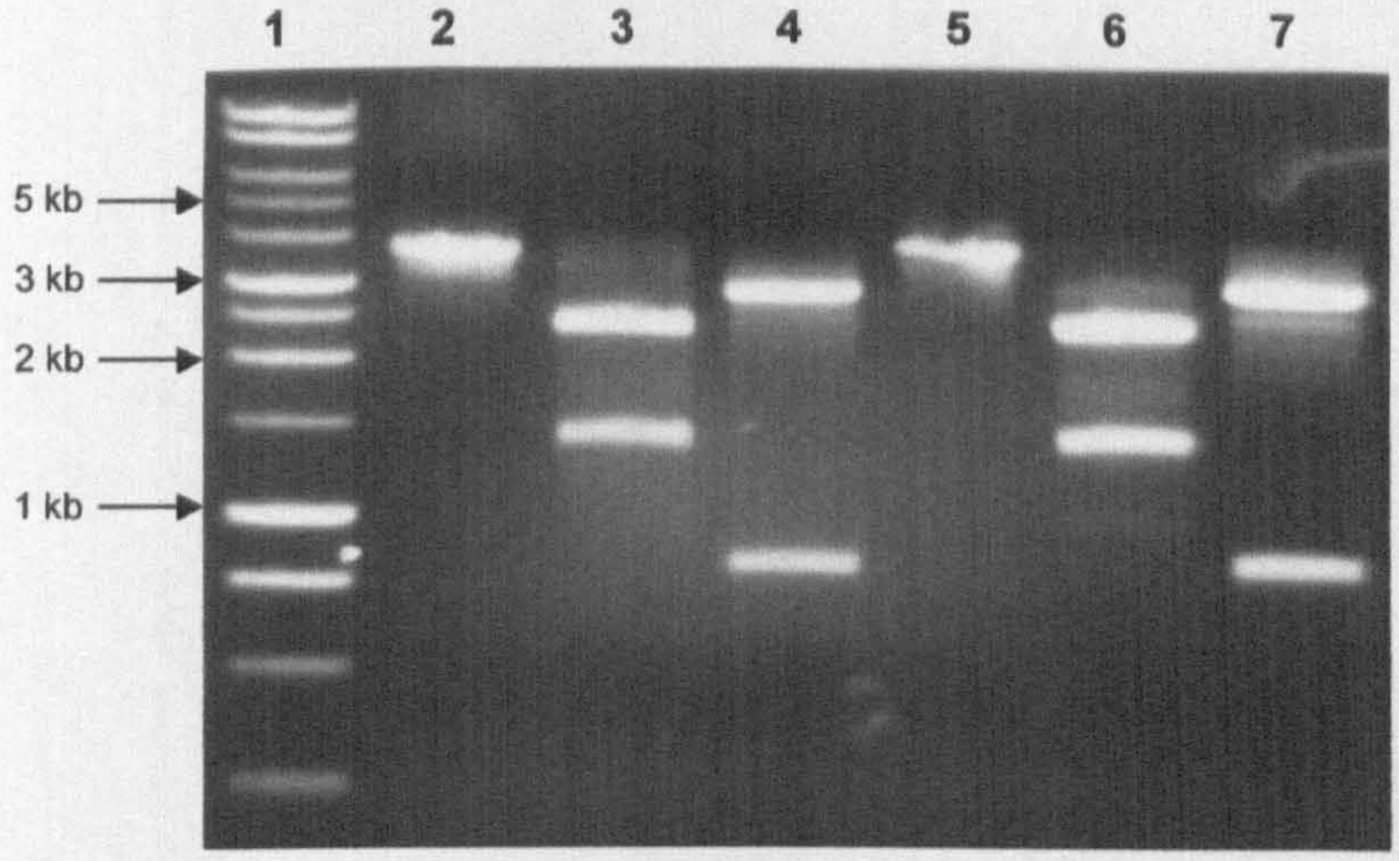


Figure 5.20: 1% (w/v) TAE agarose gel analysis of restriction digests of fragments ABK. Lanes 2-4) AK + Arm B; Lanes 5-7) BK + Arm A. Lane 1, molecular weight marker; 2 and 5, undigested; 3 and 6, *Sal*I-digested; 4 and 7, *Xho*I-digested. Results demonstrate the correct amplification of the insert with bands at the expected sizes of 3.6 kb (undigested), 2.3 kb and 1.3 kb (*Sal*I), and 2.8 kb and 0.8 kb (*Xho*I).

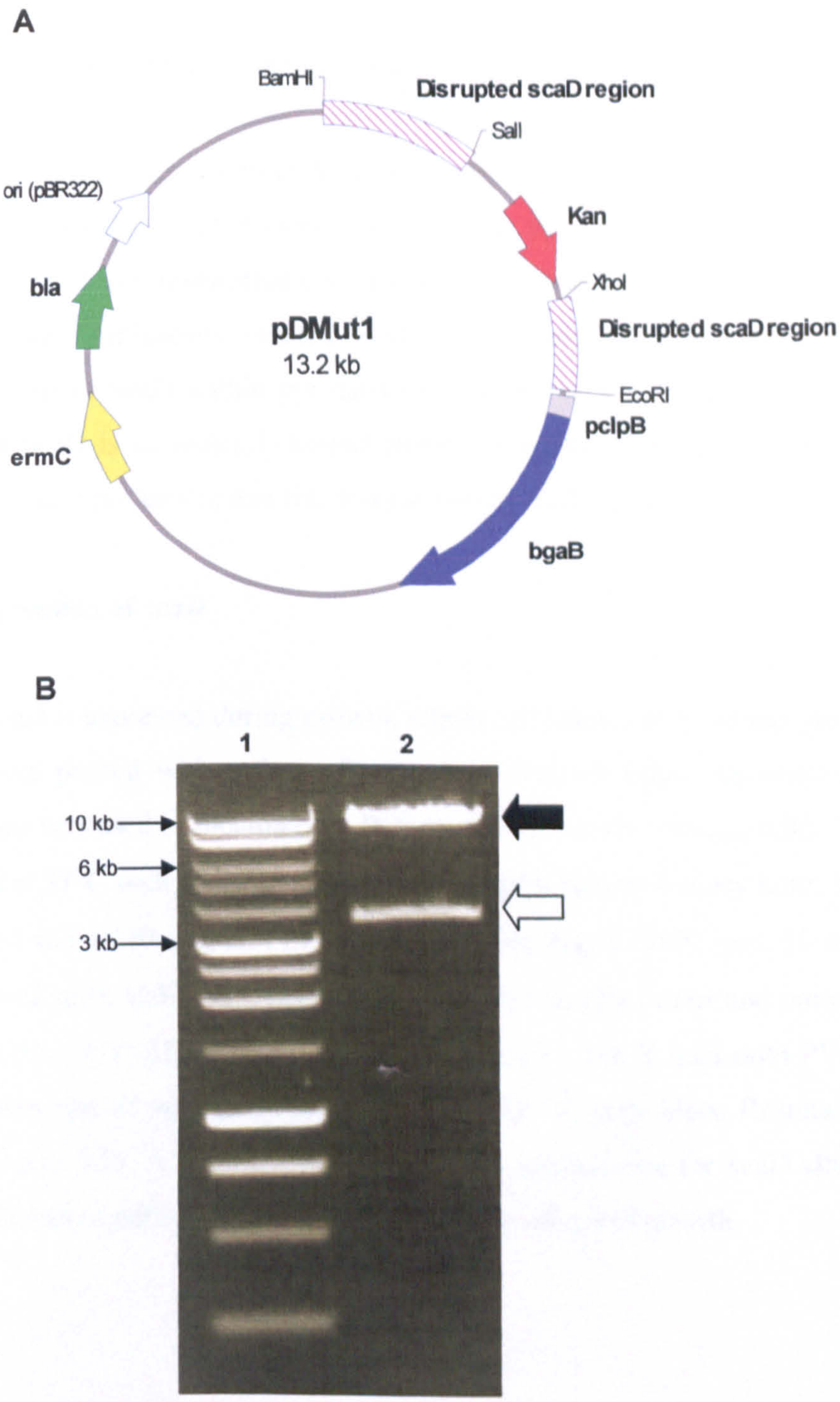


Figure 5.21: Panel A, Map of pDMut1; Panel B, 1% (w/v) TAE agarose gel analysis of pDMut1 digested with *Bam*HI and *Eco*RI. Lane 1, molecular size marker; 2, digested pDMut1. The pMAD vector is shown at 9.7 kb by the filled arrow, the 3.6 kb insert is shown by the unfilled arrow.

5.2.3. Putative identification of ScaD in the native cell walls of *S. aureus*

Native cell wall material was prepared from *S. aureus spa* (Chapter 2.11.2) and proteins separated by 17.5% (w/v) SDS-PAGE together with rScaD. Proteins were stained with Coomassie blue, as well as being transferred onto a PVDF membrane for Western blotting using anti-rScaD serum. Antibodies reacted with rScaD and identified a protein corresponding to the size of ScaD within the native cell walls of *S. aureus spa* (Figure 5.22), suggesting that ScaD is an ionically-bound protein. The anti-ScaD antibodies also cross-reacted with two other proteins in this fraction (shown by unfilled arrows).

5.2.4. Analysis of expression of *scaD*

To determine when *scaD* is expressed during growth, whole cell lysates of *S. aureus* grown to different points were probed with anti-rScaD serum in Western blots. An overnight culture of *S. aureus spa* was used to inoculate 1 l BHI to approximately OD_{600nm} 0.05. The culture was incubated at 37°C with 250 rpm shaking and samples removed every hour. The OD_{600nm} was measured and 1 OD_{600nm} unit of culture was centrifuged (5500 rpm, 10 min, 4°C) to recover bacterial cells. Cells were resuspended in 15 µl sample buffer and proteins were separated by 17.5% (w/v) SDS-PAGE. Separated proteins were blotted onto PVDF membranes, which were probed with anti-rScaD antibodies in Western blots. Results are shown in Figures 5.23 and 5.24. A fragment was seen at the expected size for ScaD after 1 h, indicating that ScaD was rapidly produced in the logarithmic phase of growth.

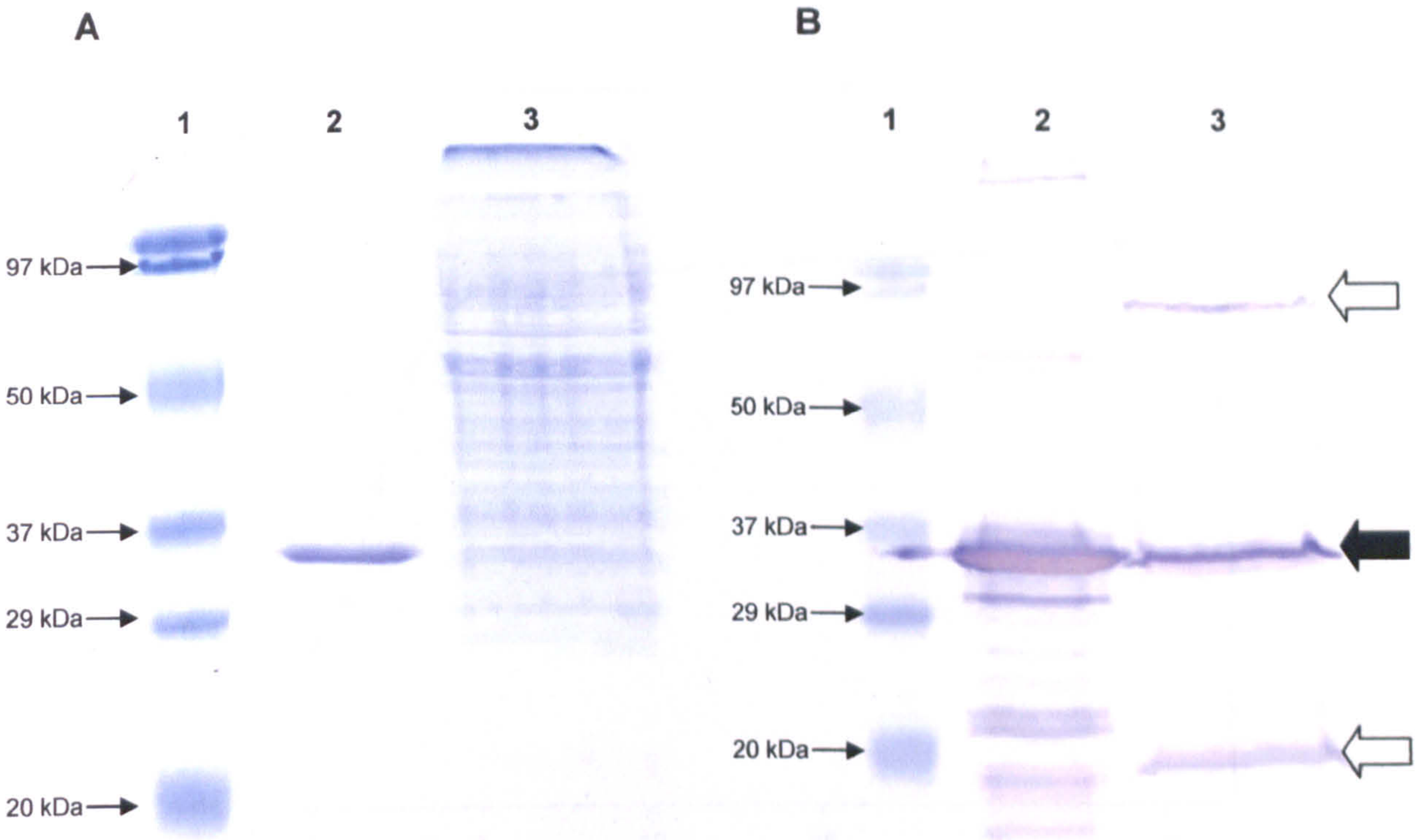


Figure 5.22: Western blot analysis using anti-rScaD antibodies. Proteins were separated by 17.5% (w/v) SDS-PAGE and either stained with coomassie blue (Panel A) or Western blotted with anti-rScaD serum (Panel B). Lane 1, molecular weight marker; Lane 2, rScaD; Lane 3, native cell wall material of *S. aureus spa*. The size of rScaD is shown by the filled arrow. Anti-rScaD antibodies also reacted with other proteins in this fraction, shown by the unfilled arrows.

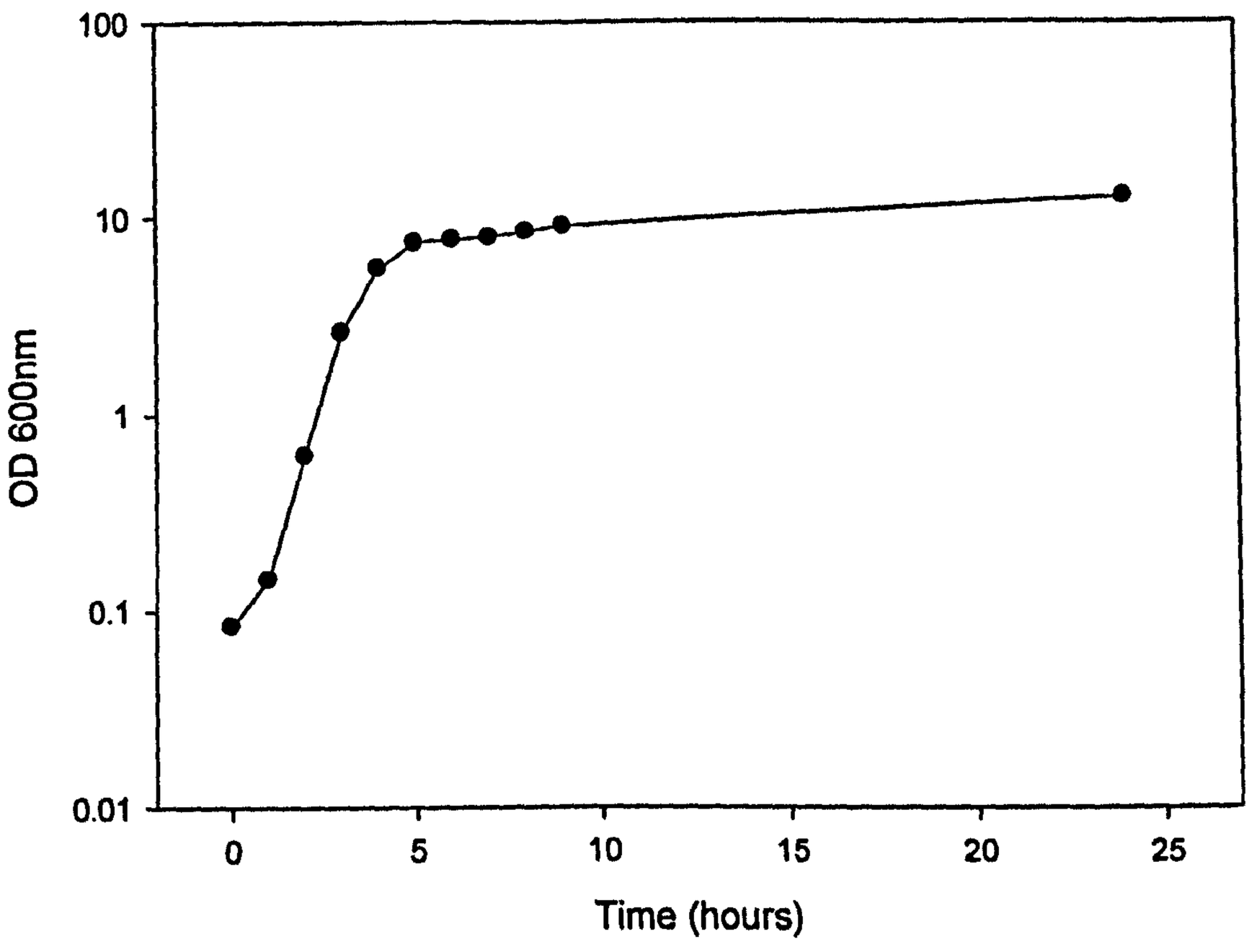


Figure 5.23: Growth of *S. aureus spa*. The strain was grown in 1 l BHI at 37°C, 250 rpm, and the OD_{600nm} measured every hour during the logarithmic phase of growth.

