An Investigation into the Function and Regulation of

GAPDH in *Staphylococcus aureus*

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by

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Abstract

Staphylococcus aureus is an important pathogen of both humans and animals. With the growing spread of MRSA strains in clinical environments and the wider community, it is imperative that we understand the basic physiology of this species to find new antimicrobial or vaccine targets.

This project focuses on the function and regulation of glyceraldehyde-3-phosphate dehydrogenase, or GAPDH, an essential component of glucose metabolism. *S. aureus* contains two GAPDH homologues; GapA, a known GAPDH protein and GapB, the function of which is undefined. Using a number of complementary approaches we have shown that GapA and GapB have reciprocal functions during glucose metabolism, and that both homologues are required during infection. We have also identified novel "moonlighting" roles for both GapA and GapB and shown that both genes are regulated by divalent metal ions. Interestingly iron regulation of *gapA* is strain variable at the transcriptional level due to sequence variation between strains, and appears to involve a *S. aureus* repeat (STAR) element locus. To our knowledge this is the first indication that these repeat elements are functional.

STAR elements are found across the *S. aureus* genome, and the number of repeats at each locus is variable between strains. Surprisingly the three STAR loci (*gapR*, *hprK* and orf_{0733}) analysed are highly conserved and maintained in the *S. aureus* genome at a level similar to that of MLST loci which suggests that they may have an important function, although transcriptional analysis failed to identify a correlation between repeat number and transcript levels. However transcriptional analysis did demonstrate that a number of STAR and non-STAR associated loci in *S. aureus,* including housekeeping genes involved in central metabolism, known transcriptional regulators and virulence factors show strain variable levels of expression, which may play a role in the significant adaptability of *S. aureus*.

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List of abbreviations

°C	degrees Celsius
ADP	adenosine diphosphate
Amp	Ampicillin
APS	ammonium persulphate
ATP	adenosine triphosphate
bp	base pair
CA-MRSA	community acquired MRSA
CC	clonal complex
CCR	carbon catabolite repression
cDNA	complementary DNA
cm	centimetres
Cm	chloramphenicol
CRISPR	clustered regularly interspersed short palidromic repeats
CRPMI	chelexed RPMI (medium)
CTAB	cetyl trimethyl ammonium bromide
Cu	copper
CV	core variable genes
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
ddH_2O	double distilled H_2O
DEPC	diethyl-pyrocarbonate
dH_2O	distilled H_2O
DNA	deoxyribonucleic acid
dNTPs	deoxyribonuleotide triphosphates
EDTA	ethylenediaminetetraacetic acid
EMRSA	epidemic MRSA
ERIC	enterobacterial repetitive intergenic consensus
Ery	erythromycin
FBP	fructose-1,6-bisphosphate
Fe	iron
g	gram
G3P	glyceraldehyde-3-phosphate
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HA-MRSA	healthcare associated MRSA
IAA	isoamyl alcohol
IgG	Immunoglobulin G
IPTG	isopropyl β-D-1-thiogalactopyranoside
Kan	kanamycin
kb	kilobase
kDa	kilodalton
l	litre
LuA	Luria Bertani agar
LuB	Luria Bertani broth
M	molar
mA	milliamps
MALDI-TOF	Matrix Assisted Laser Desorption Ionisation – time-of-flight
Mbp	megabase

mg	milligram
MGE	mobile genetic elements
min	minute
ml	millilitre
MLST	multi locus sequence typing
mM	millimolar
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	messenger RNA
MRSA	methicillin resistant *Staphylococcus aureus*
MSCRAMM	microbial surface components recognizing adhesive matrix molecules
NAD(H)	nicotinamide adenine dinucleotide (reduced)
NADP(H)	nicotinamide adenine dinucleotide phosphate (reduced)
ng	nanogram
nm	nanometer
OD	optical density
PAGE	poly-acrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PNACL	Protein and Nucleic Acid Chemistry Laboratory
PNAG	polymeric N-acetylglucosamine
pSI	pounds per square inch
PSM	Phenol-soluble modulin
PVL	Panton-Valentine leukocidin toxin
RBS	ribosomal binding site
REP	repetitive extragenic palindrome
RNA	ribonucleic acid
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute – 1640 (medium)
rRNA	ribosomal RNA
SCCmec	Staphylococcal chromosomal cassette containing *mecA*
SDS	sodium dodecyl sulphate
SMM	supplemented minimal medium (buffer)
SMMP50	supplemented minimal medium Penassay 50 (medium)
SOC	Super optimal broth with catabolite repression (medium)
Spc	spectinomycin
SSC	sodium chloride – sodium citrate (buffer)
SSD	Staphylococcal siderophore detection (medium)
ST	sequence type
STAR	*Staphylococcus aureus* Repeat
TAE	Tris base, acetic acid and EDTA containing buffer
TCA cycle	tricarboxylic acid cycle (Krebs cycle)
TEMED	N,N,N',N' - tetramethylethylenediamine
Tet	tetracycline
TIR	terminal inverted repeats
TM	Tris minimal (medium)
TSB	trypticase soy broth (medium)
TSM	Tris succinate minimal (medium)
TSST-1	toxic shoch syndrome toxin - 1
U	unit
UV	ultraviolet

V	volt
v/v	volume/volume
VISA	vancomycin intermediate-resistant *Staphylococcus aureus*
VRSA	vancomycin resistant *Staphylococcus aureus*
w/v	weight/volume
x g	times gravity
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactosidase
µg	microgram
µl	microlitre
µM	micromolar

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Chapter 1 Introduction

The aim of this project is to investigate both the regulation and function of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) homologues *gapA* and *gapB* in the pathogenic organism *Staphylococcus aureus*. This introduction will initially outline why *S. aureus* is such a successful pathogen, the evolution of the *S. aureus* genome and how this bacterium is able to adapt to changes in its environment. The remainder of the introduction will describe the importance of GAPDH proteins, their primary function in metabolism and also some additional functions that have been described in different eukaryotic and prokaryotic species.

1.1 *Staphylococcus aureus*

Staphylococcus aureus are coccus shaped, facultative anaerobes approximately 1μm in diameter, and grow in irregular clusters into yellow pigmented colonies. Many staphylococcal species, including *S. aureus, S. epidermidis, S. xylosus, S. warneri, S. hemolyticus*, and *S. saprophyticus* are common commensals of humans and animals, but only *S. aureus* and *S. epidermidis* regularly lead to infection and disease. *S. aureus* are often seen as a model Gram positive pathogen, and due to their clinical importance (see below) are one of the most extensively studied bacteria species after the Gram negative model organism *Escherichia coli* (Figure 1-1). *S. aureus* is a highly adaptable species that is extremely tolerant to environmental stress (Lowy, 1998; Plata *et al*., 2009). *S. aureus* are also one of a number of bacterial species which can form Small Colony Variants (SCV's). SCV's have a reduced growth rate and abnormal colony morphology and have been shown to be more resistant to antibiotics than wild type *S. aureus* cells (Proctor *et al*., 2006). Furthermore SCV's are able to persist asymptomatically in the host, and cause recurrent infections at a later date (Proctor *et al.,* 1995).

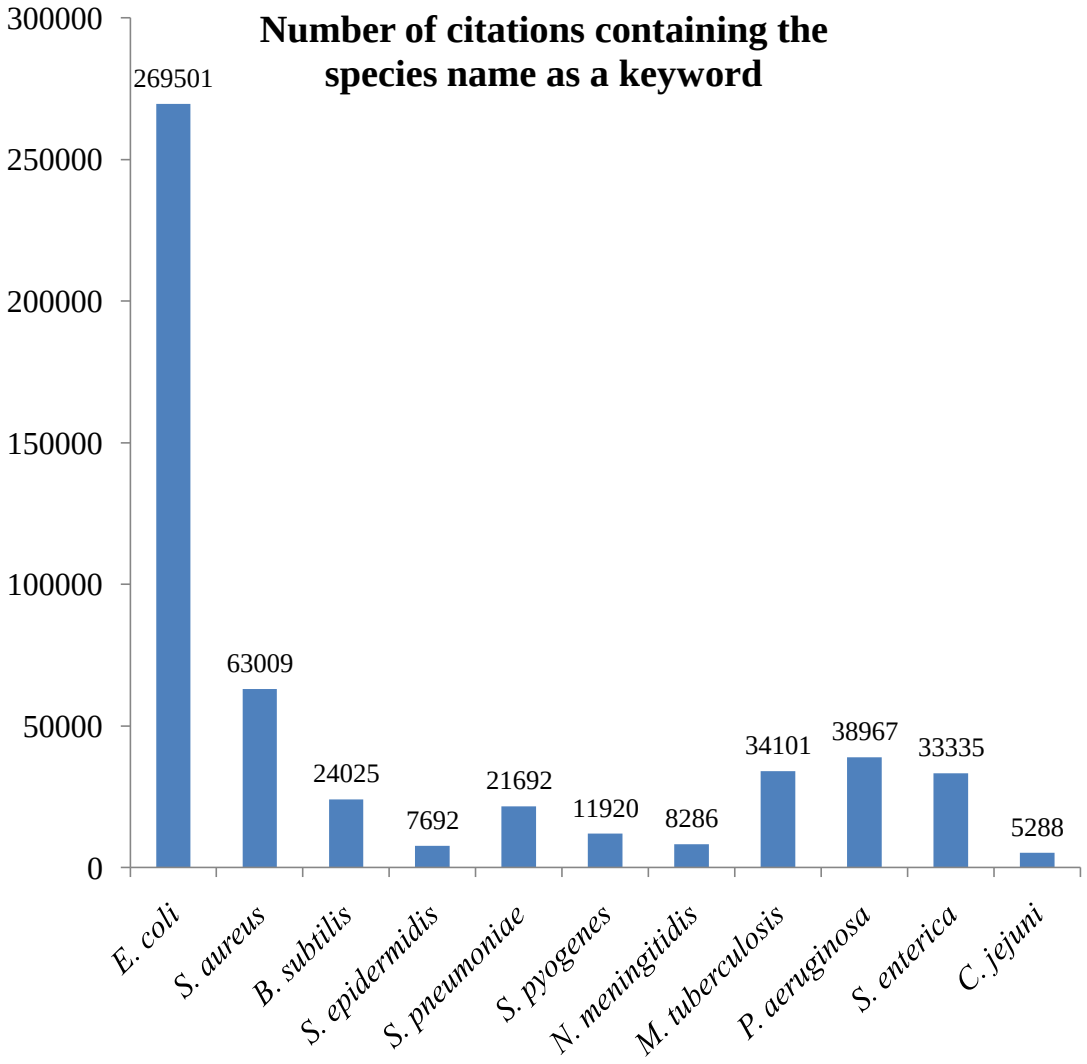


Figure 1-1. Graph showing the number of publications referring to each bacterial species between 1950 – June 2010.

Each organism name was used in a keyword search of the OVID Medline database (1950 – 4th week June 2010) to determine the number of publications in which each species is referred. After *E. coli*, the most well studied of these important bacteria species is *S. aureus* by almost 25000 publications. The gross overrepresentation of articles pertaining to *E. coli* is likely due to the use of this organism not only as a model organism but also as a common species for the cloning of DNA for many practical applications in other biological fields.

1.2 *S. aureus* as a Pathogen

S. aureus often asymptomatically colonise the skin and nasal pharynx of healthy individuals, and it is estimated that 20-30% of the human population are permanently colonised by this bacterium, with a further 50% of the population acting as intermittent carriers (Plata *et al.*, 2009). Generally *S. aureus* are tolerated by the host unless the natural balance is disturbed, at which point they can become a very successful opportunistic pathogen and cause a wide range of diseases (Figure 1-2). Depending on the site of infection and the susceptibility of the patient, these can range from mild skin and soft tissue infections such as boils, to life threatening conditions such as septicaemia and endocarditis (Lowy, 1998; Wertheim *et al*., 2005; Gordon & Lowy, 2008). In a healthy individual, superficial acute infections will often be successfully dealt with by the host immune system without further treatment. However systemic infections do occur, generally in patients who are otherwise immunocompromised, and if left untreated are likely to be life threatening. Treatment for *S. aureus* infection generally involves β-lactam antibiotics, but antibiotic resistances have become a widespread issue in dealing with *S. aureus* disease.

1.3 The MRSA problem

The widespread use of antibiotics has resulted in the emergence of antibiotic resistant strains of *S. aureus*, the most notable being Methicillin Resistant *S. aureus*, or MRSA. Not long after the introduction of the β-lactam antibiotic methicillin into clinical practice in the 1960's, *S. aureus* isolates were discovered that had developed resistance to the drug (Jevons *et al.*, 1963). The mode of action of β-lactam antibiotics is to inhibit cell wall synthesis by binding to transpeptidase (a.k.a. penicillin binding protein PBP), the enzyme responsible for cross linking petidoglycan chains in the cell wall

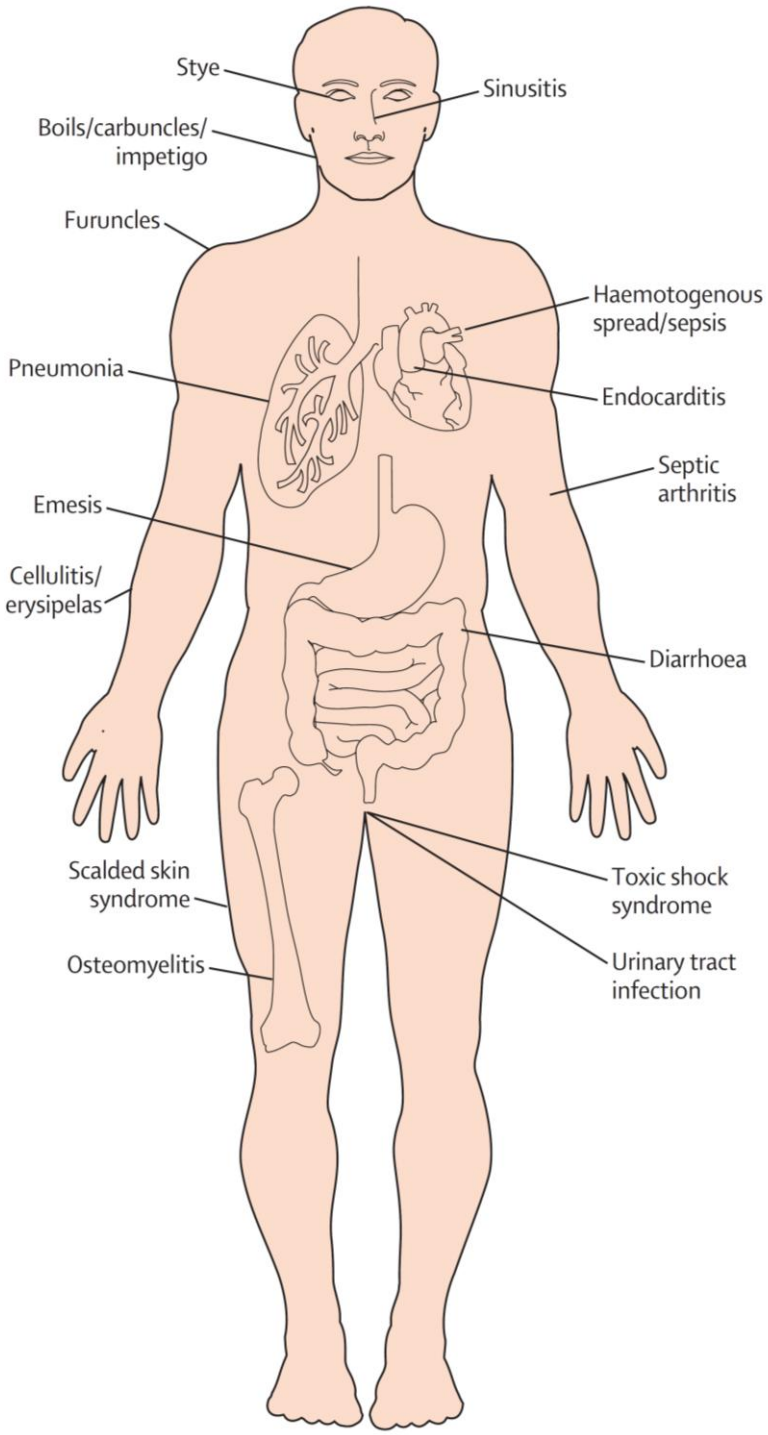


Figure 1-2. Diseases caused by *S. aureus*.

This diagram highlights a range of diseases caused by *S. aureus* infection at many different sites in the human body. The diseases included are representative of the diversity of *S. aureus* disease but are by no means an exhaustive list. Figure taken directly from (Wertheim *et al.*, 2005).

(Llarrull *et al*., 2009). Methicillin resistance is conferred by the *mecA* gene, which encodes a novel penicillin binding protein (PBP)-2', which has a reduced affinity for β-lactam antibiotics and is therefore able to remain active in the presence of methicillin (Hartman & Tomasz, 1984; Enright, 2003). The *mecA* gene is located on a mobile genetic element known as the *Staphylococcal cassette chromosome* (*SCCmec*) which has been shown to be transferred between *S. aureus* strains through horizontal gene transfer (Ito *et al*., 1999; Wielders *et al*., 2001). The element always integrates into a unique site, known as *attBssc* located within a gene of unknown function, by a similar mechanism of transfer as transposons (Ito *et al*., 1999; Plata *et al*., 2009). At least 8 *SCCmec* types have been identified; varying between 20 and 58 kb in length, and these may or may not contain additional antibiotic resistance genes (Plata *et al*., 2009).

1.3.1 Healthcare associated MRSA (HA-MRSA)

The opportunistic nature of *S. aureus* makes it a very important nosocomial, or healthcare associated pathogen, causing clinical disease in up to 2% of all patient admissions (Lindsay & Holden, 2004). While generally methicillin sensitive *S. aureus* (MSSA) infections still outnumber MRSA infections, with only 24.4% of *S. aureus* bacteraemia cases in England, Wales and Northern Ireland testing positive for methicillin resistance in 2008 (HPA report, 2008), HA-MRSA is a major burden on hospitals and care facilities across the developed world. Currently there is no conclusive evidence that MRSA are more virulent than their MSSA counterparts (Gordon & Lowy, 2008), but due to the difficulty in providing effective treatment MRSA infections may be more prolonged and require more extensive treatment.

1.3.2 Community-acquired MRSA (CA-MRSA)

Initially MRSA strains were confined to a healthcare setting, with MRSA infection causing serious disease predominantly in patients with high susceptibility, such as the very old or young, and immunocompromised patients. However since the late 1990's CA-MRSA infections have been seen to arise even though there is no link to the hospital setting and HA-MRSA strains (Witte, 2009). CA-MRSA generally appear to be more virulent than HA-MRSA, causing disease in otherwise healthy individuals. While often associated with skin and soft tissue infections, CA-MRSA can cause invasive life threatening diseases, such as necrotising pneumonia and severe sepsis (Klein *et al*., 2003; Witte, 2009). This is a major cause for concern as these strains may be more likely to spread within the general population, and the diseases caused are often very quick to take hold reducing the time available to administer effective treatment. Although the highly virulent toxin Panton-Valentine Leukocidin is often associated with CA-MRSA infection there are no definitive genetic markers for CA-MRSA strains (Feil *et al*., 2003; Lindsay *et al*., 2006; Voyich *et al*., 2006; Witte, 2009). The prevalence of CA-MRSA appears to be on the increase, but as they are now circulating in both the community and the hospital environment they are becoming more difficult to define. In the UK CA-MRSA rates increased year on year between 2000 and 2006 (Otter & French, 2008).

1.3.3 Vancomycin Resistance

The preferred method of treatment for MRSA infection is the antibiotic vancomycin, but in 2002 the first clinical vancomycin-resistant *S. aureus* (VRSA) strains were isolated in the USA (Anon, 2002a; Anon, 2002b). The VRSA phenotype is acquired through transfer of the *vanA* gene on transposon *Tn*1546, which originated in

enterococcal species (Perichon & Courvalin, 2009). There has also been an emergence of vancomycin-intermediate resistant *S. aureus* (VISA), such as the sequenced strains Mu3 and Mu50 isolated in Japan, which have an increased tolerance to vancomycin (Kuroda *et al*., 2001; Neoh *et al*., 2008). Very little is known about the acquisition of this phenotype, although whole genome comparison of *S. aureus* isolates from a single patient before and after the development of the VISA phenotype only identified 35 different point mutations, indicating that the development of resistance to vancomycin requires very little genetic change (Mwangi *et al*., 2007). Treatment options for MRSA and VRSA strains are now very limited, so new treatment or prevention methods need to be found. This is why research into the basic metabolic functions of *S. aureus* is so important, as it may lead to new targets for drugs or methods to prevent colonisation or the development of disease.

1.4 Evolution of the *S. aureus* Genome

1.4.1 Genomic arrangement of *S. aureus*

Due to the significance of *S. aureus* as a pathogen, the genomes of many clinically important strains have now been sequenced. To date the genomes of 17 *S. aureus* isolates have been fully sequenced and annotated, with over 70 more genome projects currently being undertaken (Entrez Genome Project database, NCBI). The completed *S. aureus* genomes provide a very useful tool in investigating the relationship between genetic content and virulence potential of this pathogen, as well as providing insight into its evolution. From the current data we see that the *S. aureus* genome varies between 2.74 and 2.9 Mbp in different strains, and the GC content of this species is ~33%. Comparative analysis of the sequenced *S. aureus* strains has shown that the genome is comprised of three types of sequence; the core genome, the core variable

(CV) genome and Mobile Genetic Elements (MGE's) (Lindsay *et al*., 2006; Lindsay, 2008). Approximately 38% of the proposed coding sequence in *S. aureus* currently has no known function, leaving much of *S. aureus* physiology still to be explored.

Core genes are present in 95% of *S. aureus* isolates and are highly conserved between *S. aureus* strains, usually only varying by single nucleotide polymorphisms (SNPs). They make up approximately 70% of the genome and generally encode housekeeping genes such as those involved in metabolism, DNA replication and other essential processes (Lindsay & Holden, 2006). CV genes account for between 10-12% of the genome and generally encode proteins or structures displayed on the cell surface, or regulatory proteins which control the expression of virulence factors, all of which are considered to be non-essential (Lindsay *et al*., 2006). Different *S. aureus* lineages do not have unique CV genes but unique combinations of genes, altering their specific capabilities to inhabit certain niches or cause a particular disease (Lindsay *et al*., 2006; Holden & Lindsay, 2008). MGEs are self replicating regions of DNA which can integrate into the chromosome (e.g. transposons and bacteriophage), or perform autonomous replication (e.g. plasmids). MGEs generally encode specific virulence factors and resistance genes, and their presence is highly variable between strains, even those belonging to the same lineage (Lindsay *et al*., 2006; Lindsay, 2008; Moore & Lindsay, 2001). Horizontal gene transfer primarily occurs via phage transduction in *S. aureus*, although the transfer of plasmids and transposons can occur via conjugation. However *S. aureus* do not encode the proteins required for competence and uptake via natural transformation like some bacterial species (Lindsay, 2008).

CV genes and MGEs are often referred to as the accessory genome, and provide a large amount of genetic diversity between different *S. aureus* isolates (Lindsay & Holden, 2004; Holden *et al*., 2004). The type and severity of *S. aureus* infection is largely dependent on the strain involved, as the combination of accessory genetic elements has a huge impact on its ability to invade a host and cause disease.

1.4.2 Interspersed repeats in *S. aureus* - STAR elements

Genomic variation in non-coding sequence is also important in genome evolution, as it provides sequence diversity and genome plasticity. While repetitive DNA makes up a large proportion of eukaryotic genomes, greater than 50% in the case of humans (Richard *et al*., 2008), prokaryotic genomes contain relatively little repetitive DNA in relation to their coding sequence (Ussery *et al*., 2004). Many eukaryotic repeats are considered "selfish" DNA as they have no known function, while the function of prokaryotic repeats is often much clearer. One reason for this may be that the non-functional DNA is a metabolic burden on rapidly dividing cells, resulting in selective pressure limiting its presence in the genome (Doolittle & Sapienza, 1980).

There are a number of different types of bacterial repeats, including simple sequence repeats, direct and inverted repeats and interspersed repeats (Table 1.1), many of which have been shown to be functional. A novel repeat element has been identified in *S. aureus*, although currently there is no evidence of these elements having any function. *Staphylococcus aureus* Repeat (STAR) elements are short GC rich direct repeats found in intergenic regions across the *S. aureus* genome (Cramton *et al*., 2000). They consist of 14 bp direct repeats of the consensus sequence T(G/A/T)TGTTG(G/T)GGCCC(C/A) interspersed with at least 40bp of recurring sequences (Cramton *et al*., 2000).

Table 1.1. Prokaryotic Repetitive DNA sequences and their function.

Repetitive Element	Species present (e.g.)	Specific example of function
Simple Repeats		
Mononucleotide repeats	*Neisseria meningitidis*	Poly-G tract in *porA* promoter results in variation in promoter strength by altering the spacing between the -35 and -10 boxes (van der Ende *et al*., 2000).
SSR (short direct repeat sequences)	*Haemophilus influenzae*	TA repeat between divergently transcribed open reading frames *hifA* and *hifB* alters spacing of -35 and -10 boxes, resulting in variability of their transcription levels (van Ham *et al*., 1993).
	Nessieria meningitidis	CTCTT repeat in *opa* leader peptide shifts translational frame of resulting protein, switching it on/off (Stern *et al*., 1986).
	Mycoplasma genitalium	SSR tracts in coding sequence of lipoproteins *vlpA, vlpB* and *vlpC* provide variation in protein external domains (Yogev *et al*., 1991).
MITEs		
ERIC sequences	Several enterobacterial species inc. *E. coli*.	Co-transcribed with upstream or downstream genes, regulate gene expression through altering transcript stability through conformational changes in RNA structure changing the exposure of RNaseE cleavage sites in the sequence (Hulton *et al*., 1991; De Gregorio *et al*., 2005).
NEMIS sequences	*Nesseria* species	Alter gene expression through transcript stability. TIRs form stable stem loop structure when co-transcribed with adjacent genes, which are either cleaved by or protected from RNase III (De Gregorio *et al*., 2002; De Gregorio *et al*., 2003).
Other Interspersed Repeats		
CRISPR elements	146 species to date, inc. Gram (+ve) and (–ve) spp.	Intergenic sequence of spacers derived from extra-chromosomal DNA, providing immunity from that type of element. Most likely act through expression of anti-sense RNA in a proposed RNA interference mechanism (Pourcel *et al*., 2005; Bolotin *et al*., 2005; Mojica *et al*., 2005).
REP sequences	*E. coli*, *S. typhimurium*	Regulate gene expression through altering transcript stability (Stern *et al*., 1984; Stern *et al*., 1988).
BOX elements	Streptococcal species	Co-transcribed with neighbouring genes. Modulate gene expression by forming stem loops, altering transcript stability. Orientation and composition of repeats determines whether gene is upregulated or downregulated, and the strength of this response (Martin *et al*., 1992; Knutsen *et al*., 2006).

Definitions: Short sequence repeats (SSR), Miniature Inverted repeat Transposable elements (MITEs), Enterobacterial repetitive intergenic consensus (ERIC), *Neisseria* miniature insertion sequences (NEMIS), Clustered Regularly Interspersed Short Palindromic Repeats (CRISPR), Repetitive extragenic palindrome (REP), terminal inverted repeats (TIR).

STAR elements are similar to Clustered Regularly Interspersed Short Palindromic Repeats (CRIPSR) elements, which are found in at least 148 different bacterial species (Ishino *et al*., 1987; Mojica *et al*., 2000; Godde & Bickerton, 2006) and have been shown to provide immunity to the bacterial cells from invasion by foreign DNA, possibly via RNA interference (Pourcel *et al*., 2005; Bolotin *et al*., 2005; Mojica *et al*., 2005). Although STAR elements are distinct from CRISPR elements and the *S. aureus* genome does not contain any CRISPR associated (*cas*) genes, it is possible that they perform a similar function.

STAR elements have been identified at distinct loci across all of the *S. aureus* sequenced genomes but the number of repeat units at each locus varies between strains. The mechanism of STAR element propagation is currently unknown, although it has been speculated that the repeats have been distributed throughout the genome via recombination events, possibly from a larger, hypothetical "master element" (Cramton *et al*., 2000). The maintenance of STAR elements at so many loci in the *S. aureus* genome suggest that they are more than just "selfish" DNA sequence, and have an as yet undiscovered function in the bacterial cell.

1.4.3 Tracking the evolution of *S. aureus* strains

The evolution of *S. aureus* is under the control of two opposing forces; rapid evolution through horizontal gene transfer of accessory elements, coupled with the need to retain the conserved core sequence (Lindsay & Holden, 2004). Sequence variation across the genome provides a means of tracking *S. aureus* strains both during acute outbreaks and over long term global dissemination. While short term strain differentiation is important to determine outbreak strains from non-outbreak isolates, information on the long term

evolution is important in studying the emergence and spread of *S. aureus* strains in widespread population studies (Enright, 2008).

Multi locus sequence typing (MLST) is now the standard typing scheme for tracking long term evolutionary relationships between *S. aureus* strains (Maiden *et al*., 1998; Enright *et al*., 2000). This method is based on the principle of nucleotide sequence differentiation of 7 conserved housekeeping genes. As these genes are highly conserved, strains with the same allelic profile must be closely related (Maiden *et al*., 1998; Enright *et al*., 2000). The sequencing data is easily transferable and there is now a global MLST database; an invaluable tool in tracking *S. aureus* population structure, global dissemination and evolution of *S. aureus* strains (http://saureus.mlst.net/).

MLST analysis has been useful in determining the emergence of particular HA-MRSA strains. It has been demonstrated that the majority of HA-MRSA strains are derived from 5 major clones, which have disseminated internationally (Gordon & Lowy, 2008). In the UK the two major clonal types are EMRSA-15 and EMRSA-16, although the reason for the dominance of these strains over other MSSA and MRSA strains is unclear (Moore & Lindsay, 2002; Holden *et al*., 2004). CA-MRSA strains do not appear to be restricted to specific lineages and there is currently no evidence that there are hyper virulent *S. aureus* lineages, as seen in *Neisseira meningitidis* or *Streptococcus pneumoniae*. Furthermore there does not appear to be any genetic marker or lineage particularly associated with *S. aureus* disease or carriage (Feil *et al*., 2003; Lindsay *et al*., 2006; Enright, 2008).

Unsurprisingly, strains with different host specificities tend to be separated into different evolutionary groups. Animal *S. aureus* isolates fall into 10 dominant lineages, with 61% of those studied falling into 4 ST's unique to animals (Sung *et al*., 2008). The genetic basis for host specificity is still unclear, but the distribution of a number of key virulence determinants in human isolates was found to be very different in animal isolates, including the absence of important surface adhesins involved in human colonisation (Sung *et al*., 2008).

1.4.4 *S. aureus* genetic transfer: the restriction enzyme barrier.

While genetic transfer between strains of different lineages is possible, transfer within lineages occurs at a much higher frequency (Waldron & Lindsay, 2006; Lindsay, 2010). This is due to lineage specific variation in the major restriction modification (RM) system known as SauI (Waldron & Lindsay, 2006; Cockfield *et al*., 2007). RM systems protect bacterial genomes from invasion by foreign DNA, and lineage specific RM variation means that *S. aureus* strains will recognise DNA from other lineages as "foreign" and limit transfer. The restriction enzyme subunit, encoded by *sauIhsdI*, is mutated in the laboratory strain RN4220, which has proved invaluable in cloning and genetic manipulation of *S. aureus* (Waldron & Lindsay, 2006). Interestingly this subunit is also mutated in the bovine mastitis lineage ST151, and this RM defect has resulted in this lineage being highly susceptible to resistance gene transfer from enterococci within an animal host (Sung & Lindsay, 2007). This is potentially a problem as it provides a means of resistance gene transfer, such as vancomycin resistance, from bovine enterococci isolates to *S. aureus* isolates (Sung & Lindsay, 2007). The RM barrier has been a key feature in the divergence of *S. aureus* strains as it provides a means of maintaining lineage specific variation.

1.5 Why are *S. aureus* so adaptable?

S. aureus are capable of causing such a wide range of diseases because the bacteria are so adaptable. They can inhabit a wide range of ecological niches, including most sites in the human body, and have the potential to cause disease within almost all host tissues. There are three features of *S. aureus* that allow them to be so versatile; genetic variation found between different strains of the bacteria (discussed above), the range of different virulence determinants they can produce, and the highly complex regulatory systems put in place to control the expression of their genes.

1.5.1 Virulence factors in *S. aureus*

S. aureus is capable of producing a wide range of different virulence factors (Table 1.2), although these vary dramatically between different *S. aureus* strains due to differences in the accessory genome. Virulence factors specifically improve the ability of a bacterium to infect a host, and can include specific toxins, iron-uptake systems, surface proteins, super-antigens and extracellular enzymes (Bronner *et al*., 2004; Gordon & Lowy, 2008; Plata *et al*., 2009). Table 1.2 outlines some key *S. aureus* virulence factors and how they contribute to pathogenesis. The roles that these virulence factors play are wide ranging, from initial adherence of the bacteria to the host cell, to defence against the immune system.

1.5.2 *S. aureus* can respond to a wide range of environmental stimuli

The ability to respond to changing environmental conditions is a key factor in the adaptability of any bacterium. *S. aureus* are particularly effective at this due to the wide range of regulators able to sense changes in many different stimuli. The *S. aureus* genome codes for 16 two component regulator systems although the environmental

Table 1.2 Major virulence attributes and their mode of action during *S. aureus* infection

Virulence Factor	Mode of Action	References
Secreted Virulence Factors		
α-hemolysin (*hla*)	Cytotoxic and haemolytic properties. Forms pores in the membrane of target cells, causing damage and cell death.	(Bhakdi & Tranum-Jensen, 1991)
TSST-1	Disease specific toxin of Toxic Shock Syndrome. Also superantigen (see below)	(Blomster-Hautamaa *et al*.,1986)
Extracellular enzymes, e.g. protease and lipases	Involved in invasion of host cells by breaking down cellular membrane or host defence molecules	(Archer, 1998)
Immune Modulating Agents		
Protein A (*spa*)	Anti-opsonic. Binds to the Fc domain of IgG, blocking its interaction with neutrophils and disrupting recruitment and phagocytosis	(Plata *et al.*, 2009; Laarman *et al*., 2010)
Staphylokinase (SAK)	Activates surface bound plasmin, resulting in host cell disruption and intracellular invasion. Activated plasmin is also anti-opsonic, cleaving IgG and C3b in inhibiting phagocytosis	(Rooijakkers *et al*., 2005)
Chemotaxis Inhibitory Protein of *S. aureus* (CHIPS)	Blocks neutrophil recruitment towards the chemoattractant C5a by binding to C5aR with high affinity. Limits neutrophil recruitment to the site of infection	(Postma *et al*., 2005)
PSM-like peptide toxins	Primarily associated with MRSA strains. Function to recruit, activate and subsequently lyse human neutrophils.	(Wang *et al*., 2007)
Superantigens		
e.g. Enterotoxins (A, B, C, D, E, G, Q) and TSST-1	Cross-link MHC-II molecules with T-cell receptors resulting in antigen independent T-cell proliferation and cytokine production. Weakens the immune system and allows proliferation of *S. aureus* infection	(Plata *et al*., 2009)

Table 1.2 continued

Surface associated Virulence Factors		
Fibronectin binding proteins (*fnbA* and *fnbB*)	Bind host fibronectin. Involved in adherence to host tissue. Part of MSCRAMM protein family.	(Patti *et al*., 1994; Wann *et al*.,2000)
Collagen binding protein (*cna*)	Binds host collagen. Involved in adherence to host tissue. Part of MSCRAMM protein family.	(Patti *et al*., 1992)
Eap	Originally described as fibrinogen binding protein. Also implicated in evasion of host immune system by inhibiting leukocyte recruitment and plays a role in biofilm formation	(Boden & Flock, 1992; Chavakis *et al*., 2002; Johnson *et al*., 2008)
General Virulence Attributes		
Siderophore production	Part of iron scavenging response. Uptake of iron is essential for host invasion and survival during infection.	(Dale *et al*., 2004a)
Biofilm formation	Ability of cells to form tight associations within one another and a surface, alongside production of PNAG. Protects bacteria from immune system and antimicrobial agents	(Gray *et al*., 1984; Gotz, 2002; Jones *et al*., 2001)
Carotenoid pigments	Give *S. aureus* distinct golden colour. Have antioxidant properties which help protect the bacteria from oxidant killing by the host immune system	(Liu *et al*., 2005)

Definitions: Toxic Shock Syndrome Toxin 1 (TSST-1); Immunoglobulin G (IgG); phenol-soluble modulin (PSM); Microbial surface components recognizing adhesive matrix molecules (MSCRAMM); poly-N-acetylglucosamine (PNAG) exopolysaccharide.

signal has not been elucidated for many of these (Cheung *et al*., 2004). There are also a number of transcriptional and translational regulators that specifically alter gene expression in order to combat changes in environmental conditions (Table 1.3).

What makes this system so adaptable to environmental change is the fact that the different regulons overlap. For example, *S. aureus* biofilm formation is influenced by many environmental stimuli, including glucose, pH, osmolarity, temperature and iron availability, and as such is under the control of a number of different global regulators (Gotz, 2002). The alternative transcription factor σ^B is required for the induction of biofilm formation in response to increased osmolarity (Rachid *et al*., 2000), while the metabolic regulator CcpA is essential for biofilm accumulation but does not influence attachment of cells to surfaces (Seidl *et al*., 2008). The global regulators Agr, Sae and Fur are all required for the expression of key biofilm formation proteins Eap and Emp in low iron conditions, while repression of these proteins in the presence of Fe occurs via another regulator that is currently undefined (Johnson *et al*., 2008). Therefore the action of each of these regulators is required for *S. aureus* to utilise biofilm formation to its full potential.

Not only are individual responses such as biofilm formation or exoprotein synthesis controlled by multiple regulators, these regulators can directly influence one another. For example, the *agr* system requires *sarA* in order to produce the transcriptional effector RNAIII, and *agr* can also directly influence the exoprotein regulator *sae* (Cheung & Projan, 1994; Novick & Jiang, 2003; Bronner *et al*., 2004). The control of manganese uptake via MntR directly influences the peroxide resistance (PerR) regulon

Table 1.3. Examples of some *S. aureus* global regulators and the genes they influence

Regulator	Stimulus	Examples of target genes	References
Agr	Quorum sensing – changes in cell density	Temporal expression of virulence determinant via regulatory RNAIII. Represses colonising factors, such as *fnbA* and *fnbB,* and increases expression of exoproteins such as α-hemolysin (*hla*) when cell density increases.	(Ji *et al*., 1995; Saravia-Otten *et al*., 1997; Peng *et al*., 1988; Morfeldt *et al*., 1995)
SarA	Undefined	Expression of virulence determinants. Required for RNAIII transcription and can regulate genes, e.g. represses *cna* transcription, independently of *agr*.	(Cheung *et al*., 1992; Cheung & Projan, 1994; Blevins *et al*., 1999; Karlson *et al*., 2001)
SaeRS	pH, osmolarity, oxidative stress, antimicrobial peptides	Regulation of virulence factors. Usually directly or indirectly influenced by other regulatory systems, such as *agr*. Required for the expression of adhesins Eap and Emp, and exoproteins α-hemolysin (*hla*) and coagulase (*coa*)	Giraudo *et al*., 1997; Steinhuber *et al*., 2003; Harraghy *et al*., 2005)
CcpA	Carbon availability	Control of carbon metabolism to ensure efficient utilisation of available carbon sources. Repression of gluconeogenic genes (e.g. *pckA*) and activation of glycolytic genes (e.g. *tpi, pgk*) in presence of glucose. Also controls expression of some virulence factors (e.g. *fnbB*) and alternative sigma factor B (σ^b).	(Seidl *et al*., 2006; Seidl *et al*., 2008; Seidl *et al*., 2009)
Fur	Fe	Represses genes for iron scavenging in the presence of Fe and regulates the expression of a number of virulence factors, including the haemolysins *hla, hlgC* and *hlb,* and the adhesins *eap* and *emp,* and the important virulence regulators SaeRS, Agr and Rot	(Xiong *et al*., 2000; Horsburgh *et al*., 2001b; Johnson *et al*., 2008; Johnson *et al*., 2010)
PerR	H_2O_2, Mn and Fe	Mangenese dependent repressor that can act via Fur or independently. Controls expression of the oxidative stress response regulon, and the staphylococcal ferritin proteins involved in iron storage.	(Horsburgh *et al*., 2001a; Morrissey *et al*., 2004)
MntR	Mn and Fe	Divalent metal ion dependent repressor. Controls manganese uptake via the putative transporters encoded by *mntABC* and *mntH*. Can modulate PerR and Fur regulons via control of Mn uptake	(Horsburgh *et al*., 2002b; Ando *et al*., 2003)

Definitions: Accessory gene regulator (Agr), Staphylococcal accessory regulator A (SarA), *S. aureus* exprotein regulator and sensor (SaeRS), carbon catabolite protein A (CcpA), peroxide resistance regulon repressor (PerR), manganese transport regaultor (MntR), ferric uptake regulator (Fur), repressor of toxins (Rot)

and the oxidative stress response, which includes the Fe regulator Fur (Horsburgh *et al*., 2002b). These examples highlight how interconnected the regulatory systems in *S. aureus* are and the augmentation of a single regulator in a strain could therefore dramatically influence its ability to express a wide range of different genes.

1.5.3 Iron Regulation and Fur in *S. aureus*

The concentration of extracellular iron is a key environmental signal for the regulation of virulence determinants in bacteria, as the level of free iron within a host is very low. Iron is an essential element of many biological processes, providing redox potential as a co-factor in many important enzymes (Andreini *et al*., 2008). This is due to the difference in redox potential between Fe^{3+} (ferric), which can act to oxidise substrates, and Fe^{2+} (ferrous) iron which can reduce them (Pierre & Fontecave, 1999). However this reducing power means that too much free Fe^{2+} is toxic to biological systems, through the production of the reactive oxygen species (ROS) $OH^{\bullet}$, via the Fenton reaction (shown below).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\bullet}$$

Higher eukaryotes limit Fe^{2+} by binding it to host molecules and proteins such as haem, transferrin and lactoferrin, protecting their cells from damage and limiting iron availability to invading pathogens (Bullen *et al*., 2005; Ganz & Nemeth, 2006). Low iron conditions are also a signal to the bacteria that they are within a host, and this leads to the expression of genes required for colonisation and pathogenesis (Skaar *et al*., 2004; Allard *et al*., 2006; Ratledge & Dover, 2000; Johnson *et al*., 2010).

There are a number of iron uptake systems in *S. aureus* (Figure 1-3) including the direct uptake of free Fe^{2+}, the uptake of haem (Mazmanian *et al*., 2003; Skaar *et al*., 2004; Skaar & Schneewind, 2004), the utilisation of the host iron transport protein transferrin (Lim *et al*., 1998; Modun *et al*., 1998; Modun & Williams, 1999), the production of iron chelating proteins known as siderophores (Konetschny-Rapp *et al*., 1990; Courcel *et al*., 1997; Dale *et al*., 2004a), and also the uptake of so called xenosiderophores; siderophore compounds produced by other bacterial species and "pirated" by *S. aureus* through the production of specific uptake systems such as the ferric hydroxymate uptake system *fhu* (Cabrera *et al*., 2001; Sebulsky & Heinrichs, 2001). Iron uptake in *S. aureus* is under the control of the ferric uptake regulator Fur.

Originally discovered in *E. coli*, Fur homologues have been found in a number of Gram positive and Gram negative bacteria, including *Bacillus subtilis* and *Helicobacter pylori*, and is well characterised as an Fe dependent repressor protein (Bagg & Neilands, 1987; Bsat *et al*., 1998; Bereswill *et al*., 1999; Xiong *et al*., 2000; Horsburgh *et al*.,2001b). Fur not only controls iron homeostasis genes in response to Fe, but also a wide range of virulence factors (Horsburgh *et al*., 2001b; Allard *et al*., 2006; Johnson *et al*., 2010). The classic model of Fur regulation represses the expression of target genes when bacterial intracellular Fe^{2+} levels are abundant (Figure 1-4). The Fur protein binds to Fe^{2+}, and this complex is able to bind to consensus DNA sequences known as Fur boxes, found in the promoter region of target genes, repressing gene expression at a transcriptional level (Bagg & Neilands, 1987).

Fur has also been shown to regulate genes in a number of unexpected ways (Figure 1-4B). One well characterised process is regulation of the iron-sparing response of *E.*

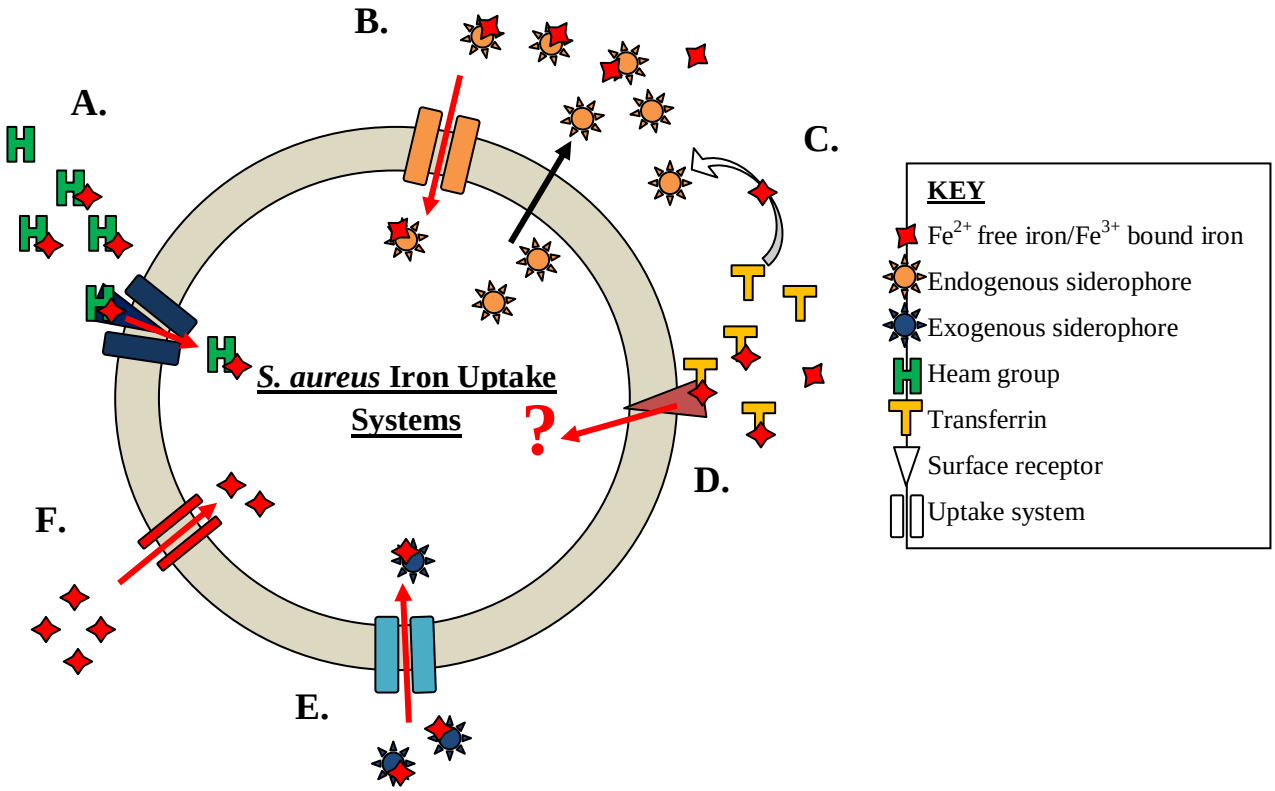


Figure 1-3. Iron uptake systems in *S. aureus*.

(A) Receptor mediated binding of haem and subsequent transport across the cell envelope by the *isd* (iron-regulated surface determinant) system. May also play a role in binding and utilisation of transferrin. (Taylor & Heinrichs, 2002; Morrissey *et al.*, 2002; Mazmanian *et al.*, 2003; Skaar & Schneewind, 2004).

(B) Endogenous siderophores, such as staphyloferrin A, aureochelin and staphylobactin, are produced and released from the bacteria. They chelate iron and are transported back into the cell (Courcel *et al.*, 1997; Dale *et al.*, 2004a; Dale *et al.*, 2004b).

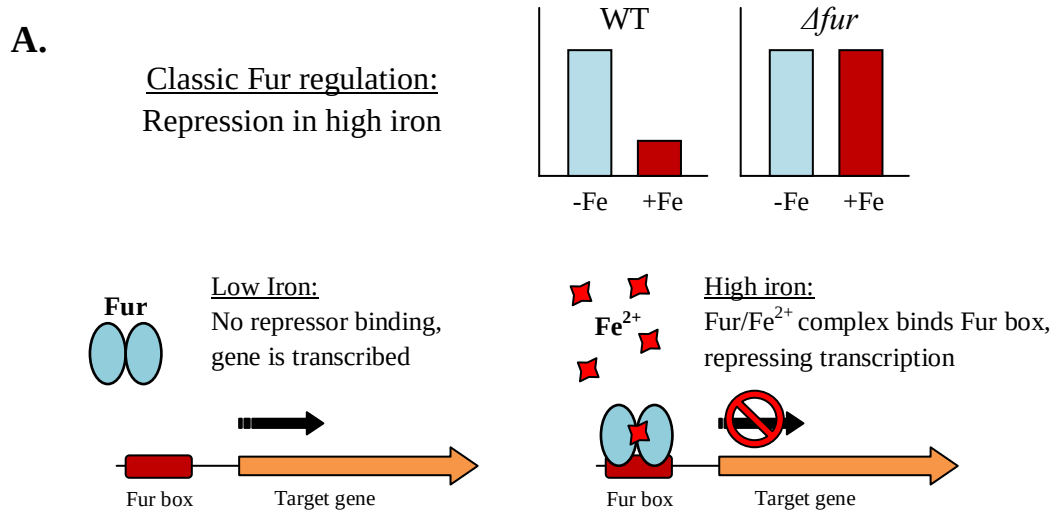
(C) The siderophore staphyloferrin A has been shown to specifically remove iron from transferrin (Park *et al.*, 2005; Modun *et al.*, 1998).

(D) Specific surface receptors (including GAPDH) bind to transferrin. Mechanism for uptake and utilisation of Fe is unknown (Modun *et al.*, 1994; Lim *et al.*, 1998; Modun & Williams, 1999; Taylor & Heinrichs, 2002).

(E) Siderophore piracy. Specific transporters, such as the *fhu* system, which can utilise siderophores produced by other bacterial species, such as ferric hydroxmates (Cabrera *et al.*, 2001; Sebulsky & Heinrichs, 2001).

(F) Direct uptake of free Fe^{2+}, most likely via FeoB.

A.



B.

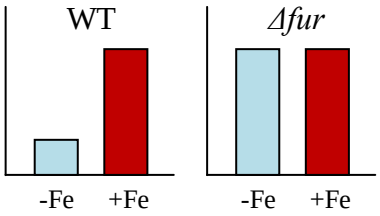
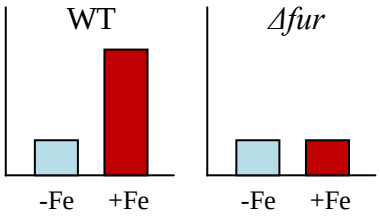
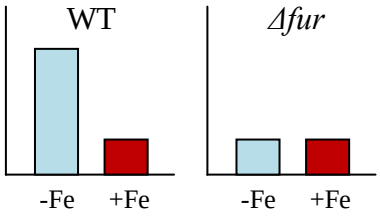
Non-classical Fur regulation	Examples of mechanism	
Repression in low iron	Fur directly binds to the *pfr* (non haem ferritin) promoter of *Helicobacter pylori* in low iron conditions, repressing transcription. Addition of Fe^{2+} reduces the efficiency of Fur, leading to transcription (Delany *et al*., 2001).	
Activation in high iron	Fur activates transcription of 3 genes encoding metalloproteins/complexes in high Fe conditions in *N. menigitidis* by directly binding to the gene operators (Delany *et al*., 2004).	
Activation in low iron	The late stages of *S. aureus* biofilm formation, at 24hours, is positively regulated by Fur in low iron conditions (Johnson *et al*., 2005).	

Figure 1-4. Iron regulation of gene via Fur.

(A) Classic Fur dependent iron regulation. In the presence of Fe, Fur binds to a consensus Fur box sequence in the promoter region of target genes, repressing transcription.

(B) Non-classical Fur dependent gene regulation, including some examples and known mechanisms.

coli, which is mediated by Fur and a small non-coding RNA (sRNA) known as RyhB (Masse *et al*., 2007). In low iron conditions, RhyB down regulates the transcription of a number of non-essential iron containing proteins through degradation of their mRNA transcripts. RhyB transcription is classically regulated by Fur, and is therefore repressed in the presence of Fe, resulting in Fur mediated expression of these iron-using proteins (Masse & Gottesman, 2002). This response is vital to ensure that in low Fe conditions the most essential Fe-containing enzymes are produced at the expense of non-essential processes. While no Gram positive RyhB homologues have been identified by sequence similarity, the sRNA FsrA of *B. subtilis* has been shown to regulate the iron-sparing response in a similar manner (Masse & Gottesman, 2002). Fur can also directly act as a transcriptional activator in the presence of Fe, and has been shown to directly repress transcription of genes in low iron conditions, indicating multiple mechanisms of transcriptional control by this important regulatory protein (Figure 1-4B; Delany *et al*., 2001; Delany *et al*., 2004).

In *S. aureus* Fur has been shown to positively regulate genes in high iron conditions, for example in the expression of *katA*, a catalase involved in the oxidative stress response (Horsburgh *et al*., 2001b). *S. aureus* Fur can also positively regulate gene expression in low iron, as demonstrated in the late stages of *S. aureus* biofilm formation (Johnson *et al*., 2005), and act to repress gene expression in low iron, as shown for the *S. aureus* ferritin FtnA (Morrissey *et al*., 2004). Currently it is unclear whether Fur acts directly or indirectly in each of these cases, but as *S. aureus* contains a number of undefined sRNA molecules and Fur modulates the expression of other global regulators such as SaeRS, Agr and Rot (repressor of toxins) (Johnson *et al*., 2010), these Fur mediated responses could involve any number of additional factors.

The *S. aureus* glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is also regulated by Fe in an unexpected manner. GAPDH surface protein expression is iron regulated in *S. aureus* and this response varies between strains (Dr. Julie Morrissey, unpublished data). Although the expression of *gapA* has been linked to Tf binding, surface expression in the majority of strains is reduced in the absence of iron, the direct opposite of what would be expected for the iron scavenging response. Therefore a key metabolic enzyme is regulated in response to iron, but the mechanism of control and the function of this response are currently undefined.

1.6 Glyceraldehyde-3-phosphate dehydrogenase

GAPDH is a metabolic enzyme that plays a key role in carbon metabolism in living systems. It is responsible for the interconversion of glyceraldehyde-3-phosphate (G3P) to 1,3-diphosphoglycerate (1,3-dPG) during glucose catabolism and biosynthesis (Figure 1-5). This enzyme is essential for the utilisation of glucose, and as such is highly conserved throughout bacteria, yeast and higher eukaryotes. A tetrameric conformation made up of 4 identical subunits is required for GAPDH enzyme activity (Figure 1-6; Pancholi & Fischetti, 1992).

1.6.1 Multiple GAPDH homologues in bacteria

While many prokaryotic and eukaryotic species contain a single GAPDH gene, which encodes an enzyme capable of catalysing both glycolytic and gluconeogenic GAPDH activity (Pancholi & Fischetti, 1992), a number of species have multiple GAPDH homologues. Some species, such as *Saccharomyces cerevisiae*, have multiple homologues that all have GAPDH activity, and appear to function in a redundant manner in carbon metabolism (Delgado *et al*., 2001). There are also three GAPDH

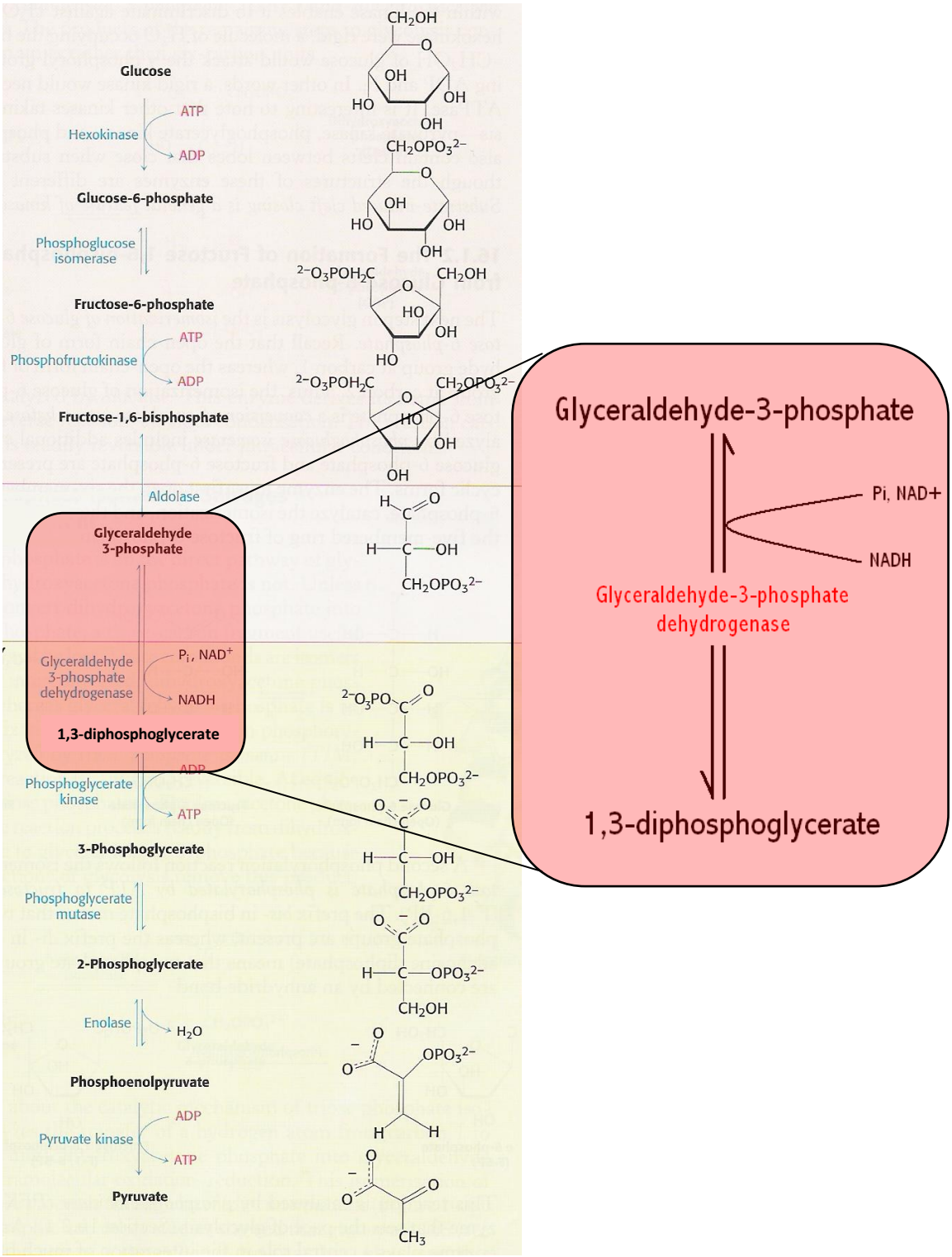


Figure 1-5. Glycolysis and gluconeogenesis.

This figure demonstrates the various enzymes and substrates involved in glycolysis (glucose conversion to pyruvate) and gluconeogenesis (pyruvate conversion to glucose). The area highlighted in red indicates GAPDH function in the pathway. This diagram was modified from Berg *et al*., (2002).

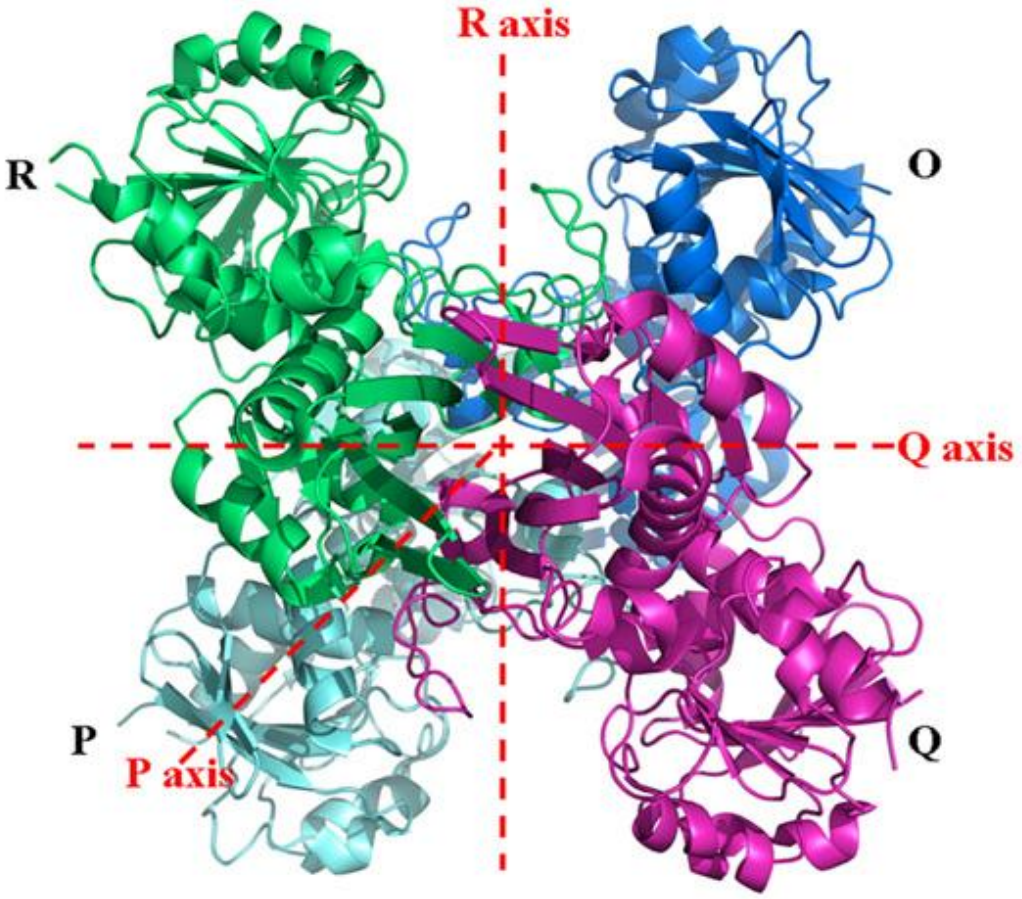


Figure 1-6. Structure of *S. aureus* GapA GAPDH protein.

This diagram demonstrates the quaternary structure of *S. aureus* GapA GAPDH protein, highlighting the interaction of four identical subunits. Figure taken directly from Mukherjee, *etal.*, (2010b).

homologues in *Escherichia coli*, however only one of these genes, *gapA*, encodes a GAPDH enzyme. The additional homologues, known as *gapB* and *gapC*, encode an erythrose-4-phosphate dehydrogenase (E4PDH) and a truncated GAPDH protein, the function of which is unknown (Zhao *et al*., 1995; Hidalgo *et al*., 1996; Seta *et al*., 1997). In this species both glycolytic and gluconeogenic GAPDH acitivity is provided by GapA, while the E4PDH GapB is involved in pyridoxine (vitamin B_6) biosynthesis (Boschi-Muller *et al*., 1997; Yang *et al*., 1998).

The Gram positive bacteria *Bacillus subtilis* also has two *gap* homologues, but in this case they both encode GAPDH proteins, each with opposing roles in glucose metabolism rather than the common scenario of one GAPDH performing both roles (Fillinger *et al*., 2000). The *B. subtilis gapA* GAPDH is only functional in glycolysis, converting G3P to 1,3-dPG, and has specificity for NAD+ as a cofactor. The *gapB* GAPDH is gluconeogenic, and specifically binds the cofactor NADP+ for conversion of 1,3dPG to G3P during gluconeogenesis (Fillinger *et al*., 2000). These proteins are also expressed in response to reciprocal stimuli, with *gapA* being expressed in response to glucose and *gapB* being repressed, and *vice versa* (Fillinger *et al*., 2000).

1.6.2 Regulation of Carbon Metabolism in Bacteria

The ability to utilise available carbon sources is a key element for bacterial survival, and the process needs to occur efficiently in order to generate both energy and key biological compounds with as little "wasted" energy and resources as possible. Many processes such as glycolysis and gluconeogenesis are directly reversible at various stages, and therefore the expression of primary and secondary carbon metabolism genes is separated to avoid the formation of a futile cycle. The regulation of carbon

metabolism primarily occurs via Carbon Catabolite Repression (CCR). This process allows bacteria to preferentially utilise the most favourable carbon source (such as glucose) over those less favourable (amino acids, TCA cycle intermediates, etc). There are two main CCR systems in bacteria, known as inducer exclusion and induction prevention (see reviews Bruckner & Titgemeyer, 2002; Warner & Lolkema, 2003; Deutscher, 2008; Gorke & Stulke, 2008). Gram negative species such as *E. coli* tend to use the inducer exclusion method, while current evidence suggests that many Gram positive species, including all known firmicutes such as *B. subtilis* and *S. aureus* utilise the induction prevention mechanism (Deutscher, 2008). Gram positive CCR is summarised in Figure 1-7.

