Environmental Regulation of Virulence Determinant Expression in *Staphylococcus aureus*

by

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Summary

Staphylococcus aureus is a highly versatile pathogen that causes a wide range of diseases. Appropriate gene expression in various niches within the human body and abiotic environments requires the sensing of environmental conditions. An important environmental parameter affecting *S. aureus* is NaCl concentration. This study investigated *S. aureus* virulence determinant regulation in the presence and absence of NaCl stress.

In the absence of NaCl stress, σ^B was found to repress *hla* transcription and protease activity, possibly via the repression of *agr* transcription. The effect of σ^B on *agr* probably occurs indirectly, although *sarA, sarH1* and *rot* are unlikely to function as intermediates in this pathway. Tn*551* mutagenesis identified numerous genes, including *lysC, ykuQ, lysA, brnQ* and *telA,* which repress *hla* transcription and protease activity by upregulating σ^B activity. These genes are clustered in the SVS (***S. aureus*** **virulence and survival**) region of the *S. aureus* genome, in which transposon insertion affected the virulence and survival of mutants isolated in numerous published screens. Other SVS region genes, including *asd, dapA, hipO, aclP* and *norQ* were also found to repress *hla* transcription and protease activity by upregulating σ^B activity.

In the presence of NaCl stress, virulence determinants (which are normally regulated by *agr*) come under the control of a novel regulatory system involving σ^B-dependent and σ^B-independent pathways. Tn*917* mutagenesis identified several genes, including *citG, opuD, yugT, oppF, ykrP, eprH, yubA, unk*1 and *unk*2, which have putative roles in the σ^B-independent pathway. The SVS region genes analysed may function in the σ^B-dependent pathway. The sensor *saeS* was found to upregulate *hla* transcription in the absence of NaCl stress and may be involved in sensing NaCl stress. The NaCl stress signal may act in concert with other parameters to allow stringent virulence determinant regulation in response to the prevailing environmental conditions.

Publications arising from this work

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Please note that I was the joint first author of this paper.

This thesis is dedicated to Andy, Joy, Bill, my parents and grandparents.

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Abbreviations

ABC	ATP-binding cassette
aht	Gene(s) affecting ***hla*** transcription
AIP	Auto-inducing peptide
Amp	Ampicillin
AP	Alkaline phosphatase
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BHI	Brain heart infusion
bp	Base pair
β-gal	β-galactosidase
cAMP	Cyclic adenosine 3', 5'-monophosphate
cfu	Colony forming units
CIAP	Calf Intestinal Alkaline Phosphatase
Cm	Chloramphenicol
°C	Degrees Celsius
dH_2O	Distilled water
DIG	Digoxigenin
DMF	N,N-dimethylformamide
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleoside-5'-triphosphate
ECL	Enhanced Chemiluminescence system
EDTA	Ethylenediamine tetra-acetic acid
Ery	Erythromycin
FSB	Frozen storage buffer
g	Grams
g	x Gravity
h	Hours
han	Gene(s) involved in repressing **Hla** activity in the presence of **NaCl**
HRP	Horseradish peroxidase
htn	Gene(s) affecting ***hla*** transcription identified by screening on BHI plates containing **NaCl**
HU	Haemolysin units
Kan	Kanamycin
kb	Kilobase pairs
kDa	Kilodaltons
kV	Kilovolts
l	Litre
LB	Luria-Bertani medium
Lin	Lincomycin
M	Molar
mA	Milliamps

Mb	Megabase
mbar	Millibars
MCS	Multiple cloning site
mg	Milligrams
min	Minutes
ml	Millilitres
M	Molar
mM	Millimolar
mRNA	Messenger RNA
MRSA	Methicillin-resistant *Staphylococcus aureus*
MU	4-Methyl umbelliferone
MUG	4-Methyl umbelliferyl-β-D-galactopyranoside
NBT	Nitroblue tetrazolium
NCBI	National Centre for Biotechnology Information
Neo	Neomycin
OD_{XYZ}	Optical density at XYZ nm
OE	Outside end
ORF	Open reading frame
PAS	Per-Arnt-Sim
PCR	Polymerase chain reaction
PBS	Phosphate buffered saline
pfu	Plaque forming units
PMSF	Phenylmethylsulfonyl fluoride
(p)ppGpp	guanosine 3',5'-bis(diphosphate) and guanosine 3'-diphosphate, 5'- triphosphate
PVDF	Polyvinylidene difluoride
Pwo	Thermostable DNA polymerase derived from *Pyrococcus woesei*
RAP	RNAIII activating protein
RNA	Ribonucleic acid
rpm	Revolutions per min
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
sec	Seconds
SFP	Staphylococcal food poisoning
Spec	Spectinomycin
SSC	Saline sodium citrate (buffer)
STM	Signature-tagged mutagenesis
SVS	*S. aureus* virulence and survival
t	Time
TAE	Tris-acetate EDTA (buffer)
Taq	Thermostable DNA polymerase derived from *Thermus aquaticus*
TCA	Trichloroacetic acid
TE	Tris-EDTA (buffer)
TEMED	N,N,N'N'-tetramethyl-ethylenediamine

Tet	Tetracycline
TIGR	The Institute of Genomic Research
Tn	Transposon
TRAP	Target of RAP
Tris	Tris(hydroxymethyl)aminomethane
TSS	Toxic shock syndrome
TSST-1	Toxic shock syndrome toxin-1
µF	Microfarad
µg	Micrograms
µl	Microlitres
µM	Micromolar
UV	Ultra violet
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
σ	Sigma factor
~	Approximately

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CHAPTER 1

INTRODUCTION

1.1 The staphylococci

Staphylococci are Gram-positive cocci of ~ 1 μm diameter that characteristically divide in more than one plane to form grape-like clusters. In 1883, this clustering prompted Ogston to introduce the word *Staphylococcus* (from staphyle, meaning bunch of grapes) to name a group of micrococci causing inflammation and suppuration (Kloos & Lambe, 1991). The genus *Staphylococcus* belongs to the low G + C content phylogenetic group of Gram-positive bacteria (Woese, 1987), and comprises 32 species and eight subspecies (Kloos & Bannerman, 1994).

The staphylococci are non-sporulating, non-motile bacteria, and are generally non-encapsulated. The presence of multiple glycine residues in the interpeptide bridge makes the cell wall peptidoglycan unique and renders it susceptible to lysostaphin. Members of the genus have complex nutritional needs, and in general require 5 - 12 essential amino acids and 2 - 3 B-group vitamins (Wilkinson, 1997). Staphylococci are catalase-positive and oxidase-negative, and are usually facultative anaerobes, capable of generating energy by respiratory or fermentative pathways.

Staphylococci are ubiquitous in the environment, and can be isolated from soil, dust, air and water. They are some of the hardiest non-sporulating bacteria known, demonstrating resistance to disinfectants, heat (*ie.* 50 °C for 30 min), desiccation, and salt, with most species capable of growth in the presence of 1.7 M NaCl or higher concentrations (Kloos & Lambe, 1991; Kloos & Bannerman, 1994).

Typically, the staphylococci are found in association with the skin, skin glands and mucous membranes of warm-blooded animals. Pathogenic staphylococci are usually identified by their ability to clot plasma by the production of coagulase (Kloos & Lambe, 1991). At present, only three species of staphylococci have been shown to be coagulase-positive. These include *S. intermedius* and *S. hyicus*, which are found in animals (Devriese *et al.*, 1978; Hajek, 1976) and *S. aureus*, which is a human pathogen.

The remaining members of the genus *Staphylococcus* are coagulase-negative and do not generally cause disease. However, a few coagulase-negative pathogenic staphylococci have been identified, such as *S. epidermidis*, although these are considered opportunistic pathogens as they infect indwelling medical devices, or following trauma or predisposing disease (Kloos & Lambe, 1991).

1.2 *Staphylococcus aureus*

Staphylococcus aureus is one of the most prevalent species in the genus *Staphylococcus*, second only to *S. epidermidis*. However, whereas *S. epidermidis* until only recently rarely caused disease, *S. aureus* is a highly versatile human pathogen and has been the focus of extensive research. The species name *aureus* (meaning 'golden') is derived from the fact that on agar, colonies of most *S. aureus* strains display yellow pigmentation due to the presence of triterpenoid carotenoids and staphyloxanthin, whereas other species form white colonies (Marshall & Wilmoth, 1981). *S. aureus* strains are alkaline phosphatase-positive and show no β-galactosidase activity. The entire genomes of a number of *S. aureus* strains have been sequenced and shown to be ~ 2.8 megabase pairs in size (Baba *et al.*, 2002). Assuming that the average gene size is 1 kb, *S. aureus* is likely to possess ~ 3,000 genes (Coulter *et al.*, 1998).

1.3 Diseases caused by *S. aureus*

S. aureus causes a range of diseases, which vary widely in seriousness, from the minor to the life-threatening. The diseases can be divided into three groups, including the superficial lesions (*eg.* small skin abscesses and wound infections), the toxinoses (*eg.* toxic shock syndrome, scalded skin syndrome and food-poisoning) and the potentially fatal systemic conditions (*eg.* bacteraemia, septicaemia, endocarditis, osteomyelitis, pneumonia and meningitis).

As the fourth most frequently isolated pathogen in hospital environments (Kloos & Bannerman, 1994), *S. aureus* is a major cause of nosocomial infections, although it also causes community-acquired infections. Infection with *S. aureus* is a common

complication following invasive hospital techniques, as skin damage allows entry into the body. For example, *S. aureus* has been identified as the main cause of postoperative wound infections, being responsible for 28 % of cases (Giacometti *et al.*, 2000). In addition, *S. aureus* frequently infects indwelling medical devices such as catheters and central venous lines, and has been shown to cause 20 % and 29 % of infections involving intravascular canulae and pacemakers respectively (Bluhm, 1985).

Toxic shock syndrome (TSS) is an acute and potentially fatal illness characterized by shock, high fever, a diffuse erythematous rash, hypotension, and involvement of multiple organ systems (Dinges *et al.*, 2000). High absorbency menstrual tampons have been identified as a risk factor for developing TSS (Reingold *et al.*, 1980), although only strains possessing the gene encoding toxic shock syndrome toxin 1 (TSST-1) are capable of causing the disease.

S. aureus causes staphylococcal food-poisoning (SFP) by contaminating food with one or more of its enterotoxins (Table 1.1). However, SFP does not require the presence of viable bacteria, and the heat-stable enterotoxins often survive the cooking process. The incidence of SFP is unknown, although it is probably the most common cause of food-poisoning in America (Dinges *et al.*, 2000). Symptoms of SFP include nausea, vomiting, abdominal pain and diarrhoea. However, SFP is a self-limiting condition that typically resolves within 24 to 48 h of onset, and is rarely fatal (Dinges *et al.*, 2000).

Bacteraemia caused by *S. aureus* generally results from local infection with dissemination into the blood stream, although it may also occur due to direct inoculation during intravenous drug abuse. Septicaemia involves the multiplication of bacteria in the blood, and often results in a secondary site of infection (*eg.* endocarditis).

S. aureus is responsible for 80 - 90 % of cases of pyogenic osteomyelitis (Dirschl & Almekinders, 1993), and can also cause septic arthritis and orthopaedic implant failure. The ability of *S. aureus* to cause bone and joint pathology has been reviewed by Nair *et al.* (2000).

The fact that 19 - 51 % of healthy individuals are carriers of *S. aureus* (Kluytmans *et al.*, 1997) has been an important factor in the spread of *S. aureus*-associated diseases by

food preparation and hospital workers. The anterior nares are the main site of carriage, although *S. aureus* is also found on the skin and in faeces (Novick, 1990; Kloos & Bannerman, 1994). The ability of the bacterium to survive in abiotic environments, and to resist common disinfection procedures has also played an important role in allowing the bacterium to become a major cause of nosocomial infection.

1.4 Treatment and prevention of *S. aureus* infections

Penicillin was introduced for the treatment of severe *S. aureus* infections in the 1940's. However, by the late 1940's the emergence of β-lactamase allowed approximately 60 % of hospital-acquired strains to become penicillin-resistant (Barber and Rozwadowska-Dowzenko, 1948). In addition, strains resistant to other commonly used antibiotics such as streptomycin, tetracycline and erythromycin were reported shortly after they began to be administered clinically (Demerec, 1948; Haight & Finland, 1952). In the 1960's, β-lactamase-resistant antibiotics, such as methicillin, were introduced. However, strains of methicillin-resistant *S. aureus* (MRSA) possessing a novel form of a penicillin binding protein (PBP2A (MecA)) with reduced affinity for methicillin rapidly emerged (Hartman & Tomasz, 1984; Jevons, 1961). Over the last decade, vancomycin has been reserved to treat *S. aureus* infections that are resistant to the other major antibiotics. However, *S. aureus* strains displaying intermediate resistance to vancomycin were identified initially in Japan (Kremery *et al.*, 1996), and subsequently in the United States and France. The first case of vancomycin-resistant *S. aureus* (VRSA) occurred in 2002 (Centres for Disease Control, 2002).

The antibiotic linezolid is effective against *S. aureus* and a number of other Gram-positive pathogens (Biedenbach & Jones, 1997). However, following its recent introduction into clinical use, *S. aureus* strains resistant to linezolid have already begun to emerge (Potoski *et al.*, 2002), and resistance to other new antibiotics is inevitable. Therefore, it is imperative that future research focuses on finding ways of preventing, in addition to curing, *S. aureus*-associated diseases. At present, mupirocin is administered topically to prevent nasal carriage of *S. aureus* by hospital staff, in order to reduce the incidence of nosocomial infection (Hudson, 1994). Numerous attempts to develop

vaccines have also been made (Foster, 1991; Schennings *et al.*, 1993; Fattom *et al.*, 1996; Menzies & Kernodle, 1996; Nilsson *et al.*, 1998).

1.5 Virulence determinants produced by *S. aureus*

Virulence determinants are bacterial products that contribute to the pathogenicity of a microorganism. The ability of *S. aureus* to cause a wide range of diseases (Chapter 1.3) can be accounted for by its ability to produce a vast repertoire of virulence determinants, which are shown in Table 1.1. The virulence determinants of *S. aureus* can be divided into three categories, including those involved in: 1) attachment (*ie.* factors involved in the attachment of *S. aureus* to cells or extracellular matrices), 2) host defence evasion (*ie.* factors that reduce or prevent phagocytosis, or that interfere with the function of anti-staphylococcal antibodies or other specific host-defence mechanisms), and 3) invasion/tissue penetration (*ie.* factors involved in attacking host cells or that degrade components of extracellular matrices) (Projan & Novick, 1997), although it is possible that some factors may have a role in more than one of these categories. However, the list of virulence determinants (Table 1.1) is not comprehensive, and it is possible that additional virulence determinants are yet to be discovered. Furthermore, many of the genes involved in the pathogenesis of *S. aureus* also have overlapping housekeeping functions. For example, whilst the peptidoglycan of *S. aureus* has the housekeeping function of conferring structure and stability, it also plays an important role in pathogenesis by provoking a harmful immune response (Kaplan & Tenenbaum, 1982). It is disputable whether such components should be included in the list of virulence determinants.

With the exception of the toxinoses, no single factor is responsible for the pathogenesis caused by *S. aureus*; disease instead occurs due to the coordinate action of multiple factors (Projan & Novick, 1997). In addition, it is important to note that some of the *S. aureus* virulence determinants are only important in particular diseases, and that no strain capable of producing all of the known virulence determinants has yet been identified.

Virulence determinant	**Gene**	**Growth phase of expression**
Attachment		
Clumping factor	*clfA*	Log phase
Fibrinogen-binding protein	*fbpA*	Log phase
Fibronectin-binding protein A	*fnbA*	Log phase
Fibronectin-binding protein B	*fnbB*	Log phase
Collagen-binding protein	*cna*	Log phase
Coagulase	*cga / coa*	Log phase
Polysaccharide/adhesin (PS/A)	-	-
Polysaccharide intracellular adhesin	-	-
Host defence evasion		
Enterotoxin A	*sea*	Constitutive
Enterotoxins B, C1-3, D, E, H	*seb-h*	Post-exponential
Toxic shock syndrome toxin-1 (TSST-1)	*tst*	Post-exponential
Staphylococcal exotoxin-like proteins	*set* 1-5	-
Exfoliative toxins A, B	*eta, etb*	Post-exponential
Protein A	*spa*	Log phase
Lipase	*geh*	Post-exponential
V8 protease (serine protease)	*sspA*	Post-exponential
Fatty acid modifying enzyme (FAME)	*fme*	Post-exponential
Panton-Valentine leukocidin	*lukPV, lukS-PV*	Post-exponential
Capsular polysaccharide types 1	*cap1 locus*	Post-exponential
Capsular polysaccharide type 5	*cap5 locus*	Post-exponential
Capsular polysaccharide type 8	*cap8 locus*	Post-exponential
Staphylokinase	*sak*	Post-exponential
Catalase	*katA*	Post-exponential
Invasion/tissue penetration		
α-haemolysin (α-toxin)	*hla*	Post-exponential
β-haemolysin (β-toxin)	*hlb*	Post-exponential
δ-haemolysin (δ-toxin)	*hld*	Post-exponential
γ–haemolysin (γ-toxin)	*hlgA, B, C*	Post-exponential
Metalloprotease (aureolysin)	*aurA*	Post-exponential
Cysteine protease	*sspB*	Post-exponential
Phospholipase C	*plc*	Post-exponential
Hyaluronidase (hyaluronate lyase)	*hysA*	Post-exponential

Table 1.1

Virulence determinants produced by *S. aureus*.

Adapted from Projan & Novick (1997). (- = not determined).

Three representative virulence determinants are considered in this thesis; surface protein A, α-haemolysin and the proteases. Protein A (Spa) is a 42 kDa cell wall protein, although it may also be released extracellularly. In both cases, protein A interacts non-specifically with the Fc portion of all subclasses of human immunoglobulin (IgG) except IgG_3 (Forsgren & Sjoguist, 1966). The function of protein A is to inhibit opsonisation and therefore prevent phagocytosis.

α-haemolysin is one of the most extensively studied *S. aureus* virulence factors, and is encoded by the gene *hla* (Gray & Kehoe, 1984). Monomers of α-haemolysin bind to cell membranes and associate to form a hexamer (or heptamer), resulting in pore formation and cell lysis (Bhakdi & Tranum-Jensen, 1991). α-haemolysin lyses a wide range of cell types, including leukocytes, platelets and fibroblasts, although erythrocytes represent the most sensitive target. Furthermore, α-haemolysin displays a wide variation in activity against erythrocytes of different species. It is at least 100-times more active against rabbit erythrocytes than those from any other species (Bhakdi *et al.*, 1984), and so rabbit blood is used to assay the toxin.

S. aureus produces three main proteases; serine protease (V8 protease) (*sspA*), cysteine protease (*sspB*) and metalloprotease (*aurA*) (reviewed by Dubin, 2002). Serine protease is synthesised as an inactive precursor that is activated by the metalloprotease. Activated serine protease then activates a cysteine protease precursor. The ability of activated serine protease to cleave IgG antibodies *in vitro* suggests that it may have a role in immune response evasion, and it has also been suggested that the proteases may provide protection against antimicrobial peptides such as the platelet microbicidal proteins (PMPs) (Yeaman *et al.*, 1994) or neutrophil defensins (Selsted *et al.*, 1993). Additionally, the proteases of *S. aureus* may play a role in the destruction of tissue proteins and therefore contribute to invasiveness (Goguen *et al.*, 1995). However, it is also possible that the proteases act as molecular scavengers to obtain utilisable nutrients from the environment, and that they are involved in regulating the stability and activity of other *S. aureus* virulence determinants.

1.6 Control of virulence determinant production

1.6.1 General mechanisms involved in coordinate virulence determinant regulation

The growth and survival of bacteria is competitive, and is dependent on the ability to adapt to environmental changes. In all bacteria, only a small proportion of genes, termed housekeeping genes, are essential for growth and survival. These are often expressed constitutively. However, the remaining genes are non-essential, and are referred to as accessory factors. Although the virulence determinants produced by *S. aureus* (Chapter 1.5) provide the bacterium with an advantage during infection, none are essential for growth and survival, and can therefore be considered accessory factors. The production of accessory factors diverts metabolic resources away from housekeeping gene expression, and so a strain which produces accessory factors constitutively would be less competitive than a strain which restricts their synthesis to the times when they are required. Therefore, bacteria have evolved a number of regulatory systems that allow gene expression to occur only when appropriate. However, the following list is not comprehensive, but instead introduces a number of important mechanisms that will be considered further in this thesis.

1.6.1.1 Signal transduction

1.6.1.1.1 Two-component sensor-regulator systems

In order for a bacterium to adapt its gene expression appropriately, it must be able to sense environmental changes. The majority of environmental stimuli do not penetrate the cell wall. Therefore, bacteria have evolved mechanisms that allow signals to be relayed from the outside to the inside of the cell. Two-component signal transduction systems detect changes in one or more environmental parameter (*eg.* temperature, osmolarity, pH, and nutrient availability), and mediate appropriate changes in gene expression. However, although the bacteria in which two-component systems have been identified are extremely diverse, ranging from soil bacteria to pathogens, their signal transduction pathways involve a common mechanism and homologous protein domains

(Nixon *et al.*, 1986: Miller *et al.*, 1989: Stock *et al.*, 1989). Similar two-component signal transduction systems have also been identified in eukaryotes.

In general, two-component systems comprise two proteins; a transmembrane sensor kinase and a transcription factor known as a response regulator. Environmental signals are detected by the extracellular or periplasmic N-terminus of the sensor kinase. This causes the sensor kinase to become autophosphorylated at a conserved histidine residue on its C-terminus. The phosphate is then transferred to a conserved aspartyl residue on the N-terminus of the response regulator, causing a conformational change that allows the C-terminal domain of the response regulator to activate or repress target gene transcription.

1.6.1.1.2 Quorum sensing

Quorum sensing systems allow bacteria to regulate gene expression in a cell density-dependent manner. A diffusible signal molecule (pheromone) is released and accumulates in the surrounding environment at concentrations directly proportional to cell density. When the concentration of the signal molecule exceeds a threshold level, the signal is transduced to make appropriate changes in gene expression. The signal molecules of Gram-positive bacteria are usually peptides (Clewell, 1993: Solomon *et al.*, 1996), which are produced by cleavage of a larger precursor molecule and which may then be further post-translationally modified. Quorum sensing systems have also been identified in Gram-negative bacteria, although their signal molecules are usually non-processed N-acyl homoserine lactones (Eberhard *et al.*, 1981).

Pseudomonas aeruginosa is a Gram-negative pathogen that mainly infects immunocompromised patients and suffers of cystic fibrosis. *P. aeruginosa* uses quorum sensing to allow the elastase-specific protease LasB to be expressed only when the bacterial population is large enough to produce sufficient amounts of LasB to cause an appreciable effect (Pearson *et al.*, 1994). This allows the bacterium to conserve metabolic resources at times when LasB production is unnecessary.

1.6.1.2 Alternative sigma factors (σ)

Many bacteria coordinately control the transcription of sets of genes in response to environmental changes through the use of sigma factors (σ). σ factors are proteins that bind to core RNA polymerase to form holoenzymes which direct transcription from specific promoters with conserved sequence motifs (reviewed by Helmann & Moran, 2002), and can be subdivided into two groups (Dombroski *et al.*, 1993). The primary σ factor is required for the transcription of housekeeping genes whose products are essential for growth. The second group comprises the alternative σ factors, which coordinate the transcription of particular sets of genes under specific cellular conditions.

The general stress response of the low-GC Gram-positive bacterium *Bacillus subtilis* is controlled by the alternative transcription factor σ^B (reviewed by Price, 2002). It is estimated that over 200 genes are directly or indirectly regulated by σ^B of *B. subtilis* in response to environmental stresses including acid, alkali, ethanol, heat, osmotic, or oxidative stress, and by energy depletion caused by starvation or entry into stationary phase (Bernhardt *et al.*, 1997; Price, 2000; Price *et al.*, 2001). In addition, σ^B is a major transcription regulator in stress resistance in *Listeria monocytogenes* (Becker *et al.*, 1998) and contributes to virulence in *Bacillus anthracis* (Fouet *et al.*, 2000).

A similar role in the general stress response of the Gram-negative bacteria *Escherichia coli* and *Salmonella typhimurium* has been suggested for RpoS (Hengge-Aronis, 1993; Loewen & Hengge-Aronis, 1994). The finding that mutation of *rpoS* in Gram-negative bacteria leads to reduced virulence in mice indicates the importance of *rpoS* in pathogenesis (Chen *et al.*, 1995; Nickerson & Curtiss, 1997, Wilson & Gulig, 1998).

1.6.1.3 DNA supercoiling

Classical models of bacterial gene regulation represent DNA as a passive substrate upon which regulatory proteins act. However, in the last four decades it has been recognised that topological changes in the DNA itself may play a role in the control of cellular processes such as transcription (Dorman, 1994). Eubacterial DNA mainly occurs as a right-handed duplex (Drlica & Riley, 1990) that forms closed loops or covalently closed

circles. In terms of DNA secondary structure, these are topologically equivalent. The DNA duplex in these loops is usually in an underwound state (Worcel & Burgi, 1972). This creates torsional tension which causes either strand separation or a distortion of the DNA helical axis in which the duplex coils around itself. Coiling of the already coiled DNA duplex is termed 'supercoiling', and is controlled by topoisomerases (*eg.* DNA gyrase) and histone-like proteins. Supercoiling of DNA in the same sense as the right-handed coiling of the duplex is referred to as 'positive supercoiling', whilst coiling in the opposite sense causes negative supercoiling.

The physical chemistry of DNA suggests that underwinding of the double helix thermodynamically favours DNA reactions that require strand separation (Drlica, 1984). These include transcription, replication and recombination. Furthermore, the possible importance of DNA supercoiling in the regulation of gene expression has been highlighted by the finding that the *E. coli* chromosome is subdivided into independently supercoiled domains (Sinden & Pettijohn, 1981). This suggests that genetic geography (*ie.* the position of a gene on the chromosome) could play a role in gene regulation. DNA supercoiling is sensitive to a wide range of environmental conditions, including osmolarity (Higgins *et al.*, 1988), anaerobiosis (Dorman *et al.*, 1988) and temperature (Goldstein & Drlica, 1984). Consequently, DNA supercoiling has the potential to coordinately regulate every gene in the bacterial cell in response to a range of environmental variables experienced during infection, and may contribute not only to environmental adaptation and survival, but also to pathogenicity. Transcription of *invA*, required for the invasive phenotype of *S. typhimurium*, is induced eight-fold by osmotic stress via DNA supercoiling (Galan & Curtiss, 1990). Additionally, DNA supercoiling regulates the expression of the alginate (*alg*) genes of *P. aeruginosa* and α-haemolysin of *E. coli* in response to osmolarity (Berry *et al.*, 1989; Carmona *et al.*, 1993).

1.6.2 Regulation of virulence determinant production in *S. aureus*

1.6.2.1 Expression of virulence determinants in *S. aureus*

The pattern of virulence determinant expression in *S. aureus* follows a coordinated program that parallels the development of an infection (Figure 1.1). The expression of

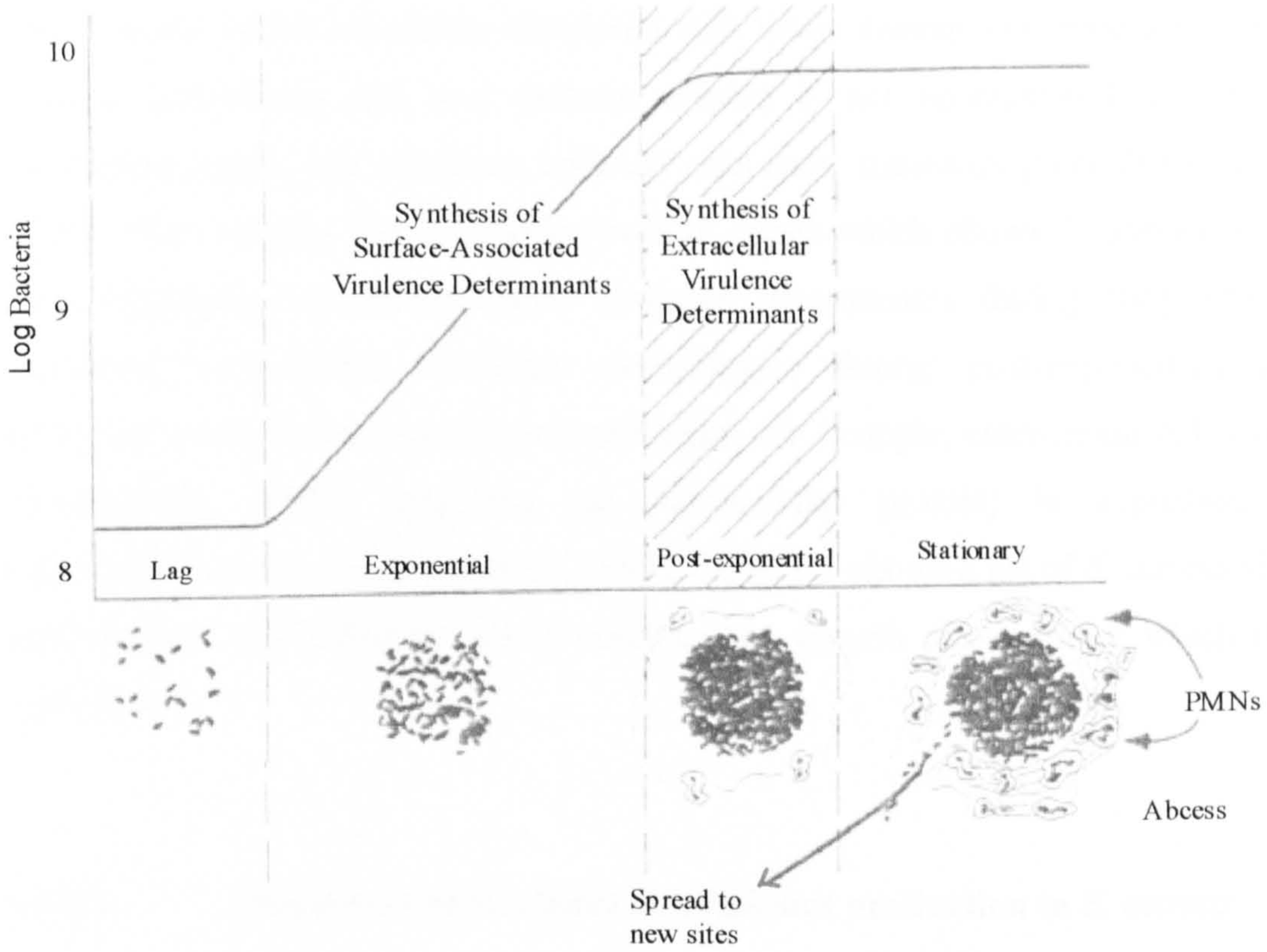


Figure 1.1

Hypothetical role of virulence determinants in *S. aureus* infection.

In this model, bacteria in lag phase would initiate the infection and would enter exponential phase, where multiplication begins, accompanied by synthesis of surface-associated virulence determinants to allow attachment to host cells. Crowding during post-exponential phase would activate a density-sensing mechanism that would stimulate the production of the extracellular virulence determinants, enabling the organisms to escape from the localised infection (abscess) during stationary phase and spread to new sites, where the cycle would be repeated.

Adapted from Projan and Novick (1997).

the surface-associated virulence determinants, which are mainly involved in attachment to host tissues, is up-regulated in early exponential phase and is sharply down-regulated in late exponential phase. In contrast, the expression of the extracellular virulence determinants, which are mainly associated with tissue damage (required for dispersal or nutrient acquisition) and host defence evasion is not up-regulated until the post-exponential phase, and continues until the cells enter stationary phase (Coleman *et al.*, 1978). This suggests that a genetic "switch" exists which allows *S. aureus* to change from expressing surface-associated virulence determinants during early growth, to expressing extracellular virulence determinants during post-exponential growth. However, a few exceptions to this model exist; for example, enterotoxin A is expressed constitutively, whilst coagulase (an extracellular protein) is expressed during logarithmic growth (Tremaine *et al.*, 1993). Table 1.1 shows a list of *S. aureus* virulence determinants, and indicates, where possible, the growth phase during which they are expressed.

1.6.2.2 Regulators of virulence determinant production in *S. aureus*

The control of virulence determinant production in *S. aureus* is mediated by a number of global regulators that have mainly been identified by transposon mutagenesis. Inactivation of these loci results in pleiotropic effects on the production of exoproteins and/or virulence determinants. These regulatory loci act either coordinately or independently, and include *agr* (Recsei *et al.*, 1986), *sar* (Cheung *et al.*, 1992) (and its homologues *rot* (McNamara *et al.*, 2000) and *sarH1-5* (Tegmark *et al.*, 2000)), as well as *sae* (Giraudo *et al.*, 1994), *arl* (Fournier & Hooper, 2000), IE3 (Cheung *et al.*, 1995) and *srrAB* (Yarwood *et al.*, 2001). A further potential global regulator, *xpr* (extracellular protein regulator), was identified in 1992 by Smeltzer *et al.*, although the *xpr* mutant was subsequently shown to contain an additional *agr* mutation (McNamara & Iandolo, 1998). The alternative sigma factor, σ^B (Wu *et al.*, 1996) has been shown to regulate virulence determinant expression, and further unknown regulators may also play a role in this complex process.

1.6.2.2.1 *agr* (accessory gene regulator)

The *agr* (accessory gene regulator) locus was identified as the site of a Tn*551* insertion affecting the expression of several virulence determinants (Recsei *et al.*, 1986, Morfeldt *et al.*, 1988; Peng *et al.*, 1988). *agr* upregulates a number of extracellular virulence determinants, including toxic-shock syndrome toxin 1 (TSST-1), several enterotoxins (*ie.* B, C and D), leukocidin, the haemolysins (*ie.* α, β, δ and γ), serine protease, metalloprotease, phospholipase C, hyaluronate lyase and lipase. In addition, *agr* represses the production of a number of cell surface-associated virulence determinants, including surface protein A and fibronectin binding protein A. The importance of *agr* in pathogenicity has been confirmed by the fact that *agr* mutants have greatly attenuated virulence in a number of animal models of infection, including arthritis, mastitis and osteomyelitis (Projan *et al.*, 1989; Collins & Tarkowski, 2000).

The chromosomal DNA around the Tn*551* insertion was sequenced by Peng *et al.* (1988), and the complete sequence of the *agr* locus determined (Kornblum *et al.*, 1990). The *agr* locus consists of two divergent transcription units (Figure 1.2) whose transcripts, RNAII and RNAIII, are transcribed from the two major promoters, P2 and P3, respectively (Kornblum *et al.*, 1990: Novick *et al.*, 1995). The 3 kb RNAII transcript encodes four proteins, designated AgrB, AgrD, AgrC and AgrA (Novick *et al.*, 1995), whilst the 0.5 kb RNAIII transcript encodes δ-haemolysin (*hld*) (Janzon *et al.*, 1989) and has two presumably untranslated ORFs at its 3' end. A third transcript, RNAI, is produced and is transcribed from the P1 promoter. However, the transcription of RNAI is weak and constitutive, and its role remains unclear (Peng *et al.*, 1988).

1.6.2.2.1.1 RNAIII as a regulatory RNA molecule

RNAIII is a 512 base transcript, and is the main effector of virulence determinant regulation by *agr* (Novick *et al.*, 1993). δ–haemolysin, the only translated product encoded by RNAIII, has no general regulatory role (Janzon & Arvidson, 1990; Novick *et al.*, 1993). For example, an insertion in the 3' region of RNAIII did not impair δ-haemolysin production, but resulted in a typical *agr* mutant phenotype (Arvidson *et al.*, 1990). However, although a nonsense mutation in codon 3 of δ-haemolysin prevented

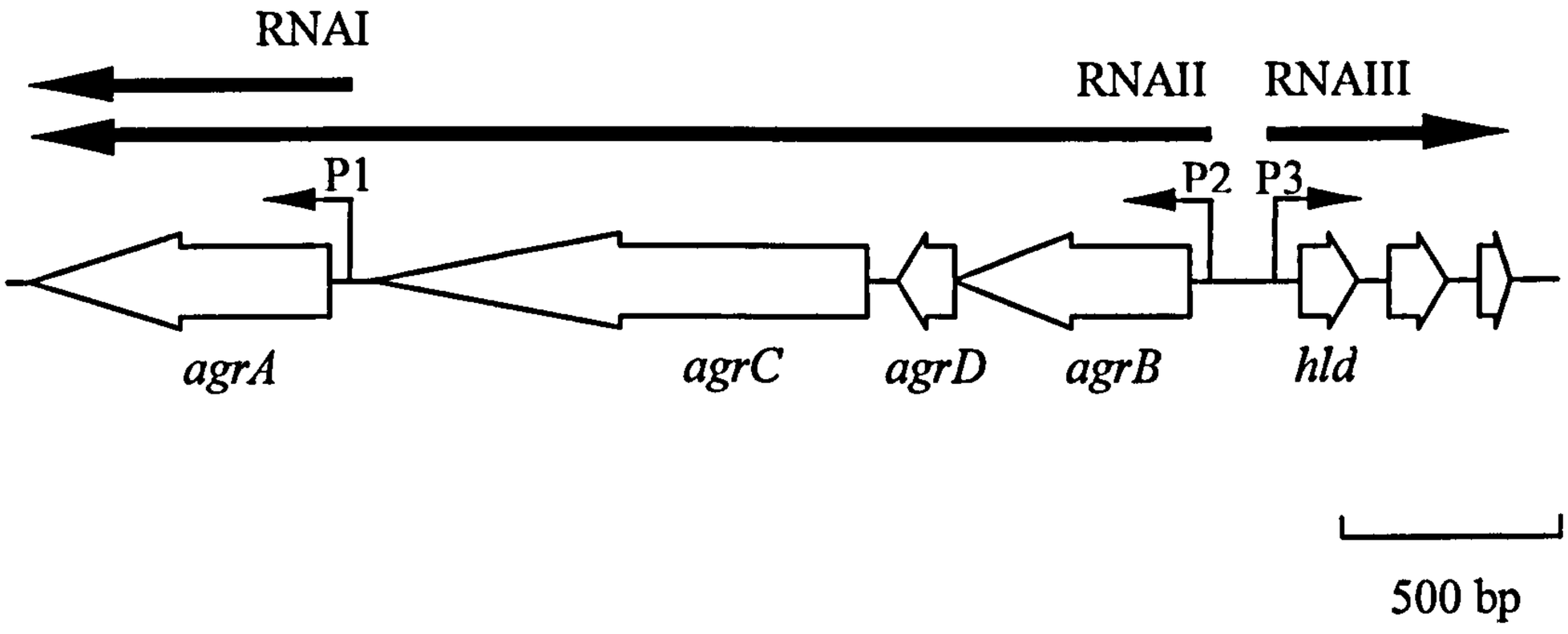


Figure 1.2

The *agr* locus of *S. aureus.*

The positions of the three *agr* promoters, P1, P2 and P3, and the length of their transcripts are indicated. The genes *agrC and agrA* encode a sensor kinase and response regulator of a two-component signal transduction system, respectively. The product of *agrD* is modified by the product of the *agrB* gene to give an octapeptide know as AIP (auto-inducing peptide). The *hld* gene encodes δ-haemolysin.

Adapted from Kornblum *et al.* (1990).

transcriptional activation of the gene encoding serine protease (Janzon & Arvidson, 1990), this probably occurred as a result of changes in RNAIII secondary structure.

RNAIII acts primarily at the level of transcription, presumably via one or more regulatory proteins (Novick *et al.*, 1993), to upregulate extracellular virulence determinant expression and repress cell surface-associated virulence determinant expression. Experiments involving deletions (Novick *et al.*, 1993) and interspecies RNAIII hybrids (Benito *et al.*, 1998, Tegmark *et al.*, 1998) have indicated that RNAIII may contain subdomains that may interact with different regulatory proteins.

However, in addition to affecting α-haemolysin (*hla*) expression at the level of transcription, RNAIII also controls *hla* translation by acting as an antisense RNA molecule. The 5' region of RNAIII contains a nucleotide sequence which is complementary to the leader region of *hla*, and which is essential for α-haemolysin translation (Novick *et al.*, 1993). In the absence of RNAIII, a stem-loop structure forms within *hla* mRNA which blocks access to the ribosome-binding site (Morfeldt *et al.*, 1995). However, the formation of a specific complex between the 5' end of RNAIII and the leader sequence of *hla* mRNA prevents the formation of the stem-loop structure (Morfeldt *et al.*, 1995). Consequently, the *hla* ribosome-binding site remains exposed, and *hla* translation occurs (Morfeldt *et al.*, 1995). It is possible that RNAIII may affect the expression of other virulence determinants at the translational level. For example, complementarity between the leader sequence of the gene encoding enterotoxin D (*sed*) and RNAIII has been demonstrated (Morfeldt, 1996).

1.6.2.2.1.2 Activation of *agr*

The main role of the P2 operon is to control RNAIII transcription in a cell density-dependent manner (Chapter 1.6.1.1.2). AgrC and AgrA are the sensor kinase and response regulator of a two-component signal transduction system respectively (Novick *et al.*, 1995). The signal molecule detected by AgrC is an octapeptide termed AIP (auto-inducing peptide) which is produced by cleavage of the 46 residue pre-peptide AgrD by AgrB (Ji *et al.*, 1995). The octapeptide is further post-translationally modified by AgrB by the introduction of a cyclic thioester bond between a conserved cysteine residue and

the C-terminal carboxyl group (Ji *et al.*, 1997). AIP is then released from the cell through AgrB, which is a transmembrane protein (Ji *et al.*, 1997).

Binding of AIP to AgrC induces autophosphorylation of a conserved C-terminal cytoplasmic histidine residue (Lina *et al.*, 1998). The phosphate group presumably then transphosphorylates the conserved N-terminal aspartyl residue of AgrA. However, although the main function of AgrA is to activate P2 and P3, purified AgrA is unable to bind to the intergenic region between the RNAII and RNAIII transcription initiation sites (Morfeldt *et al.*, 1996a). Instead, activated AgrA aids the binding of SarA to P2 and P3, resulting in an upregulation of transcription from both promoters (Cheung & Projan, 1994: Heinrichs, *et al.*, 1996) (Chapter 1.6.2.2.2).

Therefore, the P2 operon demonstrates two levels of autocatalysis; (i) it encodes its own activator (AIP), which is the ligand for the sensor kinase (AgrC) encoded by the same operon: (ii) the primary function of the signal transduction pathway is the activation of its own promoter (as well as of P3), which up-regulates production of AIP as well as the signal transduction proteins. Consequently, the net effect of this dual activation is the rapid expression of RNAIII at very high levels once the threshold cell density has been reached. The mechanism by which *agr* regulates virulence determinant expression in a density-dependent manner, and the interaction of SarA with AgrA to achieve this, is represented diagrammatically in Figure 1.3.

The *agr* system is conserved among many staphylococci (Ji *et al.*, 1997). However, regional variations in the sequences of *agrB*, *agrC* and *agrD* occur, resulting in highly variant AIP molecules (Ji *et al.*, 1997). Strains of *S. aureus* can be divided into four groups, based on the type of AIP that they produce. Within each group, mutual cross-activation of the *agr*-signalling pathway is observed. However, the AIP from one group inhibits the expression of *agr* in the other groups (Ji *et al.*, 1997). This phenomenon, known as bacterial interference, has possibly evolved to allow one strain to exclude others from an infection site, and exploitation of the AIP structure may have therapeutic potential (Ji *et al.*,1997).

However, an additional mechanism for the control of *agr* activity has been suggested by Balaban *et al.* (1995, 1998 and 2001). These authors reported that a 38 kDa protein,

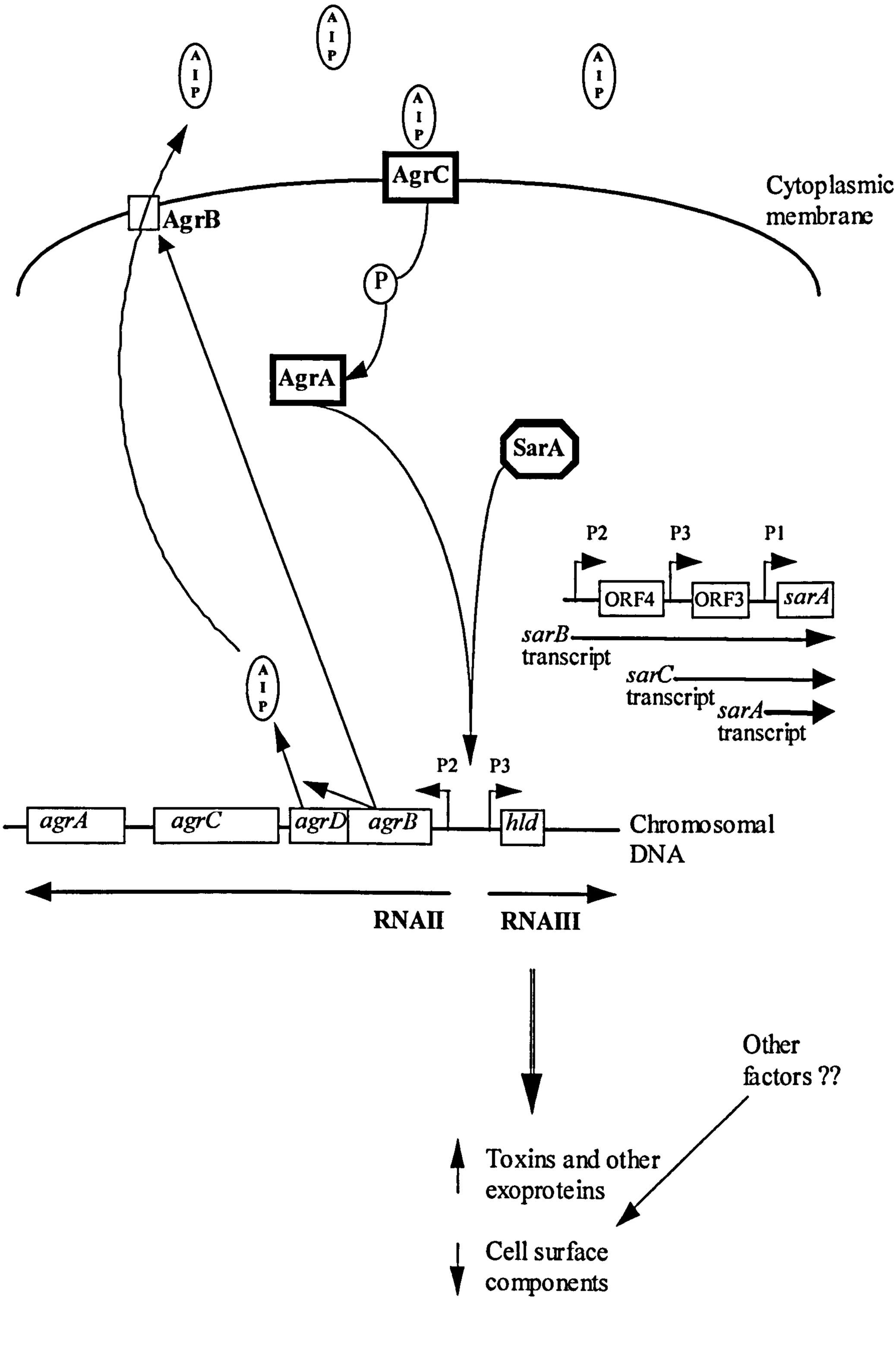

AIP
AIP
AIP
AIP
AgrC
AgrB
Cytoplasmic membrane
P
AgrA
SarA
P2
P3
P1
ORF4
ORF3
sarA
sarB transcript
sarC transcript
sarA transcript
AIP
P2
P3
agrA
agrC
agrD
agrB
hld
Chromosomal DNA
RNAII
RNAIII
Other factors ??
Toxins and other exoproteins
Cell surface components

Figure 1.3

Model of the interaction between the *agr* and *sar* gene products (depicted in bold type) which mediate changes in expression from the P2 and P3 promoters of the *agr* locus.

AgrC and AgrA are the sensor kinase and response regulator, respectively, of a two-component signal transduction system. AIP (auto-inducing peptide) is encoded within the *agrD* gene, and is modified from a 46 amino acid prepeptide to an octapeptide, by the product of the *agrB* gene. AIP is released from the cell, where it accumulates at a level proportional to cell density. When the level of AIP exceeds a critical threshold, AgrC becomes autophosphorylated and phosporylates AgrA. Activated AgrA aids the binding of SarA to P2 and P3, resulting in an upregulation of transcription from both promoters. The RNAIII transcript has been shown to upregulate extracellular virulence determinant expression and to repress the expression of surface-associated virulence determinants. Not to scale.

Adapted from Wiltshire (2000).

termed RAP (RNAIII activating protein), which is produced by both wild-type and *agr* mutant strains of *S. aureus,* induces RNAIII synthesis via the phosphorylation of a 21 kDa protein termed TRAP (target of RAP). However, the inability of other researchers to demonstrate any activation of RNAIII transcription with fractionated or unfractionated culture filtrates from the *agr*-null mutant RN6911 used by Balaban *et al.* has caused this system to be questioned (Novick *et al.*, 2000).

1.6.2.2.2 *sar* (staphylococcal accessory regulator)

The *sar* (staphylococcal accessory regulator) locus was identified as a Tn*917* insertion causing pleiotropic effects on the expression of several virulence determinants (Cheung *et al.*, 1992). Subsequent studies have shown that the *sar* locus activates the expression of the haemolysins (α, β and δ), TSST-1 and fibronectin binding protein A, but represses the expression of serine protease, metalloprotease, collagen adhesin and protein A (Chan & Foster, 1998a, Chien *et al.*, 1999; Wolz *et al.*, 2000; Cheung & Ying, 1994; Chan *et al.*, 1998; Cheung *et al.*, 1997). The importance of *sarA* in virulence has been confirmed using several animal models of infection (Booth *et al.*, 1997; Cheung *et al.*, 1994; Gillaspy *et al.*, 1995; Nilsson *et al.*, 1997).

The *sar* locus (Figure 1.3) is 1.2 kb in length, and has been mapped by Bayer *et al.* (1996). Three promoters (*ie.* P1, P3 and P2) are present, from which three overlapping transcripts (*ie. sarA*, *sarC* and *sarB*) originate respectively (Bayer *et al.*, 1996; Heinrichs *et al.*, 1996). All three *sar* transcripts have common 3' ends and encode a 372 bp ORF (*sarA*) that has been shown to be necessary for optimal α-haemolysin expression (Cheung & Ying, 1994). Upstream of *sarA* are two small ORFs (*ie.* the 39 bp ORF3 and the 18 bp ORF4). Deletion of these ORFs affects the regulatory ability of the *sar* locus, although reporter gene fusion experiments have shown that these effects are due to altered production of SarA rather than the disruption of the ORFs (Manna *et al.*, 1998). The P1 and P2 promoters are recognised by the housekeeping sigma factor σ^A (Chapter 1.6.1.2), and are mainly expressed during the early exponential phase of growth, whilst P3 is recognised by the alternative sigma factor σ^B (Chapter 1.6.2.2.8), and is induced as the cells enter the late exponential growth phase (Bayer *et al.*, 1996; Deora *et al.*, 1997; Manna *et al.*, 1998; Miyazaki et al., 1999; Bischoff *et al.*, 2001).

However, transcription from P1 is stronger than that from P2 or P3 (Cheung *et al.*, 1998).

SarA is dimeric, consists mostly of α-helices (Rechtin *et al.*, 1999), and has been shown to be a DNA-binding protein capable of binding to elements upstream of both the P2 and P3 promoters of the *agr* locus (Chein & Cheung, 1998) (Figure 1.3). Three binding sites (each consisting of two 18 bp half sites), which have a high-affinity for stable SarA dimers, have been identified upstream of the *agr* promoters (Rechtin *et al.*, 1999). By binding to these sites, SarA activates RNAII and RNAIII transcription (Chien & Cheung, 1998; Chien *et al.*, 1998; Heinrichs *et al.*, 1996), with concomitant effects on virulence determinant expression.

However, the finding that an *agr*, *sarA* double mutant displays a greater reduction in virulence determinant expression than either *agr* or *sarA* single mutant indicates that *sarA* is also able to regulate the expression of virulence determinants via an *agr*-independent mechanism (Cheung *et al.*, 1994; Booth *et al.*, 1997). This has been supported by studies which showed that the transcription of *hla*, *tst* and *fnbA* is activated by *sarA* in an *agr*-independent manner (Chan & Foster, 1998a, Chien *et al.*, 1999; Wolz *et al.*, 2000). However, there is no significant similarity between the sequence elements proposed as SarA-binding sites, indicating that SarA can bind multiple DNA targets, although they are preferably A/T-rich sequences (Arvidson & Tegmark, 2001).

One model for the mechanism by which SarA affects gene expression was originally proposed by Morfeldt *et al.* (1996b). This model is based on the ability of SarA to alter the superhelicity of DNA, and is supported by a number of findings. Firstly, Morfeldt *et al.* (1996b) have shown that the spacing between the -10 and -35 regions of the P2 and P3 promoters of *agr* is approximately 3 bp too far; this suggests that SarA may serve to overcome this spacing in order to activate *agr*. Secondly, the gene encoding collagen adhesin (*cna*) is repressed by *sarA*; the spacing of the -10 and -35 regions of the *cna* promoter is near optimal, and so SarA may act to overwind this promoter (Rechtin *et al.*, 1999). Consequently, it is possible that *sarA* may function as both an activator and a repressor.

1.6.2.2.3 The *sar* homologues

In recent years, a number of homologues of *sarA* have been identified. These include *rot* (McNamara *et al.*, 2000) and *sarH1*-5 (Tegmark *et al.*, 2000). Of these, *rot* and *sarH1* have been the most extensively researched, and are discussed below.

1.6.2.2.3.1 *rot* (repressor of toxins)

Based on the observation that protein synthesis inhibitors suppress the production of several virulence factors that are positively regulated by *agr*, Balaban & Novick (1995) suggested that protein factors must be required for RNAIII to exert its regulatory function. To identify such factors, McNamara *et al.* (2000) screened Tn*917* mutants in an *agr* background to identify mutations that restored protease and α-haemolysin production. A 498 bp ORF was identified, and its predicted 161 amino acid protein product, named Rot, was found to demonstrate a high level of similarity to SarA over the entire length of the protein. Rot has been shown to represses *hla* transcription in an *agr* background, but not in the presence of functional *agr* (McNamara *et al.*, 2000). Additionally, in an *agr* background, *rot* was shown to repress numerous other genes, including *hlb* and *sspA*, although an upregulatory effect was observed for *spa* (Said-Salim *et al.*, 2003). This indicates that *rot* may be an important global regulator which has opposing effects to *agr* on the expression of particular virulence genes (Chapter 1.6.2.2.1). Said-Salim *et al.* (2003) have suggested that the effects of *rot* in relation to those of *agr* may occur as a result of RNAIII inhibiting Rot.

1.6.2.2.3.2 *sarH1* (*sar* homologue 1)

SarH1 was originally isolated as a protein bound to the promoter regions of *hla*, *spa*, *ssp* and RNAIII in extracts from *sarA* and *agr* mutants, but not in extracts from wild-type cells (Tegmark *et al.*, 2000). SarH1 has been shown to have a strong repressive effect on *hla* and an activating effect on *spa* at the transcriptional level (Tegmark *et al.*, 2000). However, no significant effect on the transcription of *agr* or *ssp* could be identified, and the repression of *hla* by *sarH1* was only observed in a *sarA* background (Tegmark *et al.*,

2000). Northern blot analysis has identified three *sarH1*-specific transcripts, two of which share a common terminator (Tegmark *et al.*, 2000). The P1 promoter is σ^A-dependent, whilst the P2 promoter has a σ^B-dependent consensus sequence (Tegmark *et al.*, 2000). The promoter of the third transcript has not yet been identified. The P1 transcript is strongly repressed by *agr* and *sarA* (Tegmark *et al.*, 2000). This agrees with the fact that SarH1 was not bound to the *hla*, *spa*, *ssp* or RNAIII promoter regions in extracts derived from *agr* or *sarA* mutants. The gene encoding the same protein has been identified by Cheung *et al.* (2001) and designated *sarS*.

1.6.2.2.4 *sae* (*S. aureus* exoprotein expression)

The *sae* locus was discovered by Giraudo *et al.* (1994), following the isolation of a Tn*551* mutant displaying reduced β-haemolysin activity. The mutant has subsequently been shown to have diminished or null levels of α-haemolysin, DNase, coagulase and protein A (Giraudo *et al.*, 1994; Giraudo *et al.*, 1996; Goerke *et al.*, 2001). Indeed, a decrease in total extracellular protein levels was also observed, although proteases, lipase, staphylokinase and enterotoxin A were not affected (Giraudo *et al.*, 1994). Pathogenicity assays involving intraperitoneal injection into mice have shown that inactivation of *sae* results in a slower mortality rate (Giraudo *et al.*, 1996).

Cloning and sequencing of the locus has revealed two potentially co-transcribed genes (Giraudo *et al.*, 1999). These are *saeS* and *saeR*, which encode a histidine kinase and a response regulator respectively (Giraudo *et al.*, 1999) (Chapter 1.6.1.1.1). However, the actual stimuli that activate *sae* are unknown. Northern blot analysis has revealed that *sae* affects the expression of *hla*, *hlb*, *coa* and *spa* at the transcriptional level (Giraudo *et al.*, 1997). Importantly, however, *sae* has been shown to have no effect on the transcription of *agr* or *sar* (Giraudo *et al.*, 1997). Therefore, it has been hypothesised that *saeR* may exert its effect by direct interaction with target genes which are also subject to regulation by *agr* (Giraudo *et al.*, 1999). Nevertheless, a direct interaction between *sae* and *agr* cannot currently be ruled out. The production of several virulence determinants (including β-haemolysin, DNase and protease) was found to be lower in an *sae*, *agr* double mutant than the already diminished levels in either single mutant (Giraudo *et al.*, 1996). Additionally, the *sae*, *agr* double mutant demonstrated a further

decrease in virulence (Giraudo *et al.*, 1996). This suggests that there may be an additive or synergistic interaction between the two loci.

Interestingly, the effect of *sae* mutation on the *agr* phenotype is different for two virulence determinants that are normally up-regulated in an *agr* mutant; in the *sae*, *agr* double mutant coagulase expression dropped close to the null levels of the *sae* parent strain, while protein A displayed the high levels characteristic of the *agr* single mutant (Giraudo *et al.*, 1996). This suggests that the *sae* and *agr* loci interact in a complex way, and that the production of these two virulence determinants might be controlled by different mechanisms (Giraudo *et al.*, 1996).

1.6.2.2.5 *arl*

The *arl* locus comprises two genes, *arlS* and *arlR*, which encode a histidine kinase and a response regulator respectively (Fournier & Hooper, 2000) (Chapter 1.6.1.1.1). Inactivation of *arl* has been shown to reduce the production of α- and β-haemolysin, lipase, coagulase, serine protease and protein A (Fournier & Hooper, 2000; Fournier *et al.*, 2001). In addition, disruption of the *arl* locus alters biofilm formation and increases autolysis (Fournier *et al.*, 2001).

1.6.2.2.6 IE3

IE3 was defined by a Tn*551* insertion into a unique locus of the *S. aureus* chromosome (Cheung *et al.*, 1995). The locus has not been mapped or sequenced, although it has been shown to be distinct from *agr* and *sarA* (Cheung *et al.*, 1995). The IE3 transposon mutant was found to have significantly reduced levels of protein A transcription and clumping activity with fibrinogen, although α-haemolysin transcription and the level of RNAIII were slightly increased (Cheung *et al.*, 1995). In a rabbit endocarditis model, a significant decrease in both the infectivity rate and intravegetation bacterial density was observed for the IE3 mutant, compared to the parent strain (Cheung *et al.*, 1995).

1.6.2.2.7 *srrAB* (staphylococcal respiratory response)

The *srrAB* locus of *S. aureus* is a homologue of the *resDE* two-component regulator system involved in the regulation of aerobic and anaerobic respiratory metabolism in *B. subtilis* (Yarwood *et al.*, 2001). The *srrAB* locus has also been termed *srhSR* by Throup *et al.* (2001). Disruption of the locus lead to altered expression of proteins involved in energy metabolism and other metabolic processes (Throup *et al.*, 2001). These effects were generally increased under anaerobic conditions, and were accompanied by a reduction in growth rate under anaerobic conditions and attenuated growth in a murine pyelonephritis model (Throup *et al.*, 2001). The *srrAB* locus has also been shown to regulate RNAIII, protein A, and TSST-1 in response to oxygen availability (Yarwood *et al.*, 2001). This suggests that the *srrAB* locus may provide a link between respiratory metabolism, environmental sensing and virulence determinant regulation in *S. aureus*.

1.6.2.2.8 The alternative sigma factor σ^B

The role of the alternative σ-factor σ^B in regulating the general stress response of *B. subtilis* and other low-GC Gram-positive bacteria has been introduced in Chapter 1.6.1.2. The σ^B operon of *B. subtilis* (Figure 1.4) contains eight genes in the order P_A-*rsbR-rsbS-rsbT-rsbU*-P_B-*rsbV-rsbW-sigB-rsbX,* where *rsb* stands for regulator of sigma **B**, and P_A and P_B are σ^A and σ^B-dependent promoters respectively (Kalman *et al.*, 1990; Wise and Price, 1995). Two additional genes, *rsbQ* and *rsbP* reside outside the σ^B operon. In *B. subtilis*, the Rsb proteins function to regulate σ^B activity in response to signals detected by two separate pathways; the environmental stress pathway (RsbX, RsbS, RsbT, RsbR and RsbU) and the energy stress pathway (RsbQ and RsbP) (Figure 1.5) (reviewed by Price, 2002). A homologue of *B. subtilis* has been identified in *S. aureus* (Wu *et al.*, 1996; Deora *et al.*, 1997; Kullik & Giachino, 1997). However, the *S. aureus* σ^B operon only comprises the genes P_A-*rsbU*-P_B-*rsbV-rsbW-sigB* (Wu *et al.*, 1996) (Figure 1.4). Genes encoding RsbR, RsbS, RsbT, RsbX, RsbQ and RsbP are apparently not present. However, RsbW and RsbU have been shown to play a similar role in *S. aureus* as they do in *B. subtilis* (Miyazaki *et al.*, 1999; Palma & Cheung, 2001; Giachino *et al.*, 2001), although it remains unclear as to whether the remaining Rsb proteins or unknown proteins compensate for the missing components.

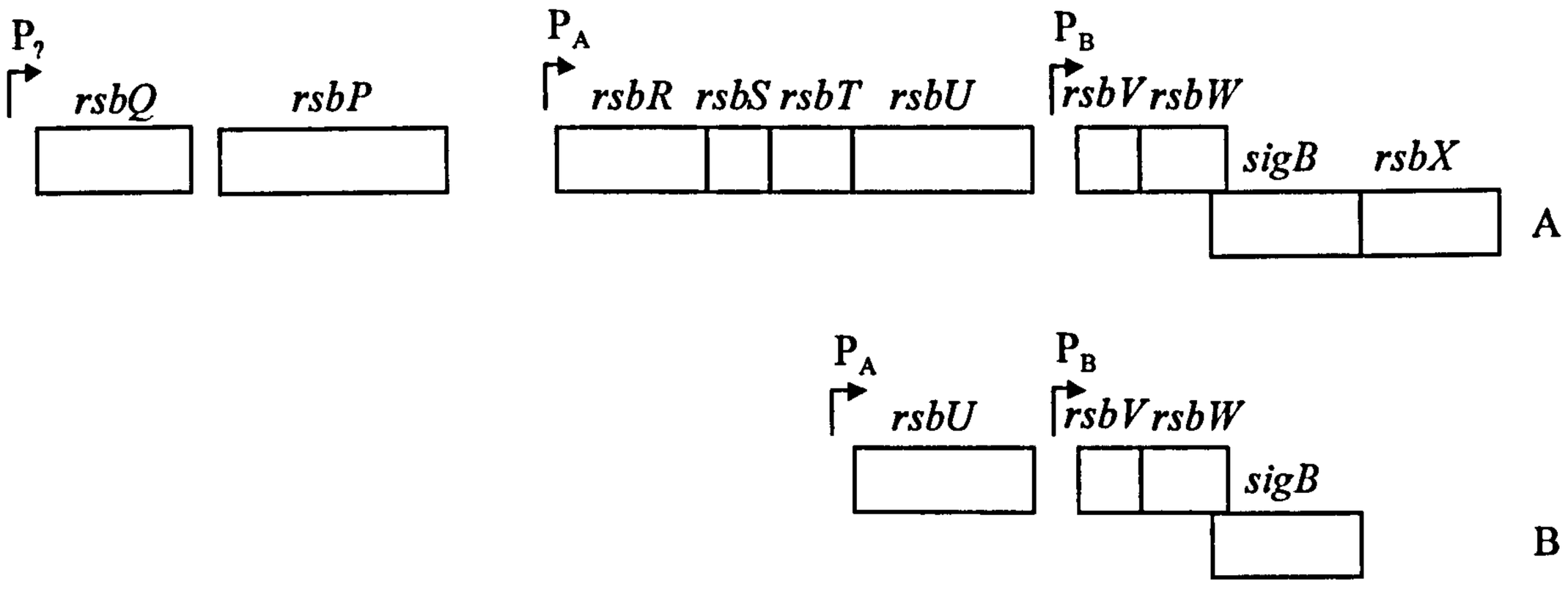


Figure 1.4

Comparison of the σ^B operons of (A) *B. subtilis* and (B) *S. aureus*.

Reproduced from Price (2002).

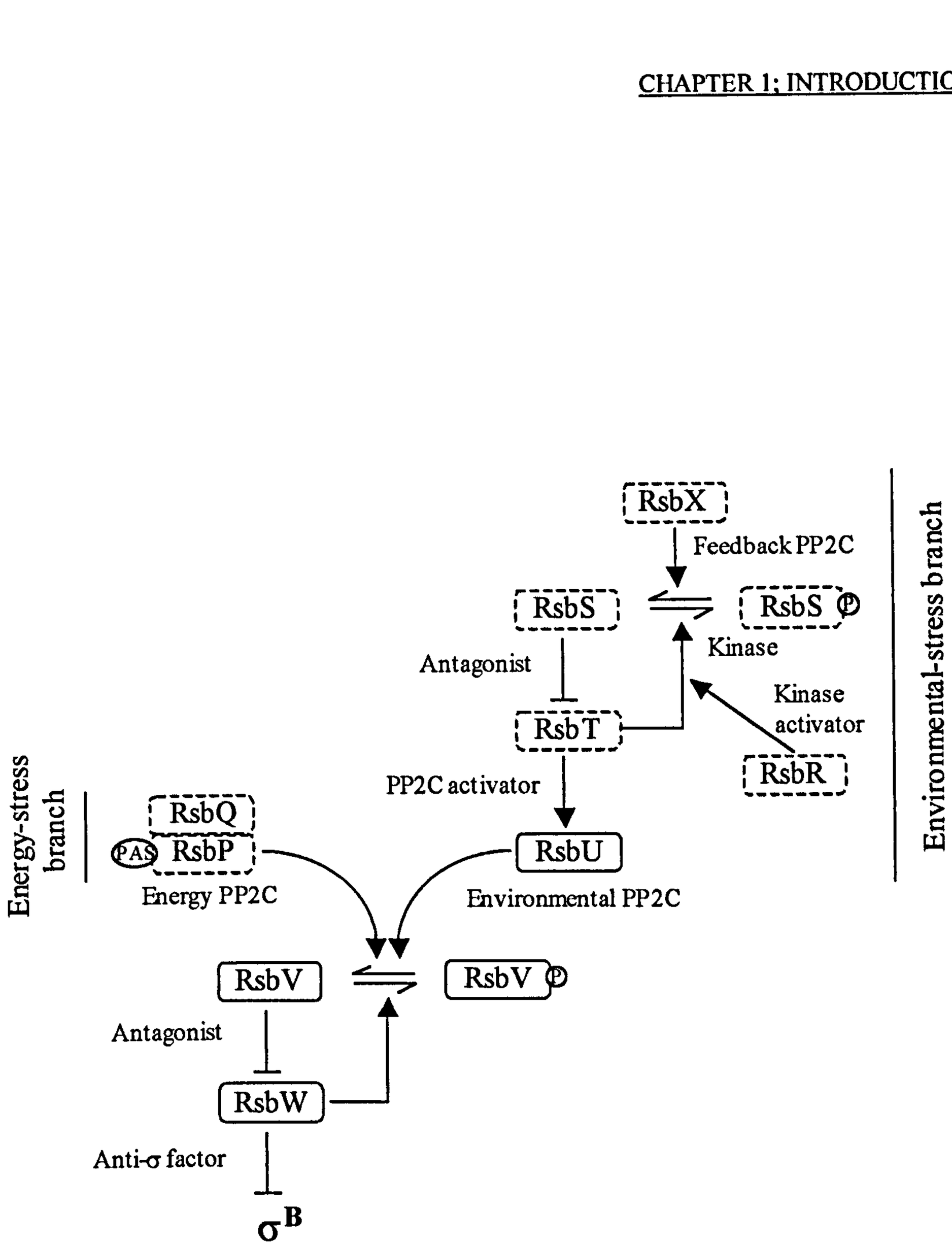

RsbX
Feedback PP2C
RsbS
RsbS P
Kinase
Antagonist
Kinase activator
RsbT
RsbR
PP2C activator
Environmental-stress branch
Energy-stress branch
RsbQ
PAS RsbP
Energy PP2C
RsbU
Environmental PP2C
RsbV
RsbV P
Antagonist
RsbW
Anti-σ factor
σB

Figure 1.5

Model of the σ^B signal-transduction network of *B. subtilis*.

Two signalling pathways converge on RsbV-P, the antagonist form found in unstressed cells. The energy-stress pathway terminates with the RsbP, which has a Per-Arnt-Sim (PAS) domain for energy-stress sensing and a PP2C phosphatase domain. RsbQ is a positive regulator presumed to act in conjunction with RsbP. The environmental-stress signalling pathway terminates with the RsbU phosphatase (PP2C), and is activated by upstream signalling elements. When activated by their respective stress, either the RsbP or the RsbU phosphatase removes the serine phosphate from RsbV-P. Dephosphorylated RsbV then binds to the RsbW anti-σ factor, forcing it to release σ^B, which can then activate the transcription of its target genes. The upstream regulators that activate RsbU include the RsbS antagonist and the RsbT kinase, which are homologues of RsbV and RsbW respectively. Unphosphorylated RsbS is thought to be the antagonist form found in unstressed cells, and this form binds the RsbT kinase. Following environmental stress, RsbS is phosphorylated by RsbT, which is then released to bind and activate the RsbU phosphatase by direct protein-protein interaction. RsbR acts as a positive regulator of σ^B activity by potentiating the activity of the RsbT kinase. The RsbX phosphatase (PP2C) fulfills a negative feed-back role by indirectly communicating σ^B protein levels.

Genes encoding only the proteins shown in boxes with solid lines have also been identified in *S. aureus*.

Adapted from Price (2002).

In *B. subtilis,* the regulation of σ^B occurs post-translationally and involves a "partner-switching" mechanism controlled by protein phosphorylation (Figure 1.5). Under normal conditions, the anti-σ-factor RsbW binds to the σ^B protein, which prevents the interaction of RNA polymerase with σ^B (Benson & Haldenwang, 1993). However, RsbV (the anti-anti-σ-factor), when dephosphorylated at a conserved serine residue, binds competitively to RsbW, resulting in the release and activation of σ^B (Dufour & Haldenwang, 1994). Control of the phosphorylation state of RsbV is modulated by dephosphorylation by RsbU and RsbP (and also by RsbW-mediated phosphorylation) in response to the conditions sensed (Wise & Price, 1995; Vijay et al, 2000; Dufour & Haldenwang, 1994). It is likely that this mechanism of control of σ^B activity is conserved in *S. aureus* (Miyazaki *et al.*, 1999; Palma & Cheung, 2001; Giachino *et al.*, 2001).

σ^B has been shown to play a role in the response of *S. aureus* to a number of environmental stresses, including hydrogen peroxide-mediated oxidative stress, exposure to acid or alkali, heat shock (54 °C), and entry into stationary phase (Kullik *et al.*, 1998; Chan *et al.*, 1998; Gertz *et al.*, 1999; Palma & Cheung, 2001; Kullik & Giachino, 1997). Indeed, the σ^B regulon of *S. aureus* has been shown to contain many components that are perceived to be important for protecting cells from various environmental stresses (Gertz *et al.*, 1999; Gertz *et al.*, 2000; Giachino *et al.*, 2001). For example, the operon involved in the biosynthesis of the yellow pigment staphyloxanthin, which protects cells from damage by environmental factors such as U.V. radiation and desiccation, has a σ^B-dependent promoter consensus sequence (Kullik *et al.*, 1998). A role for σ^B in the development of antibiotic resistance has also been demonstrated (Bischoff & Berger-Bachi, 2001; Morikawa *et al.*, 2001).

σ^B has also been shown to directly or indirectly regulate a number of *S. aureus* virulence determinants, including lipase, thermonuclease, coagulase, haemolysins, proteases and enterotoxin B (Kullik *et al.*, 1998; Nicholas et al., 1999; Cheung *et al.*, 1999; Horsburgh *et al.*, 2002; Palma & Cheung, 2001; Ziebandt *et al.*, 2001). The P3 promoter of the virulence regulator *sar* and the P2 promoter of *sarH1* have been shown to be σ^B-dependent (Deora *et al.*, 1997; Manna *et al.*, 1998; Miyazaki et al., 1999; Bischoff *et al.*, 2001). Furthermore, σ^B contributes directly to the ability of *S. epidermidis* and *S. aureus*

to form adherent biofilms (Rachid *et al.*, 2000; Knobloch *et al.*, 2001), a factor that is considered important in their ability to cause nosocomial infections (Costerton *et al.*, 1999). However, investigations involving mouse and rat pathogenicity models have been unable to show a role for σ^B in virulence (Chan *et al.*, 1998, Nicholas *et al.*, 1999), although Gertz *et al.* (2000) have questioned whether the infection models analysed so far truly reflect the natural situation in the human host.

1.6.2.3 Environmental regulation of virulence determinant expression in *S. aureus*

S. aureus is an extremely adaptable bacterium, which survives in a range of abiotic environments (Chapter 1.1) and causes disease in diverse areas of the human body (Chapter 1.3). Therefore, as well as being regulated in a growth-phase dependent manner (Chapter 1.6.2.1), the virulence determinants of *S. aureus* are regulated in response to a number of environmental parameters. Detection of environmental conditions allows *S. aureus* to determine whether it has entered the human body, to identify its location within the host, and to respond appropriately. These signals include temperature, pH, the concentration of metal ions (*eg.* magnesium, calcium and iron), and the concentration of oxygen, glucose and NaCl (Mekalanos, 1992).

Temperature is a key signal indicating entry into the host for many pathogenic bacteria (Mekalanos, 1992). Interestingly however, whereas the virulence genes of many pathogens are optimally expressed at 37 °C, in *S. aureus* RNAIII and *hla* are expressed maximally at 42 °C (Fairhead, 1998; Ohlsen *et al.*, 1997). The reason for this is unknown. pH also plays a role in virulence determinant regulation. The expression of Seb was found to be higher in BHI at pH 6.9 than at pH 5.1 (Genigeorgis & Sadler, 1966). In addition, Regassa and Betley (1993) have shown that steady-state growth under alkaline conditions (pH 8.0) virtually eliminates the expression of RNAIII, suggesting that virulence determinant expression occurs over a reasonably narrow pH range. Chan and Foster (1998a) have shown that the addition of $MgCl_2$ or $CaCl_2$ to BHI represses *tst* expression. Similarly, Fairhead (1998) showed that decreased levels of available Mg^{2+} or Ca^{2+} increase the expression of RNAIII and *hla*, suggesting that these divalent cations have important effects on virulence determinant expression. Iron

deprivation has been shown to increase RNAIII and *hla* expression (P. F. Chan, unpublished data), possibly to allow iron-acquisition in the low free iron-concentration environment of the human body. Decreased oxygen availability, but not anaerobic growth, has also been shown to increase RNAIII and *hla* expression (Fairhead 1998). This may be important in low-oxygen concentration infection sites.

Glucose downregulates the expression of RNAII, RNAIII, *hla*, *sea*, *seb*, *sec* and *geh* (Morse *et al.*, 1969; Duncan & Cho, 1972; Jarvis *et al.*, 1975; Iandolo & Shafer, 1977; Miller & Fung, 1976; Regassa *et al.*, 1991; Regassa *et al.*, 1992; Smith *et al.*, 1986). In the majority of cases, the effect of glucose was shown to occur independently of the reduction in pH caused by the accumulation of acidic end products during glucose metabolism. However, the mechanisms by which glucose affects virulence determinant expression are poorly understood. Glucose-mediated repression of *sec* transcription occurs in an *agr* mutant, and is therefore *agr*-independent (Regassa *et al.*, 1991). However, Regassa *et al.* (1992) have shown that the mechanism by which glucose represses *sec* and *hla* may also involve an *agr*-dependent pathway, although this only occurs at low pH. The effects of glucose (0.1 M) on *sec*, *hla* and RNAIII have been shown by Regassa *et al.* (1992) not to occur as a result of increased osmolarity, as their transcription was unaltered in a culture containing an iso-osmotic concentration of NaCl (0.05 M). It has been suggested that the repression of Seb by glucose involves catabolite repression (Morse *et al.*, 1969). This is interesting, as it implies a link between virulence determinant regulation and nutrition. Nevertheless, the ability of the non-metabolisable glucose analogues to inhibit *hla* and *seb* expression (Duncan & Cho, 1972; Iandolo & Shafer, 1977) suggests that the effects of glucose may involve mechanisms in addition to, or instead of, catabolite repression.

However, one of the most extensively studied environmental conditions affecting *S. aureus* is NaCl concentration. *S. aureus* is one of the most salt tolerant non-halophiles, and is capable of growth in up to 3.5 M NaCl (Scott, 1953; Armstrong-Buisseret *et al.*, 1995). This reflects the fact that high concentrations of NaCl are frequently encountered by *S. aureus* in abiotic environments and also when in association with a host. As described in Chapter 1.3, 19 - 51 % of healthy individuals are carriers of *S. aureus*, with the skin and anterior nares being the main sites of carriage. These are two particularly high-NaCl concentration niches of the human body. The concentration of NaCl has been

shown to be a maximum of 225 mM in nasal secretions and up to 170 mM in sweat as they are secreted, prior to further drying (Lorin *et al.*, 1972; Magnos, 1973)). Additionally, *S. aureus* is a common cause of food-poisoning (Chapter 1.3), especially where foods have been preserved using curing salts (Troller, 1986), as this reduces competition from less osmotolerant bacteria. For example, salt-cured ham (600 mM NaCl) caused 24 % of the food-poisoning outbreaks reviewed by Holmberg and Blake (1984).

NaCl affects *S. aureus* at two levels. Firstly, it affects general cell physiology, for example by stimulating an osmotic stress response. Secondly, the presence of NaCl alters virulence determinant expression. These two effects will be considered separately, although they may indeed be linked.

1.6.2.3.1 Effects of NaCl on the general cell physiology of *S. aureus*

1.6.2.3.1.1 The osmotic stress response

Water availability is a fundamental environmental parameter affecting the growth and survival of microorganisms. Turgor pressure (the hydrostatic pressure difference between the inside and the outside of the cell, which pushes the cytoplasmic membrane against the murein sacculus) is considered essential for viability and cell wall expansion (Holtje, 1998). Changes in external osmolarity cause water to move along the osmotic gradient, and potentially result in either swelling and bursting in hypotonic environments, or in dehydration and plasmolysis under hypertonic conditions. Therefore, it is vital that the cell possesses effective measures to counteract adverse changes in external osmolarity, and to allow a suitable level of cytoplasmic water and turgor pressure to be maintained (reviewed by Bremer & Kramer, 2000). *S. aureus* possesses a highly versatile osmotic stress response, which allows adaptation to environments containing both high and low osmolarity, and involves the accumulation of K^+ ions and a range of osmoprotective compounds.

1.6.2.1.1.1 K^+ accumulation

The initial effect of osmotic upshock in many bacteria, including *B. subtilis* and *E. coli*, is the accumulation of K^+ from environmental sources (Whatmore & Reed, 1990; Kunin & Rudy, 1991). However, *S. aureus* naturally contains high a level of cytoplasmic K^+, which is relatively unaffected by osmotic stress (Kunin & Rudy, 1991; Graham & Wilkinson, 1992). For example, the cytoplasmic concentration of K^+ in *E. coli* K10 rose from 0.6 mmol / g protein in TSB containing no added NaCl to 1.6 mmol / g protein in TSB containing 0.9 M NaCl (Kunin & Rudy, 1991). In contrast, the cytoplasmic concentration of K^+ in *S. aureus* was found to be approximately 2.5 mmol / g protein at both NaCl concentrations (Kunin & Rudy, 1991).

However, high intracellular concentrations of inorganic ions such as K^+ induce macromolecule aggregation by enhancing hydrophobic interactions, and restrict the availability of water for biochemical processes (Bremer & Kramer, 2000). The halophilic bacteria (*ie.* the *Halobacteriales* and *Haloanaerobiales*) rely on the accumulation of molar concentrations of K^+ and other ions for survival in hyperosmotic environments (2 - 5 M NaCl). Consequently, these bacteria have had to undergo evolutionary changes in essentially all of their proteins to adjust the entire cell physiology to the high ion content of the cytoplasm (Elcock & McCammon, 1998). This has severely limited the habitats of the halophilic bacteria, as they are unable to colonise environments with moderate osmolarities. However, instead of accumulating K^+ to the high levels found in the halophilic bacteria, *S. aureus* is able to grow in the presence of NaCl concentrations of up to 3.5 M (Scott, 1953; Armstrong-Buisseret *et al.*, 1995) by combining a more moderate level of cytoplasmic K^+ with the accumulation of osmoprotective compounds which can be released from the cell following a reduction in external osmolarity. This allows *S. aureus* to grow in environments of both high and low osmolarity.

1.6.2.3.1.1.2 Osmoprotectant accumulation

Osmoprotectants are compounds that relieve the growth inhibition caused by high osmolarity (Wilkinson, 1997). These include the compatible solutes, which are

compounds that can be accumulated to high amounts within the cell without inhibiting cellular processes (Wilkinson, 1997). The accumulation of compatible solutes is an almost universal response to high osmolarity, and occurs in the cells of many bacteria, fungi, plants and animals (Csonka, 1989; Sakamoto & Murata, 2000).

In general, compatible solutes are polar, highly soluble molecules, and do not usually carry a net charge at physiological pH (Bremer & Kramer, 2000). When accumulated in up to molar concentrations, they lower the osmolarity of the cytoplasm and therefore aid the restoration of a suitable cytoplasmic water volume and turgor pressure (Kempf & Bremer, 1998). Compatible solutes also stabilise proteins during periods of osmotic stress, and prevent proteins from being denatured by freezing, heating and high ionic strength (reviewed by Bremer, 2002).

The compatible solutes comprise a wide range of compounds, although the main ones accumulated by *S. aureus* in response to osmotic stress include the trimethylammonium compound glycine betaine and the amino acid L-proline (Miller *et al.*, 1991; Graham & Wilkinson, 1992; Townsend & Wilkinson, 1992; Bae & Miller, 1992; Bae *et al.*, 1993; Pourkomailian & Booth, 1992; Pourkomailian & Booth, 1994). Glycine betaine is the most potent osmoprotectant. The addition of 1 mM glycine betaine has been shown to increase the maximum stationary phase population level (OD_{580}) of *S. aureus* in defined medium containing 1.5 M NaCl from 0.05 to 0.89 (Graham & Wilkinson, 1992). The addition of 1 mM L-proline resulted in an increase to 0.48 (Graham & Wilkinson, 1992). Taurine also acts as an osmoprotectant, although it has a weaker effect; the addition of 1 mM taurine to defined medium containing 1.5 M NaCl resulted in a maximum stationary phase OD_{580} of 0.21 (Graham & Wilkinson, 1992).

Glycine betaine is also the osmoprotectant accumulated to the highest levels by *S. aureus* in response to osmotic stress. The concentration of glycine betaine, L-proline and taurine rose from 76 mM, 21 mM and 8 mM to 1,167 mM, 309 mM and 209 mM respectively upon addition of 2 M NaCl to defined medium (Graham & Wilkinson, 1992). Glycine betaine can be synthesised within *S. aureus* cells by the uptake and oxidisation of choline in response to osmotic stress (Graham & Wilkinson, 1992; Kaenjak *et al.*, 1993). However, although many other bacteria are able to synthesise compatible solutes *de novo* (Galinski & Truper, 1994; Whatmore *et al.*, 1990), no

evidence has yet been presented of a biosynthesised compatible solute in osmotically stressed *S. aureus* in the absence of exogenous osmoprotectants (Wilkinson, 1997). Instead, the accumulation of compatible solutes in *S. aureus* involves their transport into the cell from environmental sources.

S. aureus possesses two proline transporters, including a high-affinity and a low-affinity system (Townsend & Wilkinson, 1992; Bae & Miller, 1992). The low-affinity transporter has a broad substrate specificity, and its activity is stimulated by increased external osmolarity (Townsend & Wilkinson, 1992). In contrast, the high-affinity system is specific for L-proline and is involved in scavenging low concentrations of proline, possibly for nutritional purposes (Townsend & Wilkinson, 1992). *S. aureus* also possesses a high-affinity and a low-affinity glycine betaine transporter (Pourkomailian & Booth, 1992; Pourkomailian & Booth, 1994). Similar to the proline transporters, the activity of the low-affinity glycine betaine transporter is increased by high external osmolarity, whilst the high-affinity system is activated independently of osmolarity (Pourkomailian & Booth, 1992). Indeed, Pourkomailian & Booth (1994) have shown that the low-affinity glycine betaine and proline transporters may be identical.

1.6.2.3.1.1.3 Additional effects of osmotic stress on general cell physiology

Osmotic stress has been shown to induce the expression of four major proteins (with molecular masses of 50, 40, 34 and 25 kDa) in *S. aureus* (Armstrong-Buisseret *et al.*, 1995). N-terminal sequencing and database analysis were used to identify the gene encoding the 25 kDa protein. Extensive homology with *ahpC* (alkyl hydroperoxide reductase) from *E. coli* and *S. typhimurium* was demonstrated (Armstrong-Buisseret *et al.*, 1995). AhpC protects against the toxic by-products of oxidative metabolism. Significantly, this suggests that osmotic stress may cross-activate the oxidative stress response, and possibly other stress responses. Indeed, Qoronfleh *et al.* (1990) found that the majority of the 12 heat shock proteins that they identified in *S. aureus* using L-[^{35}S]methionine and SDS-PAGE were also induced by high NaCl concentrations. In *B. subtilis*, NaCl-mediated stress induces the production of both NaCl-specific stress proteins and general stress proteins via the alternative sigma factor σ^B (Chapters 1.6.1.2 and 1.6.2.2.8).

1.6.2.3.1.2 Ionic effects of NaCl on general cell physiology

In addition to providing an osmotic stress, it should be appreciated that NaCl may also affect the general cell physiology of *S. aureus* as a result of ionic stress (due to Na^+ or Cl^- ions). Vijaranakul *et al.* (1995) showed that growth in defined medium containing 2.5 M NaCl caused cells to grow more slowly, to become significantly larger (as a result of defective cell division), and to possess a less cross-linked peptidoglycan containing shorter interpeptide bridges. The charged osmolytes potassium chloride and sodium nitrite were able to cause a similar increase in cell size, although the uncharged osmolytes sucrose and sorbitol were unable to do so (Vijaranakul *et al.* 1995). This suggests that the effects observed were due to an ionic, rather than an osmotic effect, but that neither Na^+ nor Cl^- ions were specifically responsible.

1.6.2.3.2 Virulence determinant regulation in response to NaCl

NaCl has been shown to repress the transcription of *hla*, *tst*, *eta, sec* and *spa* (Chan & Foster, 1998a; Chan & Foster, 1998b; Lindsay & Foster; 1999, Ohlsen *et al.*, 1997; Sheehan *et al.*, 1992; Regassa & Betley, 1993). The concentration of NaCl used in these studies was either 1 M or 1.2 M. The expression of Seb has also been shown to be repressed in a culture containing 1.7 M NaCl (Genigeorgis & Sadler, 1966). In contrast, 1 M NaCl up-regulates the transcription of *sspA* (Lindsay & Foster, 1999). It has been suggested that the effects of NaCl on virulence determinant expression may aid the ability of *S. aureus* to survive asymptomatically as a commensal in the high-NaCl concentration niches of the skin and anterior nares (Lindsay & Foster, 1999) (Chapters 1.3 and 1.6.2.3). This is because NaCl not only represses virulence determinants which cause harm to the host, but also simultaneously upregulates *sspA*, which may possibly function to destroy preformed toxins.