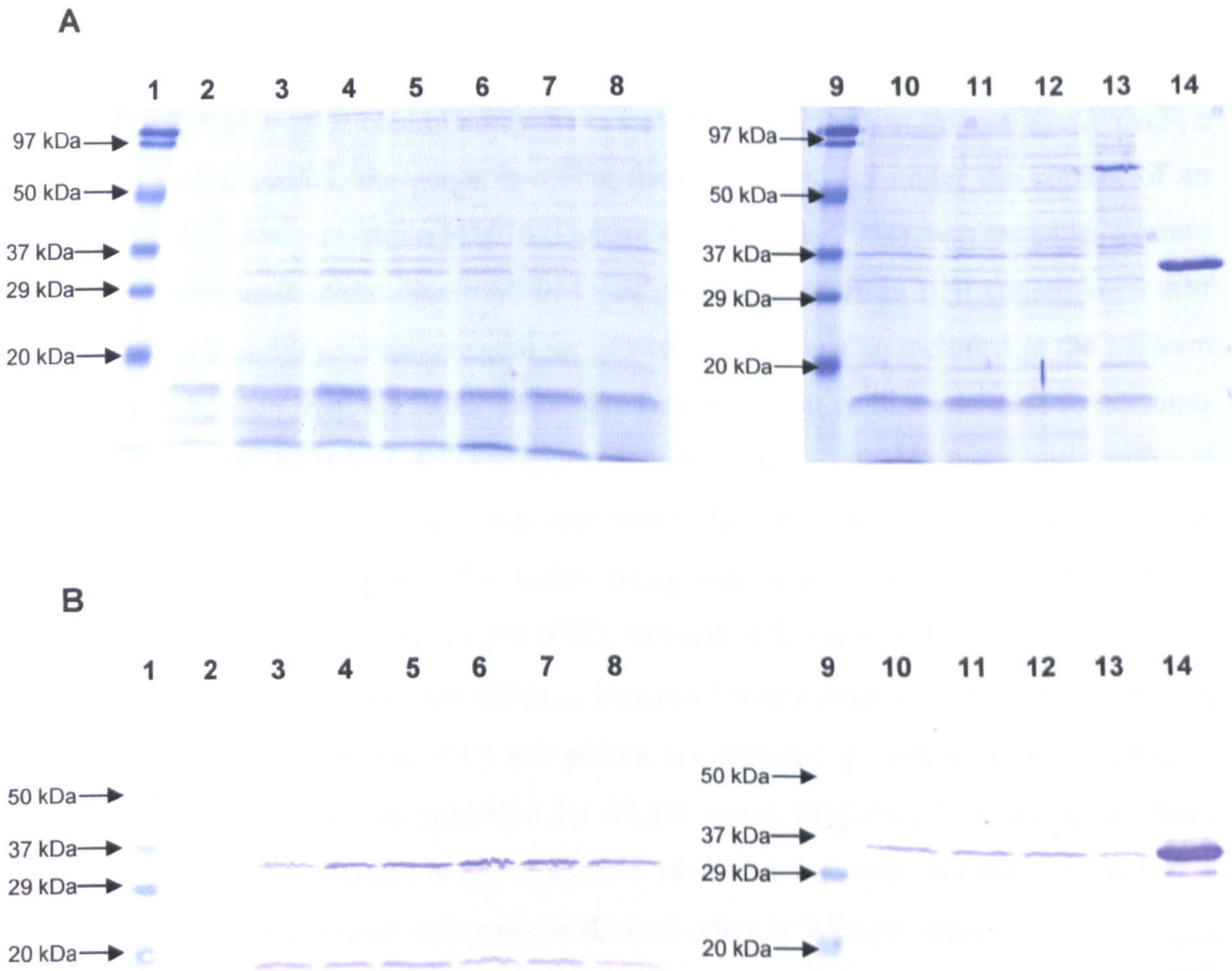


Figure 5.24: Western blot analysis using anti-rScaD antibodies. *S. aureus spa* was grown in 1 l BHI at 37°C, 250 rpm, and 1 OD_{600nm} unit of culture removed at intervals shown below. Cells were recovered by centrifugation and whole cell lysates were separated by 17.5% (w/v) SDS-PAGE. Gels were either stained with Coomassie blue (Panel A) or Western blotted with anti-rScaD serum (Panel B). Lane 1, molecular mass marker; 2, 0 h; 3, 1 h; 4, 2 h; 5, 3 h; 6, 4 h; 7, 5 h; 8, 6 h; 9, molecular mass marker; 10, 7 h; 11, 8 h; 12, 9 h; 13, 24 h; 14, rScaD.

5.2.5. Depletion of *yycFG*

To verify whether *scaD* is controlled by the essential two-component system YycG/YycF, a mutant in which *yycFG*, the genes encoding these proteins, are under the control of an IPTG-inducible promoter, strain MS014 (Dubrac and Msadek, 2004), was tested in Western blots with anti-rScaD serum. Strain MS014 was grown overnight in BHI containing 1 mM IPTG. As YycF and YycG are essential for growth, IPTG must be included in the medium to induce expression from the P*spac* promoter. This overnight culture was used to inoculate 50 ml BHI containing 10 μM IPTG to approximately OD_{600nm} 0.05. Cultures were grown at 37°C, 250 rpm, until the OD_{600nm} was approximately 1.0. Cells were then washed three times with BHI containing no IPTG before being resuspended to OD_{600nm} 0.05 in 25 ml BHI containing either no IPTG, 10 μM IPTG, 100 μM IPTG or 1 mM IPTG. Cultures were grown at 37°C, 250 rpm, and the OD_{600nm} measured every hour for 3 h. Cells were then harvested (5500 rpm, 10 min, 4°C) and pellets resuspended in sample buffer. 1 OD_{600nm} unit of pelleted culture was separated by 17.5% (w/v) SDS-PAGE, along with rScaD protein. One gel was stained with coomassie blue and one was blotted onto a PVDF membrane which was probed with anti-rScaD antibodies in Western blots.

Results are shown in Figures 5.25 and 5.26. Growth without IPTG rapidly ceased and little ScaD protein reacted with the antibodies. A small amount was seen in the Western blot analysis, however, this may be due to remaining protein from initial growth in 10 μM IPTG. The level of ScaD present increased as the amount of IPTG in the medium increased, as did growth. When MS014 was grown in 100 μM or 1 mM IPTG, growth rates were comparable to that of the wild-type and equivalent amounts of ScaD were present.

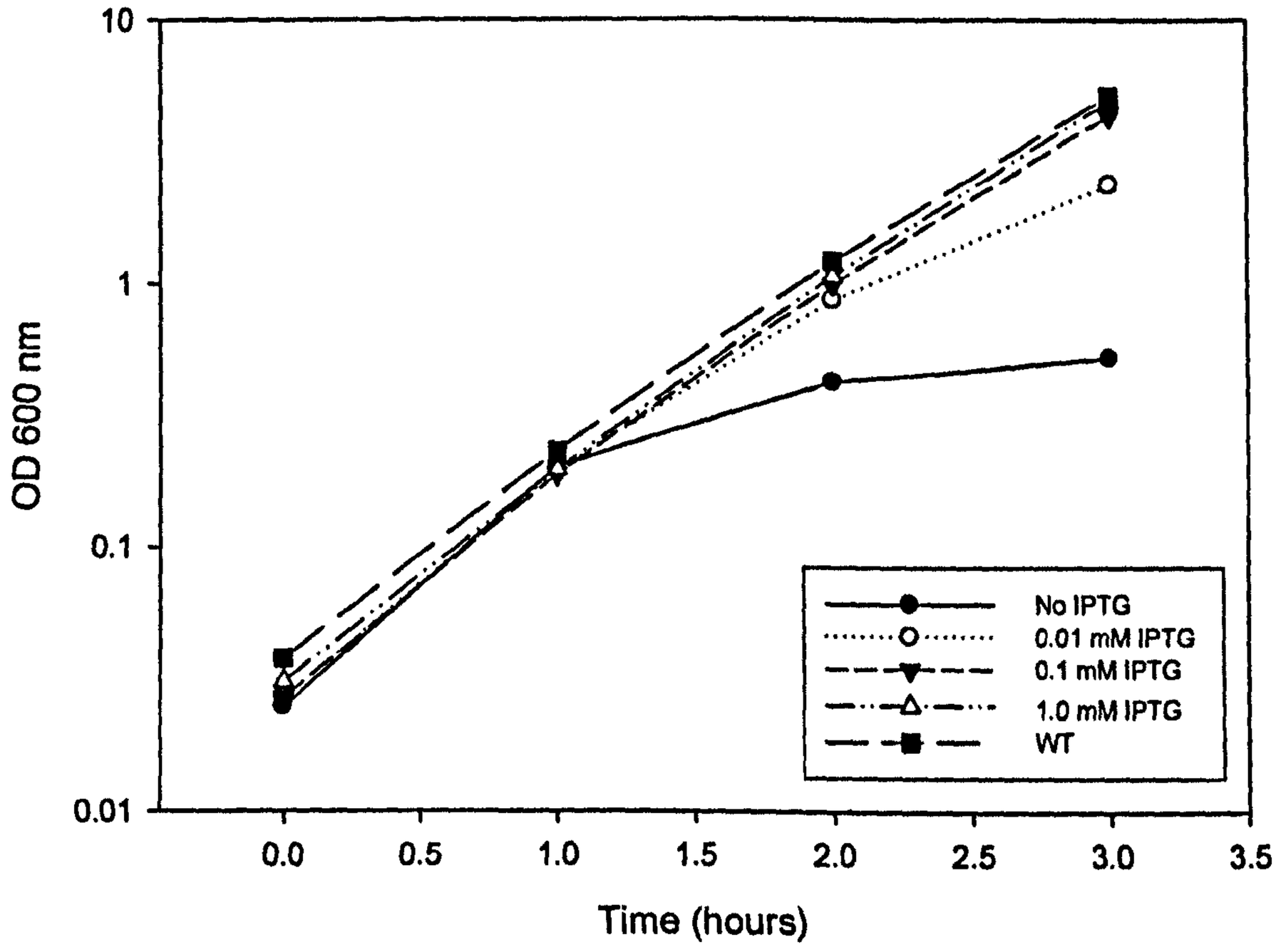
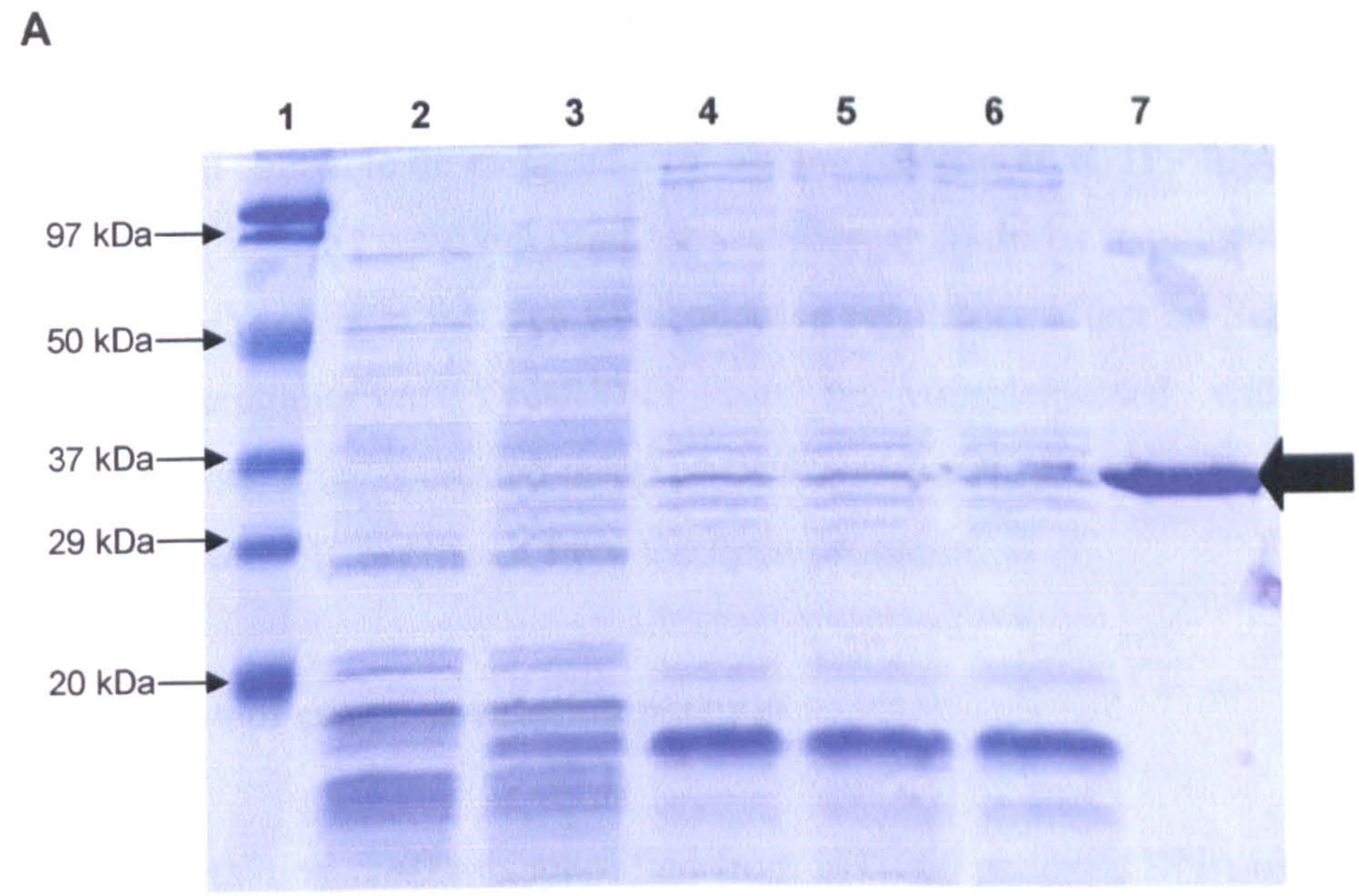


Figure 5.25: Growth of strain MS014 in depleting levels of IPTG (and wild-type RN4220). The strain was grown at 37°C, 250 rpm in IPTG concentrations of 0 – 1 mM and the optical density at 600 nm measured every hour. Concentrations of IPTG are detailed in the key.



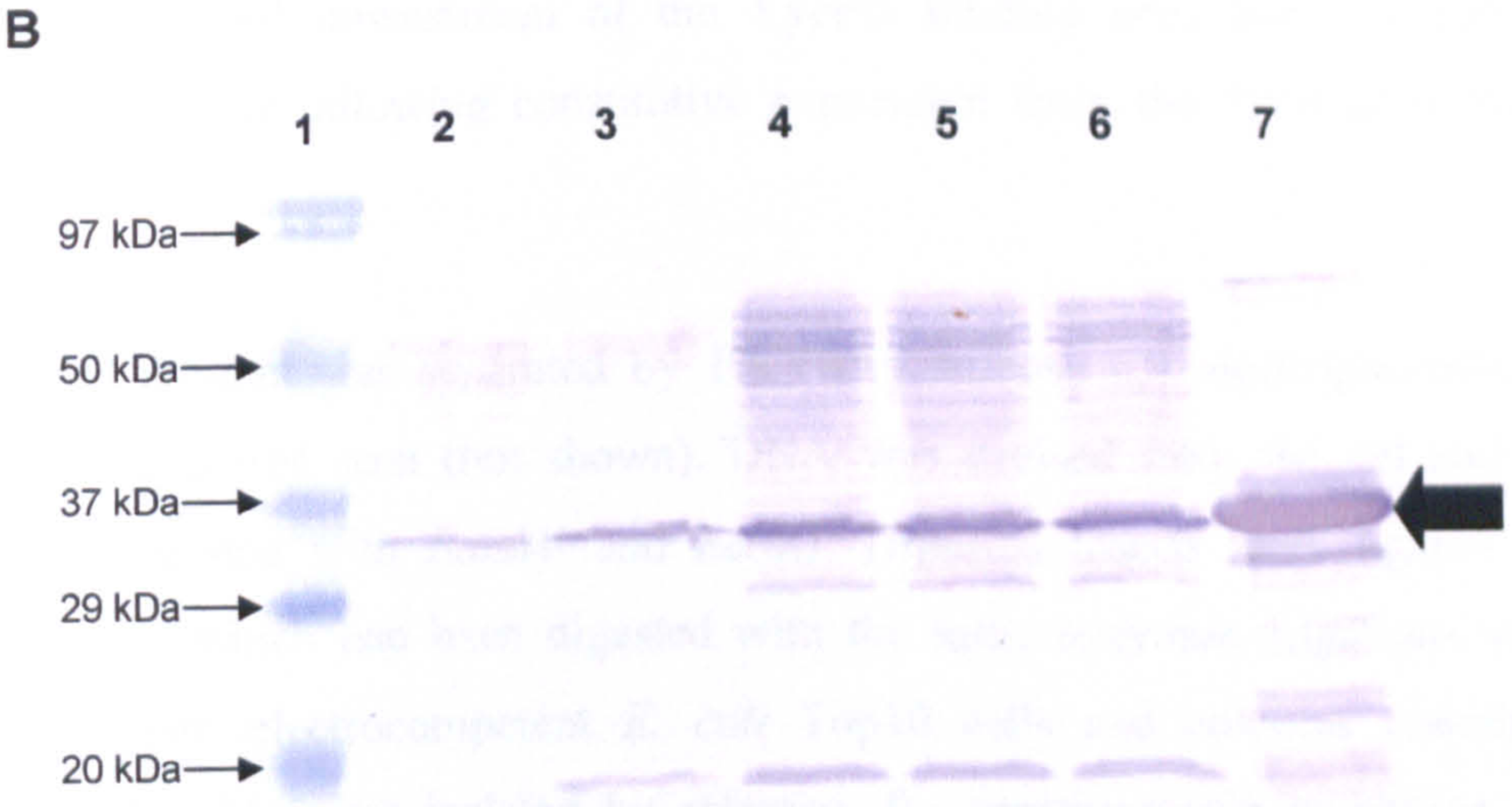


Figure 5.26: Western blot analysis of whole cell lysates of strain MS014 grown in depleting concentrations of IPTG. 1 OD_{600nm} unit of whole cells were separated by 17.5% (w/v) SDS-PAGE along with rScaD. Panel A, Coomassie-stained gel; Panel B, Western blot probed with rScaD antiserum. Lane 1, molecular weight marker; 2, no IPTG; 3, 10 μM IPTG; 4, 100 μM IPTG; 5, 1 mM IPTG; 6, RN4220; 7, rScaD. The approximate size of ScaD is highlighted by the arrows.

5.2.6. Complementation of the essential YycG/YycF two-component system with ScaD

YycG/YycF has been shown to be essential in *S. aureus* (Martin *et al.* 1999). Although not proven in this study, if ScaD were essential for viability, it could be hypothesised that the essentiality of YycG/YycF is in fact due to its positive regulatory effect on ScaD. In order to see if the essentiality of YycG/YycF can be complemented with ScaD, a complementation construct was created using pMJ8426 (Jana *et al.* 2000) (Figure 5.27), in which *scaD* is constitutively expressed from the P*pcn* promoter.