The major effector molecule in this process is not actually glucose, but an early product of glycolysis known as Fructose-1,6-bisphosphate (FBP) (Figure 1-5), which triggers HPrK kinase activity and also enhances the interaction between CcpA and HPr (Ser-P), the complex responsible for transcriptional repression of target genes (Gorke & Stulke, 2008). In *B. subtilis* the induction of glycolytic genes such as *gapA* and the glycolytic operon is also influenced indirectly by key CCR molecules (Doan & Aymerich, 2003; Zorrilla *et al*., 2007b). As glucose increases so does FBP, which binds to the glycolysis repressor CcgR preventing repression of the glycolytic operon and thus increasing transcription (Doan & Aymerich, 2003; Zorrilla *et al*., 2007a; Zorrilla *et al*., 2007b). CcpA and HPr (Ser-P) are also required for induction of glycolytic genes as they are essential for the uptake of sugars transported by the phosphotransferase system (PTS) (Ludwig *et al*., 2002). Interestingly GapA protein has also been shown to interact with both phosphorylated and non-phosphorylated HPr protein, although the functional significance of this interaction is currently unknown (Pompeo *et al*., 2007).

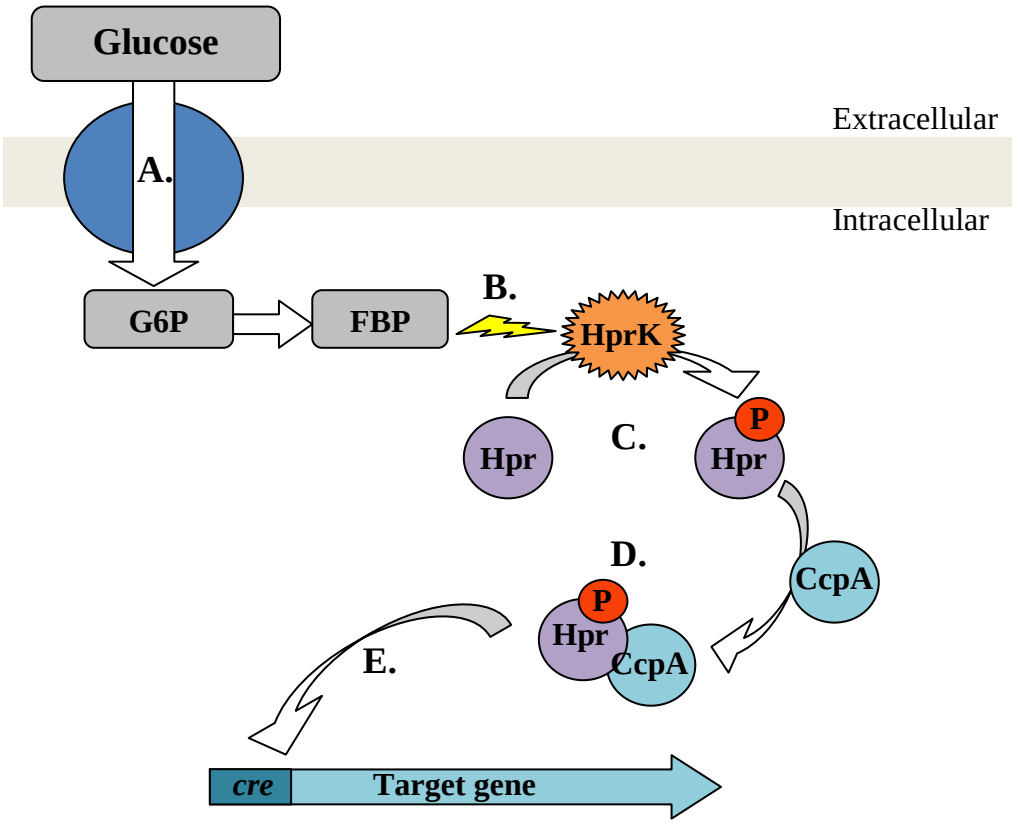


Figure 1-7. Gram positive carbon catabolite repression via induction prevention.

(A) Glucose uptake via phosphotransferase system and subsequent conversion to fructose-1,6-bisphosphate (FBP).

(B) FBP activates HprK kinase activity

(C) HprK phosphorylates Hpr protein at Ser_{46} residue

(D) Hpr(Ser-P) binds to transcriptional regulator CcpA. This interaction is enhanced by FBP.

(E) The CcpA/Hpr(Ser-P) repressor complex binds to catabolic responsive elements (*cre*) in the promoter of target genes and represses transcription.

1.6.3 GAPDH – more than just a cytoplasmic enzyme

Initially considered to only be found in the cytoplasm, it is now widely observed that GAPDH is also found localised to the surface of many bacterial cells, and some eukaryotic cells. This includes both Gram positive bacteria such as Streptococcal species (D'Costa *et al*., 2000), Gram negative bacteria such as *Escherichia coli* (Egea *et al*., 2007), yeast cells (Delgado *et al*., 2001) and also some human cells, for example macrophages (Raje *et al*., 2007).

The GAPDH found on the surface and in the cytoplasm appears to be the same molecule and in at least some bacterial and yeast species retains its glycolytic function (Winram & Lottenberg, 1996; Gil-Navarro *et al*., 1997). However, surface GAPDH also takes on a number of different functions, generally unrelated to central metabolism (Table 1.4). The most wide spread function of surface GAPDH proteins is the ability to bind to a range of different proteins. This may provide a means for pathogens to adhere to host tissue for colonisation, and it has been shown that inhibition of GAPDH surface expression results in a reduction in cellular adherence in streptococcal cells (Boel & Pancholi, 2005). This also resulted in increased phagocytic killing of the bacteria, which may suggest that the binding of a range of host proteins helps to "hide" the bacterial antigens from recognition by the host immune system (Boel & Pancholi, 2005; Terao *et al*., 2006). GAPDH expressed on the surface of human macrophages has also been shown to bind and facilitate the uptake of iron-bound transferrin on these cells (Raje *et al*., 2007).

Table 1.4. "Moonlighting" functions of GAPDH in a wide range of species.

Species	Function	Reference
Group A *Streptococci*	Binds fibronectin, lysozyme, myosin, actin, plasmin and plasminogen	(Pancholi & Fischetti, 1992; Bergmann *et al*., 2004)
	Regulates phosphorylation of human pharyngeal cell proteins	(Pancholi & Fischetti, 1997)
	Has antiphagocytic properties and is essential for evasion of neutrophils	(Boel & Pancholi, 2005; Terao *et al*., 2006)
Oral *Streptococci*	Mediates interaction with *Porphyromonas gingivalis* fimbriae	(Meada *et al*., 2004)
Staphylococci	Plasmin and transferrin binding	(Modun & Williams, 1999; Goji *et al*., 2004)
E. coli	Plasminogen and fibrinogen binding	(Egea *et al*., 2007)
Mycoplasma genitalium	Binds mucin	(Alvarez *et al*., 2003)
Lactobacillus plantarum	Binds to A and B blood group antigens	(Kinoshita *et al*., 2008)
Mycobacterium avium	Binds epidermal growth factor	(Parker & Bermudez, 2000)
Candida albicans	Binds fibronectin and laminin	(Gozalbo *et al*., 1998)
Schizosaccharomyces pombe	Promotes peroxide stress phosphorelay signalling	(Morigasaki *et al*., 2008)
Plants	Suppression of reactive oxygen species	(Baek *et al*., 2008)
Humans	Binds transferrin (macrophages only)	(Raje *et al*., 2007)
	Control of gene expression via mRNA stability	(Rodriguez-Pascual *et al*., 2008)
	Involved in oxidised protein repair	(Azam *et al*., 2008)
	Cell death	(Hara & Snyder, 2006)
	Implicated in DNA replication and repair, histone gene expression, nuclear membrane fusion, maintenance of telomere structure, nuclear RNA export, prostate cancer and viral pathogenesis	(Sirover, 1997; Sirover, 1999; Sirover, 2005)

In human cells cytoplasmic GAPDH functions in a range of processes such as DNA replication and repair, control of histone gene expression, nuclear membrane fusion and apoptosis, and protecting the cell from oxidative damage (Sirover, 1999; Azam *et al*., 2008). It is interesting to note that GAPDH is also associated with the progression of neurodegenerative disorders, including Alzheimer's disease and Huntington's disease (Mazzola & Sirover, 2004; Senatorov *et al*., 2003; Shalova *et al*., 2007). This suggests that GAPDH plays a greater role in cellular physiology than originally thought, and it is likely that prokaryotic GAPDH proteins have a wide range of additional "moonlighting" roles yet to be discovered.

1.6.4 Surface localisation of GAPDH

Surface GAPDH is one of several "anchorless proteins" found on the Gram positive cell membrane, which are so named due to the apparent lack of signalling motifs for both extracellular transport and surface attachment (Eichenbaum *et al*., 1996; Lottenberg *et al*., 1992; Gatlin *et al*., 2006). The mechanism by which anchorless proteins become surface localised is currently unclear, and it is possible they are a result of non-specific attachment of proteins released from cells during lysis. The distribution of surface GAPDH on Group A *Streptococci* cells follows that of fimbrial structures composed of M and M-related proteins, and knocking out the gene responsible for M protein expression (*mga*) also reduced GAPDH surface expression, suggesting that either *mga* or M-proteins are involved in surface GAPDH expression in this species (D'Costa *et al*., 2000). Exogenous GAPDH did not attach to the M proteins on the bacterial cell surface, which suggests that at least in this species GAPDH surface expression is specific, and secretion of the protein rather than surface attachment requires the interaction of GAPDH and M proteins (D'Costa *et al*., 2000). Furthermore the surface

associated GAPDH TDH-3 in *Candida albicans* has been demonstrated to migrate to the cell surface, suggesting that secretion of GAPDH in this species is specific and driven by the GAPDH protein itself (Delgado *et al*., 2003). GAPDH has also been found on the surface of *S. aureus* (Modun & Williams, 1999).

1.6.5 GAPDH in *S. aureus*

S. aureus has two GAPDH homologues which share approximately 44% sequence identity with one another, and have been termed *gapA* and *gapB* (Goji *et al.,* 2004). The *gapA* gene is found in an operon alongside the putative operon regulator *gapR* and 4 additional glucose metabolism enzymes, phosphoglycerate kinase (*pgk*), phosphoglycerate mutase (*pgm*), triosephosphate isomerase (*tpi*) and enolase (*eno*), as seen in several other bacterial species including *B. subtilis* (Figure 1-8; Ludwig *et al.*, 2001; Naterstad *et al.*, 2007). The *gapB* gene is located as a single open reading frame alongside genes involved in DNA replication and repair (Figure 1-8). To date the function of GapB and GapR have not been demonstrated in *S. aureus*.

The *gapA* gene has been previously shown to encode a glycolytic GAPDH protein, which is solely responsible for NAD+ dependent GAPDH activity *in vivo* in *S. aureus* (Taylor & Heinrichs, 2002; Goji *et al.*, 2004). The GapA protein is highly conserved between isolates and several groups have shown that it is surface localised, although its surface function has been disputed (Modun & Williams, 1999; Taylor & Heinrichs, 2002; Goji *et al.*, 2004). Originally found to be a staphylococcal transferrin binding protein (Tbp) identified through N-terminal sequencing, affinity purified *S. aureus* GapA protein was found to not only have NAD+ specific GAPDH activity, but to also specifically bind to transferrin (Tf) and plasmin; a process that was competitively

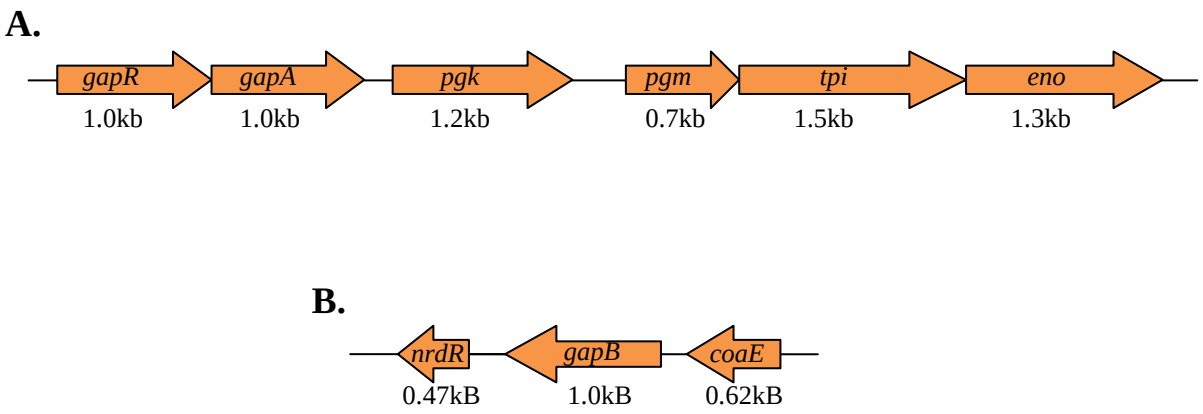


Figure 1-8. Genetic organisation of *S. aureus gapA* and *gapB*.

(A) Putative *S. aureus* glycolytic operon. Putative operon repressor (*gapR*), glyceraldehyde-3-phosphate dehydrogenase A (*gapA*), phosphoglycerate kinase (*pgk*), phosphoglycerate mutase (*pgm*), triosephosphate isomerase (*tpi*) and enolase (*eno*). The organisation of these genes is shared with other Gram positive bacteria (Ludwig *et al.*, 2001; Naterstad *et al.*, 2007).

(B) Genetic position of *S. aureus* glyceraldehyde-3-phosphate dehydrogenase B (*gapB*), alongside the adjacent open reading frames for a putative transcriptional regulator (*nrdR*) and dephospho-CoA kinase (*coaE*). These genes do not appear to form an operon.

inhibited using opposing substrates (Modun *et al.*, 1994; Modun & Williams, 1999). However a *ΔgapA* knockout mutant in strain RN6390 was found to retain whole cell Tf binding, while binding was completely lost in a *ΔstbA* (a.k.a. *frpA/isdA*) knockout mutant (Taylor & Heinrichs, 2002). There is evidence that binding of *isdA* to a Tf-HRP (horse radish peroxidase) conjugate as used in this study is non-specific, as unlabelled Tf fails to competitively inhibit this association (Morrissey *et al.*, 2002). Interestingly recombinant GapA protein cloned from a bovine mastitis isolate (referred to as GapC) has GAPDH activity but only a low affinity for Tf and plasmin binding (Goji *et al.*, 2004), whereas GapB protein does bind both Tf and plasmin. GapB protein is also surface localised in *S. aureus*, but unlike GapA this surface association does not appear to be ubiquitous in all *S. aureus* strains (Goji *et al.*, 2004). This suggests that GapB acts as a Tbp in *S. aureus*, and the lack of Tf binding in a *ΔstbA* mutant may be explained by a lack of GapB surface expression in this strain.

The enzymatic function of GapB protein is currently unknown in *S. aureus*, although it has been assumed to be a gluconeogenic GAPDH by several groups (Kohler *et al.*, 2005; Chatterjee *et al.*, 2009; Seidl *et al.*, 2009). In fact there is evidence suggesting that GapB may not be a gluconeogenic GAPDH in *S. aureus*. Gluconeogenic GapB protein purified from *B. subtilis* shows a 50-fold higher catalytic efficiency with NADP+ than with NAD+ (Fillinger *et al.*, 2000), whereas purified recombinant GapB protein from a *S. aureus* bovine mastitis isolate shows very little NAD+ dependent GAPDH activity and no NADP+ dependent GAPDH activity (Goji *et al.*,2004).

Due to the highly conserved nature of these proteins, and the fact that they are surface localised, their potential as vaccine candidates for bovine mastitis infection has been

explored (Kerro-Dego *et al.*, 2006; Perez-Casal *et al.*, 2006). The search for new treatment options and the production of a staphylococcal vaccine are crucial due to the importance of *S. aureus* as a pathogen of both humans and animals.

1.7 Project aims and objectives

GAPDH is an important metabolic enzyme that has been shown to have multiple functions in both prokaryotic and eukaryotic cells (Table 1.4), indicating that the role of this protein in general cellular physiology may be more extensive than first recognised. *S. aureus* has two GAPDH homologues, *gapA* and *gapB*, both of which are localised to the cell surface (Modun & Williams, 1999; Goji *et al.*, 2004), although the functions of these surface proteins is somewhat unclear. *S. aureus* is an important pathogen and as such understanding cellular metabolism, as well as the abundance of surface proteins, is important in identification of novel treatment and vaccine targets.

The first part of this project will focus on the metabolic functions and regulation of both *gapA* and *gapB* in order to determine whether *gapB* plays a role in carbon metabolism. Additional aims of this section are to identify potential accessory functions of both GapA and GapB outside of their metabolic roles, and to determine the impact of these proteins during infection. The second part of this project will go on to investigate novel non-glycolytic regulation of *gapA*, and to a lesser extent *gapB*, and examine how and why this regulation might occur. The final part of this project will look at a STAR repeat locus located directly upstream of the glycolytic operon and investigate its potential impact on gene expression. Additional aims of this part are to extend this investigation to two additional STAR loci, in order to determine any global patterns seen with these repeats.

1.7.1 Project objectives

- To determine the primary function of GapA and GapB in *S. aureus* carbon metabolism
- To investigate the regulation of these genes in response to glucose and additional environmental signals, and determine the influence of known regulators on transcription
- To investigate the influence of GapA and GapB during infection in a simple invertebrate model
- To determine novel "moonlighting" functions of GapA and GapB
- To investigate the transcriptional control of *gapA* and determine whether a STAR element locus located upstream impacts on expression
- Further investigate the presence of STAR elements at additional loci across the *S. aureus* genome, and attempt to determine a potential function

Chapter 2 Materials and Methods

2.1 Growth Media and Supplements

2.1.1 Sterilisation procedures

Growth media and supplements were sterilised either by autoclaving or filter sterilisation. For autoclaving, media was heated to 120°C at 15 pSI for 15 minutes. Filter sterilisation was achieved by passing the liquid through a 0.22 μm pore diameter filter sterilisation membrane; for small volumes (<50 ml) using a 25 mm syringe filter membrane (PALL Life Sciences) and for large volumes (>50 ml) using a vacuum driven Millipore membrane filtration system (Millipore).

2.1.2 Luria Bertani Broth and Agar

Luria Bertani broth (LuB) contains 1% (w/v) Tryptone (Oxoid), 0.5% (w/v) NaCl and 0.5% (w/v) yeast extract in dH_2O. The pH was corrected to 7.5 and the medium was autoclaved. For Luria Bertani agar (LuA) 1.5% Bioagar was added before autoclaving.

2.1.3 LK broth and Agar

LK broth contains 1% (w/v) Tryptone (Oxoid), 0.7% (w/v) KCl and 0.5% (w/v) yeast extract in dH_2O. For LK agar 1.5% Bioagar was added before autoclaving.

2.1.4 Trypticase Soy Broth (TSB)

TSB was produced by dissolving 3% (w/v) BBL Trypticase Soy Broth powder (BD Diagnostic Systems) in dH_2O and autoclaving. For TSB + 20% glycerol, 20% of the dH_2O used was substituted with the same volume of 100% glycerol.

2.1.5 CRPMI (Morrissey *et al.*, 2002)

The tissue culture medium RPMI-1640 (Sigma Aldrich) was used to produce iron limited growth conditions. In order to standardise the iron restriction and pH, 6% Chelex 100 (sodium form; Sigma Aldrich) was added to the medium and left stirring at 4°C overnight to chelate free metal ions. The chelex was filter sterilised from the medium, and the pH was adjusted to 8.8, before a final filter sterilisation. Before use the media was supplemented with 10% unchelexed RPMI-1640.

2.1.6 Staphylococcal Siderophore Detection (SSD) Medium (Lindsay & Riley, 1994)

Stock solutions of 20% glucose, 1.5 M Tris (pH 8.8), 20% casamino acids (Difco) and 25x Salts containing 0.68% KH_2PO_4, 1.16% NaCl and 2.3% NH_4Cl diluted in dH_2O were made and autoclaved prior to producing SSD medium. 50x supplement (1.95 mM Tryptophan, 1.6 mM Nicotinic acid and 300 μM Thiamine) was also made, filter sterilised and stored at 4°C. 1l SSD contains the following; 40 ml 25x salts, 18 ml 20% glucose, 30 ml 20% casamino acids and 20 ml 50x supplement. In order to iron restrict the medium, 1% chelex 100 (Sigma Aldrich) was added and left stirring overnight at 4°C. The medium was filter sterilised and stored at 4°C, and 50 μM $MgCl_2$ was added before use.

2.1.7 Tris succinate minimal (TSM) medium (Taylor & Heinrichs, 2002)

Stock solutions of 20% casamino acids (Difco), 1 M $MgCl_2$, 100 mM $CaCl_2$ and 25x Tris salts (14.5% NaCl, 9.25% KCl, 2.75% NH_4SO_4, 0.4% Na_2SO_4 and 0.68% KH_2PO_4 diluted in dH_2O), were made and autoclaved. Stock solutions of 4 mg/ml tryptophan, 11 mg/ml cysteine, 10 mM thiamine, 10 mM nicotinic acid, 0.5 mg/ml pantothenic acid

and 0.02 mg/ml biotin (vitamin B_7) were filter sterilised and stored at 4°C. 400 ml Tris succinate minimal media was produced by diluting 16 ml of 25x Tris salts, 4.84 g Tris base and 6.64 g succinic acid in 320 ml. The pH was adjusted to 7.4, the total volume was made up to 380 ml and the solution was autoclaved. For Tris succinate minimal agar, 1.5% Bio agar was added prior to autoclaving. Once the solution had cooled to at least 60°C, the following was added in sterile conditions; 20 ml 20% casamino acids, 2 ml 4 mg/ml tryptophan, 0.8 ml 11 mg/ml cysteine, 2 ml 10 mM thiamine, 0.4 ml 10 mM nicotinic acid, 0.4 ml 0.5 mg/ml pantothenic acid, 0.4 ml 100 mM $CaCl_2$, 0.2 ml 1 M $MgCl_2$ and 0.2 ml 0.02 mg/ml biotin. This medium was stored at 4°C.

2.1.8 Tris minimal (TM) medium

This medium was made as described for Tris succinate minimal medium, but succinic acid was omitted from the recipe.

2.1.9 Super Optimal broth with Catabolite repression (SOC) medium

SOC medium contains 2% tryptone, 1% yeast extract and 0.5% NaCl and 2.5 mM KCl diluted in dH_2O. After autoclaving, 20 mM sterile glucose and 10 mM sterile $MgCl_2$ were added, and the medium was aliquoted and stored at -20°C until use.

2.1.10 SMMP50 medium

A stock solution of SMM buffer, containing 34.2% sucrose, 0.464% maleic acid and 0.38% $MgCl_2$ diluted in dH_2O, was corrected to pH 6.5 and autoclaved. A 20 ml solution of SMMP50 was produced by adding together 11 ml SMM buffer, 8 ml Pennesay (Difco Antibiotic Medium 3, BD) and 1 ml 10% BSA. This solution was filter sterilised and stored at 4°C for up to two weeks.

2.1.11 Antibiotics and Metal Ion supplements

When required, antibiotics tetracycline (Tet), chloramphenicol (Cm) or erythromycin (Ery) were added to media to a final concentration of 10 μg/ml. Ampicillin (Amp) and spectinomycin (Spc) were added to a final concentration of 100 μg/ml, and kanamycin (Kan) was added to a final concentration of 50 μg/ml. The same antibiotic concentrations were used for both *S. aureus* and *E. coli* cultures. Unless otherwise stated, iron was supplemented by addition of 50 μM $FeSO_4$, magnesium was supplemented by addition of 25 μM $MnCl_2$ and copper was supplemented by addition of 10 μM $CuCl_2$ to the growth medium.

2.1.12 X-gal (5-bromo-4-chloro-3-indolyl-β-$_D$-galactopyranoside)

When blue/white screening of bacterial colonies was required X-gal was added to solid medium to a final concentration of 40 μg/ml. A stock solution of 40 mg/ml X-gal was produced by diluting 40 mg X-gal powder (Sigma-Aldrich) in 1 ml dimethyl formamide. Diluted X-gal was stored at -20°C.

2.2 Bacterial Storage and Growth Conditions

2.2.1 Growth Conditions

All bacterial strains were inoculated onto LuA plates unless otherwise stated, and incubated overnight at 37°C. LuB was inoculated for liquid cultures and incubated with aeration at 37°C overnight. Unless otherwise stated, iron limited conditions were achieved by growing the bacteria in CRPMI supplemented with 10% unchelexed RPMI (Morrissey *et al.*, 2002). Iron replete conditions were achieved by adding 50 μM $FeSO_4$ to the medium, and cultures were grown statically at 37°C in 5% CO_2. Where carbon source needed to be tightly controlled Tris succinate minimal medium (Taylor &

Heinrichs, 2002) and Tris minimal medium was used as either broth or agar. Agar plates were incubated at 37°C overnight and inoculated broth was incubated at 37°C with aeration.

2.2.2 Glycerol Stocks

All bacterial strains were stored in duplicate as glycerol stocks at -80°C. Glycerol stocks were produced by resuspension of either overnight liquid or plate cultures in 1-2 ml sterile TSB + 20% (v/v) glycerol, and stored in a 1.5 ml screw top microcentrifuge tube. Strains were recovered from storage by plating onto appropriate solid medium containing antibiotics if required, and grown overnight at 37°C, or 30°C if the strain contained a temperature sensitive plasmid.

2.3 Stock Buffers

2.3.1 Church Gilbert's Buffer

Church Gilbert's buffer contains 0.5 M Na_2HPO_4/NaH_2PO_4 (pH 7.4), 7% SDS and 1mM EDTA. In order to achieve the correct pH both Na_2HPO_4 and NaH_2PO_4 must be used in the correct ratio. For 1l buffer, 51.11 g Na_2HPO_4 and 21.84 g NaH_2PO_4 were used to give a pH of 7.4. Due to sedimentation of the solution at room temperature, the buffer was heated to 65°C before use.

2.3.2 10x MOPS

10x MOPS buffer was made using RNase free dH_2O for use with RNA. 0.2 M MOPS was diluted in DEPC treated dH_2O and the pH was corrected to 7.0. After this solution was autoclaved, 50 mM RNase free sodium acetate and 1 mM RNase free EDTA were added, and the solution was stored at room temperature.

2.3.3 Sephadex column elution buffer

Sephadex column elution buffer contains 10 mM Tris-HCl (pH 7.5), 1 mM EDTA and 0.1% SDS in dH_2O.

2.3.4 SDS Polyacrylamide gel electrophoresis (SDS-PAGE) buffers

For SDS PAGE Buffer A, a solution of 9.08% (w/v) Tris and 0.2% (w/v) SDS was made up in dH_2O and the pH was corrected to 8.8 before use. For SDS PAGE Buffer B, a solution of 3.02% (w/v) Tris and 0.2% (w/v) SDS was made up in dH_2O and the pH was corrected to 6.8 before use.

2.3.5 2x SDS-PAGE loading buffer

2x SDS-PAGE loading buffer contained 1 ml of 1 M Tris buffer (pH 6.8), 4 ml of 10% SDS, 1 ml of 1% Bromophenol Blue solution, 2 ml of glycerol and 200 mM DTT. This was stored at room temperature.

2.3.6 SDS Running Buffer

A 10x stock solution of SDS running buffer was made up in dH_2O containing 3.02% (w/v) Tris, 14.4% (w/v) glycine and 1% (w/v) SDS. Before use as a running buffer this was diluted 1:10 in dH_2O.

2.3.7 SDS PAGE Coomassie Brilliant Blue Stain and De-stain

0.25% (w/v) Coomassie Brilliant Blue R250 powder was diluted in a solution containing 10% (v/v) Glacial acetic acid and 45% (v/v) methanol. This was used to stain proteins in an SDS PAGE gel. SDS PAGE de-stain solution contained 7.5% glacial acetic acid and 20% methanol.

2.3.8 SDS PAGE Drying solution

A solution of 30% (v/v) methanol and 5% (v/v) glycerol was used as a drying solution for SDS PAGE gels.

2.4 Bacterial Strains and Plasmids

2.4.1 Bacterial strains

Table 2.1, Table 2.2, and Table 2.3 indicate the strains used in this study alongside their genotype or infection source, where applicable.

2.4.2 Plasmids

The plasmids used in this study can be found in Table 2.4 and Appendix Figure A-1.

2.5 DNA Manipulation

2.5.1 *S. aureus* Chromosomal Preparation (Ausubel *et al.*, 1995)

5 ml of LuB was inoculated and incubated shaking overnight at 37°C. 1.5 ml of this culture was centrifuged at 12,000 xg for 5 minutes to pellet the bacteria and the supernatant was discarded. The pellet was resuspended in 250 µl of 10 mM Tris buffer, pH 8.5, with 100 µg/ml of lysostaphin and 1 µg/ml RNase A added. This was incubated in a 37°C water bath until the cells had lysed and the mixture was clear and viscous. 2.5 µl of 20 mg/ml stock of Proteinase K was added, mixed well and then 27 µl of 10% SDS was added. Once mixed, this was incubated in a 37°C water bath for 20-30 minutes. 97 µl of 5 M NaCl and 81 µl of CTAB (Cetyl Trimethly Ammonium Bromide) preheated at 65°C was added, the tube was inverted several times and incubated at 65°C for 20 minutes. An equal volume of 24:1 chloroform:isoamylalcohol (approx. 450 µl) was added and the mixture was centrifuged at 12,000 xg for 10

Table 2.1 Defined bacterial strains used in this study

Strain	Genotype	Reference
E. coli		
TOPO 10	F⁻*mcr*AΔ (*mrr-hsd* RMS-*mcr*BC) ϕ80 *lacZ*ΔM/5Δlac-X74 *deo*R *rec*AI *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L *end*A1 *nup*G	Invitrogen
BL21 (DE3)	F⁻ *omp*T *gal dcm lon hsd*S_B(r_B^- m_B^-) λ(DE3 [*lac*I *lac*UV5-T7 *gene* 1 *ind*1 *sam*7 *nin*5])	(Studier & Moffatt, 1986)
Mach I	Δ*rec*A1398 *end*A1 *ton*A Φ80Δ*lac*M15 Δ*lac*X74 *hsd*R(r_K^- m_K^+)	Invitrogen
S. aureus		
RN4220	Restriction deficient 8325	Laboratory stock
8325-4	NTCC8325 cured of prophages	(Horsburgh *et al.*, 2001a)
Newman	Clinical MSSA isolate	(Duthie & Lorenz, 1952)
MRSA 252	EMRSA-16	(Holden *et al.*, 2004)
MRSA PM64	MRSA 252 clonal varient	(Moore & Lindsay, 2002)
Mu50	VISA strain	(Kuroda *et al.*, 2001)
SH1000	8325-4 with *rsbU* mutation repaired	(Horsburgh *et al.*, 2002a)
RF122	Bovine mastitis associated clone	(Herron *et al.*, 2002)
RN6390B	NTCC8325 cured of three prophages	(Peng *et al.*, 1988)
ATTC 12600	Wild type	(Sitthisak *et al.*, 2005)
WCUH29	Wild type	Laboratory stock
CDC8	Wild type	Dr. Jodi Lindsay

Table 2.2 Bacterial strains carrying isogenic mutations or reporter plasmids

Strain	Genotype	Reference
8325-4*Δfur*	*Δfur::Tet*	(Horsburgh *et al.*, 2001b)
8325-4*ΔperR*	*ΔperR::Kn*	(Horsburgh *et al.*, 2001a)
8325-4*ΔmntR*	*ΔmntR::Tet*	(Horsburgh *et al.*, 2002b)
8325-4*ΔgapA*	*ΔgapA::Tet*	This study
RN4220*ΔgapB*	*ΔgapA::Spc*	This study
8325-4*ΔgapB*	*ΔgapA::Spc*	This study
8325-4*ΔgapA/ ΔgapB*	*ΔgapA::Tet ; ΔgapB::Spc*	This study
8325-4*ΔgapR*	*ΔgapR::Spc*	This study
Newman *ΔgapR*	*ΔgapR::Spc*	This study
8325-4 (pSK5645)	β-lactamase reporter negative control	This study
8325-4 (pSK5645$_{lysR}$)	*lysR* reporter positive control	Dr. J. Baker, unpublished
8325-4 (pSK5645$_{BBs}$)	*gapR* reporter BB short	This study
8325-4 (pSK5645$_{BBL}$)	*gapR* reporter BB Long	This study
8325-4 (pSK5645$_{8325\text{-}4s}$)	*gapR* reporter 8325-4 short	This study
8325-4 (pSK5645$_{8325\text{-}4L}$)	*gapR* reporter 8325-4 Long	This study
8325-4 (pSK5645$_{Newmans}$)	*gapR* reporter Newman short	This study
8325-4 (pSK5645$_{NewmanL}$)	*gapR* reporter Newman Long	This study
8325-4 (pSK5645$_{PM64}$)	*gapR* reporter PM64 (Long)	This study

Table 2.3 Wild type *S. aureus* clinical isolates used in this study

Strain	Infection Source	Reference
B1203012	Septicaemia	Queens Medical Centre, Nottingham
B2202016	Septicaemia	Queens Medical Centre, Nottingham
B2503017	Septicaemia	Queens Medical Centre, Nottingham
B0903007	Septicaemia	Queens Medical Centre, Nottingham
B1003003	Septicaemia	Queens Medical Centre, Nottingham
B1703012	Septicaemia	Queens Medical Centre, Nottingham
SA R 4/7	Sputum	Queens Medical Centre, Nottingham
SA R157/7	Sputum	Queens Medical Centre, Nottingham
SA D81/7	Wound	Queens Medical Centre, Nottingham
SA D196/7	Wound	Queens Medical Centre, Nottingham
SA 4523-7	Urine	Queens Medical Centre, Nottingham
48064	CAPD* infection	NHS University Hospital's Leicester
47979	CAPD infection	NHS University Hospital's Leicester
63505	CAPD infection	NHS University Hospital's Leicester
66155	CAPD infection	NHS University Hospital's Leicester
65985	CAPD infection	NHS University Hospital's Leicester
66195	CAPD infection	NHS University Hospital's Leicester
65991	CAPD infection	NHS University Hospital's Leicester
38963	Bovine milk	(Sung *et al.*, 2008)
982BL	Bovine milk	(Sung *et al.*, 2008)
C00759	Bovine milk	(Sung *et al.*, 2008)
C123/5/05-09	Bovine milk	(Sung *et al.*, 2008)
C01865	Bovine milk	(Sung *et al.*, 2008)
C00595	Bovine milk	(Sung *et al.*, 2008)
C00704	Bovine milk	(Sung *et al.*, 2008)
C01801	Bovine milk	(Sung *et al.*, 2008)
C01719	Bovine milk	(Sung *et al.*, 2008)
C01771	Bovine milk	(Sung *et al.*, 2008)

*(CAPD = Continuous ambulatory peritoneal dialysis)

Table 2.4 Plasmids used in this study

Plasmid	Description	Reference
pCR2.1	*E. coli* T-cloning vector. Amp and Kan resistant. *lacZ* blue/white selection	Invitrogen
pGEMT	*E. coli* T-cloning vector. Amp resistant. *lacZ* blue/white selection	Promega
pDG1513	Tet cassette containing vector	(Guerout-Fleury *et al.*, 1995)
pDG1726	Spec cassette containing vector	(Guerout-Fleury *et al.*, 1995)
pSK5645	*E. coli – S. aureus* shuttle vector. β-lactamase transcriptional reporter. Amp (*E. coli*) and Cm (*S. aureus*) resistant.	(Grkovic *et al.*, 2003)
pSK5645$_{lysR}$	pSK5645 containing *lysR* promoter	This study
pSK5645$_{BBs}$	pSK5645 containing short *gapR* promoter region from strain BB	This study
pSK5645$_{BBL}$	pSK5645 containing long *gapR* promoter region from strain BB	This study
pSK5645$_{8325\text{-}4s}$	pSK5645 containing short *gapR* promoter region from strain 8325-4	This study
pSK5645$_{8325\text{-}4L}$	pSK5645 containing long *gapR* promoter region from strain 8325-4	This study
pSK5645$_{Newmans}$	pSK5645 containing short *gapR* promoter region from strain Newman	This study
pSK5645$_{NewmanL}$	pSK5645 containing long *gapR* promoter region from strain Newman	This study
pSK5645$_{PM64}$	pSK5645 containing *gapR* promoter region from strain PM64	This study
pMAD	Mutagenesis plasmid. Amp (*E. coli)* and Ery (*S. aureus*) resistance genes. Constitutive β-galactosidase (*bgaB*) for screening. Temperature sensitive Gram positive origin of replication (pE194^{ts}).	(Arnaud *et al.*, 2004)
pMAD/*ΔgapA::tet*	pMAD mutagenesis plasmid containing mutant *gapA* allele	This study
pMAD/*ΔgapB::spec*	pMAD mutagenesis plasmid containing mutant *gapB* allele	This study
pMAD/*ΔgapR::spec*	pMAD mutagenesis plasmid containing mutant *gapR* allele	This study
pLEICS03	*E. coli* based protein expression plasmid. IPTG inducible promoter and *lacI* repressor gene. Amp and Kan resistant.	Protex, University of Leicester
pLEICS03/*gapA*	pLEICS03 containing *gapA* open reading frame for protein expression	This study
pLEICS03/*gapB*	pLEICS03 containing *gapB* open reading frame for protein expression	This study
pLEICS03/*gapR*	pLEICS03 containing *gapR* open reading frame for protein expression	This study

minutes. The top layer was transferred to a new microcentrifuge tube and an equal volume of Isopropyl-alcohol (IPA) was added. This was centrifuged for 10 minutes at 12,000 xg to pellet the DNA and the supernatant was removed. The DNA was then re-suspended in 50-100 µl of sterile dH_2O.

2.5.2 Plasmid Extraction

Plasmids were extracted from *E. coli* strains using the E.N.Z.A Plasmid Mini Prep Kit I (Omega Bio-tek), following manufacturer's instructions. For Staphylococcal plasmid extractions, 100 µg/ml of lysostaphin was added to the cells alongside Buffer 1 and incubated at 37°C until the cells had lysed completely.

2.5.3 Gel electrophoresis and imaging

1% (w/v) electrophoresis grade agarose (Lonza) was added to 1x TAE (Tris-Acetate-EDTA) buffer and heated until the agarose had fully dissolved. Ethidium bromide was then added to a final concentration of 0.5 µg/ml. 5x TBE loading buffer was added to samples and loaded onto an agarose gel alongside an appropriate size standard marker. The size markers used in this study are λ/*Hin*dIII digest (Promega), ΦX174/*Hae*III digest (Promega) and Hyper ladder I (Bioline). Gels were run at 80 V until the sample had moved a suitable distance down the gel, and then samples were visualised using a UV transilluminator (Gene Genius Bio Imaging System, Syngene).

2.5.4 Restriction Digests

Restriction digests were carried out in either a 20 µl or a 50 µl total volume depending on requirements. The mixture was made up of 10% buffer (either enzyme specific or Multi-core buffer), 1% BSA (if required), a maximum of 10% restriction enzyme and

the DNA to be digested (variable depending on concentration), and this was made up to the total volume using dH_2O. Reactions were incubated at 37°C for 1-3 hours. When cloning required a single restriction site to be used, the vector was de-phosphorylated to prevent religation of the vector alone. The digested vector was incubated in 1x Antarctic phosphatase buffer alongside 5U Antarctic phosphatase for 1 hour at 37°C, followed by 15 minutes at 65°C to deactivate the enzyme. The vector was then used as normal for the ligation.

2.5.5 Gel extraction

Digested DNA fragments were separated by gel electrophoresis, and the required fragments were extracted from the gel and purified using the Zymoclean Gel DNA Recovery kit (Zymo Reseach Corp.), following manufacturer's instructions.

2.5.6 Ligation

Ligations were carried out at either a 1:1 or 3:1 ratio of insert:vector. 150 ng of vector was used, and volumes for insert and vector were calculated based on DNA concentrations. 0.1 volumes of T4 DNA ligase buffer and 400U T4 DNA ligase (New England Biolabs) were added in a total volume of 30 µl and ligations were incubated overnight at 4°C.

2.5.7 T-cloning with pCR2.1® vector or pGEM®-T

T-cloning experiments were carried out using either The Original TA Cloning® Kit (Invitrogen) or the pGEM®-T vector system (Promega), depending on the restriction enzyme requirements of subsequent experiments. In each case, PCR products were directly cloned into the vector following the manufacturer's instructions, and

recombinant plasmids were transformed into *E. coli* TOPO 10 and selected on LuA containing Amp and X-gal.

2.6 PCR reactions and purification

2.6.1 Standard PCR reaction mix

Each PCR reaction contained 5 µl 2x FailSafe™ PCR Premix-D buffer (Cambridge Bioscience) containing MgCl and dNTP's, 1 µM Forward primer, 1 µM Reverse primer, 0.5 µl KapA Taq polymerase (KAPA Biosystems), and 2.5 µl chromosomal or plasmid DNA template (100-150 ng). All reactions were assembled on ice in 0.2 ml PCR tubes.

2.6.2 Standard PCR program – amplicons up to 1 kb

An initial denaturing step at 94˚C for 3 minutes was followed by 25 cycles of a 30 second denaturing step at 94˚C, a 30 second annealing step at Tm˚C (dependant on primer), and a 30 second extension step at 62˚C. A final step at 72˚C for 3 minutes completed the program, to ensure complete elongation of the PCR product.

2.6.3 Standard PCR program – amplicons greater than 1 kb

An initial denaturing step at 94˚C for 5 minutes was followed by 25 cycles of a 60 second denaturing step at 94˚C, a 60 second annealing step at Tm˚C (dependant on primer), and a 3 minute extension step at 62˚C. A final step at 72˚C for 5 minutes completed the program, to ensure complete elongation of the PCR product.

2.6.4 High Fidelity PCR

For high fidelity PCR, the proof reading polymerase Bio-X-act Long Taq polymerase (Bioline) was used in place of KapA Taq and all extension steps were adjusted to 68˚C in the PCR programs.

2.6.5 Colony PCR

For colony PCR reactions 2.5 µl DNA was replaced with sterile dH_2O and a single *E. coli* colony was lifted from an agar plate using a sterile pipette tip, patched onto a fresh agar plate, and then added to the PCR reaction mix directly. An additional 10 minute 96°C step was added to the start of the appropriate PCR program, and the patched colony plate was incubated overnight at 37°C to produce a stock of each colony tested.

2.6.6 PCR purification

PCR products were purified using the E.N.Z.A Cycle Pure kit (Omega Biotek), following manufacturer's instructions.

2.6.7 Primers

All primers used in this study are listed in Table 2.5.

2.7 DNA sequencing and Analysis

2.7.1 Standard DNA sequencing reaction

Sequencing reactions were performed on approximately 200 ng plasmid DNA or 20-40 ng of purified PCR product (up to 1 kb). 1 µl of 3.2 µM sequencing primer and 4 µl of diluted Big-Dye (0.5 µl Big Dye V3.1, 1.75 µl sequencing buffer and 1.75 µl dH_2O) were added to the DNA template and the final volume was made up to 10 µl using

Table 2.5. Primers used in this study.

Primer name	Sequence 5'- 3' (*restriction sites*)	Enzymes	Application
M13F	TGTAAAACGACGGCCAGT		pUC based plasmid screening
M13R	CAGGAAACAGCTATGACC		pUC based plasmid screening
pSK5645seq	TCATTTAACTCTTTGGCATG		pSK5645 screening
pSK5645F	CGCTCCTGAGTAGGAC		pSK5645 screening
GapBF	GG*GGATCC*GCTAATGATAAGTAGTATTTAG	*Bam*HI	*gapR* promoter including STAR elements
GapRCF	CCC*GGATCC*CCTGACTTTAATTGGAAAAGC	*Bam*HI	*gapR* promoter excluding STAR elements
GapSRB	GG*GGATCC*GTAAATAAGGATATATCACAAC	*Bam*HI	*gapR* promoter
lysR F	ACAT*GGATCC*GCATTTCCAACTAACCCG	*Bam*HI	*lysR* β-lactamase reporter positive control
lysR R	TTGC*AAGCTT*CTTTTATTTATAGATTCAAGC	*Hind*III	*lysR* β-lactamase reporter positive control
GapRFusF	GAAATTTATTTTTTCAGTCAATTACTACTAATG		BB *gapR* reporter fusion primer
GapRFusR	CGCCTATCACTTTAAATAAAAAAGTCAGTTAAT		BB *gapR* reporter fusion primer
Gap4R	TGCAAATAATTTTACAG		*ΔgapA* mutagenesis
Gap30f	GG*TCTAGA*TTCGTACCAGCCAGAGGT	*Xba*I	*ΔgapA* mutagenesis
Gap16f	TGGTGATGCTGATAATA		*ΔgapA* mutagenesis screening
PGKSR	CAACTGGTTTATGTGGATC		*ΔgapA* mutagenesis screening
tetNxF	AA*GGCGCC*ATGCTAACATAGCATTACGG	*Nar*I	Tetracycline cassette
tetNxR	AA*GGCGCC*CGATTTAGAAATCCCTTTGAG	*Nar*I	Tetracycline cassette
GapB5F	GG*GGATCC*CCTTGAGATTGTTAGAAGA	*Bam*HI	*ΔgapB* mutagenesis fragment A
GapB5R	GG*GCTAGC*CATTAACTATTCCAAACTG	*Nhe*I	*ΔgapB* mutagenesis fragment A
GapB3F	GGG*CATATG*GCATTATTCCTACTTCTAC	*Nde*I	*ΔgapB* mutagenesis fragment B
GapB3R	GAAAACTGCTCTCTTGTG		*ΔgapB* mutagenesis fragment B
GapB2F	GAAGTGTGGGTTGTATAC		*ΔgapB* mutagenesis screening
gapB1R	GCTAACAATTGATCAACATC		*ΔgapB* mutagenesis screening
SpecF	GGG*GCTAGC*GATATAAAATAGGTACTAATC	*Nhe*I	Spectinomycin cassette
SpecR	GGG*GCTAGC*GC*CATATG*CAAGGGTTTATTG	*Nhe*I; *Nde*I	Spectinomycin cassette
GapR 3'F	AAAA*CCCGGG*CACAAGGTCAAATTGTCC	*Xma*I	*ΔgapR* mutagenesis
SpecR NoTT	GGG*GCTAGC*AAA*CCCGGG*TGTTTCCACCATTTTTTC	*Nhe*I; *Xma*I	Spectinomycin cassette, no terminator
GapR expF	TACTTCCAATCCGTGAAAGACTTATTGCAAGCACA		GapR pLEICS03 expression construct
GapR expR	TATCCACCTTTACTGTCATCTTATTCAAGTATTATCTTTGCT		GapR pLEICS03 expression construct
GapA expF	TACTTCCAATCCATGGCAGTAAAAGTAGCAATTAAT		GapA pLEICS03 expression construct

Table 2.5 continued

Primer name	Sequence 5'- 3'(*restriction sites*)	Enzymes	Application
GapA expR	TATCCACCTTTACTGTCATTATTTAGAAAGTTCAGCTAAGTA		GapA pLEICS03 expression construct
GapB expF	TACTTCCAATCCATGTCAACGAATATTGCAATTAAT		GapB pLEICS03 expression construct
GapB expR	TATCCACCTTTACTGTCAGAAGTCAGAGTTAGGCTATAAATTA		GapB pLEICS03 expression construct
gapAPF	CACAGATCTGGAAGGCCATTATAATGGCAG		GapA transcriptional probe
gapAPR	CACTCTAGAGCGGAGAAGCGTTTGTGC		GapA transcriptional probe
GapR probe F	GTGAAAGACTTATTGCAAG		*gapR* transcriptional probe
GapR probe R	GGGGCTAGCCCAGTTACAGCAACTATC		*gapR* transcriptional probe
GapB probe F	GAATGGTATTACGTATTGC		*gapB* transcriptional probe
GapB probe R	GTGCTCCAATTTGCTCAG		*gapB* transcriptional probe
HprK probe F	CACATTATGCGTCAGATAG		*hprK* transcriptional probe
HprK probe R	CCCACGTTTAACTAATTCC		*hprK* transcriptional probe
Orf_{0733} probe F	CCACCAGTGTTATTGCC		orf_{0733} transcriptional probe
Orf_{0733} probe R	CTGCATCCATCTGAATCC		orf_{0733} transcriptional probe
Hla F	CACGTATAGTCAGCTCAG		*hla* transcriptional probe
Hla R	GGTAGTTGCAACTGTACC		*hla* transcriptional probe
Fur1F	AGGTACAGTTCACTTAGATG		*fur* transcriptional probe
Fur1R	CGCGCAATAGATTTAGCAG		*fur* transcriptional probe
CsoR F	GTCTTTAATCAATTTTTGAAAA		*csoR* transcriptional probe
CsoR R	GTATAGTAAGGAATGTAAATG		*csoR* transcriptional probe
16SF	GATCCTGGGTCAGGATG		*16S* transcriptional probe
16SR	CTAGAGTTGTCAAAGGATG		*16S* transcriptional probe
HprK STARS F	CCTACTCTTACATCTCTTC		*hprK* STAR element region
HprK STARS R	GTCAATCTAGAGTAGTTAAAC		*hprK* STAR element region
Orf_{0733} STARS F	CTAGAACTTAGTACGTATC		orf_{0733} STAR element region
Orf_{0733} STARS R	CATAAATCAATGTCCTAGG		orf_{0733} STAR element region
GapRT1	CAGTTGCTCACCTTCAGC		*gapR* transcript mapping primer 1
GapRT2	CAGAACGCAGTACACGTTC		*gapR* transcript mapping primer 2 (nested)
GapBRT2	CCATGTGTCGTATCGTAATTG		*gapB* transcript mapping primer
EapR	CTTGTACCGTTCACAGTGATTG		*Eap* transcript mapping primer
GapR PE+FAM	[6FAM]CAGTCATATCCATATGTTCGCTTAAACTTCGAC		FAM labelled *gapR* transcript mapping
EapPE+FAM	[6FAM]GGCTTGTACCATTCACAGTGATTGTATATGG		FAM labelled *eap* transcript mapping

Table 2.5 continued

Primer name	Sequence 5'- 3' (*restriction sites*)	Enzymes	Application
arc up	TTGATTCACCAGCGCGTATTGTC		MLST loci
arc dn	AGGTATCTGCTTCAATCAGCG		MLST loci
aro up	ATCGGAAATCCTATTTCACATTC		MLST loci
aro dn	GGTGTTGTATTAATAACGATATC		MLST loci
glp up	CTAGGAACTGCAATCTTAATCC		MLST loci
glp dn	TGGTAAAATCGCATGTCCAATTC		MLST loci
gmk up	ATCGTTTTATCGGGACCATC		MLST loci
gmk dn	TCATTAACTACAACGTAATCGTA		MLST loci
pta up	GTTAAAATCGTATTACCTGAAGG		MLST loci
pta dn	GACCCTTTTGTTGAAAAGCTTAA		MLST loci
tpi up	TCGTTCATTCTGAACGTCGTGAA		MLST loci
tpi dn	TTTGCACCTTCTAACAATTGTAC		MLST loci
yqi up	CAGCATACAGGACACCTATTGGC		MLST loci
yqi dn	CGTTGAGGAATCGATACTGGAAC		MLST loci

dH_2O. This was cycled in a PCR machine using the following program. An initial 10 second 96˚C denaturing step was followed by 29 cycles of a 10 second denaturing step at 96˚C, a 10 second annealing step at 50˚C, and a 4 minute extension step at 60˚C. A final step at 60˚C for 5 minutes completed the program, to ensure complete elongation of the sequencing product.

2.7.2 DNA sequencing reaction clean up

To clean up the reaction 1 µl of 2.2% SDS was added to 10 µl of sequencing product, then mixed and heated to 98˚C for 5 minutes. This was allowed to return to room temperature before it was purified using a Performa DTR Gel filtration catridge (Edge BioSystems). Sequencing gels were run and analysed using an Applied Biosystems 3730 sequencer by PNACL (Protein Nucleic Acid Chemistry Laboratory) at the University of Leicester, and returned as .abi and .seq files.

2.7.3 Sequence analysis tools

DNA sequencing files (.abi) were visualised in Chromas v1.45, a program that converts the sequencing data into a readable trace with base pair calling. This program was used to extract the DNA sequence data and import it into either DNA* Edit Sequence (DNA Star), Vector NTI (Invitrogen), or Clone Manager (Sci-Ed) for more sophisticated analysis and manipulation of the sequence. Multiple sequence alignments were constructed using the Neighbour-joining algorithm. Genome wide sequence motif searches were carried out using the RSA-tools Genome-scale DNA-pattern search (http://rsat.ulb.ac.be/rsat/; van Helden, 2003; Thomas-Chollier *et al.*, 2008).

2.7.4 Multi-locus sequence typing (MLST) of strains

Primer pairs for each of the seven MLST loci, *araC, aroE, glpF, gmk, pta, tpi* and *yqiL,* were used to PCR amplify an internal fragment of each gene from a template of chromosomal DNA. PCR reactions were then directly used as a template for sequencing, using both the loci specific forward and reverse primers. Forward and reverse sequencing reactions were compared to check for sequencing errors, and sequences were trimmed down to a region recognised by the MLST database. For each strain, each sequenced loci was used to query the *S. aureus* MLST database (http://saureus.mlst.net/) to determine specific alleles, and the sequence type (ST) of the strain was determined by the specific combination of MLST loci alleles present.

2.8 Transformation and Transduction of DNA

2.8.1 Preparation of Electrocompetent *E. coli*

5 ml of LuB was inoculated with either *E. coli* TOPO 10 or *E. coli* DE3 and incubated shaking overnight at 37°C. The culture was diluted 1/100 in pre-warmed LuB and incubated shaking at 37°C to an optical density (OD) of 0.5 at 600 nm. The cells were rapidly chilled on ice and centrifuged at 4°C to pellet the cells. The cells were resuspended in 1 volume of ice cold dH_2O, and centrifuged at 4°C. This washing step was repeated, and the pelleted cells were then resuspended in 1/2 volume of ice cold 10% glycerol. This suspension was centrifuged at 4°C and the cells were then resuspended in 1/20 volume of 10% glycerol. After a final 4°C centrifugation, the electro-competent cells were resuspended in 1/200 volume of 10% glycerol and stored at -80°C.

2.8.2 Preparation of Electrocompetent *S. aureus*

5 ml of TSB was inoculated with *S. aureus* RN4220 and incubated shaking overnight at 37°C. The culture was diluted 1/50 in fresh TSB and incubated shaking at 37°C to an OD of 0.4 at 600 nm. The culture was centrifuged at 4°C and the pellet resuspended in 1 volume of 0.5 M sucrose. The suspension was centrifuged at 4°C and the pellet resuspended in 1/2 volume of 0.5 M sucrose and kept on ice for 30 minutes. The suspension was centrifuged at 4°C and the pellet resuspended in 1/100 volume of 0.5 M Sucrose and stored at -80°C.

2.8.3 Transformation of Plasmid DNA

For transformation of plasmid DNA into *E. coli*, 10 µl of plasmid was dialysed for 30 minutes to remove excess salt using a 0.02 µm nitrocellulose membrane filter (Millipore) and then added to 50 µl of electro-competent *E. coli* cells and placed in an electroporation cuvette. This was given a single pulse at 25 µF, 1.5V and 1000 Ω. 1 ml of SOC medium was added immediately after and the mixture was transferred to a universal and incubated shaking at 37˚C for 1 hour. The cells were plated onto LuA selection plates and incubated overnight at 37˚C.

For transformation of plasmid DNA into *S. aureus*, 15 µl of plasmid was dialised for 30 minutes to remove excess salt using a 0.02 µm nitrocellulose membrane filter (Millipore) and then added to 40 µl of electro-competent *S. aureus* RN4220 cells and left at room temperature for 30 minutes. This was given a single pulse at 25 µF, 2.5V and 200Ω. 900 µl of SMMP50 medium was added immediately after and the mixture was transferred to a universal and incubated shaking at 37°C for 1 hour. 100 µl of cells were plated onto LuA selection plates and incubated overnight at 37°C.

2.8.4 Phage Lysate production

5 ml of LuB with required antibiotics was inoculated with the donor strain of bacteria and cultured overnight shaking at 37°C. This culture was diluted in fresh LuB to an OD of 0.05 at 600 nm, and after addition of 0.2 ml 1 M $CaCl_2$ the culture was incubated shaking at 37°C to an OD of 0.2 at 600 nm. 10 ml of this was subcultured into 25 ml of fresh LuB + $CaCl_2$, inoculated with 1 ml of bacteriophage phi11 and incubated shaking at 37°C for a minimum of 4 hours until the culture had completely lysed. The culture was centrifuged at 3200 xg for 10 minutes to pellet any cell debris, filter sterilised and stored at 4°C. The titre of the lysate was calculated by spotting 10 µl of serially diluted lysate (10^{-1} to 10^{-6}) onto a LuA plate lawned with either the donor or the recipient bacteria and incubating overnight at 37°C.

2.8.5 Phage Transduction

The recipient bacteria was grown overnight in 20 ml LK broth with required antibiotics. This was centrifuged at 3200 xg for 10 minutes and the pellet was resuspended in 1 ml of LK broth. 500 µl of the suspension was added to 1 ml of LK broth containing 10 mM $CaCl_2$ and 500 µl donor phage lysate. As a control 500 µl of bacterial suspension was added to 1.5 ml LK broth containing 10 mM $CaCl_2$ without phage. These were incubated for 25 minutes in a 37°C water bath and a further 15 minutes in a 37°C orbital shaker. Both suspensions were centrifuged for 3200 xg for 10 minutes and the pellets were resuspended in 1 ml of 0.02 M Na-citrate and left on ice for 2 hours. 100 µl of each suspension was plated out onto LK agar plates containing required antibiotics and 0.05% Na-citrate, and incubated for 12-72 hours at required temperature.

2.9 Mutagenesis of *S. aureus* (Arnaud *et al.*, 2004).

2.9.1 Construction of pMAD/*ΔgapA::tet*

The *gapA* open reading frame and 1282 bp of flanking sequence was PCR amplified with Bio-X-act long (Bioline) from 8325-4 chromosomal DNA using primers Gap30F and PGKSR. This product was T-cloned into the vector pCR2.1 (Invitrogen) to create pCR2.1/*gapA*, and a 402 bp internal fragment of the open reading was excised by digestion with *Cla*I. Primers TetNxF and TetNxR were used to PCR amplify a terminator less tetracycline resistance cassette from plasmid pDG1513 (Guerout-Fleury *et al.*, 1995) carrying a *Nar*I restriction site at both the 5' and 3' ends. This PCR product was subsequently digested with *Nar*I and ligated into the *Cla*I site of the digested pCR2.1/*gapA* to create the plasmid pCR2.1/*ΔgapA::tet*. This was transformed into *E. coli* TOPO 10 and recombinant plasmids were screened using primer pairs M13R and TetNxR, and M13F and TetNxF. The *gapA* flanking regions were sequenced using primers M13F and M13R to confirm there were no errors before proceeding. The 3.5 kB *ΔgapA::tet* region was excised from pCR2.1 by digestion with *Bam*HI and *Sal*I, and ligated into the *Bam*HI and *Xba*I sites of the *E. coli – S. aureus* shuttle vector pMAD (Table 2.4; Appendix Figure A-1; Arnaud *et al.*, 2004) to create the plasmid pMAD/*ΔgapA::tet*. This was transformed into *E. coli* TOPO10, and as pMAD carries an intact *lacZ* gene, transformants were screened on LuA plates containing Amp, Tet and X-gal. Blue colonies were selected and the recombinant plasmid was confirmed by restriction digest and PCR before transformation into *S. aureus* RN4220 at 30°C.

2.9.2 Construction of pMAD/*ΔgapB::spc*

Primer pairs GapB5F + GapB5R and GapB3F + GapB3R were used to PCR amplify the 5' and 3' ends of the *gapB* open reading frame, alongside a total of 609 bp of flanking

sequence, from 8325-4 chromosomal DNA with Bio-X-act Long polymerase. The products were then T-cloned into vectors pGEM-T (Promega) and pCR2.1 (Invitrogen) respectively, to give plasmids pGEM-T/*gapB5'* and pCR2.1/*gapB3'*. Together these PCR products covered the entire *gapB* open reading frame with the exception of an internal 94bp deletion. A spectinomycin cassette, with its own promoter and terminator, was PCR amplified from pDG1726 (Guerout-Fleury *et al.*, 1995) using primers SpecF and SpecR, and was subsequently digested with *Nhe*I and ligated into the same site in plasmid pGEM-T/*gapB5'* to create the plasmid pGEM-T/*gapB5'-Spec*. This was transformed into *E. coli* TOPO10 and recombinants were selected for on LuA containing Amp and Spc, and confirmed by PCR. The *gapB5'-Spec* region was excised from pGEM-T by restriction digest with *Bam*HI and *Nde*I, and ligated into pCR2.1/*gapB3'* upstream of the *gapB3'* region to give the construct pCR2.1/*ΔgapB::spc.* This was transformed into *E. coli* TOPO10 and selected for on LuA containing Kan and Spc, and recombinant plasmids were confirmed by PCR using primers M13R and SpecR, and GapB3R and SpecF. Plasmid pCR2.1/*ΔgapB::spc* was sequenced using M13F and M13R primers to confirm that the flanking sequences did not contain errors before proceeding. The 2.5kB *ΔgapB::spc* was excised from pCR2.1 by digestion with *Bam*HI and *Sal*I, and ligated into the *Bam*HI and *Xba*I restriction sites of pMAD (Arnaud *et al.*, 2004) to produce pMAD/*ΔgapB::spc,* which was screened as previously described and transformed into *S. aureus* RN4220.

2.9.3 Construction of pMAD/*ΔgapR::spc*

Construction of 8325-4*ΔgapR::spc* followed the same approach as described for 8325-4*ΔgapB::spc*, with a few minor exceptions. The 5' and 3' regions of the *gapR* open reading frame, with 1766bp of flanking DNA was PCR amplified and T-cloned as

previously described using primer pairs GapSRB + GapRPR and GapR3F + GapAPR respectively to give plasmids pGEM-T/*gapR5'* and pCR2.1/*gapR3'*. A spectinomycin cassette was PCR amplified from pDG1726 (Guerout-Fleury *et al.*, 1995) using primers SpecF and SpecRNoTT, which retained the promoter but excluded the terminator. This product was digested using *Nhe*I and ligated into the same site on plasmid pGEM-T/*gapR5'* to create plasmid pGEM-T/*gapR5'*-Spec, and the *gapR5'-spec* fragment was excised from pGEM-T using the enzymes *Bam*HI and *Xma*I and subsequently ligated into these sites on plasmid pCR2.1/*gapR3'* to produce pCR2.1/*ΔgapR::spc*. This construct was confirmed and sequenced as described for pCR2.1/*ΔgapB::spc*. Plasmid pMAD/*ΔgapR::spc* was constructed and confirmed as previously described and transformed into *S. aureus* RN4220.

2.9.4 Mutagenesis of *gapA*, *gapB* and *gapR*

Mutagenesis of *ΔgapA* and *ΔgapR* were carried out directly in strain 8325-4, after phage transduction of the mutagenesis plasmids, while mutagenesis of *ΔgapB* was performed in strain RN4220 and then transduced by phage into strain 8325-4. As mutating the *gapA* loci has previously been shown to effect GAPDH activity, the mutagenesis protocol was carried out in Tris succinate minimal medium as previously described (Taylor & Heinrichs, 2002), while mutagenesis of both *gapB* and *gapR* was carried out in Luria-Bertani medium. Strains carrying the mutagenesis pMAD constructs were plated onto LuA containing selection antibiotic, Ery and X-gal, and a single colony was used to inoculate 10 ml appropriate liquid medium and incubated with aeration for 2 hours at 30°C, followed by 6 hours at 42°C. The culture was plated onto solid medium containing selection antibiotic and X-gal and incubated overnight at 42°C. Double homologous recombination events were screened for selection antibiotic resistance, Ery

sensitivity and were white when grown on media containing X-gal, as described by Arnaud *et al,* 2004 and demonstrated in Figure 2-1. Mutants were confirmed by PCR using flanking chromosomal primers (Gap16f and PGKSR for *gapA,* GapB2F and GapB1R for *gapB* and GapBF and PGKSR for *gapR*), together and in conjunction with resistance cassette primers. All primer sequences used in this procedure can be found in Table 2.5.

2.9.5 Construction of 8325-4*ΔgapA/ΔgapB*

In order to produce an 8325-4*ΔgapA/ΔgapB* double mutant, the *ΔgapB::spc* mutant allele was transduced from strain RN4220*ΔgapB* into strain 8325-4*ΔgapA* with bacteriphage phi11. Selection of transductant colonies was carried out using Tris succinate minimal medium, and double mutants were confirmed by PCR as described for the single mutants.

2.10 Northern Blot Transcriptional Analysis

2.10.1 DEPC treatment of dH_2O

In order to produce RNase free dH_2O for all solutions where RNA stability was crucial, 0.1% (v/v) DEPC (di-ethyl-pyrocarbonate) solution was added to dH_2O in a fume hood and mixed thoroughly. This was left overnight in a fume hood with the lid on loosely, and then autoclaved to destroy any residual DEPC before use. Any buffers, such as MOPS, that were required to be RNase free were made up with DEPC treated dH_2O in place of dH_2O.