Importantly, the studies introduced above also show that NaCl represses not only extracellular virulence determinants (*ie. hla, tst, eta, sec* and Seb, which are normally up-regulated by *agr*) but also a cell surface-associated virulence determinant (*ie. spa,* which is normally down-regulated by *agr*). Furthermore, the effects of NaCl on *hla, tst, sec, sspA* and *spa* have been shown to occur independently of *agr* and/or *sarA* (Chan &

Foster, 1998a; Chan & Foster, 1998b; Lindsay & Foster; 1999, Regassa & Betley, 1993). This is supported by the fact that 1 M NaCl has no appreciable effect on *sarA* or RNAIII expression (Chan & Foster, 1998a; Lindsay & Foster, 1999). Overall, this suggests that *S. aureus* virulence determinants, which are normally regulated by *agr* and *sarA*, may come under the control of a currently unknown alternative regulatory mechanism in the presence of high NaCl concentrations (Chan & Foster, 1998a).

1.6.2.3.2.1 The mechanism by which NaCl affects virulence determinant expression

A number of studies have been performed to determine whether the mechanism by which NaCl affects virulence determinant expression involves an ionic effect and/or an osmotic effect. Sheehan *et al.* (1992) showed that, when grown in TSB for up to 8 h, the uncharged osmolyte sucrose (0.44 M) represses *eta* transcription to a similar level as an iso-osmotic concentration of NaCl (0.3 M). This suggests that NaCl alters virulence determinant expression by an osmotic, rather than an ionic effect. Additionally, Ohlsen *et al.* (1997) showed that potassium chloride and sodium glutamate have similar repressive effects on *hla* transcription as NaCl, indicating that neither Na^+ nor Cl^- ions are specifically required for the effect of NaCl on virulence determinant expression. The possibility that the effect of NaCl on virulence determinant expression involves an osmotic effect is further supported by the fact that the osmoprotectants glycine betaine, L-proline and choline relieved the NaCl-induced repression of *sec* transcription (Regassa & Betley, 1993) (Chapter 1.6.2.3.1.1.2).

As shown in Chapter 1.6.1.3, the osmoregulation of virulence determinant expression in many pathogens involves changes in DNA supercoiling. The presence of high NaCl concentrations has been shown to decrease *eta* transcription and increase the negative supercoiling of *S. aureus* plasmid DNA (Sheehan *et al*, 1992). Sheehan *et al.* (1992) correlated these observations with the finding that novobiocin (which inhibits DNA gyrase, and therefore reduces negative DNA supercoiling) increases *eta* transcription. However, Chan and Foster (1998a) showed that novobiocin had no effect on the repression of *tst* by 1 M NaCl. Therefore, the involvement of DNA supercoiling in the repression of virulence determinant expression by NaCl remains debatable, although it

is possible that the effects of novobiocin, and consequently of DNA supercoiling, may be restricted to specific virulence determinant genes.

1.7 Rationale and objectives for this project

The ability to sense environmental parameters and respond appropriately is vital to the survival of *S. aureus* in abiotic environments, in infection situations, and whilst existing as a commensal. NaCl is an important environmental parameter encountered by *S. aureus*. However, the mechanisms by which *S. aureus* senses the level of NaCl in the surrounding environment and transduces this signal into changes in gene expression remain poorly understood. The objective of this project was to characterise the effects of NaCl-mediated stress on virulence determinant production and to determine the role of known regulators in this process through the use of reporter gene fusions. A further aim of this project was to identify novel genes involved in NaCl-mediated virulence determinant regulation through the use of transposon mutagenesis.

CHAPTER 2

MATERIALS AND METHODS

2.1 Media and antibiotics

2.1.1 Media

Unless otherwise stated all media were prepared using distilled water (dH_2O) and were sterilised by autoclaving for 20 min at 121 °C (15 pounds per square inch).

2.1.1.1 B2

Casein acid hydrolysate (Oxoid)	10 g l^{-1}
Yeast extract (Oxoid)	25 g l^{-1}
K_2HPO_4	1 g l^{-1}
NaCl	5 g l^{-1}

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

2.1.1.2 Brain heart infusion (BHI) (Oxoid)

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

Oxoid agar No. 1 (1 % (w/v)) was used for BHI agar.

Details of the composition of BHI are shown in Table A.1.1.

When NaCl was added to BHI, equal volumes of 2X BHI and 2X the relevant concentration of NaCl were prepared separately and mixed after autoclaving.

2.1.1.3 Luria-Bertani (LB) (Miller, 1972)

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

The pH was adjusted to 7.2 using NaOH. Oxoid Agar No. 1 (1.0 % (w/v)) was added for LB agar.

2.1.1.4 LK

Tryptone (Oxoid)	10 g l^{-1}
Yeast extract (Oxoid)	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.

2.1.1.5 Milk agar

Milk agar was made using equal volumes of 2X BHI agar and 2 % (w/v) dried skimmed milk powder (Marvel). The components were prepared separately and mixed after autoclaving.

2.1.1.6 Phage agar

Casamino acids (Oxoid)	3 g l^{-1}
Yeast extract (Oxoid)	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.

2.1.1.7 Rabbit blood agar

Blood agar base (Oxoid)	39.5 g l^{-1}

Details of the composition of blood agar base are shown in Table A.1.2.

25 ml sterile defibrinated rabbit blood (E & O laboratories) was added to 225 ml blood agar base which had been cooled to 50 °C. This gave a final rabbit blood concentration of 10 % (w/v). 6 ml was then used to overlay warm 20 ml blood agar base plates.

For plates containing added NaCl, equal volumes of 2X the relevant concentration of NaCl and 2X blood agar base/overlay were prepared separately and mixed after autoclaving and cooling.

2.1.1.8 SOB (Sambrook *et al.*, 1989)

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

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

2.1.1.9 SOC (Sambrook *et al.*, 1989)

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

2.1.2 Antibiotics

All antibiotics used in this study are listed in Table 2.1. The stock solutions were filter-sterilised (0.2 μm pore size) and stored at -20 °C. For use in agar plates, the antibiotic stock solutions were added to the media once they had cooled to below 55 °C. For use in liquid media, the antibiotic stock solutions were added just before use. Concentrations of antibiotics used for selection were as in Table 2.1, unless otherwise stated.

2.2 Buffers and stock solutions

All buffers were prepared in dH_2O and stored at room temperature. Solutions for use in microbiological work and *in vitro* DNA manipulations were sterilised by autoclaving. All of the methods in this chapter are as in Sambrook *et al.* (1989), unless otherwise stated.

2.2.1 ABT buffer

NaCl	100 mM
K_2HPO_4	60 mM
KH_2PO_4	40 mM
Triton® X-100	0.1 % (v/v)

2.2.2 ABTN buffer

ABTN buffer was made by mixing equal volumes of ABT buffer and 0.4 M Na_2CO_3.

Antibiotic	Stock Concentration (mg ml^{-1})	Working Concentration In *S. aureus* (μg ml^{-1})	Working Concentration In *E. coli* (μg ml^{-1})
Ampicillin (Amp)	50[a]	-	50
Erythromycin (Ery)	5 or 75[b]	5	300
Lincomycin (Lin)	25[c]	25	-
Kanamycin (Kan)	50[a]	50	50
Neomycin (Neo)	50[a]	50	-
Tetracycline (Tet)	5[c]	5	12.5
Spectinomycin (Spec)	150[a]	150	150
Chloramphenicol (Cm)	5[b]	5	-
Cadmium chloride ($CdCl_2$)	100[a]	250	-

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}.

2.2.3 DNA loading buffer (6X)

Bromophenol blue	0.25 % (w/v)
Xylene cyanol FF	0.25 % (w/v)
Glycerol	30 % (v/v)

2.2.4 Frozen storage buffer (FSB)

KCl	7.4 g
$MnCl_2.4H_2O$	8.9 g
$CaCl_2.2H_2O$	1.5 g
$Co(NH_3)Cl_3$	0.8 g
Potassium acetate (1 M, pH 7.5)	10 ml
Glycerol	100 ml

The constituents were made up to 1 l with sterile dH_20 and the pH adjusted to 6.4 using 0.1 M HCl. The solution was divided into 100 ml aliquots and stored at -20°C. Aliquots were defrosted as required and filter-sterilized (0.45 μM pore size) before use.

2.2.5 Haemolysin salts solution

NaCl	0.145 M
$CaCl_2$	20 mM

2.2.6 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 of PBS was adjusted to 7.4 using NaOH.

2.2.7 Phage buffer

$MgSO_4$	1 mM
$CaCl_2$	4 mM
Tris-HCl pH 7.8	50 mM
NaCl	0.59 % (w/v)
Gelatine	0.1 % (w/v)

2.2.8 QIAGEN buffers

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

2.2.9 SDS-PAGE and Western blotting solutions

2.2.9.1 5X Laemmli SDS-PAGE sample buffer

1 M Tris-HCl (pH 6.8)	2.5 ml
SDS	1 g
Glycerol (100 % (v/v))	5 ml
Bromophenol blue	0.05 g
β-mercaptoethanol (100 % (v/v))	2.8 ml

1X sample buffer was made by diluting the 5X stock 1:5 with dH_20.

2.2.9.2 SDS-PAGE gel formulations and construction of gel

The following components were added together in a 30 ml plastic universal;

12 % (w/v) Resolving gel

dH_20	3.4 ml
1.5M Tris-HCl (pH 8.8)	2.5 ml
10 % (w/v) SDS	100 µl
30 % (w/v) Acrylamide/Bis (37.5 : 1) (BioRad)	4.0 ml
10 % (w/v) Ammonium persulphate	50 µl
TEMED	5 µl

The components were mixed by gentle swirling, so as not to introduce air bubbles, and loaded into the gel casting apparatus using a plastic 10 ml syringe. A layer of water-saturated isobutanol was carefully pipetted on top of the gel to isolate it from the air. After the gel had solidified, the stacking gel was made up as follows;

Stacking gel

dH_20	6.1 ml
0.5 M Tris-HCl (pH 6.8)	2.5 ml
10 % (w/v) SDS	100 µl
30 % (w/v) Acrylamide/Bis (37.5 : 1) (BioRad)	1.3 ml
10 % (w/v) Ammonium persulphate	50 µl
TEMED	10 µl

The contents were mixed by gentle swirling and pipetted on top of the resolving gel. A plastic comb was inserted into the gel to create wells and to separate the gel from the air. After the gel had solidified, it was transferred to the gel-running tank and submerged in 1X SDS-PAGE buffer.

2.2.9.3 10X SDS-PAGE electrophoresis buffer

Glycine	144 g l^{-1}
Tris base	30.3 g l^{-1}
SDS	10 g l^{-1}

1X SDS-PAGE buffer was made by diluting 10X SDS-PAGE buffer 1:10 with dH_20.

2.2.9.4 Coomassie Blue staining solution

Coomassie Blue R-250	0.25% (w/v)
Acetic acid	10 % (v/v)
Methanol	50 % (v/v)

Coomassie stain is light-sensitive and was stored in a foil-wrapped duran.

2.2.9.5 Destain solution

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

2.2.9.6 Western blotting solutions

2.2.9.6.1 Blotting buffer

Glycine	150 mM
Tris-HCl	20 mM
Methanol	20 % (v/v)

2.2.9.6.2 20X Washing solution

1 M Tris-HCl (pH 7.4)	200 ml
NaCl	180 mg
Tween® 20	10 ml

The components were made up to 1 l with dH_20. 1X washing solution was made by diluting the 20X stock 1:20 with dH_20.

2.2.9.6.3 Blocking solution

Western blocking solution consisted of 1X Western washing solution containing 5 % (w/v) dried skimmed milk powder (Marvel).

2.2.9.6.4 Primary antibody solution

The primary antibody was diluted 1:6,000, 1:2,500 and 1:2,000 in blocking solution for anti-Hla, SspA and SarA rabbit antibodies respectively, and stored at -20 °C.

2.2.9.6.5 Secondary antibody solution

The secondary antibody was diluted 1:30,000 and 1:1,000 in blocking solution for alkaline phosphatase (Sigma) and horseradish peroxidase-conjugated antibodies respectively (Amersham Pharmacia), and made fresh for each experiment.

2.2.9.6.6 Alkaline phosphatase (AP) buffer

Tris-HCl (pH 9.5)	100 ml l^{-1}
NaCl	5.8 g l^{-1}
$MgCl_2.6H_20$	10.2 g l^{-1}

2.2.9.6.7 Colour substrate solution

10 ml AP buffer containing 200 µl NBT/BCIP solution (Roche).

2.2.9.7 Southern blotting buffers and solutions

2.2.9.7.1 Southern depurination solution

HCl	250 mM

2.2.9.7.2 Southern denaturing buffer

NaOH	0.5 M
NaCl	1.5 M

2.2.9.7.3 Southern neutralising buffer

Tris-HCl (pH 7.5)	0.5 M
NaCl	3 M

2.2.9.7.4 SSC (20X)

NaCl	3 M
Tri-sodium citrate·$2H_2O$	300 mM

The pH was adjusted to 7.0 with 1 M NaOH. 20X SSC was diluted with water to make 10X, 5X, 2X and 0.5X SSC.

2.2.9.7.5 Pre-hybridisation solution

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

2.2.9.7.6 Hybridisation solution

Digoxigenin-labelled DNA probes were diluted in prehybridisation buffer to 5 - 25 ng ml^{-1}.

2.2.9.7.7 Wash solution (2X)

SSC	2X
SDS	0.1 % (w/v)

2.2.9.7.8 Wash solution (0.5X)

SSC	0.5X
SDS	0.1 % (w/v)

2.2.9.7.9 Maleic acid buffer

Maleic acid	0.1 M
NaCl	0.15 M

The pH was adjusted to 7.5 with solid NaOH.

2.2.9.7.10 Washing buffer

Maleic acid buffer containing 3 % (v/v) Tween® 20.

2.2.9.7.11 Blocking solution

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

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

2.2.9.7.12 Antibody solution

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

2.2.9.7.13 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.

2.2.9.7.14 Colour substrate solution

Detection buffer containing 2 % (v/v) NBT/BCIP (Roche).

2.2.9.8 TAE (50X)

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.

2.2.9.9 TE

Tris-HCl	1 mM
EDTA	0.1 mM

The pH was adjusted to 7.5 using 1 M HCl before autoclaving.

2.3 Enzymes and chemicals

All chemicals and enzymes were of analytical grade and purchased from Sigma, Merck (BDH) or Fisons unless otherwise stated. All restriction enzymes, RNase A, DNase, T4 ligase, polymerases and buffers for the modification of DNA were purchased from ABI Perkin-Elmer, Life Technologies (formally Gibco BRL), Northumbria Biologicals Limited (NBL), Promega, MBI Fermentas or Roche (formally Boehringer Mannheim).

Lysostaphin (Sigma) was dissolved in 20 mM sodium acetate to 5 mg ml^{-1} and stored at -20 °C. MUG (4-methylumbelliferyl-β-D-galactopyranoside) (Sigma) was dissolved in DMSO to 4 mg ml^{-1} and stored at -20 °C. MU (4-methylumbelliferone) (Sigma) was dissolved in DMSO to 1 mM and stored at -20 °C. PMSF was dissolved to 0.25 M in 100 % ethanol and stored at -20 °C. RNase A (DNase-free) (Sigma) was dissolved in dH_2O to 10 mg ml^{-1} and stored at -20 °C. X-Gal (Sigma) was dissolved in DMF to 20 - 100 mg ml^{-1} and stored at -20 °C in a foil-wrapped 30 ml universal tube. Purified SspA (Sigma) was dissolved to 100 ng μl^{-1} in 50 mM tris, pH 7.5, and stored at - 20 °C.

2.4 Bacterial strains, plasmids and bacteriophage

2.4.1 Bacterial maintenance, culture and storage conditions

Bacterial strains used in this study are listed in Table 2.2 - 2.3.

2.4.1.1 *Staphylococcus aureus* strains

Staphylococcus aureus 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 2X 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 -20 °C and -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 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. For additional information on growth conditions for reporter gene fusion analysis and/or growth experiments, see Chapter 2.7.

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. Liquid cultures were prepared as for *S. aureus* (Chapter 2.4.1.1) using LB medium in place of BHI. 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.

Strain	Relevant Genotype / Markers	Source / Reference
8325-4	8325 cured of known prophages	Novick, 1963
SH1000	Functional *rsbU*$^+$ derivative of 8325-4	Horsburgh *et al.*, 2002
RN4220	Restriction deficient transformation recipient	Kreiswirth *et al.*, 1983
PC400	8325-4 *sigB::tet* (Tet^R)	Chan *et al.*, 1998
SH1002	SH1000 *sarA::kan* (Kan^R)	Horsburgh *et al.*, 2002
PM783	RN6390 *rot::tet* (Tet^R)	P. McNamara, personal communication
SMH1005	8325-4 *sarH1*::pAULA (Ery^R)	S. Horsburgh, personal communication
PC6911	8325-4 *agr::tet* (Tet^R)	Chan & Foster, 1998b
JL140	8325-4 *hla::ery* (Ery^R)	O'Reilly *et al.*, 1986
MJH502	SH1000 *rsbU*$^+$ *sigB::tet* (Tet^R)	Horsburgh *et al.*, 2002
PC203	8325-4 *spa::lacZ spa*$^+$ (Ery^R)	Chan & Foster, 1998a
PC161	8325-4 *sarA::lacZ sarA*$^+$ (Ery^R)	Chan & Foster, 1998a
JLA311	SH1000 *sarA::lacZ sarA*$^+$ (Ery^R)	This study
PC4030	8325-4 *sarA::lacZ sarA*$^+$ *sigB::tet* (Ery^R, Tet^R)	Chan *et al.*, 1998
JLA313	SH1000 *sarA::lacZ sarA*$^+$ *sigB::tet* (Ery^R, Tet^R)	This study
SH101F7	8325-4 *agr* (RNAIII)::*lacZ agr*$^+$ (Ery^R)	Fairhead, 1998
JLA341	SH1000 *agr* (RNAIII)::*lacZ agr*$^+$ (Ery^R)	This study
PC604	8325-4 *agr* (RNAIII)::*lacZ agr*$^+$ *sigB::tet* (Ery^R, Tet^R)	Horsburgh *et al.*, 2002
JLA343	SH1000 *agr* (RNAIII)::*lacZ agr*$^+$ *sigB::tet* (Ery^R, Tet^R)	This study
PC600	8325-4 *agr* (RNAIII)::*lacZ agr*$^+$ *sarA::kan* (Ery^R, Kan^R)	Horsburgh *et al.*, 2002
JLA345	SH1000 *agr* (RNAIII)::*lacZ agr*$^+$ *sarA::kan* (Ery^R, Kan^R)	This study
PC602	8325-4 *agr* (RNAIII)::*lacZ agr*$^+$ *sigB::tet sarA::kan* (Ery^R, Tet^R, Kan^R)	Horsburgh *et al.*, 2002
JLA347	SH1000 *agr* (RNAIII)::*lacZ agr*$^+$ *sigB::tet sarA::kan* (Ery^R, Tet^R, Kan^R)	This study
JLA511	RN4220 *hla*::*lacZ hla*$^+$ (Tet^R)	This study
JLA512	8325-4 *hla*::*lacZ hla*$^+$ (Tet^R)	This study
JLA513	SH1000 *hla*::*lacZ hla*$^+$ (Tet^R)	This study
PC322	8325-4 *hla*::*lacZ hla*$^+$ (Ery^R)	Chan & Foster, 1998a
JLA371	SH1000 *hla*::*lacZ hla*$^+$ (Ery^R)	This study
PC4044	8325-4 *hla*::*lacZ hla*$^+$ *sigB::tet* (Ery^R, Tet^R)	Horsburgh *et al.*, 2002
JLA373	SH1000 *hla*::*lacZ hla*$^+$ *sigB::tet* (Ery^R, Tet^R)	This study
PC3221	8325-4 *hla*::*lacZ hla*$^+$ *sarA::kan* (Ery^R, Kan^R)	Horsburgh *et al.*, 2002
JLA375	SH1000 *hla*::*lacZ hla*$^+$ *sarA::kan* (Ery^R, Kan^R)	This study
JLA376	8325-4 *hla*::*lacZ hla*$^+$ *sigB::tet sarA::kan* (Ery^R, Tet^R, Kan^R)	This study
JLA377	SH1000 *hla*::*lacZ hla*$^+$ *sigB::tet sarA::kan* (Ery^R, Tet^R, Kan^R)	This study
JLA378	8325-4 *hla*::*lacZ hla*$^+$ *rot::tet* (Ery^R, Tet^R)	This study
JLA379	SH1000 *hla*::*lacZ hla*$^+$ *rot::tet* (Ery^R, Tet^R)	This study
JLA380	8325-4 *hla*::*lacZ hla*$^+$ *sarH1*::pAULA (Tet^R, Ery^R)	This study

Strain Cont.	Relevant Genotype / Markers	Source / Reference
JLA381	SH1000 *hla*::*lacZ hla*$^{+}$ *sarH1*::pAULA (Tet^{R},Ery^{R})	This study
*han*1	8325-4 *han*1::Tn*917* (*unk*1::Tn*917*) (Ery^{R})	This study
*han*2	8325-4 *han*2::Tn*917* (*citG*::Tn*917*) (Ery^{R})	This study
*han*3	8325-4 *han*3::Tn*917* (*citG*::Tn*917*) (Ery^{R})	This study
*han*4	8325-4 *han*4::Tn*917* (*unk*2::Tn*917*) (Ery^{R})	This study
*han*5	8325-4 *han*5::Tn*917* (*citG*::Tn*917*) (Ery^{R})	This study
*han*6	8325-4 *han*6::Tn*917* (*citG*::Tn*917*) (Ery^{R})	This study
*han*7	8325-4 *han*7::Tn*917* (*citG*::Tn*917*) (Ery^{R})	This study
*han*8	8325-4 *han*8::Tn*917* (*citG*::Tn*917*) (Ery^{R})	This study
*han*9	8325-4 *han*9::Tn*917* (*opuD*::Tn*917*) (Ery^{R})	This study
*han*10	8325-4 *han*10::Tn*917* (*unk*1::Tn*917*) (Ery^{R})	This study
*han*11	8325-4 *han*11::Tn*917* (*yugT*::Tn*917)* (Ery^{R})	This study
*han*12	8325-4 *han*12::Tn*917* (*oppF*::Tn*917*) (Ery^{R})	This study
*han*13	8325-4 *han*13::Tn*917* (*ykrP*::Tn*917*) (Ery^{R})	This study
*han*14	8325-4 *han*14::Tn*917* (*eprH*::Tn*917*) (Ery^{R})	This study
*han*15	8325-4 *han*15::Tn*917* (*yubA*::Tn*917*) (Ery^{R})	This study
*han*16	8325-4 *han*16::Tn*917* (*yubA*::Tn*917*) (Ery^{R})	This study
JLA401	*han*1::Tn*917* (*unk*1::Tn*917*) transduced into 8325-4 (Ery^{R})	This study
JLA402	*han*2::Tn*917* (*citG*::Tn*917*) transduced into 8325-4 (Ery^{R})	This study
JLA403	*han*3::Tn*917* (*citG*::Tn*917*) transduced into 8325-4 (Ery^{R})	This study
JLA404	*han*4::Tn*917* (*unk*2::Tn*917*) transduced into 8325-4 (Ery^{R})	This study
JLA405	*han*5::Tn*917* (*citG*::Tn*917*) transduced into 8325-4 (Ery^{R})	This study
JLA406	*han*6::Tn*917* (*citG*::Tn*917*) transduced into 8325-4 (Ery^{R})	This study
JLA407	*han*7::Tn*917* (*citG*::Tn*917*) transduced into 8325-4 (Ery^{R})	This study
JLA408	*han*8::Tn*917* (*citG*::Tn*917*) transduced into 8325-4 (Ery^{R})	This study
JLA409	*han*9::Tn*917* (*opuD*::Tn*917*) transduced into 8325-4 (Ery^{R})	This study
JLA410	*han*10::Tn*917* (*unk*1::Tn*917*) transduced into 8325-4 (Ery^{R})	This study
JLA411	*han*11::Tn*917* (*yugT*::Tn*917* transduced into 8325-4 (Ery^{R})	This study
JLA412	*han*12::Tn*917* (*oppF*::Tn*917*) transduced into 8325-4 (Ery^{R})	This study
JLA413	*han*13::Tn*917* (*ykrP*::Tn*917*) transduced into 8325-4 (Ery^{R})	This study
JLA414	*han*14::Tn*917* (*eprH*::Tn*917*) transduced into 8325-4 (Ery^{R})	This study
JLA415	*han*15::Tn*917* (*yubA*::Tn*917*) transduced into 8325-4 (Ery^{R})	This study

Strain Cont.	Relevant Genotype / Markers	Source / Reference
JLA416	*han*16::Tn*917* (*yubA*::Tn*917*) transduced into 8325-4 (Ery^R)	This study
JLA417	8325-4 *han*1::Tn*917 agr::tet* (*unk*1::Tn*917*) (Ery^R, Tet^R)	This study
JLA418	8325-4 *han*2::Tn*917 agr::tet* (*citG*::Tn*917*) (Ery^R, Tet^R)	This study
JLA419	8325-4 *han*4::Tn*917 agr::tet* (*unk*2::Tn*917*) (Ery^R, Tet^R)	This study
JLA420	8325-4 *han*9::Tn*917 agr::tet* (*opuD*::Tn*917*) (Ery^R, Tet^R)	This study
JLA421	8325-4 *han*11::Tn*917 agr::tet* (*yugT*::Tn*917)* (Ery^R, Tet^R)	This study
JLA422	8325-4 *han*12::Tn*917 agr::tet* (*oppF*::Tn*917*) (Ery^R, Tet^R)	This study
JLA423	8325-4 *han*13::Tn*917 agr::tet* (*ykrP*::Tn*917*) (Ery^R, Tet^R)	This study
JLA424	8325-4 *han*14::Tn*917 agr::tet* (*eprH*::Tn*917*) (Ery^R, Tet^R)	This study
JLA425	8325-4 *han*15::Tn*917 agr::tet* (*yubA*::Tn*917*) (Ery^R, Tet^R)	This study
JLA501	RN4220 EZ::TN(tet) (Tet^R)	This study
JLA502	RN4220 EZ::TN(tet) (Tet^R)	This study
JLA503	RN4220 EZ::TN(tet) (Tet^R)	This study
JLA504	RN4220 EZ::TN(tet) (Tet^R)	This study
JLA505	RN4220 EZ::TN(tet) (Tet^R)	This study
JLA506	RN4220 EZ::TN(tet) (Tet^R)	This study
JLA507	RN4220 EZ::TN(tet) (Tet^R)	This study
JLA508	RN4220 EZ::TN(tet) (Tet^R)	This study
JLA509	RN4220 EZ::TN(tet) (Tet^R)	This study
JLA510	RN4220 EZ::TN(tet) (Tet^R)	This study
JLA514	SH1000 *hla::lacZ* hla^+, pRN3208 (Tet^R, Cd^R, Ery^R)	This study
*aht*1	SH1000 *hla::lacZ* hla^+ *aht*1::Tn*551* (*rsbU::Tn551*) (Tet^R, Ery^R)	This study
*aht*2	SH1000 *hla::lacZ* hla^+ *aht*2::Tn*551* (*ykuQ::Tn551*) (Tet^R, Ery^R)	This study
*aht*3	SH1000 *hla::lacZ* hla^+ *aht*3::Tn*551* (*ykuQ::Tn551*) (Tet^R, Ery^R)	This study
*aht*4	SH1000 *hla::lacZ* hla^+ *aht*4::Tn*551* (*telA::Tn551*) (Tet^R, Ery^R)	This study
*aht*5	SH1000 *hla::lacZ* hla^+ *aht*5::Tn*551* (*lysA::Tn551*) (Tet^R, Ery^R)	This study
*aht*6	SH1000 *hla::lacZ* hla^+ *aht*6::Tn*551* (*brnQ::Tn551*) (Tet^R, Ery^R)	This study
*aht*7	SH1000 *hla::lacZ* hla^+ *aht*7::Tn*551* (*rsbU::Tn551*) (Tet^R, Ery^R)	This study
*aht*8	SH1000 *hla::lacZ* hla^+ *aht*8::Tn*551* (*rsbU::Tn551*) (Tet^R, Ery^R)	This study

Strain Cont.	Relevant Genotype / Markers	Source / Reference
*aht*9	SH1000 *hla::lacZ hla*$^+$ *aht*9::Tn*551* (*rsbU*::*Tn551*) (Tet^R, Ery^R)	This study
*aht*10	SH1000 *hla::lacZ hla*$^+$ *aht*10::Tn*551* (*yjbH*::*Tn551*) (Tet^R, Ery^R)	This study
*aht*11	SH1000 *hla::lacZ hla*$^+$ *aht*11::Tn*551* (*yjbH*::*Tn551*) (Tet^R, Ery^R)	This study
*aht*12	SH1000 *hla::lacZ hla*$^+$ *aht*12::Tn*551* (*yjbH*::*Tn551*) (Tet^R, Ery^R)	This study
*aht*13	SH1000 *hla::lacZ hla*$^+$ *aht*13::Tn*551* (*yjbH*::*Tn551*) (Tet^R, Ery^R)	This study
*aht*14	SH1000 *hla::lacZ hla*$^+$ *aht*14::Tn*551* (*agrB*::*Tn551*) (Tet^R, Ery^R)	This study
*aht*15	SH1000 *hla::lacZ hla*$^+$ *aht*15::Tn*551* (*agrB*::*Tn551*) (Tet^R, Ery^R)	This study
*aht*16	SH1000 *hla::lacZ hla*$^+$ *aht*16::Tn*551* (*agrB*::*Tn551*) (Tet^R, Ery^R)	This study
*aht*17	SH1000 *hla::lacZ hla*$^+$ *aht*17::Tn*551* (*agrB*::*Tn551*) (Tet^R, Ery^R)	This study
*aht*18	SH1000 *hla::lacZ hla*$^+$ *aht*18::Tn*551* (*saeR*::*Tn551*) (Tet^R, Ery^R)	This study
JLA515	*aht*1::Tn*551* (*rsbU*::*Tn551*) transduced into JLA513 (SH1000 *hla::lacZ hla*$^+$) (Ery^R, Tet^R)	This study
JLA516	*aht2*::Tn*551* (*ykuQ*::*Tn551*) transduced into JLA513 (SH1000 *hla::lacZ hla*$^+$) (Ery^R, Tet^R)	This study
JLA517	*aht3*::Tn*551* (*ykuQ*::*Tn551*) transduced into JLA513 (SH1000 *hla::lacZ hla*$^+$) (Ery^R, Tet^R)	This study
JLA518	*aht*4::Tn*551* (*telA*::*Tn551*) transduced into JLA513 (SH1000 *hla::lacZ hla*$^+$) (Ery^R, Tet^R)	This study
JLA519	*aht5*::Tn*551* (*lysA*::*Tn551*) transduced into JLA513 (SH1000 *hla::lacZ hla*$^+$) (Ery^R, Tet^R)	This study
JLA520	*aht6*::Tn*551* (*brnQ*::*Tn551*) transduced into JLA513 (SH1000 *hla::lacZ hla*$^+$) (Ery^R, Tet^R)	This study
JLA521	*aht7*::Tn*551* (*rsbU*::*Tn551*) transduced into JLA513 (SH1000 *hla::lacZ hla*$^+$) (Ery^R, Tet^R)	This study
JLA522	*aht*8::Tn*551* (*rsbU*::*Tn551*) transduced into JLA513 (SH1000 *hla::lacZ hla*$^+$) (Ery^R, Tet^R)	This study
JLA523	*aht*9::Tn*551* (*rsbU*::*Tn551*) transduced into JLA513 (SH1000 *hla::lacZ hla*$^+$) (Ery^R, Tet^R)	This study
JLA524	*aht*10::Tn*551* (*yjbH*::*Tn551*) transduced into JLA513 (SH1000 *hla::lacZ hla*$^+$) (Ery^R, Tet^R)	This study
JLA525	*aht*11::Tn*551* (*yjbH*::*Tn551*) transduced into JLA513 (SH1000 *hla::lacZ hla*$^+$) (Ery^R, Tet^R)	This study
JLA526	*aht*12::Tn*551* (*yjbH*::*Tn551*) transduced into JLA513 (SH1000 *hla::lacZ hla*$^+$) (Ery^R, Tet^R)	This study
JLA527	*aht*13::Tn*551* (*yjbH*::*Tn551*) transduced into JLA513 (SH1000 *hla::lacZ hla*$^+$) (Ery^R, Tet^R)	This study
JLA528	*aht*14::Tn*551* (*agrB*::*Tn551*) transduced into JLA513 (SH1000 *hla::lacZ hla*$^+$) (Ery^R, Tet^R)	This study

Strain Cont.	Relevant Genotype / Markers	Source / Reference
JLA529	*aht*15::Tn*551* (*agrB*::*Tn551*) transduced into JLA513 (SH1000 *hla::lacZ hla*$^+$) (Ery^R, Tet^R)	This study
JLA530	*aht*16::Tn*551* (*agrB*::*Tn551*) transduced into JLA513 (SH1000 *hla::lacZ hla*$^+$) (Ery^R, Tet^R)	This study
JLA531	*aht*17::Tn*551* (*agrB*::*Tn551*) transduced into JLA513 (SH1000 *hla::lacZ hla*$^+$) (Ery^R, Tet^R)	This study
JLA532	*aht*18::Tn*551* (*saeR*::*Tn551*) transduced into JLA513 (SH1000 *hla::lacZ hla*$^+$) (Ery^R, Tet^R)	This study
*htn*1	SH1000 *hla::lacZ hla*$^+$ *htn*1::Tn*551* (*rsbU*::*Tn551*) (Tet^R, Ery^R)	This study
*htn*2	SH1000 *hla::lacZ hla*$^+$ *htn*2::Tn*551* (*rsbU*::*Tn551*) (Tet^R, Ery^R)	This study
*htn*3	SH1000 *hla::lacZ hla*$^+$ *htn*3::Tn*551* (*rsbU*::*Tn551*) (Tet^R, Ery^R)	This study
*htn*4	SH1000 *hla::lacZ hla*$^+$ *htn*4::Tn*551* (*rsbU*::*Tn551*) (Tet^R, Ery^R)	This study
*htn*5	SH1000 *hla::lacZ hla*$^+$ *htn*5::Tn*551* (*rsbU*::*Tn551*) (Tet^R, Ery^R)	This study
*htn*6	SH1000 *hla::lacZ hla*$^+$ *htn*6::Tn*551* (*rsbU*::*Tn551*) (Tet^R, Ery^R)	This study
*htn*7	SH1000 *hla::lacZ hla*$^+$ *htn*7::Tn*551* (*rsbU*::*Tn551*) (Tet^R, Ery^R)	This study
*htn*8	SH1000 *hla::lacZ hla*$^+$ *htn*8::Tn*551* (*rsbU*::*Tn551*) (Tet^R, Ery^R)	This study
*htn*9	SH1000 *hla::lacZ hla*$^+$ *htn*9::Tn*551* (*rsbU*::*Tn551*) (Tet^R, Ery^R)	This study
*htn*10	SH1000 *hla::lacZ hla*$^+$ *htn*10::Tn*551* (*lysC*::*Tn551*) (Tet^R, Ery^R)	This study
*htn*11	SH1000 *hla::lacZ hla*$^+$ *htn*11::Tn*551* (*lysA*::*Tn551*) (Tet^R, Ery^R)	This study
*htn*12	SH1000 *hla::lacZ hla*$^+$ *htn*12::Tn*551* (*lysA*::*Tn551*) (Tet^R, Ery^R)	This study
*htn*13	SH1000 *hla::lacZ hla*$^+$ *htn*13::Tn*551* (*lysA*::*Tn551*) (Tet^R, Ery^R)	This study
*htn*14	SH1000 *hla::lacZ hla*$^+$ *htn*14::Tn*551* (*rsbU*::*Tn551*) (Tet^R, Ery^R)	This study
*htn*15	SH1000 *hla::lacZ hla*$^+$ *htn*15::Tn*551* (*telA*::*Tn551*) (Tet^R, Ery^R)	This study
*htn*16	SH1000 *hla::lacZ hla*$^+$ *htn*16::Tn*551* (*telA*::*Tn551*) (Tet^R, Ery^R)	This study
*htn*17	SH1000 *hla::lacZ hla*$^+$ *htn*17::Tn*551* (*hyp*1::*Tn551*) (Tet^R, Ery^R)	This study
*htn*18	SH1000 *hla::lacZ hla*$^+$ *htn*18::Tn*551* (*saeS*::*Tn551*) (Tet^R, Ery^R)	This study
*htn*19	SH1000 *hla::lacZ hla*$^+$ *htn*19::Tn*551* (*saeS*::*Tn551*) (Tet^R, Ery^R)	This study
*htn*20	SH1000 *hla::lacZ hla*$^+$ *htn*20::Tn*551* (*lysA*::*Tn551*) (Tet^R, Ery^R)	This study

Strain Cont.	Relevant Genotype / Markers	Source / Reference
*htn*21	SH1000 *hla::lacZ hla*$^{+}$ *htn*21::Tn*551* (*pstC*::*Tn551*) (Tet^{R}, Ery^{R})	This study
JLA533	*htn*1::Tn*551* (*rsbU*::*Tn551*) transduced into JLA513 (SH1000 *hla::lacZ hla*$^{+}$) (Ery^{R}, Tet^{R})	This study
JLA534	*htn*2::Tn*551* (*rsbU*::*Tn551*) transduced into JLA513 (SH1000 *hla::lacZ hla*$^{+}$) (Ery^{R}, Tet^{R})	This study
JLA535	*htn*3::Tn*551* (*rsbU*::*Tn551*) transduced into JLA513 (SH1000 *hla::lacZ hla*$^{+}$) (Ery^{R}, Tet^{R})	This study
JLA536	*htn*4::Tn*551* (*rsbU*::*Tn551*) transduced into JLA513 (SH1000 *hla::lacZ hla*$^{+}$) (Ery^{R}, Tet^{R})	This study
JLA537	*htn*5::Tn*551* (*rsbU*::*Tn551*) transduced into JLA513 (SH1000 *hla::lacZ hla*$^{+}$) (Ery^{R}, Tet^{R})	This study
JLA538	*htn*6::Tn*551* (*rsbU*::*Tn551*) transduced into JLA513 (SH1000 *hla::lacZ hla*$^{+}$) (Ery^{R}, Tet^{R})	This study
JLA539	*htn*7::Tn*551* (*rsbU*::*Tn551*) transduced into JLA513 (SH1000 *hla::lacZ hla*$^{+}$) (Ery^{R}, Tet^{R})	This study
JLA540	*htn*8::Tn*551* (*rsbU*::*Tn551*) transduced into JLA513 (SH1000 *hla::lacZ hla*$^{+}$) (Ery^{R}, Tet^{R})	This study
JLA541	*htn*9::Tn*551* (*rsbU*::*Tn551*) transduced into JLA513 (SH1000 *hla::lacZ hla*$^{+}$) (Ery^{R}, Tet^{R})	This study
JLA542	*htn*10::Tn*551* (*lysC*::*Tn551*) transduced into JLA513 (SH1000 *hla::lacZ hla*$^{+}$) (Ery^{R}, Tet^{R})	This study
JLA543	*htn*11::Tn*551* (*lysA*::*Tn551*) transduced into JLA513 (SH1000 *hla::lacZ hla*$^{+}$) (Ery^{R}, Tet^{R})	This study
JLA544	*htn*12::Tn*551* (*lysA*::*Tn551*) transduced into JLA513 (SH1000 *hla::lacZ hla*$^{+}$) (Ery^{R}, Tet^{R})	This study
JLA545	*htn*13::Tn*551* (*lysA*::*Tn551*) transduced into JLA513 (SH1000 *hla::lacZ hla*$^{+}$) (Ery^{R}, Tet^{R})	This study
JLA546	*htn*14::Tn*551* (*rsbU*::*Tn551*) transduced into JLA513 (SH1000 *hla::lacZ hla*$^{+}$) (Ery^{R}, Tet^{R})	This study
JLA547	*htn*15::Tn*551* (*telA*::*Tn551*) transduced into JLA513 (SH1000 *hla::lacZ hla*$^{+}$) (Ery^{R}, Tet^{R})	This study
JLA548	*htn*16::Tn*551* (*telA*::*Tn551*) transduced into JLA513 (SH1000 *hla::lacZ hla*$^{+}$) (Ery^{R}, Tet^{R})	This study
JLA549	*htn*17::Tn*551* (*hyp*1::*Tn551*) transduced into JLA513 (SH1000 *hla::lacZ hla*$^{+}$) (Ery^{R}, Tet^{R})	This study
JLA550	*htn*18::Tn*551* (*saeS*::*Tn551*) transduced into JLA513 (SH1000 *hla::lacZ hla*$^{+}$) (Ery^{R}, Tet^{R})	This study
JLA551	*htn*19::Tn*551* (*saeS*::*Tn551*) transduced into JLA513 (SH1000 *hla::lacZ hla*$^{+}$) (Ery^{R}, Tet^{R})	This study
JLA552	*htn*20::Tn*551* (*lysA*::*Tn551*) transduced into JLA513 (SH1000 *hla::lacZ hla*$^{+}$) (Ery^{R}, Tet^{R})	This study
JLA553	*htn*21::Tn*551* (*pstC*::*Tn551*) transduced into JLA513 (SH1000 *hla::lacZ hla*$^{+}$) (Ery^{R}, Tet^{R})	This study
JLA554	SH1000 *aht*12::Tn*551* (*yjbH*::*Tn551*) (Ery^{R})	This study
JLA555	SH1000 *htn*10::Tn*551* (*lysC*::*Tn551*) (Ery^{R})	This study
JLA556	SH1000 *aht*2::Tn*551* (*ykuQ*::*Tn551*) (Ery^{R})	This study
JLA557	SH1000 *aht*5::Tn*551* (*lysA*::*Tn551*) (Ery^{R})	This study

Strain Cont.	Relevant Genotype / Markers	Source / Reference
JLA558	SH1000 *aht*4::Tn*551* (*telA*::*Tn551*) (Ery^R)	This study
JLA559	SH1000 *aht*6::Tn*551* (*brnQ*::*Tn551*) (Ery^R)	This study
JLA560	SH1000 *aht*1::Tn*551* (*rsbU*::*Tn551*) (Ery^R)	This study
JLA561	SH1000 *htn*17::Tn*551* (*hyp*1::*Tn551*) (Ery^R)	This study
JLA562	SH1000 *htn*18::Tn*551* (*saeS*::*Tn551*) (Ery^R)	This study
JLA563	SH1000 *aht*14::Tn*551* (*agrB*::*Tn551*) (Ery^R)	This study
JLA564	SH1000 *aht*18::Tn*551* (*saeR*::*Tn551*) (Ery^R)	This study
*seg*24	8325-4 *asd*::Tn*917* (Ery^R)	Wiltshire & Foster, 2001
JLA601	SH1000 *asd*::Tn*917* (Ery^R)	This study
*seg*10	8325-4 *dapA*::Tn*917* (Ery^R)	Wiltshire & Foster, 2001
JLA603	SH1000 *dapA*::Tn*917* (Ery^R)	This study
LES153	SH1000 *hipO*::Tn*551* (Ery^R)	Shaw, 2002
JLA605	SH1000 *hla*::*lacZ hla*$^+$ *hipO*::Tn*551* (Tet^R, Ery^R)	This study
LES154	SH1000 *aclP*::Tn*551* (Ery^R)	Shaw, 2002
JLA606	SH1000 *hla*::*lacZ hla*$^+$ *aclP*::Tn*551* (Tet^R, Ery^R)	This study
LES155	SH1000 *norQ*::Tn*551* (Ery^R)	Shaw, 2002
JLA607	SH1000 *hla*::*lacZ hla*$^+$ *norQ*::Tn*551* (Tet^R, Ery^R)	This study
MDW41	8325-4 *lysC*::*lacZ* (Ery^R)	Wiltshire & Foster, 2001
JLA608	SH1000 *lysC*::*lacZ* (Ery^R)	This study
JLA609	SH1000 *lysC*::*lacZ sigB*::*tet* (Ery^R, Tet^R)	This study
SPW2	8325-4 *lysA*::Tn*917* (Ery^R)	Watson *et al*., 1998
JLA610	SH1000 *lysA*::Tn*917* (Ery^R)	This study
JLA611	SH1000 *lysA*::Tn*917 sigB*::*tet* (Ery^R, Tet^R)	This study
J68	SH1000 *telA*::*lacZ*(Ery^R)	Lithgow & Foster, personal communication
JLA612	SH1000 *telA*::*lacZ sigB*::*tet* (Ery^R, Tet^R)	This study

Table 2.2

S. aureus strains used in this study.

Ery^R, erythromycin resistant; Tet^R, tetracycline resistant; Kan^R, kanamycin resistant; Cd^R, Cadmium resistant.

Strain	Relevant Genotype / Markers	Source / Reference
DH5α	*supE44Δ lacU169* (ϕ 80 *lacZ* ΔM15) *hsdR17* *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1	Hanahan, 1983
TOP10 One Shot™ chemically competent cells	F⁻ *mcrA* Δ(*mrr-hsd*RMS-*mcr*BC) ϕ 80 *lacZ* ΔM15 Δ*lac*X74 *rec*A1 *deo*R *ara*D139 Δ(*ara-leu*)7697 *gal*K *rps*L (Str^r) *end*A1 *nup*G	Invitrogen

Table 2.3

E. coli strains used in this study.

2.4.2 Plasmids

The plasmids used in this study are listed in table 2.4. Plasmid DNA was purified using QIAGEN plasmid kits (Chapters 2.11.2 and 2.11.3) in accordance with the manufacturer's instructions. Purified plasmid DNA was stored in TE buffer at -20 °C.

2.4.3 Bacteriophage

Bacteriophage ϕ11 (Mani *et al.*, 1993) was used for phage transduction of *S. aureus* (Chapter 2.9). This phage is a *S. aureus*-specific, temperate, transducing phage of serological group B, and requires Ca^{2+} ions for maintenance of infection in bacterial cells. ϕ11 has an approximate genome size of 45 kb (Novick, 1991).

2.5 Centrifugation

A number of different centrifuges were used for the harvesting of cells and precipitated material. These were: i) Eppendorf microfuge 5415D; maximum volume - 2 ml, maximum speed 13,200 rpm (10,000 **g**); ii) Centaur 2 centrifuge (Sanyo); maximum volume - 50 ml, maximum speed 5,000 rpm; iii) Avanti™ J25I (Beckman), maximum volumes and speeds dependent on the rotor used: JA-20; maximum volume - 50 ml, maximum speed 20,000 rpm (48,384 **g**); JA-14; maximum volume - 250 ml, maximum speed 14,000 rpm (30,074 **g**); JA-10.5; maximum volume - 500 ml, maximum speed 10,000 rpm (18,480 **g**).

All centrifugation was carried out at room temperature unless stated otherwise.

Plasmid	Relevant Genotype / Markers	Source / Reference
pMUTIN4	Promoterless transcriptional *lacZ* fusion vector (Amp^R, Ery^R)	Vagner *et al.*, 1998
pMAL23	*Hin*dIII-*Bam*HI insert in *Hin*dIII-*Bam*HI–cut pMUTIN4 (Amp^R, Ery^R)	M. Horsburgh, personal communication
pCR-II-TOPO	Vector for cloning via TOPO TA cloning method (Amp^R, Kan^R)	Invitrogen
pDG646	Vector carrying Ery cassette suitable for selection in Gram-positive bacteria (Amp^R, Ery^R)	Guerout-Fleury *et al.*, 1995
pJA3	2.9 kb fragment comprising *hla* and approximately 1 kb of flanking DNA either side, introduced into pCR-II-TOPO via TOPO TA cloning (Amp^R, Kan^R)	This study
pJA4	1.6 kb *Hin*dIII fragment containing Ery cassette from pDG646, in *Hin*dIII-cut pJA3 (Amp^R, Kan^R, Ery^R)	This study
pDG1513	Vector carrying Tet cassette suitable for selection in Gram-negative and positive bacteria (Amp^R, Tet^R)	Guerout-Fleury *et al.*, 1995
pMOD(MCS)	Cloning vector for introducing DNA into the MCS of the EZ::TN transposon (Amp^R)	Epicentre Technologies
pMOD(tet)	pMOD(MCS) containing a 1.5 kb Tet cassette fragment from pDG1513 inserted into the MCS of the EZ::TN transposon (Amp^R, Tet^R)	This study
pMC4	2.3 kb *Xba*I-*Eco*RI PCR product containing the upstream regulatory region and 5' end of *sigB* cloned into *Xba*I-*Eco*RI pAZ106 (Amp^R, Ery^R)	Chan *et al.*, 1998
pAISH1	A derivative of pMUTIN4 in which the erythromycin-resistance cassette has been replaced with a tetracycline-resistance cassette (Amp^R, Tet^R)	This study
pPF4	2.2 kb *Hin*dIII-*Sca*I fragment from pDU1212 containing the promoter and 5' coding region of the *hla* gene in *Hin*dIII / *Sca*I-cut pBK-CMV (Kan^R)	Chan & Foster, 1998a
pJA5	2.2 kb *Eco*RI-*Not*I fragment from pPF4 containing the promoter and 5' coding region of the *hla* gene in *Eco*RI / *Not*I-cut pAISH1 (Amp^R, Tet^R)	This study
pRN3208	Vector carrying Tn*551* (Amp^R, Cd^R, Ery^R)	Kornblum *et al.*, 1986

Table 2.4

Plasmids used in this project.

2.6 Determination of bacterial cell density

2.6.1 Spectrophotometric measurement (OD_{600})

To quantify the bacterial yield of a culture, spectrophotometric measurements at 600 nm (OD_{600}), 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 Direct cell counts (cfu)

An alternative method for the quantification of cell numbers involved direct 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, as in Chapter 2.4.1.1. 5 ml BHI (no antibiotics) 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 preculture was used to inoculate 100 ml pre-warmed BHI (no antibiotics, same batch as overnight preculture) in a 250 ml conical flask to an OD_{600} of 0.01. The 100 ml culture was grown at 37 °C in a Grant OLS 200 water-bath with linear shaking (equivalent to 250 rpm). For growth experiments without *lacZ* fusion analysis, culture OD_{600} was determined in duplicate over a period of 24 h. For growth experiments with *lacZ* fusion analysis, the 100 ml culture was grown to an OD_{600} of approximately 1.0 and used to inoculate a second 250 ml conical flask containing 100 ml BHI (no antibiotics, same batch as overnight preculture) to an OD_{600} of 0.01. The use of exponential-phase precultures prevented carry-over of preformed β-galactosidase from stationary phase cultures, as this would

affect *lacZ* fusion analysis results. The second 100 ml culture was then grown under the same conditions as the 100 ml preculture. At appropriate intervals over a 24 h period, culture OD_{600} was determined in duplicate and samples for β-galactosidase assays were taken (Chapter 2.8).

2.8 β-galactosidase assays using MUG as a substrate

Liquid culture samples were assayed for β-galactosidase (LacZ) production with MUG as substrate, using a method based on that developed by Youngman (1990). 100 µl culture samples were collected in 1.5 ml microfuge tubes at regular intervals from cultures grown as in Chapter 2.7. Following centrifugation (11,000 g, 3 min), supernatants were discarded, and cell-pellets were snap-frozen in liquid nitrogen and stored at -70 °C for later analysis. The cell pellets were thawed at room temperature for 5 min and resuspended in 0.5 ml ABT buffer. 50 µl of freshly prepared MUG (4 mg ml^{-1}) was added and the reactions were mixed by gently inverting the tubes. The reactions were immediately incubated at 25 °C in a water-bath for exactly 60 min. During the reaction, MUG is hydrolysed to β-D-galactopyranoside and 4-methylumbelliferone (MU) by the action of β-galactosidase. MU is a fluorescent compound and is therefore a quantifiable indicator of β-galactosidase activity. The reaction between β-galactosidase and MUG was stopped by adding 0.5 ml 0.4 M Na_2CO_3, and gently inverting the tubes to mix.

250 µl of each sample was pipetted into the top wells of a 96-well microtitre plate (Nunc). 225 µl of ABTN was added to each of the remaining wells to be used. 25 µl was removed from the 250 µl sample and diluted 1:10 by mixing with 225 µl ABTN. Serial 1:100 and 1:1,000 dilutions were then performed. 25 µl was removed from the 1:1,000 dilution well to allow a consistent well volume of 225 µl.

A fluorimeter (Victor^{2}™, Wallac) was used to measure the fluorescence of each sample (355 / 460 nm, 0.1 sec). The relationship between fluorescence and amount of MU was determined using a calibration curve (Chapter 2.8.1; Figure 2.1). This allowed the amount of MU in each 225 µl sample to be calculated using the equation

shown in Chapter 2.8.1. The amount of MU was then related to β-galactosidase activity using the equation shown below and expressed in MUG units of β-galactosidase activity. 1 MUG unit of β-galactosidase activity is defined as the amount of enzyme that catalyses the hydrolysis of 1 pmol of MUG per min, per ml of culture, per unit of optical density at 600nm (OD_{600}). The background level of β-galactosidase activity measured from 8325-4 and SH1000 control samples was deducted from the β-galactosidase activity of *lacZ* fusion strains.

$$\text{pmoles} \times (A / B) / (60 \times OD_{600} \times 0.1) = \beta\text{-galactosidase activity, } min^{-1}\ ml^{-1}\ OD_{600}^{-1}\ \text{(MUG Units)}$$

Where;

pmoles = pmoles MU (see Chapter 2.8.1 and Fig 2.1 for equation to determine amount of MU from calibration curve)

A = Volume of assay (1.05, where cell pellets were resuspended in 0.5 ml ABT buffer and had 50 µl MUG and 0.5 ml Na_2CO_3 added)

B = Volume of sample read in plate (*ie*. 0.225 where 225 µl sample read)

60 = No. min incubated at 25 °C

OD_{600} = OD_{600} of culture at given time-point

0.1 = Volume of culture sampled (*ie*. 0.1 ml)

2.8.1 β-galactosidase assay calibration curve

A calibration curve was prepared each time β-galactosidase assays were performed. These were created with the fluorescent product (MU) diluted to a range of concentrations such that the final amount of MU in the 225 µl samples was 625, 250, 125, 62.5, 25, 12.5 and 2.5 pmoles. An example of such a calibration curve is shown in Figure 2.1, for which the equation of the straight line was $y = 1494.4 + 111.74x$. For

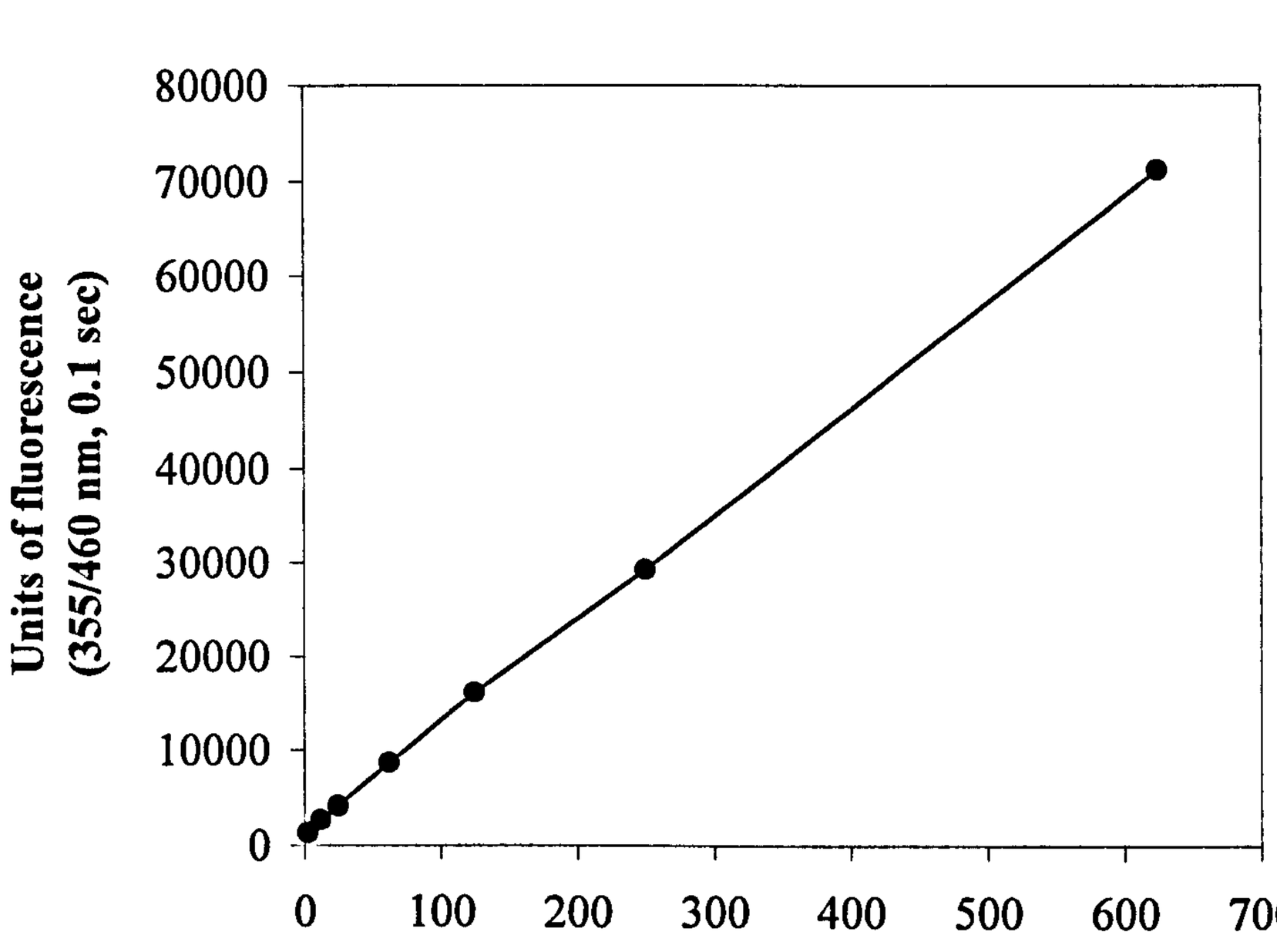


Figure 2.1

Calibration curve of amount of MU against units of fluorescence.

this example, the following equation was used to convert fluorescence readings into pmoles MU.

$$\frac{[(\text{Fluorescence}*D) - \text{Background fluorescence}] - 1494.4}{111.74} = \text{pmoles MU}$$

D = dilution of samples in microtitre plate wells (*eg.* 10 for 1:10 dilution)

2.9 Phage techniques

2.9.1 Preparation of phage lysates

S. aureus strains were used to inoculate 5 ml BHI (containing relevant antibiotics) in a 30 ml universal tube, and grown at 37 °C (unless otherwise stated) with 250 rpm rotary shaking until in log phase (OD_{600} 0.2 - 0.5). 2 - 5 ml of cells were pelleted (4,000 rpm (Centaur 2), 3 min) and resuspended in 5 ml sterile BHI. 5 ml phage buffer and 100 µl phage ϕ11 stock lysate (propagated using *S. aureus* 8325-4 or SH1000 cells) were added to give a cell:phage ratio of approximately 20:1). The tubes were then incubated at 30 °C shaking slowly (50 rpm) for 2 - 4 h, until clear. If cells failed to lyse in this time, they were left overnight (static) at 25 °C. Lysates were filter sterilised (0.2 µm pore size) and stored at 4 °C. The resulting lysates contained between 10^9 and 10^{10} pfu ml^{-1}.

2.9.2 Determination of phage titres

S. aureus 8325-4 or SH1000 was grown in 5 ml BHI at 37 °C with 250 rpm rotary shaking until log phase (OD_{600} ~ 0.5). Phage lysates were diluted in phage buffer to 10^{-7}. 100 µl of diluted phage was mixed with 50 µl of 1 M $CaCl_2$ and 400 µl of culture, and were incubated for 10 min at room temperature. 5 ml of phage top agar (cooled to 50 °C), was added to the phage mixture and used to overlay a pre-warmed (~ 60 °C) phage bottom agar plate. After setting (10 min), plates were incubated overnight at 37 °C, after which time the number of pfu ml^{-1} was determined.

2.9.3 Phage transduction

The recipient *S. aureus* strain was inoculated into 100 ml LK broth and incubated overnight at 37 °C with shaking (250 rpm). The cells were harvested by centrifugation (4,000 rpm (Centaur 2), 10 min), and resuspended in 5 ml sterile unused LK. 500 µl of cell culture was transferred to a sterile 30 ml universal tube. 500 µl of phage lysate and 1 ml LK (containing 10 mM $CaCl_2$) were then added. After gentle mixing the lysate/cell mixture was incubated statically for 25 min, followed by 15 min with shaking (250 rpm). The temperature used for incubation steps following addition of the lysate was 37 °C unless otherwise stated. 1 ml ice-cold 0.02 M sodium citrate was added and cells were harvested (5000 rpm (Centaur 2), 10 min) and resuspended in 1 ml 0.02 M sodium citrate before being left on ice for 2 h.

Using sterile glass beads, 100 µl and 200 µl aliquots of the lysate/cell mixture were spread onto 25 ml LK bottom agar plates containing 0.05 % (v/v) sodium citrate and inducing levels of Ery (0.15µg ml^{-1}) where necessary. Plates were incubated for 90 min, and overlaid with 5 ml LK top agar containing relevant antibiotics at 6X their normal selective concentration. After setting (10 min), plates were incubated for 24-72 h. Any resulting colonies were considered putative phage transductants, and were patched onto separate BHI plates containing relevant selective antibiotics to ensure that they possessed the correct resistance profile.

2.10 Transformation techniques

2.10.1 Transformation of *E. coli*

2.10.1.1 Preparation of *E. coli* competent cells (Hanahan, 1983)

A single colony of *E. coli* DH5α cells from an overnight LB agar plate was inoculated into 1 ml of SOB and the cells were dispersed by vortexing. This was used to inoculate 100 ml of pre-warmed SOB in a 1 l conical flask. The culture was incubated at 37 °C, with shaking (250 rpm), until an OD_{600} of 0.5 - 0.6 was reached. The culture was divided into 2 X 50 ml centrifuge tubes and chilled on ice for 15 min. The cells were

then harvested by centrifugation (5,000 rpm (Centaur 2), 15 min, 4 °C), and the pellet was drained (removing all traces of supernatant with a pipette). After resuspension in 33 ml of FSB (16.5 ml per pellet), the two cell suspensions were pooled and incubated on ice for 15 min. The cells were pelleted and drained as before, and resuspended in 8 ml FSB and placed on ice. 280 µl of DMSO was added, the sample was mixed by swirling and the tube was incubated on ice for 5 min. A second 280 µl volume of DMSO was added as before and the tube was incubated on ice for a further 15 min. 200 µl aliquots were pipetted into microfuge tubes, used immediately or snap-frozen using liquid nitrogen and stored at -70 °C. The level of competence of the cells was determined by transformation with a suitable control plasmid (Chapter 2.10.1.2).

2.10.1.2 Transformation of Hanahan competent *E. coli* cells by heat-shock

An aliquot of frozen Hanahan competent cells (200 µl) (Chapter 2.10.1.1) was thawed on ice in a pre-chilled microfuge tube. Up to 50 ng of DNA (in a volume of up to 20 µl) was added, and the mixture was incubated on ice for 45 min. The tube was then incubated at 42 °C for 90 sec and immediately returned to the ice for a further 2 min. 800 µl SOC was added to the cells, which were incubated for 1 hr at 37 °C with shaking at 250 rpm to allow expression of plasmid encoded antibiotic resistance markers. A 200 µl aliquot of transformed cells were spread using sterile glass beads onto an LB agar plate containing appropriate antibiotics. The remainder of the mixture was then spun down and most of the supernatant was removed so that only approximately 200 µl remained. The pellet was then resuspended in the residual volume of supernatant and the whole of the sample was spread onto an LB agar plate as before. The plates were then incubated at 37 °C for 18 - 48 h.

2.10.2 Transformation of *S. aureus*

2.10.2.1 Preparation of *S. aureus* electrocompetent cells

A single colony of *S. aureus* RN4220 was used to inoculate 10 ml B2 medium and incubated overnight at 37 °C with shaking (250 rpm). 2 ml of culture was used to

inoculate 1 l of fresh B2 in a 2 l conical flask and grown at 37 °C with shaking (250 rpm) until late log phase (OD_{600} 0.5 - 1.0). Cells were harvested by centrifugation (5,000 rpm (Centaur 2), 10 min, 4°C) and washed on ice 3X with 300 ml prechilled sterile water (4 °C). Cells were then concentrated in a series of harvesting (5000 rpm (Centaur 2), 10 min, 4 °C), and gentle resuspension steps using the following volumes of 10 % (v/v) ice-cold glycerol: i) 100 ml; ii) 50 ml; and iii) 25 ml. All of the supernatant was removed after each spin. The cells were finally resuspended in 1.25 ml 10 % (v/v) ice-cold glycerol. Cells were snap-frozen in liquid nitrogen and stored at -70 °C. The level of competence of the cells was determined by electroporation with a suitable control plasmid (Chapter 2.10.2.2).

2.10.2.2 Transformation of *S. aureus* by electroporation

5-20 µg of plasmid DNA was precipitated by isopropanol precipitation (Chapter 2.11.4), and washed twice with 70 % (v/v) ice-cold (-20 °C) ethanol, ensuring the complete removal of salt. The DNA was then resuspended in 100 µl of sterile dH_2O and transferred to a 0.1 cm gap cuvette (BioRad). 2×10^{10} - 2×10^{11} competent cells, which had been defrosted for 5 min at room temperature were then added to the cuvette. The plasmid/cell mix was electroporated at room temperature at 100 ohms, 25 µF and 1.25 kV using a BioRad Gene Pulser. Cells were recovered by adding to the cuvette 1 ml of pre-warmed B2 (containing an inducing concentration of Ery, *ie.* 0.15 µg ml^{-1}, where appropriate). Cells were then transferred to a sterile 2 ml microfuge tube and incubated at 37 °C with shaking (250 rpm) for 2 h. A 200 µl aliquot of recovered cells was spread using sterile glass beads onto a BHI plate containing appropriate antibiotics. The remainder of the mixture was then spun down and most of the supernatant was removed so that only approximately 200 µl remained. The pellet was then resuspended in the residual volume of supernatant and the whole of the sample was spread onto a BHI plate as before. The plates were then incubated at 37 °C for 18 - 48 h.

2.11 DNA purification techniques

2.11.1 Genomic DNA preparation

Genomic DNA was isolated and purified from *S. aureus* using a QIAGEN DNeasy™ kit using a method based on the manufacturer's instructions. A single colony of *S. aureus* was used to inoculate 5 ml of BHI in a 30 ml sterile universal tube and grown overnight at 37 °C with shaking at 250 rpm. 1.25 ml of cells was harvested by centrifugation (4,000 rpm (Centaur 2), 15 min). 180 µl QIAGEN Buffer B1, 10 µl RNAse A (10 mg ml^{-1}) and 5 µl lysostaphin (5 mg ml^{-1}) were added and used to resuspend the cell pellet. After incubation at 37 °C for 30 min, the protocol was continued as per the manufacturer's instructions. Genomic DNA was eluted into clean 1.5 ml microfuge tubes using two 200 µl aliquots of buffer AE, and the two eluates were pooled. Genomic DNA was concentrated by isopropanol precipitation (Chapter 2.11.4). The DNA was dissolved in 20 µl QIAGEN Buffer EB overnight at 4 °C, and stored at this temperature.

2.11.2 Small scale plasmid preparation from *E. coli*

A single colony of *E. coli* DH5α plasmid-bearing cells was used to inoculate 5 ml LB containing appropriate antibiotics. The culture was incubated overnight at 37 °C with shaking at 250 rpm, and the cells were harvested (4,000 rpm (Centaur 2), 5 min). Plasmid DNA (up to 20 µg) was isolated and purified using a QIAprep Spin Miniprep kit DNA purification system (QIAGEN) according to the manufacturer's instructions. Plasmid DNA was eluted using 50 µl of Buffer EB into a clean 1.5 ml microfuge tube and stored at -20 °C.

2.11.3 Large scale plasmid preparation from *E. coli*

Large amounts of plasmid DNA (up to 500 µg) were isolated from *E. coli* using a Plasmid Maxi kit DNA purification system (QIAGEN). A single colony of plasmid-containing cells of *E. coli* DH5α from an overnight LB agar plate containing

appropriate antibiotics was used to inoculate 5 ml LB broth containing antibiotics where necessary. The culture was grown for 8 h at 37 °C with shaking at 250 rpm. The whole culture was used to inoculate 500 ml LB (containing appropriate antibiotics), and grown overnight at 37 °C with shaking at 250 rpm. The cells were harvested by centrifugation (6,000 **g**, 15 min, 4 °C). The protocol was then continued as per the manufacturer's instructions. Purified plasmid DNA was resuspended in an appropriate volume of TE and stored at -20 °C.

2.11.4 Isopropanol precipitation of DNA

DNA was precipitated by the addition of an equal volume of isopropanol and a 1/10 volume of 3 M sodium acetate (pH 5.2). The mixture was vortexed and incubated at room temperature for 2 min. The precipitated DNA was recovered by centrifugation (11,000 **g**, 10 min) and the pellet was washed with 500 µl ice-cold 70 % (v/v) ethanol. The DNA was pelleted by centrifugation (11,000 **g**, 5 min), and the 70 % (v/v) ethanol wash step was repeated. The pellet was air-dried, and the DNA was dissolved in an appropriate volume of TE and stored at -20 °C.

2.11.5 Gel extraction of DNA using a QIAquick spin column

DNA fragments were purified from agarose gels (Chapter 2.11.5) with a QIAquick Gel Extraction kit (QIAGEN) according to the manufacturer's instructions. Purified DNA was eluted into a clean 1.5 ml microfuge tube using 50 µl of Buffer EB and stored at -20 °C.

2.11.6 Purification of PCR products using a QIAquick spin column

PCR products were purified using a QIAquick PCR Purification kit (QIAGEN), according to the manufacturer's instructions. Purified DNA was eluted into a clean 1.5 ml microfuge tube using 30 µl Buffer EB and stored at -20 °C.

2.11.7 Purification of dye-terminator sequencing reactions using a DyeEX™ spin column

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

2.12 Quantification of DNA

To quantify the concentration of DNA in a solution, spectrophotometric measurements were performed at 260 nm. An OD_{260} of 1 corresponds to approximately 50 μg ml^{-1} for double stranded DNA, and approximately 20 μg ml^{-1} for single stranded oligonucleotides. OD_{260} measurements were taken using a Shimadzu UV-2401PC spectophotometer. Alternatively, the concentration of DNA could be estimated by agarose gel electrophoresis (Chapter 2.13.5). This involved comparing the intensity of ethidium bromide stained bands to bands of molecular weight markers containing known amounts of DNA (Table 2.6).

2.13 *In vitro* DNA manipulation techniques

2.13.1 Polymerase chain reaction (PCR) techniques

2.13.1.1 Primer design

The primers used for PCR amplification were short synthetic oligonucleotides (19 - 32 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 2.5.

Primer	Primer sequence (5' → 3')	Source / Reference
OL-78	TAT CTA CCA ATC TTT GAT AAT CTC GAT AAC	Kullik & Giachino, 1997
OL-79	GCT CTA GAG TTC AAG ACA TTA GAT G	
Tn2	CTC ACA ATA GAG AGA TGT CAC CGT C	Watson *et al.*, 1998
JA-5	CCC AAG CTT GTC CCA TGA TTA GTG TTC	This study
JA-6	CGC GGA TCC ATG CAT GCT ATA TAT AC	
OL-32	CCG GTA CCC GGA TTT TAT GAC CGA TGA TGA AG	M. Horsburgh, personal communication
OL-33	CCG GTA CCT TAG AAA TCC CTT TGA GAA TGT TT	
FP-1	ATT CAG GCT GCG CAA CTG T	Epicentre Technologies
RP-1	GTC AGT GAG CGA GGA AGC GGA AG	
JA-7	CAT CGT GTC GAC CTG CAG ATA GTG TAC G	This study
JA-8	CAT CGT AGA TCT CTC TCT CCC AAA GTT G	
JA-9	CAT CGT AGA TCT CTC TCT AGC TTG AGG CAT C	This study
JA-10	CAT CGT CTC GAG GTT CAT GTA ATC ACT CCT TC	

Table 2.5

Synthetic oligonucleotides used as primers for PCR amplification of DNA fragments in this project.

Relevant restriction sites are underlined.

2.13.1.2 DNA amplification

2.13.1.2.1 Taq polymerase

Where accurate amplification was not required, standard PCR amplification reactions were performed using Taq polymerase (Promega). The following components were added, on ice, to a 0.5 ml thin-walled PCR tube.

Template DNA	100 - 500 ng
Forward primer	300 nM*
Reverse primer	300 nM*
10X Taq PCR buffer (Promega)	10 µl
$MgCl_2$	1.5 - 4.5 mM*
dNTPs	0.2 mM*
Taq DNA polymerase	0.5 µl (2.5 Units)
dH_2O	to 100 µl

*final concentration

PCR amplification was carried out using an Eppendorf 5330 Mastercycler. The lid was heated to 106 °C for the duration of the PCR amplification, and the block was pre-heated to 95 °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 sec
3) Anneal	50-55 °C; 30 sec
4) Extension	72 °C; t min (t=1 min kb^{-1} + 10 %)
5) Extension	72 °C, 20 min

PCR products were stored at -20 °C.

2.13.1.2.2 Pwo polymerase

DNA amplifications of < 3 kb that required 3'-5' proof-reading activity were performed with Pwo polymerase (Roche).

The following Master mixes were made on ice:

Master mix 1:

Template DNA	0.1 - 0.75 µg
dNTPs	0.2 mM*
Forward primer	300 nM *
Reverse primer	300 nM *
dH_2O	to 50 µl

Master mix 2:

10X PCR buffer with 20 mM $MgSO_4$	10 µl
Pwo polymerase	0.5 µl (2.5 Units)
dH_2O	to 50 µl

*final concentration

Master mix 1 and 2 were combined on ice in a 0.5 ml thin-walled PCR tube and cycling immediately commenced.

PCR amplification was performed using an Eppendorf 5330 Mastercycler. The lid was heated to 106 °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 :

Denature	94 °C; 15 sec
Anneal	45-65 °C; 30 sec
Extension	72 °C; t min (t = 45 sec kb^{-1})

Programme B :

Denature	94 °C; 15 sec
Anneal	45-65 °C; 30 sec
Extension	72 °C; t min
	(t = (45 sec kb^{-1}) + 5 sec per cycle)

Once all the cycles were complete a final step of 72 °C for 7 min was added to allow complete extension of the primers. The PCR products were stored at -20 °C.

2.13.1.2.3 Expand™ Long Template PCR system

DNA amplifications of >3 kb, that required 3'-5' proof-reading activity were performed with the Expand™ Long Template PCR system (Roche). This system is composed of a unique enzyme mix containing the thermostable Taq and Pwo polymerases, and is optimised for amplifying fragments of up to 27 kb in length.

The following Master mixes were made on ice:

Master mix 1:

Template DNA	10 - 500 ng
dNTPs	350 µM*
Forward primer	300 nM*
Reverse primer	300 nM*
dH_2O	to 25 µl

Master mix 2:

10X PCR buffer with 1.75 mM $MgCl_2$	5 µl
Expand™ polymerase	0.75 µl
dH_2O	to 25 µl

*final concentration

Master mix 1 and 2 were combined on ice in a 0.5 ml thin-walled PCR tube and cycling immediately commenced.

PCR amplification was carried out using an Eppendorf 5330 Mastercycler. The lid was heated to 106 °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 :

Denature	94 °C; 10 sec
Anneal	65 °C; 30 sec
Extension	68 °C; t min (t = 40 sec kb^{-1})

Programme B:

Denature	94 °C; 10 sec
Anneal	50-55 °C; 30 sec
Extension	68 °C; t min (t = (40 sec kb^{-1}) + 20 sec per cycle)

Once all 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.

2.13.1.2.4 Direct genomic DNA sequencing

Short (up to 700 bp) single read sequencing reactions were carried out on DNA flanking transposon insertions (Chapter 2.15) using the ABI Prism® BigDye™ Terminator Cycle Sequencing Ready Reaction kit (version 2.0) (Perkin-Elmer). Genomic DNA was prepared and concentrated as in Chapter 2.11.1, and sheared by vigorous vortexing to aid binding of the primer to the target sequence. The following were combined in a 0.5 ml thin-walled PCR tube on ice:

Genomic DNA	2.5 µg
Primer Tn2	3.2 pM (final concentration)
BigDye™ sequencing premix	16 µl
dH_2O	to 40 µl

PCR amplification was carried out using an Eppendorf 5330 Mastercycler. The lid was heated to 106 °C for the duration of the PCR amplification, and the block was pre-heated to 95 °C. Once the tubes were added the DNA was denatured by heating at 95 °C for 5 min before the following thermal cycling programme of 99 cycles was initiated:

Denature	95 °C for 30 sec
Anneal	55 °C for 20 sec
Extend	60 °C for 4 min

The PCR sequencing products were cleaned (to remove unincorporated nucleotides and primers) using a DyeEX™ Spin kit (QIAGEN) (Chapter 2.11.7). The samples were then dried in a heat-block at 55 °C for 3 h and sequenced using an ABI 377 sequencer by Dr Arthur Moir (Department of Molecular Biology and Biotechnology, University of Sheffield) and by MWG-BIOTECH.

2.13.1.2.5 PCR screening of *E. coli* cells

Putative *E. coli* transformants were patched using sterile toothpicks onto LB agar plates containing appropriate antibiotics and grown overnight at 37 °C. A small amount of each patched positive clone was transferred to a PCR tube containing the PCR mix as in Chapter 2.13.1.2.1 (minus the DNA). The PCR programme followed was as in Chapter 2.13.1.2.1 with the tubes heated to 95 °C initially for 10 min to ensure lysis of the cells and release of DNA. The PCR products were visualised by agarose gel electrophoresis (Chapter 2.13.5) and putative positive clones were chosen for further screening using small scale plasmid preparations (Chapter 2.11.2) and restriction digestions (Chapter 2.13.2).

2.13.2 DNA restriction

DNA restriction digests were performed in volumes from 20 - 100 µl. They were prepared as follows:

Restriction enzyme buffer	10 % (v/v)
Restriction enzyme(s)	up to 10 % (v/v)
DNA	up to 80 % (v/v)

Where appropriate the reaction volume was made up with dH_2O. The restriction digests were incubated at 37 °C for between 2 h and 16 h. Before digested DNA was used for further manipulation, it was purified using a QIAGEN PCR purification kit (Chapter 2.11.6) or separated using agarose gel electrophoresis (Chapter 2.13.5) and purified using a QIAquick gel extraction kit (Chapter 2.11.5).

2.13.3 Alkaline phosphatase treatment of restriction-digested plasmids

The 5' ends of plasmids digested with only one restriction enzyme were dephosphorylated using Calf Intestinal Alkaline Phosphatase (CIAP) (Promega) to reduce vector re-ligation in subsequent cloning steps. The following were added on ice:

Digested plasmid DNA	up to 5 µg
10X reaction buffer (Promega)	10 µl
CIAP	0.01 U per pmol of ends*
dH_2O	to 100 µl

*the number of picomoles of ends for a given sample of digested plasmid was calculated using the following equation;

(µg DNA / kb size of DNA) X 3.04 = pmol of ends

The mixture was incubated for 30 mins at 37 °C. A second aliquot of CIAP (0.01 U per pmol of ends) was added and the sample was incubated for an additional 30 mins at 37 °C. To stop the reaction, 2 µl of 0.5 M EDTA was added and the mixture incubated at 65 °C for 20 min. The DNA was purified and concentrated using a QIAquick PCR purification kit (Chapter 2.11.6) prior to use in a ligation reaction.

2.13.4 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;

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

The reaction was made up to 20 µl with dH_2O and incubated overnight at 14 °C. The completed ligation mix was then used to transform *E. coli* (Chapter 2.10.1).

2.13.5 Agarose gel electrophoresis

DNA fragments were separated by horizontal gel electrophoresis using various size electrophoresis tanks (Life Technologies). Appropriate volumes of agarose gel (0.8 - 4 % (w/v) dissolved in TAE by microwaving) were submerged in suitable volumes of TAE electrophoresis buffer. The gel contained 0.2 µg ml^{-1} ethidium bromide. DNA samples were mixed with $1/5^{th}$ their volume of 6X DNA loading buffer and loaded into

wells in the gel. The gel was run at 80 - 120 V for 1 - 2 h and visualised by means of a UV transilluminator at 260 nm. To estimate the sizes of DNA fragments, DNA markers were also loaded into the gel. These included pUC mix markers (MBI Fermentas), Generuler^{TM} 1 kb DNA ladder (MBI Fermentas), or λ genomic DNA double-digested with *Hin*dIII/*Eco*RI (MBI Fermentas) (Table 2.6).

2.13.5.1 Agarose gel photography

A permanent record of agarose gels was obtained by photographing the ethidium bromide-stained gels illuminated from below with UV light at 260 nm. A Kodak 203 red-orange filter, and Polaroid 667 (ASA 3000) film were used. In addition, photographs were scanned using a UMAX Powerlook 1100 for a permanent electronic record.

2.13.6 DNA cloning

2.13.6.1 Construction of a tetracycline-resistant *hla* cassette knock-out

2.13.6.1.1 Attempt to clone a 2.9 kb *hla* insert into pMUTIN4 directly

A 2.9 kb DNA fragment containing the whole 1 kb *hla* gene and approximately 1 kb of flanking DNA was PCR-amplified from 8325-4 genomic DNA using the Expand™ Long Template PCR system (Chapter 2.13.1.2.3). The primers, JA-5 and JA-6, introduced unique terminal *Hin*dIII and *Bam*HI restriction sites respectively. The PCR product was gel-extracted (Chapter 2.11.5), and both the PCR product and the vector (pMUTIN4) were cut with *Hin*dIII overnight (Chapter 2.13.2). Following PCR purification (Chapter 2.11.6), the PCR product and pMUTIN4 were cut with *Bam*HI overnight (Chapter 2.13.2). The two fragments were run on a 0.8 % (w/v) agarose gel (Chapter 2.13.5) to ensure they were of the correct size. The insert fragment and cut vector were then gel-extracted (Chapter 2.11.5), ligated (Chapter 2.13.4) and transformed into *E. coli* DH5α (Chapter 2.10.1). Transformants were selected on LB containing 50 μg ml^{-1} Amp.

DNA size markers (bp)		
pUC mix	1 kb DNA ladder	λ *Hin*dIII/*Eco*RI
1116	10,000	21,226
883	8,000	5,148
692	6,000	4,973
501	5,000	4,268
489	4,000	3,530
404	3,500	2,027
331	3,000*	1,904
242	2,500	1,584
190	2,000	1,375
147	1,500	947
111	1,000	831
110	750	564
67	500	
34	250	
26		
19		

Table 2.6

The size of DNA fragments used as size markers for agarose gel electrophoresis.

*The 3,000 bp band of 1kb generuler contains ~ 147 ng DNA per 10 µl marker.

2.13.6.1.2 Attempt to clone a 2.9 kb *hla* insert into pMUTIN4, with an intermediate stage of cloning the *hla* insert into pCR-II-TOPO

To ensure that both enzymes cut the vector DNA fully, plasmid pMAL23 (pMUTIN4 containing a *Bam*HI / *Hin*dIII insert) was cut with *Hin*dIII and *Bam*HI overnight (Chapter 2.13.2). The digest was run on a 0.8 % (w/v) agarose gel (Chapter 2.13.5) and the vector fragment was gel-extracted (Chapter 2.11.5).

A 2.9 kb *hla* insert was prepared for cloning into the vector pCR-II-TOPO (Invitrogen) by PCR amplification, as described in Chapter 2.13.6.1.1. A sample of the PCR product was run on a 1 % (w/v) agarose gel (Chapter 2.13.5) and found to have no background banding. The PCR product was therefore used directly in the TOPO cloning reaction without purification, as gel extraction can interfere with the process. The TOPO TA cloning protocol was performed, according to the manufacturer's instructions;

The following were added at room temperature;

PCR product (*hla* insert)	0.5 - 4 µl
Linearised pCR-II-TOPO	1 µl
Salt solution	1 µl
Sterile water	To 5 µl

The reaction was mixed gently and incubated at room temperature for 5 min. 2 µl of the cloning reaction was then added to a vial of TOP10 One Shot™ chemically competent *E. coli* cells (Invitrogen) on ice and mixed gently. Following incubation on ice for 30 min, the reaction was heat-shocked at 42 °C for 30 sec. The tube was then returned back to the ice for 2 min. Following addition of 250 µl of room temperature SOC, the tube was shaken at 37 °C for 1 h. 10 – 50 µl from of the transformation mix was plated onto LB agar plates containing X-gal (Chapter 2.16.2) and 50 µg ml^{-1} Amp and incubated overnight at 37 °C.

Hundreds of transformants were produced, and 5 colonies were tested. Since pCR-II-TOPO has blue / white selection capabilities, all of the colonies selected for analysis were white. Plasmid DNA was prepared by small scale plasmid preparation (Chapter

2.11.2) using LB containing 50 μg ml^{-1} Amp. Plasmid DNA was cut with *Hin*dIII and *Bam*HI together, or with *Kpn*I for 3 h (Chapter 2.13.2), and run on a 1 % (w/v) agarose gel (Chapter 2.13.5) to identify positive clones.

Using plasmid DNA prepared from a positive clone, the *hla* insert was cut from the pCR-II-TOPO backbone by cutting overnight with *Hin*dIII and *Bam*HI together (Chapter 2.13.2). The digest was run on a 0.8 % (w/v) agarose gel, and the *hla* insert was gel extracted (Chapter 2.11.5). The cut *hla* insert and pMUTIN4 vector backbone cut from pMAL23 were then ligated (Chapter 2.13.4) and transformed into *E. coli* DH5α (Chapter 2.10.1). Transformants were selected on LB containing 50 μg ml^{-1} Amp.

2.13.6.1.3 Attempt to clone a counter-selectable erythromycin-resistance cassette into pJA3

Plasmids pDG646 and pJA3 were cut with *Hin*dIII overnight (Chapter 2.13.2). Digests were separated on a 0.8 % (w/v) agarose gel (Chapter 2.13.5). The 1.6 kb erythromycin resistance cassette (from pDG646) and the 6.9 kb pJA3 band were gel extracted (Chapter 2.11.5). *Hin*dIII-cut pJA3 was alkaline-phosphatase treated to prevent vector recircularisation (Chapter 2.13.3). The vector and insert fragments were then ligated (Chapter 2.13.4) and transformed into *E. coli* (Chapter 2.10.1). Transformants were selected on LB containing 50 μg ml^{-1} Amp, and then replica plated onto LB containing 300 μg ml^{-1} Ery.

2.13.6.2 Cloning of a tetracycline-resistance cassette into pMOD(MCS)

A 1.5 kb band comprising a tetracycline-resistance cassette was PCR amplified from pDG1513 using Pwo polymerase (Chapter 2.13.1.2.2). The primers, OL-32 and OL-33, both introduced flanking *Kpn*I restriction sites. The 1.5 kb tetracycline-resistance cassette band was gel extracted (Chapter 2.11.5) from a 0.8 % (w/v) agarose gel (Chapter 2.13.5). The PCR product and the 2.5 kb vector pMOD(MCS) were then cut with *Kpn*I overnight (Chapter 2.13.2), and the fragments resolved on a 0.8 % (w/v)

agarose gel (Chapter 2.13.5). The insert and vector were gel extracted (Chapter 2.11.5), ligated (Chapter 2.13.4) and transformed into *E. coli* DH5α (Chapter 2.10.1). Transformants were selected on LB containing 12.5 μg ml^{-1} Tet. Growth at 37 °C for 48 h produced a single tetracycline-resistant colony. The colony was streaked onto an LB plate containing 50 μg ml^{-1} Amp, and following growth at 37 °C overnight was found to be ampicillin resistant. Plasmid DNA was prepared by small scale plasmid preparation (Chapter 2.11.2) using LB containing 50 μg ml^{-1} Amp. Restriction mapping of the resulting plasmid DNA was then performed using *Kpn*I, *Bam*HI and *Sca*I (Chapter 2.13.2).

2.13.6.3 Construction of the *lacZ* reporter gene fusion vector pAISH1

Inverse PCR amplification of pMUTIN4 was performed using the Expand™ Long Template PCR system (Chapter 2.13.1.2.3) to amplify a 7.7 kb fragment containing the entire sequence of pMUTIN4, except for the erythromycin-resistance cassette. The primers used, JA-9 and JA-10 introduced *Bgl*II and *Xho*I sites respectively. A 2.1 kb fragment containing a tetracycline-resistance cassette was PCR amplified from pDG1513 using the Pwo polymerase protocol described in Chapter 2.13.1.2.2. The primers used, JA-7 and JA-8 introduced *Sal*I and *Bgl*II sites respectively. The PCR products were run on a 0.8 % (w/v) agarose gel (Chapter 2.13.5) and the 7.7 kb pMUTIN4 and 2.1 kb tetracycline-resistance cassette bands were gel extracted (Chapter 2.11.5). The pMUTIN4 fragment was cut with *Bgl*II and *Xho*I overnight, whilst the tetracycline-resistance cassette was cut with *Sal*I and *Bgl*II overnight (Chapter 2.13.2). The digests were resolved on a 0.8 % (w/v) agarose gel (Chapter 2.13.5), and the *Bgl*II / *Xho*I-cut pMUTIN4 and *Sal*I / *Bgl*II-cut tetracycline-resistance cassette bands were gel extracted (Chapter 2.11.5), ligated (Chapter 2.13.4) and transformed into *E. coli* DH5α (Chapter 2.10.1). Transformants were selected on LB containing 12.5 μg ml^{-1} Tet and grown at 37 °C for 48 h. Colonies were streaked onto LB plates containing 50 μg ml^{-1} Amp and grown at 37 °C overnight to ensure that they were ampicillin resistant. Plasmid DNA was prepared by small scale plasmid preparation (Chapter 2.11.2) using LB containing 50 μg ml^{-1} Amp. Restriction mapping of the resulting plasmid DNA was then preformed using *Hin*dIII, *Cla*I / *Nco*I and *Dra*I (Chapter 2.13.2).