5.2.6.1. Construction of complementation plasmid

A 1 kb fragment containing *scaD* was amplified from SH1000 genomic DNA using primers CompF and CompR (Table 2.6). Primers were designed to allow the in-frame expression of *scaD* and *Bam*HI and *Eco*RI restriction sites were engineered to facilitate cloning. Primer CompF was designed downstream of the YycFG binding sites and upstream of the ribosome binding site, allowing constitutive expression from the P*pcn* promoter in the vector.

The amplified product was separated by 1% (w/v) agarose gel electrophoresis and the correct sized band was seen (not shown). DNA was excised from the gel and purified before being digested with *Bam*HI and *Eco*RI. Digested inserts were ligated into the pMJ8426 vector, which had been digested with the same enzymes. Ligations were then electroporated into electrocompetent *E. coli* Top10 cells and colonies containing the recombinant plasmids were isolated by selecting for spectinomycin resistance. Plasmid DNA was purified from transformants using Qiagen mini-prep kits (Chapter 2.16.5) and recombinant plasmids were digested with *Bam*HI and *Eco*RI and separated by 1% (w/v) agarose gel electrophoresis to confirm the presence of the insert (Figure 5.28 B). The recombinant plasmid containing the *scaD* insert was named pDComp and is shown in Figure 5.28 A.

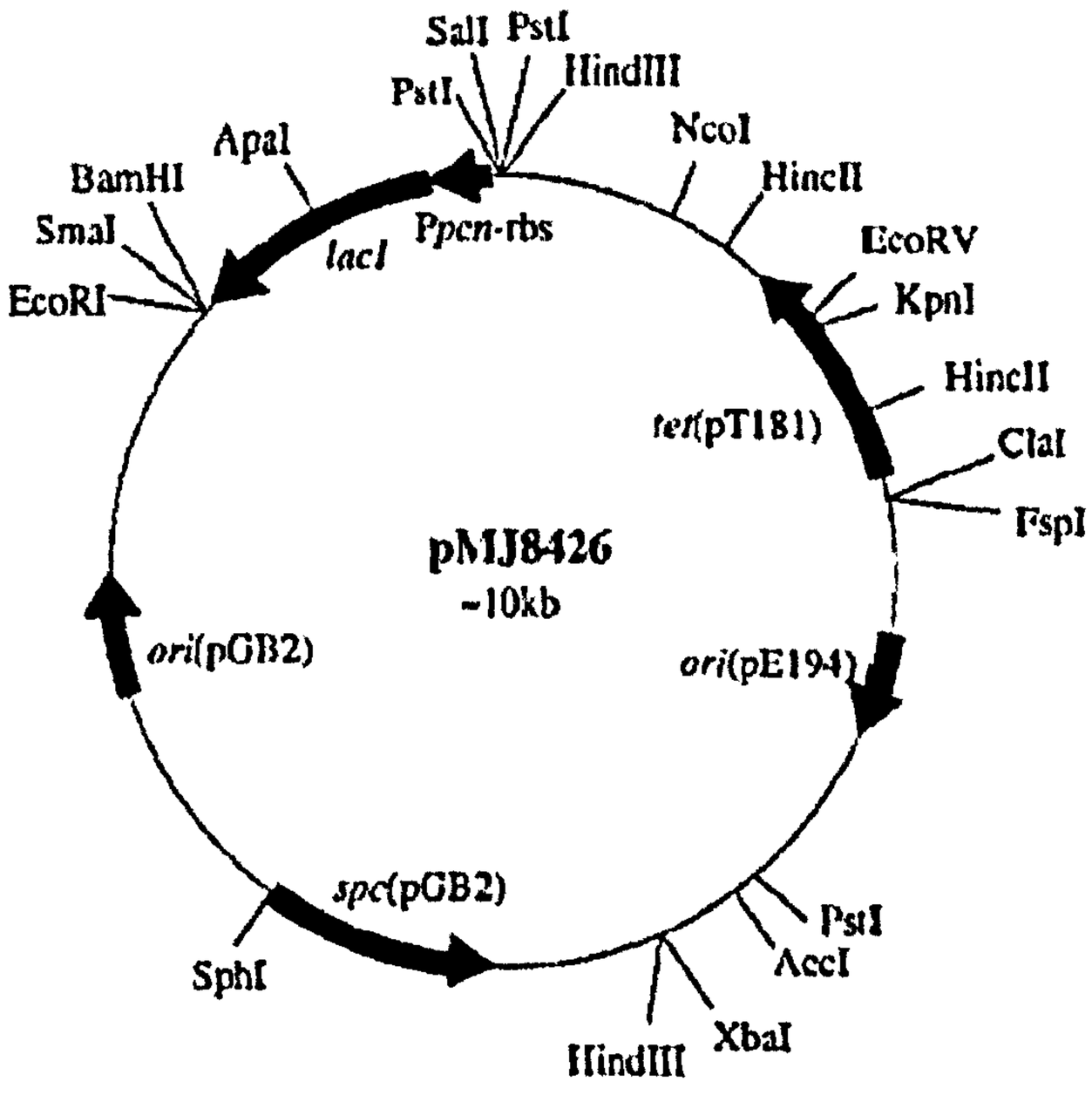
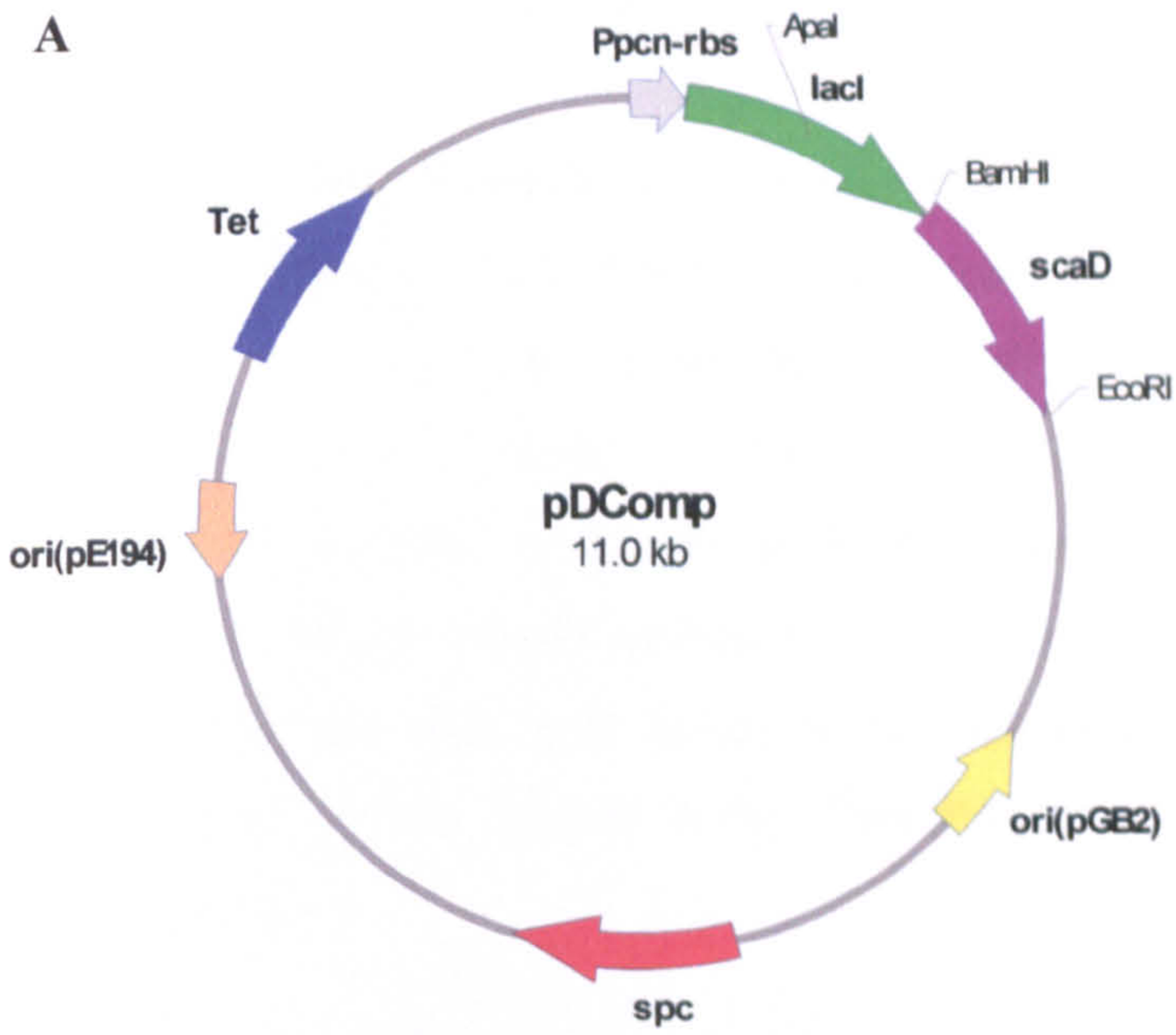


Figure 5.27: Map of pMJ8426 (reproduced from Jana *et al.* 2000). The plasmid contains a spectinomycin (*spc*) resistance gene for selection in *E. coli* and a tetracycline (*tet*) resistance gene for selection in *S. aureus*. A *lacI* gene is also present under the control of the constitutive P*pcn* promoter.



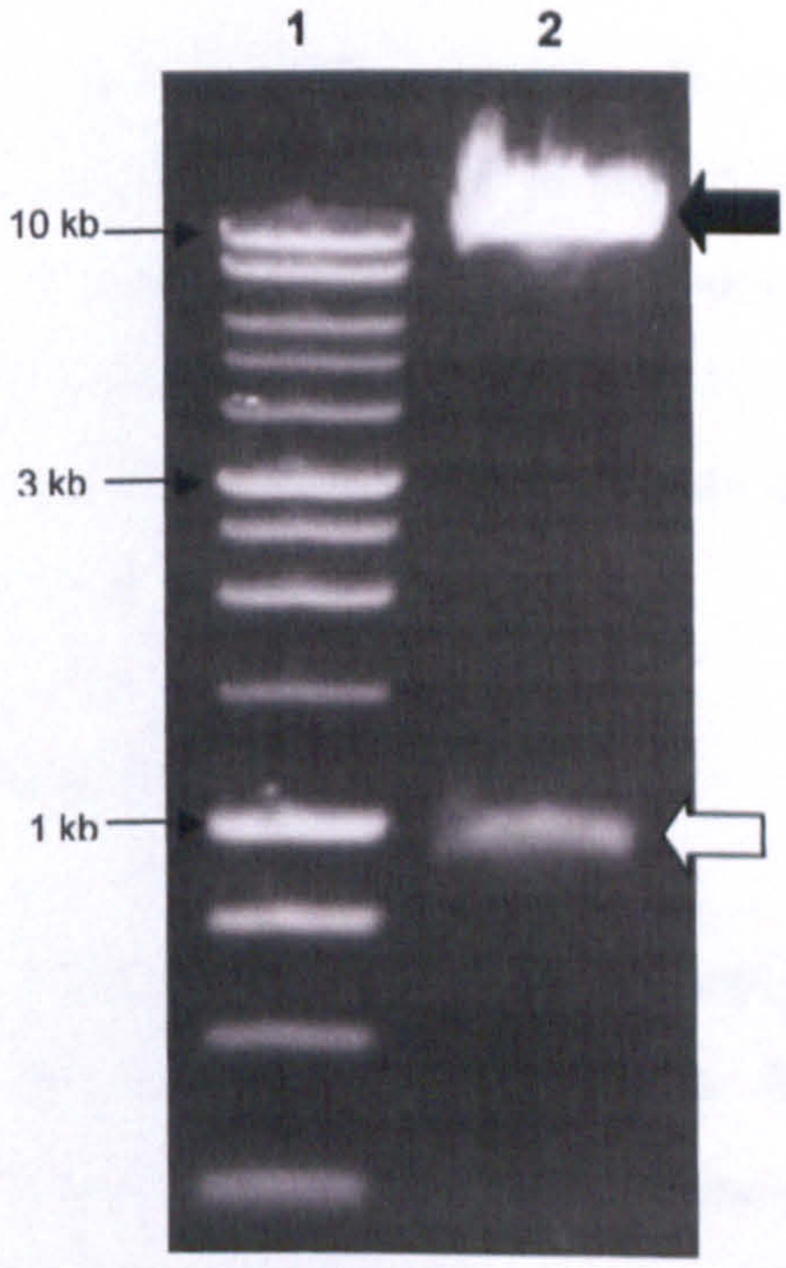


Figure 5.28: Panel A, Map of pDComp; Panel B, 1% (w/v) TAE agarose gel analysis of pDComp digested with *Bam*HI and *Eco*RI. Lane 1, molecular size marker; 2, digested pDComp. The pMJ8426 vector is shown at ~10 kb by the filled arrow, the ~1 kb insert is shown by the unfilled arrow.

5.2.6.2. Transformation of constructs into RN4220

Recombinant pDComp and pMJ8426 plasmids were transformed into competent *S. aureus* RN4220 by electroporation (Chapter 2.20.2). Positive clones were identified by selecting for tetracycline resistance. To verify the overproduction of ScaD protein in the complementation plasmid, whole cell lysates of RN4220 and RN4220 pDComp were separated by 17.5% (w/v) SDS-PAGE and proteins blotted onto PVDF membranes. Membranes were then probed with anti-rScaD antibodies in Western blots. Results showed that more protein reacted with the anti-rScaD serum at the size expected for the strain carrying the complementation plasmid (Figure 5.29). Therefore, the overproduction of ScaD in pDComp was confirmed.

5.2.6.3. Transduction of plasmids into *yycFG* mutant

Phage transduction (Chapter 2.19) was used to transfer pDComp and pMJ8426 into strain MS014, which contains *yycFG* under the control of the IPTG-inducible promoter P*spac* (Chapter 5.2.5). Transductants were selected on LK top agar containing tetracycline and positive clones were verified by purification of plasmid DNA using Qiagen midi-prep kit and restriction digestion using *Bam*HI and *Eco*RI. Digests were analysed by 1% (w/v) agarose gel electrophoresis (not shown).

5.2.6.4. Complementation of YycG/YycF with ScaD

To determine the ability of ScaD to complement the essentiality of YycG/YycF, MS014 containing pMJ8426 and pDComp were streaked onto BHI agar plates containing tetracycline both with and without IPTG. Plates were incubated overnight at 37°C and the level of growth compared. Figure 5.30 demonstrates that MS014 containing the control plasmid is unable to grow in the absence of IPTG. However, when ScaD is overproduced via the plasmid pDComp, growth occurs independently of IPTG.

To further test the complementation, strains RN4220, MS014, MS014 pMJ8426 and MS014 pDComp were grown in BHI with and without IPTG. For strains carrying a plasmid, tetracycline was also included in the medium. Strains were grown overnight in 5 ml BHI containing 1 mM IPTG and 5 μg/ml tetracycline, where appropriate. The overnight cultures were then used to inoculate 50 ml BHI containing 10 μM IPTG to approximately OD_{600nm} 0.05. Cultures were grown at 37°C until OD_{600nm} 0.5 and cells harvested by centrifugation (5500 rpm, 10 min, 4°C). Cells were washed three times with BHI, before being resuspended to OD_{600nm} 0.01 in 50 ml BHI with and without 1 mM IPTG. Cultures were then incubated at 37°C for 5 hours and the OD_{600nm} measured every hour. Figure 5.31 demonstrates that growth of MS014 is comparable to that of wild-type (RN4220) in the presence of IPTG, but growth is rapidly arrested in the absence of the inducer, when no *yycFG* is expressed. The strain containing the control plasmid pMJ8426 exhibited a similar pattern, with growth only occurring in the presence of the inducer. However, strain MS014 pDComp grew at a similar rate both with and without IPTG. The growth rate of this strain was lower than that of MS014 pMJ8426 in the presence of IPTG, however, the overproduction of ScaD in this strain complemented the essentiality of YycFG and allowed growth to occur independently of the inducer.

After 5 h growth, cells were harvested by centrifugation (5500 rpm, 10 min, 4°C) and 1 OD_{600nm} unit of each pelleted culture was resuspended in sample prep buffer. Whole cell lysates were then separated by 17.5% (w/v) SDS-PAGE. One gel was stained with Coomassie-blue and one gel was transferred onto a PVDF membrane by electroblotting. The membrane was then probed with ScaD antiserum in a Western blot. Figure 5.32 shows that strain MS014 pDComp produced a larger amount of ScaD compared to RN4220, MS014 and MS014 pMJ8426.