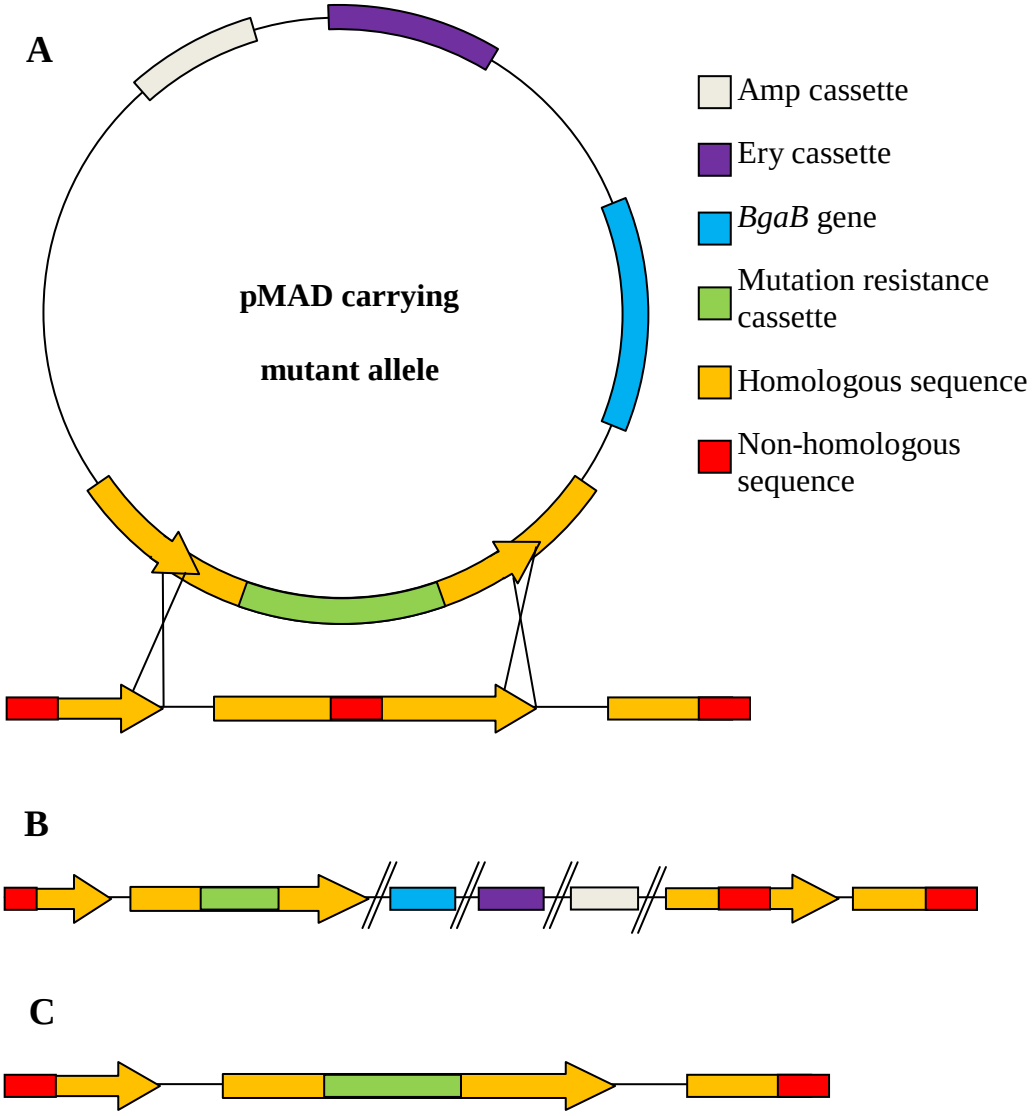


Figure 2-1 - Schematic representation of possible homologous recombination events during mutagenesis.

A) Homologous sequences within the pMAD construct and the chromosomal DNA align and recombination can occur at any point within the homologous sequences.

B) Plasmid integration - result of a single cross-over. The entire pMAD construct is introduced into the chromosome and wildtype allele is retained. Colonies will be resistant to erythromycin and will be blue.

C) Allelic exchange - result of a double cross-over. The wildtype copy of the gene is "replaced" by the mutant allele, and the rest of the pMAD construct is lost. Colonies will be white, sensitive to erythromycin and resistant to mutant selective marker.

2.10.2 RNA extraction from *S. aureus* (Schmitt *et al.*, 1990)

For iron limited and replete conditions *S. aureus* cultures were grown overnight in 10 ml CRPMI, resuspended in a fresh 10 ml of CRPMI to an OD of 0.1 at 600 nm and incubated statically at 37°C in 5% CO_2. Cultures were grown for 6 hours until growth reached late exponential phase. For controlled carbon source conditions strains were grown overnight in TSB medium, then washed and resuspended in 10 ml of TM to an OD of 0.05 at 600 nm and grown for 4 hours until growth reached mid exponential phase. Once an appropriate growth phase was reached, cells were pelleted by centrifugation and resuspended in 1 ml of RNA*later* (Ambion) and incubated overnight at 4°C. Suspensions were centrifuged for 5 minutes at 12,000 xg and the supernatant was discarded, before freezing the cells at -80°C for at least 1 hour. After thawing at room temperature the bacteria were resuspended in 400 µl of RNase free 10 mM Tris buffer, pH 8.5, with 30 µl of 10 mg/ml lysostaphin and incubated at 37°C for no longer than 15 minutes. 80 µl of 10% (w/v) SDS was added to the suspension and vortexed for 20 seconds, before addition of an equal volume of saturated phenol pH 4 (Fischer Scientific). The suspension was vortexed for a further 30 seconds and centrifuged at 12,000 xg for 10 minutes. The aqueous phase was transferred to a fresh microcentrifuge tube, an equal volume of phenol was added, and the suspension was vortexed for 30 seconds and centrifuged at 12,000 xg for 10 minutes. The aqueous phase was transferred to a clean tube and 500 µl phenol:chloroform:IAA (25:24:1) was added before a final round of vortexing and centrifugation. The aqueous phase was again transferred to a fresh tube, and the RNA was precipitated by addition of 2 volumes of cold 100% ethanol and incubation overnight at -80°C. Precipitated RNA was washed in 70% ethanol, dried and resuspended in 50 µl of DEPC treated dH_2O. RNA

concentration was determined using a Nanodrop ND-1000 spectrophotomoeter (Thermo Scientific).

2.10.3 Formaldehyde gel electrophoresis

For each denaturing formaldehyde agarose gel 1.8 g agarose was heated in 130.5 ml DEPC dH_2O until fully dissolved and 15 ml 10xMOPS (0.2 M MOPS, pH 7, 50 mM Sodium acetate, 1 mM EDTA, RNase free), 4.5 ml formaldehyde (37%) and 3 µl 10mg/ml EtBr were added before casting gel in trays and combs prewashed in IMS. 10 µg of each RNA sample was equalised to a constant volume and 5x RNA loading buffer (4 ml 10x MOPS, 3.084 ml deionised formamide, 2 ml glycerol, 720 µl 37% formaldehyde, 80 µl 0.5 M EDTA, 16 µl RNase free saturated aqueous bromophenol blue solution, 0.1 ml DEPC dH_2O) was added. Samples were heated at 65°C for 10 minutes and chilled on ice before loading into the gel, and then run at ~100V in 1x MOPS until approximately 2/3 through the gel. When necessary, 4 µl Lonza RNA size standard ladder was run alongside samples to determine transcript sizes.

2.10.4 Northern Blotting

Once run on a formaldehyde denaturing gel, the RNA was visualised using a Syngene UV imager as previously described, and where transcript sizes were required a UV ruler was placed alongside the gel to allow quantification of how far each transcript had moved through the gel. RNA was transferred onto Magna charge nylon transfer membrane (GE Water & Process Technologies) by Northern blotting overnight in 20x SSC with gel-sized filters soaked in 2x SSC. After cross linking the RNA to the membrane by exposure to 70,000 µJ cm^{-2}, the membrane was placed in a hybridisation tube and prehybridised at 65°C for at least 1 hour in Church's Gilbert's buffer.

DNA probes were synthesised by PCR using the primer pairs in Table 2.5, and purified by gel extraction as previously described. 30 ng purified PCR product was boiled in 18 µl dH_2O for 5 minutes and then rapidly cooled on ice for 5 minutes. This was added to a chilled tube containing 5 µl oligolabelling buffer, 1 µl purified BSA and 1 µl Klenow fragment of DNA *Pol1*. 2.5 µl of [α^{32}P]-labelled dCTP was added and incubated at 37°C for 1-2 hours. The labelled probe was purified using Illusta NICK Sephadex columns (GE Healthcare) and Sephadex column elution buffer, boiled on a heating block for 10 minutes and then chilled on ice for 10 minutes before being added to the prehybridised membrane and incubated rotating overnight at 65°C.

After overnight incubation with a DNA probe, the blot was rinsed in 3x SSC with 0.1% SDS that had been preheated to 65°C, and then washed in the buffer for 30 minutes rotating at 65°C. Washes were repeated at 30 minute intervals until the wash buffer was no longer radioactive. Washed membranes were removed from their hybridisation tubes and rinsed briefly in 2x SSC prewamed at 65°C, before being transferred onto a membrane sized piece of 3MM paper that had been soaked in 2x SSC. The membrane was sealed alongside the 3MM paper in cling film and transferred to an autoradiograph cassette with intensifiers. Autoradiograph film (Medical X-ray Film, FujiFilm) was added and exposed at either room temperature (very high signal) or -80°C (low signal), and subsequently developed by treatment for 1 minute in developer solution, followed by 30 seconds in neutraliser solution and a final 1 minute in fixer solution.

To quantitatively measure loading of samples, probes were stripped from each Northern blot by repeated washing with boiling 0.1% SDS, and subsequently re-probed with [α^{32}P]-labelled 16S DNA probe. Densitometric analysis of autoradiographs was

performed using ImageJ 1.41 (NCBI; http://rsbweb.nih.gov/ij/), to compare the level of expression of the test probe to that of the *16S* probe and give a quantitative measure of expression between samples.

2.11 Cloning and Expression of Recombinant Protein

2.11.1 Cloning of GapA, GapB and GapR into pLEICS03

Using expression primer pairs indicated in Table 2.5, the open reading frame of *gapA*, *gapB* and *gapR* were PCR amplified from strain 8325-4 and cloned into the *E. coli* expression plasmid pLEICS03 (Table 2.4; Appendix Figure A-1; Protex, University of Leicester). After ligation, plasmids were transformed into the *E. coli* strain Mach1 and potential transformants were screened by colony PCR using cloning primers. Positive plasmids were extracted and sequenced using both the forward and reverse cloning primers, and the plasmids were transformed into the *E. coli* protein expression strain BL21 (DE3) and screened by colony PCR to confirm successful transformation.

2.11.2 Protein expression

E. coli expression strains were grown overnight in LuB containing 50 μg/ml Kan, and 10 ml of this starter culture was then used to inoculate 500 ml of LuB + Kan and incubated at 37°C until the OD reached 0.6 at 600 nm. 100 μM IPTG (Isopropyl-β-$_{D}$-Thiogalactopyranoside) was added to the culture to induce recombinant protein expression and this was incubated overnight at 20°C. The cells were harvested by centrifugation at 3200 xg for 15 minutes at 4°C (Beckman Avanti J20-XP), resuspended in 30 ml of buffer 1 (20 mM Tris, 20 mM Imidazole and 500 mM NaCl pH 8.0) with 50 μL of Protease Inhibitor Cocktail Set III (Calbiochem) and frozen at -20°C.

2.11.3 Protein extraction

When frozen cell suspensions had thawed, 20 µL of Lysonase Bioprocessing Reagent (containing Lysozyme and Benzonase Nuclease) (Merck) was added and suspensions were incubated stirring at room temperature for 10 minutes. Sonication was used to lyse the cells and the supernatant was extracted from the cell debris by centrifugation (30 minutes, 12,000 xg, 4 °C). A pre-equilibrated 5ml Hi-Trap Nickel Sepharose (GE Healthcare) column was used to purify the proteins. The supernatant was loaded onto the column, and the recombinant proteins were eluted using a linear gradient of buffer 2 (20 mM Tris, 500 mM imidazole and 500 mM NaCl pH 8.0). Fractions were run on an SDS-PAGE gel and the protein concentrations were quantified using the Bradford Standard Assay (see below).

2.11.4 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) gel

Proteins were visualised by separating and staining a fraction of each wash and elute sample by SDS-PAGE. First a 10% separating layer was made by adding 2.7 ml SDS-PAGE Buffer A, 1.83 ml Protogel 30% acrylamide solution (Geneflow), 765 µl dH_2O, 100 µl 1% Ammonium Persulphate (APS) and 15 µl TEMED (N-N-N`-N`-tetramethylethylenediamine, Sigma Aldrich). This was used to cast a 1 mm width gel between two glass plates, previously scrubbed with SDS and rinsed in dH_2O to remove any trace of proteins. IPA was overlaid onto the gel until it had fully polymerised, and then removed and washed in dH_2O, before the addition of a 5% stacking layer. The stacking later contained 1 ml SDS-PAGE Buffer B, 616 µl dH_2O, 330 µl Protogel 30% acrylamide solution, 50 µl 1% APS and 4 µl TEMED. A comb was placed into this layer before polymerisation to cast wells in the stacking layer.

Purified protein samples were mixed in an equal volume of 2x SDS protein loading buffer and boiled for 5 minutes before loading onto the gel. 10 µl of pre-stained protein ladder (Protomarker, National Diagnostics) was loaded alongside the samples to determine approximate protein size. Gels were run in 1x SDS running buffer at a constant 20 mAmps until the loading buffer had run completely through the gel. Protein bands were visualised by staining the gel overnight in Coomassie Brilliant Blue stain, followed by washes in SDS-destain in cycles of 1 hour until clear protein bands appeared and the edges of the gel were no longer blue. Where necessary, proteins were identified by extracting a small portion of the protein band and analysing by MALDI-ToF mass spectrometry By PNACL (University of Leicester).

2.11.5 Bradford Standard Assay

Protein concentration was determined by the Bradford Standard Assay. 10 µl of protein sample was aliquoted in triplicate into a 96 well plate, alongside 10 µl aliquots of BSA solution of known concentrations (ranging from 8 µg/ml to 0.5 mg/ml). 1 part Bio-Rad protein assay dye reagent (Bio-Rad) was diluted in 4 parts ddH_2O and filtered through a 125 mm circle of Whatman paper #1. 190 µl filtered Bio-Rad reagent was added to each of the sample and standard proteins using a multi-pipette and incubated at room temperature for 5 minutes. The absorbance of each well was measured at 595 nm using a FLUOstar Omega plate reader (BMG Labtech), and a standard curve was produced from the readings for known concentrations of BSA. This was used to determine the concentrations of protein samples tested.

2.12 Enzyme Activity Assays

2.12.1 Whole cell GAPDH assay

The strains to be tested was streaked out from frozen glycerol stock to single colonies on solid medium, and used to inoculate 5ml of TSB medium. The cultures were incubated overnight, and the OD at 600 nm was then used to equalise the cultures for cell growth. Approximately 9 x 10^6 intact cells were washed in dH_2O and then resuspended in 1 ml of GAPDH assay buffer containing 50 mM Na_2HPO_4, 5 mM EDTA, 40 mM Triethanolamine, 2 mM Glyceraldehyde-3-phosphate (G3P), and 2 mM of cofactor NAD+ or NADP+. Samples were incubated in a 37°C water bath for 30 minutes, and then the cells were pelleted by centrifugation at 12,000 xg for 10 minutes. The supernatant was extracted and the production of NAD(P)H was detected by measuring the absorption of the suspension at 340 nm in a UV/visible Spectrophotometer (Pharmacia Biotech Ultraspec 2000).

2.12.2 Purified Protein GAPDH Assay

In a 96 well plate, 200 ng (10 µl of a 20 ng/µl solution) of protein was mixed with 190 µl GAPDH assay buffer containing 50 mM Na_2HPO_4, 5 mM EDTA, 40 mM Triethanolamine, 4 mM G3P, and 2 mM of cofactor NAD+ or NADP+. This was carried out in triplicate for each sample, and the plate was incubated at 37°C in a FLUOstar Omega plate reader (BMG Labtech). The absorption was measured at 340 nm after 30 minutes, and an average reading from the 3 wells was recorded for each sample.

2.13 Promoter Reporter Assays

2.13.1 Construction of *gapR* reporter strains

Two separate transcriptional reporter constructs were produced for each strain by PCR amplification of the putative *gapR* promoter region with and without the STAR element repeat region. Primer pairs GapBF + GapSRB (with STAR elements) and GapRCF + GapSRB (without STAR elements) were used to PCR amplify the *gapR* promoter region from chromosomal DNA preparations from strains BB, 8325-4, Newman and Mu50 using Bio-X-act Long polymerase. The primers introduced a *Bam*HI restriction site onto the 5' and 3' end of each fragment, and PCR products were subsequently digested and ligated into plasmid pSK5645 (Table 2.4; Appendix Figure A-1) at the *Bam*HI site. Recombinant plasmids were transformed into *E. coli* TOPO 10 by electroporation and screened by colony PCR using primers pSK5645F and GapSRB. Positive constructs were extracted from *E. coli* and sequenced using pSK5645F and pSK5645seq primers, before transformation into *S. aureus* RN4220. Plasmids were extracted from potential colonies and recombinant plasmids were confirmed by PCR and restriction digests. The plasmids were then transduced into strain 8325-4 using bacteriophage phi11, and subsequently extracted and screened using restriction digests and PCR.

2.13.2 Nitrocefin assay (Grkovic *et al.*, 2003)

Reporter strains were streaked to single colonies on LuA containing appropriate antibiotics and grown overnight at 37˚C, before resuspension of bacteria in 25 ml SSD medium to an OD of 0.05 at 600 nm. 20 μg/ml Cm was added to the culture, which was split into two 10 ml cultures, the first being iron deplete conditions and the second iron replete conditions with the addition of 50 μM $FeSO_4$. Cultures were grown for 16 hours

shaking vigorously, and OD at 600 nm was taken as a measure of growth. The samples were centrifuged for 8 minutes at 3200 xg at 22˚C. The pellet was washed in 10 ml 50 mM sodium phosphate buffer pH 6.5, and then resuspended in 700 µl 50 mM sodium phosphate buffer pH 6.5. The sample was then diluted to 1/100 using sodium phosphate buffer. 200µl of sodium phosphate buffer, 300 µl of the diluted cells and 200 µl of 125 µg/ml nitrocefin solution (Oxoid) were added to a microcentrifuge tube and incubated at 37˚C for 90 minutes. A blank was included that contained additional buffer in place of cells. The samples were centrifuged at 12,000 xg, and two 200 µl aliquots were transferred to a 96 well plate. The absorption at 490 nm was read for each sample on a FLUOstar Omega plate reader (BMG Labtech), and an average of the two aliquots was recorded for each sample. In order to correct for bacterial growth the following equation was used:

$$\left(\frac{1}{OD_{600}}\right) \times \left(\frac{Average\ OD_{490}}{Average\ blank}\right)$$

The corrected 490 nm absorbance (OD_{490}) values were then analysed and converted into a measure of enzyme units using the equation set out in Figure 2-2.

2.14 Growth curves

2.14.1 TSB growth curves

5 ml of TSB medium, with appropriate antibiotics, was inoculated and grown overnight at 37˚C with aeration. The culture was diluted to an OD of 0.05 at 600 nm in fresh TSB, with antibiotics where required, and split into 3 equal volumes. Either 0.75% glucose (v/v), 5 mM pyruvate or dH_2O were added to the culture, and these cultures were incubated at 37°C. The OD (600 nm) of the culture was taken every hour for 7 hours against a blank reading of medium only. A final reading was then taken after 24 hours growth. Each assay was repeated a minimum of 3 times from separate inoculums, and

$$\frac{\left(\frac{\Delta A}{\Delta T}\right)}{\varepsilon} \times Vf \times D \times \frac{Vt}{Vs} = Enzyme\ activity\ \mu mol\ min^{-1}$$

ΔA = Change in absorbance OD_{490nm}

ΔT = Time taken for change in absorbance (min)

ε = Molecular extinction coefficient variation

Vf = Final volume of sample in which OD_{490nm} taken (µl)

D = Dilution factor

Vt = Total volume of reaction sample (µl)

Vs = Volume of nitrocefin 125 µg/ml used (µl)

Figure 2-2. Equation for the calculation of enzyme units

Equation for converting absorbance at 490 nm into enzyme units for the nitrocefin assay, measured as µmol of nitrocefin hydrolysed per minute. Note that ΔA values were corrected for differences in growth of the bacteria as shown above. Molar extinction coefficient variation for nitrocefin = 15000 M/cm.

the results averaged and presented alongside the standard deviation of the data. P-values were derived using Student's T-Test, at 4 hours after inoculation.

2.14.2 TM growth curves

5 ml of TSB medium, with appropriate antibiotics, was inoculated and grown overnight at 37˚C with aeration. The culture centrifuged at 3200 xg for 5 minutes to pellet the cells, which were washed in 5 ml of TM medium before being resuspended to an OD of 0.05 at 600 nm in fresh TM medium with antibiotics where required. This inoculate was split into separate volumes and additional carbon sources were added as required, before incubation with aeration at 37°C. The OD (600 nm) of the culture was taken every hour for 7 hours against a blank reading of medium only. A final reading was then taken after 24 hours growth. Each assay was repeated a minimum of 3 times on separate days, and the results averaged and presented alongside the standard deviation of the data. P-values were derived using Student's T-Test, at 5 hours after inoculation.

2.14.3 5 hour Growth Assays

This assay was set up in the same way as the TM growth curve, except cultures were incubated undisturbed at 37°C with aeration. The OD of the culture was measured at 600 nm after 5 hours growth against a blank reading of medium only. Each assay was repeated a minimum of 3 times on separate days, and the results averaged and presented alongside the standard deviation of the data. P-values were derived using Student's T-Test.

2.14.4 H_2O_2 Survival Assay

Bacteria were grown overnight in TSB at 37°C with shaking in air, diluted to an OD of 0.05 at 600 nm in fresh TSB with appropriate antibiotics, and then split into duplicate cultures and incubated with shaking at 37°C for 4 h. The OD was measured at 600 nm and 0.03% H_2O_2 (v/v) was added to one of the duplicates; both were then incubated with aeration at 37°C and the growth was measured by OD at 600 nm both 1 and 2 hours after addition of H_2O_2. The survival of the H_2O_2 treated cultures was calculated as a percentage of growth of the untreated culture.

2.14.5 Biofilm Assay

TM medium + 1% glycerol was inoculated with bacteria and grown overnight at 37°C with aeration. The culture was diluted to an OD of 0.1 at 595 nm in fresh TM + 1% glycerol (with antibiotics as required), and 4 x 200 µl aliquots were added to 4 wells of a flat 96-well optical bottom plate (NUNC, Thermo scientific), alongside 4 x 200 µl of uninoculated TM + 1% glycerol medium. The OD of the plate was read in a plate reader at 595 nm, and then incubated statically at 37°C in 5% CO_2 for 24 hours. After growth the OD (595 nm) was taken again, and the wells were washed in PBS, air dried at 50 °C, and stained with safranine stain for 30 minutes. The wells were rinsed in H_2O and dried before reading in a plate reader at 490 nm. The data was corrected for variation in growth using the following equation:

$$\frac{(average\ test\ OD490) - (average\ blank\ OD490)}{(OD595\ after\ growth - starting\ OD595)} \times 0.1$$

2.15 Transcript Mapping

2.15.1 Radiolabelled Primer Extension

Radioactive Primer Labelling

Gene specific reverse primers were radiolabelled using γ-^{32}P ATP. 1 µl of γ-^{32}P ATP has a specific activity of 0.01 mCi, which is equal to 3.3 pmol of ATP. 5 pmol of gene specific primer, 2.5 µl 10x optikinase reaction buffer, 5 pmol γ-^{32}P ATP and 1 µl of 10U/µl Optikinase (Polynucleotide kinase, USB) were mixed in a 25 µl reaction volume and incubated at 37˚C for 30 minutes. The reaction was then terminated by incubation at 65˚C for 10 minutes. The reaction should result in approximately 60% of the primers being 5' end labelled with the radioisotope.

Radioactive Sequencing Ladder

PCR amplification was used to generate a template for sequencing of the *gapR* and *gapB* putative promoter region, while the *eap* promoter region was sequenced directly from a previously cloned reporter plasmid. Primers GapBF and GapSRB were used for *gapR* and primers GapB5F and GapBRT1 were used for *gapB*. PCR products were purified and template DNA concentrations were determined using a Nanodrop ND-1000 spectrophotomoeter (Thermo Scientific). A 17.5 µl master mix was produced containing 100 ng of template DNA, 2.5 µl of γ-^{32}P ATP labelled primer, 2 µl of Thermo Sequenase reaction buffer and 2 µl of Thermo Sequenase DNA polymerase (GE Healthcare), and 4 µl aliquots were subsequently added to PCR tubes containing 4µl of one of the following: ddATP, ddTTP, ddCTP and ddGTP. The four sequencing reactions were cycled on a PCR machine using the following program. An initial denaturing step at 95˚C for 30 minutes was followed by 30 cycles of a 30 second denaturing step at 95˚C, a 30 second annealing step at 50˚C, and a 1 minute extension

step at 72˚C. A final step at 72˚C for 5 minutes completed the program. To terminate the sequencing reactions 6 µl of each reaction was added to 4 µl of stop solution/loading buffer.

Radioactive Primer Extension

12 µg of total RNA was diluted in a volume of 10 µl in dH_2O and heated to 65˚C for 5 minutes, before cooling on ice. 4 µl of 5x reverse transcriptase buffer, 2 µl of 10mM dNTP's, 1.5 µl of γ-^{32}P ATP labelled primer, 1 µl of 0.1M DDT, 1 µl of Thermoscript reverse transcriptase (Invitrogen) and 0.5 µl of RNase inhibitor was added and the reaction was incubated (not cycled) in a PCR machine for 5 minutes at 65^{o}C followed by 20 minutes each at 55^{o}C, 60^{o}C and 65^{o}C and finally for 5 minutes at 85^{o}C to terminate the reaction. The reaction was concentrated by ethanol precipitation and resuspended in 6 µl of dH_2O and before addition of 4 µl of stop solution/loading buffer.

6% DNA sequencing gel

1x gel mix was prepared from 50 g of urea, 12 ml of 50% long ranger® gel solution, 5 ml of 20x glycerol tolerant buffer and made up to 100 ml with dH_2O. Before use the gel mix was filtered and all the sequencing gel equipment was washed thoroughly in IMS. The upwards facing glass plate was coated with 1 ml of Gel Slick to allow easy removal after the gel has run. 100 µl of 25% APS and 100 µl of TEMED were added to the gel mix and the gel was cast and left to polymerise overnight. After pre-running the gel for 30 minutes at a constant 90 W in 1x glycerol tolerant buffer, samples were heated to 100^{o}C for 8 minutes and 2 µl of each of the sequencing ladder reactions was loaded into the gel alongside 2 µl of primer extension product. The samples were run at a constant 50 W until the loading buffer reached the bottom of the gel. The gel was removed from

the glass plates, dried and placed in a radiograph cassette with an autoradiograph film (Medical X-ray Film, FujiFilm) and exposed at -80°C for upto 5 days.

2.15.2 FAM labelled Primer Extension

10 nM of fluorescently labelled gene specific primer (5'-FAM label) was added to 20 µg total RNA and adjusted to 20 µl using dH_2O. This was heated to 70°C for 5 minutes, rapidly chilled on ice for 10 minutes, heated to 58°C for 20 minutes and finally left to cool at room temperature for 15 minutes. 1x AMV Reverse Transcriptase buffer (Promega), 1mM dNTP mix, 40 U RNase inhibitor and 25 U of AMV Reverse Transcriptase enzyme (Promega) were added to the suspension, made up to a final volume of 30 µl with dH_2O and incubated at 42°C for 1 hour to produce gene specific cDNA. The reaction was enriched by denaturing the product at 96°C for 5 minutes, followed by addition of 40 U RNase inhibitor and 25 U of AMV Reverse Transcriptase enzyme (Promega) and a second incubation at 42°C for 1 hour. Template RNA was digested for 30 minutes at 37°C after addition of 10 ng of RNase A. The primer extension product was concentrated by ethanol precipitation and then dissolved in 9.5 µl formamide and mixed with 0.5 µl of GeneScan® size LIZ 500 or LIZ 1200 size standard (Applied Biosystems). Samples were run on an Applied Biosystems 3730 sequencer by PNACL (Protein Nucleic Acid Chemistry Laboratory) at the University of Leicester, and analysed using GeneScan® software (Applied Biosystems).

2.16 *Galleria mellonella* infection model

Galleria mellonella larvae were purchased from Vine House Farms Ltd, Spalding and were stored at 4°C. Prior to use, larvae were maintained at room temperature overnight. Bacterial strains were grown overnight at 37°C with aeration in 5 ml volumes of CY

broth (Casamino acids (10 g/l), yeast extract (10 g/l), NaCl (5.9 g/l)) supplemented with appropriate antibiotics. Bacteria were pelleted by centrifugation at 13,000 x g for 2 min and then washed and resuspended in phosphate buffered saline (PBS) to an optical density of 0.125 at 600 nm. Approximately $5x10^7$ bacteria in a 20 μl volume were injected into the larval hemolymph adjacent to the 4th pro-leg using a syringe with a 29 gauge needle and a Tridak Stepper repetitive dispenser (Intertronics, Kidlington). PBS alone was used as a negative control. Groups of 10 infected or control larvae were maintained at 37°C and viability determined every 24h over a 72h period. The results of three independent experiments were combined and survival curves were calculated using the Kaplan-Meier method. Significant differences between survival curves were calculated using the Logrank test.

Chapter 3 Metabolic Functions of the GAPDH homologues GapA and GapB in *S. aureus*

3.1 Introduction

GAPDH enzymes play an essential role in carbon metabolism in a vast number of species, ranging from simple prokaryotes to higher eukaryotes. The enzymatic function of GAPDH is the conversion of G3P to 1,3-dPG during glycolysis and also the reverse reaction of the conversion of 1,3-dPG to G3P during gluconeogenesis. Therefore the action of GAPDH is essential in both the catabolism of glucose as a carbon source, ultimately leading into the TCA cycle and energy generation, and also in gluconeogenesis, producing *de novo* glucose from a range of carbon sources in a low glucose environment.

Many species have a single GAPDH enzyme which is able to catalyse both the glycolytic and gluconeogenic GAPDH reactions. This is true in higher eukaryotes such as mammals and also several bacterial species such as s*treptococci* and some *Lactobacilli* species (Winram & Lottenberg, 1996; Naterstad *et al*., 2007). However there are also many species which have multiple GAPDH homologues. In some cases, such as the Gram positive bacterium *B. subtilis*, the homologues each have opposing roles in carbon metabolism, with one enzyme functioning in glycolysis and another in gluconeogenesis (Fillinger *et al*., 2000). In other species, such as *E. coli*, one homologue is a true GAPDH and acts in both glycolysis and gluconeogenesis, while the remaining homologues have an entirely different function (Hidalgo *et al*., 1996; Boschi-Muller *et al*., 1997).

S. aureus has two GAPDH homologues which share approximately 40% amino acid identity, known as *gapA* and *gapB*. It has been assumed that, like in the case of *B. subtilis*, GapB is a gluconeogenic GAPDH; however no biological evidence for this has been published to date (Kohler *et al*., 2005; Chatterjee *et al*., 2009; Seidl *et al*., 2009). Direct comparison of carbon metabolism between these two species does not take into account the vastly different environments they inhabit. *S. aureus* is a highly adaptable pathogenic organism that inhabits a wide range of sites within the host, whereas the non-pathogenic environmental organism *B. subtilis* is commonly found in soil, meaning the available carbon sources for the two species may be very different. As such aspects of carbon metabolism may be very varied between these species, and it is possible that in *S. aureus* GapB is not a gluconeogenic GAPDH at all.

A previous publication suggests that *S. aureus* GapB does not function as a gluconeogenic GAPDH as recombinant GapB protein cloned from a *S. aureus* bovine mastitis isolate had very little NAD+ dependent GAPDH activity and no NADP+ dependent GAPDH activity (Goji *et al*., 2004), whereas GapB protein purified from *B. subtilis* shows a 50-fold higher catalytic efficiency with NADP+ than with NAD+ (Fillinger *et al*., 2000). It has also been shown that GapB does not have glycolytic GAPDH activity *in vivo*, as NAD+ dependent GAPDH activity is completely lost in a *gapA* deletion mutant. However NADP+ dependent (gluconeogenic) activity was not tested in this study (Taylor & Heinrichs, 2002).

The primary aim of this chapter is to investigate the metabolic functions of the *S. aureus* GAPDH homologues and define their roles in the growth and metabolism of this important pathogen. Key objectives are to investigate the enzymatic properties of both

GapA and GapB *in vitro* and *in vivo*, and determine how the regulation of these proteins is affected by the availability of different carbon sources. Further objectives include determining the importance of each of the genes in virulence and pathogenesis in the *Galleria mellonella* invertebrate model of infection, and also to start investigating potential "moonlighting" functions of these proteins outside of central metabolism in *S. aureus*. The majority of the data presented in this chapter has recently been accepted for publication.

3.2 Metabolic function of recombinant GapA and GapB proteins

3.2.1 Cloning and purification of recombinant *S. aureus* GAPDH proteins

In order to investigate the enzymatic function of GapA and GapB *in vitro*, recombinant proteins were cloned and purified for each gene. Primer pairs GapAexpF + GapAexpR and GapBexpF + GapBexpR were used to PCR amplify the open reading frames of *gapA* and *gapB* respectively from strain 8325-4, which were subsequently cloned into the *E. coli* protein expression vector pLEICS03 (Protex, University of Leicester). As a negative control for subsequent assays, the glycolytic operon regulator GapR was also cloned into this vector, using primers GapRexpF and GapRexpR. GapR is a putative regulatory protein and should not have any GAPDH enzyme activity. Vector pLEICS03 contains an IPTG inducible T7 promoter located upstream of a cleavable His_6-tag sequence, which introduces an N-terminal His_6 tag to the recombinant proteins for purification. If required, this tag can be cleaved from the protein using a TEV protease.

Each of the expression constructs were cloned into the *E. coli* expression strain BL21 (DE3), and protein expression was optimised by altering both IPTG concentration, temperature and incubation time (Figure 3-1A). Optimal protein expression conditions were found to be incubation of the cultures overnight at 20°C in the presence of 100 mM IPTG. Cells were then harvested and the tagged recombinant proteins were purified from the supernatant using NiNTA affinity chromatography as described in Section 2.11.3. The purified recombinant protein fractions were run on an SDS-PAGE gel, and the protein bands were extracted and sent to PNACL (University of Leicester) for protein identification by MALDI-ToF mass spectroscopy (Figure 3-1B). Protein concentrations were then quantified using the Bradford Standard assay, and proteins were stored at -80 °C.

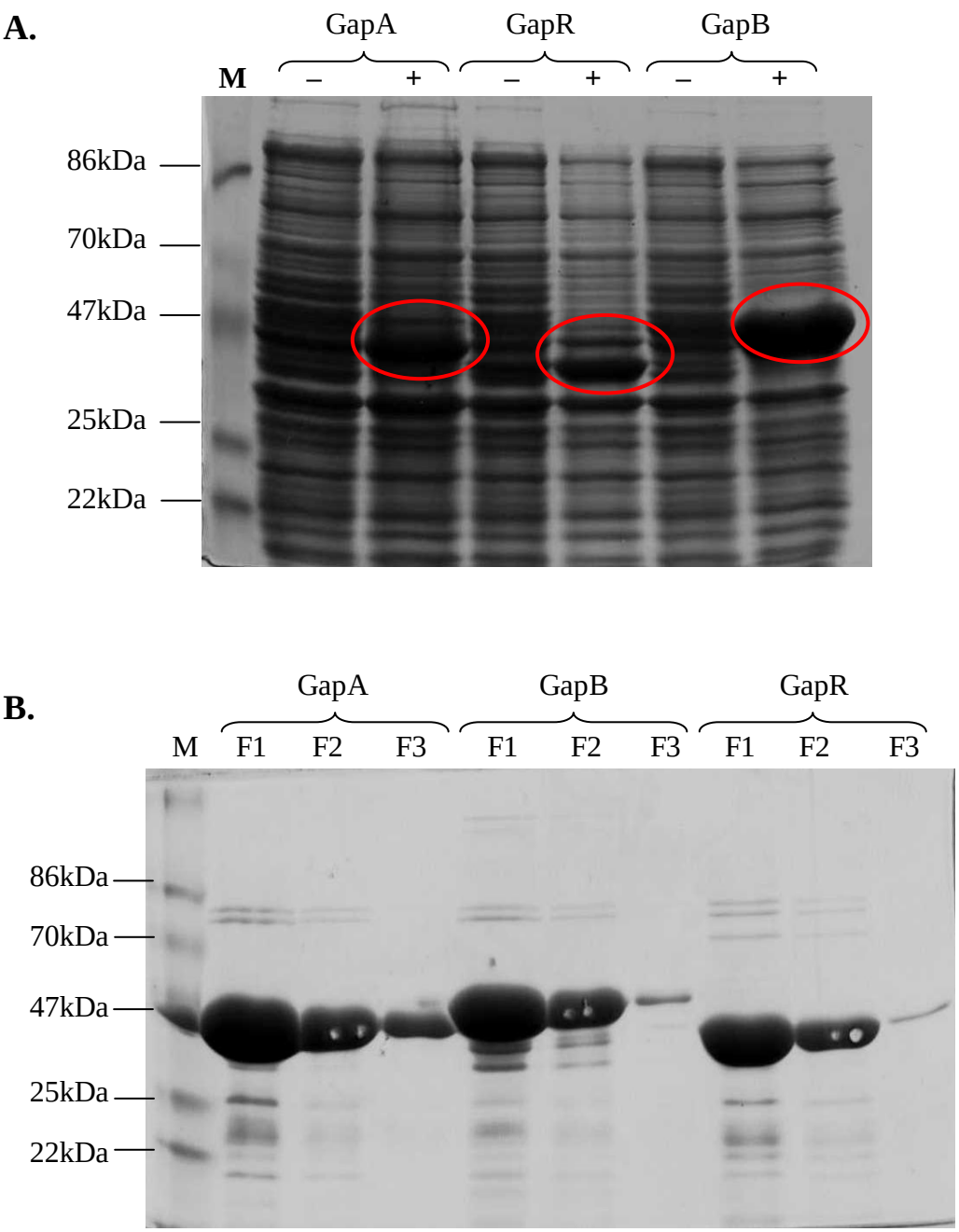


Figure 3-1. GapA, GapB and GapR recombinant protein expression

SDS-PAGE gel of (A) whole cell protein extracts from un-induced (–) and induced (+) recombinant protein constructs in *E. coli* BL21 (DE3), and (B) eluted fractions 1, 2 and 3 of purified recombinant GapA, GapB and GapR proteins. Protein expression was induced by the addition of 100 mM IPTG and cells were incubated for overnight at 20°C. Induced proteins are highlighted in red circles.

3.2.2 Recombinant GapB protein does not have NAD+ or NADP+ dependent GAPDH activity

To determine the roles of GapA and GapB in glycolysis and gluconeogenesis, the recombinant GapA, GapB and GapR proteins were assayed for GAPDH activity in the presence of either NAD+ or NADP+ as a cofactor. Enzyme activity was measured by a change in absorbance at 340 nm, which is caused by the conversion of NAD+ to NADH (or NADP+ to NADPH). The GapA protein showed very high levels of NAD+ dependent GAPDH activity, but had ~10-fold less NADP+ dependent GAPDH activity ($p < 0.001$) (Figure 3-2), showing that GapA has a reduced affinity for NADP+ and therefore has less gluconeogenic GAPDH activity than glycolytic activity. Both GapB and GapR showed no detectable GAPDH activity with either cofactor (Figure 3-2). The GAPDH activities of each of the proteins was unaffected by the removal of the His_6-tag, which indicates that the presence of the tag is not responsible for the lack of recombinant GapB enzyme activity (data not shown). These data suggest that GapA is a glycolytic GAPDH in *S. aureus*, and also appears to retain some gluconeogenic GAPDH activity, while the GapB protein does not appear to have any detectable GAPDH activity and may not function in either pathway *in vivo*.

3.3 Mutagenesis of *ΔgapA* and *ΔgapB* provides insight in to the metabolic function of GapB *in vivo*

3.3.1 Construction of the GAPDH mutants in strain 8325-4

To further investigate the function of *S. aureus* GapB *in vivo*, and examine its contribution to *S. aureus* physiology, mutations were introduced into the *gapA* and the *gapB* loci in strain 8325-4. A deletion/insertion mutagenesis strategy was chosen for disruption of each of the genes in strain 8325-4, introducing antibiotic resistance

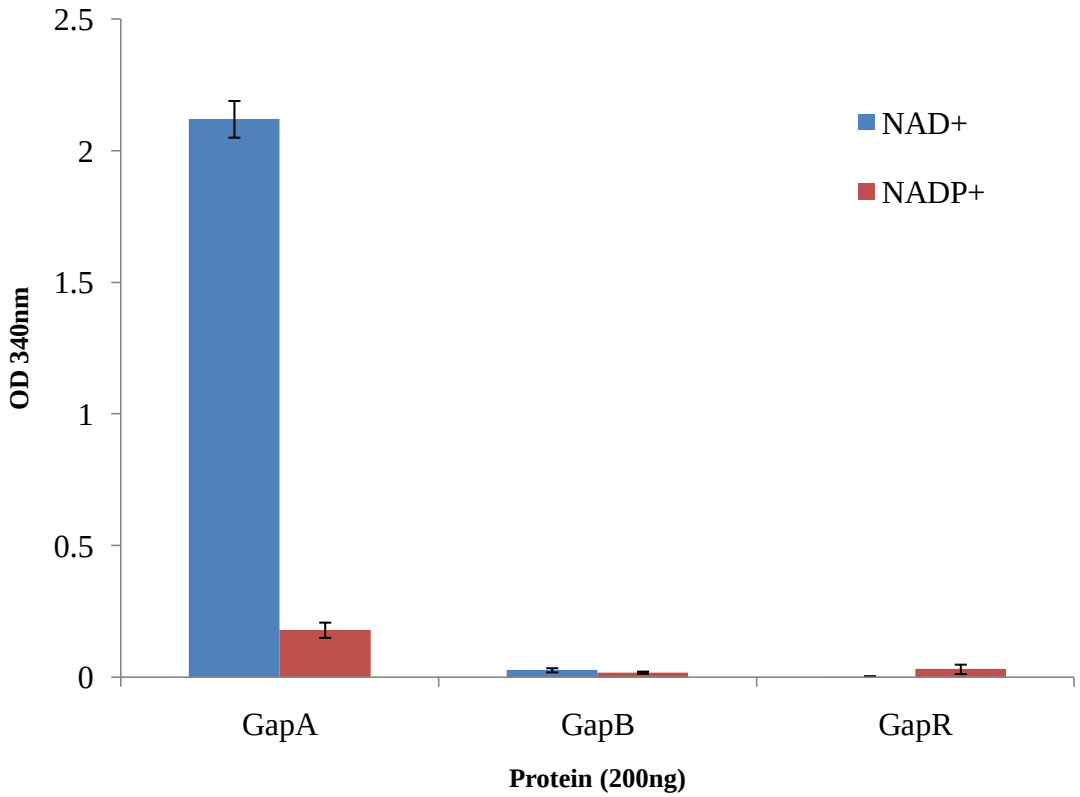


Figure 3-2. NAD+ and NADP+ dependent GAPDH activity of recombinant proteins

This graph shows the NAD+ and NADP+ dependent GAPDH activity of 200 ng of purified His_6-tagged recombinant GapA, GapB and GapR *S. aureus* proteins. Proteins were incubated alongside 4 mM G3P in the presence of either 2 mM NAD+ or 2 mM NADP+ at 37°C for 30 minutes. Enzyme activity is expressed as the change in absorbance of the reaction mixture at 340 nm, and the results are an average of 3 experiments carried out on different days. The error bars indicate the standard error of the averaged results.

cassettes into each allele to allow selection of mutant colonies. A terminator-less tetracycline cassette was introduced into the disrupted *gapA* open reading frame, which allowed the remainder of the glycolytic operon to be expressed and resulted in only the function of *gapA* being impaired. This was confirmed by northern blot analysis of the *pgk* open reading frame, located directly downsteam of *gapA*, in strain 8325-4*ΔgapA* (data not shown). As the *gapB* open reading frame does not appear to form part of an operon, a spectinomycin cassette containing both a promoter and terminator was used. The use of spectinomycin also allowed the production of a *ΔgapA*/*ΔgapB* double mutant. The *ΔgapA* mutant allele was introduced directly into strain 8325-4, as poor growth of this strain in the phage transduction medium meant that it was not possible to produce reliable phage lysate for transduction of the mutant allele. The *ΔgapB* mutant was produced in the restriction deficient strain RN4220 and then transferred into 8325-4 through phage transduction, reducing the possibility of secondary mutations in the genome. The double mutant was also produced by phage transduction of the *ΔgapB* mutation into strain 8325-4*ΔgapA*. During mutagenesis and selection, both the *ΔgapA* and *ΔgapA*/*ΔgapB* double mutants required medium lacking glucose, due to the selective pressure of disrupting glycolysis in these strains (Taylor & Heinrichs, 2002). Mutagenesis of the *gapA* and *gapB* open reading frames was confirmed by PCR in the single and double mutants (Figure 3-3).

3.3.2 Both GapA and GapB have NADP+ dependent GAPDH activity *in vivo*

In order to determine the enzymatic function of the GAPDH proteins *in vivo*, whole cell GAPDH activity assays were carried out in strains 8325-4, 8325-4*ΔgapA*, 8325-4*ΔgapB* and 8325-4*ΔgapA/ΔgapB* grown overnight in TSB medium. Strains were each tested for NAD+ and NADP+ dependent GAPDH activity after growth to stationary phase. In

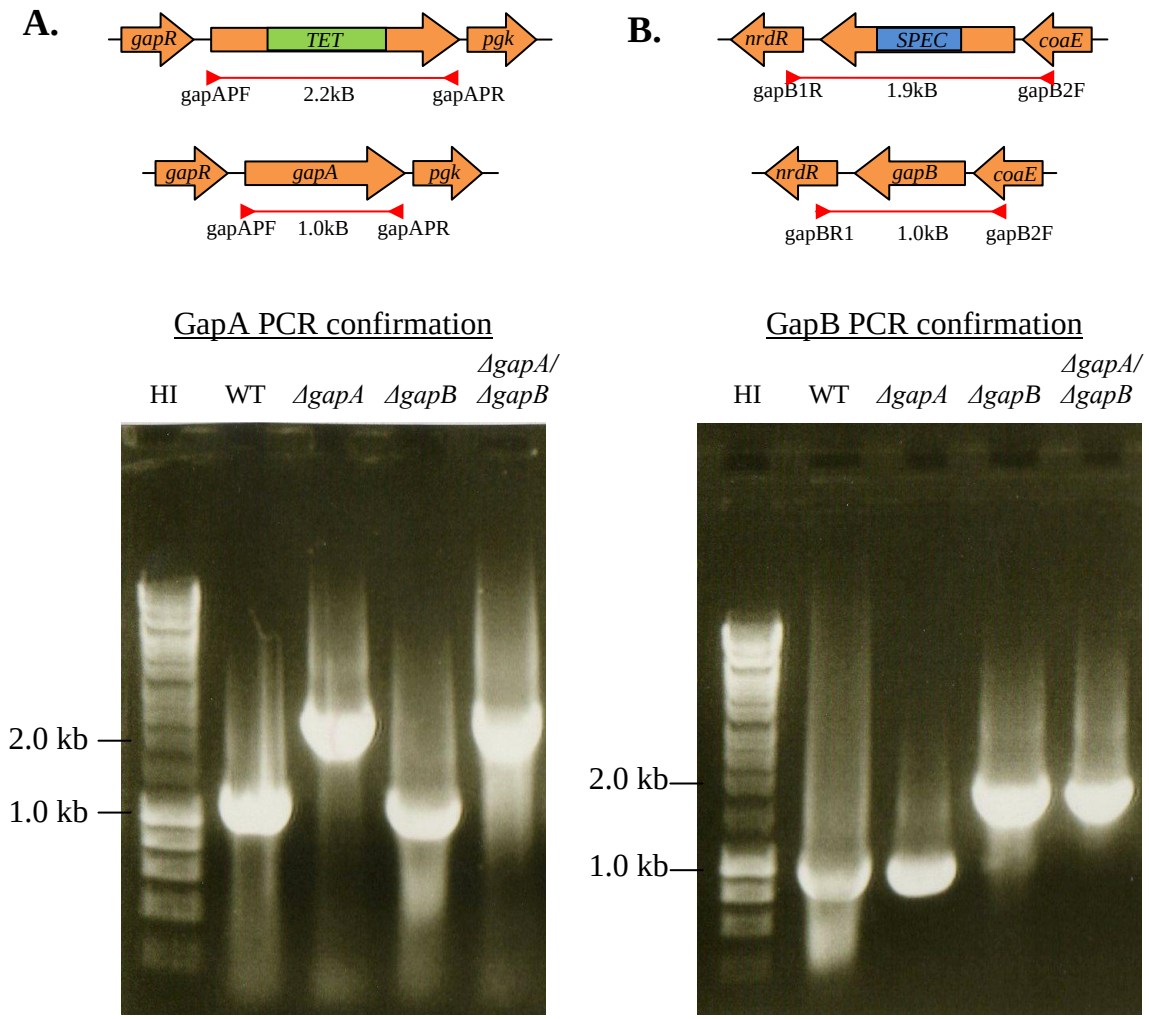


Figure 3-3. PCR confirmation of *ΔgapA*, *ΔgapB* and *ΔgapA/ΔgapB* mutagenesis in 8325-4.

PCR amplification of (A) the *gapA* open reading frame using primers GapAPF and GapAPR, and (B) the *gapB* open reading frame using primers GapB probe F and GapB probe R, in strains (1) 8325-4, (2) 8325-4*ΔgapA*, (3) 8325-4*ΔgapB* and (4) 8325-4 *ΔgapA/ΔgapB*. The sizes of the wildtype and mutant alleles are as follows: wildtype *gapA* = 1011 bp, *ΔgapA* = 2189 bp, wildtype *gapB* = 1053 bp and mutant *ΔgapB* = 1874 bp. Also included is a DNA size ladder (HI).

both the *ΔgapA* and *ΔgapA/ΔgapB* mutant strains the NAD+ dependent GAPDH activity was ~5-fold lower than that of the wild type strain ($p < 0.001$) (Figure 3-4A). NAD+ dependent GAPDH activity was not significantly different between the wild type and the *ΔgapB* mutant, or between the *ΔgapA* mutant and the double mutant (Figure 3-4A). This suggests that *in vivo*, GapA is solely responsible for NAD+ dependent GAPDH activity. Surprisingly both GapA and GapB have NADP+ dependent GAPDH activity *in vivo*. NADP+ dependent GAPDH activity is significantly lower in both the *ΔgapA* ($p < 0.001$) and the *ΔgapB* mutant ($p < 0.01$) compared to the wild type strain, by 2.5-fold and 2-fold respectively (Figure 3-4B). This is contrary to the purified protein data but shows that *in vivo* GapB has NADP+ dependent GAPDH activity that was not detected in the recombinant protein. Unexpectedly the NADP+ dependent activity in the *ΔgapA/ΔgapB* double mutant is not significantly lower than either of the single mutants, indicating that some residual NADP+ dependent GAPDH activity is still present. It is possible that another enzyme is responsible for the residual activity and may or may not play a role in gluconeogenic GAPDH function *in vivo*.

3.3.3 GapA is not responsible for both glycolytic and gluconeogenic GAPDH activity in *S. aureus*

In order to assess the role of GapA and GapB in response to changes in carbon flow, 7 hour growth curves were carried out on strains 8325-4, 8325-4*ΔgapA*, 8325-4*ΔgapB* and 8325-4*ΔgapA /ΔgapB* in the complex medium TSB with the addition of either glucose or pyruvate. TSB contains a mix of casamino acids and nitrogenous substances from the digest of casein and soybean meal, with 0.25% glucose (w/v) as the primary carbon source. Glucose and pyruvate were chosen as they both increase carbon flow

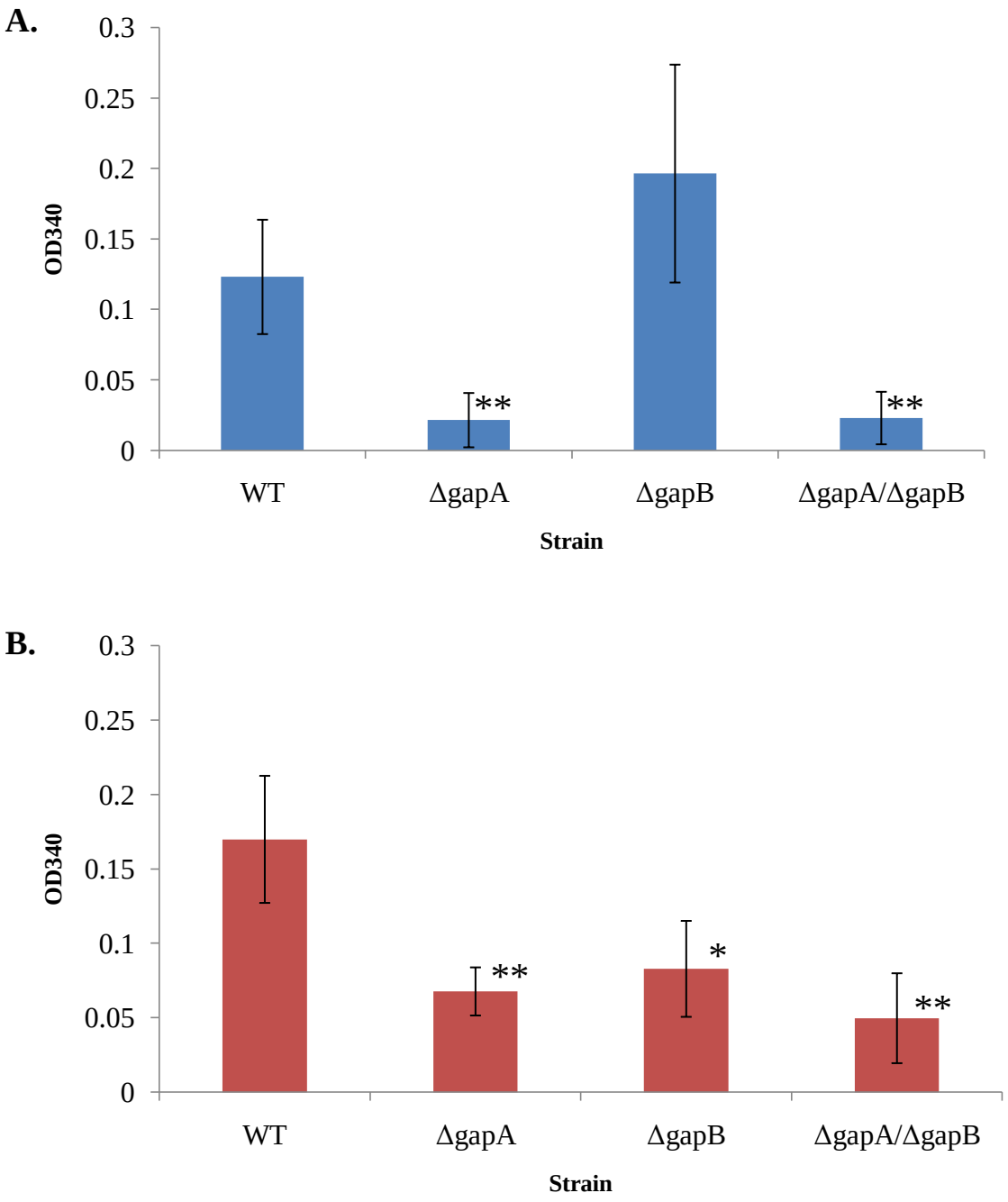


Figure 3-4. Whole cell GAPDH activity of GAPDH mutant strains.
The graphs show (A) the NAD+ dependent GAPDH activity and (B) the NADP+ dependent GAPDH activity determined from whole cell GAPDH assays of strains 8325-4, 8325-4*ΔgapA*, 8325-4*ΔgapB* and 8325-4*ΔgapA/ΔgapB*. Cells were grown to stationary phase and then assayed for GAPDH activity in the presence of 2 mM G3P and 2 mM cofactor. Enzyme activity was determined by the change in absorbance of the supernatant at 340 nm. The data presented is an average of at least 3 independent biological repeats, and the error bars indicate the standard deviation of the data. * indicates a significant difference from the wild type strain at $p < 0.01$. ** indicates a significant difference from the wild type strain at $p < 0.001$.

into the TCA cycle and increase growth rate, but the addition of pyruvate should bypass the need for a functional glycolytic pathway (Figure 3-6).

There was no significant difference between the growth of the *ΔgapB* mutant and the wild type strain in any of the growth conditions tested (Figure 3-5), which suggests that glucose utilisation is not affected in the *ΔgapB* mutant. However both the *ΔgapA* and *ΔgapA/ΔgapB* double mutants grew at a much slower rate than the wild type strain in both TSB alone and TSB + glucose, but this growth defect was significantly improved by the addition of pyruvate ($p < 0.01$) (Figure 3-5). These data suggests that in both of these strains the glycolytic pathway is disrupted but the processes downstream of glycolysis (TCA cycle, etc) are still functional, allowing the utilisation of pyruvate but not glucose.

In the presence of glucose the cells will preferentially utilise this carbon source, which may mask any potential effect the *ΔgapB* mutation has on growth of the bacteria. Therefore the 7 hour growth assays were repeated in a defined medium where the carbon source could be tightly controlled. Tris minimal (TM) medium was chosen as it contains no glucose. Instead casamino acids (1% v/v) act as both the carbon and nitrogen source for growth. In the absence of additional carbon the *ΔgapA* mutant was able to grow at a rate comparable to that of the wild type strain (Figure 3-5D). This indicates that this mutant is able to utilise secondary carbon sources and gluconeogenesis must be functional. Therefore GapA cannot be solely responsible for gluconeogenic GAPDH activity in *S. aureus*.

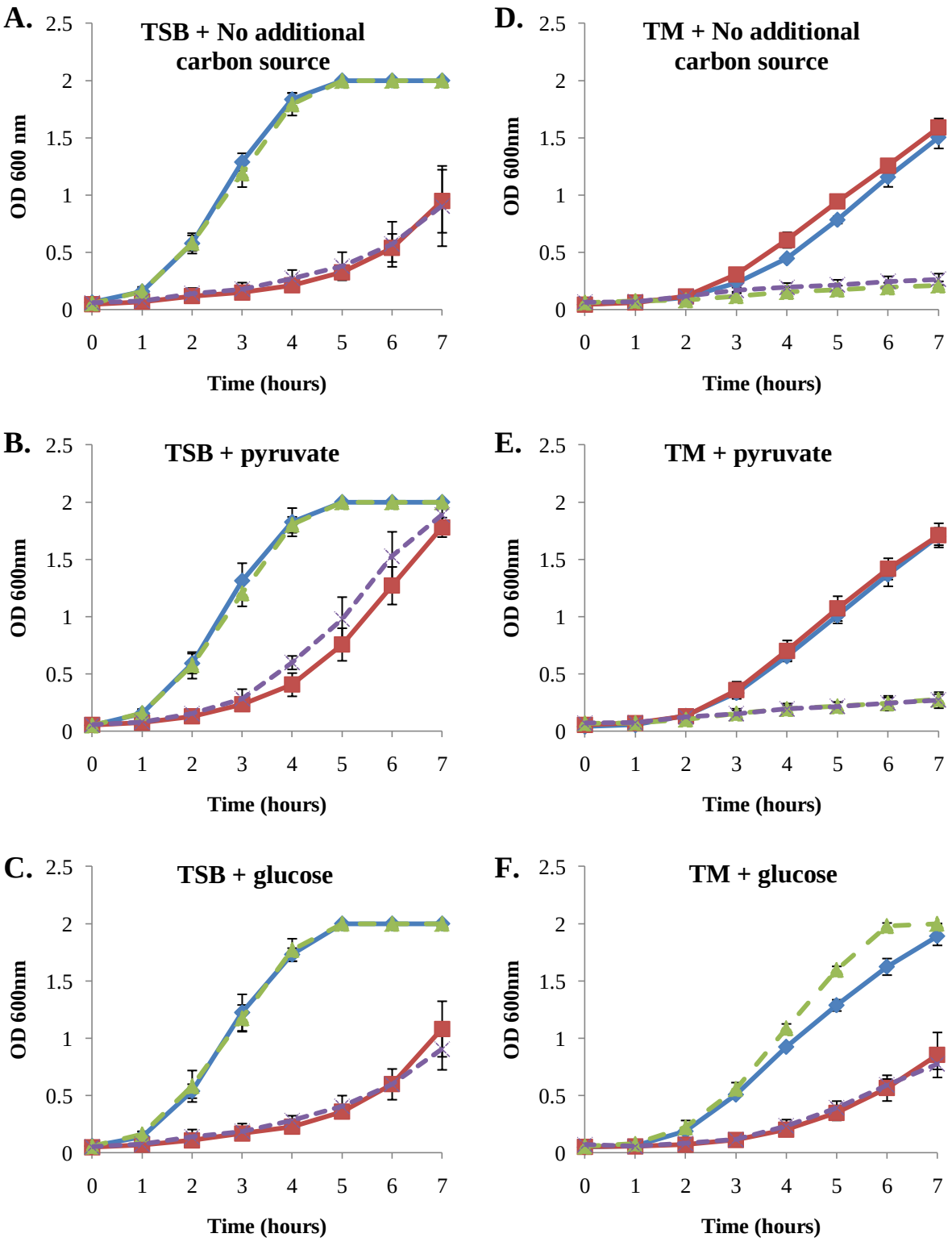


Figure 3-5. Growth of GAPDH mutant strains in TSB and TM medium.

This graph shows the growth of strains 8325-4 (—◆—), 8325-4*ΔgapA* (—■—), 8325-4*ΔgapB* (- -▲- -) and 8325-4*ΔgapA/ΔgapB* (···×···) over 7 hours in (A) the complex medium TSB, (B) TSB + 5 mM pyruvate, (C) TSB + 0.75% glucose, (D) the defined TM medium, (E) TM + 5 mM pyruvate, and (F) TM + 1% glucose. The data presented is an average of at least 3 repeat experiments carried out on different days. The error bars indicate the standard deviation of the data at each time point.

In contrast the growth of both the *ΔgapB* and the double mutant was significantly lower than the wild type in TM medium (Figure 3-5D). Furthermore the addition of 5 mM pyruvate to the medium resulted in an increase in the growth rate of the wildtype and *ΔgapA* mutant, but not the *ΔgapB* or double mutant, suggesting that a loss of *gapB* results in a loss of secondary carbon metabolism (Figure 3-5E). The addition of 1% glucose resulted in a significant increase in the growth of the *ΔgapB* mutant ($p < 0.001$), ultimately to a rate comparable to that of the wild type strain (Figure 3-5F). Conversely the addition of glucose significantly reduced the growth rate of the *ΔgapA* mutant ($p < 0.001$). Although the growth of the double mutant was enhanced, this was only to a similar level of growth seen in the *ΔgapA* mutant (Figure 3-5F).

These results show that GapA is not responsible for both glycolytic and gluconeogenic GAPDH activity in *S. aureus,* as the *ΔgapA* mutant is able to grow on casamino acids as the sole carbon source, gluconeogenesis must still be functional. In direct contrast, GapB must be involved in the utilisation of secondary carbon sources in *S. aureus* due to the inability of the *ΔgapB* mutant to grow in the absence of glucose. The fact that pyruvate did not improve growth may indicate a loss of gluconeogenesis and an inability to produce *de novo* glucose for use in other essential processes.

3.3.4 A *ΔgapB* mutant is unable to utilise a range of secondary carbon sources

To determine the functional role of GapB in carbon metabolism, the GAPDH mutant strains were tested for their ability to utilise a wider range of carbon sources.
Figure 3-6 demonstrates where a variety of different carbon sources can enter either glycolysis or gluconeogenesis. Two of the additional carbon sources included in this experiment were succinate, a TCA cycle intermediate, and glutamate, an amino acid,

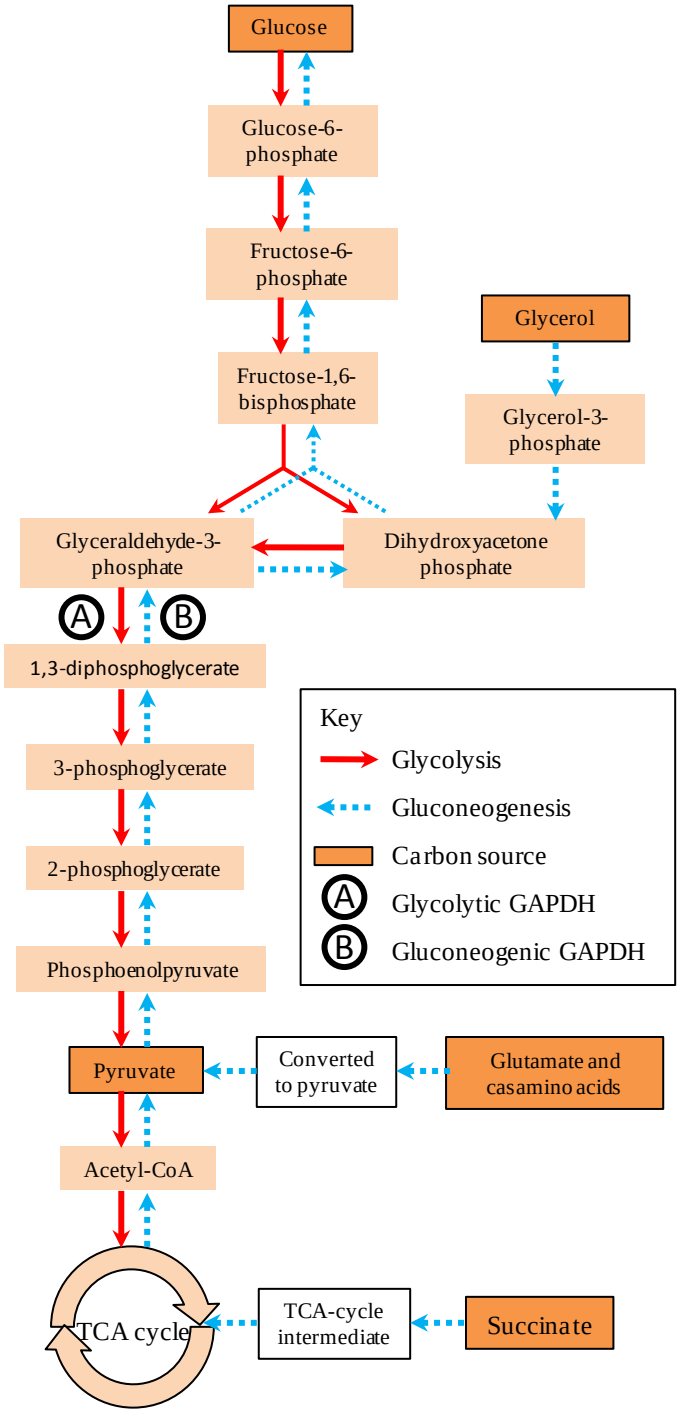


Figure 3-6. The glycolytic and gluconeogenic pathways.
A schematic representation of glycolysis and gluconeogenesis in *S. aureus*, demonstrating where various carbon sources can enter each of the pathways. Also highlighted are the points at which glycolytic (NAD+ dependent) and gluconeogenic (NADP+ dependent) GAPDH activity is required.

both of which can be used as substrates for gluconeogenesis. Glycerol was also chosen as it can enter both the glycolytic and gluconeogenic pathway through a three step conversion to G3P. By analysing the ability of the GAPDH mutants to utilise carbon sources that enter the carbon metabolic pathways at different points, this should allow us to determine which stage of carbon metabolism GapB is involved in.