2.13.6.4 Construction of an *hla::lacZ* reporter gene fusion

An *hla::lacZ* reporter gene fusion was constructed in pAISH1 using a 2.2 kb *Eco*RI / *Not*I-flanked fragment (containing the 5' region of the *hla* gene and upstream regulatory sequences) from the plasmid pPF4. Plasmids pPF4 and pAISH1 were cut overnight with *Eco*RI (Chapter 2.13.2), and the digests passed through a PCR purification column (Chapter 2.11.6). The *Eco*RI-cut pPF4 and pAISH1 were then cut overnight with *Not*I. The restriction products were resolved on a 0.8 % (w/v) agarose gel, and the 2.2 kb *Eco*RI / *Not*I-cut *hla* promoter insert band (from pPF4) and the 9.8 kb *Eco*RI / *Not*I-cut pAISH1 vector band were gel extracted (Chapter 2.11.5), ligated (Chapter 2.13.4) and transformed into *E. coli* DH5α (Chapter 2.10.1). Transformants were selected on LB containing 50 μg ml^{-1} Amp and grown at 37 °C overnight. Colonies were streaked onto LB plates containing 12.5 μg ml^{-1} Tet and grown at 37 °C overnight to ensure that they were tetracycline resistant. Plasmid DNA was prepared by small scale plasmid preparation (Chapter 2.11.2) using LB containing 50 μg ml^{-1} Amp. Restriction mapping of the resulting plasmid DNA was then preformed using *Eco*RI / *Not*I and *Kpn*I (Chapter 2.13.2).

2.14 DNA Hybridisation techniques

2.14.1 Labelling of DNA probes with digoxigenin

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, followed by purification using a QIAquick PCR purification kit (Chapter 2.11.6). The purified probe was then quantified (Chapter 2.14.2).

2.14.2 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 2.14.4) and DIG-labelled DNA was then detected immunologically, using AP-linked anti-dioxygenin antibody (Chapter 2.14.6). The spot intensities of the control and probe dilutions were compared visually to estimate the concentration of the probe.

2.14.3 Southern blotting

Agarose gel electrophoresis of ~ 1 µg samples of digested genomic DNA was performed and photographed as described in Chapters 2.13.5 and 2.13.5.1 with a 0.8 % (w/v) agarose gel. DIG-labelled λ *Hin*dIII / *Eco*RI markers were loaded into the gel to estimate the size of DNA bands following development of the blot using AP-conjugated anti-DIG antibody. The gel was soaked in Southern depurination solution for 10 min and washed in dH_2O. The gel was soaked in Southern denaturation buffer twice for 15 min and rinsed in dH_2O. The gel was neutralised by soaking in Southern neutralisation buffer twice for 15 mins. The DNA was transferred from the gel to a Hybond-N+ Extra membrane by vacuum blotting at 60 mbar for 90 mins, using 10X SSC as the transfer buffer.

2.14.4 Fixing the DNA to the membrane

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

2.14.5 Prehybridisation and hybridisation

Membranes to be probed with the DIG-labelled DNA were prehybridised for 2 h at 68 °C in pre-hybridisation solution (20 ml per 100 cm^2 of membrane). Just prior to use, the labelled probe (Chapter 2.14.1) was denatured in a microfuge tube by placing the tube 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 for 5 min at room temperature. The membrane was then washed twice in 0.5X wash solution for 5 min at 68 °C.

2.14.6 Colorimetric detection of DIG-labelled DNA

The hybridised and washed membranes were equilibrated with washing buffer for 1 min and then blocked for 30 min with gentle rocking in blocking solution. The membrane was then transferred to antibody solution containing a 1:5,000 dilution of stock anti-DIG-AP antibody (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 before 10 ml colour substrate solution 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 or spots. After the colour had developed sufficiently, the membrane was washed in 1X TE for 5 min to stop the reaction. The membrane was then air-dried, scanned using a UMAX Powerlook 1100 and stored in the dark.

2.15 Transposon mutagenesis

2.15.1 Transposon mutagenesis of 8325-4 using EZ::TN(tet)

Transposon mutagenesis of *S. aureus* 8325-4 using the Tn*5*-based EZ::TN transposition system of Epicentre Technologies was performed using the following *in vitro* and *in vivo* methods.

2.15.1.1 *In vitro* transposon mutagenesis

Genomic DNA was prepared from *S. aureus* 8325-4 as described in Chapter 2.11.1. The DNA was sheared using a 23-gauge needle (Microlance) attached to a 5 ml syringe. The reaction components were then added in the following order;

EZ::TN 10 X reaction buffer	1 µl
Sheared 8325-4 genomic DNA	200 ng
EZ::TN(tet) transposon	1 - 20 ng
dH_20	To 10 µl
EZ::TN transposase	1 µl

The reaction components were then incubated at 37 °C for 2 h. The reaction was stopped by adding 1 µl of EZ::TN 10 X stop solution and heating 70 °C for 10 min. The reaction products were then electroporated into competent RN4220 cells (Chapter 2.10.2), with selection on BHI agar plates containing 5 µg ml^{-1} Tet. Plates were incubated at 37 °C for 48 h.

2.15.1.2 *In vivo* transposon mutagenesis

EZ::TN(tet) transposomes were produced by adding the following;

100 µg ml^{-1} EZ::TN(tet) transposon DNA	5 µl
EZ::TN transposase	10 µl
100% glycerol	5 µl
dH20	To 20 µl

The components were mixed by vortexing and incubated for 30 min at room temperature. 1 µl of the reaction mixture was then electroporated into *S. aureus* RN4220 (Chapter 2.10.2). Cells were plated onto BHI agar plates containing 5 µg ml^{-1} Tet, and incubated for up to 48 h at 37 °C.

2.15.2 Transposon mutagenesis of SH1000 *hla::lacZ* using Tn*551*

JLA514 (SH1000 *hla::lacZ hla*$^+$ (Tet^R), pRN3208) (see Figure 5.7 for diagram of pRN3208) was streaked onto a BHI agar plate containing 250 µg ml^{-1} $CdCl_2$ and 5 µg ml^{-1} Ery, and grown overnight at 30 °C. A single colony was used to inoculate 100 ml BHI broth containing 250 µg ml^{-1} $CdCl_2$ and 5 µg ml^{-1} Ery, and grown overnight at 30 °C in a Grant OLS 200 water-bath with linear shaking (equivalent to 250 rpm). 3 ml of culture was removed and the cells were harvested by centrifugation (10 min, 3000 rpm, Centaur 2) and resuspended in 100 ml BHI containing 5 µg ml^{-1} Ery (which had been pre-equilibrated to either 42 or 43 °C). The culture was then grown at the relevant non-permissive temperature (*ie.* either 42 or 43 °C) in a Grant OLS 200 water-bath with linear shaking (equivalent to 250 rpm) until an OD_{600} of 0.3 - 0.4 was reached. 3 ml of culture was transferred to a fresh, pre-equilibrated 100 ml batch of BHI containing 5 µg ml^{-1} Ery, and grown at the same non-permissive temperature as before (*ie.* either 42 or 43 °C) in a Grant OLS 200 water-bath with linear shaking (equivalent to 250 rpm) for 12 h. The cells were harvested by centrifugation (10 min, 8000 rpm, Centaur 2) and resuspended in 4 ml BHI containing glycerol at a final concentration of 10 % (w/v). 500 µl aliquots were transferred to 1.5 ml microfuge tubes, snap-frozen in liquid nitrogen and stored at -70 °C.

2.15.3 Transposon library evaluation and enumeration

The efficiency of Tn*917* insertion (see Chapter 4.2.1 for source of libraries) was determined by performing 10-fold serial dilutions of cells in PBS and plating 100 µl of the 10^3 - 10^7 dilutions onto BHI plates containing either Ery and Lin, or Ery, Lin and Tet (Chapter 2.6.2). Transposon insertion frequency was calculated by comparing the number of colonies on plates containing Ery and Lin (*ie.* cells containing Tn*917*, either

inserted into the chromosome, or carried by the plasmid pLTV1) with the number of colonies on plates containing Ery, Lin and Tet (*ie.* cells containing intact pLTV1). Tn*551* insertion into libraries produced in Chapter 2.15.2 was determined in the same way as for Tn*917* insertion, except that $CdCl_2$ was used to select for the presence of pRN3208. See Figures 4.1 and 5.7 for diagrams of pLTV1 and pRN3208 respectively.

2.15.4 Transposon mutant library screening

2.15.4.1 Screen to identify genes involved in the repression of Hla in the presence of 1 M NaCl

An aliquot of Tn*917* library X or Z (see Chapter 4.2.1) was suitably diluted in PBS to give ~ 100 colonies per BHI Ery Lin plate. After spreading with sterile glass beads, the plates were incubated overnight at 37 °C. The colonies were replica plated onto rabbit blood plates containing 1 M NaCl (Chapter 2.16.1) using sterile velvets and incubated overnight at 37 °C. Non-haemolytic colonies and colonies with a zone of haemolysis of increased diameter compared to the background colonies were patched onto a rabbit blood plate containing either 1 M NaCl or no added NaCl, with 8325-4 as a control, and grown overnight at 37 °C to ensure reproducibility and NaCl-specificity of the mutant phenotype. The mutants were also patched onto BHI plates containing Tet to ensure that pLTV1 had been cured from the cells. Mutations were transduced into 8325-4 using ϕ11-mediated transduction, with selection using Ery and Lin (Chapter 2.9). 10 colonies per mutation were patched onto rabbit blood plates containing either no added NaCl or 1 M NaCl with 8325-4 as a control and grown overnight at 37 °C. Linkage of the mutant phenotype to transposon insertion was determined by considering the percentage of the transductant colonies which demonstrated the same phenotype as the original mutant.

2.15.4.2 Screen to identify genes involved in regulating *hla::lacZ* transcription

An aliquot of Tn*551* library F1 or F4 (Chapter 2.15.2) was suitably diluted in PBS to give ~ 300 colonies per BHI agar plate (containing 600 mg l^{-1} X-Gal). After spreading

with sterile glass beads, the plates were incubated overnight at 37 °C. Colonies demonstrating increased blue colouration compared to the surrounding background colonies were isolated. The plates were incubated for a further night at 37 °C, and re-screened to identify mutants demonstrating less blue colouration than the background colonies. The putative mutants were patched onto BHI agar plates containing 600 mg l^{-1} X-Gal to ensure the reproducibility of their phenotypes. The mutants were also patched onto BHI plates containing $CdCl_2$ to ensure that pRN3208 had been cured from the cells. Mutations were transduced into JLA513 (SH1000 *hla::lacZ* (Tet^R)) using ϕ11-mediated transduction, with selection using Ery and Lin (Chapter 2.9). 10 colonies per mutation were patched onto BHI agar plates containing 600 mg l^{-1} X-Gal with JLA513 (SH1000 *hla::lacZ* (Tet^R)) as a control, and grown overnight at 37 °C. Linkage of the mutant phenotype to transposon insertion was determined as in Chapter 2.15.4.1.

2.15.4.3 Screen to identify genes involved in regulating *hla::lacZ* transcription in the presence of 1 M NaCl

An aliquot of Tn*551* library F1 or F4 (Chapter 2.15.2) was suitably diluted in PBS to give ~ 100 colonies per BHI Ery Lin plate. After spreading with sterile glass beads, the plates were incubated overnight at 37 °C. The colonies were replica plated onto BHI agar plates (containing 1 M NaCl and 2.4 g l^{-1} X-Gal) using sterile velvets and incubated overnight at 37 °C. Colonies demonstrating increased blue colouration compared to the surrounding background colonies were isolated. The plates were incubated for a further night at 37 °C, and re-screened to identify mutants demonstrating less blue colouration than the background colonies. The putative mutants were patched onto either BHI agar plates (containing 1 M NaCl and 2.4 g l^{-1} X-Gal) or onto BHI agar plates (containing no added NaCl and 600 mg l^{-1} X-Gal) to determine the reproducibility and NaCl-specificity of their phenotypes. The mutants were also patched onto BHI plates containing $CdCl_2$ to ensure that pRN3208 had been cured from the cells. Mutations were transduced into JLA513 (SH1000 *hla::lacZ* (Tet^R)) as in Chapter 2.15.4.2 and linkage of the mutant phenotype to transposon insertion was determined as in Chapter 2.15.4.1 by plating onto BHI agar plates (containing 1 M NaCl and 2.4 g l^{-1} X-Gal).

2.16 Qualitative analysis of virulence determinant expression on agar plates

2.16.1 Analysis of α-haemolysin activity on rabbit blood plates

Rabbit blood agar plates were prepared as described in Chapter 2.1.1.7. *S. aureus* strains were inoculated by patching colonies onto the test plate. After overnight incubation at 37 °C, α-haemolysin activity was visualised by a zone of clearing (lysis) around the patch of bacteria.

2.16.2 Analysis of *lacZ* fusion expression on X-Gal plates

β-galactosidase activity was detected directly on solid media using the substrate X-Gal. X-Gal is hydrolysed by β-galactosidase resulting in β-D-galactopyranoside and 5-bromo-4-chloro-3-indolyl. The latter has a blue colouration and is thus a visible indicator of β-galactosidase activity. Unless otherwise stated, 100 µl 20 mg ml^{-1} X-Gal solution was pipetted onto agar plates and spread across the surface with sterile beads. The beads were then removed and the plates were dried at 50 °C for 20 min. Plates were used immediately or stored at -4 °C for up to one week.

2.16.3 Analysis of protease activity on milk plates

Milk agar plates were prepared as described in Chapter 2.1.1.5. *S. aureus* strains were inoculated by patching colonies onto the test plate. After overnight incubation at 37 °C, protease activity was visualised by a zone of clearing (proteolysis) around the patch of bacteria.

2.16.4 Plate photography

Agar plates were photographed using a Nikon Coolpix 775 digital camera. Software used was Nikon View 4.

2.17 Sequence and database analysis

2.17.1 Sequence analysis for cloning

DNA sequences for cloning were obtained from the National Centre for Biotechnology Information (NCBI) database (http://ncbi.nlm.nih.gov/), or from manufacturer's instructions (*ie.* supplied by Invitrogen and Epicentre for use of PCR-II-TOPO and pMOD(MCS) respectively). Putative open reading frames (ORFs) and restriction sites were identified in DNA sequences using the Gene Jockey II program (Biosoft) or the program Visual Cloning 2000 (Redasoft). Diagrammatic representations of DNA fragments and plasmids were prepared using Visual Cloning 2000 (Redasoft).

2.17.2 Sequence and database analysis for the identification of genes inactivated by transposon mutagenesis

Automated sequence data was edited and analysed using the Applied Biosystems 373A DNA sequencer data analysis program, and the sequence corresponding to the end of the transposon removed. The sequence was entered into a BLASTN search in the database of The Institute of Genomic Research (TIGR) (http://tigr.org/tigr-scripts/CMR2/CMRHomePage.spl) against the *S. aureus* COL genome sequence to identify the position of transposon insertion (NB; the COL genome sequence was searched because the 8325 genome has not yet been annotated). The putative functions of the inactivated genes and their flanking genes were identified by entering their amino acid sequence from the TIGR website into a BLASTP search against the sequences of all other published genomes in the NCBI BLAST (http://ncbi.nlm.nih.gov/BLAST) and/or TIGR website to identify putative homologues. The function of proteins for which homologues were identified in *B. subtilis* was further investigated using the SubtiList World-Wide Web Server v3.1 (http://genolist.pasteur.fr/SubtiList/). Genes were assigned accession numbers based on those of the COL database. The sequence of the genes inactivated by transposon mutagenesis and of flanking DNA containing surrounding genes of interest was extracted from the TIGR website and entered into a BLASTN search at the NCBI BLAST website in the *S. aureus* NCTC 8325 unfinished genome database to ensure that the sequence and position of the surrounding genes in COL is the same as that in

8325. This sequence was also inserted into the program Visual Cloning 2000 to allow a diagrammatic representation of the region and of the transposon insertion site to be made.

2.18 Protein analysis techniques

2.18.1 Growth of cultures for protein production

S. aureus was grown in 5 ml BHI in a sterile 30 ml universal tube at 37 °C with rotary shaking (250 rpm) until an OD_{600} of 1.0 was reached. The culture was used to inoculate 100 ml BHI in a 250 ml conical flask to an OD_{600} of 0.01. The second culture was grown at 37 °C in a Grant OLS 200 waterbath with linear shaking (equivalent to 250 rpm) until in stationary phase (14 h), unless otherwise stated.

2.18.2 Quantification of α-haemolysin activity in liquid assays

S. aureus was grown as in Chapter 2.18.1. Duplicate OD_{600} measurements were taken and, unless otherwise stated, 600 µl of culture was removed to a clean 1.5 ml microfuge tube and centrifuged (5,500 **g**, 1 min, 4°C). 500 µl of supernatant was transferred to a clean microfuge tube. 5 µl of PMSF was added to the supernatant and 100 µl aliquots were snap-frozen in liquid nitrogen and stored at -70°C.

Supernatant samples were defrosted at room temperature and placed on ice. Where stated, samples were concentrated and desalted using a YM-10 Centricon centrifugal filter device (Amicon Bioseparations) according to manufacturer's instructions. 100 µl supernatant was added to 900 µl haemolysin salts solution. 25 µl sterile defibrinated rabbit blood (E & O laboratories) was added and the sample mixed by inversion. Following incubation at 37 °C for 15 mins, the sample was centrifuged (5,500 **g**, 1 min, room temperature). The supernatant was transferred to a 1.5 ml plastic cuvette, and OD at 543 nm was measured (1 ml HA salts solution and 25 µl rabbit blood were incubated and centrifuged as above to create a blank). If the OD_{543} was not between

0.2 and 0.9 on the Jenway 6100 spectrophotometer, the assay was reperformed with less than 100 µl supernatant.

One haemolysin unit was defined as the amount which caused an increase in OD_{543} of 0.5 per minute due to erythrocyte lysis per OD_{600} unit of original culture, and was calculated using the equation shown below;

Haemolysin units (HU) = $(OD_{543} \times 1000) / (V \times T \times OD_{600} \times C \times 0.5)$

V = Volume of supernatant (ml)

T = Duration of incubation at 37 °C (min) (*ie.* 15)

C = Degree of concentration of supernatant using a Centricon column

2.18.3 Preparation of exoprotein and total cellular protein samples from *S. aureus* cultures

S. aureus was grown as in Chapter 2.18.1. Samples were taken at normalised volumes, such that 5 OD_{600} units of culture were removed (ie. x ml of culture removed = 5 / OD_{600}). The samples were immediately centrifuged (11,000 g, 5 min) and the supernatant removed to a clean microfuge tube. Supernatants and cell pellets were snap-frozen in liquid nitrogen and stored at -70 °C for future use.

2.18.3.1 Exoproteins

Culture supernatants (Chapter 2.18.3) were defrosted at room temperature and placed on ice. 0.1 volumes of 100 % (w/v) trichloroacetic acid (TCA) was added to each culture supernatant and mixed by vortexing. The mixture was incubated on ice for 30 min before centrifugation (11,000 g, 5 min) to recover the precipitated proteins. The supernatant was discarded and 500 µl acetone was used to wash to the pellet. Following resuspension of the pellet, the sample was centrifuged (11,000 g, 5 min) and the supernatant discarded. Washing with acetone was repeated twice more to ensure full removal of TCA. The supernatant was again removed and the pellet air-dried. The pellet

was resuspended in 50 µl 1X Laemmli SDS-PAGE sample buffer and incubated at 100 °C for 3 min before being cooled at room temperature for 3 mins. The sample was then centrifuged (5,000 g, 3 min) before 10 µl was loaded onto an SDS-PAGE gel (Chapter 2.18.4). Samples were stored at -70 °C.

2.18.3.2 Total cellular proteins

Cell pellets (Chapter 2.18.3) were resuspended in 400 µl 1.5 mM Tris-HCl (pH 6.8) and mixed by vortexing. 2 µl lysostaphin (5 mg ml^{-1}) was added to the cell suspension and the mixture was incubated at 37°C for 30 min. 100 µl 5X SDS-PAGE sample buffer was added and the sample was incubated at 100 °C for 3 min before being cooled at room temperature for 3 min. The sample was then centrifuged (5,000 g, 3 min) before 10 µl was loaded onto and SDS-PAGE gel (Chapter 2.18.4). Samples were stored at -70 °C.

2.18.4 SDS-PAGE protein analysis

Protein samples prepared from *S. aureus* were visualized using the Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) system. The gels were cast using the BioRad Mini-Protean 3® cell system according to the manufacturer's instructions. 12 % (v/v) polyacrylamide gels were used for the resolving portion of the gel and 4 % (v/v) polyacrylamide gels were used for the stacking portion of the gel (Chapter 2.2.9.2).

Protein samples were applied to the wells in the gel using a pipette. Low molecular weight protein markers (Sigma) were loaded to enable determination of the approximate sizes of proteins in the samples. The gels were electrophoresed at 200 V for approximately 45 min, or until the blue dye front of the sample buffer was at the base of the gel plates.

The gels were carefully removed from the apparatus and transferred to 50 ml Coomassie stain for 30 min at room temperature, with gentle rocking. The Coomassie stain was

removed after this time and the gels were washed 3X 5 min with 50 ml of destain solution at room temperature, with gentle rocking. After the third wash the gels were left in 50 ml destain solution until the blue bands of the proteins were apparent and the gel background was colourless. The destain was discarded and the gels were washed twice (10 min) with dH_2O at room temperature with rocking. The dH_2O was removed and replaced with 20 ml Gel-Dry™ gel drying solution (Invitrogen) for 30 min. The gel was then removed from the solution and placed between two DryEase™ minicellophane (Invitrogen) sheets which had been pre-soaked in 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 a UMAX Powerlook 1100.

2.18.5 Western blotting

2.18.5.1 Western blotting involving detection with alkaline-phosphatase conjugated secondary antibodies

Western blotting was used to estimate the proportions of specific proteins in a preparation from *S. aureus*. Specific primary antibodies were used to probe samples fixed to polyvinylidene difluoride (PVDF) nylon membranes (BioRad), and detected using alkaline phosphatase (AP)-conjugated secondary antibodies.

Exoproteins were prepared from *S. aureus* cultures (Chapter 2.18.3.1) and run on SDS-PAGE gels (Chapter 2.18.4). As the gel was running, a gel-sized piece of BioRad Immun-Blot PVDF membrane was placed in 100 % (v/v) methanol for 1 - 3 seconds. The PVDF membrane was then transferred to dH_2O for 1 - 2 mins and to blotting buffer for 5 – 10 mins. Gel-sized pieces of Whatman 3 mm filter paper were also pre-equilibrated in blotting buffer for 10 mins. Transfer of the proteins to the PVDF membrane was carried out using an LKB Bromma 2117 Multiphor II electrophoresis unit. The apparatus was assembled according to the manufacturer's instructions, and electrophoresis was performed for 1 h at x mA (x mA = blot area in cm^2 X 1.6).

The apparatus was disassembled and the PVDF membrane was placed in a container with 100 ml blocking solution. Pig serum (20 % v/v) (Sigma) was included in the blocking solution where stated. The container was rocked gently overnight at 4 °C. The membrane was then washed with 50 ml Western washing solution at room temperature for 2X 5 mins with rocking. 100 ml primary antibody solution (Chapter 2.2.9.6.4) was added and the membrane was incubated for 2 h at room temperature with gentle rocking. The primary antibody was removed and the membrane washed once for 15 min and twice for 5 min with 50 ml Western washing solution at room temperature with rocking. The membrane was then placed in 100 ml specific secondary antibody solution for 1 h with rocking at room temperature. The secondary antibody (Chapter 2.2.9.6.5) was raised against antibodies from the animal in which the primary originated (*ie.* since the primary antibodies were raised in rabbits, the secondary was goat-anti-rabbit). In addition, the secondary antibody was conjugated with AP to allow colourmetric detection.

The secondary antibody was discarded and the membrane was washed once for 15 min and twice for 5 min with 50 ml washing solution at room temperature with gentle rocking. The membrane was then pre-equilibrated with AP buffer. The AP buffer was discarded and replaced with 10 ml colour substrate solution. The blot was then placed in the dark to allow the membrane to develop. The presence of proteins bound with primary antibodies was 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 a UMAX Powerlook 1100.

2.18.5.2 Western blotting using the Enhanced Chemiluminescence (ECL) system (Amersham Pharmacia)

The ECL Western blotting detection system was used to provide a highly sensitive non-radioactive method for the detection of specific proteins.

Total cellular protein samples were prepared from *S. aureus* cultures (Chapter 2.18.3.2) and run on SDS-PAGE gels (Chapter 2.18.4). The resolved proteins were then blotted

onto PVDF membranes, probed with primary antibodies, detected with secondary antibodies and washed as in Chapter 2.18.5.1. However, the secondary antibodies used in the ECL system were horseradish peroxidase (HRP)-conjugated donkey-anti-rabbit antibodies (Chapter 2.2.9.6.5). Detection was performed according to the manufacturer's instructions, using a 5 min exposure on Kodak MXB autoradiography film. Densitometric analysis of autoradiographs was performed by using ImageMaster 3.01 software (Amersham-Parmacia).

2.18.6 N-Terminal Sequencing

Proteins under investigation were run on a 12 % (w/v) SDS-PAGE gel (Chapter 2.18.4) and blotted onto a PVDF membrane (Chapter 2.18.5) as previously described. After the transfer procedure the membrane was soaked in dH_20 for 5 min, followed by 5 min soaking in methanol at room temperature. The membrane was then stained with coomassie blue staining solution for 1 min and then destained for 3x 5 min in destain solution. The blot was then dried protein side up, on blotting paper for 1h at room temperature. The protein under investigation was then carefully removed from the membrane and sequenced by Dr Arthur Moir (Department of Molecular Biology and Biotechnology, Sheffield University) using an ABI 476A sequencer.

2.19 Murine subcutaneous lesion pathogenicity model

A single colony of *S. aureus* cells was used to inoculate 100 ml BHI in a 250 ml conical flask and grown at 37 °C with rotary shaking (250 rpm) in a waterbath. Cells were grown to stationary phase (15 h) and 50 ml of culture was harvested by centrifugation (4,000 rpm (Centaur 2), 10 min, 4 °C). The supernatant was discarded and the cells were resuspended in 50 ml PBS. The centrifugation and resuspension procedure was repeated and the cell suspension was adjusted to ~ 5 X 10^8 cfu ml^{-1}. Female 6- to 8-week old BALB/c mice were inoculated subcutaneously with 200 µl cell suspension (~ 1 X 10^8 cfu). After 7 days, the mice were euthanized with CO_2. Skin lesions were aseptically removed, snap-frozen in liquid nitrogen and stored at -70 °C. The lesions were weighed, chopped, and homogenised in a mini-blender in 2.5 ml of PBS for 2 min.

After incubation on ice for 1 hr, the lesions were homogenised for a further 2 min. The total number of bacteria recovered from the lesions was determined by direct cell counts (Chapter 2.6.2) by plating out 200 µl of 10^{-1} to 10^{-6} dilutions on BHI agar. The statistical significance of the recovery of strains compared to SH1000 was evaluated using the Student *t* test.

Handling, care and sacrificing of the mice, and the removal of lesions, was performed by Professor Eileen Ingham (Department of Biological Sciences, University of Leeds).

CHAPTER 3

ANALYSIS OF THE EFFECTS OF NaCl ON VIRULENCE DETERMINANT EXPRESSION

3.1 Introduction

The fact that *S. aureus* frequently encounters niches containing high concentrations of NaCl is reflected by its ability to grow in the presence of up to 3.5 M NaCl (Scott, 1953; Armstrong-Buisseret *et al.*, 1995). NaCl has been shown to have major effects on virulence determinant expression in *S. aureus*. When tested at a concentration of 1 M or higher, NaCl repressed the expression of *spa, hla, tst, eta, sec* and Seb, whilst transcription of *sspA* was upregulated (Chapter 1.6.2.3.2; Chan & Foster, 1998a; Chan & Foster, 1998b; Lindsay & Foster; 1999, Ohlsen *et al.*, 1997; Sheehan *et al.*, 1992; Regassa & Betley, 1993; Genigeorgis & Sadler, 1966). These virulence determinant genes are also regulated by *agr* and/or *sarA*. However, the effects of NaCl on *hla, tst, sec, sspA* and *spa* have been shown to occur independently of *agr* and/or *sarA,* and 1 M NaCl has no significant effect on RNAIII or *sarA* expression (Chapter 1.6.2.3.2; Chan & Foster, 1998a; Chan & Foster, 1998b; Lindsay & Foster; 1999, Regassa & Betley, 1993). As described in Chapter 1.6.2.3.2, this has led to the suggestion that NaCl-mediated control of virulence determinant transcription may involve a novel regulatory system (Chan and Foster, 1998a).

The alternative transcription factor σ^B regulates the general stress response in *B. subtilis* and a number of other low-GC Gram-positive bacteria (Chapters 1.6.1.2 and 1.6.2.2.8). In *S. aureus,* σ^B has been shown to control the response to a number of environmental stresses (Chapter 1.6.2.2.8; Kullik *et al.*, 1998; Chan *et al.*, 1998; Gertz *et al.*, 1999; Palma & Cheung, 2001; Kullik & Giachino, 1997). However, although σ^B has an important role in the NaCl-mediated stress response of *B. subtilis* (Voelker *et al.*, 1995; Bernhardt *et al.*, 1997), σ^B was not shown to be involved in resistance to osmotic shock in *S. aureus* by Chan *et al.* (1998).

Determining the exact role of σ^B in *S. aureus* has been impeded, however, by the presence of an 11 bp deletion in the *rsbU* gene of the genetic lineage used most

frequently for molecular and physiological analysis of *S. aureus*, 8325-4 (Kullik & Giachino, 1997). Since *rsbU* is a positive regulator of σ^B function (Chapter 1.6.2.2.8), the *rsbU* mutation produces a strong defect in σ^B activity (Giachino *et al.*, 2001). Horsburgh *et al.* (2002) constructed an *rsbU*$^+$ derivative of 8325-4, termed SH1000, by introducing intact *rsbU* from Newman into 8325-4 using a method that does not leave behind an antibiotic resistance marker. Unlike 8325-4, the phenotypic properties of SH1000 resemble those of clinical isolates, in that it is a low producer of Hla and SspA, and produces yellow colonies overnight due to pigmentation with staphyloxanthin (Horsburgh *et al.*, 2002). Therefore, whereas 8325-4 was used as the wild-type strain of choice for the initial stages of this study, use of the recently available SH1000 was later favoured to allow a more representative impression of NaCl-mediated virulence determinant regulation in *S. aureus* and the role of σ^B to be obtained.

The aims of this chapter were to analyse the effects of NaCl on virulence determinant expression and to further evaluate the role of known regulators in the NaCl-mediated stress response.

3.2 Results

3.2.1 The effect of NaCl on *spa*, *hla* and *agr*

The effects of NaCl on the representative virulence determinants *hla* and *spa*, and on the virulence regulator *agr*, were investigated to verify the published data (Chapter 1.6.2.3.2). In their study into the effects of NaCl on *hla*, *spa*, *tst* and *sarA* transcription, Chan and Foster (1998a) found that BHI medium gave the highest toxin expression and the highest growth yield of a wide range of media, and so the following growth and reporter gene fusion experiments were performed in BHI. Furthermore, Chan and Foster (1998a) found that an NaCl concentration of 1 M was the maximum which did not significantly affect growth yield, and so this concentration was chosen for the following investigations of the NaCl-mediated stress response of *S. aureus* (unless otherwise stated).

Strains PC203 (8325-4 *spa*::*lacZ*), PC322 (8325-4 *hla*::*lacZ*) and SH101F7 (8325-4 *agr* [RNAIII]::*lacZ*) were grown, as described in Chapter 2.7, in BHI containing no added NaCl (85.6 mM NaCl) and in BHI containing NaCl up to 1 M (as stated in Figure 3.1). Growth was measured spectrophotometrically as OD_{600} (Chapter 2.6.1), and *lacZ* fusion expression was determined using MUG, as in Chapter 2.8.

The presence of 1 M NaCl was found to repress *spa* transcription by approximately 7-fold at t = 1 h (*ie.* 195 MUG units in BHI containing no added NaCl compared to 28 MUG units in BHI containing 1 M NaCl) (Figure 3.1a). NaCl was also found to repress *hla*; as the concentration of NaCl was increased, transcription of *hla* decreased accordingly (Figure 3.1b). At t = 5 h, *hla* transcription was repressed (from 57,833 MUG units in BHI containing no added NaCl) by 1.4-fold in BHI containing 200 mM NaCl (*ie.* to 40,724 MUG units), 3-fold in BHI containing 400 mM NaCl (*ie.* to 19,789 units), 14-fold in BHI containing 900 mM NaCl (*ie.* to 4,040 MUG units), and was almost negligible in BHI containing 1 M NaCl (*ie.* 156 MUG units). However, transcription of *agr* (RNA III) remained relatively unaffected by the presence of 1 M NaCl, with an average 1.5-fold repression occurring throughout growth (Figure 3.1c). Cultures grown in BHI containing 1 M NaCl showed a minor reduction in growth rate and final OD_{600} compared to cultures grown in BHI alone (Figures 3.1a-c).

3.2.2 The role of σ^B in virulence determinant regulation and in the NaCl-mediated stress response

3.2.2.1 Construction of relevant *lacZ* fusion strains by transduction, and confirmation of *rsbU*$^+$ genotype of SH1000 *sigB* derivatives by PCR

In order to allow the role of σ^B in *S. aureus* to be investigated, a number of strains containing *lacZ* fusions were created by transduction. The *sarA*::*lacZ*, *agr* (RNA III)::*lacZ* and *hla*::*lacZ* fusions were transduced into SH1000 from PC161, SH101F7 and PC322 respectively using ϕ11. This produced the strains JLA311 (SH1000 *sarA*::*lacZ*), JLA341 (SH1000 *agr* (RNA III)::*lacZ*) and JLA371 (SH1000 *hla*::*lacZ*). The *sigB* mutation was then transduced from PC400 using ϕ11 into JLA311, JLA341 and JLA371. Putative transductants were patched onto BHI agar plates containing the

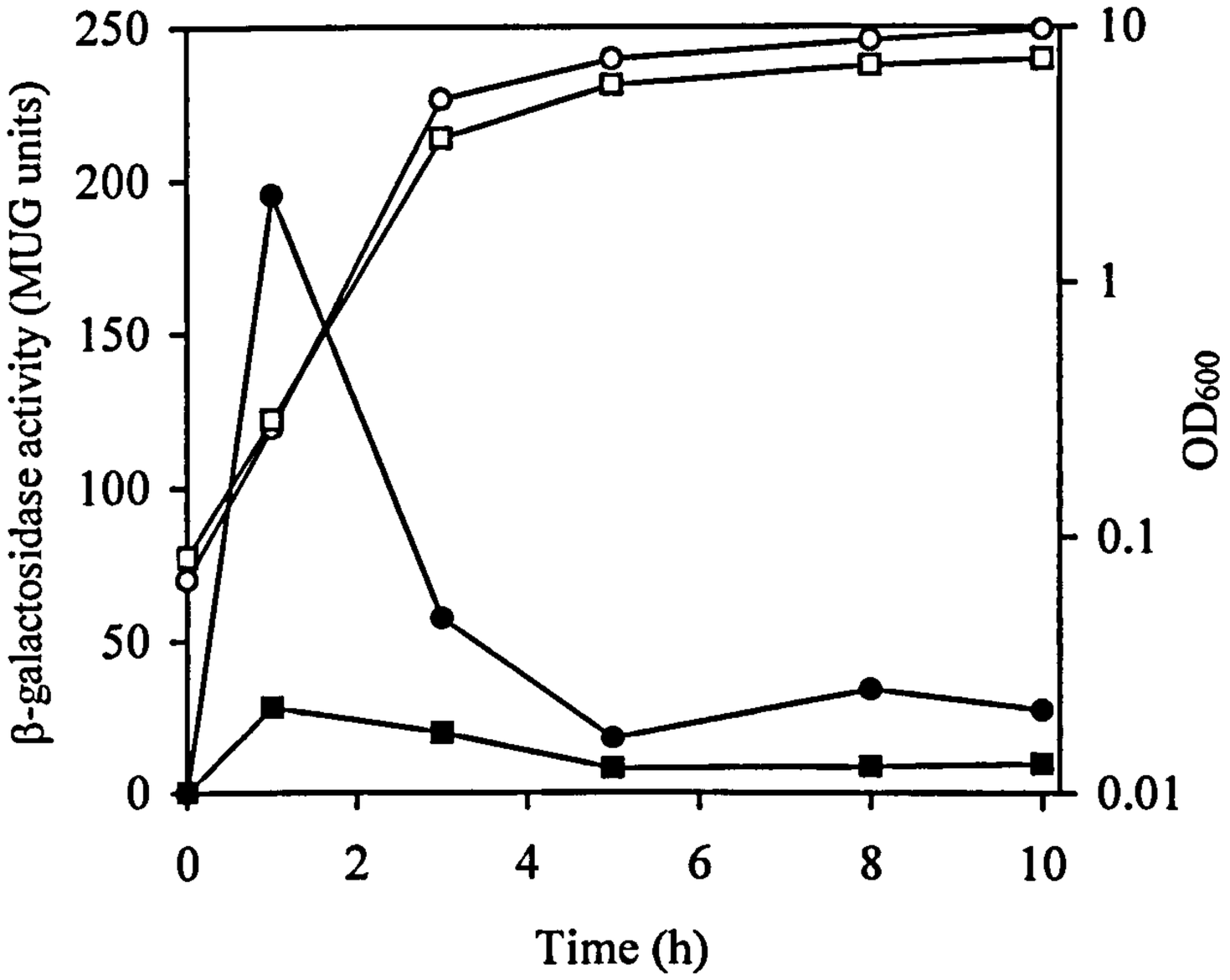


Figure 3.1a

Effect of 1 M NaCl on *spa::lacZ* transcription and growth in BHI.

S. aureus PC203 (8325-4 *spa*::*lacZ*) was grown in either BHI (○ and ●) or BHI containing 1 M NaCl (□ and ■) as described in Chapter 2.7. Growth was measured spectrophotometrically as OD_{600} (open symbols) (Chapter 2.6.1), and *lacZ* fusion expression was measured using MUG (closed symbols), as in Chapter 2.8. Results are representative of two independent experiments.

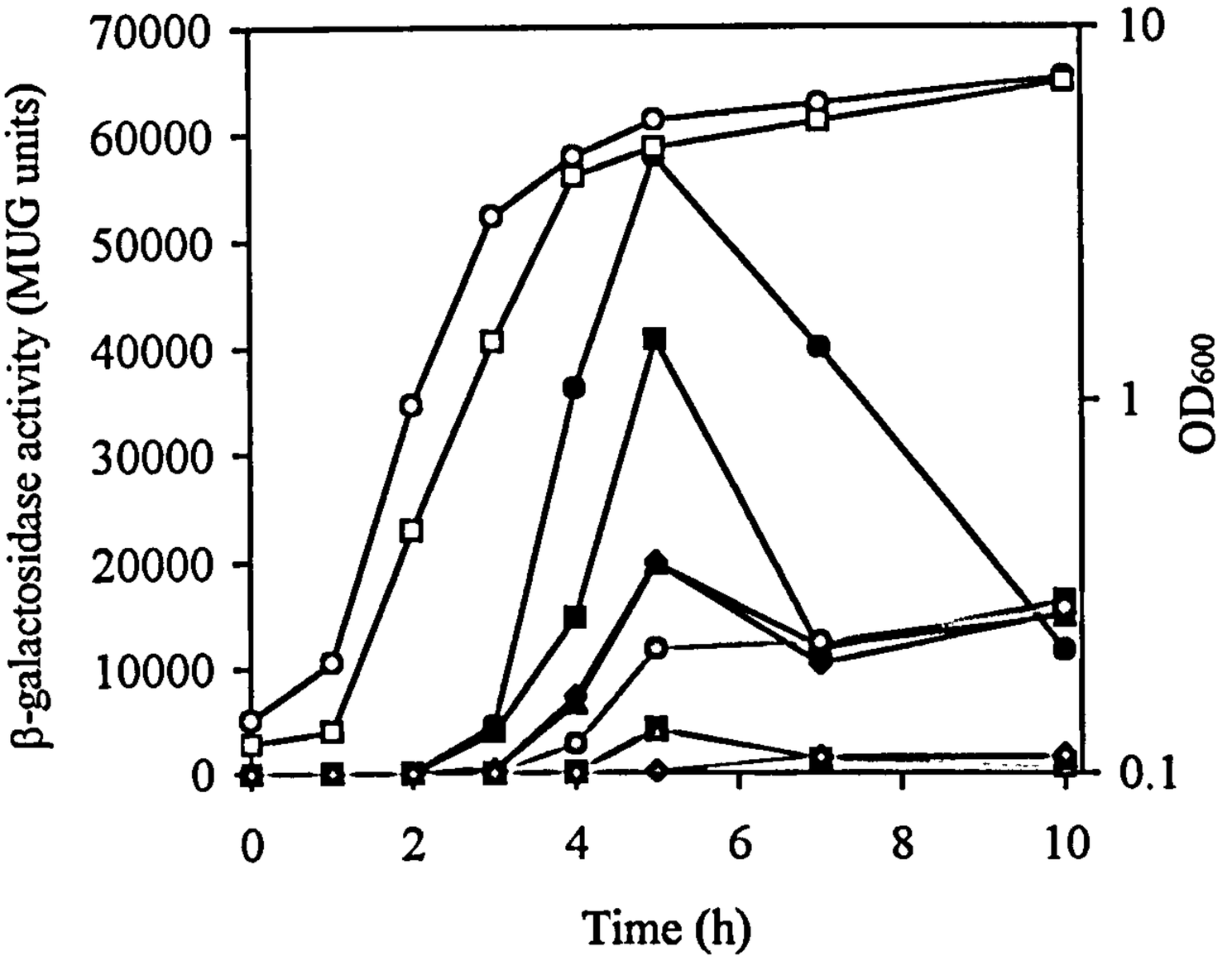


Figure 3.1b

Effect of a range of concentrations of NaCl on *hla::lacZ* transcription and growth in BHI.

S. aureus PC322 (8325-4 *hla*::*lacZ*) was grown in either BHI (○ and ●) or BHI containing NaCl at a final concentration of 200 mM (■), 400 mM (▲), 600 mM (◆), 700 mM (◍), 800 mM (▣), 900 mM (◬) or 1 M (◈ and □) as described in Chapter 2.7. Growth was measured spectrophotometrically as OD_{600} (open symbols) (Chapter 2.6.1), and *lacZ* fusion expression was measured using MUG (closed symbols), as in Chapter 2.8. Results are representative of two independent experiments.

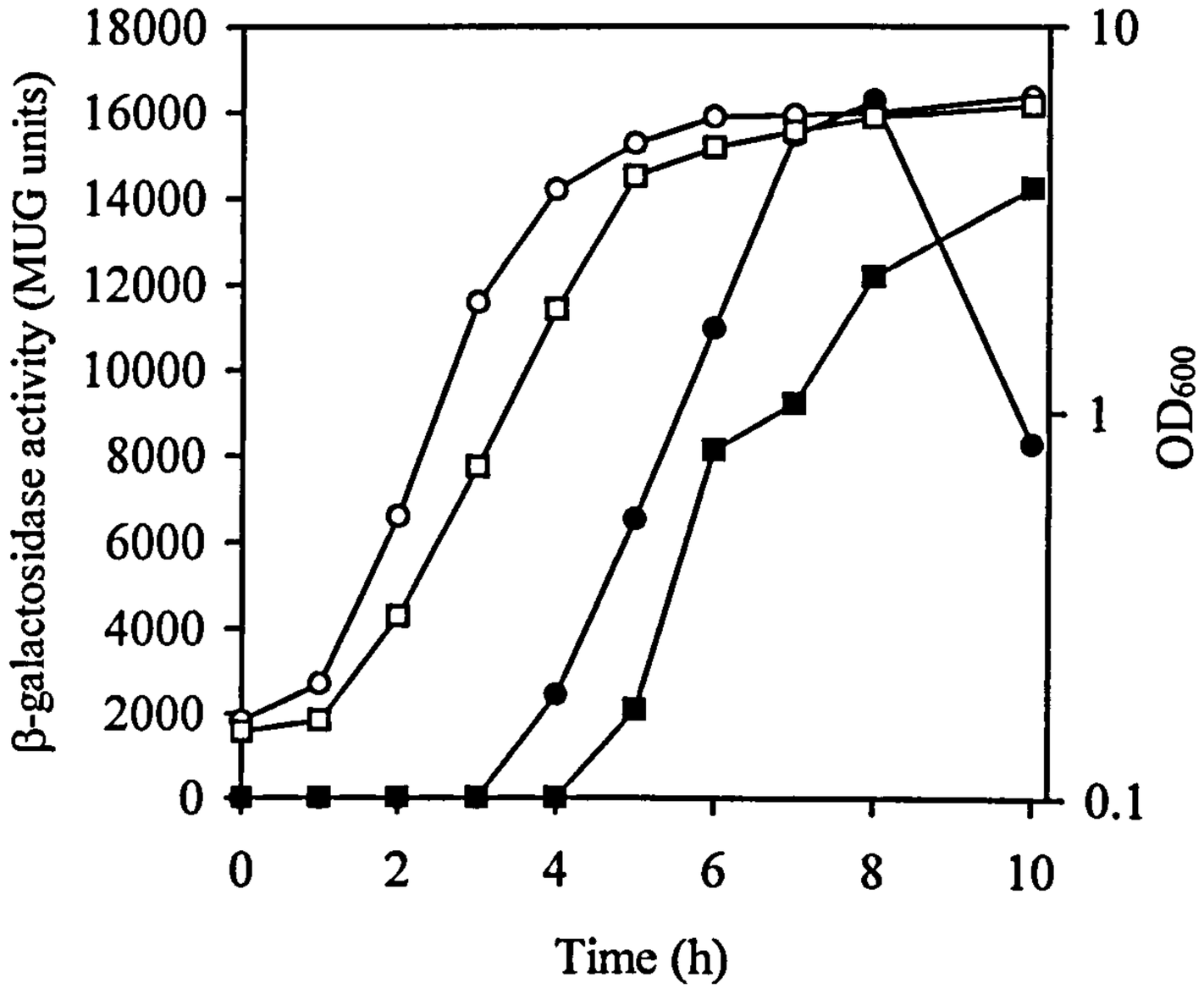


Figure 3.1c

Effect of 1 M NaCl on *agr* (RNA III)::*lacZ* transcription and growth in BHI.

S. aureus SH101F7 (8325-4 *agr* (RNA III)::*lacZ*) was grown in either BHI (○ and ●) or BHI containing 1 M NaCl (□ and ■) as described in Chapter 2.7. Growth was measured spectrophotometrically as OD_{600} (open symbols) (Chapter 2.6.1), and *lacZ* fusion expression was measured using MUG (closed symbols), as in Chapter 2.8. Results are representative of two independent experiments.

relevant antibiotics to ensure the correct resistance profile. However, due to the close proximity of *rsbU* to *sigB* in the *sigB* operon, it is possible that the mutation in *rsbU* will have been cotransduced with the *sigB* mutation into SH1000 from 8325-4 at a high frequency. Therefore, in order to ensure that the *sigB* strains produced in SH1000 were *sigB*, *rsbU*$^+$ and not *sigB*, *rsbU*, putative transductants were screened by the PCR method of Kullik and Giachino (1997). This involved using primers OL-78 and OL-79, which bound either side of the 11 bp deletion site in *rsbU* to produce a 77 bp fragment for *rsbU* strains and an 88 bp fragment for *rsbU*$^+$ strains, which could be resolved by gel electrophoresis on 4 % (w/v) agarose gels (Figure 3.2). Despite the fact that the mutations in *rsbU* and *sigB* are separated by less than 3 kb, cotransduction was found to be only 80 %, which facilitated the isolation of the *sigB, rsbU*$^+$ derivatives JLA313 (SH1000 *sarA::lacZ sigB*), JLA343 (SH1000 *agr* (RNA III)*::lacZ sigB*) and JLA373 (SH1000 *hla::lacZ sigB*).

3.2.2.2 The effect of σ^B on *hla*, *agr* and *sarA* transcription

Before the role of σ^B in the NaCl-mediated stress response was investigated, the general effects of σ^B on *hla*, *sarA* and *agr* were analysed. Initially, the effect of σ^B on the expression of an *hla::lacZ* fusion was investigated. *S. aureus* JLA371 (SH1000 *hla*::*lacZ*), PC322 (8325-4 *hla*::*lacZ*), JLA373 (SH1000 *hla*::*lacZ sigB*) and PC4044 (8325-4 *hla*::*lacZ sigB*) were grown in BHI as described in Chapter 2.7. Growth was measured spectrophotometrically as OD_{600} (Chapter 2.6.1), and *lacZ* fusion expression was determined using MUG, as in Chapter 2.8. Transcription of the *hla::lacZ* fusion was up to approximately 7.5-fold lower in JLA371 (SH1000 *hla*::*lacZ*) than in the other three strains (*ie.* 14,201 MUG units, 104,844 MUG units, 80,923 MUG units and 80,540 MUG units at t = 12 h for JLA371 (SH1000 *hla*::*lacZ*), PC322 (8325-4 *hla*::*lacZ*), JLA373 (SH1000 *hla*::*lacZ sigB*) and PC4044 (8325-4 *hla*::*lacZ sigB*) respectively) (Figure 3.3a). This suggests that σ^B represses *hla* transcription.

In order to determine the effects of σ^B on *agr*, the expression of *agr* (RNA III)::*lacZ* in *S. aureus* JLA341 (SH1000 *agr* (RNA III)::*lacZ*), SH101F7 (8325-4 *agr* (RNA III)::*lacZ*), JLA343 (SH1000 *agr* (RNA III)::*lacZ sigB*) and PC604 (8325-4 *agr* (RNA III)::*lacZ sigB*) in BHI was determined, as for the *hla::lacZ* fusion strains. A reduction

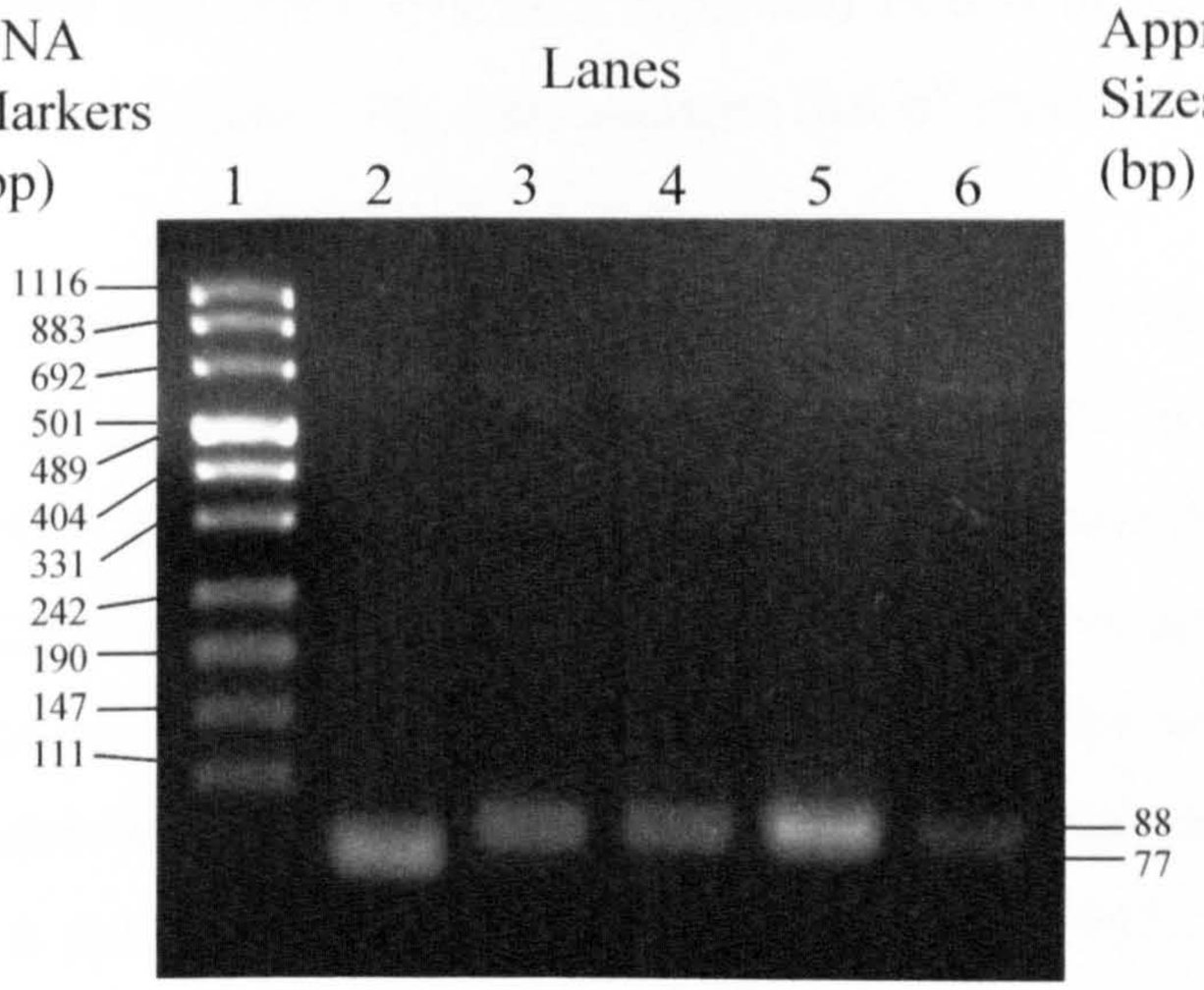


Figure 3.2
PCR to show intact *rsbU* in *lacZ* fusion strains in the SH1000 *sigB* background.

Genomic DNA was prepared using a Qiagen DNeasy™ spin column (Chapter 2.11.1). PCR amplification was based on the method of Kullik and Giachino (1997), and was performed using the general Taq polymerase protocol (Chapter 2.13.1.2.1). Primers used were OL-78 and OL-79. Products were separated by gel electrophoresis on 4 % (w/v) agarose gels.

Lane order; lane 1, pUC mix DNA size markers; lane 2, 8325-4; lane 3, SH1000; lane 4, JLA313 (SH1000 *sarA::lacZ sigB*); lane 5, JLA343 (SH1000 *agr::lacZ sigB*); lane 6, JLA373 (SH1000 *hla::lacZ sigB*).

in *agr* (RNA III)::*lacZ* expression of up to 4.6-fold was observed during growth of JLA341 (SH1000 *agr* (RNA III)::*lacZ*) compared to the other three strains (*ie.* 3,177 MUG units, 12,569 MUG units, 14,083 MUG units and 14,678 MUG units at t = 12 h for JLA341 (SH1000 *agr* (RNA III)::*lacZ*), SH101F7 (8325-4 *agr* (RNA III)::*lacZ*), JLA343 (SH1000 *agr* (RNA III)::*lacZ sigB*) and PC604 (8325-4 *agr* (RNA III)::*lacZ sigB*) respectively) (Figure 3.3b). This suggests that σ^B represses *agr* transcription.

Furthermore, whilst a 4.4-fold repression of *agr* (RNAIII) transcription by σ^B can be demonstrated by comparing the data for JLA341 (SH1000 *agr* (RNA III)::*lacZ*) and JLA343 (SH1000 *agr* (RNA III)::*lacZ sigB*), the levels of *agr* (RNAIII) transcription in SH101F7 (8325-4 *agr* (RNA III)::*lacZ*) and PC604 (8325-4 *agr* (RNA III)::*lacZ sigB*) were very similar at t = 12 h. The same is true when comparing the *hla::lacZ* transcription data of the two *sigB*$^+$ strains with their *sigB* derivatives. This suggests that *rsbU*, which is defective in 8325-4 (Kullik & Giachino, 1997), is normally involved in the repression by σ^B of *hla* and *agr* (RNAIII) by way of its role in upregulating σ^B activity. Importantly, this indicates that data obtained regarding the effects of σ^B on virulence determinant expression in the SH1000 and 8325-4 backgrounds are not directly comparable.

The P2 and P3 promoters of *agr* do not possess putative σ^B-dependent consensus sequences (Cheung *et al.*, 1999). Therefore, the repression of *agr* by σ^B probably occurs indirectly. SarA can act as an activator of *agr* expression (Chien & Cheung 1998), and the *sar*P3 promoter is σ^B-dependent (Deora *et al.*, 1997; Manna *et al.*, 1998; Miyazaki et al., 1999; Bischoff *et al.*, 2001). Therefore, the possibility that the diminished level of *agr* (RNA III) was a consequence of reduced SarA expression was investigated. *S. aureus* JLA311 (SH1000 *sarA::lacZ*), PC161 (8325-4 *sarA::lacZ*), JLA313 (SH1000 *sarA::lacZ sigB*) and PC4030 (8325-4 *sarA::lacZ sigB*), were grown in BHI, and *sarA::lacZ* expression and growth was measured as for the *hla::lacZ* fusion strains. Overall levels of *sarA* transcription in each of the four strains was found to be very similar (*ie.* 1,155 MUG units, 1,342 MUG units, 1,879 MUG units and 1,815 MUG units at t = 4 h for strains JLA311 (SH1000 *sarA::lacZ*), PC161 (8325-4 *sarA::lacZ*), JLA313 (SH1000 *sarA::lacZ sigB*) and PC4030 (8325-4 *sarA::lacZ sigB*) respectively) (Figure 3.3c). The σ^B-mediated reductions in expression of *agr* (RNA III) and *hla* are therefore likely to be SarA-independent.

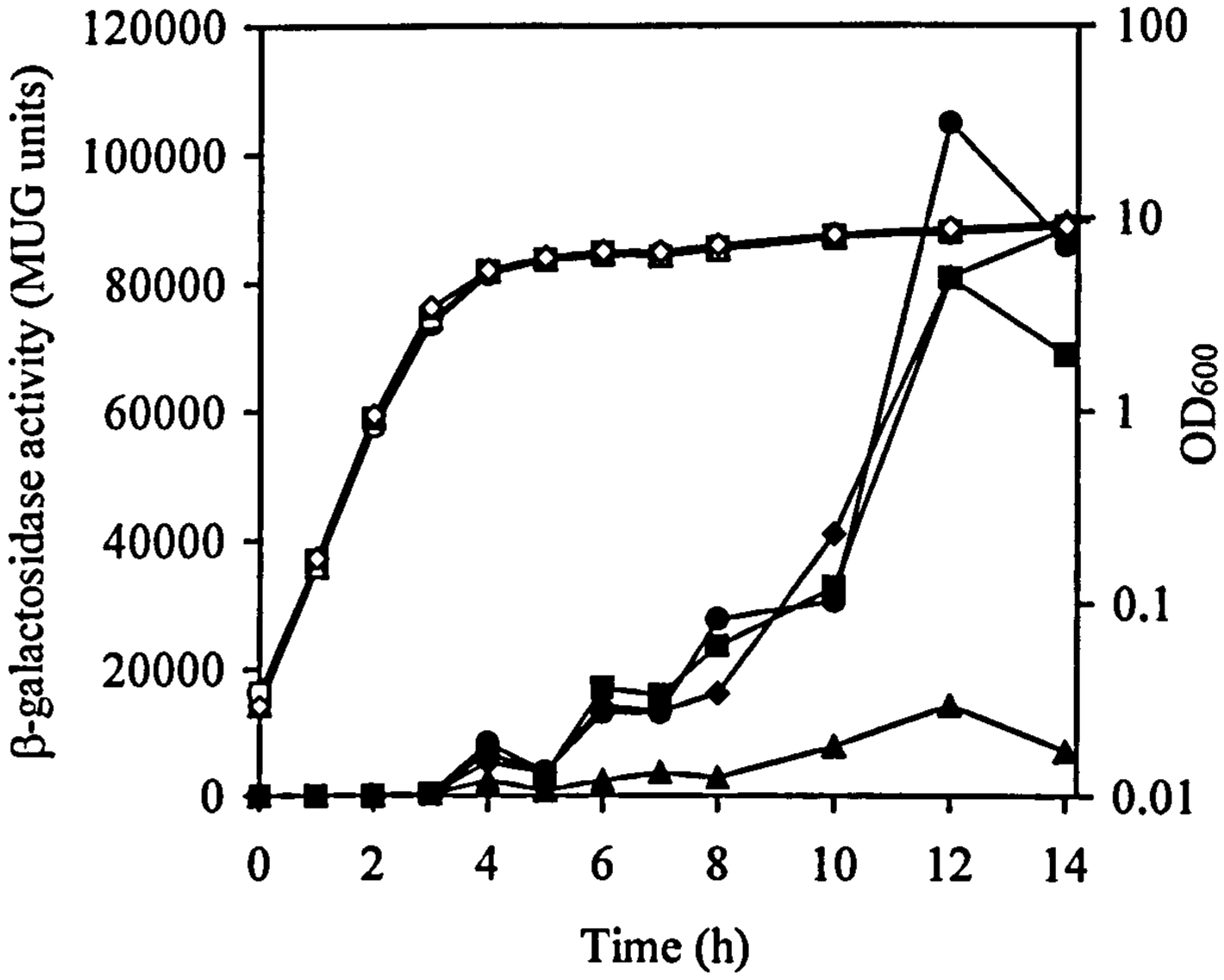


Figure 3.3a

Effect of σ^B on *hla::lacZ* transcription.

S. aureus PC322 (8325-4 *hla::lacZ*) (○ and ●), JLA371 (SH1000 *hla::lacZ*) (△ and ▲), PC4044 (8325-4 *hla::lacZ sigB*) (□ and ■) and JLA373 (SH1000 *hla::lacZ sigB*) (◇ and ◆) were grown in BHI as described in Chapter 2.7. Growth was measured spectrophotometrically as OD_{600} (open symbols) (Chapter 2.6.1), and *lacZ* fusion expression was measured using MUG (closed symbols), as in Chapter 2.8. Results are representative of two independent experiments.

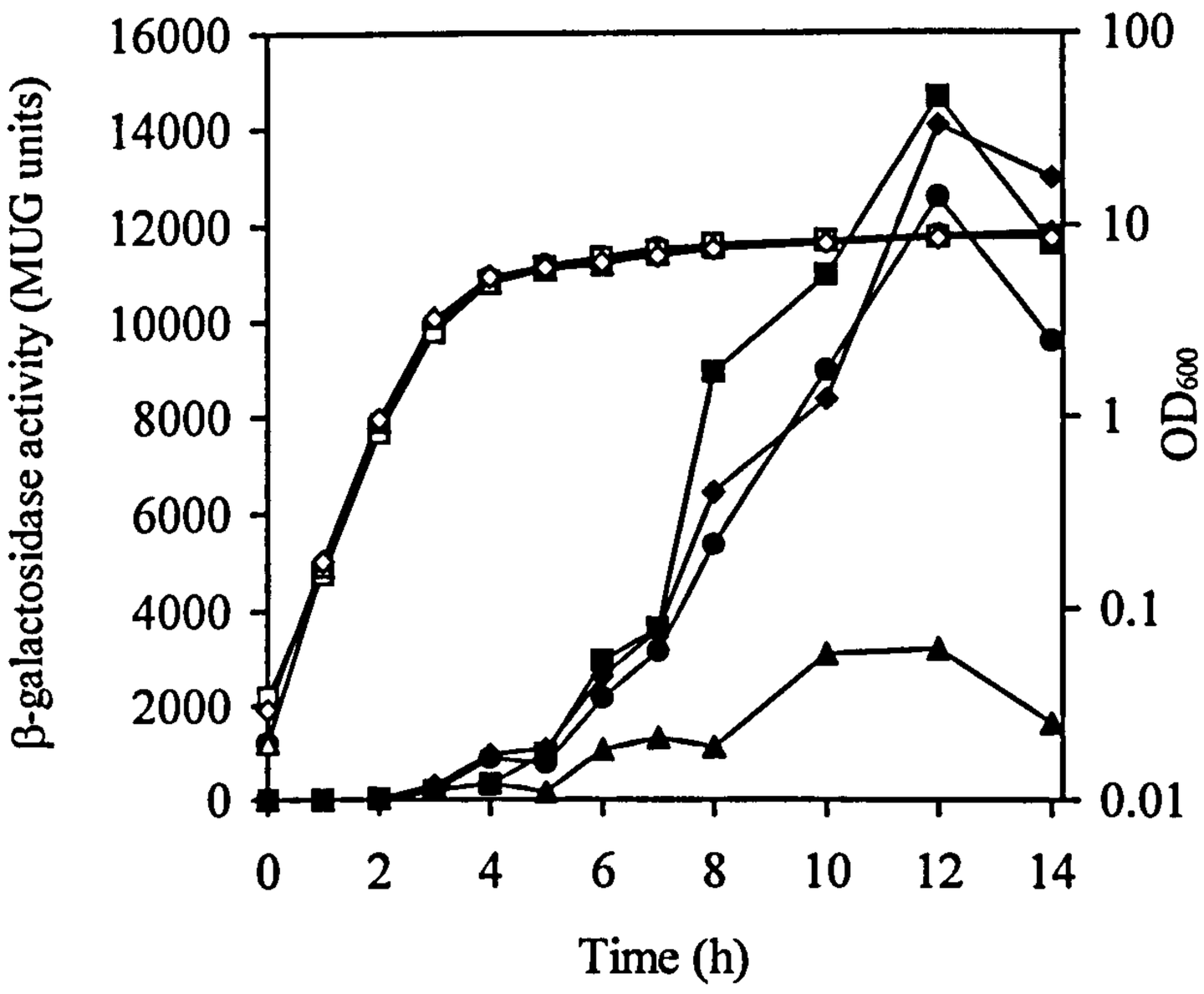


Figure 3.3b

Effect of σ^B on *agr* (RNA III)::*lacZ* transcription.

S. aureus SH101F7 (8325-4 *agr* (RNA III)::*lacZ*) (○ and ●), JLA341 (SH1000 *agr* (RNA III)::*lacZ*) (△ and ▲), PC604 (8325-4 *agr* (RNA III)::*lacZ sigB*) (□ and ■) and JLA343 (SH1000 *agr* (RNA III)::*lacZ sigB*) (◇ and ◆) were grown in BHI as described in Chapter 2.7. Growth was measured spectrophotometrically as OD_{600} (open symbols) (Chapter 2.6.1), and *lacZ* fusion expression was measured using MUG (closed symbols), as in Chapter 2.8. Results are representative of two independent experiments.

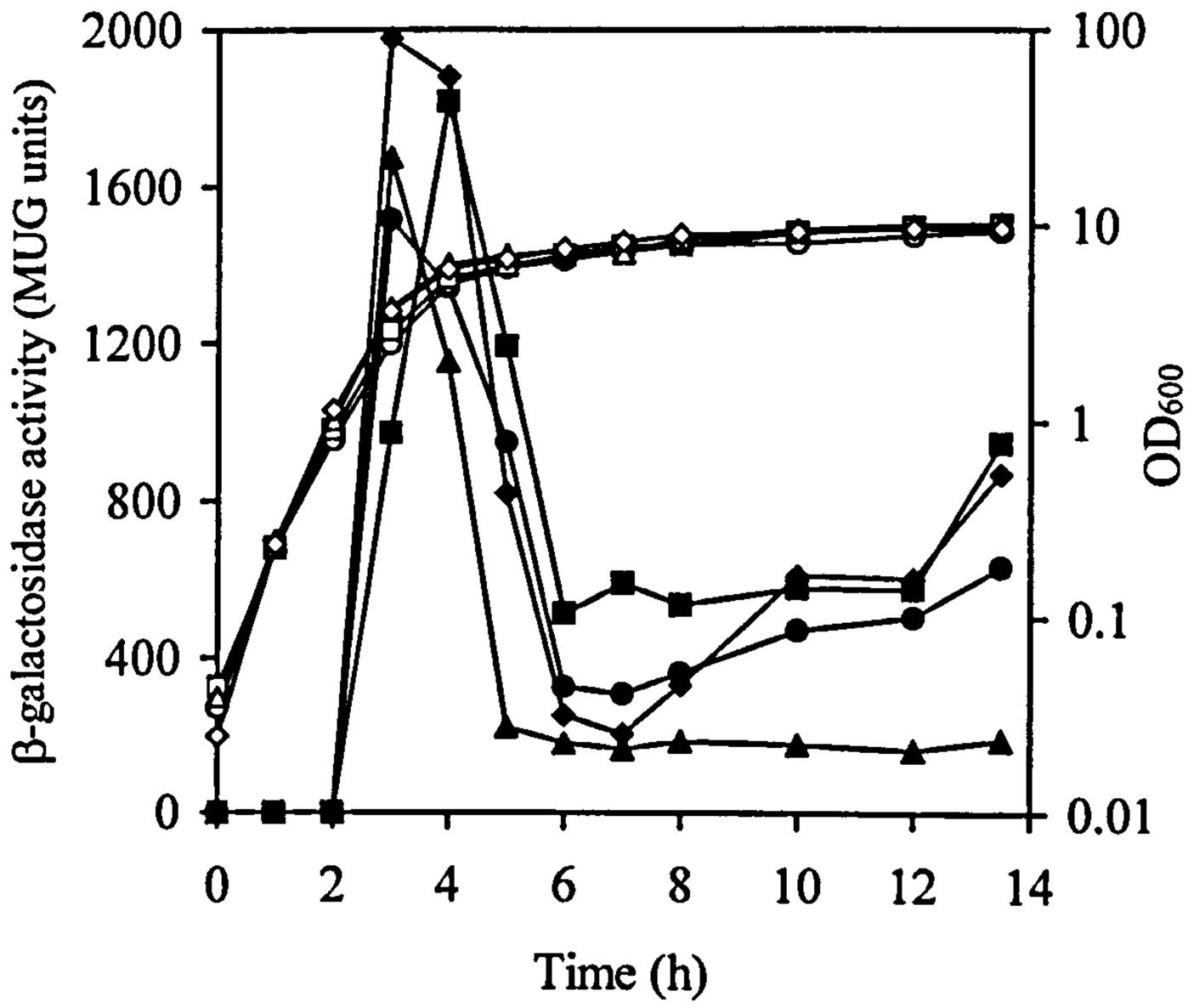


Figure 3.3c

Effect of σ^B on *sarA::lacZ* transcription.

S. aureus PC161 (8325-4 *sarA::lacZ*) (○ and ●), JLA311 (SH1000 *sarA::lacZ*) (△ and ▲), PC4030 (8325-4 *sarA::lacZ sigB*) (□ and ■) and JLA313 (SH1000 *sarA::lacZ sigB*) (◇ and ◆) were grown in BHI as described in Chapter 2.7. Growth was measured spectrophotometrically as OD_{600} (open symbols) (Chapter 2.6.1), and *lacZ* fusion expression was measured using MUG (closed symbols), as in Chapter 2.8. Results are representative of two independent experiments.

3.2.2.3 The effect of σ^B on SarA protein levels

The finding that the repression by σ^B of *agr* (RNAIII) and *hla* is likely to be SarA-independent (Chapter 3.2.2.2) contrasts with the report by Bischoff *et al.* (2001) that *sarA* transcription is increased in two *rsbU*$^+$ strains compared to their *sigB* derivatives. Consequently, the SarA protein levels during growth of strains SH1000, MJH502 (SH1000 *sigB*), 8325-4 and PC400 (8325-4 *sigB*) were determined by Western blotting. Strains were grown as in Chapter 2.18.1 and samples were removed at 3, 5, and 7 h. Total cellular proteins were prepared as in Chapter 2.18.3.2, resolved by SDS-PAGE (Chapter 2.18.4), and blotted (Chapter 2.18.5). Detection was performed with rabbit anti-SarA primary antibodies (Blevins *et al.*, 1999) using the ECL system (Chapter 2.18.5.2). 20 % (v /v) pig serum was included in the blocking solution. Densitometric analysis of autoradiographs was performed, as in Chapter 2.18.5.2. At the time-points analysed, the levels of SarA were very similar for each of the strains (Figure 3.4). Therefore, when the *lacZ* reporter assay was used or when protein levels were detected by Western blotting, the levels of SarA and temporal regulation of SarA were found to be very similar for strains 8325-4, SH1000 and their *sigB* mutant derivatives.

3.2.2.4 The effect of σ^B on *hla* and *agr* transcription in a *sarA* background

To confirm the apparent lack of a role for SarA in the overall negative effect of σ^B on the expression of *hla* and *agr* (RNA III) transcription (Chapters 3.2.2.2 and 3.2.2.3), a *sarA* mutation was introduced into *hla::lacZ* and *agr* (RNA III)::*lacZ* reporter strains by ϕ11-mediated transduction. This produced the strains JLA375 (SH1000 *hla::lacZ sarA*), JLA376 (8325-4 *hla::lacZ sigB sarA*), JLA377 (SH1000 *hla::lacZ sigB sarA*) JLA345 (SH1000 *agr* (RNA III)::*lacZ sarA*), and JLA347 (SH1000 *agr* (RNA III)::*lacZ sigB sarA*).

Strains JLA375 (SH1000 *hla::lacZ sarA*), PC3221 (8325-4 *hla::lacZ sarA*), JLA377 (SH1000 *hla::lacZ sigB sarA*) and JLA376 (8325-4 *hla::lacZ sigB sarA*) were used to analyze the effects of σ^B on *hla* expression in a *sarA* background. Strains JLA345 (SH1000 *agr* (RNA III)::*lacZ sarA*), PC600 (8325-4 *agr* (RNAIII)::*lacZ sarA*), JLA345 (SH1000 *agr* (RNA III)::*lacZ sarA*) and PC602 (8325-4 *agr* (RNAIII)::*lacZ sigB sarA*)

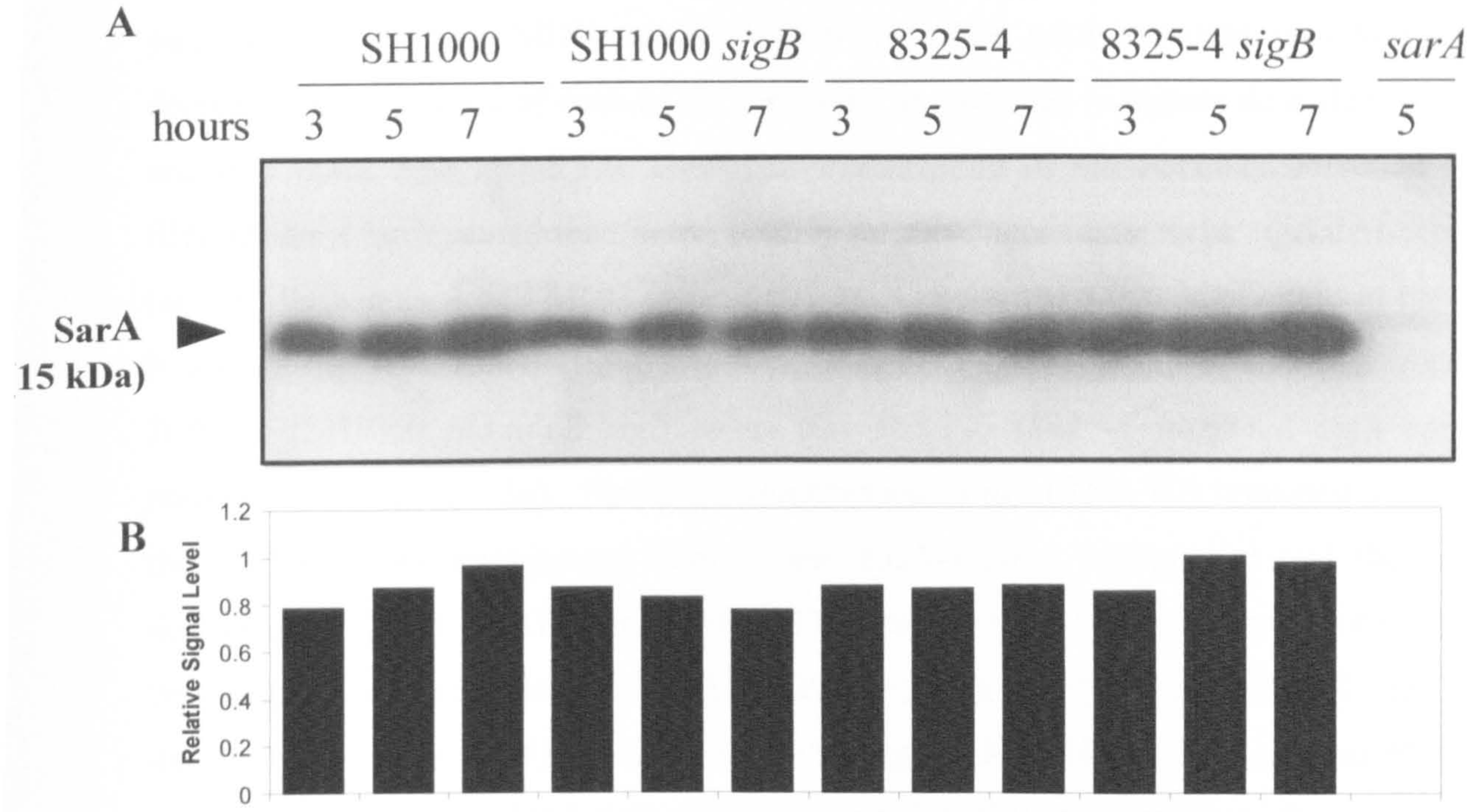


Figure 3.4

Level of SarA protein in different strains.

(A) Equivalent amounts of total cellular protein (OD_{600} of culture, 0.1), isolated after lysostaphin digestion of cells from cultures grown for 3, 5, and 7 h, were blotted and probed with purified immunoglobulin G antibodies raised against SarA (Blevins *et al.*, 1999) using the ECL system (Chapter 2.18.5.2). Strains used were SH1000, MJH502 (SH1000 *sigB*), 8325-4, PC400 (8325-4 *sigB*), and SH1002 (SH1000 *sarA*). (B) SarA signals on the blot were quantified by densitometry (Chapter 2.18.5.2). The relative signal level for each lane was compared to the maximum signal level obtained. The results are representative of three independent experiments.

were used to analyze the effects of σ^B on *agr* (RNAIII) expression in a *sarA* background. Strains were grown in BHI as described in Chapter 2.7. Growth was measured spectrophotometrically as OD_{600} (Chapter 2.6.1), and *lacZ* fusion expression was determined using MUG, as in Chapter 2.8. The *sarA* mutation was found to decrease the expression of both of the reporters, as expected (compare data shown here and in Figures 3.3a and 3.3b). However, transcription of *hla* remained lower in the SH1000 *sarA* background than in the 8325-4 *sarA* background and the *sigB* derivatives (*ie.* 74 MUG units, 1,519 MUG units, 2,072 MUG units and 1,094 MUG units at t = 12 h for strains JLA375 (SH1000 *hla*::*lacZ sarA*), PC3221 (8325-4 *hla*::*lacZ sarA*), JLA377 (SH1000 *hla*::*lacZ sigB sarA*) and JLA376 (8325-4 *hla*::*lacZ sigB sarA*) respectively) (Figure 3.5a). Similarly, transcription of *agr* (RNA III) remained lower in the SH1000 *sarA* background than in the 8325-4 *sarA* background and the *sigB* derivatives (*ie.* 102 MUG units, 2,594 MUG units, 1953 MUG units and 2549 MUG units at t = 12 h for strains JLA345 (SH1000 *agr* (RNA III)::*lacZ sarA*), PC600 (8325-4 *agr* (RNAIII)::*lacZ sarA*), JLA345 (SH1000 *agr* (RNA III)::*lacZ sarA*) and PC602 (8325-4 *agr* (RNAIII)::*lacZ sigB sarA*) respectively) (Figure 3.5b). Since the repression of *agr* (RNAIII) and *hla* transcription by σ^B was not relieved by the mutation of *sarA*, the decrease in *agr* (RNAIII) and *hla* transcription in SH1000 was not due to the activity of SarA.

3.2.2.5 Investigation of the role of Rot and SarHI as the effector of the repression of *agr* and *hla* by σ^B

In Chapters 3.2.2.2, 3.2.2.3 and 3.2.2.4, the level of SarA was shown not to be the effector of the repression of *agr* (RNAIII) and *hla* by σ^B. The sequence of the *S. aureus* genome has revealed a wide array of SarA homologues, including SarH1 (Tegmark *et al.*, 2000), and Rot (McNamara *et al.*, 2000) (Chapter 1.6.2.2.3). Rot has been shown to repress *hla* transcription in an *agr* background (McNamara *et al.*, 2000; Said-Salim *et al.*, 2003). Furthermore, *sarH1* has a σ^B-dependent promoter, has been shown to bind to the P3 promoter of the *agr* operon, and represses *hla* at the level of transcription in a *sarA* background (Tegmark *et al.*, 2000). Therefore, the possibility that either *rot* or *sarH1* might act as the intermediate between σ^B and *agr* was determined by

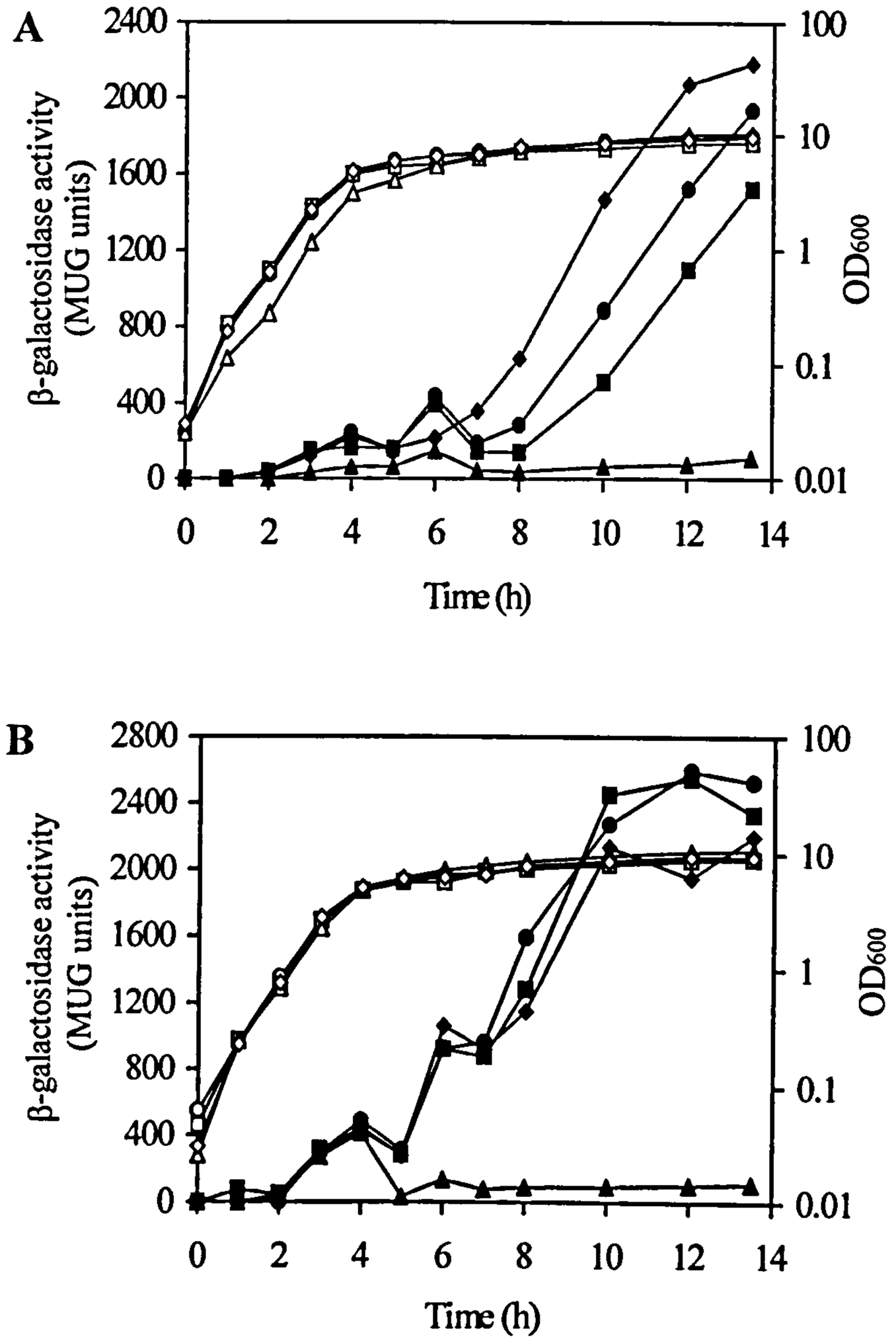


Figure 3.5

Effect of σ^B on *hla::lacZ* and *agr* (RNAIII)::*lacZ* transcription in a *sarA* background. (A) *hla::lacZ* transcription of strains PC3221 (8325-4 *hla*::*lacZ sarA*) (○ and ●), JLA375 (SH1000 *hla*::*lacZ sarA*) (△ and ▲), JLA376 (8325-4 *hla*::*lacZ sigB sarA*) (□ and ■) and JLA377 (SH1000 *hla*::*lacZ sigB sarA*) (◇ and ◆). (B) *agr* (RNA III)::*lacZ* transcription of strains PC600 (8325-4 *agr* (RNAIII)::*lacZ sarA*) (○ and ●), JLA345 (SH1000 *agr* (RNA III)::*lacZ sarA*) (△ and ▲), PC602 (8325-4 *agr* (RNAIII)::*lacZ sigB sarA*) (□ and ■) and JLA347 (SH1000 *agr* (RNA III)::*lacZ sigB sarA*) (◇ and ◆). Strains were grown in BHI as described in Chapter 2.7. Growth was measured spectrophotometrically as OD_{600} (Chapter 2.6.1) (open symbols), and *lacZ* fusion expression was determined using MUG, as in Chapter 2.8 (closed symbols). Results are representative of two independent experiments.

investigating the effects of mutation of these genes on *hla::lacZ* transcription. A cassette knock-out of *rot* was transferred from PM783 to PC322 (8325-4 *hla*::*lacZ*) and JLA371 (SH1000 *hla*::*lacZ*) by ϕ11-mediated transduction. This produced the strains JLA378 (8325-4 *hla*::*lacZ rot*) and JLA379 (SH1000 *hla*::*lacZ rot*) respectively. Similarly, the *sarH1* mutation was transduced from strain SMH1005 to JLA512 (8325-4 *hla*::*lacZ*) and JLA513 (SH1000 *hla*::*lacZ*) to create JLA380 (8325-4 *hla*::*lacZ sarH1*) and JLA381 (SH1000 *hla*::*lacZ sarH1*). Strains were grown in BHI as described in Chapter 2.7. Growth was measured spectrophotometrically as OD_{600} (Chapter 2.6.1), and *lacZ* fusion expression was determined using MUG, as in Chapter 2.8.

Transcription of *hla::lacZ* was found to be reduced by approximately 1.9-fold (t = 10 h) in the presence of the *rot* mutation in the SH1000 background (*ie.* 16,007 MUG units and 8,650 MUG units for strains JLA371 (SH1000 *hla*::*lacZ*) and JLA379 (SH1000 *hla*::*lacZ rot*) respectively) (Figure 3.6a). Therefore, Rot is unlikely to be the effector of the repression of *agr* and *hla* by σ^B, since inactivation of *rot* failed to relieve the repression of *hla::lacZ* transcription by σ^B. Furthermore, since a similar minor reduction in *hla::lacZ* transcription of approximately 1.3-fold was found to occur in the presence of the *rot* mutation in the 8325-4 background (*ie.* 111,596 MUG units and 87,285 MUG units at t = 10 h for strains PC322 (8325-4 *hla*::*lacZ*) and JLA378 (8325-4 *hla*::*lacZ rot*) respectively) (Figure 3.6b), this suggests that the effects of *rot* on *hla* occur independently of the levels of σ^B activity.

Transcription of *hla::lacZ* was found to be increased by approximately 1.6-fold (t = 10 h) in the presence of the *sarH1* mutation in the SH1000 background (*ie.* 10,148 MUG units and 16,034 MUG units for strains JLA513 (SH1000 *hla*::*lacZ*) and JLA381 (SH1000 *hla*::*lacZ sarH1*) respectively) (Figure 3.7a). Consequently, *sarH1* is also unlikely to be the mediator of the effect on *agr* (RNAIII) and *hla* by σ^B, since inactivation of *sarH1* failed to relieve the repression of *hla::lacZ* by σ^B to any significant extent. Furthermore, *hla::lacZ* transcription was found to be increased by approximately 1.5-fold at t = 10 h in the presence of the *sarH1* mutation in 8325-4 (*ie.* 53,136 MUG units and 78,541 MUG units for strains JLA512 (8325-4 *hla*::*lacZ*) and JLA380 (8325-4 *hla*::*lacZ sarH1*) respectively) (Figure 3.7b), suggesting that the effects of *sarH1* on *hla* occur independently of σ^B.