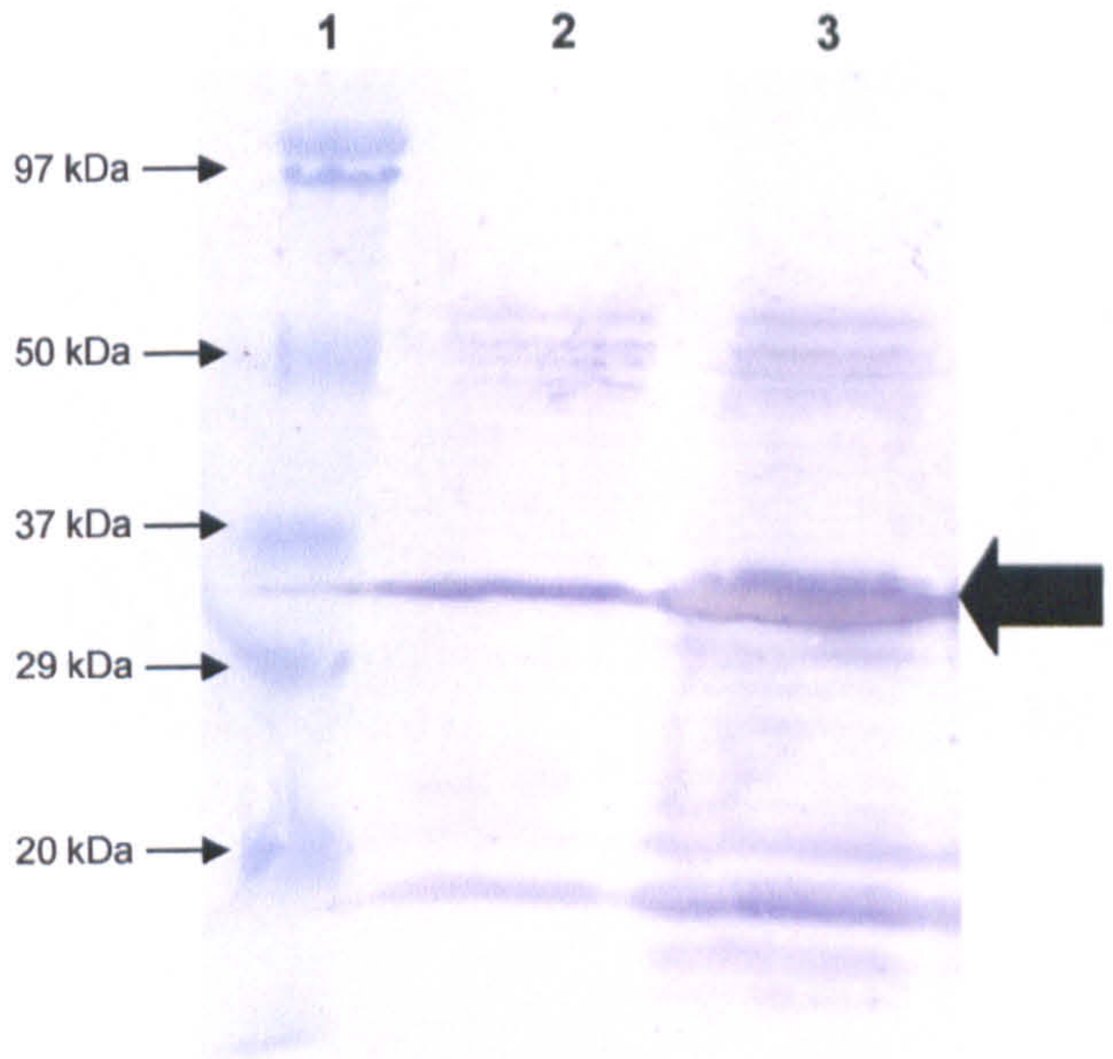


Figure 5.29: Western blot analysis of ScaD complementation strains. Whole cell lysates of RN4220 and RN4220 pDComp were separated by 17.5% (w/v) SDS-PAGE, proteins were blotted onto membranes and probed with anti-rScaD antibodies. Lane 1, molecular weight marker; 2, RN4220; 3, RN4220 pDComp. ScaD was successfully overproduced in the strain carrying the plasmid (shown by the filled arrow).

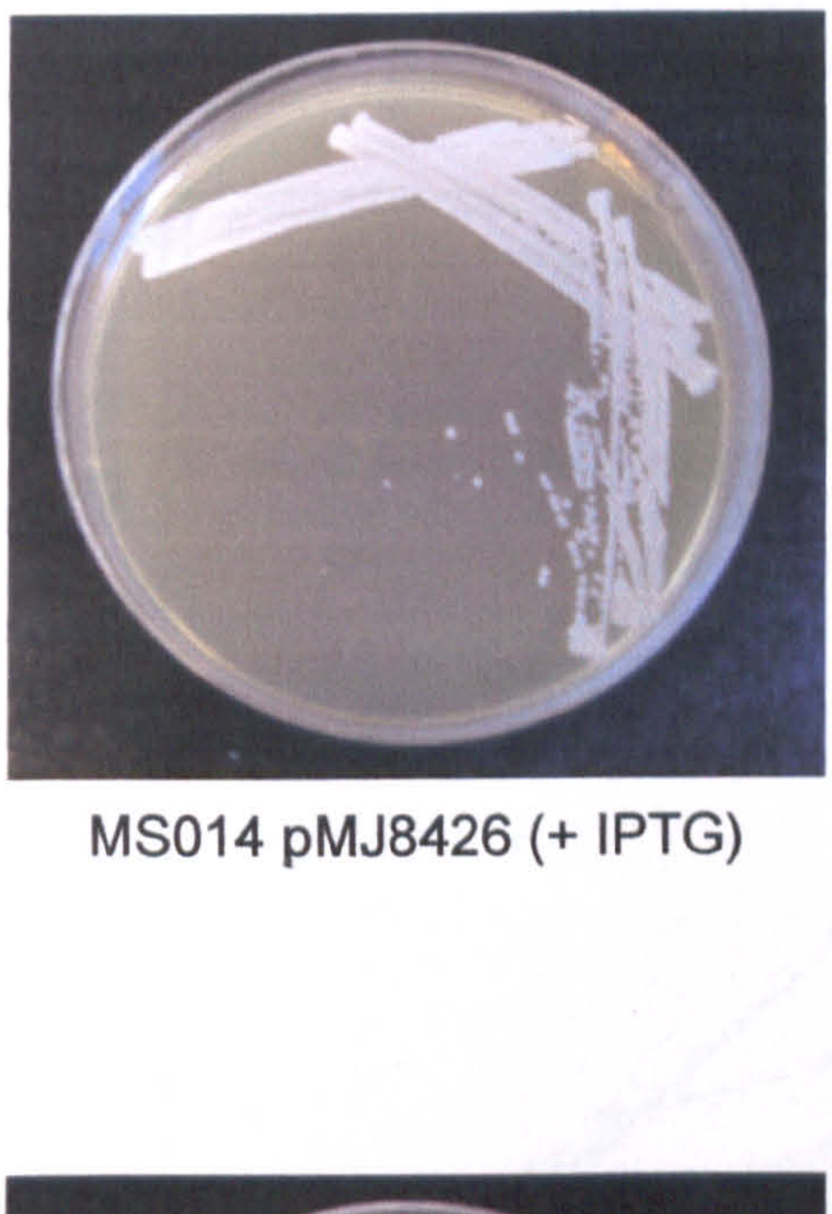


Figure 5.30: Complementation of strain MS014 with ScaD. Strains containing the ScaD complementation plasmid (pDComp) and strains containing a control plasmid (pMJ8426) were streaked onto BHI containing tetracycline both in the absence and presence of IPTG, and incubated overnight at 37°C.

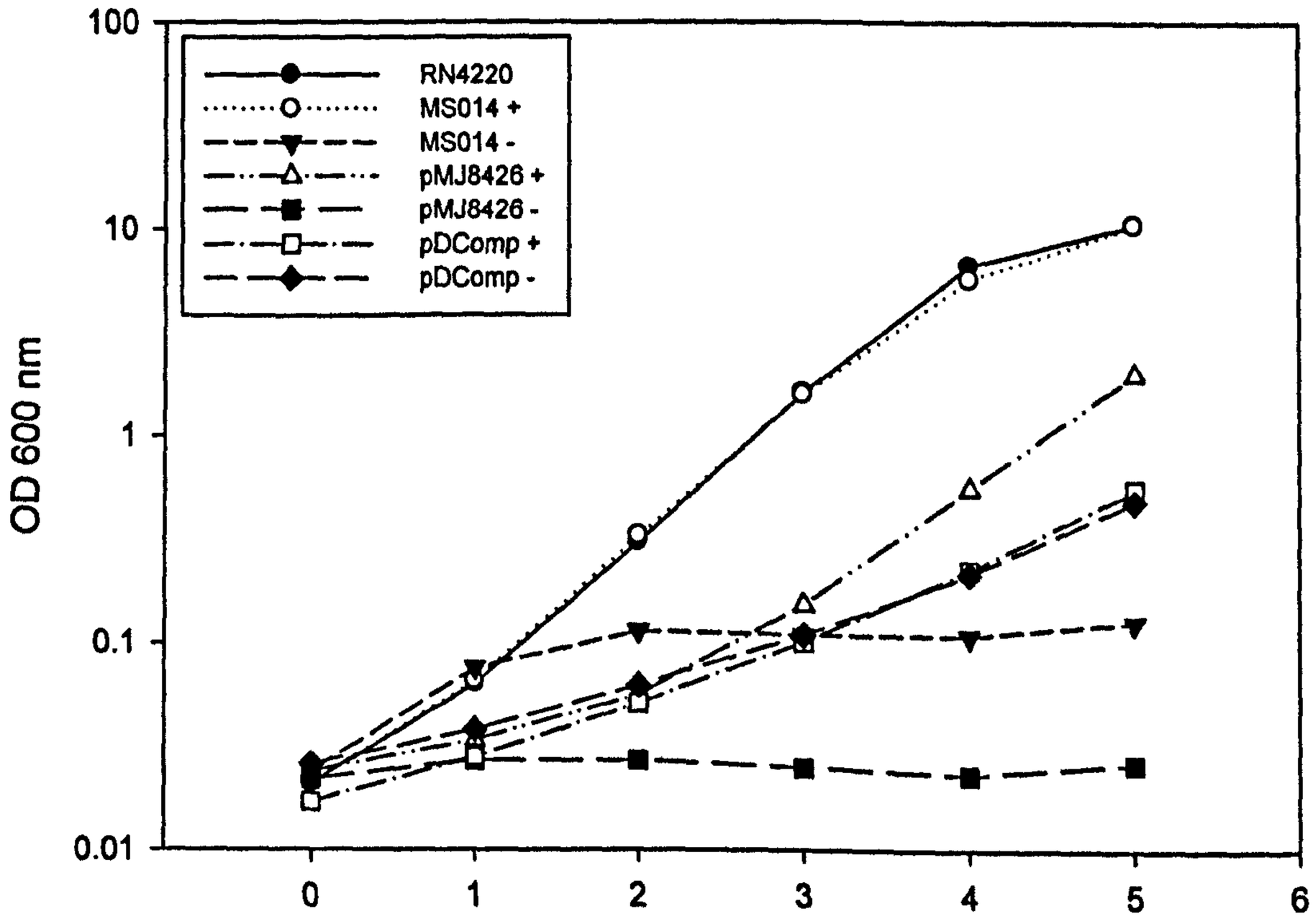


Figure 5.31: Analysis of complementation of the essential YycG/YycF in strain MS014 by overproduction of ScaD. Strains were grown in BHI both with (+) and without (-) IPTG. Strains RN4220, MS014, MS014 pMJ8426 and MS014 pDComp are as detailed in the key.

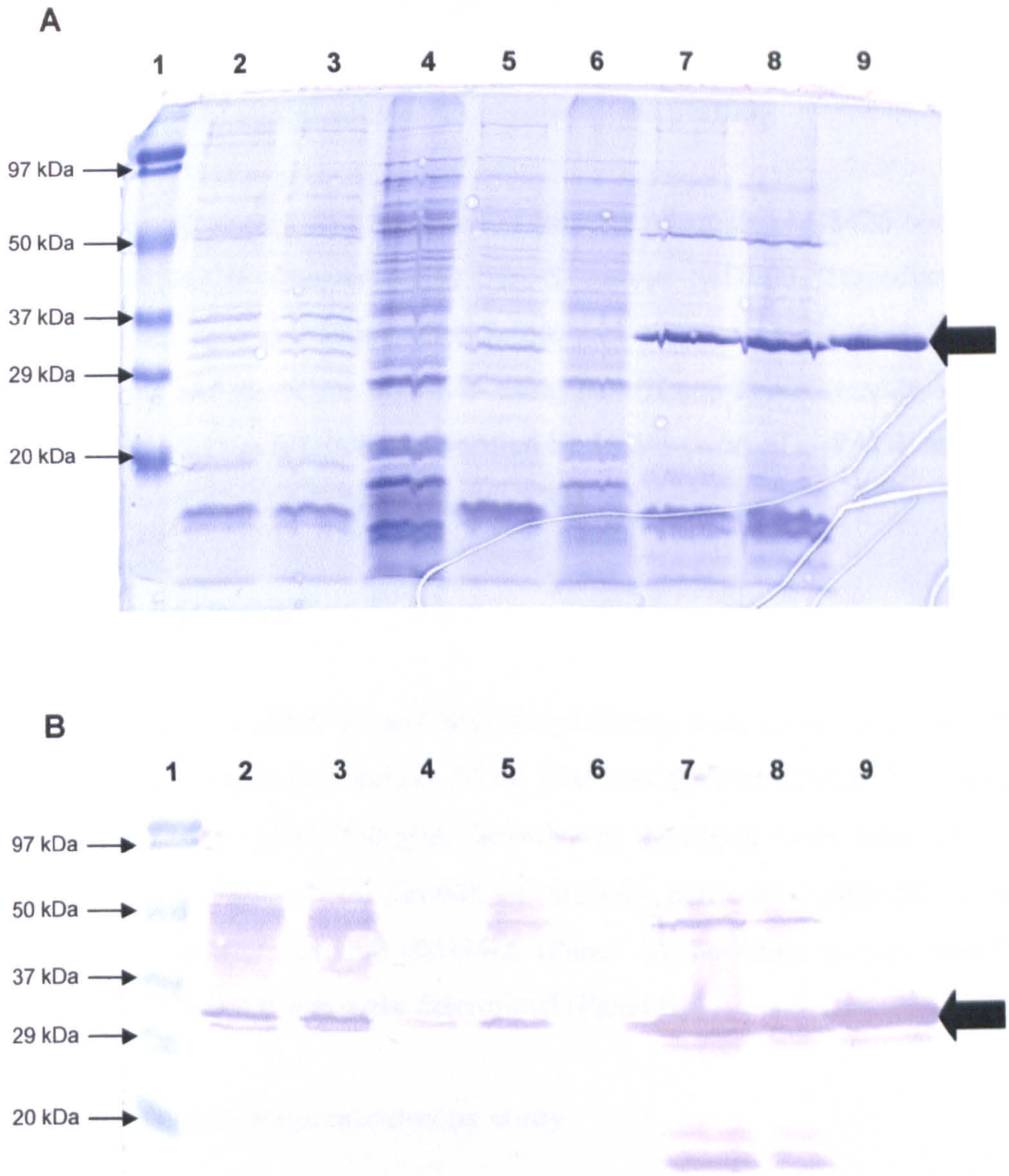


Figure 5.32: Western blot analysis of the overproduction of ScaD in MS014. 1 OD_{600nm} unit of whole cells were separated by 17.5% (w/v) SDS-PAGE along with rScaD protein. Panel A, Coomassie blue-stained gel; Panel B, western blot probed with rScaD antiserum. Lane 1, molecular mass marker; 2, RN4220; 3, MS014 (+ IPTG); 4, MS014 (no IPTG); 5, MS014 pMJ8426 (+ IPTG); 6, MS014 pMJ8426 (no IPTG); 7, MS014 pDComp (+ IPTG); 8, MS014 pDComp (no IPTG); 9, rScaD. The approximate size of ScaD is shown by the filled arrows.

5.2.7. Overproduction of ScaD in wild-type *S. aureus*

5.2.7.1. Transduction of complementation plasmids into SH1000

Phage transduction (Chapter 2.19) was used to transfer plasmids pMJ8426 and pDComp from *S. aureus* RN4220 (Chapter 5.2.6) into *S. aureus* SH1000. Transductants were selected on LK top agar containing tetracycline and positive clones were verified by plasmid preparation and restriction digestion using *Bam*HI and *Eco*RI (results not shown). Overproduction of ScaD in SH1000 was verified by 17.5% (w/v) SDS-PAGE analysis and Western blotting with rScaD antiserum (Figure 5.33).

5.2.7.2. Growth experiments

Strains SH1000, SH1000 pMJ8426 and SH1000 pDComp were grown overnight in 5 ml BHI with antibiotics, where appropriate. 50 ml BHI was then inoculated to OD_{600nm} 0.01 and cultures incubated at 37°C, 250 rpm. Growth was measured every hour for 12 h and results are shown in Figure 5.34. Growth of SH1000 pDComp displayed comparable optical densities to that of SH1000 pMJ8426 (Panel A), however, growth was notably lower when colony forming units were determined (Panel B).

5.2.7.1. Transmission electron microscopy study

Strains were assessed by transmission electron microscopy as described in Chapter 2.9.2. and results are shown in Figure 5.35. Cells containing only the control plasmid pMJ8426 (Panel B) displayed a similar morphology to the wild-type SH1000 cells (Panel A). However, overproduction of ScaD via the pDComp plasmid (Panel C) led to disruption of cell division. Asymmetric septa were formed leading to irregular cell division.