Strains 8325-4, 8325-4*ΔgapA*, 8325-4*ΔgapB* and 8325-4*ΔgapA/ΔgapB* were grown in TM medium with each additional carbon source and growth was measured after 5 hours (Figure 3-7). The *ΔgapA* mutant was able to grow in the presence of pyruvate, succinate, glutamate and in the absence of additional carbon at the same rate as the wild type. Growth was significantly inhibited in *ΔgapA* mutant compared to the wild type by the presence of both glucose ($p < 0.001$) and glycerol ($p < 0.01$). This confirms that gluconeogenesis is functional, and that glycolysis is disrupted through a loss of glycolytic GAPDH activity. Conversely the *ΔgapB* mutant was unable to grow in TM media unless glucose or glycerol was present. Glycerol enters the gluconeogenic pathway after conversion to G3P (Figure 3-6). Therefore GapB must function before this point in the pathway for glycerol to recover the growth defect in the *ΔgapB* mutant. As the role of gluconeogenic GAPDH is to convert 1, 3-dPG to G3P, the most likely function of GapB in *S. aureus* is as a gluconeogenic GAPDH.

3.3.5 *gapA* and *gapB* are reciprocally regulated in response to glucose at the transcriptional level

If the GapA and GapB proteins are functional in opposing pathways then they should only be expressed in response to their individual stimuli and not at the same time. Therefore the transcription of *gapA* and *gapB* was determined in both glycolytic and

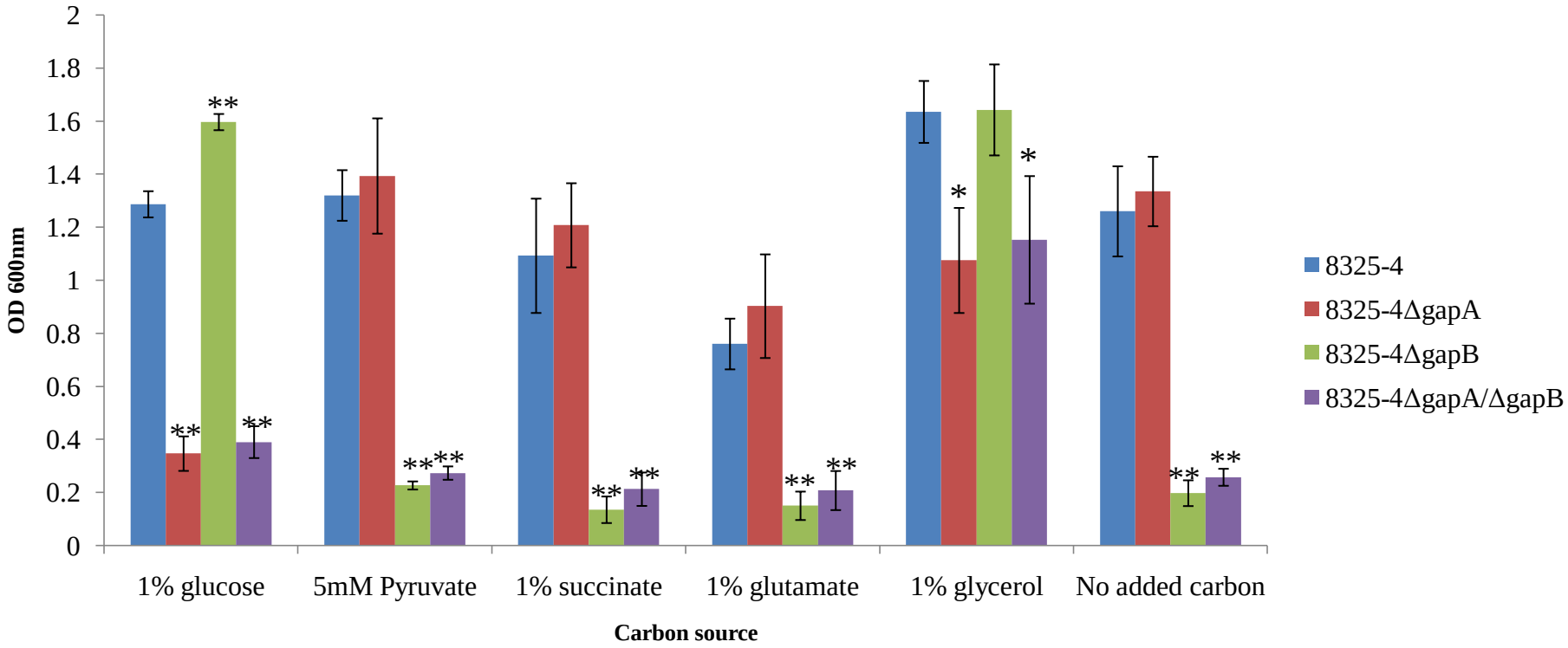
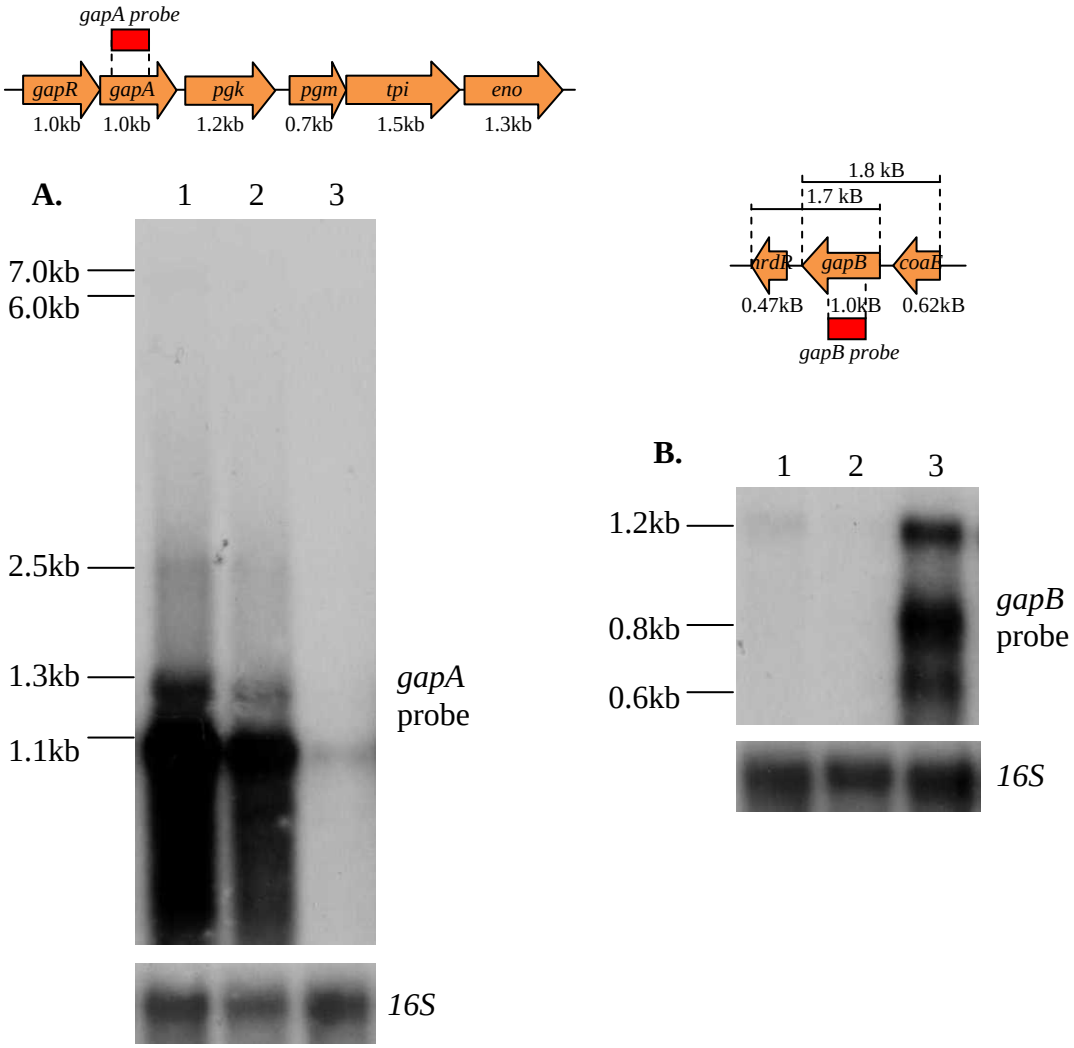


Figure 3-7. 5 hours growth assays of GAPDH mutant strain in TM + various carbon sources.

This graph shows the growth of strains 8325-4, 8325-4*ΔgapA*, 8325-4*ΔgapB* and 8325-4*ΔgapA/ΔgapB* after 5 hours in defined TM medium + various additional carbon sources. Growth was measured by the optical density of the cultures at 600 nm after 5 hours incubation at 37°C with aeration. The data presented is an average of at least 3 repeat experiments carried out on different days, and the error bars indicate the standard deviation of the results. Significant differences in growth of mutant strains compared to the wildtype in the same conditions are marked with either a * ($p > 0.01$) or ** ($p > 0.001$).

gluconeogenic conditions by northern blot. DNA probes were produced by PCR from the open reading frame of each gene using the primer pairs GapAPF and GapAPR for *gapA* and GapB probe F and GapB probe R for *gapB*, and subsequently radiolabelled with [α^{32}P]-labelled dCTP (see Table 2.4 for primer sequences). Strain 8325-4 was grown for 4 hours in glycolytic and gluconeogenic conditions before the cells were pelleted and the RNA harvested. Glycolytic conditions were achieved by addition of 1% glucose to TM medium, while TM medium alone was sufficient for gluconeogenic conditions. Addition of glucose to the medium at the start of growth results in an increase in growth rate, which alters both cell density and pH, two stimuli with the potential to effect the expression of genes. To confirm that any transcriptional differences in *gapA* and *gapB* expression in glycolytic conditions were due to the presence of glucose and not changes in cell density, growth rate or pH, 1% glucose was added to cultures at either time 0 or 1 hour prior to harvesting the cells.

Northern blots show that the *gapA* probe detects multiple transcripts between 1.1 kb and 7.0 kb in length, confirming that *gapA* is transcribed as part of the glycolytic operon in *S. aureus* (Figure 3-8A). The *gapB* probe detects 3 transcripts in gluconeogenic conditions in TM medium, however based on the sizes of the transcripts these are likely due to the degradation or post transcriptional processing of the *gapB* transcript (1.2 kb), and are not the product of co-transcription of *gapB* with adjacent open reading frames (Figure 3-8B). The transcriptional data show that *gapA* and *gapB* are reciprocally expressed in response to glucose in *S. aureus* (Figure 3-8). In the presence of glucose *gapA* transcription is induced while *gapB* transcription is repressed. This is true when glucose is added both at time 0 and 1 hour before harvesting the cells, indicating that this effect is due to the presence of glucose and not changes associated with increased



C.

	gapA probe			*gapB* probe		
Strain	8325-4 +G	8325-4 +GP	8325-4 –	8325-4 +G	8325-4 +GP	8325-4 –
Densitometry	1.08	0.97	0.15	0.08	0.07	1.09

Figure 3-8. Transcription of *S. aureus gapA* and *gapB* in glycolytic and gluconeogenic conditions.

Northern blot showing RNA transcription of (A) *gapA* and (B) *gapB* in response to glucose induction in strain 8325-4. Total RNA was extracted from cells grown for 5 hours in TM broth with 1% glucose added at time 0 (lane 1), 1% glucose added 1 hour before harvesting cells (lane 2), and without glucose (lane 3). The gel presented is representative of experiments that were repeated twice using RNA extracts from cultures grown on different days, with similar results observed each time. Blots were stripped and re-hybridised with a control probe (*16S*) to ensure equal loading of RNA in each case. Densitometry data (C) is presented as a ratio of *gapA/16S* transcript levels.

growth. In the absence of glucose *gapA* transcription is repressed while *gapB* transcription is induced. This is further evidence that GapA and GapB are active in opposing metabolic pathways.

3.3.6 The reciprocal growth response of the *ΔgapA* and *ΔgapB* mutants highlights the level of glucose required for carbon catabolite repression in *S. aureus*

Human blood glucose levels range between 3.6-5.8 mM *in vivo* (~0.06-0.1%), and even in trauma only rise to approximately 12 mM (~0.2%) (Kummer *et al*., 2007). Consequently the 1% glucose concentration used here is much higher than physiological levels. Therefore to determine the level of glucose required to switch from *gapA* expression to *gapB* expression, and thus determine whether they will have a similar effect on *S. aureus* growth *in vivo*, the GAPDH mutant strains were grown in TM for 5 hours in the presence of varied concentrations of glucose (Figure 3-9). The concentrations of glucose tested were 0.001%, 0.005%, 0.01%, 0.05%, 0.1% and 0.2%, each added to the culture at the start of growth. At 0.001% glucose the *ΔgapA* mutant and wild type strains are able to grow, but both the *ΔgapB* and the *ΔgapA/ΔgapB* mutants have a severe growth defect. As glucose concentrations are increased, the growth of the wild type and *ΔgapB* mutant also increase, while the growth of the *ΔgapA* mutant is decreased. Above 0.05% glucose the *ΔgapB* mutant grows at a comparable level to that of the wild type strain, while little or no growth can be detected in either the *ΔgapA* or the *ΔgapA/ΔgapB* double mutant strains. The point at which the bacteria switch from gluconeogenic to glycolytic metabolism is approximately 0.01% glucose, and this is likely to occur through carbon catabolite repression (CCR) (see Section 1.6.2).

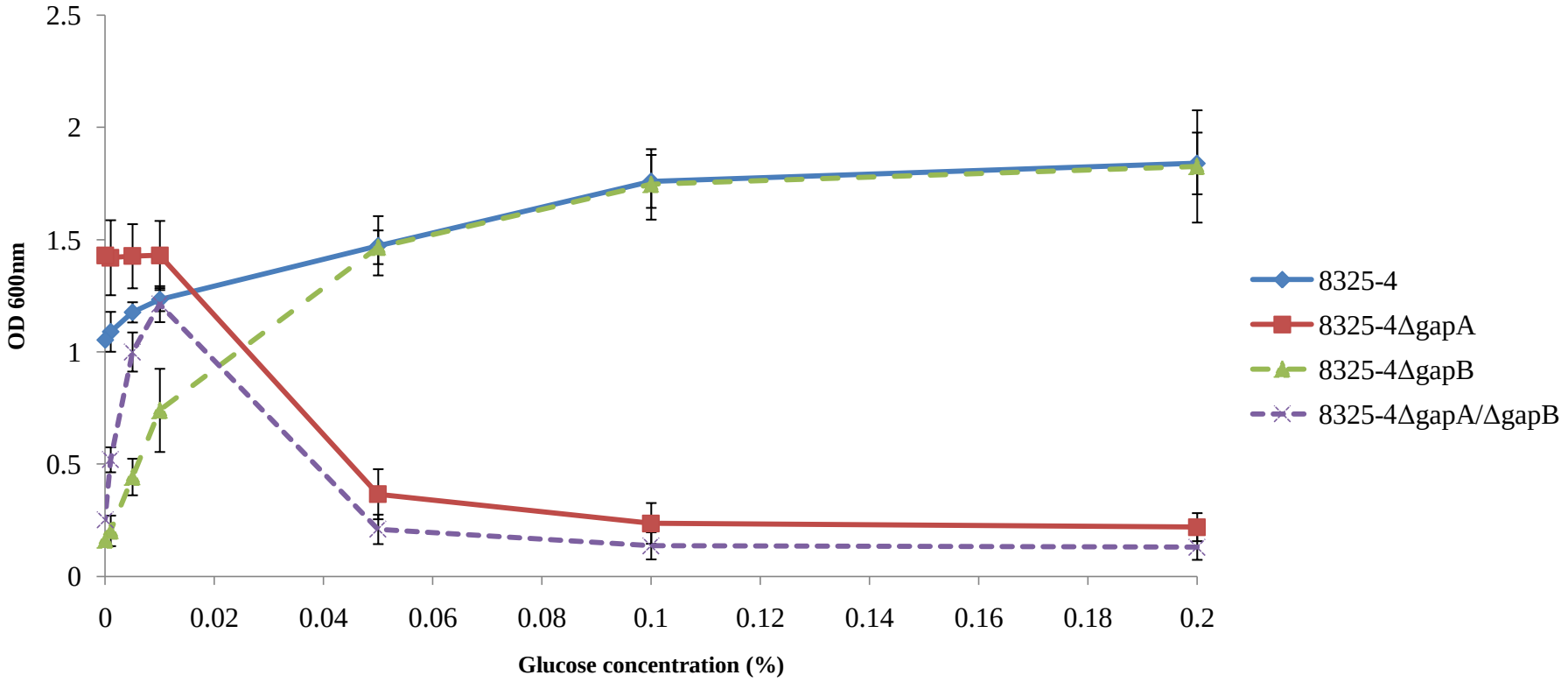


Figure 3-9. 5 hour growth of GAPDH mutants in various glucose concentrations.
The graph shows the growth of strains 8325-4, 8325-4*ΔgapA*, 8325-4*ΔgapB*, and 8325-4*ΔgapA/ΔgapB* after 5 hours incubation in TM + varied concentrations of glucose. Cultures were grown at 37°C with aeration and growth was measured as the optical density of the culture at 600 nm. Each experiment was repeated three times on different days, and presented as an average with error bars indicating the standard deviation.

As both primary and secondary carbon metabolism are disrupted in the double mutant it could be assumed that this strain would be unable to grow regardless of the concentration of glucose present. Unexpectedly there is an increase in growth as glucose concentration increases up until 0.01%. As glucose concentrations increase past this point we see a rapid decline in the growth of the bacteria in line with that of the *ΔgapA* mutant. This suggests that 0.01% glucose is sufficient for growth without the need for *de novo* glucose via gluconeogenesis. The reason that we fail to see this level of growth in the *ΔgapB* single mutant may be because the glycolytic pathway will utilise this glucose, limiting its availability for other metabolic functions. Therefore, the regulation of carbon metabolism is tightly controlled in *S. aureus* to ensure that the bacteria utilise environmental carbon sources in the most efficient way possible.

3.4 The glycolytic operon regulator *gapR* is also involved in the regulation of *gapB* expression

In *B. subtilis* the transcription of the glycolytic operon, including *gapA*, is repressed in gluconeogenic conditions by the operon regulator CggR (Doan & Aymerich, 2003; Zorrilla *et al*., 2007b). In the absence of Fructose-1, 6- bisphosphate (FBP), an early product of glycolysis, CggR binds to its own promoter region and blocks the elongation of transcription of the glycolytic operon (Doan & Aymerich, 2003). In *B. subtilis* CggR binds to a DNA consensus sequence found upstream of the operon that is not present in the rest of the genome, and as such it is speculated that this regulator only controls transcription of the glycolytic operon. Sequence analysis of the *gapR* putative promoter region shows there is no evidence of a *cggR*-like consensus binding sequence in *S. aureus*. In order to determine whether the *ccgR* homologue *gapR* plays a similar role in

S. aureus, and to assess whether this regulator also effects the transcription of *gapB*, a mutation was introduced into the *gapR* locus in strain 8325-4.

3.4.1 Construction of a *ΔgapR* mutant in *S. aureus*

An 8325-4*ΔgapR* mutant strain was produced using a similar deletion/insertion method used for the mutation of both *ΔgapA* and *ΔgapB*. A terminator-less spectinomycin cassette was introduced into the *gapR* open reading frame to allow transcription of the glycolytic operon genes located downstream, therefore not disrupting the glycolysis pathway in this strain. Transcription of the glycolytic operon in the *ΔgapR* mutant could therefore not be analysed using a *gapA* specific probe, as transcription from the spectinomycin cassette promoter would distort the results. To overcome this problem, a new probe was designed that binds to the *gapR* sequence located upstream of the spectinomycin cassette insertion (Figure 3-10). As *gapR* is proposed to act on transcription of the operon as a whole and bind to a sequence in the operon promoter region, the *gapR* specific probe should detect any effect the *ΔgapR* mutation has on transcription of the operon. Growth of the *ΔgapR* mutant was comparable to that of the wild type in each of the conditions tested confirming the glycolytic operon is functional.

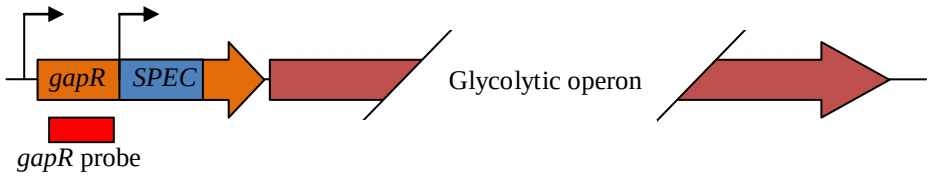
3.4.2 Transcription of both the glycolytic operon and *gapB* are influenced by GapR in *S. aureus*.

Both *gapR* and *gapB* specific DNA probes were produced by PCR using appropriate primer pairs (Table 2.4) and subsequently radiolabelled with [α^{32}P]-labelled dCTP. RNA was extracted from strains 8325-4 and 8325-4*ΔgapR* grown in TM medium for 4 hours, with glucose added at time 0, glucose added 1 hour before harvesting the cells and in the absence of additional carbon. Northern blotting was then carried out on each

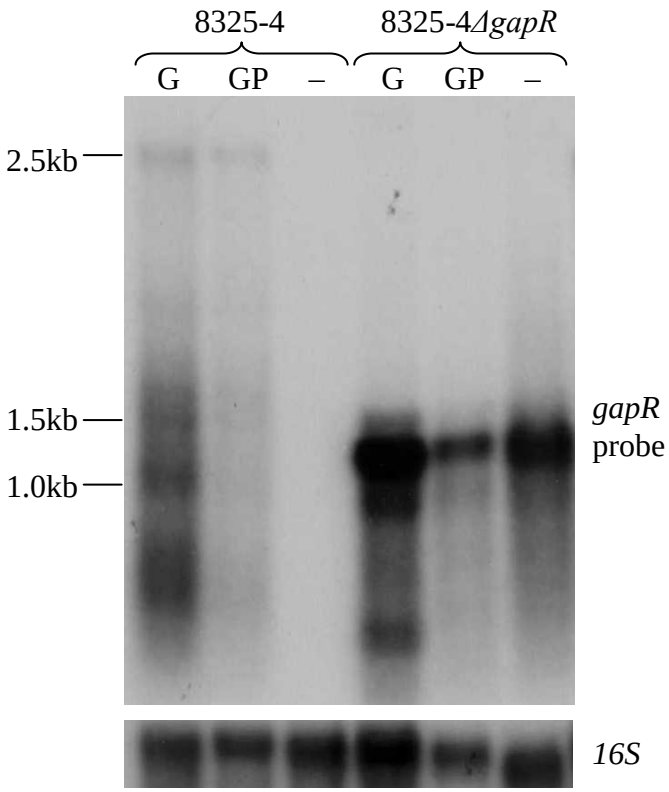
of the RNA samples to detect *gapR* and *gapB* transcription. The *gapR* probe detects 3 transcripts in the wild type strain with approximate sizes of 2.5 kb, 1.5 kb and 1.0 kb (Figure 3-10). Interestingly the level of expression is lower than that of *gapA* in *S. aureus* and the largest transcripts seen with a *gapA* probe were not detected. However the increase in response to glucose is consistent at both the *gapA* and *gapR* loci. Therefore there appears to be differential expression of the operon regulator and the glycolytic genes, even though they form a polycistronic transcript. Due to an apparent lack of a promoter upstream of *gapA*, this variable expression may be a product of post transcriptional processing of the polycistronic gene transcripts, as seen in *B. subtilis* (Ludwig *et al*., 2001).

Transcription of *gapR* increased in both glycolytic and gluconeogenic conditions in the *ΔgapR* mutant (Figure 3-10) which indicates that GapR functions as a repressor of the glycolytic operon in the absence of glucose, and also limits the level of expression in the presence of glucose. It is interesting that in the mutant the *gapR* probe only detects a single transcript, corresponding in size to the *ΔgapR*::*spec* allele, although the reason for this is unclear. The expression of *gapB* compared to the wild type was also increased in gluconeogenic conditions in the *ΔgapR* mutant and also shows a slight increase in the presence of glucose (Figure 3-11). Therefore *S. aureus* GapR also represses the transcription of *gapB* and limits its expression during both glycolysis and gluconeogenesis. This indicates that the function of GapR is not limited to control of the glycolytic operon but is also involved in regulating gluconeogenic metabolism in *S. aureus*. To our knowledge this is the first indication a CcgR-like homologue acts to regulate genes other than those of the glycolytic operon.

A.



B.



C.

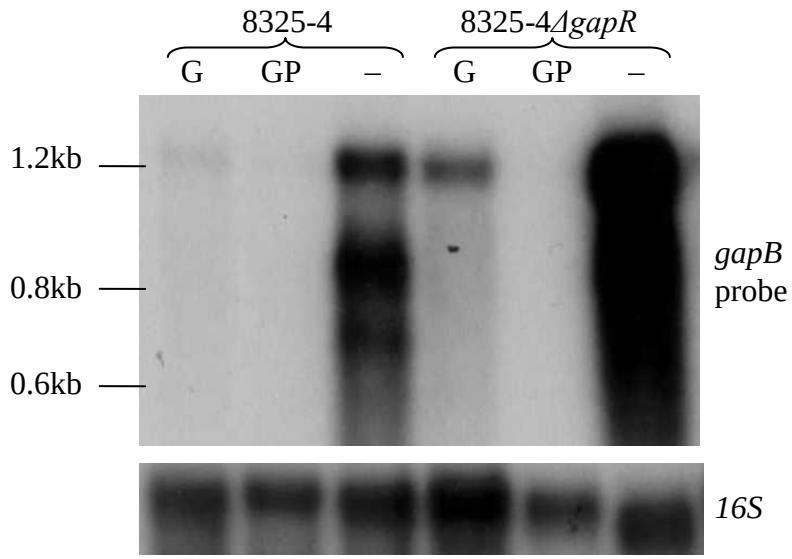
Strain	8325-4 +G	8325-4 +GP	8325-4 –	*ΔgapR* +G	*ΔgapR* +GP	*ΔgapR* –
Densitometry	0.90	0.26	0.04	0.94	0.56	0.77

Figure 3-10. Transcription of *S. aureus gapR* in response to glucose in a *ΔgapR* mutant.

(A) Schematic representation of *gapR* and the glycolytic operon, highlighting the position of the spectinomycin cassette in the *ΔgapR* mutant and the position of the *gapR* Northern blot probe.

(B) Northern blot with (C) densitometry data, showing RNA transcription of *gapR* in strains 8325-4 and 8325-4*ΔgapR* grown in TM broth with 1% glucose added at time 0 (G), 1% glucose added 1 hour before harvesting cells (GP), and without glucose (–). The gel presented is representative of experiments that were repeated twice using RNA extracts from cultures grown on different days, with similar results observed each time. Blots were stripped and re-hybridised with a control probe (*16S*) to ensure equal loading of RNA in each case.

A.



B.

Strain	8325-4 +G	8325-4 +GP	8325-4 –	*ΔgapR* +G	*ΔgapR* +GP	*ΔgapR* –
Densitometry	0.08	0.07	1.09	0.33	0.15	1.42

Figure 3-11. Transcription of *S. aureus gapB* in response to glucose in a *ΔgapR* mutant.

(A) Northern blot with (B) densitometry data, showing RNA transcription of *gapB* in strains 8325-4 and 8325-4*ΔgapR* grown TM broth with 1% glucose added at time 0 (G), 1% glucose added 1 hour before harvesting cells (GP), and without glucose (–). The gel presented is representative of experiments that were repeated twice using RNA extracts from cultures grown on different days, with similar results observed each time. Blots were stripped and re-hybridised with a control probe (*16S*) to ensure equal loading of RNA in each case.

3.4.3 Potential role of GapA and GapB in host colonisation and virulence

To determine the importance of both GapA and GapB in the wider role of host colonisation and infection, caterpillars of the Greater Wax Moth *Galleria mellonella* were infected with each of the GAPDH mutant strains. This experiment was carried out by our collaborator Dr. Alan Cockayne at the Institute of Infection, Immunity and Inflammation, University of Nottingham. In this simple infection model, pathogenesis of the bacteria is measured by the % viability of the infected caterpillars at 24, 48 and 72 hours post infection. A negative control of PBS was also included. The results show that both *gapA* and *gapB* are required for a full pathogenic phenotype of *S. aureus* in this model. Survival of the larvae infected with the *ΔgapA* mutant ($p < 0.0001$), the *ΔgapB* mutant ($p = 0.0003$) and the double mutant ($p < 0.0001$) is significantly reduced compared to infection with the wild type strain. Survival curves were calculated using the Kaplan Meier estimator, and significant differences were calculated using the logrank test. At 72 hours post infection the % viability was lower in the *Galleria* larvae infected with the wild type strain (20%) compared with larvae infected with either *ΔgapA* (93.3%) or *ΔgapB* (56.7%), showing that either colonisation or virulence was reduced in each of the mutants.

Furthermore, pathogenesis of *S. aureus* was attenuated entirely in the double mutant strain, with 100% survival of the *Galleria* seen at all time points. The loss of either *gapA* or *gapB* appears to result in a reduction in virulence in *S. aureus*, which suggests that during infection *S. aureus* relies on both primary and secondary carbon metabolism to utilise the available carbon in the host environment.

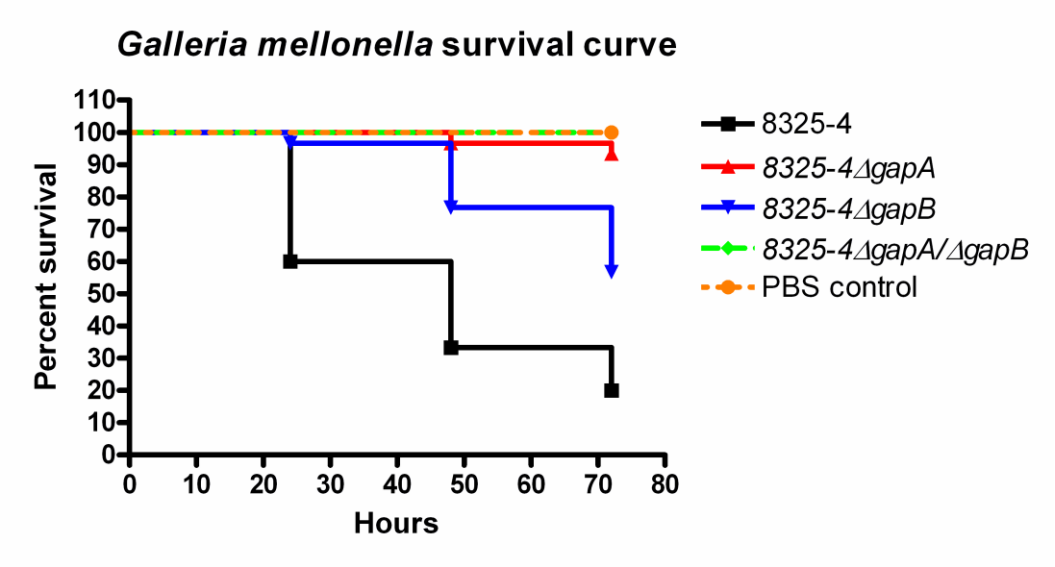


Figure 3-12. Pathogenesis of *S. aureus* GAPDH mutants during infection of *Galleria mellonella*.

This graph shows the % viability of *Galleria mellonella* caterpillars at 24, 48 and 72 hours post infection with *S. aureus* strains 8325-4 wild type, 8325-4*ΔgapA*, 8325-4*ΔgapB*, and 8325-4*ΔgapA/ΔgapB*. The data presented is the combined results for three independent experiments, in which 10 caterpillars were infected with each strain. A PBS negative control was also included, in which 100% of the caterpillars survived the duration of the experiment.

3.5 Alternative functions of GapA and GapB in *S. aureus*

In many prokaryotic and eukaryotic species GAPDH proteins have been found to take on a varied number of "moonlighting" roles, independent of their functions in carbon metabolism (for reviews see Sirover *et al*., 1999; Modun *et al*., 2000). These include, but are not restricted to, the binding of host proteins, mediation of host cell interaction, DNA replication and repair, cell signalling, and protection of the cell from the host immune system (for detailed examples see section 1.6.3). In *S. aureus* GAPDH proteins have been implicated in the iron scavenging response through binding to the host iron transport protein transferrin, and in cellular interaction through the binding of plasmin (Modun & Williams, 1999; Goji *et al*., 2004). However, due to the highly conserved nature of GAPDH proteins, it is possible that *S. aureus* GAPDH plays a number of other undefined "moonlighting" roles in this species.

3.5.1 GapA but not GapB appears to be involved in the protection of *S. aureus* from oxidative stress

Eukaryotic GAPDH has long been established as a major target of oxidative stress, with the enzyme activity of the protein being abolished through S-thiolation of Cys^{152}, a key residue in the active site (Schuppe-Koistinen *et al*., 1994; Shenton & Grant, 2003). This oxidation also conveys alternative roles to the protein, altering its capability to interact with other proteins and therefore allowing it to play unexpected roles in processes such as RNA splicing (Hwang *et al*., 2009) and signal transduction (Morigasaki *et al*., 2008). Furthermore eukaryotic GAPDH has also been implicated in the suppression of reactive oxygen species (ROS) in plants (Bustos *et al*., 2008), reactivation of oxidised proteins involved in DNA repair in mammalian cells (Azam *et al*., 2008), and even the redox-senstive control of gene expression through directly altering mRNA stability in human

cells (Rodriguez-Pascual *et al*., 2008) The link between metabolism and the oxidative stress response has also been highlighted in *Sacchromyces cerevisiae*, where the inactivation of GAPDH via oxidative stress triggers a switch in carbon flux from glycolysis to the pentose phosphate pathway in order to balance the redox state of the cell (Ralser *et al*., 2009).

Prokaryotic GAPDH proteins are also sensitive to oxidative stress, and the Cys^{151} residue in the active site of *S. aureus* GAPDH is S-thiolated in response to H_2O_2, resulting in inactivation of the protein and potentially playing a role in growth arrest in these conditions (Weber *et al*., 2004). Therefore to investigate the potential role of GapA and GapB in the oxidative stress response in *S. aureus*, strains 8325-4, 8325-4*ΔgapA*, 8325-4*ΔgapB* and 8325-4*ΔgapA/ΔgapB* were assayed for their ability to adapt to H_2O_2 induced oxidative stress in a simple assay. Cultures were grown in duplicate in TM medium + 1% glycerol until mid exponential phase, before addition of H_2O_2 to one of the cultures. This medium was chosen as the strains grow at a similar rate in these conditions. Growth of the treated and untreated cultures was measured by optical density at 600 nm 1 and 2 hours after addition of H_2O_2, and the growth of the H_2O_2 treated cells was calculated as a percentage of the growth of untreated cells (Figure 3-13). This method allows direct comparison between the strains.

The data show that both the *ΔgapA* ($p > 0.05$) and the double mutant ($p > 0.01$) are more sensitive to H_2O_2 oxidative stress than wild type *S. aureus*, while the *ΔgapB* mutant shows no significant difference in sensitivity (Figure 3-13). These data suggest that GapA, but not GapB, may be involved in protecting the cells from oxidative stress in

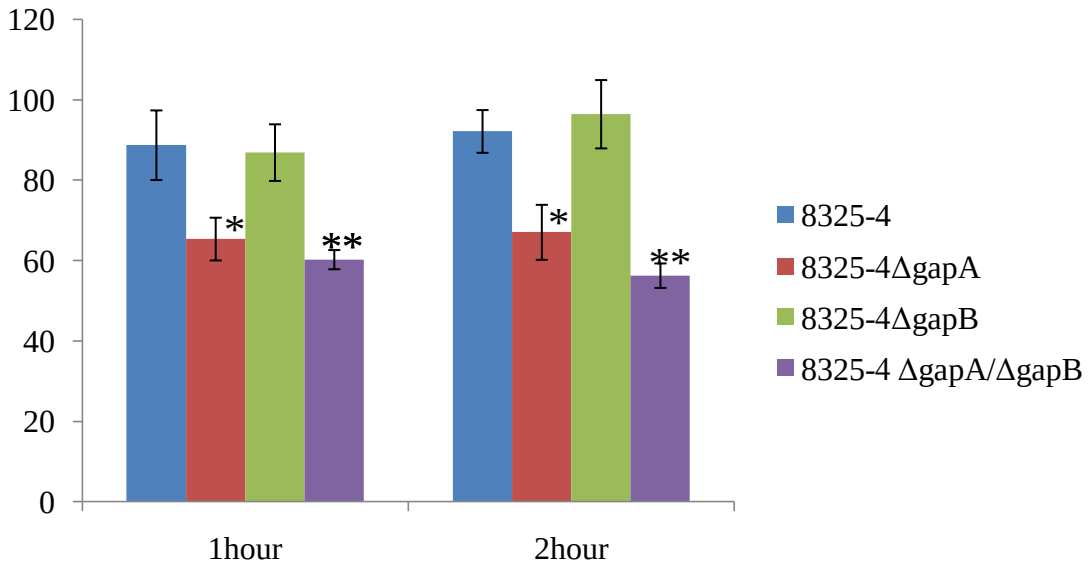


Figure 3-13. Percentage growth of GAPDH mutant strains after exposure to H_2O_2 oxidative stress.

This graph shows the % growth of *S. aureus* strains 8325-4 wild type, 8325-4*ΔgapA*, 8325-4*ΔgapB*, and 8325-4*ΔgapA/ΔgapB* after exposure to H_2O_2 induced oxidative stress compared to that of untreated cells. The data presented is an average of 3 repeats carried out on different days, and the error bars indicate the standard deviation of the results. Significant differences between the mutant strains compared to the wild type are marked with either a * ($p > 0.05$) or ** ($p > 0.01$).

S. aureus. However more detailed analysis is required to determine the functional role of GAPDH protein during the oxidative stress response.

3.5.2 Both GapA and GapB appear to be required for biofilm formation in *S. aureus*.

Upon colonisation *S. aureus* cells have the ability to form a biofilm, a process which involves the adherence and accumulation of the bacteria to a surface, followed by the production of a protective layer of poly-*N*-acetylglucosamine (PNAG) exopolysaccharide (Gotz, 2002; Stanley & Larazzera, 2004). Cells within a biofilm are more resistant to antimicrobial agents and the host immune system, making the ability to form a biofilm a key virulence determinant in *S. aureus* infection (Gray *et al*., 1984; Jones *et al*., 2001). Glucose has previously been shown to induce biofilm formation in Staphylococcal species through induction of PNAG, increasing adherence and accumulation of the biofilm (Mack *et al*., 1992; Gotz, 2002).

In human erythrocytes GAPDH and other glycolytic enzymes have even been shown to form active complexes when on the cell surface (Campanella *et al*., 2005). If this also occurs in bacteria it may provide an advantage in a biofilm as glycolysis would be able to occur at the cell surface providing ATP or reducing power to the cells without the need for uptake and export across the cell membrane. Furthermore *S. aureus* GapA and GapB have been shown to bind to host proteins such as transferrin and plasmin, and may provide a more direct role in adherence to a surface or cell-cell interaction within the biofilm (Modun & Williams, 1999; Modun *et al*., 2000; Gotz, 2002).

To determine the impact of GapA and GapB on biofilm formation in *S. aureus*, strains 8325-4, 8325-4*ΔgapA*, 8325-4*ΔgapB* and 8325-4*ΔgapA/ΔgapB* were each assayed for their ability to adhere and accumulate on 96-well polycarbonate plates over 24 hours (Figure 3-14). The results demonstrate that the *ΔgapA*, *ΔgapB* and double mutants are unable to form a biofilm in the conditions tested, while the wild type is able to biofilm at a low level. Although the assay was only carried out twice the results were consistent, providing preliminary evidence that suggests both GapA and GapB play a role in biofilm formation in this model. However further investigation is required to determine how these proteins may be involved.

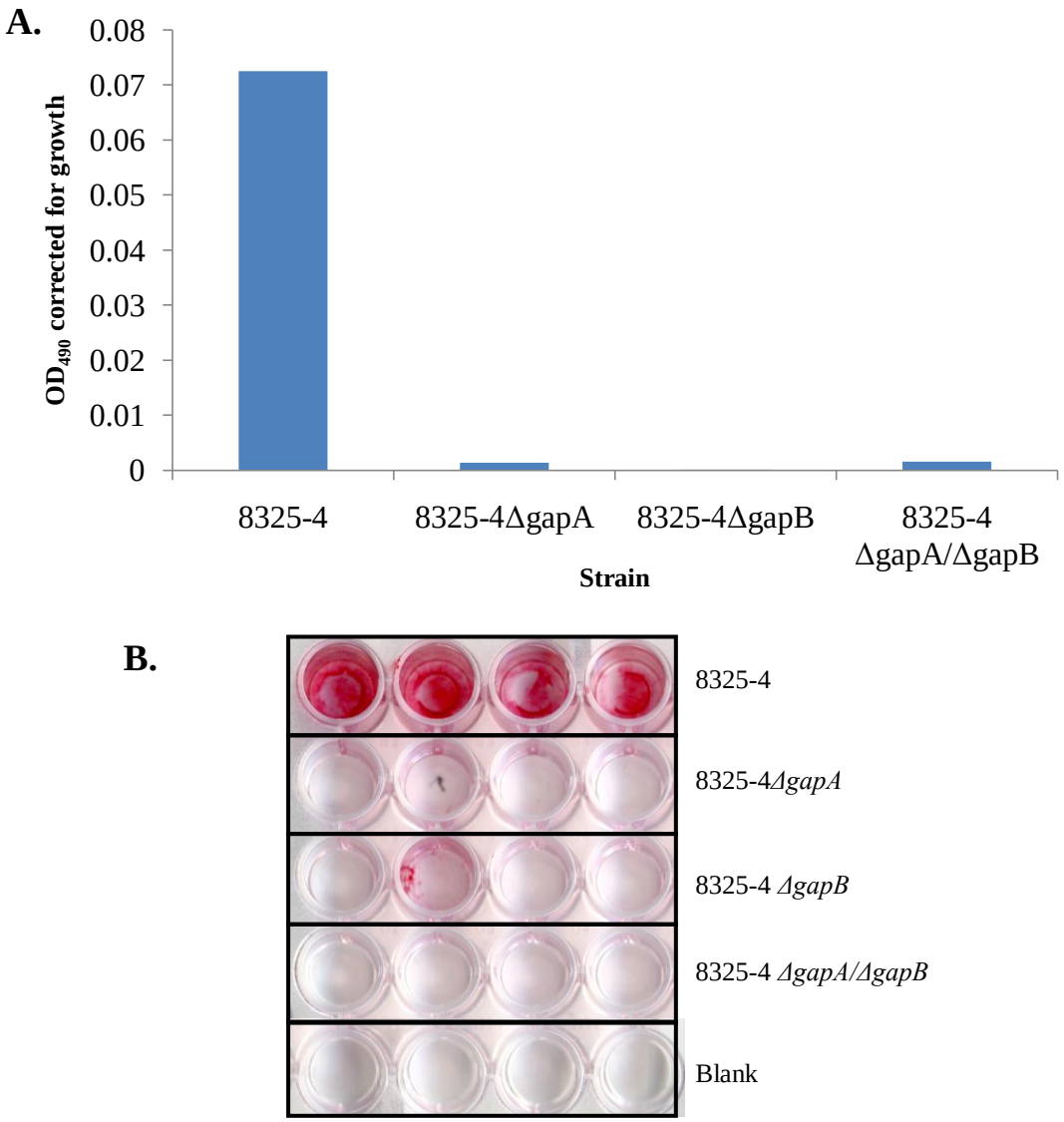


Figure 3-14. Biofilm formation in the GAPDH mutant strains.

Biofilm formation in *S. aureus* strains 8325-4 wild type, 8325-4*ΔgapA*, 8325-4*ΔgapB*, and 8325-4*ΔgapA/ΔgapB* after 24 hours static growth in TM medium + 1 % glycerol in the presence of CO_2. The data is presented as (A) a graph of data corrected to take into account any differences in growth between the strains and (B) as a picture of a biofilm plate after drying and staining of the cells. The data presented is representative of two independent biological repeats.

3.6 Discussion

The control of carbon metabolism is a deciding factor in the ability of bacteria to inhabit different ecological niches. We have shown that the GAPDH homologue GapB is essential in secondary carbon metabolism, and appears to function as a gluconeogenic GAPDH in this species. Furthermore the data demonstrates the putative glycolytic regulator GapR not only represses the transcription of the glycolytic *gapA* but also influences the level of *gapB* transcription during gluconeogenesis. To our knowledge this is the first demonstration of a role of this regulator outside of the glycolytic operon. Finally the data presented in this chapter demonstrate that both GapA and GapB are important for virulence during infection in an invertebrate model, and preliminary evidence suggests that both proteins may be involved in biofilm formation in *S. aureus,* while GapA may have an additional "moonlighting" role in the oxidative stress response.

Although previous evidence suggested that *S. aureus* GapB may not be a gluconeogenic GAPDH protein (Goji *et al*., 2004), the evidence presented in this chapter strongly suggests that this protein functions in glucose biosynthesis in this way. A *ΔgapB* mutant strain is unable to grow in the absence of either glucose or glycerol (Figure 3-7), demonstrating that it is essential in the utilisation of secondary carbon sources. Furthermore, the ability of a *ΔgapA* mutant to utilise secondary carbon sources demonstrates that gluconeogenesis must be intact in this strain, and that GapA cannot be responsible for both glycolytic and gluconeogenic activity in *S. aureus* (Figure 3-5; Figure 3-7). As glycerol enters the gluconeogenic pathway after conversion to G3P, the product of gluconeogenic GAPDH activity, the recovery of growth in the *ΔgapB* mutant by glycerol strongly suggests that GapB is a gluconeogenic GAPDH (Figure 3-6). The

function of GapB cannot occur before this point in the pathway as *S. aureus* contains all other functional enzymes required for gluconeogenesis (KEGG database, http://www.genome.jp/kegg/), most importantly those involved in the conversion of pyruvate to 1,3-diphosphoglycerate. The *ΔgapB* mutant strain also showed a significant reduction in NADP+ dependent GAPDH activity compared to the wild type strain (Figure 3-4). Sequence comparison of key amino acid residues of this protein, as determined for a number of GAPDH homologues (Fillinger *et al*., 2000), shows that *S. aureus* GapB contains the structural residues for NADP+ substrate specificity ($Ala_{32,}$ Asn_{187}) and G3P binding ($Thr_{179,}$ Thr_{208}), which is further evidence that this protein is a gluconeogenic GAPDH and not a homologue of another gluconeogenic enzyme.

Although all of the growth data and whole cell GAPDH assays strongly suggest that *S. aureus* GapB is a gluconeogenic GAPDH, recombinant *S. aureus* GapB protein does not have any detectable NAD+ or NADP+ dependent GAPDH activity (Figure 3-2). It is possible that an unidentified co-factor which is not present in the *in vitro* assay is required for either the folding of the GapB protein or substrate-GapB interactions, leading to a lack of detectable activity. Further investigation will need to be carried out to determine whether altering the conditions of the assay or addition of another cofactor or protein can stimulate gluconeogenic GapB enzyme activity. One possible test for this would be to add total *S. aureus* cell *ΔgapA/ΔgapB* mutant lysate to the protein assay and measure NAD+/NADP+ dependent activity in the presence and absence of recombinant GapB protein. It would also be useful to complement the *ΔgapA* and *ΔgapB* mutations by re-introducing the wild type alleles on a plasmid. If this rescued the mutant phenotypes of *ΔgapA* and *ΔgapB* strains (growth curve and GAPDH activity) it would further strengthen the argument that GapB is a gluconeogenic GAPDH.

Northern blot analysis demonstrates that like *B. subtilis*, the *S. aureus* glycolytic GAPDH *gapA* is co-transcribed as part of an operon alongside the operon regulator (*gapR*), which acts as a repressor of the operon in gluconeogenic conditions (Figure 3-8; Figure 3-10). Our data show that transcript levels of *S. aureus gapA* far exceed those of *gapR*, as seen by the transcripts detected by the *gapA* and *gapR* specific probes (Figure 3-8; Figure 3-10), with the largest *gapA* transcripts corresponding to the whole operon being expressed at the lowest levels, potentially indicating that they are the least stable. Differential expression between an operon regulator and the remainder of the operon is quite common, as regulatory proteins are generally not required at the same levels as the proteins they control. In *B. subtilis gapA* does not have its own promoter, and transcription initiates upstream of the *cggR* regulator gene (Fillinger *et al*., 2000), with cleavage of the transcript at the 3` end of the *cggR* coding sequence producing two transcripts of different stability, resulting in GapA protein levels being ~100 fold higher than that of the CggR repressor (Ludwig *et al*., 2001; Meinken *et al*., 2003). Our data suggest that differential transcript stability may also be involved in the expression of these genes in *S. aureus.*

Interestingly in the *ΔgapR* mutant there is a clear shift in the processing of *gapR* transcript levels, and larger transcripts are no longer present (Figure 3-10). The reason for this is unclear, as the growth of this strain in the presence of glucose clearly indicates that *gapA* and the glycolytic operon are still being expressed. The overproduction of *gapR* transcript, or simply the introduction of the mutation, may have resulted in an increase in processing of this gene and a further destabilisation of the larger transcripts of the operon.

As well as acting as a repressor of the glycolytic operon, *S. aureus* GapR also appears to repress *gapB* transcription in both glycolytic and gluconeogenic conditions (Figure 3-11). In the absence of glucose GapR limits the level of *gapB* transcription, while in the presence of glucose GapR contributes to the repression of *gapB*. This is interesting as genome sequence analysis and transcriptome comparison in *B. subtilis* showed that the GapR homologue CcgR does not regulate any genes other than those in the glycolytic operon (Doan & Aymerich, 2003). The CggR DNA target sequence has been determined in *B. subtilis* (Doan & Aymerich, 2003; Zorrilla *et al*., 2007b), but neither the *gapR* or *gapB* putative promoter sequences contain any potential homologues for GapR binding. Identification of the GapR target sequence would determine whether this regulator acts directly or indirectly to repress *gapB* transcription, and potentially help to determine its influence on the regulation of other *S. aureus* genes.

As *gapB* transcription is not fully de-repressed in the presence of glucose (Figure 3-11), it is clear that at least one other regulator is involved in carbon catabolite repression (CCR) of *gapB* in *S. aureus*. In *B. subtilis gapB* CCR is under the control of both CcpA, which binds to catabolite responsive element (*cre*) sequences in gene promoters, and a second catabolite control protein CcpN. CcpN is an additional mediator of CCR in *B. subtilis*, and has been shown to directly regulate two gluconeogenic genes, *gapB* and *pckA*, and a regulatory non-coding RNA to date (Servant *et al*., 2005; Zorrilla *et al*., 2008; Eckhart *et al*., 2009; Licht & Brand, 2009). In *S. aureus gapB* is also repressed by CcpA, and contains a putative *cre* site, although the influence of CcpN has not been tested to our knowledge (Seidl *et al*., 2009). It is likely that these regulators may directly or indirectly co-operate with GapR in the repression of *gapB* in *S. aureus*. It would be interesting to investigate the contribution of CcpN on carbon regulation in this

species, as it has been shown to play a wider role in modulating carbon flux in *B. subtilis* (Tannler *et al*., 2008).

CcpA is not only involved in the regulation of carbon metabolism in *S. aureus*, but also modulates the regulation of a large number of virulence factors (Seidl *et al*., 2006; Seidl *et al*., 2008). This is a direct link between the regulation of metabolism and the modulation of virulence in *S. aureus*. Metabolism is very important during colonisation of a host, and recent indication that metabolic enzymes may also "moonlight" as virulence factors in pathogens means that metabolism may play a more active role in the pathogenesis of *S. aureus* than first thought (for review see Pancholi & Chhatwal, 2003). Therefore we investigated the influence of both GapA and GapB on virulence in a simple invertebrate model of infection.

Caterpillars of the Greater Wax Moth *Galleria mellonella* have recently been shown to provide a useful insight into the pathogenesis of a wide range of bacterial and eukaryotic microbial infections, including *Pseudomonas aeuriginosa* (Jander *et al*., 2000), *Listeria* species (Mukherjee *et al*., 2010a), *Bacillus cereus* (Fedhila *et al*., 2006), *Burkholderia cepacia* complex (Seed & Dennis, 2008), *Aspergillus* (Reeves *et al*., 2004), *Candida albicans* (Cotter *et al*., 2000) and *S. aureus* (Peleg *et al*., 2009). This model is useful as immunity in insects such as *Galleria* share many common aspects with mammalian innate immunity, with infection model results often successfully correlating with those of similar mammalian studies (Jander *et al*., 2000; Brennan *et al*., 2002). Although *Galleria mellonella* caterpillars use trehalose rather than glucose as their primary haemolymph sugar (Wyatt *et al*., 1956), *S. aureus* can utlise this sugar (Kloos & Schleifer, 1975). *S. aureus* possess the required genes both for uptake of this

sugar (*treP*) and conversion to glucose-6-phosphate (*treC*), resulting in trehalose feeding into glycolysis only one step after that of glucose (KEGG database, http://www.genome.jp/kegg/; Figure 3-6). This also occurs before the production of FBP, the key signalling molecule in de-repression of the glycolytic operon and CCR control in *B. subtilis* (Doan & Aymerich, 2003; Warner & Lolkema, 2003: Gorke & Stulke, 2008; Zorrilla *et al*., 2007a). Therefore this sugar should influence the control of central carbon metabolism in *S. aureus* in a similar way to that of glucose.

Infection of the caterpillars with *S. aureus ΔgapA, ΔgapB* and *ΔgapA/ΔgapB* mutants showed that both GapA and GapB are important during infection in this model system. Caterpillar survival was significantly increased in both the *ΔgapA* and *ΔgapB* mutants compared to the wild type, while the *ΔgapA/ΔgapB* double mutant was attenuated for virulence in this model (Figure 3-12). Clearly GapA plays a more substantial role during infection than GapB, but both primary and secondary metabolism appear to be required for full infection capacity of *S. aureus*. This is likely to be primarily due to the ability of the bacteria to effectively utilise carbon sources and colonise the host, although there may also be other more subtle but active roles for these proteins during infection.

Preliminary evidence presented here suggests that both GapA and GapB may also play a role in biofilm formation in *S. aureus* (Figure 3-14). It has previously been shown that *S. aureus* cells growing in a biofilm utilise glucose, most probably via glycolysis, and therefore surface localisation of the glycolytic GapA protein may provide the bacteria with the ability to rapidly metabolise glucose, produce ATP and balance redox state without the need for uptake and export of substrates (Zhu *et al*., 2007). The expression

of *gapA* and the additional glycolytic genes *pgk* and *eno*, which are also surface localised, are up-regulated in a *S. aureus* biofilm compared to planktonic cells (Gatlin *et al*., 2006; Resch *et al*., 2006; Sibbald *et al*., 2006). Surface GapB could potentially play a role in balancing the redox state of the biofilm, but it is possible that the ability of both GapA and GapB to bind to other proteins may contribute to *S. aureus* cell adhesion in a biofilm (Modun & Williams, 1999; Goji *et al*., 2004). More investigation is required to determine whether the metabolic or "moonlighting" functions of these proteins affect biofilm formation in *S. aureus*, and also whether they would have a similar effect in media that better reflect *in vivo* conditions, or in greater biofilm producing strains.

Finally our evidence also suggests that GapA, but not GapB, is involved in the oxidative stress response in *S. aureus*. Both the *ΔgapA* mutant and *ΔgapA/ΔgapB* double mutant were more sensitive to H_2O_2 induced oxidative stress in a simple survival assay (Figure 3-13). GAPDH proteins have previously been linked to the protection of eukaryotic cells from oxidative stress via a number of different processes (Azam *et al*., 2008; Bustos *et al*., 2008; Rodriguez-Pascual *et al*., 2008, Ralser *et al*., 2009), and therefore it is possible that *S. aureus* GapA also shares one or more of these functions. A more quantitative H_2O_2 sensitivity assay is required to determine the viability of the cells after exposure to H_2O_2, to determine whether a loss in GapA results in increased killing of the bacteria or a delay in the oxidative stress response. Further investigation is also required to determine whether this role would occur *in vivo*. However, both the potential roles in biofilm formation and the oxidative stress response highlight that during *S. aureus* pathogenesis GAPDH proteins are likely to play a role in more than just central metabolism.

In conclusion, this chapter outlines the importance of both GapA and GapB in central metabolism in *S. aureus*, and gives some insight into the regulation of these genes. For the first time the glycolytic operon regulator GapR has been shown to function in regulating a gene involved in secondary carbon metabolism in *S. aureus*, which to my knowledge is the only indication of a CggR homologue functioning outside of the glycolytic operon. Furthermore, these data show that both GapA and GapB play a role in the pathogenesis of *S. aureus* infection in an insect model, and highlight potential "moonlighting" roles of these two proteins in both the oxidative stress response and biofilm formation. The subsequent chapter will look into the non-glycolytic regulation of *gapA*, primarily in response to extracellular iron, as this regulation suggests there may be other potential roles of this protein outside of central metabolism.

Chapter 4 Strain variable regulation of GAPDH in response to changes in environmental stimuli

4.1 Introduction

Unexpectedly for a key cytoplasmic enzyme, surface GAPDH (GapA) activity is iron regulated in *S. aureus* and this response is strain variable (Dr. Julie Morrissey, unpublished data; Figure 4-1A). The iron regulation can be grouped into two distinct classes; Class I where GAPDH activity is highest in low iron conditions (strains BB and B0903007) and Class II where GAPDH activity is highest in high iron conditions (including strains 8325-4, Newman and MRSA PM64). Western blotting has shown that in strain 8325-4 surface expression of GapA is strongly iron regulated and the protein is almost completely absent in this fraction in low iron conditions. However iron regulation of cytoplasmic GapA is less clear, with protein levels being only slightly increased in high iron conditions compared to low iron (Dr. Alan Cockayne, unpublished data; Figure 4-1B). It is unclear whether iron regulation of GapA occurs at the transcriptional or protein level, but these data do show that a key glycolytic gene is regulated by iron in a strain variable manner, and expression is also variable across different cellular fractions.

The main aim of this chapter is to investigate the iron regulation of GapA in *S. aureus*, to further our understanding of the function of this key housekeeping enzyme outside of its primary role in carbon metabolism. Key objectives include determining whether sequence variation or differences in strain background are responsible for the strain variable iron regulation of GapA, and also determining whether this variation is specific to iron. Finally this chapter will briefly investigate whether *gapB* is also iron regulated in *S. aureus*, and whether or not this response varies between *S. aureus* isolates.

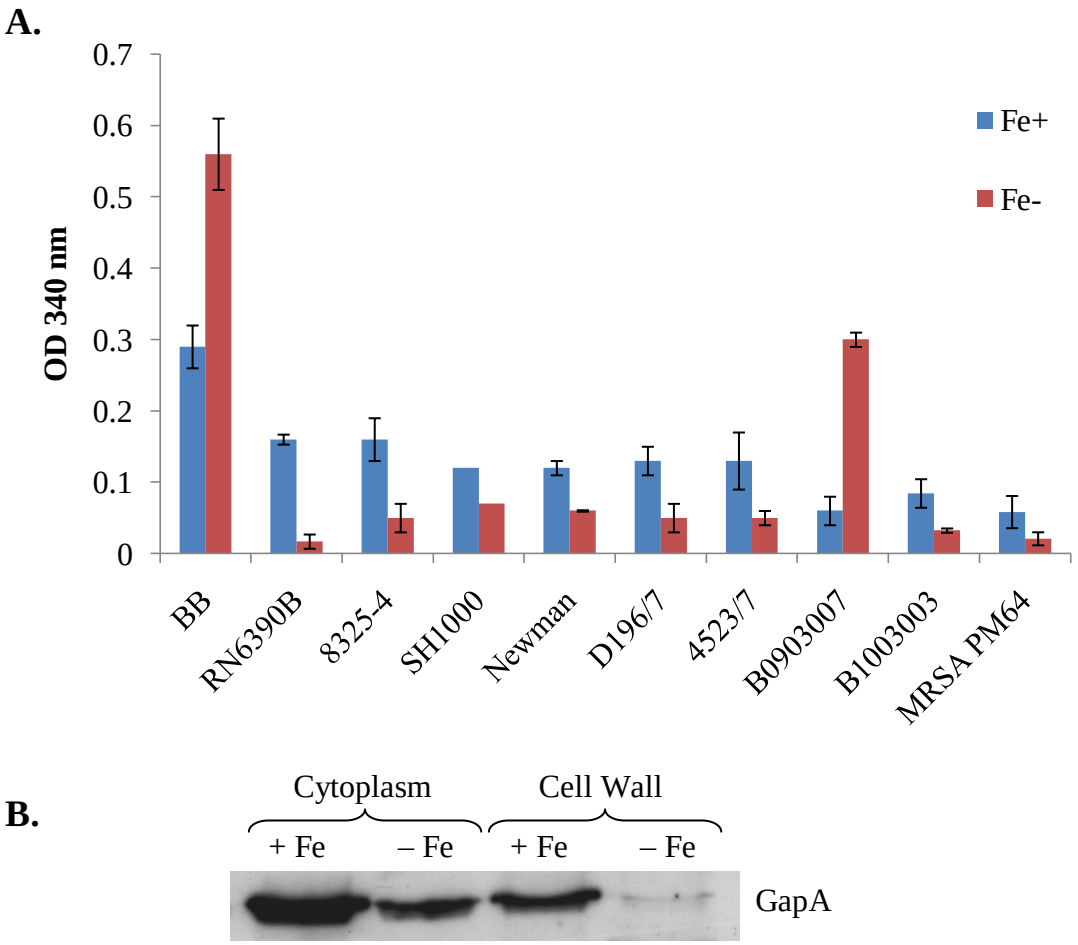


Figure 4-1. Iron regulation of the *S. aureus* GAPDH GapA is strain variable and shows differential levels of regulation between cell fractions.

(A) The GAPDH activity of *S. aureus* cells grown overnight in CRPMI medium in the presence and absence of 50 µM $FeSO_4$, washed in sodium phosphate buffer and incubated in GAPDH buffer containing G3P as substrate and NAD+ as cofactor. Activity is measured by absorbance of the reaction at 340nm after 30 minutes incubation at 37°C. Data presented is an average of three repeats carried out on different days, and errors bar show the standard deviation of the results. Data provided by Dr. Julie Morrissey (unpublished data).

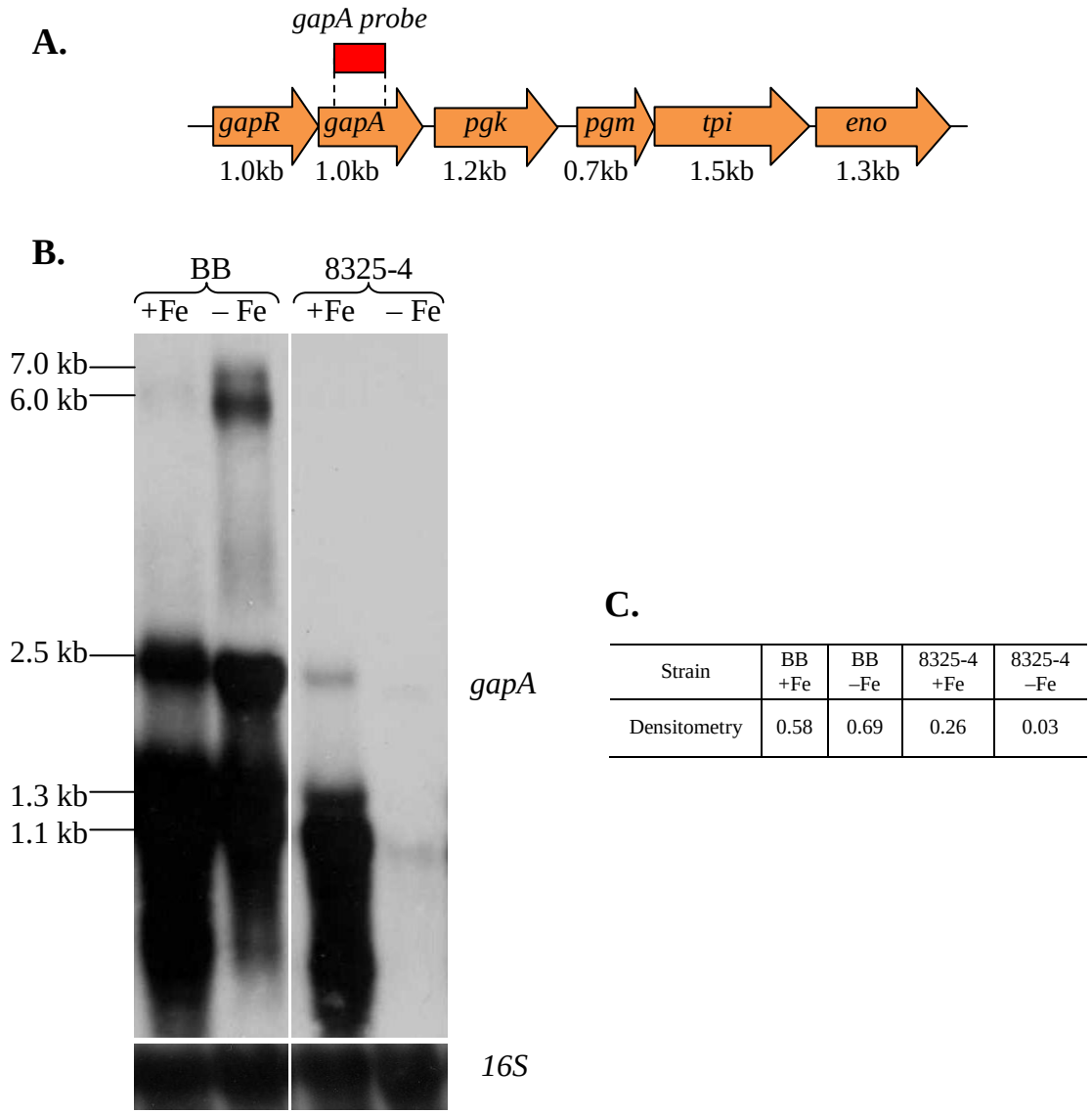
(B) Western blot showing the variation in the iron regulatory response between the cytoplasm and the cell surface of *S. aureus* strain RN6390B. After the cells were grown to stationary phase in CRPMI ± 10 µM Fe_2SO_4, protein fractions were harvested and immunoblots were carried out using anti-GapA antiserum. Equivalent samples are loaded in each lane. Data provided by Dr. Alan Cockayne, University of Nottingham (unpublished data).