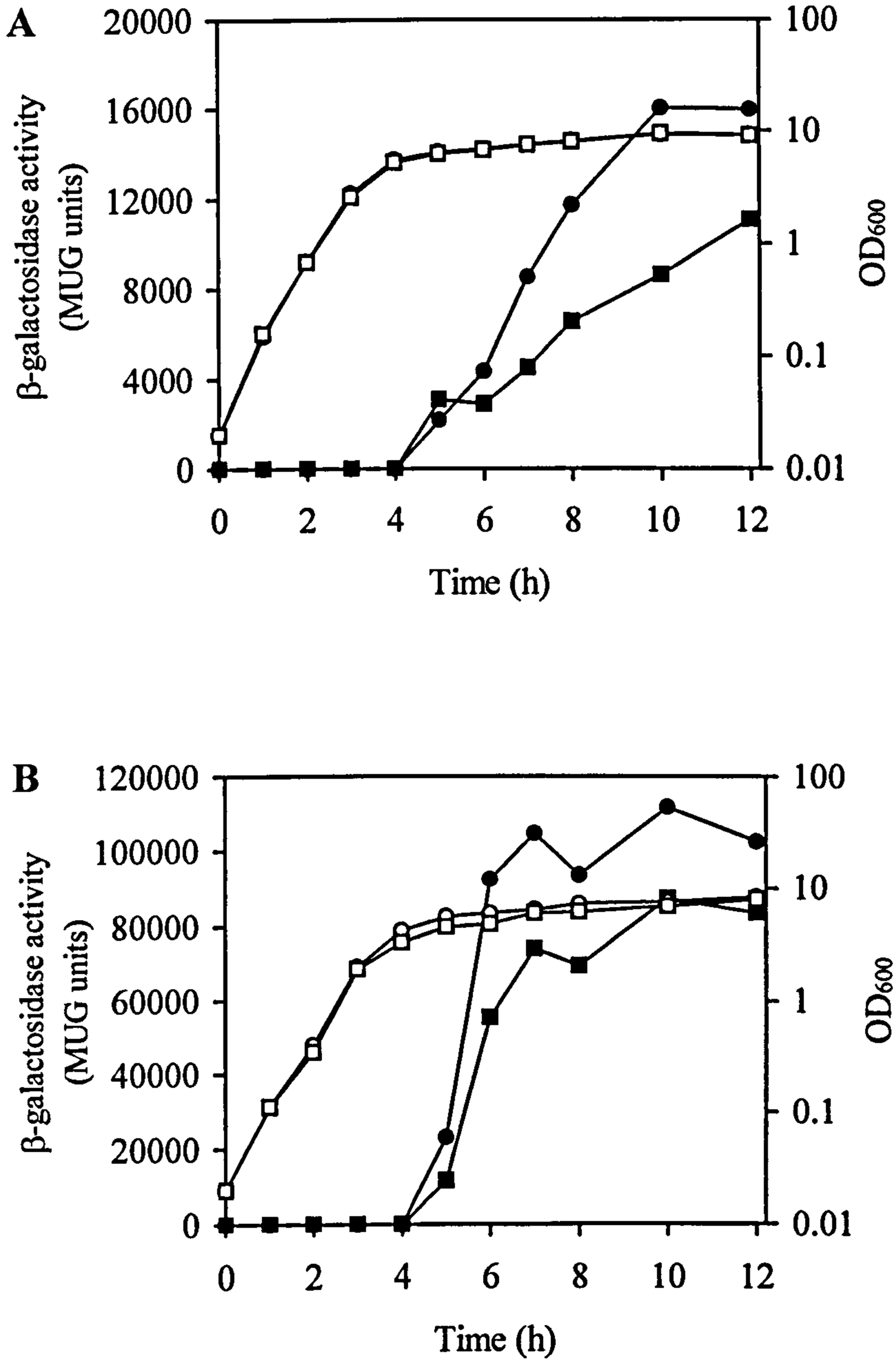


Figure 3.6

Effect of *rot* on *hla::lacZ* transcription in SH1000 and 8325-4.

(A) *hla::lacZ* transcription of strains JLA371 (SH1000 *hla*::*lacZ*) (○ and ●) and JLA379 (SH1000 *hla*::*lacZ rot*) (□ and ■). (B) *hla::lacZ* transcription of strains PC322 (8325-4 *hla*::*lacZ*) (○ and ●) and JLA378 (8325-4 *hla*::*lacZ rot*) (□ and ■). Strains were grown in BHI as described in Chapter 2.7. Growth was measured spectrophotometrically as OD_{600} (Chapter 2.6.1) (open symbols), and *lacZ* fusion expression was determined using MUG, as in Chapter 2.8 (closed symbols). Results are representative of a minimum of two independent experiments.

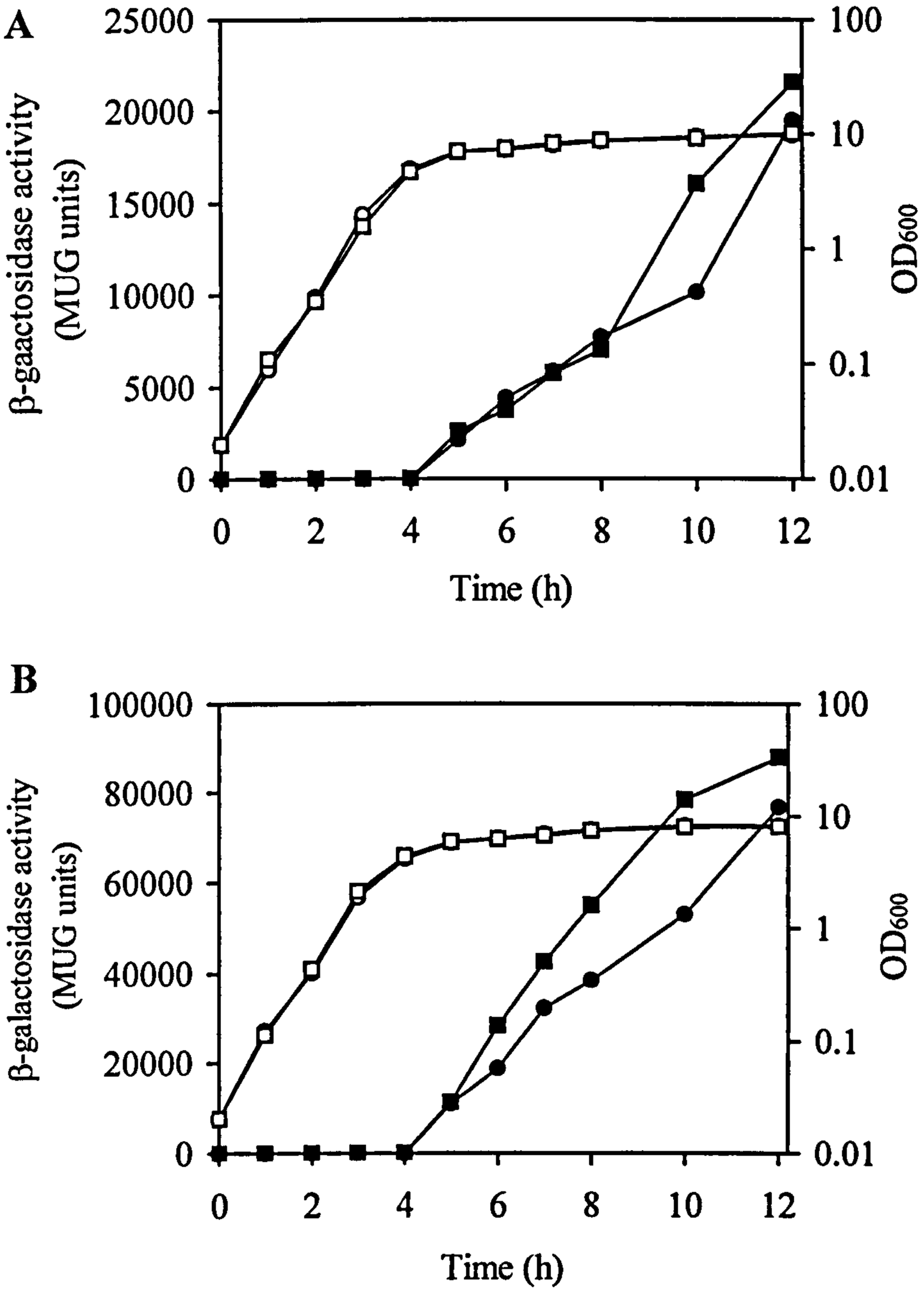


Figure 3.7

Effect of *sarH1* on *hla::lacZ* transcription in SH1000 and 8325-4.

(A) *hla::lacZ* transcription in strains JLA371 (SH1000 *hla*::*lacZ*) (○ and ●) and JLA379 (SH1000 *hla*::*lacZ rot*) (□ and ■). (B) *hla::lacZ* transcription in strains PC322 (8325-4 *hla*::*lacZ*) (○ and ●) and JLA378 (8325-4 *hla*::*lacZ rot*) (□ and ■). Strains were grown in BHI as described in Chapter 2.7. Growth was measured spectrophotometrically as OD_{600} (Chapter 2.6.1) (open symbols), and *lacZ* fusion expression was determined using MUG, as in Chapter 2.8 (closed symbols). Results are representative of two independent experiments.

3.2.2.6 The role of σ^B in virulence determinant regulation in response to NaCl-mediated stress

In order to investigate the role of σ^B in virulence determinant regulation in response to NaCl-mediated stress, JLA371 (SH1000 *hla*::*lacZ*), JLA373 (SH1000 *hla*::*lacZ sigB*), JLA341 (SH1000 *agr* (RNAIII)::*lacZ*) and JLA343 (SH1000 *agr* (RNAIII)::*lacZ sigB*) were grown in BHI containing 700 mM NaCl as described in Chapter 2.7. Growth was measured spectrophotometrically as OD_{600} (Chapter 2.6.1), and *lacZ* fusion expression was determined using MUG, as in Chapter 2.8. The results obtained were compared with Figures 3.3a and 3.3b, for which the data was produced simultaneously with this set of experiments.

In BHI containing no added NaCl (Figure 3.3a), *hla*::*lacZ* transcription was 5.6-fold lower in JLA371 (SH1000 *hla*::*lacZ*) compared to JLA373 (SH1000 *hla*::*lacZ sigB*) (*ie.* 14,201 and 80,923 MUG units respectively at t = 12 h). However, in BHI containing 700 mM NaCl (Figure 3.8a), *hla*::*lacZ* transcription was approximately 85-fold lower in JLA371 than in JLA373 (*ie.* 68 and 5,777 MUG units respectively at t = 12 h). This suggests that the level of repression of *hla* transcription by σ^B increases greatly in the presence of 700 mM NaCl.

In BHI containing no added NaCl (Figure 3.3b), *agr* (RNAIII)::*lacZ* transcription was 4.5-fold lower in JLA341 (SH1000 *agr* (RNAIII)::*lacZ*) compared to transcription in JLA343 (SH1000 *agr* (RNAIII)::*lacZ sigB*), (*ie.* 3,177 and 14,678 MUG units respectively at t = 12 h). Similarly, in BHI containing 700 mM NaCl (Figure 3.8b), *agr* (RNAIII)::*lacZ* transcription was 7.2-fold lower in JLA341 than in JLA343 (*ie.* 1,989 and 14,393 MUG units respectively at t = 12 h). This indicates that the level of repression of *agr* (RNAIII) transcription by σ^B does not increase significantly in the presence of NaCl-mediated stress.

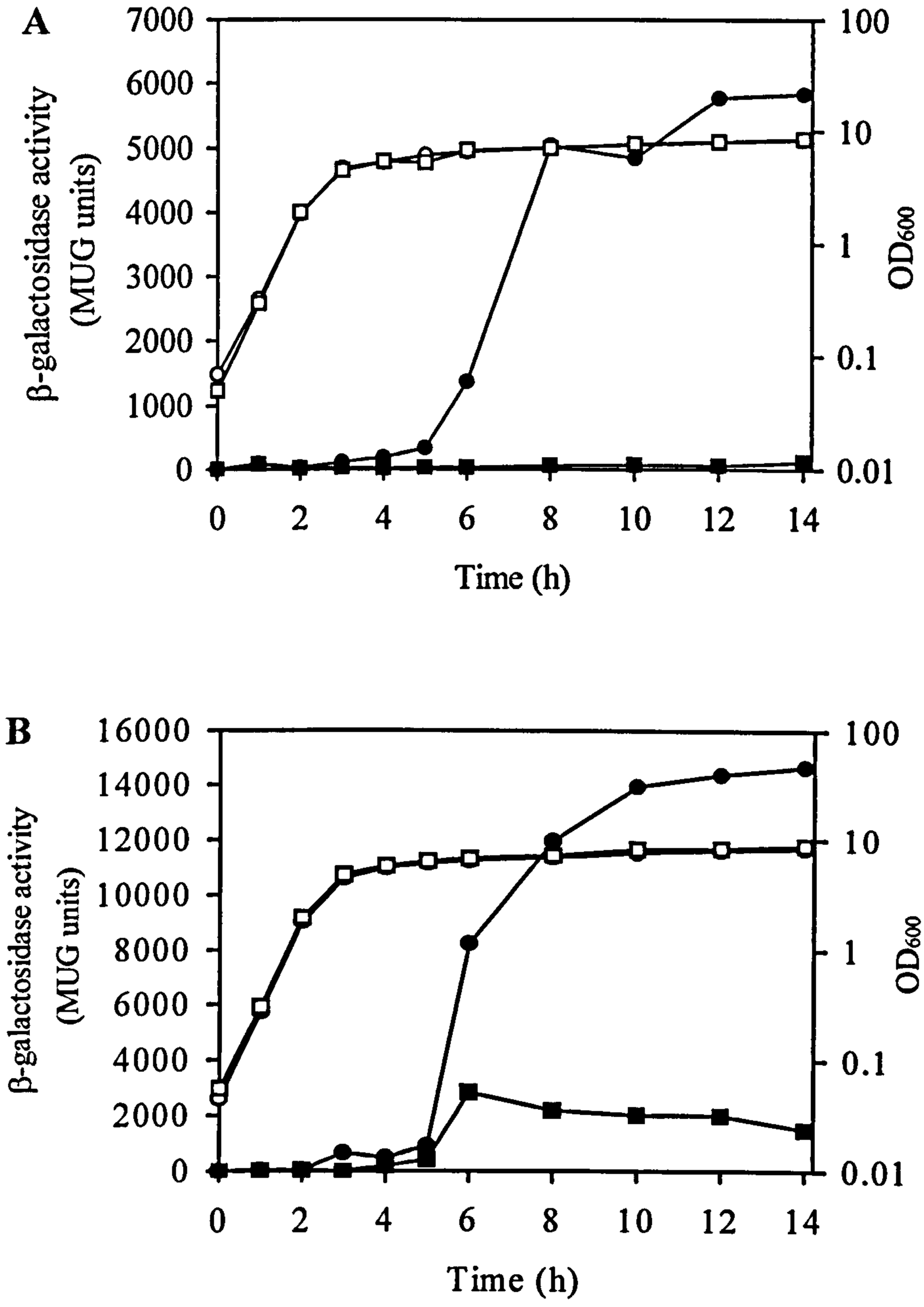


Figure 3.8

Effect of σ^B on *hla::lacZ* and *agr* (RNAIII)::*lacZ* transcription during growth in BHI containing 700 mM NaCl. (A) *hla::lacZ* transcription in strains JLA371 (SH1000 *hla*::*lacZ*) (□ and ■) and JLA373 (SH1000 *hla*::*lacZ sigB*) (○ and ●). (B) *agr* (RNAIII)::*lacZ* transcription in strains JLA341 (SH1000 *agr* (RNAIII)::*lacZ*) (□ and ■) and JLA343 (SH1000 *agr* (RNAIII)::*lacZ sigB*) (○ and ●). Strains were grown in BHI containing 700 mM NaCl as described in Chapter 2.7. Growth was measured spectrophotometrically as OD_{600} (Chapter 2.6.1) (open symbols), and *lacZ* fusion expression was determined using MUG, as in Chapter 2.8 (closed symbols). Results are representative of two independent experiments.

3.3 Discussion

Transcriptional analysis using *lacZ* reporter gene fusions showed that 1 M NaCl repressed *spa* and *hla* in *S. aureus* 8325-4. However, only a minor effect on the transcription of *agr* (RNA III) was identified. This supports the findings of Lindsay and Foster (1999) and of Chan & Foster (1998a) that 1 M NaCl represses *spa* and *hla* in an *agr*-independent manner, and confirms the suggestion of Chan and Foster (1998a) that in the presence of NaCl, a novel regulatory system might assume control of genes which are also regulated by *agr* and/or *sarA*.

However, an alternative explanation for the effects of NaCl on virulence determinant and *agr* expression might be that NaCl prevents the binding of RNAIII to its target sites. This would prevent the upregulation of extracellular virulence determinants (*eg. hla*) during the post-exponential growth phase, resulting in a low level of expression throughout growth, despite the fact that *agr* (RNA III) transcription remains unaffected. However, the finding that 1 M NaCl repressed the transcription of the cell surface-associated protein *spa* suggests that this is not the case; *spa* is normally repressed by *agr* during the post-exponential growth phase, and so if NaCl prevented RNAIII from binding to its target sites, *spa* would not be repressed after the post-exponential growth phase, and would therefore remain highly expressed. Since *spa* was found to be repressed throughout growth in the presence of 1 M NaCl, this suggests that NaCl does not interfere with RNAIII interactions, and so the effects of NaCl on the binding of RNAIII were not analysed further.

In the investigation of the effects of σ^B on *hla*, *agr* (RNAIII) and *sarA*, σ^B was found to repress *hla* transcription. This is in accordance with the findings of a number of other researchers (Cheung *et al.*, 1999; Nicholas *et al.*, 1999; Giachino *et al.*, 2001; Ziebandt *et al.*; Horsburgh *et al.*, 2002). For example, Horsburgh *et al.* (2002) have shown the level of Hla to be greatly reduced in stationary-phase exoprotein samples from SH1000 compared to 8325-4, and demonstrated a decrease in Hla activity in SH1000. σ^B has also been shown to repress several other *agr*-dependent virulence determinants, including β-haemolysin (Ziebandt *et al.*, 2001), proteases (Ziebandt *et al.*, 2001; Horsburgh *et al.*, 2002) and enterotoxin B (Ziebandt *et al.*, 2001). The mechanism for this is likely to involve repression of *agr* by σ^B, since a large reduction in *agr* (RNA III)

transcription in SH1000 was observed, compared to 8325-4 and the *sigB* derivatives. However, Cheung *et al.*, (1999) demonstrated that the transcription of RNAII and RNAIII of the *agr* locus was unaltered by mutation of *sigB*. Nevertheless, this discrepancy is probably due to the use of the *rsbU* 8325-4 derivative RN6390 in the studies by Cheung *et al.* (1999). Furthermore, the data presented here is supported by the findings of Bischoff *et al.* (2001), who showed by Northern blot and reporter gene fusion analysis that the transcription of *agr* (RNA III) was higher in the *sigB* derivatives of the *rsbU*$^+$ strains MSSA1112, Newman and GP268 compared to their *sigB*$^+$ parents.

The repression of *agr* by σ^B probably occurs indirectly, since the P2 and P3 promoters of *agr* do not possess σ^B-dependent consensus sequences (Cheung *et al.*, 1999). The most obvious candidate for mediating the reduction in *agr* levels was SarA, which is a known activator of *agr* expression (Chien & Cheung, 1998; Chien *et al.*, 1998; Heinrichs *et al.*, 1996). Furthermore, the *sar*P3 promoter has been shown to be σ^B-dependent (Deora *et al.*, 1997; Manna *et al.*, 1998; Miyazaki et al., 1999; Bischoff *et al.*, 2001). However, when a *sarA::lacZ* reporter fusion (which measures expression from all three *sar* promoters (Chan & Foster, 1998a)) was assayed or when the amount of SarA protein was determined by Western blotting in SH1000, 8325-4 and their σ^B mutant derivatives, the levels were found to be very similar. This suggests that σ^B had little or no effect on SarA under the conditions studied here. This is in accordance with the results of Blevins *et al.* (1999), who reported that SarA is expressed constitutively. In their analysis of SarA levels during different stages of growth of RN6390 and clinical isolate UAMS-1, these authors observed no variation between the strains (Blevins *et al.*, 2002). It may be possible to extend this observation to strains 8325-4, SH1000 and their *sigB* mutant derivatives. However, this finding contrasts with the observations of Bischoff *et al.* (2001). In the studies of these authors, expression of a *sarA::lux* fusion reporter strain was found to be higher in the *rsbU*$^+$ strains MSSA1112 and Newman than in the *rsbU* strain BB255 or in *sigB* derivatives of any of the three strains grown in LB. Similarly, Karlsson and Arvidson (2002) showed that in a group of 92 non-isogenic clinical isolates, strains which possessed higher σ^B-activity also demonstrated higher levels of *sarA* mRNA in BHI. Furthermore, Gertz *et al.* (2000) showed that there was decreased SarA expression in a *sigB* mutant of *S. aureus* COL grown in minimal medium by using two-dimensional electrophoresis. Alternatively, Cheung *et al.* (1999) showed that SarA levels were elevated in a *sigB* mutant of *S. aureus* RN6390. The

reason for the difference in *sarA* expression between these studies and the data presented here is unclear. However, since different strains, media, and growth conditions were used, we cannot exclude the possibility that genetic or environmental differences had an effect. Therefore, it is important to study a number of environmental conditions to determine the effect of σ^B on SarA expression.

The σ^B-dependent *sar* promoter, P3 (Bayer *et al.*, 1996; Manna *et al.*, 1998), is most active during the late exponential growth phase in strains with a wild-type *rsbU* locus (Bischoff *et al.*, 2001). SarA represses its own transcription via the proximal P1 promoter but not via the more distal P3 promoter (Chakrabarti & Misra, 2000). Therefore, the failure to observe increased expression of SarA despite the presence of a σ^B promoter may be due to the autoregulatory capacity of SarA (Blevins *et al.*, 1999; Chackrabarti & Misra, 2000) maintaining constant protein levels. It can therefore be hypothesized that under certain environmental conditions where σ^B activity increases, resulting in a concomitant reduction in SarA repression, SarA autoregulation increases and maintains the level of SarA.

Since under the conditions studied here the SarA protein level was found to be relatively constant during growth irrespective of the *rsbU* or *sigB* genotype, SarA is unlikely to be the mediator for the repression of *agr* (RNAIII) and *hla* by σ^B. Furthermore, neither of the two SarA homologues investigated, Rot or SarH1, is likely to be the missing effector, as their inactivation failed to increase the transcription of *hla*. This agrees with the findings that the transcription of *agr* is unaffected by mutation of *sarH1*, and that the repression of *hla* by *sarH1* only occurs in a *sarA* mutant (Tegmark *et al.*, 2000). Furthermore, inactivation of *rot* has been found to increase *hla* transcription only in an *agr* background (McNamara *et al.*, 2000). Therefore, in an attempt to identify the missing component(s), a transposon mutant library screen using Tn*551* was performed in Chapter 5.

Analysis of the role of σ^B in the NaCl-mediated stress response indicated that the level of repression of *hla* transcription by σ^B is greatly increased in the presence of 700 mM NaCl compared to the level of repression in the absence of added NaCl. This suggests that σ^B might play a role in regulating *hla* in the presence of NaCl-mediated stress. However, the level of repression of *agr* (RNAIII) transcription by σ^B was not

significantly increased in the presence of 700 mM NaCl. Overall this data indicates that σ^B might be involved in the *agr*-independent repression of *hla* (and possibly therefore of other virulence determinants) in BHI containing 700 mM NaCl. This conflicts with the findings of Chan *et al.* (1998), who could not identify a role for σ^B in resistance to osmotic shock in *S. aureus*. However, a possible explanation for the difference in results may be the use of the *rsbU* strain 8325-4 by Chan *et al.* (1998). Furthermore, σ^B has been shown to be required for the activation of biofilm formation in *S. aureus* and *S. epidermidis* in response to NaCl-mediated stress (Rachid *et al.*, 2000; Knobloch *et al.*, 2001). This indicates that σ^B might have a similar role in regulating the NaCl-mediated stress response in *S. aureus* as it does in *B. subtilis* (Chapters 1.6.1.2 and 1.6.2.2.8). However, due to the absence of the environmental stress pathway genes *rsbR, rsbS, rsbT* and *rsbX* from the *S. aureus* σ^B operon (Chapter 1.6.2.2.8), the mechanisms by which σ^B senses NaCl in *S. aureus* will differ from those operating in *B. subtilis*.

However, inactivation of *sigB* did not completely prevent the repression of *hla* by NaCl-mediated stress, since *hla::lacZ* transcription was reduced by 14-fold in JLA373 (SH1000 *hla::lacZ sigB*) in BHI containing 700 mM NaCl (Figure 3.8a) compared to transcription in BHI containing no added NaCl (Figure 3.3a) (*ie.* 5,777 and 80,923 MUG units respectively at t = 12 h). The data presented in Figure 3.1b and of Chan and Foster (1998a) corroborates this, since NaCl was found to repress *hla::lacZ* transcription in the *rsbU* strain 8325-4. This suggests that although σ^B may be involved in the regulation of virulence determinants in response to NaCl-mediated stress in *S. aureus*, additional regulatory components might exist. A possible explanation for this may be that NaCl is an extremely important environmental parameter affecting the survival of *S. aureus*, and consequently the mechanisms involved in sensing and responding to it will be complex. Therefore, in order to identify novel components involved in virulence determinant regulation in response to NaCl-mediated stress, transposon mutant library screens were performed using Tn*917* and Tn*551* in Chapters 4 and 5 to identify genes that are required to repress the activity or transcription of *hla* in the presence of 1 M NaCl.

CHAPTER 4

IDENTIFICATION AND CHARACTERISATION OF GENES INVOLVED IN REGULATING α-HAEMOLYSIN ACTIVITY IN RESPONSE TO NaCl

4.1 Introduction

Insights into the mechanisms of infection have been obtained by the characterisation of mutants with altered virulence determinant expression or which are attenuated in pathogenicity. Transposon mutagenesis has been used extensively in this process, and has contributed to the discovery of major virulence determinant regulators in *S. aureus*, including *agr*, *sarA* and *sae* (Recsei *et al.*, 1986; Cheung *et al.*, 1992; Giraudo *et al.*, 1994).

Transposons are mobile genetic elements that can insert into host DNA. One of the transposons used most extensively in genetic research is Tn*917* (Tomich *et al.*, 1980; reviewed in Youngman, 1990). Tn*917* was first identified in an isolate of *Enterococcus faecalis* and is a member of the Tn*3* family of replicative transposable elements (Tomich *et al.*, 1979; Shaw & Clewell, 1985). Tn*917* is plasmid-associated and has a number of features that make it particularly useful as a genetic tool. The transposon exhibits a high degree of insertional randomness in a broad host range and creates extremely stable insertional mutations (Youngman, 1990). Furthermore, Tn*917* has been modified such that it carries a promoterless *lacZ* gene that can be fused to the regulatory elements of chromosomal genes in order to give a quantitative measure of promoter activity (Perkins & Youngman, 1986). Additional modification by Camilli *et al.* (1990) produced the plasmid pLTV1 (Figure 4.1). This comprises Tn*917* carried by a highly temperature-sensitive derivative of the vector pE194Ts to allow improved recovery of chromosomal insertions (Camilli *et al.*, 1990). In addition, the transposition frequency of Tn*917* held on pLTV1 is high (Camilli *et al.*, 1990), allowing insertion libraries to be generated on a relatively small scale.

Although pLTV1 was initially constructed for use in *L. monocytogenes* (Camilli *et al.*, 1990), it has also been used in Gram-positive bacteria, including *B. subtilis* and *B. cereus* (Clements & Moir 1998; Behravan *et al.*, 2000). This system has also been utilised successfully in *S. aureus*, where it has permitted the identification of, for

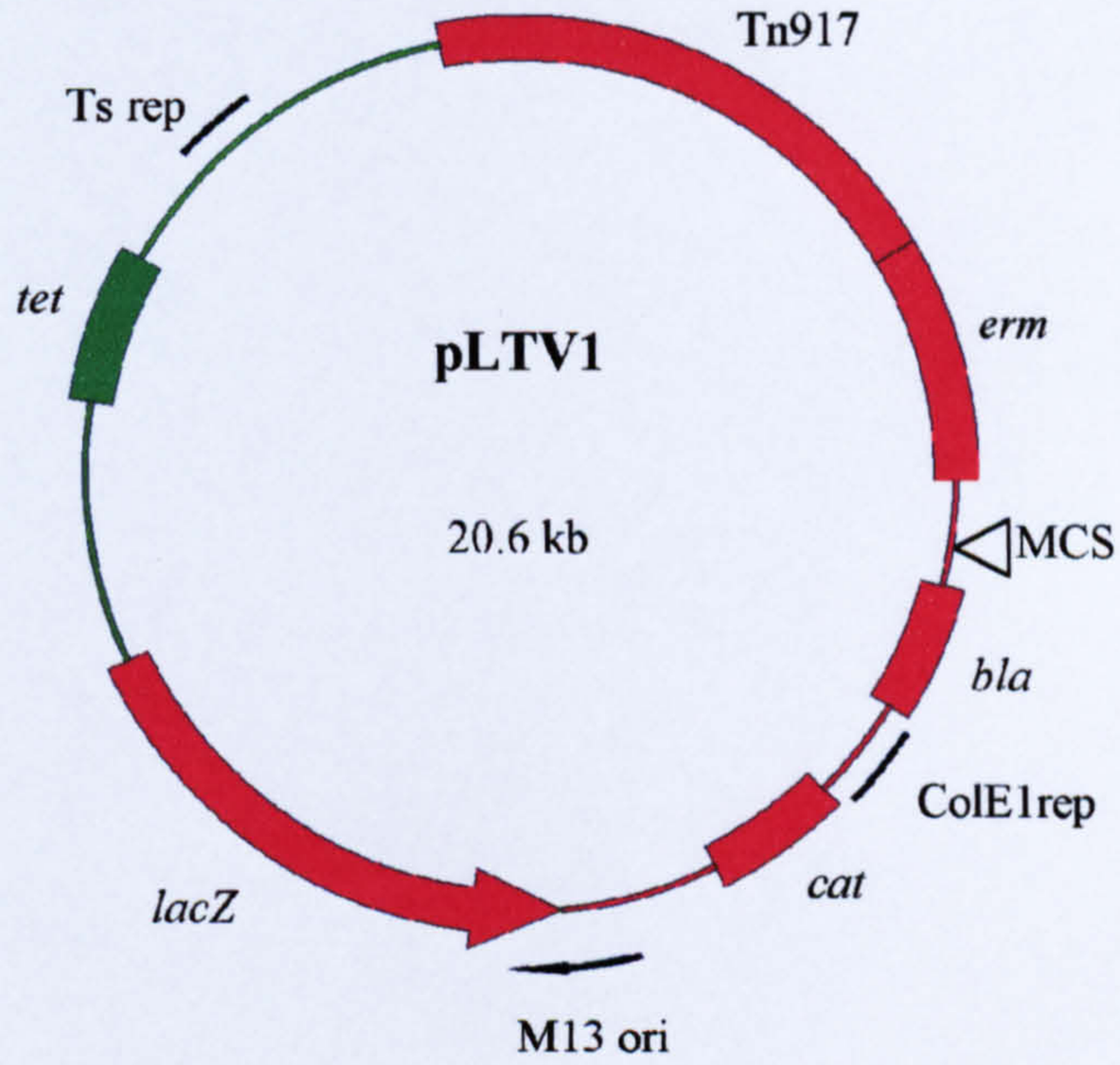


Figure 4.1

Physical map of pLTV1, the vector carrying a transposition-proficient derivative of Tn*917*.

Regions coloured in red are part of the transposable element, whilst the remainder of the vector is coloured in green. Features of this map; *erm*, erythromycin-resistance gene; MCS, multiple cloning site; *bla*, pBR322 β-lactamase gene; ColE1rep, ColE1 origin of replication; *cat*, an *S. aureus* pC194-derived chloramphenicol acetyltransferase gene; M13 ori, origin of replication for single-stranded DNA synthesis; *lacZ*, promoterless *lacZ* gene from *E. coli* with translation initiation signals derived from *B. subtilis*; *tet*, a tetracycline resistance gene from pAMα1Δ1; Ts rep, pE194Ts temperature-sensitive replicon.

Adapted from Camilli *et al.* (1990) and Youngman (1990).

example, *sarA*, *arl*, additional genes involved in virulence, and genes required for starvation survival (Cheung *et al.*, 1992; Fournier & Hooper, 2000; Coulter *et al.*, 1998; Lammers *et al.*, 2000; Vriesema *et al.*, 2000; Wiltshire & Foster, 2001; Watson *et al.*, 1998). Tn*917* has been shown to insert into the *S. aureus* genome with a relatively high degree of randomness (Coulter *et al.*, 1998), and is therefore a useful tool in the genetic analysis of *S. aureus*.

This chapter describes the use of Tn*917* in a screen to identify genes involved in regulating Hla activity in the presence of NaCl-mediated stress.

4.2 Results

4.2.1 Tn*917* mutagenesis of *S. aureus* 8325-4

The plasmid pLTV1 (Figure 4.1) is 20.6 kb in length, and comprises the transposon Tn*917* (which carries an erythromycin-resistance gene) and additional flanking sequences containing a tetracycline-resistance marker and a temperature-sensitive Gram-positive origin of replication. The copy number of pLTV1 decreases at high temperatures, with replication being totally inhibited at temperatures above 37 °C (Villafane *et al.*, 1987). Consequently, by growing cells at a temperature above 37 °C in the presence of erythromycin, cells containing a chromosomal Tn*917* insertion can be selected since resistance to erythromycin (carried by Tn*917*) will remain, whilst resistance to tetracycline (carried by pLTV1) will be lost. Two Tn*917* libraries, X and Z, were created in 8325-4 by Wiltshire and Foster (2001). The suitability of these libraries for use in screening was evaluated.

4.2.1.1 Enumeration and evaluation of Tn*917* libraries

Only libraries with > 90 % transposon insertion are considered useful for screening. Libraries with a lower percentage insertion would contain a high proportion of cells in which Tn*917* remained on pLTV1, and so screening would have to be performed on a larger scale. Using the method described in Chapter 2.15.3, libraries X and Z were

found to have a transposon insertion frequency of 94.6 % and 95 % respectively (*ie.* 2.2 X 10^9 and 5.75 X 10^8 cfu ml^{-1} of Ery resistant cells respectively, and 1.2 X 10^8 and 2.9 X 10^7 cfu ml^{-1} Ery and Tet resistant cells respectively). Libraries X and Z were therefore deemed suitable for use in screening.

4.2.2 Screen to identify mutants with altered Hla activity in response to NaCl

In Chapter 3, 1 M NaCl was shown to strongly repress *hla* transcription in BHI broth. Therefore, a transposon mutant library screen was performed to identify mutants that were still able to produce Hla on rabbit blood plates containing NaCl, as signified by a zone of haemolysis of greater diameter surrounding the colony than for the background colonies. These mutants would contain Tn*917* inserted into a gene potentially involved in repressing *hla* in the presence of NaCl-mediated stress. However, the mechanism by which *hla* transcription is lowered in the presence of NaCl might involve reduced activation of *hla* transcription in the presence of NaCl than in its absence. Therefore, mutants that were non-haemolytic were also screened for on the rabbit blood plates containing NaCl.

4.2.2.1 Screen development

4.2.2.1.1 Identification of the most appropriate concentration of NaCl to add to the rabbit blood plates

In order to determine the most suitable concentration of NaCl to add to the rabbit blood plates, plates were made at a final NaCl concentration of 85.6 mM (no added NaCl), 250 mM, 500 mM, 750 mM and 1 M (Chapter 2.1.1.7). 8325-4 from a fresh overnight BHI plate was streaked onto each of the plates in duplicate and grown overnight at 37 °C. The diameters of 3 colonies and their zones of haemolysis were measured on each plate and the average values calculated. The zones of haemolysis were found to decrease in diameter as the concentration of NaCl increased, although the diameters of the colonies remained approximately the same (Table 4.1). A final concentration of 1 M

Concentration of NaCl (mM)	Average colony diameter (mm)	Average diameter of zone of haemolysis (mm)
85.6 (no added NaCl)	0.9	2.6
250	0.9	2.4
500	0.9	1.7
750	0.9	1.6
1000	0.8	1.3

Table 4.1

The affect of NaCl on the colony diameter and Hla-mediated haemolysis of 8325-4 on rabbit blood plates.

Rabbit blood plates were made at a final NaCl concentration of 85.6 mM (no added NaCl), 250 mM, 500 mM, 750 mM and 1 M (Chapter 2.1.1.7). 8325-4 from a fresh overnight BHI plate was streaked onto each of the plates in duplicate and grown overnight at 37 °C. Growth and Hla-mediated haemolytic activity were measured as colony diameter and diameter of the zone of haemolysis respectively (mm). Average diameters were calculated after measuring 3 colonies and their zones of haemolysis per plate on duplicate plates.

NaCl was chosen at which to make the plates, as this gave a zone of haemolysis that was sufficiently reduced in size to allow mutants expressing increased levels of Hla in the presence of NaCl to be easily identified. Furthermore, since the colonies were still slightly haemolytic on the 1 M NaCl rabbit blood plates, non-haemolytic mutants could also be screened for.

4.2.2.1.2 Effect of 1 M NaCl on Hla activity

As shown in Table 4.1, the zones of haemolysis surrounding *S. aureus* colonies decreased in diameter as the concentration of NaCl in rabbit blood plates was increased. To show that this was caused solely by NaCl repressing Hla expression, and not by NaCl preventing Hla from causing haemolysis on the plates, the following control was performed. Supernatants from duplicate 12 h *S. aureus* 8325-4 cultures grown in 5 ml BHI or 5 ml BHI containing 1 M NaCl were filter-sterilised (0.2 μm pore size). The supernatants were diluted in PBS as shown in Figure 4.2, and 10 μl was spotted onto a rabbit blood plate containing no added NaCl and onto a rabbit blood plate containing 1 M NaCl. After incubation overnight at 37 °C, the supernatant of the BHI culture containing no added NaCl was as haemolytic on the rabbit blood plate containing 1 M NaCl as it was on the rabbit blood plate containing no added NaCl at each of the corresponding dilutions (Figure 4.2). This suggests that NaCl present in the plate containing 1 M NaCl had no effect on Hla activity. Furthermore, only the undiluted spots of the supernatants from the cultures grown in BHI containing 1 M NaCl caused haemolysis on either type of rabbit blood plate; the lower dilutions were non-haemolytic (results not shown). Overall, this suggests that 1 M NaCl represses the production of Hla, but does not prevent Hla from causing haemolysis on rabbit blood plates. Consequently, rabbit blood plates can be used to examine the effects of NaCl on Hla expression.

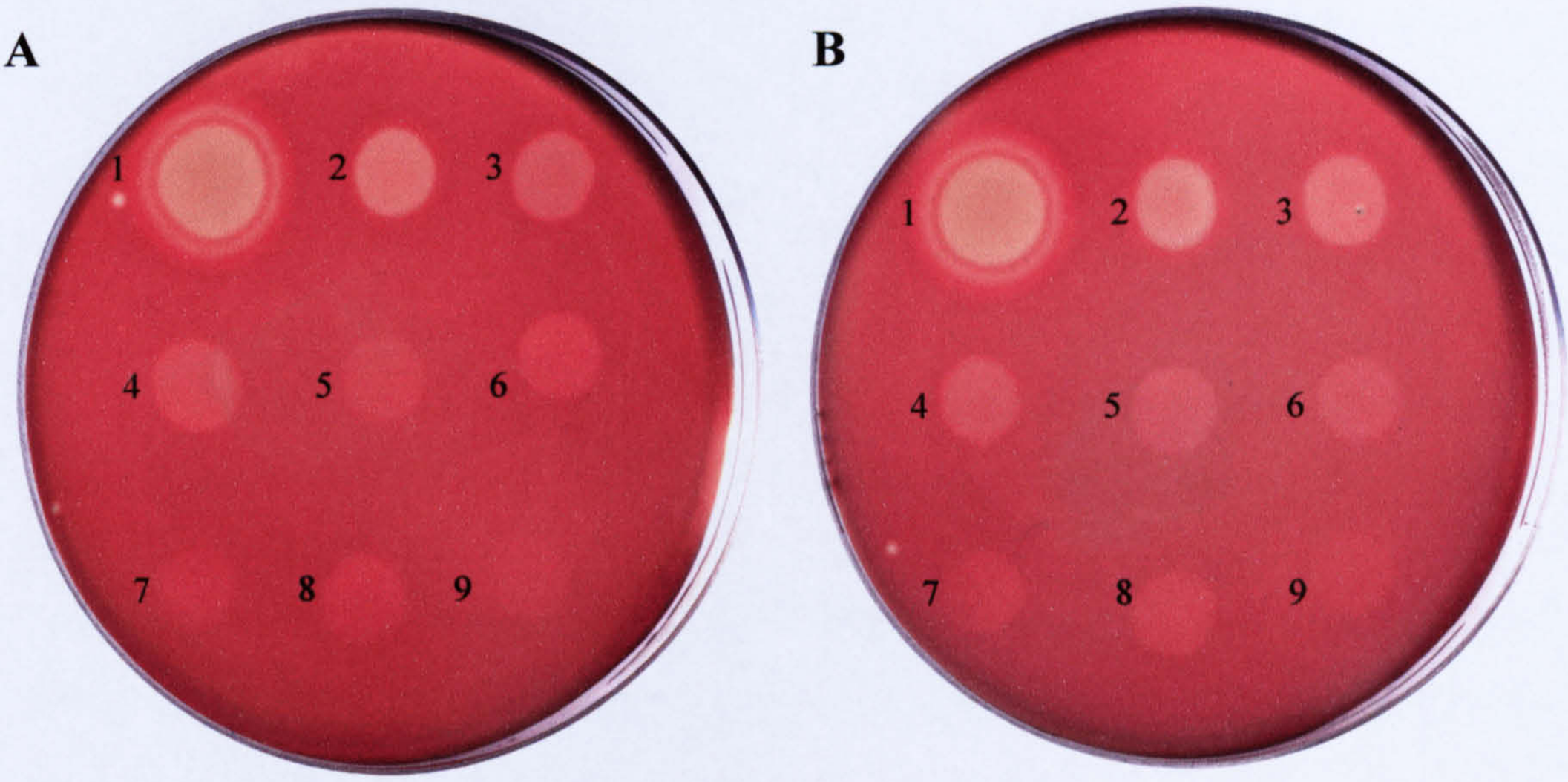


Figure 4.2

Haemolysis caused by an 8325-4 BHI culture supernatant at various dilutions on (A) a rabbit blood plate containing 1 M NaCl and on (B) a rabbit blood plate containing no added NaCl.

S. aureus 8325-4 was grown for 12 h in 5 ml BHI in a sterile universal tube at 37 °C with 250 rpm rotary shaking. Cells were removed by centrifugation (4,000 rpm (Centaur 2), 5 min), and the supernatant was filter-sterilized (0.2 μm pore size). The supernatant was then diluted using PBS and 10 μl was spotted onto a rabbit blood plate and onto a rabbit blood plate containing 1 M NaCl. Dilutions used were; 1) neat, 2) 1/25, 3) 1/50, 4) 1/75, 5) 1/100, 6) 1/125, 7) 1/150, 8) 1/175 and 9) 1/200. The plates were then incubated overnight at 37 °C. Results are representative of two independent experiments.

4.2.2.2 Primary screen

Since cells from libraries X and Z were unable to grow when plated directly from glycerol stocks onto rabbit blood plates containing 1 M NaCl, the transposon mutant libraries were first diluted using PBS to give ~ 100 colonies per plate and recovered on BHI agar plates containing Ery and Lin. After growth overnight at 37 °C, colonies were replica plated onto rabbit blood plates containing 1 M NaCl and grown overnight at 37 °C. The plates were then screened to identify non-haemolytic mutants and mutants with a zone of haemolysis of increased diameter compared to the background colonies.

~ 6,500 colonies were screened in total (~ 3,500 from library X and ~ 3,000 from library Z). 39 mutants were selected, of which 23 were non-haemolytic and 16 displayed increased haemolytic activity (Table 4.2).

4.2.2.3 Secondary screen

To ensure that the phenotypes of the Tn*917* mutants were reproducible, the strains were patched onto rabbit blood plates containing 1 M NaCl, with 8325-4 as a control. All of the mutants that were selected for their non-haemolytic phenotype displayed wild-type levels of haemolysis (results not shown) and were therefore discarded. However, all of the 16 mutants selected for increased haemolytic activity compared to the background colonies demonstrated the same mutant phenotype compared to 8325-4 and therefore passed the screen (Table 4.2).

The 16 mutants were patched onto BHI agar plates containing Ery and Lin and onto BHI agar plates containing Tet to determine whether Tn*917* was inserted into the genome of the cells or carried on pLTV1. All of the mutants were Ery resistant and Tet sensitive, and therefore possessed chromosomal Tn*917* insertions. Furthermore, when patched onto rabbit blood plates containing no added NaCl, all of the mutants displayed a similar level of haemolytic activity to 8325-4 (results not shown). This suggests that the effects of the genes mutated by Tn*917* are NaCl-specific. The inactivated genes were therefore designated *han* 1-16 (genes involved in repressing Hla activity in the presence of NaCl).

4.2.2.4 Tertiary screen

To determine whether the increased haemolytic activity of the *han* mutants compared to 8325-4 displayed on rabbit blood plates containing 1 M NaCl was caused by Tn*917* insertion or by point mutations, the Tn*917* insertions were transferred into 8325-4 via ϕ11-mediated transduction, as described in Chapter 2.9.3. For each mutation, all 10 transductants tested displayed a similar level of increased haemolytic activity as the donor mutant strain when patched onto rabbit blood plates containing 1 M NaCl (Table 4.2), and demonstrated similar levels of haemolysis to 8325-4 on rabbit blood plates containing no added NaCl (data not shown). Therefore, 100 % linkage of the mutant phenotype to the transposon insertion was demonstrated for all 16 mutants. The strain names of a representative transductant per *han* mutation are shown in Table 4.2.

A diagrammatic summary of the screening protocol and results is presented in Figure 4.3.

4.2.3 Characterisation of *han* mutants

4.2.3.1 Direct genomic DNA sequencing to identify genes inactivated by Tn*917*

To identify the genes inactivated by Tn*917* in the *han* mutants, direct sequencing of genomic DNA was performed as in Chapter 2.13.1.2.4. The primer used, Tn2, was a 25 bp oligonucleotide (Table 2.5) which binds 106 bp upstream of the *lacZ* end of the transposon. Consequently, the sequencing reaction proceeds through this end of the transposon and into the *S. aureus* genomic DNA. The sequence corresponding to the *lacZ* end of Tn*917* was removed, and the remaining data was used to identify the position of Tn*917* insertion in the *S. aureus* genome. The sequence of *S. aureus* genomic DNA is held in two main databases (Chapter 2.17.2); the NCBI BLAST website contains unannotated *S. aureus* 8325 genomic DNA sequence, whilst the TIGR website contains annotated genomic DNA sequence of strains including *S. aureus* COL. Due to its increased versatility, the TIGR database was first used to identify the position of Tn*917* insertion in the *S. aureus* COL genome. Alignments of the sequencing

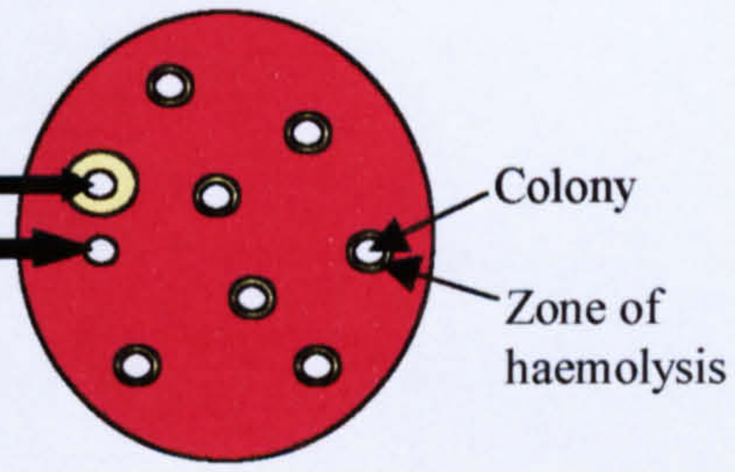
~ 6,500 Tn*917* mutants in 8325-4 background
(~ 3,500 from library X and ~ 3,000 from library Z)

↓ Cells recovered on BHI Ery Lin (37 °C overnight)

~ 100 colonies per plate

↓ Colonies replica plated onto rabbit blood plates containing 1 M NaCl (37 °C overnight)

Colonies with increased haemolysis or which were non-haemolytic were selected **(Primary screen)**



39 putative Tn*917* mutants
- 16 mutants displaying increased haemolytic activity
- 23 non-haemolytic mutants

↓ Colonies patched onto rabbit blood plates containing 1 M NaCl to ensure reproducibility of mutant phenotype (37 °C overnight) **(Secondary screen)**

16 putative mutants displaying increased haemolytic activity

↓ Mutations transduced into 8325-4 to check linkage of mutant phenotype to the transposon insertion. 10 transductants per strain patched onto rabbit blood plates containing 1 M NaCl (37 °C overnight) **(Tertiary screen)**

16 putative mutants displaying increased haemolytic activity

Figure 4.3

Diagrammatic representation of the screen performed to identify Tn*917* mutants in the 8325-4 background that were non-haemolytic or that displayed increased haemolytic activity compared to the background colonies on rabbit blood plates containing 1 M NaCl, and which may therefore contain Tn*917* insertions in genes involved in regulating Hla in response to NaCl.

reaction data with the COL DNA sequence are shown in Appendix A.2. Flanking ORFs were identified, and the putative function of the gene products were determined based on homology to proteins in other organisms (Chapter 2.17.2 and Appendix A.2.1 - A.2.9). Genes were assigned accession numbers based on those of the COL database. Finally, the COL genomic DNA sequence containing the inactivated genes and their surrounding genes of interest was extracted from the TIGR website and compared to the 8325 sequence in the NCBI BLAST database to ensure that the sequence and position of the genes in COL is the same as it is in 8325. For each region compared, the COL and 8235 genomic DNA sequence was 99 - 100 % identical (data not shown). The genes inactivated by Tn*917* are shown in Table 4.2, and are diagramatically represented with their surrounding genes of interest in Figure 4.4.

In total, 9 genes were found to have been inactivated by Tn*917* in the 16 mutants. Where a gene contained more than one insertion, Tn*917* was found to have inserted in exactly the same site for each mutation within the gene. In each case, the mutants with identical Tn*917* insertion sites were derived from the same library (Table 4.2), and are therefore likely to be siblings.

In JLA401 (8325-4 *han*1) and JLA410 (8325-4 *han*10), Tn*917* was found to have inserted into SA1896, at base 105 of the 555 bp gene. However, protein sequence analysis revealed no significant homology to a known protein (Figure A.2.1), and so a putative function for the gene could not be assigned. The gene was therefore designated *unk*1 (*unk*nown protein 1).

In JLA402 (8325-4 *han*2), JLA403 (8325-4 *han*3), JLA405 (8325-4 *han*5), JLA406 8325-4 *han*6), JLA 407 (8325-4 *han*7) and JLA408 (8325-4 *han*8), Tn*917* was found to have inserted into SA1908, at base 856 of the 1383 bp gene. Protein sequence analysis demonstrated homology to CitG, the Class-II fumarate hydratase (fumarase), of *B. subtilis* (66 % identity over 460 amino acids).

In JLA404 (8325-4 *han*4), Tn*917* was found to have inserted into SA1418, at base 124 of the 342 bp gene. However, protein sequence analysis revealed no significant homology to a known protein (Figure A.2.3), and so a putative function for the gene could not be assigned. The gene was therefore designated *unk*2 (*unk*nown protein 2).

In JLA409 (8325-4 *han*9), Tn*917* was found to have inserted into SA1384, at base 242 of the 1644 bp gene. Protein sequence analysis demonstrated homology to the glycine betaine transporter OpuD of *B. subtilis* (56 % identity over 496 amino acids).

In JLA411 (8325-4 *han*11), Tn*917* was found to have inserted into SA1551, at base 1,394 of the 1,647 bp gene. Protein sequence analysis demonstrated homology to a number of alpha-1,4-glucosidases and oligo-1,6-glucosidases, including the exo-alpha-1,4-glucosidase homolog YugT of *B. subtilis* (56 % identity over 549 amino acids).

In JLA412 (8325-4 *han*12), Tn*917* was found to have inserted into SA1414, at base 294 of the 699 bp gene. Protein sequence analysis demonstrated homology to a number of oligopeptide ABC (ATP-binding cassette) transporter ATP-binding proteins, including OppF of *Fusobacterium nucleatum* (31 % identity over 223 amino acids). Analysis of the DNA surrounding the inactivated gene identified the presence of an operon comprising three other oligopeptide ABC transporter genes. Furthermore, the gene inactivated by Tn*917* in JLA412 was found to be 4 genes downstream of the gene inactivated by Tn*917* in JLA404 (8325-4 *han*4 (*unk2*)).

In JLA413 (8325-4 *han*13), Tn*917* was found to have inserted into SA1059, at base 898 of the 1008 bp gene. Protein sequence analysis demonstrated homology to the conserved hypothetical protein YkrP of *B. subtilis* (35 % identity over 278 amino acids). Although the function of YkrP was unknown in the Subtilist database (Chapter 2.17.2), the protein sequence of SA1059 also showed homology to a putative integral membrane protein of *Streptomyces coelicolor* (25 % identity over 296 amino acids) and acetyltransferases, including the acetyltransferase nodulation protein NolL of *Mesorhizobium loti* (23 % identity over 309 amino acids).

In JLA414 (8325-4 *han*14), Tn*917* was found to have inserted into SA1265, at base 1078 of the 1242 bp gene. Protein sequence analysis demonstrated homology to the endopeptidase resistance / peptidoglycan biosynthesis protein *eprH* of *S. aureus* (100 % identity over 414 amino acids).

In JLA415 (8325-4 *han*15) and JLA416 (8325-4 *han*16), Tn*917* was found to have inserted into SA1395, at base 1202 of the 1206 bp gene. Protein sequence analysis

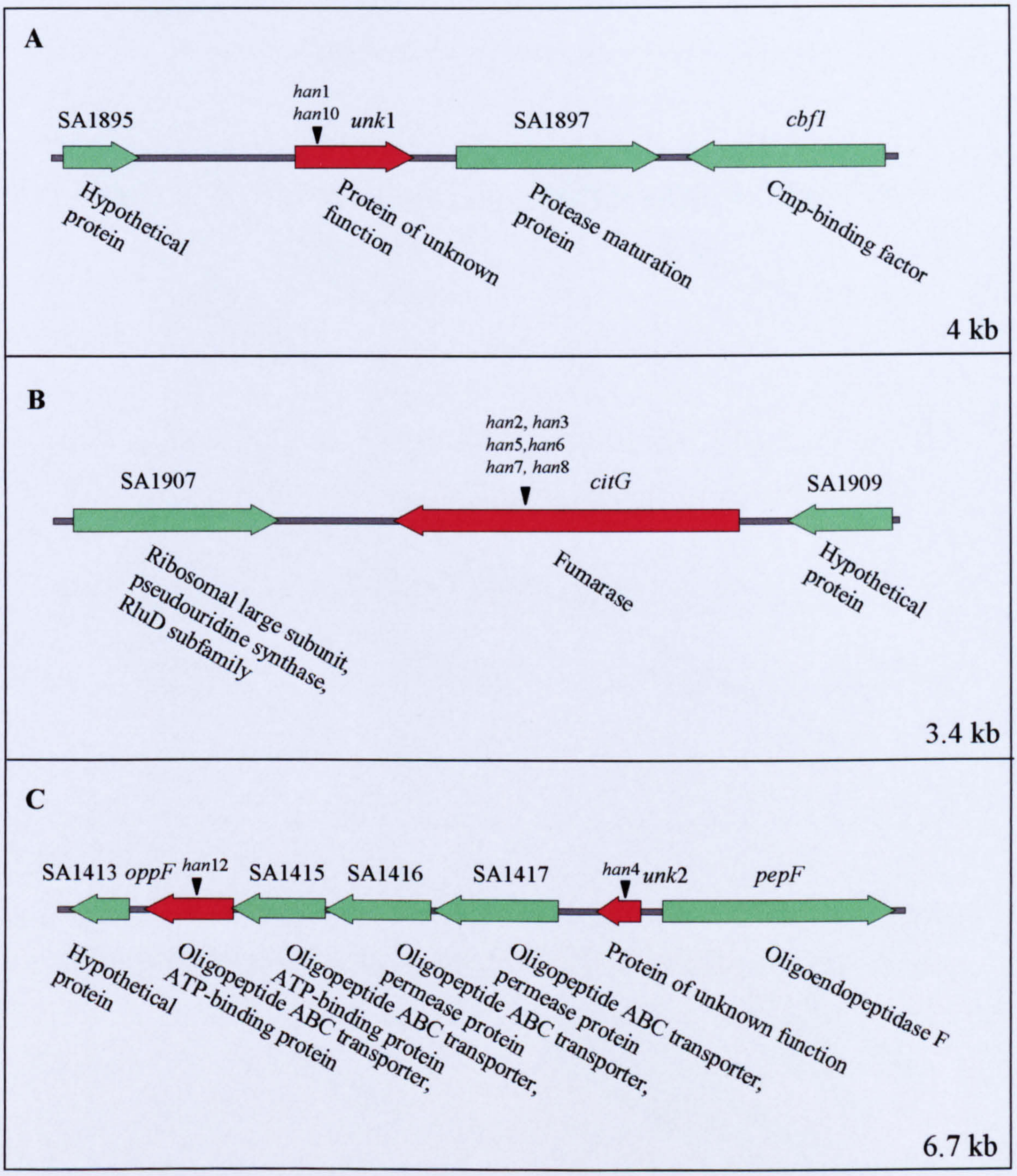


Figure 4.4 a-c

Position of Tn*917* insertions in *han* mutants, and surrounding genes of interest for (A) *han*1 and *han*10 (*unk*1), (B) *han*2, *han*3, *han*5, *han*6, *han*7 and *han*8 (*citG*), and (C) *han*12 (*oppF*) and *han*4 (*unk*2) .

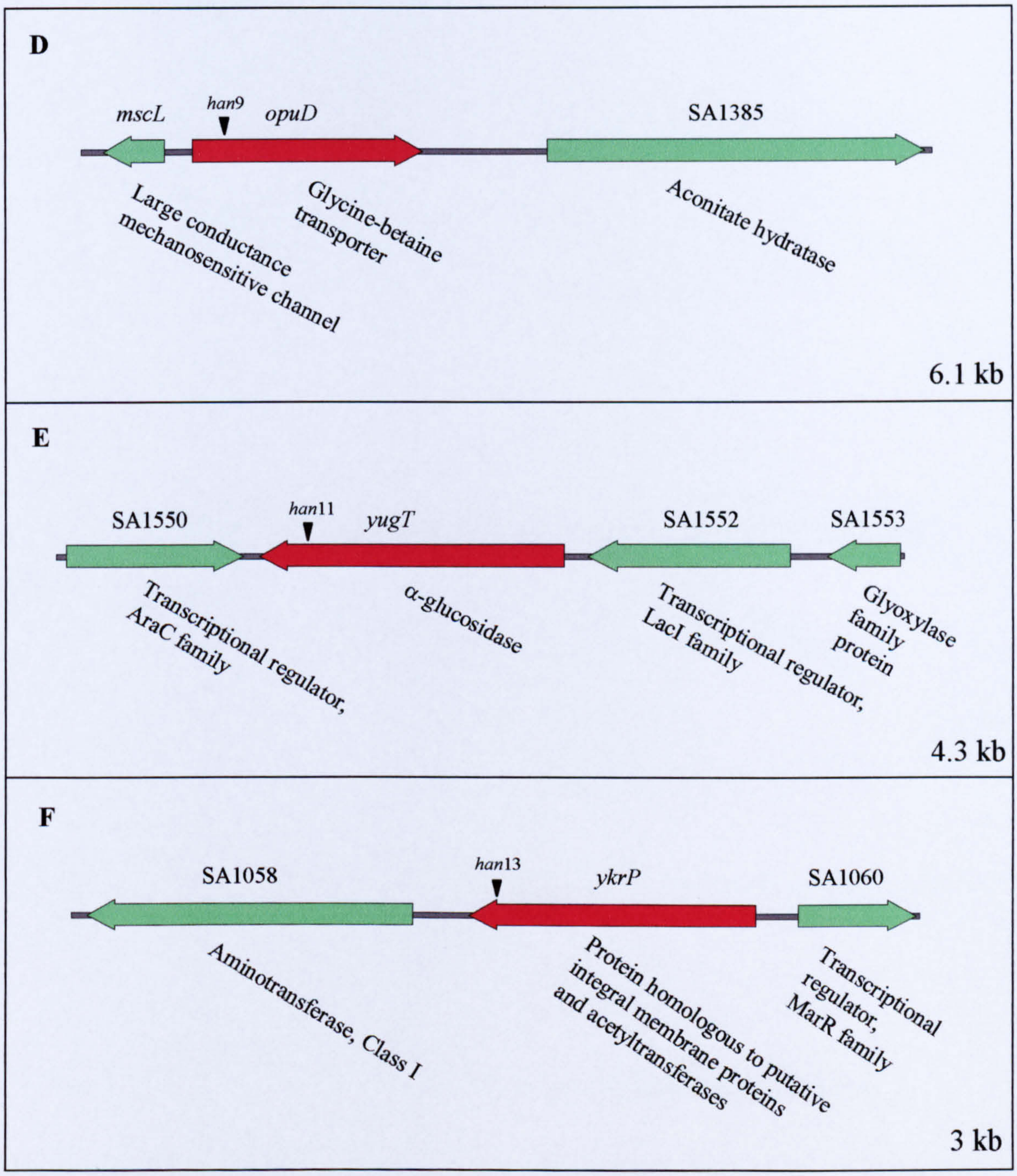


Figure 4.4 d-f

Position of Tn*917* insertions in *han* mutants, and surrounding genes of interest for (D) *han9* (*opuD*), (E) *han*11 (*yugT*), and (F) *han*13 (*ykrP*).

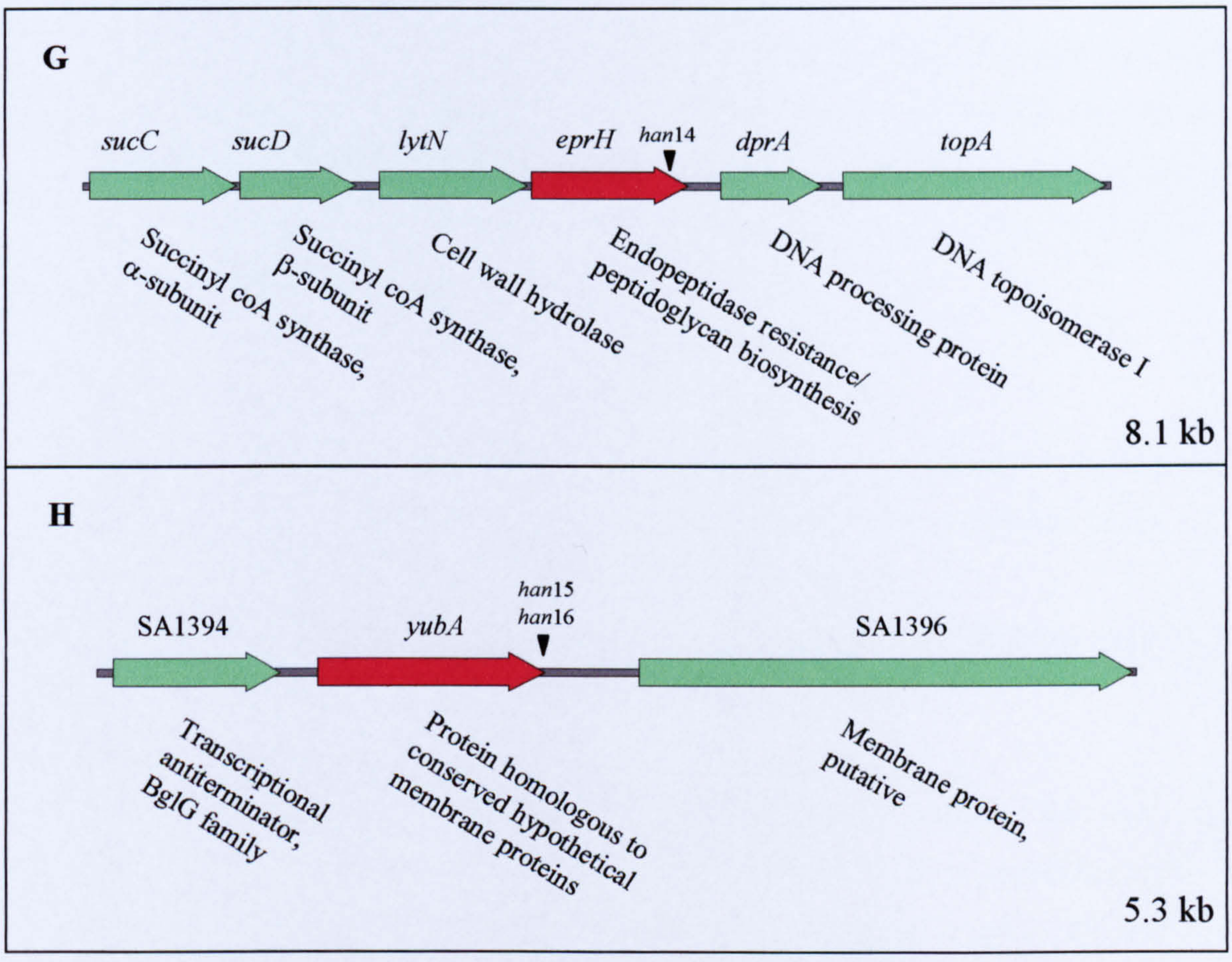


Figure 4.4 g-h

Position of Tn*917* insertions in *han* mutants, and surrounding genes of interest for (G) *han*14 (*eprH*) and (H) *han*15 and *han*16 (*yubA*).

Figure 4.4 a-h

Position of Tn*917* insertions in *han* mutants, and surrounding genes of interest.

Genes inactivated by Tn*917* are presented in red, and the position of Tn*917* insertion is indicated (▼). Flanking genes of interest are shown in green. The approximate size of each DNA fragment is shown in the bottom right-hand corner of each diagram. The information for each mutation was obtained by analysing sequence data (Chapter 2.17.2) from strains derived by transduction of the *han* mutations into an 8325-4 background (see Table 4.2 for strain names).

demonstrated homology to the conserved hypothetical protein YubA of *B. subtilis* (40 % identity over 366 amino acids). Although the function of YubA was unknown in the Subtilist database (Chapter 2.17.2), the protein sequence of SA1395 also showed homology to conserved hypothetical proteins and membrane proteins, including a conserved hypothetical membrane protein from *Listeria monocytogenes* EGD-e (38 % identity over 367 amino acids).

4.2.3.2 Analysis of the effects of *han* mutations on virulence determinant expression in the presence and absence of NaCl-mediated stress

4.2.3.2.1 Haemolytic activity of *han* mutants on rabbit blood plates containing either no added NaCl or 1 M NaCl

Photographs showing the haemolysis of a representative mutant for each gene inactivated by Tn*917*, compared to 8325-4, on rabbit blood plates containing either no added NaCl or 1 M NaCl are presented in Figure 4.5. Mutation of *unk*1, *citG* and *oppF* led to greatly increased haemolytic activity compared to 8325-4 on rabbit blood plates containing 1 M NaCl, whilst mutation of *unk*2, *opuD, yugT, ykrP, eprH* and *yubA* led to slightly increased haemolysis in the presence of 1 M NaCl. Scores for the level of haemolysis for each of the mutants, compared to 8325-4, are shown in Table 4.2. On rabbit blood plates containing no added NaCl, each of the mutants demonstrated a similar level of haemolytic activity to 8325-4.

4.2.3.2.1.1 Determination of whether the increased haemolysis of *han* mutants on rabbit blood plates containing 1 M NaCl is due to increased Hla expression, or due to the increased production of another haemolytic factor

####### 4.2.3.2.1.1.1 Construction of a tetracycline-resistant *hla* cassette knock-out

To determine whether the increased haemolytic activity of the *han* mutants on rabbit blood plates containing 1 M NaCl is due to increased Hla expression or due to the

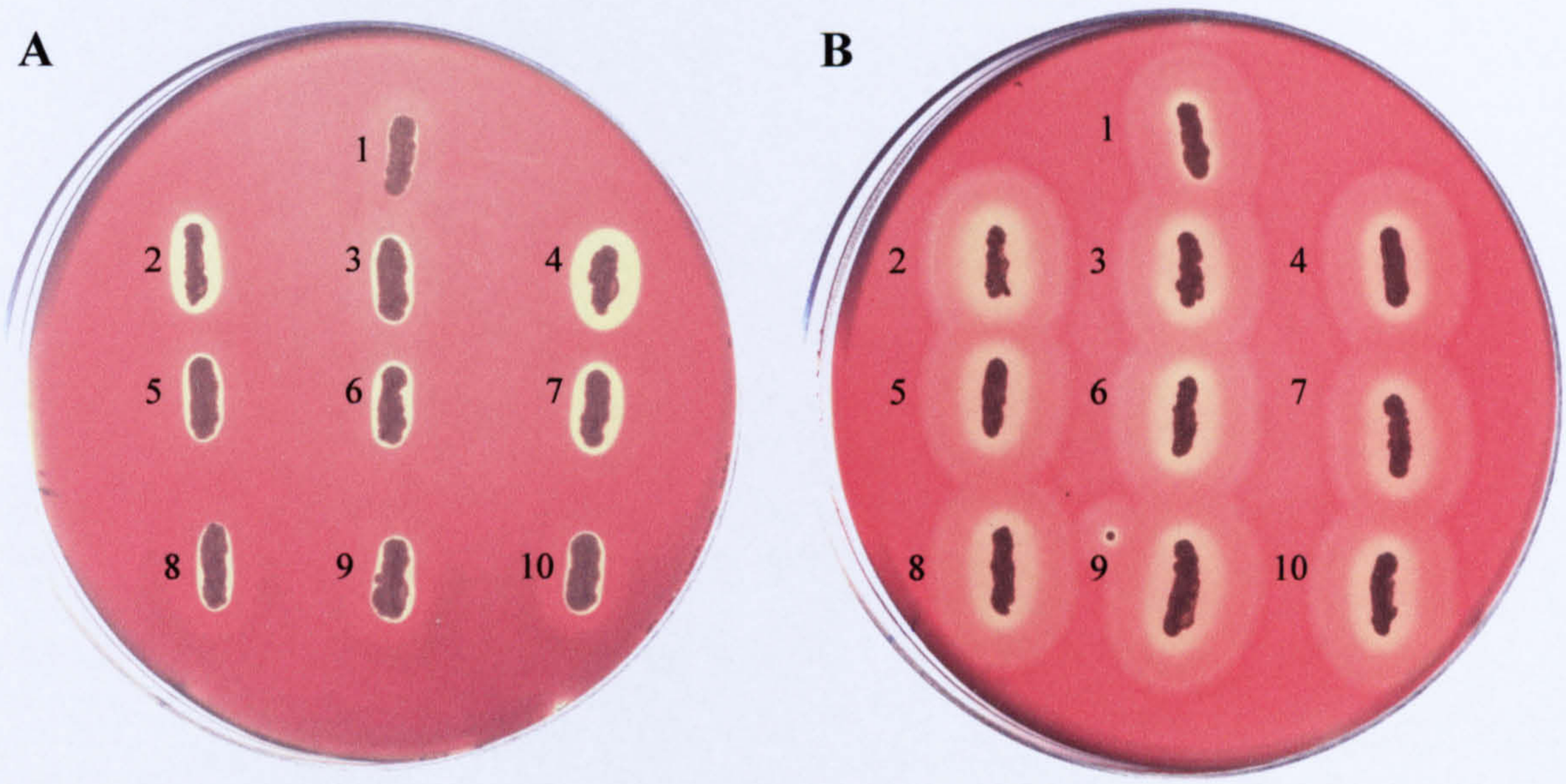


Figure 4.5

Haemolytic activity of representative *han* mutants compared to 8325-4 on (A) a rabbit blood plate containing 1 M NaCl and on (B) a rabbit blood plate containing no added NaCl.

Plates were patched with strains 1) 8325-4, 2) JLA401 (8325-4 *han*1 (*unk*1)), 3) JLA404 (8325-4 *han*4 (*unk*2)), 4) JLA402 (8325-4 *han*2 (*citG*)), 5) JLA409 (8325-4 *han*9 (*opuD*)), 6) JLA411 (8325-4 *han*11 (*yugT*)), 7) JLA412 (8325-4 *han*12 (*oppF*)), 8) JLA413 (8325-4 *han*13 (*ykrP*)), 9) JLA414 (8325-4 *han*14 (*eprH*)), and 10) JLA415 (8325-4 *han*15 (*yubA*)) and incubated overnight at 37 °C. Results are representative of two independent experiments.

han no.	Strain *no.* after transduction into 8325-4	Lib-rary	Haemolytic activity (+ 1 M NaCl)[a]			*S. aureus* gene no.[b]	Gene[c]	Function[c]	Organism	Identity
			1°	2°	3°					
1	JLA401	X	+++	+++	+++	SA1896	*unk*1	No significant homology to a known protein	-	-
2	JLA402	X	+++	+++	+++	SA1908	*citG*	Fumarase, class II	*B. subtilis*	66% (307/460)
3	JLA403	X	+++	+++	+++	SA1908	*citG*	Fumarase, class II	*B. subtilis*	66% (307/460)
4	JLA404	X	++	++	++	SA1418	*unk*2	No significant homology to a known protein	-	-
5	JLA405	X	+++	+++	+++	SA1908	*citG*	Fumarase, class II	*B. subtilis*	66% (307/460)
6	JLA406	X	+++	+++	+++	SA1908	*citG*	Fumarase, class II	*B. subtilis*	66% (307/460)
7	JLA407	X	+++	+++	+++	SA1908	*citG*	Fumarase, class II	*B. subtilis*	66% (307/460)
8	JLA408	X	+++	+++	+++	SA1908	*citG*	Fumarase, class II	*B. subtilis*	66% (307/460)
9	JLA409	X	++	++	++	SA1384	*opuD*	Glycine betaine transporter	*B. subtilis*	56% (278/496)
10	JLA410	X	+++	+++	+++	SA1896	*unk*1	No significant homology to a known protein	-	-
11	JLA411	Z	++	++	++	SA1551	*yugT*	Exo-alpha-1,4-glucosidase	*B. subtilis*	56% (308/549)
12	JLA412	Z	+++	+++	+++	SA1414	*oppF*	Oligopeptide ABC transporter ATP-binding protein	*F. nucleatum*	31% (70/223)
13	JLA413	Z	++	++	++	SA1059	*ykrP*	Conserved hypothetical protein	*B. subtilis*	35% (100/278)
14	JLA414	Z	++	++	++	SA1265	*eprH*	Endopeptidase resistance/peptidoglycan synthesis	*S. aureus*	100% (414/414)
15	JLA415	Z	++	++	++	SA1395	*yubA*	Conserved hypothetical protein	*B. subtilis*	40% (149/366)
16	JLA416	Z	++	++	++	SA1395	*yubA*	Conserved hypothetical protein	*B. subtilis*	40% (149/366)

Table 4.2

Summary of the phenotypic and sequence analysis results for the Tn*917 han* mutants.

[a] Haemolytic activity on rabbit blood plates containing 1 M NaCl, during primary (1°), secondary (2°) and tertiary (3°) screens, compared to the background colonies for the primary screen and to 8325-4 for the secondary and tertiary screens (++ = slightly increased haemolytic activity. +++ = greatly increased haemolytic activity (see Figure 4.5)).

[b] *S. aureus* gene number according to the TIGR *S. aureus* COL database (Chapter 2.17.2).

[c] Highest BLASTP hit against a named gene (Chapters 2.17.2 and A.2).

increased production of another haemolytic factor, attempts were made to construct a tetracycline-resistant *hla* cassette knock-out. This would be transduced into the erythromycin-resistant Tn*917* mutants to determine whether their mutant phenotype is dependent on the production of Hla. A cassette knock-out of *hla* and an *hla::lacZ* fusion have already been constructed (O'Reilly *et al.*, 1986; Chan & Foster, 1998a). However, since these both carry erythromycin-resistance markers, they cannot be used to determine whether the *han* genes inactivated by Tn*917* specifically affect *hla* without first constructing cassette knock-outs of the genes using a selectable marker other than erythromycin-resistance.

The initial strategy to construct a tetracycline-resistant *hla* cassette knock-out aimed to clone a *Hin*dIII / *Bam*HI-flanked 2.9 kb PCR product (containing the whole 1 kb *hla* gene and approximately 1 kb of flanking DNA) into *Hin*dIII / *Bam*HI-cut pMUTIN4. The resulting plasmid would then be cut with *Kpn*I (to remove an ~ 600 bp fragment from within *hla*) and ligated to a *Kpn*I-flanked tetracycline resistance cassette, PCR amplified from pDG1513 using primers OL-32 and OL-33.

Attempts to clone the *hla* insert into pMUTIN4 (Chapter 2.13.6.1.1) produced hundreds of ampicillin-resistant *E. coli* DH5α transformants. However, when 60 transformants were tested by colony PCR screening (Chapter 2.13.1.2.5) with the primers used to amplify the *hla* insert (*ie.* JA-5 and JA-6), none were found to contain the *hla* insert. Plasmid DNA was prepared from 5 transformants grown in LB containing 50 μg ml^{-1} Amp (Chapter 2.11.2). The plasmid DNA was cut with *Bam*HI (Chapter 2.13.2) and run on a 1 % (w/v) agarose gel (Chapter 2.13.5). The presence of a single ~ 8.6 kb band showed that all 5 transformants contained recircularised pMUTIN4 (data not shown). This suggests that either *Bam*HI or *Hin*dIII had not cut the vector DNA when it was being prepared for ligation. However, when the same pMUTIN4 stock was cut with either *Bam*HI or *Hin*dIII (Chapter 2.13.2) and run on a 1 % (w/v) agarose gel (Chapter 2.13.5), both enzymes were found to be capable of cutting pMUTIN4 (data not shown).

To ensure that both *Hin*dIII and *Bam*HI cut the vector DNA fully, plasmid pMAL23 (pMUTIN4 containing a *Hin*dIII / *Bam*HI insert) was cut with *Hin*dIII and *Bam*HI (Chapter 2.13.6.1.2). Since this removed the insert, the vector DNA was known to have been cut fully with both enzymes. Additionally, the 2.9 kb *hla* insert was cloned into

the vector pCR-II-TOPO to create the plasmid pJA3 (Chapter 2.13.6.1.2) (Figure 4.6). Construction of pJA3 was verified by restriction mapping with *Hin*dIII / *Bam*HI or with *Kpn*I (Chapter 2.13.6.1.2) (data not shown). The *hla* insert was then cut from the pCR-II-TOPO backbone using *Hin*dIII and *Bam*HI (Chapter 2.13.2). Since a discrete 2.9 kb insert band was produced, both enzymes were shown to have fully cut the *hla* insert DNA (data not shown). However, attempts to clone the *hla* insert into the pMUTIN4 vector backbone cut from pMAL23 (Chapter 2.13.6.1.2) yielded no *E. coli* DH5α transformants.

Therefore, a new cloning strategy was developed whereby construction of the *hla* cassette knock-out would be completed in pJA3. A counter-selectable erythromycin-resistance cassette would be introduced into the vector backbone of pJA3, since the kanamycin-resistance cassette present on the pCR-II-TOPO backbone is not suitable for selection in Gram-positive organisms. The *Kpn*I-flanked tetracycline resistance cassette could then be used to insertionally inactivate *hla*. However, the MCS of pCR-II-TOPO contains a *Kpn*I site, and a site for its isoschizomer Asp718. Nevertheless, these sites could be removed by digesting pJA3 with *Hin*dIII, which cuts at the start of the *hla* insert and in the MCS upstream of the *Kpn*I and *Asp*718 sites. *Hin*dIII-cut pJA3 was therefore ligated to the *Hin*dIII-flanked erythromycin-resistance cassette cut from pDG646 (Chapter 2.13.6.1.3) to create the plasmid pJA4 (Figure 4.7). Transformation of the ligation reaction into *E. coli* DH5α (Chapter 2.13.6.1.3) yielded a single erythromycin-resistant colony. Plasmid DNA was prepared from this colony by large scale plasmid preparation (Chapter 2.11.3). Restriction mapping by double-cutting with *Bam*HI and *Hin*dIII identified the correct 1.6 kb Ery cassette band, the 2.9 kb hla insert band, and the remaining 4 kb vector backbone band (Figure 4.7, restriction fragment data not shown). Restriction mapping with *Kpn*I yielded the correct 600 bp band from within *hla* (Figure 4.7, restriction fragment data not shown). However, instead of producing a single 7.9 kb band representing the remaining plasmid, an ~ 2.7 kb and an ~ 5.2 kb band were produced (Figure 4.7, restriction fragment data not shown). This suggests that *Hin*dIII had not cut at the site upstream of *Kpn*I in the pCR-II-TOPO MCS when preparing pJA3 for ligation with the Ery cassette, and consequently the *Kpn*I site had not been removed. Due to time restrictions, attempts to construct the tetracycline-resistant *hla* cassette knock-out were abandoned.

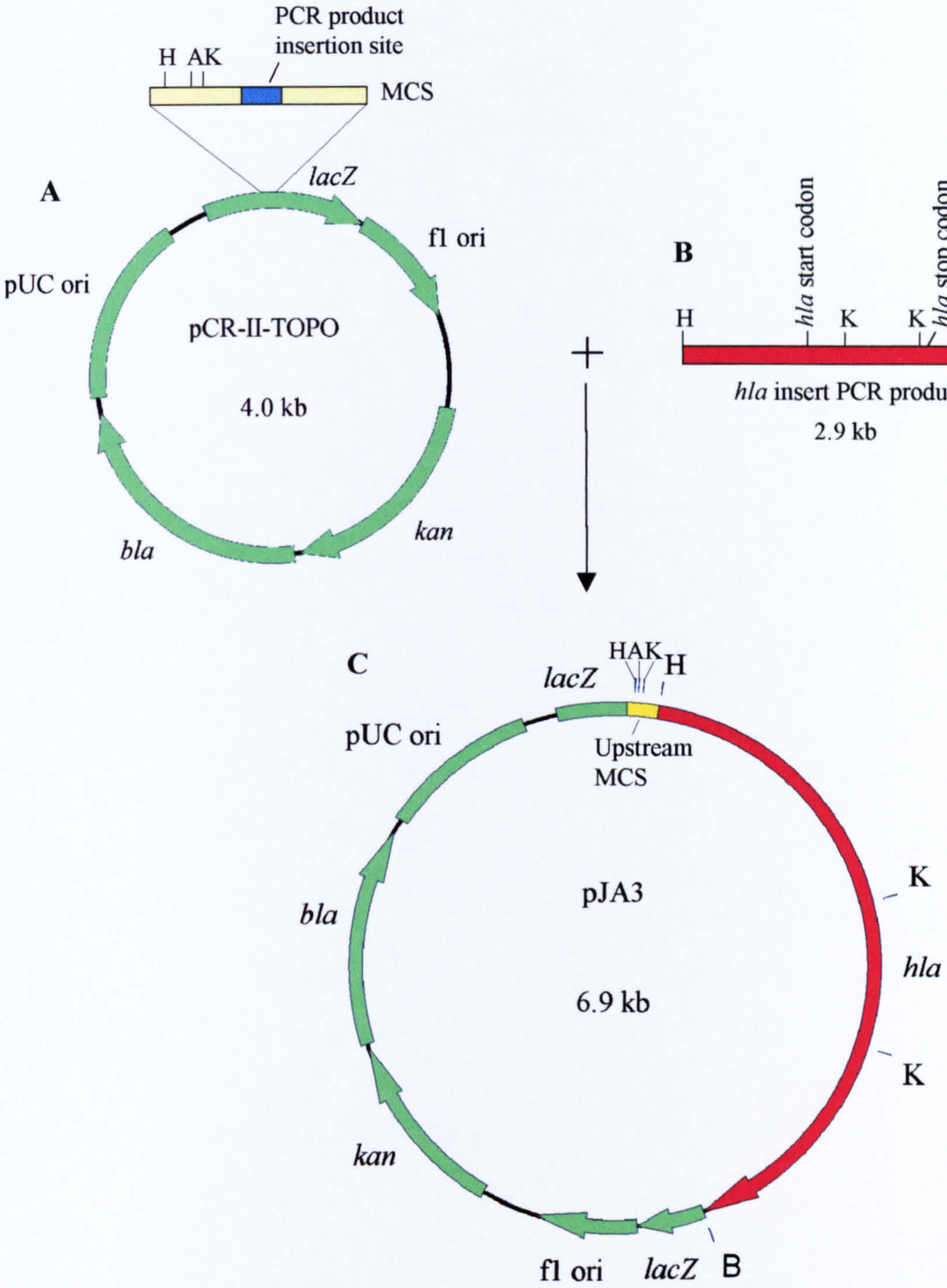
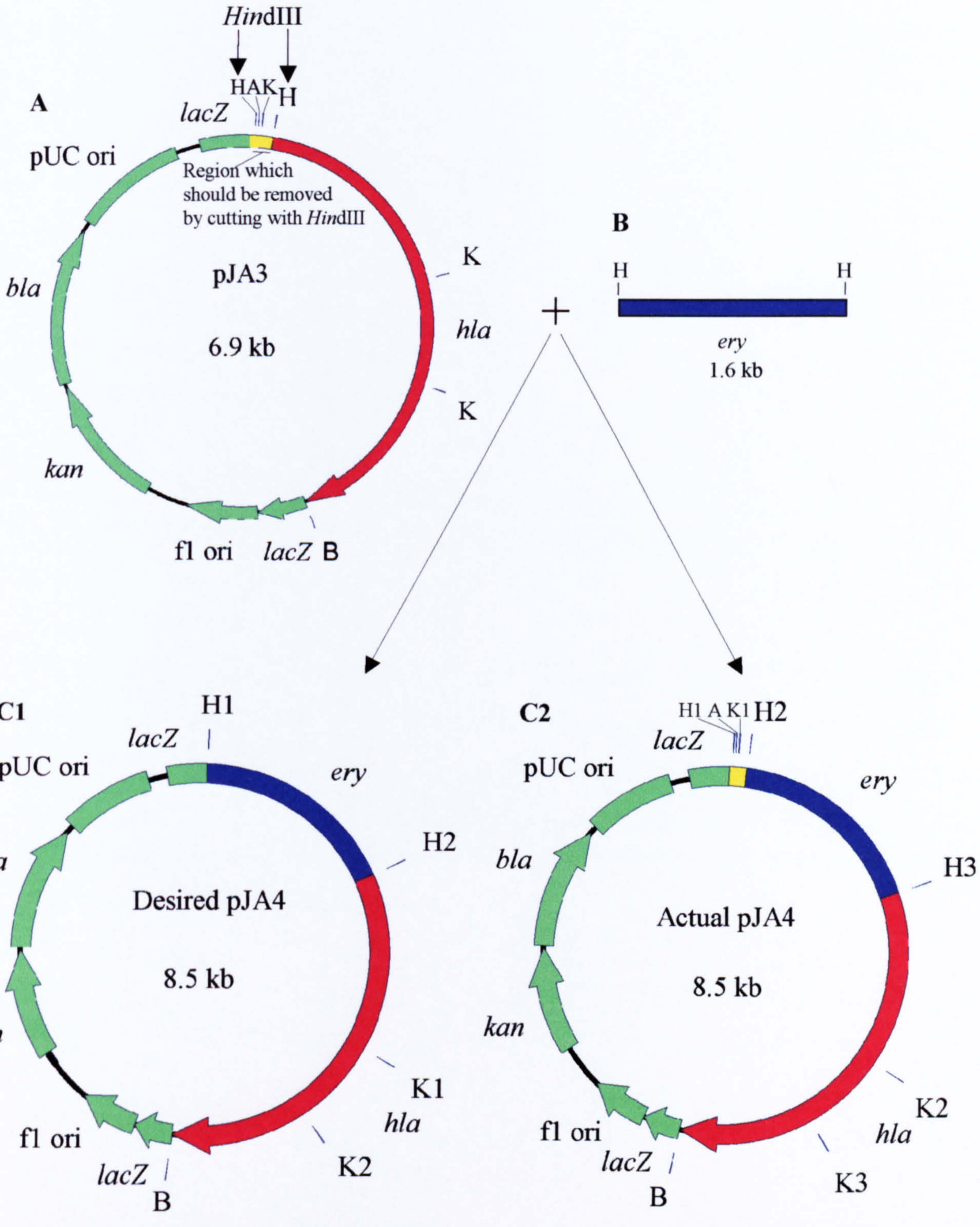


Figure 4.6

Construction of pJA3.

(A) pCR-II-TOPO and (B) a 2.9 kb *hla* insert were used to create (C) pJA3. Features of this diagram; *lacZ*, promoterless β-galactosidase gene; f1 ori, f1 origin of replication; *kan*, kanamycin resistance cassette; *bla*, β-lactamase gene; pUC ori, pUC origin of replication; *hla*, whole *hla* gene plus ~ 1 kb flanking DNA. Relevant restriction sites; A, *Asp*718; B, *Bam*H1; H, *Hin*dIII; K, *Kpn*I. The site where the *hla* insert was introduced into the pCR-II-TOPO MCS is shown in blue. Not to scale.