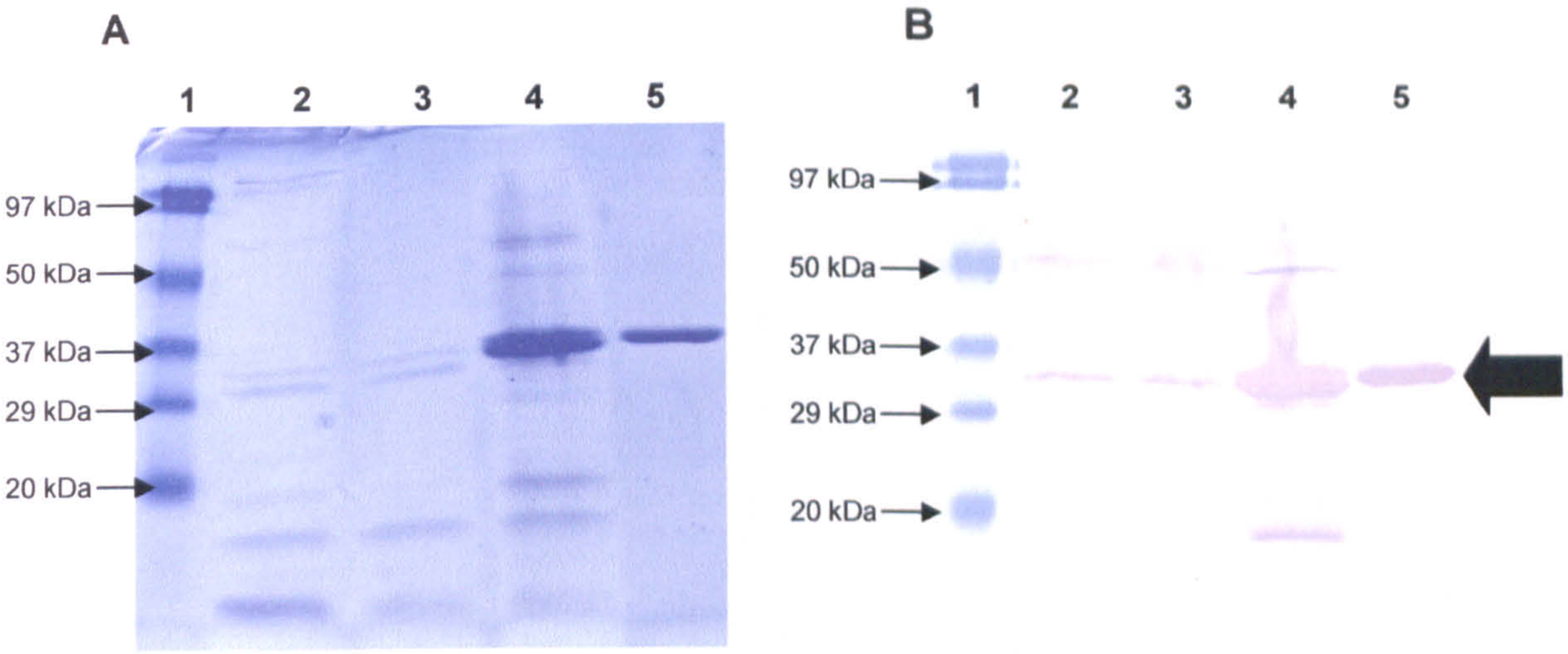
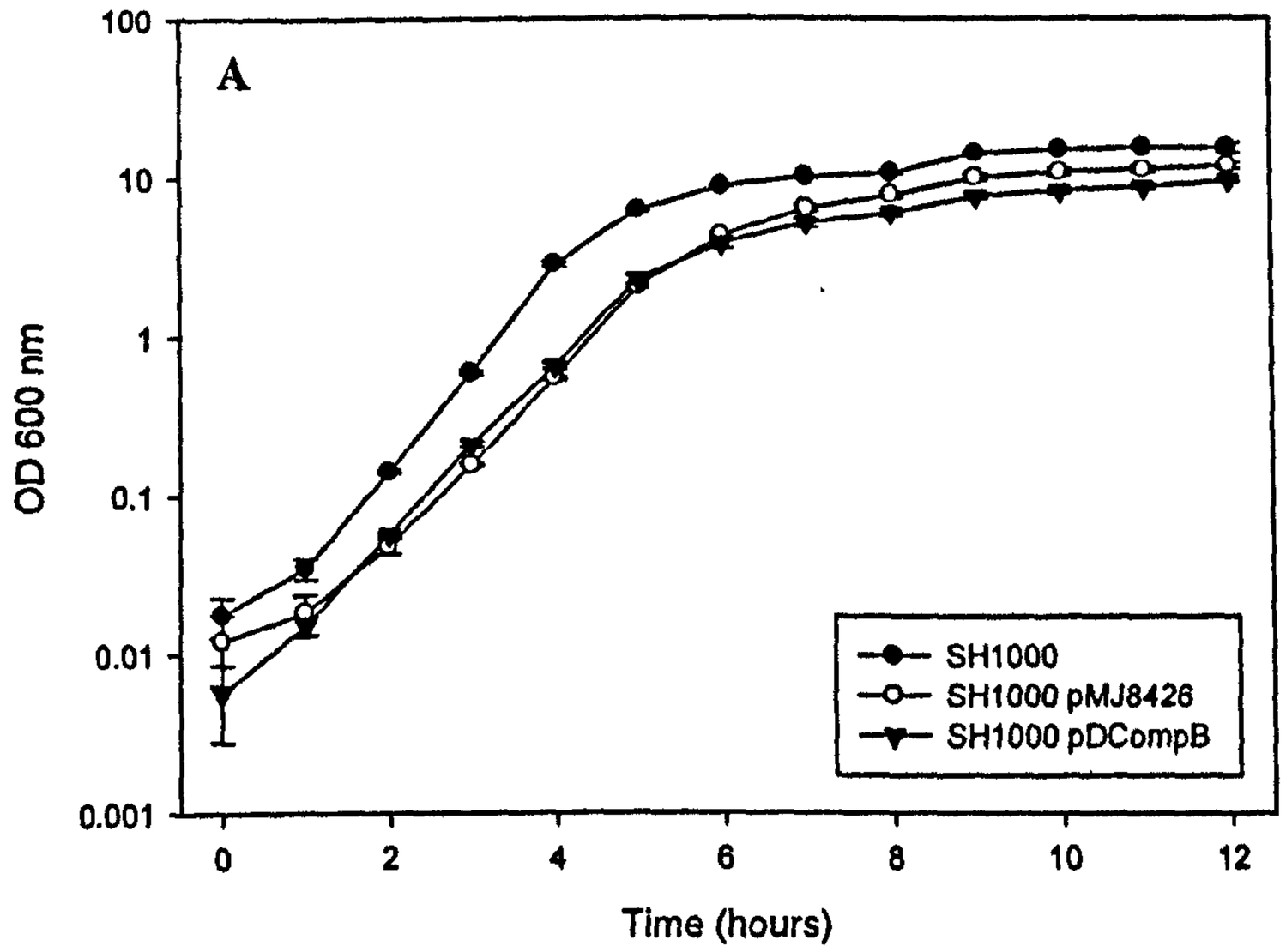


Figure 5.33: Western blot analysis of overproduction of ScaD in *S. aureus* SH1000. 1 OD_{600nm} unit of whole cells were separated by 17.5% (w/v) SDS-PAGE along with rScaD. Panel A, coomassie blue-stained gel; Panel B, western blot probed with rScaD antiserum. Lane 1, molecular weight marker; 2, SH1000; 3, SH1000 pMJ8426; 4, SH1000 pDComp; 5, rScaD. The approximate size of ScaD is highlighted by the filled arrow.



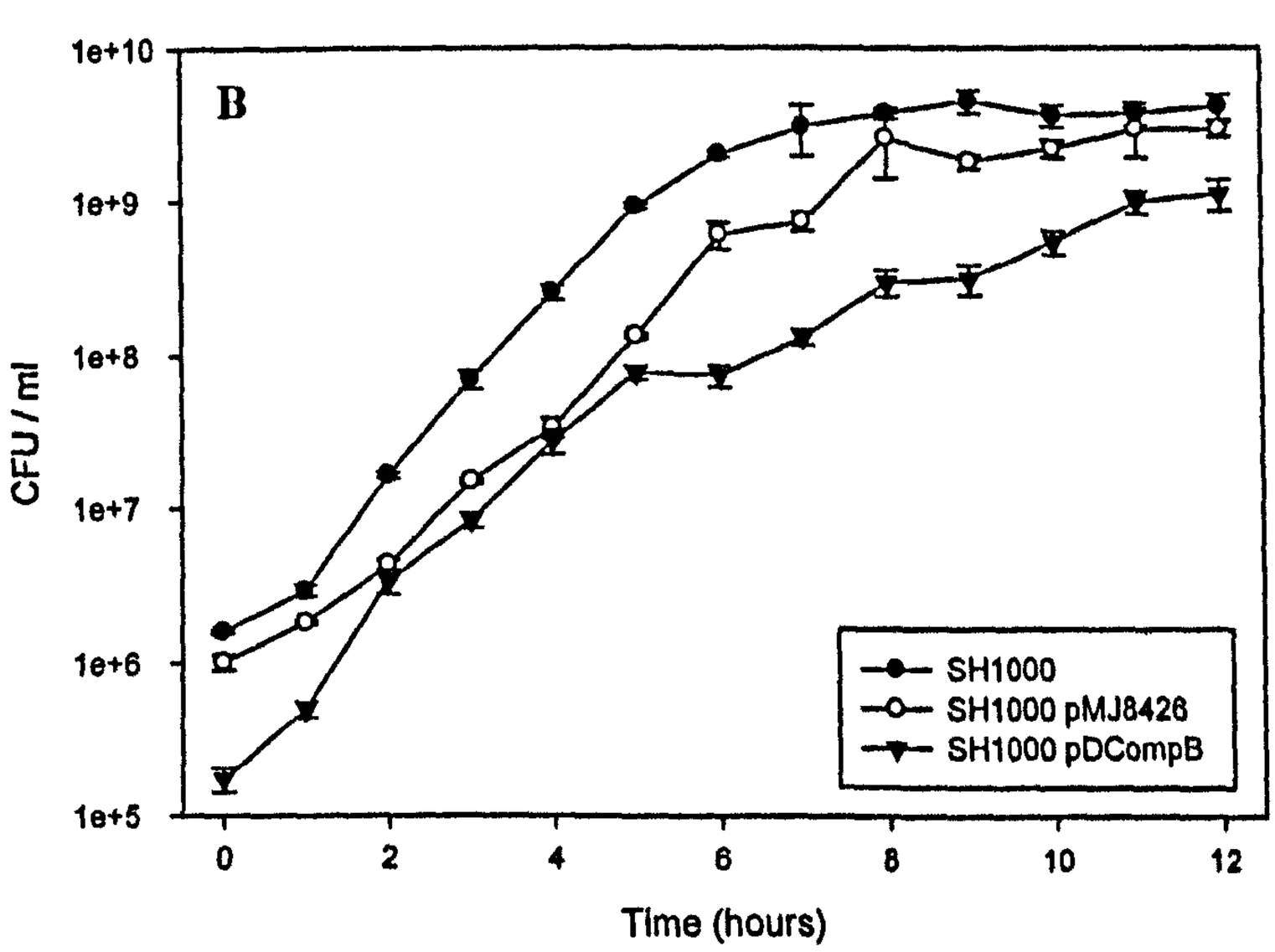


Figure 5.34: Overproduction of ScaD in *S. aureus* SH1000. Strains were grown in BHI and growth measured every hour. Panel A, optical density (OD_{600nm}); Panel B, CFU/ml. Strains are as detailed in the keys, standard error bars are shown.

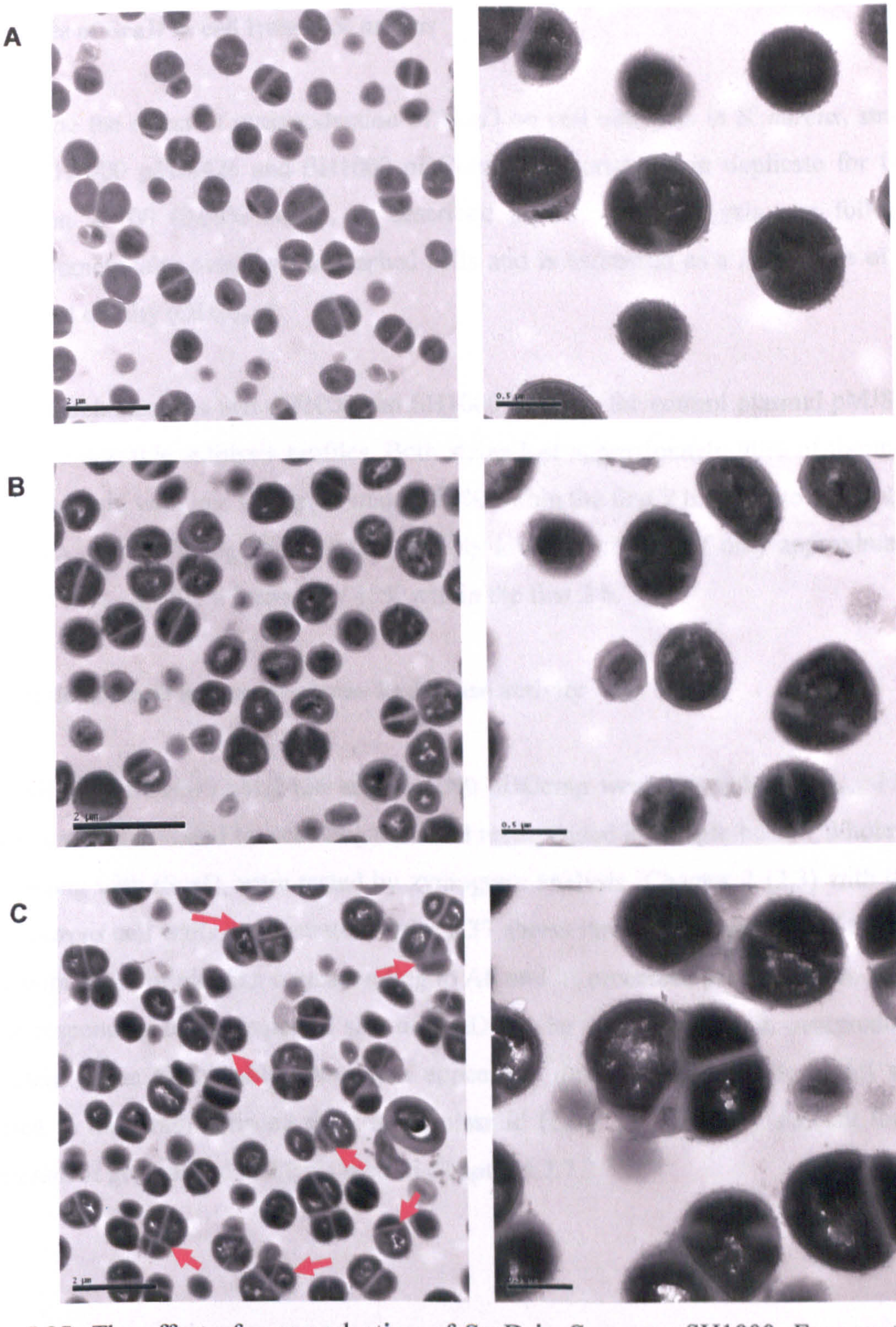


Figure 5.35: The effect of overproduction of ScaD in *S. aureus* SH1000. Exponentially growing bacteria were analysed by transmission electron microscopy. Magnification is shown by the black bars. A) SH1000; B) SH1000 pMJ8426; C) SH1000 pDComp. Asymmetric septa leading to irregular cell division are highlighted by the red arrows.

5.2.7.2. Role of ScaD in cell lysis of *S. aureus*

To determine the effect of overproduction of ScaD on cell autolysis in *S. aureus*, strains SH1000, SH1000 pMJ8426 and SH1000 pDComp were analysed in duplicate for lysis using Triton X-100 (0.05% (w/v)), as described in Chapter 2.8. Lysis was followed spectrophotometrically over time of washed cells and is expressed as a percentage of the initial optical density (OD_{600nm}).

Figure 5.36 demonstrates that SH1000 and SH1000 carrying the control plasmid pMJ8426 displayed comparable autolysis profiles. Both stains lost approximately 90% of the initial OD_{600nm} after 6 h, with loss of approximately 80% within the first 2 h. The rate of autolysis of the strain overproducing ScaD is considerably lower, with loss of only approximately 75% initial OD_{600nm} after 6 h and only 45% within the first 2 h.

5.2.7.3. Role of ScaD in peptidoglycan hydrolase activity

Strains SH1000, SH1000 pMJ8426 and SH1000 pDComp were grown to OD_{600nm} 1.0 in BHI. Cells were harvested by centrifugation and resuspended in sample buffer. Whole cell lysates, along with rScaD, were tested by zymogram analysis (Chapter 2.12.3) with 0.1% (w/v) *S. aureus* cell walls as substrate. Figure 5.37 shows the autolytic profiles of the three strains, with bands of clearing corresponding to Atl and its processed products seen. A faint band corresponding to the expected size of ScaD can be seen in the strain overproducing this protein (Lane 4). Notably, this strain appears to produce lower levels of Atl when compared to the strain carrying the control plasmid (Lane 3). This may account for the reduced rate of generalised cell lysis seen in Chapter 5.2.7.2.

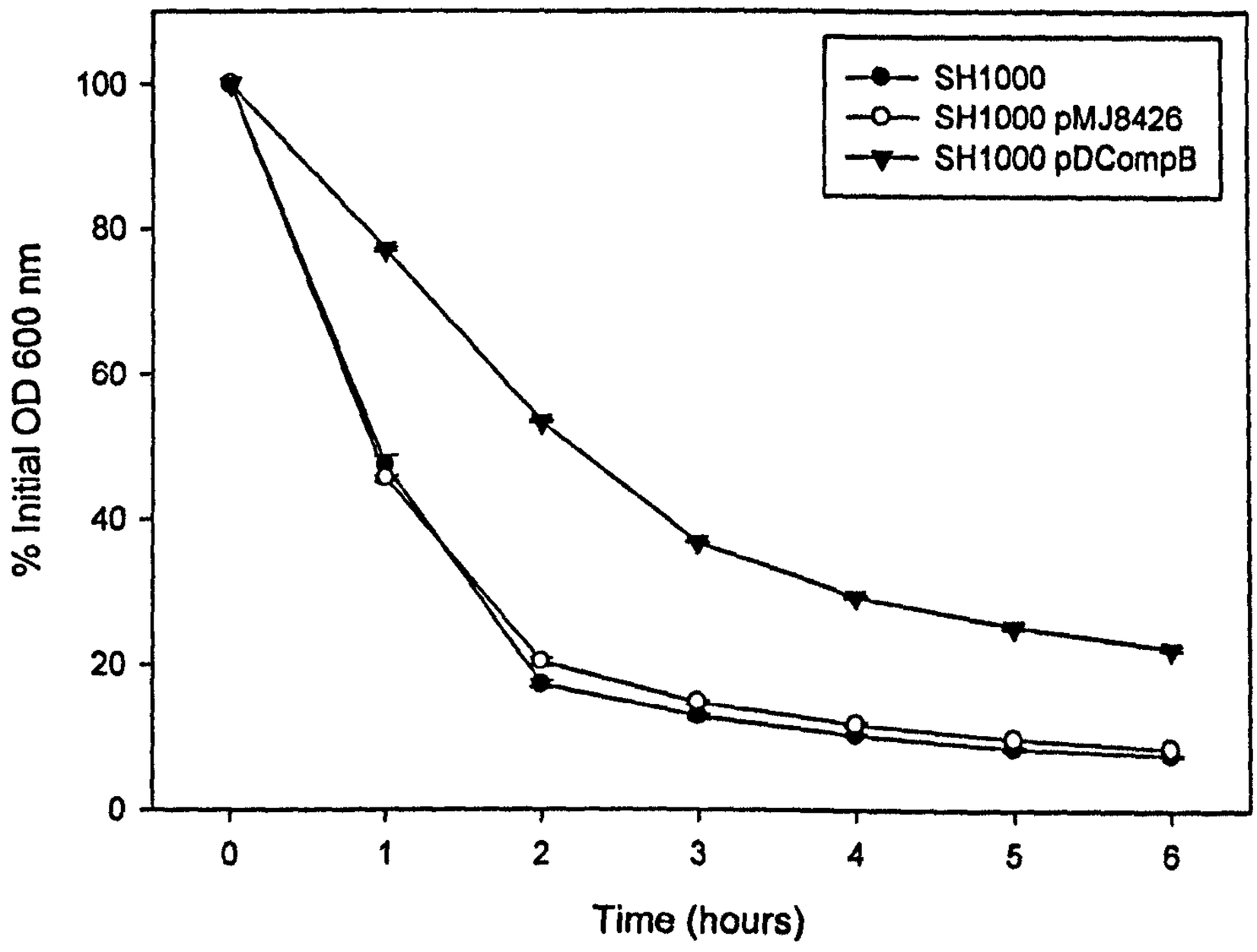


Figure 5.36: Role of ScaD in generalised cell lysis using Triton X-100 (0.05% (w/v)). Strains are as described in the key and represent means of duplicate samples.