4.2 Regulation of the glycolytic operon in response to metal ions

4.2.1 Transcription of *gapA* is strain variable in response to iron

Northern blot analysis was used to determine whether strain variable iron regulation of *gapA* occurs at the transcriptional level in the Class I strain BB and the Class II strain 8325-4. RNA was extracted from cells grown until late exponential phase in CRPMI medium in the presence and absence of 50 μM $FeSO_4$ and probed with an [α^{32}P] radiolabelled *gapA* DNA probe, produced using primers GapAPF and GapAPR (Figure 4-2A, Table 2.4). Growth of the strains was not significantly different in ± 50 μM $FeSO_4$ conditions after 6 hours static growth in CRPMI (late exponential phase).

As shown in Chapter 3, *gapA* forms part of the glycolytic operon in *S. aureus* and is co-transcribed with 4 other glycolytic enzymes and the operon regulator *gapR* (Figure 4-2A). In both BB and 8325-4 the *gapA* probe is able to detect multiple transcripts of approximate sizes 7.0, 6.0, 2.5, 1.3 and 1.1 kb (Figure 4-2B), with the largest transcript (7.0 kb) likely representing transcription of the entire operon. The results show that *gapA* is iron regulated at the transcriptional level and this regulation is strain variable (Figure 4-2B). In strain BB the larger transcripts (7.0 and 6.0 kb) are lower in high iron conditions compared to low iron, although the iron regulation of the smaller transcripts is less clear (Class I regulation). In strain 8325-4, the operon is expressed in high iron conditions, but is reduced in low iron conditions (Class II regulation). It should be noted that similar results were obtained for both 5 and 10 μM $FeSO_4$, and are not due to stress on the cell at 50 μM $FeSO_4$. Expression of *gapA* in strain 8325-4 is lower than that of BB, and as such the larger transcripts are often undetectable. It is therefore possible that Fe regulation of *gapA* involves control of transcript stability rather than transcript initiation. The difference in the level of expression between these strains is consistent



C.

Strain	BB +Fe	BB –Fe	8325-4 +Fe	8325-4 –Fe
Densitometry	0.58	0.69	0.26	0.03

Figure 4-2. Strain variable transcription of *S. aureus gapA* in high and low iron conditions

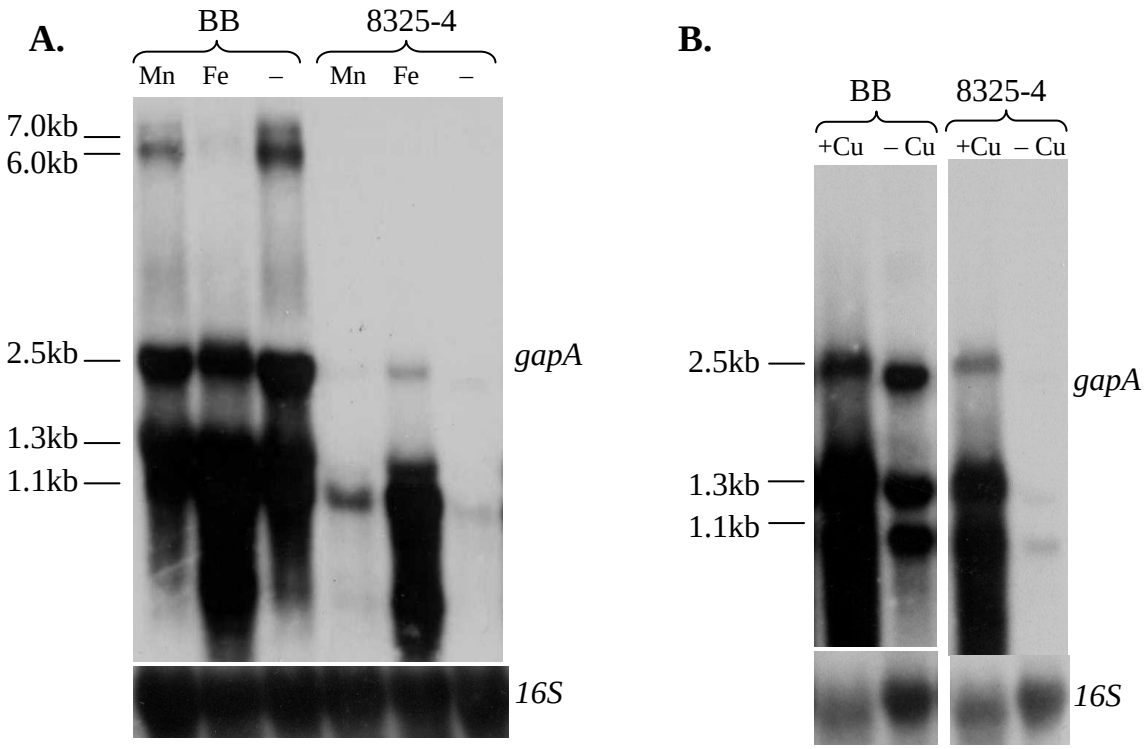
(A) Schematic representation of the glycolytic operon in *S. aureus*, including the position of the *gapA* probe. The position of transcriptional start sites and terminators has not been mapped for this operon to date.

(B) Northern blot with (C) densitometry data showing RNA transcription of *gapA* in strains BB and 8325-4 in response to iron. RNA was extracted from cells grown to late exponential phase in CRPMI ± 50μM Fe_2SO_4. The gel presented is representative of experiments that were repeated numerous times using RNA extracts from cultures grown on different days, with similar results observed each time. Blots were stripped and re-hybridised with a control probe (*16S*) to ensure equal loading of RNA in each case.

with the level of surface GAPDH activity. Northern blots using RNA harvested from strains grown to stationary phase (16 hours growth) also showed the same pattern of iron regulation as late exponential phase, indicating that there is no temporal change in expression of *gapA* in these strains (data not shown). The transcription of both *gapR* and *pgk* is also iron regulated in a strain dependent manner, which indicates that transcription of *gapA* is representative of iron regulation of the glycolytic operon as a whole (data not shown). Therefore a key "housekeeping" metabolic operon is not only iron regulated, but is also controlled in a strain dependent manner.

4.2.2 Strain variable regulation of *gapA* is specific to iron.

In order to determine whether the strain variable regulation of *gapA* is a specific response to iron, northern blot analysis was used to assess the transcriptional control of *gapA* in the presence of two other divalent transition metal ions, manganese and copper. Strains BB and 8325-4 were grown to late exponential phase in CRPMI medium, in the absence and presence of 25 μM $MnCl_2$ or 10 μM $CuCl_2$, and the RNA was harvested and probed with a radiolabelled *gapA* probe. These concentrations of metal ions were chosen as they are not toxic to the bacteria and did not affect the level or rate of growth, but normally elicit a regulatory response in *S. aureus*. Northern blot analysis shows that manganese does not have a significant effect on the transcription of *gapA* in either strain as transcription of *gapA* remains constant in CRPMI + Mn and CRPMI alone (Figure 4-3A), whereas copper increases transcription of *gapA* in both strains compared to the other experimental conditions (Figure 4-3B).



C.

Strain	BB +Mn	BB +Fe	BB – ions	8325-4 +Mn	8325-4 +Fe	8325-4 - ions	BB +Cu	BB –Cu	8325-4 +Cu	8325-4 –Cu
Densitometry	0.68	0.58	0.69	0.09	0.26	0.03	1.74	0.87	1.28	0.07

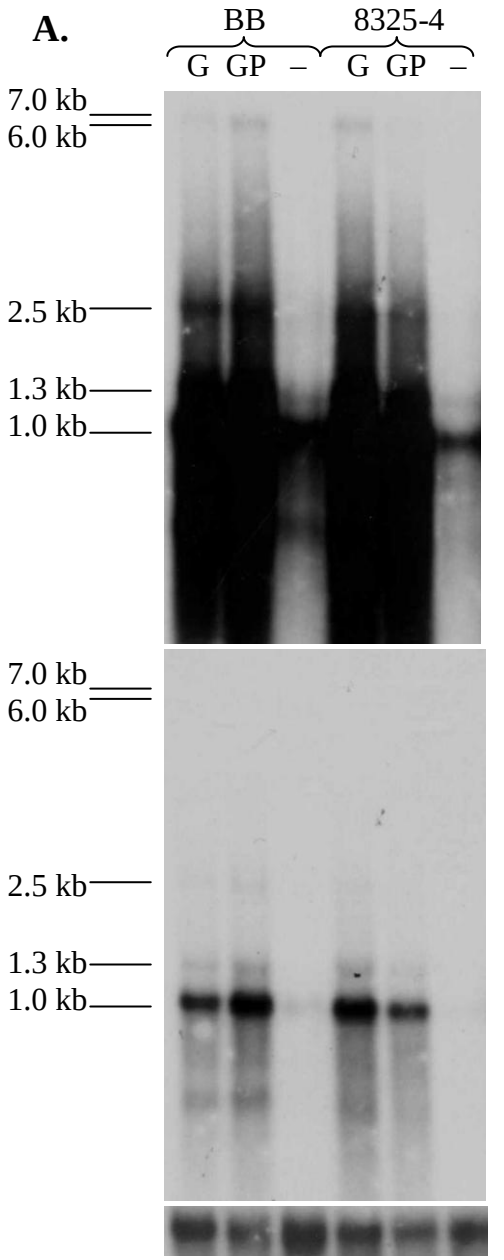
Figure 4-3. Transcription of *S. aureus gapA* is not strain variable in response to other divalent metal ions

Northern blot showing the regulation of *S. aureus gapA* in response to (A) manganese, iron and (B) copper in strains BB and 8325-4. Strains were grown in CRPMI medium with 25 μM $MnCl_2$, 50 μM Fe_2SO_4, 10 μM $CuCl_2$ or in the absence of additional metal ions to late exponential phase before RNA was harvested. Each gel is representative of multiple repeat experiments, and the *16S* control probe was used to ensure equal loading of the RNA samples, and the densitometry data (C) is presented as a ratio of *gapA/16S* transcript levels.

As previously shown in Chapter 3, *gapA* transcription is also increased in the presence of glucose in strain 8325-4. To determine whether this occurs in a strain dependent manner, strains BB and 8325-4 were grown in Tris minimal medium in the presence and absence of 1% glucose added to the cultures at either time 0 or 1 hour prior to harvesting the cells and probed for *gapA* transcription. CRPMI could not be used for this analysis as it contains 0.2% glucose, which we have already shown to be sufficient to induce glycolysis in *S. aureus* (Chapter 3). Figure 4-4 shows that in the presence of glucose transcription of *gapA* is increased in both strain BB and 8325-4, and is repressed in gluconeogenic conditions. Taken together these data show that strain variable expression of *gapA* and the glycolytic operon is specific to iron, and the strains behave in a similar manner in their response to other stimuli such as Cu and glucose.

4.2.3 The glycolytic operon is under the control of the global regulator Fur and MntR, but not PerR.

As the transcription of *gapA* is influenced by both Fe and Cu concentrations, it is possible that the glycolytic operon is under the control of global metal homeostasis regulators. As the Fe, Cu and Mn regulons overlap, *gapA* transcription was assessed in the isogenic regulator mutants 8325-4*Δfur*, 8325-4*ΔperR* and 8325-4*ΔmntR*. It is not possible to genetically manipulate strain BB, and so this experiment could not be carried out in a Class I strain. Fur is a global iron regulator, involved in the regulation of the iron scavenging response and also in the regulation of many virulence factors in *S. aureus*, while PerR is primarily involved in the regulation of the oxidative stress response and MntR is a Fe and Mn responsive regulator involved in manganese uptake (see Section 1.5.2 for details). At the time of study no Cu stress regulators had been identified to include in this experiment.



B.

Strain	BB +G	BB +GP	BB –	8325-4 +G	8325-4 +GP	8325-4 –
Densitometry	1.27	2.03	0.15	1.65	1.46	0.05

Figure 4-4. Transcription of *gapA* is increased in response to glucose in the Class I and Class II strains

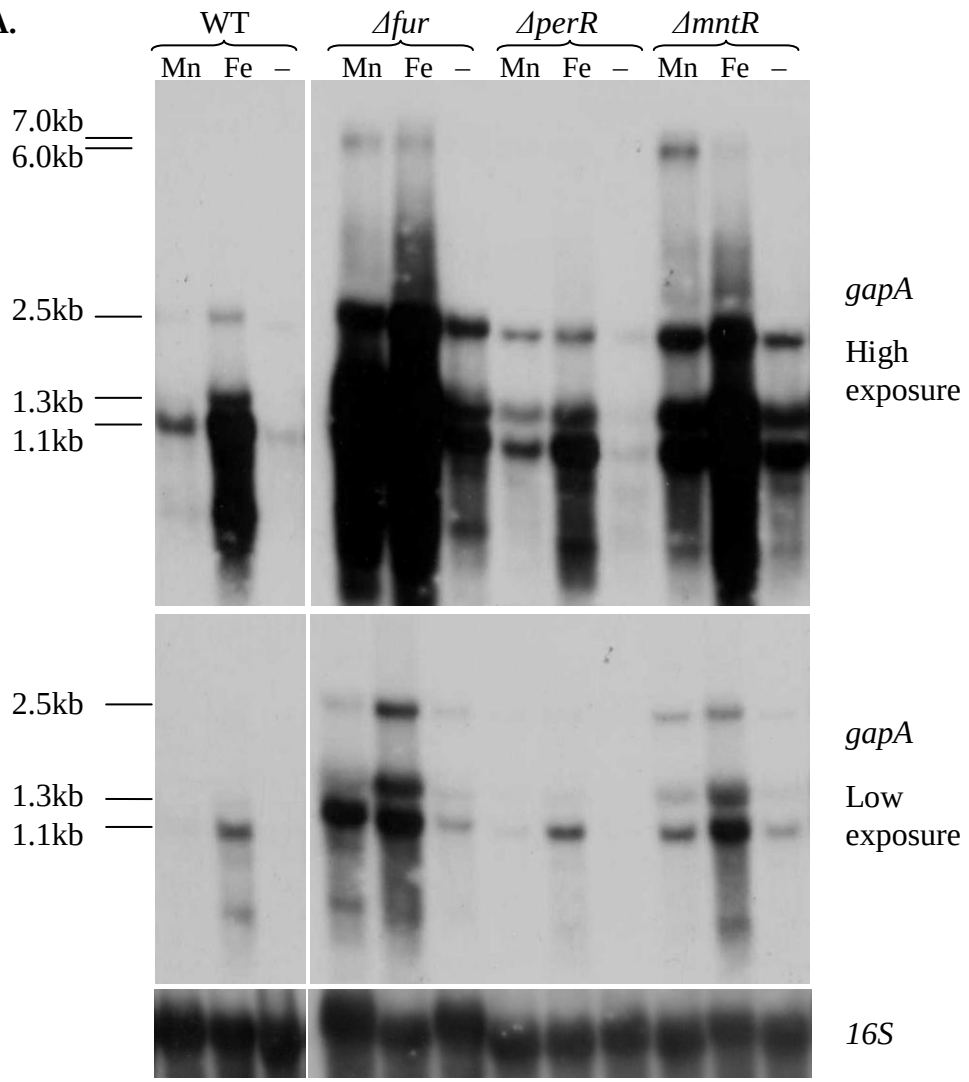
(A) Northern blot with (B) densitometry data showing the regulation of *S. aureus gapA* in response to glucose in strains BB and 8325-4. Total RNA was extracted from cells grown for 5 hours in TM broth with 1% glucose added at time 0 (G), 1% glucose added 1 hour before harvesting cells (GP), and without glucose (–). The gel presented is representative of experiments that were repeated twice using RNA extracts from cultures grown on different days, with similar results observed each time. Blots were stripped and re-hybridised with a control probe (*16S*) to ensure equal loading of RNA in each case.

RNA was extracted from the wild type and each of the mutant strains after growth to late exponential phase in CRPMI + 25μM $MnCl_2$, CPRMI + 50μM Fe_2SO_4 and CRPMI alone, and transcription of *gapA* was analysed using a radiolabelled *gapA* probe as previously described. The results show derepression of *gapA* transcription in each of the conditions tested in the *Δfur* and *ΔmntR* mutants, but not the *ΔperR* mutant (Figure 4-5). Therefore *gapA* appears to be regulated by Fur and MntR, but not via classic metal dependent repression, as both regulators repress *gapA* expression in low metal conditions as well as in the presence of divalent metal ions. The putative promoter region for the glycolytic operon (described below) does not contain binding motifs for either Fur or MntR, and therefore it is unclear whether the activity of these regulators is direct or indirect. It is possible that the control of *gapA* expression occurs via a novel mechanism for each of these two regulatory proteins.

4.3 Sequence variation of the putative *gapR* promoter region

Strain variable iron regulation of *gapA* may be a product of either sequence variation of the *gapA* promoter or variation in the strain background between the Class I and Class II strains. There does not appear to be a promoter upstream of *gapA* in *S. aureus*, and as this gene is transcribed as part of an operon the promoter is most likely to be upstream of *gapR*, as it is in *B. subtilis* (Ludwig *et al*., 2001). Bioinformatic analysis of the glycolytic operon from the publically available *S. aureus* genome sequences of strains 8325-4, COL, Newman, Mu50, JH1, MRSA252 and RF122, as well as the partial operon sequence from strain BB (GenBank accession no. AJ133520) showed that the glycolytic operon coding sequence is highly conserved between *S. aureus* strains, but surprisingly the intergenic region upstream of *gapR* is highly variable (Figure 4-6).

A.



B.

Strain	WT +Mn	WT +Fe	WT – ions	*Δfur* +Mn	*Δfur* +Fe	*Δfur* –ions	*ΔperR* +Mn	*ΔperR* +Fe	*ΔperR* –ions	*ΔmntR* +Mn	*ΔmntR* +Fe	*ΔmntR* –ions
Densitometry	0.03	0.13	0.02	0.42	0.79	0.09	0.04	0.10	0.003	0.16	0.52	0.07

Figure 4-5. Transcription of *gapA* in strain 8325-4 and isogenic regulator mutants

(A) Northern blots with (B) densitometry data showing iron regulation of *S. aureus gapA* in response to iron and manganese in the wild type strain 8325-4 and the isogenic 8325-4 regulatory mutants *Δfur, ΔperR* and *ΔmntR*. RNA was extracted from cells grown to late exponential phase in CRPMI + 50μM Fe_2SO_4, CRPMI + 25 μM $MnCl_2$ or CRPMI without additional ions. The gels presented are representative of experiments that were repeated at least twice using RNA extracts from cultures grown on different days, with similar results observed each time. Blots were stripped and re-hybridised with a control probe (*16S*) to ensure equal loading of RNA in each case.

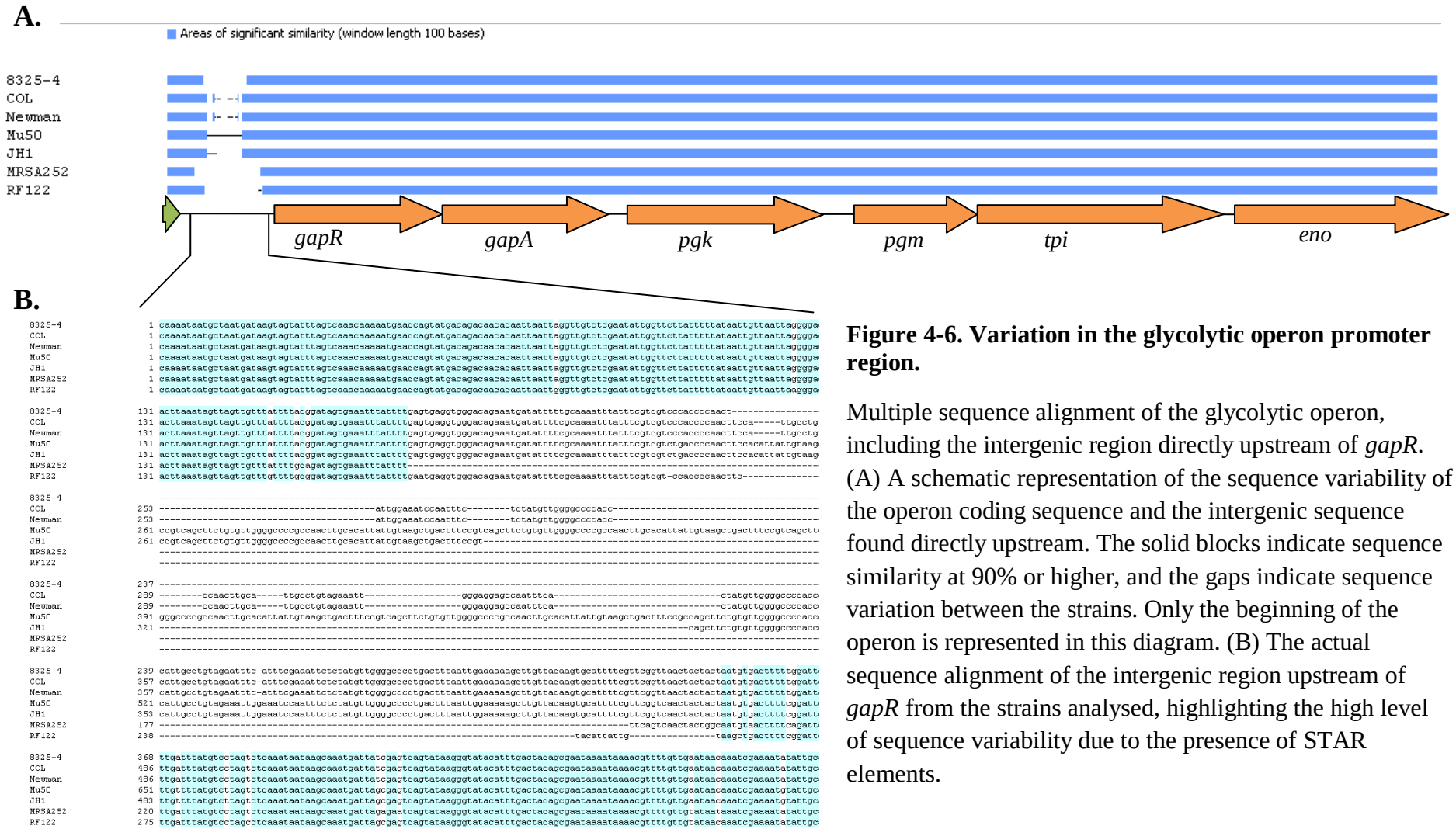


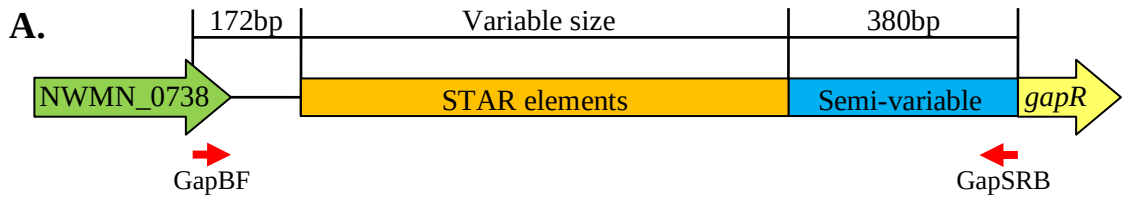
Figure 4-6. Variation in the glycolytic operon promoter region.

Multiple sequence alignment of the glycolytic operon, including the intergenic region directly upstream of *gapR*. (A) A schematic representation of the sequence variability of the operon coding sequence and the intergenic sequence found directly upstream. The solid blocks indicate sequence similarity at 90% or higher, and the gaps indicate sequence variation between the strains. Only the beginning of the operon is represented in this diagram. (B) The actual sequence alignment of the intergenic region upstream of *gapR* from the strains analysed, highlighting the high level of sequence variability due to the presence of STAR elements.

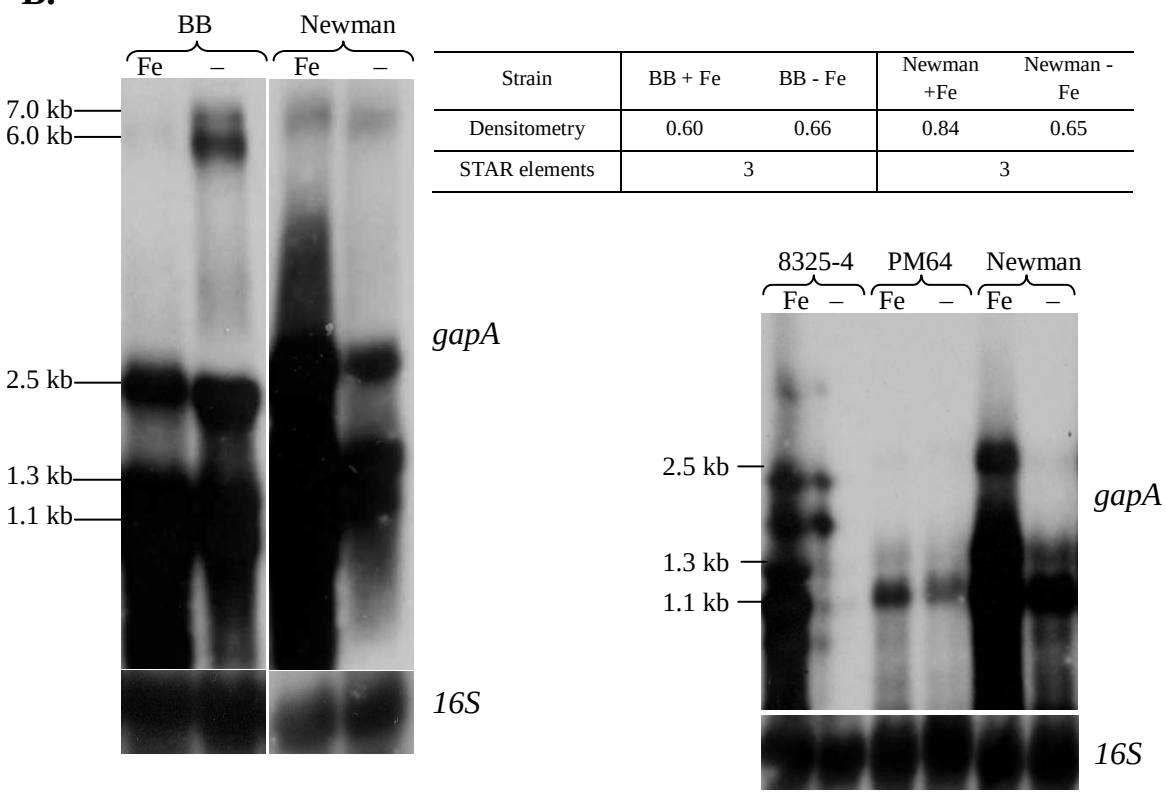
Importantly the *gapR* protein is 100% conserved and therefore the variable iron regulation is not due to differences in this regulatory protein. The sequence variation upstream of *gapR* can be subdivided into two regions (Figure 4-7). Directly upstream of the *gapR* open reading frame is a 380 bp semi-variable region. This sequence is primarily conserved between strains, although there is some sequence variation between the Class I and Class II strains. This region is preceded by a variable number of *Staphylococcus aureus* Repeat (STAR) elements (Figure 4-7). STAR elements are GC-rich interspersed direct repeats found at many loci across the *S. aureus* genome (Cramton *et al*., 2000), and are discussed further in Section 1.4.2 and in Chapter 5.

4.3.1 The *gapR* STAR elements do not appear to be directly responsible for the strain variable iron regulation of the glycolytic operon

To determine whether sequence variation could be responsible for the strain variable iron regulation of the glycolytic operon this intergenic region was compared between the Class I strain BB and the Class II strain 8325-4, alongside two additional Class II strains Newman and MRSA PM64 (Figure 4-7). Northern blot analysis shows that the number of STAR elements in this intergenic region has no correlation with the Fe regulation of *gapA*. The Class I strain BB and the Class II strain Newman each contain 3 STAR element repeats, but show different iron regulation of *gapA*. Strains 8325-4 and PM64 both show Class II iron regulation of *gapA*, but 8325-4 only contains a single repeat motif, while PM64 is devoid of STAR elements at this locus (Figure 4-7B). Therefore the number of STAR elements cannot be responsible for strain variable iron regulation of *gapA*.



B.



Strain	BB + Fe	BB - Fe	Newman +Fe	Newman - Fe
Densitometry	0.60	0.66	0.84	0.65
STAR elements	3		3	

Strain	8325-4 + Fe	8325-4 - Fe	PM64 + Fe	PM64 - Fe	Newman + Fe	Newman - Fe
Densitometry	0.88	0.16	0.25	0.21	1.01	0.58
STAR elements	1		0		3	

Figure 4-7. Comparison of *gapA* expression in strains with variation in the *gapR* intergenic region

(A) Schematic representation of the organisation of the intergenic sequence upstream of *gapR*, including the position of the primers used to amplify and sequence this region.

(B) Two northern blots with densitometry data demonstrating the variable expression levels of this operon in the Class I strain BB, and the Class II strains 8325-4, PM64 and Newman. RNA was extracted from cells grown to late exponential phase in CRPMI ± 50μM Fe_2SO_4. The gels presented are representative of experiments that were repeated at least twice using RNA extracts from cultures grown on different days, with similar results observed each time. Blots were stripped and re-hybridised with a control probe (*16S*) to ensure equal loading of RNA in each case.

However there does appear to be a correlation with the number of repeats and the level of *gapA* transcription between these strains (Figure 4-7B). In strains BB and Newman, both of which have 3 repeats, *gapA* is expressed at a similar level. Expression in strain 8325-4 which has a single repeat motif is lower than BB and Newman, and strain PM64 which is missing the repeats entirely has an even lower level of *gapA* expression than 8325-4. The potential contribution of the STAR elements on the level of expression of the glycolytic operon is examined further in Chapter 5.

4.3.2 Sequence variation of the semi-variable region of the *gapR* putative promoter appears to correlate with strain variable iron regulation

Comparison of the semi-variable region between these strains revealed that the sequence is 100% conserved between the Class II strains 8325-4 and Newman, while the Class I strain BB contains 18 bp substitutions and a single bp deletion (Figure 4-8). 12 of these substitutions, and the deletion fall within the first 70 bp at the 5' of the semi-variable region. This region was sequenced from a second Class I regulated *S. aureus* strain, B0903007, and was found to be 100% conserved with strain BB, indicating that this sequence of the semi-variable region may be responsible for strain variable iron regulation of the operon. Interestingly the first 39 bp is missing from the 5' end of this region in strain PM64 (Figure 4-8). Transcriptional analysis of this strain shows that this strain follows Class II type expression albeit at a lower level than strains 8325-4 and Newman, which may be due to loss of this sequence or the lack of STAR elements.

4.4 Mapping the transcriptional start site of the glycolytic operon

In order to determine whether sequence variation found upstream of *gapR* may be important for promoter activity, it was important to establish the position of the

Figure 4-8. Variability of the *gapR* putative promoter semi-variable region.

This is a multiple sequence alignment of the 380 bp semi-variable region located directly upstream of the *gapR* open reading frame from the Class I strain BB and the Class II strains 8325-4, Newman and MRSA 252 (MRSA PM64). Base pair changes between the strains are highlighted in red. The sequence from strains 8325-4 and Newman are identical at each position identified, while the sequence from strain BB contains 19 bp substitutions compared to these strains. Strain MRSA 252 (MRSA PM64), which has already been identified as lacking STAR elements, is missing the first 39 bp of this region, and also has several base pair changes in common with strain BB.

transcriptional start site upstream of *gapR*. Three strategies were used to attempt to map the transcriptional start site of the glycolytic operon, each with limited success. A 5' rapid amplification of cDNA ends (5'RACE) technique failed to produce any usable results, and will be discussed further in the Section 4.6.

4.4.1 Radioactive Primer Extension

Primer extension experiments are commonly used in transcript mapping of genes in many species, including *S. aureus* (Harraghy *et al*., 2008). The basic principle is to produce a single labelled cDNA molecule from total RNA extract using a reverse primer located downstream of the 5' end of the gene of interest. As the reverse transcription reaction will terminate at the 5' end of the mRNA molecule, the size of the primer extension product can be used to determine the transcriptional start site (+1) by running the product alongside a sequencing ladder (Figure 4-9). Gene specific primers GapBRT2, GapRT2 and EapR were used for the transcript mapping of the genes *gapB*, *gapR* and *eap* respectively. The +1 site of the extracellular adherence protein *eap* has previously been mapped by radioactive primer extension and was included as a positive control for this technique (Harraghy *et al*., 2008).

The +1 site of *eap* from strain Newman was accurately mapped by radioactive primer extension (Figure 4-10). The procedure was then repeated for transcript mapping of *gapB* and *gapR*, and was able to determine the +1 site for *gapB*. The *gapB* transcript originates 38 bp upstream of the *gapB* coding sequence (Figure 4-11). This is the first time the transcript start site of this gene has been mapped in *S. aureus*. The experiment was repeated twice with RNA from strains BB and Newman on different days, demonstrating reproducibility in multiple strains.

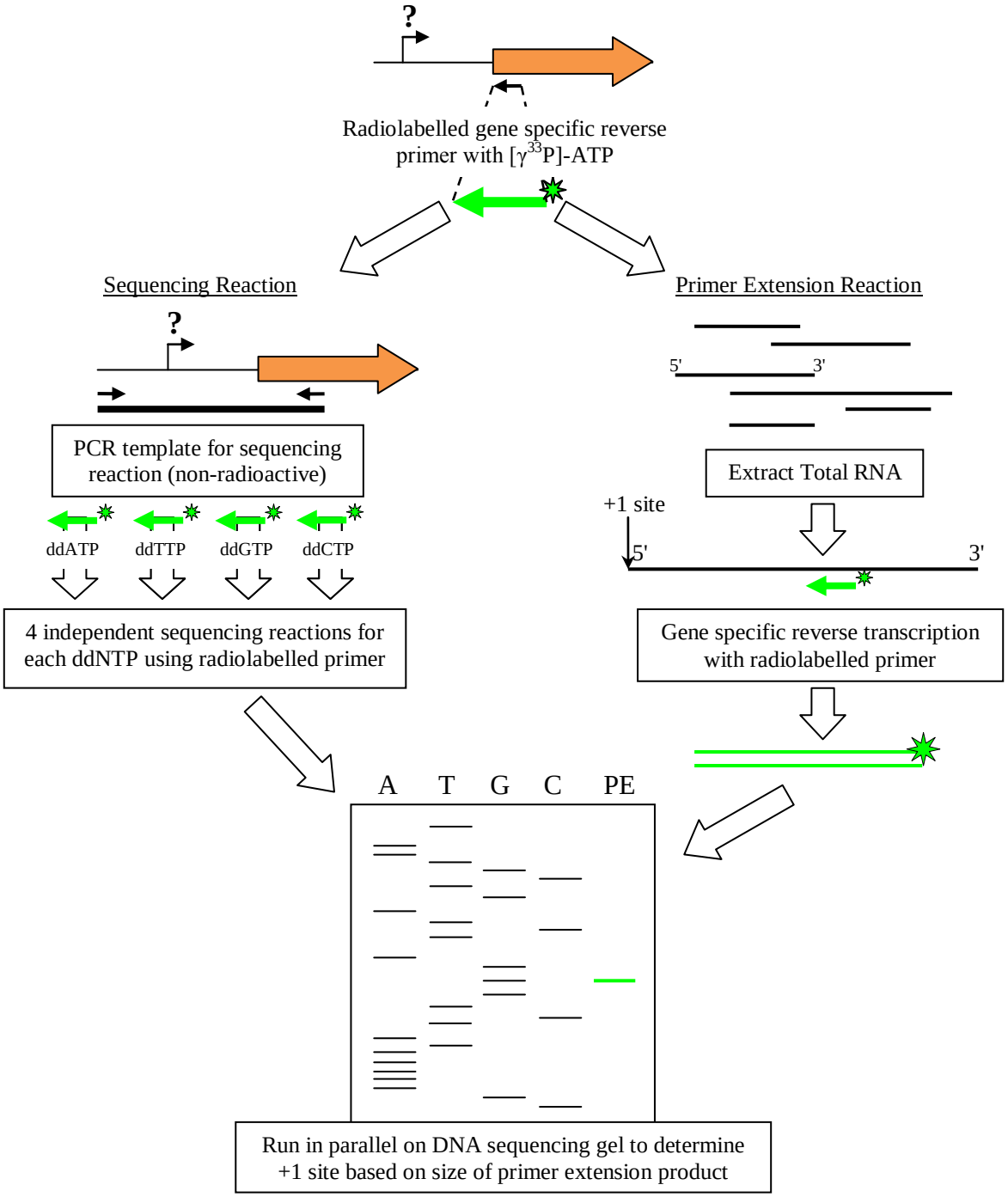


Figure 4-9. Radioactive primer extension method for transcript mapping of +1 site.

A gene specific reverse primer is radiolabelled using [γ^{33}P]-ATP and used for both sequencing of the putative promoter region and primer extension of mRNA. Primer extension using reverse transcriptase produces a radiolabelled product of specific size, between the known primer site and the unknown start of the gene transcript. The 4 independent sequencing reactions produce a sequencing ladder which begins at the known primer site. When the primer extension product and sequencing ladder are run in parallel the base with which the primer extension product aligns is revealed to be the +1 site of the gene.

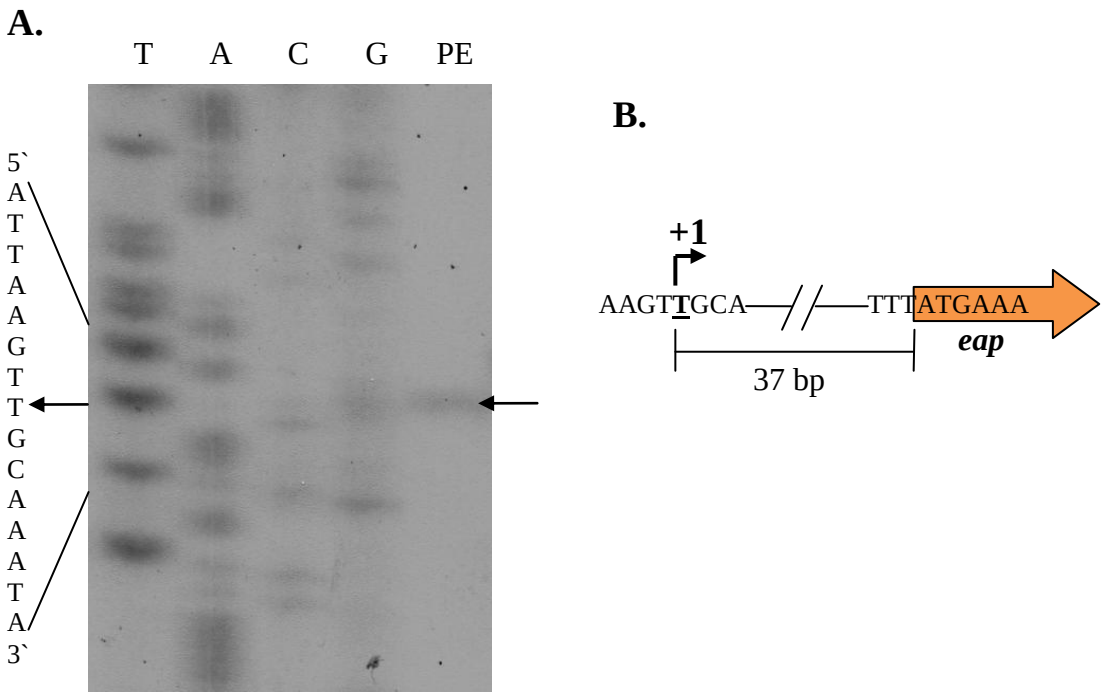


Figure 4-10. Radioactive primer extension for the *eap* transcript, confirming the position of the +1 site.

(A) Radiograph showing the position of the *eap* +1 transcriptional start site, as determined by primer extension The independent sequencing ladder for each of the base pairs is shown in the first four lanes (T, A, G, C), and can be read 5` to 3` from the top of the gel to the bottom, and the primer extension product is shown in the fifth lane (PE).

(B) A schematic representation of the position of the +1 site in relation to the coding sequence. The primer extension product aligns with the T nucleotide, which is consistent with the result published by (Harraghy *et al*., 2005).

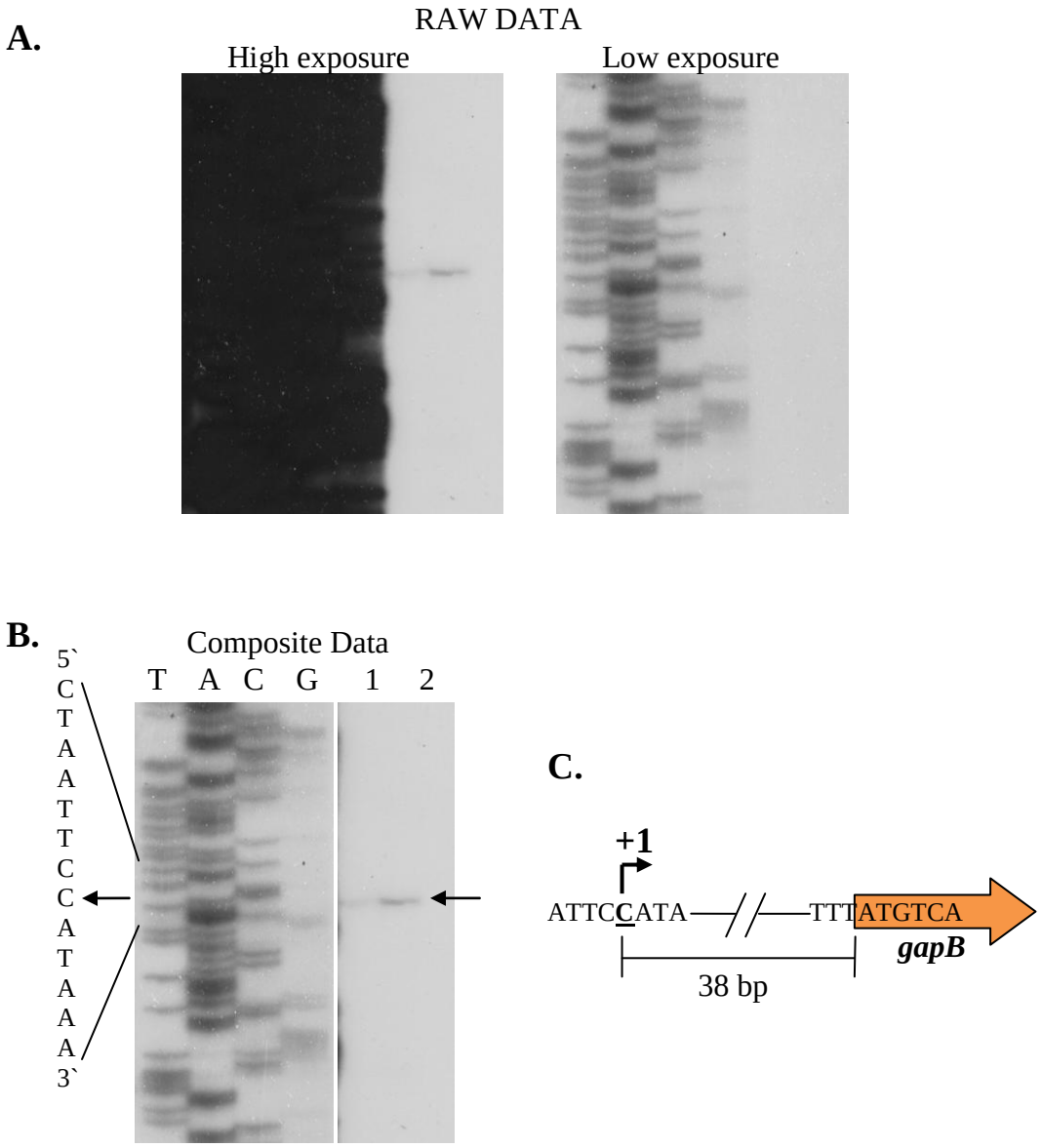


Figure 4-11. Radioactive primer extension for the *gapB* transcript, identifying the position of the +1 site.

(A) Radiographs of the *gapB* primer extension sequencing gel after high and low exposure of the results. The primer extension product is clearly visible after high exposure, but this renders the sequencing ladder unreadable. Low exposure clearly shows the ladder, by the primer extension product is difficult to detect.

(B) Composite of the *gapB* ladder and sequencing gel results to accurately determine the *gapB* +1 site, highlighted with an arrow. Each individual sequencing ladder (lanes T, A, G, C) can be read 5` to 3` from the top of the gel to the bottom, and the primer extension product is shown in lane PE.

(C) Schematic representation of the position of the +1 site upstream of *gapB*.

As sequence analysis of the putative *gapR* promoter region identified variation of the sequence between strains, it was decided that to get a true representation of the *gapR* +1 site the primer extension protocol would have to be carried out separately for strains BB, 8325-4 and PM64 (Figure 4-12). By using the primer GapRT2, approximately 140 bp upstream of the *gapR* coding region was covered in the initial tests for this promoter, and for each strain there was no indication of a primer extension product for *gapR* in this region (Figure 4-12A). This is one limitation of this technique in identifying transcriptional start sites; it only allows approximately 300 bp of sequence to be analysed, and if the primer is too far from the +1 site it will not be detected. Therefore the experiment was repeated using primer GapSRB, which originates 27 bp upstream of the *gapR* open reading frame, allowing coverage approximately ~300 bp upstream of *gapR* and encompassing almost the entire semi-variable region (Figure 4-12B).

No clear primer extension product was detected for any of the strains using either primer, even after overexposure of the blots (data not shown). Furthermore there is no evidence of a *gapR* primer extension product for any strain in the upper regions of the gel where the sequencing ladder is not fully resolved. This suggests that rather than the product being retarded in the top of the gel as it is too large to run, there is actually no product present. This procedure was repeated for each strain using fresh RNA and produced similar results. A similar fluorescent labelled primer extension technique which allows mapping of large regions of sequence in a single reaction with greater sensitivity was chosen to try and determine whether the *gapR* +1 site occurs further upstream than previously tested.

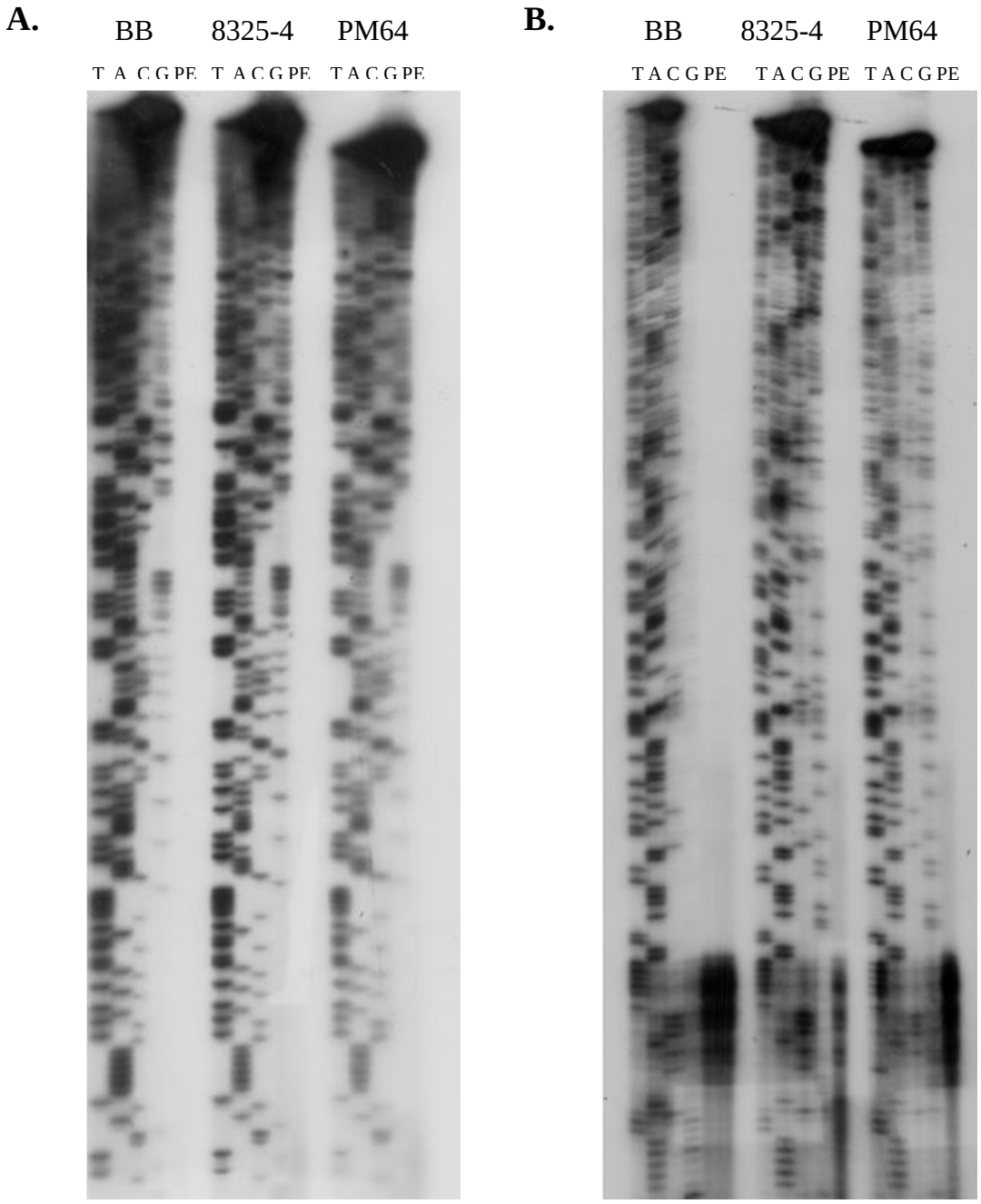


Figure 4-12. Primer extension results for *gapR*.

The above radiographs show the results for *gapR* primer extension experiments from strain BB, 8325-4 and PM64 using (A) primer GapRT2 and (B) primer GapSRB. Sequencing ladders are shown in the order of T, A, C, G and can be read 5` to 3` from the top of the gel to the bottom. No primer extension products were detected for any of the strains.

4.4.2 Fluorescent Primer Extension

Fluorescent primer extension was first described for the mapping of transcriptional start sites of *Helicobacter pylori* from promoters cloned into *E. coli* (Lloyd *et al*., 2005). The method allows a rapid determination of the +1 site of a promoter, without the use of radiation or the need for production of gene specific sequencing ladders. The principle is exactly the same as radioactive primer extension, but the use of a fluorescently labelled primer means that the primer extension product can be run using a capillary sequencer alongside a known size standard and analysed using GeneScan® software (Applied Biosystems). This allows greater resolution of the product over a much larger length of sequence (up to 1.2 kb if necessary) and the ability to determine the exact size of the primer extension product to a single nucleotide. The +1 site can be determined as the size of the fragment represents the distance between the +1 site and the gene specific primer used.

Fluorescent primer extension was carried out for both the positive control *eap* with RNA from strain Newman, and for *gapR* with RNA from strains BB and 8325-4, using primers Eap PE+FAM and GapR PE+FAM respectively (Figure 4-13; Figure 4-14). The primer extension products were run using an Applied Biosystems 3730 sequencer (PNACL, University of Leicester) alongside either a GeneScan 500 LIZ or 1200 LIZ size standard ladder (Applied Biosystems). These ladders are made up of known sized fragments covering 500 and 1200 bp respectively, to allow accurate size determination of the primer extension products down to a single nucleotide. The results for the positive control *eap* conclusively determined the +1 site for this gene, 221 bp upstream of the Eap PE+FAM primer binding site (Figure 4-13). This agrees with both the previously published +1 site and the results from the radioactive primer extension

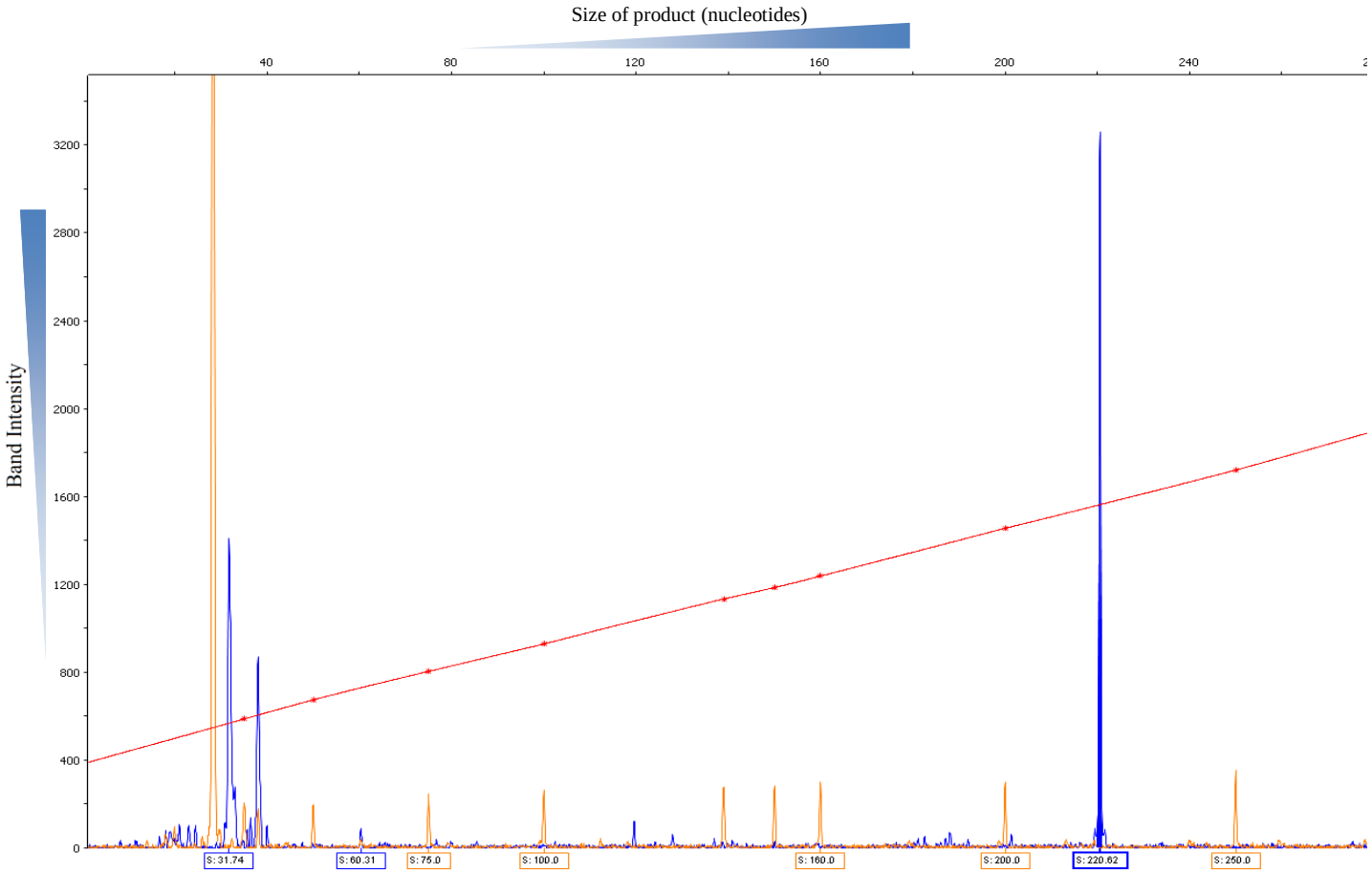


Figure 4-13. Fluorescent primer extension data identifying the position of the *S. aureus eap* +1 site.

GeneScan trace of the *eap* fluorescently labelled primer extension product (blue peaks), run alongside a GeneScan LIZ 500 ladder (orange peaks). The data was produces using primer Eap PE+FAM from RNA extracted from strain Newman. Size (in nucleotides) are marked below key peaks from both the sample and the ladder. The large blue peak (221 bp), corresponds to the +1 site previously identified for *S. aureus eap*, and the blue peak corresponding to 31 bp in length is unincorporated primer.

described above (Harraghy *et al*., 2005). A large peak situated at ~ 30 bp represents unincorporated labelled primer, while a smaller peak at ~ 60 bp is a common artefact identified in the original description of this protocol (Lloyd *et al*., 2005).

No primer extension product was detected for *gapR* using RNA from either BB or 8325-4 when using either the 500 or 1200 bp size ladder, but both unincorporated primer (~30 bp) and the unknown common artefact (~ 60 bp) were still detectable (Figure 4-14). These results suggests that no primer extension product is being produced during the reverse transcription reaction, as the peak representing primer alone is very intense compared to the *eap* positive control showing that it has not been incorporated into cDNA. This is likely to be the same reason that no product is detectable in the radioactive primer extension protocol.

Taken together these data (or lack thereof) suggest the mapping of the *gapR* +1 site is not possible via standard reverse transcriptase processes. The fact that accurate transcript mapping of both the positive control *eap* and also the GAPDH homologue *gapB* was achieved using these methods may suggest that the inability to map the glycolytic operon promoter is to due to certain properties of this DNA region, possibly due to the presence of STAR elements.

4.5 Sequence variation rather than strain background is responsible for the strain variable iron regulation of the glycolytic operon

To further investigate whether strain variable iron regulation of *gapA* is due to sequence variation of the *gapR* upstream region or differences in strain background, transcriptional reporters were used to analyse the expression of putative *gapR* promoters

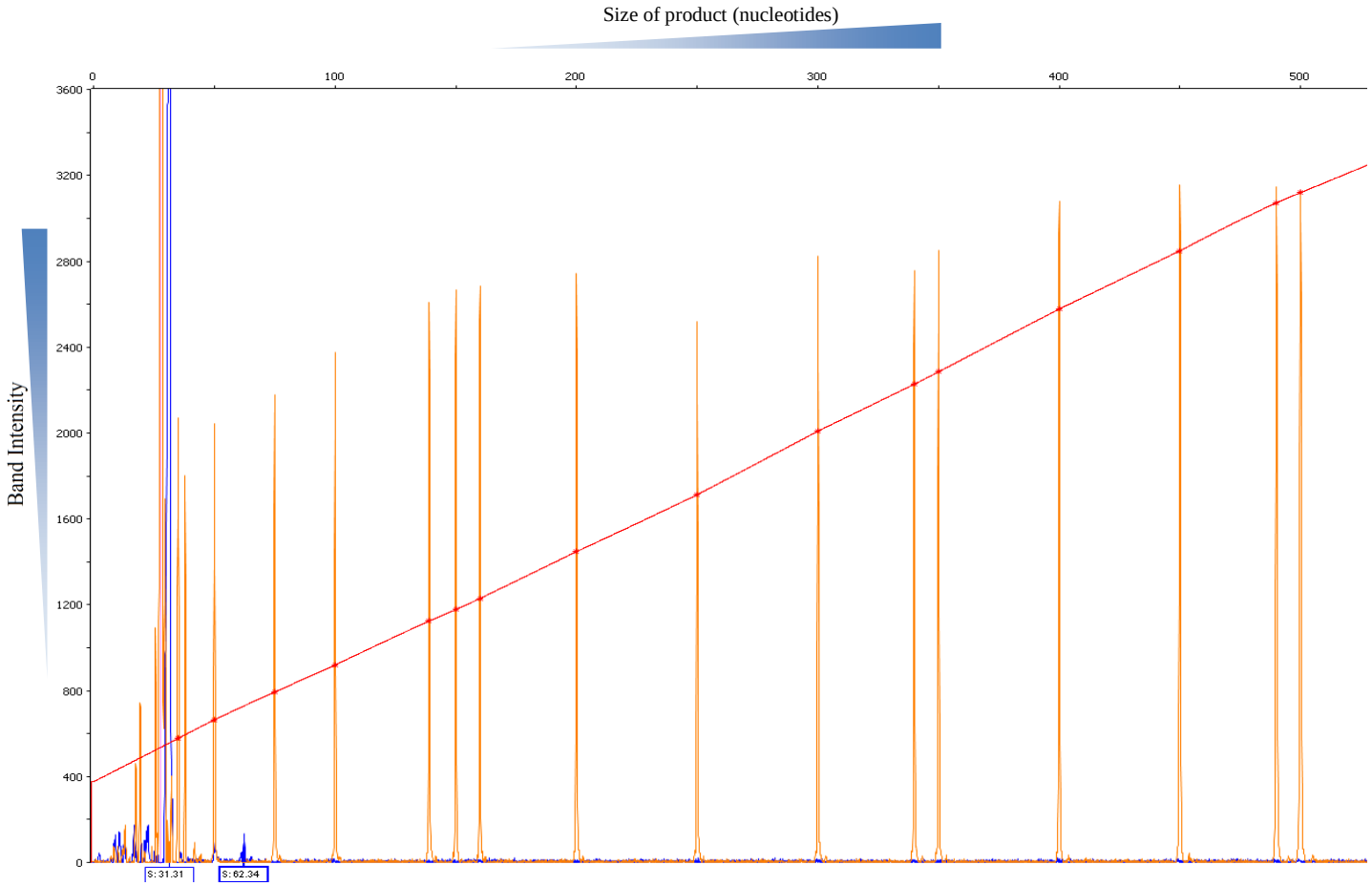


Figure 4-14. Fluorescent primer extension data showing the lack of a product for *S. aureus gapR*.

GeneScan trace of the *gapR* fluorescently labelled primer extension product (blue peaks), run alongside a GeneScan LIZ 500 ladder (orange peaks). The data was produces using primer GapR PE+FAM from RNA extracted from strain 8325-4. Similar results were obtained with RNA from strain BB. Sizes (in nucleotides) are marked below key peaks from the sample only. The large blue peak (31 bp) is unincorporated primer, and the second smaller peak (62 bp) is a common artefact seen for this method, and not a valid +1 site (Lloyd *et al*., 2005).

of different *S. aureus* strains. Although the +1 site for *gapR* transcription has not yet been mapped, this technique is still useful in determining the impact of STAR elements on *gapR* transcription as well as identifying sequences important in the regulation of this operon. The β-lactamase transcriptional reporter plasmid pSK5645 (Appendix Figure A-1), which has a promoterless *βlaZ* gene containing its own ribosomal binding site, was chosen for this study (Grkovic *et al*., 2003). Promoter activity was measured via hydrolysis of the chromogenic β-lactamase substrate nitrocefin, resulting in a rapid colour change quantitatively measured as absorbance of the solution at 490nm.

The iron regulated gene *lysR* was used as a positive control for this experiment, and empty pSK5645 vector was used as a negative control (Figure 4-15). The *lysR* promoter was cloned into pSK5645 using primers *lysR* F and *lysR* R, and both constructs were transduced into *S. aureus* strain 8325-4 for analysis. β-lactamase activity was assayed in both CRPMI and the iron depleted medium SSD (staphylococcal siderophore detection) (Lindsay & Riley, 1994), in the presence and absence of 50 μM $FeSO_4$. The results show that the positive control *lysR* is classically iron regulated, with promoter activity reduced in high iron conditions, while the negative control has no promoter activity. Although the trend in β-lactamase activity was the same in both media, the results for SSD were more reproducible and this medium was used for the remainder of the experiments.

In order to assess the role of STAR elements on expression of the operon, both short (semi-variable region only) and long (semi-variable region with STAR elements) putative promoter regions from strains BB, 8325-4 and Newman were cloned into pSK5645. Primers GapRCF and GapSRB were used for cloning the short constructs and

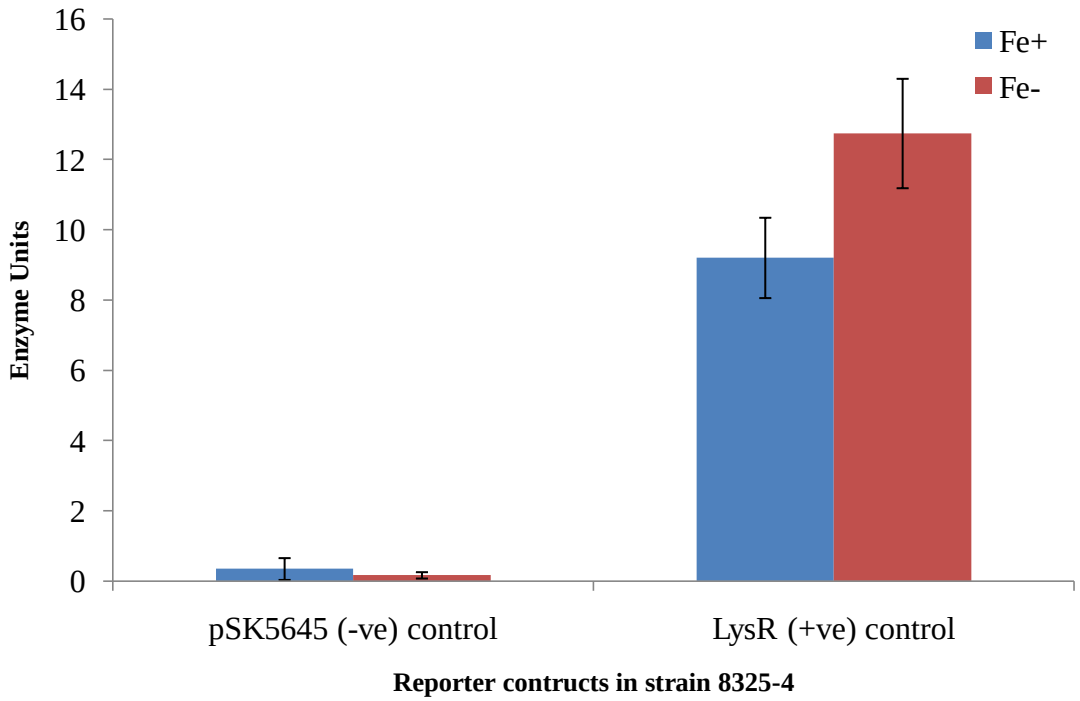


Figure 4-15. Controls for the β-lactamase reporter system.