*Bam*HI/*Hin*dIII restriction profile;
H1 - H2 = 1.6 kb
H2 - B = 2.9 kb
B - H1 = 4.0 kb

*Kpn*I restriction profile;
K1 - K2 = 0.6 kb
K2 - K1 = 7.9 kb

*Bam*HI/*Hin*dIII restriction profile;
H1 - H2 = 1.6 kb
H2 - B = 2.9 kb
B - H1 = 4.0 kb

*Kpn*I restriction profile;
K1 - K2 = 2.65 kb
K2 - K3 = 0.6 kb
K3 - A = 5.25 kb
A - K1 = 4 bp

Figure 4.7

Construction of pJA4.

(A) *Hin*dIII-cut pJA3 and (B) an erythromycin-resistance cassette (cut from pDG646 using *Hin*dIII) were used to construct (C) pJA4 (C1; diagram of desired pJA4, C2; actual pJA4 produced).

Features of this diagram; *lacZ*, promoterless β-galactosidase gene; f1 ori, f1 origin of replication; *kan*, kanamycin resistance cassette; *bla*, β-lactamase gene; pUC ori, pUC origin of replication; *ery*, erythromycin-resistance cassette from pDG646; *hla*, whole *hla* gene plus ~ 1 kb flanking DNA. Relevant restriction sites; A, *Asp*718; B, *Bam*H1; H, *Hin*dIII; K, *Kpn*I. The region which should be removed from pJA3 by cutting with *Hin*dIII are shown in (A). Not to scale.

4.2.3.2.1.1.2 Haemolytic activity of *han* mutants in an *agr* background on rabbit blood plates containing either no added NaCl or 1 M NaCl

Since attempts to construct a tetracycline-resistant *hla* knock-out were unsuccessful (Chapter 4.2.3.2.1.1.1), it was not possible to determine whether the mutant phenotype of the Tn*917 han* mutants was dependent specifically on Hla, or on another haemolytic factor. Therefore, the *agr* mutation from PC6911 (8325-4 *agr*) was transferred by ϕ11-mediated transduction to JLA401 (8325-4 *han*1 (*unk*1)), JLA402 (8325-4 *han*2 (*citG*)), JLA404 (8325-4 *han*4 (*unk2*)), JLA409 (8325-4 *han*9 (*opuD*)), JLA411 (8325-4 *han*11 (*yugT*)), JLA412 (8325-4 *han*12 (*oppF*)), JLA413 (8325-4 *han*13 (*ykrP*)), JLA414 (8325-4 *han*14 (*eprH*)) and JLA415 (8325-4 *han*15 (*yubA*)). This produced the strains JLA417 (8325-4 *han*1 (*unk*1) *agr*), JLA418 (8325-4 *han*2 (*citG*) *agr*), JLA419 (8325-4 *han*4 (*unk2*) *agr*), JLA420 (8325-4 *han*9 (*opuD*) *agr*), JLA421 (8325-4 *han*11 (*yugT*) *agr*), JLA422 (8325-4 *han*12 (*oppF*) *agr*), JLA423 (8325-4 *han*13 (*ykrP*) *agr*), JLA424 (8325-4 *han*14 (*eprH*) *agr*) and JLA425 (8325-4 *han*15 (*yubA*) *agr*) respectively. The *agr* strains were patched onto rabbit blood plates containing either no added NaCl or 1 M NaCl, with 8325-4 and PC6911 (8325-4 *agr*) as controls. After incubation overnight at 37 °C, all of the *agr han* mutants were found to be non-haemolytic on both types of rabbit blood plate (data not shown). This indicates that the increased haemolytic activity of the *han* mutants on rabbit blood plates containing 1 M NaCl is due to the increased production of an *agr*-dependent virulence determinant, and is not caused by, for example, an increase in the production of acid. Furthermore, 10 µl spots of purified 100 ng μl^{-1} SspA (Chapter 2.3) were unable to lyse rabbit blood plates containing either no added NaCl or 1 M NaCl, but were able to cause a zone of proteolysis on milk plates following incubation overnight at 37 °C (data not shown). Additionally, JL140 (8325-4 *hla*) was found to be completely non-haemolytic on a rabbit blood plate containing 1 M NaCl, whilst 8325-4 was slightly haemolytic (data not shown).

4.2.3.2.2 Quantitative measurement of haemolytic activity of *han* mutants in BHI containing either no added NaCl or 1 M NaCl

To quantitatively compare the haemolytic activity of the *han* mutants to that of 8325-4, haemolysin assays were performed using samples obtained from 8325-4, JLA401 (8325-4 *han*1 (*unk*1)), JLA402 (8325-4 *han*2 (*citG*)), JLA404 (8325-4 *han*4 (*unk*2)), JLA409 (8325-4 *han*9 (*opuD*)), JLA411 (8325-4 *han*11 (*yugT*)), JLA412 (8325-4 *han*12 (*oppF*)), JLA413 (8325-4 *han*13 (*ykrP*)), JLA414 (8325-4 *han*14 (*eprH*)) and JLA415 (8325-4 *han*15 (*yubA*)) grown in BHI containing either no added NaCl or 1 M NaCl for 14 h (Chapter 2.18.1). All of the mutants demonstrated a similar level of haemolytic activity to 8325-4 when grown in BHI containing no added NaCl; the mutants expressed between 3277 HU and 3922 HU, whilst 8325-4 expressed 3527 HU (Figure 4.8). However, attempts to measure the haemolytic activity of samples taken from strains grown in BHI containing 1 M NaCl gave readings below the level of accuracy of the spectrophotometer (data not shown).

To determine whether NaCl present in the samples interfered with the ability of Hla to lyse the rabbit erythrocytes, solid NaCl was added to a 100 µl aliquot of supernatant from 8325-4 (grown in BHI containing no added NaCl) to give a final concentration of 1 M. The sample was incubated on ice for 15 min to allow the NaCl to dissolve and interact with Hla. The haemolytic activity of this sample and of a 100 µl aliquot of untreated supernatant from the same tube was then measured. The experiment was performed in duplicate. The haemolytic activity of untreated supernatant and of supernatant supplemented with NaCl was 2769 HU and 2174 HU respectively. This suggests that NaCl may prevent Hla from lysing rabbit erythrocytes in liquid assays to a minor degree. However, the extent to which NaCl affected the haemolysin assay is not sufficient to account for the inability to detect haemolytic activity in samples taken from the *han* mutants grown in BHI containing 1 M NaCl if they were able to over-express Hla compared to 8325-4 under these conditions to the same extent as they did on rabbit blood plates containing 1 M NaCl. This indicates that the inability to measure the haemolytic activity of the samples obtained from *han* mutants grown in BHI containing 1 M NaCl was most likely due to the expression of a low level of Hla under these conditions.

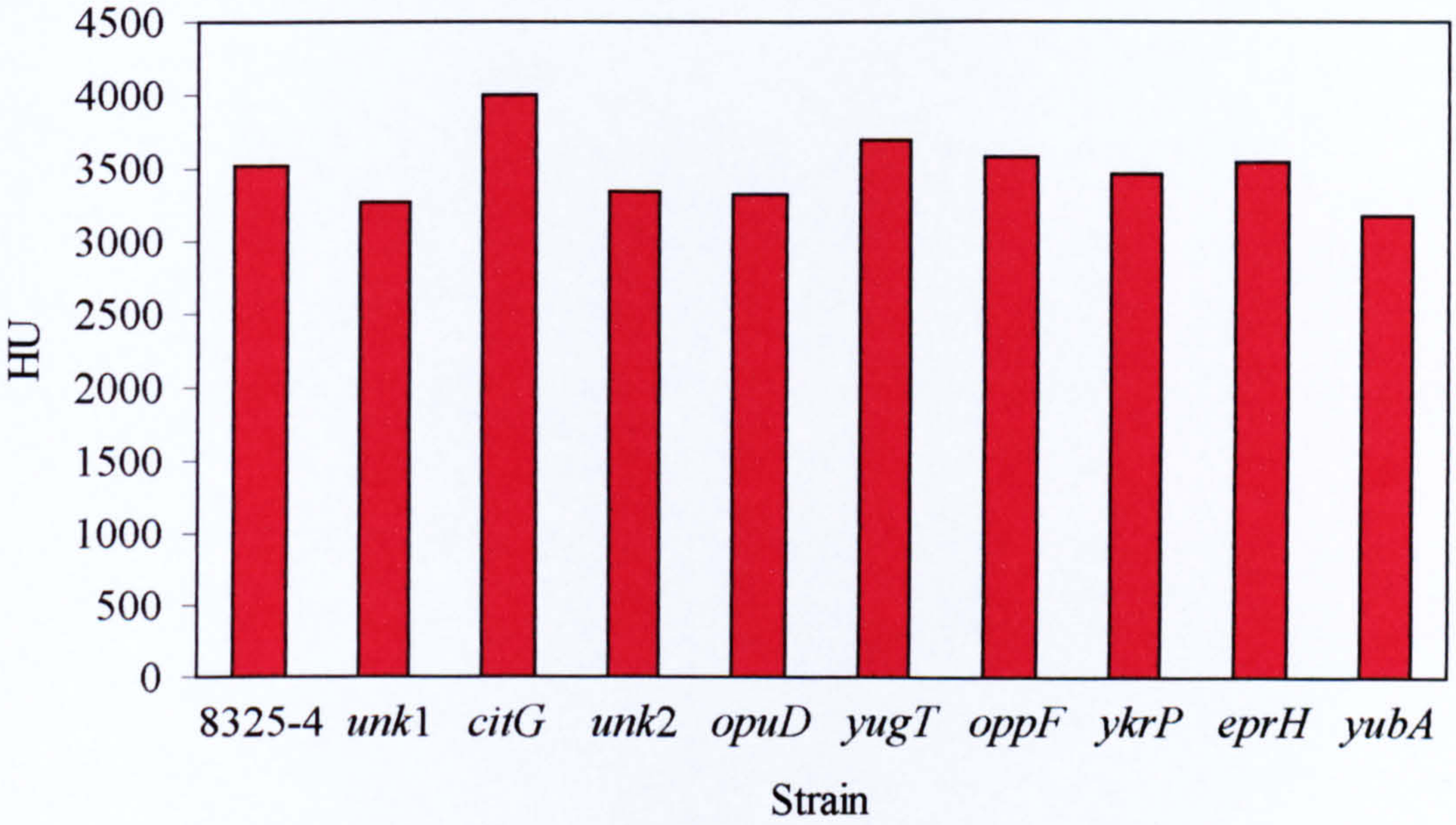


Figure 4.8

Haemolytic activity of *han* mutants and 8325-4 grown in BHI containing no added NaCl.

The haemolytic activity of samples taken from 8325-4, JLA401 (8325-4 *han*1 (*unk*1)), JLA402 (8325-4 *han*2 (*citG*)), JLA404 (8325-4 *han*4 (*unk*2)), JLA409 (8325-4 *han*9 (*opuD*)), JLA411 (8325-4 *han*11 (*yugT*)), JLA412 (8325-4 *han*12 (*oppF*)), JLA413 (8325-4 *han*13 (*ykrP*)), JLA414 (8325-4 *han*14 (*eprH*)) and JLA415 (8325-4 *han*15 (*yubA*)) grown in BHI for 14 h was measured as described in Chapter 2.18.2.

To establish whether the haemolytic activity of the *han* mutants grown in BHI containing 1 M NaCl could be quantified after desalting and concentrating the samples, 1.5 ml supernatant from cultures of 8325-4, JLA401 (8325-4 *han*1 (*unk*1)) and JLA402 (8325-4 *han*2 (*citG*)) was passed through a Centricon YM-10 centrifugal filter device (Chapter 2.18.2) until only ~ 50 µl liquid remained. Following resuspension in haemolysin salts solution to give a final volume of 300 µl, the haemolytic activity of the samples was quantified as in Chapter 2.18.2. However, assay of these samples could not reproduce the increased haemolysis of JLA401 (8325-4 *han*1 (*unk*1) and JLA402 (8325-4 *han*2 (*citG*)) compared to 8325-4 observed on rabbit blood plates containing 1 M NaCl, since 8325-4, JLA401 (8325-4 *han*1 (*unk*1)) and JLA402 (8325-4 *han*2 (*citG*)) and were found to have a haemolytic activity of 59, 39 and 31 HU respectively.

To determine whether a difference in the haemolysis of the *han* mutants and 8325-4 could be identified by using a lower concentration of NaCl, samples were taken from 8325-4, JLA401 (8325-4 *han*1 (*unk*1)) and JLA402 (8325-4 *han*2 (*citG*)) grown in BHI containing 700 mM NaCl. However, assay of these samples gave readings below the level of accuracy of the spectrophotometer (data not shown). Furthermore, samples taken from cultures grown in BHI containing 500 mM NaCl did not show a significant difference between the haemolytic activity of 8325-4, JLA401 (8325-4 *han*1 (*unk*1)) and JLA402 (8325-4 *han*2 (*citG*)). These strains had a haemolytic activity of 1039, 978 and 1112 HU respectively.

4.2.3.2.3 Determination of the expression of Hla, SspA and other exoproteins by *han* mutants in BHI containing either no added NaCl or 1 M NaCl using exoprotein gel and Western blot analysis

To determine whether the increased expression of Hla (or another factor responsible for the increased haemolytic activity of the *han* mutants, compared to 8325-4, on rabbit blood plates containing 1 M NaCl) could be demonstrated in the presence of NaCl-mediated stress in liquid culture, Western blotting (using antibodies raised against Hla and SspA) and exoprotein gel analysis was performed. Culture supernatants samples were taken from cultures of 8325-4, JLA401 (8325-4 *han*1 (*unk*1)), JLA402 (8325-4 *han*2 (*citG*)), JLA404 (8325-4 *han*4 (*unk*2)), JLA409 (8325-4 *han*9 (*opuD*)), JLA411

(8325-4 *han*11 (*yugT*)), JLA412 (8325-4 *han*12 (*oppF*)), JLA413 (8325-4 *han*13 (*ykrP*)), JLA414 (8325-4 *han*14 (*eprH*)) and JLA415 (8325-4 *han*15 (*yubA*)) grown in BHI containing either no added NaCl or 1 M NaCl after 14 h. Exoproteins were prepared as described in Chapter 2.18.3.1, separated on 12 % (w/v) SDS-PAGE gels as in Chapter 2.18.4, and probed with antibodies as in Chapter 2.18.5 where appropriate.

In all of the strains the level of Hla was decreased, whilst the level of SspA and AurA was increased, in BHI containing 1 M NaCl compared to BHI containing no added NaCl. Furthermore, the exoprotein profiles of the *han* mutants were similar to those of 8325-4 in BHI containing no added NaCl. However, no difference in the amount of Hla, SspA, or any other exoprotein expressed by the *han* mutants, compared to 8325-4, could be identified when grown in BHI containing 1 M NaCl. Examples of gels and blots for JLA401 (8325-4 *han*1 (*unk*1)), JLA404 (8325-4 *han*4 (*unk*2)) and JLA402 (8325-4 *han*2 (*citG*)), are shown in Figure 4.9.

Exoprotein gel analysis suggested that samples taken from BHI cultures containing 1 M NaCl contained significantly lower amounts of a number of proteins, including *hla*, than samples from BHI cultures containing no added NaCl (Figure 4.9a). To determine whether this is because NaCl present in the exoprotein samples interferes with the TCA precipitation of the proteins or their running on SDS-PAGE gels, NaCl crystals were added to an exoprotein sample removed from an 8325-4 culture (grown in BHI containing no added NaCl) to give a final concentration of 1 M. The sample was prepared and run on an SDS-page gel under standard conditions alongside an untreated sample removed from the same culture (Chapter 2.18.4). The intensity of the protein bands for the NaCl-treated sample was found to be similar to that of the untreated sample (Figure 4.10). Consequently, this suggests that NaCl present in the samples does not interfere with the preparation or running of the proteins. Instead, the low levels of proteins observed are probably a result of reduced protein production by the strains when grown in BHI containing 1 M NaCl.

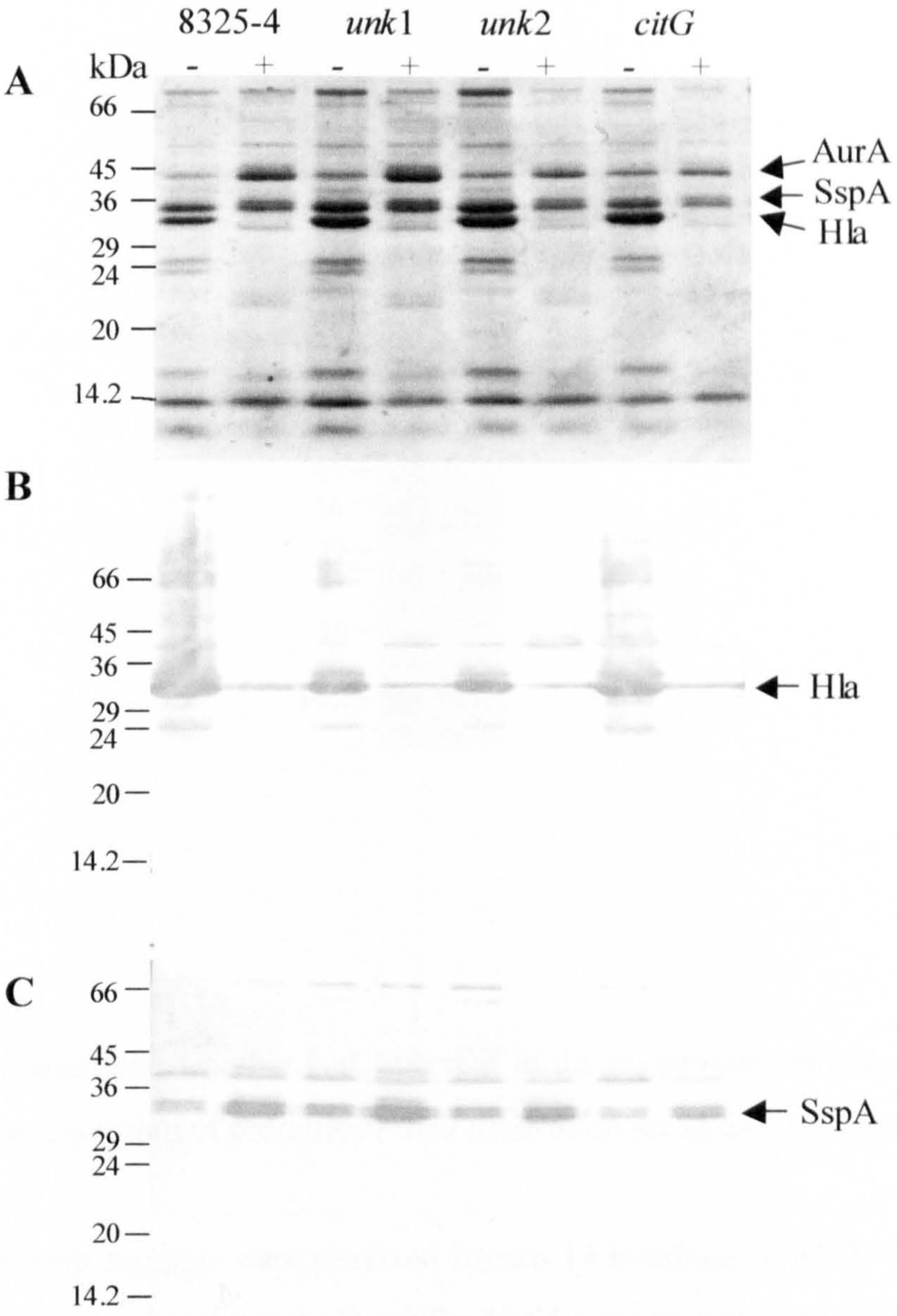


Figure 4.9

The effect of *han* mutations on the expression of Hla, SspA and other exoproteins.

(A) Exoprotein gels, (B) Hla Western blots and (C) SspA Western blots for 8325-4, JLA401 (8325-4 *han*1 (*unk*1)), JLA404 (8325-4 *han*4 (*unk*2)) and JLA402 (8325-4 *han*2 (*citG*)). Samples were removed after 14 h from cultures grown in BHI containing either no added NaCl (-) or 1 M NaCl (+) and prepared as described in Chapter 2.18.3.1. Proteins were separated on 12 % (w/v) SDS-PAGE gels as described in Chapter 2.18.4 and probed with relevant antibodies as described as in Chapter 2.18.5 where appropriate. Lanes contain exoproteins from 1.0 or 0.2 OD_{600} units of culture supernatant for exoprotein gels and Western blots respectively. Results are representative of a minimum of two independent experiments. Sizes of Sigma low molecular weight markers are shown.

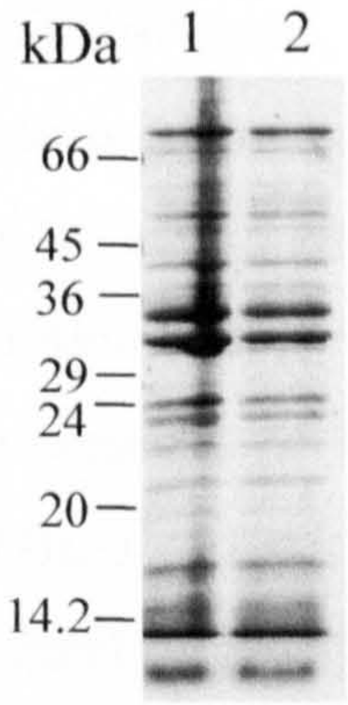


Figure 4.10

Control to determine whether NaCl present in the exoprotein samples interferes with the TCA-precipitation of proteins or their separation on SDS-PAGE gels.

Two exoprotein samples were removed from a 14 h culture of 8325-4 grown in BHI. One sample was left untreated (1), whilst NaCl crystals were added the other sample to give a final concentration of 1 M (2). The samples were prepared and separated on an SDS-PAGE gel, as described in Chapter 2.18. Lanes contain exoproteins from 1.0 OD_{600} unit of culture supernatant. Results are representative of two independent experiments. Sizes of Sigma low molecular weight markers are indicated.

4.2.3.3 Analysis of the effects of *han* mutations on growth in BHI containing either no added NaCl or 2 M NaCl

1 M NaCl has been shown to significantly repress virulence determinant expression in *S. aureus* (Chapter 1.6.2.3.2). However, 1 M NaCl only reduces the growth rate and final cell yield of *S. aureus* to a minor extent (Chapter 3.2.1), and *S. aureus* is able to grow in the presence of up to 3.5 M NaCl (Scott, 1953; Armstrong-Buisseret *et al.*, 1995). Therefore, a concentration of 2 M NaCl was used to determine the effects of *han* mutations on growth in the presence of NaCl. 8325-4, JLA401 (8325-4 *han*1 (*unk*1)), JLA402 (8325-4 *han*2 (*citG*)), JLA404 (8325-4 *han*4 (*unk*2)), JLA409 (8325-4 *han*9 (*opuD*)), JLA411 (8325-4 *han*11 (*yugT*)), JLA412 (8325-4 *han*12 (*oppF*)), JLA413 (8325-4 *han*13 (*ykrP*)), JLA414 (8325-4 *han*14 (*eprH*)) and JLA415 (8325-4 *han*15 (*yubA*)) were grown in BHI (with a 5 ml BHI preculture containing no added NaCl), in BHI containing 2 M NaCl (with a 5 ml BHI preculture containing 2 M NaCl) (NaCl-adaptation), and in BHI containing 2 M NaCl (with a 5 ml BHI preculture containing no added NaCl) (NaCl-shock) (Chapter 2.7). Growth was measured spectrophotometrically as OD_{600} at regular intervals over a 24 h period (Chapter 2.6.1). JLA401 (8325-4 *han*1 (*unk*1)), JLA404 (8325-4 *han*4 (*unk*2)), JLA411 (8325-4 *han*11 (*yugT*)), JLA412 (8325-4 *han*12 (*oppF*)), JLA413 (8325-4 *han*13 (*ykrP*)), JLA414 (8325-4 *han*14 (*eprH*)) and JLA415 (8325-4 *han*15 (*yubA*)) displayed similar levels of growth to 8325-4 under all three conditions (full data not shown). These mutants reached an OD_{600} of 8 – 8.8, 3.4 – 3.8 and 3.0 – 3.4 at t = 10 h in BHI containing no added NaCl, under NaCl adaptation and in the presence of NaCl shock respectively, whilst 8325-4 reached an OD_{600} of approximately 8.54, 3.75 and 3.40 at t = 10 h under the three respective conditions.

The growth of JLA402 (8325-4 *han*2 (*citG*)) was similar to that of 8325-4 in BHI containing no added NaCl and under NaCl adaptation, reaching an OD_{600} of 8.00 and 3.50 at t = 10 h under the two respective conditions (Figure 4.11a). However, a minor growth defect for JLA402 (8325-4 *han*2 (*citG*)) was observed in the presence of NaCl-shock (*ie.* a maximum of 1.6-fold lower growth for JLA402 at t = 9 h; 1.75 and 2.81 OD_{600} units respectively).

The growth of JLA409 (8325-4 *han*9 (*opuD*)) was similar to that of 8325-4 in BHI containing no added NaCl, reaching an OD_{600} of 8.79 at t = 10 h (Figure 4.11b).

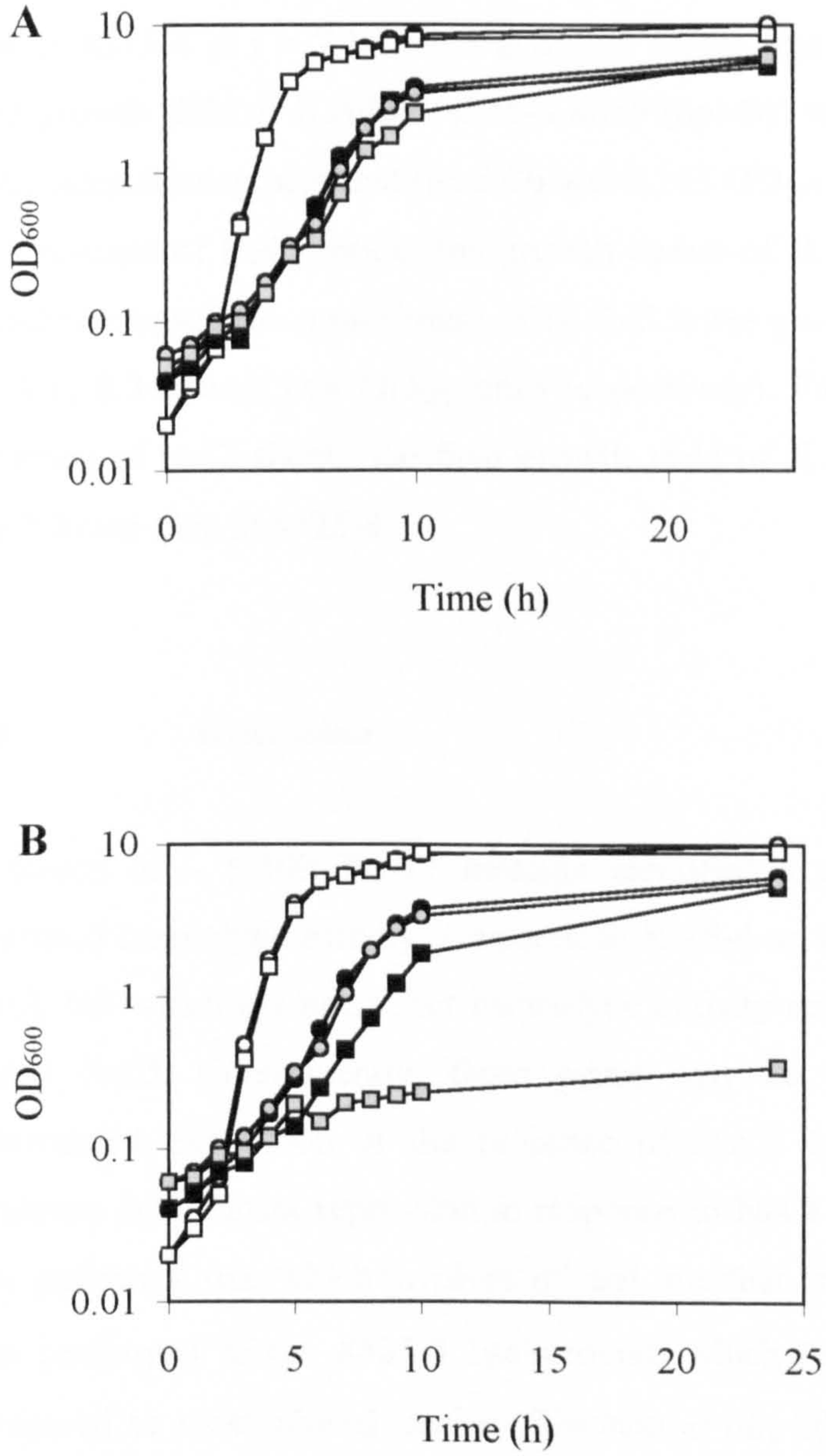


Figure 4.11a-b

Growth of (A) JLA402 (8325-4 *han*2 (*citG*)) (□, ■ and ▢) and (B) JLA409 (8325-4 *han*9 (*opuD*)) (□, ■ and ▢), compared to 8325-4 (○, ● and ◯) in BHI (with a 5 ml BHI preculture containing no added NaCl) (open symbols), in BHI containing 2 M NaCl (with a 5 ml BHI preculture containing 2 M NaCl) (NaCl-adaptation) (black symbols), and BHI containing 2 M NaCl (with a 5 ml BHI preculture containing no added NaCl) (NaCl-shock) (grey symbols). Strains were grown at 37 °C as described in Chapter 2.7. Growth was measured spectrophotometrically as OD_{600} (Chapter 2.6.1). Results are representative of two independent experiments.

However, JLA409 (8325-4 *han*9 (*opuD*)) was found to have a growth defect compared to 8325-4 under NaCl adaptation (*ie*. a maximum of 3-fold lower growth in JLA409 than in 8325-4 at t = 7 h; 0.455 and 1.29 OD_{600} units respectively). Nevertheless, the final growth yield of JLA409 (8325-4 *han*9 (*opuD*)) was similar to that of 8325-4 in the NaCl-adaptation experiment (*ie*. 5.26 and 6.165 OD_{600} units respectively at t = 24 h). In the presence of NaCl shock, the growth defect of JLA409 (8325-4 *han*9 (*opuD*)) was greatly increased (*ie*. a maximum of 16-fold lower growth in JLA409 than in 8325-4 at t = 24 h; 0.345 and 5.68 OD_{600} units respectively). Furthermore, this shows that in the presence of NaCl-shock, the final growth yield of JLA409 (8325-4 *han*9 (*opuD*)) was much lower than in 8325-4.

4.3 Discussion

A screen of ~ 6,500 Tn*917* mutants identified 9 genes, mutation of which led to increased haemolytic activity compared to 8325-4 on rabbit blood plates containing 1 M NaCl, but which did not affect haemolytic activity on rabbit blood plates containing no added NaCl. Consequently, these genes may be involved in repressing virulence determinant expression in the presence of NaCl. In Chapter 3, it was shown that virulence determinant repression in response to NaCl in *S. aureus* is likely to occur via two pathways, one which involves σ^B and one that is σ^B-independent. Since the screen was performed in the 8325-4 background, which has a reduced level of σ^B activity compared to most clinical strains (Giachino *et al.*, 2001), the genes identified here are likely to act via the σ^B-independent pathway. Furthermore, two different phenotypes were demonstrated for the mutants (either slightly increased or greatly increased haemolysis on rabbit blood plates containing 1 M NaCl). This suggests that the genes may function at different levels of the same σ^B-independent pathway, or via two separate σ^B-independent pathways.

The genes identified encode fumarase (CitG), a glycine betaine transporter (OpuD), an exo-alpha-1,4-glucosidase (YugT), an oligopeptide ABC transporter ATP-binding protein (OppF), a protein homologous to putative integral membrane proteins and acetyltransferases (YkrP), a protein involved in endopeptidase resistance and peptidoglycan biosynthesis (EprH), a conserved hypothetical membrane protein

(YubA), and two proteins for which a putative function could not be defined (Unk1 and Unk2).

Fumarase (fumarate hydratase) is a Krebs cycle enzyme involved in the interconversion of fumarate and L-malate. Interestingly, fumarase activity has been shown to increase in *Pseudomonas aeruginosa* in response to iron deprivation (Hassett *et al.*, 1997). Furthermore, fumarase activity increases in the leaves of *Phaeseoulus vulgaris* in response to NaCl-mediated stress (Hernandez *et al.*, 1993). Additionally, fumarate directly inhibits transcription of the *clcABD* operon (encoding enzymes of a chloroaromatic biodegradative pathway) in *Pseudomonas putida*, and acts as a signal molecule in the regulation of phototaxis in *Halobacterium salinarum*, and in metabolic signalling and chemotaxis in *E. coli* (reviewed by Marwan & Oesterhelt, 2000). Therefore, it is possible that in *S. aureus*, fumarate acts as a signal molecule in the NaCl-mediated stress response, and that fumarase is involved in regulating the level of this signal. Consequently, the inactivation of fumarase by Tn*917* would have interfered with the ability of *S. aureus* to signal the presence of NaCl in rabbit blood plates containing 1 M NaCl, resulting in altered Hla production.

Interestingly, other Krebs cycle enzymes have been isolated in transposon mutant library screens to identify genes involved in *S. aureus* virulence. These include oxoglutarate dehydrogenase (*odhB*) and aconitase (*citB*) (Mei *et al.*, 1997; Coulter *et al.*, 1998). Mei *et al.* (1997) suggested that because respiratory activity is essential for capsule production during some stages of *S. aureus* growth *in vitro*, and since capsule size can have significant effects on virulence, it may be possible that mutations in genes encoding Krebs cycle enzymes have an indirect effect on virulence via capsule production.

EprH is an endopeptidase resistance gene of *S. aureus*, and is involved in peptidoglycan biosynthesis (DeHart *et al.*, 1995; Sugai *et al.*, 1997; Sugai *et al.*, 1998). In the peptidoglycan of staphylococci, peptide units of individual glycan chains are covalently linked by pentapeptide-forming interpeptide chains; in *S. aureus* the interpeptide chain is composed of pentaglycine (Schleifer & Khandler, 1972; Sugai *et al.*, 1998). Certain strains of staphylococci produce glycylglycine endopeptidase, which specifically digests the gly-gly bond in the interpeptide chains of *S. aureus* peptidoglycan (Sugai *et al.*,

1997; Sugai *et al.*, 1998). Resistance of the producing strains to lysis by the enzyme involves altering the amino acid composition of the interpeptide chains by increasing the serine content and decreasing the glycine content (DeHart *et al.*, 1995; Sugai *et al.*, 1997; Sugai *et al.*, 1998). EprH has been shown to transfer serine to the interpeptide chains (Sugai *et al.*, 1997). Glycine residues are added by FemA and FemB (Sugai *et al.*, 1997; Stranden *et al.*, 1997). Major changes occur in the *S. aureus* cell wall when grown in defined medium containing 2.5 M NaCl (Vijaranakul *et al.*, 1995). These include an increased cell size and shortened peptidoglycan interpeptide bridge (Chapter 1.6.2.3.1.2). Alterations in the composition of the peptidoglycan interpeptide bridge have mainly been attributed to disturbance of the glycine addition process (Vijaranakul *et al.*, 1995). Therefore, disruption of the ability to add both glycine and serine to the interpeptide bridge in the *eprH* mutant grown on rabbit blood plates containing 1 M NaCl would have led to a significant defect in peptidoglycan structure. This would have affected the fitness of the cell, potentially leading to the altered virulence determinant production observed.

In addition, *femA* and *femB* have been identified in the pathogenicity screens of Mei *et al.* (1997), Coulter *et al.* (1998) and Lammers *et al.* (2000). Since FemA, FemB and EprH are members of the same family involved in adding amino acids to the peptidoglycan (Sugai *et al.*, 1997), this may support the possible role of EprH in *S. aureus* virulence determinant regulation identified here.

The oligopeptide transporter identified in the Tn*917* mutant library screen is a member of the ABC family of transporters that are involved in importing and exporting a range of compounds in a variety of organisms (Higgins, 1992). Oligopeptide permeases typically comprise a high affinity substrate-binding protein (which is extracellular, but bound to the membrane), two permease proteins (which form a solute-specific channel) and one or two ATP-binding proteins (which provide energy for the system and sometimes serve a regulatory role) (Saier *et al.*, 1993). Importantly, a role for oligopeptide transporters and oligopeptides in signalling and gene regulation has been established. For example, uptake of the cCF10 sex phermone via an oligopeptide permease system is involved in controlling conjugation in *E. faecalis* (Leonard *et al.*, 1996). Oligopeptide permeases have also been shown to play a role in virulence, affecting the ability of *Streptococcus pneumoniae* to adhere to eukaryotic cells (Cundell

et al., 1995). Additionally, uptake of an oligopeptide signalling molecule(s) via the *spoOK* oligopeptide transporter is involved in the induction of sporulation and competence in *B. subtilis* (Rudner *et al.*, 1991, Magnuson *et al.*, 1994; Solomon *et al.*, 1995).

Interestingly, it has been suggested that cell wall peptides can signal the onset of stationary phase and initiate sporulation in *B. subtilis* and a number of other bacteria, and that the inability of *opp* mutants to take up and accumulate these cell wall peptides may be responsible for their sporulation defects (Perego *et al.*, 1991; Saier *et al.*, 1993). An association between the accumulation of cell wall components in *Myxococcus xanthus* and sporulation has been reported (Shimkets & Kaiser, 1982). Since major changes in the structure of the *S. aureus* cell wall occur when grown in medium containing 2.5 M NaCl (Vijaranakul *et al.*, 1995), it is possible to envisage a system whereby oligopeptides released from the peptidoglycan in the presence of NaCl-mediated stress are taken up by an oligopeptide transporter and act to signal that the conditions are inappropriate for virulence determinant production. This is supported by the fact that components of oligopeptide transporters have also been isolated in transposon mutant library screens to identify genes involved in *S. aureus* pathogenesis (Mei *et al.*, 1997; Coulter *et al.*, 1998; Vriesema *at el.*, 2000). However, analysis of the genes surrounding the ATP-binding protein inactivated by Tn*917* indicates that the oligopeptide transporter isolated in this study does not posses a ligand-binding protein. This is either because the system does not require a ligand-binding protein for substrate uptake, or because the transporter is involved in efflux (Saier *et al.*, 1993). If the latter is correct, then the mechanism by which the oligopeptide transporter functions in controlling the NaCl-mediated stress response is unclear. Whether the transporter functions in oligopeptide uptake or efflux could be determined through the use of toxic peptide analogues (*eg.* tri-L-ornithine), which will prevent growth only if the system is involved in oligopeptide uptake (Barak *et al.*, 1973).

Since the construction of a tetracycline-resistant *hla* cassette knock-out proved difficult, it was not possible to demonstrate conclusively that the haemolytic factor overexpressed by the *han* mutants, compared to 8325-4, on rabbit blood plates containing 1 M NaCl was Hla. However, the haemolytic factor was shown to be an *agr*-dependent virulence determinant. Purified protease (SspA) was unable to cause haemolysis on rabbit blood

plates containing either no added NaCl or 1 M NaCl. Furthermore, an erythromycin-resistant *hla* cassette knock-out was completely non-haemolytic on a rabbit blood plate containing 1 M NaCl, whilst 8325-4 was slightly haemolytic. Overall, this suggests that the virulence determinant over-expressed by the *han* mutants on rabbit blood plates containing 1 M NaCl is likely to be Hla.

Quantification of haemolytic activity using liquid assays showed that the *han* mutants demonstrated the same level of haemolytic activity as 8325-4 when grown in BHI containing no added NaCl. However, the increased haemolytic activity of the *han* mutants compared to 8325-4 observed on rabbit blood plates containing 1 M NaCl could not be demonstrated using samples taken from BHI cultures containing 1 M NaCl. Similarly, exoprotein gels, Hla Western blots and SspA Western blots showed that the *han* mutants expressed the same level of Hla and SspA as 8325-4 when grown in BHI containing either no added NaCl or 1 M NaCl.

Analysis of the growth of the *han* mutants showed that the *unk*1, *unk2*, *yugT, oppF, ykrP, eprH* and *yubA* mutants demonstrated wild-type levels of growth in BHI containing either no added NaCl or 2 M NaCl (under NaCl-adaptation or NaCl-shock conditions). The effect of *citG* mutation on growth in the presence and absence of NaCl was also investigated. Analysis of the TIGR genome database (Chapter 2.17.2) indicates that *S. aureus* possesses only one fumarase gene, which has been inactivated here by Tn*917*. Fumarase is a Krebs cycle enzyme, and so in order to be able to metabolise and grow at all, the *citG* mutant may possibly resort to using the glyoxylate shunt, whereby isocitrate is converted to malate, thus bypassing the section of the Krebs cycle involving fumarase (Figure 4.12). Since this would result in the production of less NADH and therefore less ATP, a small reduction in growth rate would presumably occur. However, no appreciable growth defect was observed for the *citG* mutant in BHI containing no added NaCl, or under NaCl adaptation with 2 M NaCl. This agrees with the finding that inactivation of genes encoding other Krebs cycle enzymes which can be bypassed by the glyoxylate shunt (*eg.* oxoglutarate dehydrogenase) has little impact on the growth rate of *S. aureus* under unstressed conditions (Coulter *et al.*, 1998). However, the *citG* mutant was found to have a minor growth defect upon NaCl-shock in BHI containing 2 M NaCl. This suggests that a severe NaCl-mediated stress coupled with the defect in metabolism affects growth.

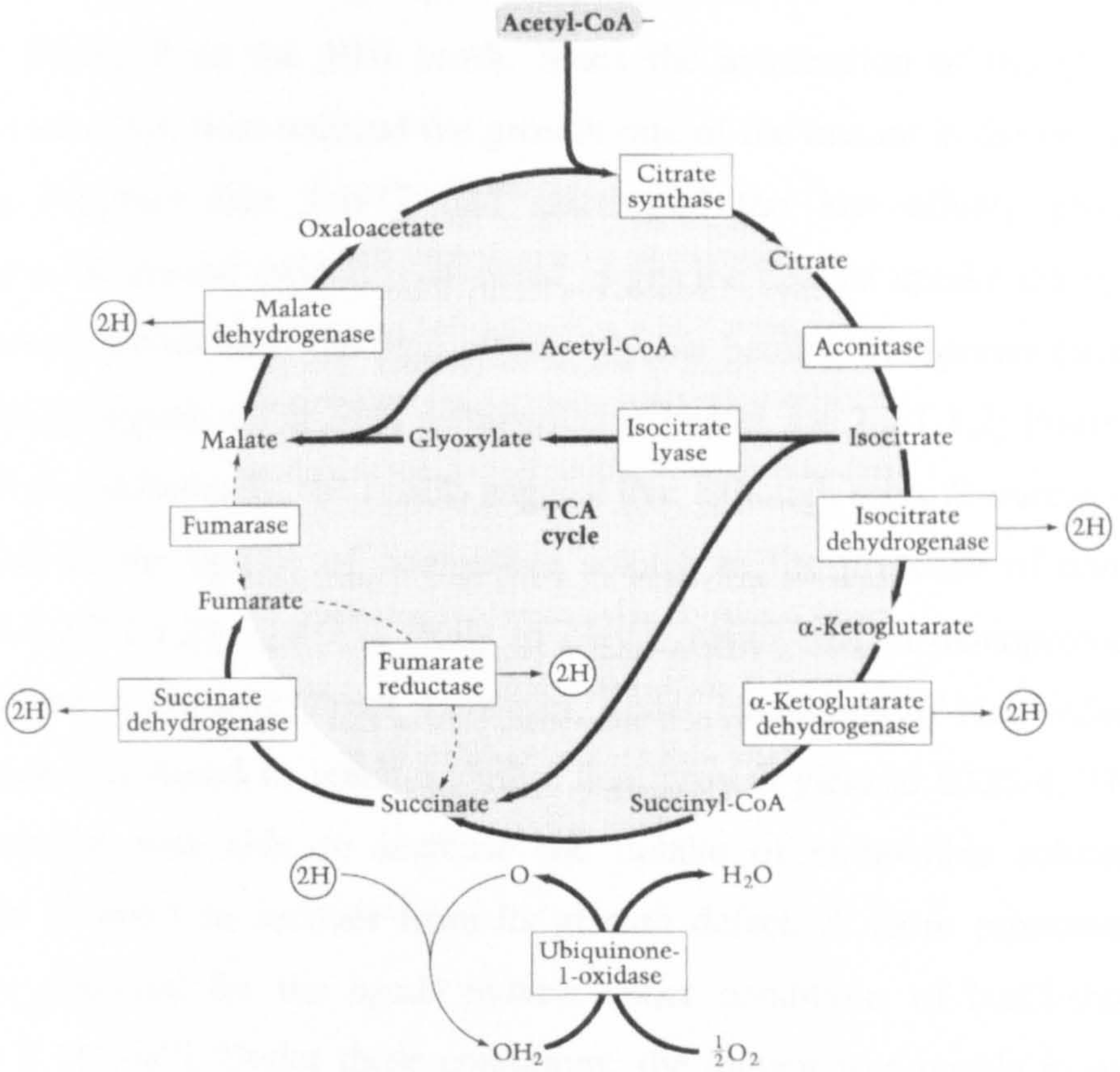


Figure 4.12

Diagram of the Krebs cycle, including the glyoxylate shunt pathway.

Reproduced from Neidhardt *et al.* (1990)

The *opuD* mutant was found to have similar growth to 8325-4 in BHI containing no added NaCl. However, a growth defect for the *opuD* mutant was observed under conditions of NaCl-adaptation in BHI containing 2 M NaCl. This probably occurred because the mutant was defective in its ability to take up glycine betaine (which relieves the growth inhibition caused by high osmolarity (Chapter 1.6.2.3.1.1.2; Pourkomailian & Booth, 1992)) from the BHI broth. Since the inactivation of the glycine betaine transporter identified here reduced the growth rate of the mutant in the presence of 2 M NaCl, this suggests that Tn*917* had inactivated the low affinity glycine betaine transporter of *S. aureus* (which is involved in glycine betaine uptake during periods of osmotic stress), rather than the high affinity glycine betaine transporter (whose activity is increased by signals other than osmolarity) (Chapter 1.6.2.3.1.1.2; Pourkomailian & Booth, 1992). Additionally, the results suggest that although other *S. aureus* transporters are involved in the uptake of compatible solutes in the presence of osmotic shock (Chapter 1.6.2.3.1.1.2), *opuD* is likely to play a major role in osmoprotection in the initial response to osmotic stress. However, under conditions of NaCl-adaptation, the *opuD* mutant was found to reach a similar final growth yield to 8325-4. This suggests that the mutant was able to increase the uptake of compatible solutes by other transporters in order to recover from its growth defect. A more pronounced growth defect was observed for the *opuD* mutant under conditions of NaCl-shock in BHI containing 2 M NaCl. Under these conditions, the mutant was unable to overcome its growth defect and reach a similar final growth yield to 8325-4 after 24 hours. This presumably occurred due to the inability of other compatible solute transporters to compensate for the inactivation of *opuD* under these conditions.

The genes identified here provide an interesting insight into the σ^B-independent regulation of virulence determinant expression in response to NaCl-mediated stress. However, the ability to analyse the altered haemolytic activity of the *han* mutants appears to be largely limited to qualitative work on solid media. It has been suggested that NaCl may be important in regulating virulence determinant expression in the development of the commensal state in the human nose and on the skin (Lindsay & Foster, 1999). Consequently, the presence of a solid surface may be required for these genes to mediate a response to NaCl. Furthermore, it is possible that additional environmental factors (*eg.* aeration) may act in concert with the NaCl-stress signal in order to stringently regulate virulence determinant expression. The fact that these

conditions would not have been the same in BHI broth as they were on rabbit blood plates may have prevented the mutant phenotypes from being reproduced in liquid culture. However, only ~ 6,500 mutants were screened, since a large number of mutants with relevant phenotypes were isolated from the colonies already screened. Complete coverage of the genome requires the screening of approximately 5 times the total number of genes present. Since *S. aureus* possesses ~2,700 genes (Baba *et al.*, 2002), complete genome coverage would involve screening ~13,500 mutants. Therefore, it is possible that further screening might permit the identification of novel mutants for which an NaCl-mediated phenotype can be reproduced in liquid culture. As the scope for further analysis of the *han* mutants was limited, these strains were not characterised further.

CHAPTER 5

DEVELOPMENT OF AN ALTERNATIVE TRANSPOSON MUTANT LIBRARY SCREENING STRATEGY

5.1 Introduction

In Chapter 4, Tn*917* mutagenesis of *S. aureus* 8325-4 was used to identify genes with a putative role in regulating haemolysin production in response to NaCl. However, the usefulness of transposon mutagenesis in *S. aureus* is restricted by a major limitation. The main transposons used for research in *S. aureus* (*ie*. Tn*917* and Tn*551*) both carry an erythromycin-resistance gene, as do the main vectors used for *lacZ* reporter gene fusion construction in *S. aureus* (*ie*. pMUTIN4 and pAZ106) (Youngman, 1990; Wu *et al.*, 1999; Vagner *et al.*, 1998; Kemp *et al.*, 1991). Furthermore, many of the cassette knock-outs of *S. aureus* genes have also been constructed using erythromycin-resistance cassettes. In this study, such resistance marker incompatibility has prevented pre-constructed erythromycin-resistant cassette knock-outs (*eg*. of *hla* (O'Reilly *et al.*, 1986)) and *lacZ* fusions (*eg*. the *hla::lacZ* fusion (Chan & Foster, 1998a)) from being used in conjunction with the Tn*917*-inactivated *han* genes. Additionally, it would be extremely useful to be able to perform transposon mutagenesis screens directly in *lacZ* reporter gene fusion strains to identify factors affecting the transcription of a specific gene, especially where the product of that gene cannot easily be assayed. Two potential solutions could allow the problem of selectable marker incompatibility between *S. aureus* transposons and *lacZ* fusions/cassette knockouts to be overcome. Firstly, alternative transposons carrying selectable markers other than erythromycin resistance could be evaluated for use in *S. aureus*. Alternatively, a *lacZ* fusion vector carrying a selectable marker other than erythromycin resistance could be constructed.

5.2 Results

5.2.1 Evaluation of the EZ::TN transposition system for use in *S. aureus*

The EZ::TN transposition system, produced by Epicentre Technologies, is based on the Tn*5* transposition system developed by Goryshin & Reznikoff (1998) and Goryshin *et al.* (2000). All information regarding this system has been obtained from the Epicentre Technologies website (http://www.epicentre.com) unless otherwise stated. Tn*5* (reviewed by Reznikoff, 1993) is a composite transposon comprising three antibiotic resistance genes flanked by two nearly identical IS*50* elements, of which IS*50*R encodes the transposase. However, it has been shown that inverted 19 bp sequences present at the outside ends of the IS*50* elements (*ie*. the OE sequences) are the only sequences required for the transposase to facilitate Tn*5* transposition (Goryshin & Reznikoff, 1998). The DNA between the 19 bp OE sequences plays no role in transposition (Goryshin & Reznikoff, 1998), and the fact that any sequence can make up this region (provided that a source of transposase is available) has been exploited in the EZ::TN transposition system. The EZ::TN transposon, carried on the cloning vector pMOD(MCS), consists of the 19 bp OE sequences flanking a MCS into which any piece of DNA (*eg*. any selectable marker) can be introduced. The transposon, either cut from the vector DNA using suitable restriction enzymes or amplified by PCR, can be incubated with purified Tn*5* transposase in order to generate a transposome. Transposition can then be achieved *in vitro* by incubating transposomes with genomic DNA fragments and introducing the products into cells by electroporation. Alternatively, electroporation of transposomes into suitable recipient cells can be used to achieve transposition *in vivo*. The position of transposon insertions can be identified in resulting mutants using sequencing primers provided by Epicentre Technologies.

A 1000-fold increase in the transposition efficiency of a prototype of the system has been achieved by engineering three mutations into the transposase gene and multiple changes into the 19 bp transposase recognition sequences (Goryshin & Reznikoff, 1998). This has allowed high frequency, independent transposon insertion into single random sites to be achieved through *in vivo* use of the prototype system in *E. coli*, *S. typhimurium* and *Proteus vulgaris* (Goryshin *et al.*, 2000). The EZ::TN system has also been used successfully for the *in vivo* transposition of the Gram-positive bacterium

Mycobacterium smegmatis (Derbyshire *et al.*, 2000). Consequently, the suitability of the EZ::TN transposition system for use in *S. aureus* was investigated. However, although versions of the EZ::TN containing tetracycline and kanamycin resistance markers have been developed by Epicentre Technologies, these are not suitable for selection in Gram-positive organisms. As a result, it was necessary to first clone an appropriate selectable marker into the EZ::TN transposon.

5.2.1.1 Insertion of a tetracycline-resistance cassette into the EZ::TN transposon

A 1.5 kb *Kpn*I-flanked tetracycline-resistance cassette, PCR-amplified from pDG1513, was cloned into the *Kpn*I-cut 2.5 kb vector pMOD(MCS), as described in Chapter 2.13.6.2 (Figure 5.1). Restriction mapping with *Kpn*I, *Bam*HI and *Sca*I gave bands of the expected size (data not shown). Furthermore, the restriction profile produced by digestion with *Sca*I indicated that the tetracycline-resistance cassette was inserted in the same orientation as the ampicillin resistance gene and origin of replication of pMOD(MCS) (data not shown). The 4kb plasmid produced was termed pMOD(tet), and the EZ::TN transposon containing the tetracycline-resistance cassette will be referred to as the EZ::TN(tet) transposon.

5.2.1.2 Generation of EZ::TN(tet) transposon free from the pMOD(MCS) backbone of pMOD(tet)

The effectiveness of the EZ::TN transposition system is dependent on the efficiency of introducing DNA into host cells by electroporation (Derbyshire *et al.*, 2000). The first stage of transposome production therefore involved producing EZ::TN(tet) transposon free from the pMOD(MCS) backbone of pMOD(tet). This should increase the efficiency of electroporation of the transposomes into *S. aureus* cells for *in vivo* transposition, due to the shorter length of the DNA fragments. Furthermore, the efficiency of electroporating *in vitro* transposition products into *S. aureus* cells will be increased due to a smaller amount of contaminating backbone DNA being present amongst the genomic DNA / transposon fragments. Two methods can be used to obtain

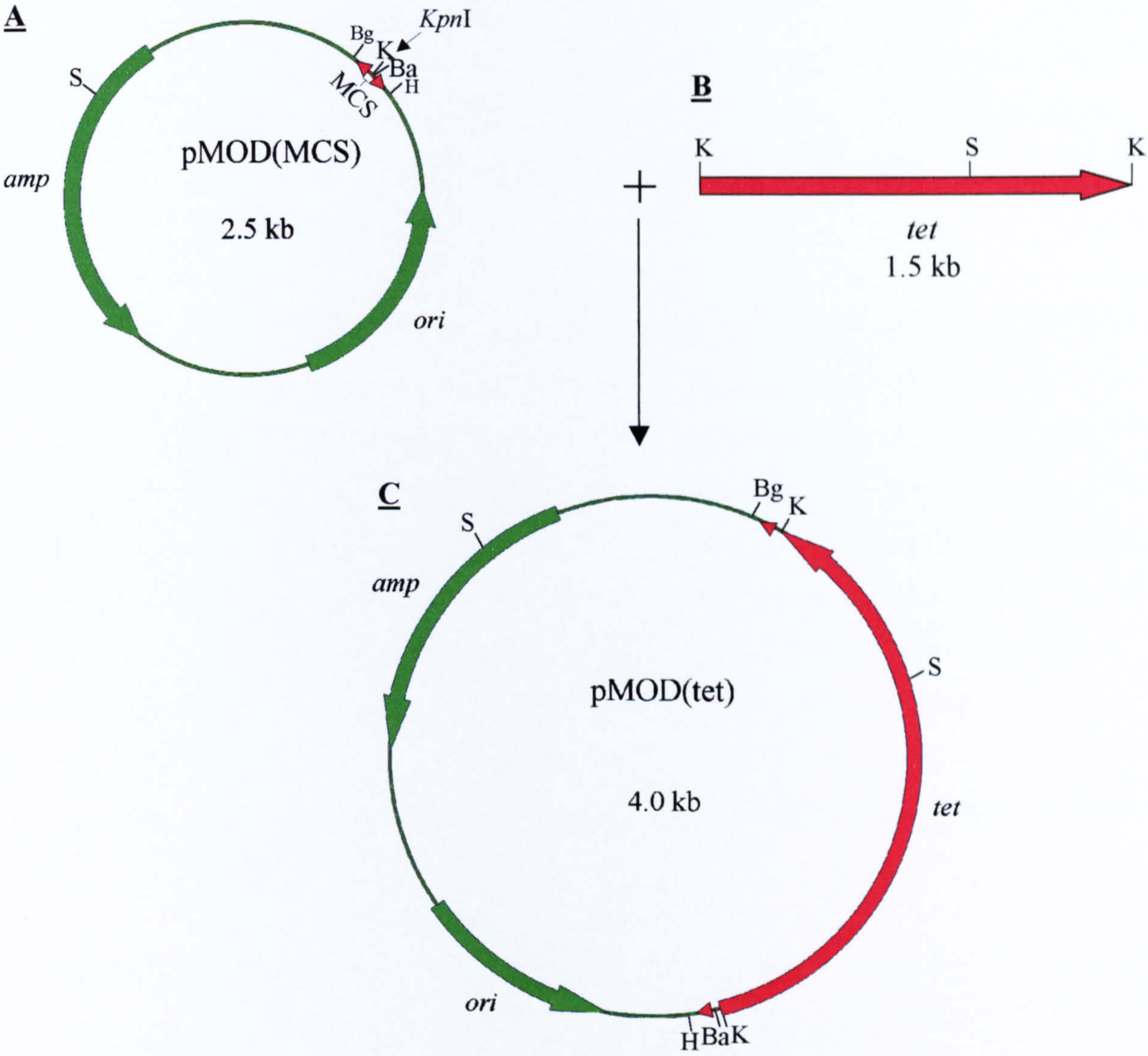


Figure 5.1

Construction of pMOD(tet). (A) *Kpn*I-cut pMOD(MCS) and (B) a *Kpn*I-cut PCR product containing the tetracycline-resistance cassette amplified from pDG1513 were used to construct (C) pMOD(tet), a vector carrying the Tn*5*-based EZ::TN(tet) transposon.

Regions coloured in red on the plasmids are part of the transposable element, whilst the remainder of the vector backbone is coloured in green. The 19 bp outside end (OE) sequences mark the ends of the transposon, and are represented by red triangles. Additional features; *ori*, origin of replication; *amp*, ampicillin-resistance gene; *tet*, tetracycline-resistance gene; MCS, multiple cloning site. Restriction sites; K, *Kpn*I; Ba, *Bam*HI; S, *Sca*I; Bg, *Bgl*I; H, *Hae*II. Not to scale.

EZ::TN(tet) transposon free from the pMOD(MCS) backbone. Firstly, the transposon can be amplified from pMOD(tet) by PCR using the primers FP-1 and RP-1 (Epicentre Technologies). However, attempts to PCR amplify the EZ::TN(tet) transposon sequence using Pwo polymerase (Chapter 5.2.1) were unsuccessful, even when pMOD(tet) was first cut with *Nde*I to prevent plasmid supercoiling from interfering with the PCR reaction (data not shown).

An alternative strategy to obtain EZ::TN(tet) transposon free from the pMOD(MCS) backbone involves cutting the transposon from the vector backbone using suitable restriction enzymes. pMOD(tet) was therefore digested overnight with *Bgl*I and *Hae*II (Chapter 5.2.1), which cut 49 bp and 54 bp outside the 19 bp OE sequences of the EZ::TN(tet) transposon respectively (Figure 5.1). The digestion products were resolved on a 0.8 % (w/v) agarose gel (Chapter 2.13.5), and the 1.7 kb excised EZ::TN(tet) transposon fragment was gel purified (Chapter 2.11.5) to reduce vector background.

5.2.1.3 *In vitro* EZ::TN(tet) transposition into *S. aureus* 8325-4 genomic DNA

The *in vitro* transposition of the EZ::TN(tet) transposon into sheared 8325-4 genomic DNA was performed as described in Chapter 2.15.1.1. The reaction buffer used contained Mg^{2+} ions required for transposase activity. The transposition products were electroporated into competent RN4220 cells (Chapter 2.10.2.2), with selection on BHI agar plates containing 5 μg ml^{-1} Tet. However, after growth at 37 °C for 48 h, no colonies were produced. The lack of transposon mutants was unlikely to have been caused by low cell competence, as electroporation of 500 ng pMC4 (*ie.* the suicide vector pAZ106 containing the upstream regulatory region and 5' end of *sigB* (Chan *et al.*, 1998)) into the RN4220 cells (Chapter 2.10.2.2) yielded approximately 50 colonies on BHI agar plates containing Ery and Lin. Since *in vitro* EZ::TN(tet) transposition into *S. aureus* 8325-4 genomic DNA was unsuccessful, the electroporation of the EZ::TN(tet) transposon into *S. aureus* RN4220 for *in vivo* transposition was attempted.

5.2.1.4 *In vivo* transposition of EZ::TN(tet) into *S. aureus* RN4220

In vivo transposition by electroporating EZ::TN(tet) transposomes into RN4220 competent cells was performed as described in Chapter 2.15.1.2. Introduction of Tn*5*-based transposomes into *E. coli* for *in vivo* transposition was found to be most efficient when the transposomes were used in the absence of Mg^{2+} (Goryshin *et al.*, 2000); this prevents transposition from occurring *in vitro* and consequently increases the number of active transposomes present in the cells. Two methods were used to limit the exposure of the transposomes to Mg^{2+} outside the cells during electroporation. Firstly, the samples were immediately electroporated following addition of the transposomes to the RN4220 cells. Alternatively, Mg^{2+} was removed from the RN4220 cell samples prior to transposome addition by adding 50 µl 0.5 M EDTA to a 100 µl aliquot of cells and washing the samples twice with 100 µl 10 % (v/v) glycerol to remove the EDTA-chelated Mg^{2+}. The cells were then resuspended in 100 µl 10 % (v/v) glycerol and used in the electroporation. *In vivo* transposition using the immediate electroporation method produced hundreds of tetracycline-resistant colonies, whilst the Mg^{2+} removal method produced none.

5.2.1.5 Southern blot to determine the randomness of EZ::TN(tet) transposition in the *S. aureus* genome

Southern blotting was performed to determine the randomness of EZ::TN(tet) transposon insertion in the tetracycline-resistant RN4220 mutants produced by *in vivo* transposon mutagenesis in Chapter 5.2.1.4. Genomic DNA was prepared from 10 randomly chosen EZ::TN(tet) mutants and from RN4220 using a QIAGEN DNeasy™ kit (Chapter 2.11.1). Genomic DNA was cut with *Sal*I or *Hin*dIII overnight. A DIG-labelled probe was prepared using the EZ::TN(tet) transposon DNA fragment cut from pMOD(tet) using *Bgl*I and *Hae*II (Chapters 5.2.1.2 and 2.14.1) (Figure 5.1). Southern blotting was performed, as described in Chapter 2.14.3.

The Southern blots (Figure 5.2) showed that in all of the mutants a single hybridising band was present which was absent in RN4220. However, in all of the mutants, the

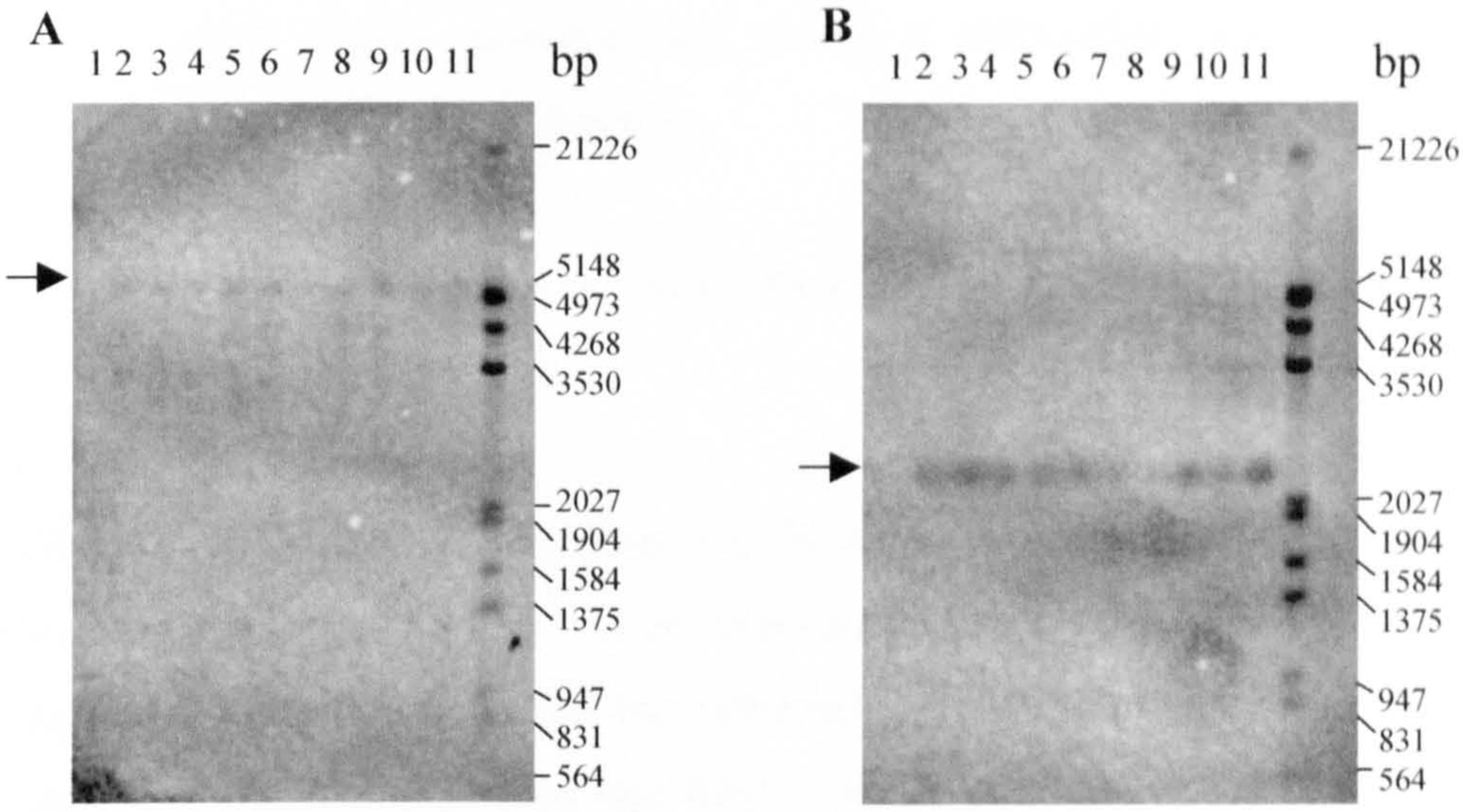


Figure 5.2

Southern blots showing the randomness of EZ::TN(tet) insertion in 10 RN4220 mutants produced by *in vivo* mutagenesis.

Position of EZ::TN(tet) insertion in (A) *Sal*I-cut genomic DNA and (B) *Hin*dIII-cut genomic DNA from 1) RN4220 and 2-11) 10 randomly chosen RN4220 EZ::TN(tet) mutants, JLA501-JLA510.

Genomic DNA was prepared using a QIAGEN DNeasy™ kit (Chapter 2.11.1) and cut with *Sal*I or *Hin*dIII overnight (Chapter 2.13.2). A DIG-labelled probe was prepared using the EZ::TN(tet) transposon DNA fragment cut from pMOD(tet) using *Bgl*I and *Hae*II (Chapters 5.2.1.2 and 2.14.1). Southern blotting was then performed, as described in Chapter 2.14.3. Sizes of DIG-labelled λ HindIII / EcoRI markers are shown.

band sizes were identical. This suggests that the EZ::TN(tet) transposon has inserted into the same position in the *S. aureus* genome in all of the mutants analysed.

5.2.2 *lacZ* fusion construction using a selectable marker other than erythromycin-resistance

5.2.2.1 Construction of a *lacZ* fusion vector carrying a tetracycline-resistance marker

Since the EZ::TN transposition system was found to be unsuitable for use in *S. aureus* (Chapter 5.2.1.5), a *lacZ* fusion vector carrying a tetracycline-resistance marker was constructed in order to overcome the selectable marker incompatibility between the erythromycin-resistant transposons and *lacZ* reporter gene fusions. The 8.6 kb plasmid pMUTIN4 (Vagner *et al.*, 1998) has routinely been used to construct *lacZ* reporter gene fusions in *S. aureus* (Chan & Foster, 1998a) (Figure 5.3). The vector carries a modified *E. coli lacZ* reporter gene that can be used to quantitatively measure gene expression in both Gram-positive and Gram-negative bacteria. There are ColE1 replication sequences and a β-lactamase resistance gene (Amp^R) for amplification in *E. coli*. An erythromycin-resistance gene is present for selection in Gram-positive bacteria.

The erythromycin-resistance cassette of pMUTIN4 was replaced with a tetracycline resistance cassette, as described in Chapter 2.13.6.3 (Figure 5.3). Restriction mapping with *Hin*dIII, *Cla*I / *Nco*I and *Dra*I produced bands of the expected size (data not shown). The resulting plasmid was termed pAISH1.

5.2.2.2 Construction of an *hla::lacZ* reporter gene fusion in pAISH1

In order to be able to perform transposon mutagenesis screens directly in an *hla::lacZ* reporter gene fusion strain, an *hla::lacZ* fusion was created in the vector pAISH1. A 2.2 kb *Eco*RI / *Not*I fragment containing the promoter and 5' coding region of *hla* was cut from the plasmid pPF4 and cloned into *Eco*RI / *Not*I-cut pAISH1, as described in Chapter 2.13.6.4 (Figure 5.4). The 2.2 kb *hla* fragment was the same as that cloned into

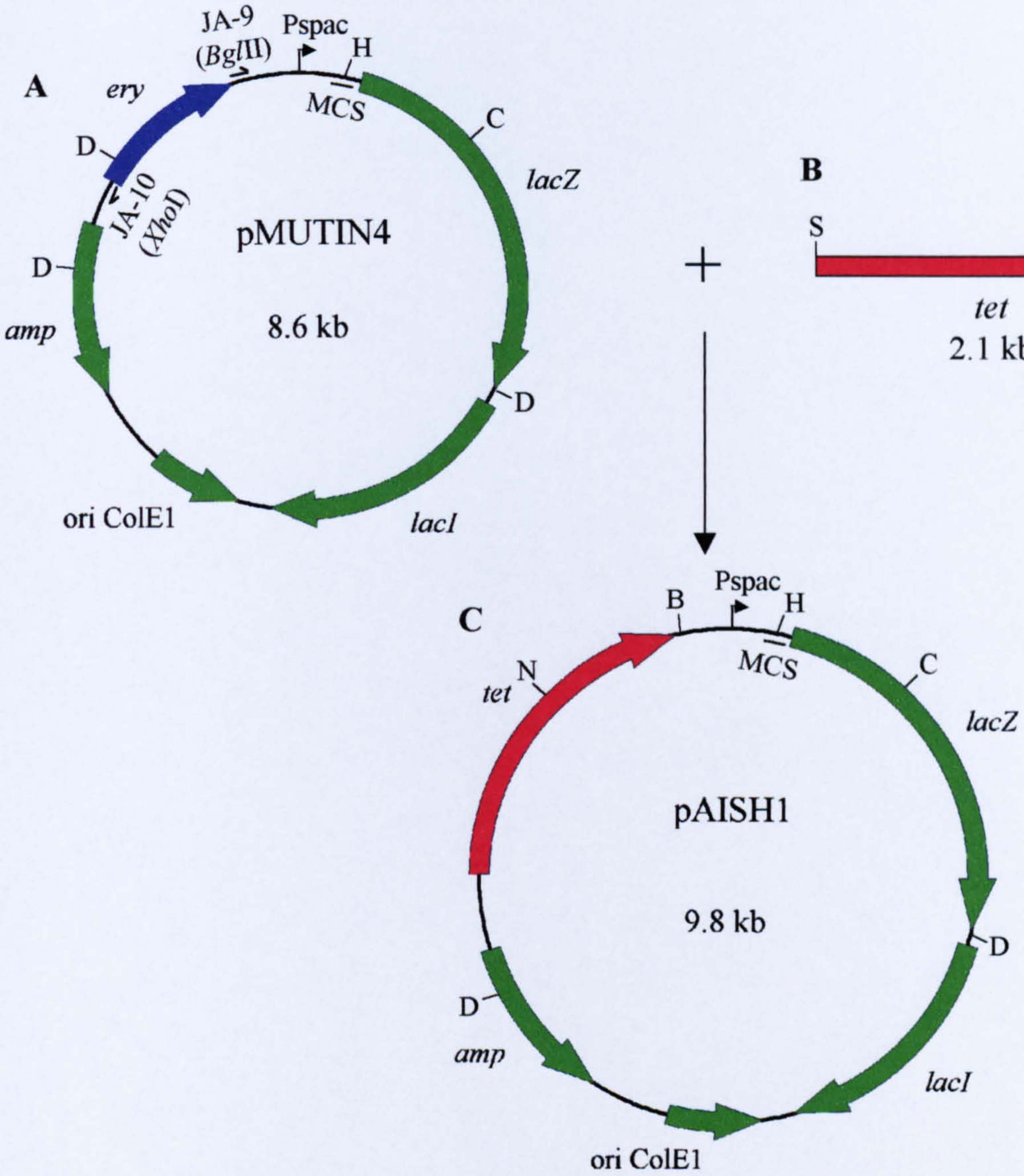


Figure 5.3 Construction of pAISH1. (A) a 7.7 kb *Bgl*II / *Xho*I-cut inverse PCR product containing the entire sequence of pMUTIN4 (except for the erythromycin-resistance cassette) and (B) a *Sal*I / *Bgl*II-cut PCR product containing a tetracycline-resistance cassette amplified from pDG1513 were used to construct (C) the vector pAISH1.

Features; Pspac, an IPTG inducible promoter; MCS, a multiple cloning site containing the unique sites *Hin*dIII, *Eco*RI, *Not*I, *Sac*II and *Bam*HI, in both pMUTIN4 and pAISH1; *lacZ*, a promoterless *E. coli* β-galactosidase gene; *lac*I, a repressor of the Pspac promoter; *ori* ColE1, a ColE1 origin of replication; *amp*, ampicillin resistance gene; *ery*, an erythromycin-resistance gene; *tet*, a tetracycline-resistance gene. Annealing sites of the PCR primers JA-9 and JA-10 are indicated. Relevant restriction sites; B, *Bgl*II; C, *Cla*I; D, *Dra*I; H; *Hin*dIII; N, *Nco*I; S, *Sal*I. Not to scale.

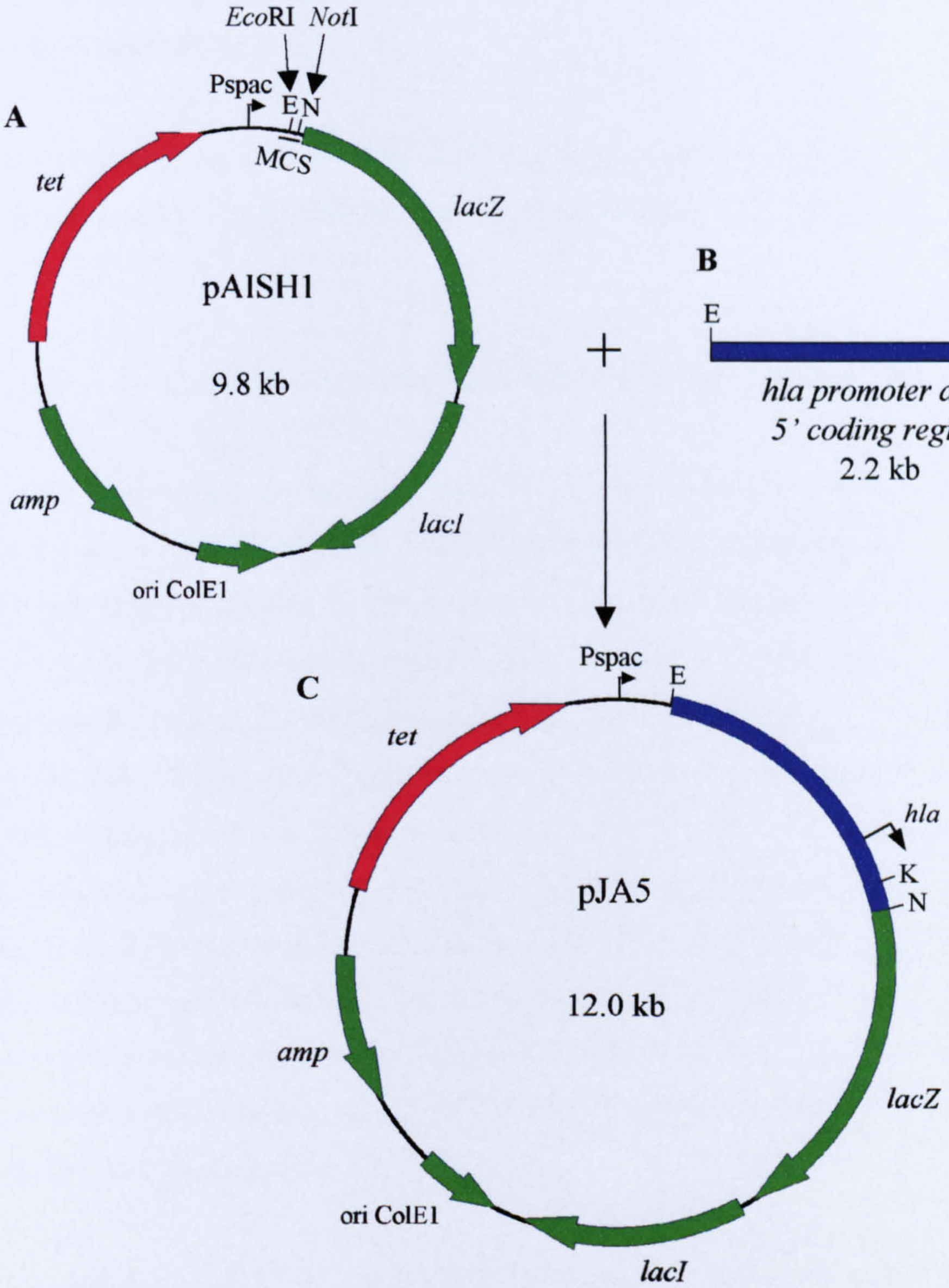


Figure 5.4

Construction of pJA5. (A) *Eco*RI / *Not*I-cut pAISH1 and (B) a 2.2 kb fragment containing ~ 400 bp of the 5' coding region of *hla* and ~ 1.8 kb of upstream sequence, cut from pPF4 using *Eco*RI and *Not*I were used to construct (C) the plasmid pJA5.

Features; Pspac, an IPTG inducible promoter; MCS, a multiple cloning site; *lacZ*, a promoterless *E. coli* β-galactosidase gene; *lac*I, a repressor of the Pspac promoter; *ori* ColE1, a ColE1 origin of replication; *amp*, ampicillin resistance gene; *tet*, a tetracycline-resistance gene. Relevant restriction sites; E, *Eco*RI; K, *Kpn*I; N, *Not*I. Not to scale.

*Eco*RI / *Not*I-cut pMUTIN4 by Chan & Foster (1998a) to construct the *hla::lacZ* fusion present in strain PC322.

Restriction mapping with *Eco*RI / *Not*I and *Kpn*I produced bands of the expected size (data not shown). The plasmid produced was named pJA5.

5.2.2.3 Construction of *hla::lacZ* fusion *S. aureus* strains using pJA5

In order to construct an *hla::lacZ* fusion in *S. aureus*, plasmid pJA5 was electroporated into *S. aureus* RN4220 (Chapter 2.10.2.2) (Figure 5.5). Transformants were selected on BHI agar plates containing X-Gal (Chapter 2.16.2) and 5 μg ml^{-1} Tet. Following growth at 37 °C for 24 h, one tetracycline-resistant, blue colony was produced. The construct was transferred into the 8325-4 and SH1000 backgrounds using ϕ11-mediated phage transduction (Chapter 2.9). Selection was performed at 37 °C overnight on BHI agar plates containing X-Gal (Chapter 2.16.2) and 5 μg ml^{-1} Tet. Genomic DNA was prepared from 8325-4, from the blue, tetracycline-resistant RN4220 colony, and from a blue, tetracycline-resistant transductant in the 8325-4 and SH1000 background (Chapter 2.11.1). Genomic DNA was cut with *Eco*RI overnight (Chapter 2.13.2). A DIG-labelled probe was prepared using the 2.2 kb *hla* promoter insert fragment cut from pPF4 with *Eco*RI and *Not*I (Chapters 2.14.1 and 2.13.6.4). Southern blotting was performed as described in Chapter 2.14.3.

The expected 5.5 kb *Eco*RI hybridising fragment for a wild-type, hla^+ strain (Figure 5.5a) was obtained for 8325-4 (Figure 5.5b). The expected 14.8 kb and 2.7 kb *Eco*RI hybridising fragments for a strain containing an *hla::lacZ* fusion next to a functional copy of *hla*, as introduced by pJA5 (Figure 5.5a), were obtained for the blue, tetracycline-resistant RN4220, 8325-4 and SH1000 samples tested (Figure 5.5b). The strains produced were named JLA511 (RN4220 *hla*::*lacZ*), JLA512 (8325-4 *hla*::*lacZ*) and JLA513 (SH1000 *hla*::*lacZ*). When patched onto rabbit blood plates (Chapter 2.16.1) alongside RN4220, 8325-4 and SH1000 and grown overnight at 37 °C, JLA511 (RN4220 *hla*::*lacZ*), JLA512 (8325-4 *hla*::*lacZ*) and JLA513 (SH1000 *hla*::*lacZ*) were found to produce levels of haemolysis identical to their respective parents (data not shown).

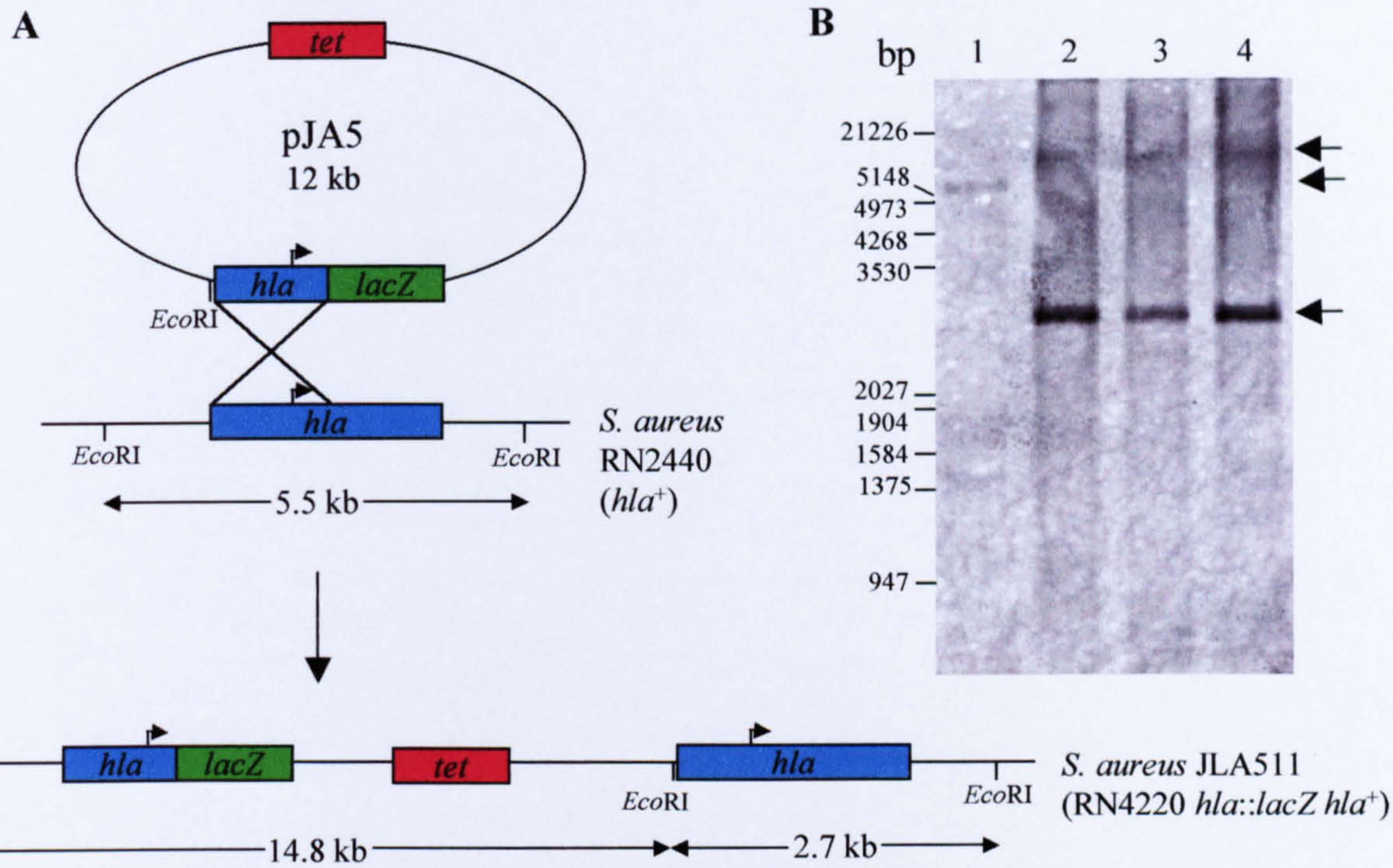


Figure 5.5

Construction of an *hla::lacZ* fusion in RN4220, 8325-4 and SH1000.

(A) Diagram showing the integration of pJA5 into the *S. aureus* RN4220 chromosome to give JLA511 (RN4220 *hla*::*lacZ*) (not to scale). The construct was transferred into the 8325-4 and SH1000 backgrounds using ϕ11-mediated phage transduction (Chapter 2.9). This produced strains JLA512 (8325-4 *hla*::*lacZ*) and JLA513 (SH1000 *hla*::*lacZ*). The sizes of the relevant *Eco*RI restriction fragments are indicated. Please note that the box representing the 2.2 kb *hla* promoter insert introduced by pJA5 comprises ~ 400 bp of the 5' coding region of *hla* and ~ 1.8 kb of upstream sequence, whereas the box representing the functional *hla* gene present on the *S. aureus* genomic DNA comprises the entire coding region of *hla* and ~ 1.8 kb of upstream sequence (B) Southern blot of *S. aureus* 8325-4 (lane 1), JLA511(RN4220 *hla*::*lacZ*) (lane 2), JLA512 (8325-4 *hla*::*lacZ*) (lane 3) and JLA513 (SH1000 *hla*::*lacZ*) (lane 4) genomic DNA cut with *Eco*RI and probed with a digoxygenin-labelled 2.2 kb *Eco*RI / *Not*I *hla* promoter insert from pPF4 (Chapters 2.14 and 2.13.6.4). The sizes of λ *Hin*dIII-*Eco*RI markers are indicated. Not to scale.

5.2.2.4 Quantitative β-galactosidase assay data produced by the *hla::lacZ* fusions constructed with tetracycline-resistant and erythromycin-resistant selectable markers

To confirm that the *hla::lacZ* fusion (Tet^R) constructed in this study produces similar β-galactosidase assay data to the published *hla::lacZ* fusion (Ery^R) created by Chan & Foster (1998a), strains JLA513 (SH1000 *hla*::*lacZ* (Tet^R)) and JLA371 (SH1000 *hla*::*lacZ* (Ery^R)) were grown in BHI as described in Chapter 2.7. Growth was measured spectrophotometrically as OD_{600} (Chapter 2.6.1), and *hla::lacZ* fusion expression was measured using MUG, as described in Chapter 2.8.

The two *hla::lacZ* fusions were found to produce very similar β-galactosidase assay data (Figure 5.6); JLA513 (SH1000 *hla*::*lacZ* (Tet^R)) and JLA371 (SH1000 *hla*::*lacZ* (Ery^R)) expressed 13,543 and 15,411 MUG units at t = 14 h respectively. The maximum difference between the two strains was 1.9-fold at t = 5 h (3,075 and 1,596 MUG units for JLA513 (SH1000 *hla*::*lacZ* (Tet^R)) and JLA371 (SH1000 *hla*::*lacZ* (Ery^R)) respectively).