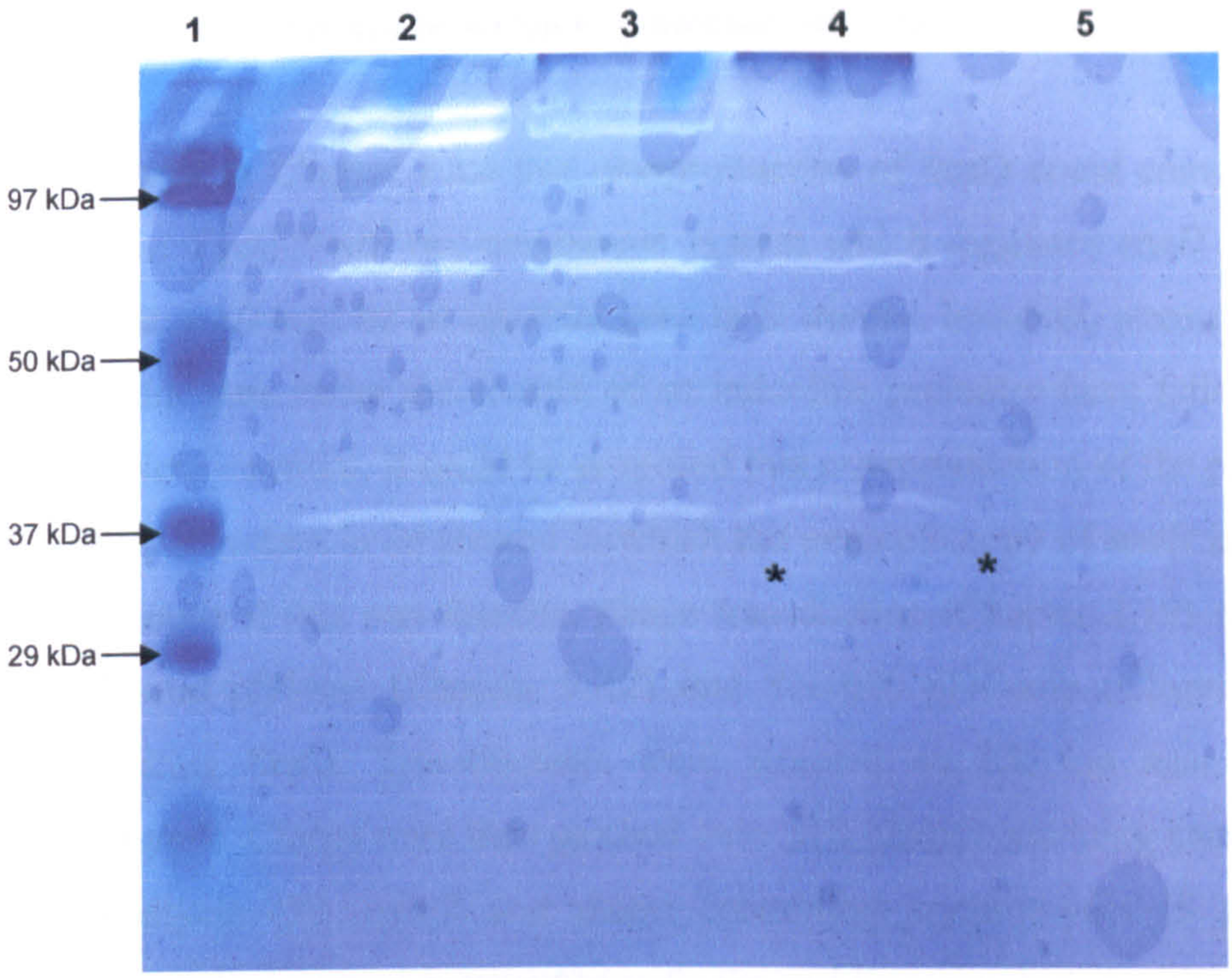


Figure 5.37: Zymography analysis of the effect of overproduction of ScaD in *S. aureus* SH1000 on the autolytic profile. Whole cell lysates and rScaD were analysed by 17.5% (w/v) SDS-PAGE renaturing gel electrophoresis containing 0.1% (w/v) purified *S. aureus* cell walls as substrate. Lytic bands demonstrating autolytic activity are seen as bands of clearing in the stained gel. Lane 1, molecular mass marker; 2, SH1000; 3, SH1000 pMJ8426; 4, SH1000 pDComp; 5, rScaD. Bands corresponding to the approximate size of ScaD are shown by the asterisks.

5.2.8. Creation of *scaD* mutant in strain complemented with ScaD

It was demonstrated in Chapter 5.2.6 that overproduction of ScaD could complement the essentiality of the YycG/YycF two-component system which regulates *scaD* expression. This suggests that *scaD* may be an essential gene in *S. aureus*, however, attempts to verify this by placing the gene under the control of an inducible promoter have failed (Chapter 5.2.1). If ScaD were essential, it could be proposed that overproduction of the protein in *S. aureus* may allow a mutant to be created in which the genomic copy of *scaD* is disrupted. In order to determine if this was feasible, phage transduction (Chapter 2.19) was used to transfer the plasmid pDMut1 (Chapter 5.2.2) into SH1000 pDComp (Chapter 5.2.6), a strain overproducing ScaD. Transductants were selected on LK top agar containing tetracycline (5 µg/ml). Clones were then patched onto BHI plates containing kanamycin (50 µg/ml) and neomycin (50 µg/ml) and plates containing erythromycin (5 µg/ml) and lincomycin (25 µg/ml). Colonies resistant to both Tet and Kan/Neo but sensitive to Ery/Lin would represent putative clones with a disrupted genomic copy of *scaD*. However, after 3 independent attempts, *scaD* could not be inactivated in a strain overproducing ScaD.

5.3 Discussion

In Chapter 4, the Sca family of amidases was characterised. One of these proteins, ScaD, was identified as being putatively important in *S. aureus* and its function was further elucidated. Molecular genetic approaches were used to attempt to determine the role of *scaD* by replacement of its native promoter with an inducible promoter, and also by insertional inactivation of the gene using a kanamycin cassette. However, numerous problems were encountered during mutagenesis, the reasons for which are not fully understood, resulting in the inability to create these mutations. Therefore, other analyses were carried out to further determine the role of *scaD*.

Western blot analysis using anti-rScaD antibodies and native cell wall material of *S. aureus* identified ScaD, suggesting that it may be ionically-bound. ScaD is expressed early during the logarithmic phase of growth. Protein corresponding to the expected size of ScaD was seen in cell lysate after just 1 hour of growth, suggesting that this enzyme is required by the cell early during the exponential phase. It has been described previously that the production of autolysins is predominantly during the logarithmic growth phase (Komatsuzawa *et al.* 1995) and is most likely due to the high rate of cell division, requiring hydrolytic enzymes to cleave nascent septa allowing separation of daughter cells. The partially mutually compensatory autolysins IsaA and SceD are also produced maximally during exponential growth and these putative lytic transglycosylases are involved in cell separation (Stapleton *et al.* 2007). The amidase Aaa/Sle1 (ScaA) is involved in separation of daughter cells during cell division, along with the major *S. aureus* autolysin AtlA (Sugai *et al.* 1994; Foster, 1995; Kajimura *et al.* 2005). The two products of *atlA*, a 62 kDa amidase and a 51 kDa glucosaminidase form a ring structure on the cell surface and, following septum formation, these two autolysins digest the peptidoglycan connecting the two daughter cells, allowing the cells to separate (Yamada *et al.* 1996).

The importance of autolysin activity during the exponential phase is further highlighted in *E. faecalis*, in which the enzymes AtlA and AtlB, which are responsible for digesting the septum during cell separation, are mutually compensatory only during the logarithmic

phase of growth (Mesnage *et al.* 2008). This suggests that peptidoglycan hydrolase activity is most crucial during this time, hence strategies are employed by the bacteria to ensure that cell separation is carried out by at least one of these enzymes. The production of ScaD so early during growth suggests that this protein may have a key function in cell physiology and may be vital for viability. In order to further elucidate the function of this enzyme during the early stages of growth, subsequent work is required, including localisation of the protein and inactivation of *scaD* to determine its role.

It has been reported previously that *scaD* is controlled by the essential two-component regulator YycG/YycF (Dubrac and Msadek, 2004). This was confirmed by Western blot analysis using anti-rScaD antibodies and a strain depleted for *yycFG*. Depletion of IPTG, resulting in reduced expression of the *yycF* and *yycG* genes, also resulted in decreased levels of ScaD being detected in Western blots. This demonstrates the direct effect of this regulator on production of ScaD. Indeed, Dubrac and Msadek (2004) identified two binding sites of the YycF regulator on the promoter region of *scaD*, indicating that *scaD* transcription is directly controlled by this regulator.

In addition to YycF, ScaD levels are also influenced by the two component system SaeRS (Rogasch *et al.* 2006). In *S. aureus* COL, SaeRS was shown to negatively influence the amount of ScaD, as mutation of *saeS* resulted in accumulation of ScaD. This effect most likely occurs at the post-transcriptional level, as transcription of *scaD* was not influenced in the mutant. Post-transcriptional regulation may occur via endonucleolytic enzyme activity which degrades mRNA or by binding of proteins to mRNA transcript to inhibit translation of the protein (Dubrac and Touati, 2002; Tang *et al.* 2004).

YycG/YycF has been demonstrated to be essential in several species, including *B. subtilis*, *S. aureus* and *S. pneumoniae* (see Chapter 5.1). However, no single protein has been identified in any strain which is directly correlated with the essentiality of YycF. In *B. subtilis*, the endopeptidases LytE and YvcE (CwlO) were demonstrated to be controlled by YycFG and their activities were demonstrated to be essential for lateral cell wall synthesis and cell elongation during growth (Bisicchia *et al.* 2007). Whilst single mutants were

constructed for both *lytE* and *yvcE*, no double mutant could be obtained, demonstrating that the enzymes are mutually functionally redundant but together are required for growth. The essential nature of their endopeptidase activity may be explained by the proposed mechanism of peptidoglycan synthesis, in which three new murein strands are synthesised from a single docking strand and attached to cross-bridges whilst the original docking strand is degraded (Höltje, 1998). This "three-for-one" model is carried out by a multi-enzyme complex in *E. coli*, consisting of transpeptidases and transglycosylases to synthesise new bonds, and endopeptidase and lytic transglycosylase autolysins which degrade the original strand. As the synthase enzymes are in front of the hydrolase enzymes within this complex, new peptidoglycan strands are constructed before old strands are degraded, hence the stress-bearing cell wall structure and shape is maintained throughout. Whilst this model has been proposed for *E. coli*, it could be speculated that a similar mechanism in the Gram-positive *B. subtilis* may give reason for the essential nature of endopeptidase activity seen by Bisicchia *et al.* (2007).

Despite the essentiality of LytE and YvcE in *B. subtilis*, it was concluded that YycFG-controlled expression of these genes was not the only reason for the essentiality of the two-component system (Bisicchia *et al.* 2007). Indeed, it has been proposed that the essentiality of the two-component system is more likely due to the global regulation of several proteins which, in combination, are essential for correct cell wall metabolism and hence viability (Dubrac *et al.* 2007).

Studies by Ng *et al.* (2004) originally attributed the essentiality of YycG/YycF in *S. pneumoniae* to its positive regulatory effect on production of the autolysin PcsB, as YycF was not required when *pcsB* was constitutively expressed. However, more recently, *pcsB* has been inactivated, confirming that it is not in fact essential for growth (Giefing *et al.* 2008). Mutants demonstrated severely defective growth, with disrupted cell morphology and aberrent septum formation, indicating that PcsB is highly important during pneumococcal growth and division. This suggests that, whilst this enzyme is not uniquely responsible for the essentiality of YycFG, it most likely contributes significantly along with other enzymes involved in cell wall metabolism.

Bioinformatic analysis of PcsB revealed the presence of a C-terminal CHAP domain, which shares 32% identity to that of ScaD. As these proteins share significant homology in their C-terminal domains, ScaD was constitutively expressed in a conditional mutant, in which *yycFG* is under the control of an IPTG-inducible promoter. Overproduction of ScaD in this strain allowed it to grow independently of IPTG, hence complementing the essentiality of *yycFG*, similar to PcsB in *S. pneumoniae*. Further analysis to confirm whether constitutive expression of *scaD* would allow the disruption of *yycF* in *S. aureus* would provide further evidence towards the role of the regulation of this gene and its effect on the essentiality of YycFG. In addition, placing *scaD* under the control of the IPTG-inducible P*spac* promoter, which was not possible during this study, would establish whether or not *scaD* itself is essential in *S. aureus*.

As PcsB and ScaD are homologues, it could be speculated that they may have similar roles in the cell physiology. It has been demonstrated that severe depletion and inactivation of PcsB resulted in aberrant cell shapes and irregular septum formation, indicating an important role in cell wall biosynthesis (Ng *et al.* 2004; Giefing *et al.* 2008). Underexpression also led to the formation of long chains of cells, a phenotype commonly associated with strains deficient in cell wall hydrolase activity (De Las Rivas *et al.* 2002; Carballido-López *et al.* 2006; Priyadarshini *et al.* 2007; Mesnage *et al.* 2008).

The role of PcsB in cell wall dynamics is further supported in studies in *S. agalactiae* (GBS). Mutants lacking *pcsB* exhibited dramatically reduced growth rates compared to wild-type strains, and grew in irregular clumps with severely disturbed morphology (Reinscheid *et al.* 2001). Unequal septa formed in different planes without subsequent cell wall separation, leading to giant and irregular-shaped cells. These results indicated that PcsB is required for cell division in GBS, either responsible for placement of the septum at the midpoint of the cell or involved in splitting of the septum. Disruption of *pcsB* was also associated with an increased susceptibility to β-lactam antibiotics, possibly due to increased uptake via a defective cell wall (Reinscheid *et al.* 2001, 2003). A similar function has been assigned to *scaD*, which has been demonstrated to be associated with resistance to

macrolide-lincosamide-streptogramin B (MLS_B) antibiotics (Martin *et al.* 2002), indicating a putative role in cell wall biosynthesis.

Although overproduction of ScaD in strains depleted for *yycFG* complemented its essentiality and allowed growth, the rate of growth of this strain was reduced when compared to the control strain containing only the plasmid with no *scaD* insert. This suggests that although ScaD may provide a vital role within the cell, unregulated expression of this autolysin may also be detrimental. To further determine this effect, ScaD was overproduced in wild-type *S. aureus* SH1000. Growth experiments revealed a markedly lower growth rate of cells overproducing ScaD compared to cells containing just the control plasmid. This reduced growth rate was most prominent when CFUs were measured rather than optical density, suggesting that whilst cells may not have been viable, they did not necessarily lyse. Interestingly, cells depleted for YycG/YycF, which would also be depleted for ScaD, were shown to die without lysis (Dubrac *et al.* 2007).

To determine the effect of ScaD overproduction on cell autolysis, strains were lysed using Triton X-100 and followed spectrophotometrically over time. Results demonstrated that the strain overproducing ScaD was more resistant to this lysis. This is again in contrast to previous studies, in which *yycFG* depletion (and hence reduction of *scaD*) led to increased resistance to lysostaphin-induced lysis (Dubrac *et al.* 2007). Although the specific role of *scaD* is unknown, it could be speculated that it is tightly regulated within the cell and that disruption of levels of the enzyme, whether increased or reduced, may lead to defective cell wall synthesis and hence result in the phenotypes seen here. Uncontrolled cell wall hydrolysis activity may lead to excessive breakage of bonds within the peptidoglycan, ultimately causing cell lysis (Chauhan *et al.* 2006). Structural muropeptide analysis of the cell wall in strains with varying levels of ScaD may further elucidate the function of this enzyme in the dynamics of the peptidoglycan.

Another possible explanation for the observed effects is that overproduction of ScaD affects production of other autolysins, disrupting the autolytic profile. When whole cell lysates of strains overproducing ScaD and control strains were analysed by zymography, it was

observed that the levels of AtlA were slightly reduced when ScaD was overproduced. The reason for this effect is not known, however, it could be speculated that excess ScaD enzyme bound to the cell wall may mean that less AtlA is able to do so. Alternatively, excess hydrolysis of peptidoglycan by ScaD may lead to the down-regulation of other autolysins. Indeed, it is speculated that a regulatory mechanism exists which down-regulates expression of autolysin genes when cell wall synthesis is disturbed (Antignac *et al.* 2007). Therefore, disruption of cell wall synthesis by excess ScaD enzyme may also have resulted in down-regulation of other autolysins, including AtlA, exacerbating the effect. AtlA is the major autolysin of *S. aureus* and is involved in cell separation (Foster, 1995; Biswas *et al.* 2006). This enzyme is also controlled by YycG/YycF, therefore, depletion of the two-component regulator in experiments by Dubrac *et al.* (2007) would also result in reduction of AtlA. The similarities of the observed phenotypes when ScaD is overproduced may hence be accounted for by the indirect effect on levels of AtlA.

Cells overproducing ScaD were analysed by transmission electron microscopy and, whilst wild-type and control strains displayed cells of regular size and shape, with evenly placed septa, cells containing excess ScaD had misplaced septa leading to asymmetrical cell division. This phenotype has been seen previously, for example, *E. coli* lacking the amidases AmiA, AmiB and AmiC produced abnormal septa, indicating a role for these enzymes in the correct orientation of septa during cell division (Priyadarshini *et al.* 2007). The *B. subtilis* endopeptidase LytE has also been proposed to function in digesting peptidoglycan from the division septum to allow separation of cells, as strains lacking this enzyme were irregular in shape and formed long chains (Carballido-López *et al.* 2006). Along with YvcE (CwlO), this endopeptidase activity is an essential function in *B. subtilis* as no viable mutants could be obtained lacking both these autolysins (Bisicchia *et al.* 2007). In *Mycobacterium tuberculosis*, overproduction of the cell wall hydrolase Rv2719c led to reduced growth with severe clumping and cell lysis (Chauhan *et al.* 2006). Overproduction of Rv2719c also disrupted the assembly of FtsZ-rings, indicating a role in cell division. It was proposed that this murein hydrolase is needed for cell wall remodelling during active growth of mycobacteria and may have a specific role following stressful growth conditions. Furthermore, ScaA (Sle1) of *S. aureus*, which shares 30% identity with ScaD, including

51% identity within the C-terminal CHAP domains, has been shown to be involved in cell separation after division (Kajimura *et al.* 2005). The homology of ScaA and ScaD may suggest that these proteins have similar functions in the cell.