β-lactamase activity of the negative control plasmid (pSK5645 alone) and the positive control promoter (*lysR*) in strain 8325-4, after 16 hours growth in SSD medium in the presence and absence of 50 μM $FeSO_4$. The data presented is an average of at least three repeat experiments carried out on different days, and the error bars indicate the standard error of the data.

primers GapBF and GapSRB were used for cloning the long constructs (Figure 4-16A). Strain MRSA PM64 does not contain STAR elements or the primer site for GapRCF and therefore a single "long" reporter construct was produced for this strain using primers GapBF and GapSRB. The recombinant plasmids were cloned into *E. coli* and sequenced before transformation into *S. aureus* strain RN4220. As the Class I strain BB is unable to accept foreign DNA, all of the reporters were introduced into the Class II strain 8325-4. However this will determine whether sequence variation in the putative promoter region or the strain background is responsible for the different classes of iron regulation of the glycolytic operon.

4.5.1 Both the semi-variable and STAR element regions are involved in *gapR* promoter activity

Each of the reporter construct strains were grown for 16 hours in SSD medium in the presence and absence of 50 μM $FeSO_4$, before the cells were washed and assayed for β-lactamase activity (Figure 4-16B). Comparison of the "short" reporters demonstrated promoter activity in the semi-variable region. The BB short reporter shows strong promoter activity which retains Class I Fe regulation even in a Class II strain background, indicating that the sequence of the *gapR* putative promoter, rather than strain background, is responsible for the strain variable iron regulation of this operon. Furthermore the iron regulation is due to control of the initiation of transcription and not differences in transcript stability between the high and low iron conditions. Interestingly the Class II short reporters (8325-4 and Newman) and the PM64 reporter demonstrate that although the semi-variable region of these strains contains a promoter, it is not regulated by iron.

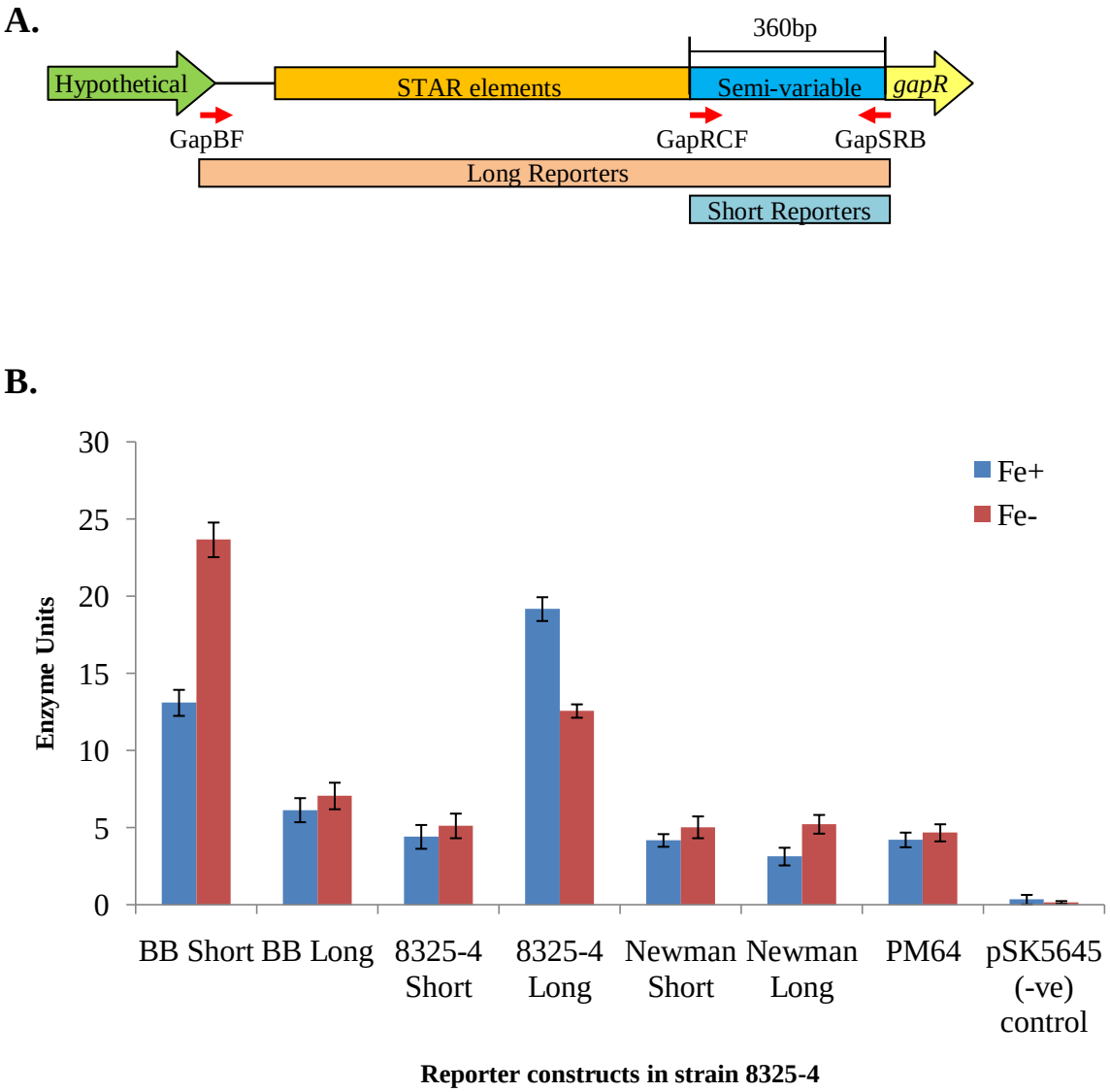


Figure 4-16. The impact of the STAR elements on expression of a *gapR* transcriptional reporter.

(A) Schematic representation of the putative *gapR* promoter regions cloned into the transcriptional β-lactamase reporter plasmid pSK5645.

(B) A graph showing the enzyme activity of β-lactamase expressed from the putative promoter of *gapR* from each of the short and long reporter constructs. Promoters were cloned into plasmid pSK5645 and expression was analysed in a common strain background of 8325-4. The data presented is an average of at least 3 repeats for each construct, and the error bars indicate the standard error of the results.

In comparison the 8325-4 “long” reporter, containing a single STAR motif, shows increased promoter activity and Fe dependent regulation (Figure 4-16B). This indicates that the STAR elements are required for Class II regulation of the operon. Unexpectedly the addition of 3 STAR motifs to the BB and Newman long reporters did not enhance the promoter activity in this way, and actually reduced the BB promoter activity to a level similar to the Class II short reporters (Figure 4-16B). These results are not representative of expression of the glycolytic operon in the actual strains, as determined at both the transcriptional and protein level, which suggests that the presence of multiple STAR element repeat motifs in the plasmid based reporter limits the levels of expression. As the presence of a single repeat motif does enhance transcription and Class II iron regulation, the reason multiple repeats fail to do this is most likely the result of the formation of secondary structures in the plasmid DNA.

4.5.2 The first 39 bp of the semi-variable region are responsible for Class I regulation of the glycolytic operon

To further investigate the position of the promoter within the semi-variable region, the STAR elements as well as the first 39 bp of the semi-variable region which are both missing in strain PM64, were deleted from the region in BB to produce a PM64-like BB reporter construct (BB Fusion, Figure 4-17A). This was achieved using a fusion PCR strategy, to produce a seamless integration of the 5' and 3' regions without the introduction of restriction enzyme sites. The BB fusion construct was cloned into pSK5645, introduced into *S. aureus* strain 8325-4, and the promoter activity was assayed as previously described. The results show that the BB fusion construct has a similar level of β-lactamase activity to that of PM64; a dramatic decrease compared to the short BB reporter construct (Figure 4-17B). Therefore strain BB also contains the

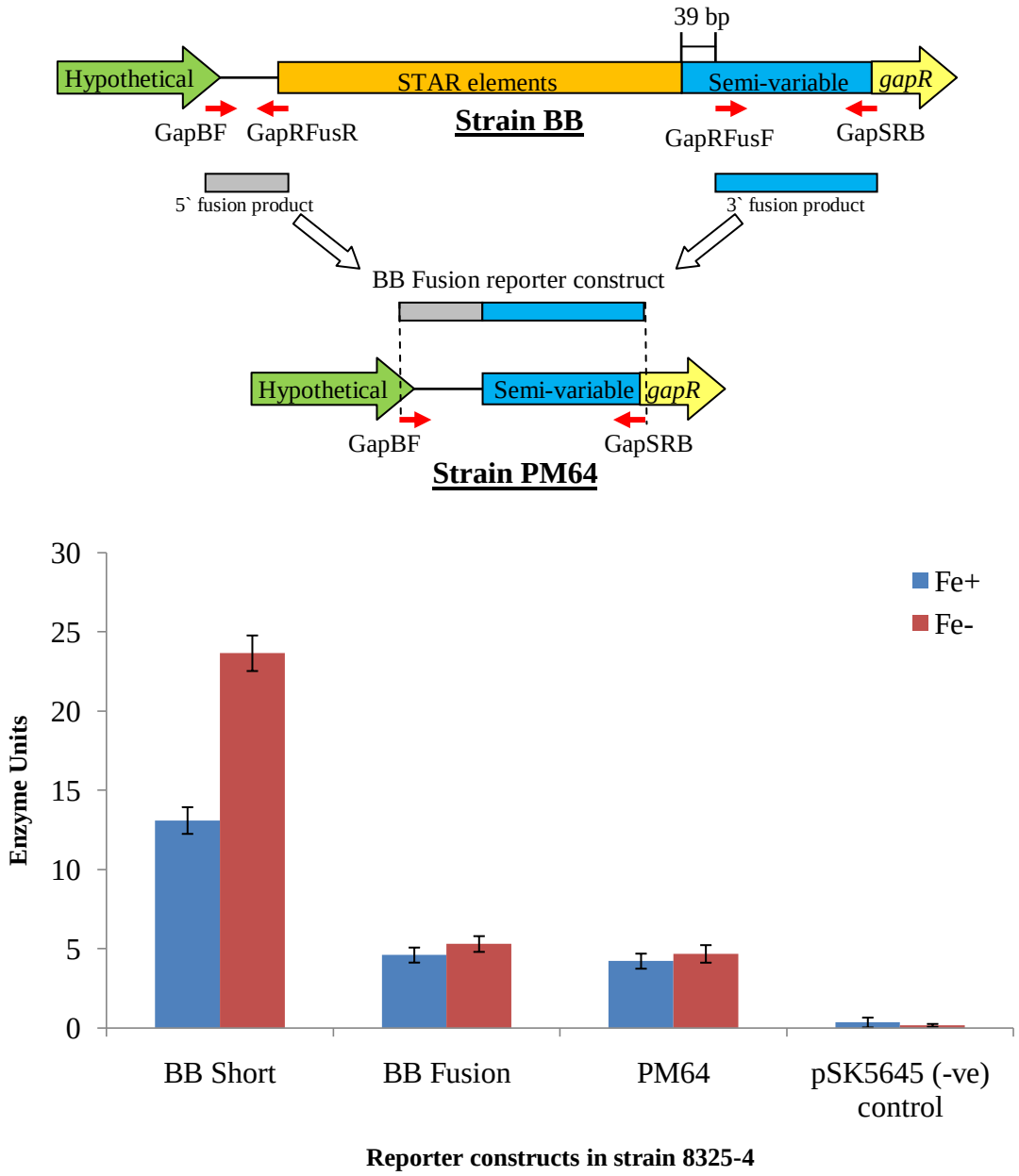


Figure 4-17. Strain BB has an iron regulated promoter in the first 39 bp of the semi variable region

(A) Strategy for the production of the BB Fusion reporter construct and (B) the enzyme activity of β-lactamase expressed from different fragments of the BB putative *gapR* promoter, and the PM64 promoter region. Promoters were cloned into plasmid pSK5645 and expression was analysed in a common strain background of 8325-4. The data presented is an average of at least 3 repeats for each construct, and the error bars indicate the standard error of the results.

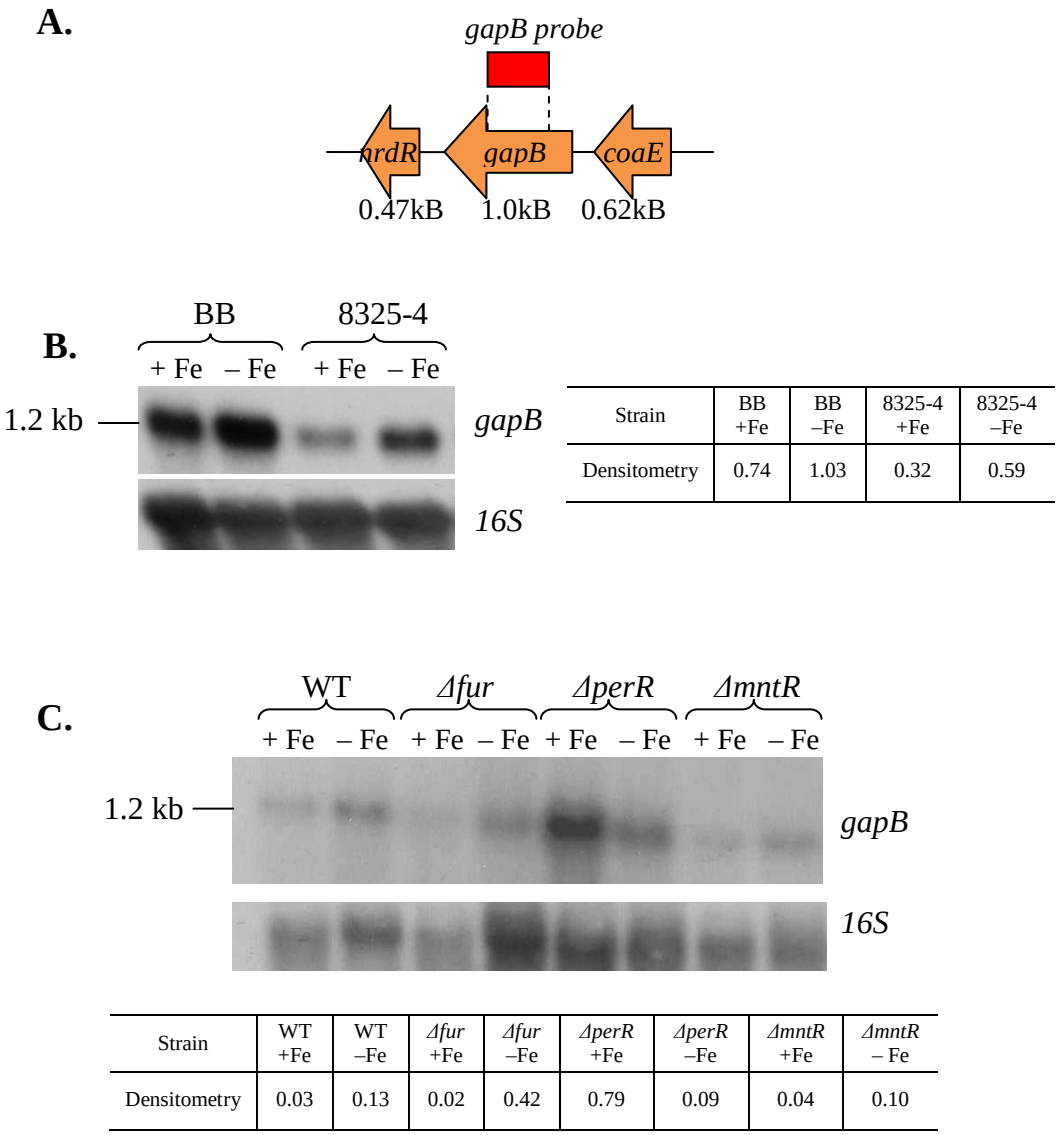
same promoter found in the Class II strains, but sequence variation within this first 39 bp of the semi-variable results in the Class I Fe regulation of the glycolytic operon, either through the introduction of a second promoter or modulation of the semi-variable region promoter.

In summary, sequence variation of the semi-variable region rather than strain background is responsible for strain dependent iron regulation of the glycolytic operon, but the STAR element region is required for full expression and iron regulation at least in the Class II strains. Furthermore there is evidence that the key sequence differences between the Class I and Class II strains fall within the first 39 bp of the semi-variable region, but without the transcriptional start sites the mechanism by which this regulation occurs remains unclear.

4.5.3 Iron regulation of the GAPDH homologue *gapB*

Previous studies have implicated both GapA and GapB as transferrin binding proteins in *S. aureus* (Modun & Williams, 1999; Goji *et al*., 2004). To determine whether *gapB* is also regulated in response to iron, *gapB* transcription in strains BB and 8325-4 grown to late exponential phase in CRPMI ± 50μM Fe_2SO_4 was analysed by northern blot. RNA was harvested and probed with a *gapB* specific [α^{32}P] radiolabelled DNA probe produced by PCR using primers GapB probe F and GapB probe R (Table 2.4; Figure 4-18A).

The *gapB* probe detected a single transcript approximately 1.2 kb in length, showing that *gapB* is monocistronic and does not form part of an operon with the genes surrounding it (Figure 4-18B). The lack of the smaller transcripts previously identified



Strain	BB +Fe	BB –Fe	8325-4 +Fe	8325-4 –Fe
Densitometry	0.74	1.03	0.32	0.59

Strain	WT +Fe	WT –Fe	*Δfur* +Fe	*Δfur* –Fe	*ΔperR* +Fe	*ΔperR* –Fe	*ΔmntR* +Fe	*ΔmntR* – Fe
Densitometry	0.03	0.13	0.02	0.42	0.79	0.09	0.04	0.10

Figure 4-18. Transcriptional of *gapB* is also iron regulated in *S. aureus*, and is regulated by PerR but not Fur or MntR.

(A) Schematic representation of the genomic organisation of the *S. aureus gapB* open reading frame, highlighting the position of the *gapB* probe.

Northern blots with densitometry data showing the regulation of *S. aureus gapB* in response to iron in (B) the wild type strains BB and 8325-4, and (C) the isogenic 8325-4 regulatory mutants *Δfur, ΔperR* and *ΔmntR*. RNA was extracted from cells grown to late exponential phase in CRPMI ± 50μM Fe_2SO_4. The gels presented are representative of experiments that were repeated at least twice using RNA extracts from cultures grown on different days, with similar results observed each time. Blots were stripped and re-hybridised with a control probe (*16S*) to ensure equal loading of RNA in each case.

when *S. aureus* are grown in gluconeogenic conditions in Tris minimal medium (see Chapter 3) is likely due to the presence of 0.2% glucose in CRPMI resulting in very low levels of *gapB* expression in this medium, which may limit processing of the transcripts or mean that the smaller transcripts cannot be detected. This data shows that transcription of *gapB* is classically iron regulated, and is reduced in the presence of iron in each of the strains tested (Figure 4-18B). This is consistent with *gapB* acting as a transferrin binding protein as part of the iron uptake system in *S. aureus*. The iron regulation of this gene is not strain variable, although the level of expression does vary between the strains, with BB showing an overall increase in *gapB* transcription compared to that of 8325-4.

As *gapB* is iron regulated, transcription of this gene was also assessed in the metal homeostasis mutants 8325-4*Δfur,* 8325-4*ΔperR* and 8325-4*ΔmntR* (Figure 4-18C). The transcription of *gapB* is repressed by PerR in response to Fe, but is not influenced by either Fur or MntR. Interestingly the promoter region of *gapB* does not contain known binding motifs for any of these regulators. The lack of Fur involvement in the regulation of a known Tf binding protein is interesting, as it may indicate novel regulation of an aspect of iron uptake in *S. aureus* that does not involve Fur. The involvement of PerR may also indicate that *gapB* has an additional role in either iron storage or the oxidative stress response, although the lack of a PerR binding site suggests an indirect or novel mechanism of control by this regulator. Therefore although both GAPDH homologues are Fe regulated in *S. aureus*, they are both influenced by independent metal homeostasis regulators in unexpected and potentially novel ways. Furthermore only *gapA* shows strain variable Fe regulation, suggesting that the Fe regulation of *gapA* and *gapB* occurs independently of one another.

4.6 Discussion

In this study we have shown that *gapA* is regulated at the transcriptional level by Fe, Cu and glucose, but that only Fe regulation occurs in a strain dependent manner. We also have evidence that *gapA* is repressed by the metal homeostasis regulators Fur and MntR, but that this regulation occurs in a non-classical way in the presence and absence of different metal ions. Although the exact position of the transcriptional start sites has remained elusive, transcriptional reporters have provided evidence that sequence variation and not differences in strain background are responsible for the strain variable iron response of *gapA* transcription, and that STAR elements are involved in transcription of the operon. Finally we have determined the transcriptional start site of the GAPDH homologue *gapB,* and shown that this gene is also regulated by Fe and the oxidative stress regulator PerR.

Transcriptional regulation of *gapA* in response to Fe occurs in a strain dependent manner, with Class I strains showing up-regulation in low iron, and Class II strains showing up regulation in high iron (Figure 4-2). The putative promoter region of the operon, located upstream of *gapR*, shows a high degree of sequence variability between strains (Figure 4-6) and contains both a semi-variable region and a variable number of STAR elements (Figure 4-7A). Through the use of transcriptional reporters we provide strong evidence that strain variable Fe regulation of *gapA* is the result of sequence variation rather than differences in the genetic background of the Class I and Class II strains, and involves both the semi-variable region and the STAR elements (Section 4.5). Figure 4-19 demonstrates a potential mechanism for the strain variable iron regulation seen in the *gapR* promoter of *S. aureus*. This mechanism has been derived

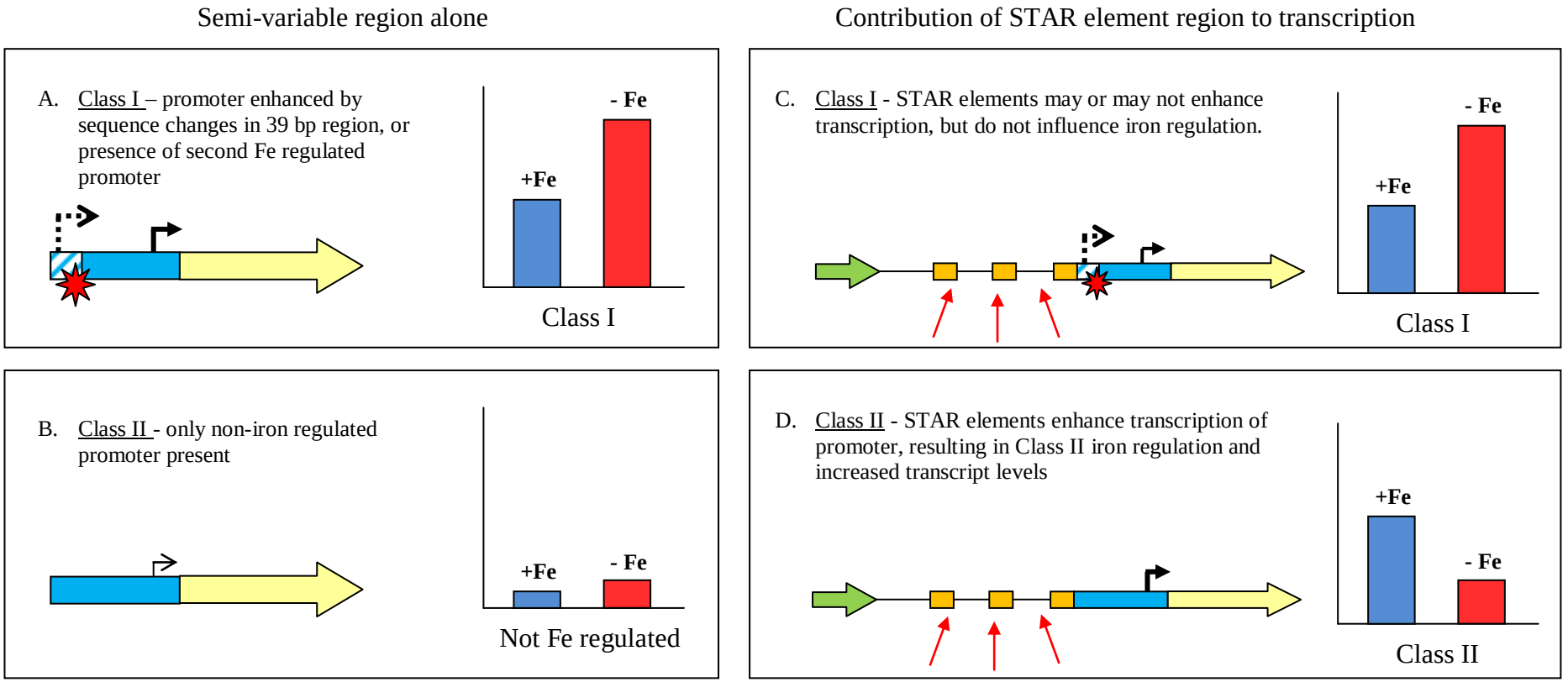


Figure 4-19. Potential mechanism for the strain variable iron regulation of the glycolytic operon.

(A) In the Class I strains the presence of an additional promoter or binding of enhancer elements in the first 39 bp of the semi-variable region results in strong transcription that is repressed in high iron conditions. (B) In the Class II strains there is only the promoter present. (C) Transcription of *gapR* is still lower in the presence of Fe, and transcription appears unaffected by the addition of STAR elements, while (D) in the Class II strains addition of the STAR elements enhances transcription of the conserved promoter resulting in Class II Fe regulation. This may also be due to the addition of another promoter.

from the data presented in this chapter as the simplest scenario for strain variable iron regulation to occur.

Class I iron regulation is dependent on the sequence of the first 39 bp of the semi-variable region (Figure 4-16; Figure 4-17). Removal of this region reveals a non-iron regulated promoter in the remainder of the semi-variable region, which is also present in the rest of the Class II strains (Figure 4-16). Therefore the sequence in the 39 bp region must either contain a second Class I Fe regulated promoter or provide a binding site for additional elements which modulate the expression of the conserved non-iron regulated promoter (Figure 4-19). Similarly Class II regulation of the operon requires the presence of the STAR elements (Figure 4-16). The addition of STAR elements to the conserved promoter in the Class II strains either modulates its transcription, or the STAR elements contain an additional Class II regulated promoter sequence (Figure 4-19).

Gene promoters can be modulated by transcriptional activators, which bind to target sequences at or around the gene operator. These activators can directly interact with transcriptional machinery such as RNA polymerase (RNAP), altering their affinity for a particular promoter and influencing transcription, as demonstrated by the catabolite activator protein (CAP) of *E. coli* (Dove *et al*., 1997; Lawson *et al*., 2004). DNA binding proteins (transcriptional enhancers), can also target specific sequences associated with gene promoters in order to bend the DNA, either enhancing or blocking the access of the transcriptional machinery to the gene promoter. These DNA targets can be found distantly upstream of the gene promoter they influence. The heavy metal-responsive regulator MerR forms one such family of proteins, and the binding of MerR to its target DNA sequence results in DNA remodelling and realignment of the -35 and

-10 sequences, promoting or blocking transcription (Summers, 1992). It is possible that both the 39 bp Class I associated sequence, or the Class II associated STAR elements, modulate the Fe regulation of the conserved *gapR* promoter via one of these mechanisms. The repetitive nature of the STAR elements and their distance from the putative promoter region may suggests that they either provide binding sites for regulatory proteins, or the sequence itself has a function via the formation of secondary hairpin structures altering the conformation of the DNA.

Without determining the exact transcriptional start site of *gapR* we are unable to determine whether the Class I semi-variable region or the STAR elements contain additional promoters. We have accurately identified the +1 site of both the positive control *eap* and the gluconeogenic GAPDH *gapB* using primer extension (Figure 4-10; Figure 4-11), but no product could be detected for *gapR* using either the radioactive or fluorescent methods, covering a total region of ~1000 bp upstream of the *gapR* coding sequence (Figure 4-12; Figure 4-14). The use of the fluorescent labelled primer extension protocol and GeneScan® technology identified that during cDNA production the labelled primer was not being incorporated and cDNA synthesis does not occur. The most likely explanation for this is that the level of *gapR* transcript is too low for detection due to transcript instability. Northern blot analysis using a *gapA* probe identifies two very faint transcripts of 7.0 kb and 6.0 kb, most likely representing transcription of the entire operon, but these are generally only detected in strains with high levels of *gapA* transcription such as BB and Newman. These large transcripts occur at a much lower level than the smaller transcripts suggesting that they are unstable. This is supported by our data which shows that *gapR* expression is lower than that of *gapA* in 8325-4 wild type cells grown in Tris minimal medium (Figure 3-15). If

this is the case then detecting the transcriptional start site may require a more sensitive method, or initial enrichment of the total RNA for mRNA transcripts.

However mapping of the *gapR* promoter using a third reverse transcriptase based mapping technique known as 5'RACE (rapid amplification of cDNA ends) provided results that may suggest the STAR elements are transcribed. This method produces a target specific cDNA product which can be T-cloned and sequenced to determine the +1 site (Frohman *et al*., 1988). The sequencing results were inconclusive for identification of the *gapR* +1 site, and in each case only 60 – 260 bp of sequence directly upstream of the GapRT2 primer site was accurately mapped to the *gapR* intergenic region, while the remainder of the sequence consisted of seemingly random DNA sequence of variable length (data not shown). The sequence shares no homology to each other or the rest of the *S. aureus* genome and their origin cannot be identified through BLAST database searches or whole genome pattern search programs. This has led us to believe that the sequences are random and not a result of non-specific primer pairing amplifying another cDNA sequence. As this method has only been used for strains containing STAR motifs, it is possible that if STAR elements are also transcribed their repetitive nature is likely to result in the formation of hairpin-like structures, as seen with other transcribed repeats, which could potentially block the reverse transcriptase machinery and prevent cDNA production. The use of temperature stable reverse transcriptase, with an optimal temperature of 65°C which could help to resolve RNA secondary structures, failed to improve primer incorporation in the fluorescently labelled primer extension method (data not shown). An alternative method of transcript mapping that could be used is S1 nuclease mapping. A known labelled RNA riboprobe which is complementary to the proposed 5' region of the target gene mRNA is produced and then added to total RNA.

The riboprobe will anneal and form a double stranded RNA molecule with the target RNA transcript, leaving overhanging riboprobe at the 5' end (before the +1 site) and overhanging RNA transcript at the 3' end. S1 nuclease is then used to digest the single stranded overhangs of the RNA dimer, leaving a labelled product which is size specific to determine where the target RNA originated, revealing the +1 site (Brans *et al*., 1997). As no cDNA production is required in this method, it may provide a more effective means of transcript mapping *gapR* than the reverse transcriptase methods previously used.

Transcriptional reporters derived from the first 39 bp of the semi-variable region or the STAR elements alone would help determine whether these sequences contain additional promoters. As the presence of multiple STAR elements in a plasmid based reporter has a negative impact on transcription which is not indicative of *in vivo* transcript levels (Figure 4-16), the presence of promoter elements within the STAR element region would need to be tested using an integrated reporter system rather than the plasmid based system used in this study. An additional promoter within the repeat elements is possible, as other interspersed bacterial repeats have been shown to be transcribed (De Gregorio *et al*., 2002; De Gregorio *et al*., 2003; Knutsen *et al*., 2006), but as the addition of STAR elements do not appear to influence the Fe regulation of the Class I strains, and the sequences associated with these repeats can vary (discussed further in Chapter 5) this does not appear to be likely.

In the majority of strains tested expression of *gapA* and the glycolytic operon is reduced in low iron conditions (Class II regulation; Figure 4-1; Figure 4-7). Although *S. aureus* GapA has been implicated as a transferrin binding protein (Tbp) (Modun & Williams,

1999), this down-regulation of transcription in Fe limited conditions is not typical of genes involved in *S. aureus* Fe uptake (Allard *et al*., 2006). However it is typical of bacterial genes regulated by the iron-sparing response. In order to preserve available iron for use in essential Fe-containing enzymes, many non-essential Fe-using proteins are down regulated in iron limiting conditions in bacteria (Masse *et al*., 2007; Gaballa *et al*., 2008). In *E. coli* the sRNA RyhB complexes with the complementary mRNA transcripts of target genes, an interaction which is stabilised by the chaperone protein Hfq, which signals the transcripts for degradation by RNase E (Geissmann & Touati, 2004; Masse *et al*., 2007). Fur modulates the iron sparing response by repressing RyhB transcription in the presence of Fe, thus relieving the repression on RyhB targeted genes and activation of their expression (Masse & Gottesman, 2002). This process has also been demonstrated in *B. subtilis*, via a distinct sRNA molecule known as FsrA (Gaballa *et al*., 2008). A similar response has been seen in *S. aureus*, and although no RyhB or FsrA homologues have been found in this species *S. aureus* contains at least 12 sRNA, the functions of many of which are yet to be defined (Friedman *et al*., 2006).

GAPDH is not a metalloprotein and does not require the incorporation of Fe into its active site, suggesting that it is not directly regulated as part of the iron-sparing response, although it is possible that the transcription in response to Fe is altered due to changes in the central metabolic pathways, such as down-regulation of key TCA cycle genes in limited Fe (Masse & Gottesman, 2002; Gaballa *et al*., 2008). However our data show that transcription of *gapA* was de-repressed in strains 8325-4*Δfur* and 8325-4*ΔmntR* compared to the wild type in the presence and absence of Fe and Mn (Figure 4-5). This suggests that *gapA* is not regulated as part of the *S. aureus* iron-sparing response, as it is not activated by Fur in high iron conditions but repressed in low iron

conditions. Therefore we have a novel regulation mechanism in which both Fur and MntR repress gene transcription in the absence of metal ions. As the putative operon promoter does not contain known sequence motifs for either *fur* or *mntR* binding it is unclear whether these regulators act directly or indirectly. The construction of a *Δfur/ΔmntR* double mutant strain may be useful in determining whether additional regulators are involved in this response. Interestingly Fe regulation of the operon is not influenced by the only other known regulator of the operon, *gapR,* (Section 3.4) as Fe regulation was not altered in a *ΔgapR* mutant (data not shown).

The glycolytic operon is also induced in the presence of Cu and glucose (Figure 4-3; Figure 4-4), but unlike Fe regulation these responses do not vary between *S. aureus* strains. As *gapA* is a glycolytic GAPDH induction of the operon by glucose is not unexpected, but in the restricted medium CRPMI, which contains 0.2% glucose, induction of the operon is limited in the absence of Fe or Cu in the Class II strains. Although we have already shown 0.2% glucose to be sufficient to switch *S. aureus* carbon metabolism to glycolysis in Tris minimal medium (see Section 3.3.6), this data suggests that iron or copper is required to increase the expression of GAPDH in CRPMI. As it is unlikely that *gapA* is controlled as part of the iron-sparing response, this may indicate that modulation of *gapA* transcription is required to control the expression of additional moonlighting functions. As previously discussed in Chapter 3, GAPDH proteins are highly sensitive to oxidative stress dependent protein damage (Schuppe-Koistinen *et al*., 1994; Shenton & Grant, 2003; Weber *et al*., 2004). Cu and Fe induce oxidative stress via the Fenton reaction, with increased levels of both metals being highly toxic to *S. aureus* (Pierre & Fontecave, 1999; Baker *et al*., 2010). In eukaryotes GAPDH protein damage has been shown to provide a rapid switch in carbon

flow from glycolysis to the pentose phosphate pathway as a means of balancing redox potential, followed by an increase in transcription to allow the cell to regain glycolytic function (Ito *et al*., 1996; Ralser *et al*., 2009). It is possible that this is also occurring in *S. aureus*. However, if *S. aureus* GapA is more directly involved in the oxidative stress response, as our H_2O_2 stress experiment suggests (see Section 3.5.1), then up-regulation in oxidative stress conditions may be required to protect the cell in a more direct manner.

Finally our data show that the gluconeogenic GAPDH *gapB* is also Fe regulated in *S. aureus*, although this response is not strain variable; transcription is reduced in response to Fe in both the Class I strain BB and the Class II strain 8325-4 (Figure 4-18). GapB has also been proposed as a Tbp in *S. aureus*, and therefore this classic Fe regulation of this gene is not unexpected (Goji *et al*., 2004). However this response occurs independently of Fur in strain 8325-4, but Fe repression occurs via PerR (Figure 4-18). There are no known PerR binding motifs in the promoter sequence of *gapB*, suggesting this regulation may be indirect. Interestingly PerR is not generally involved in the regulation of iron acquisition genes in *S. aureus*, and plays a more prominent role in the oxidative stress response and iron storage (Horsburgh *et al*., 2001a). Strangely GapB is not involved in the oxidative stress response in *S. aureus* (Figure 3-13) but is transcriptionally regulated by PerR, while GapA is involved in the oxidative stress response (Figure 3-13) and is not transcriptionally regulated by PerR. It is possible that GapB has additional moonlighting roles in *S. aureus* physiology that are yet to be uncovered related to this regulation.

In conclusion, we have demonstrated that *gapA* is iron regulated in a strain dependent manner in *S. aureus*, and have shown that in the Class II strains this regulation is repressed in a novel manner by both Fur and MntR in the presence and absence of metal ions. Furthermore we have demonstrated that strain variable expression is due to sequence variation of the putative *gapR* promoter region, and importantly have demonstrated for the first time a potential impact of STAR elements on gene transcription. These STAR elements will be examined in greater detail in the following chapter.

Chapter 5 Sequence variation in the promoter region of the glycolytic operon correlates with strain lineage

5.1 Introduction

Repetitive DNA sequences are very diverse in structure and function, and are found across both eukaryotic and prokaryotic genomes. The functional role of repetitive DNA is often much clearer in prokaryotic organisms, possibly due to selective pressure streamlining their genomes against the propagation of "selfish" DNA (Doolittle & Sapienza, 1980). Prokaryotic repeats have been shown to play a role in antigenic variation of proteins, phase variation of genes at the transcriptional and translational levels, gene regulation by mechanisms such as transcriptional stability and promoter modification, and protection of the genome from phage invasion (see Section 1.4.2).

The focus of this chapter is the *S. aureus* specific direct interspersed repetitive element known as STAR (*Staphylococcus aureus* Repeat) (Cramton *et al*., 2000). STAR elements are short GC-rich direct repeats found in intergenic regions across the *S. aureus* genome. STAR elements have been identified in some other staphylococcal species, but always at much lower frequencies than in *S. aureus* strains, and currently there is no evidence that these elements exist outside of the genus (Cramton *et al*., 2000). These elements are identified by conserved 14bp direct repeat units of T(G/A/T)TGTTG(G/T)GGCCC(C/A), which are interspersed with recurring intergenic sequence, and have been identified in variable numbers at different loci across the *S. aureus* genome (Cramton *et al*., 2000; Quelle *et al*., 2003). Currently no biological function has been described for these repeat elements, but the maintenance of these repeats at so many loci in *S. aureus* strains does suggest that they play a functional role in some aspect of cell physiology.

As discussed in Chapter 4, STAR elements are present upstream of the *S. aureus* glycolytic operon, which is a region that shows high sequence variability between different *S. aureus* strains. Preliminary evidence from the 4 strains investigated suggests a potential correlation between the number of STAR elements present at this locus and the expression level of the operon. The aims of this chapter are to further investigate the variability of the sequence of the *gapR* STAR locus in a larger number of strains, and determine whether the number of STAR elements present affects the level of transcription of the glycolytic operon. Further objectives also include expansion of this analysis to additional STAR loci to determine any global patterns in STAR element variability and functionality within the strains tested.

5.2 Structural variability of the *gapR* STAR element region

5.2.1 The *gapR* promoter region varies in structure and sequence between *S. aureus* isolates

As shown in Chapter 4 the number of STAR elements at the *gapR* locus varies between 0 and 3 repeating units in the 4 strains examined. Therefore to gain a better understanding of the variability of STAR loci in a wider number of strains, the *gapR* STAR locus was examined in a total of 28 *S. aureus* laboratory and human clinical isolates from a variety of infections and 13 *S. aureus* bovine mastitis isolates. More detailed information regarding these strains can be found in Tables 2.1 and 2.3 and Appendix Table A-1. The region directly upstream of *gapR* was amplified by PCR using the primers GapBF and GapSRB (Figure 5-1) and then sequenced for each of the 41 *S. aureus* strains. Sequence data was also retrieved for 12 more *S. aureus* strains from completed genome sequences in the NCBI database. Figure 5-1 shows the basic structure of the *gapR* region including the STAR repeat motifs, the positions of the primers used, and the size variability of the PCR product from 8 different *S. aureus* isolates.

Comparison of the DNA sequence of the *gapR* STAR locus between *S. aureus* strains revealed a large amount of variability in this region (Figure 5-2; Appendix Table A-1). This variation not only included different numbers of STAR element repeat units but also large scale structural changes, which can be categorised into 5 different structural Groups (Figure 5-2).

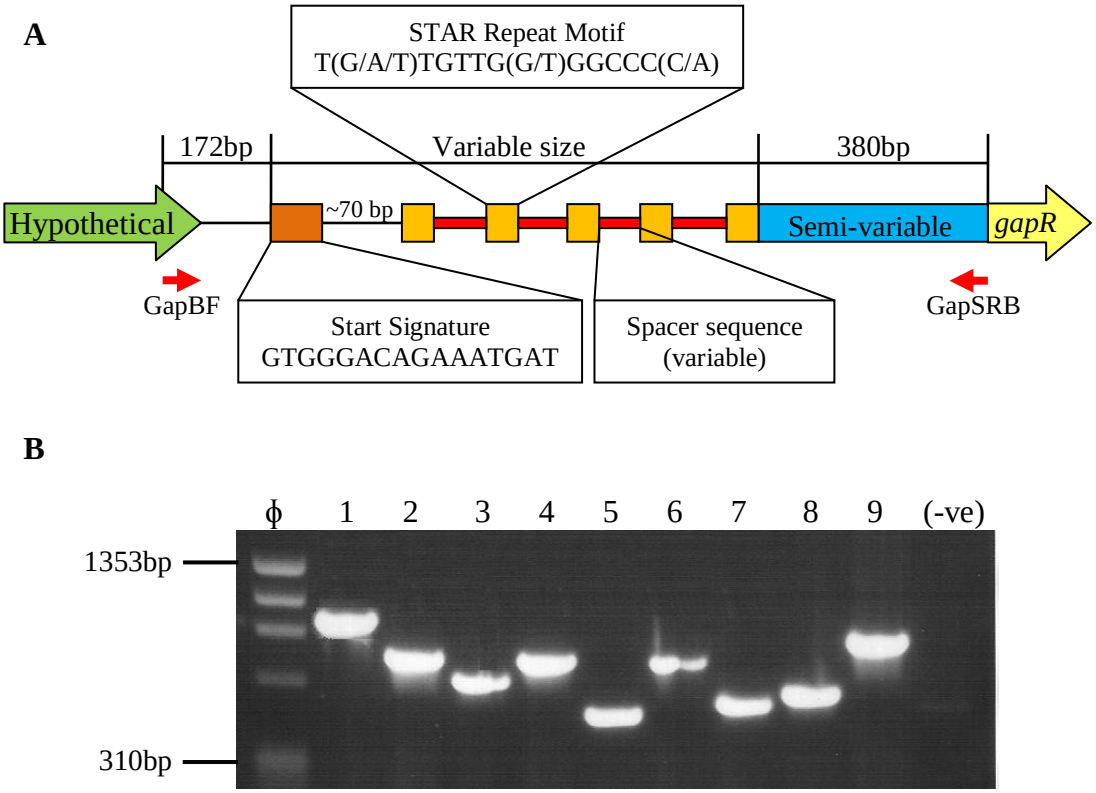


Figure 5-1. Variability of the *gapR* upstream region between different *S. aureus* strains.

(A) Schematic representation of the structure of the region directly upstream of *gapR*, including position of primers GapBF and GapSRB and STAR element motifs.

(B) PCR amplification using primers GapBF and GapSRB of the *gapR* upstream region from strains (1) Mu50, (2) BB, (3) 8325-4, (4) Newman, (5) MRSA252, (6) MRSA PM25, (7) RF122, (8) CDC8 and (9) 66195.Sizes range from approximately 900bp to 450bp. Also included are a ϕ*hae*III ladder (ϕ) and a negative control PCR.

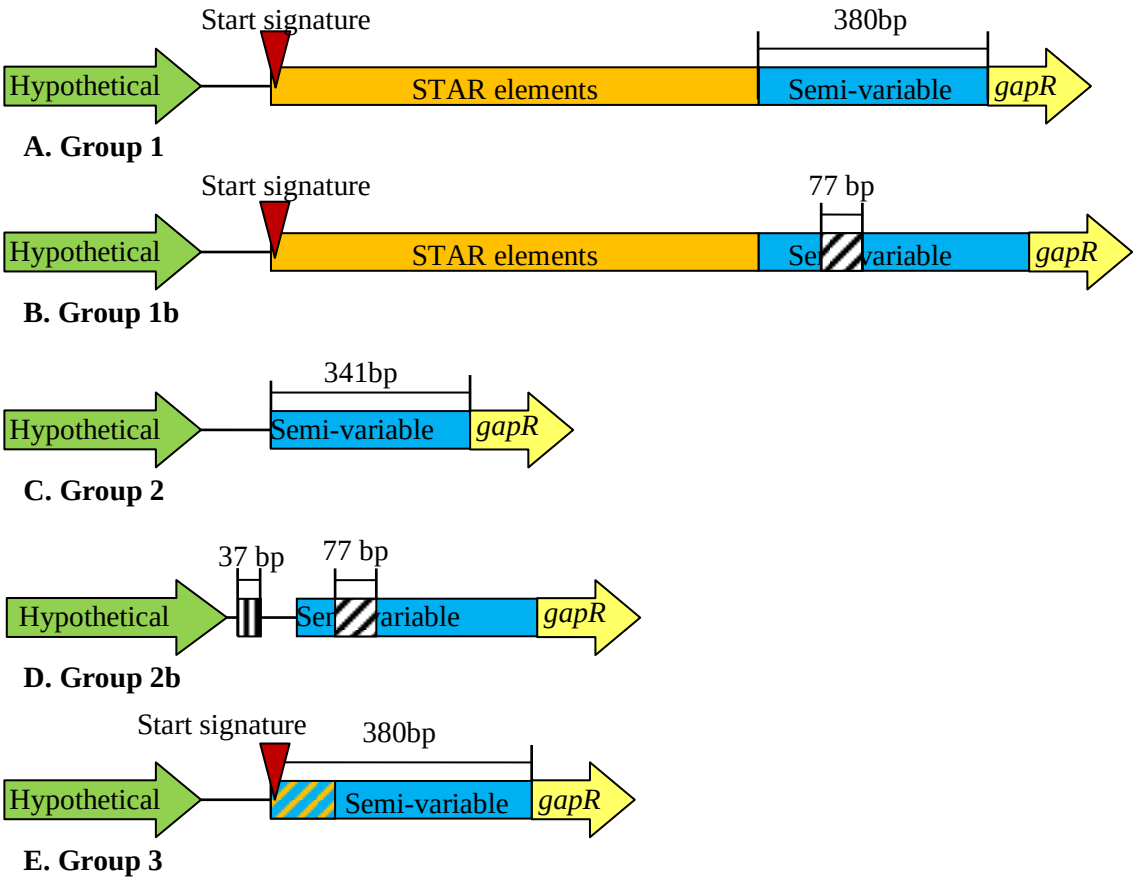


Figure 5-2. The five structural variants of the *gapR* upstream region.

Schematic representations of the 5 structural groups identified showing variation in the region upstream of *gapR*. Structure of the region from (A) Group 1, highlighting the location of the STAR elements, the semi-variable region and the surrounding conserved regions (B) Group 1b, (C) Group 2, (D) Group 2b, and (E) Group 3. STAR elements are missing in Groups 2 and 2b at the same deletion site. Groups 1b and 2b contain identical 77 bp insertions within the semi-variable region (diagonal cross-hatch). Group 2b also contains an unrelated 37 bp insertion upstream of the STAR deletion site (vertical cross-hatch). Group 3 contains the STAR start signature followed by 70 bp of sequence unrelated to the STAR elements or semi-variable regions of the other strains examined (diagonal blue and orange cross-hatch).

Group 1 – The majority of strains (33/53) fall into Group 1. In these strains the STAR locus begins 172bp downstream from the preceding open reading frame with a STAR "start signature" sequence of GTGGGACAGAAATGAT, which is slightly truncated compared to the sequence initially identified at the *hprK* STAR locus (Cramton *et al.*, 2000). The "start signature" occurs 70-72 bp upstream of the first STAR element repeat unit, which consists of the conserved sequence (G/A/T)TGTTG(G/T)GGCCC(C/A). The number of repeat units in these strains varies between 1 and 6, and the sequence spacing these repeats showed a small amount of variability. Finally the structure contains a 380 bp "semi-variable" region downstream of the repeat elements and directly upstream of the *gapR* coding region (Figure 5-1; Figure 5-2A). This region is not identical between isolates (as discussed in Chapter 4), but does share approximately 88% sequence identity. This group includes many *S. aureus* isolates from various human infection sources, a number of sequenced *S. aureus* strains, and 7 of the 12 bovine mastitis isolates analysed in this study, therefore encompassing a wide range of both human and bovine *S. aureus* strains.

Group 2 – Group 2 strains are missing the entire STAR element region, including the start signature sequence, as well as the first 39 bp of the 5' end of the semi-variable region (Figure 5-2C). All of group 2 strains identified (9/53), including the sequenced strain MRSA 252, share 100% sequence identity across this region. This structure was only identified in *S. aureus* human infection isolates, although these strains were isolated from a number of different sources including septicaemia, urine and CAPD infections (Table 2.3, Appendix Table A-1).

Groups 1b and 2b - Group 1b (2/53) and 2b (3/53) appear to be variants of groups 1 and 2 respectively. Group 1b has an insertion of 77 bp within the semi-variable region of the Group 1 structure (Figure 5-2B). This insertion occurs 300 bp upstream from the *gapR* ATG site. Group 2b is missing the STAR elements at the same position as Group 2, but also contains the same 77 bp insertion in the semi-variable region found in the Group 1b strains. Furthermore Group 2b also has a second 37 bp insertion 27bp upstream of the STAR element deletion site (368bp upstream of ATG) (Figure 5-2D). The 37bp insertion seen in Group 2b does not share any sequence similarity with the 77bp insertion. The origin of these insertion sequences is unclear, although bioinformatic analysis of the published genomes reveals that they share some similarity with other intergenic regions throughout the *S. aureus* genome. 4 of these strains were isolated from continuous ambulatory peritoneal dialysis (CAPD) infection at the same hospital over a similar time period, so evolutionary relationships between these strains may explain the similarities in these structures.

The loss of the STAR element region in both the Group 2 and Group 2b strains occurs at a consistent and precise deletion site. There are no major changes in the immediate sequence surrounding this deletion site between the Group 1 and Group 2 (or 2b) strains (Figure 5-3). Taken together these data suggests that there may be a conserved mechanism for the loss or gain of STAR elements in *S. aureus*.

Group 3 - Group 3 strains (5/53) retain the STAR start signature but there is no evidence of any STAR element repeat sequences (Figure 5-2E). In addition to this, the first 70bp of the "semi-variable" region in this group, beginning directly downstream of the STAR start sequence, shares little similarity to the semi-variable region or the

Table 5.1. Strains represented in each *gapR* structural group.

***gapR* STAR element structure**	**No. of Strains**	**Origin of strain represented**
Group 1	33	Most abundant, seen in both bovine mastitis isolates and human isolates
Group 1b	2	Found only in human CAPD isolates
Group 2	8	Found in human isolates
Group 2b	3	Found only in CAPD human isolates and strain CDC8
Group 3	5	Found only in bovine mastitis isolates

STAR element sequences identified in any of the other strains. Currently this group appears to be unique to bovine mastitis isolates, occurring in 5 of the 12 strains tested, including the sequenced strain RF122.

5.2.2 *gapR* STAR elements are made up of discreet modules and the spacer sequences vary between strains

Comparison of the spacer sequences found between the STAR element repeat motifs showed that the *gapR* STAR locus is made up of discreet modules. The spacer sequences between the interspersed STAR motifs are similar to each other but are not identical, and appear to be arranged in a conserved pattern (Figure 5-4). The spacer sequence between the STAR start signature and the first STAR motif is designated spacer A and is ~70 bp in length. Spacer B occurs between STAR motifs 1 and 2 and is 42-44 bp in length. This sequence is repeated in strains with more than three STAR motifs, such as EMRSA-15 PM25 which has 4 repeat or Mu50 which has 6. Finally the spacer separating the penultimate and final repeat motifs (spacer C), is 44-46bp in length. The sequence of spacers A, B and C share some similarities but are distinct from one another, and each of these spacer sequences can vary between different *S. aureus* strains. However in all of the strains found with multiple repeats at this locus, the spacer sequences follow this modular pattern. It appears that multiple STAR element repeats occur at the *gapR* locus through direct expansion of spacer B alongside a STAR motif, although the mechanism for this expansion is unclear.

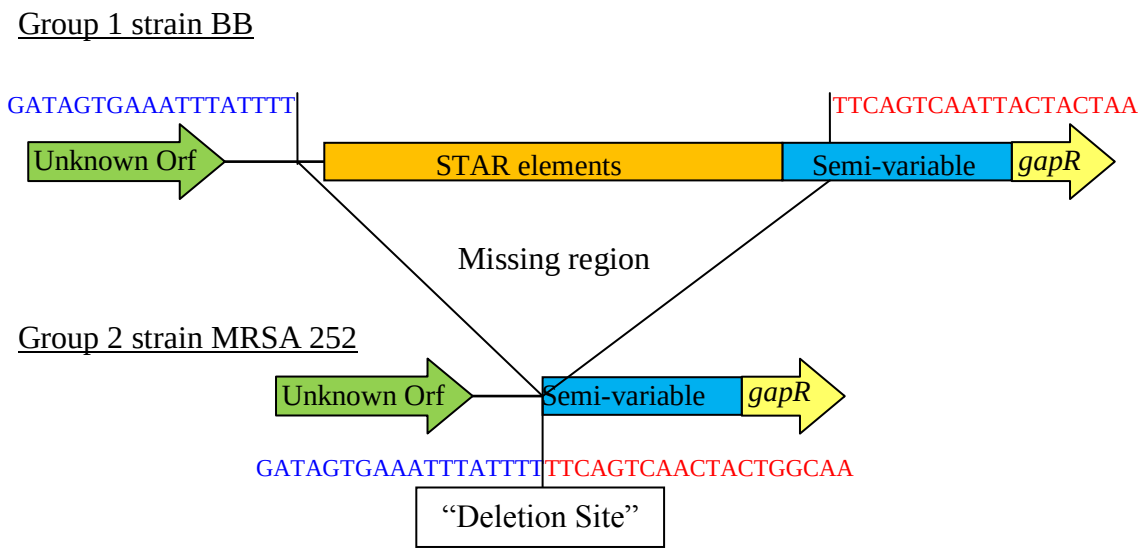


Figure 5-3. Schematic representation of the *gapR* STAR element deletion site.

Schematic comparing the *gapR* STAR locus from strain BB and MRSA252, indicating the region missing from the Group 2 strains. The conserved sequences flanking the deletion site are highlighted in each strain, demonstrating the sequence conservation in these regions between the strains. The upstream sequence (blue) is highly conserved in all strains analysed, while the downstream sequence (red) shows more variability, but still retains homology between strains.

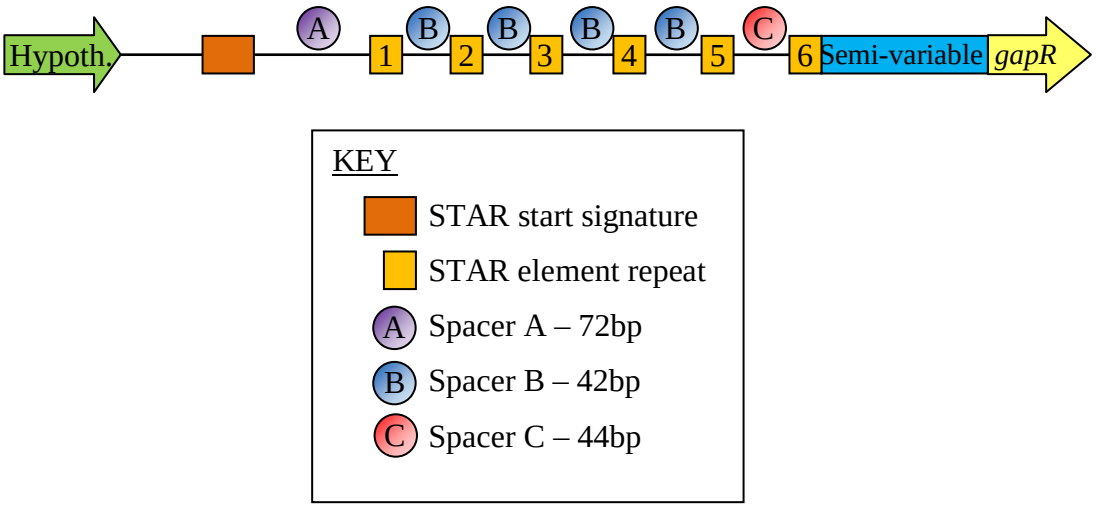


Figure 5-4. Schematic representation of the modular structure of the *gapR* STAR locus from strain Mu50.

Schematic of the *gapR* STAR locus representing the variation in the spacer sequences between the STAR repeat motifs. Strains carrying more than 3 repeat elements show a direct expansion of spacer B, as demonstrated here in Mu50.

5.3 STAR element spacer sequences correlate with strain lineage

STAR elements were originally described from the STAR locus found upstream of the *hprK* open reading frame in 12 different *S. aureus* strains, including the laboratory strain 8325-4 (Cramton *et al*., 2000). The *hprK* STAR locus has previously been the focus of a restriction based strain typing method, allowing variation in a STAR element locus to be used to determine relatedness of strains (Quelle *et al*., 2003). Using Multi-locus Sequence Type (MLST) data derived from *S. aureus* complete genome sequences we were able to determine that a similar restriction enzyme based typing method for the *gapR* STAR locus would not necessarily group the strains into their respective sequence type (ST). However, analysis of the *gapR* STAR locus sequence at the nucleotide level does suggest that strains of the same lineage have conserved spacer sequences, even when the number of repeat units varies (Figure 5-5A; Figure 5-5B), whereas spacer sequences from strains of different lineages are variable (Figure 5-5C). Therefore to investigate further, the ST and the spacer sequences from a broader selection of strains were analysed.

5.3.1 MLST sequencing of a selection of *S. aureus* strains

MLST sequencing analysis was carried out on a selection of strains representative of each *gapR* structural group. The strains chosen were BB (Group 1), 66195 and 65991 (Group 1b), 66155, 65985 and CDC8 (Group 2b), and 47979, 63505 and B2503017 (Group 2). MLST data for all of the bovine mastitis isolates (which includes Group 3) was provided by Dr. Jodi Lindsay, St George's University of London. Sequence types of the fully sequenced *S. aureus* strains was also included in the analysis, and where necessary MLST data was derived from the complete genome sequences which are publically available at NCBI.

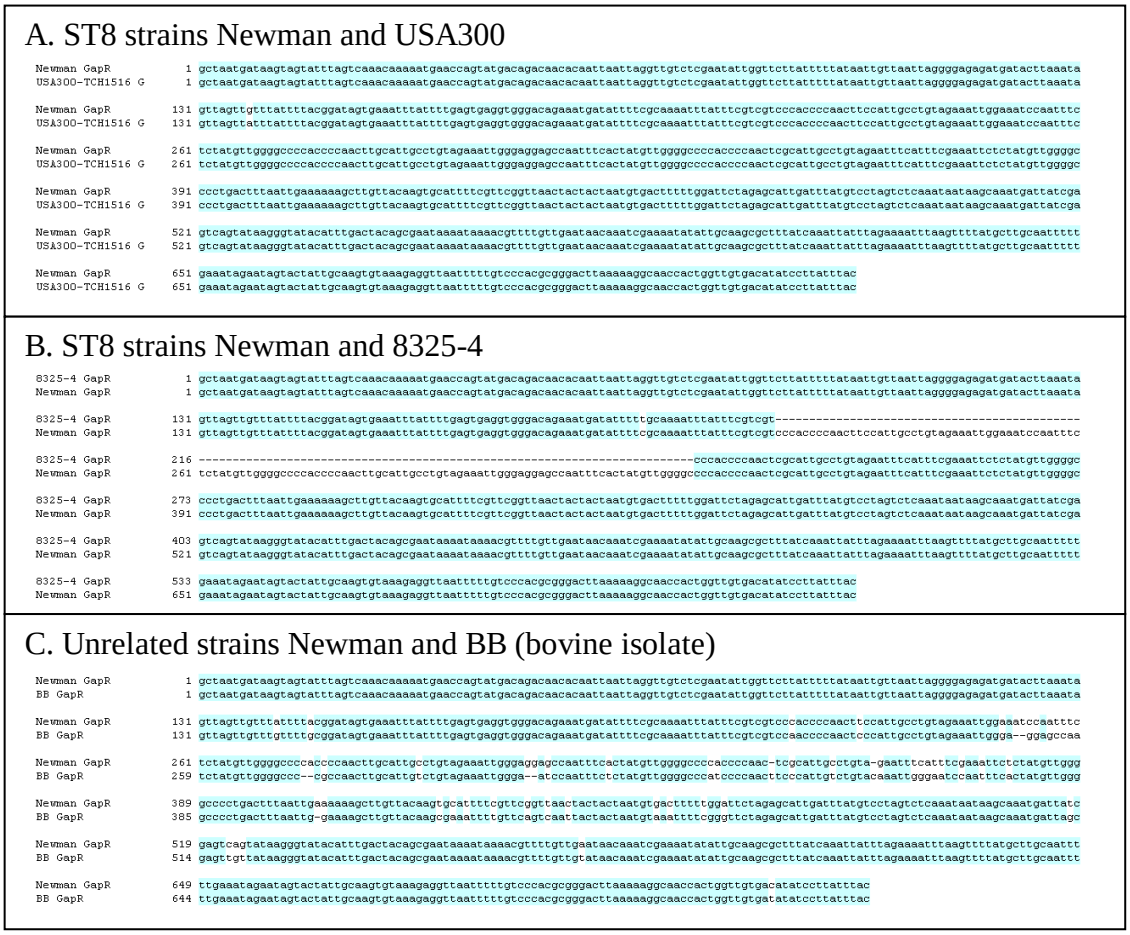


Figure 5-5. Sequence alignments indicating the conservation of *gapR* spacer sequences in related strains.

Sequence alignment of the *gapR* STAR locus from (A) the ST8 strains Newman and USA300 FRP3735, (B) the ST8 strains Newman and 8325-4 containing 3 and 1 repeat motifs respectively and (C) the unrelated strains Newman (human isolate) and BB (bovine mastitis isolate). For each of the alignments presented above, the region is comprised of the sequence between the primers GapBF and GapSRB (Figure 5-1). Conserved bases are shaded in blue.

Locus specific MLST primers (see Table 2.4) were used to PCR amplify each of the 7 *S. aureus* MLST loci, *araC, aroE, glpF, gmk, pta, tpi* and *yqiL* (Enright *et al*., 2000). At each locus variant alleles are assigned a number and an allelic profile can be built for each strain based on the combination of alleles at all 7 loci and as it is highly unlikely that two unrelated strains will have identical allelic profiles, this allows accurate typing of strain lineage (Enright *et al*., 2000). PCR products were purified and sequenced for each locus in the test strains, and this sequence was used to query the *S. aureus* MLST database (http://saureus.mlst.net/) to determine the sequence type (ST) for each strain (Figure 5-6; Table 5.2). Table 5.3 contains the ST of each strain alongside the structure and number of repeats at the *gapR* STAR locus. A phylogenetic tree of the strains was derived using the Neighbour-joining algorithm, based on the MLST profile of each strain (Figure 5-7).