5.2.3 Screen of Tn*551* mutant libraries in *hla::lacZ* SH1000 to identify genes regulating *hla* expression

In Chapter 3, it was shown that σ^B represses *agr* and *hla*, and that neither *sarA, rot* nor *sar*HI functions as an intermediate in this pathway. Since the identity of the missing effector(s) remains unknown, a transposon mutant library screen was performed in the SH1000 background to identify mutants in which *hla::lacZ* expression is increased, as determined by increased blue colouration compared to the background colonies on BHI agar plates containing X-Gal. In such mutants, the transposon might have inactivated the missing repressor, thus preventing σ^B from repressing *agr,* and consequently allowing the transcription of *hla::lacZ* to be de-repressed. Alternatively, a more complex regulatory system might occur, whereby σ^B upregulates a repressor which functions indirectly by down-regulating an activator of *agr*. Therefore, mutants that were less blue than the background colonies were also isolated in order to identify putative activators involved in the pathway. In order to allow colonies that were either

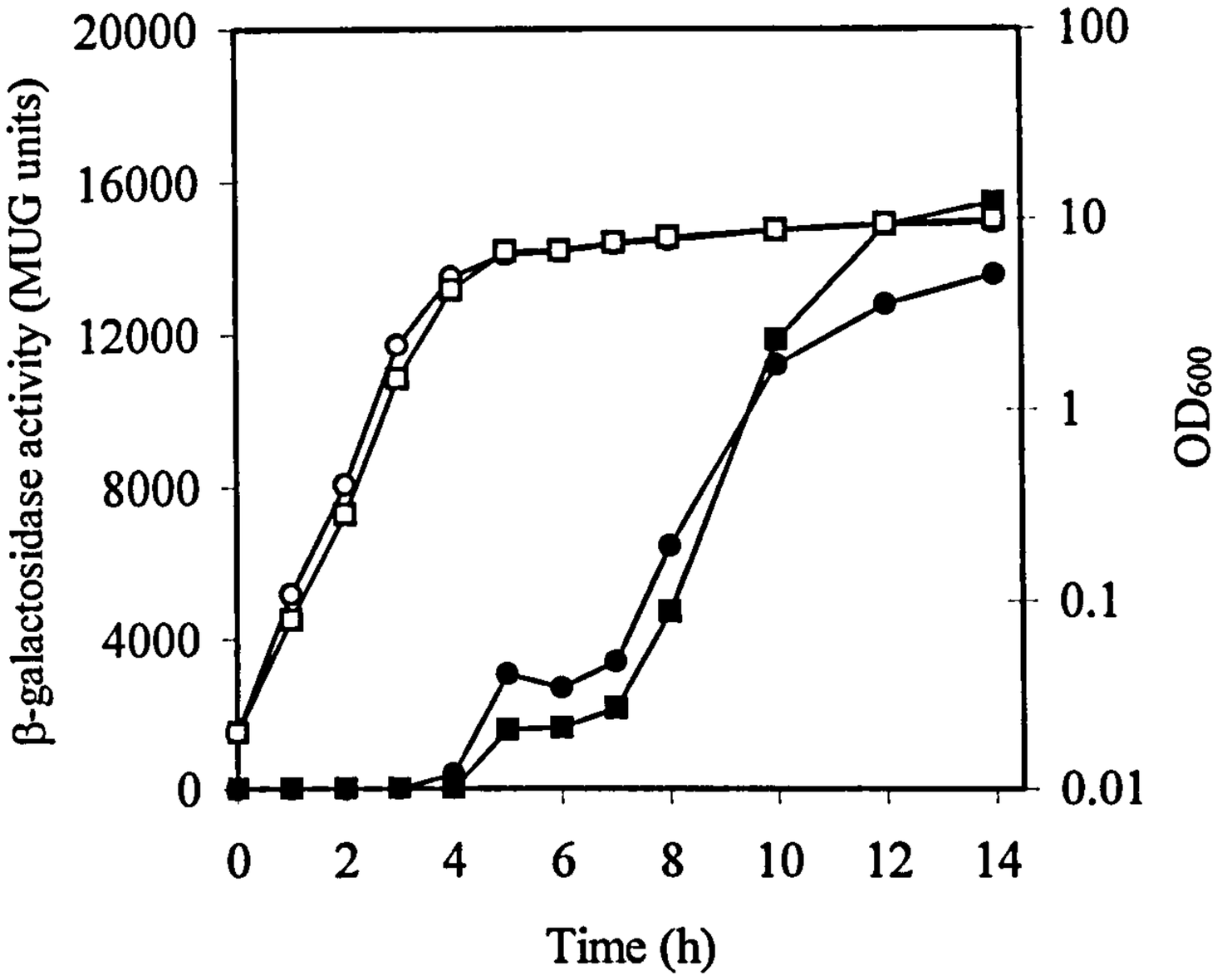


Figure 5.6

Comparison of β-galactosidase assay data produced by the *hla::lacZ* fusions constructed with tetracycline-resistant and erythromycin-resistant selectable markers.

S. aureus JLA513 (SH1000 *hla::lacZ* (Tet^R)) (○ and ●) and JLA371 (SH1000 *hla::lacZ* (Ery^R)) (□ and ■) were grown in BHI as described in Chapter 2.7. Growth was measured spectrophotometrically as OD_{600} (open symbols) (Chapter 2.6.1), and *lacZ* fusion expression was measured using MUG (closed symbols), as in Chapter 2.8. Results are representative of two independent experiments.

more or less blue than the background colonies to be screened for, a range of concentrations of X-Gal to be added to the BHI agar plates, including a final concentration of 200, 400, 600 and 800 mg l^{-1}, were tested. Colonies of JLA513 (SH1000 *hla*::*lacZ* (Tet^R)) demonstrated a suitable level of blue colouration to allow both more and less blue Tn*551* mutants to be identified on BHI agar plates containing a final concentration of 600 mg l^{-1} X-Gal (data not shown).

The transposon used in the screen performed in Chapter 4 was Tn*917*, carried by the plasmid pLTV1. However, this version of Tn*917* carries a promoterless *lacZ* gene, and is consequently unsuitable for screening in an *hla::lacZ* background. Therefore, the transposon Tn*551* was used. Tn*551* is a 5.3 kb transposon which was first identified in *S. aureus* on the plasmid p1258 (Mitsuhashi *et al.*, 1963). Like Tn*917*, Tn*551* is a member of the Tn*3* transposon family. Indeed, the nucleotide sequence of Tn*551* was found to be 99.8 % identical to that of unmodified Tn*917*, and the genetic organisation of the two transposons was also found to be the same (Wu *et al.*, 1999). Tn*551* has been used extensively in *S. aureus*, where it has facilitated the identification of the global regulators *agr* and *sae* (Recsei *et al.*, 1986; Giraudo *et al.*, 1994). Tn*551* has also been used in *S. aureus* for chromosome and plasmid mapping (Pattee *et al.*, 1977).

The delivery vector used to introduce Tn*551* into *S. aureus* in this study was the plasmid pRN3208 (Figure 5.7), a 20 kb derivative of p1258 (Kornblum *et al.*, 1986). The section of pRN3208 originating from p1258 carries a cadmium-resistance gene and a β-lactamase gene, and Tn*551* itself contains an erythromycin-resistance gene. In addition, pRN3208 has a temperature-sensitive replicon which only allows replication up to ~ 30 °C. Consequently, by growing cells at a temperature above 30 °C, cells containing a chromosomal Tn*551* insertion can be selected, since resistance to erythromycin (carried by Tn*551*) will remain, whilst resistance to cadmium (carried by pRN3208) will be lost.

5.2.3.1 Tn*551* mutagenesis of SH1000 *hla::lacZ*

The plasmid pRN3208 was introduced into JLA513 (SH1000 *hla::lacZ* (Tet^R)) by ϕ11-mediated transduction (Chapter 2.9), with selection on BHI agar plates containing 250

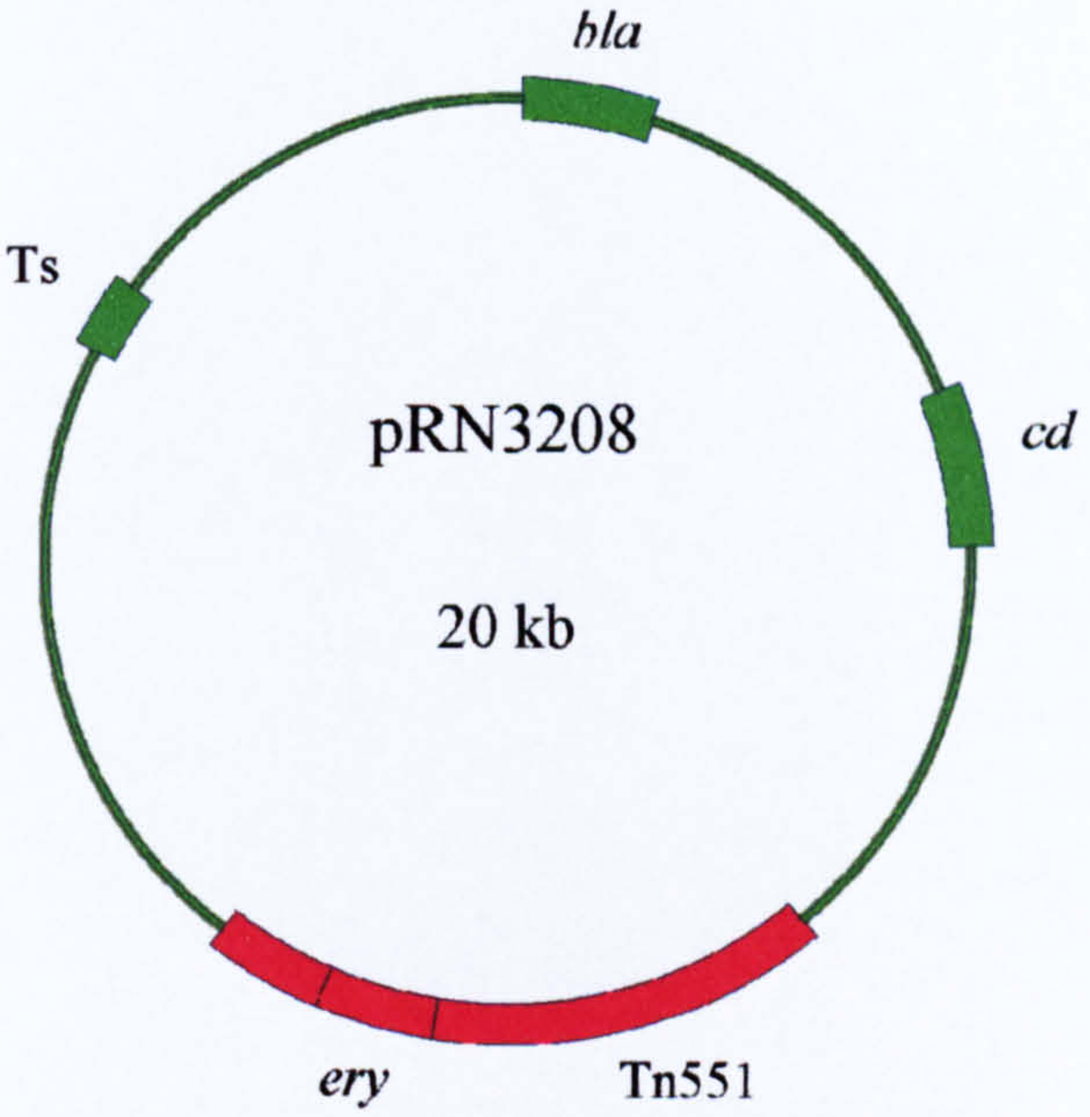


Figure 5.7

Physical map of pRN3208, the vector carrying Tn*551*.

Regions coloured in red are part of the transposable element, whilst the remainder of the vector is coloured in green. Features of this map; *bla*, β-lactamase gene; *cd*; cadmium-resistance gene; *ery*, erythromycin-resistance gene; Ts, a temperature-sensitive replicon.

Adapted from Fairhead (1998).

μg ml^{-1} $CdCl_2$. Transductants were also patched onto BHI agar plates containing 5 μg ml^{-1} Ery and 5 μg ml^{-1} Tet. All incubation stages were performed at 30 °C. The resulting strain was named JLA514 (SH1000 *hla::lacZ* (Tet^R), pRN3208). Transposon mutagenesis of this strain was performed as described in Chapter 2.15.2.

5.2.3.1.1 Enumeration and evaluation of Tn*551* libraries in SH1000 *hla::lacZ*

Four Tn*551* libraries were created using two different plasmid-curing temperatures (*ie.* either 42 or 43 °C). Libraries F1 and F2 were cured of pRN3208 at 42 °C, whereas F3 and F4 were grown at a non-permissive temperature of 43 °C. However, enumeration (Chapter 2.15.3) showed that only libraries F1 and F4 had a Tn*551* insertion frequency > 90 % and were therefore deemed suitable for screening (Table 5.1).

5.2.3.2 Primary screen

Libraries F1 and F4 were diluted using PBS to give ~ 300 colonies per plate, and spread onto BHI agar plates containing 600 mg l^{-1} X-Gal. Following growth overnight at 37 °C, the plates were screened to identify mutants that were more blue than the background colonies. The plates were then incubated for a further night at 37 °C and re-screened to identify mutants that were less blue than the background colonies.

In total, ~ 8,400 colonies were screened (~ 4,050 from library F1 and ~ 4,350 from library F4). 158 mutants were selected, of which 62 were more blue than the background colonies, and 96 were less blue (Table 5.2a).

5.2.3.3 Secondary screen

To ensure that the phenotypes of the Tn*551* mutants were reproducible, the strains were patched onto BHI agar plates containing 600 mg l^{-1} X-Gal, with JLA513 (SH1000 *hla::lacZ* (Tet^R)) as a control. 4 of the more blue Tn*551* mutants and 46 of the less blue mutants displayed wild-type levels of blue colouration, and were therefore discarded

Library	Plasmid curing temperature (°C)	Mean cfu ml^{-1} Ery-resistant	Mean cfu ml^{-1} Ery and Cd-resistant	% Tn*551* insertion
F1	**42**	**7.50×10^{11}**	**1.10×10^{9}**	**98.5**
F2	42	3.20×10^{10}	1.25×10^{10}	60.9
F3	43	4.10×10^{8}	1.15×10^{8}	72.0
F4	**43**	**3.45×10^{10}**	**6.50×10^{8}**	**98.1**

Table 5.1

Enumeration of Tn*551* libraries in SH1000 *hla::lacZ*.

Mean cfu ml^{-1} were calculated from the results of at least two experiments where colonies were plated onto BHI agar plates containing either erythromycin (Ery) or erythromycin and cadmium chloride (Ery and Cd) (Chapter 2.15.3). Details of the libraries used for screening are shown in bold. Results are representative of two independent experiments.

(results not shown). The remaining 58 more blue and 50 less blue mutants demonstrated reproducible phenotypes compared to JLA513 (SH1000 *hla::lacZ* (Tet^R)), and therefore passed the screen (Table 5.2a). These mutants were patched onto BHI plates containing Ery and Lin and onto BHI plates containing $CdCl_2$ to determine whether Tn*551* was inserted into the genome of the cells or carried on pRN3208. All of the mutants were Ery resistant and $CdCl_2$ sensitive, and therefore possessed chromosomal Tn*551* insertions. 13 more blue and 5 less blue representative mutants were selected for further analysis. The genes inactivated in these mutants were designated *aht* 1-18 (genes affecting ***hla*** transcription).

5.2.3.4 Tertiary screen

To determine whether the phenotypes displayed by the *aht* mutants on BHI agar plates containing 600 mg l^{-1} X-Gal were caused by Tn*551* insertion or by point mutations, the Tn*551* insertions were transferred into JLA513 (SH1000 *hla::lacZ* (Tet^R)) via ϕ11-mediated transduction, as described in Chapter 2.9. For each mutation, all 10 transductants tested displayed a similar level of increased or decreased blue colouration to the donor mutant strain when patched onto BHI agar plates containing 600 mg l^{-1} X-Gal with JLA513 (SH1000 *hla::lacZ* (Tet^R)) as a control (Table 5.2). Therefore, 100 % linkage of the mutant phenotype to the transposon insertion was demonstrated for all 18 mutants analysed. The strain names of a representative transductant per *aht* mutation are shown in table 5.2a.

A diagrammatic summary of the screening protocol and results is presented in Figure 5.8.

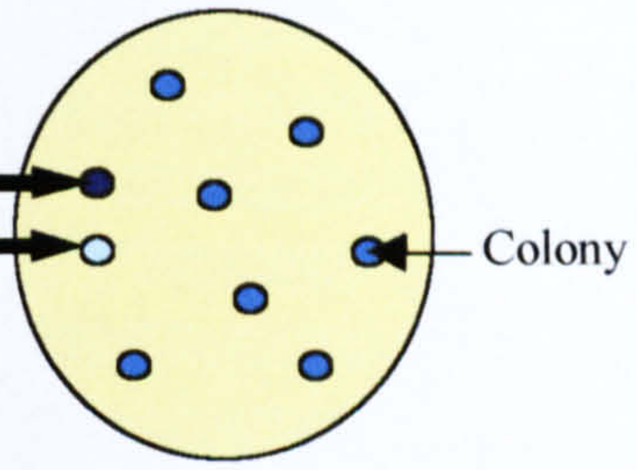
5.2.3.5 Direct genomic DNA sequencing to identify genes inactivated by Tn*551*

To identify the genes inactivated by Tn*551*, direct sequencing of genomic DNA was performed as in Chapter 2.13.1.2.4. The primer was the same as that used for sequencing the genomic DNA of the Tn*917 han* mutants in Chapter 4.2.3.1 (*ie.* primer

~ 8,400 Tn*551* mutants in SH1000 *hla::lacZ* background
(~ 4,050 from library F1 and ~ 4,350 from library F4)

↓ Plating onto BHI plates containing 600 mg l^{-1} X-Gal
(37 °C for up to 48 h)

~ 300 colonies per plate



158 putative Tn*551* mutants
- 62 mutants displaying increased blue colouration
- 96 mutants displaying decreased blue colouration

↓ Colonies patched onto BHI plates containing 600 mg l^{-1} X-Gal to ensure reproducibility of mutant phenotype (37 °C overnight)
(Secondary screen)

108 putative Tn*551* mutants
- 58 mutants displaying increased blue colouration
- 50 mutants displaying decreased blue colouration
- **18 representative mutants selected for further analysis**

↓ Mutations transduced into JLA513 (SH1000 *hla::lacZ* (Tet^R))
to check linkage of mutant phenotype to the transposon insertion.
10 transductants per strain patched onto BHI plates containing
600 mg l^{-1} X-Gal (37 °C overnight)
(Tertiary screen)

18 representative putative Tn*551* mutants
- 13 mutants displaying increased blue colouration
- 5 mutants displaying decreased blue colouration

Figure 5.8

Diagrammatic representation of the screen performed to identify Tn*551* mutants in the JLA513 (SH1000 *hla::lacZ* (Tet^R)) background that displayed either increased or decreased blue colouration compared to the background colonies on BHI agar plates containing 600 mg l^{-1} X-gal, and which may therefore contain Tn*551* insertions in genes involved in mediating the regulation of *hla*.

Tn2). The sequences of Tn*917* and Tn*551* are 100 % identical in the region where primer Tn2 anneals.

Database analysis was used to identify the Tn*551*-inactivated genes and the genes surrounding them in the *S. aureus* COL genome, as described in Chapter 4.2.3.1. Alignments of the sequencing reaction data against the COL DNA sequence are shown in Appendix A.2.10 – A.2.20. Homology of the products of the inactivated genes to other proteins is also shown in Appendix A.2.10 – A.2.20. As described in Chapter 2.17.2, the sequence of the region of COL genomic DNA containing the transposon-inactivated gene and surrounding genes of interest was extracted from the TIGR website and compared to the 8325 sequence in the NCBI BLAST database to ensure that the sequence and position of the genes in COL is the same as it is in 8325. For each region compared, the COL and 8235 genomic DNA sequence was 99 - 100 % identical, unless otherwise stated (data not shown). The genes inactivated by Tn*551* are listed in Table 5.2a, and are diagrammatically represented with their surrounding genes of interest in Figure 5.10. In total, 8 genes were found to have been inactivated by Tn*551* in the 18 mutants.

5.2.3.5.1 Mutations resulting in increased *hla::lacZ* expression

In JLA515 (SH1000 *hla::lacZ aht*1), JLA521 (SH1000 *hla::lacZ aht*7), JLA522 (SH1000 *hla::lacZ aht*8) and JLA523 (SH1000 *hla::lacZ aht*9), Tn*551* was found to have inserted into intergenic DNA, at base 2123979 of the *S. aureus* COL genome. The insertion is 238 bp downstream of *pemK*. However, the start codon of SA2057 is at base 2123864 of the *S. aureus* COL genome, 115 bp downstream of the Tn*551* insertion site. Therefore, it is possible that the Tn*551* insertion might be in a region regulating the transcription of SA2057, or that an alteration in the structure of the DNA caused by the presence of Tn*551* might affect the expression of SA2057. Protein sequence analysis showed that SA2057 demonstrated homology to RsbU, the upregulator of σ^B activity of *S. aureus* COL (99 % identity over 333 amino acids). The sequence of the region flanking the transposon insertion, represented diagrammatically in Figure 5.10, was found to be 99 % identical in 8325 and COL, except for the 11 bp deletion in *rsbU* of 8325, as discussed in Chapter 3.1 (data not shown).

In JLA516 (SH1000 *hla::lacZ aht*2) and JLA517 (SH1000 *hla::lacZ aht*3), Tn*551* was found to have inserted into SA1432 at base 256 of the 717 bp gene. Protein sequence analysis demonstrated homology to the tetrahydrodipicolinate acetyltransferase YkuQ of *S. aureus* 8325-4 (100 % identity over 239 amino acids).

In JLA518 (SH1000 *hla::lacZ aht*4), Tn*551* was found to have inserted into SA1441 at base 881 of the 1134 bp gene. Protein sequence analysis demonstrated homology to the tellurite resistance protein TelA of *R. sphaeroides* (33 % identity over 342 amino acids).

In JLA519 (SH1000 *hla::lacZ aht*5), Tn*551* was found to have inserted into SA1435 at base 1224 of the 1263 bp gene. Protein sequence analysis demonstrated homology to the diaminopimelate decarboxylase LysA of *S. aureus* 8325-4 (100 % identity over 421 amino acids).

In JLA520 (SH1000 *hla::lacZ aht*6), Tn*551* was found to have inserted into SA1443 at base 1221 of the 1341 bp gene. Protein sequence analysis demonstrated homology to the branched-chain amino acid carrier protein BrnQ of *S. aureus* RN450 (84 % identity over 429 amino acids).

In JLA524 (SH1000 *hla::lacZ aht*10) and JLA525 (SH1000 *hla::lacZ aht*11), Tn*551* was found to have inserted into SA1006 at base 762 of the 804 bp gene. Additionally, in JLA526 (SH1000 *hla::lacZ aht*12) and JLA527 (SH1000 *hla::lacZ aht*13), Tn*551* was found to have inserted into SA1006 at base 159. Protein sequence analysis demonstrated homology to the hypothetical protein YjbH of *B. subtilis* (31 % identity over 261 amino acids). However, the function of YjbH was unknown in the Subtilist database.

5.2.3.5.2 Mutations resulting in decreased *hla::lacZ* expression

In JLA528 (SH1000 *hla::lacZ aht*14), JLA529 (SH1000 *hla::lacZ aht*15), JLA530 (SH1000 *hla::lacZ aht*16) and JLA531 (SH1000 *hla::lacZ aht*17), Tn*551* was found to have inserted into SA2023 at base 331 of the 621 bp gene. Protein sequence analysis

demonstrated homology to the accessory gene regulator protein AgrB of *S. aureus* 8325-4 (99 % identity over 189 amino acids).

In JLA532 (SH1000 *hla::lacZ aht*18), Tn*551* was found to have inserted into SA0766 at base 414 of the 684 bp gene. Protein sequence analysis demonstrated homology to the response regulator SaeR of *S. aureus* (100 % identity over 228 amino acids).

The characterisation of these mutants is described in Chapter 5.2.5.

5.2.4 Screen of a Tn*551* mutant library in SH1000 *hla::lacZ* to identify mutants with altered *hla* transcription in response to NaCl

As described in Chapter 4, screening of Tn*917* mutant libraries identified 16 putative 8325-4 mutants demonstrating increased haemolytic activity compared to 8325-4 on rabbit blood plates containing 1 M NaCl. However, it was not possible to determine whether the haemolytic factor for which activity was affected in the mutants was *hla* in particular, as the published *hla* cassette knock-out and *hla::lacZ* reporter gene fusion (O'Reilly *et al.*, 1986; Chan & Foster, 1998a) cannot be used in conjunction with the Tn*917* mutations because the selectable markers of all of these constructs are erythromycin-resistance cassettes (Chapter 4.2.3.2.1.1.1). Therefore, the approach presented here involved screening Tn*551* mutants in the JLA513 (SH1000 *hla::lacZ* (Tet^R)) background in order to identify genes specifically affecting the transcription of *hla* in response to NaCl. Colonies were isolated which demonstrated either increased or decreased *hla::lacZ* transcription, as determined by either increased or decreased blue colouration compared to the background colonies on BHI agar plates containing 1 M NaCl.

In order to allow mutants that were either more or less blue than the background colonies to be screened for, a range of concentrations of X-Gal to be added to the BHI agar plates containing 1 M NaCl was tested. This included a final X-Gal concentration of 0.4, 0.8 and 2.4 g l^{-1}. JLA513 (SH1000 *hla*::*lacZ* (Tet^R)) demonstrated a suitable level of blue colouration to allow both more and less blue Tn*551* mutants to be

identified on BHI agar plates containing 1 M NaCl and a final concentration of 2.4 g l^{-1} X-Gal (data not shown).

5.2.4.1 Primary screen

Cells from libraries F1 and F4 were unable to grow when plated directly from glycerol stocks onto BHI plates containing 1 M NaCl. Therefore, the transposon mutant libraries were first diluted using PBS to give ~ 100 colonies per plate and recovered on BHI agar plates containing Ery and Lin. Following growth overnight at 37 °C, colonies were replica plated onto BHI agar plates (containing 1 M NaCl and 2.4 g l^{-1} X-Gal) and grown overnight at 37 °C. The plates were then screened to identify mutants that were more blue than the background colonies. Following incubation for a further night at 37 °C, the plates were re-screened to identify mutants that were less blue than the background colonies.

In total, ~ 6,250 colonies were screened (~ 3,750 from library F1 and ~ 2,500 from library F4). 27 mutants were selected, of which 25 were more blue than the background colonies, whilst 2 were less blue (Table 5.2b).

5.2.4.2 Secondary screen

To ensure that the phenotypes of the Tn*551* mutants were reproducible, the strains were patched onto BHI agar plates (containing 1 M NaCl and 2.4 g l^{-1} X-Gal), with JLA513 (SH1000 *hla::lacZ* (Tet^R)) as a control. 5 of the more blue Tn*551* mutants and 1 of the less blue mutants displayed wild-type levels of blue colouration (results not shown), and were therefore not studied further here. The remaining 20 more blue mutants and 1 less blue mutant demonstrated reproducible phenotypes compared to JLA513 (SH1000 *hla::lacZ* (Tet^R)), and therefore passed the screen (Table 5.2b).

The 21 mutants that passed the secondary screen were patched onto BHI agar plates containing Ery and Lin and onto BHI agar plates containing $CdCl_2$ to determine whether Tn*551* was inserted into the genome of the cells or carried on pRN3208. All of the

mutants were Ery resistant and $CdCl_2$ sensitive, and therefore possessed chromosomal Tn*551* insertions.

The mutant displaying decreased blue colouration on BHI agar plates containing 1 M NaCl and 2.4 g l^{-1} X-Gal demonstrated a wild-type level of colouration when patched onto a BHI agar plate containing 600 mg l^{-1} X-Gal. However, when patched onto BHI agar plates containing 600 mg l^{-1} X-Gal, all of the mutants selected for increased blue colouration demonstrated a similar degree of blue colouration as they did on BHI agar plates containing 1 M NaCl and 2.4 g l^{-1} X-Gal. This suggests that the effects of the genes mutated by Tn*551* which lead to increased blue colouration are not NaCl-specific. However, it was decided to further characterise the mutants and identify the genes inactivated, as they could potentially still provide a useful insight into the NaCl-mediated regulation of virulence determinant expression in *S. aureus*. Furthermore, the mutants displayed altered *hla::lacZ* transcription on BHI agar plates containing no added NaCl. Consequently, the genes identified might add to the list of genes mediating the repression of *agr* and *hla* by σ^B, as screened for by virtue of altered *hla::lacZ* transcription on BHI agar plates in Chapter 5.2.3. The genes inactivated in the mutants isolated on BHI agar plates containing 1 M NaCl and 2.4 g l^{-1} X-Gal were designated *htn* 1-21 (genes affecting *hla* transcription identified by screening on BHI plates containing NaCl).

5.2.4.3 Tertiary screen

To determine whether the phenotypes displayed by the *htn* mutants on BHI agar plates (containing 1 M NaCl and 2.4 g l^{-1} X-Gal) and on BHI agar plates (containing 600 mg l^{-1} X-Gal) were caused by Tn*551* insertion or by point mutations, the mutations were transferred into JLA513 (SH1000 *hla::lacZ* (Tet^R)) via ϕ11-mediated transduction, as described in Chapter 2.9. For the mutations leading to increased blue colouration, all 10 transductants tested displayed a reproducible mutant phenotype compared to the donor mutant strain when patched onto BHI agar plates (containing 1 M NaCl and 2.4 g l^{-1} X-Gal) and onto BHI agar plates (containing 600 mg l^{-1} X-Gal) (Table 5.2b). Therefore, 100 % linkage of the mutant phenotype to the transposon insertion was demonstrated for all 20 mutants displaying increased blue colouration. However, for the mutation

causing decreased blue colouration (*ie. htn*21), all 10 of the transductants displayed wild-type levels of blue colouration when patched onto BHI agar plates (containing 1 M NaCl and 2.4 g l^{-1} X-Gal) and on BHI agar plates (containing 600 mg l^{-1} X-Gal). Since 0 % linkage of the mutant phenotype to the transposon insertion was demonstrated, *htn*21 failed the tertiary screen. The strain names of a representative transductant per *htn* mutation are shown in Table 5.2b.

A diagrammatic summary of the screening protocol and results is presented in Figure 5.9.

5.2.4.4 Direct genomic DNA sequencing to identify genes inactivated by Tn*551*

To identify the genes inactivated by Tn*551*, direct sequencing of genomic DNA was performed as in Chapter 2.13.1.2.4, using the primer Tn2. Database analysis was used to identify the Tn*551*-inactivated genes and the genes surrounding them in the *S. aureus* COL genome, as described in Chapter 4.2.3.1. Alignments of the sequencing reaction data against the COL sequence are shown in Appendix A.2.10 -A.2.20. Homology of the products of the inactivated genes to other proteins is also shown in Appendix A.2.10 - A.2.20. As described in Chapter 2.17.2, the sequence of the region of COL genomic DNA containing the transposon-inactivated gene and surrounding genes of interest was extracted from the TIGR website and compared to the 8325 sequence in the NCBI BLAST database to ensure that the sequence and position of the genes in COL is the same as it is in 8325. For each region compared, the COL and 8325 genomic DNA sequence was 99 - 100 % identical, unless otherwise stated (data not shown). The genes inactivated by Tn*551* are listed in Table 5.2b, and are diagrammatically represented with their surrounding genes of interest in Figure 5.10. In total, 6 genes were found to have been inactivated by Tn*551* in the 20 mutants.

In JLA533 (SH1000 *hla::lacZ htn*1), JLA534 (SH1000 *hla::lacZ htn*2), JLA535 (SH1000 *hla::lacZ htn*3), JLA536 (SH1000 *hla::lacZ htn*4), JLA537 (SH1000 *hla::lacZ htn*5), JLA538 (SH1000 *hla::lacZ htn*6), JLA539 (SH1000 *hla::lacZ htn*7), JLA540 (SH1000 *hla::lacZ htn*8), JLA541 (SH1000 *hla::lacZ htn*9) and JLA546 (SH1000

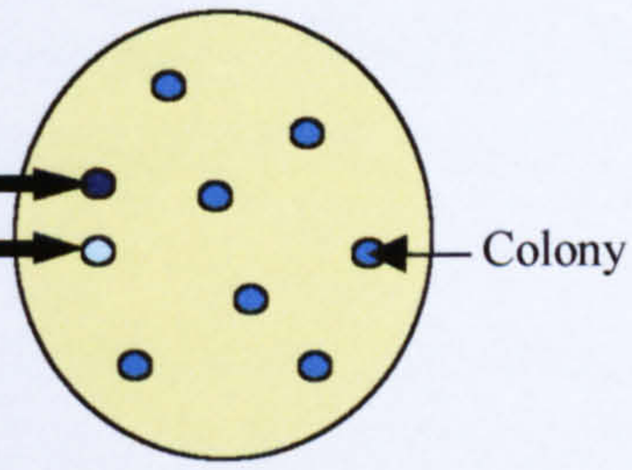
~ 6,250 Tn*551* mutants in SH1000 *hla::lacZ* background
(~ 3,750 from library F1 and ~ 2,500 from library F4)

↓ Cells recovered on BHI Ery Lin

~ 100 colonies per plate

↓ Colonies replica plated onto BHI plates containing 1 M NaCl and 2.4 g l^{-1} X-Gal (37 °C for up to 48 h)

Colonies with increased or decreased blue colouration compared to the background colonies were selected
(Primary screen)



27 putative Tn*551* mutants
- 25 mutants displaying increased blue colouration
- 2 mutants displaying decreased blue colouration

↓ Colonies patched onto BHI plates containing 1 M NaCl and 2.4 g l^{-1} X-Gal to ensure reproducibility of mutant phenotype (37 °C overnight)
(Secondary screen)

21 putative Tn*551* mutants
- 20 mutants displaying increased blue colouration
- 1 mutant displaying decreased blue colouration

↓ Mutations transduced into JLA513 (SH1000 *hla::lacZ* (Tet^R)) to check linkage of mutant phenotype to the transposon insertion. 10 transductants per strain patched onto BHI plates containing 1 M NaCl and 2.4 g l^{-1} X-Gal (37 °C overnight)
(Tertiary screen)

20 putative Tn*551* mutants displaying increased blue colouration

Figure 5.9

Diagrammatic representation of the screen performed to identify Tn*551* mutants in the JLA513 (SH1000 *hla::lacZ* (Tet^R)) background that displayed either increased or decreased blue colouration compared to the background colonies on BHI agar plates containing 1 M NaCl and 2.4 g l^{-1} X-gal, and which may therefore contain Tn*551* insertions in genes involved in regulating *hla* transcription in response to NaCl.

*hla::lacZ htn*14), Tn*551* was found to have inserted into base 2123979 of the *S. aureus* COL genome. The position of insertion is identical to that in JLA515 (SH1000 *hla::lacZ aht*1), JLA521 (SH1000 *hla::lacZ aht*7), JLA522 (SH1000 *hla::lacZ aht*8) and JLA523 (SH1000 *hla::lacZ aht*9) (Chapter 5.2.3.5). The *htn* mutants therefore also possess a Tn*551* insertion that may affect the transcription of *rsbU*.

In JLA542 (SH1000 *hla::lacZ htn*10), Tn*551* was found to have inserted into SA1428 at base 328 of the 1203 bp gene. Protein sequence analysis demonstrated homology to the type II aspartokinase, LysC, of *S. aureus* 8325-4 (99 % identity over 401 amino acids).

In JLA543 (SH1000 *hla::lacZ htn*11), JLA544 (SH1000 *hla::lacZ htn*12), JLA545 (SH1000 *hla::lacZ htn*13) and JLA552 (SH1000 *hla::lacZ htn*20), Tn*551* was found to have inserted into SA1435 at base 1224 of the 1263 bp gene. The position of insertion is identical to that in JLA519 (SH1000 *hla::lacZ aht*5) (Chapter 5.2.3.5). The *htn* mutants therefore also possess a Tn*551* insertion in *lysA*.

In JLA547 (SH1000 *hla::lacZ htn*15) and JLA548 (SH1000 *hla::lacZ htn*16), Tn*551* was found to have inserted into SA1441 at base 881 of the 1134 bp gene. The position of insertion is identical to that in JLA518 (SH1000 *hla::lacZ aht*4) (Chapter 5.2.3.5). The *htn* mutants therefore also possess a Tn*551* insertion in *telA*.

In JLA549 (SH1000 *hla::lacZ htn*17), Tn*551* was found to have inserted into base 1466658 of the *S. aureus* COL genome. The insertion is 198 bp downstream of SA1452 (encoding the type 2 phosphatidic acid phosphatase Pap2) and 593 bp upstream of SA1451 (the DNA-binding response regulator ArlR). It is possible that an alteration in the structure of the DNA caused by the presence of Tn*551* might affect the transcription of these genes. However, when the DNA sequence between *arlR* and *pap*2 was entered into a BLASTX search in the NCBI BLAST website (Chapter 2.17.2), 100 % identity to a 57 amino acid hypothetical protein of the *S. aureus* strains Mu50 and N315 was demonstrated within the region indicated in Figure 5.10g. However, no homology to a protein of known function could be shown. The position of Tn*551* insertion is at base 51 of this gene. It is most likely that the mutant phenotype of JLA549 (SH1000 *hla::lacZ htn*17) is caused by Tn*551*-inactivation of the hypothetical protein, which will be referred to as Hyp1.

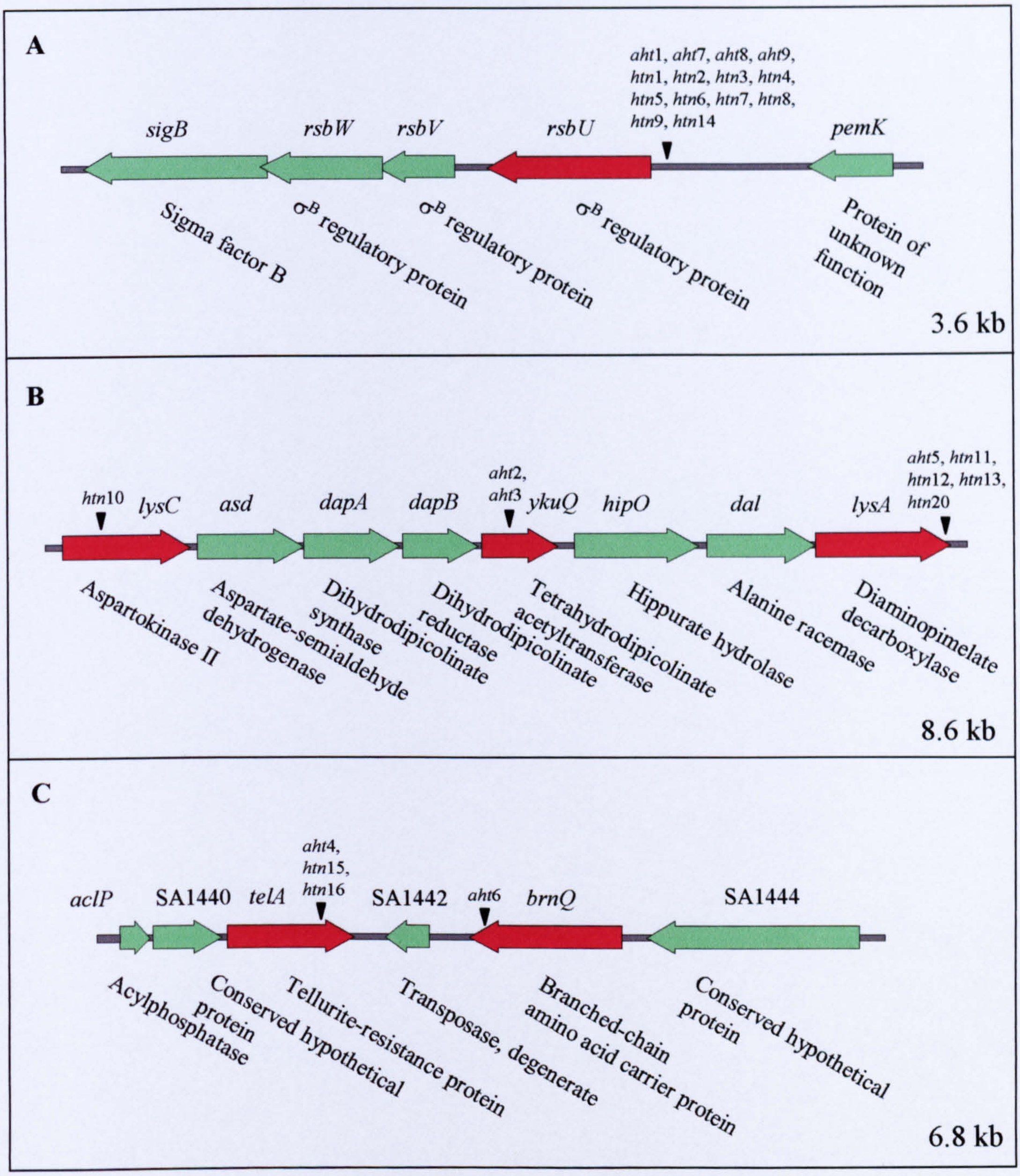


Figure 5.10 a-c

Position of Tn*551* insertions and surrounding genes of interest for (A) *aht*1, *aht*7, *aht*8, *aht*9, *htn*1, *htn*2, *htn*3, *htn*4, *htn*5, *htn*6, *htn*7, *htn*8, *htn*9 and *htn*14 (*hyp*1), (B) *htn*10 (*lysC*), *aht*2 and *aht*3 (*ykuQ*) and *aht*5, *htn*11, *htn*12, *htn*13, *htn*20 (*lysA*), and (C) *aht*4, *htn*15 and *htn*16 (*telA*) and *aht*6 (*brnQ*).

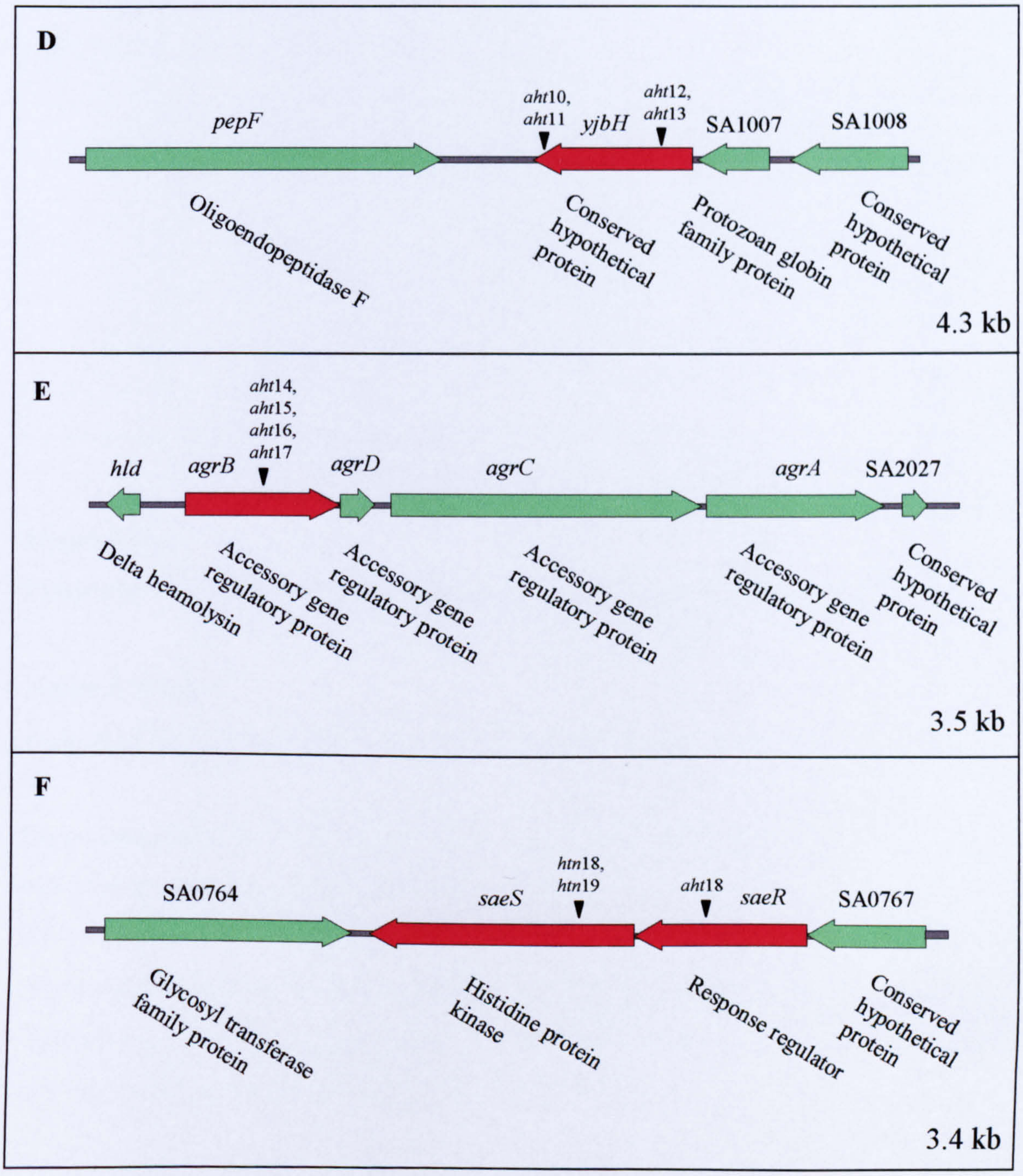


Figure 5.10 d-f

Position of Tn*551* insertions and surrounding genes of interest for (D) *aht*10, *aht*11, *aht*12 and *aht*13 (*yjbH*), (E) *aht*14, *aht*15, *aht*16 and *aht*17 (agrB), and (F) *htn*18 and *htn*19 (*saeS*), and *aht*18 (*saeR*).

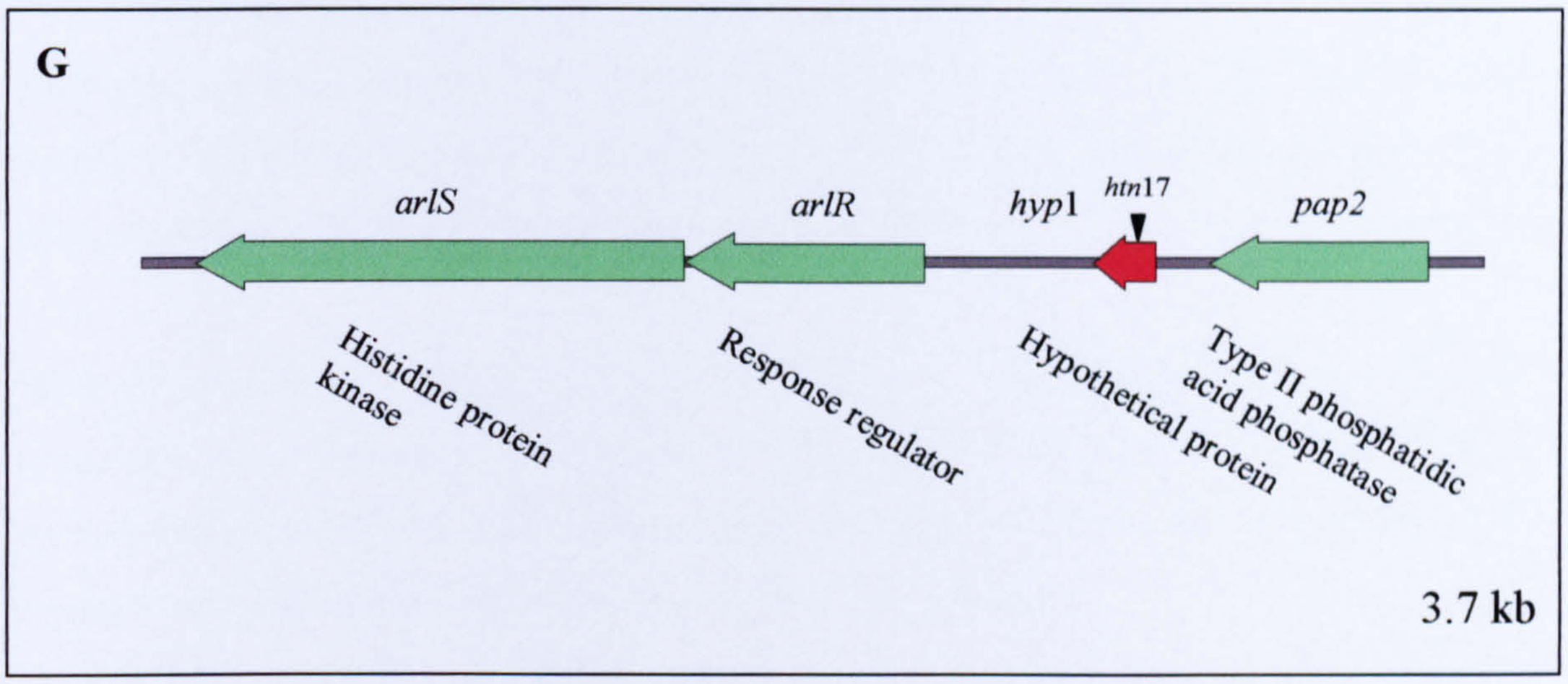


Figure 5.10 g

Position of Tn*551* insertions and surrounding genes of interest for *htn*17 (*hyp*1).

Figure 5.10 a-g

Position of Tn*551* insertions in *aht* and *htn* mutants, and surrounding genes of interest.

Genes inactivated by Tn*551* are presented in red, and the position of Tn*551* insertion is indicated (▼). Flanking genes of interest are shown in green. The approximate size of each DNA fragment is shown in the bottom right-hand corner of each diagram. The information for each mutation was obtained by analysing sequence data (Chapter 2.17.2) from strains derived by transduction of the *aht* and *htn* mutations into JLA513 (SH1000 *hla::lacZ* (Tet^R)) (see Tables 5.2a and 5.2b for strain names).

In JLA550 (SH1000 *hla::lacZ htn*18) and JLA551 (SH1000 *hla::lacZ htn*19), Tn*551* was found to have inserted into SA0765 at base 238 of the 1053 bp gene. Protein sequence analysis demonstrated homology to the histidine protein kinase SaeS of *S. aureus* Newman (89 % identity over 353 amino acids).

Many of the mutants isolated in this screen contain a transposon insertion in either the same base or in the same operon as an *aht* mutant (Chapter 5.2.3). Therefore, a representative mutant for each of the genes identified in the *aht* and *htn* screens was characterised, and collectively referred to as the Tn*551* mutants.

5.2.5 Characterisation of the Tn*551* mutants

5.2.5.1 *hla::lacZ* transcription in the Tn*551* mutants in the presence of either no added NaCl or 1 M NaCl

5.2.5.1.1 *hla::lacZ* transcription of Tn*551* mutants on BHI plates containing X-gal and either no added NaCl or 1 M NaCl

Photographs showing the blue colouration of a representative clone for each Tn*551* mutant, compared to JLA513 (SH1000 *hla::lacZ* (Tet^R)), on BHI agar plates containing either 1 M NaCl and 2.4 g l^{-1} X-gal or no added NaCl and 600 mg l^{-1} X-gal are presented in Figure 5.11. Scores for the level of blue colouration of the mutants on both types of plate are shown in Table 5.2. Blue colouration was estimated on a scale of 0 - 6.

Wild-type JLA513 (SH1000 *hla::lacZ* (Tet^R)) demonstrated a level of blue colouration of 3 on BHI containing no added NaCl and 600 mg l^{-1} X-gal, and 1 on BHI containing 1 M NaCl and 2.4 g l^{-1} X-gal. JLA526 (SH1000 *hla::lacZ aht*12 (*yjbH*)) was found to have a level of 4 on both types of plate. JLA542 (SH1000 *hla::lacZ htn*10 (*lysC*)), JLA516 (SH1000 *hla::lacZ aht*2 (*ykuQ*)), JLA519 (SH1000 *hla::lacZ aht*5 (*lysA*)), JLA518 (SH1000 *hla::lacZ aht*4 (*telA*)) and JLA520 (SH1000 *hla::lacZ aht*6 (*brnQ*)) were found to have a level of 5 on both types of plate. Additionally, JLA515 (SH1000 *hla::lacZ aht*1 (*rsbU*)) was found to have a level of 6 on both types of plate. These

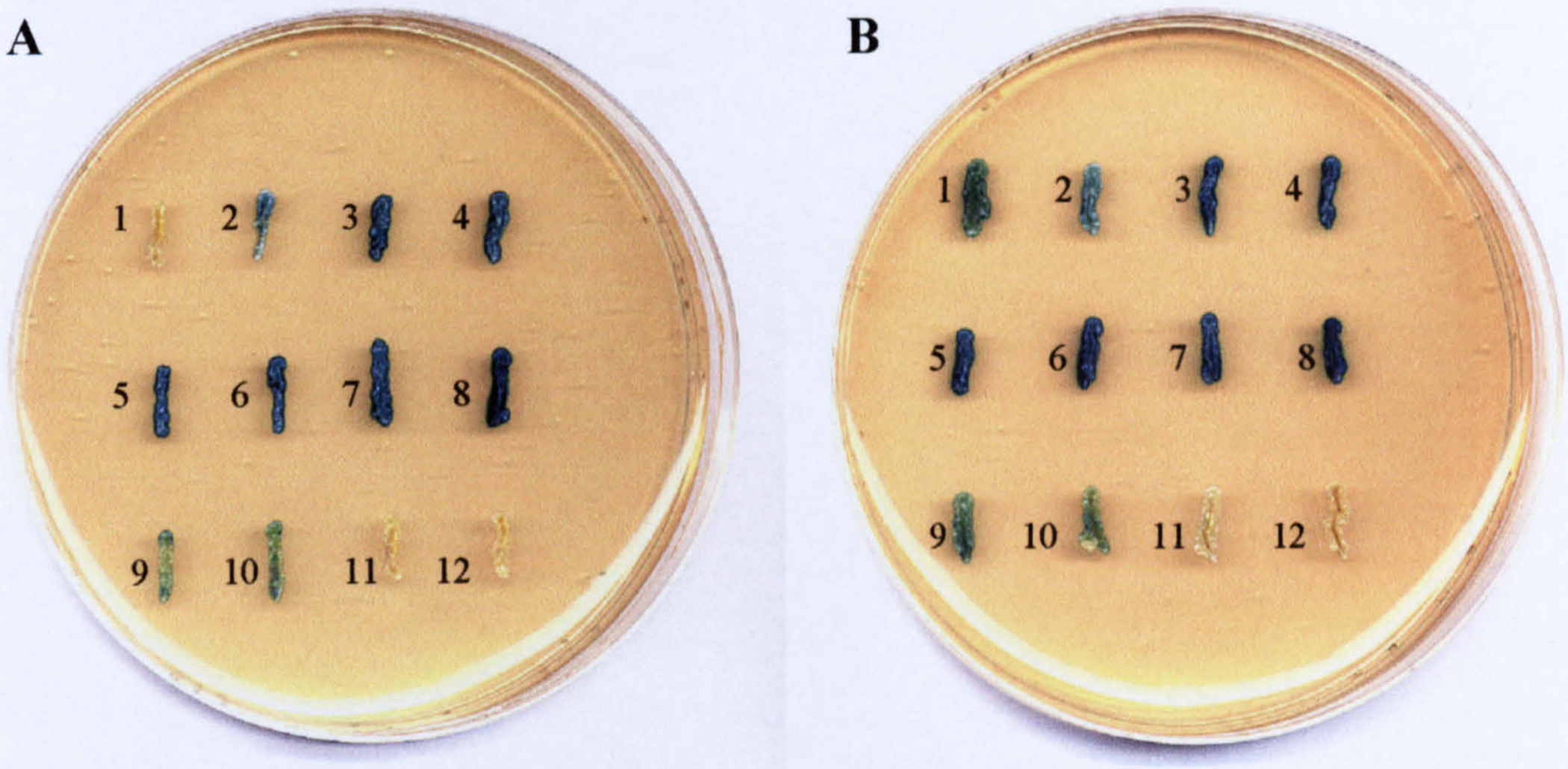


Figure 5.11

hla::lacZ transcription of representative *aht* and *htn* mutants, compared to JLA513 (SH1000 *hla::lacZ* (Tet^R)), as determined by blue colouration on (A) a BHI agar plate containing 1 M NaCl and 2.4 g l^{-1} X-gal, and on (B) a BHI agar plate containing no added NaCl and 600 mg l^{-1} X-gal.

Plates were patched with strains 1) JLA513 (SH1000 *hla::lacZ* (Tet^R)), 2) JLA526 (SH1000 *hla::lacZ aht*12 (*yjbH*)), 3) JLA542 (SH1000 *hla::lacZ htn*10 (*lysC*)), 4) JLA516 (SH1000 *hla::lacZ aht*2 (*ykuQ*)), 5) JLA519 (SH1000 *hla::lacZ aht*5 (*lysA*)), 6) JLA518 (SH1000 *hla::lacZ aht*4 (*telA*)), 7) JLA520 (SH1000 *hla::lacZ aht*6 (*brnQ*)), 8) JLA515 (SH1000 *hla::lacZ aht*1 (*rsbU*)), 9) JLA549 (SH1000 *hla::lacZ htn*17 (*hyp*1)), 10) JLA550 (SH1000 *hla::lacZ htn*18 (*saeS*)), 11) JLA528 (SH1000 *hla::lacZ aht*14 (*agrB*)), and 12) JLA532 (SH1000 *hla::lacZ aht*18 (*saeR*)) and incubated overnight at 37 °C. Results are representative of two independent experiments.

A

aht no.	Strain *no.* after transduction into JLA513 (SH1000 *hla::lacZ*)	Lib-rary	Blue colouration (No added NaCl)[a]			Blue colour-ation (1 M NaCl)	*S. aureus* gene no.[b]	Gene[c]	Function	Organism	Identity
			1°	2°	3°						
1	JLA515	F1	6	6	6	6	SA2057[d]	*rsbU*	Regulator of sigma factor σ^B	*S. aureus* COL	99% (332/333)
2	JLA516	F1	5	5	5	5	SA1432	*ykuQ*	Tetrahydrodipicolinate acetyltransferase	*S. aureus* 8325-4	100% (239/239)
3	JLA517	F1	5	5	5	5	SA1432	*ykuQ*	Tetrahydrodipicolinate acetyltransferase	*S. aureus* 8325-4	100% (239/239)
4	JLA518	F1	5	5	5	5	SA1441	*telA*	Tellurite resistance protein	*R. sphaeroides*	33% (115/342)
5	JLA519	F1	5	5	5	5	SA1435	*lysA*	Diaminopimelate decarboxylase	*S. aureus* 8325-4	100% (421/421)
6	JLA520	F1	5	5	5	5	SA1443	*brnQ*	Branched-chain amino acid carrier protein	*S. aureus* RN450	84% (361/429)
7	JLA521	F4	6	6	6	6	SA2057[d]	*rsbU*	Regulator of sigma factor σ^B	*S. aureus* COL	99% (332/333)
8	JLA522	F4	6	6	6	6	SA2057[d]	*rsbU*	Regulator of sigma factor σ^B	*S. aureus* COL	99% (332/333)
9	JLA523	F4	6	6	6	6	SA2057[d]	*rsbU*	Regulator of sigma factor σ^B	*S. aureus* COL	99% (332/333)
10	JLA524	F4	4	4	4	4	SA1006	*yjbH*	Hypothetical protein	*B. subtilis*	31% (81/261)
11	JLA525	F4	4	4	4	4	SA1006	*yjbH*	Hypothetical protein	*B. subtilis*	31% (81/261)
12	JLA526	F4	4	4	4	4	SA1006	*yjbH*	Hypothetical protein	*B. subtilis*	31% (81/261)
13	JLA527	F4	4	4	4	4	SA1006	*yjbH*	Hypothetical protein	*B. subtilis*	31% (81/261)
14	JLA528	F1	1	1	1	0	SA2023	*agrB*	Accessory gene regulator	*S. aureus* 8325-4	99% (188/189)
15	JLA529	F1	1	1	1	0	SA2023	*agrB*	Accessory gene regulator	*S. aureus* 8325-4	99% (188/189)
16	JLA530	F1	1	1	1	0	SA2023	*agrB*	Accessory gene regulator	*S. aureus* 8325-4	99% (188/189)
17	JLA531	F1	1	1	1	0	SA2023	*agrB*	Accessory gene regulator	*S. aureus* 8325-4	99% (188/189)
18	JLA532	F1	0	0	0	0	SA0766	*saeR*	Response regulator	*S. aureus*	100% (228/228)

B

htn no.	Strain *no.* after transduction into JLA513 (SH1000 *hla::lacZ*)	Lib-rary	Blue colouration (1 M NaCl)[a]			Blue colour-ation (no added NaCl)[a]	*S. aureus* gene no.[b]	Gene[c]	Function[c]	Organism	Identity
			1°	2°	3°						
1	JLA533	F4	6	6	6	6	SA2057[d]	*rsbU*	Regulator of sigma factor σ^B	*S. aureus* COL	99% (332/333)
2	JLA534	F4	6	6	6	6	SA2057[d]	*rsbU*	Regulator of sigma factor σ^B	*S. aureus* COL	99% (332/333)
3	JLA535	F4	6	6	6	6	SA2057[d]	*rsbU*	Regulator of sigma factor σ^B	*S. aureus* COL	99% (332/333)
4	JLA536	F4	6	6	6	6	SA2057[d]	*rsbU*	Regulator of sigma factor σ^B	*S. aureus* COL	99% (332/333)
5	JLA537	F4	6	6	6	6	SA2057[d]	*rsbU*	Regulator of sigma factor σ^B	*S. aureus* COL	99% (332/333)
6	JLA538	F4	6	6	6	6	SA2057[d]	*rsbU*	Regulator of sigma factor σ^B	*S. aureus* COL	99% (332/333)
7	JLA539	F4	6	6	6	6	SA2057[d]	*rsbU*	Regulator of sigma factor σ^B	*S. aureus* COL	99% (332/333)
8	JLA540	F4	6	6	6	6	SA2057[d]	*rsbU*	Regulator of sigma factor σ^B	*S. aureus* COL	99% (332/333)
9	JLA541	F4	6	6	6	6	SA2057[d]	*rsbU*	Regulator of sigma factor σ^B	*S. aureus* COL	99% (332/333)
10	JLA542	F1	5	5	5	5	SA1428	*lysC*	Aspartokinase II	*S. aureus* 8325-4	99% (400/401)
11	JLA543	F1	5	5	5	5	SA1435	*lysA*	Diaminopimelate decarboxylase	*S. aureus* 8325-4	100% (421/421)
12	JLA544	F1	5	5	5	5	SA1435	*lysA*	Diaminopimelate decarboxylase	*S. aureus* 8325-4	100% (421/421)
13	JLA545	F1	5	5	5	5	SA1435	*lysA*	Diaminopimelate decarboxylase	*S. aureus* 8325-4	100% (421/421)
14	JLA546	F4	6	6	6	6	SA2057[d]	*rsbU*	Regulator of sigma factor σ^B	*S. aureus* COL	99% (332/333)
15	JLA547	F1	5	5	5	5	SA1441	*telA*	Tellurite resistance protein	*R. sphaeroides*	33% (115/342)
16	JLA548	F1	5	5	5	5	SA1441	*telA*	Tellurite resistance protein	*R. sphaeroides*	33% (115/342)
17	JLA549	F1	2	2	2	2	-	*hyp*1	Hypothetical protein	*S. aureus* Mu50	100 % (57/57)
18	JLA550	F1	2	2	2	2	SA0765	*saeS*	Histidine protein kinase	*S. aureus* Newman	89% (316/353)
19	JLA551	F1	2	2	2	2	SA0765	*saeS*	Histidine protein kinase	*S. aureus* Newman	89% (316/353)
20	JLA552	F1	5	5	5	5	SA1435	*lysA*	Diaminopimelate decarboxylase	*S. aureus* 8325-4	100% (421/421)

Table 5.2

Summary of the phenotypic and sequence analysis results for the (A) *aht* and (B) *htn* Tn*551* mutants.

[a] *hla::lacZ* transcription, as determined by blue colouration on BHI agar plates (containing either 600 mg l^{-1} X-Gal or 1 M NaCl and 2.4 g l^{-1} X-gal) during primary (1°), secondary (2°) and tertiary (3°) screens. Blue colouration was compared to the background colonies for the primary screen and to JLA513 (SH1000 *hla::lacZ* (Tet^R)) for the secondary and tertiary screens. Blue colouration scale = 0 - 6, where JLA513 (SH1000 *hla::lacZ* (Tet^R)) = 3 on the BHI agar plate containing 600 mg l^{-1} X-Gal and 1 on the BHI agar plates containing 1 M NaCl and 2.4 g l^{-1} X-gal (see Figure 5.11)).

[b] *S. aureus* gene number according to the TIGR *S. aureus* COL database (Chapter 2.17.2).

[c] Highest BLASTP hit against a named gene (Chapter 2.17.2 and Appendix A.2.10 - A.2.20).

[d] Gene for which Tn*551* insertion is not within the ORF, but may be in a regulatory region controlling transcription.

mutants therefore demonstrated a higher level of *hla::lacZ* transcription than JLA513 (SH1000 *hla::lacZ* (TetR)) on both types of plate.

JLA549 (SH1000 *hla::lacZ htn*17 (*hyp*1)) and JLA550 (SH1000 *hla::lacZ htn*18 (*saeS*)) displayed a level of blue colouration of 2 on both types of plate, and therefore demonstrated a higher level of *hla::lacZ* transcription than JLA513 (SH1000 *hla::lacZ* (TetR)) on BHI containing 1 M NaCl and 2.4 g l^{-1} X-gal and a lower level of *hla::lacZ* transcription on BHI containing no added NaCl and 600 mg l^{-1} X-gal. JLA528 (SH1000 *hla::lacZ aht*14 (*agrB*)) displayed a level of blue colouration of 1 on BHI containing no added NaCl and 600 mg l^{-1} X-gal, and a level of blue colouration of 0 on BHI containing 1 M NaCl and 2.4 g l^{-1} X-gal, and therefore demonstrated a lower level of *hla::lacZ* transcription than JLA513 (SH1000 *hla::lacZ* (TetR)) on both types of plate. JLA532 (SH1000 *hla::lacZ aht*18 (*saeR*)) displayed a level of blue colouration of 0 on both types of plate, suggesting that *hla::lacZ* was either not being transcribed, or was being transcribed to an extremely low level in this mutant.

5.2.5.1.2 *hla::lacZ* transcription of Tn*551* mutants in BHI broth containing either no added NaCl or 1 M NaCl

JLA542 (SH1000 *hla::lacZ htn*10 (*lysC*)), JLA516 (SH1000 *hla::lacZ aht*2 (*ykuQ*)), JLA519 (SH1000 *hla::lacZ aht*5 (*lysA*)), JLA518 (SH1000 *hla::lacZ aht*4 (*telA*)) and JLA520 (SH1000 *hla::lacZ aht*6 (*brnQ*)) demonstrated a similar level of blue colouration to each-other on BHI agar plates containing no added NaCl and 600 mg l^{-1} X-gal (Chapter 5.2.5.1.1). The level of blue colouration of the mutants observed on BHI agar plates containing no added NaCl and 600 mg l^{-1} X-gal remained similar when patched onto BHI agar plates containing 1 M NaCl and 2.4 g l^{-1} X-gal (Chapter 5.2.5.1.1). On both types of plate, the level of blue colouration of these Tn*551* mutants was higher than that of JLA513 (SH1000 *hla::lacZ* (TetR)) (Chapter 5.2.5.1.1). In order to quantitatively measure the level to which *hla::lacZ* transcription was increased in the mutants, the representative mutant JLA542 (SH1000 *hla::lacZ htn*10 (*lysC*)) and JLA513 (SH1000 *hla::lacZ* (TetR)) were grown in BHI containing either no added NaCl or 700 mM NaCl, as described in Chapter 2.7. Growth was measured

spectrophotometrically as OD_{600} (Chapter 2.6.1), and *hla::lacZ* fusion expression was measured using MUG, as described in Chapter 2.8.

In BHI containing no added NaCl (Figure 5.12), *hla::lacZ* transcription was found to be up to 3.5 times greater in JLA542 (SH1000 *hla::lacZ htn*10 (*lysC*)) than in JLA513 (SH1000 *hla::lacZ* (TetR)) (*ie.* 12,028 and 3,398 MUG units respectively at t = 7 h). However *hla::lacZ* transcription was found to be very similar in JLA542 (SH1000 *hla::lacZ htn*10 (*lysC*)) and JLA513 (SH1000 *hla::lacZ* (TetR)) when grown in BHI containing 700 mM NaCl (Figure 5.12) (*ie.* 191 and 193 MUG units respectively at t= 7h). This suggests that the increased *hla::lacZ* transcription observed for JLA542 (SH1000 *hla::lacZ htn*10 (*lysC*)) on BHI agar plates containing 1 M NaCl and 2.4 g l^{-1} X-gal could not be reproduced in liquid culture. Due to time constraints, the levels of *hla::lacZ* transcription in the other Tn*551* mutants were not quantified using MUG.

5.2.5.2 Determination of the expression of Hla, SspA and other exoproteins by the Tn*551* mutants in BHI containing either no added NaCl or 1 M NaCl using exoprotein gel and Western blot analysis

To allow the effects of the Tn*551* mutations on the expression of virulence determinants and other exoproteins to be analysed in the absence of β-galactosidase production by the *hla::lacZ* fusion, the Tn*551* mutations present in JLA526 (SH1000 *hla::lacZ aht*12 (*yjbH*)), JLA542 (SH1000 *hla::lacZ htn*10 (*lysC*)), JLA516 (SH1000 *hla::lacZ aht*2 (*ykuQ*)), JLA519 (SH1000 *hla::lacZ aht*5 (*lysA*)), JLA518 (SH1000 *hla::lacZ aht*4 (*telA*)), JLA520 (SH1000 *hla::lacZ aht*6 (*brnQ*)), JLA515 (SH1000 *hla::lacZ aht*1 (*rsbU*)), JLA549 (SH1000 *hla::lacZ htn*17 (*hyp*1)), JLA550 (SH1000 *hla::lacZ htn*18 (*saeS*)), JLA528 (SH1000 *hla::lacZ aht*14 (*agrB*)), and JLA532 (SH1000 *hla::lacZ aht*18 (*saeR*)) were transferred into SH1000 via ϕ11 mediated transduction. This produced the strains JLA554 (SH1000 *aht*12 (*yjbH*)), JLA555 (SH1000 *htn*10 (*lysC*)), JLA556 (SH1000 *aht*2 (*ykuQ*)), JLA557 (SH1000 *aht*5 (*lysA*)), JLA558 (SH1000 *aht*4 (*telA*)), JLA559 (SH1000 *aht*6 (*brnQ*)), JLA560 (SH1000 *aht*1 (*rsbU*)), JLA561 (SH1000 *htn*17 (*hyp*1)), JLA562 (SH1000 *htn*18 (*saeS*)), JLA563 (SH1000 *aht*14 (*agrB*)) and JLA564 (SH1000 *aht*18 (*saeR*)) respectively. Strains were grown in BHI containing either no added NaCl or 1 M NaCl, as described in Chapter 2.18.1. Culture

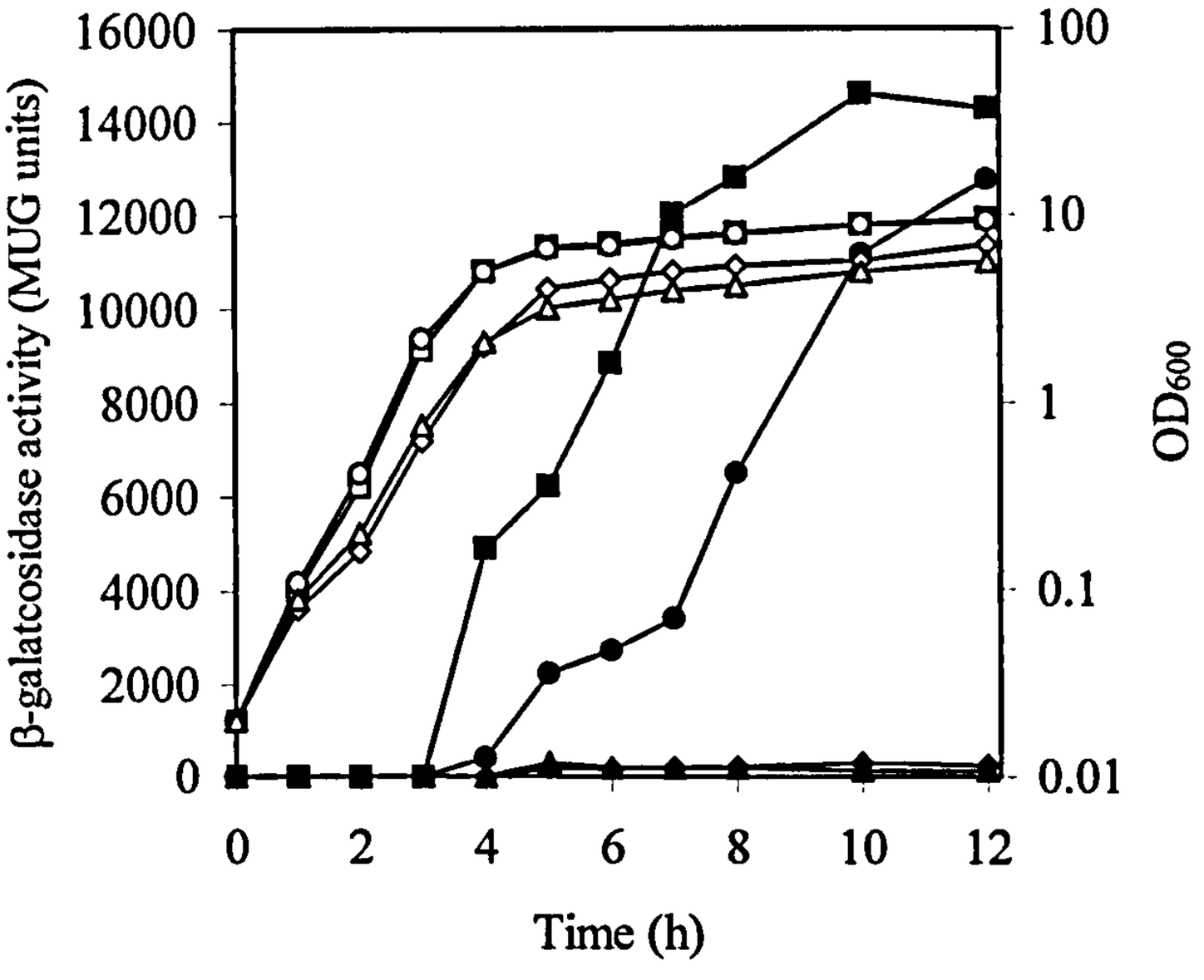


Figure 5.12

The effect of *lysC* on *hla::lacZ* transcription in BHI containing either no added NaCl or 700 mM NaCl.

S. aureus JLA513 (SH1000 *hla::lacZ* (TetR)) (○ and ●) and JLA542 (SH1000 *hla::lacZ htn*10 (*lysC*)) (□ and ■) were grown in BHI containing no added NaCl as described in Chapter 2.7. Additionally, JLA513 (SH1000 *hla::lacZ* (TetR)) (△ and ▲) and JLA542 (SH1000 *hla::lacZ htn*10 (*lysC*)) (◇ and ◆) were grown in BHI containing 700 mM NaCl. Growth was measured spectrophotometrically as OD_{600} (open symbols) (Chapter 2.6.1), and *lacZ* fusion expression was measured using MUG (closed symbols), as in Chapter 2.8. Results are representative of two independent experiments. N.B. ▲ and ◆ merge with baseline.

supernatants were removed after 14 h and exoprotein samples prepared as described in Chapter 2.18.3.1. Proteins were separated on 12 % (w/v) SDS-PAGE gels as described in Chapter 2.18.4. For Western blots, membranes were blocked with 20 % (v/v) pig sera to reduce non-specific binding, and probed with relevant antibodies as described in Chapter 2.18.5.

The level of Hla and SspA was greater in JLA554 (SH1000 *aht*12 (*yjbH*)), JLA555 (SH1000 *htn*10 (*lysC*)), JLA556 (SH1000 *aht*2 (*ykuQ*)), JLA557 (SH1000 *aht*5 (*lysA*)), JLA558 (SH1000 *aht*4 (*telA*)), JLA559 (SH1000 *aht*6 (*brnQ*)) and JLA560 (SH1000 *aht*1 (*rsbU*)) than in SH1000 when grown in BHI containing no added NaCl (Figure 5.13). The difference was most pronounced in JLA555 (SH1000 *htn*10 (*lysC*)), JLA556 (SH1000 *aht*2 (*ykuQ*)), JLA558 (SH1000 *aht*4 (*telA*)), JLA559 (SH1000 *aht*6 (*brnQ*)), and JLA560 (SH1000 *aht*1 (*rsbU*)).

The level of Hla and SspA was lower in JLA561 (SH1000 *htn*17 (*hyp*1)), JLA562 (SH1000 *htn*18 (*saeS*)), JLA563 (SH1000 *aht*14 (*agrB*)), and JLA564 (SH1000 *aht*18 (*saeR*)) than in SH1000 when grown in BHI containing no added NaCl (Figure 5.14). Indeed, (SH1000 *htn*18 (*saeS*)), JLA563 (SH1000 *aht*14 (*agrB*)), and JLA564 (SH1000 *aht*18 (*saeR*)) expressed virtually no detectable Hla.

However, an altered level of Hla and SspA compared to SH1000 could not be demonstrated for any of the Tn*551* mutants when cultures were grown in BHI containing 1 M NaCl (data not shown).

5.2.5.3 Analysis of the effects of the Tn*551* mutations on growth in BHI containing either no added NaCl or 2 M NaCl

To determine the effects of the Tn*551* mutations on growth, JLA554 (SH1000 *aht*12 (*yjbH*)), JLA555 (SH1000 *htn*10 (*lysC*)), JLA556 (SH1000 *aht*2 (*ykuQ*)), JLA557 (SH1000 *aht*5 (*lysA*)), JLA558 (SH1000 *aht*4 (*telA*)), JLA559 (SH1000 *aht*6 (*brnQ*)), JLA560 (SH1000 *aht*1 (*rsbU*)), JLA561 (SH1000 *htn*17 (*hyp*1)), JLA562 (SH1000 *htn*18 (*saeS*)), JLA563 (SH1000 *aht*14 (*agrB*)) JLA564 (SH1000 *aht*18 (*saeR*)) and SH1000 were grown in either BHI containing no added NaCl or in BHI containing 2 M

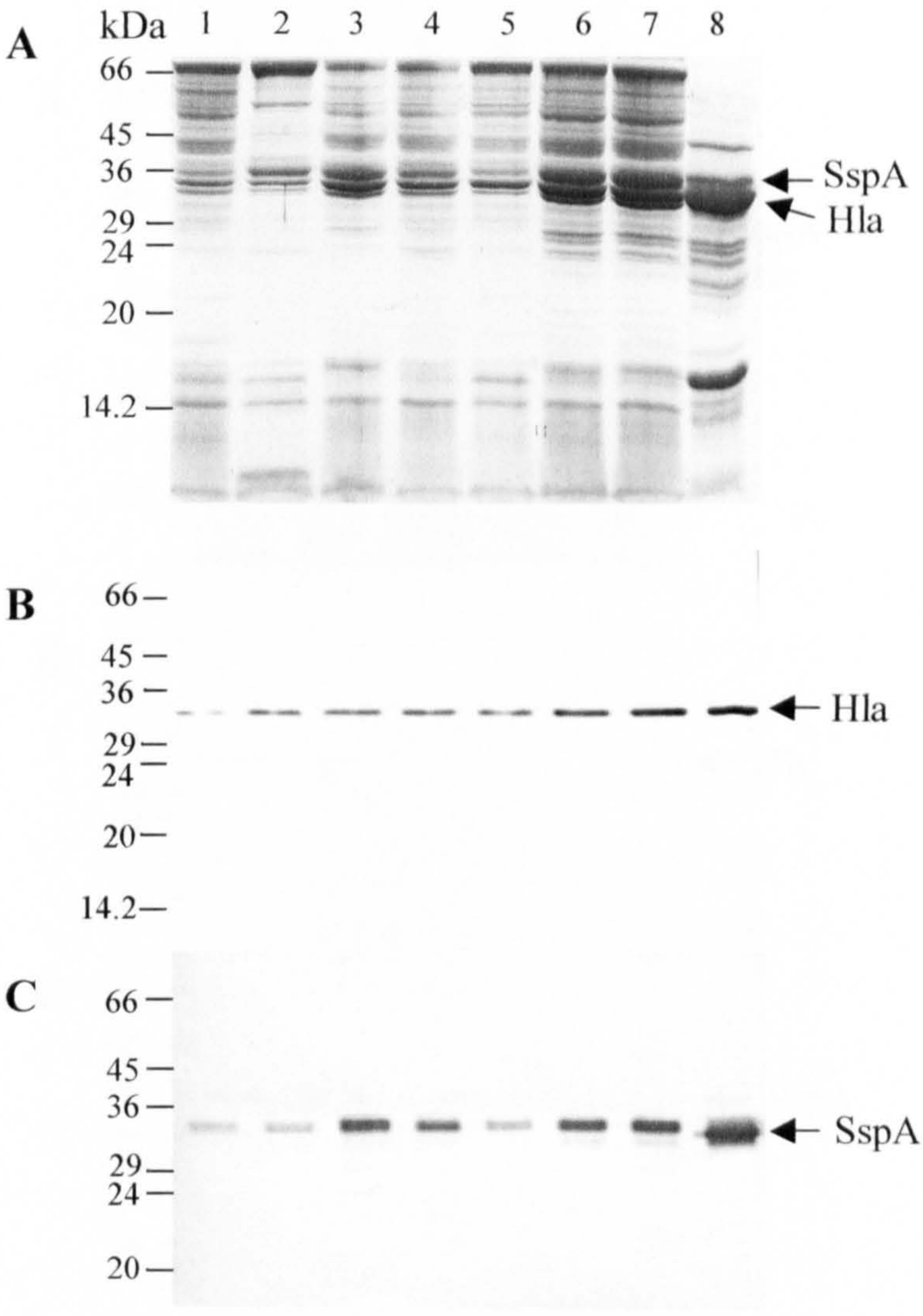


Figure 5.13

The effect of the Tn*551* mutations on the production of Hla, SspA and other exoproteins. (A) Exoprotein gel, (B) Hla Western blot and (C) SspA Western blot for 1) SH1000, 2) JLA554 (SH1000 *aht*12 (*yjbH*)), 3) JLA555 (SH1000 *htn*10 (*lysC*)), 4) JLA556 (SH1000 *aht*2 (*ykuQ*)), 5) JLA557 (SH1000 *aht*5 (*lysA*)), 6) JLA558 (SH1000 *aht*4 (*telA*)), 7) JLA559 (SH1000 *aht*6 (*brnQ*)) and 8) JLA560 (SH1000 *aht*1 (*rsbU*)). Culture supernatant was removed after 14 h from cultures grown in BHI containing no added NaCl and exoprotein samples prepared as described in Chapter 2.18.3.1. Proteins were separated on SDS-PAGE gels as described in Chapter 2.18.4. For Western blots, membranes were blocked with 20 % (w/v) pig sera and probed with relevant antibodies as described as in Chapter 2.18.5.1. Lanes contain exoproteins from 1.0 or 0.2 OD_{600} units of culture supernatant for exoprotein gels and Western blots respectively. Results are representative of a minimum of two independent experiments. Sizes of Sigma low molecular weight markers are shown.

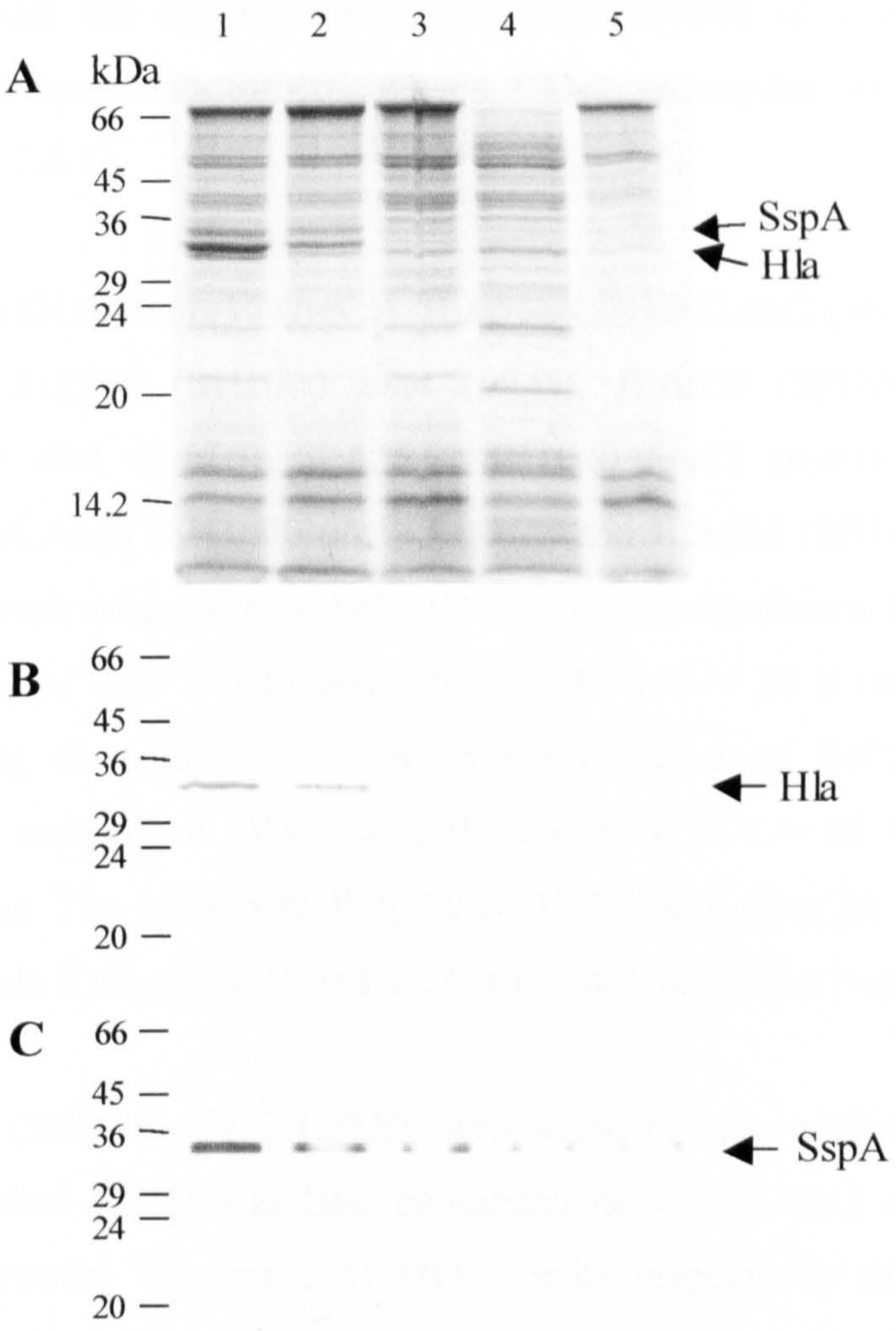


Figure 5.14

The effect of the Tn*551* mutations on the production of Hla, SspA and other exoproteins. (A) Exoprotein gel, (B) Hla Western blot and (C) SspA Western blot for 1) SH1000, 2) JLA561 (SH1000 *htn*17 (*hyp*1)), 3) JLA562 (SH1000 *htn*18 (*saeS*)), 4) JLA563 (SH1000 *aht*14 (*agrB*)), and 5) JLA564 (SH1000 *aht*18 (*saeR*)). Culture supernatant was removed after 14 h from cultures grown in BHI containing no added NaCl and exoprotein samples prepared as described in Chapter 2.18.4. For Western blots, membranes were blocked with 20 % (w/v) pig sera and probed with relevant antibodies as described as in Chapter 2.18.5.1. Lanes contain exoproteins from 1.0 or 0.2 OD_{600} units of culture supernatant for exoprotein gels and Western blots respectively. Results are representative of a minimum of two independent experiments. Sizes of Sigma low molecular weight markers are shown.