The observed effect on cell division may explain the inability to inactivate the chromosomal *scaD* in a strain overproducing ScaD via the plasmid pDComp. It was hypothesised that if *scaD* was constitutively transcribed via the P*pcn* promoter in the plasmid pDComp, then the genomic copy of the gene may be inactivated, which was not possible by conventional mutagenesis. However, this could not be achieved during this study, which may be explained by the defective phenotype caused by overproduction of ScaD. As mentioned previously, the defects caused by overproduction of the protein are comparable to that seen in several studies where amidases were inactivated, suggesting that the uncontrolled expression of *scaD* is as hazardous to the cell as underexpression.

Whilst the true role of *scaD* remains to be determined, it can be speculated that the function of this likely amidase is most likely implicated in the division and separation of cells, and in the biosynthesis of the cell wall. Further studies are required, including placing *scaD* under the control of an inducible promoter, to fully establish the role of this putatively important amidase. Analysis of the effects of levels of ScaD in an *atlA* mutant would also resolve the question of whether any of the observed effects are due to indirect influence on the amounts of the major staphylococcal autolysin.

Chapter 5 – Summary

- ScaD is an ionically bound cell wall protein produced early during logarithmic growth.
- Expression of *scaD* is controlled by the essential two-component system YycF/YycG; depletion of this regulator leads to a decrease of ScaD.
- The essentiality of YycF/YycG can be complemented by overproducing ScaD in a conditional *yycFG* mutant.
- Overproduction of ScaD in *S. aureus* SH1000 resulted in a growth defect with asymmetical septum formation during cell division.
- Cells overproducing ScaD demonstrated increased resistance to cell lysis.

Chapter 6

General Discussion

6.1 Vaccines against opportunistic pathogens

Gram-positive pathogens cause significant morbidity and mortality world-wide, and are common causes of nosocomial disease, which constitute a significant economic burden due to increased costs of hospital care. In particular, patients undergoing surgery, immunocompromised individuals and the elderly represent key groups at risk of nosocomial infections. In Europe and the USA, 5-10% of patients develop infections during their hospital stay, and this rises to almost 50% in critically ill patients in intensive care units (Datamonitor, 2008). Therefore, development of vaccines to target these pathogens is highly desirable in order to reduce incidence of disease and associated healthcare costs. In particular, the potential emergence of strains of *S. aureus* which are resistant to all available antibiotics poses a serious threat, prompting the need for novel prophylactic and therapeutic measures.

Immunological approaches based on vaccination or the use of prophylactic/therapeutic monoclonal antibodies are attractive alternatives to antibiotics. Current approaches for *S. aureus* have targeted specific antigens. For example, Aurexis® (tefibazumab) consists of a humanised monoclonal antibody (MAb) that recognises the *S. aureus* MSCRAMM ClfA (Patti, 2004). This MAb was protective in animal models of *S. aureus* infection and was well-tolerated and effective in human trials (Domanski *et al.* 2005; Weems *et al.* 2006). StaphVAX™ (Nabi) is a bivalent polysaccharide and protein-conjugated vaccine, directed against the two most prevalent *S. aureus* capsular polysaccharides, types 5 and 8 (Jones, 2002). However, despite a significant decrease in *S. aureus* bacteraemia in the first 10 months after vaccination during phase III clinical trials, StaphVAX™ failed to elicit significant protection after one year (Fattom *et al.* 2004). Aurograb® (NeuTec Pharma) is a recombinant antibody preparation against peptides based on the ATP

binding subunit of an ABC transporter associated with strains of MRSA (Burnie *et al.* 2000). Antibodies were shown to reduce bacterial counts in mouse organs and further development of this prophylaxis by NeuTec Pharma is ongoing. It is proposed that this immunotherapeutic approach may be used in combination with vancomycin antibiotic therapy against MRSA infections (NeuTec Pharma).

In streptococci, prophylactic approaches primarily target the surface-located M protein (Fischetti, 1989; McMillan *et al.* 2004b). Whilst antibodies against the amino terminus of this protein have been both opsonic and protective, vaccines based on M proteins are hindered by the high antigenic variability, with over 100 *emm*-types reported (McMillan *et al.* 2004b). In order to circumvent these difficulties, vaccines have been developed which consist of several M protein serotypes. For example, a 7-valent conjugative vaccine (PVC-7), known as Prevnar, is currently in use in the USA and Europe to combat *S. pneumoniae* infections (Leibovitz, 2008). Although this vaccine has proved effective in reducing the incidence of invasive pneumococcal diseases, acute otitis media, community-acquired respiratory infections and nasopharyngeal colonisation, an increase in replacement serotypes of this pathogen has also occurred, creating concerns regarding the development of replacement disease against which Prevnar would be ineffective (McGee, 2007; Leibovitz, 2008).

The ideal vaccine would induce antibodies to prevent the key stages in the bacterial infective process, including inhibiting bacterial adherence, promoting opsonophagocytic killing by leukocytes and neutralising the toxic exoproteins produced by the pathogen (Lee and Pier, 1997). Due to the complexity of staphylococcal and streptococcal infections and the multitude of virulence determinants produced by these organisms, a single-component vaccine, such as those described above, is unlikely to be effective. Incorporation of several peptides into a multi-valent vaccine can confer higher levels of protection and may also protect against multiple strains or species (McMillan *et al.* 2004a).

A single vaccine against a pathogen may provide significant benefits to those at risk. However, these benefits must also be commercially viable. Whilst a vaccine against *S. aureus* would be considered highly valuable for people undergoing dialysis or surgery, its use could be limited by effective alternative infection prevention methods, such as hand hygiene and patient screening. Therefore, a vaccine protective against several pathogens would have greater potential in a wider range of patient groups. Indeed, a single vaccine against *S. epidermidis* would be of limited use but could prove valuable for patients with prosthetic implants if combined with a *S. aureus* antigen (Datamonitor, 2008). Moreover, a vaccine targeted against several virulence components would be less likely to induce resistant strains, such as those observed during vaccination with the PCV-7 (Prevnar) (McGee, 2007; Leibovitz, 2008).

Peptide based vaccines have the advantage of being able to choose minimal sequences necessary to generate an immune response, and peptides may be linked to carrier molecules to increase this immunogenicity (McMillan *et al.* 2004a). In my current study, putative immunogenic protein domains were identified which were cross-reactive between five pathogenic species, *S. aureus*, *S. epidermidis*, *S. pneumoniae*, *S. agalactiae* and *S. pyogenes*. Thirteen families of conserved domains were identified and alignment of these regions led to the identification of three domains which were particularly highly conserved within these species, IsdA-c (amino acids 215-310), SdrD-n (amino acids 30-200) and ScaB-c (amino acids 156-265). Further analysis demonstrated that antibodies against these domains were opsonic during infection by *S. aureus* and *S. agalactiae*, and that IsdA C-terminus polypeptide showed promise in protecting against infection by these pathogens in a mouse model of septic arthritis.

SdrD is structurally related to ClfA, which has also been demonstrated to provide effective passive protection (Aurexis®) (Patti, 2004). Antibodies against full-length SdrD have also been identified previously to mediate complement-dependent phagocytosis of *S. aureus* (Stranger-Jones *et al.* 2006). Whilst IsdA is not essential for *S. aureus* growth, its requirement for nasal colonisation is a predisposing factor for nosocomial infections, making it a suitable target for prophylactic development (García-Lara *et al.* 2005; Clarke

et al. 2006). Furthermore, IsdA is associated with *S. aureus* infection and confers resistance to hydrophobic fatty acids, antimicrobial peptides of the host cell, thereby promoting survival of *S. aureus* on human skin (Clarke *et al.* 2006; 2007). ScaB is a novel protein identified during this immunoscreen, which is part of a large family of surface proteins in staphylococci (Pourmand *et al.* 2006). The conserved CHAP domain of this protein is also found in several species, including *Streptococcus*, *Enterococcus*, *Lactococcus* and *Bacillus* species (Rigden *et al.* 2003; Pourmand *et al.* 2006). Targeting the specific domains within these proteins which are antigenic and cross-reactive provides the opportunity to develop a specific vaccine which incorporates several antigenic polypeptides, thereby providing effective protection against this family of pathogens. Identification of further immunogenic targets within these Gram-positive pathogens may permit the development of an effective multi-valent vaccine which could successfully contribute to the decrease in incidence of disease.

6.2 An essential autolysin in *S. aureus*?

Peptidoglycan hydrolases represent a vast and diverse group of enzymes which are involved in some of the key processes of bacterial physiology, including cell separation during division. The importance of these enzymes in maintaining growth and physiology is highlighted by the defects seen when key peptidoglycan hydrolases are inactivated. For example, inactivation of *pcsB* in *S. pneumoniae* resulted in severely defective growth with disrupted cell morphology and aberrant septum formation, indicating that this enzyme plays a crucial role during pneumococcal growth and division (Giefing *et al.* 2008). The key contribution of peptidoglycan hydrolases to bacterial survival is also highlighted by the functional redundancy of this group of enzymes. A process may be performed by several enzymes, and individual enzymes may be involved in numerous processes (Smith *et al.* 2000). This ensures that hydrolytic activity occurs when needed under all circumstances and conditions, and may provide a means of adapting to the various environments encountered by the bacteria during infection by being able to modify the peptidoglycan.

Whilst the importance of autolysins is evident, to date no single enzyme has been found to be essential. In *B. subtilis*, endopeptidase activity carried out by LytE and YvcE (CwlO) was demonstrated to be essential for lateral cell wall synthesis and cell elongation during growth (Bisicchia *et al.* 2007). This requirement may be attributed to the "three-for-one" mechanism of cell wall synthesis, in which transpeptidases and transglycosylases synthesise new bonds, and endopeptidase and lytic transglycosylase autolysins degrade the original strand (Höltje, 1998).

Gene products that are essential for cell growth under all conditions are primary targets for antimicrobial therapy because their inhibition would be fatal for the cell (García-Lara *et al.* 2005). In particular, surface proteins represent promising vaccine targets as they have key roles in the interaction between pathogen and host cells. The identification of a cell wall-associated peptidoglycan hydrolase enzyme which was essential for viability would represent a promising candidate for prophylaxis due to its surface location and requirement for survival of the pathogen. Although not essential for viability, several important autolysins have been identified which are immunogenic during infection and represent putative vaccine targets, for example, PcsB of *S. pneumoniae* (Giefing *et al.* 2008), IsaA and SceD of *S. aureus* (Stapleton *et al.* 2007) and AtlE of *S. epidermidis* (Pourmand *et al.* 2006).

Enzymes involved in peptidoglycan structural dynamics have previously been demonstrated to be essential for growth of *S. aureus*, for example, MurB, a UDP-*N*-acetylenolpyruvylglucosamine reductase which acts early during peptidoglycan biosynthesis (Matsuo *et al.* 2003). Several effective antibiotics have targeted peptidoglycan biosynthesis (e.g. glycopeptides, β-lactams, phosphomycin), hence the essentiality of MurB during cell wall synthesis suggests it could be a suitable new target for drug development (García-Lara *et al.* 2005).

The use of a peptidoglycan hydrolase as a therapeutic agent against *S. aureus* infection has been demonstrated with the endopeptidase lysostaphin, which cleaves the cross-linking pentaglycine bridges in the cell walls of staphylococci. Application of lysostaphin

formulated in a cream was effective in eradicating *S. aureus* nasal colonisation in a cotton rat model (Kokai-Kun *et al.* 2003). Furthermore, lysostaphin successfully treated aortic valve endocarditis in a rabbit model of infection (Climo *et al.* 1998; Patron *et al.* 1999). In addition, phage-encoded peptidoglycan hydrolases, including the muramidase Cpl-1 and the amidase Pal, have demonstrated effective treatment of *S. pneumoniae* infection, protecting mice from bacteraemia and death (Jado *et al.* 2003; Loeffler *et al.* 2003). Intraperitoneal therapy with the pneumococcal autolysin LytA was also shown be an effective treatment in reducing β-lactam-resistant *S. pneumoniae* infection in a mouse peritonitis-sepsis model, suggesting a novel putative treatment regimen for antibiotic-resistant pathogens (Rodríguez-Cerrato *et al.* 2007).

As the key roles of peptidoglycan hydrolases are further elucidated, it could be speculated that amongst this important group of enzymes there may indeed be one without which the bacteria cannot survive. As autolysins are involved in the fundamental processes of cell growth, their contribution to bacterial survival and pathogenicity is considerable. Aberrant cell separation due to lack of specific autolytic activity may result in impaired dissemination in the host and thus reduced virulence (Stapleton *et al.* 2007). However, whether a single essential autolysin does in fact exist in *S. aureus*, or any other species, remains to be determined.

6.3 Future work

The three conserved domains identified by immunoscreening displayed potential as immunogenic targets. Further work to determine their use is required, including assessment of the opsonophagocytic abilities of antiserum against each polypeptide in several bacteria. Protection studies using multiple proteins would also determine the protective capacity of these components in a multi-valent vaccine. Finally, identification of additional domains or proteins which may be immunogenic in the species studied through further screening would provide additional putative components for a vaccine targeted against a range of Gram-positive pathogens.

In order to establish the essential nature of ScaD, it is necessary to complete the mutagenesis attempted during this study, placing the *scaD* gene under the control of an inducible promoter. Once this is achieved, work to further elucidate the role of this peptidoglycan hydrolase is possible, including phenotypic analysis of strains depleted for ScaD.

Further work to determine the overlapping functions of the Sca family of autolysins is required, including expression studies to confirm the conditions under which each gene is optimally expressed. Solubilisation of the recombinant proteins would allow more detailed analysis of the activity of these enzymes.

As *S. aureus* is an opportunistic and ubiquitous pathogen carried either continuously or intermittently by up to 80% of the population (Foster, 2004), levels of antibodies against this pathogen are likely to be high. Therefore, it could be speculated that high levels of antibodies may not necessarily be protective. Indeed, despite inducing high levels of antibodies in patients after immunisation, against the two most common capsular polysaccharides of *S. aureus*, the vaccine StaphVAX™ (Nabi) failed to significantly protect against *S. aureus* bacteraemia (Fattom *et al.* 2004). Therefore, it may not be feasible to develop a vaccine in this manner, or it may be necessary to target very specific components in order to induce an effective level of protection. The organism itself does not want the host to develop an immune response, therefore, key proteins involved in virulence may not be immunogenic. This may give reason for the lack of reactivity seen for ScaD, as this protein failed to bind antibodies in human serum and displayed a relative lack of reactivity during rabbit immunisation. It could be speculated that monoclonal antibodies against ScaD may be more effective and further work to determine this is required. My study has identified putative vaccine components and further insight may lead to the rational development of immunogenic approaches for the control of *S. aureus* and other important human pathogens.

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Appendix

S. aureus antigen	Species	Homologous proteins	% identity	% similarity	Homologous protein (aa length)	Region of *S. aureus* protein with homology (aa length)
SA0609	*S. epidermidis*	SE0750	25	47	43-238 (195)	45-204 (159)
(SdrD)	ATCC 12228		27	45	35-159 (124)	70-184 (114)
			27	47	676-723 (47)	637-683 (46)
			33	48	1137-1163 (26)	715-741 (26)
			29	45	1118-1163 (45)	915-962 (47)
		SE0245	29	48	1-190 (190)	16-199 (183)
			21	43	130-354 (224)	45-274 (229)
			25	45	96-259 (163)	27-204 (177)
			35	54	618-654 (36)	959-992 (33)
			31	45	304-335 (31)	698-732 (34)
		SE0331	60	74	601-825 (224)	902-1123 (221)
			27	44	54-1025 (971)	47-1039 (992)
			30	58	1-203 (203)	1-201 (201)
		SE0185	28	46	4-185 (181)	17-185 (168)
			36	48	187-226 (39)	1024-1062 (38)
		SE2319	25	41	9-82 (73)	36-108 (72)
			37	54	34-69 (35)	685-721 (36)
	S. pneumoniae	SP_0648	23	47	7-171 (164)	18-196 (178)
	TIGR4		32	52	672-708 (36)	390-429 (39)
			30	44	676-718 (42)	542-580 (38)
			20	46	1879-1917 (38)	1044-1082 (38)
			50	62	963-978 (15)	187-202 (15)
			46	61	252-264 (12)	451-463 (14)
		SP_1174	21	41	232-312 (80)	46-126 (80)
		SP_2190	22	41	330-474 (144)	49-180 (131)
			33	46	628-672 (44)	986-1028 (42)
			43	68	627-642 (15)	875-890 (15)
		SP_2216	23	43	7-184 (177)	33-204 (171)
			30	51	348-388 (40)	528-570 (42)
	S. agalactiae	GBS0016	21	44	15-182 (167)	29-198 (169)
	NEM316	GBS1529	21	42	134-597 (463)	60-521 (460)
			45	46	1259-1278 (19)	975-994 (19)
	S. pyogenes	SP1937	23	47	88-179 (91)	58-150 (92)
	MGAS8232		44	55	420-437 (17)	1009-1026 (17)
SA0856	*S. epidermidis*	SE0750	26	45	3-199 (196)	12-204 (192)
(ClfA)	ATCC 12228		26	42	849-996 (147)	173-321 (148)

		SE0245	28	44	1-114 (114)	3-102 (99)
			26	37	158-202 (44)	163-207 (44)
			28	44	589-638 (49)	229-270 (41)
		SE0331	28	44	225-597 (372)	163-542 (379)
			38	54	1003-1055 (52)	881-932 (51)
		SE0185	27	44	3-110 (107)	2-105 (103)
	S. pneumoniae	SP_0057	33	51	1111-1137 (26)	186-211 (25)
	TIGR4	SP_2190	43	60	669-691 (22)	498-518 (20)
			37	44	81-109 (28)	256-284 (28)
	S. agalactiae	GBS0016	23	42	70-215 (145)	29-178 (147)
	NEM316	GBS1356	26	42	461-521 (60)	393-459 (66)
			28	44	777-812 (35)	256-293 (37)
			25	50	1297-1328 (31)	416-447 (31)
			27	48	690-724 (34)	276-304 (28)
			30	46	623-659 (36)	373-411 (38)
		GBS1529	25	46	319-597 (278)	229-491 (262)
SA2511	*S. epidermidis*	SE0331	46	62	1011-1053 (42)	976-1017 (41)
(FnbA)	ATCC 12228		33	45	729-790 (61)	28-93 (65)
			40	54	42-63 (21)	842-862 (20)
	S. pneumoniae	SP_0648	30	59	2176-2221 (45)	961-1007 (46)
	TIGR4		22	43	1805-1919 (114)	196-310 (114)
			21	43	1826-1902 (76)	605-678 (73)
			22	51	1812-1856 (44)	823-867 (44)
		SP_0057	26	43	1021-1162 (141)	151-300 (149)
			30	50	1263-1312 (49)	964-1015 (51)
			50	61	958-975 (17)	521-538 (17)
		SP_2190	24	41	565-693 (128)	344-463 (119)
			30	44	239-286 (47)	146-194 (48)
	S. pyogenes	SP1937	25	50	196-257 (61)	405-464 (59)
	MGAS8232		32	58	481-510 (29)	714-744 (30)
SA2150	*S. epidermidis*	SE0750	27	48	1092-1124 (32)	1076-1108 (32)
(SasB/FmtB)	ATCC 12228		27	41	531-580 (49)	1823-1872 (49)
			22	42	26-243 (217)	1301-1524 (223)
			25	44	28-183 (155)	1631-1801 (170)
			22	43	24-211 (187)	488-664 (176)
			32	50	1091-1139 (48)	887-936 (49)
			30	41	934-986 (52)	1346-1396 (50)
			27	37	608-705 (97)	2261-2360 (99)
			26	56	191-220 (29)	2141-2170 (29)