5.3.2 Correlation of *gapR* STAR locus structure and strain lineage

The MLST data shows that the Group 2 and Group 3 strains, which do not contain STAR elements at the *gapR* locus, fall into distinct evolutionary lineages as compared to the Group 1 strains (Table 5.3; Figure 5-7). MLST sequence types can be further sorted into clonal complexes to determine common ancestry between ST's, which is useful in determining evolution and long term epidemiology of strains. A Clonal Complex (CC) is defined as a group of ST's which each have at least 5 common MLST alleles with at least one other member of the CC. Interestingly, all of the Group 2 strains (ST30, ST36, ST34, novel ST B), which are 100% conserved at the *gapR* STAR locus, fall into CC30 (Table 5.3). No examples of strains from CC30 have been identified outside of Group 2 in this study, so the loss of STAR elements in these strains may have

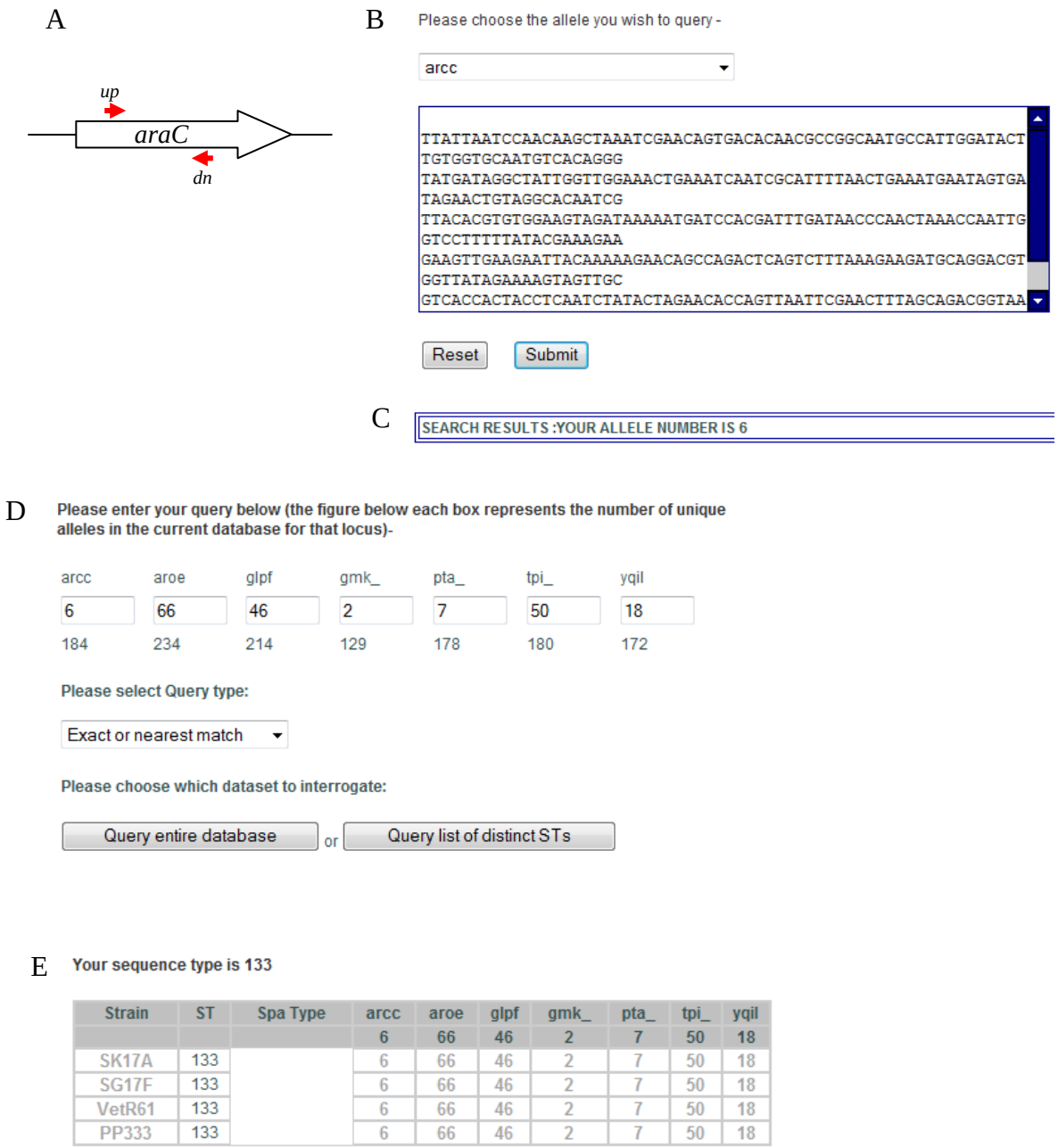


Figure 5-6. Demonstration of MLST typing in *S. aureus*.

(A) PCR amplify and sequence each MLST loci with *up* and *dn* primers. (B) Trim sequence to region specified, and use to search MLST database for (C) the allele number of each loci. (D) Input each allele number for strain and search MLST database. Results (E) give the ST type of the strain and any other strains in this ST in the database. Screen shots taken from http://saureus.mlst.net/.

Table 5.2. MLST data for strains typed in this study.

Strain	*araC*	*aroE*	*glpF*	*gmk*	*pta*	*tpi*	*yqiL*	ST
BB	6	66	46	2	7	50	18	**133**
66195	19	23	15	2	19	20	15	**59**
65991	19	23	15	2	19	20	15	**59**
66155	10	14	8**	13	10	3	2	**A ***
65985	10	14	8**	13	10	3	2	**A***
CDC8	10	14	8	6	10	3	2	**45**
47979	8	2	2	2	6	3	2	**34**
63505	8	2	2	2	3	3	2	**B***
B2503017	2	2	2	2	6	3	2	**30**

Sequence types marked with a * indicate novel sequence types identified in this study. The *glpF* alleles marked with a ** indicate a novel allelic variant identified in this study, which varies from *glpF* allele type 8 by a single nucleotide. Novel ST's are currently undefined by the MLST database, and as such have been designated A and B.

Table 5.3. Combined data for the *gapR* STAR element region, strain origin and Sequence Type (ST) for each strain tested.

Strain	*gapR* STAR structure	No. of STAR repeats	Strain origin	Sequence Type	Clonal Complex
8325-4	1	1	Laboratory	8	8
Newman	1	3	Human	8	
USA300 FRP	1	1	Human CA-MRSA	8	
USA300 TCH1317	1	3	Human CA-MRSA	8	
COL	1	3	Human MRSA	250	
TW20	1	3	Human MRSA	239	
Mu50	1	6	Human VRSA	5	5
N315	1	6	Human MRSA	5	
Mu3	1	6	Human	5	
ED98	1	4	Poultry	5	
JH1	1	3	Human VSSA	105	
JH9	1	3	Human VISA	105	
MSSA476	1	2	Human MSSA	1	1
Mw2	1	1	Human CA-MRSA	1	
C01865	1	2	Bovine	1	
30375	1	4	Bovine	188	188
BB	1	3	Bovine	133	133
C01719	1	3	Bovine	771	
C01771	1	3	Bovine	771	
MRSA PM25	1	2	Human EMRSA	22	22
C00595	1	2	Bovine	97	97
C00704	1	1	Bovine	97	
66195	1b	4	Human CAPD	59	59
65991	1b	4	Human CAPD	59	
MRSA252	2	0	EMRSA	36	30
MRSA PM64	2	0	EMRSA	36	
B2503017	2	0	Human septicaemia	30	
47979	2	0	Human CAPD	34	
63505	2	0	Human CAPD	B	
CDC8	2b	0	Human	45	45
66155	2b	0	Human CAPD	A	
65985	2b	0	Human CAPD	A	
RF122	3	N/A	Bovine mastitis	151	151
38963	3	N/A	Bovine mastitis	151	
982BL	3	N/A	Bovine mastitis	151	
C00759	3	N/A	Bovine mastitis	151	
C123/5/05-09	3	N/A	Bovine mastitis	151	

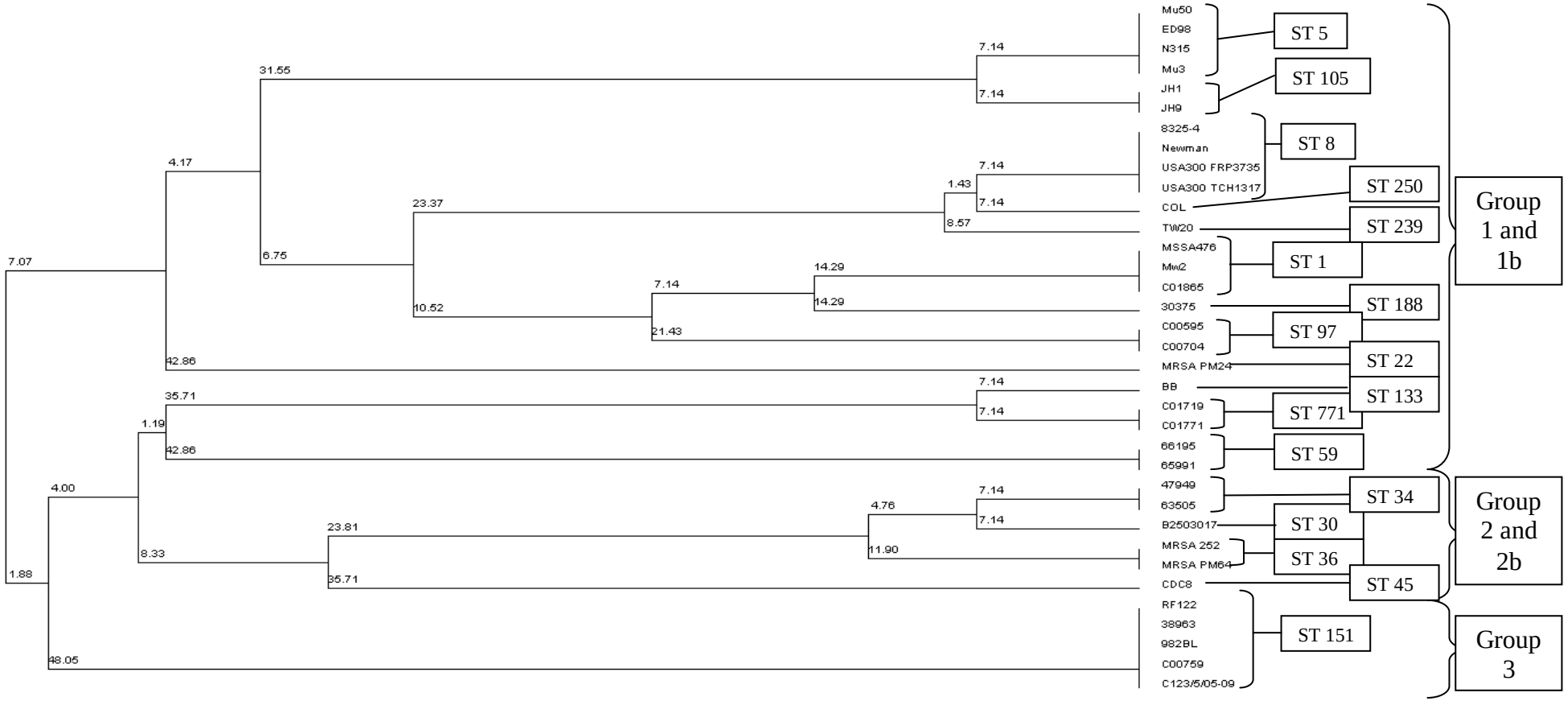


Figure 5-7 Neighbour-joining tree of *S. aureus* strains based on MLST.

The above Neighbour-joining tree was derived from the MLST profiles of each of the *S. aureus* tested in this study, using the *S. aureus* MLST database phylogenetic tree tool at http://saureus.mlst.net/. The *gapR* structural groups of the strains is also highlighted.

occurred in a common ancestor of these strains and been maintained as the ST's diverged from one another. This is also observed in the Group 3 strains, which have a partial deletion of the STAR elements which has only been identified in strains from ST151 (CC151). As the remaining region is 100% conserved between the ST151 strains, this again suggests that the deletion occurred early in the evolution of this sequence type and has been maintained in subsequent isolates.

Interestingly the Group 1b and Group 2b strains, which have additional sequences compared to Group 1 and 2, fall into distinct clonal complexes with very different allelic profiles; the Group 1b strains are from ST59, which is in CC59, and the Group 2b strains are from ST45 and novel ST A , which are both in CC45. This may suggest that either a common ancestor of these strains carried the insertion sequences, and the STAR elements were lost/gained as the ST's diverged from each other, or that these additional insertion sequences have been incorporated into each lineage at the same site in the DNA as independent events. Together this data suggests that either the *gapR* STAR locus differences occurred in ancient lineages and have been maintained at a level equal to that of CC in strains, or that sequence insertions or deletions occur at specific hotspots within the repeat region.

5.3.3 Correlation of *gapR* STAR spacer sequences and strain lineage

The Group 1 strains, which contain variable numbers of STAR elements, belong to a wide range of ST's and CC's, and therefore the structure alone does not correlate with any particular lineage. However our data shows sequence conservation across the *gapR* STAR locus, specifically that of the spacer sequences, strongly correlates with the lineage of the strains as defined by MLST analysis (Figure 5-7). For example in strains

from ST5, the STAR element spacer sequences remain consistent between isolates even though the number of repeat motifs can vary. We have confirmed the conservation of spacer sequences within an ST in all strains tested here, with the exception of those in ST97. The spacer sequences from the ST97 strains C00595 and C00704 are still highly conserved, but they are not 100% identical.

In order to visualise the relationship between all of the strains with respect to the variability of the *gapR* STAR locus, a phylogenetic tree was created using the Neighbour-joining algorithm from the DNA sequence between primer sites GapBF and GapSRB from strains representative of each ST identified (Figure 5-8). The Neighbor-joining bootstrap method was chosen because it provides a simple means of pairwise alignment of the sequences, and allows construction of a branched tree, with branch length representing evolutionary relationships. This tree clearly clusters strains into the same groups as those seen for MLST relationships (Figure 5-7; Figure 5-8). The Group 3 strains, in ST151 cluster away from the rest of the strains tested, while the Group 2 strains from CC30 also cluster together in a branch distinct from the other strains. This is further evidence that the *gapR* STAR locus is maintained within distinct evolutionary lineages.

5.3.4 STAR spacer sequences are distinct at different loci within *S. aureus* strain, but still correlate with strain lineage

To further investigate the link between STAR spacer sequences and MLST lineage of strains, different STAR loci needed to be tested. An RSAT (Regulatory sequence analysis tools) DNA pattern search was used to identify STAR element repeat motifs across entire *S. aureus* genomes. The motif TNTGTTGNGGCCCN was queried against

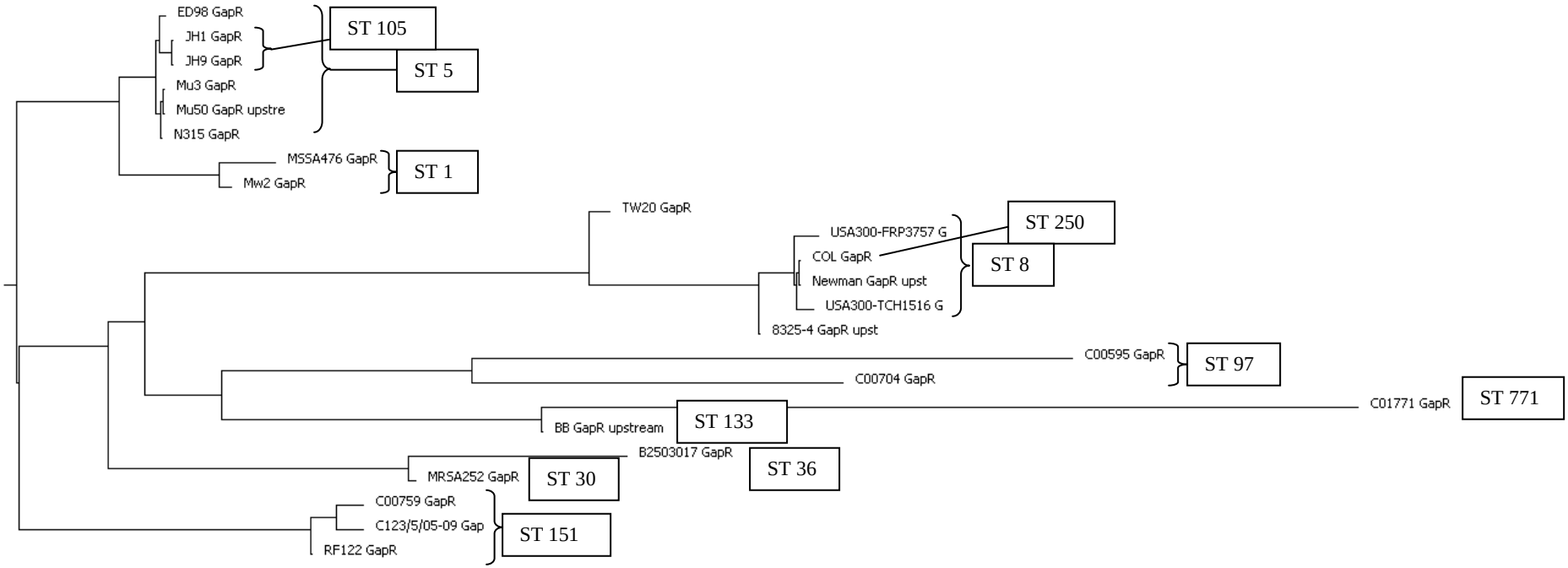


Figure 5-8. Phylogenetic tree of *gapR* STAR element region from different *S. aureus* ST's

The phylogenetic tree above was derived from the sequence of the *gapR* STAR element locus from each of the strains, using the Neighbour-joining algorithm. The tree demonstrates that the sequence of the STAR element locus is conserved between strains from the same lineage, and strains cluster into their ST's as derived by MLST analysis.

the entire genome sequences of strains Newman, Mu50, MRSA252 and RF122 using RSA-tools Genome-scale DNA-pattern search (http://rsat.ulb.ac.be/rsat/; van Helden, 2003; Thomas-Chollier *et al*., 2008), to identify loci with STAR elements in intergenic regions up to 1000 bp upstream of their open reading frame. A second pattern search was also carried out with the motif GTGGGACAGAAAATGAT, to identify loci which also had evidence of the STAR element "start signature" alongside the STAR element repeats (Appendix Figure A-2 to A-5). The Hpr kinase/phosphorylase gene *hprK* and a gene of unknown function NWMN_0733, referred to as orf_{0733} in this study, were chosen for further analysis as they both contain STAR element repeat motifs preceded by a start signature, and variable numbers of repeat motifs were identified in the different strains.

Interestingly both of these loci are linked to carbon metabolism in *S. aureus*. HprK catalyses the phosphorylation of the phosphocarrier protein Hpr, which functions in modulating carbon catabolite repression in Gram positive bacteria (Titgemeyer & Hillen, 2002). The function of orf_{0733} is yet to be determined, but the protein contains putative nucleotide binding domains and due to its position in a putative operon it may function as a transcriptional regulator. The next open reading frame in this operon is also undefined, but it contains a domain similar to the CofD-YvcK superfamily, and YcvK has been proposed to play a role in carbon metabolism in *B. subtilis* (NCBI conserved domain search), which may mean that the orf_{0733} regulates an undefined aspect of carbon metabolism in *S. aureus*.

The STAR element regions upstream of *hprK* and orf_{0733} were PCR amplified and sequenced from a selection of *S. aureus* strains using primer pairs HprK STARS F +

HprK STARS R and Orf_{0733} STARS F + Orf_{0733} STARS R respectively (Figure 5-9; Figure 5-10). The strains chosen for this analysis can be found in Table 5.4, and were chosen as they included at least 2 examples, where possible, of strains from each lineage identified previously. The sequence of each of these STAR element regions was also determined from the complete genome sequences of 12 *S. aureus* strains publically available at NCBI.

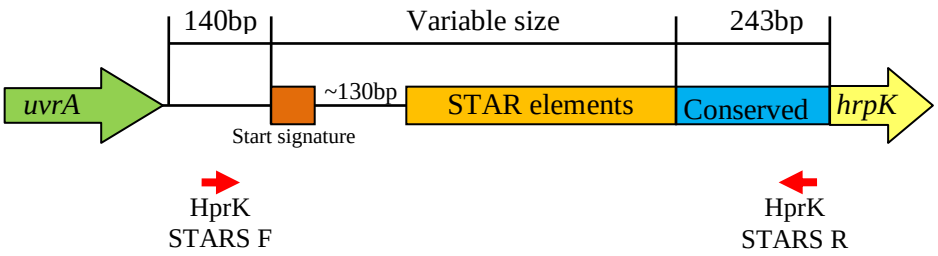
The sequence data from the *hprK* STAR locus and the orf_{0733} STAR locus from each strain was analysed to determine the number of repeats in each strain and the similarity in spacer sequences between strains. Analysis of the *hprK* region as a whole identifies some key differences in this locus compared to the *gapR* locus (Figure 5-9). The STAR "start signature" sequence is present at the *hprK* STAR locus, but it occurs ~130bp before the first STAR element repeat motif rather than ~70bp seen at the *gapR* locus. The *hprK* STAR locus from each strain has a conserved Group 1-like arrangement, and repeat units range between 1 and 3 sequence motifs in any given strain (Table 5.4). Interestingly, there are no major differences in the structure of this STAR locus in the bovine ST151 isolates tested or the CC30 strains, which may mean that this locus is structurally more stable than the *gapR* locus. Comparison of the *hprK* STAR spacer elements shows that the sequence of these spacers differs from the spacers found at the *gapR* locus within each strain, and do not appear to follow the same modular arrangement seen at the *gapR* locus. Multiple repeats are not necessarily the product of a direct expansion of module B (Figure 5-4); there can be different spacer elements within the repeats of the locus as shown for other STAR loci previously (Cramton *et al*., 2000).

Table 5.4. STAR element repeat units at the *hrpK* and orf_{0733} loci.

Table showing the number of STAR element repeat units identified at the *hprK* and orf_{0733} loci in various *S. aureus*. Strains marked with a * were subsequently used for transcriptional studies

Strain	***gapR* STAR group**	**No. of *gapR* STAR element repeats**	**No. of *hprK* STAR element repeats**	**No. of orf_{0733} STAR element repeats**
BB *	1	3	1	5
C00704	1	1	1	4
C01719	1	3	1	5
C01771	1	3	1	4
30375	1	4	1	3
66195	1b	4	2	3
65991	1b	4	2	3
C00595	1	2	--	3
C00759	3	0	1	4
C123/5/05-09	3	0	1	6
8325-4 *	1	1	3	7
Newman *	1	3	3	5
USA300 TCH1516	1	3	3	5
USA300 FRP3735	1	1	3	5
Mu50 *	1	6	3	6
N315	1	6	3	6
Mu3	1	6	3	6
MSSA476	1	2	2	3
Mw2	1	1	2	3
JH1	1	3	3	5
JH9	1	3	3	5
COL	1	3	2	2
RF122 *	3	0	1	5
MRSA252 *	2	0	2	3
TW20	1	3	2	4
ED98	1	4	3	3

A



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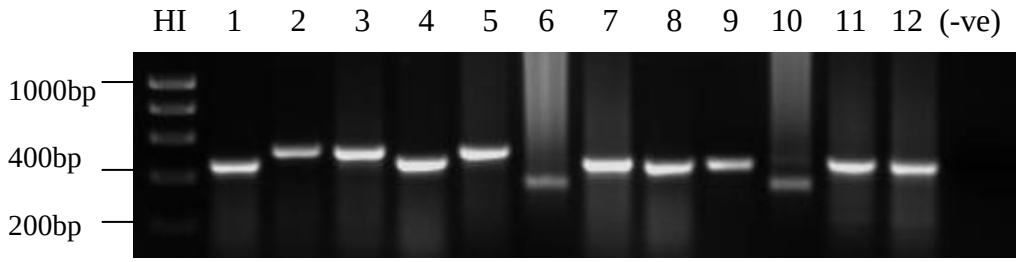


Figure 5-9. Variability of the *hprK* STAR element region between *S. aureus* strains.

(A) Schematic representation of the structure of the region directly upstream of *hrpK*, including position of primers HprK STARS F and HprK STARS R.

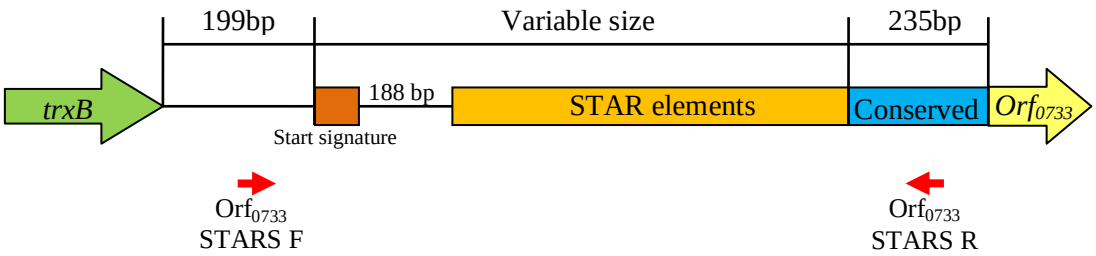
(B) PCR amplification of the *hprK* STAR element region, using primers HprK STARS F and HprK STARS R, from *S. aureus* strains (1) BB, (2) 8325-4, (3) Newman, (4) MRSA252, (5) Mu50, (6) RF122, (7) C00704, (8) C01719, (9) C01771, (10) C00759, (11) 66195 and (12) 65991. Sizes range from approximately 350bp to 550bp. Also included are a DNA size ladder (HI) and a negative control PCR.

Overall the *orf*$_{0733}$ STAR element locus follows the same structure found at the *hprK* and *gapR* Group 1 STAR loci, with no major structural changes identified between strains and no examples of a total loss of repeat elements (Figure 5-10). The STAR start signature sequence is found 188 bp upstream of the first STAR element repeat motif and the number of repeat motifs varies between 2 and 7 in the different strains. Like *hprK*, the spacers of this STAR locus do not appear to follow the simple modular structure of the *gapR* locus. There are some examples of common spacer sequences between this locus the *hprK* and *gapR* loci, but these are not necessarily consistent within a particular strain.

Correlation with lineage

Although there is evidence that both the *hrpK* and the *orf*$_{0733}$ STAR loci are more structurally stable than the *gapR* STAR locus, the spacer sequences at these loci still strongly correlate with strain lineage. This can be demonstrated by using the Neighbour-joining bootstrap method to create a phylogenetic tree for the *hprK* and *orf*$_{0733}$ STAR loci using the sequence for the entire region determined above (Figure 5-11; Figure 5-12). In both cases the strains cluster into the same groups determined by MLST lineage; the only exception being that the RF122 *orf*$_{0733}$ STAR locus clusters alongside the ST 8 strains rather than clustering separately. Therefore, we have evidence that three separate repeat regions in the *S. aureus* genome are highly conserved within evolutionary lineages and are maintained at a similar level to MLST loci. This is highly unusual as intergenic and repetitive DNA sequence would be expected to have a much higher mutation rate than conserved housekeeping genes. What is also interesting is that within a particular strain, the spacer sequences are distinct at each STAR locus examined but they are still maintained and correlate with strain lineage. This suggests

A



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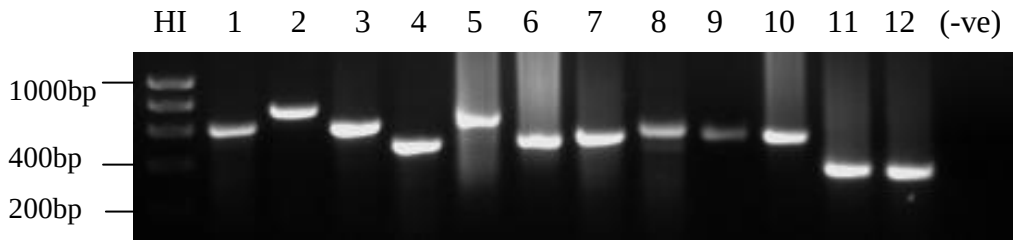


Figure 5-10. Variability of the *orf*$_{0733}$ STAR element region between *S. aureus* strains.

(A) Schematic representation of the structure of the region directly upstream of *orf*$_{0733}$, including position of primers Orf$_{0733}$ STARS F and Orf$_{0733}$ STARS R.

(B) PCR amplification of the *orf*$_{0733}$ STAR element region, using primers Orf$_{0733}$ STARS F and Orf$_{0733}$ STARS R, from *S. aureus* strains (1) BB, (2) 8325-4, (3) Newman, (4) MRSA252, (5) Mu50, (6) RF122, (7) C00704, (8) C01719, (9) C01771, (10) C00759, (11) 66195 and (12) 65991. Sizes range from approximately 400bp to 750bp. Also included are a DNA size ladder (HI) and a negative control PCR.

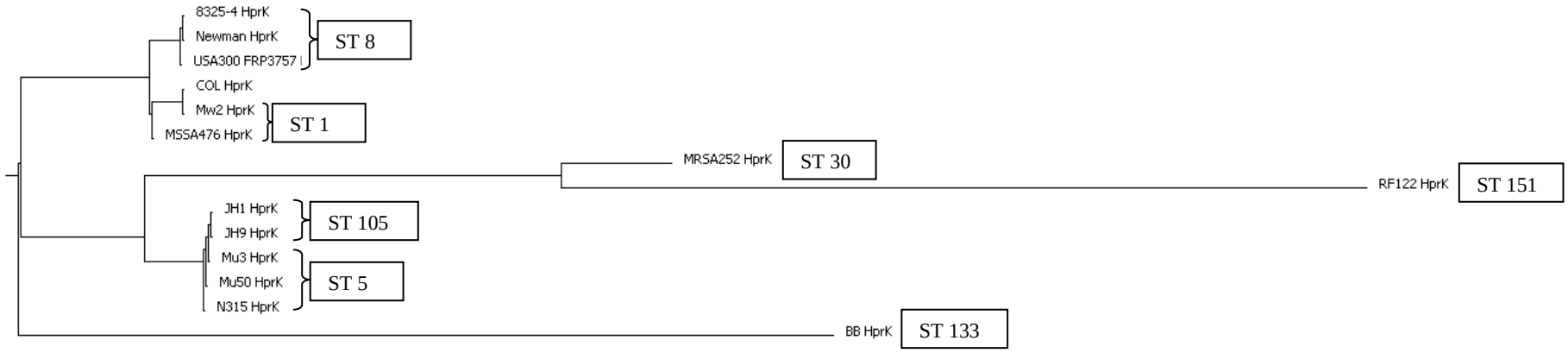


Figure 5-11. Phylogenetic tree of *hprK* STAR element region from different *S. aureus* ST's

The phylogenetic tree above was derived from the sequence of the *hprK* STAR element locus from each of the strains (between primers HprK STARS F and HprK STARS R), using the Neighbour-joining algorithm. The tree demonstrates that the sequence of the STAR element locus is conserved between strains from the same lineage, and strains cluster into their ST's as derived by MLST analysis.

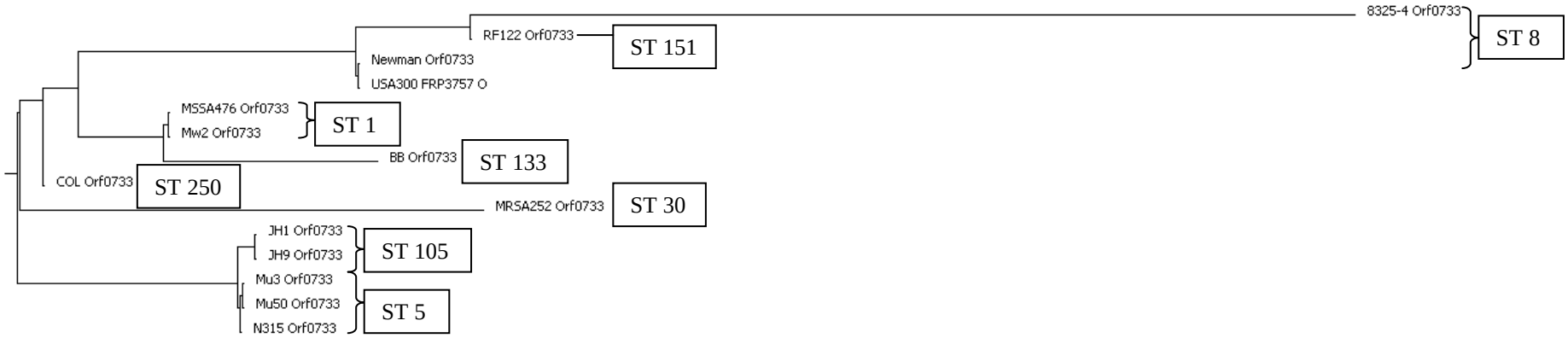


Figure 5-12. Phylogenetic tree of *orf$_{0733}$* STAR element region from different *S. aureus* ST's

The phylogenetic tree above was derived from the sequence of the *orf$_{0733}$* STAR element locus from each of the strains (between primers Orf$_{0733}$ STARS F and Orf$_{0733}$ STARS R), using the Neighbour-joining algorithm. The tree demonstrates that the sequence of the STAR element locus is conserved between strains from the same lineage, and strains cluster into their ST's as derived by MLST analysis. The only exception appears to be RF122 (ST 151), which clusters alongside ST8 in this case.

that the repeats motifs are not readily transferred between STAR loci in *S. aureus*. The conserved nature of the STAR element loci, and the fact that these repeat elements are maintained throughout the *S. aureus* genome suggests that they have a functional role in the cell, which could be the driving force behind this conservation.

As previously described in Section 1.4.2, interspersed repeat elements in other bacterial species have been shown to play a functional role in the regulation of transcription (De Gregorio *et al*., 2002; De Gregorio *et al*., 2003; De Gregorio *et al*., 2005; Knutsen *et al*., 2006), phase variation of genes in response to particular stimuli (Yogev *et al*., 1991) or protection of the genome from phage invasion (Bolotin *et al*., 2005; Mojica *et al*., 2005). Preliminary evidence from strains BB, 8325-4, Newman and MRSA PM64 suggests that the level of transcription of the glycolytic operon correlates with the number of STAR elements present at the *gapR* STAR locus found upstream (Section 4.3.1). To further investigate this, transcription of the open reading frames downstream of each of the STAR loci were examined in different strains containing variable numbers of STAR motifs.

5.4 Do STAR elements have an effect on gene transcription?

Transcription of *gapA*, *hprK* and orf_{0733} was assessed from 6 *S. aureus* strains carrying different numbers of repeats to determine if the number of STAR elements at a given locus correlated with gene expression levels. Northern blotting was chosen to analyse the transcription from total RNA extracts of each strain rather than to use gene expression reporters. The main advantage of using Northern blotting is that this method will take into account any post transcriptional processing of the transcripts, which may

also be influenced by the STAR elements, and give an accurate representation of the transcript levels in the cell in the conditions used.

5.4.1 Transcription of STAR associated genes

Total RNA was extracted from strains 8325-4, MRSA252, Newman, BB, Mu50 and RF122 grown to late exponential phase in the iron restricted medium CRPMI in the presence and absence of 50 μM Fe_2SO_4 to give iron restricted and iron replete conditions. The strains were chosen as together they provided a representation of the full range of variability in the number of STAR repeat units at the *gapR*, *hprK* and orf_{0733} STAR loci (Table 5.5). [α^{32}P] radiolabelled DNA probes were produced, as outlined in the materials and methods, for *gapA*, *hprK* and orf_{0733} using the following primer pairs; *gapA* using primers GapAPF and GapAPR, *hprK* using primers HprK probe F and HprK probe R, and orf_{0733} using primers Orf_{0733} probe F and Orf_{0733} probe R (see Table 2.4).

Transcription of gapA

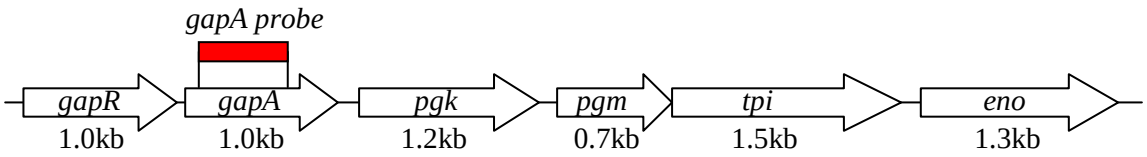
Northern blot analysis reveals 5 transcripts (7.0 kb, 6.0 kb, 2.5 kb, 1.3 kb and 1.0 kb) using a *gapA* probe, which are repressed by the presence of Fe in strain BB and repressed in the absence of Fe in the remaining strains (Figure 5-13). The variable iron regulation of this operon is discussed further in Chapter 4. Surprisingly, the level of transcription of this operon does not correlate with the number of STAR element repeat motifs present in each strain. The transcription of the operon in strain Mu50, which has 6 STAR elements, is in fact lower than that of MRSA252, which has 0 STAR elements. Interestingly the Group 3 strain RF122, which has a partial loss of STAR elements, has an expression level similar to that of Newman, which has 3 repeat elements.

Table 5.5. Strains used for transcriptional analysis of STAR associated loci

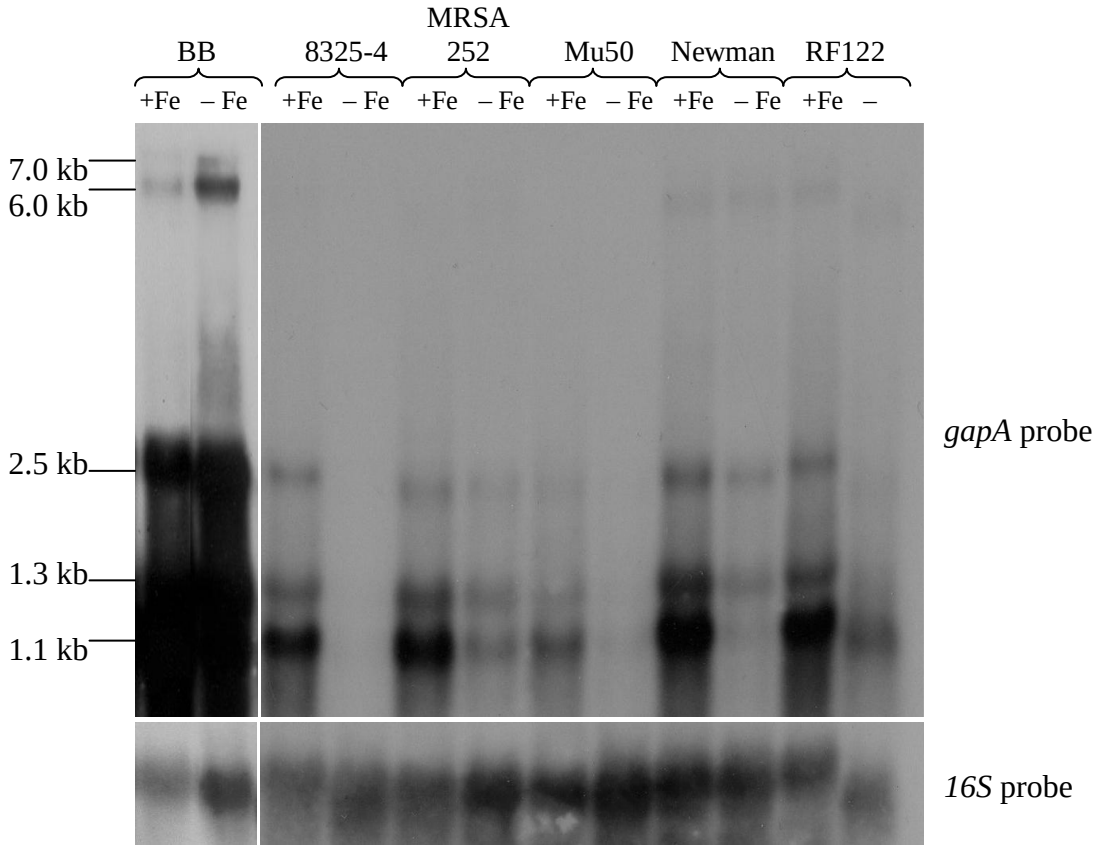
Table showing the number of STAR element repeat motifs at the *gapR*, *hprK* and orf_{0733} STAR loci and the sequence type of each of the strains used for transcriptional analysis of STAR associated genes.

Strain	**No. of *gapR* STAR motifs**	**No. of *hprK* STAR motifs**	**No. of orf_{0733} STAR motifs**	**Sequence Type (ST)**
BB	3	1	5	133
8325-4	1	3	7	8
MRSA 252	0 (total loss)	2	3	36
Mu50	6	3	6	5
Newman	3	3	5	8
RF122	0 (partial loss)	1	5	151

A



B



C

Strain	BB +Fe	BB – Fe	8325-4 +Fe	8325-4 –Fe	252 +Fe	252 – Fe	Mu50 + Fe	Mu50 – Fe	Newman + Fe	Newman – Fe	RF122 + Fe	Rf122 – Fe
Densitometry	0.48	0.61	0.35	0.06	0.30	0.14	0.15	0.07	0.32	0.16	0.43	0.29
No. of STAR motifs	3		1		0		6		3		0	

Figure 5-13. Transcription of *S. aureus gapA* in multiple strains.

(A) Schematic representation of the *gapR* glycolytic operon, indicating open reading frame sizes and the position of the *gapA* Northern blot probe.

(B) Northern blot showing RNA transcription of *gapA* in strains BB, 8325-4, MRSA252, Mu50, Newman and RF122 in response to iron. All RNA extracts were taken from cells grown in CRPMI ± 50μM Fe_2SO_4 and harvested at late exponential phase. The *16S* probe is included to show that RNA was loaded equally in each lane, and the gel presented is representative of two biological repeats.

(C) Densitometry data for the above Northern blot, presented as a ratio of *gapA:16S* transcript levels, alongside the number of STAR motifs in each strain

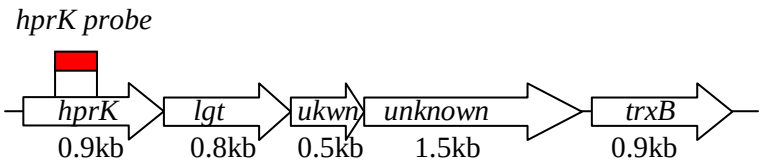
Transcription of HprK

The *hprK* gene has been proposed to be the first unit of a putative operon (Figure 5-14A), containing the prolipoprotein diacylglyceryl transferase gene *lgt*, which functions in prolipoprotein biosynthesis, two hypothetical open reading frames of unknown function, and the thioredoxin reductase gene *trxB* (Wang *et al*., 2004). The *hprK* probe detects multiple transcripts, with approximate sizes of 5.0 kb, 2.5 kb, 2.25 kb and 1.2 kb (Figure 5-14B), and these transcripts appear to be iron regulated in a similar way to *gapA*. Based on the sizes, the 5.0 kb transcript may be from transcription of the entire operon, while 1.2 kb is likely to be the *hprK* open reading frame alone, possibly alongside untranslated 5' RNA sequence. The transcriptional level of this operon does not correlate with the number of STAR element repeat motifs present upstream (Figure 5-14B). Strain Newman shows a very high level of transcription, while strains 8325-4 and MRSA252 both show low levels of transcription, even though all three of these strains carry 3 repeat motifs at the *hprK* STAR locus. BB and RF122 have an intermediary level of transcription of this locus compared to these strains, and in both cases only contain a single repeat unit.

Transcription of orf_{0733}

The orf_{0733} gene is also part of a putative operon (Figure 5-15A) alongside two more hypothetical open reading frames located directly downstream of orf_{0733} (Wang *et al*., 2004). The orf_{0733} probe was able to detect multiple transcripts, with approximate sizes of 3.8 kb, 3.0 kb and 1.25 kb (Figure 5-15B). These transcript sizes appear to be quite large for the predicted operon, and the 3.8 kb transcript may suggest a fourth gene located downstream, *clpP*, is also co-transcribed with orf_{0733}. As previously seen, the transcription of the operon was variable between different *S. aureus* strains, but this

A



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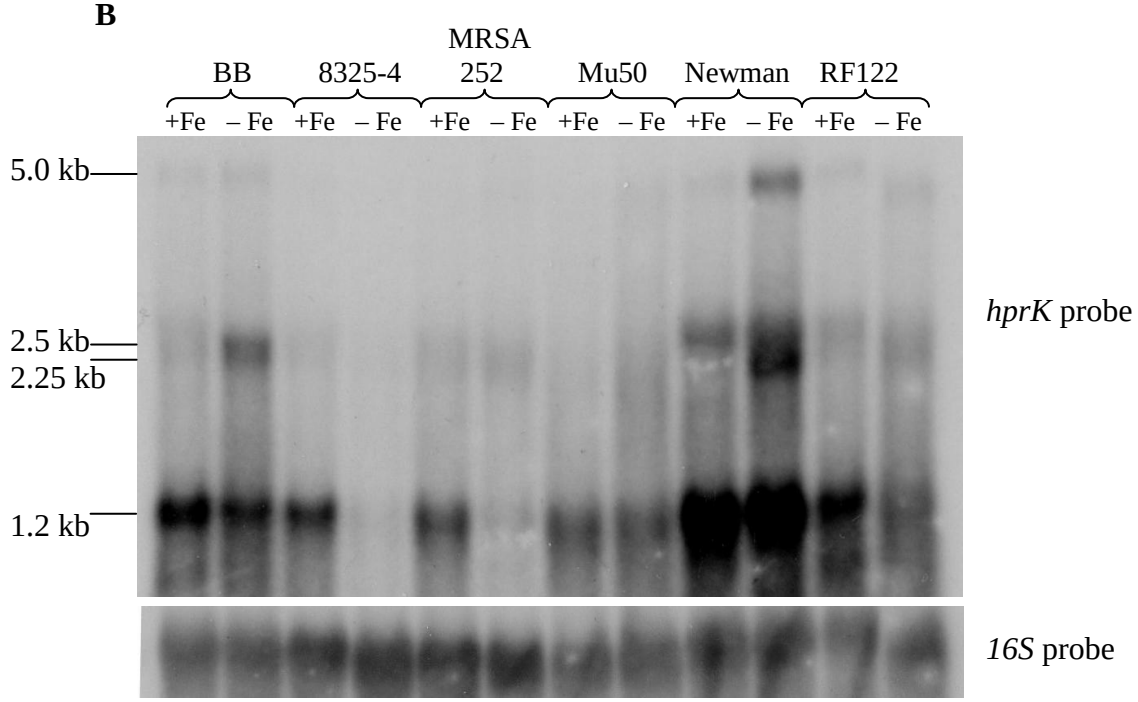


C

Strain	BB +Fe	BB – Fe	8325-4 +Fe	8325-4 –Fe	252 +Fe	252 – Fe	Mu50 + Fe	Mu50 – Fe	Newman + Fe	Newman – Fe	RF122 + Fe	Rf122 – Fe
Densitometry	0.64	0.79	0.37	0.13	0.39	0.23	0.50	0.63	0.96	1.19	0.83	0.69
No. of STAR motifs	1		3		2		3		3		1	

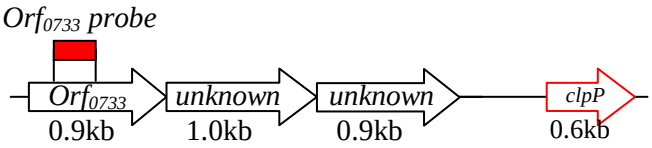
Figure 5-14. Transcription of *S. aureus hprK* in multiple strains.

(A) Schematic representation of the putative *hprK* operon, as predicted by (Wang *et al*., 2004), indicating open reading frame sizes and positioning, as well as the position of the *hprK* probe.

(B) Northern blot showing RNA transcription of *hprK* in strains BB, 8325-4, MRSA252, Mu50, Newman and RF122 in response to iron. All RNA extracts were taken from cells grown in CRPMI ± 50μM Fe_2SO_4 and harvested at late exponential phase. The *16S* probe is included to show that RNA was loaded equally in each lane, and the gel presented is representative of two biological repeats.

(C) Densitometry data for the above northern blot, presented as a ratio of *hprK:16S* transcript levels, alongside the number of STAR motifs in each strain

A



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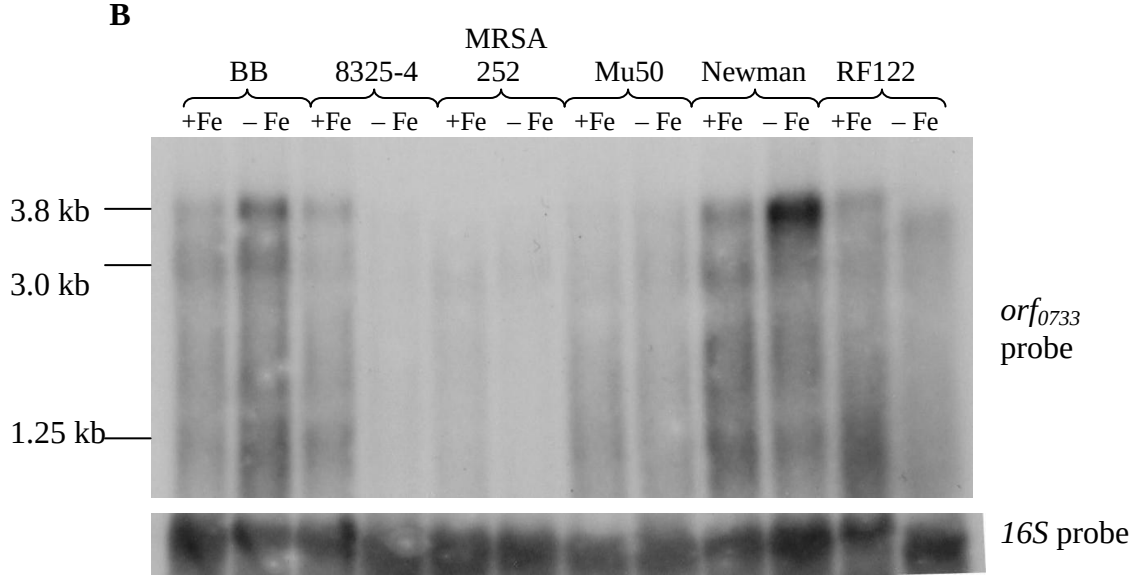


C

Strain	BB +Fe	BB – Fe	8325-4 +Fe	8325-4 –Fe	252 +Fe	252 – Fe	Mu50 + Fe	Mu50 – Fe	Newman + Fe	Newman – Fe	RF122 + Fe	Rf122 – Fe
Densitometry	0.28	0.54	0.21	0.05	0.06	0.05	0.13	0.18	0.45	0.61	0.36	0.25
No. of STAR motifs	5		7		3		6		5		5	

Figure 5-15. Transcription of *S. aureus orf$_{0733}$* in multiple strains

(A) Schematic representation of the putative *S. aureus orf$_{0733}$* operon, as predicted by (Wang *et al*., 2004), indicating the size and positioning of the open reading frames, and the position of the *orf$_{0733}$* probe. The *clpP* open reading frame is included in red, as the size of the 3.8 kb transcript suggests that it may also be co-transcribed as part of this operon.

(B) Northern blot showing RNA transcription of *orf$_{0733}$* in strains BB, 8325-4, MRSA252, Mu50, Newman and RF122 in response to iron. All RNA extracts were taken from cells grown in CRPMI ± 50μM Fe_2SO_4 and harvested at late exponential phase. The *16S* probe is included to show that RNA was loaded equally in each lane, and the gel presented is representative of two biological repeats.

(C) Densitometry data for the above northern blot, presented as a ratio of *orf$_{0733}$:16S* transcript levels, alongside the number of STAR motifs in each strain.

variability did not correlate with the number of STAR element repeat units present at this locus (Figure 5-15B). The operon is also iron regulated in a strain dependent manner, and this does not correlate with variability in the STAR element repeat tract.

What does appear to be interesting is that in general the level of orf_{0733} transcription between the strains follows the same pattern seen for both *gapR* and *hprK*, with very low expression in strains 8325-4, MRSA252 and Mu50, medium levels in RF122 and BB and high levels in strain Newman. We know that the glycolytic operon has a key function in carbon metabolism in growing bacterial cells, while *hprK* is involved in the regulation of metabolism. The other members of the *hprK* operon are also important in aspects of cell growth and survival, and the orf_{0733} operon represents a set of genes with unknown functions in *S. aureus* which may be involved in carbon metabolism. Therefore taken together we have a set of genes, some with key housekeeping functions, showing a wide range of variability in their level of expression between different *S. aureus* isolates.

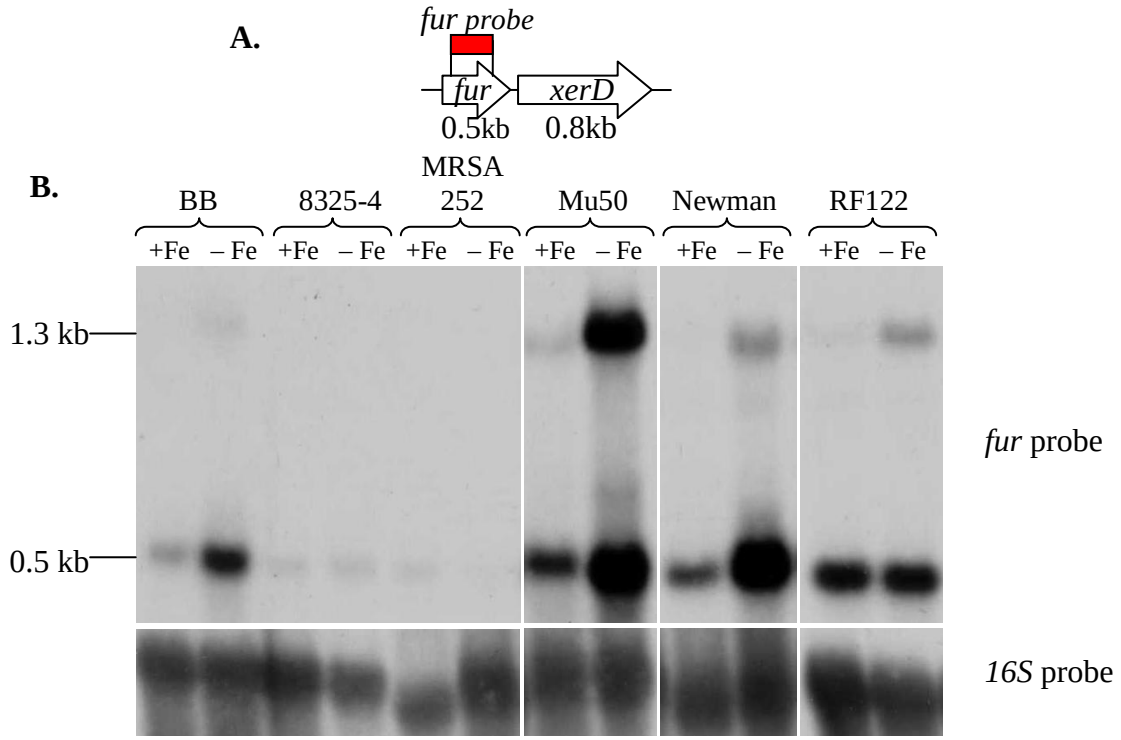
5.4.2 Transcription of non STAR-associated loci

One simple explanation for the transcriptional strain variability of the STAR element associated loci is that global RNA levels vary between these strains, and overall the expression of a wide variety of genes will follow this pattern. To test this theory, northern blot analysis was used to determine the expression of non STAR-associated genes; the global iron regulator *fur*, the α-hemolysin *hla,* and the copper regulator *csoR*. *Fur* was chosen as all 3 STAR associated loci appear to be regulated by iron, and there is evidence that at least *gapA* is regulated by Fur (Section 4.2.3). Fur is not only a major contributor to the regulation of genes in response to iron (see Section 1.5.3), but it is

also iron regulated itself, and therefore if the strain variable levels of transcription are related to iron regulation, transcription of *fur* is likely to follow the same pattern. The α-hemolysin gene *hla* was chosen as it is also iron regulated, but the *hla* protein is a surface associated virulence factor with a function unrelated to that of the STAR-associated genes studied (Menestrina *et al*., 2001). The copper responsive regulator *csoR* was also chosen for further study, as this gene is regulated by copper concentrations but not iron (Dr. J. Baker, unpublished data).

Fur – the Ferric-uptake Regulator

Primers Fur 1F and Fur 1R were used to produce a 313 bp [α^{32}P] radiolabelled probe from within the *fur* open reading frame, and this was used to determine transcription levels of Fur in strains BB, 8325-4, MRSA252, Mu50, Newman and RF122 grown to late exponential phase in CRPMI +/- 50μM Fe_2SO_4. This probe detects two transcripts of 1.3 kb and 0.5 kb (Figure 5-16), which represent the transcription of *fur* and the *fur/xerD* operon (Johnson *et al*., 2010). Unexpectedly the level of expression of this important global regulator varies quite dramatically between isolates, even those closely related to one another (ST8 strains 8325-4 and Newman). Although the level of *fur* expression does vary between the strains tested, this does not correlate with the variable levels of expression seen at the STAR-associated loci. For example, expression of STAR associated genes has been consistently low in strain Mu50, but this strain shows the highest level of *fur* transcription. Also, strains BB and Newman have generally shown similar expression levels of the STAR associated loci, but *fur* is clearly expressed at a lower level in BB compared with Newman. Therefore the variability in expression of the STAR associated loci is probably not due to a global variation in the iron regulatory response in different strains. Nucleotide sequence analysis from



C.

Strain	BB +Fe	BB – Fe	8325-4 +Fe	8325-4 –Fe	252 +Fe	252 – Fe	Mu50 + Fe	Mu50 – Fe	Newman + Fe	Newman – Fe	RF122 + Fe	Rf122 – Fe
Densitometry	0.06	0.16	0.03	0.03	0.02	0.02	0.18	0.58	0.09	0.32	0.13	0.16

Figure 5-16. Transcription of *S. aureus fur* in multiple strains.

(A) Schmatic representation of the *fur/xerD* operon, as determined by (Johnson *et al.*, 2010).

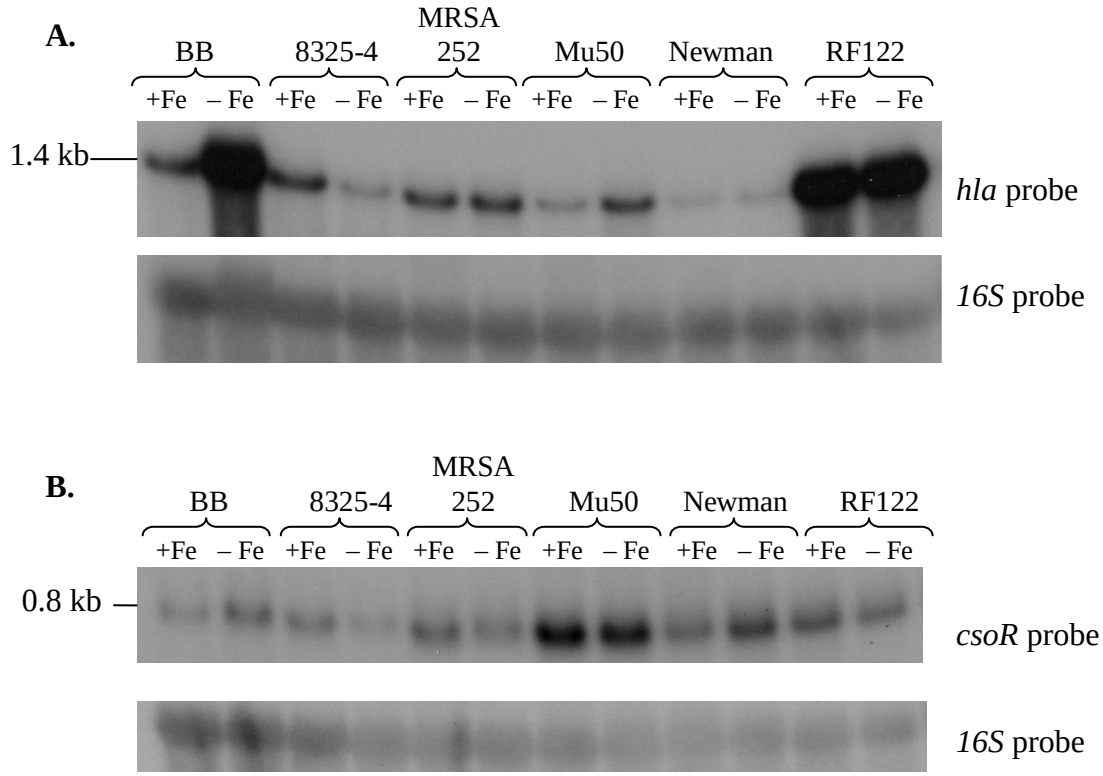
(B) Northern blot showing RNA transcription of *fur* in strains BB, 8325-4, MRSA252, Mu50, Newman and RF122 in response to iron. All RNA extracts were taken from cells grown in CRPMI ± 50μM Fe_2SO_4 and harvested at late exponential phase. The *16S* probe is included to show that RNA was loaded equally in each lane, and the gel presented is representative of two biological repeats.

(C) Densitometry data for the above Northern blot, presented as a ratio of orf_{0733}*:16S* transcript levels.

available *S. aureus* genome sequences shows that there are no sequence differences in either the promoter or coding region of the *fur* gene to account for this strain variable expression. This result is interesting as the *fur* transcript provides another example of strain variable regulation of a key gene in different strains of *S. aureus*. The fact that the expression of *fur* does not correlate with the expression of the STAR-associated loci also indicates that the variable expression levels are not due to global differences in RNA levels between the strains, but that the expression level of these loci are independently controlled.

Hla and CsoR

[α^{32}P] radiolabelled probes were produced for *hla* and *csoR* (see Table 2.4 for primers) and used to measure transcription levels of the two genes in strains BB, 8325-4, MRSA252, Mu50, Newman and RF122 grown to late exponential phase in CRPMI +/- 50μM Fe_2SO_4. A single 1.4 kb transcript was detected for *hla* and a single 0.8 kb transcript was detected for *csoR*. The *hla* gene is repressed by iron in the majority of strains tested, while there is no significant iron regulation of *csoR* (Figure 5-17). Transcription levels of both of these loci are variable between different *S. aureus* strains, but this regulation does not correlate with the levels of expression seen at the previous loci examined. Interestingly, the level of expression of *hla* was much higher in the bovine mastitis isolates BB and RF122 than any of the human isolates tested. Expression of *hla* in strain Newman is much lower than the other strains, which is a stark contrast to the high levels of transcription seen in this strain at the STAR-associated loci. The transcriptional level of the regulator *csoR* was very low in all of the strains, but strain Mu50 showed up to a 10-fold higher level of transcription compared



C.

Strain	BB +Fe	BB – Fe	8325-4 +Fe	8325-4 –Fe	252 +Fe	252 – Fe	Mu50 + Fe	Mu50 – Fe	Newman + Fe	Newman – Fe	RF122 + Fe	Rf122 – Fe
hla densitometry	0.72	1.83	0.90	0.30	0.65	0.75	0.33	0.80	0.17	0.28	2.28	3.33
csoR densitometry	0.39	0.70	0.74	0.54	1.36	1.13	3.53	4.16	2.38	2.98	2.34	1.71

Figure 5-17. Transcription of *S. aureus hla* and *csoR* in multiple strains

Northern blot showing RNA transcription of (A) *hla* and (B) *csoR* in strains BB, 8325-4, MRSA252, Mu50, Newman and RF122 in response to iron. All RNA extracts were taken from cells grown in CRPMI ± 50μM Fe_2SO_4 and harvested at late exponential phase. The *16S* probe is included to show that RNA was loaded equally in each lane, and the gels presented are representative of two biological repeats.

(C) Densitometry data for the *hla* and *csoR* Northern blots, presented as a ratio of *gene:16S* transcript levels.

to the other strains. Mu50 is a strain which had previously shown very low levels of transcription of *hla* and the STAR associated loci.

Therefore we have evidence that a wide variety of genes are expressed at variable levels in different *S. aureus* isolates. This variable regulation occurs in key metabolic genes such as the glycolytic operon, global regulators such as *fur* and virulence factors such as *hla*, but each of these systems is not expressed at equivalent levels between strains. Instead the expression level appears to be independent at each locus and not due to overall global changes in RNA production. In order to visualise this, the densitometry data for each Northern was coloured to represent the level of transcription in each strain relative to one another (Table 5.6). Together these data suggest that each *S. aureus* strain has the ability to adapt to its environment in a unique way, and that variability in the transcript levels of genes may be another means of strains diverging from one another to exploit unique biological niches.

Table 5.6. Densitometry data for the transcription of the above loci.

The shading in this table represents the relative transcript level of each gene between the different strains. This is not representative of the transcription level of each locus relative to one another

Strain and condition	***gapA***	***hprK***	***orf*$_{0733}$**	***fur***	***hla***	***csoR***
BB + Fe	0.48	0.64	0.28	0.06	0.72	0.39
BB - Fe	0.61	0.79	0.54	0.16	1.83	0.70
8325-4 + Fe	0.35	0.37	0.21	0.03	0.90	0.74
8325-4 - Fe	0.06	0.13	0.05	0.03	0.30	0.54
MRSA252 + Fe	0.30	0.39	0.06	0.02	0.65	1.36
MRSA252 - Fe	0.14	0.23	0.05	0.02	0.75	1.13
Mu50 + Fe	0.15	0.50	0.13	0.18	0.33	3.53
Mu50 - Fe	0.07	0.63	0.18	0.58	0.80	4.16
Newman + Fe	0.32	0.96	0.45	0.09	0.17	2.38
Newman - Fe	0.16	1.19	0.61	0.32	0.28	2.98
RF122 + Fe	0.43	0.83	0.36	0.13	2.28	2.34
RF122 - Fe	0.29	0.69	0.25	0.16	3.33	1.71

5.5 Discussion

STAR elements are highly abundant in *S. aureus*, and vary in both sequence and structure between loci and between strains. However, we have shown that the spacer sequences at three independent STAR loci are conserved within strain lineages at a similar level to that of MLST. Although the number of STAR motifs does not correlate with the level of transcription of associated genes, we have also demonstrated that the expression of a number of different *S. aureus* genes are strain variable, and that this expression is independent at each locus.

The main aim of this chapter was to analyse the variability of the *gapR* STAR locus in detail in a number of *S. aureus* strains. This repeat region was found to exhibit strain variability in the structure of the region, the number of repeat motifs present and also the sequences spacing the STAR motifs (Figure 5-2; Figure 5-5; Figure 5-8). The *gapR* STAR elements appear to be modular, based on sequence variation of the spacers within the locus in a particular strain, and follow an A-B_n-C structure. Multiple repeats appear to occur due to a direct expansion of spacer B alongside a STAR motif (Figure 5-4). The modularity of the *gapR* STAR locus is reminiscent of the modular structure seen in the streptococcal repeat element BOX (Martin *et al*., 1992). BOX elements are made up of three subunits and generally follow the structure A-B_n-C, although some may be found in an inverted orientation, and some lack the B subunit entirely (Martin *et al*., 1992; Koeuth *et al*., 1995; Knutsen *et al*., 2006). The modular structure of these elements is important, as they are involved in fine tuning the expression of upstream or downstream genes, and the arrangement and orientation of the modules in relation to one another has a direct effect on the control of gene expression (Martin *et al*., 1992; Koeuth *et al*., 1995; Knutsen *et al*., 2006). Although we are yet to identify a specific

function for the *gapR* STAR elements, it is possible that their structural organisation and orientation will prove important in any roles they do have, as it does with other functional repeats.