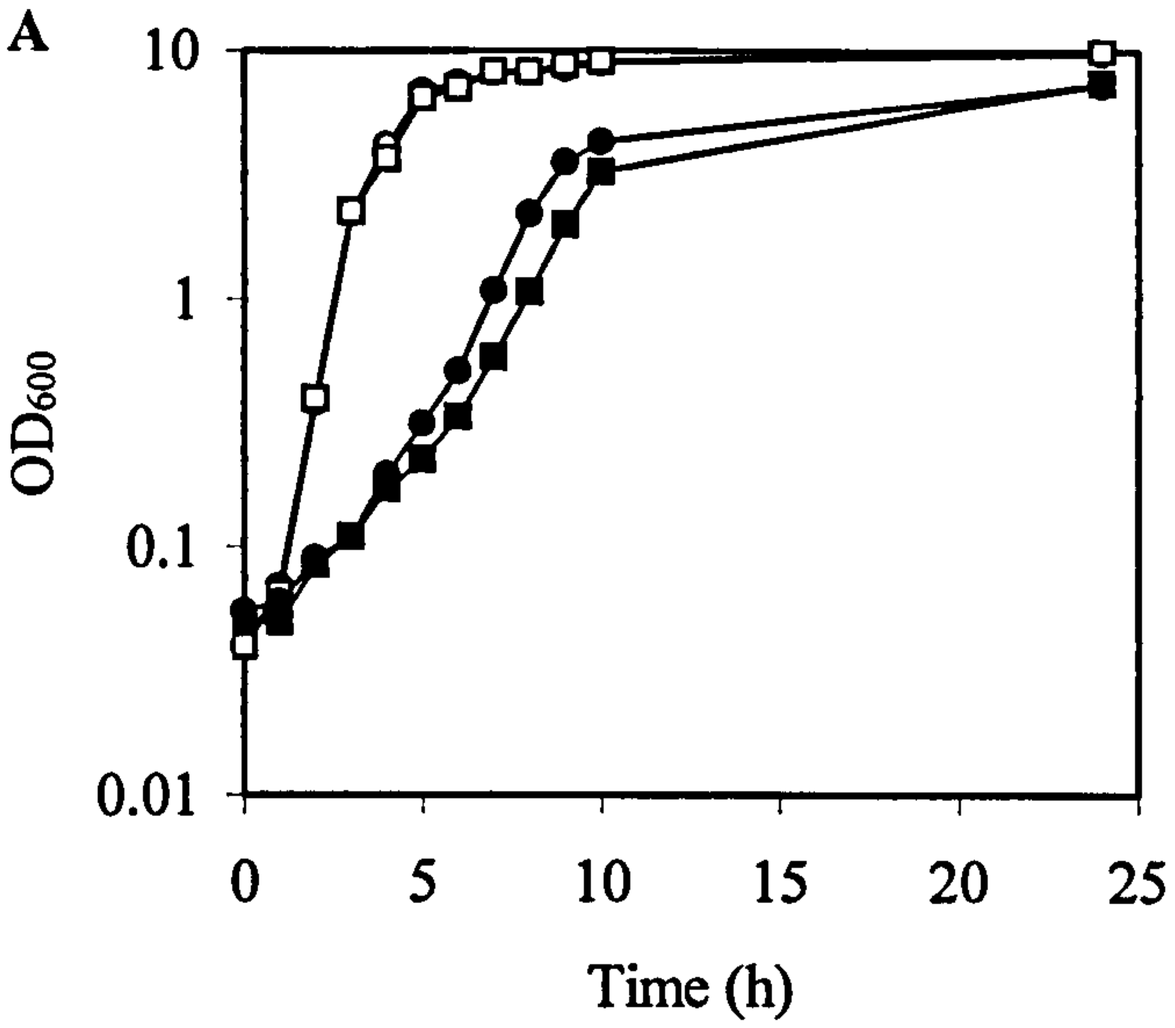
NaCl under the NaCl-shock conditions described in Chapter 4.2.3.3. Growth was measured spectrophotometrically as OD_{600} at regular intervals over a 24 h period (Chapter 2.6.1).

JLA555 (SH1000 *htn*10 (*lysC*)), JLA556 (SH1000 *aht*2 (*ykuQ*)), JLA557 (SH1000 *aht*5 (*lysA*)), JLA558 (SH1000 *aht*4 (*telA*)), JLA559 (SH1000 *aht*6 (*brnQ*)), JLA560 (SH1000 *aht*1 (*rsbU*)), JLA561 (SH1000 *htn*17 (*hyp*1)), JLA562 (SH1000 *htn*18 (*saeS*)), JLA563 (SH1000 *aht*14 (*agrB*)) and JLA564 (SH1000 *aht*18 (*saeR*)) displayed similar levels of growth to SH1000 under both conditions tested. The mutants reached an OD_{600} of 8.33 - 9.45 and 3.02 – 4.89 at t = 10 h (full data not shown) in BHI containing no added NaCl and in the presence of NaCl-shock respectively, whilst SH1000 reached an OD_{600} of 8.97 and 4.28 at t = 10 h under the two respective conditions. The growth of JLA559 (SH1000 *aht*6 (*brnQ*)) is shown in Figure 5.15a as an example (OD_{600} = 9.01 and 3.22 at t = 10 h under the two respective conditions).

JLA554 (SH1000 *aht*12 (*yjbH*)) was found to have slightly slower exponential phase growth than SH1000 in BHI containing no added NaCl (*ie.* a maximum of 1.9 fold lower growth; 1.2 and 2.23 OD_{600} units respectively at t = 3 h) (Figure 5.15b). Nevertheless, JLA554 (SH1000 *aht*12 (*yjbH*)) reached a similar growth yield to SH1000 after 5 h (OD_{600} = 6.74 and 6.78 respectively). However, JLA554 (SH1000 *aht*12 (*yjbH*)) was found to have a significant growth defect under NaCl-shock conditions (a maximum of 8.3-fold lower growth in JLA554 than in SH1000; 0.425 and 3.54 OD_{600} units respectively at t = 9 h).

5.2.5.4 Analysis of the effects of *saeS* and *saeR* on pathogenicity in a murine subcutaneous abscess model

JLA562 (SH1000 *htn*18 (*saeS*)) and JLA564 (SH1000 *aht*18 (*saeR*)) have been shown to produce less Hla and SspA than SH1000 (Chapters 5.2.5.1.1 and 5.2.5.2). The effects of *saeS* and *saeR* on virulence were determined using a murine subcutaneous abscess model of infection, as described in Chapter 2.19. 10 mice were inoculated per strain (cfu = 9.2×10^7 for SH1000, 4.10×10^7 for JLA562 (SH1000 *htn*18 (*saeS*)) and 6×10^7 for JLA564 (SH1000 *aht*18 (*saeR*))). The number of cfu recovered per lesion are shown



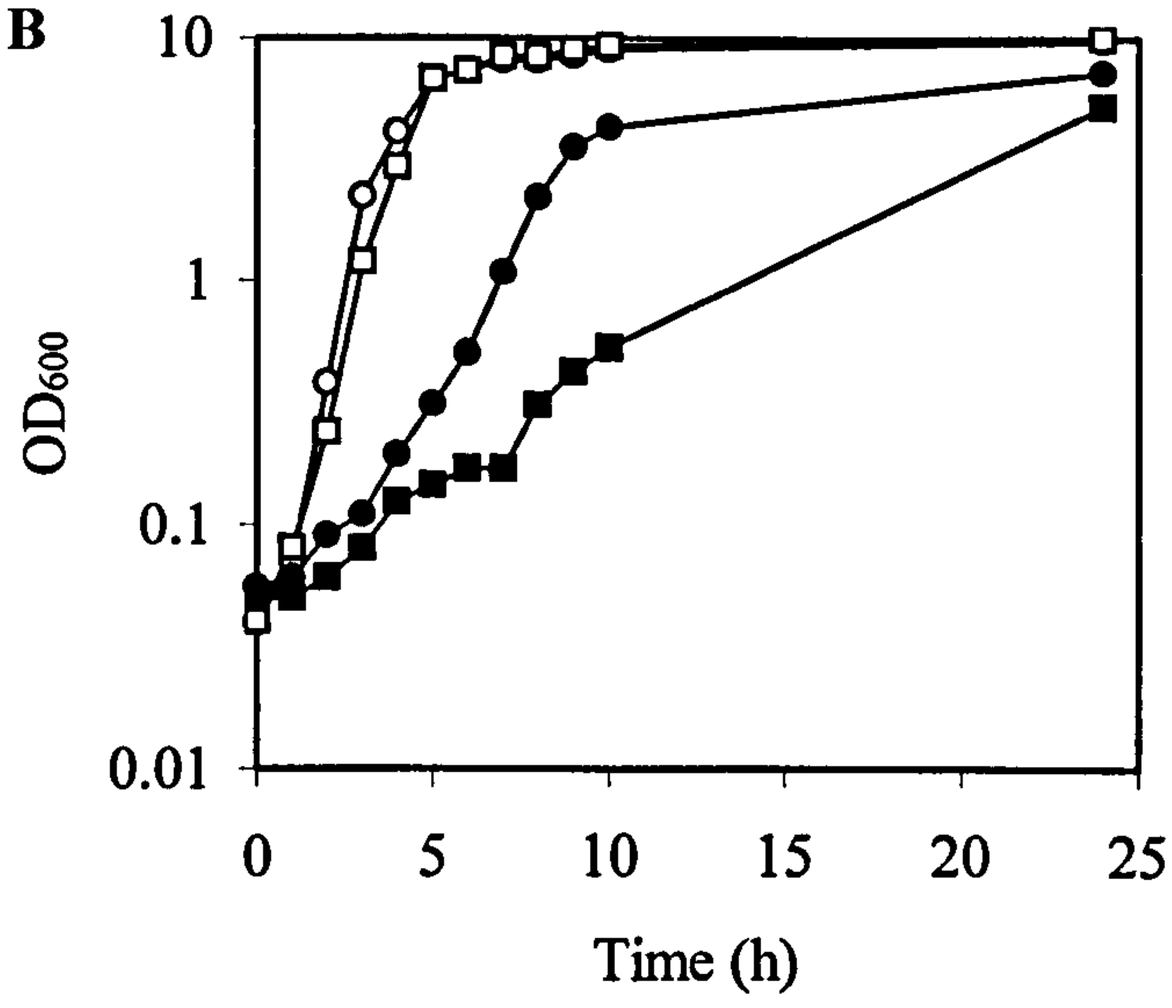


Figure 5.15

Growth (OD600) of (A) JLA559 (SH1000 *aht*6 (*brnQ*)) (□ and ■) and (B) JLA554 (SH1000 *aht*12 (*yjbH*)) (□ and ■) compared to SH1000 (○ and ●) in BHI containing no added NaCl (open symbols) and in BHI containing 2 M NaCl under NaCl-shock conditions (closed symbols). Strains were grown at 37 °C as described in Chapter 2.7. Results are representative of two independent experiments.

in Figure 5.16. The statistical significance of the recovery of strains was evaluated using the Student *t* test with a 5 % confidence limit. P values of 0.0122 and 0.0115 were obtained for JLA562 (SH1000 *htn*18 (*saeS*)) and JLA564 (SH1000 *aht*18 (*saeR*)) respectively, suggesting that the attenuation of the mutants is statistically significant.

5.3 Discussion

In this Chapter, possible methods to overcome the problem of selectable marker incompatibility between the commonly used *S. aureus* transposons (*ie.* Tn*917* and Tn*551*) and pre-constructed *lacZ* fusions and knockouts were investigated. During evaluation of the EZ::TN(tet) transposition system for use in *S. aureus*, transposon mutants were obtained by the *in vivo* transposition method, but not by *in vitro* transposition. This may have occurred because the efficiency of the system is limited by the ability to electroporate DNA into host cells. Using the *in vitro* method, the large fragments of target genomic DNA containing the EZ::TN(tet) transposon would have been electroporated into *S. aureus* RN4220 cells at a significantly lower frequency than the EZ::TN(tet) transposome alone, as used in the *in vivo* transposition method. Alternatively, the ability of the genomic DNA fragments containing the transposon to recombine with the host DNA following electroporation into the cells may have limited the success of the *in vitro* transposition method. This could be tested by performing the *in vitro* transposition method using pMC4 instead of genomic DNA, as pMC4 has previously been shown to be capable of recombining with host DNA in *S. aureus* (Chan *et al.*, 1998).

However, Southern blotting of the transposon mutants obtained by *in vivo* transposition suggested that the EZ::TN(tet) transposon had inserted into the same position in all of the colonies analysed. This may have been caused either by a defect in the EZ::TN(tet) transposome or by the presence of a hotspot for insertion in the *S. aureus* genome. A prototype of the EZ::TN transposition system has been used to produce random insertions in *E. coli*, *S. typhimurium* and *Proteus vulgaris* (Goryshin *et al.*, 2000), and the EZ::TN system has been used successfully in *Mycobacterium smegmatis* (Derbyshire *et al.*, 2000). However, the EZ::TN transposition system is Tn*5*-based, and hotspots for Tn*5* insertion have been identified using pBR322 as a target (Nag *et al.*,

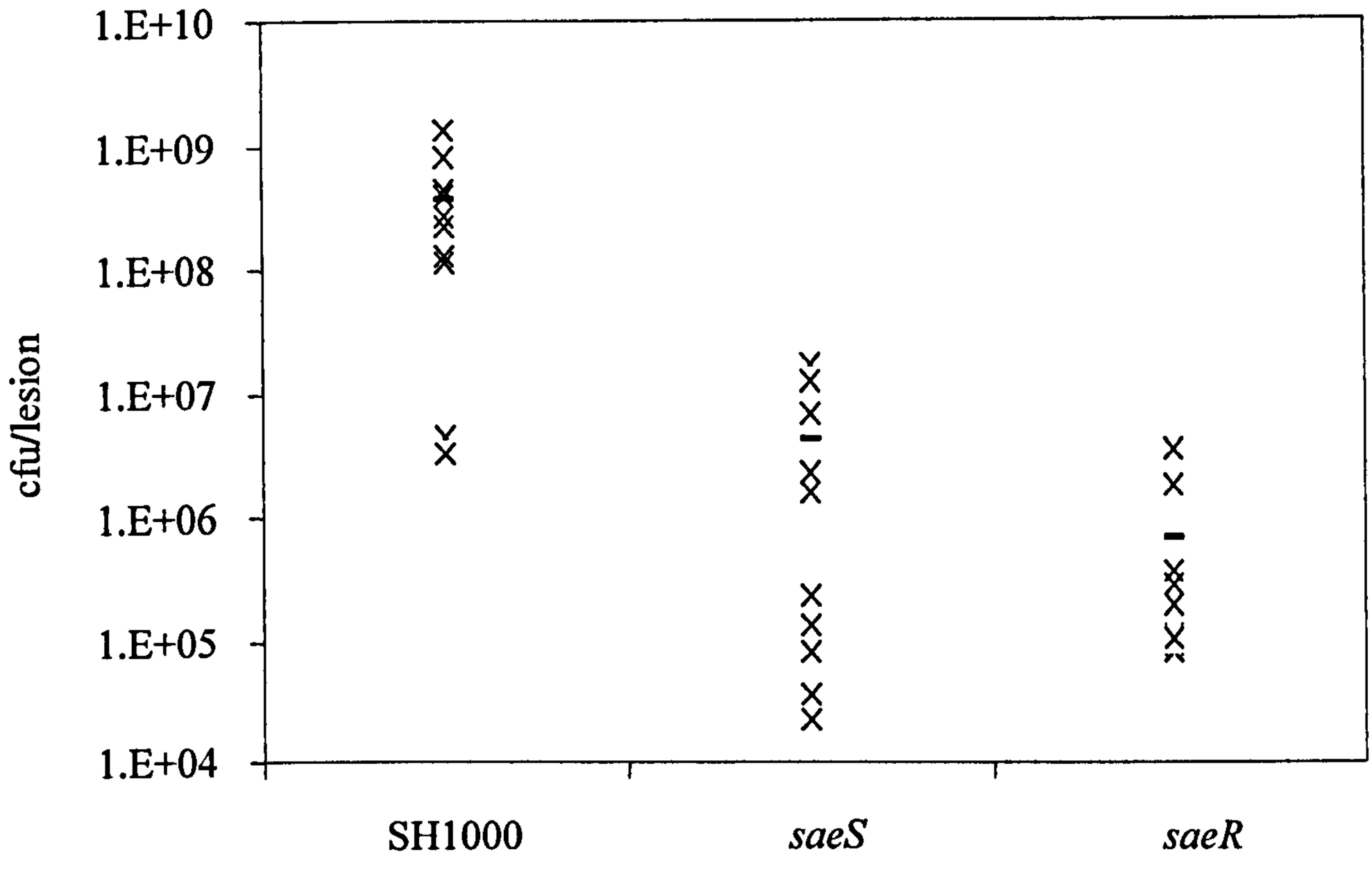


Figure 5.16

The effect of *saeS* and *saeR* on pathogenicity in a murine subcutaneous abscess model of infection.

10 female 6- to 8-week old BALB/c mice were inoculated per *S. aureus* strain, as described in Chapter 2.19. After 7 days, the mice were euthanized with CO_2, skin lesions were aseptically removed and homogenised, and viable bacteria were counted after dilution and growth on BHI agar plates. Crosses represent the cfu/lesion for individual mice, whilst bars represent the mean cfu/lesion for each *S. aureus* strain.

1985). It has been noted that Tn*5* inserts preferentially into G/C-rich DNA, and that additional features such as degenerate DNA sequences or particular topological structures may direct Tn*5* to preferred sites (reviewed by Berg *et al.*, 1989). For example, the frequency of Tn*5* transposition has been shown to be reduced in strains lacking DNA gyrase or topoisomerase (Isberg & Syvanen, 1982; Sternglanz *et al.*, 1981). An initial insight into whether a particular DNA sequence and/or putative topological conformation is present which creates a strong hotspot for insertion in this area of the *S. aureus* genome could be obtained by sequencing the DNA flanking the EZ::TN(tet) transposon insertions.

In order to allow erythromycin-resistant transposon-mediated mutations to be used in conjunction with *lacZ* fusions without encountering the problem of resistance marker incompatibility, the vector pAISH1 was constructed. This vector carries a tetracycline-resistant selectable marker, and was used to construct an *hla::lacZ* fusion. As shown in Figure 5.5, the resulting *hla::lacZ* fusion strains contain an intact copy of *hla* plus a truncated copy transcriptionally fused to the *lacZ* gene. The *hla::lacZ* fusion is stably inserted into the *S. aureus* chromosome and therefore does not require maintenance by the addition of antibiotics, which may alter toxin production (Doss *et al.*, 1993). Furthermore, the *hla::lacZ* fusion is in single copy so as not to titrate-out regulatory elements. The tetracycline-resistant *hla::lacZ* fusion was found to give very similar β-galactosidase assay data to the published erythromycin-resistant *hla::lacZ* fusion constructed in pMUTIN4 by Chan & Foster (1998a). This is to be expected, since the two vectors, pAISH1 and pMUTIN4, differ only in terms of their Gram-positive selectable marker. Furthermore, identical *hla* promoter inserts were used to produce the *lacZ* fusions in both plasmids.

A screen of ~ 8,400 Tn*551* mutants in the SH1000 *hla::lacZ* background identified 6 genes which may mediate the repression of *agr* and *hla* by σ^B. These genes were designated *aht* (genes affecting *hla* transcription). Mutants containing Tn*551* insertions affecting the transcription of *agrB* and potentially of *rsbU* were also isolated, suggesting that the screen was capable of identifying suitable genes. Mutants containing Tn*551* insertions in genes encoding tetrahydrodipicolinate acetyltransferase (YkuQ), diaminopimelate decarboxylase (LysA), a tellurite-resistance protein (TelA), a branched-chain amino acid carrier protein (BrnQ), and a conserved hypothetical protein

(YjbH) were isolated by virtue of their increased blue colouration on BHI X-gal plates compared to the wild-type. Therefore, the genes inactivated by Tn*551* in these mutants may have a repressive effect on *hla*, and it is possible that these genes may be upregulated by σ^B in order to repress *agr/hla*. The repression of *agr* by σ^B has also been shown to result in a reduction in protease transcription (Horsburgh *et al.*, 2002). A screen of Tn*551* mutants in SH1000 to identify genes involved in this pathway by virtue of increased protease activity compared to background colonies identified genes including *lysA*, *telA* and *brnQ* (Shaw, 2002), thus supporting the data presented here. However, in order to verify the role of the *aht* genes in the repression of *agr* and *hla* by σ^B, it would be necessary to determine whether their transcription is affected by σ^B (Chapter 6.2.3) and whether they affect the transcription of *agr* (*eg.* by performing Northern blots).

A mutant containing a Tn*551* insertion in the response regulator *saeR* was also identified in the screen to identify *aht* genes, and was non-blue on BHI X-gal. The two-component sensor/regulator system *saeS/saeR* is known to activate the transcription of *hla* and of several other virulence determinants, including *hlb*, *coa* and *spa* (Giraudo *et al.*, 1994; Rampone *et al.*, 1996; Giraudo *et al.*, 1997; Goerke *et al.*, 2001). However, Giraudo *et al.* (1997) have shown that the *sae* locus has no effect on *agr* transcription, and so the effects of *saeR* on virulence determinant expression are likely to occur independently of *agr*.

A screen of ~ 6,250 Tn*551* mutants to identify genes involved in regulating *hla* transcription in response to NaCl revealed 5 genes. The genes were designated *htn* (genes affecting ***hla*** transcription identified by screening on BHI plates containing NaCl), and encode aspartokinase II (LysC), diaminopimelate decarboxylase (LysA), a tellurite-resistance protein (TelA), a hypothetical protein (Hyp1) and a sensor kinase (SaeS). The screen was performed in the σ^B-proficient SH1000 *hla::lacZ* background, and was therefore capable of identifying genes constituting both the σ^B-dependent and σ^B-independent pathways involved in repressing *hla* in response to NaCl (Chapter 3.3). None of the genes identified here were found in the Tn*917* screen performed in the σ^B-deficient 8325-4 background in Chapter 4. Therefore, it is possible that the genes identified in this Chapter may be involved in the σ^B-dependent pathway. Indeed, mutants carrying Tn*551* insertions potentially affecting *rsbU* transcription were isolated

in this screen. However, it is also possible that differences in the ability of Tn*917* and Tn*551* to insert within different regions of the genome may have caused different genes to be identified in the two screens.

Interestingly, *ykuQ*, *lysC* and *lysA* are all located within the *dap* operon (Figure 5.10b). The *dap* operon consists of 8 genes, six of which are involved in the biosynthesis of the amino acids lysine, methionine, threonine and isoleucine (Wiltshire & Foster, 2001). Since these amino acids derive the majority of their carbon atoms from L-aspartate, they are referred to as the aspartate family of amino acids. The two "common-pathway" enzymes aspartokinase II (*lysC*) and aspartate semialdehyde dehydrogenase (*asd*) are involved in the biosynthesis of all four aspartate family amino acids. However, dihydrodipicolinate synthase (*dapA*), dihydrodipicolinate reductase (*dapB*), tetrahydrodipicolinate acetyltransferase (*ykuQ*) and diaminopimelate decarboxylase (*lysA*) are only involved in the biosynthesis of lysine. The two remaining genes of the *dap* operon, which do not have a role in aspartate family amino acid biosynthesis, are *hipO* and *dal*. These genes encode hippurate hydrolase and alanine racemase respectively. Hippurate hydrolase cleaves benzoylglycine (hippuric acid) into benzoic acid and glycine (Hani & Chan, 1995). Alanine racemase interconverts L-alanine and D-alanine, providing D-alanine for bacterial cell wall synthesis (Wasserman *et al.*, 1984).

Since many of the *aht* and *htn* mutants contained Tn*551* insertions in either the same genes or in the same operons as each other, representative clones from both screens were characterised together to determine their phenotypes in both the presence and absence of NaCl-mediated stress. In the SH1000 *hla::lacZ* background, mutants containing Tn*551* insertions in *ykuQ*, *lysC*, *lysA*, *telA*, *brnQ*, and *yjbH* demonstrated a similar level of blue colouration on BHI X-gal plates containing no added NaCl as they did on BHI X-gal plates containing 1 M NaCl. On both types of plate, the level of blue colouration of the mutants was greater than that of the wild-type. Since the phenotype of these mutants appears to be NaCl-independent, it is unclear how these genes may function in the regulation of *hla* transcription in response to NaCl. However, since the mutants were more blue than the wild-type on BHI X-gal containing no added NaCl, this suggests that they act as repressors of *hla* transcription in the absence of NaCl-mediated stress. Therefore, it is possible that the genes may serve to repress *hla*

transcription from a maximal level to a submaximal level in the absence of NaCl-mediated stress, and that this level of repression is increased further in order to produce an even lower level of *hla* transcription in the presence of NaCl-mediated stress. Therefore, inactivation of the genes using Tn*551* would result in *hla* transcription returning to the maximal level in both the presence and absence of NaCl, causing the mutants to have a higher level of *hla::lacZ* transcription than the wild-type under both conditions. This effect was observed on the BHI X-gal plates. In Chapter 3, σ^B was shown to repress *hla* to a submaximal level in the absence of added NaCl, and to increase this level of repression even further in the presence of NaCl-mediated stress. This would further suggest that the genes may be part of the pathway involved in the σ^B-dependent repression of *hla* in response to NaCl. This could be confirmed by using *lacZ* reporter gene fusions to the genes to determine whether their transcription is altered in the presence or absence of NaCl and whether or not their transcription is affected by σ^B in the presence of NaCl.

Whereas the degree of blue colouration of wild-type SH1000 *hla::lacZ* was lower on BHI X-gal plates containing 1 M NaCl than it was on BHI X-gal plates containing no added NaCl, the *saeS* mutant demonstrated a similar level of blue colouration on both types of plate. This may suggest that the inactivation of *saeS* by Tn*551* has prevented the mutant from sensing NaCl and repressing *hla* transcription appropriately. Indeed, although the *saeS/saeR* two-component sensor-regulator system is known to activate the transcription of a number of virulence determinant genes (Giraudo *et al.*, 1994; Rampone *et al.*, 1996; Giraudo *et al.*, 1997; Goerke *et al.*, 2001), details of the environmental signals which *sae* regulates these genes in response to have not yet been published. The data presented here indicates that *sae* may be important in sensing NaCl in order to allow virulence determinants to be expressed to an appropriate level. This could be confirmed by determining whether the level of phosphorylation of SaeS is reduced in the presence of NaCl.

However, the ability to confirm the putative roles of *ykuQ, lysC, lysA, telA, brnQ, yjbH, saeR* and *saeS* in the NaCl-mediated regulation of *hla* has been limited by the fact that exoprotein gels, Western blots, and the quantification of *hla::lacZ* transcription using MUG assays showed that the phenotypes of the mutants displayed on BHI X-gal plates containing 1 M NaCl could not be reproduced in liquid cultures of BHI supplemented

with NaCl. This effect was also demonstrated by the *han* mutants isolated in Chapter 4. Again, this suggests that a number of environmental conditions (which are different in liquid culture and on plates) may interact with the NaCl-stress signal in order to allow virulence determinant expression to be stringently regulated.

Nevertheless, the phenotypes displayed by the Tn*551* mutants on BHI X-gal plates containing no added NaCl were found to be reproducible in liquid cultures of BHI. Exoprotein gels and anti-Hla Western blots showed that mutation of *ykuQ, lysC, lysA, telA, brnQ, yjbH* and *rsbU* in the SH1000 background led to an increase in the level of Hla compared to wild-type SH1000 during growth in BHI containing no added NaCl. MUG assays showed that *hla::lacZ* transcription was up to 3.5 X greater in the *lysC* mutant than in wild-type *hla::lacZ* SH1000 in BHI containing no added NaCl. Additionally, mutation of *hyp*1, *saeS, agrB* and *saeR* resulted in a reduction in the level of Hla compared to the wild-type in BHI containing no added NaCl. Consequently, the level of Hla expression by the mutants in BHI containing no added NaCl was found to match the degree of blue colouration of the mutants in the SH1000 *hla::lacZ* background compared to the wild-type on BHI X-gal plates containing no added NaCl. Additionally, exoprotein gels and anti-SspA Western blots showed that the mutations had a similar effect on the level of SspA in BHI containing no added NaCl as was observed for the level of Hla. This suggests that the Tn*551*-inactivated genes may be involved in the regulation of virulence determinants in addition to *hla*.

Analysis of the effects on growth of the Tn*551* mutations in the SH1000 background indicated that the *ykuQ, lysC, lysA, telA, rsbU, hyp*1, *saeS, agrB* and *saeR* mutants displayed levels of growth similar to the wild-type in BHI containing either no added NaCl or under NaCl-shock conditions in BHI containing 2 M NaCl. However, *yjbH* was found to have a slightly lower exponential phase growth rate than SH1000 in BHI containing no added NaCl. A significant growth defect was also observed for the *yjbH* mutant under NaCl-shock conditions. The *brnQ* mutant was not found to have a significant growth defect under either condition. However, a *brnQ*::Tn*917* mutant in the RN450 background has previously been shown to have an extremely long lag phase (60 to 70 h compared to the 6 h lag phase of the parent strain) in defined medium containing 2.5 M NaCl (Vijaranakul *et al.*, 1997; Vijaranakul *et al.*, 1998). However, differences in media, growth conditions and the *S. aureus* background used may account for the

phenotypic differences observed between the *brnQ* mutant isolated here and the published mutant strain.

The use of a murine subcutaneous abcess model showed that the mutation of *saeS* and *saeR* in the SH1000 background led to statistically significant attenuation of virulence. This agrees with the finding of Giraudo *et al.* (1996) that an *saeR*::Tn*551* mutant had reduced virulence compared to the parent strain following intraperitoneal injection into mice. In addition, the lower P value obtained for the attenuation of the *saeR* mutant suggests that the mutation of *saeR* leads to greater attenuation than the inactivation of *saeS*. This is in accordance with the levels of blue colouration of the two mutants in the SH1000 *hla::lacZ* background on BHI X-gal plates and the results of exoprotein gels and Western blots using anti-Hla antibodies, which suggest that the *saeR* mutant expresses lower levels of Hla than the *saeS* mutant. Since mutations in *saeR* are polar on *saeS*, this suggests that genes in addition to *saeS* may be able to interact with *saeR* in order to regulate virulence determinant expression.

Interestingly, many of the *aht* and *htn* genes have also been isolated in published transposon mutagenesis screens performed to identify genes involved in virulence. This is discussed in Chapter 6, and adds further weight to the finding that these genes are involved in virulence determinant regulation.

CHAPTER 6

CHARACTERISATION OF SVS REGION GENES

6.1 Introduction

In Chapter 5, two Tn*551* mutant library screens performed to isolate genes involved in the regulation of *hla* in the presence and absence of NaCl-mediated stress were described. In particular, mutants containing a Tn*551* insertion in genes encoding aspartokinase II (*lysC*), tetrahydrodipicolinate acetyltransferase (*ykuQ*), diaminopimelate decarboxylase (*lysA*), a tellurite-resistance protein (*telA*) and a branched-chain amino acid transporter (*brnQ*) were found to demonstrate increased *hla::lacZ* transcription on BHI X-gal plates containing either no added NaCl or 1 M NaCl. These genes are clustered together within a particular region of the *S. aureus* genome (*ie.* between gene numbers SA1428 and SA1445) (Table 5.2) (Figure 6.4). Indeed, the genes *lysC, ykuQ* and *lysA* form part of the *dap* operon, which is involved in the biosynthesis of the aspartate family amino acids lysine, threonine, isoleucine and methionine. The genes present within the 8-gene *dap* operon, and their functions, are discussed in Chapter 5.3.

Interestingly, many genes within and around this region of the *S. aureus* genome have been repeatedly identified in published transposon mutagenesis screens performed to identify *S. aureus* genes involved in either pathogenesis, virulence determinant regulation or starvation survival (Table 6.1). Screening with Tn*917-lacZ* in a model system mimicking *S. aureus* bacteraemia showed that *lysC*, *asd* (aspartate-semialdehyde dehydrogenase) and *dapA* (dihydrodipicolinate synthase) are induced by growth in serum (Wiltshire & Foster, 2001). A Tn*917*-based STM screen to identify genes affecting growth and survival in multiple infection environments found that the inactivation of *asd, ykuQ* and *lysA* leads to attenuation (Coulter *et al.*, 1998). The genes *asd* and *lysA* were also isolated in a screen to identify genes important for *S. aureus* survival and virulence through the use of Tn*917*-based STM in a murine model of bacteraemia (Mei *et al.*, 1997). Lammers *et al.* (2000) showed that *lysC, asd*, *dapA* and *lysA* are important in bovine mastitis by screening Tn*917-lacZ* libraries to identify genes that are expressed during growth in milk. The genes *lysC*, *asd*, *dapB*, *lysA* and

Gene	SA No.	This study	Wiltshire *et al.* (2001)	Coulter *et al.* (1998)	Mei *et al.* (1997)	Lammers *et al.* (2000)	Vriesema *et al.* (2000)	Watson *et al.* (1998)	Shaw (2002)	Fairhead (1998)
lysC	SA1428	*	*			*	*		*	
asd	SA1429		*	*	*	*	*			
dapA	SA1430		*			*				
dapB	SA1431						*			
ykuQ	SA1432	*		*					*	
hipO	SA1433								*	
lysA	SA1435	*		*	*	*	*	*	*	
aclP	SA1439								*	
telA	SA1441	*						*	*	
brnQ	SA1443	*					*		*	*
norQ	SA1445								*	*

Table 6.1

Genes within hotspot region identified in transposon mutant library screens. SA numbers represent the gene number according to the TIGR *S. aureus* COL database. Wiltshire *et al.* (2001); a screen performed using Tn*917-lacZ* in a model system mimicking *S. aureus* bacteraemia to identify genes induced by growth in serum. Coulter *et al.* (1998); a Tn*917*-based STM screen to identify genes affecting growth and survival in multiple infection environments. Mei *et al.* (1997); a screen to identify *S. aureus* virulence genes through the use of Tn*917*-based STM in a murine model of bacteraemia. Lammers *et al.* (2000); a screen involving the use of Tn*917-lacZ* to identify genes important in bovine mastitis. Vriesema *et al.* (2000); a screen using Tn*917-lacZ* to identify genes whose expression is increased upon interaction with human endothelial cells. Watson *et al.* (1998); a Tn*917* screen to identify genes involved in starvation survival or recovery. Shaw (2002); a Tn*551* screen to identify genes involved in the repression of *agr* and proteases by σB. Fairhead (1998); a Tn*551* screen to identify genes involved in the regulation of *agr*.

brnQ were isolated in a screen of Tn*917-lacZ* mutants to identify genes whose expression is increased upon interaction with human endothelial cells (Vriesema *et al.*, 2000). A Tn*551* library screen to identify genes involved in the repression of *agr* and proteases by σ^B by virtue of increased protease activity on milk plates identified *lysC, ykuQ, hipO* (hippurate hydrolase), *lysA, aclP* (acylphosphatase), *telA, brnQ* and *norQ* (nitric-oxide reductase) (Shaw, 2002). The genes *brnQ* and *norQ* were isolated in a Tn*917* library screen to identify genes involved in the regulation of *agr* (Fairhead, 1998). In addition, *lysA* and *telA* were isolated in a Tn*917*-based screen to identify genes involved in starvation survival (Watson *et al.*, 1998).

Consequently, it is possible that this region of the genome may represent a cluster of genes of fundamental importance to the virulence and survival of *S. aureus*. The region will therefore be referred to as the SVS (***S.*** ***aureus*** virulence and survival) region. However, it may be the case that it is an alteration in DNA topology caused by transposon insertion within the SVS region, rather than the loss of actual gene function, that causes the phenotypes. Alternatively, it is possible that a hotspot for transposon insertion is present within the SVS region and that the phenotypes of the mutants might be an artifact, thus causing the genes to be mis-represented in the transposon mutagenesis screens. Therefore, representative transposon mutants were analysed in order to characterise this region of the genome more fully.

6.2 Results

6.2.1 Characterisation of virulence determinant expression in mutants containing transposon insertions within the SVS region

In Chapter 5, the inactivation of *lysC, ykuQ, lysA, telA* and *brnQ* by Tn*551* in the SH1000 *hla::lacZ* background led to increased blue colouration on BHI X-gal plates. To determine whether transposon insertions anywhere within the SVS region are able to cause increased *hla::lacZ* transcription on BHI X-gal plates, a representative clone for each available transposon-inactivated SVS region gene was analysed. The effect of the transposon insertions on protease expression was also investigated.

In preparation for these experiments, the *asd* and *dapA* mutations were transferred from *seg*24 and *seg*10 respectively to SH1000 by ϕ11-mediated transduction. This produced the strains JLA601 (SH1000 *asd*) and JLA603 (SH1000 *dapA*). The *hipO*, *aclP* and *norQ* mutations were transduced into JLA513 (SH1000 *hla*::*lacZ*) from LES153, LES154 and LES155 respectively to produce JLA605 (SH1000 *hla*::*lacZ hipO*), JLA606 (SH1000 *hla*::*lacZ aclP*) and JLA607 (SH1000 *hla*::*lacZ norQ*).

6.2.1.1 *hla::lacZ* transcription on BHI X-gal plates

To estimate the level of *hla::lacZ* transcription in the SVS region mutants, JLA513 (SH1000 *hla*::*lacZ*), JLA373 (SH1000 *hla*::*lacZ sigB*), PC322 (8325-4 *hla*::*lacZ*), JLA542 (SH1000 *hla*::*lacZ htn*10 (*lysC*)), JLA516 (SH1000 *hla::lacZ aht*2 (*ykuQ*)), JLA605 (SH1000 *hla*::*lacZ hipO*), JLA519 (SH1000 *hla::lacZ aht*5 (*lysA*)), JLA606 (SH1000 *hla*::*lacZ aclP*), JLA518 (SH1000 *hla::lacZ aht*4 (*telA*)), JLA520 (SH1000 *hla::lacZ aht*6 (*brnQ*)) and JLA607 (SH1000 *hla*::*lacZ norQ*) were patched onto BHI X-gal plates containing 600 mg l^{-1} X-gal and grown overnight at 37 °C.

The results of this experiment are shown in Figure 6.1. The level of blue colouration of the strains was estimated on a scale of 0 - 6, as in Chapter 5.2.5.1.1. Wild-type JLA513 (SH1000 *hla*::*lacZ*) demonstrated a level of blue colouration of 3. JLA542 (SH1000 *hla*::*lacZ htn*10 (*lysC*)), JLA516 (SH1000 *hla::lacZ aht*2 (*ykuQ*)), JLA605 (SH1000 *hla*::*lacZ hipO*), JLA519 (SH1000 *hla::lacZ aht*5 (*lysA*)), JLA606 (SH1000 *hla*::*lacZ aclP*), JLA518 (SH1000 *hla::lacZ aht*4 (*telA*)), JLA520 (SH1000 *hla::lacZ aht*6 (*brnQ*)) and JLA607 (SH1000 *hla*::*lacZ norQ*) demonstrated a level of 5. JLA373 (SH1000 *hla*::*lacZ sigB*) and PC322 (8325-4 *hla*::*lacZ*) were found to have a level of 6.

6.2.1.2 Protease activity on milk plates

To qualitatively estimate the level of protease expression in the SVS region mutants, SH1000, MJH502 (SH1000 *sigB*), 8325-4, JLA555 (SH1000 *htn*10 (*lysC*)), JLA601 (SH1000 *asd*), JLA603 (SH1000 *dapA*), JLA556 (SH1000 *aht*2 (*ykuQ*)), LES153 (SH1000 *hipO*), JLA557 (SH1000 *aht*5 (*lysA*)), LES154 (SH1000 *aclP*), JLA558

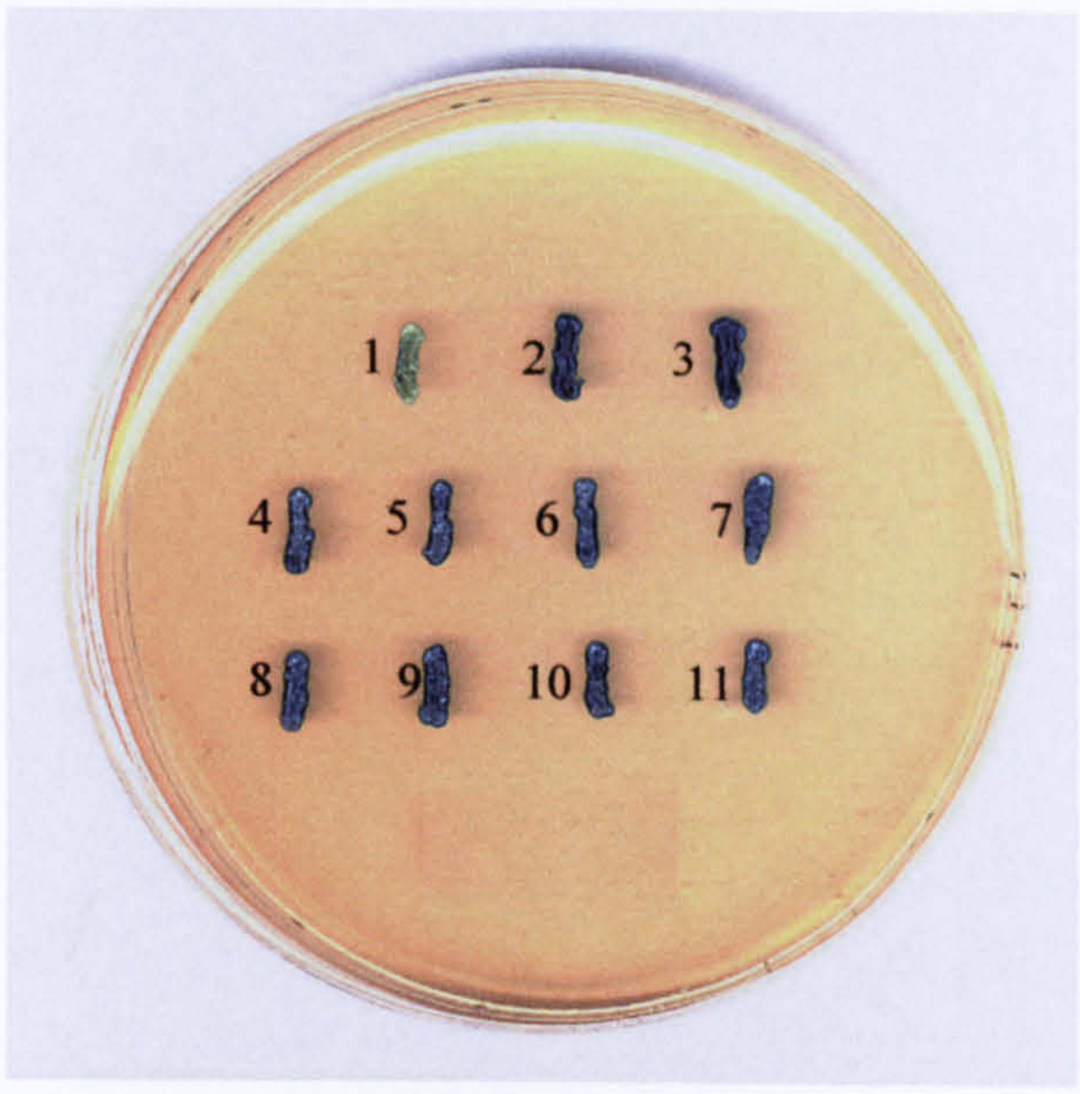


Figure 6.1

hla::lacZ transcription of SVS region mutants, compared to JLA513 (SH1000 *hla::lacZ*), as determined by blue colouration on BHI X-gal plates.

Strains 1) JLA513 (SH1000 *hla*::*lacZ*), 2) JLA373 (SH1000 *hla*::*lacZ sigB*), 3) PC322 (8325-4 *hla*::*lacZ*), 4) JLA542 (SH1000 *hla*::*lacZ htn*10 (*lysC*)), 5) JLA516 (SH1000 *hla::lacZ aht*2 (*ykuQ*)), 6) JLA605 (SH1000 *hla*::*lacZ hipO*), 7) JLA519 (SH1000 *hla::lacZ aht*5 (*lysA*)), 8) JLA606 (SH1000 *hla*::*lacZ aclP*), 9) JLA518 (SH1000 *hla::lacZ aht*4 (*telA*)), 10) JLA520 (SH1000 *hla::lacZ aht*6 (*brnQ*)) and 11) JLA607 (SH1000 *hla*::*lacZ norQ*) were patched onto BHI agar plates containing 600 mg l^{-1} X-gal and incubated overnight at 37 °C. Results are representative of two independent experiments.

(SH1000 *aht*4 (*telA*)), JLA559 (SH1000 *brnQ*)) and LES155 (SH1000 *norQ*) were patched onto milk plates (Chapter 2.16.3) and incubated overnight at 37 °C.

The results of this experiment are shown in Figure 6.2. Wild-type SH1000 demonstrated little visible proteolytic activity. 8325-4, JLA555 (SH1000 *htn*10 (*lysC*)), JLA601 (SH1000 *asd*), JLA603 (SH1000 *dapA*), JLA556 (SH1000 *aht*2 (*ykuQ*)), LES153 (SH1000 *hipO*), JLA557 (SH1000 *aht*5 (*lysA*)), LES154 (SH1000 *aclP*), JLA558 (SH1000 *aht*4 (*telA*)), JLA559 (SH1000 *brnQ*)) and LES155 (SH1000 *norQ*) were found to be proteolytic. However the level of proteolytic activity of these mutants was slightly less than that demonstrated by MJH502 (SH1000 *sigB*).

6.2.2 Direct genomic DNA sequencing and *hla::lacZ* transcription of *htn*21 (SH1000 *hla::lacZ htn*21)

The mutant *htn*21 was isolated in the screen of Tn*551* mutants in the JLA513 (SH1000 *hla::lacZ*) background performed to identify genes involved in the regulation of *hla* in the presence of NaCl-mediated stress (Chapter 5.2.4). *htn*21 demonstrated less blue colouration than the background colonies on a BHI X-gal plate containing 1 M NaCl and 2.4 g l^{-1} X-gal (Chapter 5.2.4). However, this phenotype was not reproduced following transduction of the mutation into JLA513 (SH1000 *hla::lacZ*) to create the strain JLA553 (SH1000 *hla::lacZ htn*21) (Chapter 5.2.4).

Direct sequencing of *htn*21 genomic DNA and analysis of the resulting data was performed as in Chapter 4.2.3.1. Alignment of the sequencing reaction data against the COL sequence is shown in Appendix A.2.21. Tn*551* was found to have inserted into SA1423 at base 832 of the 924 bp gene. Homology of the product of the inactivated gene to other proteins is shown in Appendix A.2.21. Homology to the phosphate ABC transporter permease PstC of *Halobacterium* sp. NRC-1 was demonstrated (50 % identity over 253 amino acids).

JLA513 (SH1000 *hla::lacZ*), *htn*21 (SH1000 *hla::lacZ htn*21), and JLA553 (SH1000 *hla::lacZ htn*21 (*pstC*)) were patched onto BHI X-gal plates containing 600 mg l^{-1}

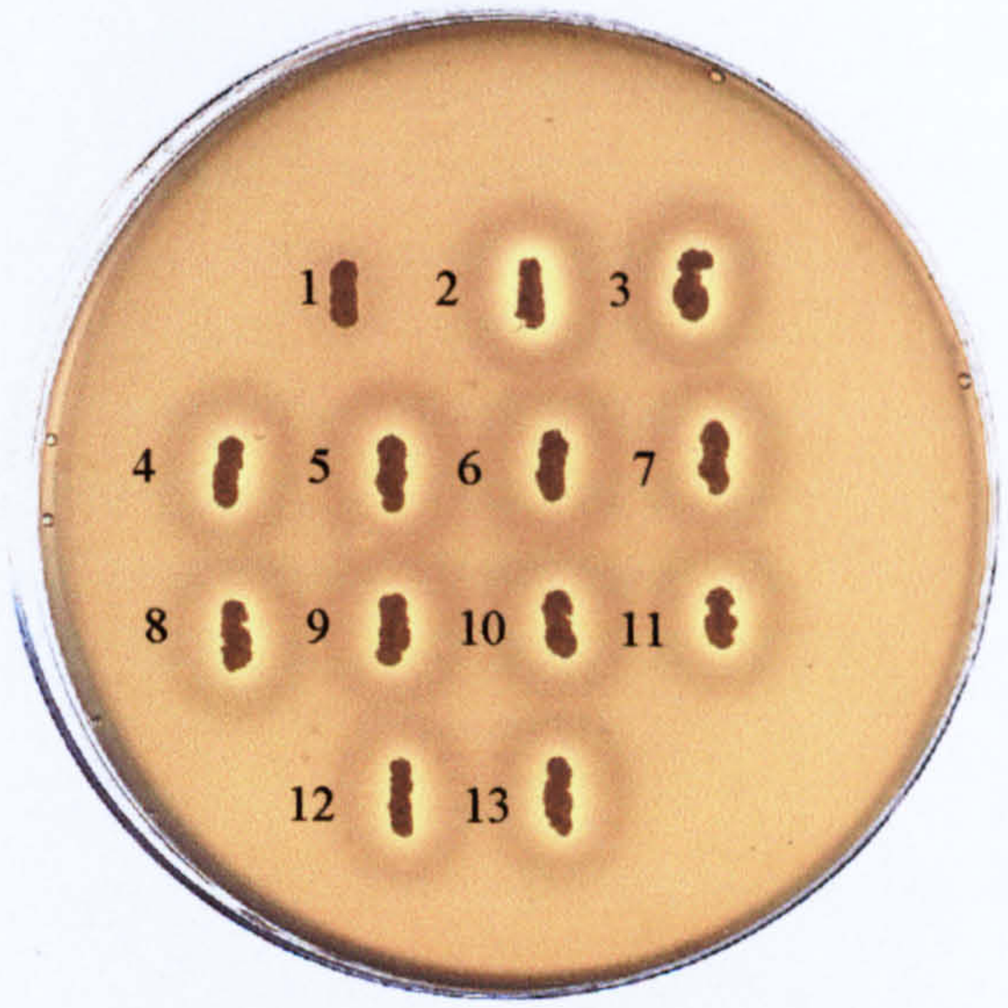


Figure 6.2

Protease activity of SVS region mutants, compared to SH1000, on a milk plate.

Strains 1) SH1000, 2) MJH502 (SH1000 *sigB*) 3) 8325-4, 4) JLA555 (SH1000 *htn*10 (*lysC*)), 5) JLA601 (SH1000 *asd*), 6) JLA603 (SH1000 *dapA*), 7) JLA556 (SH1000 *aht*2 (*ykuQ*)), 8) LES153 (SH1000 *hipO*), 9) JLA557 (SH1000 *aht*5 (*lysA*)), 10) LES154 (SH1000 *aclP*), 11) JLA558 (SH1000 *aht*4 (*tel*A)), 12) JLA559 (SH1000 *brnQ*)) and 13) LES155 (SH1000 *norQ*) were patched onto a milk plate (Chapter 2.16.3) and incubated overnight at 37 °C. Results are representative of two independent experiments.

X-gal. The level of blue colouration of the strains is shown in Figure 6.3. This gives a qualitative estimate of *hla::lacZ* transcription in the strains.

The mutant *htn*21 (SH1000 *hla::lacZ htn*21) was found to be non-blue, whilst JLA553 (SH1000 *hla::lacZ htn*21 (*pstC*)) demonstrated a level of blue colouration similar to the wild-type. Therefore, mutation of *pstC* does not result in the increased blue colouration shown by the SVS mutants analysed in Chapter 6.2.1.1. The genes for which inactivation by Tn*551* is known to cause increased blue colouration in the JLA513 (SH1000 *hla::lacZ*) background are between SA1426 and SA1445. Therefore, *pstC* (SA1423) represents a possible boundary upstream of these genes whereby Tn*551* insertion does not result in the phenotype demonstrated by the other SVS region mutants. Similarly, in Chapter 5.2.5.1.1, the inactivation of *hyp*1 was shown to cause JLA549 (SH1000 *hla::lacZ htn*17 (*hyp*1)) to demonstrate less blue colouration than JLA513 (SH1000 *hla::lacZ*) (*ie.* blue colouration scores of 2 and 3 respectively). Consequently, *hyp*1 (next to SA1452) represents a possible downstream border of the SVS region. A diagram of the SVS region, which is approximately 35 kb in length, is shown in Figure 6.4.

6.2.3 The effect of σ^B on *lysC*, *lysA* and *telA* transcription

In Chapter 6.2.1.1, inactivation of genes within the SVS region by Tn*551* was shown to result in increased *hla::lacZ* transcription. A possible explanation for this phenotype is that the normal function of the SVS region genes is to act as intermediates in the repression by σ^B of *agr* (and also of *hla*). Therefore, transposon-mediated inactivation of the SVS genes would prevent σ^B from repressing *agr*, thus allowing *hla* transcription to be derepressed. In order to test this hypothesis, *lacZ* reporter gene fusion assays were used to determine whether σ^B regulates the transcription of the three representative SVS region genes *lysC*, *lysA* and *telA*.

The *lysC::lacZ* reporter gene fusion was transduced from MDW41 (Wiltshire & Foster, 2001) into SH1000 and MJH502 (SH1000 *sigB*) by ϕ11-mediated transduction. This produced the strains JLA608 (SH1000 *lysC::lacZ*) and JLA609 (SH1000 *lysC::lacZ*

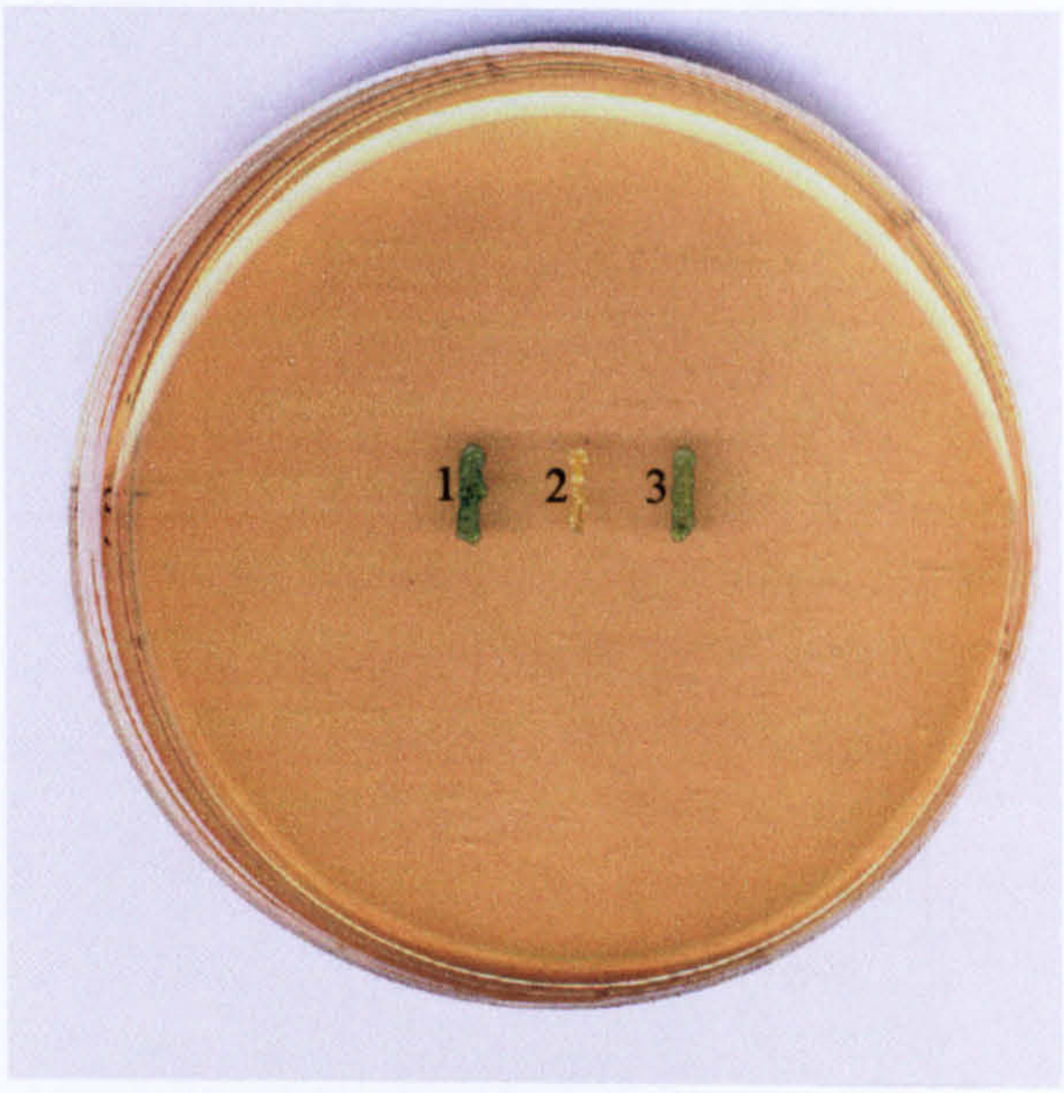


Figure 6.3

hla::lacZ transcription of *S. aureus* strains, as determined by blue colouration on BHI X-gal plates.

Strains 1) JLA513 (SH1000 *hla*::*lacZ*), 2) *htn*21 (SH1000 *hla::lacZ htn*21) (isolated from primary screen in Chapter 5.2.4.1) and 3) JLA553 (SH1000 *hla::lacZ htn*21 (*pstC*)) (created by transduction of *htn*21 into JLA513 (SH1000 *hla::lacZ*) in Chapter 5.2.4.3) were patched onto a BHI agar plate containing 600 mg l^{-1} X-gal and incubated overnight at 37 °C. Results are representative of two independent experiments.

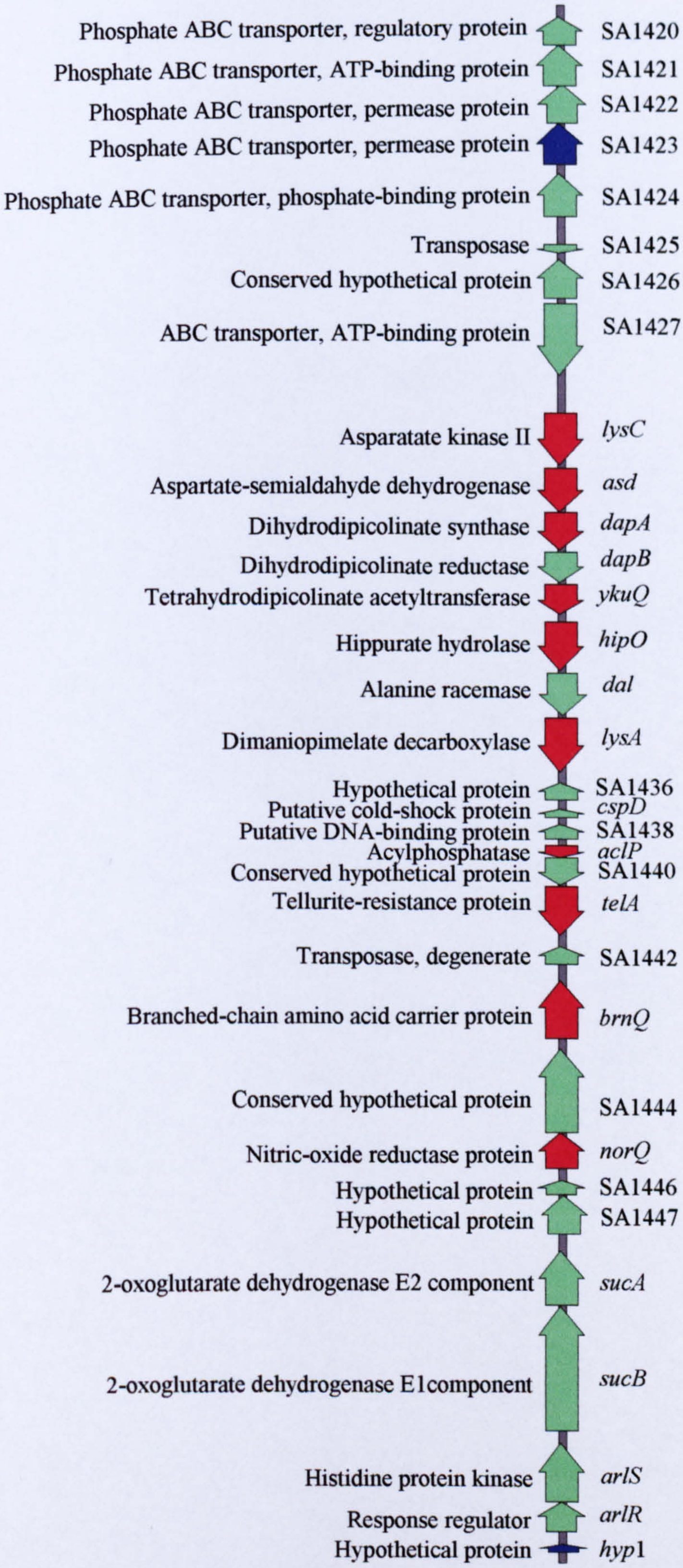

Phosphate ABC transporter, regulatory protein
SA1420
Phosphate ABC transporter, ATP-binding protein
SA1421
Phosphate ABC transporter, permease protein
SA1422
Phosphate ABC transporter, permease protein
SA1423
Phosphate ABC transporter, phosphate-binding protein
SA1424
Transposase
SA1425
Conserved hypothetical protein
SA1426
ABC transporter, ATP-binding protein
SA1427
Asparatate kinase II
lysC
Aspartate-semialdahyde dehydrogenase
asd
Dihydrodipicolinate synthase
dapA
Dihydrodipicolinate reductase
dapB
Tetrahydrodipicolinate acetyltransferase
ykuQ
Hippurate hydrolase
hipO
Alanine racemase
dal
Dimaniopimelate decarboxylase
lysA
Hypothetical protein
SA1436
Putative cold-shock protein
cspD
Putative DNA-binding protein
SA1438
Acylphosphatase
aclP
Conserved hypothetical protein
SA1440
Tellurite-resistance protein
telA
Transposase, degenerate
SA1442
Branched-chain amino acid carrier protein
brnQ
Conserved hypothetical protein
SA1444
Nitric-oxide reductase protein
norQ
Hypothetical protein
SA1446
Hypothetical protein
SA1447
2-oxoglutarate dehydrogenase E2 component
sucA
2-oxoglutarate dehydrogenase E1component
sucB
Histidine protein kinase
arlS
Response regulator
arlR
Hypothetical protein
hyp1
1 kb

Figure 6.4

The 35 kb SVS region of the *S. aureus* genome.

The genes displayed in red have been repeatedly identified in transposon mutant library screens as being involved in *S. aureus* virulence and survival. Transposon-mediated inactivation of these genes has been shown to result in increased blue colouration in the JLA513 (SH1000 *hla::lacZ*) background on BHI X-gal plates containing 600 mg l^{-1} X-gal. Genes for which transposon-mediated inactivation has been shown not to result in this phenotype are represented in blue. Genes for which no known transposon mutants were available are shown in green.

This diagrammatic representation of the SVS region was created by extracting the DNA sequence of the entire region from the *S. aureus* COL database in the TIGR website (Chapter 2.17.2). This sequence was compared to the *S. aureus* 8325 sequence in the NCBI BLAST database. The two sequences were shown to be ~ 99 % identical (data not shown). Protein functions and gene names were based on the protein sequence analysis shown in Appendix A.2.10-21, and on information from the COL database in the TIGR website. Genes have been assigned numbers based on those of the COL genome.

sigB) respectively. SPW2 contains Tn*917* inserted into *lysA* (Watson *et al.*, 1998). The version of Tn*917* used to create SPW2 was carried on the vector pLTV1 and contains a promoterless *lacZ* gene (Chapter 4.1). In SPW2, Tn*917* is inserted in the correct orientation to create a *lacZ* fusion to the *lysA* promoter (Watson *et al.*, 1998). The Tn*917*-mediated *lacZ* fusion was transduced from SPW2 to SH1000 and to MJH502 (SH1000 *sigB*) to produce JLA610 (SH1000 *lysA::*Tn*917*) and JLA611 (SH1000 *lysA::*Tn*917 sigB*) respectively. The *telA::lacZ* reporter gene fusion was transduced from J68 (Lithgow & Foster, unpublished) to MJH502 (SH1000 *sigB*) to produce JLA612 (SH1000 *telA::lacZ sigB*).

JLA608 (SH1000 *lysC::lacZ*), JLA609 (SH1000 *lysC::lacZ sigB*), JLA610 (SH1000 *lysA::*Tn*917*), JLA611 (SH1000 *lysA::*Tn*917 sigB*), J68 (SH1000 *telA::lacZ*) and JLA612 (SH1000 *telA::lacZ sigB*) were grown in BHI, as described in Chapter 2.7. Growth was measured spectrophotometrically as OD_{600} (Chapter 2.6.1), and *lacZ* reporter gene fusion expression was measured using MUG, as described in Chapter 2.8.

The transcription of the *lysC::lacZ* fusion in JLA609 (SH1000 *lysC::lacZ sigB*) was 1.4-fold higher than in JLA608 (SH1000 *lysC::lacZ*) at t = 10 h (*ie.* 685 and 486 MUG units respectively) (Figure 6.5a). Similarly, the transcription of the *lysA::lacZ* fusion in JLA611 (SH1000 *lysA::*Tn*917 sigB*) was 0.9-fold lower than in JLA610 (SH1000 *lysA::*Tn*917*) at t = 10 h (*ie.* 881 and 978 MUG units respectively) (Figure 6.5b). The transcription of the *telA::lacZ* fusion in JLA612 (SH1000 *telA::lacZ sigB*) was 0.96-fold lower than in J68 (SH1000 *telA::lacZ*) at t = 10 h (*ie.* 1611 and 1675 MUG units respectively) (Figure 6.5c). This suggests that σ^B is unlikely to regulate *lysC*, *lysA* or *telA*.

6.2.4 The effect of transposon insertions within the SVS region on σ^B activity

In Chapter 6.2.3, σ^B was shown not to regulate the transcription of three representative genes within the SVS region. Therefore, the normal roles of the SVS region genes are unlikely to involve functioning as intermediates in the repression by σ^B of *agr* (and also of *hla*). Alternatively, however, the SVS region genes may act upstream of

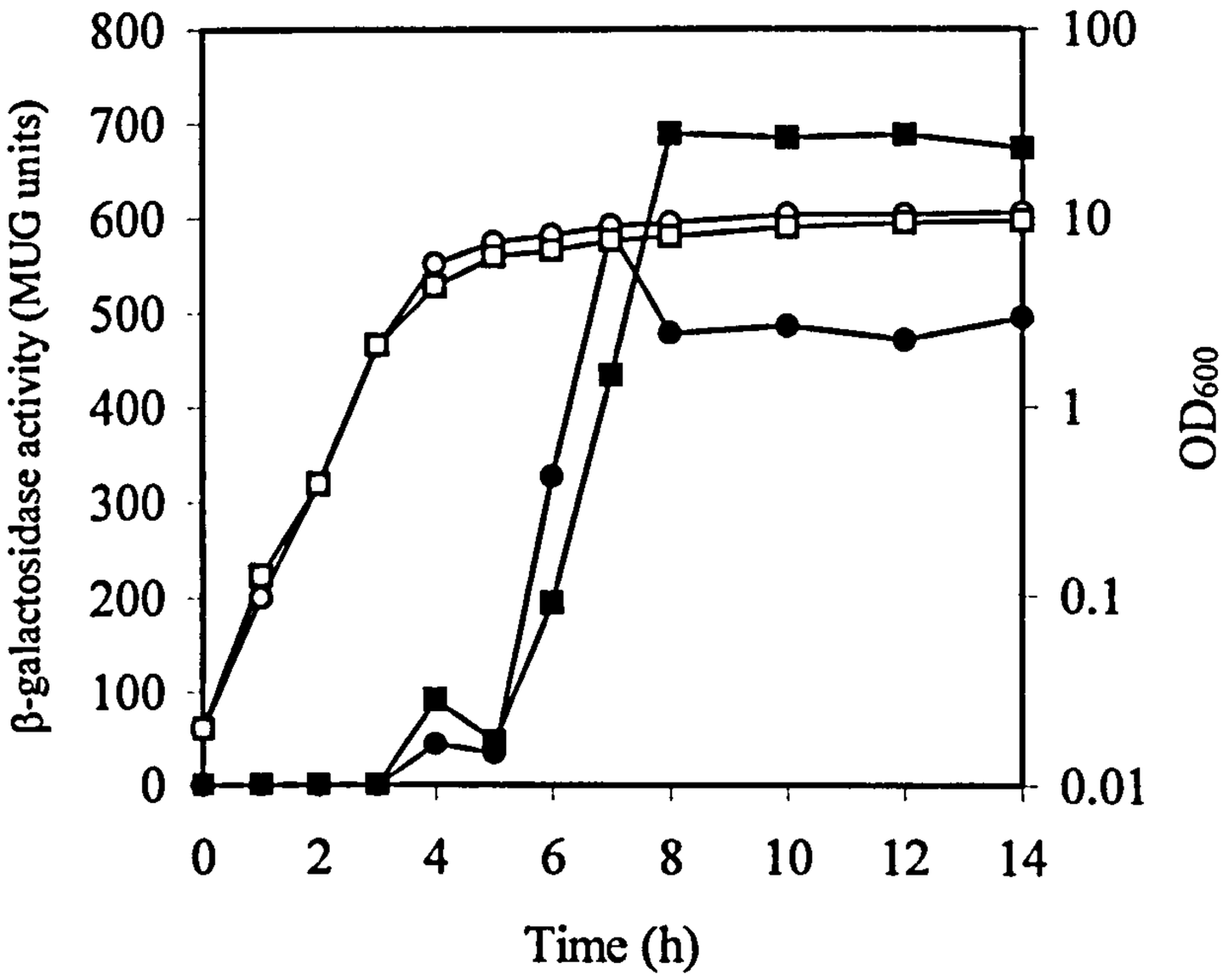


Figure 6.5a

The effect of σ^B on *lysC::lacZ* transcription.

S. aureus JLA608 (SH1000 *lysC::lacZ*) (○ and ●) and JLA609 (SH1000 *lysC::lacZ sigB*) (□ and ■) were grown in BHI as described in Chapter 2.7. Growth was measured spectrophotometrically as OD_{600} (open symbols) (Chapter 2.6.1), and *lacZ* fusion expression was measured using MUG (closed symbols) as in Chapter 2.8. Results are representative of two independent experiments.

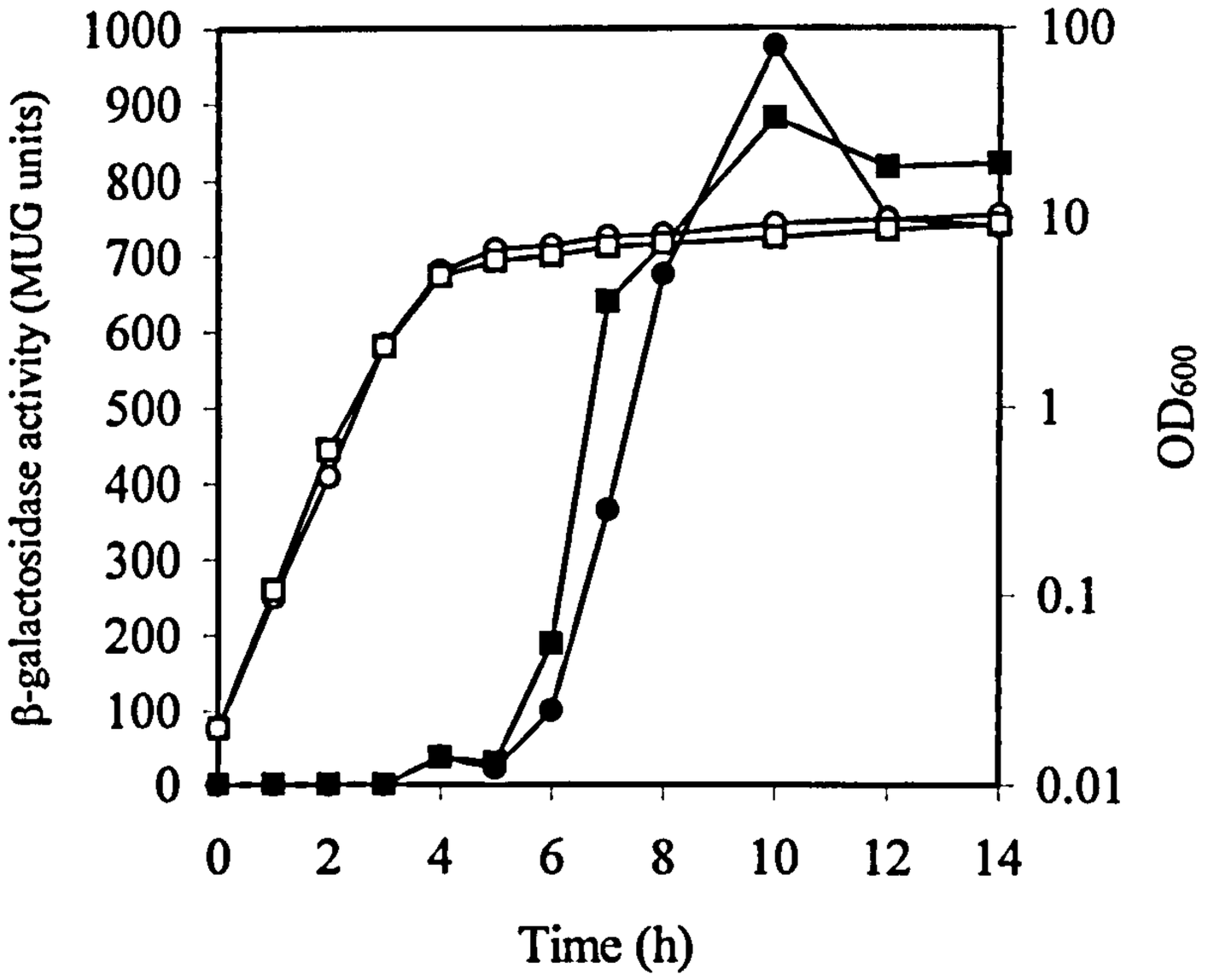


Figure 6.5b

The effect of σ^B on *lysA::lacZ* transcription.

S. aureus JLA610 (SH1000 *lysA::*Tn*917*) (○ and ●) and JLA611 (SH1000 *lysA::*Tn*917 sigB*) (□ and ■) were grown in BHI as described in Chapter 2.7. Growth was measured spectrophotometrically as OD_{600} (open symbols) (Chapter 2.6.1), and *lacZ* fusion expression was measured using MUG (closed symbols) as in Chapter 2.8. Results are representative of two independent experiments.

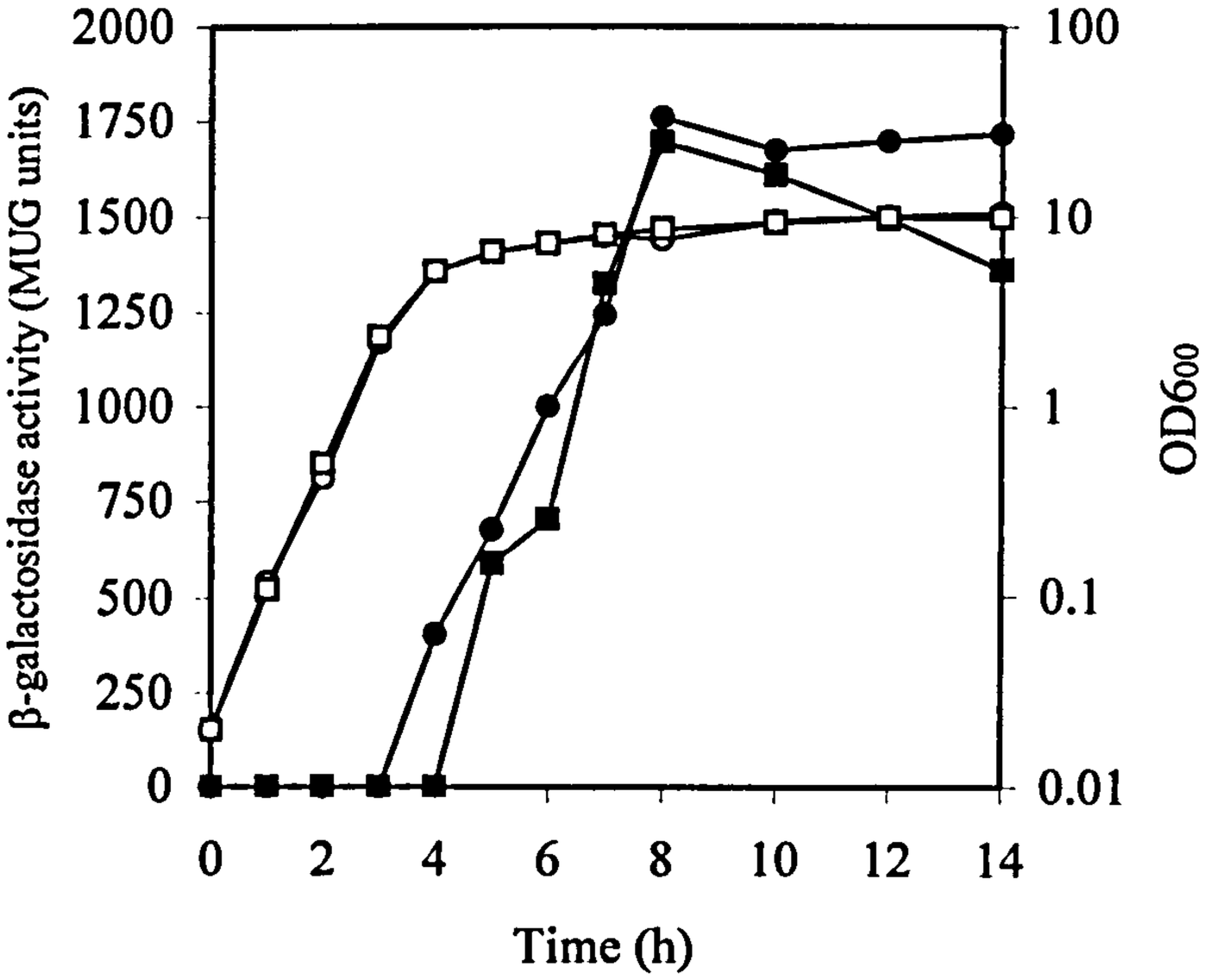


Figure 6.5c

The effect of σ^B on *telA::lacZ* transcription.

S. aureus J68 (SH1000 *telA::lacZ*) (○ and ●) and JLA612 (SH1000 *telA::lacZ sigB*) (□ and ■) were grown in BHI as described in Chapter 2.7. Growth was measured spectrophotometrically as OD_{600} (open symbols) (Chapter 2.6.1), and *lacZ* fusion expression was measured using MUG (closed symbols) as in Chapter 2.8. Results are representative of two independent experiments.

this pathway and themselves activate σ^B, thus allowing σ^B to repress *agr* and *hla* through unknown intermediates. Inactivation of the SVS region genes by transposon insertion would therefore prevent σ^B from being activated. Consequently, *agr* would be derepressed, allowing *hla* to be transcribed at a high level. In order to test this hypothesis, the effects of the SVS region genes on σ^B activity were examined. As described in Chapter 1.6.2.2.8, σ^B is mainly regulated at the level of activity of the SigB protein, rather than at the level of transcription. Although no assay to directly measure the activity of σ^B is available, the level of σ^B activity can be estimated from the expression of two σ^B-dependent markers. These are the production of alkaline shock protein 23 (Asp23) (Kullik *et al.*, 1998; Gertz *et al.*, 1999; Miyazaki *et al.*, 1999; Giachino *et al.*, 2001), and the yellow pigment staphyloxanthin (Kullik *et al.*, 1998; Giachino *et al.*, 2001). The *asp*23 gene has a single σ^B-dependent promoter (Kullik *et al.*, 1998; Gertz *et al.*, 1999; Miyazaki *et al.*, 1999), and *sigB* mutants lack staphyloxanthin, resulting in the loss of the characteristic yellow colony colouration of *S. aureus* (Kullik *et al.*, 1998; Giachino *et al.*, 2001).

6.2.4.1 The use of Asp23 production as a measure of σ^B activity

To determine the effects of Tn*551*-inactivation of the SVS region genes on σ^B using Asp23 expression as a marker of σ^B activity, SH1000, MJH502 (SH1000 *sigB*), and the representative SVS region mutants JLA555 (SH1000 *htn*10 (*lysC*)), JLA558 (SH1000 *aht*4 (*tel*A)) and JLA559 (SH1000 *brnQ*)) were grown in BHI for 14 h as described in Chapter 2.18.1. Total cellular protein samples were prepared as in Chapter 2.18.3.2, and separated on SDS-PAGE gels as in Chapter 2.18.4.

Although the molecular mass of Asp23 is predicted to be 19.2 kDa, Asp23 was found to have an apparent molecular mass of 23 kDa on protein gels (Kullik *et al.*, 1998). An ~ 21 kDa band was identified which was present in large amounts in SH1000 but which was weakly expressed in MJH502 (SH1000 *sigB*), JLA555 (SH1000 *htn*10 (*lysC*)), JLA558 (SH1000 *aht*4 (*tel*A)) and JLA559 (SH1000 *brnQ*)) (Figure 6.6). N-terminal sequencing (Chapter 2.18.6) of this protein revealed the amino acid sequence VDNNXAXQAYDXQ. The assigned amino acids showed 100 % identity to the sequence of Asp23 of *S. aureus* (Chapter 2.17.2).

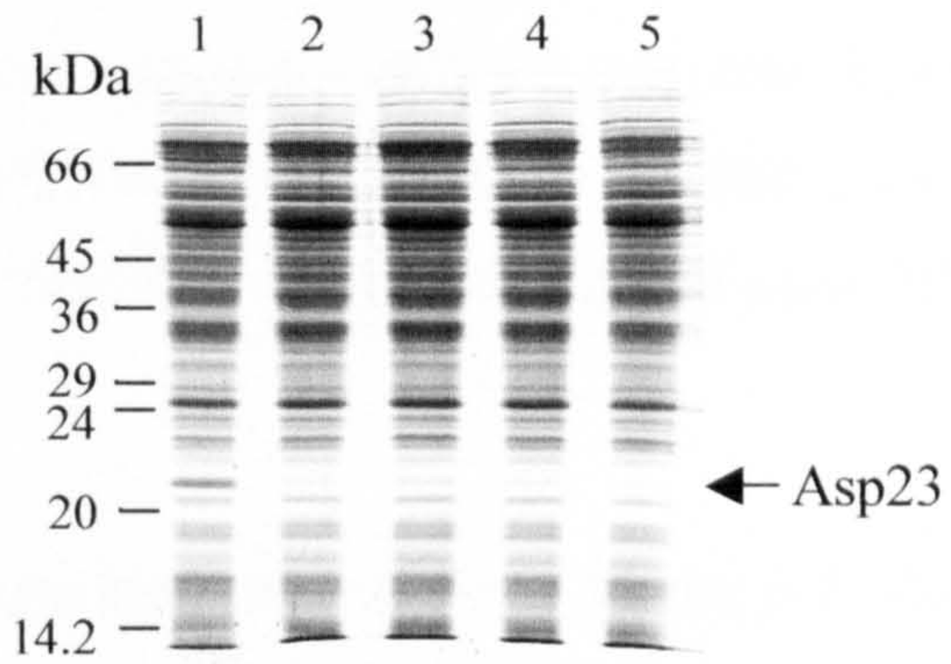


Figure 6.6

The effect of SVS region genes on σ^B activity, as determined by the production of Asp23.

Strains 1) SH1000, 2) MJH502 (SH1000 *sigB*), 3) JLA555 (SH1000 *htn*10 (*lysC*)), 4) JLA558 (SH1000 *aht*4 (*tel*A)) and 5) JLA559 (SH1000 *brnQ*)) were grown in BHI for 14 h as described in Chapter 2.18.1. Total cellular protein samples were prepared as in Chapter 2.18.3.2, and separated on 12 % (w/v) SDS-PAGE gels as in Chapter 2.18.4. Lanes contain total cellular proteins from 0.1 OD_{600} unit of culture. Results are representative of two independent experiments. Sizes of Sigma low molecular weight makers are shown.

6.2.4.2 The use of staphyloxanthin production as a measure of σ^B activity

To determine the effect of Tn*551*-inactivation of the SVS region genes on σ^B using staphyloxanthin production as a marker of σ^B activity, SH1000, MJH502 (SH1000 *sigB*), JLA555 (SH1000 *htn*10 (*lysC*)), JLA601 (SH1000 *asd*), JLA603 (SH1000 *dapA*), JLA556 (SH1000 *aht*2 (*ykuQ*)), LES153 (SH1000 *hipO*), JLA557 (SH1000 *aht*5 (*lysA*)), LES154 (SH1000 *aclP*), JLA558 (SH1000 *aht*4 (*tel*A)), JLA559 (SH1000 *brnQ*)) and LES155 (SH1000 *norQ*) were patched onto BHI agar plates and incubated overnight at 37 °C.

The results of this experiment are shown in Figure 6.7. The degree of staphyloxanthin production can be visualised as the level of yellow colouration of the strains. All of the SVS region mutants produced less staphyloxanthin than wild-type SH1000, as shown by paler colouration. No yellow colouration was observed for MJH502 (SH1000 *sigB*).

6.2.5 The role of DNA topology in the SVS phenotype

Transposon insertion into genes of apparently unrelated function within the SVS region has been shown to result in almost identical mutant phenotypes (Chapters 6.2.1 and 6.2.4). Therefore, it is possible that the phenotypes demonstrated by the SVS region mutants result from a general alteration in DNA topology caused by the physical insertion of transposon DNA within the SVS region, rather than the loss of actual gene function. To determine whether the insertion of exogenous DNA within the SVS region can cause an increase in protease activity similar to that resulting from transposon insertion, SH1000, JLA555 (SH1000 *htn*10 (*lysC*)) and JLA608 (SH1000 *lysC::lacZ*) were patched onto a milk plate and grown overnight at 37 °C. Whereas in JLA555 (SH1000 *htn*10 (*lysC*)) *lysC* has been inactivated by Tn*551*, JLA608 (SH1000 *lysC::lacZ*) possesses a functional copy of *lysC* and contains an additional exogenous promoterless β-galactosidase gene fused to a second *lysC* promoter.

JLA608 (SH1000 *lysC::lacZ*) did not demonstrate the same increase in protease activity compared to SH1000 as JLA555 (SH1000 *htn*10 (*lysC*)) (Figure 6.8).

Figure 6.7

The effects of SVS region gene mutation on σ^B activity, as determined by production of the yellow pigment staphyloxanthin.

Strains 1) SH1000, 2) MJH502 (SH1000 *sigB*), 3) JLA555 (SH1000 *htn*10 (*lysC*)), 4) JLA601 (SH1000 *asd*), 5) JLA603 (SH1000 *dapA*), 6) JLA556 (SH1000 *aht*2 (*ykuQ*)), 7) LES153 (SH1000 *hipO*), 8) JLA557 (SH1000 *aht*5 (*lysA*)), 9) LES154 (SH1000 *aclP*), 10) JLA558 (SH1000 *aht*4 (*telA*)), 11) JLA559 (SH1000 *brnQ*)) and 12) LES155 (SH1000 *norQ*) were patched onto BHI agar plates and incubated overnight at 37 °C. Results are representative of two independent experiments.

Figure 6.8

Determination of whether the phenotypes resulting from transposon insertion within the SVS region are caused by alterations in DNA topology.

Strains 1) SH1000, 2) JLA555 (SH1000 *htn*10 (*lysC*)) and 3) JLA608 (SH1000 *lysC::lacZ*) were patched onto a milk plate and grown overnight at 37 °C. Results are representative of two independent experiments.

6.3 Discussion

The genes *lysC* (SA1428), *asd* (SA1429), *dapA* (SA1430), *ykuQ* (SA1432), *hipO* (SA1433), *lysA* (SA1435), *aclP* (SA1439), *telA* (SA1441), *brnQ* (SA1443) and *norQ* (SA1445) are clustered together within the SVS region of the *S. aureus* genome, and have been repeatedly identified in transposon mutant library screens as being involved in *S. aureus* virulence and survival. The inactivation of these genes was found to result in increased *hla::lacZ* transcription to a similar level in each of the mutants. However, Tn*551*-mediated inactivation of the genes encoding the phosphate ABC transporter permease protein PstC (SA1423) and the hypothetical protein Hyp1 (immediately upstream of SA1551) did not increase *hla::lacZ* transcription. Therefore, *pstC* (SA1423) and *hyp*1 (immediately upstream of SA1551) represent the possible upstream and downstream borders of the SVS region. However, transposon mutants containing insertions between SA1424 – SA1427 and SA1446 – SA1551 were not available. Consequently, the actual boundaries within which Tn*551* insertion causes the phenotypes displayed by the tested SVS region mutants could not be determined. Furthermore, it would be also important to investigate the effects of inactivation of *dapB*, *dal*, SA1436, *cspD*, SA1438, SA1440, SA1442 and SA1444 on *hla::lacZ* transcription to determine whether inactivation of all of the genes within the SVS region results in the same phenotype. However, no transposon mutants containing insertions within these genes were available to be tested.

In addition to increasing *hla::lacZ* transcription, inactivation of *lysC*, *asd, dapA, ykuQ, hipO, lysA, aclP, tel*A, *brnQ* and *norQ* was found to increase protease activity and decrease the activity of σ^B. Therefore, it is possible that a function of the genes within the SVS region is to activate σ^B activity, allowing σ^B to repress *agr* (and *hla*, proteases and perhaps other *agr*-upregulated virulence determinants). However, the effect of inactivation of the representative SVS region genes on the level of *hla::lacZ* transcription, and on the activity of proteases and σ^B, was slightly less than that resulting from the inactivation of *sigB*. This suggests that a level of redundancy exists in the interaction of the SVS region genes with σ^B.

As described in Chapter 1.6.2.2.8, *B. subtilis* possesses a number of genes involved in upregulating σ^B activity (*ie. rsbP, rsbQ, rsbR, rsbS, rsbT* and *rsbX*) that are absent from

the *S. aureus* genome (Kalman *et al.*, 1990; Wise and Price, 1995; Wu *et al.*, 1996). In *B. subtilis*, the Rsb proteins regulate σ^B activity in response to signals detected by two separate pathways; RsbP and RsbQ constitute the energy stress pathway, whilst RsbS, RsbT, RsbR and RsbX form part of the environmental stress pathway (reviewed by Price, 2002). It is possible that the SVS region genes may replace the functions of the missing *rsb* genes. Alternatively, it is possible that the pathway(s) by which σ^B is activated in *S. aureus* are fundamentally different to those in *B. subtilis,* and that the SVS region genes play a role in upregulating σ^B activity via a novel mechanism. It is conceivable that the SVS region genes may be involved in activating σ^B activity in *S. aureus* in response to different signals to those in *B. subtilis*, as the environmental conditions encountered by the soil bacterium *B. subtilis* and the human pathogen *S. aureus* will presumably differ greatly.