		SE0245	23	42	31-216 (175)	1565-1747 (182)
			25	45	44-177 (133)	909-1030 (121)
			23	43	50-249 (199)	1302-1516 (214)
			29	48	148-346 (198)	1849-2047 (198)
		SE0185	27	41	165-245 (80)	2243-2326 (83)
			25	44	42-183 (141)	1301-1452 (151)
			30	50	96-134 (38)	2142-2181 (39)
			25	45	442-510 (68)	1604-1682 (78)
		SE0331	25	40	314-562 (248)	205-468 (263)
			21	41	49-242 (193)	1349-1539 (190)
			24	46	146-271 (125)	2240-2358 (118)
			26	41	395-447 (52)	902-954 (45)
			23	45	67-173 (106)	1910-2030 (120)
			22	41	15-125 (110)	1911-2015 (104)
			47	70	786-802 (16)	1242-1258 (16)
			31	48	779-807 (28)	1342-1370 (28)
			26	51	984-1031 (47)	1912-1960 (48)
			39	51	737-769 (32)	271-302 (31)
	S. pneumoniae	SP_0648	30	47	58-148 (90)	710-795 (85)
	TIGR4		22	46	1230-1382 (152)	1788-1940 (152)
			25	47	67-175 (108)	806-919 (113)
			25	41	47-174 (127)	1161-1302 (141)
			21	41	54-180 (126)	1462-1591 (129)
			32	46	524-583 (59)	1240-1299 (59)
			27	48	464-506 (42)	562-602 (40)
			26	41	335-436 (101)	273-373 (100)
			27	50	98-156 (58)	1591-1650 (59)
			28	42	274-325 (51)	1192-1242 (50)
			29	50	471-512 (41)	1358-1400 (42)
			40	59	1820-1841 (21)	1183-1204 (21)
			29	44	297-371 (74)	204-264 (60)
			42	57	2169-2195 (26)	1289-1316 (27)
			22	50	2145-2199 (54)	1437-1494 (57)
			32	44	1559-1592 (33)	2059-2092 (33)
			23	45	2068-2118 (50)	1981-2031 (50)
		SP_0057	25	40	73-309 (236)	2240-2459 (219)
			25	43	548-664 (116)	1308-1424 (116)
			27	45	975-1131 (156)	539-695 (156)
			22	46	549-658 (109)	1770-1884 (114)
			30	44	550-658 (108)	1921-2037 (116)
			23	41	40-250 (210)	1325-1531 (206)
			23	44	96-270 (174)	1607-1781 (174)
			27	45	56-193 (137)	1657-1799 (142)
			25	41	120-312 (192)	1607-1801 (194)
			28	53	585-639 (54)	1190-1243 (53)
			21	43	43-207 (164)	577-731 (154)
			28	50	104-181 (77)	785-860 (75)

			24	42	43-160 (117)	909-1027 (118)
			27	45	546-639 (93)	917-1012 (95)
			23	47	539-634 (95)	1631-1732 (101)
			27	50	270-337 (67)	897-960 (63)
			32	52	602-663 (61)	1597-1665 (68)
			38	53	1143-1168 (25)	2274-2299 (25)
			28	46	1217-1266 (49)	1334-1381 (47)
			27	37	154-193 (39)	1069-1108 (39)
			34	60	128-150 (22)	1140-1162 (22)
		SP_2216	25	42	13-241 (228)	509-746 (237)
			23	42	11-225 (214)	797-1025 (228)
			22	42	36-234 (198)	1673-1873 (200)
		SP_2190	24	43	243-438 (195)	1596-1791 (195)
			23	41	396-494 (98)	1596-1697 (99)
			28	42	466-528 (62)	1604-1666 (62)
		SP_1282	25	42	200-345 (145)	1750-1884 (134)
	S. agalactiae	GBS0016	21	41	19-277 (258)	1271-1528 (257)
	NEM316		22	45	19-154 (135)	1932-2069 (137)
		GBS1356	35	60	1413-1440 (27)	1903-1930 (27)
			29	62	1524-1558 (34)	828-864 (36)
			28	50	1563-1594 (31)	2140-2171 (31)
			25	37	659-745 (86)	2278-2362 (84)
			31	46	785-825 (40)	1203-1240 (37)
		ScpB	25	44	1029-1119 (90)	2240-2339 (99)
			23	42	18-160 (142)	1373-1505 (132)
			39	60	592-619 (27)	2150-2177 (27)
		GBS1529	22	44	118-370 (252)	433-680 (247)
			35	60	48-67 (19)	1447-1466 (19)
	S. pyogenes	SP1937	24	48	32-127 (95)	936-1030 (94)
	MGAS8232		28	48	68-131 (63)	2298-2361 (63)
SA1806	*S. epidermidis*	SE0750	23	43	35-287 (252)	1609-1842 (233)
(SasC/FmtB	ATCC 12228		24	44	41-209 (168)	1101-1263 (162)
homologue)			22	42	1111-1210 (99)	1985-2086 (101)
			29	49	532-580 (48)	1903-1952 (49)
			29	54	1107-1142 (35)	1339-1375 (36)
			23	42	759-822 (63)	1088-1149 (61)
		SE0245	22	41	95-344 (249)	1522-1782 (260)
			28	42	295-346 (51)	1094-1150 (56)
			27	45	503-542 (39)	1405-1442 (37)
			37	58	688-710 (22)	320-343 (23)
		SE0185	25	41	66-142 (76)	38-117 (79)
			31	60	469-508 (39)	820-864 (44)
			40	63	486-507 (21)	1121-1142 (21)
			37	66	470-496 (26)	1451-1476 (25)
		SE0331	23	41	70-196 (126)	1139-1264 (125)

			26	40	85-172 (87)	33-122 (89)
			24	54	1001-1033 (32)	276-308 (32)
			26	45	445-482 (37)	317-358 (41)
	S. pneumoniae	SP_0648	21	44	2079-2213 (134)	1134-1260 (126)
	TIGR4		21	43	71-156 (85)	1796-1882 (86)
			21	41	295-387 (92)	1076-1171 (95)
			21	52	85-145 (60)	1191-1255 (64)
			41	76	859-875 (16)	680-696 (16)
		SP_0057	24	48	548-638 (90)	1522-1609 (87)
			24	43	1116-1251 (135)	557-701 (144)
			24	44	530-628 (98)	1622-1717 (95)
			33	71	35-55 (20)	250-270 (20)
			21	41	499-621 (122)	714-841 (127)
			25	40	1008-1080 (72)	1127-1197 (70)
			31	63	135-156 (21)	1284-1305 (21)
		SP_2216	25	47	25-276 (251)	1661-1916 (255)
			26	40	39-245 (206)	1339-1550 (211)
		SP_2190	21	41	104-353 (249)	675-917 (242)
	S. agalactiae	GBS0016	24	42	39-301 (262)	1569-1827 (258)
	NEM316		24	41	45-322 (277)	1734-2005 (271)
			22	41	46-315 (269)	1400-1667 (267)
		GBS1356	26	42	108-245 (137)	776-909 (133)
			25	42	792-913 (121)	435-561 (126)
			26	40	177-319 (142)	733-861 (128)
			22	46	273-383 (110)	1580-1695 (115)
			23	43	792-855 (63)	1668-1732 (64)
			30	50	1125-1166 (41)	1178-1219 (41)
		GBS1529	24	45	198-283 (85)	29-112 (83)
			35	57	1269-1310 (41)	2146-2185 (39)
			23	50	706-810 (104)	20-126 (106)
			22	46	1094-1198 (104)	20-126 (106)
			23	45	838-942 (106)	20-126 (106)
			22	48	934-1042 (108)	20-126 (106)
			24	41	453-643 (190)	348-533 (185)
			22	51	115-193 (78)	48-126 (78)
			28	48	447-481 (34)	1120-1154 (34)
			28	43	54-137 (83)	393-468 (75)
SA2668	*S. epidermidis*	SE0750	23	42	11-240 (229)	19-246 (227)
(SasF)	ATCC 12228	SE0331	27	51	605-633 (28)	440-468 (28)
	S. pneumoniae	SP_0648	21	46	21-196 (175)	16-186 (170)
	TIGR4		26	41	2034-2153	205-320 (115)

					(119)	
	S. pyogenes	SP_1937	25	40	50-174 (124)	86-223 (137)
	MGAS8232					
SA2676	*S. epidermidis*	SE0185	29	49	35-88 (53)	1350-1406 (56)
(SasA)	ATCC 12228		27	48	497-549 (52)	580-629 (49)
		SE0750	21	46	55-310 (255)	1326-1582 (256)
			30	45	41-192 (151)	81-226 (145)
			21	43	145-326 (181)	97-270 (200)
		SE0245	22	41	106-241 (135)	85-215 (130)
			23	51	272-346 (74)	83-162 (79)
			26	40	282-371 (91)	521-613 (92)
			57	64	504-517 (13)	2200-2213 (13)
	S. pneumoniae	SP_0057	21	43	1111-1311 (200)	2062-2249 (187)
	TIGR4		25	40	22-117 (95)	63-154 (91)
			26	43	28-117 (89)	116-216 (100)
			25	48	31-117 (86)	733-828 (95)
			36	50	682-716 (34)	254-289 (35)
			22	49	34-117 (83)	1320-1406 (86)
			24	41	270-339 (69)	492-563 (71)
			22	41	52-117 (65)	1464-1530 (66)
			25	45	236-275 (39)	1014-1053 (39)
			38	77	1109-1126 (17)	1038-1055 (17)
			29	51	236-266 (30)	253-283 (30)
	S. agalactiae	GBS1356	25	43	533-607 (74)	1507-1585 (78)
	NEM316	GBS1529	32	54	638-697 (59)	684-743 (59)
			25	47	113-190 (77)	490-568 (78)
			25	53	139-199 (60)	686-749 (63)
			30	49	500-550 (50)	688-740 (52)
SA1138	*S. epidermidis*	SE0185	28	52	62-113 (41)	564-616 (42)
(SasJ/IsdB)	ATCC 12228	SE0750	21	45	39-213 (174)	445-622 (177)
		SE0245	21	41	2-418 (416)	6-423 (417)
		SE2272	21	51	650-682 (32)	587-619 (32)
			32	48	22-96 (74)	225-300 (75)
			36	57	230-262 (32)	387-418 (31)
	S. pneumoniae	SP_0648	34	46	1-154 (154)	1-153 (153)
	TIGR4		40	48	805-830 (15)	578-604 (26)
			31	63	882-903 (21)	344-365 (21)
		SP_2190	33	57	145-175 (20)	55-87 (22)
	S. agalactiae	GBS0016	23	46	64-183 (119)	492-606 (114)
	NEM316		23	50	70-148 (78)	288-363 (75)
		GBS1356	26	42	119-262 (143)	446-597 (151)

		ScpB	22	45	12-162 (150)	19-159 (140)
			31	55	595-618 (23)	238-266 (28)
			27	51	693-721 (28)	495-523 (28)
SA1140	*S. epidermidis*	SE0185	22	45	2-98 (96)	9-100 (91)
(SasE/IsdA)	ATCC 12228		30	50	164-205 (41)	278-316 (48)
		SE0750	25	48	51-150 (99)	215-312 (97)
			34	46	123-215 (92)	222-305 (83)
			24	44	102-216 (114)	37-157 (120)
			32	41	608-663 (55)	241-298 (57)
		SE0245	25	45	2-159 (157)	11-163 (152)
			34	57	178-225 (47)	259-309 (50)
			21	48	276-332 (56)	256-315 (59)
		SE0331	31	50	170-236 (66)	254-326 (72)
			35	56	985-1021 (36)	279-317 (38)
	S. pneumoniae	SP_0648	24	42	57-171 (114)	205-309 (104)
	TIGR4	SP_0057	27	42	29-94 (65)	258-316 (58)
	S. agalactiae	GBS0016	30	42	247-329 (82)	252-326 (74)
	NEM316	GBS1356	31	45	131-207 (76)	253-326 (73)
	S. pyogenes	SP1745	29	48	1111-1184 (73)	252-352 (100)
	MGAS8232		23	47	341-382 (41)	69-110 (41)
SA2509	*S. epidermidis*	SE0750	21	41	4-144 (140)	6-146 (140)
(FnbB)	ATCC 12228	SE0245	27	44	4-133 (129)	2-136 (134)
			22	43	405-485 (80)	424-497 (73)
		SE0331	23	42	148-853 (705)	33-751 (718)
			26	50	109-218 (109)	30-136 (106)
			44	60	1011-1053 (42)	898-939 (41)
			40	54	42-63 (21)	778-798 (20)
		SE0185	23	46	138-243 (105)	28-144 (116)
			22	44	148-214 (66)	493-559 (66)
	S. pneumoniae	SP_2216	28	54	338-371 (33)	652-686 (34)
	TIGR4		25	41	60-145 (85)	31-117 (86)
		SP_0648	29	48	2136-2187 (51)	466-519 (53)
			21	49	916-1040 (124)	22-135 (113)
			30	59	2176-2221 (45)	883-929 (46)
			23	53	1798-1858 (60)	376-436 (60)
			25	58	1687-1739 (52)	221-271 (50)
			25	59	801-827 (26)	717-743 (26)
			58	75	421-432 (11)	446-457 (11)
			28	56	803-827 (24)	681-705 (24)
			42	52	890-910 (20)	565-585 (20)
		SP_0057	27	43	8-148 (140)	6-146 (140)
			27	48	1097-1125 (28)	331-359 (28)

		SP_1174	26	45	685-793 (108)	28-144 (116)
		SP_2190	25	43	386-480 (94)	33-120 (87)
			21	41	298-390 (92)	49-142 (93)
	S. agalactiae	GBS1356	35	48	616-716 (100)	772-869 (97)
	NEM316		21	45	111-256 (145)	45-189 (144)
		ScpB	28	44	24-117 (93)	50-140 (90)
			33	48	476-520 (44)	316-355 (39)
		GBS0016	26	42	240-322 (82)	34-117 (83)
			23	41	138-227 (89)	33-119 (86)
			30	51	397-428 (31)	654-686 (32)
			39	57	277-309 (32)	341-370 (29)
		GBS1529	23	45	1188-1281 (93)	16-110 (94)
			21	44	809-908 (99)	35-135 (100)
SA2418	*S. pneumoniae*	SP_0648	25	46	43-157 (114)	198-304 (106)
(SBI)	TIGR4		28	53	2042-2073 (31)	353-384 (31)
		SP_0057	21	41	755-862 (107)	274-386 (112)
			28	43	532-628 (96)	159-239 (80)
			27	60	552-581 (29)	233-265 (32)
			30	53	258-283 (25)	105-130 (25)
		SP_1174	30	53	688-767 (79)	243-316 (73)
			28	47	670-761 (91)	202-290 (88)
			29	49	456-525 (69)	162-233 (71)
		SP_1282	22	43	221-343 (122)	111-235 (124)
	S. agalactiae	GBS0016	26	51	125-218 (93)	212-305 (93)
	NEM316					
	S. pyogenes	SP1937	26	40	88-173 (85)	147-230 (83)
	MGAS8232					
SA1781	*S. epidermidis*	SE0185	25	47	48-149 (101)	278-365 (87)
(SasI)	ATCC 12228		29	52	191-245 (54)	278-331 (53)
			21	46	129-175 (46)	656-702 (46)
		SE0750	25	42	158-247 (89)	656-738 (82)
		SE0245	25	47	243-305 (62)	643-701 (58)
			21	44	145-222 (77)	282-363 (81)
		SE0331	30	53	606-644 (38)	591-628 (37)
			32	48	87-161 (74)	652-721 (69)
			22	52	971-1017 (46)	285-334 (49)
			31	43	378-409 (31)	139-170 (31)
			23	47	147-237 (90)	277-358 (81)
			27	46	60-111 (51)	284-335 (51)
			23	48	247-310 (63)	288-348 (60)
	S. pneumoniae	SP_0648	23	45	1823-1956 (133)	288-422 (136)

	TIGR4		30	52	1795-1864 (69)	679-743 (64)
			33	44	2188-2232 (44)	850-893 (43)
			27	50	1458-1493 (35)	435-470 (35)
	S. pyogenes	SP1937	29	43	25-106 (81)	681-763 (82)
	MGAS8232		33	51	78-132 (54)	278-336 (58)
SA2666	*S. epidermidis*	SE0750	28	48	1185-1328 (143)	326-482 (156)
	ATCC 12228		46	57	688-715 (27)	179-204 (25)
		SE2319	26	44	55-323 (268)	338-616 (278)
		SE0433	40	55	162-265 (103)	508-616 (108)
		SE0331	31	47	689-827 (138)	376-505 (129)
			25	45	105-216 (111)	234-340 (106)
			34	53	545-570 (25)	111-136 (25)
			33	44	193-225 (32)	173-206 (33)
	S. pneumoniae	SP_2216	32	44	282-389 (107)	503-616 (113)
	TIGR4		27	44	1367-1449 (82)	362-439 (77)
			29	45	249-296 (47)	164-206 (42)
			26	50	1813-1866 (53)	384-439 (55)
			26	52	1678-1715 (37)	408-445 (37)
		SP_2190	30	46	141-221 (80)	238-307 (69)
	S. agalactiae	GBS0016	34	47	330-444 (114)	497-616 (119)
	NEM316		24	41	26-134 (108)	338-445 (107)
			35	45	272-308 (36)	164-200 (36)
		GBS1306	26	44	44-137 (93)	721-813 (92)
	S. pyogenes	SP1937	31	47	247-532 (285)	349-622 (273)
	MGAS8232					

Table A.1: Families of cross-reactive antigens, based on sequence homologies and representation of each screened genome.