Three of the five structural variants identified at the *gapR* STAR locus showed either a total or partial loss of the STAR elements from the region (Figure 5-2). Interestingly, the Group 2 and Group 2b strains lose the STAR elements at exactly the same position, and the surrounding DNA sequence is undisturbed compared to that of the Group 1 and Group 1b strains (Figure 5-3). This is similar to another class of bacterial repeats known as Enterobacterial repetitive intergenic consensus (ERIC) sequences (see Section 1.4.2 for more detail), which have been identified across the eubacterial kingdom (Versalovic *et al*., 1991). ERICs are not necessarily present at the same loci in different species, but the sequence surrounding an inserted ERIC remains unchanged, indicating a precise insertion or deletion event via a mechanism distinct from classic transposition mechanisms (Sharples & Lloyd, 1990; Lupski & Weinstock, 1992). It is unclear whether there is a conserved mechanism for the loss or gain of STAR elements or whether the deletion site merely acts as a hotspot for STAR element translocation. The partial loss of elements seen in strains such as RF122 (Group 3), does not occur at this deletion site, and may represent a different mechanism of repeat propagation, or an error in repeat translocation in an ancestral strain that has been propagated in subsequent generations. In each case these structural groups were maintained within distinct evolutionary lineages (Table 5.3; Figure 5-7), indicating that the loss or gain of STAR elements at the *gapR* locus may have occurred in early ancestors of these lineages and been retained in subsequent isolates.

This analysis was extended to two more STAR loci, found upstream of *hprK* and orf_{0733}. Each of these STAR loci appeared to be more structurally stable than *gapR*, and there was no evidence of a loss of the STAR elements in either case in any of the 27 strains tested. However each locus was still variable in the number of motifs present and the sequence of the spacers between strains (Table 5.4; Figure 5-11; Figure 5-12). It is interesting to note that at the *hprK* and orf_{0733} STAR loci the spacer sequences between the STAR motifs do not follow the same modular A-B_n-C pattern seen at the *gapR* locus, as additional unique spacers can be found within the STAR locus. Therefore it is possible that the addition of repeats may not be as simple as direct expansion of the internal spacer-STAR sequences at all loci. However, alignment of the sequence of each individual STAR locus as a complete unit from different strains still correlates with their evolutionary relationships (Figure 5-11; Figure 5-12). This shows that both the *hprK* and orf_{0733} STAR loci repeats are still maintained in *S. aureus* at the same level as the *gapR* STAR locus.

Sequence variation at each of the STAR loci strongly correlates with the evolutionary lineage of the strain tested, as defined by MLST analysis (Figure 5-7; Figure 5-8; Figure 5-11; Figure 5-12). At a particular STAR locus, strains belonging to the same ST contain identical spacer sequences between the interspersed STAR motifs, even when the number of repeat units varies (Figure 5-5). However, the spacer sequences are distinct at each STAR locus, even within a particular strain. Due to the repetitive nature of STAR elements it has previously been suggested that homologous recombination between repeats occurs as a means of large scale genomic rearrangements (Cramton *et al*., 2000), or could provide a simple means of propagating these repeats at different loci throughout the genome. As the spacers are distinct between unrelated strains and at

different STAR loci within a strain (Figure 5-5), homologous recombination cannot be occurring at a high frequency between STAR loci either intergenomically or intragenomically, as this would result in gene conversion and the emergence of one particular spacer sequence variant at all loci. Therefore the actual means of dissemination of the STAR elements throughout the *S. aureus* genome are not clear.

The fact that these repeat elements are so conserved is interesting. Mutation rates in the coding region of highly conserved genes housekeeping genes is very slow, and therefore the combined difference between strains at these loci provide an indication of how long ago strains diverged from one another. The emergence of new alleles in *S. aureus* has been shown to occur at a 15-fold higher frequency by point mutation than by recombination, although homologous recombination events contribute to the long term evolution of this species (Feil *et al*., 2003). However a non-functional intergenic region of sequence containing repetitive elements that can be found throughout the genome would be expected to evolve at a more rapid rate than the conserved functional MLST loci, most likely via homologous recombination between conserved repeat motifs, but this does not appear to be the case with the STAR elements. The highly conserved nature of these repeat elements suggests that they have a functional role. Unlike eukaryotic genomes which can contain more than 50% repetitive DNA (Richard *et al*., 2008), prokaryotic genomes are very streamlined as the propagation of non-functional "selfish" DNA is a burden to the rapidly dividing organisms and selected against (Doolittle & Sapienza, 1980; Ussery *et al*., 2004). Other repeat elements in bacteria have functions in cell physiology, such as transcriptional control (Knutsen *et al*., 2006) and protection of the microbial genome against foreign DNA (Bolotin *et al*., 2005; van der Oost *et al*., 2009; Karignov & Hannon, 2010). It is interesting to note that although

other species of staphylococci do contain STAR elements, there are far fewer STAR loci across their genomes compared to that of *S. aureus* (Cramton *et al*., 2000). The conservation of STAR elements in the highly adaptive, pathogenic organism *S. aureus* compared to less invasive and commensal organisms such as *S. epidermidis* or *S. carnosus* may further indicate that the STAR elements do play a role in cell physiology.

Due to their proximity to open reading frames, in particular to highly transcribed genes, one possible functional role for STAR elements could be in transcriptional control. Therefore the transcription of genes associated with each of the STAR element loci was examined in a range of strains (Figure 5-13; Figure 5-14; Figure 5-15). Although the structural variability of the STAR loci does not appear to correlate with gene expression levels (Figure 5-13), the variability of these repeats may be important in another, as yet undefined, function. Through the use of transcriptional reporters we have already shown that the presence of STAR elements is required for Class II Fe regulation of the glycolytic operon (Section 4.5.1), and therefore a role in transcriptional regulation cannot be ruled out without further study.

The transcription level of all three STAR-associated loci was found to be strain variable, which is of particular interest as at least two of these genes, *gapA* and *hprK*, are highly conserved and considered housekeeping genes in *S. aureus*. Transcription of three non-STAR associated loci, *fur*, *hla* and *csoR* (Figure 5-16; Figure 5-17) indicate that strain variable transcription of genes is common in *S. aureus*, but the level of expression of each gene is not always at the same relative level in each strain (Table 5.6). Therefore we have a number of STAR- and non STAR-associated genes which

show strain variable levels of expression in *S. aureus* which cannot be explained as simply the result of variation in global RNA levels.

It has long been established that different *S. aureus* isolates have different capacities to inhabit certain environments and cause certain diseases. For example, some strains are better adapted as commensals while others are very invasive (e.g. PVL toxin producing strains). The gene content is generally thought to be the primary factor in the capabilities of a strain, but the way that a strain utilises the genes it has could be just as important. For example small differences in the expression of key metabolic genes, such as *gapA* and *hprK*, may give one strain an advantage over another in a particular environment, allowing it to preferentially colonise that particular niche. In addition, the level of expression of a particular virulence factor may affect the host's immune response during infection, allowing *S. aureus* cells expressing high levels of virulence factor to invade the host more aggressively but also for the immune system to recognise and destroy the bacteria faster. It is interesting that the α-hemolysin gene *hla* is expressed at a higher level in the bovine isolates RF122 and BB compared with the human isolate strains, and this may be partially responsible for the host specificity of these strains (Figure 5-17). It is possible that expression of *hla* at such a high level in humans will trigger an overt immune response and the invading species will be quickly destroyed, or that bovine infection pathogenesis simply requires higher levels of α-hemolysin activity.

The expression levels of each of the 6 genes examined may be independently controlled, but as we have seen strain variable expression can also occur for transcriptional regulators, which will have an impact on the expression of entire

regulons. In the case of *fur* transcription, strain variable expression levels may alter iron homeostasis mechanisms and specific virulence factors in *S. aureus*, which in turn is likely to influence both colonisation and virulence of a particular strain (Horsburgh *et al*., 2001b; Morrissey *et al*., 2004; Johnson *et al*., 2005). The expression of the global virulence regulator *sae* has previously been reported to vary between strains (Steinhuber *et al*., 2003). It is very possible that the interplay between different global regulators on gene expression will be influenced by their relative expression levels, potentially providing a fine tuning mechanism for the specific expression of different genes in individual *S. aureus* strains. Therefore the differential expression levels of a set of conserved genes may be a useful tool to aid the elucidation of specific markers of invasiveness and virulence of different *S. aureus* strains.

It is important to note that although transcriptional levels of the genes analysed vary greatly between strains, this difference may not be translated to the protein level. As the use of northern blotting only looks at transcript levels at a single time point, it is possible that the strain variability is due to differences in RNA turnover, and actual translated protein levels may not show as much variation. By quantitatively measuring gene expression at the protein level a more accurate picture of strain variability could be produced, which may shed further insight into the functional significance of these strain differences. However this does not detract from the significance of key metabolic and regulatory genes being controlled differently in a number of *S. aureus* strains.

In conclusion, STAR elements appear to be highly variable in both sequence and structure at different loci across the *S. aureus* genome, but at each locus they are conserved within *S. aureus* lineages, indicating selective pressure to retain STAR

elements in particular intergenic regions at a low rate of change. Our evidence suggests that the number of STAR element repeat motifs does not directly influence the level of transcription of downstream genes. However, other experiments indicate that they do have a function. As discussed in Chapter 4, the presence of STAR elements in a plasmid based reporter system appeared to negatively influence expression as the number of repeats increased, and in the absence of the STAR element region the *gapR* reporters for strains 8325-4 and Newman showed little or no expression, suggesting that at least some part of the STAR element region is required for *gapR* transcription in these strains.

Chapter 6 Conclusions and Future Work

There is increasing evidence that GAPDH proteins are important, not only in central carbon metabolism, but also in a number of other aspects of cellular physiology in both prokaryotic and eukaryotic cells. Understanding their functional significance in the colonisation and virulence of pathogens may provide novel targets for treatments or vaccines. The primary aim of this thesis was to investigate the function and regulation of two GAPDH homologues in the important pathogen *Staphylococcus aureus*. The data presented in this work not only identifies the primary metabolic function of both GapA and GapB, but also demonstrates novel moonlighting functions and novel non-glycolytic regulation of both GAPDH homologues. An additional aim of this project was to investigate a STAR repeat locus located directly upstream of the glycolytic operon and determine whether it had an impact on gene expression. The data demonstrate that this STAR locus appears to be required for iron regulation of the glycolytic operon in at least some strains, which is the first indication that these repeat elements are functional in *S. aureus*. Although we have not identified a global function for these repeat elements to date, we are able to demonstrate that they are highly conserved at multiple loci across the *S. aureus* genome. Finally we also show that the transcriptional level of a number of genes, including metabolic enzymes and key global regulators, is variable between different *S. aureus* strains, which may play a role in the adaptability of *S. aureus* strains.

We have shown that in *S. aureus* the GAPDH homologue GapA is essential for the utilisation of glucose via glycolysis, while GapB is essential in the utilisation of secondary carbon sources (Figure 3-5; Figure 3-7). Although we are unable to conclusively demonstrate that GapB is a gluconeogenic GAPDH at the protein level, all

of the metabolic and transcriptional data strongly suggest that this is its function *in vivo*. It is unclear why the recombinant GapB protein is not enzymatically active, but it is possible that *in vitro* the protein is unable to fold properly or form the tetramer required for enzyme activity due to the lack of an unidentified co-factor found *in vivo*. Purification of native GapB protein, as demonstrated for *B. subtilis* (Fillinger *et al*., 2000), rather than recombinant protein may be required to demonstrate enzyme activity *in vitro*. It would also be useful to complement the GAPDH mutants by re-introducing functional *gapA* and *gapB* alleles back into the mutant strains, to confirm the mutant phenotypes observed are due to a loss of these genes and not polar or secondary mutations. This is particularly important in testing whole cell GAPDH activity in the *ΔgapB* mutant, as wild type *gapB* protein should restore NADP+ dependent GAPDH activity and would further strengthen the evidence that it is a gluconeogenic GAPDH protein.

One area of interest that has previously been overlooked in *S. aureus* is the role of carbon metabolism during infection. While the role of the central metabolic regulator CcpA in *S. aureus* infection has been examined, transcriptional data shows that this regulator influences a multitude of virulence factors as well as carbon metabolism genes (Seidl *et al*., 2006; Seidl *et al*., 2008; Seidl *et al*., 2009). Our GAPDH mutant strains therefore provided an opportunity to assess the impact of individual carbon metabolism pathways during *S. aureus* pathogenesis. Through the use of an invertebrate infection model, we have clearly shown that both GapA and GapB are required for *S. aureus* pathogenesis (Figure 3-12), which strongly suggests that *S. aureus* need to utilise both primary and secondary carbon sources during infection.

From the data presented here it is impossible to say exactly how the loss of these pathways influences infection, but it is possible that the loss of glycolysis and/or gluconeogenesis decreases the growth of the bacteria within the host, limiting either colonisation or accumulation during infection. It would be interesting to fluorescently tag these *S. aureus* GAPDH mutant strains via the introduction of a constitutive GFP gene on a plasmid vector in order to track them during infection in the *Galleria* caterpillars, as has been shown for other bacterial species (Champion *et al*., 2009). This would allow a visual indication of the growth and colonisation of the bacteria over the course of infection, and determine how each of the mutations influences the growth and/or survival of the bacteria *in vivo*.

The *Galleria mellonella* infection model shares similarities with mammalian innate immunity and often provides correlative data to studies in mammalian systems (Brennan *et al*., 2002). However it does not provide a means to test different types of infection. As carbon source availability may vary between different sites within a mammalian host, it would be useful to extend these infection studies to a mammalian system, such as a mouse model. This would allow us to determine the relative importance of carbon metabolism during different infections, such as abscess formation, device related infection, respiratory infection and systemic infection. The use of GFP tagged strains could also be used in this system, to determine whether colonisation or clearing of the bacteria by the immune system varies between different sites of infection for the mutant strains (Zhao *et al*., 2001).

Although the most profound phenotypic effect of the *ΔgapA* and *ΔgapB* mutations is the disruption of carbon metabolism, the mutagenesis strategy used results in loss of the

GapA and/or GapB proteins across all cellular fractions. It would be useful to assess the influence of GAPDH surface expression during infection independently of GAPDH cytoplasmic enzyme activity, by preventing GAPDH surface localisation. This would allow a distinction between the primary role of these proteins during carbon metabolism and potential surface associated moonlighting roles. The impact of surface localisation of the GAPDH proteins was demonstrated in Group A *Streptococci* by introducing a mutation into the hydrophobic tail of GAPDH, preventing its surface expression but retaining its cytoplasmic activity (Boel *et al*., 2005). This technique provided useful information regarding the direct impact of surface GAPDH in bacterial adherence and immune evasion, and could potentially be used with *S. aureus* GAPDH if the residues for surface attachment could be determined. Investigation into the mechanism of surface localisation of GAPDH proteins was beyond the scope of this project, and as yet it is unclear how these proteins attach to the surface of *S. aureus*.

It has previously been proposed that the surface attachment of anchorless proteins such as GAPDH may be a result of adherence of exogenous protein released from neighbouring cells via lysis (Scott & Barnett, 2006), although in Group A *Streptococci* at least, GAPDH appears to be specifically released from the cell (Boel *et al*., 2005). Furthermore, provoked lysis of *Lactobacillus platarum* cells does not result in an increase in surface GAPDH levels in this species, but surface protein levels do correlate with changes in membrane permeability during growth of the bacteria (Saad *et al*., 2009). To determine whether *S. aureus* GAPDH is specifically surface expressed, the ability of recombinant tagged proteins to re-associate with unlabelled *S. aureus* cells could be analysed. If the protein is able to associate with the bacteria, the next step may be to utilise the tagged protein to "pull down" *S. aureus* proteins from total cell lysate to

determine which structures GAPDH proteins can attach to on the cell surface. If the protein is unable to associate with the bacteria this would suggest that *S. aureus* GAPDH proteins are specifically released and reattached to the cell surface, opening the possibility for the discovery of a novel surface expression system in this pathogen. Recently it has been shown that *S. aureus* produce membrane vesicles which contain a number of cytoplasmic proteins including some involved in metabolism, cellular processing and gene expression (Lee *et al*., 2009). Although the *S. aureus* GAPDH proteins were not identified in this study, this phenomenon cannot be overlooked in potentially playing a role in surface localisation of anchorless proteins.

As well as demonstrating key roles in carbon metabolism, we have also demonstrated novel moonlighting roles for both GapA and GapB. Bacterial surface associated GAPDH proteins are commonly found to bind to host proteins, and *S. aureus* GapA and GapB are no exception (Modun & Williams, 1999; Goji *et al*., 2004). Due to this property, the impact of each of these proteins on *S. aureus* biofilm formation was analysed. Both proteins appear to be required for biofilm formation in strain 8325-4, although the role they play in this process is unknown (Figure 3-14). As the growth of each of the strains tested was comparable in the liquid medium used, the differences in biofilm formation between the wild type and mutant strains cannot be the result of cell growth. However it is possible that a loss of GAPDH surface localisation means that the ability to produce ATP or reducing power at the cell surface is decreased, which may be important in either the adherence or accumulation of cells within the biofilm structure. During biofilm formation it is also possible that the binding activity of GapA and GapB is required for cell-cell adherence or adhesion of the cells to a surface. If this is the case, the addition of GapA and GapB specific antibodies may block protein interactions and

provide an interesting means of determining the role of GAPDH binding activity in a *S. aureus* biofilm (Sun *et al*., 2005).

An additional moonlighting role was also demonstrated for GapA in the oxidative stress response. Strains with a *⊿gapA* mutation were more sensitive to H_2O_2 introduced to a growing culture compared to wild type cells (Figure 3-13). It is unclear whether a loss of GapA results in increased killing of the cells or a delay in the oxidative stress response. The ability to resist oxidative stress is important for bacterial survival within neutrophils, and contributes to the persistence of some *S. aureus* infections (Voyich *et al*., 2005). Therefore it would be interesting to conduct intracellular survival assays with the GAPDH mutant strains to determine whether GapA plays an active role in this process. A previous study has shown that during phagocytosis a number of metabolic genes are unregulated, suggesting a wider role for metabolism in intracellular survival and persistence, particularly in CA-MRSA infections (Voyich *et al*., 2005). Thiol-oxidation of GAPDH in *S. cerevisae* has shown that inactivating GAPDH causes a very rapid shift in carbon metabolism from glycolysis to the pentose phosphate pathway to balance redox potential of the cell (Ralser *et al*., 2009). Through examining the end products of metabolism it would be possible to determine whether this same shift occurs in *S. aureus*. Both eukaryotic and prokaryotic GAPDH proteins undergo S-thiol oxidation in response to oxidative stress, and it is possible that like its role in carbon metabolism, this oxidative response is another conserved function of GAPDH proteins (Schuppe-Koistinen *et al*., 1994; Shenton & Grant, 2003: Weber *et al*., 2004).

The expression of both *gapA* and *gapB* is influenced by glucose concentrations at the transcriptional level and growth data suggests that very low glucose levels are required

to shift from gluconeogenic metabolism to glycolytic metabolism in *S. aureus* (Figure 3-8; Figure 3-9). Due to their reciprocal metabolic functions during carbon metabolism, the up-regulation of *gapA* and down regulation of *gapB* in the presence of glucose was not unexpected (Figure 3-8). However the fact that both genes are influenced by the glycolysis regulator GapR was surprising. For the first time we have demonstrated that the genes *gapR, gapA, pgk, tpi, pgm,* and *eno* are transcribed as an operon in *S. aureus* as seen in other species, and that GapR acts as a negative regulator of this operon in the absence of glucose (Figure 3-8; Figure 3-10). Furthermore we have shown that GapR also represses the transcription of *gapB* in both glycolytic and gluconeogenic conditions (Figure 3-11). To our knowledge this is the first time that any GapR homologue has been shown to regulate a gene outside of the glycolytic operon. The mechanism by which GapR functions in the regulation of either *gapA* or *gapB* is not clear at present. The *B. subtilis* GapR homologue CggR directly binds to a unique target sequence and blocks transcriptional elongation of the glycolytic operon (Doan & Aymerich, 2003; Zorrilla *et al*., 2007b), but sequence homology searches failed to identify a putative binding sequence for GapR in *S. aureus*. Through the use of DNAse footprinting of the *gapR* and *gapB* promoter regions it may be possible to determine a *S. aureus* GapR binding motif and demonstrate whether this protein acts to regulate gene transcription directly or indirectly in *S. aureus*. Microarray analysis of the *ΔgapR* mutant could be used to determine additional genes that are regulated by GapR in *S. aureus*. This technique could also be utilised for the *ΔgapA* and *ΔgapB* mutant strains to determine whether disruption to the carbon utilisation pathways results in a shift in the transcriptional profile of *S. aureus* strains.

The regulation of *gapA* and *gapB* transcription is not only under the influence of glucose but also divalent metal ions. The transcription of *gapB* is repressed in the presence of Fe and appears to be under the control of the regulator PerR (Figure 4-6). *S. aureus* recombinant GapB protein has been shown to bind transferrin *in vitro*, and it is possible this Fe regulation relates to this function (Goji *et al*., 2004). The transcription of *gapA* is up-regulated in response to Cu (Figure 4-3) but interestingly shows strain variable expression in response to Fe; Class I strains have reduced expression in the presence of Fe, and Class II strains have reduced expression in the absence of Fe (Figure 4-2). The induction of *gapA* transcription in response to Cu may be a product of the potential role of this protein in the oxidative stress response, as the primary method of Cu toxicity is inducing oxidative stress. Additional divalent metals that could also be included for further study are manganese and calcium, to determine whether these also influence expression of *gapA* and the glycolytic operon.

The transcriptional iron regulation of *gapA* concurs with the levels of GAPDH activity at the cell surface and also the amount of surface localised protein in similar conditions. However cytoplasmic levels of GAPDH do not show such dramatic iron regulation, with levels in low iron conditions only slightly reduced compared to high iron conditions in 8325-4 (Figure 4-1; Figure 4-2). This suggests that Fe regulation of GAPDH occurs at two levels; first during transcription and again at either transport or attachment to the cell surface. This may indicate a unique method of differentially regulating surface expression of proteins while maintaining relatively consistent cytoplasmic levels, and requires further investigation which is beyond the scope of this project. One possible explanation for Fe regulation of the transcript and surface protein, but not the cytoplasmic protein, is that there is a threshold for intercellular protein

expression. Up to a point cytoplasmic GAPDH accumulates, providing similar levels in high and low iron conditions despite varied transcription. Past this threshold protein is transported and expressed on the cell surface. Therefore surface expression will greatly vary with changes in transcription, but intercellular levels will remain relatively constant. Potentially this could maintain constant levels of GAPDH in the cytoplasm regardless of Fe concentration to allow efficient glycolytic activity and glucose utilisation, but provide a means of regulating surface expression to modulate secondary functions of GapA protein (Modun *et al*., 2000). Many surface associated anchorless proteins are highly expressed housekeeping proteins in the cytoplasm of *S. aureus*, involved in central metabolism, biosynthesis of molecular components and DNA/RNA synthesis and repair (Gatlin *et al*., 2006). It would be interesting to see whether the surface expression of additional anchorless proteins follows a similar pattern of differential expression at different cellular compartments.

Transcription of *gapA* is repressed by the global regulators Fur and MntR in the presence and absence of divalent metal ions in the Class II strains (Figure 4-5). This is potentially a novel mechanism of transcriptional control for each of these two regulators, although the lack of DNA binding motifs in the putative *gapR* promoter suggests that their action may be indirect. Fur has been shown to regulate the expression of a number of other global regulators, including Sae, Agr and Rot, and therefore it may be useful to determine the impact of these regulators on *gapA* transcription (Johnson *et al*., 2010). A promoter pull down experiment could be used to determine any *S. aureus* regulatory proteins that directly associate with the glycolytic operon promoter region.

It is important to note that all of the transcriptional data regarding *gapA* and *gapB* environmental regulation has been derived through northern blotting. Although this method has proved very useful in determining transcript sizes and differences in stability between transcripts, it is not the most effective means of quantitatively determining gene expression levels. In addition the way in which this technique was utilised only provided a snapshot of gene expression at a particular time point, and perhaps more time points should have been included from different growth phases of *S. aureus*. To further strengthen the transcriptional data presented here additional methods would need to be considered. QRT-PCR (quantitative real-time PCR) would be a logical choice to be used in conjunction with northern blotting. This method not only provides a quantitative measurement of gene expression but can be more easily used to monitor gene expression over time. Coupled with the ability to determine transcript size and relative stability gained through northern blotting, this provides a powerful tool in analysing bacterial gene expression and would be a useful next step in analysing the transcription of *gapA* and the glycolytic operon in *S. aureus*.

Through the use of transcriptional reporters I have shown that sequence variation of the putative *gapR* promoter region is responsible for this strain variable response (Figure 4-8; Figure 4-16). Furthermore there is evidence that the STAR elements found upstream of the *gapR* coding sequence are required for Fe regulation of transcription in the Class II strains (Figure 4-16). To my knowledge this is the first demonstration of a function for these repeats. Initial observations also suggested that the number of STAR elements may correlate with the level of transcription of the associated genes (Figure 4-7). Although Northern blot analysis of the expression of the glycolytic operon and two additional STAR associated loci, *hprK* and orf_{0733}, in strains with greater numbers of

repeat elements failed to confirm this correlation we cannot rule out a function for these repeats in *S. aureus* (Figure 5-13; Figure 5-14; Figure 5-15). To further determine the impact of STAR elements on transcription an integrated reporter is required, as the addition of multiple STAR motifs in a plasmid based reporter resulted in a loss of promoter expression. The integrated reporter could be used to determine whether the STARs alone contain promoter elements, and also determine the impact of the elements on gene transcription in a quantitative manner. Many available integrated reporter systems recombine into the promoter region of the gene of interest (Horsburgh *et al*., 2001b). A limitation of this type of system is that STAR elements occur throughout the *S. aureus* genome, and it is possible that a reporter will integrate into the promoter region of another gene, or that homologous recombination will result in the loss or gain of repeats in the reporter. One way to avoid this problem might be to integrate the reporter construct, with variable STAR elements, into a pseudogene or gene with limited impact on the *S. aureus* phenotype. One such gene is a lipase precursor known as *geh*, which has previously been used as an integration site due to a bacteriophage *attB* site within its coding region (Luong & Lee, 2007; Geiger *et al*., 2008).

Although the STAR elements have not been shown to perform a specific function, the non-repeating sequences of these repeat elements are lineage specific at each of the loci tested. This suggests that although the number of STAR elements can vary between strains of the same lineage at a particular locus, the STAR motifs do not frequently interchange between different STAR loci within a strain, nor between similar STAR loci in different strains (Section 5.3). It would be useful to expand the analysis of the STAR element loci in a wider variety of *S. aureus* strains, and also in multiple isolates of the same lineage, to further our understanding of the evolution of this repeat element.

The abundance of these repeat elements in the *S. aureus* genome alongside their conserved nature suggests that they are functional in some aspect of cell physiology. It is important to determine whether these repeat elements are transcribed in *S. aureus*. There is evidence that at least some STAR elements are present in the leader region of some mRNAs, but it is not clear whether this is true of all STAR element loci or whether this transcription is functionally significant (Geissmann *et al.*, 2009). A different transcript mapping procedure could be used to determine whether the STAR loci are transcribed, as outlined in Section 4.6, but a simpler method may be to probe total *S. aureus* RNA for the presence of STAR element specific sequences using northern blotting or QRT-PCR. A STAR specific probe was previously used in Southern blotting to determine the abundance of STAR loci in different *S. aureus* strains (Cramton *et al*., 2000). The major factor limiting the accuracy of this type of experiment is the size and specificity of the STAR probe. If the probe is too small it is likely to bind to non-STAR specific sequences, potentially providing false positive results. Even if results were obtained using this method, another more specific experiment would still need to be used to confirm the transcription of these repeats. Whether or not the STAR elements are transcribed, it is possible that regulatory proteins, such as transcriptional enhancers or RNAP-associated transcriptional machinery bind to the STAR locus sequences. To determine whether this is the case, DNA pull down experiments could be used to identify any staphylococcal proteins that directly associate with STAR motif DNA. This could potentially shed light on any functional significance of the STAR repeat loci.

Although transcriptional analysis of the STAR-associated loci did not determine any correlation between the level of gene expression and the number of STAR motifs

present, the data do highlight strain variation in the level of expression of the genes (Figure 5-13; Figure 5-14; Figure 5-15). By extending this analysis to three additional loci that are not associated with STAR elements (*fur, csoR* and *hla*) we can clearly see that the level of expression of a wide range of genes from different functional groups are expressed at variable levels in different *S. aureus* strains (Figure 5-16; Figure 5-17). Transcript levels are not relative at each locus in a particular strain, i.e. expression is not uniformly high or low at all loci in a particular strain, which shows that this variability is not due to global changes in RNA transcription. Rather there appears to be co-ordinated regulation of transcription levels at each locus. An important next step would be to analyse the expression levels of these genes at the protein level to determine whether strain variable transcription actually results in phenotypic strain differences. Such an analysis would also provide data on strain variation in post-transcriptional regulation in *S. aureus*. As global regulators such as Fur and CsoR appear to be expressed at variable levels in different strains (Figure 5-16; Figure 5-17), it is very likely that this will influence the expression of entire gene regulons. It has previously been shown that expression of the global regulator *sae* is varied between *S. aureus* isolates (Steinhuber *et al*., 2003), and therefore relative expression levels of particular regulators may provide an additional means of fine tuning the expression of certain genes during infection in response to particular stimuli.

This study highlights that the regulation of genes in *S. aureus* is highly complex and must be tightly controlled at a number of levels for gene products to perform all of their physiological functions. Transcription of both *gapA* and *gapB* is under the influence of multiple environmental signals and the resulting proteins are involved in several functions within the cell (Figure 6-1). As the primary function of each of these genes

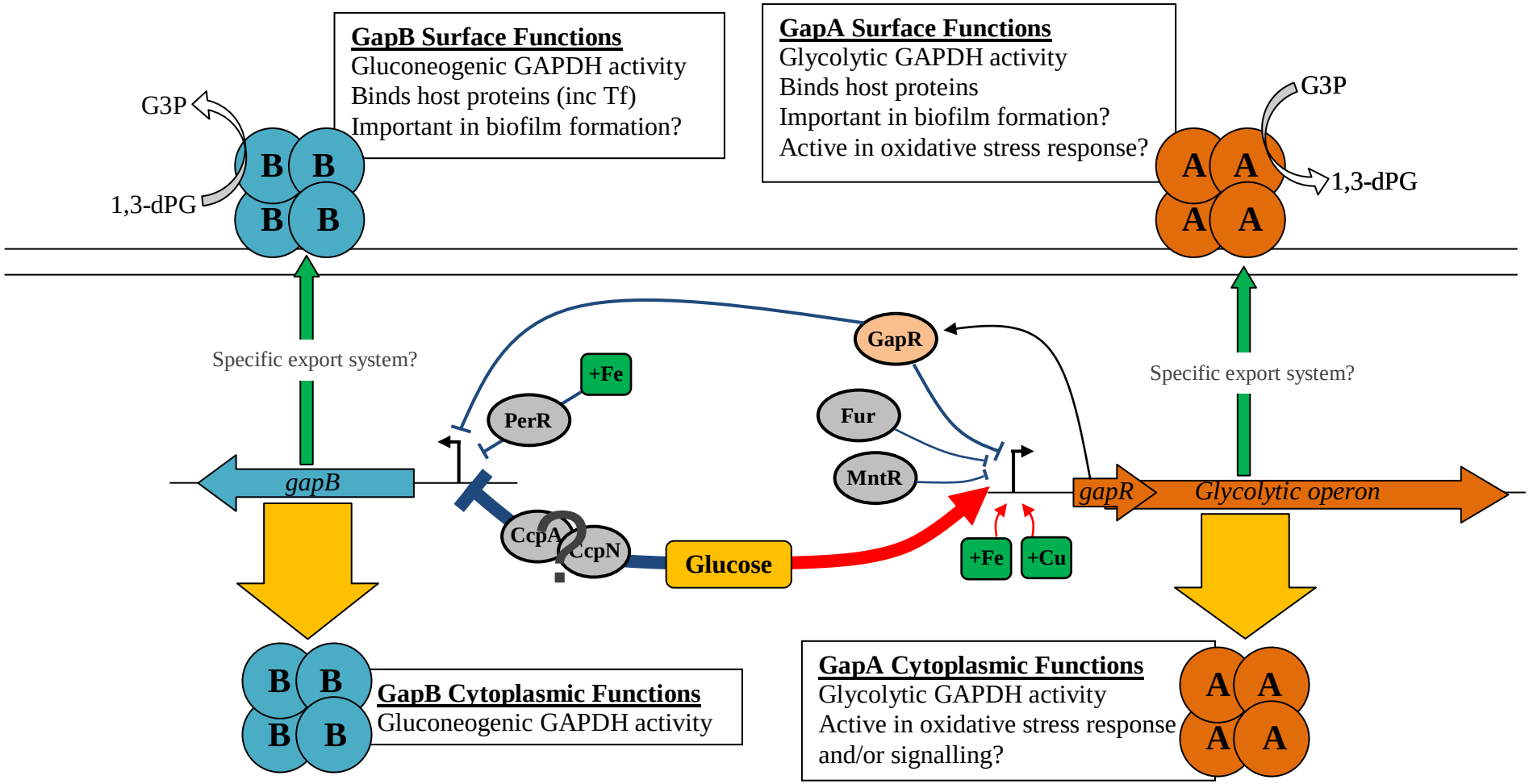


Figure 6-1. A proposed model for the regulation and function of the *S. aureus* GAPDH homologues GapA and GapB.
This model highlights the key factors influencing the expression of *gapA* and *gapB* genes in *S. aureus* and links these regulatory stimuli to the localisation and function of the proteins. The larger the connecting arrow, the greater influence this factor has on the system (i.e. glucose has the largest influence *gapA* and *gapB* expression). The majority of the proteins produced are retained in the cytoplasm to function during carbon metabolism. Secondary stimuli such as Fe and Cu are required to fine tune gene expression and may specifically regulate secondary functions at the cell surface.

lies in carbon metabolism, it is likely that carbon source is the signal with the highest priority; no matter what additional signals are present, *gapA* will only be expressed in the presence of glucose and *gapB* in the absence of glucose. The control of this regulation will occur via catabolite control genes such as *gapR*, *ccpA* and *ccpN*, although the exact role each of these regulators plays is not yet clear. In addition, both GAPDH homologues have secondary functions, and therefore the genes must also be expressed in response to additional stimuli such as iron and copper (Figure 6-1). It is possible that these stimuli are required to fine-tune the expression of *gapA* and *gapB* in order to influence their availability for supplemental functions such as host protein binding or oxidative stress resistance. The control of secondary GAPDH functions may also alter the level of surface localised protein, providing a means of modulating surface functions while cytoplasmic enzyme activity remains relatively consistent.

In conclusion, the *S. aureus* GAPDH homologues play a key role in central metabolism as well as additional unrelated moonlighting roles, and it is clear that they are both important during *S. aureus* infection. Understanding the functions of these fundamental proteins during infection may provide insight into new treatment or prevention methods for *S. aureus* infection. In addition STAR elements are maintained across the genome within independent *S. aureus* lineages, strongly suggesting that these elements may be functional in *S. aureus*. The abundance of these elements in this species compared to other coagulase negative staphylococci may help to explain the adaptability and variability in the evolution of this important opportunistic pathogen.

Appendix

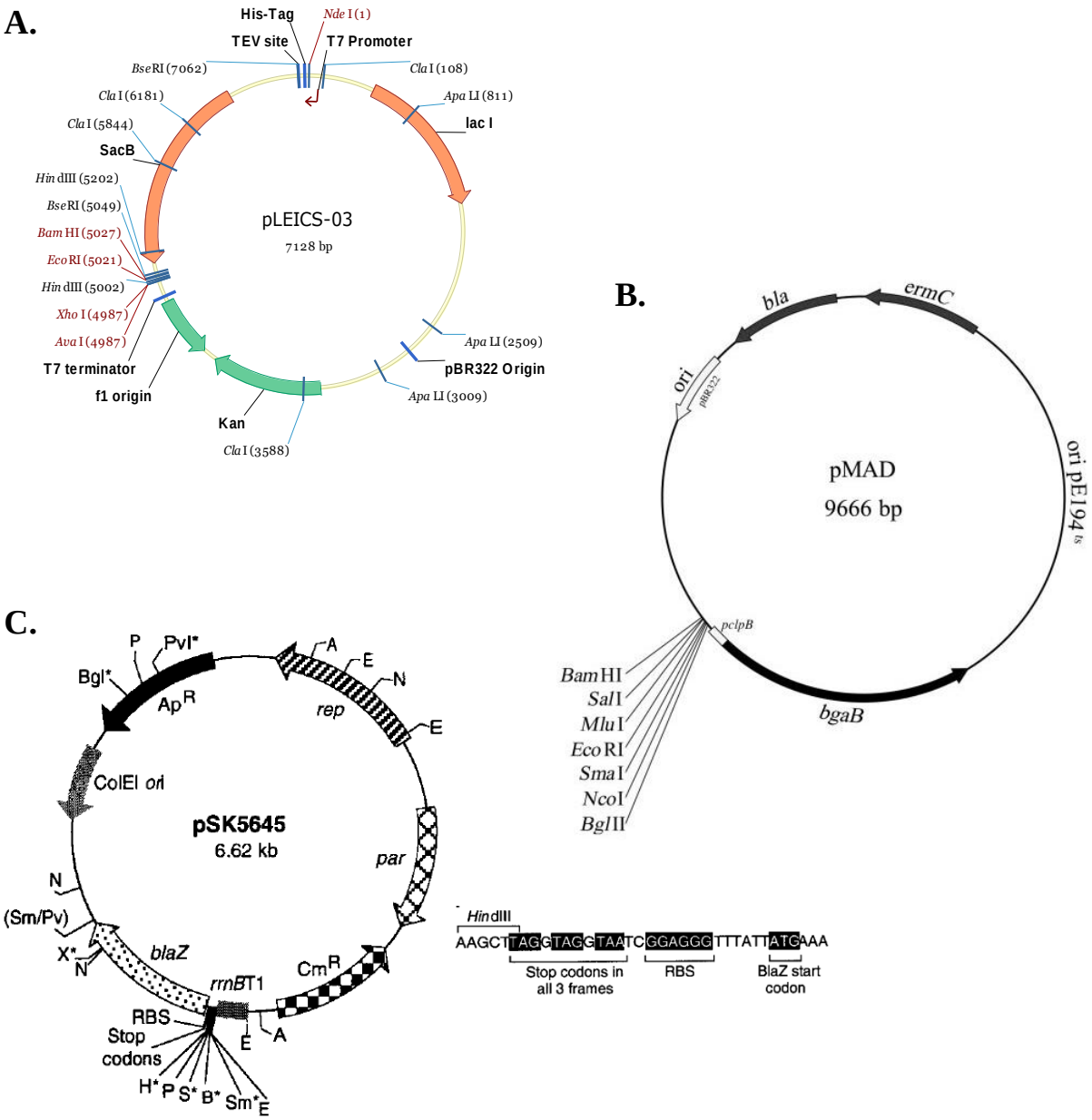


Figure A-1. Plasmids used in this study.

(A) Protein expression plasmid pLEICS-03, including Amp and Kan resistance genes, an IPTG inducible promoter and *lacI* repressor gene. Figure supplied by Protex (University of Leicester).

(B) Mutagenesis plasmid pMAD, including Amp and Ery resistance genes and a constitutive β-galactosidase (*bgaB*). The Gram positive origin of replication (pE194^{ts}) is temperature sensitive. Map taken from Arnaud *et al.*, (2004).

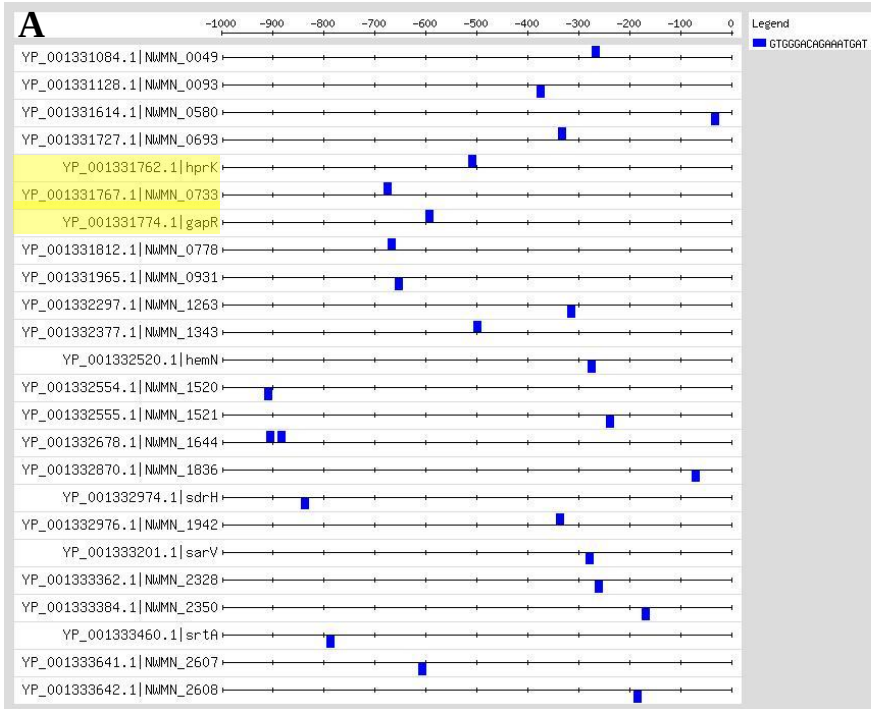
(C) Reporter plasmid pSK5645 including Amp and Cm resistance genes and a promoterless β-lactamase (*blaZ*) with ribosomal binding site and the DNA sequence surrounding the *blaZ* start codon. Figure taken from Grkovic *et al.*, (2003).

Table A.1 – Strains used in this study, including lineage and STAR analysis where possible.

Strain	*gapR* STAR Structure	No. *gapR* STARs	No. *hprK* STARS	No. orf_{0733} STARS	Strain origin	Sequence Type	Clonal Complex
8325-4	1	0	3	7	Human	8	
Newman	1	3	3	5	Human	8	
USA300 FPR3757 [1]	1	1	3	5	Human	8	
USA300 TCH1317 [1]	1	3	3	5	Human	8	8
RN6390B	1	1	--	--	Human	8	
COL[1]	1	3	2	2	Human	250	
TW20[1]	1	1	2	4	Human	239	
BB	1	3	1	5	Bovine	133	
C01719	1	3	1	5	Bovine	771	133
C01771	1	3	1	4	Bovine	771	
Mu50	1	6	3	6	Human	5	
N315[1]	1	6	3	6	Human	5	
Mu3[1]	1	6	3	6	Human	5	5
ED98[1]	1	4	3	3	Poultry/Human	5	
JH1[1]	1	3	3	5	Human	105	
JH9[1]	1	3	3	5	Human	105	
MSSA476[1]	1	2	2	3	Human	1	
Mw2[1]	1	1	2	3	Human	1	1
C01865	1	2	--	--	Bovine	1	
MRSA PM25	1	2	--	--	Human	22	22
C00595	1	2	--	3	Bovine	97	
C00704	1	1	2	4	Bovine	97	97
C01801	1	1	--	--	Bovine	97	
ATTC 12600	1	1	--	--	Human	--	--
WCUH29	1	3	--	--	Human	--	--
B1203012	1	1	--	--	Human	--	--

Strain	*gapR* STAR Structure	No. *gapR* STARs	No. *hprK* STARS	No. orf_{0733} STARS	Strain origin	Sequence Type	Clonal Complex
B2202016	1	1	--	--	Human	--	--
SA R4/7	1	2	--	--	Human	--	--
SA D81/7	1	2	--	--	Human	--	--
SA D196/7	1	3	--	--	Human	--	--
B0903007	1	3	--	--	Human	--	--
B1703012	1	5	--	--	Human	--	--
30375	1	4	1	3	Bovine	188	188
66195	1b	4	2	3	Human	59	59
65991	1b	4	2	3	Human	59	
MRSA 252	2	0	2	3	Human	36	
MRSA PM64	2	0	2	3	Human	36	
B2503017	2	0	--	--	Human	30	30
47979	2	0	--	--	Human	34	
63505	2	0	--	--	Human	B[2]	
SA4523-7	2	0	--	--	Human	--	--
SA R157/7	2	0	--	--	Human	--	--
48064	2	0	--	--	Human	--	--
B1003003	2	0	--	--	Human	--	--
CDC8	2b	0	--	--	Human	45	
66155	2b	0	--	--	Human	A[2]	45
65985	2b	0	--	--	Human	A[2]	
RF122	3	N/A	1	5	Bovine	151	
38963	3	N/A	--	--	Bovine	151	
982BL	3	N/A	--	--	Bovine	151	151
C00759	3	N/A	1	6	Bovine	151	
C123/5/05-09	3	N/A	1	--	Bovine	151	

[1] Indicates only sequence available for these strains. [2] Indicates sequence types undefined by MLST database at present.



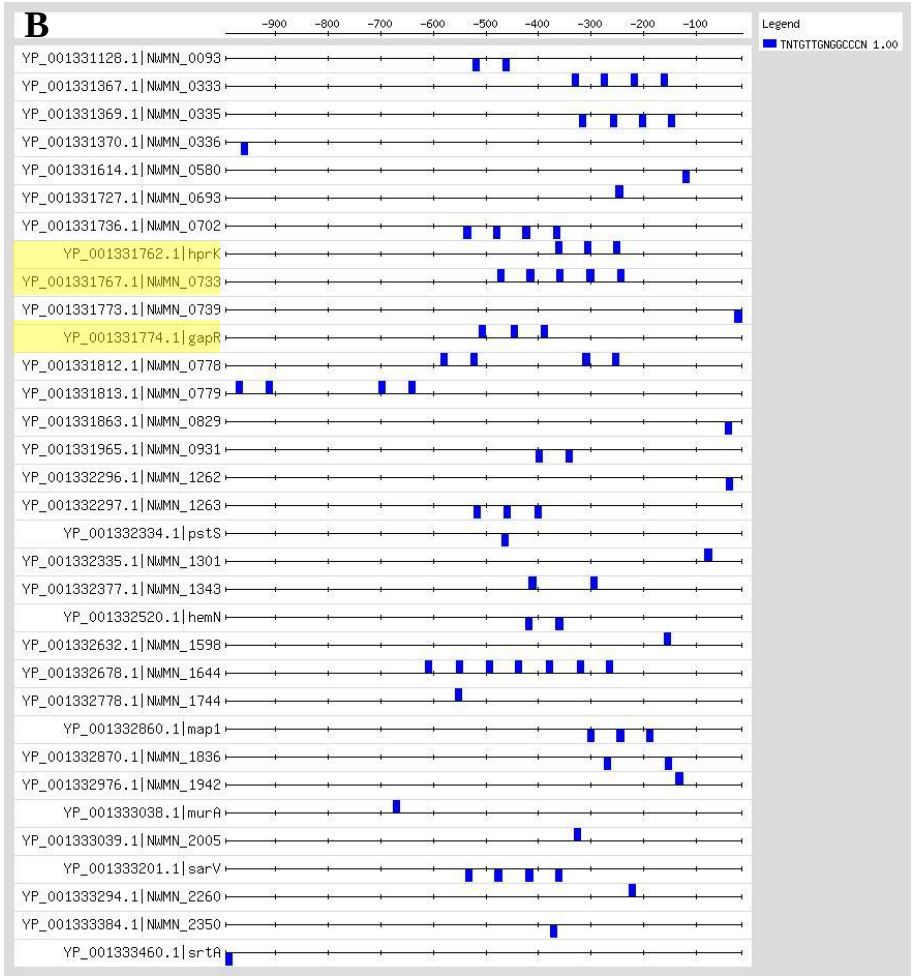
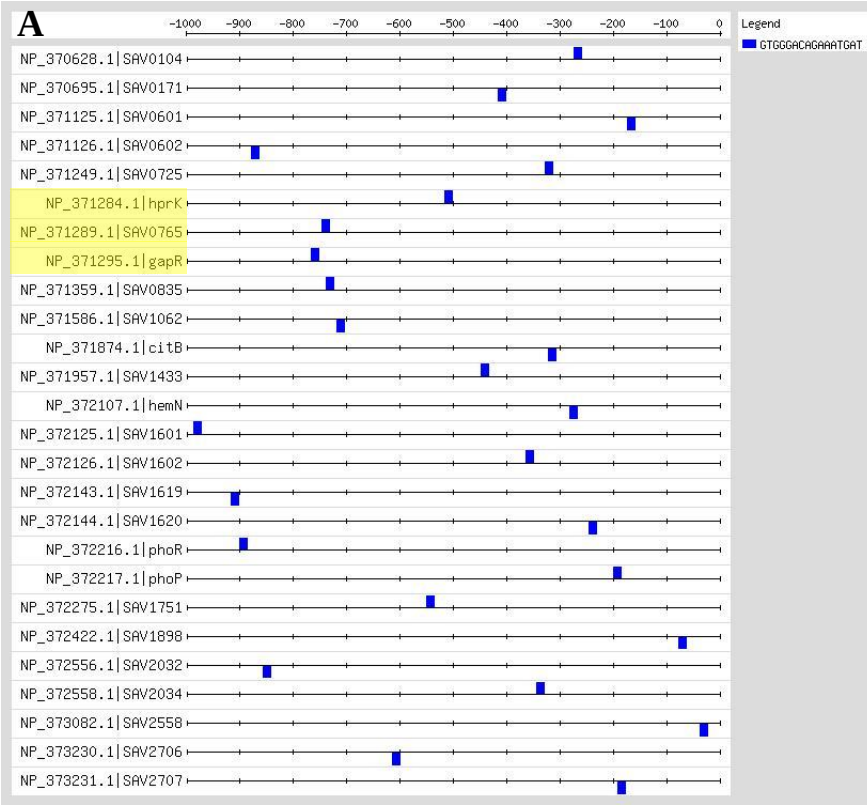


Figure A-2. STAR element RSAT data for strain Newman.

Identification of (A) STAR start signature sequences and (B) STAR element repeat motif sequences up to 1000 bp upstream of open reading frames in *S. aureus* strain Newman using the RSA-tools Genome-scale DNA-pattern search (http://rsat.ulb.ac.be/rsat/). The *hprK*, *gapR* and orf_{0733} loci are highlighted in yellow.



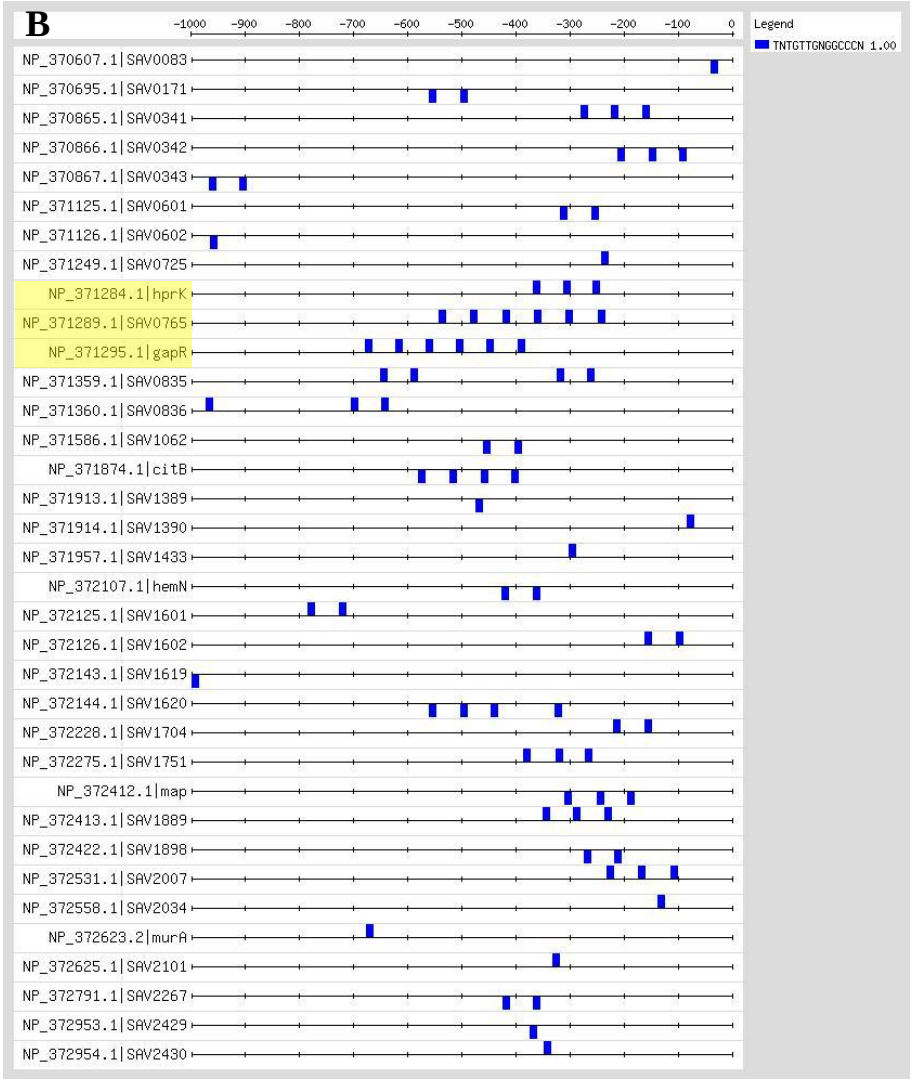
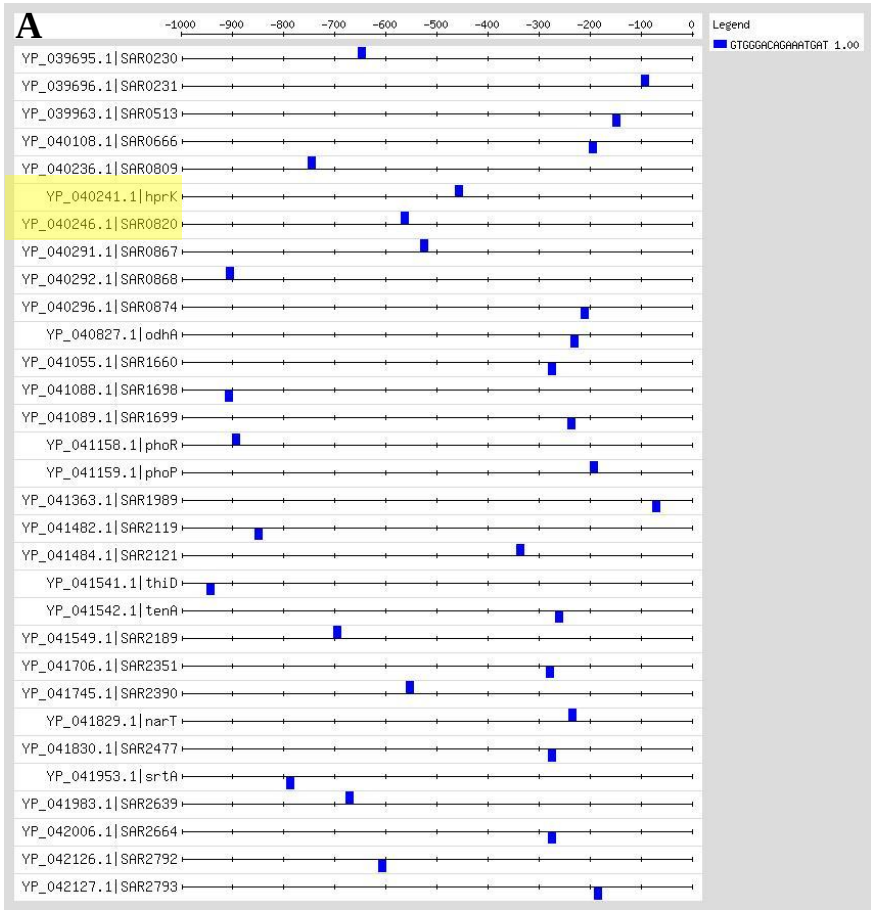


Figure A-3. STAR element RSAT data for strain Mu50.

Identification of (A) STAR start signature sequences and (B) STAR element repeat motif sequences up to 1000 bp upstream of open reading frames in *S. aureus* strain Mu50 using the RSA-tools Genome-scale DNA-pattern search (http://rsat.ulb.ac.be/rsat/). The *hprK*, *gapR* and orf_{0733} (SAV0765) loci are highlighted in yellow.



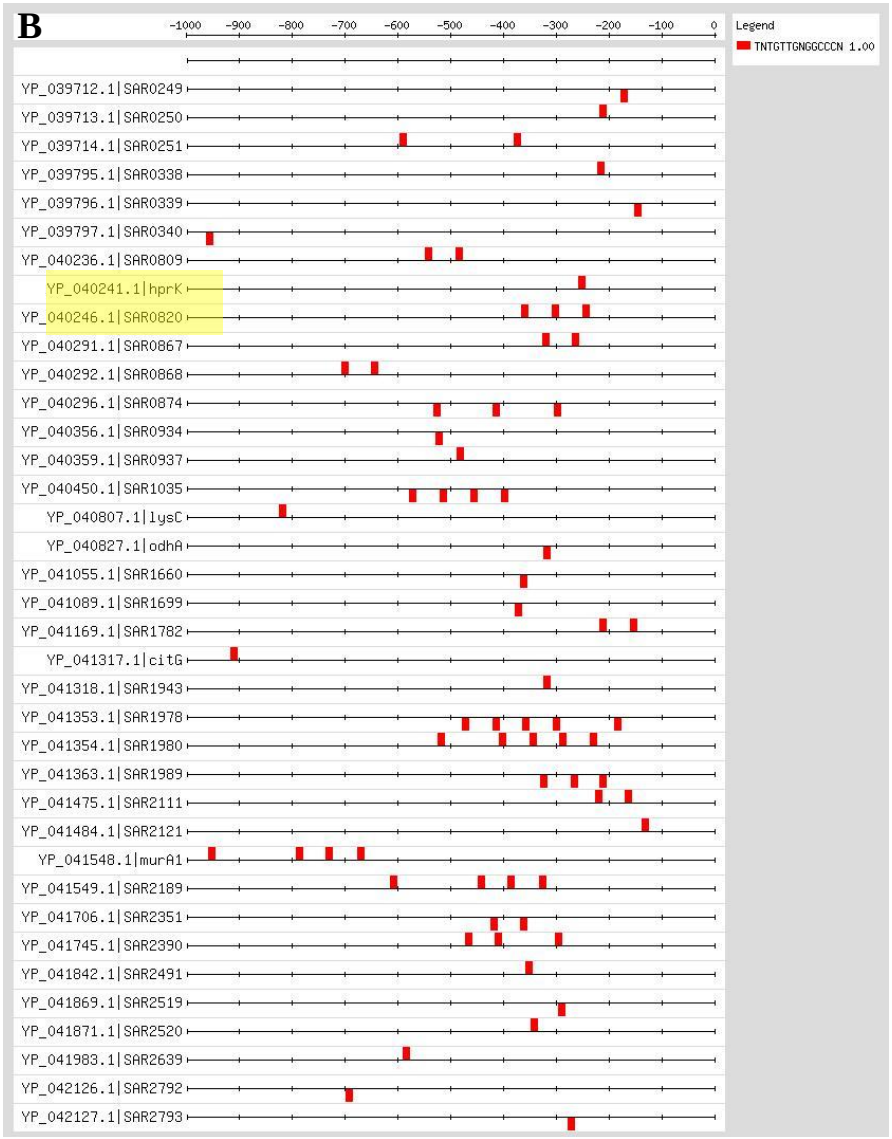
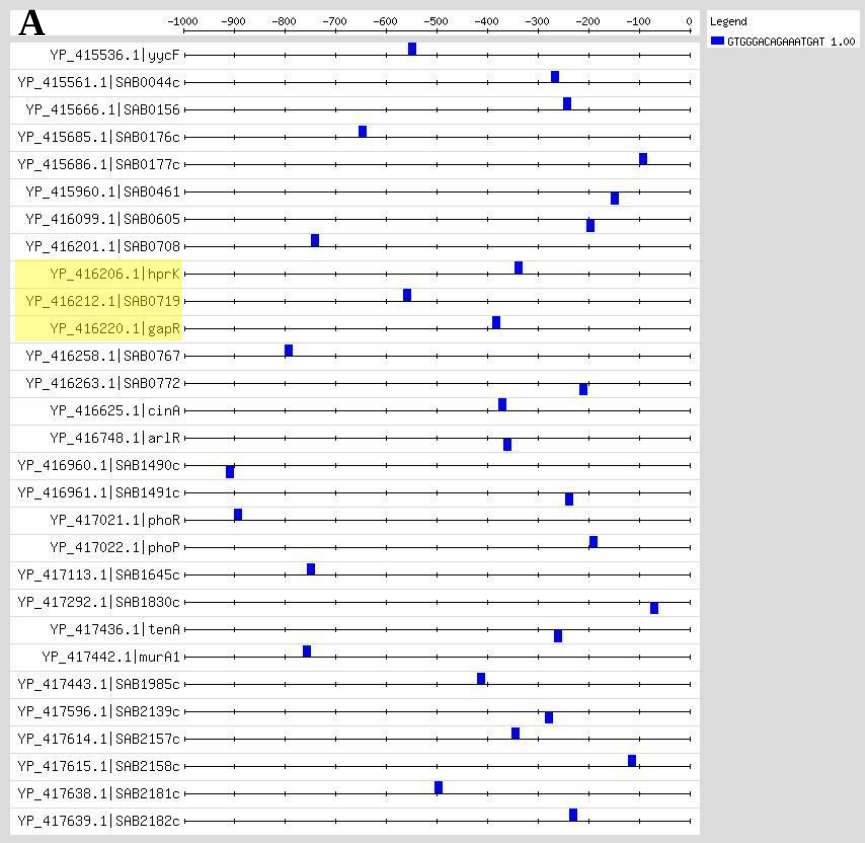


Figure A-4. STAR element RSAT data for strain MRSA 252.

Identification of (A) STAR start signature sequences and (B) STAR element repeat motif sequences up to 1000 bp upstream of open reading frames in *S. aureus* strain MRSA 252 using the RSA-tools Genome-scale DNA-pattern search (http://rsat.ulb.ac.be/rsat/). The *hprK* and orf_{0733} (SAR0820) loci are highlighted in yellow.



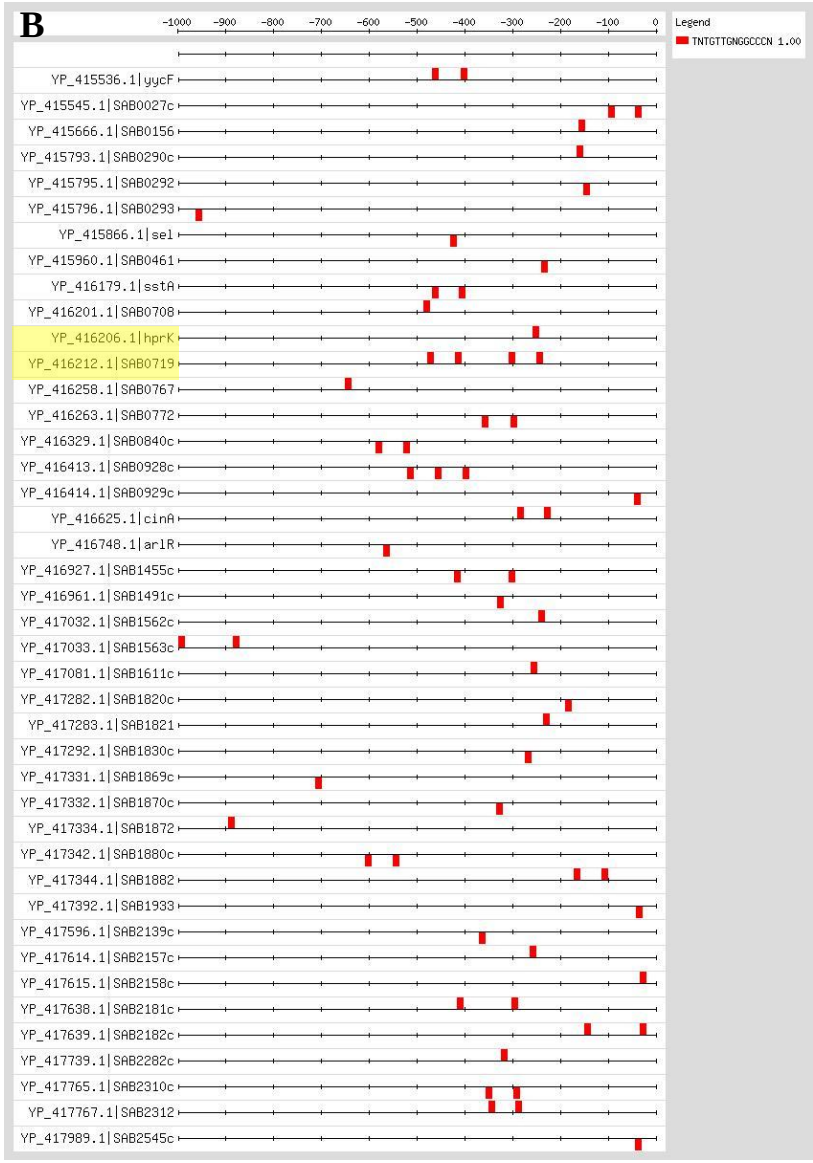


Figure A-5. STAR element RSAT data for strain RF122.

Identification of (A) STAR start signature sequences and (B) STAR element repeat motif sequences up to 1000 bp upstream of open reading frames in *S. aureus* strain RF122 using the RSA-tools Genome-scale DNA-pattern search (http://rsat.ulb.ac.be/rsat/). The *hprK*, *gapR* and orf_{0733} (SAB0719) loci are highlighted in yellow.

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