The SVS region includes the 8-gene *dap* operon which is involved in the biosynthesis of the aspartate family amino acids (Chapter 5.3; Wiltshire & Foster, 2001). The branched-chain amino acid transporter *brnQ* (Vijaranakul *et al.*, 1997; Vijaranakul *et al.*, 1998) is also present within the SVS region. Furthermore, although *telA* has been implicated in tellurite-resistance, it has been proposed that this might occur as a secondary effect of a particular metabolic function rather than through the action of a distinct resistance determinant (Taylor, 1999). Indeed, a *telA* mutant was isolated in a Tn*917* mutant library screen to identify genes involved in stationary-phase amino acid starvation survival (Watson *et al.*, 1998). Therefore, a number of genes within the SVS region may have roles in amino acid biosynthesis and uptake. *S. aureus* and other bacteria adapt to amino acid limitation via the stringent response (reviewed by Cashel & Rudd, 1987). This involves the synthesis of (p)ppGpp by *rel* (Gentry *et al.*, 2000; Cassels & Knowles, 1995). (p)ppGpp inhibits RNA polymerase, resulting in a rapid reduction in the synthesis of housekeeping gene mRNA to conserve the limited stock of amino acids (Vogel & Jensen, 1994). Conversely, in *E. coli*, (p)ppGpp has been shown to upregulate the synthesis of proteins involved in amino acid biosynthesis or uptake (Barker *et al.*, 2001). An additional way to provide a supply of amino acids during amino acid starvation could be to upregulate the expression of virulence determinants (*eg.* haemolysins and proteases) that could potentially scavenge amino acids from the host or surrounding environment. This may explain why, in the SVS region mutants containing transposon insertions within genes involved in amino acid biosynthesis/uptake (which

would have possessed a lower pool of their respective amino acid(s)), α-haemolysin and protease expression was increased.

However, the SVS region genes (including the *dap* operon genes, *brnQ* and *telA*) have been shown to upregulate σ^B activity. Therefore, the increased expression of these amino acid biosynthesis/uptake genes following amino acid starvation would potentially lead to an increase in σ^B activity, resulting in a concomitant increase in the repression of *agr* and virulence determinant expression. Consequently, the pathway(s) by which inactivation of genes with putative functions in amino acid biosynthesis/uptake within the SVS region might lead to increased virulence determinant expression in *S. aureus* remain unclear. Additional work to determine the effects of amino acid starvation on the expression of these genes in *S. aureus* is required. It would also be useful to determine whether genes located outside of the SVS region that have a putative role in amino acid biosynthesis/uptake or other functions have similar effects on *hla* transcription and the activity of proteases and σ^B.

Furthermore, is it important to note that although a number of genes within the SVS region have putative functions in amino acid biosynthesis/uptake, some of the genes (*eg. norQ* and *aclP*) are likely to have roles in unrelated processes. However, the inactivation of these genes results in almost identical mutant phenotypes to those displayed by the amino acid biosynthesis/uptake SVS region mutants. This raises two questions. Firstly, are the phenotypes displayed by the SVS region mutants artifacts? It is possible that the increased *hla::lacZ* transcription observed is caused by a defect in the *hla::lacZ* reporter gene fusion of the mutants. However, this is extremely unlikely since the distance between *hla* (SA1173) and the SVS region is too great for such a defect to have been co-transduced with the transposon insertions when creating the strains in the SH10000 *hla::lacZ* background to verify the mutant's phenotypes (Chapters 5.2.3.4 and 5.2.4.3) (*ie. hla* is ~ 250 kb away from the SVS region and ϕ11 is only capable of transducing up to 45 kb of DNA (Novick, 1991)). Nevertheless, it is possible that the mutations affect the stability or activity of β-galactosidase, causing an inaccurate estimate of *hla* transcription to be obtained. However, the altered protease and σ^B activity of the mutants was demonstrated using methods that did not rely on *lacZ* reporter gene fusion expression. Furthermore, the mutant phenotypes are unlikely to be caused by insertion of the specific sequences of Tn*551* or Tn*917* within the *S. aureus*

genome, since the insertion of Tn*551* into *pstC* and *hyp*1 did not result in increased *hla::lacZ* transcription.

Interestingly, attempts to create a collection of mutants containing transposon insertions in all of the genes of the *S. aureus* genome identified the region between genes SA1400 and SA1455 as a hotspot for the insertion of Tn*551* and Tn*917* (Horsburgh, 2002). This area contains the SVS region. Consequently, the SVS region genes will have been over-represented in the pool of transposon mutants screened in this study. However, the SVS region mutants display an altered phenotype (*ie.* increased *hla::lacZ* and protease expression and reduced σ^B activity) and are therefore valid mutants regardless of the presence of the hotspot. No reason why this region acts as a hotspot could be identified in studies by Horsburgh (2002).

The second question posed by the finding that Tn*551* or Tn*917*-mediated inactivation of genes of possibly unrelated function within the SVS region results in almost identical phenotypes is; are the phenotypes caused by the loss of actual gene function, or by a more general effect such as a change in DNA topology resulting from the physical insertion of transposon DNA within the region? A strain containing a promoterless *lacZ* gene inserted after the *lysC* promoter did not display the same increased level of protease activity as the *lysC*::Tn*551* mutant. This suggests that the insertion of the DNA fragment carrying the promoterless *lacZ* gene was unable to cause a change in DNA topology that results in the mutant phenotype. Therefore, it is likely that the mutant phenotype is caused by loss of gene function. However, to verify this more conclusively, mutants containing intergenic transposon insertions in the SVS region could be analysed to determine whether they possess the mutant phenotype. However, no such mutants were available. Alternatively, complementation could be used to show that the return of the function of the inactivated SVS region genes results in restoration of the wild-type phenotype. Complementation of the *dap* operon, *brnQ* and *telA* mutations are currently being performed (J. Lithgow, personal communication).

CHAPTER 7

GENERAL DISCUSSION

7.1 Introduction

This study focussed on the pathways by which the virulence determinants of *S. aureus* are regulated in the presence and absence of NaCl-mediated stress. The experimental results obtained have been discussed in detail at the end of each chapter. However, models of the regulatory pathways that control virulence determinant expression under the two conditions, based on the findings of this study and of published research, are proposed here to provide an overview of the key points.

7.2 *S. aureus* virulence determinant regulation in the absence of NaCl-mediated stress

Previous studies have shown that in the absence of NaCl-mediated stress, *agr* upregulates the extracellular virulence determinants (*eg. hla* and *sspA*), and represses the surface-associated virulence determinants (Recsei *et al.*, 1986, Morfeldt *et al.*, 1988; Peng *et al.*, 1988). The *agr* locus is upregulated by *sarA* (Chien & Cheung, 1998; Chien *et al.*, 1998; Heinrichs *et al.*, 1996) and positively autoregulates itself via the autoinducing peptide (AIP) (Ji *et al.*, 1995, Lina *et al.*, 1998). This study showed that σ^B indirectly represses the transcription of *agr*. σ^B was also shown to repress *hla* transcription, possibly as a result of reduced *agr* expression. It has been suggested that the increased transcription of *hla* observed in a *sigB* background of *S. aureus* RN6390 occurred via an elevated level of SarA (Cheung *et al.*, 1999). However, in this study, transcriptional and Western blot analysis showed that SarA is not an intermediate in the repression by σ^B of *agr* or *hla*. Additionally, the *sarA* homologues *sarH1* and *rot* are unlikely to function in this pathway. The genes mediating the repression of *agr* and *hla* by σ^B therefore remain unknown.

Tn*551* mutagenesis identified a number of genes that upregulate σ^B activity, and which consequently repress *hla* transcription and protease activity. These genes include *lysC*, encoding aspartokinase II; *ykuQ*, encoding tetrahydrodipicolinate acetyltransferase; *lysA*, encoding diaminopimelate decarboxylase; *brnQ*, encoding a branched-chain amino acid carrier protein; and *telA*, encoding a tellurite-resistance protein. The genes are clustered together within the SVS region of the *S. aureus* genome. Transposon insertions within this region have been found to affect the virulence and survival of *S. aureus* in a large proportion of published transposon mutant library screens (Wiltshire & Foster, 2001; Coulter *et al.*, 1998; Mei *et al.*, 1997; Lammers *et al.*, 2000 Vriesema *et al.*, 2000; Shaw, 2002; Watson *et al.*, 1998). Other genes within the SVS region were also found to upregulate the activity of σ^B and to repress the activity of proteases and the transcription of *hla*. These genes include *asd,* encoding aspartate-semialdehyde dehydrogenase; *dapA,* encoding dihydrodipicolinate synthase; *hipO,* encoding hippurate hydrolase; *aclP,* encoding acylphosphatase; and *norQ*, encoding nitric-oxide reductase.

Many of the genes within the SVS region have putative roles in amino acid biosynthesis/uptake. However, it is unclear whether these genes affect virulence determinant expression via the stringent response. In addition, the functions of many other genes within the region are predicted to be unrelated to amino acid accumulation. However, the finding that all of the available mutants with transposon insertions in SVS region genes displayed similar phenotypes was unlikely to be an artifact or due to transposon insertion altering DNA topology within the region. The pathway(s) by which transposon insertions within the SVS region affect virulence determinant expression remain undetermined.

In the absence of NaCl-mediated stress, other environmental signals may affect virulence determinant expression via σ^B (possibly through the SVS region genes), or via σ^B-independent pathway(s). In this and other studies, the sensor/regulator system *saeS/saeR* has been shown to upregulate *hla* (Giraudo *et al.*, 1994; Giraudo *et al.*, 1996; Goerke *et al.*, 2001). This is likely to occur independently of *agr*, since Giraudo *et al.* (1997) have shown that *sae* has no effect on *agr* transcription.

The proposed regulatory pathways controlling virulence determinant expression in the absence of NaCl-mediated stress are represented diagramatically in Figure 7.1.

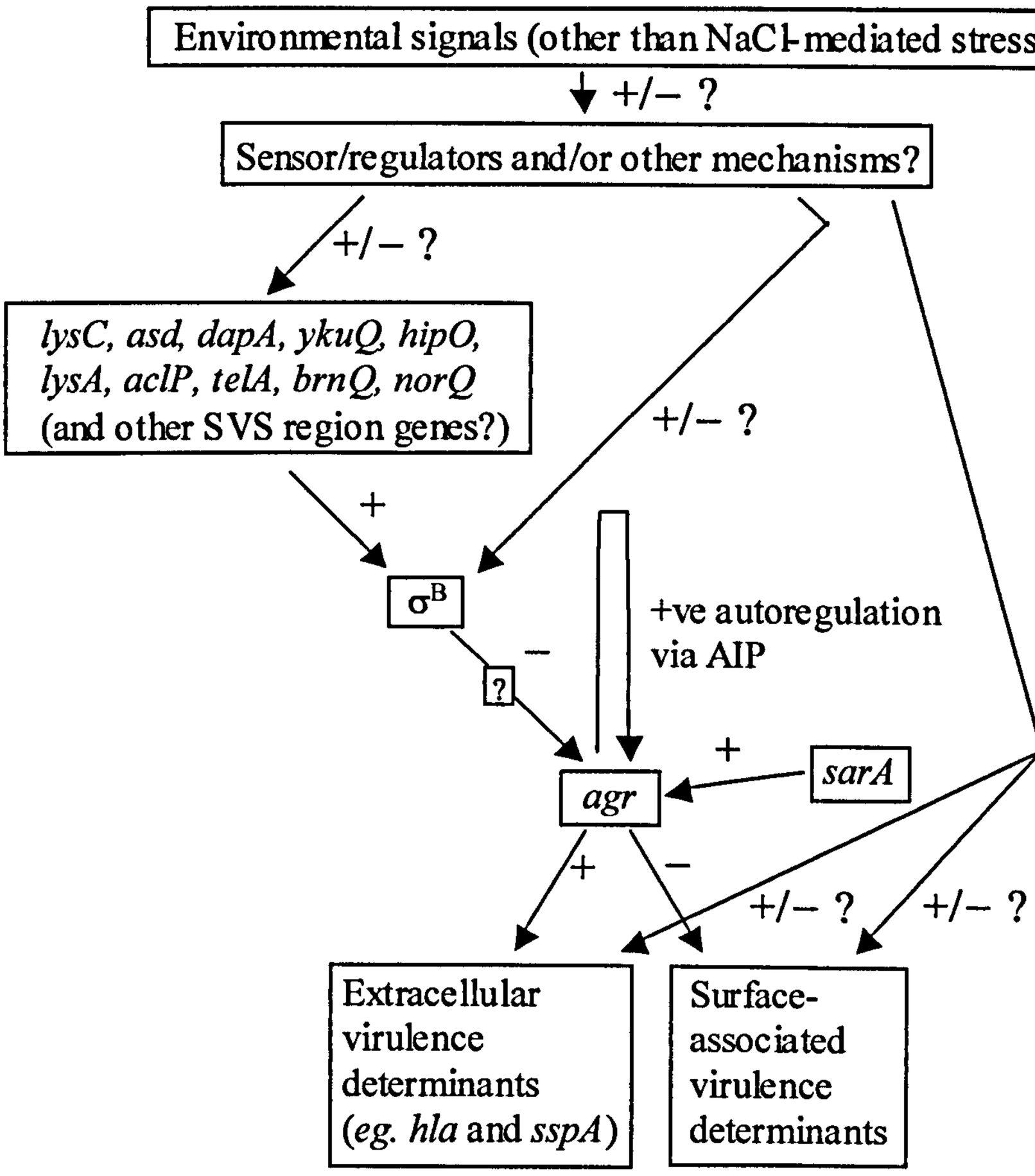


Figure 7.1

Proposed model for the regulation of *S. aureus* virulence determinants in the absence of NaCl-mediated stress (+ and - = positive and negative regulation respectively, AIP = autoinducing peptide).

7.3 *S. aureus* virulence determinant regulation in the presence of NaCl-mediated stress

In this study, *lacZ* fusion analysis showed that 1 M NaCl represses the transcription of *hla* and *spa*, but has no significant effect on *agr* transcription. This agrees with the findings of Chan & Foster (1998a) and Lindsay and Foster (1999), and suggests that virulence determinants, which are usually regulated by *agr*, might come under the control of a novel regulatory system in the presence of a high concentration of NaCl. Indeed, the repression of *hla* transcription by σ^B increased in the presence of 700 mM NaCl. This indicates that σ^B plays a role in virulence determinant regulation in response to NaCl in *S. aureus*. However, *hla* was repressed by 700 mM NaCl in a *sigB* background. Overall, this suggests that the regulation of virulence determinant transcription in response to NaCl in *S. aureus* occurs via both σ^B-dependent and σ^B-independent pathways.

Tn*917* mutant library screening identified a number of novel genes involved in regulating virulence determinant expression in response to NaCl, including *citG*, encoding fumarase; *opuD*, encoding a glycine betaine transporter; *yugT*, encoding exo-alpha-1,4-glucosidase; *oppF*, encoding an oligopeptide ABC transporter ATP-binding protein; *ykrP*, encoding a protein homologous to putative integral membrane proteins and acetyltransferases; *eprH*, encoding a protein involved in endopeptidase resistance and peptidoglycan biosynthesis; *yubA*, encoding a conserved hypothetical membrane protein; and *unk*1 and *unk*2, encoding proteins for which putative functions could not be defined. Tn*917*-mediated inactivation of these genes partially de-repressed the activity of an *agr*-dependent haemolytic factor (probably Hla) in the presence of 1 M NaCl, but had no effect in the absence of added NaCl. These genes are likely to be involved in the σ^B-independent pathway by which virulence determinants are regulated in response to NaCl, rather than the σ^B-dependent pathway, as the screen was performed in the 8325-4 background. As discussed in Chapter 3.1, 8325-4 possesses a defect in *rsbU*, a gene involved in the upregulation of σ^B activity (Kullik & Giachino, 1997).

The SVS region genes tested were found to have putative roles in the σ^B-dependent regulation of virulence determinant expression in response to NaCl. It is possible that the genes upregulate σ^B to a greater extent in the presence than in the absence of NaCl-

mediated stress. σ^B would therefore be able to repress *hla* to a greater extent than in the absence of the NaCl signal, perhaps via a novel regulator which assumes control in the presence of NaCl of otherwise *agr*-regulated virulence determinants. It is possible that the σ^B-dependent and σ^B-independent pathways converge on a common regulator in order to alter virulence determinant expression in response to NaCl, or that the two pathways mediate their effects via two completely separate mechanisms. Lindsay and Foster (1999) have shown that NaCl upregulates *sspA*. It is unknown whether the genes isolated in the transposon mutagenesis screens are involved in this process.

The NaCl-stress signal is probably detected via sensor/regulator system(s) and/or other sensory mechanism(s). For example, Tn*551*-mediated inactivation of *saeS*, the sensor component of a sensor/regulator system, was found to prevent *hla* transcription from being repressed appropriately in the presence of NaCl. It is therefore possible that *saeS* may play a role in sensing NaCl. The fact that the phenotypes displayed by the transposon mutants on agar plates supplemented with NaCl could not be reproduced in liquid culture containing added NaCl is interesting. This suggests that *S. aureus* evaluates and responds to the NaCl signal in relation to additional environmental parameters (*eg.* aeration) in order to allow virulence determinant expression to be regulated in a sensitive, sophisticated manner.

The proposed regulatory pathways controlling virulence determinant expression in the presence of NaCl-mediated stress are represented diagramatically in Figure 7.2.

7.4 Concluding remarks

This study investigated the regulation of *S. aureus* virulence determinant expression in the presence and absence of NaCl-mediated stress. The role of known regulators was assessed, and novel regulatory components were identified by transposon mutagenesis. Under both conditions, virulence determinant regulation was found to be complex, involving a number of putative pathways. This highlights the fact that the stringent control of virulence determinant expression in response to the prevailing conditions is of fundamental importance to this highly versatile pathogen.

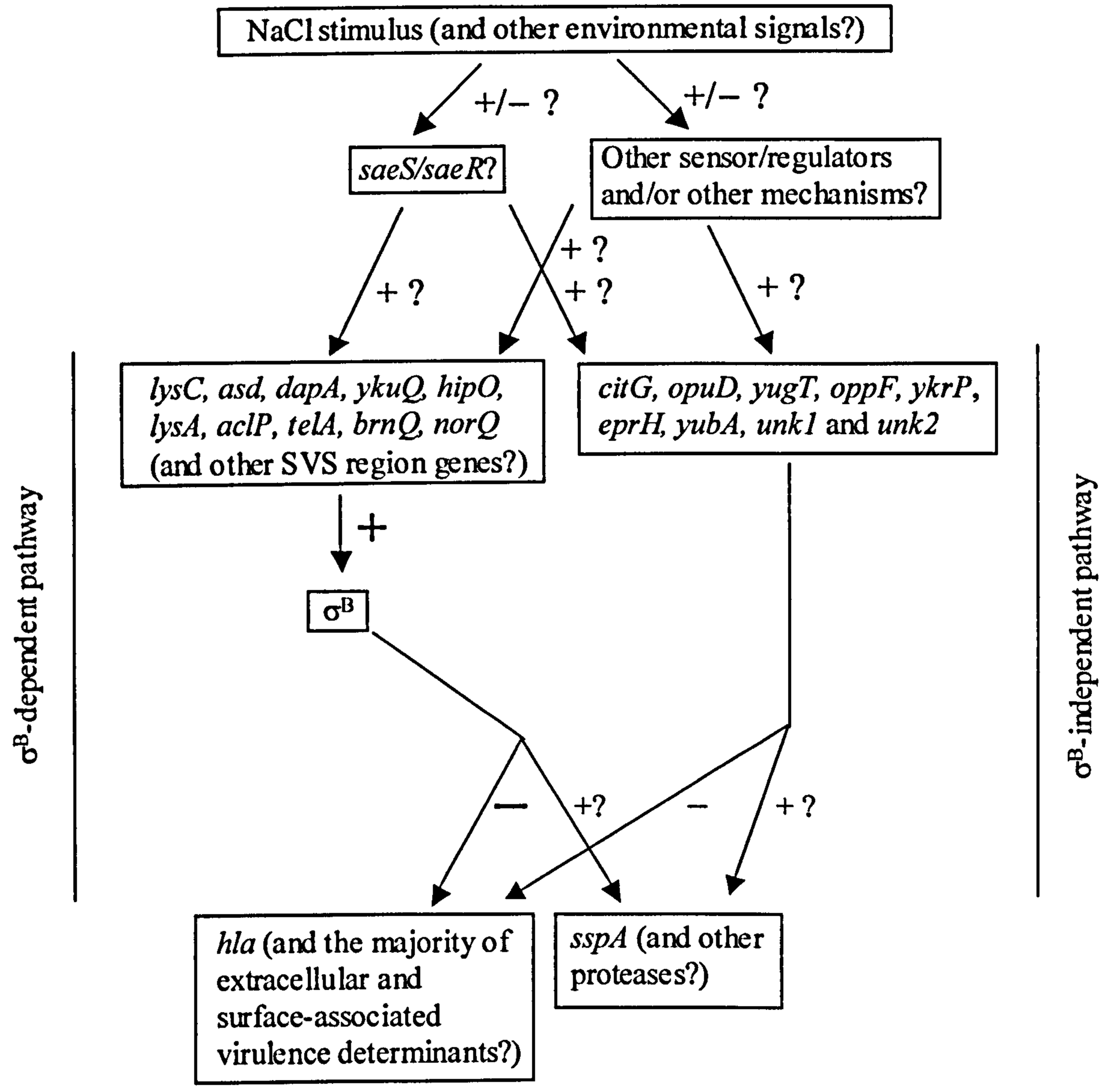


Figure 7.2

Proposed model for the regulation of *S. aureus* virulence determinants in the presence of NaCl-mediated stress (+ and - = positive and negative regulation respectively. + and - = increased level of positive and negative regulation compared to that occurring in the absence of NaCl-mediated stress respectively).

7.5 Future directions

During this study, transposon mutant library screening isolated novel genes with putative roles in the regulation of *S. aureus* virulence determinants in the presence and absence of NaCl-mediated stress. However, the ability to further analyse the roles of these genes in the NaCl-mediated stress response is limited by the fact that the phenotypes displayed by the mutants in the presence of NaCl are not reproducible in liquid culture and are therefore difficult to quantify. However, it may be possible to identify proteins that bind to the *hla* promoter in the presence of NaCl by affinity chromatography.

Experiments to further characterise the roles of the SVS region genes in virulence determinant regulation in the absence of NaCl-mediated stress could be performed. For example, whether the SVS region genes tested control σ^B activity by acting upstream or downstream of *rsbU* could be determined by Northern blot analysis. Furthermore, although the phenotypes of many SVS region mutants were analysed in this study, mutations in a number of SVS region mutants were unavailable during this project. The effects of these genes on σ^B activity and the expression of *hla* and proteases could be examined to determine whether all of the genes within the region have the same function.

Whether the phenotypes of the SVS region mutants are caused by lowered amino acid pools could be investigated by determining whether the phenotypes can be complemented by the addition of the respective amino acid(s). It would also be interesting to ascertain whether genes involved in amino acid biosynthesis/uptake that reside outside of the SVS region have the same effects of σ^B activity and virulence determinant expression.

Finally, complementation by introducing a functional copy of the genes/operons inactivated in the mutants could be used to further clarify whether the mutant phenotypes are caused by loss of gene function or alterations in DNA topology. Complementation of the *dap* operon, *brnQ* and *telA* mutations is currently being performed by J. Lithgow (Sheffield University).

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APPENDIX

A.1 Growth media

A.1.1 BHI

Component	Weight ($g\ l^{-1}$)
Calf brain infusion solids	12.5
Beef heart infusion solids	5.0
Proteose peptone	10.0
Glucose	2.0
Sodium chloride	5.0
Di-Sodium phosphate	2.5

Table A.1.1

List of ingredients of BHI (Oxoid)

A.1.2 Blood agar base

Component	Weight ($g\ l^{-1}$)
Proteose peptone	15.0
Liver digest	2.5
Sodium chloride	5.0
Yeast extract	5.0
Agar	12.0

Table A.1.2

List of ingredients of Blood agar base (Oxoid)

A.2 Sequence analysis

The following Figures show the alignments of genomic DNA sequencing reaction data with *S. aureus* COL DNA to identify the site of transposon insertion (Chapter 2.17.2). Genes were assigned numbers according to the *S. aureus* COL database. Results of BLASTP searches to identify putative homologues (Chapter 2.17.2) are also shown. Please note that the sequences corresponding to the terminus of the transposon have been removed from the sequencing reaction data. Sequence data therefore only shows *S. aureus* genomic DNA sequence. Putative homologues have been allocated NCBI accession numbers, and where possible, the top 5 hits are shown. Hits against *S. aureus* proteins are shown only where the function of the protein has been determined by experimentation, rather than by homology to other proteins. Identity = percentage and number of identical amino acids.

Figure A.2.1

JLA401 (8325-4 *han*1 *(unk*1*)*)

Identities = 100 % (215/215)

```
Query:     215 CTCCTTTCTTCTTATATAACAATTCTTCTAACATGATATGTTACTATTGAATTACTGAAC 156
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1950039 CTCCTTTCTTCTTATATAACAATTCTTCTAACATGATATGTTACTATTGAATTACTGAAC 1950098

Query:     155 CTGAGTTAGTTATAATCTAACTTATATTGAAAAGAGATGAGGCGTAAGATATGTTTTTAT 96
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1950099 CTGAGTTAGTTATAATCTAACTTATATTGAAAAGAGATGAGGCGTAAGATATGTTTTTAT 1950158

Query:      95 GTAAAAGACAAATTGATATCAATGCACGATTTGGTTTGCCTAGAATTGCATTTATGAGTG 36
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1950159 GTAAAAGACAAATTGATATCAATGCACGATTTGGTTTGCCTAGAATTGCATTTATGAGTG 1950218
```


```
Query:      35 CAGTTGCAACCATCATTATGTTTTTAGTTAGTTAT 1
               ||||||||||||||||||||||||||||||||||
Sbjct: 1950219 CAGTTGCAACCATCATTATGTTTTTAGTTAGTTAT 1950253
```

Tn*917* was found to have inserted into the same base in JLA410 (8325-4 *han*10 (*unk*1)) as in JLA401 (8325-4 *han*1 *(unk*1)).

The transposon insertion was in SA1896. BLASTP hits against the protein sequence of SA1896 are shown in the table below.

Accession no.	Protein	Organism	Identity	Expect
NC_002570	BH1182, unknown	*B. halodurans*	27 % (48/175)	1e-11
NC_003210	lmo2218, unknown	*L. monocyt-ogenes* EGD-e	22 % (39/175)	8e-09
NC_003212	lin2321, unknown	*L. innocua*	26 % (47/180)	2e-07
NC_004193	Conserved hypothetical protein	*O. iheyensis*	23 % (42/182)	6e-07

Figure A.2.2

JLA402 (8325-4 *han*2 (*citG*))

Identities = 100 % (229/229)

```
Query:     229 CTGGTTTATAAACATTCAATTCAAAGTTACCTTGTGAACTTGCGAAGCCAACAACTGTAT 170
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1963868 CTGGTTTATAAACATTCAATTCAAAGTTACCTTGTGAACTTGCGAAGCCAACAACTGTAT 1963927

Query:     169 CATTACCCATTACTTGGACTGCAACCATTGTTAACATTTCACATTGTGTAGGATTAACTT 110
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1963928 CATTACCCATTACTTGGACTGCAACCATTGTTAACATTTCACATTGTGTAGGATTAACTT 1963987

Query:     109 TACCAGGCATAATTGATGAACCTGGTTCATTTTCAGGGATAGAAATTTCTGCCAAACCAG 50
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1963988 TACCAGGCATAATTGATGAACCTGGTTCATTTTCAGGGATAGAAATTTCTGCCAAACCAG 1964047
```

Tn*917*

```
Query:      49 CTCGTGGCCCTGAAGCCAACCATCTCACATCATTAGCAATTTTCATTAA 1
               ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1964048 CTCGTGGCCCTGAAGCCAACCATCTCACATCATTAGCAATTTTCATTAA 1964096
```

Tn*917* was found to have inserted into the same base in JLA403 (8325-4 *han*3 (*citG*)), JLA405 (8325-4 *han*5 (*citG*)), JLA406 (8325-4 *han*6 (*citG*)), JLA407 (8325-4 *han*7 (*citG*)), JLA408 (8325-4 *han*8 (*citG*)) as in JLA402 (8325-4 *han*2 (*citG*)).

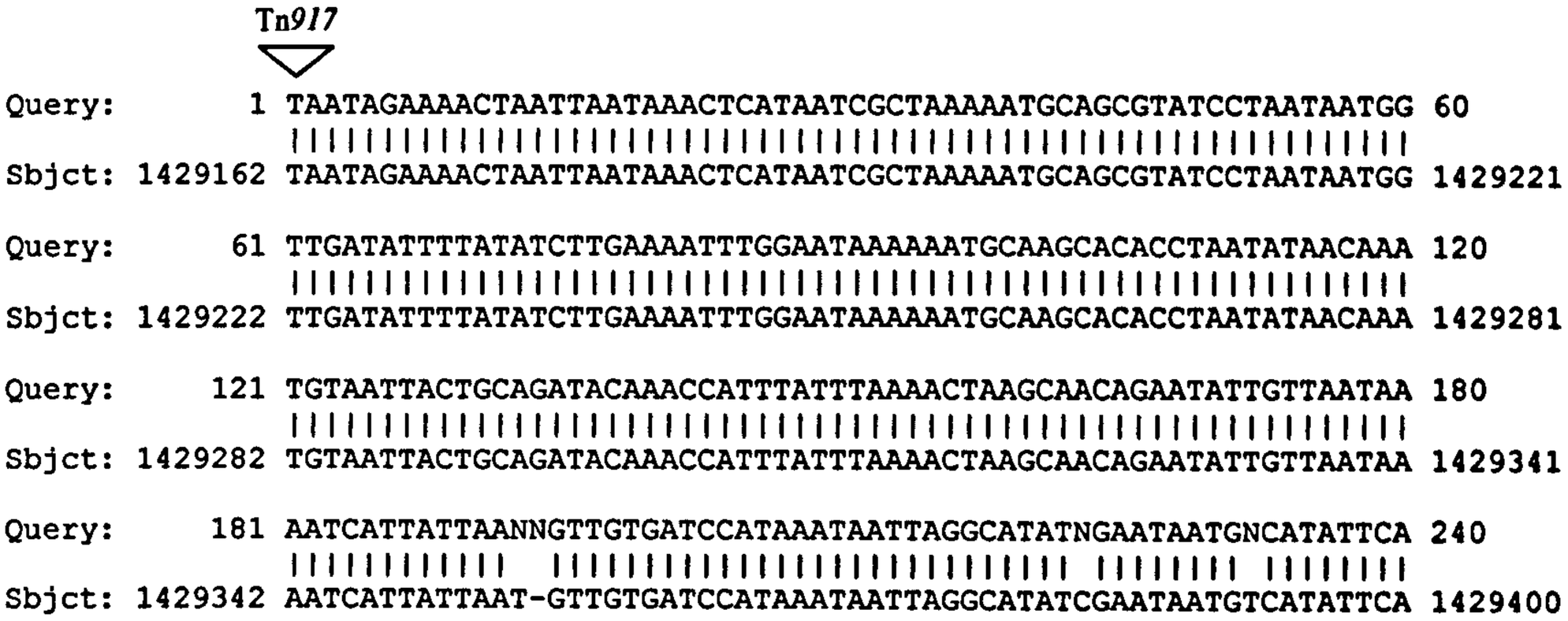
The transposon insertion was in SA1908. BLASTP hits against the protein sequence of SA1908 are shown in the table below.

Accession no.	Protein	Organism	Identity	Expect
NC_000964	Fumarate hydratase, Class-II (fumarase), CitG	*B. subtilis*	66 % (307/460)	e-176
NC_000921	Fumarase	*H. pylori* J99	61 % (284/461)	e-163
NC_003210	Similar to fumarate hydratase	*L. monocyt-ogenes* EGD-e	63 % (289/456)	e-162
NC_002163	Fumarate hydratase, Class II (fumarase)	*C. jejuni*	60 % (280/461)	e-161
NC_000913	Fumarate hydratase class II (Fumarase), FumC	*E. coli* K12	60 % 277/458	e-157

Figure A.2.3

JLA404 (8325-4 *han*4 (*unk*2))

Identities = 98 % (236/240)



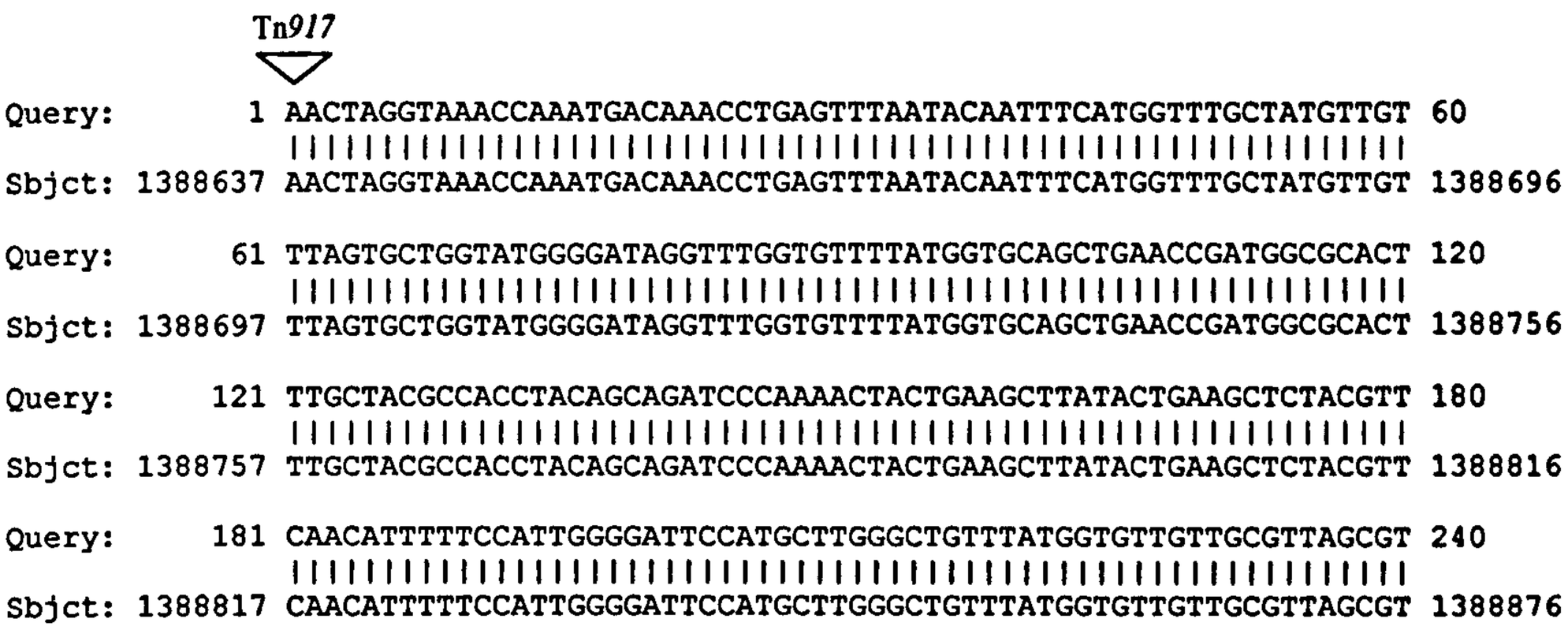
The transposon insertion was in SA1418. BLASTP hits against the protein sequence of SA1418 are shown in the table below.

Accession no.	Protein	Organism	Identity	Expect
NC_003902	Conserved hypothetical protein	*X. campestris* pv. *campestris* str. ATCC33913	40 % (20/50)	3.1
NC_003919	Conserved hypothetical protein	*X. axonopodis* pv. *citri* str. 306	40 % (20/50)	3.1
NC_004193	Hypothetical protein	*O. iheyensis*	32 % (25/78)	6.9
NC_003551	Uncharacterized protein	*M. kandleri AV19*	36 % (23/63)	9.0
NC_003902	Conserved hypothetical protein	*X. campestris* pv. *campestris* str. ATCC33913	40 % (20/50)	3.1

Figure A.2.4

JLA409 (8325-4 *han*9 (*opuD*))

Identity = 100 % (240/240)



The transposon insertion was in SA1384. BLASTP hits against the protein sequence of SA1384 are shown in the table below.

Accession no.	Protein	Organism	Identity	Expect
NC_000964	Glycine betaine transporter OpuD	*B. subtilis*	56 % (278/496)	e-150
NC_003212	Glycine betaine transporter BetL	*L. innocua*	56 % (285/506)	e-141
NC_003210	Glycine betaine transporter BetL	*L. monocyt-ogenes* EGD-e	48 % (284/506)	e-144
NC_004116	Transporter, BCCT family protein	*S. agalactiae 2603V/R*	51 % (265/518)	e-122
NC_003995	BCCT, BCCT family transporter	*B. anthracis* A2012	48 % (243/504)	e-116

Figure A.2.5

JLA411 (8325-4 *han*11 (*yugT*))

Identity = 100 % (211/211)

```
Query:     211 AAATTAACTTTGGCATCATAATTAAATAGCTTTACTTCTCCATGGCTTAAATCAAATGGT 152
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1586876 AAATTAACTTTGGCATCATAATTAAATAGCTTTACTTCTCCATGGCTTAAATCAAATGGT 1586935

Query:     151 ACAGTTAATTCTGCTTCGTGGTTAGTAAGATTACCTACAATAAGAACTTGCTTTTCATTT 92
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1586936 ACAGTTAATTCTGCTTCGTGGTTAGTAAGATTACCTACAATAAGAACTTGCTTTTCATTT 1586995

Query:      91 AATGTTCTCGTGTACGCAAAAACTTGTGAATTTTCAGCATCTACTAAATCAAATTGACCA 32
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1586996 AATGTTCTCGTGTACGCAAAAACTTGTGAATTTTCAGCATCTACTAAATCAAATTGACCA 1587055

                                        Tn917
                                          ▽
Query:      31 TATACGTATACATCATTAGACTTTCTTAATT 1
               |||||||||||||||||||||||||||||
Sbjct: 1587056 TATACGTATACATCATTAGACTTTCTTAATT 1587086
```

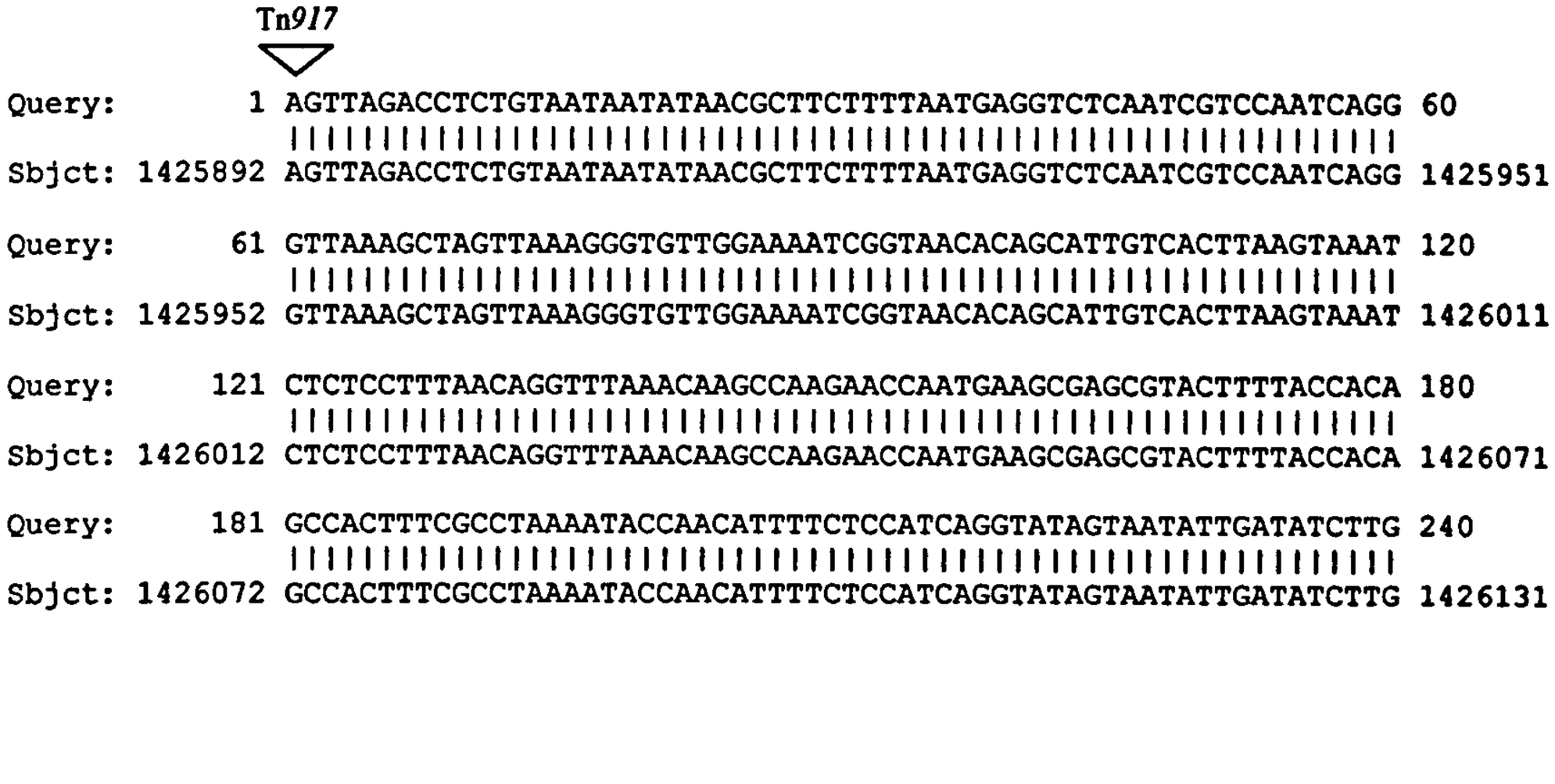
The transposon insertion was in SA1551. BLASTP hits against the protein sequence of SA1551 are shown in the table below.

Accession no.	Protein	Organism	Identity	Expect
NC_003366	Exo-alpha-1,4-glucosidase	*C. perfringens*	59 % (329/555)	e-188
NC_000964	Exo-alpha-1,4-glucosidase homolog YugT	*B. subtilis*	56 % (308/549)	e-180
NC_002570	Oligo-1,6-glucosidase	*B. halodurans*	53 % (296/556)	e-173
NC_003210	Similar to oligo-1,6-glucosidase	*L. monocyt-ogenes EGD-e*	47 % (261/554)	e-154
NC_000964	Oligo-1,6-glucosidase	*B. subtilis*	48 % (268/555)	e-152

Figure A.2.6

JLA412 (8325-4 *han*12 (*oppF*))

Identity = 100 % (240/240)



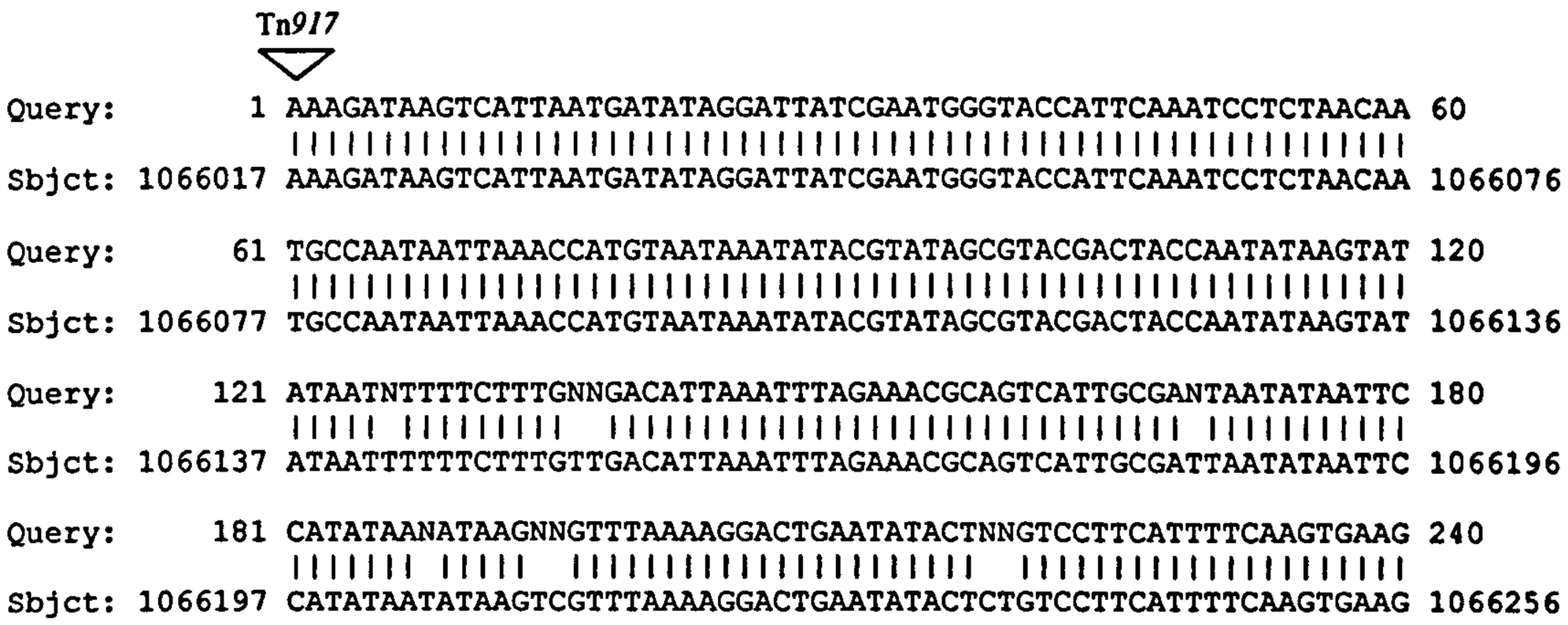
The transposon insertion was in SA1414. BLASTP hits against the protein sequence of SA1414 are shown in the table below.

Accession no.	Protein	Organism	Identity	Expect
NC_003305	Oligopeptide ABC transporter, ATP binding protein	*A. tumefaciens* str. C58	37 % (81/218)	1e-32
NC_000921	ABC transporter, ATP-binding protein	*H. pylori* J99	34 % (87/249)	1e-32
NC_004113	ABC transporter ATP-binding protein	*T. elongatus BP-1*	33 % (80/242)	2e-30
NC_003047	Putative ATP-binding protein of oligopeptide ABC transporter	*S. meliloti*	37 % (82/218)	5e-32
NC_003454	Oligopeptide ABC transporter ATP-binding protein OppF	*F. nucleatum ATCC 25586*	31 % (70/223)	5e-30

Figure A.2.7

JLA413 (8325-4 *han*13 (*ykrP*))

Identity = 96 % (231/240)



The transposon insertion was in SA1059. BLASTP hits against the protein sequence of SA1059 are shown in the table below.

Accession no.	Protein	Organism	Identity	Expect
NC_003210	Similar to *B. subtilis* YkrP protein	*L. monocyt-ogenes* EGD-e	40 % (113/281)	3e-56
NC_000964	Conserved hypothetical protein YkrP	*B. subtilis*	35 % (100/278)	1e-49
NC_003888	Putative integral membrane protein	*S. coelicolor A3(2)*	25 % (75/296)	5e-26
NC_002678	Acetyltransferase, nodulation protein NolL	*M. loti*	23 % (73/309)	1e-09
NC_003030	O-actetyl transferase related protein	*C. acetobutylicum*	23 % (71/296)	6e-08

Figure A.2.8

JLA414 (8325-4 *han*14 (*eprH*))

Identity = 100 % (228/228)

```
Query:     228 AGTTAAATATGCAATTATCTAGTATTAATAATAGAATTAGTAAAACCGAAGAACTAATAT 169
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1274971 AGTTAAATATGCAATTATCTAGTATTAATAATAGAATTAGTAAAACCGAAGAACTAATAT 1275030

Query:     168 TTGAAGATGGACCTGTTTTGGATTTAGCTGCTGCTTTATTTATATGTACTGATGATGAAG 109
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1275031 TTGAAGATGGACCTGTTTTGGATTTAGCTGCTGCTTTATTTATATGTACTGATGATGAAG 1275090

Query:     108 TTTATTATCTATCAAGTGGATCAAATCCGAAATATAATCAGTATATGGGTGCATATCATC 49
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1275091 TTTATTATCTATCAAGTGGATCAAATCCGAAATATAATCAGTATATGGGTGCATATCATC 1275150
                                                                Tn917
                                                                ▽
Query:      48 TACAATGGCATATGATAAAATATGCAAAATCACATAATATTAATAGGT 1
               |||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1275151 TACAATGGCATATGATAAAATATGCAAAATCACATAATATTAATAGGT 1275198
```

The transposon insertion was in SA1265. BLASTP hits against the protein sequence of SA1265 are shown in the table below.

Accession no.	Protein	Organism	Identity	Expect
AB015195.1	EprH	*S. aureus* MW2	100 % (414/414)	0.0
AJ250765.1	Alanine adding enzyme MurN	*S. pneumoniae*	26 % (110/416)	2e-30
NC_003028	Beta-lactam resistance factor	*S. pneumoniae*	26 % (110/416)	2e-30
NC_004116	Beta-lactam resistance factor	*S. agalactiae* 2603V/R	24 % (102/416)	7e-28
NC_002737	Putative peptidoglycan branched peptide synthesis protein, alanine adding enzyme	*S. pyogenes* M1 GAS	24 % (101/416)	6e-27

Figure A.2.9

JLA415 (8325-4 *han*15 (*yubA*))

Identity = 100 % (239/239)

```
Query:     239 TTTCACCAAATATCATGGGTAAAACACTTAAGATTCATCCACTTACAATCATTTTCATTT 180
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1404538 TTTCACCAAATATCATGGGTAAAACACTTAAGATTCATCCACTTACAATCATTTTCATTT 1404597

Query:     179 TACTGTGTGCAGGCAAATTGCTTGGTATTGTAGGCGTTATTTTAGGTATTCCGGGATATG 120
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1404598 TACTGTGTGCAGGCAAATTGCTTGGTATTGTAGGCGTTATTTTAGGTATTCCGGGATATG 1404657

Query:     119 CTATTTTAAAAGTATTAGTTACTCATTTATTCCAATTATTTAAACGTCGATACAATCGTT 60
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1404658 CTATTTTAAAAGTATTAGTTACTCATTTATTCCAATTATTTAAACGTCGATACAATCGTT 1404717
```

Tn*917*

```
Query:      59 TCTATGGTAATGATGTAGGTGAATATGATATTAAAGAAAGTAATAAAATAGT 1
               ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1404718 TCTATGGTAATGATGTAGGTGAATATGATATTAAAGAAAGTAATAAAATAGT 1404769
```

Tn*917* was found to have inserted into the same base in JLA416 (8325-4 *han*16 (*yubA*)) as in JLA415 (8325-4 *han*15 (*yubA*)).

The transposon insertion was in SA1395. BLASTP hits against the protein sequence of SA1395 are shown in the table below.

Accession no.	Protein	Organism	Identity	Expect
NC_003210	Conserved hypothetical membrane protein	*L. monocyt-ogenes* EGD-e	38 % (142/367)	3e-79
NC_000964	Conserved hypothetical protein YubA	*B. subtilis*	40 % (149/366)	4e-79
NC_003212	Conserved hypothetical membrane protein	*L. innocua*	38 % (142/367)	2e-78
NC_003995	UPF0118, Domain of unknown function DUF20	*B. anthracis A2012*	35 % (132/376)	3e-62
NC_004116	Membrane protein, putative	*S. agalactiae* 2603V/R	30 % (120/390)	2e-56

Figure A.2.10

JLA515 (SH1000 *hla::lacZ hla*$^+$ *aht*1 (*rsbU*))

Identity = 93 % (224/240)

Tn*551*

```
Query:       1 CTGTAGATTATACTTTTAAGCTNNNNNNNNCTTTAGGATGGAATNTCTAAAGCCTAAAAA 60
               ||||||| ||||||||||||       ||||||||||||||  ||||||||||||||
Sbjct: 2123979 CTGTAGATGATACTTTTAAGCTAAAAAAAACTTTAGGATGGAATACCTAAAGCCTAAAAA 2124038

Query:      61 ACTTACNACATCCTTTTATAATAAAAAATATGAATATCATANCTGGTATTTGCTAAAATC 120
               |||||  ||||||||||||||||||||||||||||||||| ||||||||||||||||||
Sbjct: 2124039 ACTTAAGACATCCTTTTATAATAAAAAATATGAATATCATAACTGGTATTTGCTAAAATC 2124098

Query:     121 TTTGTTNATAAGAAAATTCTTCGTNCAGAATTTAGAAATAGTTATAATTATTATAAAGTT 180
               |||||| |||||||||||||||| ||||||||||||||||||||||||||||||||||
Sbjct: 2124099 TTTGTTTATAAGAAAATTCTTCGTACAGAATTTAGAAATAGTTATAATTATTATAAAGTT 2124158

Query:     181 ATTGNCTTTTATATCAATATTTTATTTATCTCTGAAAAATACATATAATAGACGCCTAAT 240
               |||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 2124159 ATTGTCTTTTATATCAATATTTTATTTATCTCTGAAAAATACATATAATAGACGCCTAAT 2124218
```

Tn*917* was found to have inserted into the same base in JLA521 (SH1000 *hla::lacZ hla*$^+$ *aht7* (*rsbU*)), JLA522 (SH1000 *hla::lacZ hla*$^+$ *aht*8 (*rsbU*)), JLA523 (SH1000 *hla::lacZ hla*$^+$ *aht*9 (*rsbU*)), JLA533 (SH1000 *hla::lacZ hla*$^+$ *htn*1 (*rsbU*)), JLA534 (SH1000 *hla::lacZ hla*$^+$ *htn*2 (*rsbU*)), JLA535 (SH1000 *hla::lacZ hla*$^+$ *htn*3 (*rsbU*)), JLA536 (SH1000 *hla::lacZ hla*$^+$ *htn*4 (*rsbU*)), JLA537 (SH1000 *hla::lacZ hla*$^+$ *htn*5 (*rsbU*)), JLA538 (SH1000 *hla::lacZ hla*$^+$ *htn*6 (*rsbU*)), JLA539 (SH1000 *hla::lacZ hla*$^+$ *htn*7 (*rsbU*)), JLA540 (SH1000 *hla::lacZ hla*$^+$ *htn*8 (*rsbU*)), JLA541 (SH1000 *hla::lacZ hla*$^+$ *htn*9 (*rsbU*)) and JLA546 (SH1000 *hla::lacZ hla*$^+$ *htn*14 (*rsbU*)) as in JLA515 (SH1000 *hla::lacZ hla*$^+$ *aht*1 (*rsbU*)).

The transposon insertion was in a putative regulatory region of SA2057. BLASTP hits against the protein sequence of SA2057 are shown in the table below.

Accession no.	Protein	Organism	Identity	Expect
CAA71065.1	Sigma factor σ^B regulation protein RsbU	*S. aureus* COL	99 % (332/333)	e-178
AF359562.1	Sigma factor σ^B regulation protein RsbU	*S. epidermidis*	84 % (282/333)	e-155
BAA19307.1	Sigma factor σ^B regulation protein RsbU	*B. subtilis*	37 % (126/332)	2e-54
AAN75461.1	RsbU	*L. monocytogenes*	39 % (129/330)	e-57

Figure A.2.11

JLA516 (SH1000 *hla::lacZ hla*$^+$ *aht2* (*ykuQ::Tn551*))

Identity = 98 % (217/221)

```
Query:     221 AATATATAAGTGATGCTAAAA-GTCTACACCAATAAAAGTATATTTAAATNGTAATTTTG 163
               ||||||||||||||||||||| |||||||||||||||||||||||||| |||||||||
Sbjct: 1444528 AATATATAAGTGATGCTAAAAAGTCTACACCAATAAAAGTATATTTAAATGGTAATTTTG 1444587

Query:     162 AAGGCATCACATATCCAGAAAGTTTAAAGTATTTGGTTCANAACAATCTAAAGTAATCT 103
               ||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||
Sbjct: 1444588 AAGGCATCACATATCCAGAAAGTTTAAAGTATTTGGTTCAGAACAATCTAAAGTAATCT 1444647

Query:     102 TTTGTGAAGCGGATGATTGAAAACCTTTTTACGAAGCATATGGTAGTCAATTCGAAGATA 43
               |||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||
Sbjct: 1444648 TTTGTGAAGCGGATGATTGGAAACCTTTTTACGAAGCATATGGTAGTCAATTCGAAGATA 1444707

                                                          Tn551
                                                           ▽
Query:      42 TAGAAATTGAAATGGATCGTCGCAATTCTGCTATTCCATTAA 1
               |||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1444708 TAGAAATTGAAATGGATCGTCGCAATTCTGCTATTCCATTAA 1444749
```

Tn*917* was found to have inserted into the same base in JLA517 (SH1000 *hla::lacZ hla*$^+$ *aht3* (*ykuQ::Tn551*)) as in JLA516 (SH1000 *hla::lacZ hla*$^+$ *aht2* (*ykuQ::Tn551*)).

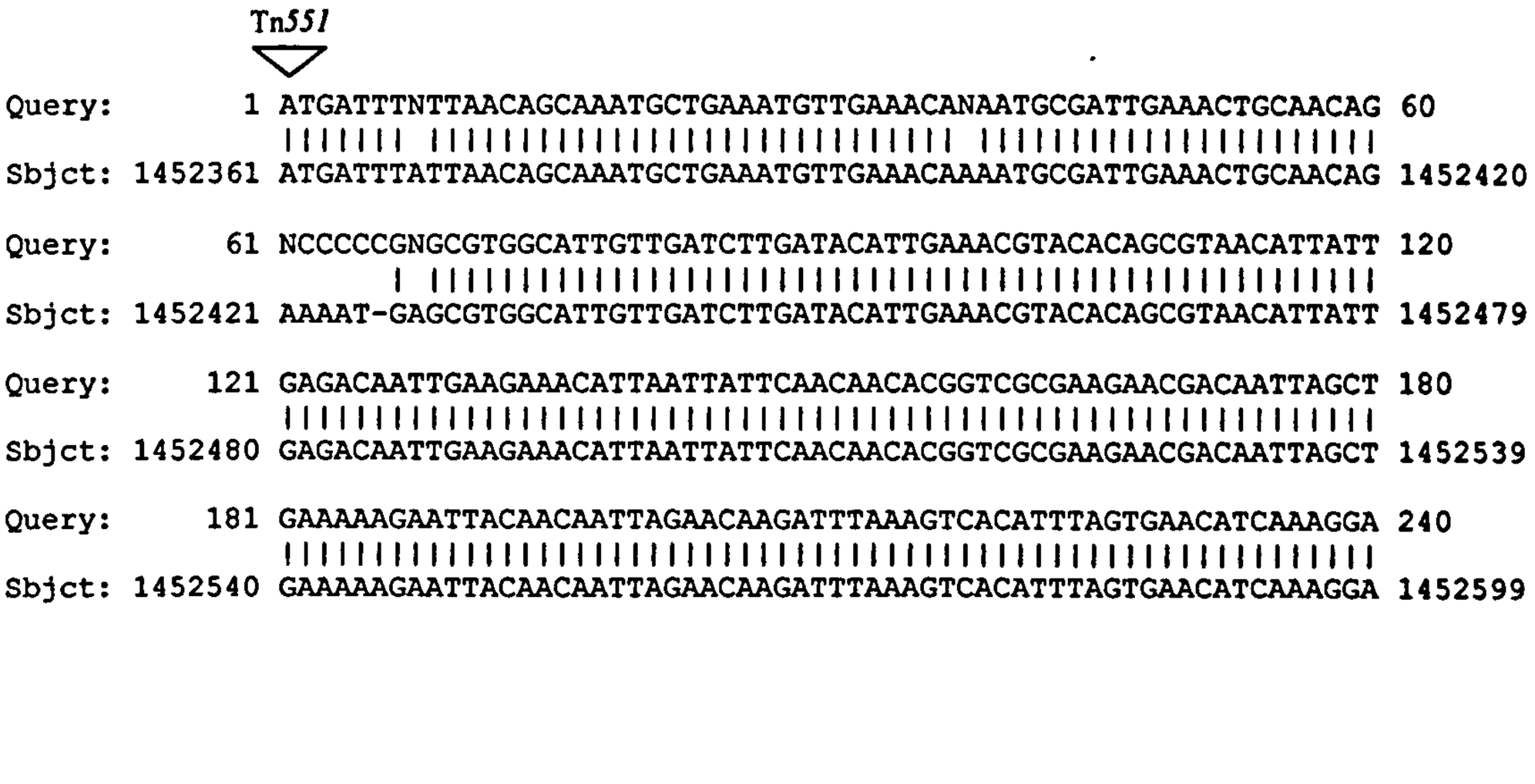
The transposon insertion was in SA1432. BLASTP hits against the protein sequence of SA1432 are shown in the table below.

Accession no.	Protein	Organism	Identity	Expect
AF306669.1	Tetrahydrodipicolinate acetyltransferase YkuQ	*S. aureus* 8325-4	100 % (239/239)	e-120
F6_9866	Tetrahydrodipicolinate succinylase homolog YkuQ	*B. subtilis*	60 % (142/233)	2e-64
NP_470347.1	Similar to tetrahydro-dipicolinate succinylase	*L. innocua*	57 % (136/236)	9e-63
BAB81615.1	Probable tetrahydro-dipicolinate succinylase	*C. perfringens str. 13*	58 % (133/228)	3e-60
AAM24087.1	Tetrahydrodipicolinate N-succinyltransferase	*T. tengcongensis*	55 % (126/229)	6e-58

Figure A.2.12

JLA518 (SH1000 *hla::lacZ hla*$^+$ *aht*4 (*telA*))

Identity = 96 % (231/240)



Tn*917* was found to have inserted into the same base in JLA547 (SH1000 *hla::lacZ hla*$^+$ *htn*15 (*telA*)) and JLA548 (SH1000 *hla::lacZ hla*$^+$ *htn*16 (*telA*)) as in JLA518 (SH1000 *hla::lacZ hla*$^+$ *aht*4 (*telA*)).

The transposon insertion was in SA1441. BLASTP hits against the protein sequence of SA1441 are shown in the table below.

Accession no.	Protein	Organism	Identity	Expect
NC_004461	Tellurite resistance protein	*S. epidermidis*	66 % (248/375)	e-107
NC_004668	Tellurite resistance protein	*E. faecalis*	50 % (171/342)	4e-75
AAB93480.1	Tellurite resistance protein TelA	*R. sphaeroides*	33 % (115/342)	6e-50
AAA98154.1	Tellurite resistance protein, TelA	*K. aerogenes*	28 % (106/370)	1e-36
AE015943.1	Tellurite resistance protein	*C. tetani*	27 % (101/365)	9e-36

Figure A.2.13

JLA519 (SH1000 *hla::lacZ hla*$^+$ *aht*5 (*lysA*))

Identity = 98 % (201/205)

```
Query:     205 TGTAACTATAGCTGGAAAATTATGTGAGTCTTGGTGATTATCATTATTAAAGACGCTAAA 146
               |||||||||||||||||||||||||||||||| |||||| ||||||||||||||||||||
Sbjct: 1448609 TGTAACTATAGCTGGAAAATTATGTGAGTCT-GGTGAT-ATCATTATTAAAGACGCTAAA 1448666

Query:     145 TTACCTTCATCAGTCAAACGTGGAGACCTATCTTGCTATATTATCAACTGGTGCATATCA 86
               |||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||
Sbjct: 1448667 TTACCTTCATCAGTCAAACGTGGAGAC-TATCTTGCTATATTATCAACTGGTGCATATCA 1448725

Query:      85 TTACTCTATGCCATCCAATTACAATCAAATGCAAAAGCCTTCTGTGTTTTTCTTAAAAGA 26
               |||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1448726 TTACTCTATGGCATCCAATTACAATCAAATGCAAAAGCCTTCTGTGTTTTTCTTAAAAGA 1448785

                                    Tn551
                                    ▽
Query:      25 TGGCAAAGCACGTGAAGTTATAAAG 1
               |||||||||||||||||||||||||
Sbjct: 1448786 TGGCAAAGCACGTGAAGTTATAAAG 1448810
```

Tn*917* was found to have inserted into the same base in JLA543 (SH1000 *hla::lacZ hla*$^+$ *htn*11 (*lysA*)), JLA544 (SH1000 *hla::lacZ hla*$^+$ *htn*12 (*lysA*)), JLA545 (SH1000 *hla::lacZ hla*$^+$ *htn*13 (*lysA*)) and JLA552 (SH1000 *hla::lacZ hla*$^+$ *htn*20 (*lysA*)) as in JLA519 (SH1000 *hla::lacZ hla*$^+$ *aht*5 (*lysA*)).

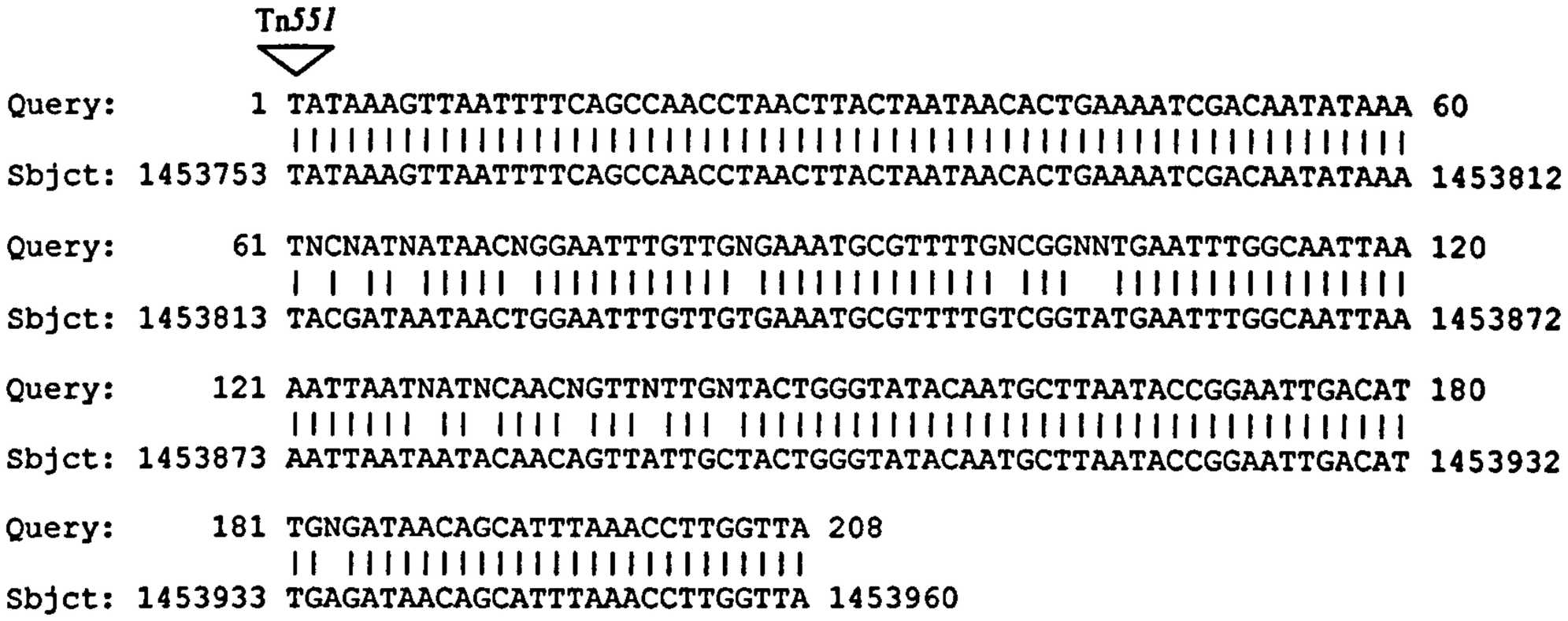
The transposon insertion was in SA1435. BLASTP hits against the protein sequence of SA1435 are shown in the table below.

Accession no.	Protein	Organism	Identity	Expect
AF306669_8	Diaminopimelate decarboxylase LysA	*S. aureus* 8325-4	100 % (421/421)	0.0
BAA12662.1	Diaminopimelate decarboxylase LysA	*B. subtilis*	49 % (210/423)	e-115
AH1318	Diaminopimelate decarboxylase homolog LysA	*L. mono-cytogenes* EGD-e	46 % (200/427)	e-106
AAM23511.1	Diaminopimelate decarboxylase	*T. tengcongensis*	48 % (206/426)	e-106

Figure A.2.14

JLA520 (SH1000 *hla::lacZ hla*$^{+}$ *aht6* (*brnQ*))

Identity = 93 % (194/208)



The transposon insertion was in SA1443. BLASTP hits against the protein sequence of SA1443 are shown in the table below.

Accession no.	Protein	Organism	Identity	Expect
AAC38146.1	Branched-chain amino acid carrier protein BrnQ	*S. aureus* RN450	84 % (361/429)	e-150
NP_764645.1	Branched-chain amino acid carrier protein BrnQ	*S. epidermidis*	74 % (333/447)	e-155
NP_246812.1	BrnQ	*P. multocida*	47 % (209/436)	3e-90
G97940	Branched-chain amino acid transport system II carrier protein BrnQ	*S. pneumoniae*	38 % (168/437)	1e-64
C69596	Branched-chain amino acid transporter BraB	*B. subtilis*	39 % (172/440)	5e-62

Figure A.2.15a

JLA524 (SH1000 *hla::lacZ hla*$^{+}$ *aht*10 (*yjbH*))

Identity = 99 % (202/203)

```
                    Tn551
                    ▽
Query:        1 ATCTGGATATTTAAGTTTTTCAATTTTTTGTTGAATGGCTAACTTCTTTAANTCTTTGTT 60
                ||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||
Sbjct: 1015395 ATCTGGATATTTAAGTTTTTCAATTTTTTGTTGAATGGCTAACTTCTTTAACTCTTTGTT 1015454

Query:       61 TAAAAGTTTTTCTGGCCATTCATAAATAGTAAGTAATTCTTCCATCGTTACAAGTTGTTG 120
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1015455 TAAAAGTTTTTCTGGCCATTCATAAATAGTAAGTAATTCTTCCATCGTTACAAGTTGTTG 1015514

Query:      121 TTGCTGTATATAAGTTTCTAATTTAGGAGGAAGATTCTTTTCGATAGGTTTACCCATCAA 180
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1015515 TTGCTGTATATAAGTTTCTAATTTAGGAGGAAGATTCTTTTCGATAGGTTTACCCATCAA 1015574

Query:      181 TTCATTAATTATATAAGTATAGA 203
                |||||||||||||||||||||||
Sbjct: 1015575 TTCATTAATTATATAAGTATAGA 1015597
```

Tn*917* was found to have inserted into the same base in JLA525 (SH1000 *hla::lacZ hla*$^{+}$ *aht*11 (*yjbH*)) as in JLA524 (SH1000 *hla::lacZ hla*$^{+}$ *aht*10 (*yjbH*)).

The transposon insertion was in SA1006. BLASTP hits against the protein sequence of SA1006 are shown in the table below.

Accession no.	Protein	Organism	Identity	Expect
H69843	Hypothetical protein YjbH	*B. subtilis*	31 % (81/261)	7e-28
AB1553	*B. subtilis* YjbH protein homolog lin0963	*L. innocua*	29 % (75/253)	4e-27
AD1195	*B. subtilis* YjbH protein homolog lmo0964	*L. mono-cytogenes EGD-e*	28 % (73/253)	2e-26
NP_655103.1	Hypothetical protein	*B. anthracis*	26 % (76/288)	5e-23

Figure A.2.15b

JLA526 (SH1000 *hla::lacZ hla*$^{+}$ *aht*12 (*yjbH*))

Identity = 99 % (207/210)

```
Query:     210 ACAGTCACAAATCATTGATTCTGTAATAATATCTCTTTTAGGTATGATTTCATTTTGCAT 151
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1015788 ACAGTCACAAATCATTGATTCTGTAATAATATCTCTTTTAGGTATGATTTCATTTTGCAT 1015847

Query:     150 TAAATGTATAAATCGTTCN-CACGTACACGACCTTGTAACTCAGCTGCTTTATAAGCTAG 92
               |||||||||||||||||  ||||||||||||||||||||||||||||||||||||||
Sbjct: 1015848 TAAATGTATAAATCGTTCGACACGTACACGACCTTGTAACTCAGCTGCTTTATAAGCTAG 1015907

Query:      91 GNCGATGTTATCAAAGTTGGATGTACTTTGAGCTTGGCATTTCGTTAANACTTTTAACGA 32
               | ||||||||||||||||||||||||||||||||||||||||||| ||||||||||||
Sbjct: 1015908 GGCGATGTTATCAAAGTTGGATGTACTTTGAGCTTGGCATTTCGTTAATACTTTTAACGA 1015967
```

Tn*551*

```
Query:      31 AGGATTTAATATATGTCTGATACGTATATAT 1
               ||||||||||||||||||||||||||||||
Sbjct: 1015968 AGGATTTAATATATGTCTGATACGTATATAT 1015998
```

Tn*917* was found to have inserted into the same base in JLA527 (SH1000 *hla::lacZ hla*$^{+}$ *aht*13 (*yjbH*)) as in JLA526 (SH1000 *hla::lacZ hla*$^{+}$ *aht*12 (*yjbH*)). The gene inactivated, SA1006, is the same as that inactivated in JLA524 (SH1000 *hla::lacZ hla*$^{+}$ *aht*10 (*yjbH*)).

The transposon insertion was in SA1006. BLASTP hits against the protein sequence of SA1006 are shown in Figure A.2.15a.

Figure A.2.16

JLA528 (SH1000 *hla::lacZ hla*$^+$ *aht*14 *(agrB)*)

Identity = 98 % (222/225)

```
Query:     225 GAAATAACTTAGATCATATTCAATTTTTGCAAGTACGATTAGGGATGCAGGTCTTAGCTA 166
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 2083190 GAAATAACTTAGATCATATTCAATTTTTGCAAGTACGATTAGGGATGCAGGTCTTAGCTA 2083249

Query:     165 AAAATATAGGTAAATTAATTGTTATGTATACTATTGCCTATATTTTAAACATTTTTCTGT 106
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 2083250 AAAATATAGGTAAATTAATTGTTATGTATACTATTGCCTATATTTTAAACATTTTTCTGT 2083309

Query:     105 TTACGTTAATTACGAATTTAACATTTTATTTAATAAGAAGACATGNACATGGTGCACATN 46
               |||||||||||||||||||||||||||||||||||||||||||| ||||||||||||
Sbjct: 2083310 TTACGTTAATTACGAATTTAACATTTTATTTAATAAGAAGACATGCACATGGTGCACATG 2083369
```

Tn*551*

```
Query:      45 CACCTTCNTCTTTTTGGTGTTATGTAGAAAGTATTATACTATTTA 1
               ||||||| ||||||||||||||||||||||||||||||||||||
Sbjct: 2083370 CACCTTCTTCTTTTTGGTGTTATGTAGAAAGTATTATACTATTTA 2083414
```

Tn*917* was found to have inserted into the same base in JLA529 (SH1000 *hla::lacZ hla*$^+$ *aht*15 *(agrB)*), JLA530 (SH1000 *hla::lacZ hla*$^+$ *aht*16 *(agrB)*) and JLA531 (SH1000 *hla::lacZ hla*$^+$ *aht*17 *(agrB)*) as in JLA528 (SH1000 *hla::lacZ hla*$^+$ *aht*14 *(agrB)*).

The transposon insertion was in SA2023. BLASTP hits against the protein sequence of SA2023 are shown in the table below.

Accession no.	Protein	Organism	Identity	Expect
P21545	Accessory gene regulator protein AgrB	*S. aureus* 8325-4	99 % (188/189)	1e-77
AF173933_1	Accessory gene regulator protein AgrB	*S. lugdunensis*	50 % (101/202)	4e-45
CAA89193.1	Accessory gene regulator protein AgrB	*S. epidermidis*	52 % (99/188)	3e-39
AF346718_1	Accessory gene regulator protein AgrB	*S. caprae*	43 % (47/109)	3e-15
NP_346723.1	Accessory gene regulator protein B	*C. acetobutylicum*	27 % (47/171)	3e-08

Figure A.2.17

JLA532 (SH1000 *hla::lacZ hla*$^{+}$ *aht*18 (*saeR*))

Identity = 97 % (212/218)

The transposon insertion was in SA0766. BLASTP hits against the protein sequence of SA0766 are shown in the table below.

Accession no.	Protein	Organism	Identity	Expect
NP_373916.1	Response regulator SaeR	*S. aureus*	100 % (228/228)	e-119
AE014209_12	DNA-binding response regulator	*S. agalactiae* 2603V/R	46 % (104/224)	3e-49
NP_344631.1	DNA-binding response regulator	*S. pneumoniae*	46 % (105/227)	5e-49
AF430807_10	VanRE	*E. faecalis*	39 % (91/230)	2e-41
NP_655198.1	REC, cheY-homologous receiver domain	*B. anthracis* A2012	41 % (92/223)	6e-41

Figure A.2.18

JLA542 (SH1000 *hla::lacZ hla*$^{+}$ *htn*10 (*lysC*))

Identity = 98 % (222/226)

```
Query:     226 GAACAATTAATTGTCGTTGTAAGTGCTATGGGTAACACAACAGATCAATTAATGACGAAT 167
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1440703 GAACAATTAATTGTCGTTGTAAGTGCTATGGGTAACACAACAGATCAATTAATGACGAAT 1440762

Query:     166 GTATCAACCTTGACTAAAGCACCAAAACAACAAGAACTGGCATTATTATTGACAACCGGA 107
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1440763 GTATCAACCTTGACTAAAGCACCAAAACAACAAGAACTGGCATTATTATTGACAACCGGA 1440822

Query:     106 GAGCAACAAACTGTATCTTATTTATCAATGGTATTAAATGATATCGGNATGAATGCCANN 47
               |||||||||||||||||||||||||||||||||||||||||||| ||||||||||
Sbjct: 1440823 GAGCAACAAACTGTATCTTATTTATCAATGGTATTAAATGATATCGGTATGAATGCCAAA 1440882
```

Tn*551*

```
Query:      46 GCAATGACTGGCTATCAAGCGGGTATTAAAACCATTGGCNATCATT 1
               |||||||||||||||||||||||||||||||||||| ||||||
Sbjct: 1440883 GCAATGACTGGCTATCAAGCGGGTATTAAAACCATTGGCCATCATT 1440928
```

The transposon insertion was in SA1428. BLASTP hits against the protein sequence of SA1428 are shown in the table below.

Accession no.	Protein	Organism	Identity	Expect
AF306669_1	Aspartokinase II LysC	*S. aureus* 8325-4	99 % (400/401)	0.0
NP_229318.1	Aspartokinase II LysC	*T. maritima*	40 % (159/397)	3e-81
NP_464760.1	Aspartokinase II alpha subunit	*L. monocytogenes*	41 % (167/398)	1e-73
NP_419660.1	Aspartokinase	*C. crescentus* CB15	38 % (159/416)	1e-69

Figure A.2.19

JLA549 (SH1000 *hla::lacZ hla*$^{+}$ *htn*17 (*hyp*1))

Identity = 100 % (214/214)

```
Query:     214 CTAAGTAATAACGTTGATAAAATAATGCAAAATAATCCACCAATAATGCCGGCAATAATA 273
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1466872 CTAAGTAATAACGTTGATAAAATAATGCAAAATAATCCACCAATAATGCCGGCAATAATA 1466931

Query:     274 TCTGTTGGATAATGTACACCTAGATATACACGTGATATGGAAATCAATAAAATCATAGCT 333
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1466932 TCTGTTGGATAATGTACACCTAGATATACACGTGATATGGAAATCAATAAAATCATAGCT 1466991

Query:     334 GCACATAACCCTATAAGAATACCTTTTGAATTACCTTGATTTAATCGATTTAATAGATAG 393
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1466992 GCACATAACCCTATAAGAATACCTTTTGAATTACCTTGATTTAATCGATTTAATAGATAG 1467051

Query:     394 ATACCACTTCCAAAATATGCAGTTGATCCCATAGCATGACCGCTAGGAAAACTAAATCCT 453
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1467052 ATACCACTTCCAAAATATGCAGTTGATCCCATAGCATGACCGCTAGGAAAACTAAATCCT 1467111
```

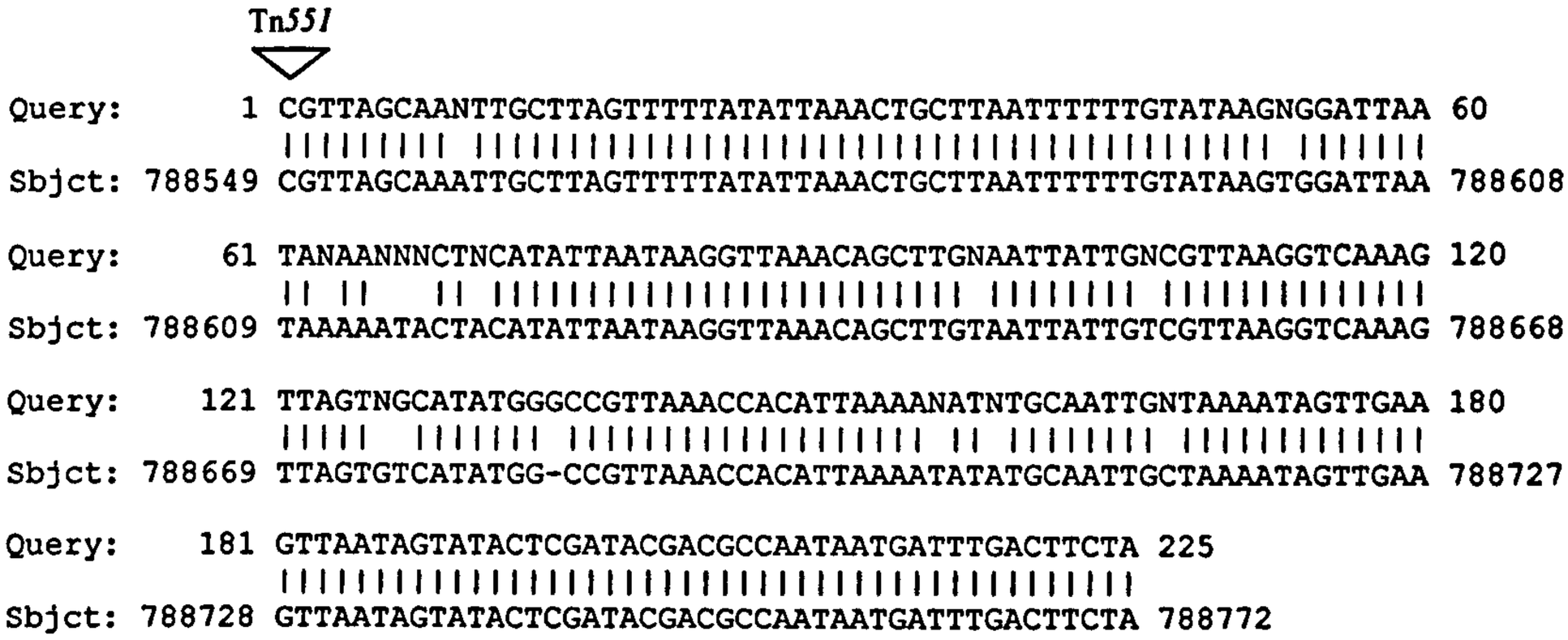
BLASTX hits against the DNA sequence flanking the transposon insertion are shown in the table below.

Accession no.	Protein	Organism	Identity	Expect
NP_371940.1	Hypothetical protein	*S. aureus* Mu50	100 % (57/57)	5e-30
NP_374530.1	Hypothetical protein	*S. aureus* N315	100 % (57/57)	5e-30

Figure A.2.20

JLA550 (SH1000 *hla::lacZ hla*$^+$ *htn*18 (*saeS*))

Identity = 93 % (210/225)



Tn*917* was found to have inserted into the same base in JLA551 (SH1000 *hla::lacZ hla*$^+$ *htn*19 (*saeS*)) as in JLA550 (SH1000 *hla::lacZ hla*$^+$ *htn*18 (*saeS*)).

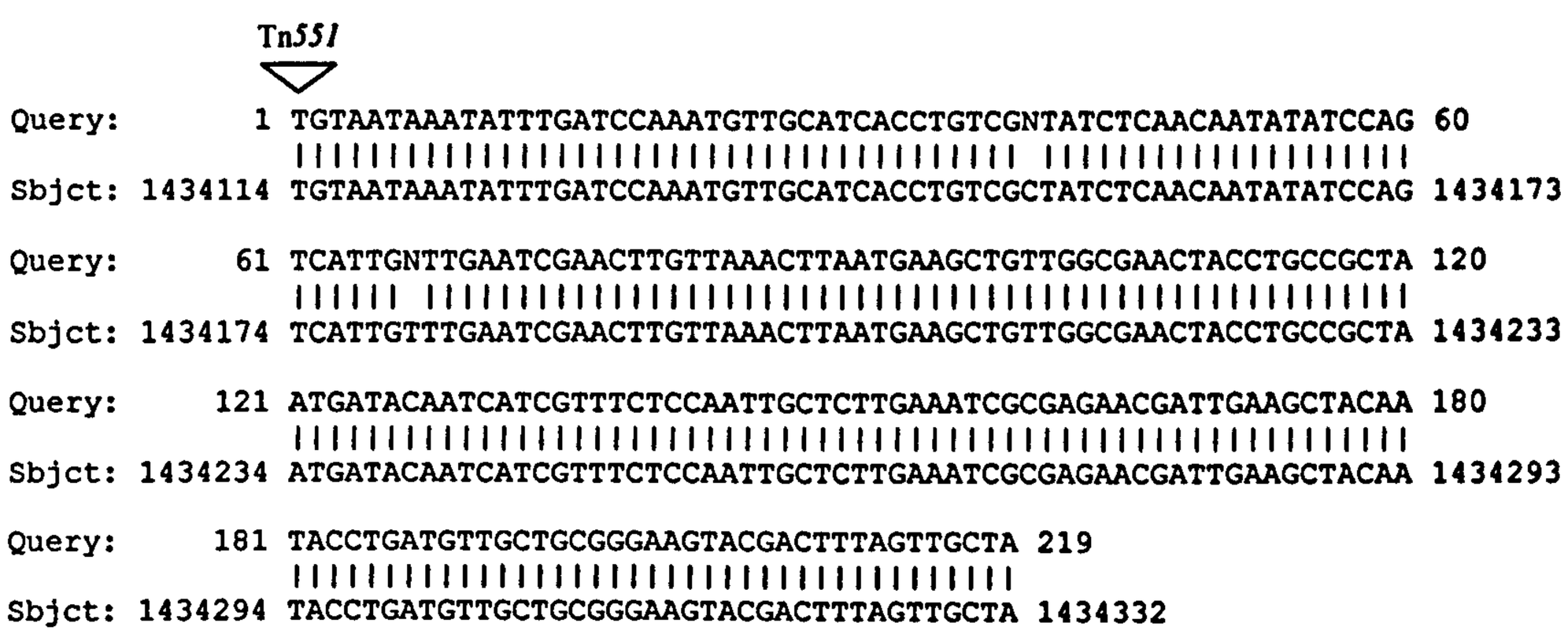
The transposon insertion was in SA0765. BLASTP hits against the protein sequence of SA0765 are shown in the table below.

Accession no.	Protein	Organism	Identity	Expect
AF129010_2	Histidine protein kinase SaeS	*S. aureus* Newman	89 % (316/353)	e-147
NP_344632.1	Sensor histidine kinase	*S. pneumoniae*	33 % (117/347)	9e-42
NP_687428.1	Sensor histidine kinase	*S. agalactiae* 2603V/R	33 % (115/348)	2e-39
NP_624089.1	Sensory transduction histidine kinases	*T. tengcongensis*	29 % (97/331)	1e-30
A27650	Phosphate response regulator histidine kinase PhoR	*B. subtilis*	30 % (72/239)	2e-25

Figure A.2.21

***htn*21 (SH1000 *hla::lacZ hla*$^{+}$ *htn*21 (*pstC*))**

Identity = 99 % (217/219)



The transposon insertion was in SA1423. BLASTP hits against the protein sequence of SA1423 are shown in the table below.

Accession no.	Protein	Organism	Identity	Expect
NP_279517.1	Phosphate ABC transporter permease PstC	*Halobacterium* sp. NRC-1	50 % (127/253)	1e-53
BAC00817.1	Phosphate transport system permease ABC transporter protein	*R. sphaeroides*	46 % (120/257)	2e-49
NP_070186.1	Phosphate ABC transporter, permease protein PstC	*A. fulgidus*	47 % (119/251)	6e-49
BAC09717.1	Permease protein of phosphate ABC transporter	*T. elongatus* BP-1	42 % (113/264)	2e-47
NP_540905.1	Phosphate transport system permease protein PstC	*B. melitensis*	44 % 116/260 (116/260)	5e-46